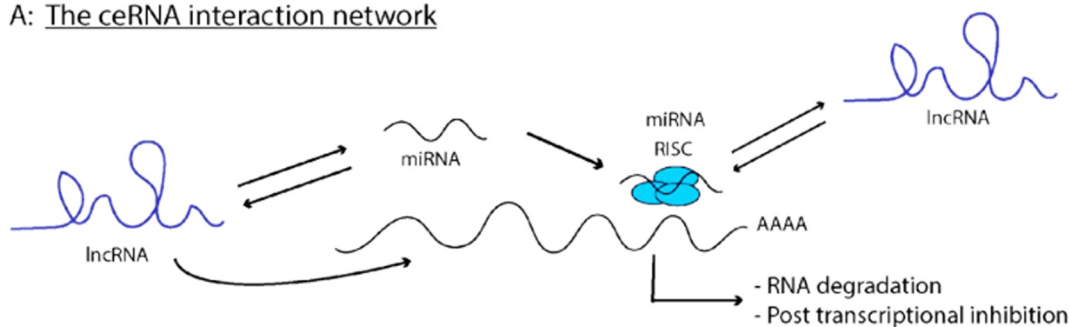
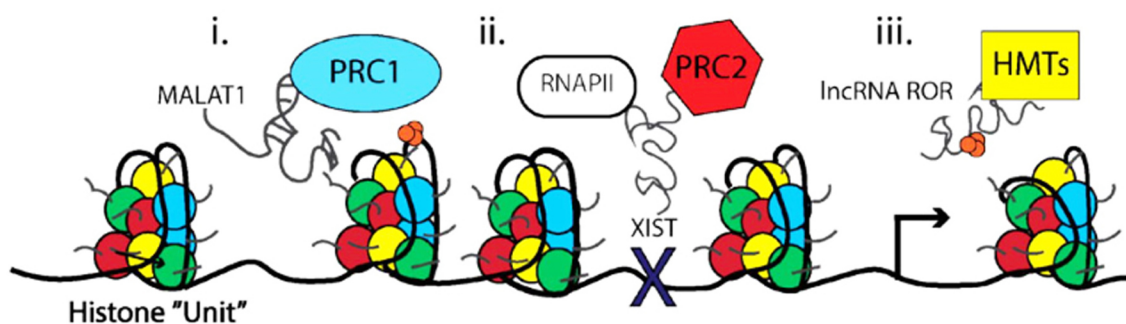


# Journal of Cancer Metastasis and Treatment

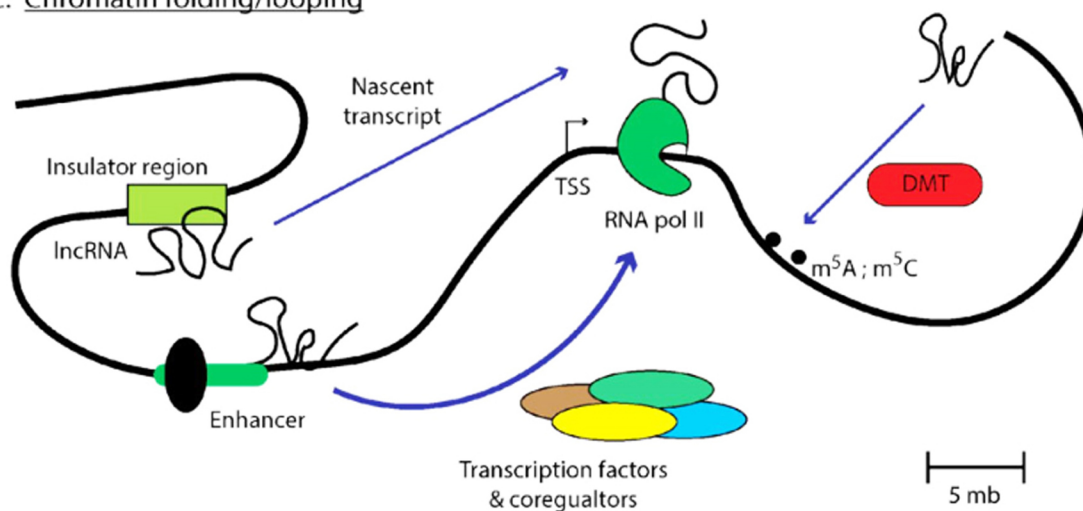
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## B: Chromatin modifiers and transcriptional repressors



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Original Article

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# Impact of previous anti-angiogenesis treatment in nivolumab-treated advanced non-small cell lung cancer

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## Abstract

**Aim:** To investigate how previous systemic therapy such as anti-angiogenesis can influence cancer immunotherapy for non-small cell lung cancer (NSCLC).

**Methods:** A total of 134 patients with advanced NSCLC who were treated with nivolumab were retrospectively reviewed. Correlation between status of prior anti-angiogenesis treatment and clinical characteristics were determined. Impact of prior anti-angiogenesis on therapeutic outcome of nivolumab was investigated for tumor efficacy such as progression-free survival (PFS).

**Results:** Sixteen patients were treated with at least one anti-angiogenesis agent prior to nivolumab. The prior use of anti-angiogenesis agent was associated with stage IV disease, non-squamous histology, and two or more lines of systemic therapy. Median PFS was significantly shorter in the prior anti-angiogenesis group than in no prior anti-angiogenesis group (8.3 vs. 11.3 weeks, log-rank  $P = 0.006$ ). Multivariate analyses demonstrated that only prior anti-angiogenesis status was associated with worse PFS. There is also a slight trend for worse disease control rate ( $P = 0.101$ , Fisher's exact test) and overall survival ( $P = 0.200$ , log-rank) in prior anti-angiogenesis group.

**Conclusion:** This retrospective study suggests that prior anti-angiogenesis treatment negatively impacts the therapeutic outcome of immunotherapy in advanced NSCLC.



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**Keywords:** Non-small cell lung cancer, nivolumab, angiogenesis, immunotherapy

## INTRODUCTION

Systemic treatment for advanced cancer had been primarily cytotoxic chemotherapy until modern systemic modalities were recently developed. Now we know that targeted therapies for selected advanced cancer such as oncogene-driven malignancy provide better outcomes than traditional chemotherapy. For instance, small molecule kinase inhibitors are available for advanced non-small cell lung cancer (NSCLC) with a somatic mutation in the catalytic domain of epidermal growth factor receptor gene (EGFR) or gene rearrangement in anaplastic leukemic kinase gene (ALK)<sup>[1-4]</sup>. More recently, inhibitors for immune checkpoints that negatively regulate anti-cancer immunity have become clinically available with improved survival outcome for the treatment of advanced NSCLC, head/neck, melanoma, bladder, and renal cell carcinomas<sup>[5-11]</sup>.

The mortality rate for lung cancer, however, has not changed dramatically over the last several decades<sup>[12]</sup>. Although recently developed cancer immunotherapy, such as anti-PD-1 therapy, has made a significant impact on daily practice for advanced NSCLC, most patients who are treated with such agents still succumb to the disease within five years<sup>[13]</sup>. Continued efforts to enhance activity of cancer immunotherapy are required to further improve outcome.

Recently researchers have been conducting clinical trials to determine if the combination of immunotherapy and other treatments may have additive clinical activity in this disease. Anti-angiogenesis agents such as bevacizumab have been developed and achieved regulatory approval for several cancer types<sup>[14,15]</sup>. These agents are also being investigated in various diseases in combination with immunotherapy<sup>[16]</sup>. Rationale for the combination is that suppression of neoangiogenesis, remodeling on distorted microvasculature, and resultant improved tumor perfusion are expected to enhance anti-cancer immunity<sup>[16]</sup>. Because bevacizumab has a relatively long half-life (approximately 20 days) and lasting biological effect<sup>[15]</sup>, previous anti-angiogenesis treatment might positively influence the efficacy of anti-cancer immunotherapy. Several studies have indicated that withdrawal of anti-angiogenesis agents results in an increase in tumor aggressiveness due to rebound angiogenesis in the tumor microenvironment<sup>[17,18]</sup>. We therefore conducted a retrospective study to determine if prior use of anti-angiogenesis therapy can impact progression-free survival in advanced NSCLC patients who were treated with anti-PD-1 therapy.

## METHODS

### Patient selection

A total of 801 advanced and metastatic NSCLC patients were registered at University of Kansas Cancer Center between January 2015 and June 2016. Review of their medical records identified 141 patients who were treated with at least one dose of the anti-PD-1/PD-L1 inhibitors at University of Kansas Cancer Center. A majority ( $n = 133$ ) of patients were treated with nivolumab alone, whereas others were treated with nivolumab and atezolizumab ( $n = 1$ ), atezolizumab alone ( $n = 1$ ), pembrolizumab alone ( $n = 4$ ), or other investigational agent alone ( $n = 2$ ). All of these agents were intravenously given every two weeks (nivolumab) or every three weeks (atezolizumab and pembrolizumab) according to standard dosing schedules.

Because most patients were treated with nivolumab ( $n = 134$ ), we decided to focus on patients who received it for recurrent or metastatic disease. They were grouped based on presence or absence of previous anti-angiogenesis treatment which included bevacizumab and ramcicrumb. None of the patients received other anti-angiogenesis agents prior to nivolumab. Information about clinical demographics was collected as well. The two groups (prior anti-angiogenesis vs. no prior anti-angiogenesis) were compared for the differences in clinical demographics and outcome. Tumor response was determined according to Response Evaluation Criteria in Solid Tumors (RECIST) 1.1 criteria, and disease control rate (DCR) was defined as the sum of

complete response, partial response, and stable disease rates. Due to retrospective analysis, repeat imaging to confirm response was not always performed. Progression-free survival (PFS) was determined by duration from the start of nivolumab to disease progression or death of any cause. Definition of disease progression for the purpose of determining PFS was based on RECIST 1.1 criteria and/or on the clinical grounds (i.e., clinical progression without formal radiologic assessment if patients were unable to perform re-staging).

### Statistical analysis

The Kaplan-Meier curves were applied and the differences were assessed using the log-rank test. Univariate and multivariable Cox proportional hazard models were used in order to assess the effects of variable(s) on PFS of the patients. Association between anti-angiogenesis treatment and other clinical features were carried out using Chi-squared or Fisher's exact test. JMP software version 14 (SAS Institute, Cary, NC, USA) was used to perform statistical analyses. For all statistical tests, significance was considered to be achieved when two-sided *P* value was less than 0.05. This study was reviewed and approved by University of Kansas Medical Center Institutional Review Board.

## RESULTS

Patient characteristics according to previous anti-angiogenesis treatments are shown in Table 1. Of the 134 patients who received nivolumab, the individual dose was 3 mg/kg or 240 mg flat for 30 and 104 patients, respectively. Sixteen patients received at least one dose of anti-angiogenesis agents prior to nivolumab. They were previously treated with bevacizumab alone ( $n = 11$ ), ramucirumab alone ( $n = 4$ ), or both ( $n = 1$ ). The number of doses for anti-angiogenesis agents ranged between one and 13 with a median of six. In seven of those, no other systemic therapy was given between anti-angiogenesis regimen and nivolumab. Of the 134 patients, seven patients completed PD-L1 immunohistochemistry with tumor material. Only two were tested positive ( $\geq 1\%$ ).

As of June 10, 2017, a total of 31 patients are still being treated with nivolumab; one in the prior anti-angiogenesis and 30 in the no prior anti-angiogenesis group. Because of the inherent limitation of retrospective review, many patients were lost to follow-up after progression on nivolumab. Only six patients in the no prior anti-angiogenesis group received an anti-angiogenesis agent after progression on nivolumab, whereas none did in prior anti-angiogenesis group.

Patients in the prior anti-angiogenesis group had significantly higher likelihood of having stage IV disease, non-squamous histology, and two or more lines of systemic therapy prior to nivolumab as compared to the no anti-angiogenesis group. The difference in histology is expected because current regulatory approval for bevacizumab, which is used in most patients in this group, is indicated for only non-squamous NSCLC. There was no pseudoprogression in either group.

PFS and overall survival (OS) were investigated according to known prognostic factors as well as prior anti-angiogenesis status. Kaplan-Meier analyses demonstrated that the prior anti-angiogenesis group had a statistically shorter PFS as compared to the no prior anti-angiogenesis group, whereas no other factors demonstrated statistical difference (log rank  $P = 0.006$ , Figures 1 and 2A). Multivariate analysis for PFS showed that previous anti-angiogenesis remained statistically significant when other factors are being considered [Table 2]. There is no dose-response relationship between the number of doses of anti-angiogenesis agent and PFS [Figure 2B]. There is a trend in favor of the no anti-angiogenesis group in OS [Table 2] and DCR [Table 3], although the difference was not significant.

## DISCUSSION

Discovery of immune checkpoints and development of agents to enhance T cell function has led to a drastic change in the management of advanced cancer, resulting regulatory approvals for several immunotherapy

**Table 1. Patient characteristics and correlation with previous anti-angiogenesis treatment (n = 134)**

Characteristics	Anti-angiogenesis n (%)		Total	P value
	Yes	No		
Total	16 (100)	118 (100)	134	
Age				0.173
< 70	10 (63)	92 (78)	102 (76)	
≥ 70	6 (37)	26 (22)	32 (24)	
Stage at diagnosis				0.001
III	0 (0)	45 (38)	45 (34)	
IV	16 (100)	73 (62)	89 (66)	
Histology				0.002
Nonsquamous	15 (94)	63 (53)	78 (58)	
Squamous	1 (6)	55 (47)	56 (42)	
Sex				0.427
Male	11 (69)	67 (57)	78 (58)	
Female	5 (31)	51 (43)	56 (42)	
ECOG Performance Status				0.360
0-1	14 (88)		103 (77)	
2+	2 (12)		31 (23)	
EGFR status				1.000
Positive	0 (0)		5 (4)	
Negative/unknown	16 (100)		129 (94)	
No. of nivolumab doses				0.208
Range (median)	1-35 (4.5)		1-59 (5)	
Dose of nivolumab				0.523
240 mg flat	2 (12)	28 (24)	30 (22)	
3 mg/kg	14 (88)	90 (76)	104 (78)	
Reason for discontinuation				0.408**
PD/Death	15 (94)	74 (63)	89 (66)	
AE	0 (0)	8 (7)	104 (78)	
Lost follow-up	0 (0)	5 (4)	89 (66)	
Ongoing	1 (7)	30 (25)	31 (23)	
Others	0 (0)	1 (1)	1 (1)	
No. of systemic chemotherapy lines				< 0.0001
1	6 (38)	103 (87)	109 (81)	
2+	10 (62)	15 (13)	25 (19)	

\*Mann-Whitney *U* test; \*\*Among those who discontinued nivolumab, there was no significant correlation between pre-angiogenesis status and frequency of PD/death. ECOG: eastern cooperative oncology group; EGFR: epidermal growth factor receptor; PD: progressive disease; AE: adverse events

agents. Researchers are looking to potentiate T cell-mediated anti-tumor activity by adding agents with different mechanisms of action. For instance, cytotoxic chemotherapy, targeted agents, and anti-angiogenesis agents are being combined with anti-PD-1/PD-L1 inhibitors in ongoing clinical trials<sup>[16]</sup>. Except for one regimen which was recently approved via the accelerated approval process and still needing larger confirmatory studies<sup>[19]</sup>, no combination regimen including immunotherapy is indicated for any human cancer. Patients with advanced cancer definitely require further development in systemic treatment which exceeds the current efficacy of single agent immunotherapy.

Targeting tumor neoangiogenesis has been extensively investigated over the last few decades. Several agents have achieved regulatory approval in the treatment of advanced cancer<sup>[15,20-25]</sup>. In contrast to vascular endothelial growth factor receptor (VEGFR) kinase inhibitors for renal cell and hepatocellular carcinomas<sup>[20-23]</sup>, monoclonal antibodies directed against VEGF/VEGFR are indicated for several cancer types only in combination with systemic chemotherapy<sup>[15,24]</sup>. For the treatment of advanced NSCLC, bevacizumab and ramucirumab are approved when combined with carboplatin-based regimens or docetaxel, respectively<sup>[15,24]</sup>. No anti-angiogenesis agent as monotherapy is indicated for NSCLC. Several studies with anti-angiogenesis agents have resulted in unexpected severe toxicity and a detrimental outcome for squamous NSCLC patients<sup>[26,27]</sup>. These findings indicate that anti-angiogenesis needs to be not only given in selected populations (i.e., non-squamous) but combined with agents with other mechanisms of action, because anti-angiogenesis by itself has only modest activity.

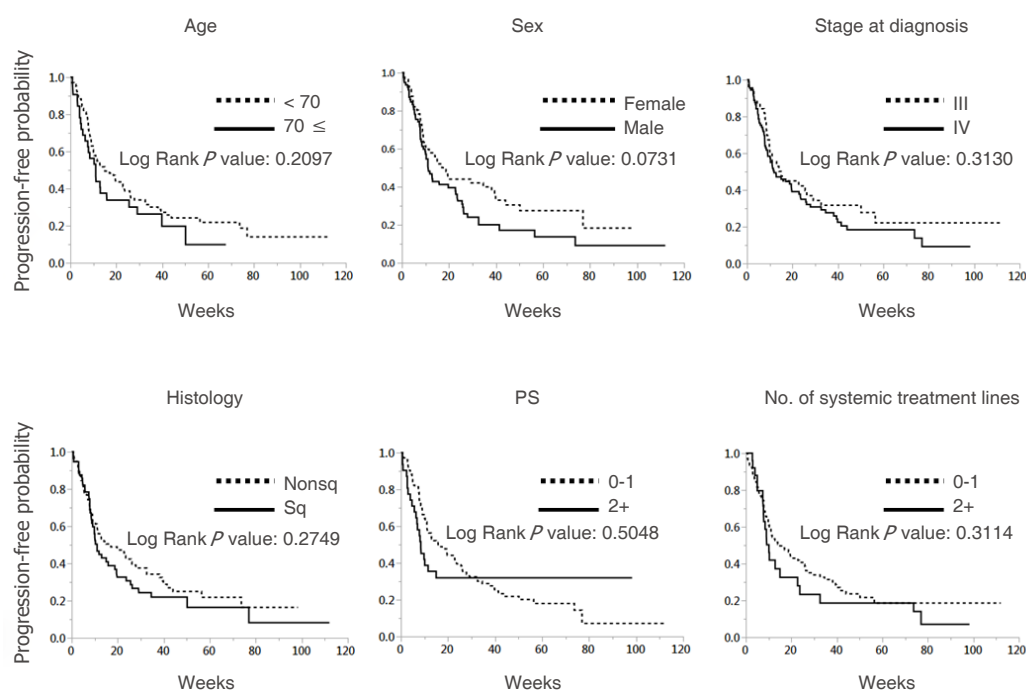
**Table 2. Univariate and Multivariate analyses for prognostic factors on PFS and OS in NSCLC patients treated with nivolumab**

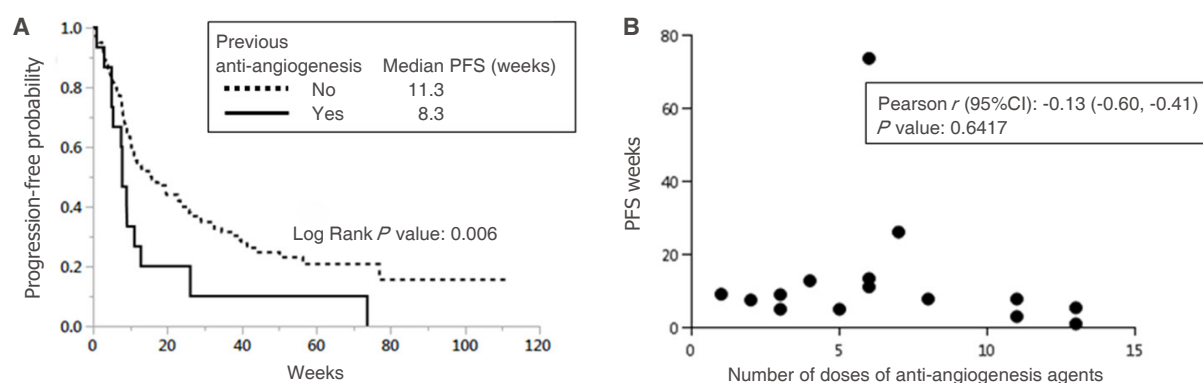
Factor	PFS		OS	
	Univariate analysis HR (95%CI) P value	Multivariate analysis HR (95%CI) P value	Univariate analysis HR (95%CI) P value	Multivariate analysis HR (95%CI) P value
Age (< 70 vs. ≥ 70)	0.882 (0.698-1.136) 0.31909	0.938 (0.729-1.228) 0.633	0.858 (0.659-1.146) 0.288	0.952 (0.710-1.277) 0.744
Stage at diagnosis (III vs. IV)	0.829 (0.652-1.038) 0.104	0.849 (0.651-1.099) 0.216	0.988 (0.766-1.258) 0.923	0.959 (0.724-1.271) 0.772
Histology (nonsquamous vs. squamous)	0.939 (0.762-1.163) 0.561	0.841 (0.659-1.077) 0.168	0.788 (0.621-1.000) 0.050	0.727 (0.549-0.964) 0.027
Sex (female vs. male)	0.810 (0.650-1.002) 0.052	0.826 (0.660-1.026) 0.084	0.829 (0.645-1.054) 0.127	0.817 (0.636-1.048) 0.107
Performance Status vs. 2+)	0.902 (0.710-1.172) 0.425	0.877 (0.680-1.155) 0.340	0.767 (0.591-1.019) 0.066	0.716 (0.538-0.952) 0.028
No. of systemic treatment (0-1 vs. 2+)	0.848 (0.668-1.100) 0.205	0.967 (0.733-1.303) 0.820	0.893 (0.679-1.212) 0.451	0.983 (0.705-1.371) 0.919
Previous anti-angiogenesis (yes vs. no)	1.466 (1.087-1.913) 0.014	1.444 (1.009-2.029) 0.044	1.277 (0.867-1.771) 0.200	1.506 (0.979-2.316) 0.074

**Table 3. Best objective response according to prior anti-angiogenesis treatment**

	Total n (%)	Prior anti-angiogenesis agent		P value
		Yes	No	
ORR (CR + PR)	134 (100)	16 (100)	118 (100)	1.00
Non-ORR	11 (8)	1 (6)	10 (8)	
SD	123 (92)	15 (94)	108 (92)	
PD	43 (32)	2 (13)	41 (35)	
NE	40 (30)	7 (44)	33 (28)	0.101
DCR (CR + PR + SD)	40 (30)	6 (38)	34 (29)	
Others (PD + NE)	54 (40)	3 (19)	51 (43)	
	80 (60)	13 (81)	67 (57)	

ORR: overall response rate; CR: complete response; PR: partial response; SD: stable disease; NE: not evaluable; DCR: disease control rate

**Figure 1.** Progression-free survival according to clinical characteristics. Progression-free survival curves were plotted according to six clinical characteristics. log-rank tests were used for statistical analysis



**Figure 2.** Impact of previous anti-angiogenesis treatment on progression-free survival. A: Progression-free survival curves were plotted according to prior anti-angiogenesis treatment; B: Relationship between number of prior anti-angiogenesis doses and progression-free survival on nivolumab

In addition to their modest clinical activity, the use of anti-angiogenesis agents in advanced cancer raised other concerns for researchers. Preclinical studies demonstrated that use and subsequent withdrawal of anti-VEGF agents could develop rebound tumor vascularization<sup>[17]</sup>. Others also reported induction of angiogenesis-related cytokines and epithelial-mesenchymal transition which enhance cancer invasiveness and eventual metastasis<sup>[28-31]</sup>. Clinical studies in patients with colorectal cancer also showed that continuation of bevacizumab beyond first progression was associated with prolonged overall survival, suggesting a detrimental withdrawal effect of anti-angiogenesis in humans as well<sup>[32]</sup>.

Despite the abovementioned negative aspects for anti-angiogenesis agents, preclinical studies demonstrated therapeutic synergism between anti-angiogenesis and immunotherapy<sup>[33,34]</sup>. Targeting VEGF enhanced IFN $\gamma$ -mediated upregulation of PD-L1 which in turn led to disease relapse in glioblastoma models. This negative effect of anti-angiogenesis treatment was nullified by dual blockade of the VEGF and PD-1/PDL1 signaling<sup>[34]</sup>. Supported by these preclinical observations, combination strategy using anti-angiogenesis agents and immune checkpoint inhibitors are actively tested in a number of clinical trials<sup>[35]</sup>.

In this retrospective study, 16 (11.9%) of 134 patients who were treated nivolumab received anti-angiogenesis agents previously. This infrequent use of anti-angiogenesis agents in the first-line systemic therapy seems consistent with the study reported by Zhu *et al.*<sup>[36]</sup>, where only 21.2% of stage IV NSCLC patients in their large SEER-Medicare analysis received bevacizumab in the first-line systemic therapy.

This study also showed that previous use of anti-angiogenesis agents was associated with significantly worse PFS. Overall response rate (ORR) and OS in the prior anti-angiogenesis group were also inferior to those in the no prior anti-angiogenesis group, although the differences were not statistically significant. Despite a relatively small number of patients in the prior anti-angiogenesis group, univariate and multivariate analyses demonstrated that prior anti-angiogenesis status is a poor prognostic factor independently for PFS. This detrimental effect of prior anti-angiogenesis on nivolumab treatment might be explained by withdrawal effect of anti-angiogenesis as discussed above. Consistent with this study, there are other similar clinical observations reported in the literature. A small retrospective study of 16 patients with glioblastoma reported a disappointing clinical effect when nivolumab was given after progression on bevacizumab<sup>[37]</sup>. A recent case series revealed that three patients with renal cell carcinoma with two or more lines of systemic anti-angiogenesis treatment developed rapid disease progression while on nivolumab treatment<sup>[38]</sup>. These patients received prior VEGFR TKIs prior to initiation of nivolumab. Moreover, in the pivotal phase III trial which led to Food and Drug Administration (FDA) approval of nivolumab for renal cell carcinoma, the difference in OS between the nivolumab and the control arms was not statistically significant when patients with two or more previous anti-angiogenesis agents were selected for subset analysis<sup>[11]</sup>. Although these observations,



including this study, are still hypothesis-generating, the potential negative effect of prior anti-angiogenesis treatment warrants further investigation.

Retrospective observational studies such as this always have limitations. Various unappreciated biases exist in all retrospective studies. For instance, several patients in each group have never undergone formal re-staging but were considered as clinical progression which determined PFS. This single institution retrospective study needs to be validated by larger prospective and/or retrospective studies. Subset analyses within prior anti-angiogenesis group showed that there was no correlation between PFS and number of doses of anti-angiogenesis agents [Figure 2B] or interval from the last administration of anti-angiogenesis agent to first dose of nivolumab (data not shown). We acknowledge that these subset analyses require a larger sample size in order to establish clinical significance.

This retrospective study suggests that preceding anti-angiogenesis treatment has detrimental effect on subsequent treatment outcome of immunotherapy in NSCLC. This phenomenon might be associated with rebound tumor angiogenesis due to withdrawal of anti-angiogenesis treatment. This hypothesis needs to be confirmed by studies with a larger patient sample.

## **DECLARATIONS**

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### **Authors' contributions**

Concept, design, clinical studies, data acquisition, data analysis: Komiya T

Literature search: Komiya T, Huang CH, Neupane P, Williamson SK

Statistical analysis: Komiya T, Chalise P

Manuscript preparation, manuscript editing, and manuscript review: Komiya T, Huang CH, Neupane P, Williamson SK, Chalise P

### **Data source and availability**

Data and survey materials are available upon request from the corresponding author.

### **Financial support and sponsorship**

None.

### **Conflicts of interest**

The authors have no conflict of interest.

### **Patient consent**

Informed consent was exempted by institutional IRB.

### **Ethics approval**

All procedures performed in studies involving human participants were in accordance with the ethical standards of the institutional and/or national research committee and with the 1964 Helsinki declaration and its later amendments or comparable ethical standards. Approval from institutional IRB was obtained.

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Review

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# A primer on recent developments in cancer immunotherapy, with a focus on neoantigen vaccines

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## Abstract

Cancer immunotherapy has now been conclusively shown to be capable of producing durable responses for a substantial number of patients. Adoptive cell transfer and checkpoint blockade therapies in particular both demonstrate that antigen-specific immune responses can be dramatically effective, even in previously refractory late stage disease. Such developments, together with advances in technology, have strongly encouraged revisiting the concept of neoantigen vaccines. Here we introduce basic ideas in the field to allow investigators from diverse backgrounds to understand these developments, grasp current issues, and contribute to further progress.

**Keywords:** Immunotherapy, cancer vaccine, immunoinformatics, precision medicine, combination therapy, theoretical models, systems biology

## INTRODUCTION

In the late 1800s, Coley<sup>[1]</sup> pursued investigations of cancer regression in the context of bacterial disease. It has been clear since then that the immune system plays an important role in cancer. Over the ensuing century, strong arguments were put forward for both why cancer immunotherapy should work and why it should not, occasionally by the same investigator<sup>[2]</sup>. The past decade has seen dramatic progress in cancer immunotherapies, such as checkpoint blockade, adoptive cell transfer, and vaccines. The success came on



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two fronts: complete durable patient response was achieved in a substantial fraction of patients in the clinic, and the mechanism of action was T-cell antigen-specific. This spurred confidence that therapy approaching a “cure” was at hand, based on a rational extrapolation of current knowledge. The immune system is inextricably linked to both the phenomenon of cancer and its treatment. This represents a paradigm shift, where cancer is no longer seen as just a collection of aberrant cells, but rather a systemic disease.

While this new vista continues to capture the public imagination worldwide, we have learned enough over the years to understand that cancer immunotherapy, in its current form, is not a panacea. The central challenge facing cancer immunotherapies and neoantigen vaccines in particular is understanding resistance.

Integrating immunology and cancer research, already two of the most complex topics in biomedicine, is an interdisciplinary effort, drawing from fields such as biology, pharmacology, chemistry, physics, engineering, statistics, and mathematics. Our main aim in this primer is to lower the barrier to entry for readers who are not specialists in immunotherapy. We focus on neoantigen vaccines, which in some ways represent T-cell based cancer immunotherapy in its most elementary form. We also address general issues, enabling readers to quickly grasp other immunotherapies and future developments.

### **Background on the immune system and cancer**

We embark first on a brief tour of immunology, with the caveat that the specifics and even the broad outlines may shift as the field advances. Many of the features described below have bearing on possible cancer resistance mechanisms.

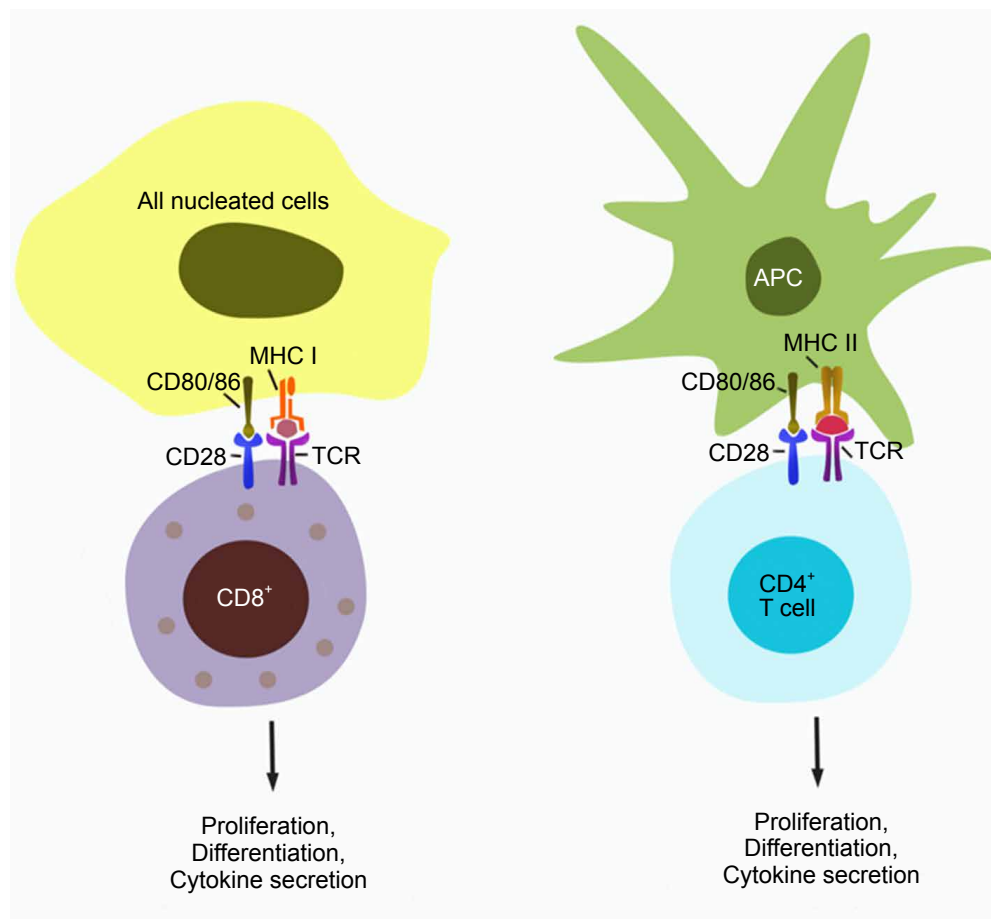
In brief, the requirements for an effective immune system include mechanisms to recognize foreign invaders, the means to trigger and coordinate a potentially complex attack (“expansion”), then return to equilibrium (“contraction”), while not attacking normal tissue. This rests critically on the ability to distinguish self from non-self. In vertebrates, robust response also leads to the development of immune memory. Immunotherapy can be viewed as an attempt to shift the equilibrium point in a complex system that can actively amplify or suppress its effects.

Cancer cells can evade the immune system through a variety of routes, such as being viewed as self, hijacking suppressive mechanisms that prevent damage, attacking or subverting immune system agents, or simply growing at a rate beyond the capacity of an often aged and weakened immune system.

The vertebrate immune system is broadly divided into two arms. Innate immunity<sup>[3]</sup> is encoded in the germline, while adaptive (“acquired”) immunity<sup>[4]</sup> is mediated by B and T lymphocytes that undergo processes of diversification and selection. T cell selection relies on processes of central tolerance (at the thymus) and peripheral tolerance (on mature circulating T cells)<sup>[5]</sup>. The two arms interact, with some cell types having a role in both arms.

In the adaptive system, T cells play the key role in recognizing pathology via antigens. The core of this task involves three parts: a presenter (major histocompatibility complex molecule, MHC), an antigen fragment (peptide), and a recognizer (T cell receptor, TCR). Elaborate processes of MHC expression and maturation, antigen processing, peptide MHC loading, and generation of mature naive T cells through the thymus underlie their formation and interaction<sup>[6-9]</sup>.

Antigen recognition takes place when a receptor on a T cell encounters a cell presenting a cognate peptide-MHC (pMHC) complex on its surface. If a CD28 co-stimulatory receptor on the T cell simultaneously binds with CD80 or CD86 expressed on the presenting cell, an activation signal is propagated on the cytosolic side of the TCR, leading to cell proliferation, differentiation, and secretion of cytokines. A lack of a co-stimulatory signal leads to a hypo-responsive state known as T cell anergy<sup>[10]</sup>. Inhibitory checkpoint molecules “put the



**Figure 1.** T cell activation. CD8<sup>+</sup> T cells inspect the surface of cells they encounter and are activated if a T cell receptor binds to a presented pMHC-I complex, leading to downstream processes including proliferation, differentiation, and cytokine secretion. CD4<sup>+</sup> T cells are similarly activated when binding pMHC-II complexes presented by professional APCs such as dendritic cells. A co-stimulating signal from CD28/CD80 (86) binding is required for full activation; its absence leads to T cell anergy. APC: antigen-presenting cell; MHC: major histocompatibility complex; TCR: T cell receptor

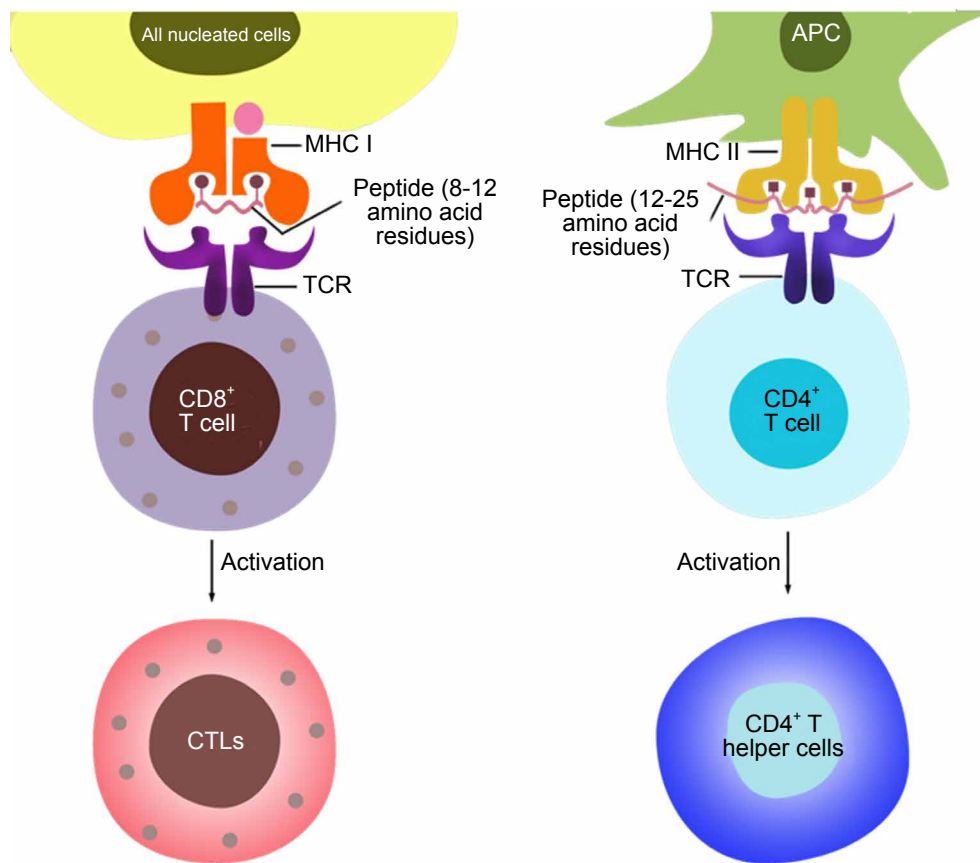
brakes” on adaptive immunity, where for example cytotoxic T-lymphocyte associated protein 4 (CTLA-4) competes with CD80/86 in binding CD28, thus suppressing activation<sup>[11]</sup>.

The detection limit for such T cell triggering is impressively low (four pMHC per TCR cluster)<sup>[12]</sup>. Note that the vast majority of the 10<sup>4</sup> presented peptides *in vivo* are in fact normal “self” peptides, with only a few from foreign antigens, if any<sup>[13]</sup>.

MHC molecules and T cells come in two subtype pairs [Figure 1]. MHC class I (MHC-I) is normally expressed in all nucleated cells and presents intracellular (endogenous) antigen fragments. The pMHC-I complexes are recognized by CD8<sup>+</sup> T cells, which are then activated and differentiate into cytotoxic T cells (CTLs) with direct cell killing capability. MHC class II (MHC-II) is expressed in “professional” antigen presenting cells (APCs), including dendritic cells, and presents exogenous antigens that have been engulfed by the APC. The resulting pMHC-II complexes are recognized by CD4<sup>+</sup> T cells, which can differentiate e.g. into T helper cells whose primary role is to activate other immune system components.

The loaded peptides in the case of MHC-I are typically 8 to 12 residues in length and are loaded into a groove that is closed on both ends. The MHC-II-loaded peptides range in length from 12 to 25 residues and are loaded into a groove that is open on both ends [Figure 2]<sup>[6,7,14]</sup>.





**Figure 2.** Differences in recognition and downstream processes between  $CD8^+$  and  $CD4^+$  T cells.  $CD8^+$  T cells recognize pMHC-I complexes, where the peptide is a fragment from an endogenous protein typically 8 to 11 AAs in length, which occupies a groove that is closed on both ends.  $CD4^+$  T cells recognize pMHC-II complexes, where the peptide is derived from cells or antigens engulfed by the APC and is typically longer, 12 to 25 AAs in length. The MHC-II groove is open on both ends. After activation,  $CD8^+$  T cells differentiate into cytotoxic T lymphocytes (CTLs), whereas  $CD4^+$  T cells differentiate e.g. into T helper cells, depending on receipt of further cytokine signals. APC: antigen-presenting cell; MHC: major histocompatibility complex; TCR: T cell receptor

Cells that do not express MHC-I on their surface are considered aberrant by the immune system. In normal environments, these are eliminated by natural killer cells, which are innate lymphoid cells with recognition receptors encoded in the germline<sup>[15]</sup>.

Both the presentation (MHC) and recognition (TCR) components are highly diverse, although MHC diversity only appears at the population level. In humans, the MHC is known as the human leukocyte antigen (HLA) complex. Each individual inherits six MHC-I alleles from three loci, HLA-A, -B and -C (i.e. two parental alleles from each locus), and similarly, six MHC-II alleles from HLA-DP, -DQ, and -DR loci. Note that the HLA nomenclature was revised in 2010<sup>[16]</sup>. As of Oct. 2015, there were 10,297 class I and 3543 class II known alleles<sup>[17,18]</sup>. Hence, the pMHC binding profile (“HLA peptidome”) varies broadly between individuals.

On the recognition side, there are in principle at least  $10^{15}$  possible TCR variants. The number of T-cells in any individual is on the order of  $10^{12}$ , and the number of clonotypes possibly around  $10^{7[19,20]}$ . The processes of receptor diversification and negative selection for immune self-tolerance is largely completed during youth. The TCR repertoire shows a linear loss of naive T cell diversity with age<sup>[21]</sup>, although more subtle characterizations can be made<sup>[22]</sup>. Such age-related changes have been hypothesized to contribute to cancer susceptibility, although their impact is not yet clear<sup>[23]</sup>. The TCR repertoire continues to be a subject of intense research, which we touch on further below. The impact of age-related changes more generally is discussed in the context of checkpoint blockade therapy by Elias *et al.*<sup>[24]</sup>.



The idea that one clonal TCR recognizes one specific antigen has been supplanted by the notion that TCRs are cross-reactive. A discussion of how TCRs must be cross-reactive in principle is given by Sewell<sup>[25]</sup>. Indeed, TCR recognition that straddles the self/non-self boundary (e.g. between self and microbial peptides) underlies the theory of molecular mimicry, whereby bacterial antigens do not provoke attack or conversely may lead to autoimmune disease<sup>[26]</sup>. The specific mechanisms are now being worked out<sup>[27,28]</sup>. Similarly, mutant tumor proteins may avoid immunogenicity by being cross-reactive with self-proteins.

Each individual's immune system will also have peptides that it cannot recognize, which can be characterized as “holes” or “blind spots”. These can arise both from gaps in presentation (lack of peptide-MHC binding) or recognition (absence from the TCR repertoire)<sup>[29]</sup>. A vaccine based solely on an antigen in such a hole will not work for that individual. Such phenomena are seen in the context of microbial immunity<sup>[30,31]</sup>. The concept of original antigenic sin<sup>[32]</sup> states that such a hole can paradoxically be created by initial exposure to an antigen, as the immune system does not mount a novel response when it encounters a slight variant.

Immunological research continues to reveal new features. Activated CD8<sup>+</sup> T cells were found to require cross presentation, i.e. co-stimulation by dendritic cells that can present exogenous antigens on MHC-I, for full induction of cytotoxic response<sup>[33,34]</sup>. The CD4 lineage was resolved into four lines<sup>[35]</sup> and then a plastic set of more<sup>[36]</sup>. Some CD4<sup>+</sup> T cells can acquire cytotoxic activity<sup>[37,38]</sup> (i.e. not only CD8<sup>+</sup> T cells can be cytotoxic). More recently, a “second touch hypothesis”<sup>[39]</sup> suggests that the high-level picture for polarization of T cells may not yet be complete. New immune cell subtypes continue to be discovered<sup>[40]</sup>. As one consequence, mathematical modeling of the immune system is likely to remain a difficult endeavor for some time.

We mention here briefly the once-dominant view of cancer as an autonomous genetic disease, as captured by the original “hallmarks of cancer”<sup>[41]</sup>. The cancer phenotype arises as a result of selection pressure on genome mutations, leading to acquisition of limitless growth and survival potential, with genome instability as an “enabling characteristic”. These mechanisms also underlie cancer's uncanny ability to acquire additional phenotypes such as eliciting immune tolerance and angiogenesis. A recent proposal that epigenetics alone may be sufficient to generate the hallmarks of cancer<sup>[42]</sup> may, amongst other things, alter our understanding of the time scales involved in tumor response<sup>[43]</sup>.

The careful examination of tumor cell evolution and its therapeutic implications are in its beginning stages<sup>[44-47]</sup>. Principles such as antagonistic pleiotropy<sup>[48]</sup>, where reproductive fitness in youth is played off against fitness in old age, are also sometimes raised as setting fundamental biological limits.

For further background on cancer and immunology, the reader can consult reference books<sup>[49,50]</sup>, a three-volume series<sup>[51]</sup>, and a broad history from a contrarian perspective<sup>[52]</sup>.

## RECENT DEVELOPMENTS IN CANCER IMMUNOTHERAPY

### Modalities of T-cell based immunotherapy

The design of currently popular T-cell based immunotherapies can be described as follows:

- Release the brakes: checkpoint blockade<sup>[53]</sup>;
- Boost instruction, via antigens: cancer vaccines;
- Boost instruction, via cell transfer, bypassing presentation: adoptive dendritic cell therapy<sup>[54,55]</sup>;
- Boost recognition, via cell transfer, bypassing instruction: adoptive T cell therapy<sup>[56]</sup>;
- Boost recognition, via cell transfer, bypassing instruction and MHC restriction: adoptive chimeric antigen receptor T-cell (CAR-T) therapy<sup>[57,58]</sup>.

All of these therapies are based on T cells. Checkpoint blockade therapy is distinguished by not targeting cancer, relying instead on the host immune system training (or having already trained) itself to target tumors.

At the other end of the spectrum, CAR-T therapy does not rely on the host immune system for tumor killing. These span so-called active to passive therapies. Passive therapies do not necessarily induce immune memory, although T cell proliferation may allow extended response. The various immunotherapies can be visualized in an informative hierarchy<sup>[59]</sup>. The 2014 Society for Immunotherapy of Cancer (SITC) primer provides an unhurried perspective on many of these developments<sup>[60]</sup>.

While the current wave of immunotherapies was heralded by dendritic cell therapy (sipuleucel-T)<sup>[55]</sup>, the most notable breakthrough was probably the development of anti-CTLA4 checkpoint blockade, which utilizes antibodies to block receptors that inhibit T cell activation. This treatment allowed some of the first demonstrations in humans of the therapeutic efficacy of neoantigen-specific T cells<sup>[61]</sup>. Checkpoint therapies based on programmed cell death protein 1 (PD-1) and programmed death-ligand 1 (PD-L1) blockade have further demonstrated improved efficacy with reduced toxicity.

### Impacts of immunotherapy on standard practice

The mainstream acceptance of cancer immunotherapy has stimulated efforts to modify clinical trial reporting<sup>[62]</sup>, with the introduction of “immune-related” adverse events (irAE) and response criteria (irRC). Progression criteria must now allow for pseudo-progression, i.e. the appearance of growing or new lesions that indicate T cell infiltration. A call for “assay harmonization” seeks to reduce variability in cellular immune response reporting. Survival criteria must account for time-dependent hazard ratios, with agent-specific delays in Kaplan-Meier survival curve separation ranging from four to eight months.

Clinical trial design itself is evolving, a process that began in response to targeted therapies (precision oncology) and is now accelerating<sup>[63]</sup>. This has seen the advent of expansion cohorts, and platform, bucket, adaptive<sup>[64]</sup>, and seamless trials. It will be increasingly important to understand the cohort and trial design to interpret results.

We note in passing the recent reports of hyperprogression<sup>[65]</sup>. Tumor size has been observed to dramatically increase with anti-PD-1/PD-L1 treatment, although whether this is more than a statistical fluctuation has been questioned<sup>[66]</sup>. It is nevertheless safe to say immunotherapies behave differently than previous standard therapies.

The effort to go beyond tumor cell-based staging has begun with the proposal of an Immunoscore<sup>[67]</sup>, which quantifies the density of CD3<sup>+</sup> and CD8<sup>+</sup> T cells in solid tumors. Due to its prognostic value, it has been proposed to augment traditional tumor size/nodal status/distant metastasis (TNM) staging<sup>[68]</sup>.

Recent advances have triggered a reconsideration of the effect of conventional therapies (surgery, chemotherapy, radiation) and of molecularly targeted therapies<sup>[69,70]</sup>. Oncogenes such as Myc have been found to also regulate immune response. When such oncogenes are inactivated, immune response is restored and plays a role in the subsequent “oncogene withdrawal”<sup>[71]</sup>. Chemotherapy perhaps surprisingly also appears to rely in part on the immune system for cytotoxic effect<sup>[72]</sup>.

Cancer immunotherapies can in principle have much milder side effects compared to radiotherapy and chemotherapy. In practice, they are associated with their own spectrum of adverse events<sup>[73,74]</sup>. In particular, cytokine release syndrome (“cytokine storm”) can lead to organ failure and death. Both treatment efficacy and adverse events are associated with proliferative and persistent cellular responses, which can vary significantly between individuals, thus requiring careful monitoring<sup>[75]</sup>. Adverse events associated with neoantigen vaccines appear to be relatively mild, compared to adoptive cell transfer, checkpoint blockade, and tumor-associated antigen (TAA) vaccine therapies<sup>[76]</sup>.

## NEOANTIGEN VACCINES

### Introduction

We now turn to neoantigen-based cancer vaccines. The objective of a vaccine is to introduce a small amount of material to instruct T and B cells to eliminate invaders that present the cognate antigen<sup>[77]</sup>. Vaccines in general can be prophylactic (preventative) or therapeutic (cure or control of observable disease). Current neoantigen vaccines are therapeutic, with the goal of restoring immune surveillance of a tumor that has likely already evolved to evade the immune system (e.g. through immunoediting; see below).

Cancer cells are genomically unstable<sup>[41,78]</sup>, which leads to the expression of novel proteins due to non-synonymous mutations. Many of these are likely to be immunogenic and are termed neoantigens. Vaccines that precisely target such neoantigens (also known as tumor-specific antigens, or TSAs) would prime an immune response that rejects tumors while sparing normal tissues, leading to optimal therapies with mild if any toxicity. A timeline that traces the foundations of this idea back to 1943 is provided by Coulie *et al.*<sup>[79]</sup>.

### Types of antigen-based cancer vaccines

Prior to the advent of next-generation sequencing, cancer vaccines were developed based on TAAs or cancer germline antigens. These self-antigens are overexpressed in tumors, or normally expressed only during development but re-expressed in tumors. Vaccines targeting these can be produced in advance at lower cost and applied across a range of tumors that share expression of the target. As the targets are self-antigens, such vaccines are possibly limited by self-tolerance and adverse events. Tumor resistance mechanisms, many of which are shared with neoantigen vaccines, are also a prominent concern<sup>[80,81]</sup>. Another class of targets are shared tumor neoantigens, which are commonly found across a subtype of tumor. As in TAAs, the vaccine can be produced beforehand, and treatment progress can be easily followed, as the neoantigen epitopes (i.e. recognized peptide fragments) are typically well known. Such epitopes however may not be the most effective for any given tumor.

With massively parallel sequencing and MHC binding and functional prediction software tools, the key hurdle to developing personalized neoantigen vaccines can now be overcome. Vaccines custom designed for each patient represents a paradigm shift in cancer treatment<sup>[82]</sup>.

Some of the strengths and weaknesses of the neoantigen vaccine approach are summarized in Table 1 and are discussed further below.

### Vaccine formulation and administration

In addition to the selection of epitopes, a number of other considerations can strongly influence the success or failure of neoantigen vaccines. Cancer vaccines can be formulated as whole cells, peptides/proteins, RNA, DNA<sup>[80]</sup>, and glycolipids<sup>[83]</sup>. Vaccines are typically formulated as peptides, due to ease of construction and low cost, although these are often observed to be weakly immunogenic. They can be modified to enhance delivery to immune cells and improve pMHC binding stability<sup>[84]</sup>. Synthetic long peptides require dendritic cell processing, argued as essential for durable response<sup>[85]</sup>. Protein vaccines are more immunogenic but have a higher risk of anaphylaxis. The robust discussion about designing and assessing peptide vaccines has been reviewed by Kumai *et al.*<sup>[86]</sup>. DNA vaccines introduce DNA coding for antigenic fragments into host cells, where they are expressed and lead to the presentation of epitopes via the MHC-I pathway. They are generally safe, stable, and easy to produce at low cost, although currently weakly immunogenic. The vaccine or delivery vehicle itself can be attacked by the host immune system<sup>[87]</sup>. RNA vaccines can encode several epitopes on a single molecule, can trigger the innate immune system, and are not at risk of integrating into the genome<sup>[88-90]</sup>. Whole cell vaccines that employ weakened or killed tumor cells can trigger immune response with the entire complement of tumor antigens, without specific instruction of the immune system, reducing time and expense. They may however induce immune response to self-proteins.

**Table 1. Strengths and weakness of the neoantigen vaccine approach**

Strengths	Weaknesses
Precise targeting	Need for tumor biopsy (in general)
Mild adverse events	Need to overcome tumor defenses
Few constraints on dosage	Slow induction of immune response
Better profile than TAA vaccines	May not be applicable to tumors with few mutations
No need for T cell extraction and <i>ex vivo</i> growth	Unreliable epitope binding prediction algorithms
Many opportunities to optimize/combine formulations	Time lag from biopsy to vaccine
Multi-epitope designs can compensate for inaccurate binding predictions, tumor heterogeneity and evolution	Cost
Induction of antigen spreading and immune memory can cope with occult disease	

Other considerations are choice of carrier, delivery vehicle<sup>[91]</sup> (including bacteria<sup>[92]</sup> or viral vectors<sup>[93]</sup>), and administration route (intravenous, intratumoral, subcutaneous, intra-lymph node, nasal, ingested). Further afield, cancer vaccine engineering has emerged to offer benefits such as lymph node targeting, reduced systemic toxicity, elimination of *ex vivo* expansion requirements, and controlled release of immunomodulators while ignoring suppressive signals<sup>[94-96]</sup>.

### Neo-epitope binding prediction

Neoantigen vaccines are produced by first inspecting the patient's tumor for immunogenic peptides, specifically epitopes<sup>[97]</sup>. TCRs recognize linear epitopes, i.e. a continuous fragment of an antigen. Note that pMHC binding is a necessary but not sufficient condition for immunogenicity.

The neo-epitope selection problem can thus be reduced to finding mutant peptides that bind well to the patient's MHC alleles. This is amenable to computational treatment and is one of the most prominent applications of machine learning to immunology<sup>[98]</sup>. The realization that such bioinformatic approaches can reveal a "gold mine" of targets and that neoantigen vaccines were feasible can be traced back to a 2008 paper<sup>[99]</sup>.

A simplified neo-epitope selection pipeline can be described as follows:

- Perform exome sequencing of tumor and normal tissue to identify non-synonymous single nucleotide variants and generate an initial list of candidate genes;
- Perform RNA-Seq to confirm expression;
- Use informatics tools to predict neoantigen-derived peptides that bind to the patient's set of HLA alleles;
- Filter candidates based on survival or growth function ("driver genes");
- Choose the top 10 or 20 epitopes.

Proteasomal cleavage predictions<sup>[100]</sup> can also be incorporated into the workflow, although the predictive value is rather low, due to the lack of sufficient training data<sup>[98]</sup>.

Numerous excellent reviews of the available tools are available<sup>[82,98,101]</sup>. The Immune Epitope Database<sup>[102]</sup> is probably the most prominent epitope database and analysis resource, freely available on the Web. TANTIGEN<sup>[103]</sup> is a database of tumor-tissue derived antigens with experimentally validated HLA binding. Step-by-step instructions on the use of a prominent suite of tools is available<sup>[104]</sup>. Mutant Peptide Extractor and Informer<sup>[105]</sup> is a web-based tool that attempts to integrate best practices and simplify neo-epitope analysis and selection for non-bioinformaticians (limited to MHC-I epitopes). ImmunoNodes<sup>[106]</sup> is a software framework for building complex immunoinformatics workflows, such as those for neo-epitope selection.

Amongst other challenges, prediction of MHC-II peptide binding lags behind MHC-I prediction, partly due to the greater length of loaded peptides that interact in flanking regions with highly polymorphic alleles.

Also, binding data does not exist for many less common MHC alleles, which has given rise to “pan MHC” algorithms with somewhat reduced performance. Therapeutic strategies based on so-called promiscuous epitopes that bind to several MHC alleles may place less stringent requirements on the accuracy of binding predictions<sup>[107]</sup>.

The extent to which epitope binding scores are a good surrogate for immunogenicity remains unclear. Peptide binding stability rather than affinity has been proposed as a better predictor of immunogenicity<sup>[29]</sup>. Numerous factors can affect antigen presentation and recognition processes, such as pH, inflammation, and peptide post-translational modifications<sup>[108]</sup>.

Many of the structural aspects of peptide-MHC binding and TCR recognition are reviewed by Hudrisier and Gairin<sup>[109]</sup>. Important aspects of the problem formulation can be found e.g. in the references cited by Meydan *et al.*<sup>[14]</sup>. A recent examination of empirical TCR-pMHC kinetic constants measured in three-dimensional assays suggests these may not accurately reflect dynamics in a two-dimensional context, such as T cell scanning of the APC surface<sup>[110]</sup>. This could suggest that some of the data underlying current epitope binding prediction algorithms needs to be re-measured.

In general, while immunogenic antigens tend to have high binding scores, the converse does not hold<sup>[111]</sup>. In addition, indels and gene fusions are typically not chosen, due to the difficulty of predicting binding. Snyder and Chan<sup>[101]</sup> caution that current prediction tools on their own are not ready for routine clinical use.

### Choice of epitope candidates

In tumors with a large number of mutations, the candidate filtering step is essential to avoid being overwhelmed by false positives<sup>[112]</sup>. Mass spectrometry has been effectively used for this task by identifying MHC-bound peptides<sup>[113]</sup>. Indeed, it can be used to generate candidates on its own<sup>[114,115]</sup>. There remain possible issues with sensitivity and translation into a clinical setting<sup>[112]</sup>. Combining functional analysis and T cell detection via multimers can help in the search for tumor rejection epitopes<sup>[116]</sup>. Proximity ligation assays can assess whether an antigen is presented *in situ*, although this requires a mutant-specific antibody<sup>[117]</sup>. Another approach tests epitopes experimentally in MHC-transgenic mice<sup>[118,119]</sup>. Further work is necessary to validate the efficacy of such workflows<sup>[120]</sup>. An interesting suggestion is that PD-1<sup>+</sup> peripheral blood cells are enriched in tumor neoantigens, from which candidate epitopes can be derived<sup>[121]</sup>.

There is a general exhortation to prioritize genes that target essential tumor “driver” functions such as growth and survival. This however may not be too helpful, as only a small percentage of neoantigens are of this type in e.g. melanoma<sup>[112]</sup>, the vast majority being “passenger” mutants not associated with cell transformation. Efforts to expand and/or refine the list of functional cancer genes may help in this regard<sup>[122,123]</sup>.

Current vaccine strategy employs several epitopes to address tumor heterogeneity and reduce acquisition of resistance, while also compensating for the imperfect predictive value of pMHC binding tools. The phenomena of immunodominance<sup>[124-127]</sup> and T cell cross-reactivity<sup>[128]</sup> suggests that simply increasing the number of epitopes in a vaccine may not be advisable, as a suboptimal epitope may interfere with the others in a dominance hierarchy, and auto-immunity remains an issue. Indeed, pioneering efforts in cancer epitope selection<sup>[113,129]</sup> found possible instances of immunodominance. Initial experience with long peptides on the other hand suggested this may not be an issue<sup>[130]</sup>. Further work is required to understand how to choose the number of epitopes to include in a vaccine, which could be e.g. cancer type-specific. The thinking behind many current vaccine approaches is examined by Kumai *et al.*<sup>[131]</sup> who also describe four steps to developing cancer vaccines and five ways of monitoring the response.

Initial effort e.g. in adoptive cell transfer was focused on MHC-I restricted epitopes to elicit direct tumor cell killing. Attention has now shifted to MHC-II restricted epitopes, in part due to the fuller realization that



CTLs require CD4<sup>+</sup> help<sup>[132-135]</sup>. Indeed, adoptive cell transfer of CD4<sup>+</sup> T cells was enough to induce tumor regression in a mouse model of melanoma<sup>[136]</sup> and in a human patient<sup>[137]</sup>.

### Initial human trials

The personalized neoantigen vaccine strategy has begun to reach the clinic with the recent reports from two Phase I trials<sup>[76,138]</sup>. These trials, on patients with advanced melanoma, demonstrate that such therapies are safe and induce a targeted neoantigen-specific response as designed. Ott *et al.*<sup>[76]</sup> enrolled 6 patients who had no evidence of disease after surgery, with 4 remaining tumor-free after 25 months. Sahin *et al.*<sup>[138]</sup> enrolled 13 patients, and 8 patients remained tumor free after 23 months. The time to develop personalized vaccines (weeks to months) remains a key obstacle, especially for patients with advanced disease.

In an apparent pattern, both trials utilized MHC-I binding scores to select neo-epitopes (Sahin *et al.*<sup>[138]</sup> combined these predictions with MHC-II binding scores). The vaccines were then seen to activate CD4<sup>+</sup> T cells, possibly because MHC-II binding is less restrictive<sup>[139]</sup>. In more detail, Ott *et al.*<sup>[76]</sup> selected neo-epitopes using predicted binding to HLA-A and HLA-B, and employed long peptides (15-30 amino acids) in several pools targeting up to 20 neoantigens for five priming and two boosting vaccinations injected subcutaneously. They observed CD4<sup>+</sup> response almost immediately and a peak response generally at 16 weeks, and found two to four immunogenic peptides per patient. Sahin *et al.*<sup>[138]</sup> ranked mutant epitopes on a combination of predicted MHC-I and MHC-II binding, plus high expression and variant allele frequency, and chose 10 epitopes per patient. Two synthetic RNAs were used to encode five 27mer peptides with the single nucleotide variant (SNV) at position 14. Patients were treated with at least eight and up to twenty neo-epitope vaccine doses injected into inguinal lymph nodes, and T cells were developed against at least three mutations per patient, with the majority being exclusively CD4<sup>+</sup> responses. Neo-epitope specific CD8<sup>+</sup> T cells expanded within two to four weeks.

### WHO BENEFITS?

We now return to a more general discussion of immunotherapies. The seminal studies in checkpoint blockade therapy have primarily been done in melanoma and lung cancer. A survey of solid tumor types that have been studied with immunotherapy, with an emphasis on understudied cancers, has been made by Young<sup>[140]</sup>. In terms of number of clinical trials, breast cancer tops the list.

In an emerging consensus, the cancers that are best indicated for immunotherapy are slow growing, exhibit high mutational load and low burden at the start of therapy, and are inflamed<sup>[141]</sup>. This suggests immunotherapies may be more effective in the early stage disease setting. A correlation has been observed in checkpoint blockade between somatic mutations per megabase and objective response rates<sup>[111]</sup>. Mutagen induced cancers such as melanoma and lung cancer subtypes with high mutational loads were some of the first to show durable complete response. Estimating mutational load using custom reduced gene panels<sup>[142]</sup> or pre-existing ones<sup>[143]</sup> may aid quick assessments within the clinic. Cancer types are characterized by a wide range of mutational loads<sup>[144]</sup>.

Recent work has sharpened focus on mismatch-repair deficiency as a biomarker to identify patients who can benefit from PD-1 blockade, independent of tissue type<sup>[145]</sup>. The immunological phenotype of microsatellite instability-high (MSI-H) colorectal tumors in particular may be unique<sup>[146]</sup>. In a literature review of anti-PD-1 clinical trials, atypical responses appeared in all cancer types except tumors with mismatch-repair deficiency and head and neck squamous cell carcinoma<sup>[147]</sup>.

While checkpoint inhibitors are often presumed to exacerbate the symptoms of patients with inflammatory/autoimmune diseases, anecdotal reports suggest this may not be the case<sup>[74]</sup>.

## RESISTANCE AND ESCAPE

The fact that immunotherapies fail to produce durable responses in a majority of patients has spurred intensive investigations of resistance. Both primary resistance, where no beneficial response is observed, and secondary resistance, where initial benefit is followed by relapse, are observed.

Before proceeding, we first ask why neoantigen vaccines work at all, as they would appear catastrophically prone to failure due to antigen loss. Such loss has been seen in checkpoint blockade therapy, where both chromosomal deletion of clonal neoantigens and negative selection of tumor subclones were observed<sup>[148]</sup>.

Accumulating evidence suggests part of the answer lies in the phenomenon of antigen spreading, aka cascade<sup>[149]</sup>. T cells are initially “instructed” by the vaccine epitopes, but effector activity need not be limited to these. This hypothesis suggests that the role of the vaccine is to nucleate immunity to an iteratively growing cascade of antigens, the epitopes of which are then committed to T cell memory. This could be key to a durable response that can also target new tumor mutations. The time required to generate such a cascade could also explain the lag often seen between vaccine administration and objective response. A related idea suggests that the initial vaccine-induced attack reverses immuno-suppressive mechanisms, allowing preexisting CTLs that already recognize other tumor neoantigens to be unleashed in a cascade<sup>[79]</sup>.

Cancers can resist therapy by circumvention of each of the immune system roles mentioned previously:

- Disrupt presentation;
- Inhibit MHC-I expression<sup>[150-152]</sup>;
- Disrupt dendritic cell trafficking to lymph nodes (i.e. T cell priming);
- Disrupt peptide processing;
- Disrupt recognition (prevent T-cell trafficking from lymph nodes back to tumor<sup>[153]</sup>, exploit holes in TCR repertoire);
- Exploit immune suppressive mechanisms.

In addition, tumor cells employ explicit defense mechanisms, e.g. downregulation of pro-apoptotic pathways, and counterattack by secreting FasL death ligands, resulting in CTL death<sup>[153]</sup>.

The tumor microenvironment (TME), i.e. the nearby cellular, vascular, and extracellular matrix environment remodeled by the tumor, is the focus of much research into resistance<sup>[154]</sup>. Here tumors are seen to induce host self-protective mechanisms, through recruitment of suppressive cells such as MDSCs<sup>[155]</sup>, regulatory T cells<sup>[156]</sup>, and tumor associated macrophages<sup>[157]</sup>. The tumor creates an immune privileged site, akin to the eye and brain, that excludes T cells<sup>[158]</sup>. Recent work provides a detailed picture of effector T cell exclusion based on a  $\beta$ -catenin signaling mechanism<sup>[159]</sup>.

The TME is a metabolically demanding place, with competition for oxygen and nutrients<sup>[160-162]</sup>. Tumor cells can outlast T cells through the induction of T cell anergy or exhaustion, part of a class of phenomena termed T cell dysfunction<sup>[10,162,163]</sup>. There also remains the possibility that the tumor simply grows faster than an often aged and weakened immune system can eliminate it. A careful 2011 discussion of the TME in which CTLA4 operates is given by Quezada *et al.*<sup>[164]</sup>.

As antigen-specific vaccines seek to activate the adaptive system, harnessing the innate immune system, and in particular natural killer (NK) cells<sup>[165-167]</sup>, would appear to be an attractive complementary approach. Unlike naive CD8<sup>+</sup> T cells that require time to acquire cytotoxic activity, NK cells are “ready to kill”<sup>[168]</sup>. NK and dendritic cells engage in mutual activation, and the former can “edit” the latter population<sup>[169]</sup>. Inhibitory receptors that bind MHC-I allow NK cells to recognize and eliminate cells that do not present MHC-I, thus closing one avenue of tumor cell escape. The activating receptor NKG2D has attracted particular interest, as



its ligands (NKG2DL) are commonly expressed by tumors. Tumor cells however can also express NKG2D and hijack NKG2DL signaling to drive stem-cell like behavior<sup>[170]</sup>. NK cells participate in tumor-induced polarization, acquiring a pro-tumorigenic and pro-angiogenic phenotype<sup>[171]</sup>.

Dammeijer *et al.*<sup>[172]</sup> provide a thorough review of primary and secondary resistance mechanisms and treatment options for re-sensitizing tumors. Guo *et al.*<sup>[80]</sup> provide a compact review of the wide variety of immunosuppressive mechanisms employed by tumors. Chen and Mellman<sup>[173]</sup> describe these mechanisms in the context of the cancer immunity cycle. A concise table of many elements that underlie tumor escape is given by Accolla and Tosi<sup>[174]</sup>. Seliger<sup>[150]</sup> and Seliger *et al.*<sup>[175]</sup> review MHC-I and MHC-II-based evasion mechanisms, respectively. A report of HLA allele-specific risk of metastasis in papillary thyroid cancer<sup>[176]</sup> provides evidence that MHC allele status impacts cancer progression.

### Frameworks for understanding tumor-immune system interactions

Reducing therapeutic resistance is closely tied to our understanding of how cancer arises in the context of the immune system, which we briefly discuss here. The primary framework for understanding the interplay between cancer and the immune system is known as immunoediting<sup>[177]</sup>. This posits that selection pressure from the immune system “edits” the tumor, forcing it to find a custom response to the local and systemic state of the immune system in order to escape immune pressure after many years of genetic changes.

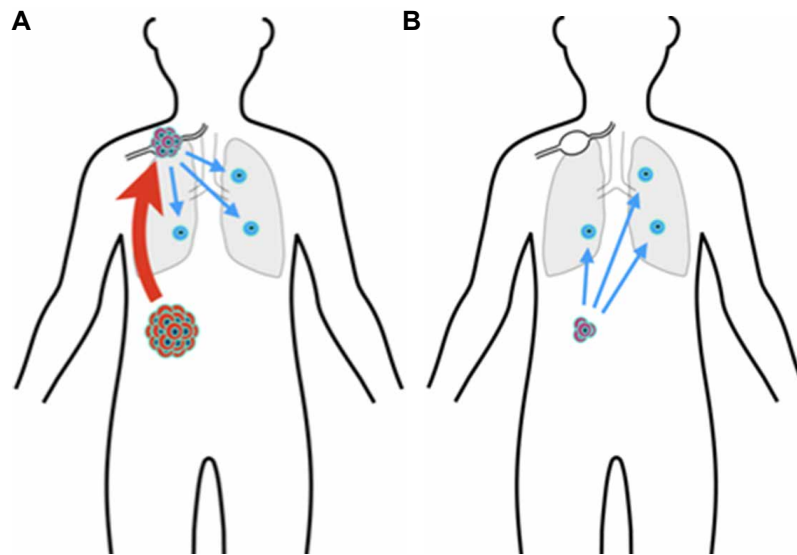
Therapeutic success is often defined by reduction in the incidence and impact of metastatic cancer. The origin and nature of metastases is a dynamic research area, with much remaining to be discovered. Do metastases represent dissemination of cancer cells from a primary tumor in late stage disease (Halsted-Meyer theory)<sup>[178]</sup>, or do they reflect the outgrowth of pre-existing cells that disseminated early on [Figure 3]<sup>[179]</sup>? TNM staging<sup>[180]</sup> encourages the former perspective. Weichselbaum and Hellman<sup>[181]</sup> posit the existence of cancers with intermediate metastatic potential. The hypothesis of cancer dormancy posits that tumor cells may disseminate early and are forced into dormancy in order to survive immune surveillance<sup>[182,183]</sup>. From this perspective, one goal of immunotherapy is to keep such cells dormant, as opposed to attempting to eliminate them all<sup>[184,185]</sup>. Such topics have been covered in a chapter-length review, including the different kinds of dormancy, the role of circulating tumor cells and of innate and adaptive immune cells, and ideas for keeping dormant tumor cell indolent<sup>[186]</sup>. It is evident that this research area is both difficult and in its early stages.

### NEXT STEPS

A number of authors have sought to identify the most urgent and interesting near-term trends. Whiteside *et al.*<sup>[187]</sup> and Hoos<sup>[188]</sup> foresee a focus on, amongst other topics, understanding PD-1 nonresponders, targeting the tumor microenvironment, improving therapy of tumors with few mutations or low tumor infiltrating lymphocyte count, better tumor and patient assessments, combination therapies, and biomarkers of response. This includes the proposed acknowledgment that stable chronic disease (“functional cure”) is a worthwhile endpoint.

Combination therapy is hailed as possibly the best way to increase the percentage of responders. Current examples of combination therapy tend to have a reactive character, applying an additional therapy in response to failure of an initial one. A strong call was issued in 2015 for increased funding of trials to study how to combine molecularly targeted and immuno-therapies<sup>[189]</sup>.

In general, it is hoped that progress can be made through examination of rational combinations<sup>[172]</sup>. The meaning of “rational synergy” has been dissected in the context of cytotoxic drug combinations<sup>[190]</sup>. The diversity of currently available modalities may allow combinations where treatments are carefully scheduled to act as e.g. “mutual adjuvants”. Adjuvants<sup>[191-193]</sup> continue to be topics of active research, with the line



**Figure 3.** Origin of metastases. (A) TNM staging, closely related to Halsted-Meyer theory of breast cancer progression, suggests that remote metastases arise late in the progression of the primary tumor, with disseminating cells first traveling to the lymph nodes; (B) the cancer dormancy hypothesis suggests that tumor cells disseminate early to remote sites and are then forced into a dormant state by immune surveillance. These two alternatives can be distinguished in part by examining cell genomes to trace cell lineages

between adjuvant and therapeutic agent blurring. New adjuvants and combinations thereof show promise and are proposed as the focus of clinical trials<sup>[194]</sup>.

Improved outcomes are being reported for combined checkpoint therapies, though at the cost of more adverse events<sup>[195,196]</sup>. Even those withdrawing from combination treatment due to severe adverse events may still be receiving benefit<sup>[197]</sup>. Emens *et al.*<sup>[198]</sup> present a list of clinical development priorities to push forward the state of the cancer immunotherapy art.

The literature on combination therapies is expanding rapidly. Dunn and Rao<sup>[199]</sup> have reviewed the combination of epigenetics and immunotherapies. Increasing attention is being paid to traditional targets of the innate immune system. Expression of endogenous viral peptides<sup>[200]</sup> and dsRNA have been shown to lead to innate and adaptive responses. The role of microbes, both as commensal microbiota that are modulatory targets<sup>[201]</sup> and as therapeutic agents<sup>[202]</sup>, are subjects of active research. The universe of immunomodulator targets is rapidly growing<sup>[203]</sup>, expanding the scope of possible combinations.

We note that therapy modalities in combination are not necessarily additive, e.g. the combination of chemotherapy (tyrosine kinase inhibitor) and a TAA vaccine required careful scheduling to avoid failure in a mouse model<sup>[204]</sup>. A literature review of combination (mostly targeted) therapies in metastatic renal cancer expresses both the promise and challenges of extracting benefit from such studies<sup>[205]</sup>. Careful reporting as captured e.g. in the Consolidated Standards of Reporting Trials (CONSORT) guidelines<sup>[206]</sup> will be essential for trial data to have maximum value.

## MONITORING AND MODELING

Immunological research is increasingly driven by the ability to gather data, often in a high throughput manner. This opens new vistas that will allow therapy to be properly targeted and monitored, and may eventually alter the character of therapy itself.

The ease with which data can now be generated highlights the responsibility to employ best practices in experimental and trial design, data acquisition (including patient metadata), and downstream analysis. The

accumulation of high quality datasets should ideally go hand in hand with the ability to model the data, with the ultimate goal of defining optimal interventions to reach a desired outcome (e.g. disease stabilization or cure)<sup>[77]</sup>.

An initial goal is the discovery of prognostic and predictive biomarkers. These can be used for treatment selection<sup>[207]</sup>, e.g. high PD-L1 expression level for pembrolizumab treatment<sup>[208]</sup>. At a more rigorous level, biomarkers indicate system state of the immune system, cancer, or both. The importance of prospective studies for data collection and analysis has long been emphasized. REMARK guidelines (REporting recommendations for tumour MARKer prognostic studies) attempt to capture the minimal information needed to objectively assess the import of a given biomarker study<sup>[209]</sup>. This baseline however is still commonly not met<sup>[210]</sup>.

The field of immune system-related prognostic and predictive biomarkers is complex and rapidly advancing. Urgent efforts are now being made to translate current knowledge and capabilities to the understanding of baseline immunity and response monitoring, and thence the choice of predictive biomarkers<sup>[68,211]</sup>. Magnetic resonance imaging (MRI) based biomarkers of response to immunotherapy have been recently proposed<sup>[212]</sup>. Systemic immune response coordinated across tissues has been observed to be essential to tumor rejection<sup>[213]</sup>. The fraction of tumor-infiltrating partially exhausted cytotoxic T lymphocytes (peCTLs) correlates with response to anti-PD-1 monotherapy, with a low fraction indicating the use of combination checkpoint blockade therapy<sup>[214]</sup>. A possible implication is that checkpoint blockade therapy is most effective when the immune system has already mounted a tumor-specific if suppressed response.

TCR repertoire profiling shows promise in immune monitoring and perhaps response prediction<sup>[215,216]</sup>. Checkpoint blockade is seen to induce diversification of T cell receptor repertoire<sup>[217]</sup>, which has been suggested as a biomarker for PD-1 inhibitor disease stabilization<sup>[218]</sup>. The assessment of TCR repertoire diversity is becoming increasingly accessible<sup>[219]</sup>. Important choices such as library preparation method, in-house versus service provider, output data type (raw and/or analyzed), and the use or not of unique molecular identifiers must first be matched to project goals<sup>[220]</sup>. Basic features of the T cell receptor repertoire are still being revealed, e.g. unexpectedly biased distributions of TCR receptors (CDR3 sequence similarity networks) that change in stereotyped ways with aging, immunization, and antigen selection<sup>[221]</sup>. Progress has been reported in developing statistical means of “reading” T cell memory, as relates e.g. to cytomegalovirus status and HLA typing<sup>[222]</sup>.

As we dissect components and interactions in more detail, the research enterprise can begin to embrace variation to learn better from animal models<sup>[223,224]</sup> and humans<sup>[225,226]</sup>, including with respect to age<sup>[227]</sup>. Data sharing can help ensure technical advances are employed towards broad evidence-based progress<sup>[228]</sup>. In this regard, standards for reporting neo-antigens, HLA alleles, and TCR repertoires may need to be developed.

Adoption of high throughput technologies such as massively parallel sequencing, immunosequencing, microarrays, mass cytometry, and DNA-barcoded pMHC multimers has led to the advent of systems immunology<sup>[20]</sup>. Rather than dissect mechanistic relationships between a few actors, systems methods attempt to capture the behavior of the immune system as a whole. The resulting descriptions tend to have a multi-scale (hierarchical) character in both space and time<sup>[229,230]</sup>. The wide variety of available modeling formalisms and applications has been surveyed by Narang *et al.*<sup>[231]</sup>.

Mathematical modeling has begun to impact the clinic through efforts to optimize dosage and timing (“schedule optimization”), which have gained a foothold in chemotherapy<sup>[232,233]</sup> and radiotherapy<sup>[234,235]</sup>. There is now a rich literature on the modeling of immunotherapy<sup>[236,237]</sup>. As an example, modeling the kinetics of the immune response<sup>[238]</sup> reveals the possibility that a proper choice of schedule can summon a robust T cell

response, overcoming what appears to be tumor resistance. To increase their impact, such models may need to integrate into Bayesian adaptive trials<sup>[63]</sup>.

One theme borrowed from the physics community is to develop simplified abstract models. Such models can generate powerful predictions, derived from the concept of universality<sup>[239]</sup>. The observation of unexpected dynamical patterns in the immune system such as oscillations over several days<sup>[240]</sup> suggests that phenomenological models have an important role to play.

Detailed mechanistic models employ knowledge at the molecular or cell level to explain and predict phenomena<sup>[241]</sup>. A recent attempt to model the cancer-immune system interaction using 12 immune cell types and 13 cytokines plus cancer cells finds steady state “basins” corresponding to escape, elimination, and equilibrium phases in immunoediting, while also finding oscillatory states<sup>[242]</sup>. The interested reader is referred to volumes focused on cancer<sup>[243]</sup> and the immune system<sup>[244]</sup>. A textbook on computational immunology has recently been released<sup>[245]</sup>.

From an artificial intelligence perspective, therapy can be viewed as planning in the presence of uncertainty. The idea that the immune system can be “steered” has been demonstrated by proof-of-concept in silico work<sup>[246]</sup>. Cancer cells can be treated as adversaries in a game theory context<sup>[247]</sup>. In the clinical trials arena, reinforcement learning approaches promise a model-free approach to sequential treatment selection<sup>[248,249]</sup>.

## CONCLUSION

After a long history of doubt and failure, checkpoint blockade therapy has opened the door for cancer immunotherapy<sup>[250]</sup>. With this key modality now accepted, the full weight of technological progress can be brought to bear. New tools provide windows through which the process of disease and treatment can be viewed. Their integration will allow increasingly sophisticated descriptions of immune system and tumor state. Neoantigen cancer vaccines in particular are beneficiaries of this new environment and are poised to lead the way to more precise and effective therapies.

While neoantigen vaccines can now be created with workflows that are increasingly standardized and almost routine, many challenges lie ahead to elicit their true potential. Foremost is gaining a better understanding of primary and secondary resistance. This can be viewed in the light of theories in which cancer cells and the immune system train each other, for better or worse, over decades.

Combination therapies are now pursued as the next step forwards. The examination of all possible protocols may however become infeasible or at least inefficient. Principled methods will need to be developed to systematically identify promising approaches and learn from both successes and failures. This complexity is also an opportunity to formalize therapy as a strategy and not simply an application of magic bullets. Over the longer term, this promises growth in novel interventions that integrate technology, data, models, and algorithms as part of an interdisciplinary biomedical science.

## DECLARATIONS

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### Conflicts of interest

There are no conflicts of interest.

### Patient consent

Not applicable.

### Ethics approval

Not applicable.

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Commentary

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# Immunotherapy of cancer is a part of biotherapy

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## INTRODUCTION

The terms immunotherapy of cancer and biotherapy of cancer have been used interchangeably in the past. Strictly speaking, biotherapy or biological therapy is more appropriate and is now considered the 4th modality of cancer therapy. It can be effective when used alone or in combination with surgery, radiation or chemotherapy. To put biotherapy into a better perspective, it is important to clarify a historical misconception associated with immunotherapy. The term biological response modifiers (BRMs), which had been widely used in the 1970s, referred to agents or approaches, whose modes of action involve the host's own biological responses. Biological substances and BRMs work through many different mechanisms in the biotherapy of cancer. These mechanisms involved for each substance/modifier may be one or several of the following: (1) to increase the host's antitumor response through augmentation or restoration of effector mechanisms or decrease a component of the host response that is deleterious (such as with immune checkpoint inhibitors, e.g. anti-CTLA-4)<sup>[1]</sup>; (2) to augment host defenses through the administration of certain immune cells, natural biological substances, or synthetic derivatives thereof as effectors (direct or indirect) of antitumor responses; (3) to enhance the host responses using modified tumor cells or other types of vaccines to stimulate greater immune responses or increase the sensitivity of tumor cells *in vivo*; (4) to increase the maturation, differentiation or dormancy of tumor cells; (5) to interfere with growth-promoting factors or signaling pathways of tumor cells concerning proliferation, migration/invasion, apoptosis, and angiogenesis; (6) to use biological molecules to target and bind to cancer cells or immune cells to induce greater effective cytostatic/cytotoxic antitumor activity; and (7) to use biological molecules to modify the tumor microenvironment or the host immune system such as allowing effector T cells or natural killer (NK) cells to effectively target and eradicate tumor cells. Thus, one can envisage biotherapy with immune



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modulatory properties, as well as direct cytolytic, cytostatic growth-inhibitory, or maturational effects on tumor cells. This is, in part, the reason why cancer biotherapy provides a much broader spectrum of antitumor action than cancer immunotherapy.

This article is an updated version of the commentary entitled, “Cancer biotherapy: more than immunotherapy” by Oldham<sup>[2]</sup> published in *Cancer Biother Radiopharm* 2017;32:111-4.

Biotherapy of cancer can be effective against clinically apparent, even bulky cancer, and treatment should not be restricted to situations where the tumor mass is imperceptible. Thus, a clinical trial designed for cancer biotherapy can be similar to other modalities as long as one measures both the specificity and activity of biological response affected by these approaches. Nevertheless, the specificity of biotherapy often requires individualized testing and therapy, one important aspect of biotherapy that is different from chemotherapy.

It should be stated at this onset that the literature addressing the concepts highlighted in this paper is immense and will not be exhaustively reviewed here. Instead, we provide a commentary on immunotherapy vs. biotherapy of cancer from both the historical and future perspectives with an overview of the current trends in research focusing particularly on recent cellular, vaccine and targeting strategies that have real potential for patients.

## HISTORICAL PERSPECTIVES<sup>[3]</sup>

The use of chemical and biological compounds to modulate biological responses has been under active investigation for more than five decades. While various chemical, bacterial extracts and viruses have been found to modulate immune responses in experimental animals, and to a much less extent in humans, these nonspecific immune modulators have not been highly effective as therapy for human cancers. Molecular biologists have developed many new technologies in the isolation of genes and their subsequent transcription and translation into protein production, yielding high levels of purity. These processes make virtually unlimited quantities of purified biological products available for both experimental and therapeutic use. *In vitro* assays of biological activity (bioassays or functional assays) were intensely developed and used to define and quantify the activity of a given biological molecule in the 1980s, and the paradigm of cancer research and therapy has changed substantially. These assays, such as flow cytometry, enzyme-linked immunosorbent assays, immunoprecipitation, immunoblotting, immunohistochemistry, human leukocyte antigen (HLA) typing, epitope prediction, tetramer assays, detection of circulating cancer cells, cytotoxicity assays, CRISPER gene-editing<sup>[3]</sup>, humanized mice and liquid biopsy have allowed the precise determination of identity, activity and specificity of these molecules or cells as part of cancer therapeutics. Some of them also provided the monitoring assays for the patients before, during and after treatment.

Since the early 1970s, inbred or syngeneic animals were used experimentally because it was realized that the variability in cancer behavior could be due to the differences in major histocompatibility complex (MHC) among out-bred animals<sup>[4]</sup>. Therapeutic manipulations using these syngeneic animals with transplantable tumors met with challenges, since they were very different from animals with naturally occurring cancers. Thus, the relevance of these animal models for cancer in humans was questionable. As opposed to transplantable cancers arising from carcinogenic stimuli in a particular organ starting from one cell or a few cells, naturally occurring cancers have been gone through a prolonged period of latency, before they are pathologically diagnosed as malignancies. In humans, these initial tumor foci may be in a benign or dormant state for various lengths of time ranging from 1% to 30% of human lifespan before there is a clinical evidence of cancer. Dissemination of these cells from the primary lesion may occur anytime during the development of the primary tumor. Subsequently, growth and metastasis may occur over periods of months to years from the primary or secondary lesions. Although we have learned a great deal about the basic biology of tumorigenesis and cancer pathophysiology from experimentally induced cancers, such as the importance of

MHC molecules in cancer and transplantation<sup>[5]</sup>, they have been considered highly artificial in many ways. For instance, a high dose of carcinogen may result in cancer in a given strain of syngeneic mice in a short time. Transplantable tumors developed in such a way have been maintained *in vitro* and *in vivo* intermittently for years. When these tumor cells were used in transplantable models by injecting cells into young, syngeneic animals, thereby circumventing the influences of environmental and genetic factors that are indeed operative in human situations, results obtained from these studies have only most remote relevance to cancers in humans. Thus, these transplantable tumors are simply not analogous to clinical cancers and the conclusions drawn from them are less likely to be applicable to human cancers.

Early immunotherapy experiments produced a dogma that immunological manipulations could only work when the tumor cell mass was imperceptible ( $< 10^8$  cells), which presented real problems for clinical immunotherapy, because the tumor mass at clinical diagnosis or after surgery is at least two orders of magnitude greater than  $10^8$  cells. Despite the obvious difficulties with experimental models and translation to humans, clinicians began large-scale immunotherapy trials in the 1970s. The results of initial, small, uncontrolled trials were often reported as positive. However, when large, randomized, controlled studies were conducted to confirm the efficacy of a particular immunotherapy regimen in a particular type of cancer, some of the controlled studies were positive and but most yielded marginal or negative results. Therefore, overall cancer immunotherapy developed a bad image among physicians, chemotherapists in particular, by the end of the 1970s.

Looking back, there were a number of reasons that could be considered for the failure of cancer immunotherapy to establish itself as a major treatment modality. One of the reasons was a lack of definition for highly purified immunotherapeutic agents. For instance, any of the nonspecific approaches using complex chemicals and poorly defined bacterial and viral extracts to stimulate the immune response of patients often made the interpretation of results difficult. Consequently, there were problems in reproducibility of the results generated even by members of the same research group. Thus, molecular definition of the molecules in question, such as immune modulators, lymphokines/cytokines, tumor antigens and antibodies, became the hot subject for many investigators to pursue. Another reason was the general lack of understanding of the immune responses in cancer patients then, such as the rule of MHC-restriction<sup>[6]</sup>, definition of T cell receptor, and interactions among components at the region of immunological synapse<sup>[7]</sup>. Immunotherapy is not an appropriate term for the modern use of biologic substances and BRMs in medicine. Biological control mechanisms should be envisioned on a much broader basis than the immune system. Immunotherapy remains a subcategory of biotherapy, but growth and differentiation (maturation) factors, cytokines, angiogenic inhibitors, and recently identified immune checkpoint inhibitors, and synthetic derived molecular analogues are indeed much broader than immunotherapy.

Certain specific developments over the past thirty years led to biotherapy becoming the 4th modality of cancer treatment<sup>[8,9]</sup>. Advances in molecular biology have given scientists the greater capacity to clone individual genes and produce large quantities of highly purified gene products as medicines. The proteins resulting from the cloned genes have a level of purity and homogeneity on a par with drugs and can be produced in unlimited amounts. They can be analyzed alone or in combination with other gene products as to achieve their optimal effects in cancer biotherapy. Additionally, progress in genomic and gene mapping science, nucleic acid sequencing and translation, protein synthesis, isolation and purification of the biological products, as well as in mass culture of cells with the use of bioreactors has given the scientific community the power to identify of new biological molecules, modify nucleic acids and proteins at the nucleotide or amino acid level to manipulate, optimize their biological activity, and use clinically. The elucidation of the human genome and the encoded products have considerably broadened the opportunities for the advancement of cancer biotherapy. Along with tumor cell vaccines and non-specific immune stimulators such as bacillus Calmette-Guerin, adoptive cell therapy and monoclonal antibodies (mAbs) are two popular biotherapeutic approaches used clinically. Some of their specific components such as chimeric antigen receptor (CAR)-T

cells, gamma delta T ( $\gamma\delta$ T), and immune checkpoint inhibitors in the form of mAbs are currently still under active investigation (see “Future perspectives” below).

### **Adoptive cell-based therapy**

A variety of effector cells including NK<sup>[10]</sup>, lymphokine-activated killer (LAK)<sup>[11,12]</sup>, cytokine-induced killer (CIK)<sup>[13]</sup>, tumor-infiltrating lymphocyte (TIL)<sup>[14]</sup>, dendritic cell (DC)<sup>[15]</sup> and antigenic peptide pulsed-DC expanded cytotoxic lymphocyte (CTL)<sup>[16]</sup>, and  $\gamma\delta$ T<sup>[17]</sup> cells have been used as part of adoptive cell-based immunotherapies for different human cancer types, with varying degrees of efficacies obtained in the past. Most important developments in the cell-based immunotherapy in recent years include (1) the rapid expansion methods for NK and TILs using stimulatory or feeder cells transfected with the genes of continuous 4-1BB co-stimulatory signals<sup>[18,19]</sup>; and (2) the development of engineered T cell receptor (TCR)-T cells<sup>[20]</sup> and CAR-T cells<sup>[21]</sup>, modes of T cell adoptive cell immunotherapy with impressive clinical results that had not been achieved previously. Apart from its ease for expansion, potent killing effect and requirement of only one *in vivo* administration, another advantage of CAR-T therapy is independent of TCR recognition. In other words, its tumor killing should be effective for patients in whom the surface HLA-class I expression on cancer cells was deficient or lost<sup>[22,23]</sup>, or whose tumors were drug-resistant<sup>[24]</sup>, as long as the patient's tumor cells could all be detected by the CAR-T cells to be infused<sup>[24,25]</sup>. It is well known that the expression of HLA class I in cancer cells of patients with the advanced stage or under the influence of treatment tends to become deficient or lost totally<sup>[22,23,26]</sup>, one way for tumor cells to escape from the host immune surveillance. Of note, these modes of adoptive cell transfer are considered personalized immunotherapies, as patients' own immune cells are processed, expanded, and infused back to the individual patients.

### **Monoclonal antibodies**

The discovery of hybridoma technology in the 1970s for the production mAbs was another major technical advance<sup>[27]</sup>. The limitation of the use of polyclonal antibodies have been the inability to generate reproducible, high-titer, specific antibodies, and to precisely define the antigenic molecules identified with such polyclonal antibodies. The development of mAbs each with its fine specificity has largely circumvented the problems associated with polyclonal antibodies, allowing an alternative way, other than molecular cloning, to produce a variety of biologicals of therapeutic grade. Furthermore, processes to be able to “chimerize” or “humanize” murine mAbs have produced therapeutic antibodies to be used in the clinical treatment of cancer and autoimmune patients possible with low immunogenicity. mAbs are highly specific for the antigens on the tumor cells and immune cells. In addition, these mAbs and genetic sequencing testing allow for the individual tailoring of treatment to each patient, now known as “precision medicine”. To be specific for cancer patients, such tailoring is called “precision oncology”.

### **FUTURE PERSPECTIVES**

There is no doubt that we now have more powerful tools and technologies for improving cancer therapy. Cancer biotherapy provides additional approaches which may work effectively in combination with surgery, radiation, targeted therapy or chemotherapy. It may work effectively through mAbs in directing radioisotopes selectively to the tumor cells and with chemotherapy, and other cytostatic and cytotoxic molecules as immunoconjugates in directing those molecules to the tumor bed, enhancing selectivity and biological activity. It may also work more effectively through a combination treatment of both innate and adaptive cellular therapy as compared with single cell therapy alone<sup>[28,29]</sup>. Thus, biotherapy offers the great hope to cancer patients for selective treatment to enhance therapeutic/toxic ratio and at the same time lessen the problem of nonspecific toxicity, a major impediment to the development of more effective cancer treatment.

The coming decade will have many opportunities to pursue new approaches in cancer treatment. Basic scientists and physician/scientists requiring special training and expertise will use new techniques in the laboratory and clinic. Currently, the medical oncologist trained in the administration of chemotherapy drugs is not well prepared to administer biological substances for cancer treatment. Biotherapy uses biological



substances that are often active in association with the immune system. The diversity of the immune system is best understood by clinical immunologists and cell biologists who are well suited to assist in the translation of these approaches to the clinic. This concept was first put forwarded in 1977 by the Nobel laureate Sir Peter Medawar<sup>[30]</sup>.

“The cure for cancer is never going to be found. It is far more likely that each tumor in each patient is going to present a unique problem for which laboratory workers and clinicians between them to work out a unique problem.”

Cancer classification and biology have largely been embedded in the minds of pathologists and transmitted through textbooks of medicine to medical students who become clinicians at later dates. These concepts classify cancers categorically according to the tissue origin and biological features. Despite the laboratory observations that phenotypic analysis and even the genotype of cancer biology confer great diversity within cancers of same histological type, we continue to evolve new therapeutics as if all breast cancers, all lung cancers, and all colorectal cancers are similar. However, this is fundamentally and biologically incorrect. There has never been a technology that allowed cancer biologists to understand cancer on an individualistic basis. Now it is possible to generate antibodies and type tumors specifically, leading to the generation of cocktails of antibodies or immune conjugates to respond to diversity inherent in cancer biology<sup>[31]</sup>. Below are the most recently developed innovative strategies to cancer biotherapy which are listed under the following three subtitles, each being involved with the cells and agents mentioned in the “Historical Perspectives” section, namely CAR-T cells and immune checkpoint inhibitors, anti-PD1 and anti-PDL1.

### **Immunotargeting cancer stem cells and metastasis**

Metastases, often resistant to conventional therapy, are the major cause of death from cancer or the treatment failure. In most cancer patients, metastases have already taken place at the time of diagnosis. Most recently, the successful identification of two cellular entities, namely cancer stem cells (CSCs) and metastatic cancer stem cells (mCSCs) with the expression of CXCR4<sup>[21,32]</sup>, both constituted very small proportions of cells within a given tumor, has stimulated a new direction for investigations as to how to eradicate or control of these two cell types. Of note, the CXCR4-positive mCSCs with metastatic potential constitute much lower numbers than the tumorigenic CSCs in the given tumor<sup>[32,33]</sup>. This is because both entities are considered the root causes of cancer (tumorigenesis), with the latter being the cause of both tumorigenic and metastatic activities. The predominant subpopulations of cells within a tumor belong to so called non-CSCs which are heterogeneous with more than one differentiated phenotype. These non-CSCs are believed to be more sensitive to be killed by conventional therapies, such as radiation and chemotherapy. Expression of surface antigens such as ALDH, CD44, EpCAM, or CD133, which distinguish CSCs from non-CSC tumor cells and normal counterpart cells, together with CSC immunogenicity and relatively low toxicity of immunotherapies, makes immunotargeting of CSCs/mCSCs a promising approach for cancer biotherapy<sup>[32-35]</sup>. The approaches to target and eliminate CSCs include using NK, DCs, T cells, mAbs, and bispecific antibodies. A case in point, Her2- specific T cells from glioblastoma multiforme (GBM) patients were constructed by genetic transfer of Her2-specific CAR<sup>[36]</sup>. These Her2-specific CAR-T cells showed cytotoxicity against Her2-positive targets *in vitro* and secreted immunostimulatory Th1 cytokines. The Her2-specific CAR-T cells were able to kill *in vitro* autologous CD133-positive GBM stem cells expressing Her2, which were found to be resistant to current conventional therapies. Adoptive transfer of Her2-specific CAR-T cells prepared in such a way resulted in prolonged regression of autologous orthotopic GBM xenografts<sup>[36,37]</sup>. These findings confirm the Her2-specific CAR-T cells targeted and eradicated Her2-positive tumor cells and their putative cancer stem cells. Furthermore, NK<sup>[38]</sup> and CIK with or without DCs<sup>[39]</sup> were found to effectively kill stem-like cancer cells. Incidentally, synergistic targeting of breast CSCs by human  $\gamma\delta$ T cells and cytotoxic CD8<sup>+</sup> T cells in combination has also been reported<sup>[28]</sup>. Further technical refinements along this line of investigations are currently underway in a number of laboratories.



We may have to design the strategies of targeting metastasis at two levels, one to prevent metastasis of the primary tumor by targeting CSCs, and another to target the established metastasis through CSCs and mCSCs. In addition to various cell-based immunotherapies such as CAR-T cells, much of *in vitro* and *in vivo* studies or clinical trials in the identification of various biological agents including a number of small molecules and botanical nutraceuticals. For example, being a potent BRM itself, withaferin-A, a withanolide extracted from the Indian winter cherry *Withania somnifera*, was found to be able to selectively block certain signaling pathways involved in the proliferation/migration/apoptosis/angiogenesis/antioxidant in the two types of CSC entities<sup>[34,40]</sup>. In contrast, those of the non-CSC and normal cell counter parts were relatively not affected by withaferin-A. Many botanical and many other biological and synthetic compounds are currently being under active investigation with regard to their targeting potentials on CSCs and/or mCSCs of a variety of tumors.

### Immunotargeting the tumor microenvironment

Investigation into targeting the tumor microenvironment is also becoming one of the major cancer biotherapeutic strategies in the recent years<sup>[41,42]</sup>. The tumor microenvironment includes infiltration of carcinoma-associated fibroblasts such as myofibroblasts and mesenchymal stem cells, infiltration of inflammatory cells such as T cells, macrophages, DC cells, NK cells, myeloid derived suppressor cells, regulatory T cells, and infiltration of blood cells such as blood endothelial cells and lymphatic endothelial cells, and non-cellular components for remodeling of extracellular matrix, *etc.* The recognition of the importance of tumor microenvironment in cancer progression has indeed led to a shift from a cancer-centered view of cancer development to the concept of a complex cancer microenvironment or an ecosystem. In a tumor microenvironment, various cellular and molecular components are as influential as cancer cells themselves for cancer progression including dissemination<sup>[41]</sup>. One feature of such a microenvironment is that minor changes in a single component noted above may cause a reorganization of the whole system. Consequently, the interference with any element of the tumor microenvironment provides an opportunity to tip off the balance of the ecosystem or counteract the cancer progression. The use of an inhibitor of checkpoint molecules, namely humanized mAb anti-CTLA-4 or anti-PD1<sup>[43,44]</sup>, or CAR-T cells<sup>[42]</sup> in combination with chemotherapy leading to some encouraging clinical results may therefore be considered as the successful stories of targeting the tumor microenvironment.

### Neoantigen/RNA mutanome vaccines

Clearly, T cells can be generated, induced and manipulated in a similar way a mAbs for specific cellular therapy<sup>[20,30]</sup>. Surely enough, thanks to the cutting edge technologies of prediction and identification of target epitopes for peptide design, very impressive clinical results was recently obtained by two groups, Harvard Medical School, Boston, USA and Biopharmaceutical New Technologies corporation/medical Center of Gutenberg University, Mainz, Germany, using personalized neoantigen<sup>[45]</sup> and RNA mutanome<sup>[46]</sup> vaccines respectively, for patients with melanoma. A few months after vaccination, some of these patients achieving partial responses found to have recurrent disease were treated with anti-PD1 therapy, and encouragingly experienced complete tumor regression<sup>[45]</sup>. The personalized vaccine therapies in both studies could induce *de novo* T-cell clones that reacted with multiple individual-specific neoantigens or mutated gene products, and recognized endogenously processed antigens, and hence autologous tumor cells. Such induced immunogenicity could therefore have better chances of targeting a diversity of cancer clones per patient with a high response rate, addressing tumor heterogeneity as well as minimizing the tumor escape by loss of antigen. These two innovative studies with different preparations of vaccines again demonstrate exciting examples of precision oncology/medicine.

### IMMUNOTHERAPY IS INCLUDED IN BIOTHERAPY

Biotherapy is constituted more broadly to include all the factors described above. To take advantage of the opportunities available through biotherapy, major structural changes are necessary in our system

of translation of developmental therapies from a concept to the laboratory and then to the clinic. We cannot afford to develop biological substances in a protracted, expensive, unidimensional manner of drug development. We have a large number of biological substances, and the current system of access and opportunity for patients, the system of funding research, our method of government regulation, and our reimbursement system for the developmental therapies must undergo major structural changes. We are now faced with the reality of many more opportunities for effective cancer biotherapy than the mechanisms by which these opportunities can be tested and brought into clinical reality.

Development of new therapeutic programs have functioned under a format in which a new drug is brought to the clinic through phase I clinical trial for toxicity followed by phase 2 for activity with the assumption that short-term effects on cancer, i.e. response rate. It will ultimately lead, if positive, to survival benefits, including overall survival and progression-free survival. Although this paradigm has been useful in developing chemotherapeutic drugs, there is much to suggest that we should now broaden our concept of developmental therapeutics to include the idea of cancer control, as cancer biotherapy becomes more utilized. As analogue to the treatment of chronic diseases such as diabetes mellitus, it is likely that through the use of biotherapeutic agents, we may achieve a long-term control of cancer growth and dissemination without eradication of cancer, i.e. to live with tumor<sup>[47,48]</sup>. This is often associated with the induction of long-term memory T cells and/or tumor dormancy. The combined use of DC vaccines, inhibitors (chimeric/humanized mAbs) of immune checkpoint molecules, such as CTLA-4, or PD1 and chemotherapy on cancer patients, resulted in survival benefits<sup>[1,43,44]</sup>.

## CONCLUSIONS

The individualization/personalization of cancer treatment represents the major challenge of the next decade<sup>[20,30,45,46]</sup>. Clearly, cancer can be characterized on an individual basis and therapy developed for individual patients. However, bringing this individualized approach to the clinic and merging it with a more general approach of cancer treatment is a major challenge. One strategy would be to reduce the bulk of cancer through a more generalized approach, such as surgery, radiotherapy, chemotherapy or targeted therapy, with application of more specific approach to eradicate or control residual cancer using some form of biotherapy. Included are manipulation of tumor microenvironment, targeting cancer stem cells, to enhance T cells infiltration and access to the tumor, augmentation of MHC expression for adequate presentation of tumor peptide antigens, generated by the treatments. These strategic approaches, while conceptually pleasing, are difficult to bring into the clinic for individual patients, because of the labor intensiveness, cost and complicated nature of a multidimensional therapeutic program.

An additional feature is that many of the more specific approaches to cancer treatment, notably, engineered TCR-T and CAR-T cell therapies<sup>[49,50]</sup>, and most recently personalized neoantigen peptide or RNA mutanome vaccines<sup>[45,46]</sup> are patient-specific and developed in good tissue practice (GTP) or good manufacturing practice (GMP) laboratories that are remote from clinical trial site. These logistical issues alongside the governmental regulatory issue are complicated and costly. With the initial success for CD19/CD20-positive leukemia/lymphoma with CAR-T cell therapy and for melaonoma with neoantigen vaccines, biotech/biopharma companies and university hospitals/medical centers have both put their great efforts as one of the top priorities in attempts to bring this type of novel approach to the clinic for other hematological malignancies as well as many other types of solid tumors<sup>[20,37,49-52]</sup>.

The major advantages of most cancer biotherapy including cell-based immunotherapeutic strategies are low or acceptable toxicity, and the ability to target defined molecules, signaling pathways, or cell subpopulations. On the other hand, biotherapy is more effective in some type of cancers and often needs to be companied by conventional strategies such as surgery/chemotherapy. Furthermore, good equipped laboratories including a wet research lab and a government-certified GTP/GMP facility, and at the same time close collaborations

between basic scientists and clinical oncologists will require for the success implementation of a cancer biotherapy program. Despite these challenges, it is becoming a fascinating treatment mode in the fighting cancer and its further development in the near future is anticipated.

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Both authors contributed equally to this commentary article.

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Review

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# Bacteria in cancer therapy: beyond immunostimulation

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## Abstract

Currently, conventional therapies in cancer are improving; chemotherapy, radiotherapy and surgery have increased survival significantly. New therapies have arisen with the same goal; immunotherapy has appeared as a promising option in the fight against cancer stimulating the immune system by inducing innate and adaptive responses. These responses include release of pro-inflammatory cytokines, making the immune system capable to eliminate or protect against multiple tumors. Nowadays, many of these therapies are being used in clinical settings, such as checkpoint inhibitors, monoclonal anti cytotoxic T-Lymphocyte associated protein 4 (CTL-4) and programmed death protein 1 (PD1), with inspiring results; however, they may decrease immunotolerance, limiting their use. At the same time, chemotherapy works by passive transport across the cell membrane, limiting its capacity to penetrate in tumor cells. For these reasons, bacteria employment represents one of the best candidates for cancer treatment. They can surpass these barriers with their selective colonization which also has an oncolytic effect by increasing proliferation and immunostimulation in the tumor environment. Attenuated strains, such as *Mycobacterium bovis*, *Clostridium*, *Salmonella typhimurium* and *Listeria monocytogenes* have been studied showing promising results in experimental models. However, their application in clinical trials has shown the need to maximize their therapeutic effect. Genetic engineering and synthetic biology are necessary to prove the scope that this novel approach has against cancer due to implications of cancer therapy and public health.

**Keywords:** Bacterium, cancer, selective colonization, *salmonella*, *clostridium*, *listeria*



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## INTRODUCTION

At present, cancer has one of the highest morbidity and mortality rates worldwide, nationwide and statewide<sup>[1,2]</sup>. It comes from the growth of uncontrolled and invasive malignant cells with DNA mutations capable of producing multiple diseases<sup>[3]</sup>. Most of these malignant neoplasms have the same etiopathogenesis. However, the diversity on the anatomic location, histologic origin, immunologic characteristics and intrinsic spreading capacity (intertumoral heterogeneity)<sup>[4]</sup>, and different genomic alterations inside the same tumor (intratumoral heterogeneity)<sup>[5]</sup> have shown the need for specific biomarkers and individualized therapy to improve patient prognosis.

Currently, conventional therapy such as surgery, chemotherapy, radiotherapy, or mixed therapy have increased survival rates worldwide against cancer in different subtypes<sup>[6,7]</sup>. However, these practices produce many adverse effects and have shown a limited tumor penetrance<sup>[8]</sup>. The role of the immune system has been studied in order to find a therapeutic approach with equivalent therapeutic potency and controlled damage to healthy tissue; which gave rise to immunotherapy as a novel treatment<sup>[9]</sup>.

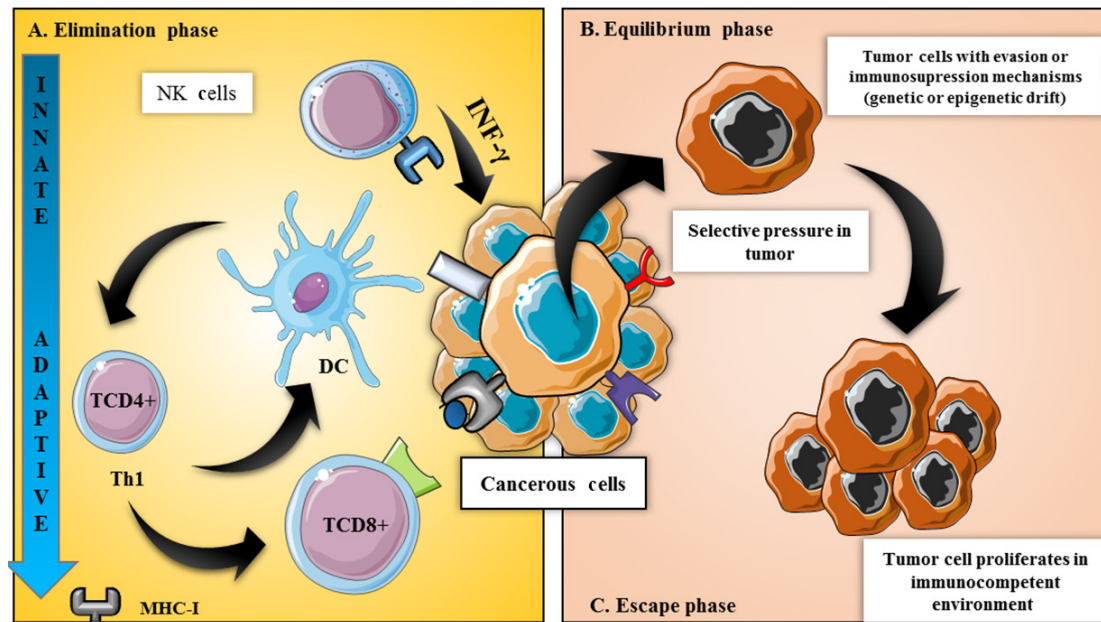
Nowadays, many of these therapies are being used in clinical settings, including the checkpoint inhibitors monoclonal antibodies anti cytotoxic T-Lymphocyte associated protein 4 (CTLA-4) and programmed death protein 1 (PD1). They have been shown to increase survival in patients with metastatic melanoma<sup>[10]</sup> but their mechanism of action decreases immunotolerance with systemic administration. The latter may cause autoimmune adverse effects, limiting its use only for specific patients<sup>[11]</sup>. In the last few decades, experimental studies and clinical trials have been aimed to assess bacteria therapeutic functions<sup>[12-15]</sup>. Bacteria selective replication within the tumor microenvironment gives them antitumor effect and minimizes systemic adverse effects. On the other hand, expression of multiple ligands, immunostimulants, cytokines and tumor antigens can be achieved through gene manipulation to increase the therapeutic effect against specific tumors.

Cancer causes many physical and psychological effects to the patients and their families, but it also increases state expenditures. For these reasons, evaluation of these novel therapies in clinical settings has great importance. This review brings the basic science principles in genetics, immunology, and microbiology that gave rise to this therapeutic approach, in addition to its latest experimental and clinical advances.

## THE BEGINNING: GENETIC AND IMMUNOLOGIC BASIS IN CARCINOGENESIS

Carcinogenesis begins as a result of multiple genomic alterations within a cell. They come from a prolonged exposure to different mutagens<sup>[16]</sup>, adverse epigenetic factors<sup>[17]</sup>, as well as chronic infections<sup>[18]</sup>. These alterations increase proliferation and affect cell cycle through gene functioning<sup>[19]</sup>, in proto oncogenes<sup>[20]</sup> and tumor suppressor genes<sup>[21]</sup>, causing different mutations<sup>[22]</sup>. They ultimately modify the cell physiology making a mutated cell capable to generate its own mitogenic signals, resist against growth inhibitory signals, and acquires its own blood vessels. In advanced stages it can even invade and metastasize<sup>[3]</sup>.

The role of the immune system in tumor surveillance comes from the response to multiple oncogenic viruses and other infecting agents that can induce a chronic inflammatory environment leading to carcinogenesis<sup>[23,24]</sup>. Identification of tumor-specific antigens (TSA) induces an immune response on carcinogenesis at an early stage<sup>[24]</sup>. Tumor cells generate multiple modified surface proteins, decreasing immunologic tolerance as carcinogenesis progresses, and many TSA are expressed<sup>[25]</sup>. The immune system can recognize and eliminate abnormal cells, in a continuous and bidirectional pathway between innate and adaptive immunity, which is called “*Immunosurveillance*”<sup>[26]</sup>. Natural killer (NK) cells<sup>[27]</sup> and cytotoxic



**Figure 1.** Immunosurveillance vs. immunoediting: key points in regulation of immune system in tumor progression/regression. Immunoediting comprises 3 phases: (A) elimination: when the tumor cells begins to proliferate, an inflammatory response is induced by the injured tissue. This causes the migration of cells from the immune system, orchestrating the innate immune response; (B) the equilibrium phase: in which this continuous process produces a selective pressure in these cells that can cause genetic or epigenetic rearrangement, causing certain cells to evade these immunological effector mechanisms; (C) the escape phase, when cells that have evaded these mechanisms also gained uncontrolled growth ability. DC: dendritic cells; MHC: major histocompatibility complex; NK: natural killers

T lymphocytes (T-CD8<sup>+</sup>) are the main mediators in this process<sup>[28]</sup>; macrophages associated to tumors, dendritic cells (DC), naïve T cells, αβT-cell receptor (TCR)-expressing T cells, γδT-cells and regulatory T cells (T-reg) FOXP3<sup>+</sup> also participate in the immune response towards the tumor. They interact with tumor cells, while some act inhibiting their growth and others stimulate it, composing the tumor microenvironment<sup>[29]</sup>.

NK cells are considered the main part of the innate immunity against tumors. They recognize and eliminate neoplastic cells effectively<sup>[30,31]</sup>; but are not confined to the innate immune system. They also act with the adaptive immunity by working as T-lymphocyte response modulators<sup>[32]</sup>. Damage associated molecule patterns (DAMPs) are released from tumor cell elimination mediated by NK cells<sup>[33]</sup>, increasing DC maturation<sup>[34]</sup> and presentation to T-CD8<sup>+</sup> lymphocytes on major histocompatibility complex (MHC)-1 molecules<sup>[35]</sup>. Once activated, NK cells and T-CD8<sup>+</sup> lymphocytes induce activation, proliferation and recruiting of other cells to the tumor site<sup>[36]</sup>. This is achieved through the release of cytokines such as interferon gamma (IFN-γ), granulocyte and macrophage colonies stimulating factor (GM-CSF) and tumor necrosis factor (TNF)<sup>[37]</sup>. IFN-γ carries important functions such as direct inhibition of tumor growth, macrophage activation, and increases Th1 expression among T-CD4<sup>+</sup> lymphocytes. This represents their major role in modulating cellular response against tumors<sup>[38]</sup>. T-CD8<sup>+</sup> lymphocytes require the expression of tumor antigens on MHC-1 molecules and co-stimulatory signals in the tumor site in order to function appropriately<sup>[39]</sup>.

Cancer may become clinically detectable in advanced stages explained by the mechanisms in which tumor cells evade immune surveillance<sup>[40]</sup>. This theory started with the “immunoediting” process<sup>[41]</sup>, where the immune system works inversely: making an immunosuppressed environment that favors tumor growth. This process is composed by three phases: elimination, equilibrium, and escape<sup>[Figure 1]</sup>, being the elimination phase a homologous mechanism from those seen in immunosurveillance<sup>[42]</sup>.

Once the tumor cell has escaped the elimination phase, it enters the equilibrium phase<sup>[43]</sup>. This phase consists in the destruction of cells expressing tumor antigens in their MHC-1 molecules by T-CD8<sup>+</sup><sup>[44]</sup>. Following this, less immunogenic cellular clones will be immunoselected and more aggressive tumor cells will grow, directing them to the escape phase<sup>[43]</sup>. Although evasion of the immune system is not an isolated event; it also includes an immunological adaptation process. During this process an immunosuppressed microenvironment comes with recruitment T-reg FOXP3<sup>+</sup><sup>[45]</sup> and release of immunomodulatory molecules such as transforming growth factor  $\beta$  (TGF $\beta$ ), prostaglandin E2 (PGE2), indoleamine 2,3 dioxygenase (IDO), adenosine, and interleukin-10 (IL 10); also with decreased expression of co-stimulatory proteins and increased expression of inhibitor molecules such as CTL-4/CD28 and PD-1/PD-L1, called checkpoints<sup>[46]</sup>.

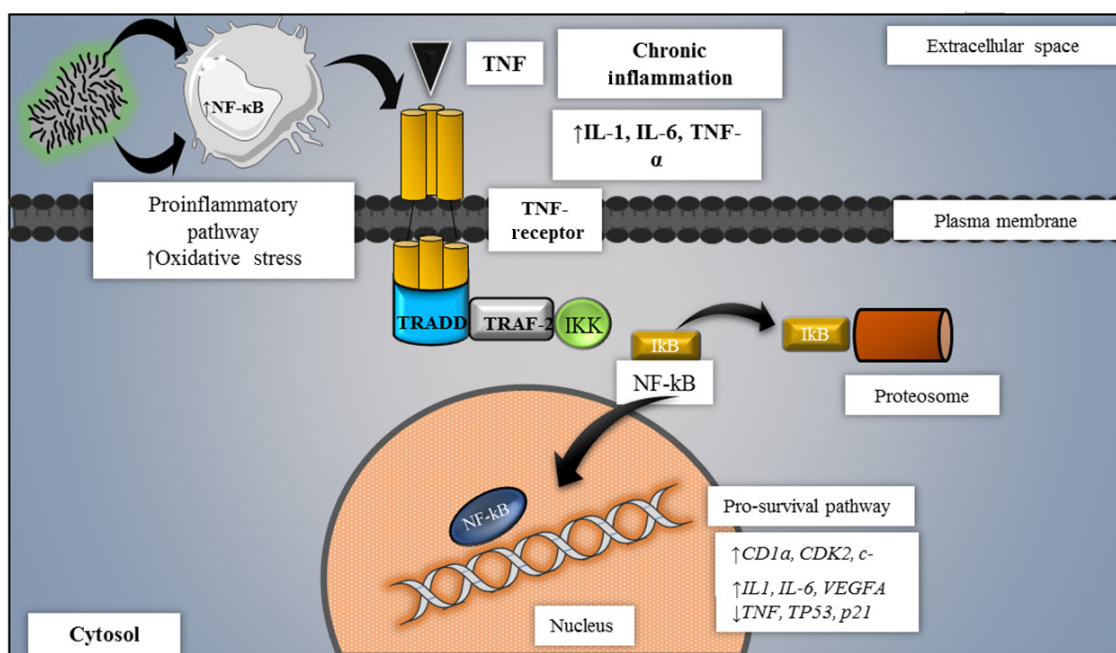
The discovery of these processes has led to research looking for novel immunologic therapies against cancer<sup>[47]</sup>. Most of this therapy approaches have been dedicated to increase active or passive immune responses. Others have tried to modify tumor cells to increase recognition by the immune system<sup>[48]</sup>. Despite of this, only few immunotherapies have achieved a response strong enough to be clinically effective<sup>[49]</sup>. For these reasons, using bacteria to potentiate response has become a promising strategy.

### INCOMING BACTERIOLOGY: ENEMIES OR ALLIES?

Chronic infection with biological agents represents a risk factor associated with cancer, with viral agents leading in this field<sup>[50]</sup>. Bacteria have been associated with cancer because of their effect on cell cycle, and their capability to evade the immune system and cause immunosuppression through chronic infections<sup>[51-53]</sup>. Bacterial infections stimulate phagocyte activity and increase oxidative stress on neighboring cells. The latter causes the release of reactive oxygen (ROS) and nitrogen (RNS) species such as peroxynitrite (ONOO<sup>-</sup>), reactive hydroxyl group (OH<sup>•</sup>) and other free radicals that damage cell membranes and DNA, affecting enzymatic activity and gene expression<sup>[54]</sup>. Among DNA alterations mediated by oxidative stress, the most common includes the formation of 8-oxoguanine and/or 8-2'-desoxyguanosine. These modified nucleotides are caused by deregulated and repetitive metabolism, and lead to mutagenesis by inhibiting or enabling expression of altered genes<sup>[55]</sup>. Chronic inflammation is considered carcinogenic<sup>[56]</sup> by activation and preservation of nuclear factor  $\kappa$ B (NF- $\kappa$ B) [Figure 2] which modulates gene expression related to cell cycle<sup>[57,58]</sup>, apoptosis<sup>[59,60]</sup>, proinflammatory cytokines, angiogenic processes<sup>[58]</sup>, invasion and metastasis<sup>[61,62]</sup>.

Infectious agents can act directly on the genome of their carrier and promote carcinogenesis by inactivation of tumor suppressor genes or mitotic stimulation. For example, chronic infections with *Helicobacter pylori* (*H. pylori*) carrying *CagA* positive virulence factor, causes mutations on p53 protein and *adenomatous polyposis coli* (APC) tumor suppressor genes; it can also induce loss of deleted in colorectal carcinoma (DCC) gene and microsatellite instability<sup>[52]</sup>. Cases of infection by *Mycobacterium tuberculosis* affect tissue structure, generating a fibrotic scar that will probably increase the risk of carcinogenesis by blocking the lymphatic flow that decreases activated leucocyte depuration and increases risk for metastatic deposits. *Mycobacterium tuberculosis* can also modulate tumor immunity together with the frequent co-infection with the human immunodeficiency virus (HIV), promoting survival of the bacillus and inhibiting INF- $\gamma$  secretion with increase in TNF- $\alpha$  secretion<sup>[63]</sup>.

Epidemiologic studies support a relationship between bacteria and cancer. *Salmonella typhi* chronic carrier state is related to gallbladder cancer<sup>[64]</sup>, *Streptococcus bovis*, found in bacteremia complications and infective endocarditis, is related to colorectal tumors<sup>[53]</sup>, *H. pylori*, known by its relationship with gastric adenocarcinoma, is also related to esophageal cancer<sup>[52]</sup>, and *Chlamydia pneumoniae* has been considered as an etiological factor in patients with lung cancer<sup>[51]</sup>.



**Figure 2.** Carcinogenesis molecular mechanisms associated to chronic inflammation. Chronic inflammatory cascade is carcinogenic by the activation of the NF- $\kappa$ B pathway. This leads to the degradation of such proteins, allowing that NF- $\kappa$ B enter the nucleus to mediate the transcription of specific cell cycle-related genes while genes responsible for apoptosis are downregulated. IKK: I kappa B kinase; NF- $\kappa$ B: nuclear factor kappa B; TNF: tumor necrosis factor; TRADD: tumor necrosis factor receptor type 1-associated DEATH domain protein; TRAF: TNF receptor-associated factor; I $\kappa$ B: I-kappa-B

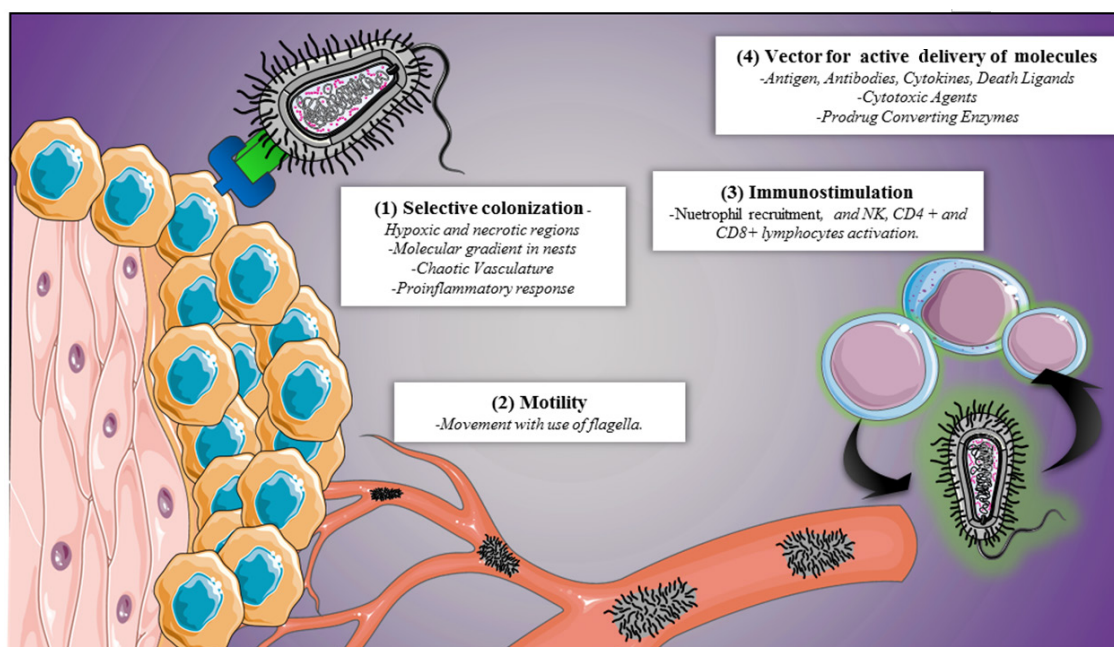
### Learning from Coley's toxin

Human carcinogenesis is not related to all bacteria<sup>[65]</sup>. Some bacterial properties work through mechanisms that stimulate the immune system and are capable to potentiate defenses against malignancy<sup>[66]</sup>. Bacteria's role against cancer was recognized in the 19th century, when an American oncologist, Dr. William Coley observed tumor regression in patients with acute bacterial infections<sup>[67]</sup>. After this observation, he decided to administer inactivated *Streptococcus pyogenes* and *Serratia marcescens*<sup>[68]</sup> - in a mixture he called Coley's toxin - to a patient with an inoperable sarcoma, inducing tumor regression and curing the patient<sup>[69-71]</sup>. Furthermore, it was used in cases with carcinomas, lymphomas, melanomas and myelomas, having significant results<sup>[72]</sup>.

The Coley's toxin mechanism of action became a key finding for immunotherapy<sup>[73]</sup>. It is composed of *gram-negative bacterial endotoxin* (*Serratia marcescens*), a lipopolysaccharide released from the bacterial cell membrane that was considered a prototype for pathogen associated molecular patterns (PAMPs). This compound induces the secretion of TNF, IL-2, INF- $\alpha$  and IL-12<sup>[74]</sup> from the immune system. Being IL-12 the most important in both innate and adaptive immunities since it stimulates T-CD4<sup>+</sup> Th1 cells development, and increases NK/NKT and TCD8<sup>+</sup> lymphocytes pathway mechanisms<sup>[75]</sup>.

These pathways require *Preexistent Immunization* in order to gain antineoplastic activity. This comes from expression of IL-12 receptors only on activated T cells<sup>[76]</sup>, explaining its major effectiveness in patients with previously sensitized T cells<sup>[76]</sup>. On the other hand, bacterial intrinsic properties could also be used against tumors, such as *Streptokinase* from *Streptococcus pyogenes* cases, an enzyme considered as one of the active agents in Coley's toxin. This enzyme has anti-angiogenic effects, suppressing new vessel formation and decreasing tumor growth and invasion<sup>[77]</sup>. Despite the fact that some clinical trials have shown effectiveness with this therapy, others have not shown any success, presenting multiple reasons for treatment failures<sup>[78]</sup>. High doses of IL-12 used as support treatment with other cytokines have produced an immunologic response with high toxicity and its employment has been cancelled<sup>[74]</sup>.





**Figure 3.** Bacterial therapeutic mechanisms. Multiple bacterial features that can be used to make novel therapies against cancer. NK: natural killer

## BACTERIA SUPPORTING THE FIGHT AGAINST CANCER: A CROSSROAD FOR GENETICS, IMMUNOLOGY AND MICROBIOLOGY

### Aiming for the perfect bacterium

Limited penetration in tumor tissue is considered a challenge for conventional therapy. This happens to chemotherapy and other specific biological therapies. They all depend on passive transport of the molecules into the tumor, limiting their efficacy and increasing their risk for toxicity<sup>[8]</sup>. On the other hand, bacterial therapy works through mechanisms against cancer that cannot be achieved with standard conventional methods, becoming a great prospect<sup>[79]</sup>.

The main issue with therapeutic uses of microorganisms against cancer in the 19th century was the adverse effects associated to immunity, such as fever, septic shock, and death<sup>[80,81]</sup>. Development in genetic engineering has led to use genetically modified bacteria- decreasing their pathogenicity- as cancer therapy<sup>[82]</sup>. Their accessible genome manipulation make bacteria the best candidates among other microorganisms<sup>[83]</sup>. Giving them the ability to enter cancerous tissue<sup>[82,84]</sup>, selecting tumor cells following specific chemical signals in their microenvironment<sup>[85,86]</sup> and acting as vectors for molecule transportation<sup>[87,88]</sup> assuming the fact they can be controlled from outside [Figure 3]<sup>[89-92]</sup>.

The “artificial medical bacteria” also have a role in the diagnostic process (detecting molecules or tumor markers related to certain diseases), therapeutic decision making (detection of chemical stimuli and production of therapeutic agents) and most importantly, can be controlled<sup>[93]</sup>. Synthetic biology has been used to design and build biologic machineries based on vehicles. Bacteria compounds integrated on genes, proteins and molecules coming from multiple origins can affect their security and therapeutic effect<sup>[94]</sup>. Systemic administration of these compounds would be better. Less concentration would be needed and multiple agents could be made without requiring neither formulation nor purification processes to amplify their effect<sup>[93]</sup>.

### Selective colonization in cancerous tissue

Blood supply in cancerous tissue is insufficient, which results in acidity, deprivation of nutrients and presence of hypoxic areas<sup>[95]</sup>. Hypoxia is more associated with expression of malignant phenotypes

characterized by genomic instability, angiogenesis and metastatic qualities<sup>[96]</sup>; leading to new approaches against this feature<sup>[97-100]</sup>. Strict anaerobic bacteria with spore germination qualities, such as *Clostridium*, cannot proliferate in highly oxygenated environments, restricting their colonization to hypoxic and necrotic regions of the tumor<sup>[101]</sup>. This is affected by tumor morphology, with central necrotic areas and well perfused cells in the periphery, allowing anaerobic bacteria to proliferate in the center. Following this, the immune system gets activated and makes a peripheral ring of immune cells prepared to eradicate the tumor completely<sup>[102]</sup>.

Facultative anaerobic bacteria such as *Salmonella* act differently. They are capable to identify and penetrate into tumors by detecting chemotactic factors including molecular gradients of serine, aspartate, and ribose<sup>[86,103]</sup>. Necrotic cancer cells release these compounds after being exposed to hypoxia for prolonged periods of time<sup>[103]</sup>. A strain of obligate anaerobic *Salmonella* has been associated with antitumor features by replacing the *asd* gene with recombinant technology. Making the gene expressed only with hypoxia-inducible promoters<sup>[104]</sup> to maximize selective colonization. Selective colonization consists of the bacteria's ability to be confined to the tumor chaotic vasculature<sup>[105]</sup>. An increase in cytokines production such as TNF- $\alpha$  is observed in response to primary colonization of bacteria that leads to a secondary colonization<sup>[106]</sup>. In addition to this, auxotrophic microorganisms-capable to grow in environments with nutrients produced only in tumor nests- have been synthesized with mutations generated from null alleles lacking biological capability<sup>[107,108]</sup>.

Recently, bacterial motility has shown to be critical in tumor colonization. Many bacteria have flagella that work with consumption of energy<sup>[109]</sup>. Bacteria use this feature to migrate and stay for longer periods of time on places distal to tumor vasculature, in contrast to passive transport with chemotherapy<sup>[83]</sup>. In addition to this, differences between diffusion and pressure gradients limit movement of molecules by passive transport and most of this happens on poorly perfused tumor areas<sup>[105]</sup>.

### Immunostimulation in tumor microenvironment

There is no bacterium capable of completely inhibiting tumor growth just through colonization<sup>[110]</sup>. However, it represents an important prospect for cancer treatment as an immunostimulator or as a vector for therapeutic components that can be released inside a tumor<sup>[111,112]</sup> [Table 1].

The main theory for this approach comes from the bacterial intrinsic ability to immunostimulate after colonizing tumor tissue. They can proliferate inside the tumour where an increased activity of the immune system has been observed. Neutrophils, T CD8<sup>+</sup> and CD4<sup>+</sup> cells recruitment, cytokine and chemokine release, potentiate immune response with no effect on the surrounding healthy tissue<sup>[130]</sup>. This approach has shown better results than conventional therapy since it can affect healthy and cancerous tissue altogether.

Bacteria have one of the largest genomes that exist. They can express multiple therapeutic transgenes and increase immune activity with cytokines and tumor antigens presentation<sup>[131]</sup>. They can transfer those genes to eukaryotic cells and get expressed or repressed<sup>[132,133]</sup>. Systemic administration of cytokines such as IL-2, IL-8, and CCL21A1 may show certain limitations related to their short half-life and adverse effects<sup>[134]</sup>. Their manufacture is highly expensive and they lack tumor orientation, which may cause severe systemic inflammatory reactions restricting their clinical use<sup>[134]</sup>. In contrast, gene modified bacteria are manufactured with low expenses, can be directed to specific tumor tissue, and may be easily eradicated with antibiotics<sup>[114,135]</sup>. Bacteria *in situ* cytokine production may benefit those with difficult DNA recombination methods and/or protein instability in production and purification. To achieve oncolytic activity genes are introduced to increase cytokine expression and promote tumor regression<sup>[113]</sup>.

Antitumor activity can be achieved without significant toxicity and related to inflammatory cell infiltration such as granulocytes, T lymphocytes and NK cells. Induction of intratumor production of



**Table 1. Pre-clinical studies for evaluation of molecular antitumor effects made by genetic engineering bacteria**

Bacterium	Molecule	Most relevant results	Reference
<i>Salmonella typhimurium</i>			
VNP20009	CCL21	Increased intratumoral production INF $\gamma$ , CXCL9 and CXCL1	Loeffler <i>et al.</i> <sup>[187]</sup>
VNP20009	LIGHT (TNFSF14)	Prominent reduction in tumor growth was observed. Evidenced with an inflammatory infiltrate (B lymphocytes, CD4 <sup>+</sup> , CD8 <sup>+</sup> in models treated with this bacterium	Loeffler <i>et al.</i> <sup>[113]</sup>
VNP20009	IL-18	Inhibition of tumor growth was observed. Evidenced with a leukocytic infiltrate (especially NK cells) and increased secretion of INF- $\gamma$ , TNF- $\alpha$ , IL-1 $\beta$ and GM-CSF	Loeffler <i>et al.</i> <sup>[114]</sup>
VNP20009	FASL	Significant reduction of tumor size was observed in primary tumors and lung metastases, increasing neutrophil recruitment	Loeffler <i>et al.</i> <sup>[115]</sup>
VNP20009	TRAIL	TRAIL expression increased tumor cells apoptosis dependent on caspase 3 and 8	Ganai <i>et al.</i> <sup>[116]</sup>
<i>S. choleraesuis</i>	Endostatine	Inhibition of tumor growth was observed in 40%-70%. Evidenced with a decrease in intratumoral microvasculature, VEGF expression and increase in T CD8 <sup>+</sup> lymphocyte recruitment	Lee <i>et al.</i> <sup>[117]</sup>
<i>S. choleraesuis</i>	Thrombospondin	Selective colonization was observed in a 1000:1 to 10000:1 ratio with respect to liver and spleen. Evidenced with inhibition of tumor growth and increase in survival by angiogenic effects	Lee <i>et al.</i> <sup>[118]</sup>
<i>Nula phoP/phoQ LH430</i>	RNAi-STAT3	RNAi inhibited significantly tumor growth, the number of metastatic lesions decreased, increased survival rate in animal models	Zhang <i>et al.</i> <sup>[119]</sup>
<i>S. typhiTy21</i>	VEGFR-2	Vaccination for this molecule showed inhibition of tumor growth, decreased metastasis growth and prevented new spontaneous metastasis, increasing survival rate in models	Niethammer <i>et al.</i> <sup>[120]</sup>
<i>aroA SL7207</i>	PSA-CtxB*	This vaccine administration conjugated with <i>Salmonella</i> showed protective effects by reducing tumor size in 8-14 days since its inoculation. This mechanism depends on T CD8 <sup>+</sup> lymphocyte activity and a prototype of the <i>E. coli</i> Hemolysin secretion system	Fensterle <i>et al.</i> <sup>[121]</sup>
<i>Clostridium</i>			
<i>C. beijerinckii</i>	NR	Nitroreductase activity increased <i>in vitro</i> antitumor activity of CB in 1954, by a factor of 22	Lemmon <i>et al.</i> <sup>[122]</sup>
<i>C. beijerinckii</i>	Cytosine deaminase	Tumor cells sensitivity to 5-fluorocytosine increased by 500 times	Fox <i>et al.</i> <sup>[123]</sup>
<i>C. sporogenes</i>	IL-12	Increased selective secretion of INF- $\gamma$ with effects on tumor growth, without signs of toxicity	Zhang <i>et al.</i> <sup>[124]</sup>
<i>C. novyi-NT</i>	AC anti-HIF $\alpha$	A heterologous gene transfer was satisfactory in this bacterium. Showing increased antibody secretion (with adhesion capacity and specificity)	Groot <i>et al.</i> <sup>[125]</sup>
<i>Listeria monocytogenes</i>			
<i>Lm-LLO-E7</i>	HPV16-E7*	This therapy induced regression in 75% of tumors expressing E7 antigen. This response depends on TCD4 <sup>+</sup> and TCD8 <sup>+</sup> lymphocytes and INF $\gamma$ secretion	Gunn <i>et al.</i> <sup>[126]</sup>
<i>ADX531-164</i>	HER-2/neu (Human)*	An increase in TCD8/Tregs ratio was observed with this therapy. It also prevented more breast tumor formation and delayed more metastasis growth than other vaccines based on this bacterium	Shahabi <i>et al.</i> <sup>[127]</sup>
<i>LM-LLO-Mage-b/2nd</i>	MAGE-b*	The most effective vaccine for breast tumors, decreasing number of metastasis by 96%, correlating to a strong CD8 <sup>+</sup> lymphocytic response in spleen after restimulation with antigen use	Kim <i>et al.</i> <sup>[128]</sup>
<i>Lm-LLO-HMW-MAA-C</i>	HMW-MAA	This therapy immunization prevented tumor growth not only in models that expressed the antigen, but in melanoma, renal carcinoma and breast carcinoma. TCD4 <sup>+</sup> and TCD8 <sup>+</sup> lymphocytes were needed to achieve this	Maciag <i>et al.</i> <sup>[129]</sup>

\*Antigen expressed on tumor

cytokines<sup>[114,136,137]</sup>, including IL-18, is important to enhance cytokine production in T lymphocytes and NK cells, to increase MHC-1 expression, and to favor differentiation of Th1 CD4<sup>+</sup> cells; leading to an immune response mediated by NK cells, macrophages, and T CD8<sup>+</sup> cells<sup>[114,138]</sup>.

Bacteria induce expression of ligands in cancerous cells with antitumor activity. For example, the FAS ligand (FASL), member of TNF family, enhances chemotaxis and IL-23 production from dendritic cells with T cell proliferation<sup>[115]</sup>. TNF related to apoptosis inducing ligand (TRAIL) protein expression has been achieved in models with breast cancer<sup>[116]</sup>, gastric cancer<sup>[139]</sup> and melanoma by employment of controlled bacteria<sup>[140]</sup>.

An interesting fact about cancer prognosis is the advanced stage by the time it is diagnosed, decreasing patient survival. Therefore, bacteria have been employed to work as vaccine vectors. These vaccines would increase tumor antigen expression on cancerous cells. Among these: prostatic specific antigen (PSA) in prostate cancer<sup>[121]</sup>, C-rapidly accelerated fibrosarcoma in pulmonary adenoma<sup>[141]</sup>, and alpha-fetoprotein ( $\alpha$ -FP) for hepatocellular carcinoma<sup>[142]</sup>. They can work by inducing an adaptive immune response to protect against these tumors. APCs recognition of these antigens is followed by a specific T-CD8<sup>+</sup> cell proliferation with immunologic memory, in contrast to systemic administration of antibodies or adoptive T cell leading to loss of immunotolerance and healthy tissues affected<sup>[143]</sup>.

### Other use as therapeutic vectors

Expression of hemolytic toxins could be achieved in tumors resistant to conventional therapy. Cytolysin A (*ClyA*) with *E. coli* K-12 use<sup>[144]</sup> is an example of these. Transcription factors could be induced in cases with *S. Typhimurium* JRG5356 where genes for *HlyE* activation are expressed so pore-forming cytolysins are made by activating the FF+20 promoter<sup>[85]</sup>. On the other hand, inhibition of angiogenetic processes with TSP-1 or endostatin genes could be used to decrease capillary density and reduce expression of vascular endothelial growth factors (VEGF)<sup>[117,118]</sup>.

Lastly, protein repression could also be induced using RNA interference (RNAi). Bacteria carrying plasmids such as *pSi-Stat3* are capable of changing specific portions of DNA and increase expression of small interference RNA (siRNA)<sup>[145]</sup> or short hairpin RNA (shRNA). All of these cause degradation of specific mRNA sequences leading to a dysfunctional tumor gene expression<sup>[146,147]</sup>.

## POTENTIAL OF BACTERIA UTILIZATION IN ONCOLOGY

Research on bacteria employment against malignant tumors in human subjects is expanding in diagnostics (for their selective colonization and external control) and therapeutics (for their antitumor effect). The next sections will discuss experimental and clinical evidence supporting bacteria utilization against cancer.

### Bacteria utilization as cancer diagnostic method and to monitor therapeutic efficacy

Bacteria utilization is not limited to the therapeutic scope but also to diagnostic methods. Developments in genetic engineering have shown expression of bacterial genes that can be detected and monitored externally by fluoroscopy<sup>[148]</sup>, magnetic resonance imaging (MRI)<sup>[90]</sup> and positron emission tomography (PET)<sup>[149]</sup> scan. These genes can code for light-emitting proteins, such as luciferase and green fluorescent protein (GFP), making them observable in real time under low light image processing; and also under micro-to-micro fluorescent microscopes<sup>[150]</sup>. *E. coli* bacterium remains as a prototype carrying PLITE201 plasmid that codes for luxCDABE protein<sup>[151]</sup> giving luminescent features. This bacterium also carries the pMW211 plasmid that codes for dsred protein<sup>[152]</sup> making cancerous cells recognizable in their exact localization by turning them luminescent without any invasive approach. *Salmonella typhimurium* and *Vibrio cholerae* remain under study for their utilization in colon and breast cancer diagnosis, respectively<sup>[153]</sup>.

MRI is routinely used for tumor diagnosis and treatment evaluation. *Magneto spirillum* is a bacterium employed in this radiologic study. It consists of a microaerophilic microorganism with magnetic properties on its magnetosome which contains magnetic crystals formed mainly of magnetite (Fe<sub>3</sub>O<sub>4</sub>) covered by a lipid bilayer membrane<sup>[154]</sup>. Experiments with AMB-1 strains of this bacterium have shown positive contrast features in T1-enhanced imaging when they were cultured under iron deprived (FeCl<sub>3</sub>) conditions<sup>[90]</sup>. Contrast was intensified with expression of *MagA* gene. This gene codes for an iron transporter that gets positively regulated in presence of low iron concentrations<sup>[155]</sup>. *In vitro* experiments with colon carcinoma models in HT-29 human subjects did not show any evidence of toxicity and tumor necrosis was observed

on both histologic slides and MRI<sup>[156]</sup>. Employment of these magnetic features implies new advances with clinical use potential.

Regarding PET scans, other modified strains of *E. coli*: *E. coli* Nissle (*EcN*) 1917<sup>[152]</sup> along with pyrimidine nucleotide analogs have been considered for diagnosis of breast tumors. Increased local accumulation of radio-isotopes has shown a positive correlation with the number of bacteria containing radioactive drugs. These bacteria selectively colonized tumors, making them detectable via PET. Other bacterium employed for these studies was Salmonella VNP20009-TK. The latter has had similar results with a positive correlation between intratumor bacteria and fialuridine sequestration (FIAU), a radio-marked nucleoside analog used for tumor identification<sup>[157]</sup>.

Other bacteria diagnostic features can be used in oncology. This includes their employment as probiotics in cancer screening<sup>[158]</sup>. Use of *EcN* with modified genetic circuits enhances detection of focal metastasis in urine samples. Two principles were applied: first, bacteria produced a luminous signal that can be detected through imaging techniques; and second, LacZ enzymatic activity on a substrate composed of luciferine and galactose (LuGal) results in luciferine traveling into the circulatory system and serving as a colorimetric indicator with fluorescent or luminescent features; these features could be detected in urine samples<sup>[158]</sup>.

### Current perspective in bacteria based therapy in medicine

Recently, bacterial strains with therapeutic characteristics against cancer have been discovered. *Mycobacterium bovis* (BCG) is considered a prototype. It is an obligate anaerobic, acid-alcohol-resistant, facultative intracellular and non-motile bacterium that has been employed in the past for tuberculosis vaccine manufacturing<sup>[159]</sup>. For more than 30 years it has been utilized in bladder cancer patients as immunotherapy. A decrease in tumor recurrence has been observed with this therapy along with its well tolerated adverse effects<sup>[160]</sup>. Even though the first-choice treatment for non-muscle invasive bladder cancer (NMIBC) is still transurethral resection for bladder tumor (TURBT), a high recurrence (50%-70%) and progression rates (10%-20%) after two years have been observed with this procedure<sup>[161]</sup>. In these cases, intravesical instillation with BCG is one of the main therapeutic options, decreasing long term appearance of distant metastasis<sup>[162]</sup>; also an increase in global survival in 5 years with long term maintenance of this therapy has been observed<sup>[163]</sup>. However, its long term use may have adverse effects such as drug induced cystitis, hematuria, and systemic toxicity<sup>[164]</sup>. Despite of this, BCG is still considered the standard treatment for NMIBC after transurethral resection for bladder tumor has been performed in patients with intermediate and high risk of progression or recurrence<sup>[12]</sup>.

The mechanism behind these benefits has not been clarified, but the antitumoral effects of BCG are considered to come from the immune response<sup>[165,166]</sup>. Once urothelial cells or macrophages internalize the bacillus, they induce an immune response with secretion of TNF- $\alpha$ , IL-6, IL-10, INF- $\gamma$ , FEC-GM, CC and expression of CXC chemokine receptors<sup>[167-169]</sup>; this stimulates recruitment of neutrophils, macrophages, T-CD4<sup>+</sup> cells and increases expression of MHC-I, MHC-II and IL-2 receptors<sup>[170-172]</sup>. TRAIL is one of the main mediators in bacteria based therapy. This ligand appears to be upregulated in response to INF- $\gamma$ , causing urothelial cell death<sup>[173,174]</sup>. BCG remains as reference for novel cancer therapies in development such as vaccines, and also for nonbacterial therapies, having similar efficacy and reliability (NCT02010203). Next sections discuss the most important bacteria used for these goals, going from their experimental research to current clinical evidence [Table 2].

### Clostridium: heading to tumor specificity

Necrosis and hypoxia in tumor tissues make them resistant to conventional therapies<sup>[106]</sup>, therefore, research on *Clostridium* began because of its natural anaerobic features<sup>[101]</sup>. In regards to utilization of this

**Table 2. Clinical evidence evaluating the safety, tolerance, adverse and therapeutic effects of bacteria against cancer**

Reference	Bacterium/ compound	Metodology	Clinical phase	Results
Nemunaitis et al. <sup>[175]</sup>	Samonella TAPET-CD	Open clinical trial that included 3 patients with solid and/or metastatic tumors, 5-FU sensitive, without any response to conventional therapies. With intratumor administration of bacteria	Phase I	A favorable response was observed in 2 patients at their injection site. Bacterial CD dependent conversion of 5-FC to 5-FU. Presented adverse effects not related to therapy
Toso et al. <sup>[110]</sup>	Salmonella VNP20009	Open clinical trial that included 24 patients with metastatic melanoma and one patient with renal cell carcinoma to assess safety, tolerability and clinical response	Phase I	From the 25 patients treated with VNP20009, none experimented an objective tumor regression. Dose-limiting toxicity was associated to TNF- $\alpha$ and IL1- $\beta$ secretion, despite the majority of adverse effects showed reversibility
Schmitz-Winnenthal et al. <sup>[176]</sup>	S. typhiTy21/ Anti-VEGFR-2 (VXM01)	Ramdomized, double-blind clinical trial to assess safety, tolerability, and clinical and immunologic responses in 45 patients with locally advanced stage IV pancreatic cancer	Phase I	Treatment was well toleraed in all applied doses. No dose-limiting toxicity was found. There was an effector T lymphocyte dependent response and a decrease in tumor perfussion in patients with preexisting immunologic memory
Roberts et al. <sup>[13]</sup>	Clostridium novyi-NT	Clinical trial including 1 patient with retroperitoneal leiomyosarcoma and received intratumoral administration of spores in a metastatic lesion on shoulder	Phase I	Extensive tumor destruction was observed, compatible with necrosis. By day 4 after administration, biopsy showed absence of viable tumor cells. By day 55, presented with a pathologic fracture. Therapy improved his quality of life
Maciag et al. <sup>[185]</sup>	Lm-LLO-E7	Non ramdomized clinical trial to assess safety of the therapy in 15 patients with advanced stage cevrical cancer, refractory or recurrent	Phase I	LI patients presented adverse effects, including severe (grade 3) in 6 patients (40%). At the end of the study, 2 patients died, 5 developed disease progression, 7 showed stable disease and partial tumor response was observed in one patient
Le et al. <sup>[177]</sup>	ANZ-100/ CRS-207	Open multicentric clinical trial to assess safety and induction of immune system in two groups: 1) ANZ-100 = 9 patients with colorectal cancer (6), pancreatic cancer (2), and melanoma (1). 2) CRS-207 = 17 patients with pancreatic cancer (7), mesothelioma (5), lung cancer (3) and ovarian cancer (2)	Phase I	In both groups, therapy was well tolerated with self-limited adverse effects. In group 1, no dose-limiting toxicity was found with ANZ-100 administration, and was related to NK cell (CD38) activation and increase in MCP-1, MIP-1 $\beta$ and INF $\gamma$ secretion. In group 2, CRS-207 was well tolerated. The majority of observed adverse effects were grade 2. Like IN group 1, an increase in proinflammatory cytokines was observed. CRS-207 induced a specific response dependent on T cells towards mesotheline and listeriolysin-O
Le et al. <sup>[178]</sup>	CRS-207	Ramdomized multicentric clinical trial to assess safety and clinical response in 90 patients with stage IV pancreatic adenocarcinoma and administration of Cy/GVAX+CRS-207 (A) in contrast to Cy/GVAX only (B)	Phase II	The mean follow-up was 3.4 months. The global survival rate was higher in patients treated with Cy/GVAX+CRS-207 ( $n = 61$ ) than those treated with GVAX/Cy only ( $n = 29$ ) (HR: 0.59; IC 95%: 0.36-0.97, $P = 0.02$ ). Nevertheless, increase in T CD8+ lymphocytic specific response to mesothelin was associated to a higher global survival rate, independent on treatment group

gram-positive, obligate anaerobe, spore forming bacteria to developa therapy against cancer in tumors with necrosis associated to bad prognosis<sup>[179-181]</sup>, *Clostridium novyi* is one of the most studied. After a deletion of its  $\alpha$ -toxine gene, *Clostridium novyi-NT* becomes capable to colonize selectively; in addition to diminished adverse effects because of its decreased exotoxin production<sup>[82]</sup>. It was used in experimental models with colorectal cancer, renal carcinoma<sup>[99]</sup>, gliomas<sup>[182]</sup>, and sarcomas<sup>[13]</sup> to observe its selective colonization, immune cell infiltration, and cytokine release leading to tumor tissue necrosis<sup>[125]</sup>. Phase I clinical trials were initiated on one patient with retroperitoneal leiomyosarcoma presenting multiple metastasis and refractoriness to conventional therapy. After intratumoral application-preferred over systemic administration to decrease adverse effects - the tumor located in this patient right shoulder regressed with an extensive necrotic area; and medically managed adverse effects<sup>[13,183]</sup>. Further clinical trials are currently in patient recruitment (NCT01924689).

Genetic therapy was employed to increase the oncolytic effects of this strain and presented promising results<sup>[122,184,185]</sup>. *Clostridium sporogenes*<sup>[13]</sup> was utilized because of its tumor directed features. Genes derived from *E. coli* serve for nitroreductase (NR) and cytosine deaminase (CD) codification<sup>[185,186]</sup>. These enzymes metabolize cytotoxic drugs inside tumors, having *in vivo* antitumoral effects.

*Clostridium* spores have low immunogenicity and can colonize multiple organs after systemic administration<sup>[187]</sup>. However, once they germinate, they induce an inflammatory response with infiltration of immune cells with oncolytic effects<sup>[99]</sup>. These strains have been employed in genetic engineering as vectors for cytokines secretion such as TNF- $\alpha$ <sup>[188]</sup>, IL-12<sup>[124]</sup> and IL-2<sup>[189]</sup>, achieving high concentrations inside tumor tissue without systemic toxicity. Also *C. novyi-NT* and *C. sporogenes* increase secretion of specific antibodies against hypoxia inducing factor-1 (HIF-1), main component observed in hypoxia response regulation inside tumors<sup>[125]</sup>.

### **Salmonella: multi-use bacterium**

*Salmonella enterica serovar typhimurium* (*S. typhimurium*) is one of the most studied bacterium for its adaptative qualities leading to new strains with bacterial engineering showing antitumor activity<sup>[119]</sup>. In the beginnings of the 21st century, phase I clinical trials were conducted to show their efficacy with gene modification via deletion in *msbB* and *purI* genes. The *msbB* gene is required for lipid A synthesis and its deletion was made to reduce TNF- $\alpha$  related toxicity, preventing septic shock<sup>[190]</sup>. On the other hand, by deletion of *purI* gene, the bacterium became able to colonize tumors more selectively. All of this made the strains depend on purine external sources for survival restricting their growth to areas with substantial cell renewal<sup>[191]</sup>. Tumor tissues with their purine rich activities would be perfect regions for their selective colonization<sup>[191]</sup>. *Salmonella typhimurium* VNP20009 is one of the main strains in experimental studies originated from this theory.

This study results showed the maximum tolerated dose of this bacteria, its toxicity limit dose, and adverse effects by increasing production of proinflammatory cytokines. The observed adverse effects included thrombocytopenia, anemia, persistent bacteremia, hyperbilirubinemia, nausea, vomit, elevated alkaline phosphatase and hypophosphatemia. However, tumor colonization was detected only in 3 patients, and no tumor regression was observed<sup>[110]</sup>. Despite the fact that the study did not show promising results, it was the start line for prospect studies to find doses that could be adjusted for efficiency and tumor localization and for other therapeutic features.

In order to increase this bacterium therapeutic effect, a study was initiated to use them as vectors in tumor gene therapy<sup>[192]</sup>. A pilot study was performed with an attenuated and gene modified *Salmonella* strain with expression of *E. coli* CD, called suicide prodrug-activating enzyme<sup>[193]</sup>. These genes were integrated in VNP20009 chromosome through Donnenberg and Karper method resulting in TAPET-CD strain<sup>[175]</sup>. The mechanism of action of this enzyme consists in conversion of 5-fluorocytosine (5-FC, antifungal agent with limited cytotoxicity) to 5-fluorouracil (5-FU, cytotoxic antimetabolite capable of producing cellular apoptosis)<sup>[193]</sup>. No promising results were obtained 2 out of 3 treated patients did not present any tumor regression but an improvement of their disease was observed<sup>[175]</sup>.

Another example from *S. typhimurium* is strains would be A1-R, which currently is on preclinical studies against different cancerous tissues such as prostate<sup>[14]</sup>, pancreatic<sup>[194,195]</sup>, glioma<sup>[196]</sup>, colorectal<sup>[197]</sup>, and ovary<sup>[198]</sup>. *S. typhimurium* A1-R colonization seems to be more selective and effective than other strains and less toxic than VNP20009 strain. It also has safer systemic administration than *C. novyiNT*<sup>[199]</sup>. Therefore, clinical trials for this strain are coming. A1-R is a gene modified strain, auxotrophic for leucine and arginine by nitroguanidine mutagenesis (NTG)-preventing healthy tissue invasion. It was utilized in animal models with prostatic cancerous cells PC3 and also in humans showing tumor regression, inhibition and prevention of cancer<sup>[14]</sup>.

Bacteria therapeutic use has been confirmed in cancer models with stem cell characteristics. This represents the only method capable to reduce *in vivo* tumor sizes in relation to chemotherapy (5-FU in monotherapy, cisplatin and gemcitabine). The efficacy increased when combined with 5-FU<sup>[195]</sup>. *S. typhimurium* A1-R



could induce cell entrance from G0/G1 to S/G2/M and reduced significant portion of cells in quiescent state, making them sensible to chemotherapy<sup>[200,201]</sup>.

Other approach in gene modification of *Salmonella* was the study of strains to decrease or inactivate gene expression. This inactivation could be achieved with utilization of iRNA<sup>[119,202]</sup>. *S. typhimurium* LH340 strain was made with deletion on popP/phoQ operon required for its virulence resulting in its attenuation<sup>[203,204]</sup>. The signal transducer and activator of transcription protein-3 (STAT3) is the goal with these therapies. Confirmation of its role in immune system depression<sup>[205]</sup> and expression of target genes such as *VEGF*, *Cyclin D1*, *Cyclin D2*, *c-Myc*, *p53*, *Bcl-XL*, *Bcl-2*, *Mcl-1* and *Survivin* have been observed<sup>[206]</sup>. A relationship between inhibition of these genes expression and suppression of tumor growth was found<sup>[207]</sup>.

Strains with expression of iRNA for Stat3 suppressed tumor growth significantly, reduced metastasis and increased survival in experimental models with prostate<sup>[119]</sup> and hepatocellular carcinoma<sup>[202]</sup>. These tumors are usually highly vascularized and angiogenesis inhibition through plasmids required for endostatin codification (SL/pEndostatin) may increase efficacy to the novel therapy<sup>[133]</sup>. By introducing Stat3, (SL/pEndo-Si-Stat3) more antitumor effects were observed. These effects were related to angiogenesis inhibition and increase in TCD8<sup>+</sup> lymphocyte proliferation, NK cytotoxicity and T-regs proliferation. The later came from inhibition probably by stimulation of INF- $\gamma$  and TNF- $\alpha$  secretion with significant decrease in TGF- $\beta$  concentrations<sup>[202]</sup>.

In clinical settings *Salmonella typhi* Ty21a is one of the new therapy prospects. It was studied to find a vaccine to prevent typhoid fever<sup>[173]</sup>. The bacterium was introduced to cancer therapy strategies with the VXM01 vaccine. This is an oral vaccine made of live attenuated strains of *S. typhi ty21a* capable to induce a T cell response; it also contains a plasmid that codes for VEGFR2 and plays an important role in tumor angiogenesis<sup>[208]</sup>. It can also induce both humoral and cellular responses<sup>[176]</sup> observed in experimental models with melanoma, colorectal cancer and lung cancer. Suppression of primary tumor growth and metastatic lesions mediated by T-CD8<sup>+</sup> cells activity was observed in these models<sup>[117]</sup>. In clinical settings, it was recently evaluated on 45 patients with stage IV pancreatic cancer and it showed the importance of preexisting immunologic memory for effector T cells to achieve an antiangiogenic effect<sup>[176]</sup>.

Clinical trials have shown that *Salmonella* still lacks therapeutic efficacy and selective tumor colonization but could be considered as a multi-use bacterium for its diverse features. It can work as a vector, and a better inducer of antitumoral response because of its efficient type III secretion system<sup>[209]</sup>. Prospecting studies should be focused on this objective with specific molecules for each cancer type, getting major effectiveness.

### **Listeria monocytogenes: the perfect antigenic vector**

*Listeria monocytogenes* (Lm) is a gram positive, facultative intracellular bacterium<sup>[131]</sup>. Over the last few decades multiple studies have shown that it can work cancer therapeutic agent with multiple effect or mechanisms<sup>[210]</sup>. It can be used against primary and metastatic tumors in an immune-privileged microenvironment. The latter helps its selective colonization and favors their elimination with ROS production<sup>[211]</sup>. In addition to this, Lm decreases T-regs cells and immunomodulation molecules such as TGF $\beta$  and IL10 in tumor microenvironment<sup>[212]</sup>. However, main feature of Lm consists on selectively infecting APCs favoring self-antigen and heterolog antigens processing and presentation<sup>[213]</sup>. These characteristics make Lm to be considered as a valuable immunostimulant agent.

Intracellular life cycle of Lm favors its use as an immunotherapeutic agent. Once infection has occurred, Lm strongly activates innate immunity with the release of proinflammatory cytokines such as IL-2, IL-6, IL-



12, and TNF- $\alpha$ ; and increases expression of co-stimulant molecules in APCs surfaces leading to maturation and activation of high affinity T cells<sup>[214]</sup>. After internalization by phagocytes, Lm is capable to escape from phagolysosomes using its virulence factor called listeriolysin O (LLO)<sup>[215]</sup>. It works as a hemolysin that perforates the phagosomal membranes of the bacterium could escape into the cytosol. Once in the cytosol, they can replicate and secrete its antigens<sup>[216]</sup>. This mechanism makes antigen processing and presentation to be via both class I and II MHC molecules<sup>[217]</sup> inducing potent specific responses from both T-CD4<sup>+</sup> and T-CD8<sup>+</sup> cells<sup>[218]</sup>.

These features of Lm have been studied with genetic engineering looking for recombinant strains capable to secrete tumor antigens<sup>[219]</sup>. They could be employed as live vectors through vaccines to potentiate cellular response and overcome immunotolerance towards certain types of cancers<sup>[131]</sup>. This could be achieved with insertion of plasmids encoding the tumor antigen<sup>[126]</sup>, or by their integration in the bacterial chromosome<sup>[220]</sup>. These antigens would be expressed as chimeric proteins along with Lm virulence factors<sup>[221]</sup> such as LLO or actin assembly inducing-protein (ActA)<sup>[222]</sup>. Lm uses ActA for motility and intercellular propagation and its immunogenic features increase the immune response towards tumor antigens with poor immunogenicity<sup>[223]</sup>. These experimental studies were oriented to measure efficacy in recently developed vaccines. Among these vaccines, Lm-LLO-E7 was studied for cervical cancer models<sup>[224]</sup>, Lm-her2-neu for metastatic breast cancer<sup>[211]</sup>, Lm-LLO-PSA for prostate cancer<sup>[225]</sup>, Lm-MPFG for hepatocellular carcinoma<sup>[226]</sup> and LM-Kras for pancreatic ductal adenocarcinoma<sup>[227]</sup> and others; all of them reporting suppression in growth and even regression<sup>[228]</sup>.

Lm utilization as live vector could induce systemic disease in immunocompromised individuals limiting its use for human vaccines<sup>[221]</sup>. Different strains have been cultured with specific gene deletions to guarantee their safety<sup>[229-232]</sup>. Among these new strains, only XFL-7 and Lm $\Delta$ actA/ $\Delta$ plcB have been used in clinical trials. The XFL-7 strain was created with chromosomal deletion in its *Prfa* gene. This gene codes for an activating transcription factor needed for bacterial virulence factor expression. In order to increase its expression, a complementation of a multicopy plasmid with a heterolog gene was introduced<sup>[231]</sup>. The Lm $\Delta$ actA/ $\Delta$ plcB strain was made with a deletion of its virulence genes *ActA* and *inlB*-used for surface proteins codification that favors cell invasion-to prevent capture from non-phagocytic cells and reduce hepatic damage<sup>[232]</sup>.

The first clinical trial to assess safety with Lm administration in cancer patients utilized attenuated strains as vaccines, specially Lm-LLO-E7<sup>[126]</sup>. The latter was made from XFL-7 strains to express E7 oncoantigen from human papilloma virus serotype 16 (HPV16). This vaccine was also designed to treat cervical cancer<sup>[15]</sup>, and other tumors induced by HPV16 such as oropharyngeal cancer<sup>[224]</sup>. In this open, nonrandomized, uncontrolled study, Maciag et al.<sup>[185]</sup> assessed safety and viability of Lm-LLO-E7 via intravenous administration with intervals of 21 days. Doses of  $1 \times 10^9$ ,  $3.3 \times 10^9$  or  $1 \times 10^{10}$  Colony-Forming Units (CFU) were administered to 15 patients with invasive cervical carcinoma in advanced stages and refractory to conventional therapy. Despite the fact that all the patients presented systemic adverse effects in the study (fever, vomit, headache, muscle aches, tachycardia, hypotension, anemia) most of them were alleviated during the first 12-h post dose, responding to symptomatic treatment whenever necessary<sup>[15]</sup>.

Safety of Lm-LLO-E7 administration in humans is still under study with insertion of plasmid encoded resistance to chloramphenicol required for bacterial survival *in vivo*<sup>[233]</sup>. Phase II clinical trials to assess efficacy and safety in patients with oropharyngeal cancer (NCT01598792) were suspended after a patient developed systemic listeriosis following vaccination<sup>[234]</sup>. This shows the need for a new attenuation, especially for their use on immunocompromised patients.

The *Listeria* strain LmΔactA/ΔplcB with application of two vaccines called ANX-100 and CRS-207 has been studied<sup>[177]</sup>. ANX-100 consisted of a vector without antigen that was administered to 9 patients with colon cancer and hepatic metastasis from colon cancer and demonstrated its safety and tolerability to a dose of  $1 \times 10^8$  CFU. It induced an antitumor inflammatory response. CRS-207 consisted of a modified strain to express mesothelin, which is an overexpressed antigen that is frequently found in multiple solid tumors, including mesothelioma, pancreatic adenocarcinoma, non-small cell lung carcinomas and ovarian cancer<sup>[235-237]</sup>. Phase I clinical trials in patients with these characteristics showed their efficacy and tolerability to a dose of  $1 \times 10^9$  CFU<sup>[177]</sup>. Seven patients were treated during these trials. Six patients had increased survival in 15 months, showing treatment efficacy. But 3 patients with high survival rates had been treated with GVAX previously. This vaccine was designed to increase GM-CSF expression for its ability to induce cellular immunity against tumor antigens. Phase II clinical trials were performed posteriorly<sup>[178]</sup>. They evaluated the safety and efficacy of the combined treatment with GVAX and cyclophosphamide (GVAX/Cy) with CRS-207 in contrast to exclusive administration of GVAX/Cy in patients with pancreatic cancer. Reports showed a global survival rate of 6.1 months in patients treated with GVAX/Cy+CRS-207, more than patients treated with GVAX/Cy exclusively (HR: 0.59; 95% CI: 0.36-0.97,  $P = 0.02$ ).

Based on these results, current research is focusing on efficacy evaluation of vaccines based on Lm attenuated strains along with other immunological or conventional therapies. Among these, combining LM-LLO-E7 with anti-PD1 antibodies<sup>[238]</sup>, or using the strain as adjuvant therapy after chemotherapy against cervical cancer (NCT02853604). There was also found that combination of CRS-207 strain with an IDO1 inhibitor increases immunotherapeutic effects in ovarian and peritoneal cancer treatment (NCT02575807); which could be used as adjuvant therapy after chemotherapy for malignant pleural mesothelioma (NCT01675765).

### Other bacteria under study

Research for bacteria use in cancer treatment is not limited to the cited genres. *Lactococcus lactis* NK34, generally used as a probiotic, showed significant antitumor activity against lung, colorectal, gastric and breast cancers on *in vitro* models<sup>[239]</sup>. These effects appear to be mediated by an increase in tumor expression of p21 and p53 leading to apoptosis<sup>[240,241]</sup>. Intratumor *Streptococcus pyogenes* was employed in pancreatic cancer models and complete tumor regression was observed and associated to cytokine release and immune cell infiltration<sup>[242]</sup>. Recently, *Bacillus subtilis* and *Bifidobacterium infantis* are being included in preclinical studies to find more evidence supporting bacteria as life-saving prospects<sup>[243-245]</sup>.

### CONCLUSION

The main advantage of bacterial therapy is its selective colonization in tumor tissue decreasing its toxicity. This direct oncolytic effect resides on proliferation and immunostimulation that take place in cancerous tissues. Despite lacking significant effects in initial models and multiple adverse effects, it has overcome these barriers. Development in genetic engineering has led to better therapeutic effects and the reinforcement of therapies with molecules such as cytokines, tumor antigens, drug metabolizing enzymes, death receptors, and even RNA interference. Promising results have been observed with these therapies during clinical trials. Research is beginning to determine their use as main, or supportive therapy in contrast to conventional therapy against cancer. Their toxicity, antitumor effect, and their long half-life represent critical variables to consider in future research protocols and clinical trials. However, microorganisms versatility remains a feature that may show encouraging results in the future [Table 3] with significant improvements in cancer diagnosis and treatment.

**Table 3. Current clinical trials to evaluate bacteria use in cancer treatment**

Bacterium	Indication	Clinical phase	NCT identification
<i>Clostridium novyi-NT</i>	Patients with malignant solid tumors refractory to treatment	Phase I	NCT01924689
<i>Lm-LLO-E7</i>	Patients with grade 2 cervical intraepithelial neoplasia	Phase II	NCT01116245
	Patients with non-small cell lung carcinoma, HPV positive	Phase II	NCT02531854
	Patients with anorectal carcinoma	Phase II	NCT02399813
	Patients with HPV positive oropharyngeal cancer	Phase II	NCT02002182
	Patients with high risk of locally advanced cervical cancer	Phase III	NCT02853604
<i>CRS-207</i>	Adults with previously treated pancreatic adenocarcinoma	Phase II	NCT02004262
	Patients with malignant pleural mesothelioma	Phase I	NCT01675765
	Patients with ovarian or peritoneal cancer	Phase I/II	NCT02575807
<i>ADU-623</i>	Patients with astrocytic tumors	Phase I	NCT01967758
<i>ADX531-142</i>	Patients with prostate cancer	Phase I/II	NCT02325557

HPV: human papilloma virus

## DECLARATIONS

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Case Report

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# Pancreatic neuroendocrine tumor liver metastasis in a patient with previously diagnosed pancreatic adenocarcinoma: an unexpected diagnosis

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## Abstract

Locally advanced pancreatic carcinoma has an usually poor prognosis despite multimodal approaches and sequential chemotherapy. The authors present a case of a long-term survivor with stage III pancreatic adenocarcinoma achieving partial response after a multimodal approach including local and systemic treatments. However, three years after diagnosis and amidst several episodes of cholangitis, hepatic metastasis were suspected. Despite pancreatic adenocarcinoma being the obvious culprit for metastatization, a hepatic biopsy was considered at that time given a stable primary disease and presenting three years since the initial diagnosis. At this point, a biopsy could have specific diagnostic, prognostic and therapeutic implications and after it was performed, an unexpected diagnosis of pancreatic neuroendocrine tumor was made. Therefore, we urge clinicians to consider hepatic biopsy in similar cases - generally when it may change prognosis and treatment strategies - and perform histological confirmation of metastatic disease whenever feasible, even if the answer may seem obvious at first impression.

**Keywords:** Neoplasm metastasis, pancreatic cancer, neuroendocrine tumors, biomarkers, survivorship, chemoradiotherapy

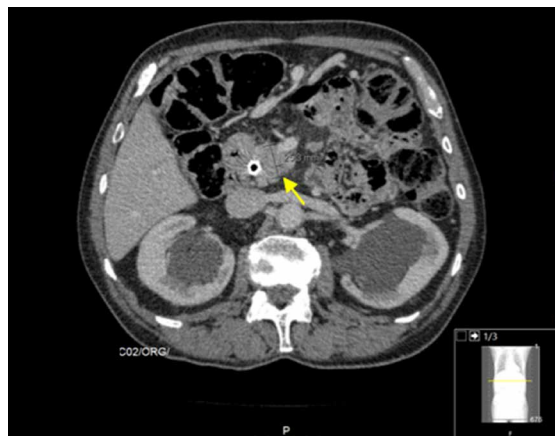
## INTRODUCTION

Pancreatic cancer is becoming increasingly relevant, since it is one of the most lethal cancers, estimated to surpass breast cancer deaths by 2017<sup>[1]</sup>, which are considerable, and is being contemplated as a public health issue<sup>[1,2]</sup>. At the time of diagnosis, most patients have either locally advanced or metastatic disease, thus not



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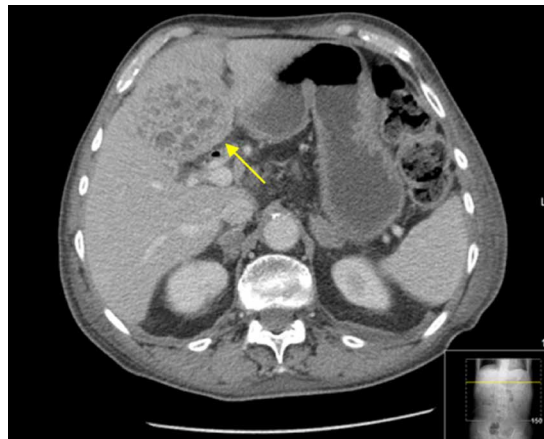
**Figure 1.** Computed tomography scan at diagnosis (2013). Shown is the pancreatic mass (25.3 mm, yellow arrow), a histologically proven pancreatic adenocarcinoma. A metal stent was placed to relieve symptoms such as jaundice

meeting surgical criteria - the only treatment offering the potential for a cure<sup>[3,4]</sup>. Alternatively, the patients may be candidates for systemic palliative treatment, if clinically compatible, or best supportive care. In general, the estimated 5-year survival is 5%<sup>[4]</sup>. More than 85% of all solid pancreatic neoplasms are ductal adenocarcinoma<sup>[3]</sup>. On the other hand, pancreatic neuroendocrine tumors are considered rare<sup>[5]</sup>, although with a reported increase in incidence in the last decades<sup>[6]</sup>. Although little is known about the epidemiology of metastization in this disease, the liver is the preferential site metastatic disease<sup>[7]</sup> and treatment algorithms are available<sup>[8]</sup>. The authors present a case of a long-term survivor with an unresectable pancreatic adenocarcinoma, stage III with a later diagnosis of liver metastasis of pancreatic neuroendocrine origin.

## CASE REPORT

A 69-year-old male patient, who was initially evaluated at another institution, presented with new-onset cholestatic jaundice and involuntary weight loss. He had a history of benign prostatic hyperplasia, osteoporosis, chronic gastritis and hiatus hernia, but he was not taking any prescription medications. The patient was diagnosed in July 2013 with a 25-mm mass at the head of the pancreas, with superior mesenteric artery invasion and regional node metastization by computed tomography (CT) scan [Figure 1]. He underwent a biopsy and subsequently was diagnosed with unresectable pancreatic ductal adenocarcinoma, thus considered clinically stage III (cT4N + M0), according to the 7th edition of the AJCC cancer staging manual criteria.

A metallic biliary stent was placed to reduce jaundice [Figure 1]. The patient was referred to best supportive care by the attending physician at that time, and he approached our institution for a second opinion. At this point, the patient was relieved of cholestatic symptoms and was considered Eastern Cooperative Oncology Group (ECOG) score 0; therefore, systemic treatment was proposed. The patient accepted the proposed treatment and began palliative single-agent weekly Gemcitabine 1000 mg/m<sup>2</sup>, for 6 months, after which a CT scan was performed in December 2013 showing stable disease (SD). The patient's carbohydrate antigen (CA) of 19.9 was not considered indicative of disease as it was consistently within normal range values. The multidisciplinary group decision was to further treat with chemoradiotherapy (CRT). The patient started continuous infusional 5-fluorouracil and radiation therapy (RT) was performed concomitantly (50.4 Gray; 28 fractions, 5 x/week, according to Intensity Modulated RT), for 5-and-a-half weeks. The CT scan after CRT in June 2014 showed SD. After this treatment, the patient re-started Gemcitabine until August 2014, after which he began regular clinical and imagiological follow-up. The patient was, shortly after, admitted for cholangitis but fully recovered after antibiotics and fluid resuscitation. The hepato-biliary group re-appreciated the case in August 2015, but still considered it to be unresectable and the patient remained in follow-up. In February



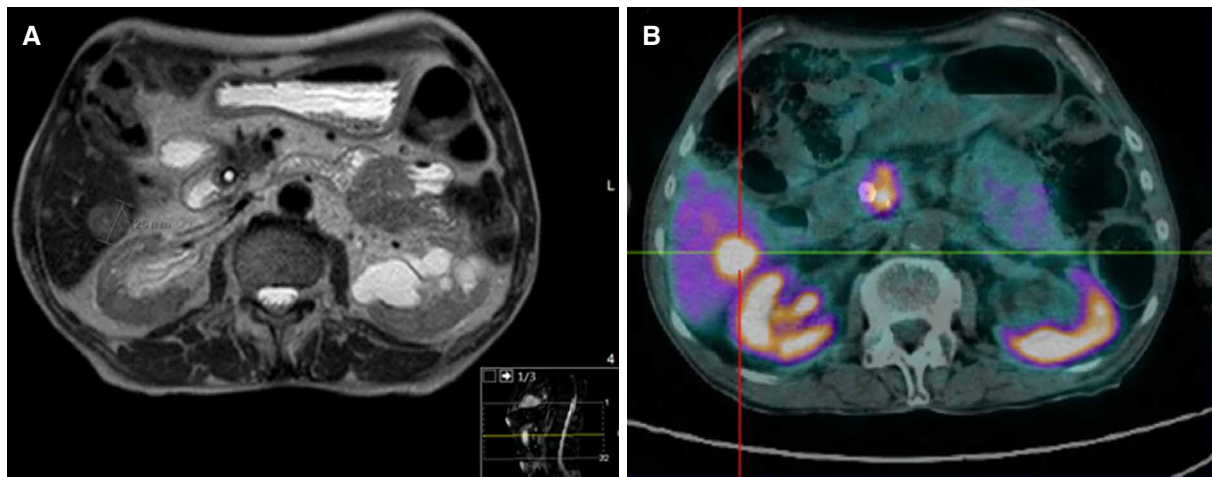
**Figure 2.** Computed tomography scan performed when the patient was admitted for cholangitis. A liver abscess (yellow arrow) was diagnosed. It was later percutaneously drained and *E. coli* was identified

2016 the patient complained of right hypochondrial pain, nausea and vomiting, generalized pruritus and fever. Abdominal ultrasound revealed evident *de novo* hepatic lesions and elevation of acute inflammatory parameters. The patient was admitted for cholangitis and started on antibiotics and supportive therapy. A CT scan identified an intrahepatic abscess which was drained percutaneously [Figure 2]. A Gram-negative bacterium, *Escherichia coli*, was identified in blood cultures as well as in the drained pus and the patient was discharged after full recovery.

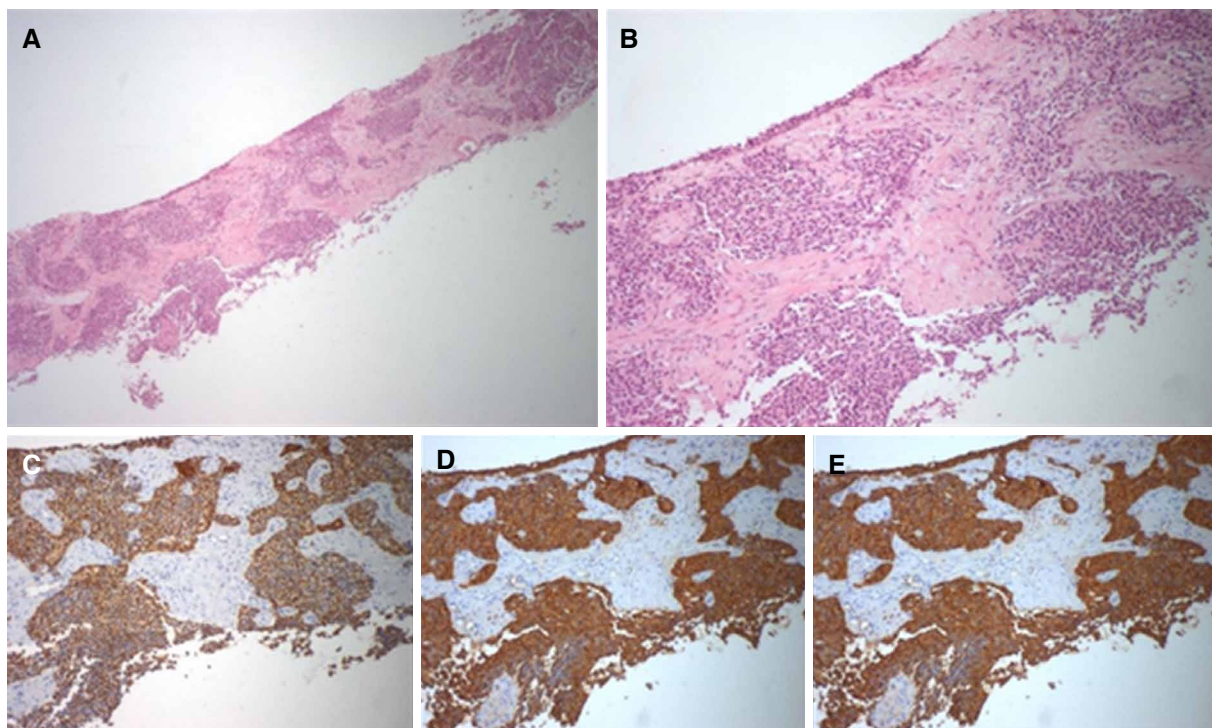
A CT scan was performed in May 2016, and identified several *de novo* small nodular masses (the biggest at 18 mm) on hepatic segment VI, highly suggestive of metastazition. The known cephalopancreatic lesion of 15 mm [a Sustained Partial Response according to Response Evaluation Criteria In Solid Tumors (RECIST) criteria, three years after the initial pancreatic adenocarcinoma diagnosis]. Additionally, the prostate was measured, revealing a transversal diameter of 50 mm with a hypervascular peripheral nodule. An osteoporotic fracture of L3 was diagnosed, but no diagnostic workup was performed at this point. A magnetic resonance imaging (MRI) scan was requested to further characterize the findings, but in the meantime the patient was again admitted with a cholangitis diagnosis. The MRI revealed heterogeneous hepatic steatosis and multiple bilobar hypervascular solid hepatic nodules (the largest at hepatic segment VI with 26 mm, Figure 3A). Pancreatic adenocarcinoma metastasis was suspected at this point.

The patient's ECOG score was 0. Considering the possible differential diagnosis (namely metachronous metastazition from pancreatic cancer, prostatic cancer or unknown primary malignancy, primary hepatocarcinoma or even non-malignant causes such as hepatic abscesses), each with different therapeutic and prognostic approaches, the lesions were biopsied. In August 2016 the histological exam showed cells with uniform nucleus, round to oval, with fine chromatin and absent or inconspicuous nucleolus; ample and eosinophilic cytoplasm. The immune-histochemical study of the hepatic lesions revealed AE1/AE3+, chromogranin+, synaptophysin+, HepPar1-, CK903- and CK7- with a Ki67 index of 14% revealing hepatic involvement by a neuroendocrine tumor [Figure 4].

The initial diagnosis of the pancreatic mass was reviewed at our institution and confirmed pancreatic adenocarcinoma without neuroendocrine differentiation. These findings were supported by performing a Positron Emission Tomography <sup>68</sup>Gallium [Figure 3B]. Shortly after the diagnosis and full staging, the patient fell and developed a femoral fracture which considerably affected his performance status; he was mostly bedridden. Treatment of the neuroendocrine tumor was no longer feasible. Unfortunately, the patient's health then further deteriorated, and he eventually succumbed to hepatic failure due to progressive extensive liver metastazition.



**Figure 3.** Imagiological and functional evaluation at the time of diagnosis of the liver metastasis (2016) (A) MRI showing histologically confirmed neuroendocrine tumor liver metastasis (26 mm) and (B) functional and radiological evaluation with PET  $^{68}\text{Ga}$ -DOTANOC showing high uptake in a hepatic lesion in segment VI; the remaining parenchyma was heterogeneous, without focal uptake; additionally, high uptake was noted on the head of pancreas (suggesting the primary tumor) and one regional lymphatic node. PET: positron emission tomography; MRI: magnetic resonance imaging



**Figure 4.** Biopsy of the liver mass (2016). (A) hepatic parenchyma with involvement of solid epithelial neoplasia (40x); (B) cells with uniform nucleus, round to oval, with fine chromatin and absent or inconspicuous nucleolus; ample and eosinophilic cytoplasm (100x); immune-histochemical study of the hepatic lesions revealing AE1/AE3+ (C), chromogranin+ (D) and synaptophysin+ (E)

## DISCUSSION

The authors want to focus mainly on three points: (1) pancreatic adenocarcinoma biological behavior; (2) when to consider a hepatic metastatic lesion biopsy when a primary cancer is already identified and (3) metastatic pancreatic neuroendocrine tumor biological behavior.

Regarding the first point, pancreatic adenocarcinoma appears to have very different biological behaviors and diverse responses to treatment that are evident in the clinical practice. Specifically in the locally advanced



uresectable setting, no matter what treatment strategy is decided upon, the average survival for these patients remains disappointingly low - less than one year<sup>[4]</sup>. Individual factors may have prognostic implications, such as non-functioning Lewis enzyme, since almost 10% of patients have normal CA 19.9 levels<sup>[4]</sup>, which is actually associated with longer survival<sup>[1]</sup>, as in this case; nonetheless, this is infrequently seen in clinical practice. The patient described here is an example of a long-term survivor patient with a progressively smaller pancreatic adenocarcinoma mass (probably due to ongoing RT lethal effects) with an apparent aggressive disease at diagnosis, with a mostly clinically silent cephalopancreatic lesion with vascular invasion.

Considering the second point, in spite of pancreatic adenocarcinoma being the obvious diagnosis for hepatic metastization, some aspects should prompt a biopsy decision (*vs.* assuming origin from the previously diagnosed primary tumor): The time interval between the primary cancer diagnosis and metastasis diagnosis (a gap of more than three years), the current partial response status of primary disease (making less probable the presence of progressive disease elsewhere), and other confounding and competing possible causes such as other malignancies - a risk that in general increases with age-prostate cancer, unknown primary or even non-malignant causes, such as hepatic abscesses, in light of previous episodes of organized pyogenic cholangitis with need of percutaneous drainage. At this point, we cannot exclude that the possibility that the initial pancreatic tumor could have had neuroendocrine foci that later developed. We can speculate that it is possible that the initial biopsy did not include those components or a second primary tumor arose independently - either way, the histological characterization of the lesion was considered useful, since it could have different diagnostic, prognostic and therapeutic implications, especially since it can be a mostly safe and ambulatory procedure. For example, in much-discussed breast cancer cases, even though performing a biopsy of suspected metastases is recommended in guidelines, it is not always performed in routine oncology practice - most often due to costs and/or invasiveness of the procedure<sup>[9]</sup>.

Lastly, the third point: focus on pancreatic neuroendocrine tumor is generally considered to have a better prognosis than pancreatic adenocarcinoma, but this naturally varies according to tumor location, staging, and metastization pattern among other individual factors. Such rare tumors should ideally be managed in reference centers dedicated to diagnosing and treating them<sup>[8]</sup>.

Of note, the simultaneous diagnosis of pancreatic adenocarcinoma and neuroendocrine tumor is indeed very rare<sup>[10]</sup>. In this particular case report, unexpectedly, the patient actually died due to hepatic failure that developed relatively quickly, and which impeded any possibility of systemic treatment.

Therefore, we conclude that patients should not be denied a treatment opportunity, if clinically compatible, solely based on their advanced disease status, especially if based on theoretically low expectations of tumor response or predicted prognosis. On the other hand, tumors perceived as less aggressive may prove fatal if not timely and effectively dealt with. We urge clinicians to consider hepatic biopsy in similar situations - generally when it may change prognosis and treatment strategies - even if the answer may seem obvious at first.

## DECLARATIONS

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### Authors'contributions

Literature search: Fontes-Sousa M

Drafting and writing the manuscript: Fontes-Sousa M



Manuscript's revision and supervised: Magalhães H, Machado M

Article read and approved: Fontes-Sousa M, Magalhães H, Machado B, Sousa O, Machado M

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### Conflict of interests

The authors have no conflict of interest.

### Patient consent

Not applicable.

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Not applicable.

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Review

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# Significance of peritoneal lavage cytology based on genetic signatures in gastric cancer

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## Abstract

Peritoneal metastasis is the most common pattern of recurrence and the most frequent cause of death after surgery in patients with gastric cancer. Peritoneal free cancer cells disseminated from the primary lesion site have been considered the main cause of peritoneal metastasis. Peritoneal lavage cytological examination (PLC) has been shown to be an independent predictor of gastric cancer relapse after curative resection and poor overall survival. However, the conventional cytological examinations have high rates of false-positive and false-negative findings. To improve the sensitivity, molecular-based methods using reverse transcriptase polymerase chain reaction have been developed for detecting cancer cells in peritoneal wash fluids of patients with gastric cancer. We performed a PubMed search for articles describing PLC in gastric cancer. Relevant articles were reviewed and data on available outcomes elaborated. The clinical roles and attributes of PLC in gastric cancer were reviewed, and its future application to this disease is discussed.

**Keywords:** Gastric cancer, peritoneal lavage cytology, genetic detection, reverse transcriptase polymerase chain reaction, carcinoembryonic antigen

## INTRODUCTION

Gastric cancer is the most common malignancy worldwide and the second leading cause of cancer-related death<sup>[1]</sup>. Despite the development of surgical techniques and new therapeutic strategies, the outcome of patients with advanced gastric cancer is still unsatisfactory<sup>[2]</sup>. Peritoneal dissemination is the most common pattern of metastasis or recurrence, and is the most frequent cause of death after surgery in patients with gastric cancer. Intraperitoneal free cancer cells exfoliated from the cancer-invaded serosa has been



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considered the main cause of peritoneal dissemination<sup>[3]</sup>. Therefore, cytological examination of peritoneal lavage fluid (PLF) obtained at the time of surgery has been considered a useful tool to detect free cancer cells. The peritoneal lavage cytological examination (PLC) has been regarded as a feasible and indeed, the most effective method to predict peritoneal recurrence and survival in patients with gastric cancer<sup>[4-6]</sup>. The Japanese Gastric Cancer Association suggests that the presence of free cancer cells in the peritoneal cavity should be considered an independent prognostic factor in patients with gastric cancer<sup>[7]</sup>. In addition, positive PLC is defined as distant metastasis in the seventh edition of the American Joint Committee on Cancer Staging<sup>[8]</sup>. Therefore, patients with positive PLC most likely will not derive a benefit from surgical procedure, and should be offered systemic chemotherapy or palliative therapy. Recent progress in systematic chemotherapy has resulted in the improvement of prognosis and has allowed the introduction of conversion surgery for select patients who respond effectively to the chemotherapy. However, there are still many issues to address, as critical evidence regarding the timing of conversion, optimal chemotherapy regimen(s), and period of chemotherapy does not exist at present.

Conventional cytology to detect cancer cells in PLF has been performed routinely<sup>[4]</sup>. However, the fact that peritoneal recurrence can be detected in approximately 10% of patients with negative PLF cytology suggests that this cytological examination might not be sufficient for the detection of free cancer cells and prediction of peritoneal spread<sup>[9]</sup>. A more sensitive method for detecting free cancer cells in the peritoneal cavity is urgently needed. The ability to predict micrometastasis development would significantly advance the therapeutic approach to gastric cancer. Over the last few decades, many investigators have proposed the use of molecular diagnostic methods, such as reverse transcription-polymerase chain reaction (RT-PCR) targeting various clinical fields, including detection of free cancer cells<sup>[3,10,11]</sup>. Hence, in an effort to achieve early detection, the analysis of a patient's "genetic signature" using PLF after curative surgery has been employed in recent years, especially in gastric cancer. In this review, we discuss the current evidence and future perspectives of PLC for gastric cancer.

## METHODS

PubMed was searched for English articles using the medical subject headings "gastric cancer", "peritoneal lavage", and "peritoneal washing". Relevant articles from clinical trials and case reports since 1989 were included, as well as background articles relevant to the disease processes of interest.

## BACKGROUND OF PLC

To detect free cancer cells and to predict peritoneal metastasis, conventional PLC performed on PLF obtained during surgery has been broadly used<sup>[4,12]</sup>. PLC is currently examined via Papanicolaou staining and assessed by a cytopathologist. Positive free cancer cells in PLC have been shown to be an important and independent prognostic factor in patients with gastric cancer<sup>[12]</sup>. Thus, PLC has been recommended in the Japanese Classification of Gastric Carcinoma from 1998 onward<sup>[13]</sup>. However, conventional PLC is positive in only 59% of patients with macroscopical peritoneal disease<sup>[14]</sup>. Additionally, the conventional cytology in patients without any macroscopic peritoneal metastasis after curative surgery shows much lower sensitivity (5%-15%)<sup>[15,16]</sup>. Meanwhile, levels of traditional tumor markers associated with gastric cancer in PLF have been calculated to obtain greater sensitivity. Carcinoembryonic antigen (CEA) is a glycoprotein found in colon cancer; it plays a role in cell adhesion<sup>[17]</sup>. Although CEA is not sufficient with regard to diagnostic sensitivity and specificity for early gastric cancer, guidelines suggest that serum CEA levels should be measured to predict recurrent gastric cancer<sup>[18]</sup>. It has been reported that CEA levels in PLF accurately predict peritoneal recurrence after a curative resection of gastric cancer<sup>[19]</sup>. The addition of immunohistochemical CEA measurement to conventional cytology resulted in increased sensitivity (26%)<sup>[20]</sup>. Combined analysis of CEA with other principal gastric tumor markers, such as CA72-4 or CA125, has been shown to enhance the accuracy for predicting peritoneal relapse<sup>[21,22]</sup>. Regardless, the CEA measurement in PLF still has an about

20% false negative for peritoneal dissemination<sup>[9]</sup>. Thus, there is still a need for more sensitive methods of PLC with lower false-positive and false-negative rates.

## GENETIC DETECTION OF PLC

The greater sensitivity of RT-PCR analysis has made it possible to detect micrometastasis in the basis of cancer-tissue-specific messenger RNA (mRNA) expression in peripheral veins, lymph nodes, bone marrow, and the peritoneal cavity<sup>[23]</sup>. Molecular diagnosis using RT-PCR is generally reflected to be a more sensitive and quantitative method than conventional cytology for the detection of micrometastasis in PLC<sup>[3,10]</sup>. Thus, RT-PCR analysis of PLF should have clinical significance in the diagnostic evaluation of suspected peritoneal metastasis and the development of therapeutic strategies. Based on a range of studies, there is a robust correlation between the results of RT-PCR analysis of PLF and prognosis after curative operation in patients with advanced gastric cancer [Table 1]. In this section, we will discuss selected molecular markers of PLC including those based on genetic approaches.

### Carcinoembryonic antigen

Studies conducted over the last couple of decades have demonstrated the usefulness of measuring CEA mRNA to detect micrometastasis in the peritoneal cavity. Nakanishi *et al.*<sup>[24]</sup> were the first to describe the high sensitivity for detecting free cancer cells through RT-PCR amplification of CEA mRNA. Positive rate of analysis through RT-PCR was elevated to 20% than that of cytology alone<sup>[24]</sup>. Subsequent to their study, many further studies examining CEA mRNA in PLF as the target molecular marker in gastric cancer were published<sup>[11,15,25-32]</sup>. For instance, one study using RT-PCR for CEA mRNA showed a detection rate of free cancer cells of 28%, with a 14% higher detection rate than for PLC<sup>[33]</sup>. A recent prospective study of quantitative CEA mRNA detection in PLF using the most desirable cutoff value of CEA mRNA of 0.1 by ROC curve analyses found that the positive rates for CEA mRNA were 45.7% and 50.0% in T3 and T4 patients, respectively. Among the CEA mRNA-positive patients, 55.0% induced peritoneal metastasis. In contrast, only 3.0% of patients who were negative for CEA mRNA had peritoneal relapse, 84.6% of the positive rate of CEA mRNA in PLF from patients with peritoneal dissemination for the period of postoperative surveillance. CEA mRNA was shown to be only an independent prognostic factor in multivariate analysis with peritoneal recurrence-free survival<sup>[30]</sup>.

Nevertheless, the sensitivity of the CEA RT-PCR assay for detecting peritoneal micrometastasis is still insufficient. Moreover, false positive results, caused by expression of CEA in no malignant cells such as mesothelial cells and lymphocytes, remains a key problem of this technique<sup>[34]</sup>. To overcome these problems, a recent study showed that a novel and rapid molecular method of diagnosis using the technique of transcription-reverse transcriptase concerted reaction (TRC) has been developed<sup>[35,36]</sup>. A prospective study at multiple institutions to examine the clinical benefit of TRC diagnosis with PLF from gastric cancer patients was carried out. Accordingly, TRC can be a prognostic factor for the prediction of patient outcome and peritoneal metastasis of gastric cancer with serosa-infiltrating tumors. On the other hand, another paper showed that CEA mRNA index (CmRI) (CEA mRNA/porphobilinogen deaminase mRNA  $\times$  10,000) values in PLF may be a useful tool for reflecting the response of peritoneal relapse to induction chemotherapy and that the advantage of conversion gastric surgery could be predicted by CmRI values<sup>[37]</sup>.

### Cytokeratin

Keratins are intermediate filament proteins which are closely related with the structural integrity of epithelial cells. Recently, several studies have identified cytokeratin-20 (CK-20) as one of the potential cancer-related biomarkers for the detection of peritoneal free cancer cells for the patients with gastric cancer<sup>[38,39]</sup>. CK-20 has been used as a factor with CEA in a multiple-marker analysis for the detection of peritoneal micrometastasis<sup>[40]</sup>. In a recent study, real-time quantitative RT-PCR analysis of the CEA and/or CK20 transcripts in PLF was

**Table 1. List of published studies regarding the genetic diagnosis of peritoneal lavage cytology in gastric cancer**

Study	Year	Marker	Detection method	Number of patients	Main results
Nakanishi <i>et al.</i> <sup>[24]</sup>	1997	CEA	RT-PCR	48	RT-PCR is more sensitive for detection of free carcinoma cells in the peritoneal cavity than conventional cytology
Fujimura <i>et al.</i> <sup>[53]</sup>	1998	Trypsinogen	RT-PCR	30	Trypsinogen-1 mRNA was positive for the patient, who did not show macroscopic or cytological peritoneal dissemination
Yonemura <i>et al.</i> <sup>[25]</sup>	2001	MMP-7	RT-PCR	152	Improved the sensitivity for peritoneal dissemination in combination with cytology
Kodera <i>et al.</i> <sup>[34]</sup>	2002	CEA	RT-PCR	90	PCR positive was a significant independent prognostic factor, but CY positive was not
Sugita <i>et al.</i> <sup>[39]</sup>	2003	CEA, CK20	RT-PCR	129	In cases with negative cytology, patients with PCR-positive findings in PLF had a poorer outcome than those with negative PCR
Mori <i>et al.</i> <sup>[66]</sup>	2004	Multiple marker	Microarray	179	Correlation with disease-free survival and immunocytochemical cytology
Wang <i>et al.</i> <sup>[15]</sup>	2005	CEA	RT-PCR	40	The technique of RT-PCR was more sensitive than conventional PLC in the detection of peritoneal free cancer cells and the prediction of peritoneal recurrence
Kodera <i>et al.</i> <sup>[38]</sup>	2005	CK20	RT-PCR	195	Not sufficiently sensitive compared with CEA
Ohashi <i>et al.</i> <sup>[35]</sup>	2007	CEA	TRC method	112	TRC has a diagnostic power almost equivalent to qRT-PCR but with the advantage of ultra-rapid detection
Da <i>et al.</i> <sup>[57]</sup>	2007	Telomerase activity	TRAP assay	60	Correlation with high proliferating activity of gastric cancer
Hiraki <i>et al.</i> <sup>[45]</sup>	2011	Aberrant gene methylation	Methylation-specific PCR	107	Methylation analysis along with a cytological examination might therefore improve the positive detection of cancer cells in PF of gastric cancer
Horikawa <i>et al.</i> <sup>[62]</sup>	2011	CD44, CD45, EpCAM	RT-PCR	147	CD44 mRNA of magnetically separated CD45EpCAM+ cell fraction of PLC is useful for predicting high-risk individuals among gastric cancer patients with stage II and III
Takata <i>et al.</i> <sup>[41]</sup>	2014	CEA, CK20	RT-PCR	104	CEA and CK20 PCR results could predict peritoneal recurrence after curative surgery
Li <i>et al.</i> <sup>[51]</sup>	2014	CEA, MMP-7	RT-PCR	116	CEA and MMP-7 transcripts in PLF could effectively predict peritoneal recurrence
Jeon <i>et al.</i> <sup>[43]</sup>	2014	CEA, MAGE	RT-PCR	117	MAGE expression was determined to be the most important prognostic factor for recurrence
Tokuhisa <i>et al.</i> <sup>[47]</sup>	2015	Exosomal miRNAs	Agilent Human miRNA microarrays and qRT-PCR	24	miRNA expression profiles can indicate the status of peritoneum in GC patients
Miwa <i>et al.</i> <sup>[49]</sup>	2017	FBXO50	The ABI StepOnePlus Real-Time PCR System and TaqMan Copy Number Assay	200	FBXO50 expression related with recurrence after curative gastrectomy and shorter overall survival

CEA: carcino-embryonic antigen; CK20: cytokeratin 20; TRAP assay: telomeric repeat amplification protocol assay; RT-PCR: reverse transcription polymerase chain reaction; qRT-PCR: quantitative reverse transcription polymerase chain reaction; TRC: transcription reverse-transcription concerted

presented to be useful for predicting peritoneal metastasis in patients after curative resection for gastric tumor. The sensitivities of combined CEA and/or CK20 mRNA levels were 86.4% and 81.5%, respectively, clearly increased compared with that of each marker alone. In the patients with a curative resection, the survival rate of the PCR-positive was significantly lower than that of PCR-negative in the gastric cancer patients with a curative surgery. Additionally, the level of CEA or CK20 mRNA was an independent prognostic factor for overall survival rate<sup>[40]</sup>. Another prospective study also found that CEA and CK20 RT-PCR results could predict peritoneal recurrence after curative surgery<sup>[41]</sup>.

### Melanoma associated gene

Melanoma associated gene (*MAGE*) has been said to be a cancer-specific marker responsible for the suppression of apoptosis and carcinogenesis<sup>[42]</sup>. RT-PCR of gastric cancer shows that the *MAGE* genes are



highly expressed than that of other markers<sup>[43]</sup>. Although the rate of expression differs in accordance with subtype, expressions of at least one of *MAGE-4*, *MAGE-6*, *MAGE-8*, *MAGE-9*, *MAGE-10*, and *MAGE-12* genes were as high as 82% in gastric cancerous specimen<sup>[44]</sup>. Furthermore, previous studies reported that *MAGE* was not expressed in normal gastric tissue<sup>[44]</sup>. These results suggest that *MAGE* has been a candidate as a novel targeted gene for the prediction of survival in patients with gastric cancer, and is expected to be a therapeutic target due to its specific expression. A recent report in trial comparing the two markers CEA and *MAGE* demonstrated that superior specificity and important association with peritoneal metastasis were revealed in *MAGE* RT-PCR than in CEA RT-PCR after long-term follow-up, and *MAGE* RT-PCR results were shown to be the most significant survival factor for peritoneal relapse in patients with gastric cancer after curative surgical procedure<sup>[43]</sup>.

### Gene methylation

To identify micrometastasis in salivary rinses for head-and-neck cancer patients and pleural effusion for several cancers, cancer-specific gene methylation has been commonly investigated. Thus, aberrant gene methylation in PLF may predict peritoneal recurrence in gastric cancer. A previous study evaluated whether methylation in the PLF by quantitative methylation-specific PCR analysis affects peritoneal metastasis after surgery in the patients in which the depth of invasion of the primary lesion was beyond the muscularis propria<sup>[45]</sup>. Twelve-fold enhanced risk of peritoneal relapse in patients with positive methylation was shown compared with in those with negative methylation by the combined assessment of the 6 genes (*BNIP3*, *CHFR*, *CYP1B1*, *MINT25*, *RASSF2*, and *SFRP2*). Additionally, positive methylation rate in patients with peritoneal metastasis or positive PLC was increased up to 75% by the combined assessment of the 6 genes, whereas the rate in gastric cancer patients with the depth of cancer invasion beyond the muscularis propria (that is, tumor involves the subserosa, tumor penetrates the serosa, and tumor invasion of adjacent structures present) was 20%.

### Exosomal miRNAs

MicroRNAs (miRNAs) are small non-coding RNAs that serve as posttranscriptional regulators of gene expression and have an essential role in the control of many biological processes<sup>[46]</sup>. A recent study investigated the diagnostic potential of exosomal miRNA profiles in peritoneal fluid for the prediction of peritoneal dissemination in gastric cancer<sup>[47]</sup>. The miRNA content of exosomes isolated from malignant ascites and peritoneal lavage fluid of gastric cancer patients was examined by miRNA microarray technology. Significant high expressions of miR-21 and miR-1225-5p were found in patients with T4-stage cancer than that in T1- to T3-stage patients, suggesting that profiling of miRNAs in peritoneal lavage fluid may be used for the prediction of a peritoneal premetastatic phenotype in gastric cancer and may provide more effective preventive and curative measures.

### FBXO50

F-box proteins, which are the substrate-recognition subunits of SKP1-cullin 1-F-box protein E3 ligase complexes, play essential roles in a variety of cellular processes through ubiquitylation which lead to the degradation of target proteins<sup>[48]</sup>. F-box only proteins (FBXOs) are key subclass of F-box proteins organized in accordance with the existence of specific substrate recognition domains. Expression levels of FBXO50 mRNA in gastric cancer tissues from 200 patients were investigated, and the level of FBXO50 expression was significantly correlated with positive peritoneal lavage cytology<sup>[49]</sup>. FBXO50 would be another new candidate tool of PLC for detecting micrometastasis in gastric cancer.

### Other genetic markers

Besides the markers described above, numerous different markers to detect micrometastasis including various aspects of biological activity in gastric cancer are known. The genetic alteration of proteinases,

which are closely associated with cancer invasion, has been regarded as one of the useful tools in the early detection of peritoneal metastasis. Matrix metalloproteinase 7 (MMP-7), also called matrilysin, is a familiar member in the MMP family due to its excessive proteolytic activity for a broad range of molecules and is selectively produced from gastric cancer cells<sup>[50]</sup>. Moreover, a previous study found that an MMP-7 RT-PCR assay of PLF detected cancer cells at densities of as low as < 10 cells/sample and was an independent predictor of peritoneal recurrence<sup>[25]</sup>. A quantitative RT-PCR analysis of the CEA and MMP-7 transcripts in the PLF effectively predicted peritoneal relapse in gastric cancer in multivariate analysis, and combination analysis of them enhanced the sensitivity and specificity compared with conventional PLC (71.1% and 74.6%, respectively)<sup>[51]</sup>. Trypsin is a member of the serine protease family which consists of 3 trypsinogen genes (*trypsinogen 1, 2 and 4*) and has a potential role in cancer invasion<sup>[52]</sup>. As a major digestive enzyme, trypsin has high proteolytic activity, and its unsuitable activation may result in peritoneal dissemination of infiltrative gastric cancer. Trypsinogen may be a good candidate for the early detection of peritoneal recurrence in gastric cancer, because trypsinogen-1 mRNA was positive in a patient who did not show macroscopic or cytological peritoneal dissemination<sup>[53]</sup>. Th17 cells have been identified as having a distinct Th cell lineage and have been found in several types of human cancers, including gastric cancer<sup>[54]</sup>. Increasing evidence suggests that IL-17 promotes tumor growth through angiogenesis and inflammation. On the other hand, it contributes to the reduction of tumor growth by promoting dendritic cells, cytotoxic T lymphocyte, and NK cells. Patients with high expression of IL-17 mRNA detected by real-time RT-PCR in peritoneal lavage showed significantly prolonged survival compared with patients with low expression of IL-17 mRNA in peritoneal lavage, suggesting that low IL-17 gene expression in PLF may correlate with cancer development and poor prognosis in patients with gastric cancer<sup>[55]</sup>. Telomerase is a ribonucleoprotein polymerase that adds TTAGGG repeats to telomeric ends. Telomerase regulates cellular immortality and is reactivated in approximately 85% of human malignancies<sup>[56]</sup>. A recent study using a telomeric repeated amplification protocol - enzyme-linked immunosorbent assay found that telomerase activity in PLF can be detected in patients with peritoneal metastasis, and found the positive rate of telomerase activity was significantly associated with the positive rate of telomerase activity and the presence of peritoneal recurrence, although these methods were not superior to conventional cytology by itself<sup>[57]</sup>.

### Other candidates for genetic marker in PLC

The approaches mentioned above focus on the detection of already known genetic changes, whereas full genome sequencing can be used for the detection of new candidates, and expression profiling may provide the detection of previously unknown markers for PLC. With regard to peritoneal metastasis, malignant features of tumor cells such as altered expression of growth factors, immuno-insufficiency, decreased intercellular adhesion, increased cell-to-matrix adhesion, and resistance to apoptosis are considered to be pivotal characteristics. The results of this comprehensive gene expression analysis of gastric cancer with peritoneal metastasis may provide new insight into the detection of micrometastasis in PLF. A previous study using a global analysis of the differential gene expression showed the relative mRNA levels of genes expressed in gastric cancer cell lines established from primary tumors and of other cell lines established from metastasis to the peritoneal cavity<sup>[58]</sup>. Twenty-four genes including *CD44*, *dopa decarboxylase (DDC)*, *keratin family genes*, *aldehyde dehydrogenase*, *CD9* and *IP3 receptor type 3* were up-regulated while 17 genes including *CD4*, *IL4 Stat*, *IGFBP2*, and *histon deacetylase 3* were down-regulated in the metastatic cell lines based on results of a high-density cDNA microarray method<sup>[58]</sup>. Among them, the precise roles of DCC in peritoneal metastasis have been investigated. DDC is an enzyme for the metabolism of dopamine, and is also responsible for the production of neurotransmitters, such as serotonin<sup>[59]</sup>. DCC was one of these upregulated genes. DDC-specific RT-PCR may become a novel marker for peritoneal dissemination of gastric cancer<sup>[60]</sup>. CD44-positive gastric cancer cells have been said to show properties of self-renewal and the capability to generate differentiated progeny, in line with the CSC<sup>[61]</sup>. CD44 mRNA of separated CD45 EpCAM-positive cell fraction of peritoneal washes using the Auto-MACS system may be a useful genetic marker for predicting high-risk individuals among stage II and III gastric cancer patients<sup>[62]</sup>. Phenotype L3-

phosphoserine phosphatase (L3-PP) is also one of the highly-expressed genes that have been analyzed by high-density microarray. L3-PP encodes the phosphatase of phosphoserine and has been said to be involved in amino acid synthesis<sup>[63]</sup>. The enhancement of the activity of L3-PP has been found according to the increased cell multiplication and frequency of mitosis<sup>[63]</sup>. It has been reported that L3-PP overexpression of L3-PP in gastric cancer cells obtained from peritoneal metastasis by RT-PCR has been shown to be closely associated with peritoneal recurrence of gastric cancers<sup>[64]</sup>. Combined RT-PCR analysis of CEA with L3-PP resulted in the reduction of false negative CEA mRNA and increased sensitivity of peritoneal metastasis detection from 71.4% to 85.7%. Another study performed global analysis on differential gene expression of a scirrhous gastric cancer cell line (OCUM-2M) and its derivative sublines with high potential for metastasis to the peritoneal cavity (OCUM-2MD3) in a nude mouse model<sup>[65]</sup>. Twelve genes including *rab32*, *trefoil factor 1*,  *$\alpha$ -1-antitrypsin*, and *gelactin4*, were up-regulated by applying a high-density oligonucleotide array method. Besides, RT-PCR was performed in 16 representative PLF samples to classify genes specific to cytology-positive samples<sup>[66]</sup>. The usefulness as markers for minimal resonant disease in 99 PLF sample was examined using 5 genes finally selected-CK20, *FABP1*, *MUC2*, *TFF1*, and *TFF2*. Positive findings which were highly specific to fatal cases (91%-100%) were found by nested RT-PCR using the 5 genes. With high specificity, the combined use of these 5 genes resulted in identifying 6 out of 20 (30%) additional patients with all kinds of early relapse.

Consequently, these genomic profiling findings suggest the critical importance of setting up a basis upon which to establish not only improved molecular understanding, but also better targeted strategies for gastric cancer treatment.

## CLINICAL APPLICATION

Up to the present, effective therapies for peritoneal metastasis have not been established. A previous study found that a new oral fluorinated pyrimidine agent (S-1), used as a postoperative monotherapy, did not show superior effect in survival in patients with macroscopic peritoneal tumor compared with patients with positive cytology<sup>[67]</sup>. To improve survival, it is essential to identify high-risk patients at an earlier phase of peritoneal metastasis. Several experimental studies have shown that micrometastases are more responsive to chemotherapy than visible metastatic tumors<sup>[68,69]</sup>. Thus, in addition to make an accurate diagnosis, molecular diagnosis using RT-PCR analysis has an important role in starting chemotherapy before the development of macrometastasis. A phase II clinical trial for evaluating the prognostic impact of postoperative S-1 monotherapy in gastric cancer patients with CEA mRNA positivity was carried out. Accordingly, the 3-year survival did not show the significant difference between the study population and the historic control (67.3% vs. 67.1%, respectively), suggesting that S-1 may delay cancer relapse but not always eradicate micrometastases<sup>[70]</sup>.

Because micrometastases are more susceptible to chemotherapy than macroscopic disease, neo-adjuvant chemotherapy would theoretically has a benefit in this subgroup of patients, because micrometastases are more susceptible to chemotherapy than macroscopic disease<sup>[71]</sup>. Positive cytology may serve as a guide to continuing chemotherapy or changing the mode of therapy. Preoperative chemotherapy protocols may select patients more likely to benefit from resection. A previous study analyzed the genetic diagnosis using PLF for detecting patients at high risk for peritoneal recurrence and for evaluating the clinical response to intraperitoneal chemotherapy in patients with gastric cancer<sup>[66]</sup>. From nineteen patients with advanced gastric cancer who underwent staging laparoscopy and intraperitoneal chemotherapy (MMC 20 mg on day 1; CDDP 20 mg on days 1-5) before surgical resection or systemic chemotherapy (docetaxel 60 mg/m<sup>2</sup> on day 1; CDDP 10 mg/m<sup>2</sup> on days 1-5; 5-fluorouracil 350 mg/m<sup>2</sup> on days 1-5), specimens of PLC were collected and were subjected to RT-PCR. All patients except for one who showed lower level of RT-PCR and finally revealed negative outcome, and all but one patient who showed an values level in the period of treatment

died of recurrence, suggesting that evaluation of genetic changes using RT-PCR analysis can provide the practical information for detecting free cancer cells in the peritoneal cavity with high sensitivity and for selecting patients at high risk of peritoneal metastasis, leading to the prediction of chemotherapeutic efficacy for these patients.

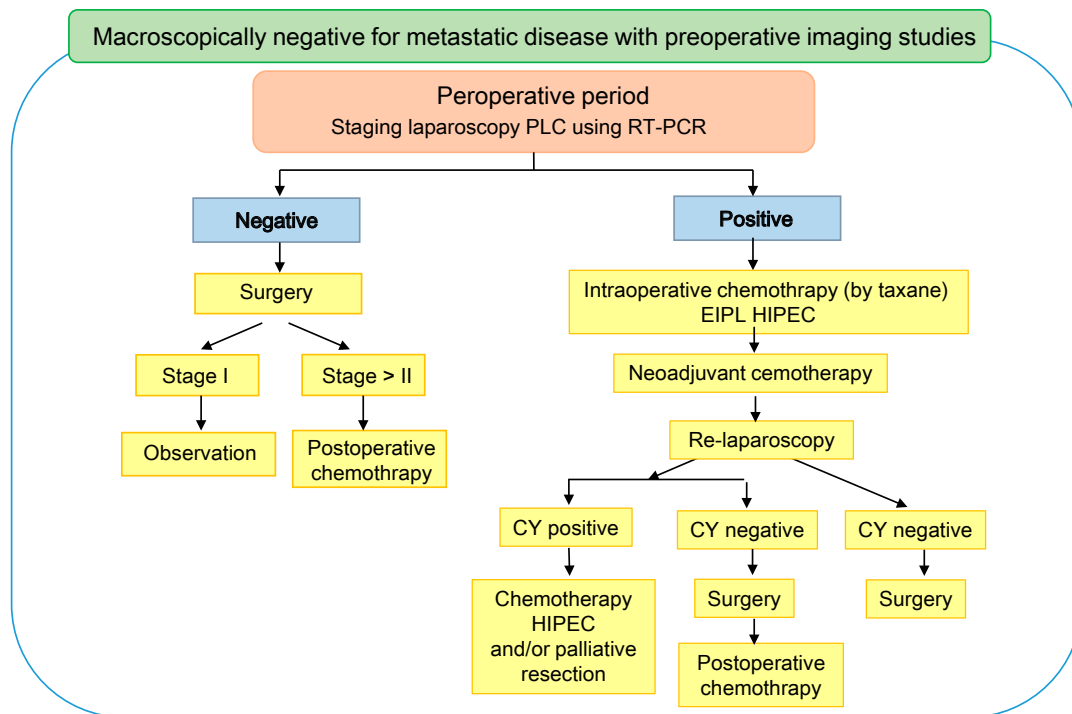
Extensive intra-operative peritoneal lavage (EIPL) therapy, i.e. extensively repeated dilution and complete suction, serve as a very simple and non-aggressive prophylactic treatment for peritoneal metastasis of gastric cancer patients with peritoneal free cancer cells<sup>[72]</sup>. Yamamoto *et al.*<sup>[73]</sup> described that the peritoneal relapse rate of the patients with EIPL therapy was significantly lower than that of the patients without EIPL therapy. Although intra-peritoneal free cancer cells were detected immediately after curative surgery using RT-PCR analysis, no cancer cells were identified in the PLF after EIPL therapy<sup>[73]</sup>. A recent study using ultra-rapid quantitative RT-PCR has shown that the number of free cancer cells in PLF was serially diluted  $3.8 \times 10^5 \pm 1.4 \times 10^5$  to  $2.8 \pm 1.5$  cells/100 mL by 6 to 8 L of saline. Notably, CEA mRNA disappeared completely from the PLF after seven to nine washes. Intraperitoneal chemotherapy followed by EIPL using an ultra-rapid detection method may be acceptable for patients with free cancer cells in PLF after curative operation<sup>[74]</sup>.

In a recent, the eligibility criteria for randomized controlled trials of neo-adjuvant chemotherapy or hyperthermic intraperitoneal chemotherapy in locally advanced gastric cancer included the presence of positive PLC at the staging laparoscopy<sup>[75]</sup>. Recently previous study presented 103 patients with gastric cancer who underwent staging laparoscopy and peritoneal metastasis was confirmed. Among them, 68 patients received the intravenous and intraperitoneal paclitaxel plus oral S-1 as induction chemotherapy. PLF of these patients was repetitively collected via intraperitoneal access ports. When a second laparoscopy showed negative PLC, gastrectomy was considered. Significant prolonged survival of patients with CmRI values that had once reduced to < 100 was identified by conversion gastrectomy. The OS of patients with a preoperative CmRI value < 100 was significantly improved compared with that of those with a preoperative CmRI value > 100 among patients who underwent conversion gastrectomy<sup>[37]</sup>.

Based on these findings, we propose a treatment strategy for gastric cancer patients with positive PLC using RT-PCR in [Figure 1](#).

## FUTURE PERSPECTIVES OF PLC BY GENETIC TECHNIQUES

Although numerous studies have presented that molecular analysis using RT-PCR may be useful for the detection of free cancer cells, there are still several obstacles for realizing the clinical application of genetically diagnosed PLC as a routine service. Namely, time-consuming, expensive, and relatively arduous techniques compared with conventional cytology are pointed out, and the sensitivity is broadly variable between laboratories; furthermore, procedures for quantitative assessment of free cancer cells are lacking. Recently, experimental studies proposing rapid, accurate, more standardized, and cost-effective detection methods have been reported. As described above, TRC can be a rapid and quantitative diagnostic technique to target CEA mRNA because it does not require cDNA synthesis and the reactions of amplification, and detection occur in a single tube, and take less than 1 h<sup>[36,76]</sup>. The reverse transcription-loop-mediated isothermal amplification (RT-LAMP) technique is a promising candidate to reduce the time requirement. In fact, there are several practical advantages to the RT-LAMP technique: it requires only simple reaction procedures, the compact incubator or turbidimeter equipment costs less than \$5000, and needs less than 1 h to obtain the final results<sup>[10]</sup>. Among patients with negative cytology, those with a positive RT-LAMP reaction had a shorter survival than those with negative RT-LAMP reaction results. The RT-LAMP method may be an alternative method to determine the necessity or feasibility of surgery. Searching for a specific diagnostic marker for peritoneal metastasis by a rapid PCR method may help patients avoid redundant surgery and determine adequate preoperative chemotherapy.



**Figure 1:** Treatment strategy for the patient with positive PLC. PLC: peritoneal lavage cytology; RT-PCR: reverse transcription-polymerase chain reaction; EIPL: extensive intra-operative peritoneal lavage; HIPEC: hyperthermic perioperative chemotherapy

As previously stated, numerous efforts have been made to gain the detection rate of intraperitoneal free cancer cells. The main purpose of these studies must principally be an enhancement of the sensitivity of PLC. To eliminate diagnostic errors and the misunderstanding of molecular diagnostic results for the sake of determining the best treatment plan, combined multiple markers would be practical for diagnosis of micrometastasis. Novel markers should also be sought. Multimarker PCR would be more clinically useful in getting expanded broad genetic profile in the near future, but this has yet to be investigated.

It is important to determine which genes should be analyzed for clinical decision making. Because personalized cancer genome analysis become more accepted and feasible, the genetic analysis of individual gastric tumors may provide insight into which tumor markers are the most sensitive for detection. Recently, The Cancer Genome Atlas Research Network (TCGA) advocated a novel classification system based on a genomic and molecular basis dividing gastric cancer into four major subtypes<sup>[77]</sup>. These subtypes include Epstein Barr Virus-infected tumors (EBV), microsatellite instability-associated tumors (MSI), genomically stable tumors (GS) and chromosomally unstable/chromosomal instability (CIN). EBV reveals mutations in PIK3CA and amplifications of JAK-2, PD-L1/2 as well as hypermethylation. MSI demonstrate multiple mutations including PIK3CA, ERBB3, HER2, EGFR in addition to MLH1 silencing. GS is related with CDH1 and RHOA mutations while CIN tumors harbor focal amplification of receptor tyrosine kinases in addition recurrent TP53 mutations. It is plausible that the relation of these genetic markers with peritoneal metastasis can be clarified on the basis of these molecular subtypes, which will lead to a future promising new candidate genetic markers in PLC for detecting intraperitoneal micrometastasis and a guide to new targeting agents. PLC should be considered as not just a survival predictor, but an important factor which can determine diagnosis and treatment of advanced gastric cancer after curative resection. Detection of molecular changes in PLF during chemotherapy, resulting in chemoresistance, could offer a promising way to shift the course of chemotherapy at the appropriate time as well as to find new therapeutic targets.



## CONCLUSION

In conclusion, new genetic technologies are improving the detection of micrometastasis in the peritoneum, although conventional cytology is still the gold standard for PLC. The development of genetic PLC based on comprehensive genomic analysis could help us to identify patients who should be treated completely with multimodal therapy in addition to radical surgery, and will be very relevant to all sorts of clinical decision-making.

## DECLARATIONS

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### Authors' contributions

Designed this review: Yashiro M, Matsuoka T

Wrote and edited the manuscript: Matsuoka T

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There are no conflicts of interest.

### Patient consent

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Not applicable.

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Review

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# Gastric cancer: prevention and treatment of peritoneal metastases

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## Abstract

Gastric cancer is an aggressive malignancy that may metastasize through the bloodstream to the liver, through lymphatics to regional lymph nodes, or by penetration of the peritoneal lining of the stomach to result in seeding of the abdominal and pelvis surfaces. Peritoneal metastases are the most common mode of cancer dissemination. Technologies to prevent or treat peritoneal metastases from advanced gastric cancer are presented in this manuscript. The world's literature, both recent and over the past three decades, was reviewed in order to identify publications that present information regarding gastric cancer peritoneal metastases. Over one dozen randomized controlled trials to test perioperative chemotherapy for prevention of peritoneal metastases were reviewed. All of the trials performed with regional chemotherapy during or shortly after gastrectomy were positive. The clinical data regarding the treatment of peritoneal metastases diagnosed at the time of primary cancer resection or in follow-up were reviewed. Neoadjuvant intraperitoneal and systemic chemotherapy shows that some long-term survivors occur after these treatments were combined with cytoreductive surgery and gastrectomy. Similar treatments are advocated for primary gastric cancer with cytology positive for gastric cancer but no visible implants. Surgery for gastric cancer should be combined with perioperative systemic and regional chemotherapy in order to maximally benefit patients with this disease by reducing the negative impact of peritoneal metastases on survival.

**Keywords:** Hyperthermic intraperitoneal chemotherapy, normothermic intraoperative intraperitoneal chemotherapy, early postoperative intraperitoneal chemotherapy, intraperitoneal chemotherapy, gastric cancer, peritoneal metastases, carcinomatosis



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## INTRODUCTION

Gastric cancer is the fourth most common cancer in the world with a 5-year survival rate of 25%<sup>[1,2]</sup>. In follow-up, a large percentage of gastric cancer patients will develop peritoneal dissemination (up to 40%) which results in a less than 5% 5-year survival rate<sup>[3-5]</sup>. In primary gastric cancer, peritoneal metastases are a common finding present in 5%-20% of patients undergoing gastrectomy<sup>[6]</sup>. The peritoneum is the most common location of first recurrence in about half of patients<sup>[7]</sup>. Although the standard of care for treatment of primary gastric cancer involves surgery, intravenous chemotherapy and radiotherapy, specific treatments for peritoneal metastases are poorly defined. Possible treatments include neoadjuvant systemic chemotherapy (NAC), neoadjuvant intraperitoneal and systemic chemotherapy (NIPS), cytoreductive surgery (CRS) and perioperative chemotherapy which may include hyperthermic intraperitoneal chemotherapy (HIPEC) and/or early postoperative intraperitoneal chemotherapy (EPIC)<sup>[8]</sup>. CRS and HIPEC/EPIC is already considered standard of care for selected patients with appendiceal peritoneal metastases, peritoneal mesothelioma, and a limited extent of peritoneal metastases from colorectal carcinomatosis<sup>[9-11]</sup>. For gastric cancer with peritoneal metastases, current treatment recommendations remain controversial. The following is an attempt to summarize the role and efficacy of NAC, NIPS, CRS and HIPEC and/or EPIC as prevention or treatment for peritoneal metastases of gastric cancer.

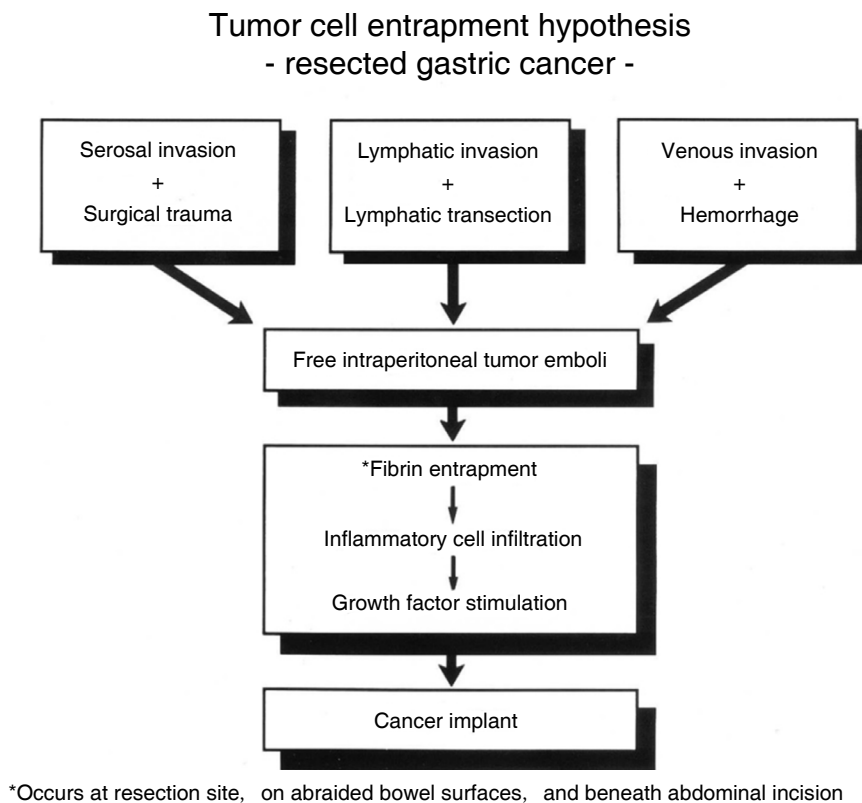
## PREVENTION OF PERITONEAL METASTASES USING PERIOPERATIVE INTRAPERITONEAL CHEMOTHERAPY

Surgical treatment failure with resection site and intraabdominal tumors are the most common sites of first recurrence in gastric cancer after potentially curative resection<sup>[12-14]</sup>. Regardless of neoadjuvant chemotherapy or postoperative adjuvant treatment, this local-regional progression occurs<sup>[15]</sup>. The peritoneal surfaces and liver remain the major sites of recurrence with a reduced local progression when extended lymphadenectomy as compared to limited surgery is used<sup>[16-18]</sup>.

Although confined to the abdomen, peritoneal seeding has an adverse impact on survival<sup>[19-22]</sup>. Sources of recurrence after curative resection are (1) spontaneous spreading from the primary tumor; and (2) surgical trauma causing scattering of cancer cells during the surgical procedure. If serosal surface invasion has occurred within the primary tumor, then spontaneous dissemination is more common and patients are frequently found to have viable intraperitoneal cancer cells (positive cytology)<sup>[19,21-23]</sup>. Tumor cells can also seed the intraabdominal cavity during surgery according to the tumor cell entrapment hypothesis [Figure 1]. During cancer resection, there is transection of lymphatic channels, close margins of resection, and tumor-contaminated blood spillage. Marutsuka *et al.*<sup>[24]</sup> identified free cancer cells in peritoneal lavage samples in patients' initial cytology negative approximately 70 min after dissection of lymph node metastases. Takebayashi *et al.*<sup>[25]</sup> showed that gastrectomy spilled viable cancer cells into the peritoneal space in 24 of 57 patients. They concluded that surgery induces peritoneal metastases. Arita *et al.*<sup>[26]</sup> determined that large amounts of intraoperative hemorrhage increased the risk of peritoneal recurrence. This may support the contention that cancer cells are present in large numbers within blood lost from the gastric cancer specimen. These iatrogenically disseminated tumor cells adhere spontaneously within minutes and vascularization is facilitated by fibrin entrapment and the wound healing process. Cytokines, such as growth factors important for wound healing, may also propel tumor progression. The tumor cell entrapment hypothesis explains part of the pathogenesis of resection site and distant peritoneal metastases. It is the theoretical basis for adjuvant perioperative intraperitoneal chemotherapy<sup>[27]</sup>.

### Perioperative timing of intraperitoneal chemotherapy

The tumor cell entrapment hypothesis suggests that intraperitoneal chemotherapy must be administered perioperatively in order to access the tumor cells prior to entrapment within fibrin and conversion into cancer implants within adhesive scar tissue. If intraperitoneal chemotherapy is delayed until after the formation of adhesive scars, it will have uneven distribution and lack direct contact with viable cancer cells.



**Figure 1.** The tumor cell entrapment hypothesis suggests three mechanisms for microscopic residual cancer cells in patients having an R-0 gastrectomy. (From Sethna *et al.*<sup>[27]</sup> with permission)

## PREVENTION PROTOCOLS USING PERIOPERATIVE CHEMOTHERAPY WITH GASTRECTOMY

Perioperative intraoperative chemotherapy can eliminate progression of peritoneal implantation after curative surgery, however, it cannot treat residual disease within lymph nodes. Therefore, an adequate lymphadenectomy is essential. Intraperitoneal chemotherapy enters the peritoneal nodule by simple diffusion so it only penetrates to 1 or 2 mm<sup>[28]</sup>. It is not effective in lymph nodes. Also, peritoneal nodules larger than 1 or 2 mm have ineffective drug delivery and all visible nodules must be removed prior to treatment.

## LITERATURE REGARDING PERIOPERATIVE INTRAPERITONEAL CHEMOTHERAPY FOR ADVANCED T-STAGE PRIMARY GASTRIC CANCER

There have been randomized and non-randomized trials about adjuvant perioperative intraperitoneal chemotherapy compared to surgery alone for resectable primary gastric cancer without peritoneal spread. Sugarbaker *et al.*<sup>[7]</sup> published a meta-analysis in 2003 of articles published in English. Xu *et al.*<sup>[29]</sup> published a similar study in 2004. Yan *et al.*<sup>[30]</sup> published a summary of randomized control trials about adjuvant intraperitoneal chemotherapy for resectable gastric cancer in 2007. Feingold *et al.*<sup>[31]</sup> published the most recent summary of non-randomized and randomized studies in English of CRS and HIPEC and/or EPIC in gastric cancer.

Yan *et al.*<sup>[30]</sup> selected 10 of 13 randomized controlled trials that were judged to be of fair quality to be used in the meta-analysis. There was a survival benefit associated with HIPEC [hazard ratio (HR) 0.060; 95% CI 0.43-0.83;  $P = 0.002$ ] or HIPEC with EPIC (HR 0.45; 95% CI 0.29-0.68;  $P = 0.0002$ ). There was a marginal benefit with normothermic intraoperative intraperitoneal chemotherapy but no significant improvement in survival with EPIC alone or delayed postoperative intraperitoneal chemotherapy [Figure 2].

**Table 1. Reports of patients with gastric peritoneal metastases treated by cytoreductive surgery and hyperthermic intraperitoneal chemotherapy**

References	Year	No. of patients	Anticancer agent during HIPEC	Median survival (months)	1-year survival (%)	3-year survival (%)	5-year survival (%)
Fujimoto <i>et al.</i> <sup>[20]</sup>	1997	48	MMC	16	54	41	31
Hirose <i>et al.</i> <sup>[38]</sup>	1999	17	MMC-cisplatin-etoposide	11	44	--	--
Rossi <i>et al.</i> <sup>[39]</sup>	2003	13	MMC-cisplatin	15	--	--	--
Glehen <i>et al.</i> <sup>[40]</sup>	2004	49	MMC	10.3	48	--	16
CC-0 or CC-1		25		21.3	74.8	--	29.4
Hall <i>et al.</i> <sup>[34]</sup> CC-0	2004	34	MMC	--	--	--	--
				11.2	45		
Yonemura <i>et al.</i> <sup>[32]</sup> CC-0	2005	107	MMC-cisplatin-etoposide	11.5	--	--	6.5
		47		15.5	--	--	27
Scaringi <i>et al.</i> <sup>[41]</sup> CC-0	2008	32	MMC-cisplatin	6.6	--	--	--
		8		15			
Glehen <i>et al.</i> <sup>[33]</sup> CC-0*	2010	159	Various	9.2	43	18	13
		85		15	61	30	23

From Sugarbaker *et al.*<sup>[43]</sup> with permission. CC-0: complete macroscopic cytoreduction; CC-1: residual tumor nodules < 5 mm; MMC: mitomycin C; HIPEC: hyperthermic intraperitoneal chemotherapy

Although there may be a survival benefit, perioperative intraperitoneal chemotherapy can increase morbidities. Even the most experienced peritonectomy centers that remove all macroscopic disease and then administer intraperitoneal chemotherapy have a higher morbidity and cost<sup>[32-34]</sup>. Yan *et al.*<sup>[30]</sup> discussed an association of improved overall survival with HIPEC with or without EPIC after resection of advanced gastric primary cancer, however, with EPIC there was an associated greater risk for intraabdominal abscess ( $P = 0.003$ ) and neutropenia ( $P = 0.007$ ). Yu *et al.*<sup>[35]</sup> also saw an increased risk of intra-abdominal abscess with the use of EPIC compared to the control arm. Intraperitoneal chemotherapy does have less systemic toxicity as compared to systemic chemotherapy. Although individual studies did not show a significant difference in neutropenia between treatment arms, the meta-analysis demonstrated a significantly higher risk of neutropenia in the intraperitoneal chemotherapy arm<sup>[30]</sup>.

Most of the randomized studies were completed in Asia and it is unknown if they can be compared with disease in Western areas. Perioperative chemotherapy may be of greater benefit in Western patients with more advanced disease and less lymph nodes dissected. Data does suggest a role of HIPEC with or without EPIC to improve overall survival for advanced primary gastric cancer with advanced T-stage and no peritoneal metastases. A prospective multi-institutional randomized controlled trial with well-defined eligibility criteria, interventions and end-points is currently in progress in France (D2 resection ± HIPEC) in locally advanced gastric carcinoma, GASTRICHIP, ClinicalTrials.gov Identifier: NCT01882933.

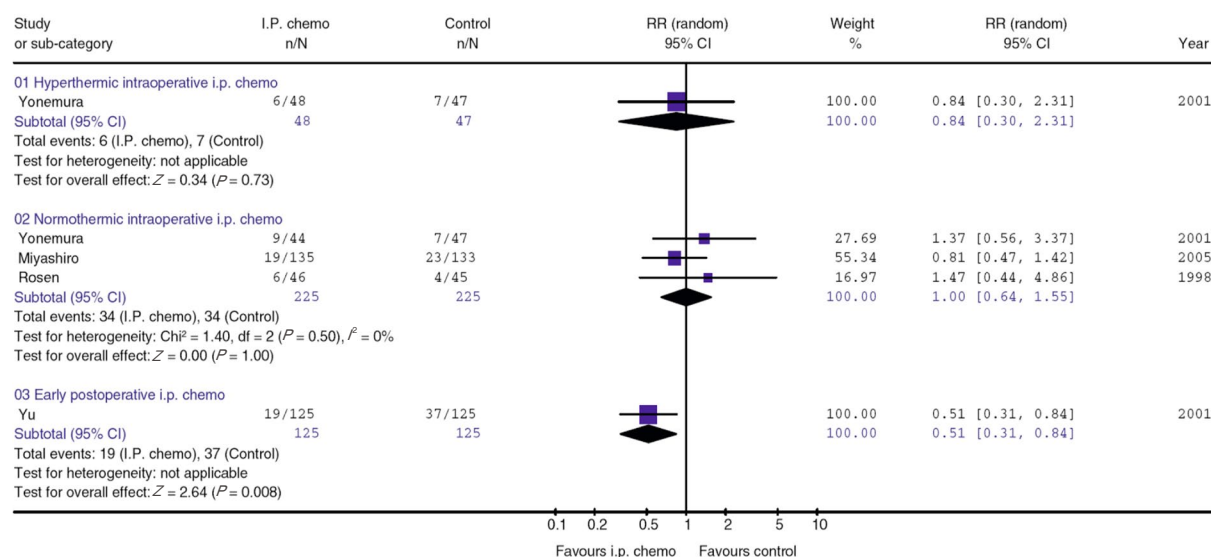
## TREATMENT PROTOCOLS FOR GASTRIC CANCER WITH PERITONEAL METASTASES

Gastric cancer with peritoneal metastases has been considered a terminal condition. Prospective studies had a median survival of less than 6 months<sup>[36]</sup>. Although response rates to systemic chemotherapy regimens have improved, there has not been a similar reflection in survival rates<sup>[37]</sup>. There may be some effective palliation of gastric cancer resections in patients with peritoneal metastases, however there is no long-term improvement in survival.

## CYTOREDUCTIVE SURGERY AND HIPEC AS AN EFFECTIVE STRATEGY

There is potential for long-term survival for patients with gastric cancer and peritoneal metastases with the combined use of CRS and HIPEC. There are single institutional data and phase II studies that support use of this strategy [Table 1]<sup>[31-34,38-41]</sup>. Glehen *et al.*<sup>[33]</sup> studied 159 patients with a median follow-up of 20.4 months. There was a median overall survival of 9.2 months but the 5-year survival rate was 13%. Although CRS and HIPEC in gastric cancer with peritoneal metastases is less effective than with other

Comparison: 01 Adjuvant intraperitoneal chemotherapy versus control  
Outcome: 02 Local-regional recurrence



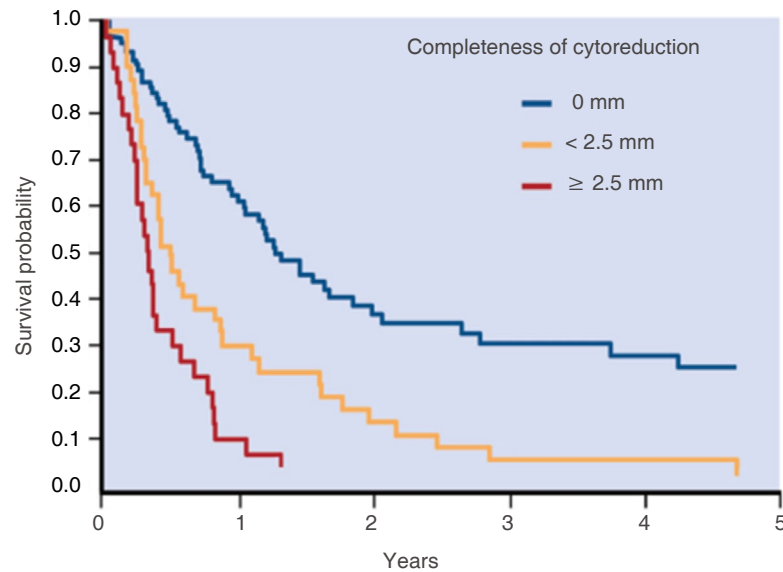
**Figure 2.** Forest plot of the relative risk (RR) of the local-regional recurrence with adjuvant intraperitoneal (IP) chemotherapy versus controls for advanced gastric cancer. The studies were analyzed according to the regimens of intraperitoneal chemotherapy used. The estimate of the RR of each individual trial corresponds to the middle of the squares and horizontal line gives the 95% confidence interval (CI). On each line, the numbers of events, expressed as a fraction of the total number randomized, are shown for both treatment groups. For each subgroup the sum of the statistics, along with the summary RR, is represented by the middle of the solid diamonds. A test of heterogeneity between the trials within a subgroup is given below the summary statistics. (From Yan *et al.*<sup>[30]</sup> with permission)

gastrointestinal malignancies, there were a few long-term survivors. Gastric cancer is a more aggressive disease. Gastric cancer patients with peritoneal metastases treated with CRS and HIPEC were the only patients that reported a 5-year survival<sup>[39-43]</sup>.

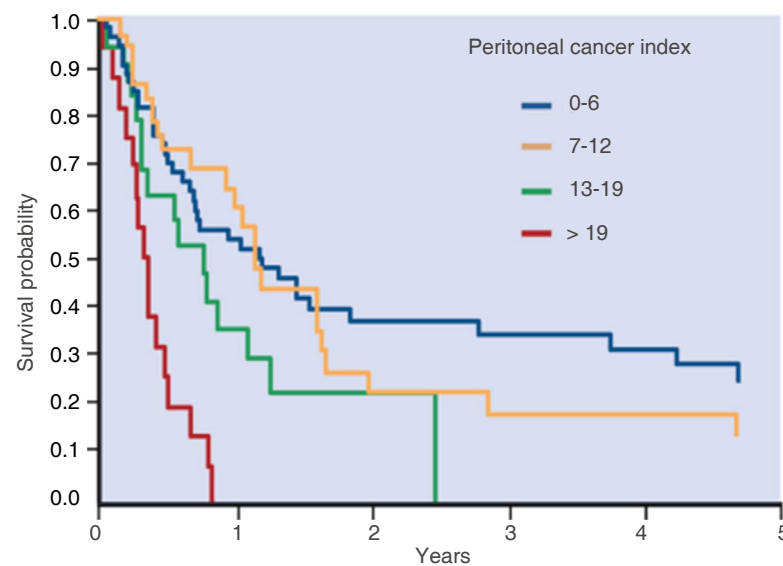
These studies have shown that strict patient selection criteria are necessary. The extent of peritoneal metastases as measured by Sugarbaker's peritoneal cancer index (PCI) significantly influences survival and is correlated with the completeness of cytoreduction<sup>[44]</sup>. Cytoreductive surgery must reduce the residual disease to a minimum for intraperitoneal chemotherapy to be effective (due to minimal chemotherapy penetration). Glehen *et al.*<sup>[33]</sup> demonstrated a 5-year survival of 23% with median survival of 15 months in patients after a complete macroscopic resection [Figure 3]. Yonemura *et al.*<sup>[45]</sup> demonstrated a similar 27% 5-year survival rate and 15.5-month median survival. Hall *et al.*<sup>[34]</sup> reported a 11.2-month overall survival after CRS and HIPEC with mitomycin C, however there was no patient alive after 2 years who had residual disease at CRS. CRS with no residual disease burden is essential for effective HIPEC. HIPEC with macroscopic disease burden does not improve survival more than 6 to 8 months. HIPEC can have morbidity and therefore should not be used for patients with bulky residual disease<sup>[46]</sup>. Palliative use for ascites may always be considered<sup>[45,47]</sup>.

Even if cytoreduction is incomplete, HIPEC is less useful for patients with high burden of peritoneal metastatic disease as measured by PCI. Glehen *et al.*<sup>[33]</sup> showed that one of the strongest prognostic factors was extent of carcinomatosis. When the PCI was greater than 12, despite a complete cytoreduction there were no survivors greater than 3 years [Figure 4]. Fujimoto *et al.*<sup>[20]</sup> reported 40%-50% 5-year survival for limited peritoneal metastases but only an 18% 1-year survival for patients with extensive peritoneal metastases. Cytoreduction with HIPEC in gastric cancer patients with a greater than 12 PCI score may be contraindicated.

Yang *et al.*<sup>[46]</sup> has provided the first and only phase III study regarding CRS and HIPEC in gastric cancer presenting with peritoneal metastases. They used cisplatin (120 mg) and mitomycin C (30 mg) in 6000 mL of normal saline at 43°C for 60-90 min. Median follow-up was 32 months and 97.1% (33 of 34) of patients



**Figure 3.** Overall survival of 159 patients treated by cytoreductive surgery and hyperthermic intraperitoneal chemotherapy according to completeness of cytoreductive surgery. (From Glehen *et al.*<sup>[33]</sup> with permission)



**Figure 4.** Overall survival of 159 patients treated by complete cytoreductive surgery according to extent of peritoneal metastases assessed by the peritoneal cancer index. (From Glehen *et al.*<sup>[33]</sup> with permission)

after CRS died as compared to 85.3% (29 of 34) of CRS with HIPEC patients who died. Median survival was 6.5 months (95% CI 4.8-8.2 months) after CRS as compared to 11 months (95% CI 10.0-11.9 months) in CRS with HIPEC group ( $P = 0.046$ ). There was similar morbidity between the groups. The independent predictors in a multivariate analysis for improved survival were synchronous peritoneal metastases, CC 0-1 cytoreduction, more than 6 cycles of systemic chemotherapy, and no adverse events. Glehen *et al.*<sup>[48]</sup> suggested that HIPEC should be reserved for patients with limited peritoneal carcinomatosis. Also, the prognostic factors analyzed by Yang *et al.*<sup>[46]</sup> suggests that it should be restricted to a limited patient population.

#### Role of laparoscopy in patient selection for CRS and HIPEC

Laparoscopy has been suggested as an important modality for selecting patients for aggressive treatments with CRS and HIPEC. Even today with high technology radiology studies, diagnostic laparoscopy remains



as an important tool to detect disease below a size threshold of approximately 1 cm<sup>[49]</sup>. If a gastric cancer patient is found to have macronodular small bowel disease or would otherwise not be able to be completely cytoreduced, HIPEC would not be warranted, and the morbidity of exploratory laparotomy could be avoided. Laparoscopy may establish that patients have very limited peritoneal metastases ( $PCI \leq 6$ ) and should be considered for CRS and HIPEC<sup>[50,51]</sup>. Recent randomized trials suggest that neoadjuvant chemotherapy should be used for gastric cancer patients free of peritoneal disease<sup>[52]</sup>. Laparoscopy may exclude patients with peritoneal metastases who would not benefit from aggressive neoadjuvant chemotherapy that is unlikely to improve their survival.

## NEOADJUVANT INTRAPERITONEAL AND SYSTEMIC CHEMOTHERAPY

In medically fit patients with gastric cancer with peritoneal metastases systemic chemotherapy may be recommended. Chemotherapy can provide palliation, improve survival, and improve quality of life compared to best supportive care in patients with metastatic disease. However, the benefits of systemic chemotherapy in gastric cancer patients with peritoneal metastases may be reduced when compared to metastatic disease at other sites. Preusser *et al.*<sup>[53]</sup> demonstrated that an aggressive systemic chemotherapy regimen can have a 50% response rate in advanced gastric cancer, however this less effective in patients with peritoneal metastases. Ajani *et al.*<sup>[54]</sup> used neoadjuvant chemotherapy and reported the failure of the regimen was most common in patients with peritoneal metastases. Systemic chemotherapy alone for primary gastric cancer with peritoneal metastases is a disappointing plan of management.

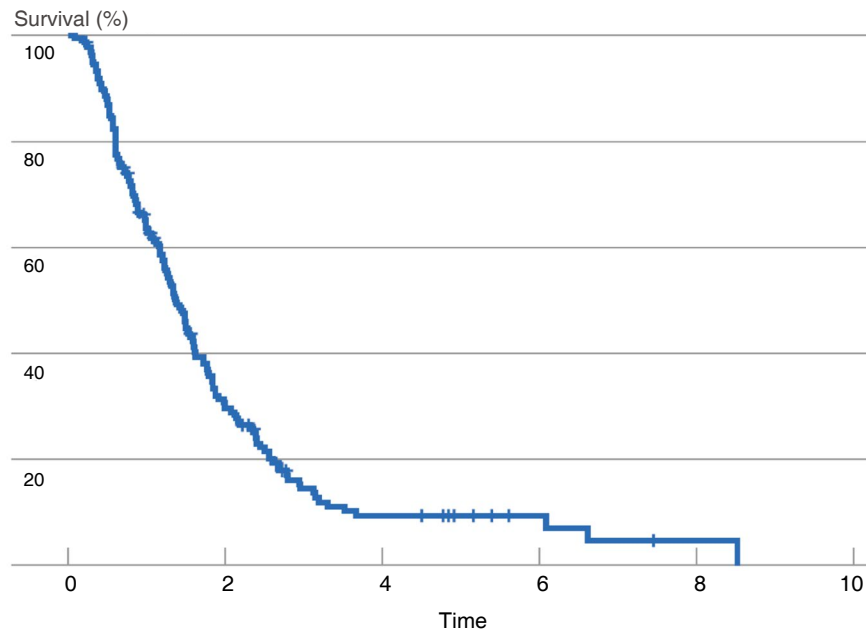
Neoadjuvant chemotherapy for gastric cancer can be modified for patients with peritoneal seeding by combining systemic and intraperitoneal chemotherapy. Chemotherapy may gain access to small peritoneal cancer nodules via the systemic circulation and by diffusion from a chemotherapy solution within the peritoneal cavity. Yonemura *et al.*<sup>[55]</sup> proposed a prospective phase II study to establish the efficacy and assess toxicities of NIPS chemotherapy in patients with gastric cancer with peritoneal metastases. They identified patients with peritoneal metastases by laparoscopy, laparotomy with biopsy or cytology from ascites. To qualify for NIPS, patients must have: (1) proven peritoneal seeding by histology or cytology; (2) no hematogenous or remote lymph node metastases; (3) be less than or equal to 65 years; (4) have an Eastern Clinical Oncology Group score of 2 or less; (5) adequate bone marrow, liver, cardiac, and renal function; and (6) no other severe medical comorbidities or synchronous malignancies.

Qualifying patients had a peritoneal port system (Bard Port, C.R. Bard Inc., USA) inserted into the abdominal cavity under local anesthesia with the tip placed within the cul-de-sac of Douglas.

### Chemotherapy regimen

Prior to administration of chemotherapy, 500 mL of saline was instilled into the peritoneal cavity and fluid was removed for cytology. Taxotere 40 mg and carboplatin 150 mg were used for intraperitoneal chemotherapy in addition to 1000 mL of saline over 30 min. Methotrexate 100 mg/m<sup>2</sup> and 5-fluorouracil 600 mg/m<sup>2</sup> in 100 mL of saline over 15 min were administered intravenously the same day. This regimen was administered weekly for two cycles. After the second cycle, peritoneal wash cytology was again performed. If cytology was positive, neoadjuvant chemotherapy was continued for 2 more cycles. Peritoneal cytology testing is repeating after the fourth cycle and the process is continued as long as cytology is positive.

If cytology became negative, upper endoscopy, laparoscopy and computed tomography scan was performed. If tumors showed no demonstrable change, then 2 more cycles were administered. The number of NIPS chemotherapy cycles was controlled by the effect on the primary cancer and peritoneal cytology. Complete cytoreduction was required for prolonged survival in prior studies that examined peritoneal metastases. Therefore, the goal of the NIPS regimen was complete or near complete response of metastases on small bowel surfaces [Figure 5].



**Figure 5.** Overall survival in gastric cancer patients with peritoneal carcinomatosis. (From Canbay *et al.*<sup>[58]</sup> with permission)

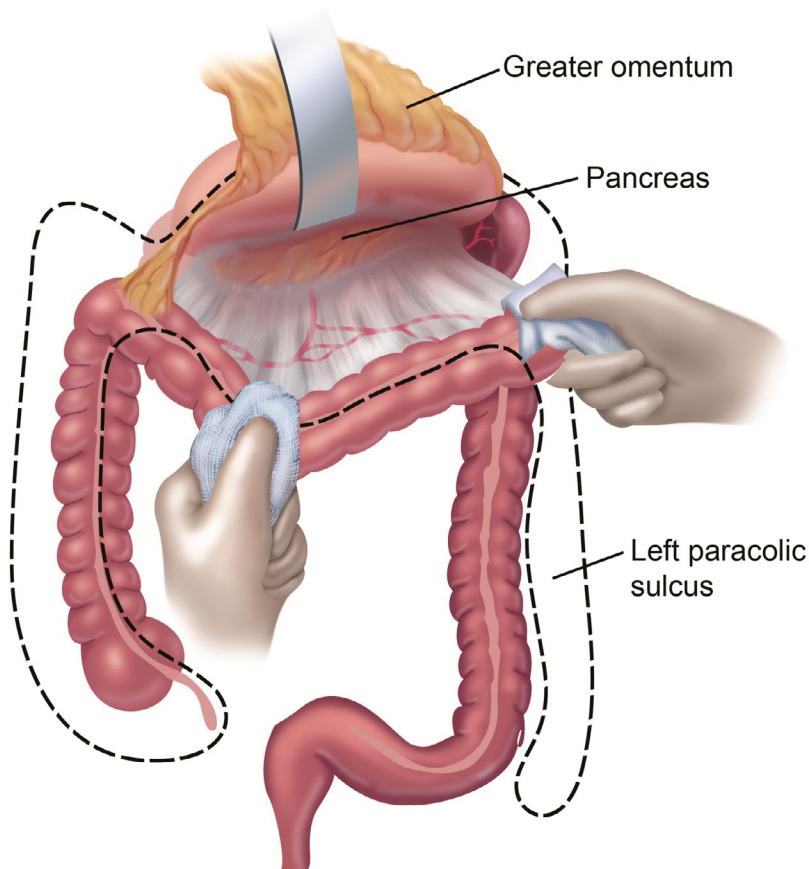
## SURGERY FOR GASTRIC CANCER WITH PERITONEAL METASTASES AFTER NIPS

Gastrectomy and peritonectomy were performed if peritoneal wash cytology became negative or there was a partial response to neoadjuvant chemotherapy. If peritoneal metastases on small bowel surfaces were eliminated by NIPS, there was a possibility that gastrectomy and parietal peritonectomy could achieve a complete cytoreduction. Patients with progressive disease or who continue to have positive cytology despite 4 to 6 cycles of NIPS were not candidates for surgery.

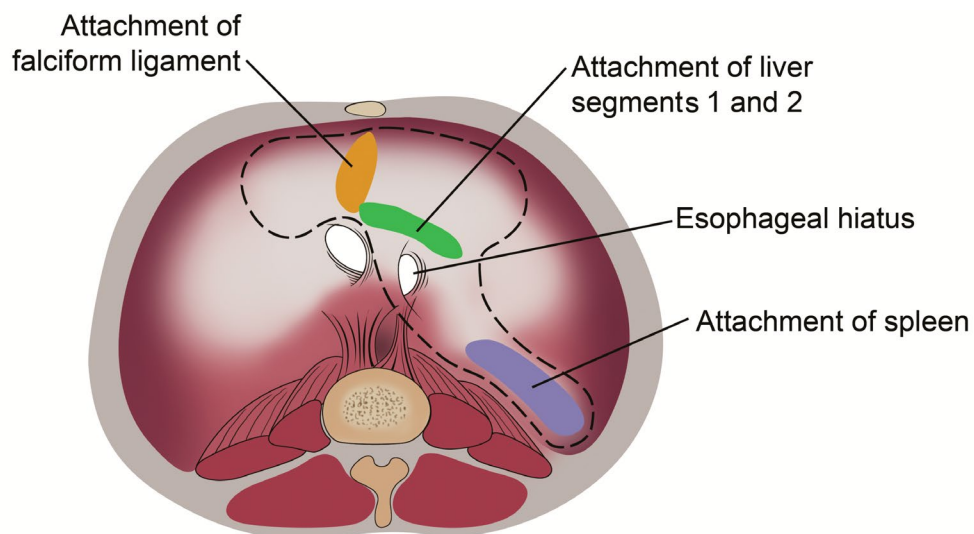
Sugarbaker<sup>[56]</sup> and Yonemura *et al.*<sup>[57]</sup> reported the use of peritonectomy for peritoneal metastases to cytoreduce the peritoneal surface and facilitate total resection of all disease associated with the primary gastric cancer. Peritonectomies required for gastric cancer have been described<sup>[7]</sup>. The epigastric peritonectomy includes any prior midline abdominal scar with the preperitoneal epigastric fat pad, xiphoid process, round and falciform ligaments. The anterolateral peritonectomy removes the greater omentum with the anterior layer of peritoneum from the transverse mesocolon, peritoneum of the right paracolic gutter along the appendix, and the peritoneum in the right subhepatic space. Sometimes the peritoneum of the right and left paracolic gutter must also be removed [Figure 6]. The subphrenic peritonectomy takes the peritoneal surfaces from the medial half of the right and left hemidiaphragm as well as the left triangular ligament [Figure 7]. The omental bursa peritonectomy starts with cholecystectomy and then removes the peritoneal covering of the porta hepatis, hepatoduodenal ligament, and floor of the omental bursa including the peritoneum overlying the pancreas [Figure 8]. If tumor was within the cul-de-sac, a pelvic peritonectomy was also performed and electroevaporative surgery strips the peritoneum from the pouch of Douglas. Sometimes, the pelvic peritonectomy will necessitate removal of the rectosigmoid colon [Figure 9]. Some or all of these visceral resections and parietal peritonectomies were performed to completely remove visible disease.

## RESULTS AFTER NIPS

Canbay *et al.*<sup>[58]</sup> analyzed 194 patients treated by NIPS. Average age was  $51.5 \pm 12.6$  years. One hundred and four patients had primary gastric cancer and 90 patients had recurrent PM. Peritoneal fluid cytology was

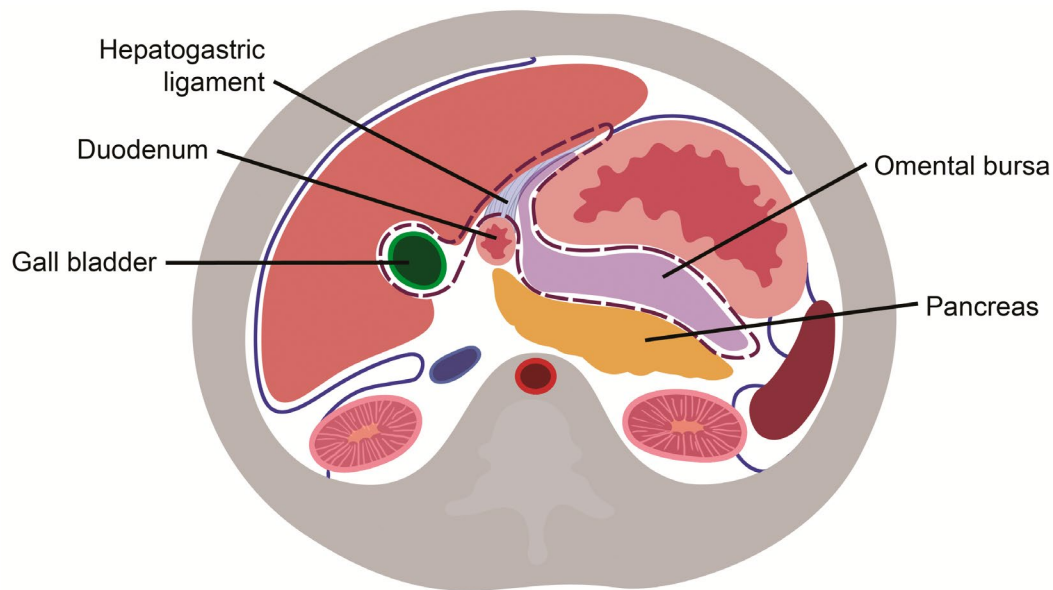


**Figure 6.** Anterolateral peritonectomy (From Sugarbaker *et al.*<sup>[7]</sup> with permission)

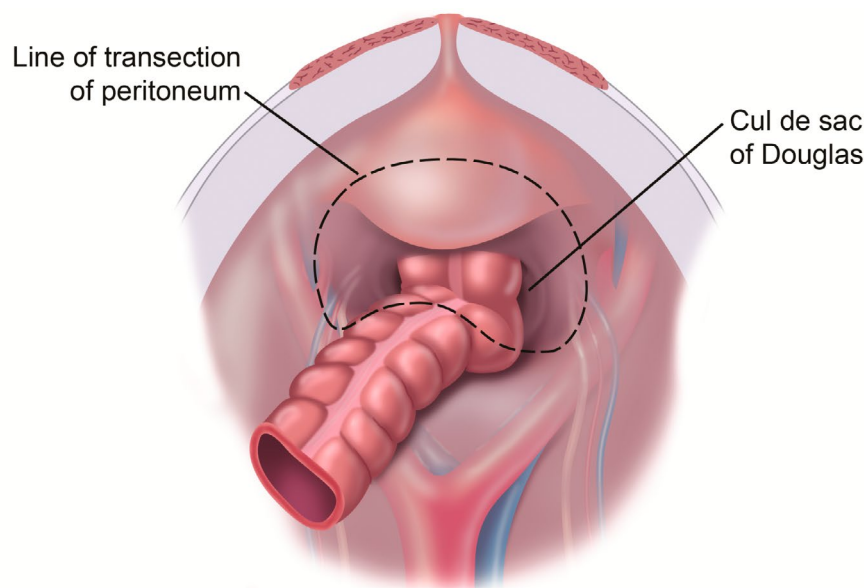


**Figure 7.** Subphrenic peritonectomy (From Sugarbaker *et al.*<sup>[7]</sup> with permission)

positive in 137 patients prior to NIPS chemotherapy and 152 patients were negative cytology after treatment. Complete or near-complete response was seen in 51 patients (26%). After induction treatment, 152 (78.3%) patients who showed negative cytology underwent CRS and HIPEC.



**Figure 8.** Omental bursa peritonectomy (From Sugarbaker *et al.*<sup>[7]</sup> with permission)



**Figure 9.** Pelvic peritonectomy (From Sugarbaker *et al.*<sup>[7]</sup> with permission)

Operative interventions were total gastrectomy ( $n = 94$ ), subtotal colectomy ( $n = 68$ ), small bowel resection ( $n = 44$ ). Left and right subdiaphragmatic peritonectomy and pelvic peritonectomy was complete in 44, 31, and 61 patients, respectively. In 102 (67.7%) patients, of the 152 cytoreductions a complete cytoreduction was recorded.

Figure 5 demonstrates survival of the 194 patients. Median survival was 18 months for all 194 patients. For those who had received surgical intervention, median survival was 15.8 months vs. 9.7 months for patients who did not have an operation. There was a significant survival difference ( $P < 0.001$ ,  $Z = 20.98$ ) between patients who underwent operative intervention vs. those who did not. There was a higher median survival of 20.5 months for patients who received a complete cytoreduction vs. 10.9 months for those who did not have a complete cytoreduction. There was no difference between primary and recurrent disease after cytoreduction

with a median survival of 18.0 vs. 17.4 months, respectively. If patients did not receive an operation, median survival was similar for primary and recurrent disease as well, 9.6 vs. 8.2 months, respectively.

Another effort to use neoadjuvant intraperitoneal chemotherapy to control peritoneal metastases prior to gastrectomy was presented by Kitayama *et al.*<sup>[59]</sup>. They used a combination of intraperitoneal and intravenous paclitaxel along with S-1. Repeated laparoscopy was performed to assess response and gastrectomy was used selectively on patients who showed shrinkage of their peritoneal nodules as well as negative peritoneal cytology at a repeat laparoscopy. After a median number of chemotherapy cycles of 5, gastrectomy was performed in 34 of the 64 patients. Sixty-five percent of these patients had an R0 resection. Median survival time and 1-year overall survival of the gastrectomized patients was 26.4 months and 82%, respectively. Those 30 patients who did not receive gastrectomy had a median survival of 12.1 months and a 26% 1-year survival. Kitayama *et al.*<sup>[59]</sup> concluded that salvage gastrectomy after intravenous and intraperitoneal paclitaxel was promising even for patients with gastric cancer and peritoneal metastases with ascites.

Fujiwara *et al.*<sup>[60]</sup> reported on 18 patients with primary gastric cancer and peritoneal metastases treated with NIPS. After combined intraperitoneal and systemic chemotherapy, 14 patients showed negative peritoneal cytology and no macroscopic peritoneal metastases. The median survival time of his entire group was 24.6 months and there was no treatment-related mortality.

#### **Neoadjuvant systemic chemotherapy vs. NIPS to date**

Clinical trials comparing the beneficial effects of systemic chemotherapy using modern regimens versus NIPS chemotherapy have not occurred. No doubt, in both treatments, those patients who have a resolution of their peritoneal metastases and then go on to have a successful R0 gastrectomy have a superior outcome. Al-Batran *et al.*<sup>[61]</sup> used neoadjuvant systemic chemotherapy followed by surgical resection in patients with limited metastatic gastric or gastroesophageal junction cancer. A small number (4 of 60, 6.7%) had peritoneal metastases as an isolated site of metastatic disease. Nevertheless, the strategy of neoadjuvant systemic chemotherapy prior to resection of all clinical evidence of disease was similar to the NIPS strategy. In their arm B, 36 of 60 (60%) of patients proceeded to surgery. Overall survival of the patients who proceeded to surgery was 31.3 months and 15.9 months for the other patients. These results are similar to the benefits of NIPS followed by cytoreductive surgery. Comparative studies at some time in the future are indicated.

#### **Adverse events from NIPS and cytoreductive surgery**

The adverse events related to combined therapies NIPS, cytoreductive surgery and then HIPEC may be less than that anticipated for a complex treatment that requires up to 6 months for completion. Problems with the intraperitoneal port are much less than in prior reports of long-term intraperitoneal chemotherapy for ovarian cancer<sup>[62]</sup>. In this report there were many catheter-related complications, most of which were caused by the extensive peritoneal adhesions. The intraperitoneal ports were placed after a major surgical intervention and only 42% of patients completed all 6 cycles of intraperitoneal chemotherapy. In contrast, catheter-related complications were rare in patients having NIPES because the ports were placed prior to any surgical intervention. Adverse effects grade 3 and 4 were reported in 9% of patients in the multi-institutional study reported by Yonemura *et al.*<sup>[63]</sup> in 2012. All of these side effects were from chemotherapy and not catheter-related.

In the 194 patients reported by Canbay *et al.*<sup>[58]</sup> in 2014, the most common chemotherapy-related grade 3 or 4 adverse events were bone marrow suppression and diarrhea. Bone marrow suppression occurred after 3 courses in 3 patients, after 5 courses in 3 patients, and after 6 courses in 4 patients. Less common adverse events were port site infection ( $n = 2$ ) and renal failure ( $n = 1$ ).

Prior reports of extensive cytoreductive surgery plus HIPEC following multiple cycles of intraoperative chemotherapy showed an increased morbidity primarily a result of fistula<sup>[64]</sup>. In the multi-institution report



of NIPS, cytoreductive surgery plus HIPEC, the grade 3 and 4 complications were 21% and mortality was 3.7%. These morbidity and mortality statistics are approximately the same as reported for cytoreductive surgery plus HIPEC in the absence of NIPS<sup>[65,66]</sup>.

### **Palliative benefits to all patients with cancerous ascites**

In the publication by Canbay *et al.*<sup>[58]</sup>, there was improvement in symptoms for the 78 patients who had ascites. These benefits occurred in patients with primary gastric cancer and also in patients with recurrent disease. Cunliffe<sup>[67]</sup> hypothesized that peritoneal metastases are nourished via ascites as well as blood supply. Therefore, peritoneal implants should be treated via a combined intraperitoneal and intravenous approach. Intravenous chemotherapy has minimal effects on peritoneal metastases and intraperitoneal chemotherapy alone has a less than 30% effect on ascites<sup>[34-36,53,54]</sup>. The bidirectional chemotherapy (intraperitoneal and intravenous) have a response rate of 57% with 100% resolution of ascites.

### **Chemotherapy agents selected for NIPS**

According to the study by Morgan *et al.*<sup>[68]</sup>, the maximum tolerated dose (MTD) of intraperitoneal taxotere is 125 mg/m<sup>2</sup> with no grade 3 or 4 toxicities at doses below 80 mg/m<sup>2</sup>. Fushida *et al.*<sup>[69]</sup> showed an absence of hematological toxicities after intraperitoneal taxotere at 45 mg/m<sup>2</sup> given once per week. The MTD of intraperitoneal carboplatin is 500 mg/m<sup>2</sup> and 300 mg/m<sup>2</sup> dose was reported as safe in Japanese ovarian cancer patients<sup>[70,71]</sup>. This study safely used taxotere 40 mg/m<sup>2</sup> and carboplatin 150 mg/m<sup>2</sup> combined. The combined use of systemic and intraperitoneal chemotherapy had no deaths and reasonable morbidity and was effective for ascites.

In summary, NIPS should be considered in gastric cancer patients with peritoneal metastases. It has maximal benefits for small volumes of peritoneal surface metastases and is reliable treatment for symptomatic ascites. Bidirectional chemotherapy may be the preferred strategy for preoperative chemotherapy of gastric carcinomatosis.

### **Management of primary gastric cancer with positive peritoneal cytology**

The survival of primary gastric cancer patients with positive peritoneal cytology in the absence of macroscopic peritoneal dissemination is very nearly the same<sup>[72]</sup>. The 5-year survival rate of cytology-positive but peritoneal metastases negative patients is 2%. Coccolini *et al.*<sup>[73]</sup> performed a systematic review and meta-analysis concerning the effects of intraperitoneal chemotherapy and peritoneal lavage on this group of patients. Coccolini *et al.*<sup>[73]</sup> concluded that 2- and 5-year overall survival in patients with free cancer cells without carcinosis is increased by intraperitoneal chemotherapy. Peritoneal lavage further increases these survival rates and also it further decreases the peritoneal recurrence rate.

Shimada *et al.*<sup>[74]</sup> in 2001 reported in a comparative non-randomized trial about the effects of intraoperative peritoneal lavage either associated or not with intraperitoneal chemotherapy for gastric cancer with free peritoneal cancer cells. Patients treated by intraoperative peritoneal lavage followed by intraperitoneal chemotherapy showed improved survival compared to patients treated by surgery alone or by surgery plus intraperitoneal lavage.

Kuramoto *et al.*<sup>[75]</sup> reported the results of a comparison between three groups of patients (total 88 patients) with advanced gastric cancer with positive cytology at peritoneal lavage but without peritoneal metastases undergone to surgical resection alone or surgical resection associated either to intraperitoneal chemotherapy or to intraperitoneal chemotherapy plus peritoneal lavage. The 5-year survival rate of the patients who had intraperitoneal chemotherapy plus peritoneal lavage was 43.8%, in the intraperitoneal chemotherapy group was 4.6% and in the surgery alone group was 0% ( $P = 0.0001$ ). The median survival time of the intraperitoneal chemotherapy plus peritoneal lavage group, intraperitoneal chemotherapy group, and surgery alone group were 35, 16, and 15 months, respectively. The multivariate analysis showed that the peritoneal lavage is the only significant factor affecting the prognosis.

## FUTURE PROSPECTS

Beyond the scope of this view are many promising new directions for prevention and treatment of peritoneal metastases from gastric cancer. Targeted therapies may be of great value for subsets of patients, such as those who are HER2-positive (ToGA Study)<sup>[76]</sup>. Also, pressurized intraperitoneal aerosol chemotherapy may substitute for NIPS in preparing patients for subsequent potentially curative resection of all clinically evident disease<sup>[77]</sup>. As these new treatments develop controlled trials comparing the new strategies will be necessary.

## DECLARATIONS

### Authors' contributions

Sugarbaker PH contributed solely to the paper.

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### Conflicts of interest

There are no conflicts of interest.

### Patient consent

Not applicable.

### Ethics approval

Not applicable.

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Original Article

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# Gastric cancer treated with pressurized intraperitoneal aerosol chemotherapy: revising an option for peritoneal carcinomatosis

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## Abstract

**Aim:** Gastric cancer is the cancer with the highest rate of peritoneal metastatization and this type of spread is associated with a higher death rate compared to distant organ metastasis. The systemic chemotherapy has a minimal effect in peritoneal metastasis so new types of treatment have emerged. The authors revised the main studies done in pressurized intraperitoneal aerosol chemotherapy (PIPAC) and presented the main conclusions.

**Methods:** A PubMed search was conducted focusing on PIPAC in gastric cancer. The MeSH database was searched with the terms: "Gastric cancer [MeSH] and intraperitoneal aerosol chemotherapy".

**Results:** Seven studies were analyzed. All the studies performed the technique with aerosol of doxorubicin and cisplatin. All cases were well tolerated, with minor adverse effects. Patients presented resolution of their abdominal symptoms and regression of macroscopic carcinomatosis. Cytoreductive surgery or hyperthermic intraperitoneal chemotherapy could be performed in some patients with good response to PIPAC. The peritonitis caused by the chemotherapy was well tolerated.

**Conclusion:** PIPAC can induce remission in end-stage and resistant disease with acceptable side effects, good safety levels for patients and health professionals, and quality of life improvement.



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**Keywords:** Pressurized intraperitoneal aerosol chemotherapy, gastric cancer, peritoneal carcinomatosis

## INTRODUCTION

Gastric cancer accounts for 6.8% of all cancers and it is the fifth most common cancer worldwide. Moreover, it is the third leading cause of death associated with cancer<sup>[1]</sup>. Gastric cancer has three ways to spread through the body: neoplastic cells could use the lymphatic system to spread to the lymph nodes, the blood stream to spread to distant organs, and the dissemination to peritoneal cavity. This last type of spread is called peritoneal metastatization. Gastric cancer is the cancer with the highest rate of peritoneal metastization and this type of spread is associated with a higher death rate compared to distant organ metastasis<sup>[2]</sup>. Without treatment, the median survival of these patients is 3-5 months.

Gastrectomy combined with D2 lymph node dissection remains the standard of care to manage gastric cancer in advanced stages, however, peritoneal metastases still needs to be optimized. The systemic chemotherapy has a minimal effect in peritoneal metastasis because the barrier between blood and peritoneum do not allow a high concentration of drug in the peritoneum<sup>[3]</sup>. An alternative to systemic chemotherapy consists in surgical removal of affected tissue combined with perioperative chemotherapy that includes: extensive intraoperative peritoneal lavage, neoadjuvant intraperitoneal/systemic chemotherapy, hypertermic intraperitoneal chemotherapy (HIPEC), laparoscopic HIPEC and early postoperative intraperitoneal chemotherapy. The problems with these techniques are the need of complete cytoreduction in surgery and they are appropriate only for selected patients<sup>[4]</sup>. Moreover, this treatment is hindered by significant risks and side effects with a 30-day mortality rate of 5% in referral centers<sup>[5]</sup>.

Recently, a new alternative therapy has emerged: pressurized intraperitoneal aerosol chemotherapy (PIPAC). This method can only be applied by laparoscopy and it is performed under general anesthesia. In this case, the chemotherapy is dispersed as pressurized aerosol into the peritoneal cavity by minimal invasive techniques, and left acting during 30 min. After this time, the gas is aspired. The recommendation is 3 applications within 3 months. The most frequent adverse effects are fever, abdominal pain and nausea. Complications like infections, herniation or adhesion are uncommon due to minimally-invasive procedure [Figure 1].

## METHODS

A PubMed search was conducted focusing on PIPAC in gastric cancer. The MeSH database was searched with the terms: "Gastric cancer [MeSH] and intraperitoneal aerosol chemotherapy".

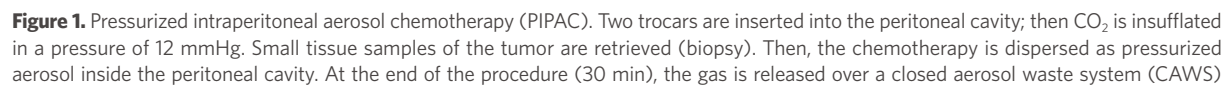
A total of 5 articles were collected. One study was excluded because it is written in Chinese. Then, 3 articles were added because they were recent and pertinent. Ultimately, 7 studies were included in the analysis.

## RESULTS

The main results of the studies are listed in Table 1<sup>[6-12]</sup>.

Nadiradze *et al.*<sup>[6]</sup> demonstrated that PIPAC is well tolerated but has no effect in patients with synchronous malignant pleural effusion. Twenty-four patients were included in the study, and 60 PIPAC were performed: 71% of the patients had repeated the procedure; no procedure-related mortality was reported; the mean survival time was 15.4 months; and objective tumor response was observed in 50% of the patients.

Hübner *et al.*<sup>[7]</sup> had used as exclusion criteria for PIPAC the thrombosis of portal vein, intestinal occlusion and some clinical condition that could be a contra-indication for capnoperitoneum. Fifty-eight patients



Tempfer<sup>[8]</sup> reported some studies, only one with gastric cancer patients. The main emphasis was ovarian cancer. The reported gastric cancer patient was the same patient described in Solass *et al.*<sup>[9]</sup> study.

Teixeira Farinha *et al.*<sup>[10]</sup> followed 42 patients that underwent PIPAC and evaluated their quality of life during the treatment time and main symptoms.

Alyami *et al.*<sup>[12]</sup> evaluated the postoperative outcome of 164 procedures of PIPAC using the peritoneal cancer index.

Intraperitoneal chemotherapy is associated to local toxicity due to high drug concentration in peritoneal cavity and the repeated delivery, which leads to chemical peritonitis and a systemic inflammatory response.

**Table 1. Main conclusions of the studies**

Authors	Year	Patients	Aerosol	Conclusion
Nadiradze <i>et al.</i> <sup>[6]</sup>	2016	24 patients with peritoneal metastases from gastric cancer resistant to systemic chemotherapy and with no option for cytoreductive surgery and HIPEC	Doxorubicin 1.5 mg/m <sup>2</sup> followed by cisplatin 7.5 mg/m <sup>2</sup>	Follow-up: 248 days; median survival time: 15.4 months; survival after follow-up time: 13 patients; objective tumor response in 12 patients; complete histological regression in 6 patients
Hübner <i>et al.</i> <sup>[7]</sup>	2017	58 patients with peritoneal disease from digestive cancer that was persistent or progressive after prior standard surgical and/or medical treatment	Doxorubicin 1.5 mg/m <sup>2</sup> in combination with cisplatin 7.5 mg/m <sup>2</sup>	Intraoperative event rate: 11%; deaths after the procedure: 1 patient
Tempfer <sup>[8]</sup>	2015	1 patient with peritoneal disease from gastric cancer after gastrectomy and 2 chemotherapy lines	Doxorubicin 1.5 mg/m <sup>2</sup> with cisplatin 7.5 mg/m <sup>2</sup>	Survival of 109 days; the patient developed liver and bone metastases but with no evidence of peritoneal metastases
Solass <i>et al.</i> <sup>[9]</sup>	2014			PIPAC had no negative impact on patients' overall quality of life or in main symptoms; there was no worse quality of life in PIPAC patients with high intraperitoneal tumor load
Teixeira Farinha <i>et al.</i> <sup>[10]</sup>	2017	42 patients: 21 patients with chemoresistant isolated peritoneal carcinomatosis from gynecological origin, 14 patients from colorectal origin and 3 from gastric origin	Not mentioned	7 patients obtained objective radiological tumor regression; 8 patients obtained objective major histological regression
Girshally <i>et al.</i> <sup>[11]</sup>	2016	9 patients with advanced peritoneal disease no candidates for primary cytoreductive surgery and HIPEC	Doxorubicin 1.5 mg/m <sup>2</sup> followed by cisplatin 7.5 mg/m <sup>2</sup>	63.5% of patients presented complete regression of symptoms; peritoneal cancer index improved in 64.5% of patients
Alyami <i>et al.</i> <sup>[12]</sup>	2017	73 patients with non-resectable peritoneal carcinomatosis (26 from gastric cancer)	Cisplatin 7.5 mg/m <sup>2</sup> followed by doxorubicin 1.5 mg/m <sup>2</sup>	

PIPAC: pressurized intraperitoneal aerosol chemotherapy; HIPEC: hyperthermic intraperitoneal chemotherapy

No significant renal toxicity was documented in these studies, however a low-grade liver toxicity was reported in a quarter of patients in Nadiradze *et al.*<sup>[6]</sup> study.

Hübner *et al.*<sup>[7]</sup> concluded that no learning curve was observed because the operation time did not decrease over time. Some minor complications were observed during this study such as constipation, ileus, transitory neutropenia, urinary retention and wound complications. Looking to these effects, the procedure seems to be safe. Only one patient died due to cardiac arrhythmia.

Tempfer *et al.*<sup>[8]</sup> reported that delivering chemotherapy as an aerosol did not represent a risk to health care workers so, it could be used safely in the clinical setting. Moreover, the quality of life improved over 5-6 months.

Solass *et al.*<sup>[9]</sup> achieved 2 complete remissions and all 3 cases had tumor response. The mean survival of the 3 patients was 288 days, and the gastric cancer patients died 109 days after the procedure.

Teixeira Farinha *et al.*<sup>[10]</sup> concluded that PIPAC had no undesirable impact on quality of life of patients with peritoneal carcinomatosis. A shorter hospital stay was associated with patients with better scores at baseline in quality of life. Nondigestive and digestive symptoms remained unchanged after repeated treatments.

Girshally *et al.*<sup>[11]</sup> concluded that patients with extensive peritoneal disease that were treated with PIPAC as neoadjuvant therapy had worse prognosis than those treated primarily with cytoreductive surgery and HIPEC in limited disease. However, when the cytoreductive surgery and HIPEC were not possible due to extensive disease, PIPAC was successful in diminishing the tumor burden and allowed forward procedures.

Alyami *et al.*<sup>[12]</sup> found that symptoms related to peritoneal carcinomatosis like ascites, pain or transit disorders were decreased during PIPAC. Some major complications occurred in 9.7% of the patients and 5 died within 30 days of the PIPAC procedure.

Searching in the ClinicalTrials.gov registry, we found 1 clinical trial completed in Germany (PIPAC-GA01), but 4 more trials recruiting: 1 in Italy, 1 in Singapore, 1 in Germany and the last 1 in 14 countries. PIPAC-GA01 is a clinical trial with 35 patients where cisplatin and doxorubicin will be applied in 3 single doses in 6 weeks intervals. The safety and efficacy in terms of the clinical benefit rate will be accessed, but no results were published yet.

At this stage, it is not possible to define indications and contraindications for PIPAC. The authors think that this method could be a good option for patients who have done systemic chemotherapy with severe side effects, patients with renal failure, hepatic failure or patients with cardiac toxicity. On the other hand, it is no option for patients with end-stage disease or malignant pleural effusion.

PIPAC was tested mainly in ovarian cancer, gastric cancer and colon cancer, and it seems feasible in most patients with refractory carcinomatosis of various origins. There were no consistent studies comparing what type of cancer will benefit the most with this technique.

This procedure could be a new palliative treatment option because it may increase quality of life. The next step should be the appliance of this technique in patients in an early stage of peritoneal carcinomatosis to access the efficacy.

## DECLARATIONS

### Authors' contributions

Concept and design of the review, article analysis and interpretation: all authors

Drafting the manuscript: Macedo F, Ladeira K

Manuscript revising: Longatto-Filho A, Martins SF

Approved the final version to be published: all authors

### Data source and availability

All the information analysed during the current study are available in the Pubmed Repository (<https://www.ncbi.nlm.nih.gov/pubmed/>).

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None.

### Conflicts of interest

There are no conflicts of interest.

### Patient consent

Not applicable.

### Ethics approval

Not applicable.

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Review

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# Therapeutic targets against gastric cancer stem cells interacting with tumor microenvironment

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## Abstract

Gastric cancer (GC) is a major cause of cancer-related deaths worldwide. The existence of cancer stem cells (CSCs) is known to be the main reason for resistance to anticancer agents as well as for the development of distant metastases. Although CSCs themselves harbor self-renewal and differentiation abilities, the tumor microenvironment that surrounds CSCs provides secreted factors and supports angiogenesis and is thus responsible for the maintenance of their CSC properties. The current review provides information regarding the impact of the tumor microenvironment on gastric CSCs, which will support the development of novel therapeutic strategies for targeting gastric CSCs.

**Keywords:** Gastric cancer stem cells, stem cell markers, tumor microenvironment, gastric cancer treatment

## INTRODUCTION

Although the proportion of individuals with gastric cancer (GC) has declined for decades, GC continues to be a major cause of cancer-related deaths worldwide<sup>[1-3]</sup>. Despite improvements in the treatment of GC, the clinical outcome of patients with advanced GC after curative resection is still poor, which is mainly due to recurrence and metastasis<sup>[4]</sup>. Therefore, new treatment options for this disease must be developed.

Recent evidence has increasingly indicated that the heterogeneity of the tumor is a consequence of cancer stem cells (CSCs), which are deeply involved in tumor progression and metastasis<sup>[5-7]</sup>. Malignant tumors have been reported to exhibit obvious histologic heterogeneity. In 1937, Furth *et al.*<sup>[8]</sup> demonstrated that a single leukemia cell could cause systemic disease in recipient mice. However, it took a long time for the concept of



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CSCs to be widely recognized. CSCs of acute myelogenous leukemia (AML) were first identified by Bonnet and Dick<sup>[9]</sup> in 1997, and they also determined that the CD34+ CD38- fraction of AML tumor cells enhances tumorigenicity after continuous transplant into immunodeficient mice. CSCs have subsequently been found in various types of solid tumors<sup>[10-12]</sup>. Gastric CSCs (GCSCs) have been vigorously investigated in studies using GC cell lines and primary GC tissues<sup>[13-15]</sup>.

The current review provides recent evidence for the regulation of GCSCs in the tumor microenvironment and for GCSC-targeted treatments.

## MARKERS OF GCSCS

### CD44

CD44 was first identified as a potential GCSC marker in a study using GC cell lines. The CD44-positive fraction in these GC cell lines showed the ability to form spheroids *in vitro* and demonstrated tumorigenicity *in vivo* when injected into the stomach wall or when injected subcutaneously into immunodeficient mice<sup>[16]</sup>. Furthermore, a combination of the cell surface markers CD44 and CD24 has been examined in GC cell lines and primary GC tissues from five patients using fluorescence-activated cell sorting. The authors of that study found that the CD44+/CD24+ fraction demonstrated a higher tumorigenicity compared with the CD44-/CD24- fraction when injected into immunodeficient mice. Therefore, not only do these cells have the ability to self-replicate and produce differentiated offspring, the combined expression of CD44+/CD24+ acts as a putative GCSC marker<sup>[17]</sup>. CSCs were isolated from the peripheral blood of GC patients using the cell surface markers CD44 and CD54, and tumors similar to the original human tumor were generated when the cells were injected into immunodeficient mice. The same cells differentiated into gastric epithelial cells *in vitro* and self-renewed *in vivo* and *in vitro*. These results suggest that the combination of CD44+/CD54+ can also be used as a potential cell surface marker for GCSCs<sup>[18]</sup>. Epithelial cell adhesion molecule (EpCAM) and CD44 have also been identified as CSC markers in various types of tumors. The EpCAM+/CD44+ fraction from human GC tissues grew into tumors in immunodeficient mice, maintained a differentiated phenotype and reproduced the morphological and phenotypical heterogeneities of the original gastric tumors. These cells acquired greater tolerance to anticancer agents than other subtypes of cells<sup>[19]</sup>.

### Lgr5

Lgr5 has received substantial attention as a new GCSC marker. Initially, Lgr5 was identified in stem cells within hair follicles, the small intestine, large intestine and stomach<sup>[20,21]</sup>. Lgr5+ stem cells in the intestinal crypts are interspersed among terminally differentiated Paneth cells, which act as guardians of the stem cells by providing essential niche signals<sup>[22]</sup>, but the role of Lgr5+ cells in the stomach is not fully understood. In addition, Notch signaling regulates gastric antral Lgr5 stem cell function. An analysis of gastric organoids revealed that Notch signaling is intrinsic to the epithelium and that it regulates growth. Furthermore, in one study, *in vivo* Notch manipulation affected the efficiency of organoid initiation from glands and single Lgr5-GFP stem cells, which indicates the regulation of stem cell function by Notch. Moreover, the authors of that study showed that, compared with control stem cells, stem cells in which Notch signaling was activated competed more effectively for niche spots, as they rapidly spread within the stem cell niche<sup>[23]</sup>. More recently, Lgr5-positive chief cells were defined as a major cell-of-origin of gastric cancer. That study revealed Lgr5 expression in a subpopulation of chief cells in mouse and human corpus glands. Using a non-variegated Lgr5-2A-CreERT2 mouse model, the authors demonstrated that the division of these Lgr5-positive cells depended on the occurrence of Wnt signaling at the time of injury. It has become clear that Lgr5-positive cells generate all the cells that form the stomach tissue and that they are able to repair wounds within the stomach. Additionally, it was also found that gastric cancer developed when cancer-associated genes were activated in Lgr5-positive stem cells. This suggests that tissue stem cells are necessary for the repair and regeneration of the injured stomach might change to CSCs<sup>[24]</sup>. As described above, LGR5 acts as a GCSC marker of gastric cancer progression.

### CD133

One study examined the expression of three putative CSC markers, including ATP-binding cassette sub-family B member 1, ATP-binding cassette sub-family G member 2, and CD133, in 90 human GC tissue samples and three human GC cell lines. The authors concluded that the expression levels of these markers in GC varied with the degree of differentiation, while poorly differentiated GC expressed high levels of these markers. Furthermore, CD133 expression in GC cells could be divided into two forms: luminal expression in the gland and cytoplasmic expression. A multivariate analysis revealed that the expression of CD133 in the cytoplasm was an independent prognostic factor in GC<sup>[25,26]</sup>.

### Other GCSC markers

In addition, aldehyde dehydrogenase 1 (ALDH1) has been identified as a marker of GCSCs. ALDH1+ cells derived from a diffuse-type GC cell line had a higher tumorigenic capacity *in vitro* and *in vivo* compared with ALDH1- cells and were capable of self-renewal and the generation of heterogeneous cell populations. Moreover, regenerating islet-derived family member 4 (REG4) was overexpressed in ALDH1+ GCSCs, and ALDH1 and REG4 expression were down-regulated by transforming growth factor- $\beta$  (TGF- $\beta$ ), which correlated with a reduction in the GCSC population and tumorigenicity<sup>[27,28]</sup>. CD90+ cells, which possessed a greater ability to initiate tumors *in vivo* compared with CD90- cells, could re-establish the cellular hierarchy of tumors from single-cell implantation, which demonstrates their self-renewal properties. In addition, previous studies on chemo-resistance revealed that ERBB2 was overexpressed in approximately 20%-25% of the gastric primary tumor models, which correlated with the higher level of CD90 expression in these tumors<sup>[29,30]</sup>. Moreover, trastuzumab treatment could decrease the CD90+ population in these tumor masses and could suppress tumor growth when combined with traditional chemotherapy. Taken together, this evidence suggests that CD90 may be another potential candidate marker of GCSCs<sup>[30]</sup>. The CD71-fraction of GC cells was enriched after treatment with 5-fluorouracil and accumulated during the G0/G1 cell cycle phase. This cell subtype also exhibited high drug resistance to conventional chemotherapy, which demonstrates its stem cell-like properties. Limiting dilution and serial transplantation assays revealed that the CD71- cell fraction had higher tumorigenicity than the CD71+ cell fraction<sup>[31]</sup>.

More recently, new tissue stem cell markers have been proposed. Lrig1, which is a marker of proliferative and quiescent stem cells in the skin and intestine, is a marker of gastric corpus epithelial progenitor cells that are capable of repopulating the damaged oxyntic mucosa via differentiation into normal gastric lineage cells in the mouse stomach. Lineage labelling using Lrig1-CreERT2/+; R26R-YFP/+ (Lrig1/YFP) or R26R-LacZ/+ (Lrig1/LacZ) mice demonstrated that the Lrig1-YFP-marked cells were gastric progenitor cells<sup>[32]</sup>. Likewise, Mist1 is a marker of quiescent stem cells in the gastric corpus isthmus. Mist1-positive stem cells serve as a cell-of-origin for intestinal-type GCs, and have the combination of Kras and Apc mutations; Mist1-positive cells are also the cell-of-origin of diffuse-type GCs when E-cadherin expression is lost<sup>[33]</sup>. Potential GCSC markers are summarized in Table 1.

### GCSC REGULATION IN THE TUMOR MICROENVIRONMENT

The tumor microenvironment consists of various types of cells including immune cells, endothelial cells, and fibroblasts, in addition to the extracellular matrix, and has a large impact on tumor progression<sup>[34,35]</sup>. Cancer cells remodel their microenvironment through the secretion of growth factors and proteases, while stromal cells also affect cancer cells through the secretion of soluble factors such as matrix metalloproteinases, TGF- $\beta$  1, Wnt ligands, bone morphogenetic proteins, stromal cell-derived factor 1 and exosomes<sup>[36-38]</sup>. Tissue stem cells are located beside the surrounding environment termed a “stem cell niche” where they play critical roles in tissue homeostasis by maintaining their ability to self-renew and differentiate<sup>[39,40]</sup>.

In the tumor microenvironment, myofibroblasts, which are also known as cancer-associated fibroblasts (CAFs), share characteristics with smooth muscle cells and fibroblasts. CAFs enhance tumor progression through the secretion of soluble factors such as growth factors and cytokines in various tumor types<sup>[41-43]</sup>.

**Table 1. Gastric cancer stem cell markers**

Marker	General function	Significance	Therapeutic targets	References
CD44	Cell adhesion molecule, hyaluronic acid receptor	Tumorigenicity, spheroid formation, chemoresistance	Glutathione metabolism (CD44v)	[16,28,58]
CD24/CD44	Cell adhesion molecule	Tumorigenicity		[17]
CD54/CD44	Cell adhesion molecule	Tumorigenicity, hierarchical organization		[18]
Lgr5	Wnt target gene, restriction to the crypt base	Tumorigenicity	Notch-mTOR signal miR-132	[21,23,24,46,59-62]
Lrig1	Regulatory factor of cell cycle	Tumorigenicity	Not shown	[32]
Mist1	Transcriptional regulator	Tumorigenicity	Not shown	[33]
EpCAM/CD44	Cell adhesion molecule	Tumorigenicity, phenotypical heterogeneity, chemoresistance	Not shown	[19]
ALDH1	Detoxifying enzyme	Tumorigenicity, phenotypical heterogeneity	Not shown	[27,28]
CD90	Immunoglobulin superfamily	Tumorigenicity, trastuzumab reduce the CD90 <sup>+</sup> population	CD90	[29,30]
CD71	Transferrin receptor	Tumorigenicity, chemoresistance, tumor cell invasion	Not shown	[31]
CD133	Pentaspans transmembrane glycoprotein	Poorly differentiated gastric cancer, independent prognostic factor	CD133	[25,26,56,63]

One study showed that CAFs significantly increased the number of spheroid colonies, the expression level of CSC markers and the fraction of side population cells in scirrhous GC cell lines. The influence of CAFs was significantly inhibited by TGF- $\beta$  inhibitors, but not by fibroblast growth factor receptor or cMet inhibitors. These findings suggest that CAFs might promote CSC properties in scirrhous GC through TGF- $\beta$  signaling<sup>[44]</sup>. IL-17B induced the expression of the self-renewal-related genes Nanog, Sox2, and Oct4 in mesenchymal stem cells and promoted tumor progression. After treatment with exogenous IL-17B, the supernatant from cultured mesenchymal stem cells promoted the proliferation and migration of GC cells. This suggests that IL-17B might promote the production of soluble factors by mesenchymal stem cells, which leads to GC progression<sup>[45]</sup>.

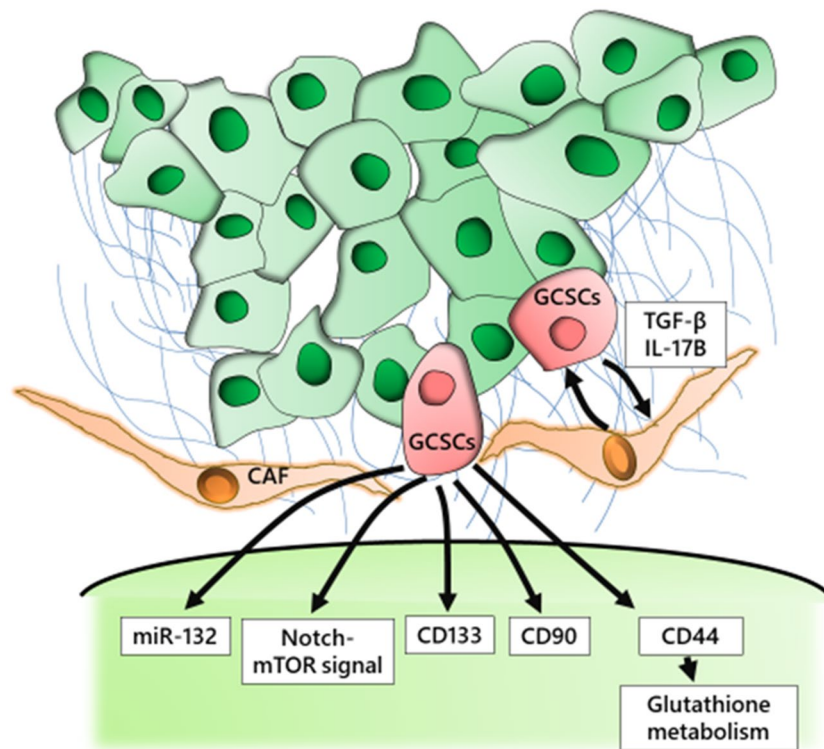
A recent compelling study demonstrated that nerves help to regulate both normal and neoplastic stem cell dynamics in the gastrointestinal stem cell niche. The authors of that study utilized a series of Dclk1-CreERT mouse models to show that acetylcholine from nerves and from Dclk1<sup>+</sup> tuft cells, which acted as intermediary niche cells to coordinate neural input to help regulate subsequent stem cell activity, induced nerve growth factor in gastric epithelial cells; this in turn promoted neuron expansion and tumorigenesis<sup>[46]</sup>.

## CURRENT TREATMENT OF GC AND THE POTENTIAL FOR TARGETING GCSCS

Surgical resection is currently the only curative modality to eliminate GC. Endoscopic screening has become widespread, however, GCs are frequently diagnosed at an advanced stage, when the clinical outcome is still poor. Even after curative surgery, patients with advanced GC still experience recurrence, which implies that undetectable GC cells exist in the blood at the time of surgery. Based on this possibility, definitive evidence has been found that multimodal treatments consisting of surgery with neoadjuvant chemotherapy, adjuvant chemotherapy, or chemoradiation would improve the poor outcomes compared with surgery alone.

In recent years, several molecular-targeted agents have been investigated in various combinations with conventional treatment as a first-line chemotherapy against advanced GC. The Trastuzumab for Gastric Cancer (ToGA) trial revealed that trastuzumab, a recombinant monoclonal antibody against HER2 (also known as ERBB2), combined with fluoropyrimidine plus cisplatin provided a significant survival advantage compared with fluoropyrimidine plus cisplatin alone in patients with HER2-positive advanced GC<sup>[29,47,48]</sup>. The ramucirumab for patients with previously treated advanced gastric or gastro-esophageal junction





**Figure 1.** GCSCs in the microenvironment and the activated pathway in GCSCs. GCSCs: Gastric cancer stem cells; CAF: cancer-associated fibroblasts; TGF: transforming growth factor; IL: interleukin

adenocarcinoma (RAINBOW) trial showed that the combination of ramucirumab and paclitaxel significantly improved overall survival compared with placebo plus paclitaxel and that this combination could be regarded as a new standard second-line chemotherapy for patients with advanced GC<sup>[49,50]</sup>.

Immune checkpoint blockade is new topic in cancer therapy. The immune checkpoint pathways, which basically maintain self-tolerance and limit collateral tissue damage during anti-microbial immune responses, can be co-opted by cancer to evade immune destruction<sup>[51]</sup>. Nivolumab is a human monoclonal IgG4 antibody that blocks the human programmed cell death-1 (PD-1) receptor. Preliminary data from a double-blinded, randomized, phase III trial (ONO-4538/BMS-936558) demonstrated the efficacy of nivolumab as salvage treatment as a third- or later line of treatment in 493 patients with advanced gastric or gastroesophageal junction cancer compared with placebo (NCT02267343). Finally, a clinical study demonstrated that nivolumab was effective as the salvage treatment for pretreated advanced GC with significantly improved clinical outcomes compared with the placebo<sup>[52]</sup>.

To develop a treatment strategy to target GCSCs, we must select critical molecules that regulate the biological characteristics of CSCs [Figure 1]. Several molecules have been investigated as possible targets including those associated with specific signaling pathways, cell surface markers, and microenvironmental factors. We previously used K19-Wnt1/C2mE mice, a transgenic GC mouse model, to demonstrate that the CD44 variant isoform (CD44v), one of the cell surface markers of GCSCs, contributed to the defense against reactive oxygen species by stabilizing the glutamate-cystine transporter subunit xCT and promoting the synthesis of the primary intracellular antioxidant glutathione<sup>[53,54]</sup>. Moreover, we found that CD44v expression was up-regulated in these gastric tumor cells. We also showed that the inhibition of the cystine transport system xc(-) with sulfasalazine, an inhibitor of xCT-dependent cystine transport, suppressed the progression of gastric tumors in these transgenic mice<sup>[55]</sup>. Our findings suggest that targeted therapy against the CD44v-xCT system may provide a strategy for the targeting of CD44v positive GCSCs. CD133 was a potential therapeutic

target for antibody-drug conjugates (ADC), which was proven by binding mouse anti-human CD133 monoclonal antibody to highly cytotoxic monomethyl auristatin F, ultimately inducing apoptosis in cancer cells with high levels of CD133 expression<sup>[56]</sup>. However, a recent study demonstrated that the hierarchical organization that involves CSCs and non-CSCs may be reversible through epigenetic gene regulation, which suggests that therapeutic strategies that target GCSCs themselves might be insufficient to eliminate cancer cells<sup>[57]</sup>.

## CONCLUSION

Molecular-targeted agents have been developed as a new treatment strategy and have been applied to various types of solid tumors. These developed agents have been assessed in diverse combinations with conventional chemotherapy as a treatment against advanced tumors including GC. However, the success of molecular-targeted agents for GC has been limited, and the prognosis of patients with advanced GC is still poor. Based on accumulating evidence, GCSCs are deeply involved in GC progression. Moreover, the tumor microenvironment that surrounds GCSCs forms the CSC niche and allows the stem cells to give rise to a hierarchy of proliferative and non-GCSC cells. Targeting the critical pathways and molecules between GCSCs and their environment may therefore represent a promising therapeutic strategy, and may provide a complementary approach to conventional therapies that target the malignant cells themselves. This review describes recent progress and evidence concerning the markers of GCSCs, related molecules within the GCSC niche and treatment targets. Further elucidation of the molecular mechanisms of GCSC regulation may lead to the development of novel treatment strategies that target GCSCs.

## DECLARATIONS

### Authors' contributions

Writing manuscript: Uchihara T, Ishimoto T, Yonemura A, Baba H

Organized data: Uchihara T, Ishimoto T

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None.

### Conflicts of interest

There are no conflicts of interest.

### Patient consent

Not applicable.

### Ethics approval

Not applicable.

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Review

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# Microenvironment in the pathogenesis of gastric cancer metastasis

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## Abstract

Tumor tissues contain cancer cells, other cellular and non-cellular components. Tumor microenvironments consist of cancer cells and various types of stromal cells, cancer associated fibroblasts, bone marrow-derived cells, endothelial cells, and hematopoietic cells, mainly tumor-associated macrophages and tumor-infiltrating lymphocytes. Increasing recent evidence has demonstrated that alteration of tumor microenvironments is deeply implicated in tumor progression and metastasis in gastric cancer (GC) patients. Recent investigations have provided insights into the molecular mechanisms of the interaction between tumor cells and tumor microenvironments. Interactions between cancer cells and their microenvironment with cytokines and microRNA in extracellular vesicles, such as the exosome, can have a substantial impact on tumor characteristics. Alterations in the tumor microenvironment may play a crucial role in facilitating the progression of tumor cells and metastasis, as well as the activation of cell signaling pathways, which are associated with GC cell proliferation and invasion by genetic or epigenetic alterations. In this review, significant molecular insights into the tumor microenvironment, which consist of cancer associated fibroblasts, bone marrow-derived cells, tumor-associated macrophages and tumor-infiltrating lymphocytes; the interactions between cancer cells and their microenvironment; and the clinical impacts of alterations of GC microenvironments will be discussed.

**Keywords:** Tumor microenvironments, cancer associated fibroblasts, bone marrow-derived cells, tumor-associated macrophages, tumor-infiltrating lymphocytes

## INTRODUCTION

Gastric cancer (GC) is the fourth most commonly diagnosed cancer worldwide<sup>[1]</sup>. Surgical resection with lymph node dissection is the most effective treatment for resectable GC; the standard surgical procedure for



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GC is reportedly gastrectomy with D2 dissection<sup>[2-4]</sup>. However, distant metastasis or disseminated relapses are experienced even after curative resection. Multidisciplinary treatment, perioperative chemotherapy and radiotherapy are other treatment options, and immunonutritional support is effective supportive care for GC. Prognosis of GC patients has been improved by multidisciplinary treatment. However, some patients experience recurrence after curative resection with perioperative therapy. Patients with unresectable GC also suffer from tumor progression and metastasis, even if they are treated with stronger therapeutic agents.

Trastuzumab and ramucirumab [targeting human epidermal growth factor receptor 2 (HER2) and vascular endothelial growth factor receptor 2, respectively] have been approved for treating GC<sup>[5-7]</sup> and these molecular targeting agents improve GC patients' survival; however, many molecular targeting drugs that have entered clinical trials for GC failed. Therefore, more effective treatment, which targets other mechanisms, should be sought for these patients. The efficacy and safety of nivolumab, a human immunoglobulin G-4 monoclonal antibody inhibitor of programmed death-1, were confirmed in GC patients<sup>[8]</sup>. Recent studies have revealed that programmed death-ligand-1 (PD-L1) expression is associated with the microenvironments in GC. The Cancer Genome Atlas project demonstrates that GC cases can be divided into four subtypes: tumors positive for Epstein-Barr virus (EBV), tumors with microsatellite instability (MSI), genomically stable (GS) tumors and tumors with chromosomal instability (CIN). EBV positive tumors are associated with phosphatidylinositol 4,5-bisphosphate 3-kinase catalytic subunit alpha mutations, extreme DNA hypermethylation, and amplification of Janus kinase 2 (JAK2), PD-L1 and PD-L2. Tumors with MSI exhibit elevated mutation rates. GS tumors are enriched for the diffuse histological variant and mutations of Ras homolog gene family member A (RHOA) or fusions involving RHO-family GTPase-activating proteins. Tumors with CIN show marked aneuploidy and focal amplification of receptor tyrosine kinases<sup>[9]</sup>. Identification of these subtypes can prove to be valuable for developing strategies for targeted therapies for cancer.

Increasing recent evidence has shown that tumor microenvironments of GC, as well as gene expression pattern, are deeply implicated in tumor progression and metastasis. Tumor microenvironments consist of cancer cells and various types of stromal cells, bone marrow-derived cells (BMDCs), mast cells (MCs), tumor-infiltrating lymphocytes (TILs), tumor-associated macrophages (TAMs) and cancer associated fibroblasts (CAFs). Interactions between cancer cells and their microenvironment with cytokines and microRNA (miRNA) in extracellular vesicles, such as the exosome, can have a substantial impact on tumor characteristics. Alterations in the tumor microenvironment may play a crucial role in facilitating the progression of tumor cells as well as the activation of cell signaling pathways by the genetic or epigenetic alteration of cancer cells.

In this review, important molecular insights into clinical impacts of the tumor microenvironment of GC will be discussed.

## BONE MARROW-DERIVED CELLS

BMDCs which are recruited to tissue injury sites are thought to represent a potential source of malignancy. Chronic infection with *Helicobacter felis* induces the intensity of bone marrow-derived inflammation and repopulation of the stomach with BMDCs. Metaplasia and dysplasia to intra-epithelial cancer progress through the recruitment and accumulation of BMDCs<sup>[10]</sup>. *Helicobacter pylori* infection leads to development of chronic inflammation as well as the recruitment and accumulation of BMDCs in the gastric epithelial mucosa. Nearly 20%-25% of dysplastic lesions include cells that originate from the bone marrow<sup>[11,12]</sup>. Bone marrow cells may be associated with GC initiation and proliferation.

Recent studies have demonstrated the mechanism of BMDCs. Infection of gastrointestinal epithelial cells by *Helicobacter pylori* stimulates the migration of BMDCs. The nuclear factor-kappa B (NF- $\kappa$ B) signaling

pathway may play a major role in the ability of infected epithelial cells to induce BMDC migration<sup>[13]</sup>. Bone marrow-derived myofibroblasts secrete high levels of murine IL-6 and hepatocyte growth factor (HGF), which activate transforming growth factor- $\beta$ 1 (TGF $\beta$ 1) and signal transducers and activators of transcription (STAT3) in GC cells. Bone marrow-derived myofibroblasts that increase IL-6/HGF and cancer cell-derived TGF $\beta$ 1 mediate the interactions between bone marrow-derived myofibroblasts and GC cells, which regulate promotion of tumorigenesis and cancer stemness<sup>[14]</sup>. NF- $\kappa$ B is regulated by miRNAs. miRNA-155-5p downregulation induces BMDCs to acquire a GC mesenchymal stem cell (MSC)-like phenotype. The function depends on NF- $\kappa$ B p65 activation. This mechanism is the cancer associated MSC remodeling in the tumor microenvironment<sup>[15]</sup>. The association between BMDCs and chemokines has been analyzed to evaluate the chemotaxis-stimulating factor from diffuse-type GC cells using BMDCs in an *in vitro* assay. CXCL1 from GC cells stimulates the recruitment of BMDCs into tumor stroma via CXCR2 signaling of BMDCs<sup>[16]</sup>.

Some markers, including CD13, CD15, CD73, CD140b, CD144, CD146, CD164 and CD271, have been used to identify MSCs<sup>[17,18]</sup>. Neural crest nerve growth factor receptor (CD271) was reported as a marker of bone marrow-derived stromal cells<sup>[18,19]</sup>. The role of bone marrow-derived stromal cells expressing CD271 has been evaluated in GC patients. CD271 expression in stromal cells is significantly associated with macroscopic type-4 cancers, diffuse type tumors and depth of invasion. In one study, patients ( $n = 279$ ) with CD271-positive stromal cells had a worse prognosis than patients with CD271-negative stromal cells<sup>[20]</sup>.

Some studies have demonstrated that the interaction between BMDCs and GC cells is an important factor in cancer development and progression and may be associated with the survival of GC patients.

## MAST CELLS

MCs play an important role in immunity and defend the body against viruses and bacteria. MCs are also known for causing uncomfortable symptoms due to their release of histamine and other mediators which cause allergic responses. MCs constitute a long-lived, heterogeneous cellular population originating from the bone marrow<sup>[21]</sup>. Mast cell density (MCD) in GC was found to be higher than that in the control. Moreover, MCD in well-differentiated adenocarcinoma was higher than that in poorly-differentiated adenocarcinoma<sup>[22]</sup>. MCs were recognized by their modulatory activities in inflammation and angiogenesis. Tryptase was used as a marker for MCs. The expression of tryptase and Foxp3 were positively correlated. Infiltration of MCs was found to be significantly associated with an advanced stage of GC<sup>[23]</sup>. MCs density increased with an increase in malignancy grade and was highly correlated with the extent of angiogenesis<sup>[24]</sup>. However, increased tryptase expression was associated with better survival outcome in early-stage GC patients after surgical resection. These opposing effects may indicate the possibility of two mast cell phenotypes (MC1 and MC2)<sup>[25]</sup>. MCs were associated with angiogenesis and tumor progression in diverse tumors<sup>[21]</sup>, however contradictory results were also reported in other cancers<sup>[25,26]</sup>. The relation between MCs and GC remains largely unknown and further investigations regarding MCs, including other factors, are needed.

## TUMOR-INFILTRATING LYMPHOCYTES

TILs consist of T cells, B cells, and NK cells. The subset of T cells is represented by CD8+ cytotoxic T cells, CD4+ T helper cells, CD45RO+ memory T cells, FOXP3+ regulatory T cells and NK cells. These cells can infiltrate stroma and tumor cells, and are considered a manifestation of the host immune response against tumor cells<sup>[27]</sup>.

Thirty-one studies (incorporating 4,185 patients) were conducted to evaluate the relationship between TILs and GC prognosis. The amount of CD8+, FOXP3+, CD3+, CD57+, CD20+, CD45RO+, Granzyme B+ and

T-bet<sup>+</sup> lymphocytes is a significant advantage to survival; moreover, the amount of CD3<sup>+</sup> TILs in the intra-tumoral compartment is the most significant prognostic marker [pooled hazard ratio (HR) = 0.52; 95% confidence interval (CI) = 0.43-0.63;  $P < 0.001$ ]. CD4<sup>+</sup> TILs are not statistically associated with prognosis. FOXP3<sup>+</sup> TILs show bidirectional prognostic roles, which have a positive effect in the intra-tumoral compartment (pooled HR = 1.57; 95% CI = 1.04-2.37;  $P = 0.033$ ) and a negative effect in the extra-tumoral compartment (pooled HR = 0.76; 95% CI = 0.60-0.96;  $P = 0.022$ )<sup>[28]</sup>.

Stromal TIL-positivity was significantly associated with GC patient survival in multivariate analysis. High densities of intratumoral-TIL has a tendency to be a favorable outcome indicator for GC patient survival<sup>[29]</sup>. The prognostic impact of TILs has also been evaluated for GC patients with microsatellite instability-high (MSI-H). Higher densities of both intratumoral CD8<sup>+</sup> and FOXP3<sup>+</sup> TILs are significantly associated with longer survival<sup>[30]</sup>. The prognostic impact of TILs has also been evaluated for patients with EBV-associated GC. EBV-associated GCs are more prevalent in CD8<sup>+</sup> and FOXP3<sup>+</sup> cell infiltration than EBV-negative GCs. CD8 expression and Foxp3 expression cell infiltration are associated with longer overall survival, whereas PD-L1 expression correlates with shorter overall survival<sup>[31]</sup>.

Most recent studies have focused on the significant association between PD-L1 expression and TILs. PD-L1 expression is associated with high densities of TILs, mismatch repair deficiency and EBV positivity, but is not a prognostic factor<sup>[32]</sup>. PD-L1 expression alone on tumor cells is not associated with survival of GC patients; however, patients with positive PD-L1 expression on a high density of TILs have a significantly shorter 5-year overall survival than those with negative PD-L1 expression. PD-L1 expression on TILs is an independent prognostic factor<sup>[33]</sup>. It is associated with the corresponding immunoscore, which is quantified by the number of high-density areas of CD3<sup>+</sup> and CD8<sup>+</sup> TILs, both in the tumor regions and compartments of MSI-H advanced GC<sup>[34]</sup>. The levels of immunosuppressive protein expression PD-L1, cytotoxic T-lymphocyte antigen 4 (CTLA-4), and indoleamine 2,3-dioxygenase (IDO) in tumors and the densities of immune cells [CD3(+), CD4(+), CD8(+), or PD-1(+)] within the tumor microenvironment have been evaluated by immunohistochemical analysis. PD-L1 positive expression and a high-CD3 tumor microenvironment are favorable prognostic markers in GC<sup>[35]</sup>. Another study has also demonstrated that PD-L1 expression alone is not associated with overall survival; however, PD-L1-/CD8 high type is an independent indicator for longer overall survival compared with PD-L1+/CD8 high. Adaptation of a recent molecular classification<sup>[9]</sup> based on EBV, MSI, E-cadherin and p53 showed no significant survival differences in this study. EBV<sup>+</sup> and MSI-H GCs are associated with PD-L1+/CD8 high, and the PD-L1/CD8 status is associated with their prognostic significance in stage II/III GCs<sup>[36]</sup>. Recent studies have revealed that PD-L1 expression was significantly associated with GC patient prognosis only under the interaction between PD-L1 and TILs.

## TUMOR-ASSOCIATED MACROPHAGES

TAMs play crucial roles in microenvironments. The polarization of macrophages into tumor-suppressive M1 or tumor-promoting M2 types is established in the microenvironment of GC<sup>[37]</sup>. TAMs represent up to 50% of the tumor and are mainly M2 macrophages in invasive cancers. M2 macrophages support proliferation, invasion and metastasis by upregulating diverse growth factors, cytokines and extracellular matrix (ECM)-remodeling molecules, such as CCL2, CXCL12, CXCR4, TGF $\beta$ , VEGF, PDGF, COX-2 and matrix metalloproteinases (MMPs)<sup>[38]</sup>. TAMs interact with T cells during tumor progression. M1 macrophages direct T cells towards Th1 tumoricidal responses. M1 macrophages are induced by NK cells that are produced by interferon- $\gamma$  (IFN- $\gamma$ ) to amplify anti-tumor activity<sup>[39,40]</sup>. TAMs are identified by immunohistochemistry with the anti-CD68 antibody (pan-macrophage). Especially, M2 macrophages are identified with the anti-CD163 antibody (M2 macrophage).

A meta-analysis of 12 studies ( $n = 1388$  patients) was conducted to evaluate the relationship between TAMs and GC prognosis. High total TAM infiltration levels in GC patients are associated with worse overall

survival (HR = 1.70, 95% CI = 1.39-2.09;  $P < 0.001$ ). A similar result was demonstrated for M2 macrophage infiltration (HR = 1.71, 95% CI = 1.19-2.45;  $P = 0.004$ ). In contrast, elevated M1 macrophage density in GC patients is associated with better overall survival (HR = 0.46, 95% CI = 0.33-0.65;  $P < 0.001$ ). This meta-analysis demonstrated that the numbers of infiltrating M2 macrophages and total TAMs may be negative prognostic factors for GC patients, while infiltrating M1 macrophages may be associated with a favorable survival rate<sup>[41]</sup>.

The mechanisms of GC progression affected by TAMs have been investigated. Macrophages induce the capillary formation of lymphatic endothelial cells. Co-culture with GC pretreated macrophages upregulate lymphangiogenic factors, including inflammatory cytokines, MMPs, adhesion molecules and vascular endothelial growth factor-C. Interaction between lymphatic endothelial cells and macrophages may be an important initial step in tumor lymphangiogenesis developing lymph node metastasis<sup>[42]</sup>. The high density of CD163+ TAMs (M2 macrophage) is an independent prognostic marker heralding prolonged disease-free survival. The prognostic implication of CD163+ TAMs might be determined by the proportional balance of TAMs and TILs in MSI-H GCs<sup>[43]</sup>.

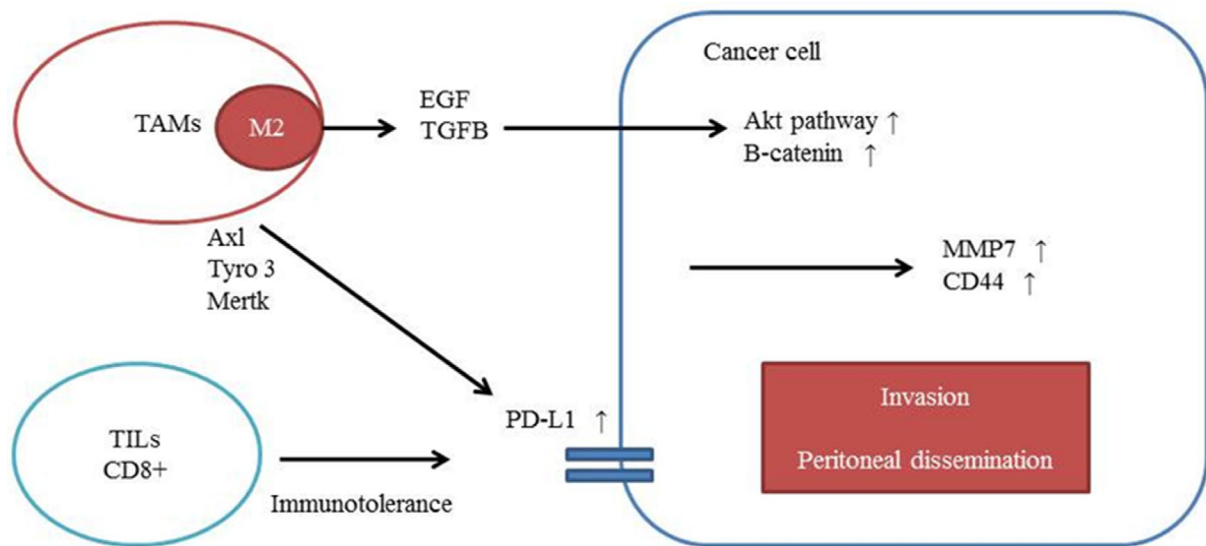
Redox adaptation enables cancer cells to survive under increased oxygen species (ROS) stress. ROS produced by TAMs triggers CD44 expression through the suppression of miR-328 in GC cells<sup>[44]</sup>. Cluster of differentiation 44 (CD44) is a major adhesion molecule and a broadly distributed cell surface receptor for hyaluronic acid<sup>[45]</sup>. The *CD44* gene is located on chromosome 11p13 and contains 20 exons, 10 of which are expressed in the standard form (CD44s)<sup>[46]</sup>. CD44 isoforms, containing variant exon 6 (CD44v6), are identified by alternative splicing of at least 12 exons<sup>[47]</sup>. CD44 variant isoform (CD44v) is one of the cell surface markers of GC stem cells<sup>[48]</sup>. Furthermore, CD44v contributes to defense against reactive ROS by stabilizing the glutamate-cystine transporter subunit xCT, and promoting the synthesis of the primary intracellular antioxidant glutathione<sup>[49]</sup>.

Epithelial-mesenchymal transition (EMT), which is induced by TAMs, may play a key role in cancer metastasis. M2 macrophages secrete epidermal growth factor or TGF $\beta$ , which stimulates EMT<sup>[50,51]</sup>. Epidermal growth factor activates the AKT pathway, which regulates  $\beta$ -catenin translocation. MMP7 and CD44, which are  $\beta$ -catenin downstream genes, are involved in macrophage-activated GC cell invasion<sup>[52]</sup>. E-cadherin and vimentin expression are markers of EMT. E-cadherin expression in GC cells co-cultured with TAMs is decreased, while vimentin expression in GC cells co-cultured with TAMs is increased<sup>[53]</sup>. Bmi1 is identified as a cancer stem cell marker, and M2 macrophages upregulate Bmi1 expression through miRNA-30e\* suppression in GC<sup>[54]</sup>.

Recent studies have revealed the relationship between TAM infiltration and PD-L1 expression. TAM receptors (Tyro3, Axl and Mertk) upregulate the expression of PD-L1 in a breast cancer cell line. According to the data, M2 macrophages might activate PD-L1 expression in tumor cells. IFN- $\gamma$  is secreted by inflammatory cells and is associated with differentiation of macrophages. IFN- $\gamma$  was found to facilitate PD-L1 expression in tumor cells<sup>[55,56]</sup>. In GC, IFN- $\gamma$  might also influence the relationship between M2 macrophage infiltration and PD-L1 expression in tumor cells. TAM infiltration is also associated with the upregulation of PD-L1 expression in GC cells [Figure 1]<sup>[57]</sup>.

## CANCER ASSOCIATED FIBROBLASTS

Tumor tissues contain cancer cells, other cellular and non-cellular components. The tumor stroma acts as an essential microenvironment of the cancer cells, which includes many types of non-cancerous cells and the ECM. Stromal fibroblasts are the major cellular constituents of the tumor stroma and are often called CAFs. CAFs contribute to the stiffening and remodeling of the stromal ECM, thereby offering an appropriate field for cancer cell invasion<sup>[58,59]</sup>. Cancer cells induce the conversion of these various types of cells into



**Figure 1.** The mechanisms of GC progression affected by TAMs. M2 macrophages secrete EGF or TGFβ. EGF activates the AKT pathway, which regulates β-catenin translocation. MMP7 and CD44 are involved in macrophage-activated GC cell invasion. TAM receptors (Tyro3, Axl and Mertk) upregulate the expression of PD-L1. GC: gastric cancer; TAM: tumor-associated macrophage; EGF: epidermal growth factor; TGFβ: transforming growth factor-β1; MMP: matrix metalloproteinase; PD-L1: programmed death-ligand-1; TIL: tumor-infiltrating lymphocyte

CAFs. CAFs acquire the properties of myofibroblasts, including expression of smooth muscle alpha-actin<sup>[60]</sup>. Formation of myofibroblasts is associated with fibrosis and increases the risk of cancer. Many other studies have demonstrated that fibroblasts often have a position at center stage, orchestrating and actively participating in the transformation process. They are also spectators in the tumorigenic process<sup>[61]</sup>. Fibroblast activation protein-α (FAP) is a protein expressed in fibroblasts, such as CAFs, which are major components of the tumor microenvironment. FAP is potentially associated with GC patient survival<sup>[62]</sup>.

Recent investigations have demonstrated that interleukins, such as IL1A, IL1B, IL-6 and IL-17B, produced by CAFs, are associated with cancer progression and metastasis. In CAFs isolated from human diffuse-type GCs, inflammatory cytokines, such as IL1A, IL1B, and tumor necrosis factor (TNF), secreted by diffuse-type GC, induce rhomboid 5 homolog (RHBDF2) expression. RHBDF2 promotes cleavage of TGFβ receptor 1 (TGFβR1) and motility of CAFs in response to TGFβ1. These highly motile CAFs induce diffuse-type GCs to invade the ECM and lymphatic vessels. IL1A, IL1B and TNF status is associated with shorter overall survival of diffuse-type GC patients<sup>[63]</sup>. IL-6 is also associated with CAFs; it has been produced by CAFs isolated from GC. The migration and EMT of GC cells are enhanced by CAFs through the secretion of IL-6, which activates JAK2/STAT3 pathway in GC cells. Inhibiting the JAK2/STAT3 pathway with a specific inhibitor markedly attenuates these phenotypes in GC cells induced by CAFs<sup>[64]</sup>. IL-17B increases the expression of stemness-related genes Nanog, Sox2 and Oct4 in MSCs, and the tumor promoting effect is enhanced. The condition medium from cultured MSCs after being treated with exogenous recombinant interleukin-17B (rIL-17B) promotes the proliferation and migration of GC cells. rIL-17B also activates the NF-κB, STAT3, β-catenin pathway in MSCs and the progression of GC is induced by IL-17B activating MSCs<sup>[65]</sup>.

miRNAs are a class of non-coding small RNA molecules, and expression of miRNAs in CAFs regulates an essential role in the communication between tumor cells and CAFs, as well as the expression of a number of target genes<sup>[66]</sup>. A miRNA microarray analysis from GC revealed the different expression of miRNA between CAFs and normal fibroblasts (NFs). In the study, four miRNAs were increased (miRNA-34b, 301a 106b and 93) and seven miRNAs were decreased (miRNA-483-3p, 26a, 7g, 148a, 145, 424 and 214) in CAFs compared with NFs. The expression of miRNA-106b is upregulated in CAFs established from patients with GC, and the expression level of miRNA-106b is associated with poor prognosis of GC patients. CAFs with downregulated miRNA-106b could significantly inhibit GC cell migration and invasion by targeting the phosphatase and



tensin homolog<sup>[67]</sup>. In CAFs, miRNA-143 overexpression is derived from diffuse type GC compared with NFs. miRNA-143 promotes GC cell invasion by regulating the expression of collagen type III in CAFs, and miRNA-143 expression serves as a prognostic marker of GC<sup>[68]</sup>. The expression of miRNA-200b is downregulated by CAFs. miRNA-200b downregulates zinc finger E-box-binding homeobox expression and upregulates E-cadherin expression in GC cells to repress tumor cell invasion and peritoneal dissemination<sup>[69]</sup>. Expression of miRNA-328 mediated by macrophages regulates CD44 signaling and may promote tumor progression by enhancing ROS defense<sup>[44]</sup>.

At least 20% of CAFs originate from the bone marrow and are derived from MSCs. Those MSC-derived CAFs were recruited to the tumor in a TGF- $\beta$ - and stromal-derived factor (SDF)-1 $\alpha$ -dependent manner in mouse models of inflammation-induced gastric dysplasia (21316604). SDF-1 $\alpha$  produced by myofibroblasts promotes gastric epithelial proliferation, partly through CXCR4-positive gastric tissue stem progenitor cells, and plays a key role in gastric carcinogenesis<sup>[11]</sup>. CXCL12 is also associated with CAF-induced cancer progression. CXCL12 and Twist1 expression are correlated in CAFs present in gastric tumor specimens. Ectopic expression of Twist1 in NFs suppresses premature senescence, whereas Twist1 attenuation accelerates senescence in CAFs<sup>[70,71]</sup>.

Interleukins and miRNAs produced by CAFs are associated with cancer progression and metastasis of GC cells. Interactions between cancer cells and CAFs with interleukins or miRNAs can have a substantial impact on tumor characteristics and alteration of signaling pathways associated with proliferation and invasion of GC cells.

## CHEMOKINES

Fibroblasts are a major component of the tumor stroma, and activated fibroblasts regulate solid tumor progression. The interaction between cancer cells and CAFs by chemokines has been suggested to be important for the progression of GC. Chemokines, more than 40 of which have been identified, are 8-10-kDa secreted proteins with 20%-70% homology in structure. They share the common functional activity as being chemotactic for leucocytes. Pro-inflammatory stimuli, such as IL-1, TNF- $\alpha$ , lipopolysaccharide or pathogens produce inflammatory chemokines, which determine the migration of inflammatory cells. Chemokines bind to G protein-coupled receptors on leukocytes and stem cells, and they function through guanine nucleotide-binding proteins to control intracellular signaling that promotes the migration ability toward the chemokine source<sup>[72]</sup>.

CXCR4 and CXCR7 are important chemokine receptors that share the same ligand CXCL12. The association of CXCR4 and CXCL12 with GC patient prognosis was evaluated in a meta-analysis [CXCR4; 13 studies ( $n = 1936$  patients) and CXCL12; 38 studies ( $n = 5807$  patients)]. High expression of CXCL12 is associated with reduced overall survival in GC patients (HR 2.08; 95% CI = 1.31-3.33;  $P = 0.0002$ )<sup>[73]</sup>. CXCR4 expression is associated with shorter overall survival (HR 2.63; 95% CI = 1.69-4.09;  $P < 0.001$ )<sup>[74]</sup>.

Many studies have demonstrated that CXCR4 is the major chemokine receptor expressed in diverse cancer cells<sup>[75]</sup>. Previous studies had shown that the CXCL12/CXCR4 axis plays an important role in invasion and metastasis. CXCL12, which is a ligand for CXCR4, activates the CXCR4 and attracts circulating CXCR4-expression cells to peripheral tissues by regulating a wide variety of downstream signal pathways related to proliferation, migration, chemotaxis and cell survival<sup>[76]</sup>. CXCR4 levels in GC are also significantly higher than those in the normal mucous membrane. CXCR4 expression is significantly related to poor differentiation, high tumor stage and lymph node metastasis<sup>[77,78]</sup>. CXCR4 activates actin polymerization to induce cell motility and the EMT after binding its ligand CXCL12<sup>[79]</sup>. CXCL12 rapidly and strongly phosphorylates Akt. Mammalian target of rapamycin (mTOR) pathways, which are located downstream of Akt, activate p70S6K (S6K) and eukaryotic initiation factor 4E binding protein 1 (4E-BP1). CXCL12/CXCR4

activation also mediates integrin  $\beta 1$  clustering at the cell surface and promotes the invasive ability of GC cells<sup>[80]</sup>. In addition, CXCL12/CXCR4 upregulates the expression of MMP-2 and MMP-7 to assist EMT<sup>[81]</sup>. Runt-related transcription factor 2 (RUNX2) directly binds to the promoter region of the gene coding area for the chemokine receptor CXCR4 to enhance its transcription, as well as that of CXCL12. RUNX2 is a regulator of embryogenesis and development, and promotes the invasion and metastasis of GC by transcriptionally upregulating the chemokine receptor CXCR4<sup>[82]</sup>.

CXCR7 is expressed in embryonic neuronal and heart tissue, some hematopoietic cells, and the activated endothelium<sup>[83]</sup>. It is a receptor specific to SDF-1, and SDF-1 expression is strongly chemotactic for lymphocytes. It is also associated with lymph nodes in GC patients<sup>[84]</sup>. CXCR7 expression is upregulated in GC tissues. Overexpression of CXCR7 promotes cell proliferation, migration and invasion<sup>[85]</sup> and it is associated with peritoneal dissemination and poor prognosis<sup>[86]</sup>.

Other chemokine receptors are also associated with GC progression and survival. CXCR1 is a class-A, rhodopsin-like G-protein-coupled receptor, which takes charge of cellular signal transduction and is targeted as a drug receptor<sup>[87]</sup>. CXCR1 functions as a high-affinity receptor for IL-8, which is a major mediator of inflammatory responses and tumorigenesis<sup>[88]</sup>. High expression of CXCR1 is associated with poorer overall survival in stage II and III GC patients. Importantly, stage II GC patients with higher CXCR1 expression have been shown to significantly benefit from 5-fluorouracil (5-FU) based adjuvant chemotherapy<sup>[89,90]</sup>. CXCR2 expression strongly correlates with CXCR4 expression. CXCR2 expression changes according to the activity of CXCR4 signaling. CXCR4 and CXCR2 cross-activate each other to promote the metastasis of GC<sup>[91]</sup>. The co-expression of CXCR2 and IL-22 receptor 2 is associated with poor prognosis in GC. CXCR2 is involved in complex mechanisms and roles, and indicates a poor outcome in GC<sup>[92]</sup>.

Analysis of the expression levels of CXCR4 and CXCR7 revealed that these chemokine receptors are associated with the activation of the oncogenic pathway in GC. CXCL12, which is the ligand of CXCR4 and CXCR7 chemokines, is associated with poor prognosis in GC patients.

## MATRIX METALLOPROTEINASES

MMPs are a family of endogenous calcium- and zinc-dependent proteolytic enzymes. Cancer cells in a microenvironment escape from the primary lesion through the surrounding ECM and intravasate into the lumina of blood vessels during metastatic progression. MMPs probably contribute to metastasis by secretion of pro-angiogenic and ECM-remodeling factors<sup>[93]</sup>. MMPs are capable of degrading ECM proteins and drive the loss of the basement membrane. The activation of MMPs and urokinase-type plasminogen activator (uPA) are required for expression of transcription factor Snail-1, which finally inhibits E-cadherin<sup>[94]</sup>. MMPs degrade most ECM components, and regulate other enzymes, chemokines and even cell receptors. Twenty-three types of MMPs have been described so far<sup>[95]</sup>. MMP-7, named matrilysin, is a distinct family member with proteolytic activity against a wide range of biomolecules including proteoglycans, laminin, fibronectin, casein and, more importantly, basement membrane collagen type IV. It is recognized as pivotal in the MMP family because it activates other MMPs (e.g. MMP-2 and MMP-9) for ECM degradation<sup>[96]</sup> and possesses the highest activity in the MMP family<sup>[97]</sup>. MMP-7 regulates the activity of other biomolecules and MMP-7 degrades ECM protein. MMP-7 may play a central role in the stromal invasion of GC cells during the formation of peritoneal dissemination<sup>[98]</sup>. MMP-9 is known as type-IV collagenase or gelatinase B<sup>[99]</sup>.

Systematic reviews and meta-analyses have demonstrated the prognostic effects of MMP2, MMP7 and MMP9 in GC patients<sup>[100]</sup>. A meta-analysis of 10 studies (incorporating 1669 patients) was conducted to evaluate the relationship between MMP-2 and GC prognosis. Overexpression of MMP-2 is associated with TNM stage, depth of invasion, lymph node metastasis and distant metastasis. Overexpression of MMP-2 significantly predicts poor overall survival of GC patients (HR = 1.92, 95% CI = 1.48-2.48;  $P < 0.001$ )<sup>[101]</sup>.

A meta-analysis of nine studies (incorporating 1208 patients) was conducted to evaluate the relationship between MMP-7 and GC prognosis. Higher MMP-7 expression is associated with deeper invasion [pooled odds ratio (OR) = 3.20; 95% CI = 1.14-8.96;  $P = 0.026$ ], higher TNM stage (pooled OR = 3.67; 95% CI = 2.281-5.99;  $P < 0.001$ ), lymph node metastasis (pooled OR = 2.84; 95% CI = 1.89-4.25;  $P < 0.001$ ), and distant metastasis (pooled OR = 3.68; 95% CI = 1.85-7.29;  $P < 0.001$ ), but not with histological grade. Higher MMP-7 expression is associated with significantly shorter overall survival (HR = 2.01, 95% CI = 1.62-2.50,  $P < 0.001$ )<sup>[102]</sup>. Ten studies with 1478 patients were included to perform a meta-analysis of the survival results to evaluate the relationship between MMP-9 and GC prognosis. Overexpression of MMP-9 tends to be associated with lymph node metastasis (OR = 1.91, 95% CI = 1.40-2.59;  $P < 0.05$ ) and presence of vascular invasion (OR = 2.64, 95% CI = 1.52-4.59;  $P < 0.05$ ). MMP-9 overexpression is associated with shorter overall survival of GC patients (HR = 1.69, 95% CI = 1.29-2.23;  $P < 0.001$ )<sup>[103]</sup>.

Many factors that stimulate MMP expression have been reported in diverse cancer cells, such as interleukins<sup>[104-106]</sup>, epidermal growth factor<sup>[107]</sup>, fibroblast growth factor<sup>[108,109]</sup> and NF- $\kappa$ B<sup>[110]</sup>. IL-1 $\alpha$  induces MMP-1 in the stimulation of dermal fibroblasts of human melanoma cells<sup>[104]</sup>. MMP-9 and MMP-14 mRNA levels are selectively increased in response to EGFR activity in ovarian tumor cells<sup>[107]</sup>. FGF and STAT3 (Ser-727) are involved in the signaling leading to MMP-7 expression in prostate cancer<sup>[109]</sup>. In GC, IL-17A is involved in the pathology of inflammatory diseases and the tumor microenvironment. It could promote the invasion of GC cells by activating the NF- $\kappa$ B pathway, and subsequently upregulating the expression of MMP-2 and MMP-9<sup>[106]</sup>. IL-10-stimulated macrophages also induce MMP-2 and MMP-9 activities in gastric and colorectal cancer cell lines<sup>[105]</sup>. Overexpression of HER2 is also associated with MMPs. HER2 overexpression is not only closely associated with tumor growth but is also related to tumor invasion. HER2 knockdown results in the downregulation of the expression of MMP-9<sup>[111]</sup>.

## EXOSOMES IN THE TUMOR MICROENVIRONMENT

Previous studies demonstrated that CAFs and cancer cells communicate by secreting a variety of cytokines, chemokines and ECM<sup>[112]</sup>. The mechanism underlying the communication among CAFs, NFs and cancer cells has been investigated. Recent extracellular vesicle assessments have demonstrated that cancer cells interact with the neighboring cells via soluble factors secreted into the extracellular space<sup>[113]</sup>. Extracellular vesicles can be classified into three main types according to size and biogenesis: exosomes (30-100 nm), microvesicles (100-1000 nm), and oncosomes (1-10  $\mu$ m)<sup>[114]</sup>. These three extracellular vesicle types play roles in cancer biology through vesicular transport.

Recent studies have demonstrated that exosomes are associated with GC progression and metastasis. GC cell-derived exosomes induce injury of peritoneal mesothelial cells through apoptosis and mesothelial-to-mesenchymal transition, resulting in mesothelial barrier destruction and peritoneal fibrosis. GC-derived exosomes can facilitate peritoneal dissemination and a novel mechanism for GC peritoneal metastasis has been identified<sup>[115]</sup>. Next-generation sequencing technology provides more complete data and allows even deeper analyses of RNA transcriptomes. Exosomes from different GC cell lines and an immortalized normal gastric mucosal epithelial cell line were extracted and the amounts of exosomal proteins and RNAs were evaluated. According to the miRNA profiles of exosomes, miRNA-21-5p and miRNA-30a-5p were two of the most abundant sequences<sup>[116]</sup>. In another study, exosomal miRNA profiles in peritoneal fluid of peritoneal dissemination in GC were investigated. miRNA-21 was also identified as having the highest signal intensity and another five miRNAs (miRNA-1225-5p, miRNA-320c, miRNA-1202, miRNA-1207-5p and miRNA-4270) were identified<sup>[117]</sup>.

EGFR in exosomes secreted from GC cells can be delivered into the liver. The translocated EGFR on the plasma membrane of liver stromal cells activates HGF. The upregulated paracrine HGF, which binds the c-MET receptor on the migrated cancer cells, promotes liver metastases to favor the development of a

**Table 1. The association between the tumor microenvironments and survival of GC patients**

Factors	Marker	HR	95% CI	P value	Year	Journal
CAFs	IL1A, IL1B and TNF	1.41	1.11-1.78	0.004	2017	<i>Gastroenterology</i> <sup>[63]</sup>
BMDCs	CD271	1.82	1.08-3.07	0.025	2015	<i>Br J Cancer</i> <sup>[20]</sup>
TILs	CD3+ TILs (intra-tumoral)	0.52	0.43-0.63	< 0.001	2017	<i>Oncotarget</i> <sup>[28]</sup>
	FOXP3+ TILs (intra-tumoral)	1.57	1.04-2.37	0.033		
	FOXP3+ TILs (extra-tumoral)	0.76	0.60-0.96	0.022		
TAMs	Total TAM	1.71	1.19-2.45	0.004	2016	<i>Genet Mol Res</i> <sup>[41]</sup>
	M1 macrophage	0.46	0.33-0.65	< 0.001		
	M2 macrophage	1.71	1.39-2.09	< 0.001		
Chemokines	CXCR4	2.63	1.69-4.09	< 0.001	2014	<i>Tumour Biol</i> <sup>[74]</sup>
	CXCL12	2.08	1.31-3.33	0.002	2017	<i>Br J Cancer</i> <sup>[73]</sup>
MMPs	MMP-2	1.92	1.48-2.48	< 0.001	2014	<i>Cancer Biother Radiopharm</i> <sup>[101]</sup>
	MMP-7	2.01	1.62-2.50	< 0.001	2015	<i>PLoS One</i> <sup>[102]</sup>
	MMP-9	1.69	1.29-2.23	< 0.001	2014	<i>Hepatogastroenterology</i> <sup>[103]</sup>

GC: gastric cancer; HR: hazard ratio; CI: confidence interval; CAF: cancer associated fibroblast; IL: interleukin; TNF: tumor necrosis factor; BMDC: bone marrow-derived cell; TIL: tumor-infiltrating lymphocyte; TAM: tumor-associated macrophage; MMP: matrix metalloproteinase

liver-like microenvironment<sup>[118]</sup>. GC cell-derived exosomes stimulate the activation of the NF-κB pathway in macrophages, which promotes cancer progression in the tumor microenvironment<sup>[119]</sup>. The expression of miRNAs in exosomes of the peripheral blood has been investigated. High expression of miRNA-221 is associated with poor clinical prognosis in GC patients. Exosomes originating from BMDCs, which were transfected with miRNA-221 mimics, promote proliferation, invasion, migration and adhesion to the matrix of GC cell lines<sup>[120]</sup>. CD97 knockdown reduces the metastatic capacity of GC cells. Exosomes or conditioned medium from the SGC-L cells (GC cell line) activate proliferation and invasion as compared with that from SGC-L/CD97-knockdown cells. Exosomal CD97 is associated with CD55, CD44v6, α5β1 and CD31, and the exosome-dependent CD97 plays a role in premetastatic niche formation<sup>[121]</sup>.

Investigating exosomal miRNA secretion provides novel insight into communication among microenvironments of cancer cells. Dysregulation of miRNAs in CAFs, NFs and cancer cells can affect the secretory phenotype of cancer cells.

## CONCLUSIONS AND PERSPECTIVE

This review describes the importance of the microenvironment of GC for cancer progression and metastasis. Major components of the microenvironments of GC consist of BMDCs, TILs, TAMs and CAFs. GC cells are also affected by these components, with chemokines and miRNA in extracellular vesicles such as the exosome. The accumulation of BMDCs is associated with *Helicobacter pylori* infection. Cytokines and growth factors secreted by BMDCs lead to cancer progression and metastasis. The amount of CD3+ TILs in the intra-tumoral compartment is the most significant prognostic marker. PD-L1 expression was significantly associated with the prognosis of GC patients owing to the interaction between PD-L1 and TILs. Increased levels of total TAM and M2 macrophage infiltration in GC patients are associated with worse overall survival. In contrast, elevated M1 macrophage density is associated with better overall survival. M2 macrophages might activate PD-L1 expression in tumor cells. Interleukins and miRNAs produced by CAFs are associated with cancer progression and metastasis of GC cells. CXCR4 and CXCL12 are associated with prognosis of GC patients. CXCL12 strongly activates the Akt pathway and upregulates the expression of MMP-2 and MMP-7 to assist EMT. These MMPs are capable of degrading ECM proteins and trigger the loss of the basement membrane.

The microenvironments of GC are associated with lymphatic invasion, vascular invasion, lymph node metastasis and survival of GC patients [Table 1]. The interaction between GC cells and the microenvironments of GC is increasingly being recognized, and the microenvironments of GC, as well as GC cells, may have become a target of anticancer strategies. Future studies may investigate whether inhibitors of the interaction between GC cells and the microenvironments improve GC patient prognosis in preclinical studies. Much research in the field of microenvironments of GC and the accumulation of molecular biological investigation are important for improving the management of GC and overcoming this disease in the future.

## DECLARATIONS

### Authors' contributions

Drafting of the manuscript: Sawayama H

Critical revision of the manuscript for important intellectual content: Ishimoto T, Baba H

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### Conflicts of interest

There are no conflicts of interest.

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Not applicable.

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Not applicable.

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**Topic: How does the prostate cancer microenvironment affect the metastatic process and/or treatment outcome?**

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# Current challenges and opportunities in treating hypoxic prostate tumors

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## ABSTRACT

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Hypoxia is a well-established characteristic of prostate tumors and is now recognised as a major contributory factor to both tumor progression and increased resistance to therapy. One strategy to target hypoxic tumor cells is the development of hypoxia-activated prodrugs (HAPs), which are activated in low oxygen environments. Several HAPs have been developed but despite encouraging results from preclinical studies many of these have performed disappointingly in clinical trials. In the developing era of precision medicine, it is clear that more strategic deployment of these agents is required, based on reliable methods that can identify patients who will benefit from HAP treatment, either alone or in combination with other drugs. This review discusses the primary limitations of using HAPs to treat hypoxic tumors and explains how these challenges can be addressed. In particular, it emphasises the importance of tumor imaging and identification of reliable biomarkers for measuring hypoxia and monitoring cellular response to treatment in individual patients. Developing predictive assays for clinical use will be paramount in demonstrating the patient impact and effectiveness of HAPs for personalised medicine.

## INTRODUCTION

A large body of evidence now exists to show that hypoxia occurs in most solid tumors and can have a major influence on treatment response<sup>[1-3]</sup>. Under hypoxic stress, cells respond in a number of ways

which are primarily mediated through hypoxia-inducible factors (HIFs)<sup>[4]</sup>. When cellular oxygen levels are normal HIF $\alpha$  subunits are degraded by the proteasome following hydroxylation by prolyl hydroxylase domain (PHD) proteins and poly-ubiquitination by the von Hippel-Lindau tumor suppressor, which is the substrate recognition component of an E3-ubiquitin ligase. When



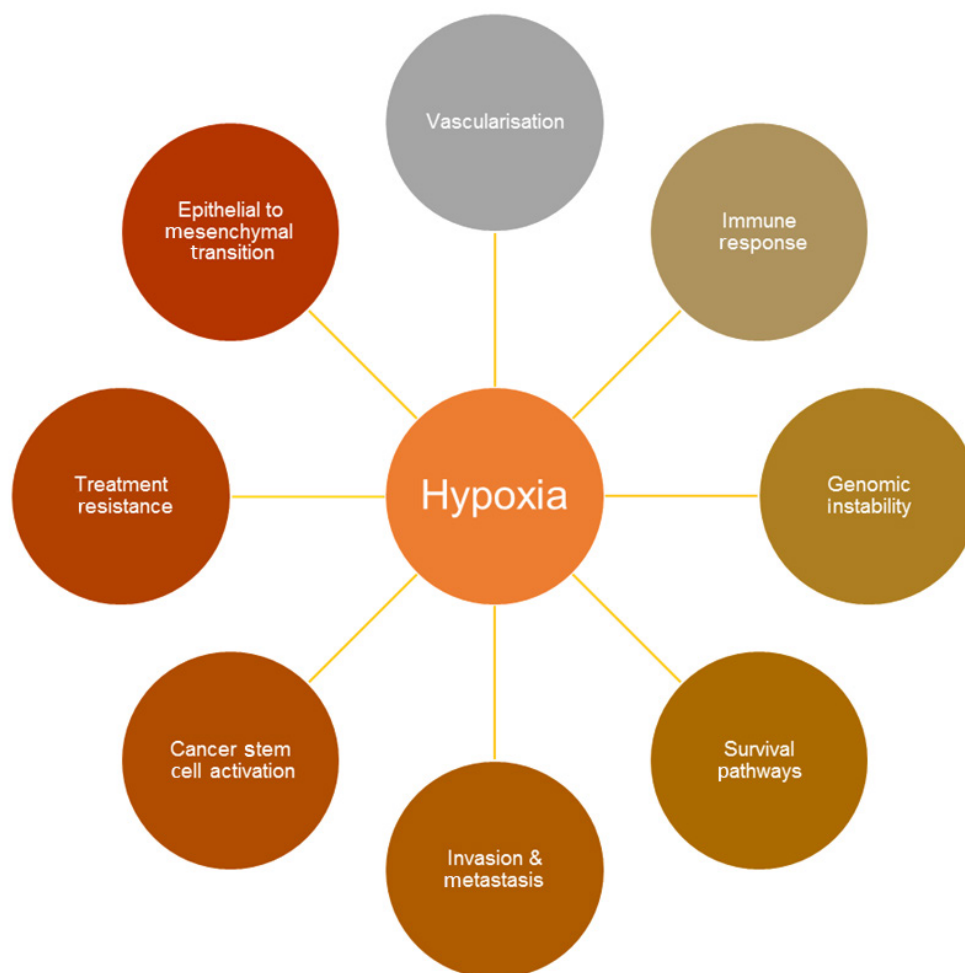
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**Figure 1:** Hypoxia impacts upon a number of important pathways which can promote tumor growth and progression

oxygen levels are low, the PHD enzymes become inactive, thereby reducing the degradation of HIF $\alpha$ . The stabilised HIF $\alpha$  molecules translocate to the nucleus, form dimers with constitutively expressed HIF $\beta$  subunit, and bind to DNA to initiate gene transcription in response to the hypoxic environment<sup>[6]</sup>. HIF-independent hypoxia responses have also been described, including adaptive mechanisms regulated by mTOR signalling<sup>[6]</sup>, p38 MAPK<sup>[7]</sup> and NF- $\kappa$ B<sup>[8]</sup>. It is therefore clear that a complex network of cellular and molecular signalling occurs when cells are exposed to hypoxic stress<sup>[9,10]</sup>.

This is important during cancer progression, because accelerated proliferation of cancer cells can result in abnormal vascularization, unstable blood flow and reduced O<sub>2</sub> diffusion within a solid tumor, causing hypoxic regions to develop. This is significant because tumor hypoxia has been shown to cause numerous molecular and genetic changes within cells which promote cell survival and drive tumor development [Figure 1]<sup>[9,10]</sup>.

Table 1 shows the reported values from different studies on various cancers, demonstrating that the oxygen level in normal tissues can vary from approximately 4%-6% oxygen depending on the tissue; the normal prostate has one of the lowest reported median oxygen levels (~4%)<sup>[3]</sup>. Normal physiological stress responses to a reducing level of oxygen probably occur between 1% and 3% although the exact level is difficult to define and may well depend on multiple factors including the tissue under investigation. In tumors, oxygen levels are frequently reported at well below 1% indicating that tumor cells are exposed to severe hypoxic stresses. The proportion of the cells exposed to these extreme hypoxic stresses will vary across the tumor and can also be modified by responses to treatment.

Untreated prostate tumors are known to be very hypoxic (~0.3% oxygen)<sup>[3,4]</sup>, which is > 12 times lower than oxygen levels found in the normal prostate<sup>[3,11]</sup>. Prostate tumor hypoxia has been implicated as a causative factor in malignant progression<sup>[12,13]</sup>, genetic

**Table 1: Reported values of the partial pO<sub>2</sub> in human tumors and corresponding normal tissues**

Tumor type	<i>n</i>	Median tumor pO <sub>2</sub>	Median % oxygen	<i>n</i>	Median normal tissue pO <sub>2</sub>	Median % oxygen	Fold pO <sub>2</sub> decrease <sup>a</sup>	Reference
Brain (6)	104	13	1.7	104	26	3.4	2	[11]
Head and neck cancer (13)	592	10	1.3		ND	5.9	4.5	[11]
	30	12.2	1.6	14	40	5.3	3.3	[88]
	23	14.7	1.9	30	43.8	5.8	3	[89]
	65	14.6	1.9	65	51.2	6.7	3.5	[90]
Lung cancer	6	14.3	1.9		ND	5.6	3	[91]
	20	16.6	2.2		42.8	5.6	2.6	[92]
Breast cancer (10)	212	10	1.3	212	52	6.8	5.2	[11,93]
Pancreatic cancer	7	2.7	0.4	7	51.6	6.8	19.1	[94]
	1	2	0.3				22.7	[95]
Prostate cancer	59	2.4	0.3	59	30	3.9	12.5 <sup>b</sup>	[96]
	55	4.5	0.6		ND		6.7 <sup>b</sup>	[97]
	10	9.4	1.2	2	26.2	3.4	2.8 <sup>c</sup>	[98]
Melanoma	18	11.6	1.5	20	40.5	5.3	3.5	[99]
Rectal carcinoma	14	32	4.2		52	6.8	1.6	[100]
	15	19	2.5		52	6.8	2.7	[101]
Sarcoma (14)	283	14	1.8	283	51	6.7	3.6	[11]

The data in the table is adapted with permission from a review by McKeown (2014). The number of studies included for each tumor type is indicated by the number in the “tumor type” column. Other data are from single studies, as referenced. <sup>a</sup>Fold reduction of tumor vs. normal tissue is based on all the data presented in the table (except prostate; see below); <sup>b</sup>fold reduction calculated on contemporaneous measurements in the psoas muscle; <sup>c</sup>data from a pilot study that included values from the “normal” prostate of two bladder cancer patients. ND: not determined; pO<sub>2</sub>: pressure of oxygen

instability<sup>[14]</sup>, endothelial-to-mesenchymal transition<sup>[15,16]</sup> and selection of cells with diminished apoptotic potential and a greater invasive potential<sup>[17,18]</sup>. These plethora of changes means that the presence of hypoxia has significant implications for cancer therapy<sup>[11,19]</sup>. Indeed, as far back as the 1950s, it was realised that hypoxia is an underlying cause of resistance to radiotherapy<sup>[20,21]</sup>. Since then it has been consistently shown that high levels of hypoxia significantly correlate with increasing clinical stage and can predict biochemical failure following radiotherapy<sup>[22]</sup>. Recent studies have shown that hypoxic conditions significantly enhances exosome secretion in a HIF-1α-dependent way<sup>[23]</sup>. Exosomes are microvesicles containing a cargo of signature proteins, lipids, nucleic acid and metabolites that can contribute to the remodelling of the tissue microenvironment<sup>[24,25]</sup>. In prostate cancer models they have been shown to mediate angiogenesis, cell stemness and activation of the surrounding tumor stroma<sup>[26]</sup>. Similarly, hypoxia has been linked with increased resistance to chemotherapeutic drugs<sup>[27,28]</sup>. Therefore, hypoxia is clearly a significant obstacle to the effective treatment of tumors, so it is a viable therapeutic strategy to directly target hypoxic tumor cells in an attempt to improve treatment<sup>[27,29,30]</sup>. Although such a strategy has yet to establish clinical acceptance, one of the most promising translational approaches for patient treatment is based on the use of bioreductive drugs<sup>[31,32]</sup>. These are now more commonly known as hypoxia activated prodrugs (HAPs) or, in the

case of the metabolically distinct anthraquinone-derived compounds, unidirectional HAPs (uHAPs). This review will focus on the therapeutic potential of these compounds in targeting hypoxic tumor cells, although the molecular targeting of hypoxia factors such as HIF is an equally valid strategy for targeting hypoxia and is reviewed elsewhere<sup>[30,33]</sup>.

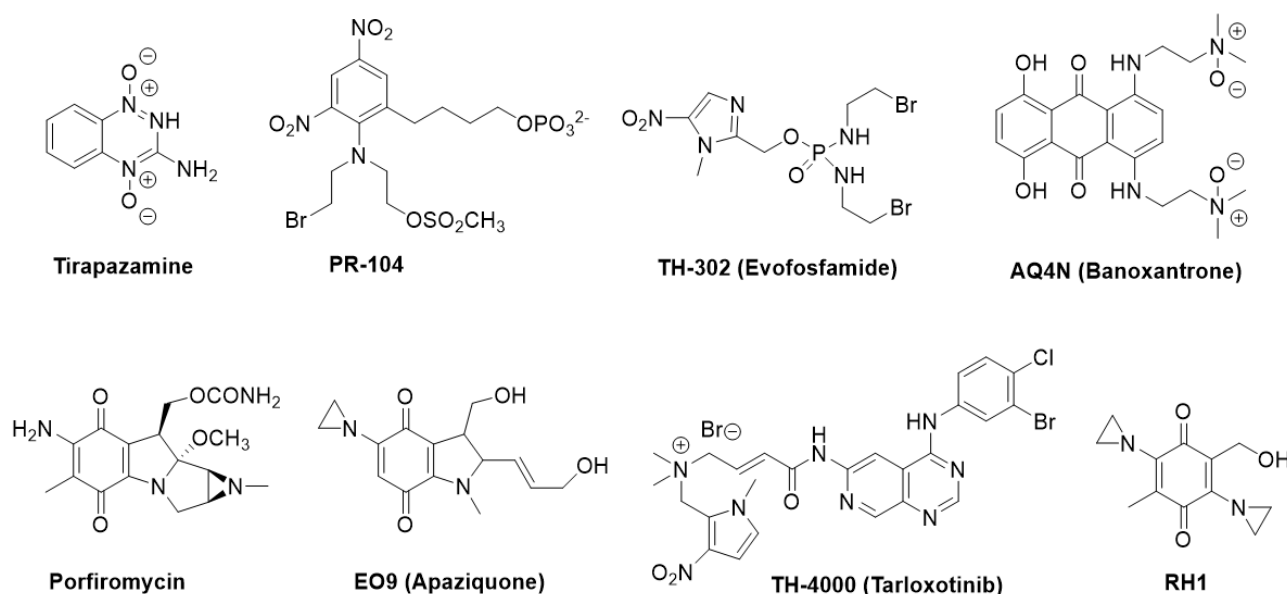
The concept underpinning the use of HAPs is well-established and several recent reviews exist, which we refer to for further understanding<sup>[32,33]</sup>. When oxygen levels are very low HAPs or uHAPs are reduced to covalently-binding active cytotoxins or release DNA-damaging radicals<sup>[31,32]</sup>. Thus the incorporation of a HAP into a treatment regime should be an ideal approach to specifically target tumor cells, particularly as hypoxia is rare in normal tissues<sup>[34]</sup>. Other properties for an effective HAP, discussed throughout this review, include (1) the ability to reach hypoxic cells, (2) pharmacological features which allow it to be metabolised effectively, and (3) exertion of a lasting, targeted effect on the tumor<sup>[32]</sup>. With these in mind several compounds have been developed and tested *in vitro*, *in vivo* and in patients with different cancers [Table 2 and Figure 2].

However, although encouraging results have been obtained from preclinical studies many of the HAPs listed in Table 2 have not been realised in clinical trials. Currently, only a few of these molecules are being

**Table 2: HAPs which have been tested in human clinical trials**

Prodrug	Company or institution	Chemical class	Mechanism of cytotoxicity	References
Tirapazamine (SR 4233)	SRI International/NCI	Aromatic N-oxide	Complex DNA damage	[102,103]
Apaziquone (E09)	Spectrum	Quinone	DNA interstrand crosslink	[104,105]
Evofofamide TH-302	Threshold	Nitroaromatic	DNA interstrand crosslink	[106-108]
Tarloxitinib	Threshold and University of Auckland	Nitroaromatic	Pan-HER inhibitor	[109]
TH-4000			Tyrosine kinase inhibitor	
PR-104	Proacta and University of Auckland	Nitroaromatic	DNA interstrand crosslink	[110,111]
Banoxantrone (AQ4N)	KuDOS/Novacea	Aliphatic N-oxide	DNA intercalator and topo II inhibitor	[35]
Porfiromycin	Vion Pharmaceuticals	Quinone	DNA interstrand crosslink	[112,113]
RH1	CRUK	Quinone	DNA interstrand crosslink	[114]

NCI: National Cancer Institute; CRUK: Cancer Research UK; HER: human epidermal growth factor receptor; HAPs: hypoxia-activated prodrugs

**Figure 2:** Chemical structures of HAPs that have been under clinical evaluation. HAPs: hypoxia-activated prodrugs

actively pursued, whereas the clinical development of others has been discontinued<sup>[31,32]</sup>. It has become clear that future large HAP clinical registration trials need to incorporate biomarkers of hypoxia to identify patients who would benefit from this type of treatment. Furthermore, in some clinical trials involving HAPs, later retrospective analyses were carried out and showed that specific cohorts treated did have a significant survival advantage. Thus, as with many cancer therapies there is a requirement to stratify patients for a number of different factors including importantly hypoxia. As **Table 1** shows, there is considerable variation in tumor hypoxia between patients, meaning not every patient will show the same response to HAP therapy. Nonetheless, a proof-of-principle study has demonstrated that in patients with different tumor types, AQ4N was activated selectively in hypoxic regions in human solid tumors to AQ4 the hypoxia-activated metabolite of AQ4N and

a potent DNA intercalator and topo II poison<sup>[35]</sup>. This phase I study, has been vital to the identification of the potential clinical efficacy of this prodrug.

Furthermore, tumor heterogeneity will also mean that not all cancer cells will have the innate capacity to be targeted in the same way or to the same extent, as the HAP may not be effectively metabolised to the same degree across the tumor micro-environment. Another difficult question to address clinically is also whether the reductases that are identified as capable of activating the HAPs in preclinical models are present in all hypoxic cells within a heterogenous tumor. Most HAPs (including nitroaromatics, quinones and benzotriazine di-oxides) are activated via a mechanism that begins with one-electron reduction by flavin-dependent oxidoreductases to generate a metabolite which can be readily back-oxidised during fluctuating oxygen tensions; this might be a

contributing factor to resistance mechanisms under acute hypoxia but not chronic fractions of solid tumors<sup>[31]</sup>. HAPs such as AQ4N that rely on an aliphatic tertiary amine N-oxide are activated via two-electron reduction that is catalysed by CYP isoforms<sup>[36-41]</sup> is not oxygen sensitive and hence a more persistent cell killing effect may be observed; the more metabolically stable, deuterated analogue of AQ4N, OCT1002 is described further below.

## COMBINATION TREATMENT WITH HAPS

It is clear from clinical results thus far that an increased understanding of how HAPs are activated in different tumor types is required in order to develop reliable predictors of tumor sensitivity to this type of treatment. Moreover, as with most chemotherapeutic drugs, it is unlikely that monotherapy with any given HAP will prove to be wholly effective. A more realistic scenario is that susceptible tumors can be treated with combinatorial therapy which includes a HAP. In the preclinical setting, enhanced anticancer activity has been demonstrated by combining chemotherapy with HAPs. In prostate cancer, synergistic effect has e.g. been achieved using doxorubicin or docetaxel in combination with TH-302, supporting HAPs with cycle-active chemotherapy to treat aggressive forms of prostate cancer<sup>[42]</sup>.

In a clinical context, several HAPs have been investigated in combination with conventional cytotoxic chemotherapy or radiotherapy<sup>[43,44]</sup>. Although some patients have benefitted from the combination therapy, the results of these trials have at large been disappointing as reflected upon by Hunter *et al.*<sup>[31]</sup>. However, with the increasing knowledge we have gained, especially over the past decade, perhaps other combination drugs that address molecular targets, oncogenic drivers and exploit DNA damage response (DDR) pathways will pave the way for the next generation of HAPs.

For example, there is evidence to suggest that DDR induced by hypoxia is altered from the classical pathways induced by damaging agents<sup>[45]</sup>. There are possibly several reasons for this and include repression of DNA repair in hypoxic conditions. Treatment is complicated further by several reports indicating that DNA repair under hypoxia is defective or abnormal and hence may not respond to exposure of the bio-reduced metabolites of the HAPs that have undergone clinical evaluation.

The complex nature of a heterogeneous tumor is likely

to result in a number of alterations and include (1) alteration of the catalytic activity of drug-metabolizing enzymes that are responsible for HAP bioconversion, and (2) the DDR may be differently regulated in different types of cells, e.g. a hypoxic cell and a hypoxia-located cancer stem cell. Some evidence indicate that hypoxia-induced DDR under more extreme hypoxia (< 0.1%) occurs in the absence of detectable single- or double-strand breaks and in a background of repressed DNA repair (Olcina & Hammond). In this regard, it could be important in the future to explore how DNA-targeted metabolites derived from HAPs can be used to exploit changes in DDR influenced by hypoxia.

It is likely that the single electron-reduced HAPs could be sensitive to changes in DDR. HAPs such as tirapazamine and PR-104A that are reduced to DNA-reactive metabolites via one-electron reduction have been shown to be more potent in cancer cells harbouring DDR pathways that include dysfunctional homologous recombination repair (HRR)<sup>[46,47]</sup>. Exploitation of dysfunctional HRR genes in hypoxic tumors require the discovery of biomarkers that can help to predict a better response to HAPs, however there has been very little systematic effort to discover and fully unravel the potential of such biomarkers<sup>[31]</sup>. This is in part due to the nature of such research, complicated by tumor reoxygenation that often occurs as a result of spontaneous changes in blood flow and therapy with subsequent impact on DDR pathways<sup>[48]</sup>. An example of how improved understanding of the DDR machinery provides an opportunity for combination therapy was demonstrated by Lindquist *et al.*<sup>[49]</sup> who investigated the potential for inhibiting DNA double strand break repair in hypoxic cells by targeting DNA-dependent protein kinase (DNA-PK). BCCA621C, a DNA-PK inhibitor was shown to be able to radiosensitize NCI-H460 cells under hypoxic but not normoxic conditions using a range of clinically relevant ionising radiation doses. There is also evidence that Chk1, ATM, ATR and poly (ADP-ribose) polymerase (PARP) are affected by hypoxia<sup>[48]</sup>. In regard to the latter, several PARP inhibitors are under clinical evaluation and information from these trials will provide key information on how HAPs could be used in combination with PARP inhibition (PARPi) alone or with additional radiotherapy. Preclinical data have shown that PARPi can be used as a radiosensitizing agent, which may increase the efficacy of radiotherapy in prostate cancer<sup>[50]</sup>. Recently, Hammond and co-workers have shown that olaparib and radiotherapy combination therapy had significant effect in hypoxic lung cancer xenografts but limited efficacy in less hypoxic tumors<sup>[51]</sup>. It is possible this effect was due to hypoxia-induced contextual synthetic cell-killing events<sup>[52]</sup>. The nature of the tumor microenvironment has an impact on treatment



outcome. Veliparib has been shown to potentiate PC-3 but not DU-145 tumors to radiotherapy, which may be correlated with higher levels of hypoxia in PC-3 tumors compared with DU-145 tumors<sup>[53]</sup>. These studies did not include pharmacokinetics of either olaparib or veliparib and hence the distribution of the PARP inhibitors within the tumor microenvironment is unknown. It is tempting to speculate that improved delivery of the PARP inhibitors to the hypoxic fractions or inclusion of an appropriate HAP could lead to an enhanced therapeutic index.

## USE OF OCT1002 TO IMPROVE EXISTING THERAPY

Research in our own labs have focused on how uHAPs can improve androgen deprivation therapy (ADT) for prostate tumors. Most HAPs are reduced in single-electron reduction steps, a process which is reversible if oxygen levels increase. However, AQ4N<sup>[54]</sup>, its deuterated analogue OCT1002 (OncoTherics Ltd)<sup>[55]</sup> and AQ4N analogues with potential to covalently adduct DNA/topo II<sup>[56]</sup> can be considered uHAPs. These compounds are alkylaminoanthraquinone di-*N*-oxides, which are irreversibly bioactivated via a two-step, two electron reduction to form the reduction products (AQ4 and OCT1001, respectively). These are metabolically stable, highly toxic DNA-affinic reduction products which exist independent of any further change in oxygenation. OCT1002 differs from AQ4N through highly selective deuterium substitution of the 12 hydrogen atoms contained within the two *N*-oxide side chains<sup>[55]</sup>. This results in superior intracellular persistence of the activated form OCT1001, since deuteration slows cytochrome P450 metabolism, alters subcellular localisation and sequestration properties, thereby contributing to an enhanced intracellular persistence of the activated drug as described for other drugs<sup>[57,58]</sup>. Consequently, it is predicted that OCT1002 should be an improved analogue and is therefore under extensive preclinical evaluation.

A recent study has investigated how OCT1002 may be combined with existing therapies for prostate cancer to prevent ADT resistance and progression to castrate resistant prostate cancer (CRPC)<sup>[55]</sup>. It was shown that OCT1002 has a hypoxia-dependent anti-tumor effect in androgen-sensitive LNCaP prostate tumor xenografts and the effect can be markedly enhanced when combined with bicalutamide, an ADT drug which inhibits androgen signaling by targeting the androgen receptor (AR). The study also showed that it could block significantly the molecular changes caused by bicalutamide alone. This is consistent with

previous studies in the same model which showed that bicalutamide induces hypoxia through vascular collapse<sup>[15,57]</sup> resulting in molecular changes that included evidence of endothelial to mesenchymal transition and increased metastasis to the lungs within 4 weeks<sup>[15]</sup>. These hypoxia-induced responses may help explain why patients treated solely with ADT often relapse; the hypoxic stress selects for resistant cells which survive to establish a tumor with a more malignant phenotype. Along with other studies investigating the link between hypoxia ADT on tumors<sup>[59,60]</sup>, this lends weight to the idea that drug-induced hypoxia may in fact drive prostate cancer progression and that HAPs may be a valuable way to address this issue.

This is timely work as the idea of combinatorial drug treatment has gained considerable traction in recent years. In particular, recent results from the CHARTED<sup>[61]</sup> and STAMPEDE<sup>[62]</sup> clinical trials have revealed that use of docetaxel in combination with ADT improved relapse-free survival in patients with high-risk localised prostate cancer, proving that combining ADT with other types of drug can benefit prostate cancer sufferers. Since hypoxia is a major factor in developing ADT resistance, it makes sense to combine ADT with HAPs or uHAPs as a therapeutic strategy. However, as discussed above the absolute requirement for patient derived evidence-led decision making during clinical development of various HAPs demonstrates that translating these compounds into clinically accepted drugs needs careful consideration of tumor micro-environment and related hypoxic status. It requires both improved understanding of the action of these agents, as well as methods with which to clearly identify tumors which will be sensitive to HAPs. We still need improved ways to predict which patients will respond to which drugs. Making the right decisions on whether to use HAPs require increased knowledge about the hypoxic mechanisms which drive prostate cancer progression in order to improve patient stratification in the clinic. This means developing accurate, sensitive ways to identify tumors that are likely to be susceptible to hypoxic targeting.

## DETECTION OF PRODRUG CONVERSION AND PREDICTION OF RESPONSES TO HAPS

The key to ascertaining or indeed stratifying a prostate tumor for sensitivity to hypoxia targeting through HAP treatment requires a multi-pronged approach which has to take into consideration multiple aspects. Importantly this requirement provides an opportunity to bring new technologies and innovations to bear in order to really elucidate the effectiveness of the



drug from molecular profiling to potentially single cell functional analysis. Thus here we consider approaches aimed at developing novel and functional assays for tumor stratification.

Many hypoxia-targeting small molecules, for example, [(18)F]FAZA, [(18)F]FMISO, [(18)F]EF5, and [(123)I]IAZA, have been shown to accrue selectively in hypoxic cells. These positron emission tomography molecular contrast agents have been extensively applied in clinical hypoxia imaging, including cancer<sup>[63]</sup>. However the outstanding challenge is to multiplex these imaging readouts with the delivery and conversion of prodrug in the same tumor and package the acquisition and analysis algorithms such that they offer pragmatic solutions for advancing our understanding of HAP bioavailability and conversion.

Many bioactive molecules have chromophores<sup>[64]</sup> thus offering the prospect for tracking target interactions through methods such as steady-state fluorescence readouts, or determining fluorescence quenching properties and fluorescence lifetime measurements for detecting drug tethering to target. Fluorescence life-time and quenching analyses can lead to a unique means for dissecting sub-resolution molecular interactions *in situ*<sup>[65]</sup>. For instance, recent spectroscopic investigations show molecular properties of doxorubicin change due to alterations in the local environment, such as when the drug is encapsulated to nanoparticles. Thus we suggest that fluorescence imaging provides a powerful tool for investigating drug delivery in tumor cells and tissue, and further allows for the linking of multi-scalar features of drug design, stability and metabolism together with the complexities imposed by the biological system including tissue penetration and drug-target interactions.

All these fluorescent modalities are very much applicable for the uHAPs such as AQ4N and OCT1002 which are fluorescent due to the anthraquinone chromophore and detectable *in vitro* and *in vivo*<sup>[55,66]</sup> and also retained in tissue even after snap-freezing of xenograft material. Cryosections of frozen xenograft tumor tissue slices were examined for AQ4 fluorescence and distribution by fluorescence microscopy, alongside HPLC/mass spectroscopy analysis<sup>[67]</sup>. To extend the concept further, the efficiency of drug-target interaction of the prodrug is driven by not only pharmacokinetic factors but a host of parallel cellular status and events that are required to elicit the sought pharmacodynamic responses, which are also heterogeneously expressed through the tumor. Hence the requirement for *in vivo*

pharmacodynamics readouts, such as that provided by a truncated 53BP1 double-strand reporter, recently shown to accentuate the approach for *in situ* single cell analysis of cancer therapeutics<sup>[68]</sup>. Applying this PK-PD linked imaging at the single cell would provide the evidence and mechanisms essential for the development of HAP therapeutic strategies that address changing patterns of target presentation in different cellular microenvironments, and prostate tissue architecture.

## BH3 PROFILING TO PREDICT CAPACITY FOR CELL DEATH AT THE SINGLE CELL LEVEL

The primary action of the AQ4N and OCT1002 metabolites is through DNA damage and subsequently apoptosis. Despite much research into the molecular pathways that regulate cell death, the signalling networks involved are so complex that molecular profiling of key pro-and anti-apoptotic players alone does not provide the predictive capability needed to assess chemo-responsiveness<sup>[69]</sup>. Thus, functional BH3 profiling would lead to the derivation of cell death fingerprinting, determining the sensitivity thresholds for apoptosis between and within heterogeneous cancer cell populations. The underlying principle of BH3 profiling is that mitochondrial depolarization or subsequent processes such as BAK/BAX oligomerisation or cytochrome release following BH3 peptide exposure serves as a functional biomarker for cellular response to pro-apoptotic cues. A recent technology innovation has led to the development and implementation of novel nano-tools (cross-linked stapled peptides) to aid the understanding of apoptotic responses using flow and image cytometry<sup>[70,71]</sup>. Feasibility studies have shown that BH3-derived peptides alkylated with azobenzene cross-linkers have the ability to induce detectable physiological changes paralleling the early events in apoptotic cell death. The objective now is to establish a validated BH3-profiling pipeline suitable for sample stratification, using these peptide BH3 pathway inducers and sensitizers<sup>[72]</sup>. In short, BH3 profiling provides a functional readout for the primed apoptotic state of a heterogeneous population of cells, again which can be directly linked to drug bio-reduction and retention at the single cell level.

## MOLECULAR PROFILING AND BIOINFORMATIC ANALYSIS

The drive towards personalised medicine depends on the discovery of biomarkers which can allow molecular stratification of patients. Such information

is likely to reside in the vast arrays of data detailing the specific genetic characteristics of individual prostate tumors which has been gathered by genomic profiling in recent years. Comprehensive bioinformatics analyses of this data has revealed that a wide molecular diversity exists in human cancer, including prostate tumors (TCGA Network, 2015)<sup>[73]</sup>. Such tumor heterogeneity may help explain why patients presenting with pathologically similar tumors can have very different responses to the same course of treatment. For example, primary prostate cancers exhibit a wide variability in AR activity, with increased AR-dependent signalling linked to gene mutations in SPOP and FOXA1 (TCGA Network, 2015)<sup>[73]</sup>. Knowing whether a tumor carries these mutations or not can help determine the most appropriate ADT approach for a patient and subsequent tracking of those gene mutations can inform adaptive drug administration. Likewise, knowing the mutational status of the AR gene itself will be critical in helping predict treatment outcome. For instance, enzalutamide cannot bind to an abnormal splice variant of the AR called AR-V7, so patients harbouring this mutation would be unlikely to respond to that particular drug, further emphasising the need to stratify patients by molecular profiles. Indeed, recent research has shown that AR-V7 can be detected in patient blood samples and efforts to validate this screening for clinical application are under way<sup>[74]</sup>.

In a similar manner, it is possible to probe this data for hypoxic markers, allowing researchers to identify key patterns which may allow patient stratification based on hypoxic indices. Hypoxic gene signatures with prognostic potential have been identified in various cancers, such as breast<sup>[75]</sup>, head and neck<sup>[76]</sup> and laryngeal cancer<sup>[77]</sup>, each study highlighting how expression of genes related to hypoxia can be used to predict outcome. In a prostate cancer setting, a combination of these signatures was subsequently used to categorise hypoxic status of a total of 271 radical prostatectomy samples from two independent cohorts in a study which showed that biochemical relapse was associated with indices of tumor hypoxia, genomic instability, and genomic subtypes based on multivariate analyses<sup>[78]</sup>. Patients with a low percentage of genome alteration and low hypoxia had the best outcome, whereas those with high levels of both measures had the worst. Another study investigated gene expression in prostate tumor biopsies staining positive for hypoxia marker pimonidazole and also identified a signature of hypoxic response genes which correlated with tumor aggressiveness<sup>[79]</sup>. These studies demonstrate the value of genetic profiling of hypoxic status to help

stratify patients for treatment, which possibly could include hypoxia targeting in selected groups. As data on clinical samples and patient outcome continues to be collected and archived in data repositories like The Cancer Genome Atlas, these genetic signatures can be continually refined by bioinformatic analysis to identify the most reliable markers of prostate tumor hypoxia.

In addition to tumor analysis, non-invasive biomarkers which can be measured in biofluids are also an attractive option for clinical use. In this regard, microRNAs have generated much excitement as potentially valuable markers of prostate progression and treatment response. These small RNA molecules are much more stably preserved than other RNA species in clinical samples, including fresh and fixed tissues, serum and urine, and can be readily detected using highly specific and sensitive PCR-based assays. miRNAs are important regulators of cell function and many of them are aberrantly expressed in prostate cancer<sup>[80,81]</sup>. Of these, miR-210 has been identified as a key regulator of hypoxia<sup>[82,83]</sup> and has been implicated in prostate cancer progression<sup>[84]</sup>. Significantly, serum levels of miR-210 have been shown to be elevated in prostate cancer patients compared to benign prostatic hyperplasia controls<sup>[85]</sup>, as well as in metastatic CRPC patients who did not respond to treatment<sup>[86]</sup>. The goal is that miR-210 and other related miRNAs can be used as a panel of serum biomarkers that will reflect extent of tumor hypoxia.

It is therefore clear that any strategies for treating prostate cancer must embrace molecular profiling as a means to stratify patients and also monitor response to treatment. Since hypoxia plays such a fundamental role in prostate cancer progression, examining the altered expression of genes involved in hypoxia-related pathways, as well as network analysis of their interactions, will be an important consideration in developing precision medicine for individual patients.

## CONCLUSION

A major challenge in cancer therapy is to develop therapeutic agents that selectively target tumor cells. One avenue towards the development of more selective cancer therapies is to exploit the unique physiological properties of solid tumors using prodrug approaches. Hypoxia generated as a result of a poor and inefficient neovasculature is a characteristic feature of many solid tumors and is associated with the development of an aggressive phenotype and resistance to radiotherapy and chemotherapy. Whilst problematical for conventional therapies, hypoxia is

regarded as a valid target for drug development and a series HAPs have been developed over a period of 30-40 years with eight HAPs reaching clinical evaluation. Currently, no HAP has reached the market and this is somewhat perplexing given the overwhelming evidence of solid tumors containing significant levels of acute and chronic hypoxia. If patients were molecularly stratified for treatment based on their tumor hypoxia signature including analysis of reductase expression, it is possible that the HAPs in combination with chemotherapy or radiotherapy would have resulted in improved treatment outcomes. Prostate tumors are considerably hypoxic as discussed in this review, which poses some unique challenges to effective treatment of aggressive forms of this disease with standard therapies such as docetaxel and/or radiotherapy. Clinical trials carried out with AQ4N have been promising, demonstrating safe administration of a uHAP that rapidly distributes throughout the body and penetrates into hypoxic regions where it is bio-reduced to a persistent DNA-affinic topo II-targeting metabolite. The deuterated AQ4N analogue OCT1002 offers great potential in the treatment of prostate cancer, for example in the combination with ADT. In prostate cancer, uHAPs could also be used in combination with PARP1 inhibitors in patients whose tumors harbour DDR deficiencies. Much progress is being made on how best to utilise PARP1 inhibitors but prior analysis of tumor heterogeneity and target expression is vital for clinical success. For example, a recent phase 2 trial that concerned patients with metastatic prostate cancer benefitted from whole-exome sequencing and transcriptome analysis on DNA from fresh-frozen tumor-biopsy samples prior to treatment. In this study, understanding of DNA defects enabled clinicians to select patients suitable for the PARP inhibitor olaparib to ensure better treatment outcome<sup>[87]</sup>. Finally, the emergence of genetic and hypoxic signatures and the ability to image and analyse the heterogeneity of prostate tumors provides new opportunities for employing HAPs and uHAPs in combination with molecularly-targeted agents and/or radiotherapy.

## DECLARATIONS

### Authors' contributions

The authors contributed equally to the manuscript writing and editing.

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None.

### Conflicts of interest

Professor Rachel Errington is a co-inventor of OCT 1002

(New compounds and uses thereof [CA2881324A1]) and non-executive director of Biostatus Ltd which is the current assignee. Rachel Errington is a shareholder of Oncotherics Ltd.

### Patient consent

Not applicable.

### Ethics approval

Not applicable.

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Review

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# Pharmacogenomics in colorectal cancer: current role in clinical practice and future perspectives

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## Abstract

The treatment scenario of colorectal cancer (CRC) has been evolving in recent years with the introduction of novel targeted agents and new therapeutic strategies for the metastatic disease. An extensive effort has been directed to the identification of predictive biomarkers to aid patients selection and guide therapeutic choices. Pharmacogenomics represents an irreplaceable tool to individualize patients treatment based on germline and tumor acquired somatic genetic variations able to predict drugs response and risk of toxicities. The growing knowledge of CRC molecular characteristics and complex genomic makeup has played a crucial role in identifying predictive pharmacogenomic biomarkers, while supporting the rationale for the development of new drugs and treatment combinations. Clinical validation of promising biomarkers, however, is often an issue. More recently, a deeper understanding of resistance mechanisms and tumor escape dynamics under treatment pressure and the availability of novel technologies are opening new perspectives in this field. This review aims to present an overview of current pharmacogenomic biomarkers and future perspectives of pharmacogenomics in CRC, in an evolving scenario moving from a single drug-gene interactions approach to a more comprehensive genome-wide approach, comprising genomics and epigenetics.

**Keywords:** Colorectal cancer, pharmacogenomics, RAS, BRAF, microsatellite instability, dihydropyrimidine dehydrogenase, UDP-Glucuronosyltransferase A1, epidermal growth factor receptor, vascular endothelial growth factor, DNA methylation



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## INTRODUCTION

Colorectal cancer (CRC) is one of the leading causes of cancer-related deaths in the western world and ranks third among the most frequent malignancies in both men and women<sup>[1]</sup>. Although still unsatisfactory, the median overall survival (OS) of patients with metastatic CRC (mCRC) has notably increased in the past 20 years, reaching around 30 months in recent phase III clinical trials<sup>[2,3]</sup>, thanks to the introduction of innovative medical and surgical treatment strategies. The availability of new drugs and treatment combinations, both in terms of cytotoxic chemotherapy regimens and new targeted therapies, has been crucial in order to reach this result. However, patients' outcome and response to treatment can be highly heterogeneous, thus an extensive effort has been directed towards the identification of reliable predictive biomarkers to aid clinical management of patients and identify subgroups more likely to benefit from different treatment strategies.

Pharmacogenomics represents an irreplaceable tool in order to tailor patients treatment to an individualized approach based on germline and somatic acquired genetic variations able to predict drugs response and risk of toxicities<sup>[4]</sup>. Moving from early studies exploring the genetic bases of individual predisposition to severe toxicities from chemotherapy agents [i.e. 5-fluorouracil (5-FU) or irinotecan] in mCRC patients, the introduction of targeted agents such as anti-epidermal growth factor receptor (EGFR) drugs, has prompted the discovery of predictive molecular biomarkers (i.e. RAS mutational status) which are now tested as part of routine clinical practice<sup>[5]</sup>. Over time, additional mechanisms of primary and secondary resistance to targeted agents have emerged as promising novel predictive biomarkers and potentially actionable target of treatment, although validation is still an issue in most cases, and many steps forward have been made in the biological understanding and molecular characterization of CRC<sup>[6]</sup>. Finally, new perspectives have been recently opened following innovative results of immunotherapy treatment, and the development of new analytical techniques which allow dynamic tumor profiling and a sensitive detection of coexisting alterations underlying tumor heterogeneity, such as liquid biopsy<sup>[7]</sup>.

In this review, we present an overview of current pharmacogenomic biomarkers validated in clinical practice and future perspectives of pharmacogenomics in CRC [Tables 1 and 2], in an evolving scenario moving from a single drug-gene interactions approach to a more comprehensive genome-wide approach, comprising genomics and epigenetics.

## CURRENT PHARMACOGENOMIC BIOMARKERS IN CLINICAL PRACTICE

### RAS

EGFR signaling pathway plays a crucial role in the regulation of cellular responses to growth signals and its constitutive activation is one of the main actor promoting CRC growth and proliferation through the KRAS/RAF/MAPK and the PI3K/AKT/mTOR axes<sup>[8]</sup>. EGFR inhibitors are nowadays well-established therapeutic agents incorporated into standard care for mCRC<sup>[9,10]</sup>. To date, two anti-EGFR monoclonal antibodies have been approved by the Food and Drug Administration (FDA) and European Medicines Agency (EMA) for the treatment of mCRC: Cetuximab (Erbix<sup>®</sup>, Merck KGaA/Lilly USA) and Panitumumab (Vectibix<sup>®</sup>, Amgen Inc). At the time when the efficacy of these drugs was first proven in advanced lines of treatment<sup>[11,12]</sup>, no predictive biomarker was available, although a subgroup effect on the activity of these agents was evident. KRAS is a small GTPase member of the RAS protein family<sup>[13]</sup>, and somatic gene mutations can lead to its constitutive activation resulting in independent cell proliferation and survival<sup>[14]</sup>. KRAS mutations, more frequently involving exon 2<sup>[15]</sup>, can be found in approximately 40% to 50% of mCRCs. The identification of KRAS exon 2 (codons 12 and 13) mutations as a negative predictive marker of response to anti-EGFRs represented the turning point on biomarker selection for anti-EGFR treatment.

First evidence of the negative predictive role of KRAS exon 2 mutation came from retrospective series<sup>[16]</sup> and was then confirmed through *post-hoc* analyses of randomized phase III trials<sup>[11,17-20]</sup>. Moving from these data, in 2008 FDA and EMA restricted the use of anti-EGFR drugs to patients with KRAS exon 2 wild-

**Table 1. Summary of main presented biomarkers**

Biomarker	Type of alteration	Frequency in CRC	Approved for clinical practice	Predictive value	Ref.
KRAS	Exon 2 (codons 12 and 13), exon 3 (codons 59 and 61) and exon 4 (codons 117 and 146) mutations	40%-50% mCRC	Y	Resistance to anti-EGFRs	[5]
NRAS	Exon 2 (codons 12 and 13), exon 3 (codons 59 and 61) and exon 4 (codons 117 and 146) mutations	3%-5% mCRC	Y	Resistance to anti-EGFRs	[5]
BRAF	V600E mutations	8%-10%	Y (prognostic value, Lynch Sdr screening in MSI-H)	Resistance to anti-EGFRs (accumulating evidence)	[5]
MSI	MMR-D (MSI-H)	20% stage I-II, 12% stage III, 4%-5% stage IV	Y (Lynch Sdr screening, prognostic value in early stage CRC)	Response to immune-checkpoint inhibitors (mCRC) Lack of efficacy of 5-FU adjuvant therapy in stage II (low evidence)	[5,81,100,101]
DPYD	DPYD*2A (IVS14+1G>A)	1%-2% heterozygous (caucasian population)	Y	5-FU severe toxicity	[9,120]
UGT1A1	UGT1A1*28	45% heterozygous 10% homozygous (caucasian population)	Y	Irinotecan severe toxicity	[9,10]
HER2	HER2 amplification	5% RAS WT mCRC	N	Resistance to anti-EGFRs Response to anti-HER2 treatment	[133-135]
PI3K	Exon 9 and 20 hotspot mutations	10%-18%	N	Resistance to anti-EGFRs	[5]
CIMP	Aberrant DNA hypermethylation at select CpG islands	10%-15%	N	Response to 5-FU adjuvant therapy Potential resistance to anti-EGFRs Potential sensitivity to demethylating agents	[161]
MGMT	MGMT promoter hypermethylation	40% mCRC	N	Response to alkylating agents	[172]

Y: yes; N: no; CRC: colorectal cancer; mCRC: metastatic CRC; EGFR: epidermal growth factor receptor; 5-FU: 5-fluorouracil; MSI-H: high microsatellite instability

type (WT) tumors. However, in the same year, the possible existence of additional predictive biomarkers of resistance to anti-EGFR treatment was highlighted by an independent meta-analysis<sup>[21]</sup> showing a low sensitivity for *KRAS* exon 2 mutations in predicting acquired resistance to anti-EGFRs. Shortly after, rare *RAS* activating mutations in exon 3 (codons 59 and 61) and exon 4 (codons 117 and 146) of *KRAS* and exons 2, 3, and 4 of *NRAS* (codons 117 and 146), were reported as novel negative predictive markers<sup>[22,23]</sup>. Outcome data from the extended *RAS* analyses in the large randomized phase III PRIME trial, comparing FOLFOX with or without panitumumab as first-line treatment in mCRC patients, provided definitive evidence in this regard. In this study, patients with any *RAS* mutation in their tumors showed a worse outcome when treated with panitumumab [hazard ratio (HR) for progression free survival (PFS) = 1.31 ( $P = 0.008$ ,  $P$  for interaction  $< 0.002$ ); HR for OS = 1.21 ( $P = 0.04$ ,  $P$  for interaction = 0.001)]<sup>[24]</sup>. Following this evidence, results of all recent randomized trials with anti-EGFR-based therapies were retrospectively re-evaluated according to the extended *RAS* mutational status<sup>[25-27]</sup> and several meta-analyses were performed. Data were consistent across different chemotherapy backbones, anti-EGFR agents and lines of therapy, showing no improvement in outcome results, both in term of PFS and OS, with the addition of anti-EGFRs in tumors harboring any *RAS* mutation ( $P > 0.05$ )<sup>[28]</sup>. Notably, in the selected extended *RAS* WT population efficacy results from the addition of anti-EGFR treatment were highly improved<sup>[29]</sup>. Based on these results, the use of anti-EGFRs has been currently restricted to *RAS* WT (exons 2, 3, and 4 of each *KRAS* and *NRAS*) tumors<sup>[30]</sup>, and regulatory authorities recommend that every patient being considered for anti-EGFR therapy must receive *RAS* mutational testing including *KRAS* and *NRAS* codons 12, 13 of exon 2; 59, 61 of exon 3; and 117 and 146 of exon 4, performed only in highly qualified and certified laboratories<sup>[5]</sup>.



**Table 2. Promising future pharmacogenomics biomarkers**

Biomarker	Description	Potential predictive value	Ref.
CMS1	Microsatellite instability immune (14%): - high TML - MSI - CIMP+ - BRAF mutation - strong immune activation - right sided	Response to anti-VEGF	[181-186]
CMS2	Canonical (37%): - epithelial signature - WNT- $\beta$ -catenin and MYC activation - CIN - left sided	Response to anti-EGFRs Response to anti-HER2 Chemo-sensitivity	[181-186]
CMS3	Metabolic (13%): - metabolic dysregulation	-	[181-186]
CMS4	Mesenchymal (23%): - TGF- $\beta$ activation - stromal invasion - angiogenesis	Resistance to anti-EGFRs Lack of benefit from 5-FU and oxaliplatin	[181-186]
Liquid biopsy	Mutational analysis of circulating tumor DNA	Identification of predictive mutations for targeted treatments at baseline Dynamic monitoring Early detection of secondary resistance	[187-191]
MiRNA	Micro RNA: noncoding single-stranded RNA molecules, < 200 nucleotides, with post-transcriptional regulatory functions	Response/resistance to chemotherapy and targeted agents	[195]

TML: tumor mutational load; EGFR: epidermal growth factor receptor; 5-FU: 5-fluorouracil; MSI: microsatellite instability; TGF: transforming growth factor; VEGF: vascular endothelial growth factor

More recently, KRAS mutations have been shown to be associated with suppressed Th1/cytotoxic immunity in CRC, irrespective of mismatch repair (MMR) status, tumor location, neoantigen load and transcriptional subtype, with a differential effect modulated by the underlying tumor consensus molecular subtypes (CMS, discussed more extensively in section 4)<sup>[31]</sup>. These findings may have a role in explaining the heterogeneity of treatment response and outcomes in RAS mutated tumors and provide a rationale for novel treatment strategies in these patients.

## BRAF

The serine/threonine protein kinase BRAF is another player in the EGFR-mediated signaling pathway which is well-known to be implicated as an oncogenic driver in CRC. In normal cells, MEK, ERK and RAF are part of a tyrosine kinase signaling cascade activated by RAS, which affects cell proliferation, growth and differentiation, and regulates key cellular function such as apoptosis, cell migration and survival<sup>[32]</sup>. Mutations in *BRAF* can be found in approximately 8%-10% of CRCs<sup>[33]</sup>, the majority of which (about 80%) involve the substitution of glutamic acid for valine at residue 600 within the protein kinase domain (V600E). BRAF constitutive activation resulting from V600E mutation promotes signaling transduction through the MEK-ERK-MAP kinase pathway even in absence of RAS-mediated signals. RAS and BRAF V600E mutations, as they work through the same pathway, are considered mutually exclusive, and their concomitant detection is extremely rare (< 0.001%)<sup>[34]</sup>.

The negative prognostic value of *BRAF* V600E mutation in mCRC has been extensively described in several univariate and multivariate models. Life expectancy for this subgroup of patients is poor when compared to *BRAF* WT ones. When retrospectively evaluated, in fact, metastatic *BRAF*-mutated patients were showed to have a median OS ranging from 10 to 19 months across multiple series, even when treated with association therapies<sup>[35-38]</sup>. Additionally, *BRAF* V600E-mutated tumors share distinct clinicopathological features: they are more frequent in women, elderly, and are often right-sided; they more often present a mucinous histology, poor differentiation and high microsatellite instability (MSI-H); more often are diagnosed as advanced disease with preferential spread to lymph nodes and peritoneum<sup>[39-41]</sup>. When oligo-metastatic liver

disease is radically resected, *BRAF*-mutated tumors tends to relapse early with extra-hepatic lesions<sup>[42,43]</sup>. A specific carcinogenesis pathway<sup>[44]</sup> and a distinct gene signature<sup>[45]</sup> have also been associated with *BRAF* V600E mutation. More recently, gene expression analyses allowed to identify two different *BRAF* V600E subtypes in a large cohort of *BRAF* V600E mutated patients unselected for tumor stage: the BM1 subtype characterized by KRAS/AKT activation, mTOR/4EBP deregulation and EMT, and the BM2 subtype characterized by cell cycle and checkpoint pathway deregulation<sup>[46]</sup>. In contrast with *BRAF* V600E mutation, metastatic tumors harboring rare mutations of *BRAF* codons 594 and 596 (less than 1% of CRCs) have been shown to have different prognosis and clinical outcome. These rare mutations are associated with a non-mucinous histology, a rectal primary tumor location, microsatellite stability, and lack of peritoneal disease. Moreover, no negative prognostic impact was observed although in a small series of patients (median OS 62.0 vs. 12.6 months; HR, 0.36; 95% CI, 0.20-0.64;  $P = 0.002$  for *BRAF* 594 or 596 mutant vs. *BRAF* V600E)<sup>[47]</sup>. Similar results on the impact and characteristics of *BRAF* nonV600E mutations were confirmed in a recent retrospective evaluation of a large cohort of patients<sup>[48]</sup>.

Although still debated, growing evidence is accumulating on the role of *BRAF* mutations as a negative predictive marker for anti-EGFR agents activity. Retrospective series showed that the response rate to anti-EGFR treatment with or without chemotherapy was significantly lower in *BRAF*-mutated vs. WT patients<sup>[22,23,49]</sup>. On the other hand, *BRAF* V600E mutation failed to demonstrate its predictive value in several sub-group analyses of phase III trials, possibly because of the small number of *BRAF*-mutated patients and lack of statistical power<sup>[24,50]</sup>. More recently, two meta-analyses showed a lack of improvement in PFS and OS in patients with *BRAF*-mutated mCRCs when treated with either cetuximab- or panitumumab-containing regimens compared to chemotherapy alone<sup>[51,52]</sup>. Additionally, a retrospective evaluation of the randomized phase III FIRE-3 trial, comparing FOLFIRI plus cetuximab or bevacizumab as first-line treatment in KRAS exon 2 WT mCRC patients, confirmed poorer survival outcomes for *BRAF*-mutated tumors irrespective of cetuximab and bevacizumab administration<sup>[53]</sup>. Based on these data, it appears that anti-EGFRs do not demonstrate a clear outcome benefit in *BRAF*-mutated tumors, and their use should be restricted to patients with no alternative therapeutic options. Notably, however, in FIRE-3 cetuximab arm a small subgroup of *BRAF*-mutated tumors achieving an early tumor shrinkage  $\geq 20\%$  (9/17) showed significantly longer median PFS (9.0 vs. 1.9 months, log-rank test  $P = 0.002$ ; HR = 0.14) and OS (29.8 vs. 5.9 months, log-rank test  $P = 0.047$ ; HR = 0.3) than those not achieving it<sup>[53]</sup>. Despite the limitations due to the retrospective nature of this evaluation and the small patients numbers, these results highlight a significant heterogeneity among *BRAF*-mutated mCRCs warranting further investigation.

While FOLFOXIRI plus bevacizumab represents the most promising treatment option in the first-line setting for clinically selected *BRAF*-mutated patients<sup>[2,54]</sup>, outcomes are still unsatisfactory. An extensive effort has been made in the last few years aiming to develop possible effective anti-*BRAF* strategies for mCRC patients. In contrast to melanoma, the use of *BRAF* inhibitors, such as vemurafenib and dabrafenib, as single-agents did not show significant activity in *BRAF*-mutated mCRC<sup>[55]</sup>. Dual blockade of *BRAF* and alternative survival pathways, such as MEK and EGFR, have been tested as well in clinical trials without convincing results<sup>[56-58]</sup>. Promising results are coming instead from a triple inhibition strategy combining *BRAF*-inhibitors, MEK-inhibitors and EGFR-inhibitors<sup>[59,60]</sup>. An additional strategy under study to increase the activity of dual targeted *BRAF* inhibition is its association with standard cytotoxic chemotherapy, such as the combination of vemurafenib with cetuximab plus irinotecan which have been explored in the SWOG 1406 trial with encouraging results<sup>[61]</sup>. Moreover, several other promising strategies designed to overcome resistance pathways to *BRAF*-inhibitors are currently under investigation<sup>[62,63]</sup>. Final results from ongoing trials are warranted to improve targeted treatment options for *BRAF*-mutated patients.

### Microsatellite Instability

MMR is a highly conserved DNA repair mechanism that ensures genomic integrity by correcting mispaired or unpaired bases which have escaped the proofreading activity of DNA polymerases during DNA replication

and recombination, as well as repairing some forms of DNA damage. The loss of MMR proteins activity leads to an accumulation of DNA replication errors, a phenomenon known as MSI, characterized by high frequency of frameshift mutations in microsatellite DNA which translates into a high somatic mutational burden in MMR-deficient (MMR-D) cells (mutator phenotype)<sup>[64]</sup>.

The prevalence of MSI in CRC depends on the stage of the disease. Approximately 20% of CRCs in stage I-II, 12% in stage III and 4%-5% in stage IV, are deficient in one or more DNA MMR proteins, with one-quarter of these resulting from Lynch syndrome (LS), an autosomal dominant condition characterized by germline mutations in genes coding for MMR proteins (i.e. *MLH1*, *MSH2*, *MSH6*, *PMS2* or *EPCAM*)<sup>[65]</sup>. The vast majority (circa 80%-90%) of sporadic MSI cases are due to hypermethylation of the *MLH1* gene promoter<sup>[66,67]</sup>, associated with a high CpG island methylation phenotype (CIMP+) and about 30% harbor a *BRAF* V600E mutation<sup>[6,68]</sup>. The remaining cases of sporadic MSI can be explained mainly by the presence of multiple somatic mutations in the MMR genes without an identifiable germline MMR mutation ("double somatic" MSI cases)<sup>[69]</sup>, found to be associated with a higher frequency of somatic mutations in *PIK3CA*<sup>[70]</sup>. According to the recent CMS classification MSI is associated with CMS1<sup>[6,71]</sup>. MSI detection is currently based on two different approaches: immunohistochemical staining (IHC) for *MLH1*, *MSH2*, *MSH6*, and *PMS2* on tumor samples to identify the loss of protein expression which characterizes MMR deficiency as a surrogate for MSI<sup>[72]</sup>; DNA MSI testing through a polymerase chain reaction (PCR)-based approach evaluating specific panels of microsatellite markers<sup>[73]</sup>. If either MSI or MMR deficiency is detected, further evaluation is recommended to rule out LS, rather than sporadic MSI. Of note, recently new computational approaches based on the evaluation of next generation sequencing (NGS) data have been proposed as a tool for MSI assessment<sup>[74-77]</sup>, as well as the evaluation of mutational burden on circulating cell-free tumor-DNA testing as a surrogate marker of mismatch repair deficiency or microsatellite instability in patients with CRC<sup>[78]</sup>.

MSI-H CRCs are characterized by distinct clinical and pathological features such as right-sided colon location, early-stage at diagnosis, prominent lymphocytic infiltrate, poor differentiation and mucinous histology<sup>[79]</sup>. When diagnosed in the metastatic setting, MSI-H mCRCs arise more frequently in women and in elderly; presenting often with synchronous metastases involving peritoneum, lymph nodes and lung rather than liver. Notably, distinct patterns characterize inherited and sporadic MSI-H mCRCs<sup>[80]</sup>. In addition to LS screening, in patients with early-stage (especially stage II) CRCs, MMR status provides important prognostic and predictive information, with MMR deficiency being associated with both a good prognosis and apparently a lack of efficacy from fluorouracil treatment, although data regarding whether or not MSI status predicts response to adjuvant chemotherapy in this setting has been controversial<sup>[81-85]</sup>. The most solid data derive from the analyses of the ACCENT database investigating the impact of MSI in stage II and III CRCs treated with surgery vs. surgery followed by 5-FU-based adjuvant therapy across 17 different trials. Stage II and III patients with MSI tumors showed better outcome with surgery alone compared to those with microsatellite stable (MSS) tumors. Conversely, stage III patients showed a significant survival benefit from the addition of 5-FU adjuvant therapy after surgery both in case of MSS and MSI tumors<sup>[84]</sup>. To date, adjuvant chemotherapy is not recommended for patients with low risk stage II MSI-H tumors due to their excellent prognosis, while stage III patients should receive adjuvant treatment irrespective of MSI status. Of note, MSI etiology (germline vs. sporadic) seems to affect the predicted benefit from 5-FU, as Sinicrope *et al.*<sup>[86]</sup> showed, in a retrospective evaluation of stage II and III CRC patients who received either adjuvant 5-FU or placebo, that individuals with MSI-H CRCs due to germline mutations (i.e. LS) had an improved disease free survival (DFS) with 5-FU compared to those with sporadic MSI-H tumors. The role of MSI as a predictive marker with modern combination regimens, such as FOLFOX and FOLFIRI, has less evidence<sup>[87-89]</sup>, and although an MSI-H status was retrospectively shown to predict improved DFS with adjuvant irinotecan and 5-FU (IFL regimen) in the CALGB (Alliance) 89803 trial, these results were inconsistently demonstrated in other exploratory analyses<sup>[90,91]</sup>. In the metastatic setting, recent data suggest a greater activity of irinotecan in MSI-H mCRC and better outcomes in favor of bevacizumab treatment compared to anti-EGFRs<sup>[92]</sup>. Indeed, vascular endothelial growth factor (VEGF) is known to play a crucial

role in tumor microenvironment immuno-modulation and anti-angiogenic treatment has been proposed as an effective modality to potentiate immunotherapy<sup>[93]</sup>. No definitive evidence is available on the prognostic role of MSI-H in mCRC; recent data suggest no statistically significant difference in OS between MSI-H and MSS mCRCs, although a trend toward a worse OS has been reported for MSI-H<sup>[94]</sup>. Some studies suggest the correlation with *BRAF* mutational status as a potential confounding factor affecting the estimation of MSI-H impact on survival in mCRC<sup>[95]</sup>. However, the prognostic role of *BRAF* in these tumors is still object of debate and in a recent analysis *BRAF* V600E mutation was not associated with a worse survival in MSI-H CRC<sup>[80]</sup>. Additionally, a possible negative prognostic effect of immune checkpoint expression in MSI-H CRCs have been recently reported, which seems to be able to counterbalance the positive effect of tumor-infiltrating cytotoxic T-cell lymphocytes in these tumors<sup>[96]</sup>.

MSI assessment has lately gained a prominent role in the metastatic setting due to the recent groundbreaking success of immunotherapy with checkpoint inhibitors in MMR-D mCRCs which has opened a new era in the treatment of MSI-H tumors. In the phase II KEYNOTE 016 trial, pembrolizumab demonstrate its activity in 28 MSI-H mCRC patients with refractory disease, significantly improving response rate (RR), disease control rate (DCR), median PFS and OS compared to MSS patients (RR: 50% vs. 0% and DCR 89% vs. 16%, respectively; HR for PFS = 0.135,  $P < 0.001$ , HR for OS = 0.247,  $P = 0.001$ )<sup>[97,98]</sup>. The combination of ipilimumab (an anti-CTLA4) and nivolumab (an anti-PD1), under investigation in the phase II CHEKMATE142 trial, showed as well significant results with a recently reported RR of 31.1% (95% CI, 20.8-42.9) in patients receiving nivolumab ( $n = 74$ ) and 55% (95% CI, 45.2-63.8) in those receiving ipilimumab plus nivolumab ( $n = 119$ ), and remarkable 12 months PFS rate and 12 months survival rate (50% and 73% respectively, for nivolumab monotherapy; 71% and 85% respectively, for nivolumab plus ipilimumab)<sup>[99,100]</sup>. Responses were irrespective of tumor *RAS* and *BRAF* mutational status, immune cell PD-L1 expression or clinical history of LS. Notably, both pembrolizumab and ipilimumab/nivolumab showed a trend towards a plateau in the tail of patients' survival curves, suggesting the possibility of long term responders similar to the previous experience with immunotherapy in melanoma. Following these striking results, FDA approval was granted for the use of checkpoint inhibitors pembrolizumab (Keytruda®, Merck & Co., Inc.)<sup>[101]</sup> and nivolumab (Opdivo®, Bristol-Myers Squibb)<sup>[100]</sup> in the treatment of MSI-H or MMR-D mCRC.

Despite the clinical success of anti-CTLA4 and PD-L1/PD-1 inhibitors, however, only a subset of selected patients exhibits durable responses, suggesting that a broader view of cancer immunity is required. A complex set of dynamic tumor, host and environmental factors modulate the strength and timing of immune anticancer response, and several key immunoregulatory pathways have been identified and involved in the definition of an immune signature to predict responses to immunotherapy<sup>[102-105]</sup>. Alongside the ongoing extensive effort to identify additional predictive biomarkers<sup>[106,107]</sup>, understanding the mechanisms limiting immunotherapy efficacy, both in terms of innate and acquired resistance, represents a challenge which needs to be addressed in order to improve treatment outcomes and develop new actionable strategies<sup>[108-110]</sup>.

### Dihydropyrimidine dehydrogenase

Fluoropyrimidine analog 5-FU and its pro-drug capecitabine represent the backbone of chemotherapy treatment for colorectal cancer<sup>[10]</sup>. The mechanism of action of these drugs is based on thymidylate synthase (TYMS) inhibition through the formation of a ternary complex between the active metabolite 5-fluoro-2-deoxyuridine-5-monophosphate (5-FdUMP), TYMS and 5,10-methylenetetrahydrofolate, leading to the suppression of DNA synthesis<sup>[111]</sup>. The rate-limiting enzyme for 5-FU catabolism is the enzyme dihydropyrimidine dehydrogenase (DPD), responsible for the inactivation of more than 80% of the administered dose of 5-FU<sup>[112]</sup>.

Up to one-third of patients treated with these agents experience severe (and in 0.5%-1% of cases lethal) toxicities including myelosuppression, mucositis and diarrhea<sup>[113]</sup>. Functional DPD gene (*DPYD*) variants

leading to a decreased enzymatic activity have been found to correlate with the risk of 5-FU and capecitabine severe toxicities in several pharmacogenetic studies. Over 30 single nucleotide polymorphisms (SNPs) in the *DPYD* gene have been studied over the last 20 years, although many of these variants did not appear to have any functional effect. Among the most well-known, the c.2846 A>T and c.1679 T>G variants, alongside the G>A mutation (DPYD\*2A) of the invariant splice site in exon 14 (IVS14+1G>A), coding for a truncated protein with no enzymatic activity, have been consistently associated with decreased DPD activity and a 4-fold increase of risk of developing 5-FU related toxicities<sup>[114]</sup>. DPYD\*2A is the most frequent SNPs in the Caucasian population, nevertheless its incidence is low (about 1%-2% for the heterozygote genotype) and shows substantial ethnic variations. Homozygous for DPYD\*2A have been associated with cases of lethal toxicities in patients treated with fluoropyrimidine-based chemotherapy<sup>[115,116]</sup>. More recently a large meta-analysis from Meulendijks *et al.*<sup>[117]</sup> confirmed the predictive role for drug-related toxicities for four *DPYD* variants: DPYD\*2A, c.2846A>T, c.1679 T>G and c.1236G>A/haplotype B3. Data from retrospective pharmacogenetic analyses from the Italian adjuvant TOSCA trial confirm the role of DPYD\*2A as a risk factor for fluoropyrimidine-related toxicities<sup>[118]</sup>. Additionally, a prospective study enrolling 2,038 patients candidate to receive a fluoropyrimidine-based chemotherapy demonstrated the feasibility and cost-effectiveness of upfront DPYD\*2A genotyping before treatment start. DPYD\*2A variant allele carriers were treated with a reduced dose-intensity leading to a significant reduction of the risk of grade  $\geq 3$  toxicity (28% vs. 73% in historical controls,  $P < 0.001$ ) and a reduction of drug-induced death from 10% to 0%<sup>[119]</sup>. The low frequencies of the aforementioned risk alleles, however, cannot fully explain the estimated risk of DPD-linked fluoropyrimidine-related adverse events, underlining the complex multi-level modulation of DPD activity, involving both transcriptional and post-transcriptional mediators, and the need to investigate additional *DPYD* risk variants. Nevertheless, available data support the role of *DPYD* testing as a pre-treatment screening in patients undergoing 5-FU and capecitabine treatment in order to improve the safety of fluoropyrimidine-based therapies and potentially allow genotype-guided dose adaptations, as recently recommended by the clinical pharmacogenetics implementation consortium<sup>[120]</sup>.

Evidence on the role of DPD deficiency as a toxicity biomarker led the FDA to include a warning annotation on the label of fluorouracil for patients with low or absent DPD activity, recommending to withheld or permanently discontinue fluorouracil in patients with evidence of acute early-onset or unusually severe toxicity, which may indicate near complete or total absence of DPD activity. On the other hand, latest published ESMO clinical practice guidelines on metastatic colorectal cancer management suggest for the first-time pre-treatment DPYD testing as an option<sup>[9]</sup>. This indication, however, is focused on those patients who experience severe 5-FU toxicity before 5-FU re-introduction and routine testing is not recommended, despite the authors stating that patients with known partial DPD deficiency benefit from dose adaptation of 5-FU/capecitabine therapy to avoid severe toxicity, while in patients with complete DPD deficiency fluoropyrimidines should be avoided and an alternative treatment offered. The lack of recommended standardized assessment techniques represents an additional issue to the introduction of routine DPD testing.

The predictive role of genetic variants in other key genes involved in the folate pathway, such as TYMS and 5,10-methylenetetrahydrofolate reductase, has not been validated and their use in clinical practice is not recommended.

### UDP-Glucuronosyltransferase A1

Irinotecan, a topoisomerase I inhibitor, is another key drug in the chemotherapy treatment of mCRC, which can be used as a monotherapy or in combination with 5-FU and/or other agents in different treatment lines<sup>[9,10]</sup>. This agent is administered as a pro-drug which is metabolized to its active form, SN-38, via carboxylation. SN-38 catabolism and excretion are subsequently dependent on conversion to its inactive form, SN-38G, operated by hepatic UDP-Glucuronosyltransferases (UGT) such as UGT1A1<sup>[121]</sup>. Additionally, the pharmacokinetics of irinotecan involves several other enzymes, such as CYP3A4, which control its



metabolism modulating the available dose of the active drug. A genetic variation in these enzymes can affect tolerability and toxicity profile in patients.

Up to 36% of patients treated with irinotecan-containing regimens experience severe and potentially life-threatening adverse events, such as neutropenia and diarrhea<sup>[122]</sup>. Variations in the UGT1A1 activity have been shown to be associated with irinotecan-induced toxicities. The most common gene variants are the UGT1A1 \*1 and \*28 alleles, representing 98%-99% of all variants in the Caucasian population. The \*28 variant, responsible for Gilbert syndrome, is characterized by the presence of an extra TA repeat in the promoter of the *UGT1A1* gene which is associated with a remarkably reduced enzymatic activity and correlates with higher incidence of drug-related adverse events due to a slower catabolism of SN-38G<sup>[123]</sup>. In USA, about 45% of the population is heterozygous for the \*28 allele (\*1/\*28) while around 10% carries a homozygous genotype for this variant. The frequency increases in the African population and is lower in South-East Asian and Pacific populations. The role of UGT1A1 genotyping has been evaluated in several clinical trials, and two large meta-analyses including nearly 2000 patients confirmed that carriers of the UGT1A1 \*28/\*28 genotype were at a higher risk for neutropenia compared to WT \*1 patients even at a low irinotecan dosage (80-145 mg/m<sup>2</sup>)<sup>[124]</sup>, while carriers of the \*28 allele were at risk of severe diarrhea at doses above 125 mg/m<sup>2</sup><sup>[125]</sup>. Consistently, genotyping analyses of patients treated with 5-FU and irinotecan within the randomized phase III Nordic IV trial<sup>[126]</sup> and the randomized phase III TRIBE trial<sup>[127]</sup>, confirmed the association between the UGT1A1\*28/\*28 genotype and higher risk of neutropenia. Subsequent meta-analyses most recently supported once again the role of UGT1A1\*28 as predictive of irinotecan-related severe toxicities, as well as the role of additional variants such as UGT1A1\*6, a missense variant frequent in the Asian population<sup>[128,129]</sup>. Finally, a recent dose-finding and pharmacokinetic study suggests that irinotecan treatment dose should be individualized based on UGT1A1 genotype. Results from this study, in fact, show that the maximum tolerated dose of irinotecan, administered as an intravenous infusion every 3 weeks, was 850, 700, and 400 mg in patients bearing the \*1/\*1, \*1/\*28, and \*28/\*28 genotypes, respectively<sup>[130]</sup>.

Based on available data the latest ESMO guidelines suggest UGT genotyping as an option in patients with a suspicion of UGT1A1 deficiency and when the administration of a dose of irinotecan >180 mg/m<sup>2</sup> is planned<sup>[9]</sup>. On the other hand, the National Comprehensive Cancer Network guidelines version 2.2017 states that irinotecan should be used with caution and at a decreased dose in patients with Gilbert syndrome or elevated serum bilirubin, but routine genotyping of UGT SNPs is not recommended<sup>[10]</sup>. It has to be noted, however, that FDA has modified irinotecan label to include a toxicity warning for the UGT1A1\*28 polymorphism, suggesting an initial dose reduction when treating patients carrying the UGT1A1\*28 homozygous allele.

## EMERGING BIOMARKERS OF SPECIAL INTEREST

### HER2

Although tumor *RAS* WT status is, as previously described, a crucial prerequisite for anti-EGFRs activity in mCRC, several patients with *RAS* and *BRAF* WT tumors still do not benefit from anti-EGFR treatment. Based on preclinical data and retrospective evaluations, additional mechanisms of primary resistance to anti-EGFR agents have been identified over time in *RAS* WT mCRC, including human epidermal growth factor receptor 2 (*HER2/neu*) amplification. *HER2* is a member of the EGRF family which regulates key cellular processes such as proliferation and apoptosis through the activation of the *RAS/RAF/ERK* and the *PI3K/PTEN/AKT* signalling pathways. *HER2* role as a driver oncogene in CRC and as potential biomarker for targeted treatment in the metastatic setting has recently been the object of great interest.

First data were reported in 2011 when *HER2* amplification (which can be found in approximately 5% of *RAS* WT mCRCs), was detected in a subset of *KRAS/NRAS/BRAF/PIK3CA* WT cetuximab-resistant patient-derived xenografts. Following this first evidence, a proof-of-concept study in the subgroup of *HER2*-amplified

xeno-patients demonstrated a significant tumor regression after combined treatment with HER2 and EGFR blockade<sup>[131]</sup>. These results were subsequently challenged in an Italian phase II clinical trial, the HERACLES study. More than 1000 mCRC cases were analysed in order to identify strict criteria for the definition of *HER2* amplification<sup>[132]</sup> in the dedicated HERACLES diagnostic. Afterwards, the activity of an HER2 double blockade with trastuzumab and lapatinib was evaluated in chemorefractory mCRC patients with *HER2*-positive tumors. Initial results of the study have been published, showing a 30% objective response rate (95% CI, 14-50), with one patient achieving a complete response, and a 44% stable disease rate (95% CI, 25-63)<sup>[133]</sup>. Of note, none of the 15 patients (56%) evaluable for response to anti-EGFRs achieved an objective response to previous treatment with either cetuximab or panitumumab, supporting the role of *HER2* amplification as a mechanism of primary resistance to anti-EGFR targeted agents. Moving from such promising results, a second cohort of the study has enrolled patients to treatment with a combination of trastuzumab-emtansine (TDM1) and pertuzumab, and patients experiencing disease progression after treatment with trastuzumab and lapatinib are receiving TDM1 monotherapy within the HERACLES Rescue trial. New results from these studies are highly anticipated.

Confirmatory results on HER2 as a possible target in mCRC came also from the phase II MyPathway trial, and retrospective series confirmed data on *HER2* as a possible predictive biomarker of resistance to anti-EGFRs<sup>[134]</sup>. Additionally, *HER2* amplification detected on tissue or on circulating tumor DNA (ctDNA) was identified as a possible mechanism of acquired resistance in *HER2* negative, *RAS/BRAF* WT, patients progressed during anti-EGFR treatment<sup>[135]</sup>. Of note, a randomized phase II trial, the S1613 study, has been recently opened to explore the efficacy of trastuzumab and pertuzumab compared to cetuximab and irinotecan in pre-treated anti-EGFR naïve mCRC patients carrying a tumor with *HER2/neu* amplification<sup>[136]</sup>.

Supported by a strong preclinical rationale and confirmatory clinical data *HER2* testing might be soon implemented in clinical practice for patients with mCRC candidate to receive anti-EGFR and/or anti-HER2 treatments.

### **Anti-EGFR agents: other biomarkers of primary and acquired resistance**

Alongside *HER2* amplification, several other mechanisms of primary resistance to anti-EGFR targeted treatment have been identified so far, including phosphatidylinositol-3-kinase catalytic subunit alpha (*PIK3CA*) mutations (exon 9 and 20 hotspot mutations), *MET* amplification, *FGFR1* and *PDGFRA* mutations, loss of *PTEN* function and low *EGFR* copy number<sup>[137]</sup>. However, the routine use of these biomarkers in clinical practice cannot be recommended at present, and further prospective validation of their predictive role is warranted. Nevertheless, different combined strategies and novel targeted agents aimed to overcome primary resistance to anti-EGFRs are currently under investigation, such as the combination of anti-EGFR agents with mammalian target of rapamycin (mTOR) inhibitors<sup>[138]</sup>. Recently, a panel of genomic alterations (the PRESSING panel) comprising activating mutations of the MAPKs or PI3K/AKT axis, *HER2* amplification or mutations, *MET* amplification and *NTRK/ROS1/ALK/RET* rearrangements, have been tested in an interesting retrospective case-control study aiming to dissect primary resistance to anti-EGFR treatment, demonstrating the negative predictive impact of these mutations in *RAS/BRAF* WT mCRCs treated with anti-EGFRs<sup>[139]</sup>. The study included 47 cases (patients resistant to anti-EGFR-containing regimens) and 47 controls (patients who responded to single agent anti-EGFRs or to a combination of irinotecan with anti-EGFRs if previously clearly irinotecan refractory). Aforementioned genomic alterations were reported in 20 (42.6%) cases and 1 (2.1%) control ( $P < 0.001$ ), meeting the primary endpoint of the study. Additionally, primary tumor right-sidedness was found to be associated with resistance to anti-EGFRs, confirming recent literature evidence, and the combined evaluation of PRESSING panel and primary tumor location demonstrated the best predictive accuracy. These results open promising perspectives on the clinical application of a more comprehensive molecular characterization of *RAS/BRAF* WT mCRCs to further improve and refine patients selection.

Secondary resistance to anti-EGFRs is often dependent on clonal selection induced by targeted treatment pressure. Emerging mutations in the RAS/RAF/MAPK signaling pathway can be detected after disease progression in tumor biopsies from previously *KRAS* wild-type tumors and multiple mutations can coexist at the same time in the same sample<sup>[140]</sup>. This seems to be the result of the amplification of pre-existing minor sub-clones, suggested by a significant overlap in the genetic events associated with primary and acquired resistance<sup>[141]</sup>. Moving from these data, several trials are currently exploring different approaches to multiple targeted inhibition based on the emergence of selected resistance drivers, such as the combination of anti-EGFRs with MEK or MET inhibitors. Mutations in the ectodomain of EGFR represent an additional mechanism of resistance limited to the acquired setting<sup>[142,143]</sup>. Notably, a subset of mutations including *EGFR* S492R as well as other acquired mutations recently identified (S464L, G465R and I491M) appears to confer resistance to cetuximab but not panitumumab. The binding epitopes of cetuximab and panitumumab on EGFR, in fact, overlap but are not identical<sup>[144,145]</sup>. Retrospective analyses from the ASPRECT trial, comparing panitumumab to cetuximab in chemorefractory mCRC patients, revealed that EGFR S492R mutations occurred in 1% vs. 16% of patients treated with panitumumab and cetuximab, respectively<sup>[146]</sup>. The possible rationale for using panitumumab after the detection of these mutations as a mechanism of resistance to cetuximab still need further validation. Other strategies to overcome acquired resistance to anti-EGFRs include treatment with novel antibodies targeting different epitopes of the EGFR ectodomain, which can increase receptor internalization and degradation such as MM-151<sup>[147]</sup> and Sym004<sup>[148]</sup>.

### VEGF pathway

Angiogenesis plays a key role in CRC development and progression, and VEGF is a key regulator in both physiological and pathological angiogenesis. Therapeutic agents targeting VEGF/VEGFR signaling (i.e. bevacizumab, aflibercept, ramucirumab and regorafenib) proved to be effective across different treatment lines in mCRC and contributed greatly to improve patients' survival in recent years<sup>[9,10]</sup>. However, despite extensive efforts to identify predictive biomarkers for antiangiogenic therapies in the last decade, no predictive marker is available in clinical practice yet<sup>[149]</sup>. The complexity of the angiogenesis signaling network and the overlap between various angiogenic factors, in fact, represent a challenge to pharmacogenomic biomarkers discovery.

In 2012, Bates *et al.*<sup>[150]</sup> retrospectively analyzed CRC tumor samples from the phase III bevacizumab E3200 trial to explore the predictive value on treatment outcomes of VEGF165b, a VEGF splice isoform. Despite not reaching a statistical significance, patients with a lower level of VEGF165b appeared to benefit more from bevacizumab treatment. Focusing on a different candidate marker, recently published data demonstrated that patients treated with first-line bevacizumab-containing regimens had a significantly longer PFS when affected by *Homeobox B9* (*HOXB9*)-negative tumors compared with those with *HOXB9*-positive tumors (18.0 vs. 10.4 months,  $P = 0.048$ ). *HOXB9* is known as a highly conserved homeobox transcription factor gene which drives neoplastic transformation and tumor progression exerting an anti-apoptotic effect and promoting tumor cell invasion. The authors demonstrated, both with preclinical and clinical data, that transcription factor *HOXB9* mediates resistance of CRC to bevacizumab modulating a complex network of alternative pro-angiogenic and pro-inflammatory secreted factors<sup>[151]</sup>. A prospective validation of these promising results is highly anticipated. In another interesting analysis, NOTCH1 expression has been proposed as a detrimental prognostic factor in mCRC patients treated with chemotherapy plus bevacizumab<sup>[152]</sup>. Of note, a phase Ib trial is ongoing exploring safety and preliminary efficacy of a bispecific antibody targeting VEGF and the NOTCH ligand DLL4 (OMP-305B83) in combination with FOLFIRI as second-line treatment in mCRC<sup>[153]</sup>. Finally, a novel emerging player in the angiogenesis regulatory pathways is the protein apelin (APLN). APLN signaling takes part in multiple physiological functions including angiogenesis, and interacts at different levels with key mechanisms regulating cell growth, survival and apoptosis. Recent preclinical data based on the analysis of tumor-derived endothelial cells from patients receiving bevacizumab showed that APLN mRNA levels are significantly associated with treatment response. In fact, APLN levels were high

in non-responders and low in patients who benefitted from bevacizumab ( $P = 0.0001$ )<sup>[154]</sup>. All these potential biomarkers, however, still need validation.

As novel anti-angiogenic agents have entered clinical practice in recent years, the interest was directed to identify specific biomarkers for each compound. A retrospective analysis of ctDNA from liquid biopsies collected from about 350 patients treated with regorafenib in the CORRECT trial was performed to investigate the impact of *KRAS*, *PIK3CA* and *BRAF* mutations on regorafenib efficacy. Results were consistent with previous data and confirmed that the benefit from regorafenib on survival and treatment outcomes was irrespective of *KRAS* and *PIK3CA* mutational status<sup>[155]</sup>. The analysis according to *BRAF* mutational status, on the other hand, was not feasible due to the small number of *BRAF*-mutated patients. Data on *RAS*, *BRAF* and sidedness as biomarkers in patients treated with aflibercept in the VELOUR trial have been recently presented as well. No significant interactions according to *RAS* and *BRAF* status were found in this analysis, although a trend for better outcomes was observed for *BRAF*-mutated tumors treated with aflibercept in comparison with the control arm (mOS 10.3 vs. 5.5 months, respectively, HR 0.42; 95% CI, 0.16-1.09;  $P = 0.08$ )<sup>[156]</sup>. Similar results were observed in patients treated with ramucirumab within the RAISE trial. In fact, the ramucirumab favorable treatment effect was similar between *RAS*-mutated and all *RAS/RAF* WT tumors; however, the benefit was more notable in *BRAF*-mutated tumors both for OS (HR 0.54; 95% CI 0.25-1.13) and PFS (HR 0.55; 95% CI 0.28-1.08)<sup>[157]</sup>. Additionally, Tabernero *et al.*<sup>[158]</sup> assessed the correlations of a series of baseline marker levels (including VEGFR-2 immunohistochemistry in tumor tissue) with clinical outcomes in the RAISE patients population. Only VEGF-D circulating serum levels were found to be statistically significant with higher levels of this soluble factor ( $\geq 115$  pg/mL) associated with improved ramucirumab efficacy in comparison with placebo<sup>[158]</sup>.

Several SNPs in different genes involved in VEGF signaling pathway have been investigated over time. Results from a large meta-analysis including 158 SNPs and 1348 patients enrolled in five phase III randomized trials suggested an association between VEGFA rs699946 and VEGFR-2 rs11133360 polymorphisms and improved PFS in bevacizumab-treated patients<sup>[159]</sup>. Unfortunately, additional promising retrospective findings on different candidate SNPs of VEGF/VEGFR pathway genes were not prospectively validated in a dedicated study<sup>[160]</sup>.

## DNA methylation

Over the last decade, evidence on the role of the epigenome in CRC has been largely explored and it is now recognized that among thousands of epigenetic alterations which can be present in each tumor, a small subgroup may be considered a driver event in CRC development<sup>[161]</sup>. Different epigenetic mechanisms, in fact, can play a key role in carcinogenesis, such as DNA methylation, nucleosome positioning, histone modifications and non-coding RNAs expression<sup>[162]</sup>. Technological advances have considerably increased our ability to detect a wide number of epigenetic alterations which can eventually have a role as clinical biomarkers for early detection, prognostic stratification and treatment efficacy prediction in CRC patients. Of note, recently the availability of more refined genome-wide mapping technologies, highlighted that the function of DNA methylation can vary depending on its context, underlining a deep complexity that warrants further evaluations<sup>[163]</sup>.

Aberrant DNA methylation is the most extensively studied epigenetic mechanism in CRC. Global DNA hypomethylation is currently considered a common feature of CRC; on the other hand, however, evidence on the role of CpG islands DNA hypermethylation in promoting CRC by silencing the expression of tumor suppressor genes led to the identification of the CpG Island Methylator Phenotype (CIMP), consisting in a subset of CRCs characterized by distinct epidemiological, histological and molecular features and prognosis<sup>[164]</sup>. CIMP+ tumors are associated with female gender and older age, show more frequently a right-sided colon location, a high incidence of *BRAF* V600E mutation and MSI-H status as a consequence of *MLH1*



epigenetic silencing through promoter DNA hypermethylation, diploid copy number and absence of TP53<sup>[165]</sup>. CIMP status has been proposed as a promising prognostic marker for CRCs, however, several studies reported contradictory results, possibly due to the overlap between the CIMP+ phenotype and the MSI-H phenotype, associated in 30%-50% of cases with *BRAF* mutation<sup>[166]</sup>. The lack of global consensus in defining CIMP+ tumors, together with these controversial results, has hindered the uptake of CIMP as a relevant biomarker in clinical practice and further studies are warranted to explore its predictive and prognostic value<sup>[167]</sup>.

Long interspersed nucleotide element-1 (LINE-1) methylation measured by pyrosequencing has been shown to correlate with global DNA methylation levels<sup>[168]</sup>. LINE-1 is a retrotransposon related to key CRC features involved in the carcinogenesis process: LINE1 hypomethylation is associated with 18q loss of heterozygosity (LOH); whereas an inverse correlation has been demonstrated between LINE-1 hypomethylation, CIMP-H and MSI-H status. LINE-1 methylation levels have been reported to impact CRC prognosis with hypomethylation conferring poor prognosis in terms of overall mortality (OM) and colorectal cancer-specific mortality<sup>[169]</sup>. Additionally, LINE-1 hypomethylation in MSS/CIMP+ stage II and III CRC has been showed to predict benefit from adjuvant chemotherapy with oral fluoropyrimidines<sup>[170]</sup>. These data suggest that DNA demethylation may play, as well, a crucial role in CRC development, prognosis and response to treatment. Although promising, however, these findings need further validation.

The DNA repair gene O6-methylguanine-DNA methyltransferase (*MGMT*) has recently gained attention and has been object of several studies. This gene encodes a DNA repair protein which removes alkylating groups from O6-guanine and is involved in protecting cells against damages from alkylating agents. *MGMT* has been shown to undergo epigenetic silencing by promoter hypermethylation in more than 40% of mCRCs<sup>[171]</sup>. The loss of *MGMT* gene expression impairs the ability of DNA repair mechanisms to remove alkyl groups, potentially enhancing the cytotoxic effects of alkylating drugs, such as dacarbazine and temozolomide. On these bases, several phase II clinical trials<sup>[172]</sup> evaluating the efficacy of alkylating agents in mCRC have been conducted with promising results. In these studies, *MGMT* methylation has been used as a predictive biomarker for patients' selection, supporting a possible role for this novel marker in clinical practice.

In an era in which immuno-oncology is revolutionizing cancer treatment strategies, novel possible relevant implications of aberrant DNA methylation come from its tight connection with the immune cells system. To date, immune-checkpoint inhibitors (ICI) have shown striking results in selected cancer types, although only a minority of patients are sensitive to these drugs. *De novo* DNA methylation has been recently reported to have a central role in maintaining a T cell exhaustion status that contributes to resistance to ICI treatment<sup>[173]</sup>. On the other hand, previous studies demonstrated that DNA demethylating drugs can enhance CTLA-4 blockade-mediated T cell responses<sup>[174]</sup>. Moreover, treatment of epithelial cancer cell lines (including CRC cell lines) with demethylating agents, i.e. 5-azacitidine, has been reported to promote a significant enrichment of immunomodulatory pathways<sup>[175]</sup>. As a possible explanation, cryptic transcription of thousands of treatment-induced non-annotated transcriptional start sites (TINATs) may contribute to cancer immunogenicity through the translation of novel potential antigenic proteins, as recently shown by Brocks and colleagues in their work exploring DNA methyltransferases inhibitors (DNMTi) treatment consequences on epigenetic and genome-wide transcription<sup>[176]</sup>. Overall, this growing evidence supports a strong immunomodulatory effect of DNA demethylating agents in cancer cells, and the rationale to combine these drugs with immunotherapy in cancer patients. Based on these premises, a deeper understanding of the interplay between epigenetic modifications, cancer cells and immune cells could reveal novel potential strategies to enhance ICI treatment efficacy and overcome primary and acquired resistance mechanisms to immunotherapy.

Finally, aberrant DNA methylation may exert a direct effect modulating well-established molecular pathways in CRC. Notably, *EGFR* promoter DNA methylation has been reported to occur in 58% of primary colon



tumors and to be strongly correlated with shorter patients' PFS and OS (PFS 2.4 vs. 7.4 months,  $P < 0.0001$ ; OS 6.1 vs. 17.8 months,  $P < 0.0001$ )<sup>[177]</sup>. On the other hand, Khambata-Ford *et al.*<sup>[178]</sup> discovered that patients with overexpression of epiregulin (EREG) and amphiregulin (AREG), two EGFR ligands, are more likely to achieve disease control when treated with cetuximab and show a significantly longer PFS. These data have been confirmed by Jacobs *et al.*<sup>[179]</sup> showing a significant association between cetuximab response and AREG/EREG expression. In a recent work, EREG and AREG expression has been found to have a strong inverse correlation with methylation and to be inversely associated with right-sided tumor location, CIMP-H status and BRAF mutation<sup>[180]</sup>. Additionally, the authors reported that treatment with hypomethylating agents (i.e. azacitidine) increased EREG expression, and that a CIMP-H status was associated with shorter PFS outcomes, also in *BRAF/NRAS* WT patients. Based on these data, promoter DNA methylation may be the main regulatory mechanism of AREG/EREG expression, which may explain, at least in part, the association between right-sided tumor location, CIMP-status and anti-EGFR treatment response in mCRC. DNA methylation may, then, partially account for primary anti-EGFRs resistance, supporting the rationale to explore the possible synergistic treatment effect of demethylating agents in combination with anti-EGFR drugs.

Despite promising evidence, the complexity and heterogeneity of epigenetic alterations in CRC still represent a considerable challenge, which needs to be further addressed in order to identify reliable biomarkers and translate current knowledge into actionable therapeutic strategies.

## FUTURE PERSPECTIVES

### CRC consensus molecular subtypes

In recent years, great advances have been made in understanding the complexity of tumor biology and genetic landscape underlying tumor development and response to treatment. In 2015 an international consortium developed the Consensus Molecular Subtypes, which classifies CRC into four distinct biological groups, based on gene expression signatures and correlated with distinct genetic, epigenomic, transcriptomic, microenvironmental, prognostic and clinical features<sup>[181]</sup>. CMS1 (microsatellite instability immune, 14%) tumors are associated with high tumor mutational load (TML), microsatellite instability, hypermethylation status (CIMP+), *BRAF* mutation, and strong immune activation. The CMS2 (canonical, 37%) subtype is characterized by an epithelial signature, marked WNT- $\beta$ -catenin pathway and MYC signaling activation. CMS3 (metabolic, 13%) tumors feature metabolic dysregulation; and CMS4 (mesenchymal, 23%) a prominent transforming growth factor (TGF)- $\beta$  activation, stromal invasion and angiogenesis. Samples with mixed features (13%) are considered to represent a transition phenotype or intratumoral heterogeneity. CMS subgroups show a strong prognostic value independent of tumor stage, with CMS4 associated with worse survival. Moreover, retrospective analyses of clinical trials have suggested a potential predictive value for CMS subtypes, including a better outcome following bevacizumab treatment for CMS1<sup>[182]</sup>, and a lack of benefit from oxaliplatin<sup>[183]</sup> and anti-EGFRs (irrespective of *RAS* mutational status)<sup>[184]</sup> for the mesenchymal-like phenotype. Although not yet implemented in clinical practice, this classification system has the potential to better inform clinicians of prognosis and therapeutic response, and to guide novel therapeutic strategies with subtype-based targeted interventions<sup>[6]</sup>. In fact, data have been published from very recent preclinical studies exploring models of CMS in large panels of CRC cell lines, primary cultures and patient-derived xenografts (PDX), with the aim of developing "adapted" classifiers optimized for pre-clinical research and investigate specific drug sensitivity of individual CMS<sup>[185,186]</sup>. Results from these studies show interesting initial findings highlighting subtype-dependent response profiles, with a different sensitivity to chemotherapy (either 5-FU or oxaliplatin)-induced apoptosis between CMS2 and CMS4, which relates to the *in vivo* efficacy of chemotherapy in PDX models where a delay in outgrowth of CMS2, but not CMS4 xenografts, was observed. Additionally, a strong response to anti-EGFRs and HER2 inhibitors was observed in the CMS2 subtype. Indeed, a deeper understanding of the unique drug-sensitivity profile of each CMS subtype and the possibility of performing high-throughput *in vitro* and *in vivo* drug screening using PDX technology have the potential to greatly advance precision medicine in CRC.

### Liquid biopsy

Another field of major interest is the rapid development of liquid biopsies technology and the analysis of ctDNA as a more comprehensive and less invasive approach to pharmacogenomic profiling in CRC patients<sup>[187,188]</sup>. Allowing large-scale genomic profiling and being able to capture the molecular heterogeneity of different tumor sub-clones coexisting in the same patients, these techniques are expected to play a pivotal role in improving patients stratification and selection for targeted treatments. Moreover, the possibility to perform serial testing over time represents a valid opportunity to guide treatment strategies through an early detection of the emergence of treatment resistance and a dynamic tumor molecular profiling<sup>[189]</sup>. Indeed, data from repeated ctDNA analyses have been able to show the emergence of *RAS* and/or *BRAF* mutations during treatment with anti-EGFRs in *KRAS* WT patients, closely dependent on treatment exposure, with a dynamic increase during EGFR blockade followed by a rapid decline after treatment withdrawal<sup>[190]</sup>. Recently, a large study on genomic profiling through liquid biopsy analyzing next generation sequencing data from cell-free DNA of 1397 CRC patients, confirmed the reliability of this methodology in detecting genomic alterations when compared with corresponding tissue-based sequencing. Additionally, results of this study highlighted the possibility of detecting the development of multiple distinct concomitant mechanisms of resistance after targeted treatment with anti-EGFRs in the same subject, proving that ctDNA sequencing can generate a valuable insight into tumor heterogeneity and therapeutic resistance<sup>[191]</sup>. Although still needing extensive investigations and prospective validation, liquid biopsy approaches to profile tumor dynamics and response to treatment and to guide rechallenge strategies based on detection of circulating genomic alterations are currently under investigation in several clinical trials.

### MiRNAs

Finally, noncoding RNAs represent an evolving field in cancer diagnosis and prognosis, and several studies have suggested their possible role as treatment target in different diseases<sup>[192,193]</sup>. miRNAs are noncoding single-stranded RNA molecules, less than 200 nucleotides in length, with a post-transcriptional regulatory function involved in the modulation of a broad range of biological processes comprising cellular signaling, metabolism, proliferation and differentiation<sup>[194]</sup>. The role of several miRNAs has been implied in CRC evolution and progression, moreover different miRNAs have been identified as predictive of treatment response to standard chemotherapy (i.e. miR-429 and miR-148a with 5-FU) and targeted agents (i.e. miR-7 and miR-375 with anti-EGFRs)<sup>[195]</sup>. Although promising these findings still need validation; nevertheless, the possible clinical application of miRNAs as biomarkers or as a potential target of treatment in CRC deserves further investigation. Of note, new strategies are currently under study to develop miRNA based inference methods to extensively infer drug-disease causal relationship (miRDDCR) to assist in experimental design for drug discovery and disease treatment<sup>[196]</sup>.

### CONCLUSION

In the era of precision medicine, optimizing therapeutics and drugs combination for a narrow subset of patients based on patients' and tumors genetic makeup is of paramount importance in order to improve outcomes and minimize unrequired toxicities. The field of pharmacogenomics is constantly growing, and with the availability of new technologies it has been moving beyond candidate gene approaches and genome-wide association studies towards a comprehensive evaluation of genomic and epigenomic markers to drive treatment choices and optimize targeted therapies. Several biomarkers have entered clinical practice so far, and many more are currently being tested in clinical trials. Biomarker discovery and validation however still encounter many issues, due often to the small subsets of patients bearing selected alterations, the retrospective nature of most studies and the difficulty in proving the cost-effectiveness of a specific novel marker. Implementing biomarker-driven clinical trials and prospective pharmacogenomic profiling in clinical research, possibly integrating companion diagnostic tests since the early stages of novel drug development, is thus a priority for future research. Finally, dynamic profiling of tumor genomics under treatment pressure will play a critical role in uncovering acquired mechanism of resistance and directing personalized treatment strategies.

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### Authors' contributions

Manuscript drafting: Battaglin F, Puccini A

Directly provided contributions, read and approved the final manuscript: all authors

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### Patient consent

Not applicable.

### Ethics approval

Not applicable.

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Commentary

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# Targeting adenosine receptor 2B in triple negative breast cancer

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In the review “Role of adenosine in tumor progression: focus on A2B receptor as potential therapeutic target”, Sorrentino and Morello make a compelling case for considering adenosine 2B receptor (A2BR) as a target in cancer therapy (*J Cancer Metastasis Treat* 2017;3:127-38). A large body of evidence has accumulated suggesting A2BR to play an active role in tumor immune suppression and metastasis. Thus, this commentary will discuss the intriguing possibility of targeting A2BR in specific breast cancers that express high levels of A2BR and attract infiltrating immune cells.

## TRIPLE NEGATIVE BREAST CANCER IS SUSCEPTIVE TO IMMUNE MODULATION

Triple negative breast cancer (TNBC) is an aggressive subtype of breast cancer that disproportionately affects younger women and those of African origins, compared with Caucasians<sup>[1,2]</sup>. TNBC is devoid of the three receptors that classify and define most mammary cancers: estrogen receptor (ER), progesterone receptor (PR) and human epidermal growth factor receptor 2 (HER2)<sup>[3]</sup>. The lack of these receptors reduces the efficacy of targeted therapies for this cancer type, limiting treatment options to chemotherapeutic agents, ionizing radiation and surgery. TNBC patients are therefore in dire need for novel targeted therapies.

Breast cancer has long been thought of as a non-immunogenic malignancy. However, a growing body of evidence suggests that this is not the case for all breast cancers. Tumor-infiltrating lymphocytes (TILs) are the most widely studied immune cells and include T cells and B cells. TILs are part of a larger category of infiltrating immune cells that include natural killer (NK) cells, macrophages, neutrophils, dendritic cells,



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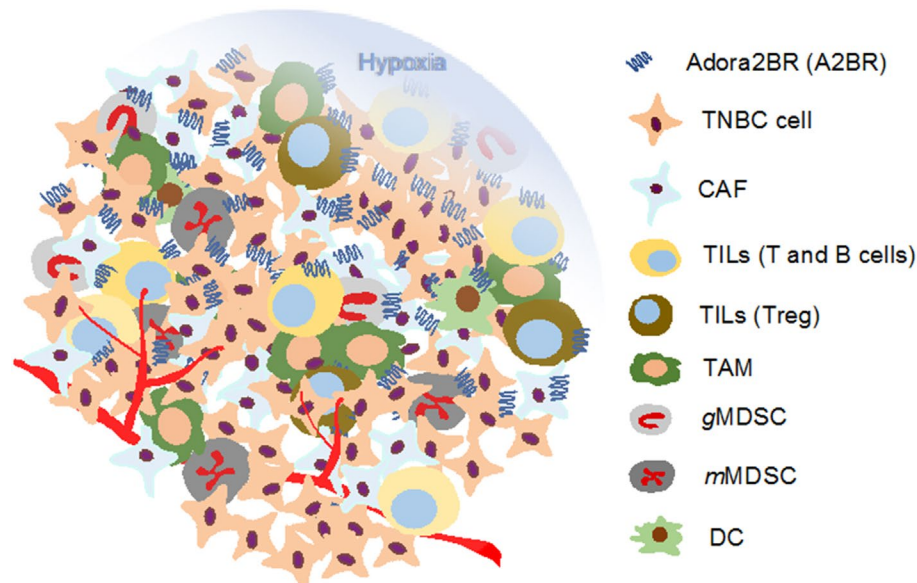
mast cells and other white blood cells. In breast cancer, TILs play an important role in mediating positive responses to chemotherapy and improving clinical outcomes. Specifically, in patients with HER2-positive breast cancer and TNBC, large adjuvant studies have shown that higher levels of TILs in primary biopsies were associated with prolonged overall survival (OS) and fewer recurrences, independent of therapy<sup>[4-6]</sup>. Similar results were also obtained in patient cohorts treated with neoadjuvant therapy. Here, increased levels of TILs in primary biopsies correlated with a higher pathological response rate (pCR)<sup>[7-9]</sup>. On the other hand, tumor-associated macrophages (TAMs) that derive from peripheral blood monocytes are recruited to the TNBC tumor microenvironment and undergo activation that leads to the secretion of inhibitory cytokines, the reduction of effector functions of TILs and the promotion of regulatory T cells (Treg)<sup>[10]</sup>. High levels of TAMs are associated with distant metastasis in TNBC in humans and can be blocked by targeting the chemokine ligand 5 (CCL5) in a mouse model<sup>[11,12]</sup>. A growing body of evidence suggests that tumor-infiltrated immune cells from myeloid origin (myeloid-derived suppressor cells, MDSCs) differentiate into cells that promote tumor progression and metastasis in addition to their immunosuppressive role<sup>[13,14]</sup>. In a TNBC mouse model it was demonstrated that while monocytic (m)MDSCs infiltrated primarily the primary tumor, granulocytic (g)MDSCs homed to metastases in the lung<sup>[15]</sup>. In humans, gMDSCs were found to increase with neoadjuvant breast cancer therapies in patients showing no pathologic responses<sup>[16]</sup>. Collectively, this suggests that a group of TNBC can benefit from targeted immunotherapies. How can this TNBC patient cohort be identified?

TNBC is a heterogeneous breast cancer. Based on 3247 gene expression profiles, 21 breast cancer data sets have been analyzed that resulted in subtyping of TNBC which has been proven useful to decipher responses of TNBC patients to neoadjuvant therapies<sup>[17,18]</sup>. For example, patients in the basal-like 1 (BL 1) subgroup showed the highest pathological complete response of 41% compared to the basal-like 2 (BL 2) and the luminal androgen receptor (LAR) subgroup, 18% and 29%, respectively<sup>[17,18]</sup>. In addition, classifying then a TNBC cohort (587 patients) in three groups based on the amount of immune cell infiltration in the tumor, allowed to examine an immune signature comprising B- and T-cell markers that include immune-suppressive as well as immune-activating genes in these TNBC subtypes. This analysis revealed that out of all 587 TNBC cases, the ones correlating highest with the immune signature, were found mostly in the BL1 subtype. Interestingly, the M subgroup showed a strong negative correlation (Spearman, -0.95)<sup>[17]</sup>. As the BL1 subtype is characterized by elevated cell cycle and DNA response genes, it may be that the higher mutation rate of this TNBC subtype causes aberrant proteins expression that in turn attracts immune infiltrates. In aggregate, this suggests that TNBC patients subtyping by gene expression studies in conjunction with histopathological tissue analyses should be useful for selecting patient cohorts benefitting from immunotherapy.

## ADENOSINE RECEPTOR 2B EXPRESSION PLAYS AN IMPORTANT ROLE IN THE TUMOR MICROENVIRONMENT

Four subtypes of G-protein - coupled adenosine receptors exist, designated Adora1 (A1R), Adora2a, (A2AR), Adora2b (A2BR), or Adora3 (A3R), and are classified according to utilization of pertussis toxin - sensitive (A1 and A3) or - insensitive (A2A and A2B) pathways<sup>[19]</sup>. In the tumor microenvironment, many cell types express A2BR, especially under hypoxic conditions [Figure 1]<sup>[20]</sup>. In neutrophils A1R has a higher affinity for adenosine compared to A2AR or A2BR, and therefore at earlier stages of inflammation, lower local concentrations of adenosine promoted neutrophil recruitment, while later high concentrations of adenosine limit neutrophil recruitment through action of A2AR or A2BR<sup>[21]</sup>. In dendritic cells (DCs), although other adenosine receptors are expressed, A2BR mediates the differentiation of DCs that behave unlike myeloid DCs as they display impaired allostimulatory activity and express high levels of angiogenic, pro-inflammatory, immune suppressor and tolerogenic factors, including VEGF, IL-8, IL-6, IL-10, COX-2, TGF- $\beta$  and IDO. Furthermore, A2BR-mediated differentiation of DCs promoted lung tumors in mice<sup>[22]</sup>. Human





**Figure 1.** Expression of A2BR on cells in the tumor microenvironment. The tumor microenvironment is very heterogeneous. Besides cancer cells, cancer-associated fibroblasts (CAFs), many different immune cells can infiltrate a tumor, such as tumor infiltrating lymphocytes (TILs), tumor associated macrophages (TAMs), granulocytic and monocytic myeloid-derived suppressor cells (g and mMDSCs) and dendritic cells (DCs). While current studies suggest that in TNBC numbers of TILs positively correlate with good patient outcome, TAMs and MDSC do not

T cells predominantly express A2AR and A2BR, in addition to A1 and A3 receptors. The cAMP-elevating signaling through A2AR or A2BR in T cells results in inhibition of T-cell receptor-triggered activation of T cells and of many effector functions, including proliferation, expansion and secretion by T cells of important anti-tumor cytokines such as IFN- $\gamma$  and TNF- $\alpha$ <sup>[23]</sup>. Studies in *Adora2b*<sup>-/-</sup> mice revealed that lack of A2BR critically diminished regulatory T-cell (Treg) populations, underscoring the important role of A2BR in T-cell differentiation<sup>[24]</sup>. A2AR as well as A2BR are also expressed on macrophages. Similarly, as found in DCs or T-cells, only A2BR plays a predominant role in the adenosine-dependent differentiation of macrophages. Once activated, macrophages express T-cell suppressing arginase, indoleamine-2,3-dioxygenase and TGF- $\beta$  and display reduced T cell stimulation which promotes tumor progression<sup>[25]</sup>. The adenosine binding to A2BR results in expansion of the MDSCs pool in tumors and accelerated tumor growth in mice<sup>[26]</sup>. MDSCs-expressing A2BR have been successfully targeted with anti-A2BR therapy, suggesting that TNBC patients may benefit from such therapy as well, because they promote TNBC progression<sup>[15,16]</sup>. In mouse models pharmacological blockade of A2BR reduces tumor burden by activating DCs and improving CXCR3-dependent T cell tumor infiltration in bladder and breast cancer<sup>[27,28]</sup>. Extensive work in mouse melanoma models has demonstrated that pharmacological A2BR blockade in combination with dacarbazine reduced tumor growth and significantly increased the number of CD8<sup>+</sup> T-cells decreases the number of cancer associated fibroblasts this way contributing to decreased melanoma tumor burden<sup>[26,29]</sup>. In summary, A2BR is an abundant protein in the tumor microenvironment.

## ADENOSINE RECEPTOR 2B FUNCTIONING IN TNBC

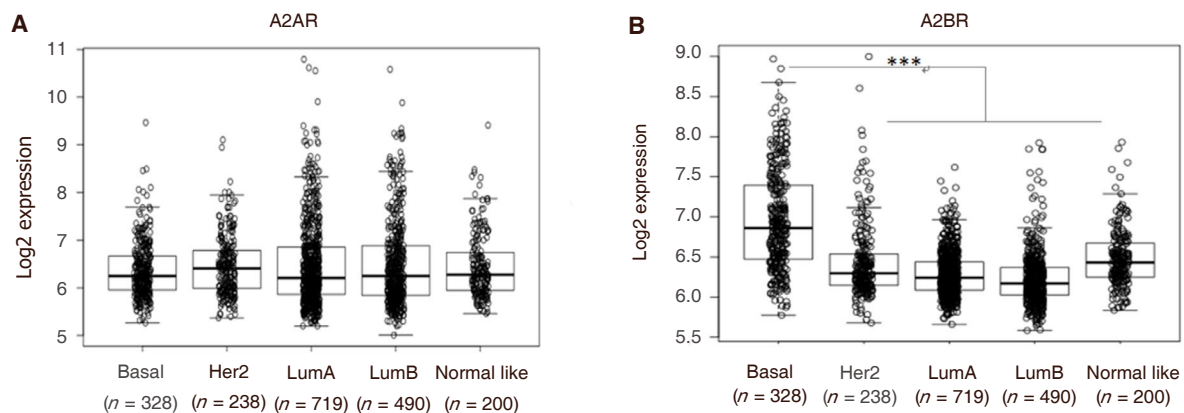
In breast cancer A2AR and A2BR expression varies significantly among breast cancer subtypes. For example, while A2AR expression levels seem similarly expressed among Pam50 subtypes within the METABRIC data set (Molecular Taxonomy of Breast Cancer International Consortium), A2BR expression is significantly higher in basal cancers compared to the other subtypes, such as Her2, LumA and LumB [Figure 2]<sup>[30]</sup>. Expression patterns were confirmed in TCGA (The Cancer Genome Atlas) as well (data not shown; <http://cancergenome.nih.gov>). Comparing survival among breast cancer patients defined by the Pam50 gene expression, showed that basal-like breast cancers with higher A2BR expression showed shorter



**Table 1. Comparison of AR2A and AR2B expression and survival in basal like breast cancers**

	OS log rank P-value	Median OS low/high expression	Hazard ratio	DMFS Log rank P-value	Median DMFS low/high expression	Hazard ratio
AR2A	0.012	40.8/97.5	0.52 (0.31-0.87)	0.0008	18/97.5	0.42 (0.25-0.71)
AR2B	0.011	95.1/41	1.96 (1.15-3.32)	0.0004	102.6/23	2.16 (1.127-3.67)

Overall survival (OS) and distant metastasis free survival (DMFS) were compared and median survival calculated using km plotter. For OS 241 patients and for DMFS 242 patients were analyzed



**Figure 2.** Comparison of A2AR (A) and A2BR (B) expression among Pam50 breast cancer subtypes in the METABRIC. A2BR is significantly higher expressed in basal like breast cancer compared to other breast cancer subtypes ( $***P = 3.9e-11$ ). Gene expression data from The Cancer Genome Atlas (TCGA) and Molecular Taxonomy of Breast Cancer International Consortium (METABRIC) were downloaded from the Gene expression Omnibus database [GEO: GSE62944] and Synapse software platform (syn1688369; Sage Bionetworks, Seattle, WA, USA), respectively

OS and distant metastasis free survival (DMFS) with a median survival for high expressors of 41 and 23 months, respectively. However, patients that expressed high levels of A2BR had a median OS of 95.1 months and a DMFS of 102.6 months, respectively [Table 1]. This is in contrast to high A2AR expression which seems to prolong overall survival in the basal breast cancer group [Table 1]. All in all, these findings suggest a functional difference between these two receptors in basal-like breast cancer. The term basal-like breast cancer is often used as a surrogate for identifying the aggressive TNBC subtype. Close to 80% of the basal like breast cancers are TNBC<sup>[31]</sup>. As TNBC is defined by lacking ER, PR and HER2, the basal subtype, is characterized by a distinct gene expression signature comprising strong expression of basal markers such as cytokeratins 5,6 and 17<sup>[32]</sup>.

Evidence already exists that blocking adenosine signaling may be a valuable option in treating TNBC. The A2BR ligand adenosine is produced in sequential action of CD39 and CD73 degrading ATP. Both are surface receptors expressed on cancer cells and like A2BR, induced by oxygen deprivation (hypoxia). In contrast to CD-39, CD73, also known as 5'-nucleotidase, is similar to A2BR, higher expressed in the ER-negative breast cancer population compared to the ER-positive cancers (METABRIC data base;  $P = 3.6e-14$ ). This suggests a close co-operation of the two receptors in TNBC progression. In fact, mouse models have clearly demonstrated that CD73 expression promotes resistance to TNBC to anthracyclins and poor prognosis<sup>[33]</sup>. This has now been confirmed in human patients as data from the BIG-02-98 study conclude that high levels of CD73 expression on epithelial tumor cells positively associates with reduced DMFS and OS and negatively correlates with tumor immune cell infiltration (Spearman's  $r = -0.50$ ,  $P < 0.0001$ ). Patients with high levels of CD73 and low levels of tumor-infiltrating leukocytes had the worse clinical outcome<sup>[34]</sup>. This suggests that adenosine signaling in TNBC associates with poor patient survival and that targeting CD73 or A2BR may provide a

promising immunotherapeutic option for a group of TNBC patients. Regulatory T-cell depletion has been recently been shown to potentiate the inhibition of the immune checkpoint in claudin-low breast cancers, a subgroup of breast cancer that is largely found within the TNBC group of patients<sup>[35]</sup>.

Besides suppressing immune responses in TNBC, some studies suggest a A2BR immune independent function in breast cancer progression. For example, adenosine stimulates proliferation and migration of human TNBC cells through A2BR-mediated stimulation of adenylyl cyclase/PKA and a PLC-dependent Ca(2+) signal<sup>[36,37]</sup>. Selective pharmacological activation of A2BR promoted tumor cell chemotaxis *in vitro* and metastasis *in vivo* using a syngeneic TNBC mouse model (4T1.2 cells). In contrast, the A2BR antagonist PSB1115 reversed significantly both phenotypes. As 4T1.2 cells express exclusively A2BR, the authors concluded that expression on A2BR on cancer cells contributes to breast cancer metastasis<sup>[38]</sup>. Mittal *et al.*<sup>[39]</sup> confirmed these findings by showing that inhibition of A2BR *in vivo*, using the 4T1.2 mouse model was independent of CD4<sup>+</sup> or CD8<sup>+</sup> T-cells and/or natural killer cells in this setting. A synthetic lethality screen identified a pharmacological axis that identifies A2BR as a target gene of the transcription factor Fos-related antigen-1 that promotes TNBC metastasis. In this model, both RNAi silencing and pharmacological inhibition of A2BR inhibited filopodia formation and invasive activity of TNBC cells and correspondingly reduced tumor outgrowth in the lungs in an immune-compromised mouse model<sup>[40]</sup>.

## FUTURE DIRECTIONS

Tumor hypoxia is an unavoidable byproduct of fast and aggressive growing tumors, and the hypoxic response is quite robust in TNBC compared to other subtypes<sup>[41]</sup>. Deprivation of oxygen induces the accumulation of extracellular adenosine in tumors providing abundant ligand for adenosine receptors, such as A2BR<sup>[25]</sup>. A2BR expression is higher in basal-like breast cancers compared to other breast cancer subtypes [Figure 2] and A2BR is a major player in immune suppression, metastasis and relapse in TNBC. Therefore, A2BR provides an attractive target for treating TNBC, for which currently no targeted therapies exist. In particular the combination of immune checkpoint inhibitors together with A2BR agonists should be considered as viable treatment option, as checkpoint inhibitors show promising results in phase 1/2 clinical trials in TNBC<sup>[42]</sup>. Besides presenting a viable drug target, A2BR may also serve as a prognostic biomarker in TNBC. More studies need to be done to test this hypothesis. Not unlike in other drug targeting strategies, more research is necessary to develop molecular and pathological parameters upfront that define appropriate patient cohorts that should be tested for anti-A2BR therapies. For example, TNBC subtyping shows how heterogeneous TNBC subtypes are. In addition, analyses are necessary to determine A2BR antagonistic effects on TILs in TNBC and patient outcome. In summary, based on current research, A2BR may present a viable drug candidate in a defined cohort of TNBC breast patients.

## DECLARATIONS

### Authors' contributions

Wrote the manuscript: Neumann CA

Developed the METABRIC expression and analysis tool: Levine K, Oesterreich S

### Financial support and sponsorship

None.

### Conflicts of interest

There are no conflicts of interest.

### Patient consent

Not applicable.

## Ethics approval

Not applicable.

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Review

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# The magnitude of benefit from adding taxanes to anthracyclines in the adjuvant settings of breast cancer: discussion of large trials and meta-analyses

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## Abstract

The taxanes family of chemotherapy, which includes paclitaxel and docetaxel, has been incorporated in the adjuvant breast cancer treatments since 1990s. Sequential and concurrent use of taxanes was investigated with anthracyclines in many adjuvant early breast cancer randomized clinical trials. Results from taxanes trials showed inconsistent benefits. However, several meta-analyses showed significant survival benefit of adding taxanes. In this review article, data were collected and summarized from eleven large randomized trials and three meta-analyses to show and discuss the magnitude of benefit of taxanes-anthracyclines combination compared to anthracyclines only adjuvant regimens in early breast cancer. This article aims at providing the oncologists with a well-organized, inclusive and updated evidence.

**Keywords:** Breast cancer, adjuvant chemotherapy, taxanes, anthracyclines

## INTRODUCTION

Adjuvant chemotherapy represents an integral part in the care of breast cancer patients. It has been shown that it significantly reduces the risk of recurrence and the risk of death from breast cancer<sup>[1]</sup>. Adjuvant chemotherapy in breast cancer has passed through six main eras; the cyclophosphamide, methotrexate, fluorouracil (CMF)-era, anthracyclines-era, taxanes-era, dose-dense era, combination with targeted therapy era, and recently the individualized use of chemotherapy based on genetic testing. This article focuses on the taxanes-era; discussing the large randomized trials (which included more than 1000 patients) [Table 1] and



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**Table 1. The 8 large taxanes trials which compare adjuvant taxanes-anthracyclines to the standard of care regimens: (4 AC, 6 FAC, 6 FEC, and 6 oral-CMF)**

Trial name	CALGB 9344	NSABP-B28	BCIRG-001	PACS-01	E 2197	GEICAM 9906	GEICAM 9805	AGO
Year of publication	2003	2005	2005	2006	2008	2008	2010	2014
Number of patients	3121	3060	1480	1999	2882	1246	1060	2011
Control arm	4 × AC (escalating dose of A)	4 × AC	6 × FAC	6 × FEC	4 × AC	6 × FEC	6 × FAC	6 × FEC or 6 × CMF-oral
Taxanes arm	4 AC → 4 P	4 AC → 4 P	6 × TAC	3 FEC → 3 D	4 × AT	4 FEC → 8 weekly P	6 × TAC	4 EC → 4 D
Taxanes dose	Paclitaxel 175 mg/m <sup>2</sup>	Paclitaxel 225 mg/m <sup>2</sup>	Docetaxel 75 mg/m <sup>2</sup>	Docetaxel 100 mg/m <sup>2</sup>	Docetaxel 60 mg/m <sup>2</sup>	Paclitaxel 100 mg/m <sup>2</sup> /W	Docetaxel 75 mg/m <sup>2</sup>	Docetaxel 100 mg/m <sup>2</sup>
LN status for eligible patients	Positive	Positive	Positive	Positive	66% -ve LNs and the rest are 1-3 +ve LNs	Positive	Negative	1-3 +ve LNs
% of patients completed full taxanes course	92%	75%	91%	96%	94%	99.5%	94.5%	81.2%
Statistically significant benefit	DFS OS	DFS	DFS OS	DFS OS	Negative	DFS	DSF	EFS OS
Absolute 5-year DFS difference	5%	4%	7%	5.2%	0%	6.4%	4.8%	5-year EFS 2.5%
Absolute 5-year OS difference	3%	NA	6%	4%	NA	NA	NA	1.7%
Subgroup who get significant benefit	ER -ve	Not affected by ER status	+ve 1-3 LNs	+ve 1-3 LNs & age U 50 years	DFS with ER/PR -ve ER+/PR-	ER -ve Her2 -ve Also, depend on LNs status and tumor size	No difference among all patients' subsets	ER +ve plus KI67 U 20%
NF or neutropenia % with taxanes arm	Granulocytopenia ≤ 500 was 16 %	NF 3%	NF 25%	NF 11.2%	Grade III neutropenia 26%	NF 9.5%	NF 9.6%	NF 3.7% (G-CSF prophylaxis was allowed)

AC: doxorubicin-cyclophosphamide; EC: epirubicin-cyclophosphamide; FAC: doxorubicin-cyclophosphamide-5-fluorouracil; TAC: docetaxel-doxorubicin-cyclophosphamide; FEC: epirubicin-cyclophosphamide-5-fluorouracil; CMF: cyclophosphamide, methotrexate, fluorouracil; OS: overall survival; DFS: disease free survival; EFS: event-free survival; G-CSF: granulocyte-colony stimulating factor; ER: estrogen receptor; PR: progesterone receptor; LN: lymph node; NF: neutropenia with fever; NA: not available

meta-analyses, to provide a well-organized and appealing summary. The primary endpoints of taxanes trials differed, overall survival (OS) is being the primary endpoint in some while disease free survival (DFS) in others. We start by discussing OS trials followed by DFS, as OS is considered the most valuable outcome in adjuvant cancer trials.

## RANDOMIZED TRIALS SHOWED SIGNIFICANT OS BENEFIT FROM ADDING TAXANES

Four large randomized controlled trials showed statistical significant OS benefit from adding taxanes to anthracyclines in the adjuvant settings of early-stage breast cancer. Those trials are discussed below.

### CALGB-B 9344 trial

This landmark trial was conducted by the Cancer and Leukemia Group B (CALGB) from USA and published in 2003. It randomized 3121 breast cancer patients with positive axillary lymph nodes (LNs) after surgery to receive 4 cycles of doxorubicin-cyclophosphamide (AC) regimen followed by either 4 cycles of paclitaxel (175 mg/m<sup>2</sup>) vs. placebo. Adding paclitaxel to AC resulted in 5-year DFS of 70% compared to 65% in AC only arm, furthermore it resulted in 5-year OS of 80% vs. 77% in AC only arm<sup>[2]</sup>. It concluded that escalating the doxorubicin dose did not add a significant benefit, but adding paclitaxel resulted in a statistical significant advantage in both DFS and OS compared to non-paclitaxel arm.

### BCIRG-001 trial

The Breast Cancer International Research Group (BCIRG) trial was published in 2005. BCIRG-001 compared 6 cycles of doxorubicin-cyclophosphamide-5-fluorouracil (5FU) regimen (FAC) vs. 6 cycles of docetaxel-doxorubicin-cyclophosphamide (TAC) regimen in 1480 breast cancer patients with positive LNs after surgery. Ninety-one percent of the patients completed the full TAC course despite the fact that there was no routine use of granulocyte-colony stimulating factor (G-CSF) primary prophylaxis. It reported 5-year OS of 87% in the TAC arm compared to 81% in the FAC arm ( $P = 0.008$ ), and 5-year DFS of 75% in the TAC arm compared to 68% in the FAC arm ( $P = 0.001$ ). Also, there were 25% of patients in the TAC arm developed neutropenia with fever (NF) vs. 2% in the FAC arm<sup>[3]</sup>.

It examined the concurrent use of taxanes-anthracyclines rather than sequential administration which was the case in both the NSABP and CALGB trials. Docetaxel, the second member in taxanes family was used unlike the CALGB and NSABP trial, which is going to be discussed later.

An update of the BCIRG-001 trial was published in 2013, and showed a maintained DFS and OS advantage, after 10-year of follow-up, in favor of the TAC arm. Ten-year OS was 76% vs. 69% in the TAC and FAC arm ( $P = 0.002$ ), respectively. In subgroup analysis, TAC improved DFS relative to FAC irrespective of the nodal, hormone receptor, and HER2 status. Grade 3-4 heart failure occurred in 3% in the TAC arm vs. 2% in the FAC arm, and it caused death in 2 patients in the TAC arm and 4 patients in the FAC arm<sup>[4]</sup>.

### PACS-01 trial

This is a French trial that was published in 2006 and randomized 1999 breast cancer patients with positive nodes to 3 cycles of adjuvant docetaxel ( $100 \text{ mg/m}^2$ ) after 3 cycles of epirubicin-cyclophosphamide-5FU (FEC) regimen (FEC-D arm) compared to 6 cycles adjuvant FEC. Five-year DFS in the FEC-D arm was 78.4% vs. 73.2% in the FEC only arm ( $P = 0.11$ ). Five-year OS was 90.7% in the FEC-D arm compared to 86.7% in the FEC arm ( $P = 0.14$ ). It is noteworthy that G-CSF primary prophylaxis was not allowed in this trial and grade 3-4 neutropenia was 11.2% in the FEC-D vs. 8.4% ( $P = 0.03$ ). Also, cardiac toxicity was less in the FEC-D arm when compared to the FEC arm ( $P = 0.03$ ). Patients with 1-3 positive nodes as well as patients aged 50 years or more had better DFS in subgroup analyses<sup>[5]</sup>.

### WSG-AGO trial

WSG-AGO Trial was published in 2014 from Germany where it randomized 2011 eligible patients to receive either adjuvant 6 cycles FEC regimen (or oral-cyclophosphamide-epirubicin-5FU, which is also known as the oral-CMF, which was received in 9 % of this arm) vs. 4 cycles of adjuvant EC followed by 4 cycles docetaxel  $100 \text{ mg/m}^2$  (EC-D arm). It included only patients with 1-3 positive level I/II axillary LNs (pN1) disease, and the results showed that 5-year event-free survival (EFS) was 87.3% in the FEC/CMF arm compared to 89.8% in the EC-D arm ( $P = 0.038$ ), and 5-year OS was 92.8% in the FEC/CMF arm compared to 94.5% in the EC-D arm ( $P = 0.034$ ). Primary G-CSF prophylaxis was allowed, and NF occurred in 3.7% in the EC-D arm vs. 2.1% in the FEC/CMF arm. It was noted that patients with estrogen receptor (ER) positive tumors plus  $\text{Ki-67} \geq 20\%$  had the most benefit from adding taxanes in subgroup analyses<sup>[6]</sup>.

## RANDOMIZED TRIALS SHOWED ONLY SIGNIFICANT DFS BENEFIT FROM ADDING TAXANES

### NSABP-B28 trial

The National Surgical Adjuvant Breast and Bowel Project (NSABP-B28) trial, which was published in 2005, is one of the landmark adjuvant taxanes' trials. It included 3060 patients with early breast cancer and positive axillary (LNs), then the eligible patients were randomized to receive either 4 cycles AC (AC arm) or 4 cycles AC followed by 4 cycles paclitaxel (AC-T arm). This trial was characterized by using a higher dose of paclitaxel which is  $225 \text{ mg/m}^2$  without primary G-CSF prophylaxis. There was a DFS benefit in the AC-T arm compared to the AC arm, where 5-year DFS was 76% in the AC-T arm compared to 72% in the AC arm ( $P$

= 0.007). There was no significant OS benefit from adding taxanes to anthracycline according to NSABP-B28 trial. It is noted that only 75% of the patients in the AC-T arm completed the full AC-T course, and this could be the reason for the absence of OS benefit. It is important to remember that there were 7 deaths which could be attributed to chemotherapy in the AC-T arm. However, it was recorded that only 3% of the patients in the AC-T arm developed febrile neutropenia, and 18% had grade III neurotoxicity in the same arm<sup>[7]</sup>.

### **GEICAM-9906 trial**

The Spanish Breast Cancer Research Group published its special trial GEICAM-9906 in 2008, and it used weekly paclitaxel regimen, however it used only 8 weeks of paclitaxel instead of 12 weeks. It randomized 1246 node positive patients to two arms; the first one received 6 cycles adjuvant FEC and the second arm received 3 cycles adjuvant FEC followed by 8 cycles of weekly paclitaxel 100 mg/m<sup>2</sup> (FEC-P). There was a statistically significant difference in DFS from adding weekly paclitaxel to FEC when compared to adjuvant FEC alone, as 5-year DFS was 78.5% in FEC-P compared to 72.1% ( $P = 0.006$ ). But, this benefit was accompanied by increase in NF of 9.5% vs. 5.1%. DFS benefit depended on the number of positive LNs and tumor size, also it was better with HER2 negative patients and patients with ER negative tumors based on subgroup analyses of this trial<sup>[8]</sup>.

### **GEICAM-9805 trial**

Another Spanish trial (GEICAM-9805) was published in 2010 which investigated the benefit of adding adjuvant taxanes in node-negative breast cancer patients, and its arms were identical to the BCIRG-001 arms. But, unlike the BCIRG trial it allowed primary G-CSF prophylaxis in its TAC arm which greatly declined the rate of NF in contrast to the BCIRG study where the NF risk was high. Interestingly, it showed a significant DFS benefit in node-negative patients. In this study, 1060 node-negative patients were randomized to receive 6 cycles adjuvant FAC vs. 6 cycles adjuvant TAC. The results showed that the 5-year DFS was 90.1% in TAC arm compared to 85.3% in the FAC arm ( $P = 0.03$ ). NF occurred in 9.6% with TAC vs. 2.3% in the FAC arm ( $P \leq 0.001$ ). It is important to note that the overall grade 3-4 toxicity from TAC was significantly higher than those with FAC (28.2% vs. 17%;  $P < 0.001$ )<sup>[9]</sup>.

## **RANDOMIZED TRIALS WHICH DID NOT SHOW BENEFIT FROM ADDING TAXANES**

### **Intergroup trial E-2197**

One of the negative taxanes' trials is the North American Breast Cancer Intergroup Trial (E 2197) that was published in 2008 and compared 4 cycles adjuvant AC to 4 cycles adjuvant concurrent doxorubicin-docetaxel (60 mg/m<sup>2</sup>) AT-arm. It included 2882 high-risk negative nodes breast cancer patients and those with 1-3 positive nodes. Also, primary G-CSF prophylaxis was not allowed in this trial. The results showed that 5-year DFS was 85% in both arms of the study ( $P = 0.78$ ), however in subgroup analyses there was a trend of better DFS in patients with ER/progesterone receptor (PR) negative ( $P = 0.02$ ) and those with ER positive/PR negative ( $P < 0.01$ ). Grade 3 neutropenia was 26% in AT arm vs. 10% in AC arm ( $P < 0.05$ ). There are some explanations why this trial was negative; one of those possible reasons is that the negative nodes patients constituted 66% of the study population. Secondly, the lower dose of docetaxel which was 60 mg/m<sup>2</sup>. Lastly, the short overall taxanes course as it was only 4 cycles of AT<sup>[10]</sup>.

### **TACT trial**

The UK-TACT trial, published in 2009, is another example of negative taxanes' trials which did not show a statistically significant benefit of adding taxanes to anthracyclines in adjuvant treatment of breast cancer, and there are two possible explanations for this negative result. The first one is that it included both node positive and node negative patients whereas the most of positive taxanes trials included only patients with positive LNs. The second reason is that the control arm received a long course which is 8 cycles of either FEC regimen or 4 cycles FEC followed by another 4 cycles of CMF, in contrast to both the NSABP and BCIRG trials in which the control arm received only 4 cycles of AC and 6 cycles of FAC respectively<sup>[11]</sup>. Another negative trial was MA-21 trial from Canada, which also included node negative patients and the control arm

**Table 2. The 5-year mortality and recurrence for the Cochrane meta-analysis 2007**

Cochrane meta-analysis 2007 results	With taxanes	Without taxanes
5-year mortality in low-risk patients	5%	6%
5-year mortality in high-risk patients	21.6%	26%
5-year recurrence in low-risk patients	11.5%	14%
5-year recurrence in high-risk patients	30.3%	36%
Neutropenic fever	13%	0.56%

**Table 3. The 8-year mortality and recurrence for the EBCTCG overview 2012**

EBCTCG overview 2012	With taxanes	Non-taxanes	Absolute difference	P value
8-year mortality	21.1%	23.9%	2.8%	0.0005
8-year recurrence	30.2%	34.8%	4.0%	0.000001

EBCTCG: Early Breast Cancer Trialists' Collaborative Group

was the oral CEF regimen. Besides the fact that it was comparing CEF to a dose-dense taxane containing regimen<sup>[12]</sup>.

## META-ANALYSES

There are three meta-analyses that demonstrated benefit from adding taxanes to anthracyclines in the adjuvant settings of breast cancer. All these meta-analyses confirmed that adding taxanes significantly increases OS compared to anthracyclines-only adjuvant regimens.

The first meta-analysis was from Cochrane data base in 2007<sup>[13]</sup>, and it included about 21,000 patients from 12 trials with a median follow-up of 60.4 months. It showed that the hazards ratio (HR) of OS was 0.81 favoring the addition of taxanes ( $P < 0.00001$ ). The HR for DFS was also 0.81 favoring the addition of taxanes ( $P < 0.00001$ ). However, it did not show which patients' subgroup demonstrated more benefit from adding taxanes. Table 2 summarizes the results.

The second meta-analysis came from Italy and was published in 2008<sup>[14]</sup>. It included 22,900 patients from 13 trials and it showed a significant DFS and OS benefit from adding taxanes to anthracycline in the adjuvant therapy for breast cancer. The absolute 5-year DFS difference was 5% between taxanes and non-taxanes adjuvant regimens, and 5-year OS difference was 3%. What is important in this meta-analysis is that it found that adding taxanes did not result in benefit for patients with ER positive and those with  $\geq 4$  positive LNs. It also concluded that sequential administration of adjuvant taxanes-anthracyclines is better than concurrent administration of both agents.

The last and the largest meta-analysis was conducted by the Early Breast Cancer Trialists' Collaborative Group (EBCTCG)<sup>[15]</sup> and published in 2012. It collected data of 100,000 patients from 123 trials and showed that adding taxanes resulted in a small but significant OS benefit compared to non-taxanes regimens. It showed also that all subgroups of patients had the benefit from adding taxanes. The results are shown in Table 3.

## CONCLUSION AND RECOMMENDATIONS

Several randomized clinical trials were conducted to investigate the role of adding taxanes to anthracyclines. Some of these trials established both OS and DFS, whereas other trials did not show any advantage from adding taxanes. Subsequent meta-analyses confirmed the clinical benefit from adding taxanes to anthracyclines in the adjuvant breast cancer chemotherapy protocols.

There are four trials which showed a statistical significant OS advantage from adding taxanes: BCIRG-001 trial, CALGAB-9344 trial, PACS-01 trial, and AGO trial. The highest OS benefit was reported in the

BCIRG-001 trial of 6% at 5 years. BCIRG-001 trial compared adjuvant TAC vs. FAC regimens in node positive early breast cancer patients, with subgroup analysis of patients with 1-3 positive nodes showing the largest OS benefit from adding taxanes. However, the incidence of NF was 25% in the TAC arm which is considered a limitation against its use. Nonetheless, G-CSF prophylaxis was not allowed in this trial and likely contributed to the high incidence of NF. Therefore, it is reasonable to prescribe G-CSF whenever TAC is considered for adjuvant chemotherapy in breast cancer to minimize NF risk.

Other regimens which were associated with significant OS benefit are 4 cycles of andriamycin-cyclophosphamide followed by 4 cycles of paclitaxel (AC-P) regimen in CALGAB-9344 trial, 3 cycles of 5FU-epirubicin-cyclophosphamide followed by 3 cycles docetaxel (FEC-D) regimen in PACS-01 trial, and 4 cycles of epirubicin-cyclophosphamide followed by 4 cycles docetaxel (EC-D) regimen in AGO trial. Subgroup analyses of each trial demonstrated the following; AC-P regimen was more beneficial to patients with ER negative tumors, whereas the EC-D regimen gave better results for ER positive patients. The FEC-D regimen was better for patients with 1-3 positive LNs and those who aged 50 years or more.

Three randomized trials reported significant DFS with adding taxanes to anthracyclines in the adjuvant settings of breast cancer. These trials are NSABP-B28, GEICAM-9906 and GEICAM-9805. It is noteworthy that most of the taxanes trials were conducted on patient with node-positive disease, whereas 3 trials showed negative results in node-negative patients: UK-TACK trial, MA-21 trial, and E-2197 trial. However, TAC regimen resulted in a significant DFS advantage for node-negative patients in GEICAM-9805 trial. Therefore, TAC regimen might be considered for node-negative breast cancer patient. Another important advantage of TAC regimen over AC-P regimen is that the short overall duration which is only 6 cycles of chemotherapy, whereas AC-P is a total 8 cycles. Moreover, The AC-P regimen which was used in NSABP-B28 trial did not result in a significant OS benefit because of the high dose of Paclitaxel that lead to 25% of patient did not complete the chemotherapy course.

There were 3 meta-analyses which investigated the role of adding taxanes to anthracyclines in the adjuvant setting of breast cancer, and all reported a significant OS benefit from adding taxanes. EBCTCG meta-analysis, which is the largest meta-analysis in this area, showed that all patient subgroups had a significant improvement of OS from adding taxanes. The Italian meta-analysis showed that adding taxanes was not beneficial to ER-positive patients and those with 4 or more positive axillary LN metastases. Nonetheless, Cochrane database meta-analysis did not report which patient subgroup had the greatest OS benefit from adding taxanes.

The positive impact of adding taxanes to anthracyclines in treating breast cancer can be explained by the different mechanisms of action at both the cellular and molecular levels. Such combination helps to overcome drug resistance of both agents if used separately. Anthracyclines works by intercalating into DNA, disrupting topoisomerase-II-mediated DNA repair and generating free radicals which trigger apoptotic pathways of cell death<sup>[16]</sup>. Whereas, taxanes works by binding to microtubules, preferentially to  $\beta$ -tubulin, and stimulate phosphorylation of  $\beta$ -tubulin which leads to stabilization of microtubules by the prevention of depolymerization. The stabilized microtubules interfere with mitotic spindle formation during the cell division and leads to cell death.

The genes that are involved in the action of doxorubicin at the cellular level are those capable of the oxidation reaction (NADH dehydrogenases, nitric oxide synthases, xanthine oxidase) and those capable of deactivating the free radicals such as glutathione peroxidase, catalase, and superoxide dismutase. Also, genes which are involved in the topoisomerase-II pathway of Doxorubicin action include the enzymes involved in the DNA repair and cell cycle control such as *TOP2A*, *MLH1*, *MSH2*, *TP53*, and *ERCC2* genes. Whereas, the main genes involved in the action of Paclitaxel are the  $\beta$ -tubulin and c-erb 2. However, both anthracyclines-



taxanes share a common mechanism of drug resistance which may explain the failure of this combination in the adjuvant setting of breast cancer. Multidrug resistance-1/P-glycoprotein over-expression and the breast cancer resistance protein are responsible for resistance to both drug categories. Better understanding of drug resistance may help to optimize such combination.

## DECLARATIONS

### Authors' contributions

Corresponding author/principal investigator, prepares and collects the scientific material of the paper, and writes the first draft of the manuscript: Elzaafarany OH

Co-author, reviews and edits the manuscript: Abusanad A

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There are no conflicts of interest.

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Not applicable.

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Original Article

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# Nodal involvement and p16-staining in upper alveolar ridge and hard palate cancer

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## Abstract

**Aim:** Upper alveolar ridge and hard palate squamous cancer is an infrequent malignancy. We evaluated factors associated with neck involvement and with p16-staining.

**Methods:** Head and neck squamous-cell carcinoma (SCC) patients who went to Head and Neck Department between 1997 and 2011 were screened, and 73 resected upper alveolar ridge and 5 hard palate SCC were selected. Tumors with available tissue were stained with p16 immunohistochemistry.

**Results:** Median age was 64.4 years, 55.1% were female, and 73.1% were in clinical stage IV. Neck dissections were performed in 24 and pathologically confirmed node metastases were found in 19 (24.3%). Cervical recurrence was found in 18 patients (23.1%) and was associated with histological grade ( $P = 0.037$ ). Three (7.3%) of 41 lesions were positive for p16 and tended to be younger ( $P = 0.067$ ). Lymphovascular invasion was associated with shorter disease-free survival (DFS) ( $P = 0.026$ ) and overall survival (OS) ( $P = 0.021$ ). Larger cT ( $P = 0.019$ ), perineural invasion ( $P = 0.039$ ) and neck dissection ( $P = 0.010$ ) were associated with shorter OS. Neck node involvement tended to have shorter DFS (31% vs. 48.7%,  $P = 0.278$ ) and OS (25.1% vs. 48.5%,  $P = 0.340$ ), and neck recurrence tended to have shorter OS (9.3% vs. 52.3%,  $P = 0.064$ ).

**Conclusion:** Neck involvement and recurrence are frequent in this location. P16-positive cases were present in 7.3% and tended to be associated with younger age.



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**Keywords:** P16, head, neck, maxilla, prognosis, survival

## INTRODUCTION

Squamous cell carcinomas (SCC) of the hard palate and upper alveolus ridge are relatively rare<sup>[1]</sup>. Prognostic factors and neck management in head and neck SCC (HNSCC) have been extensively studied in series of tongue or floor of mouth SCC, or on series with a mixture of SCC tumor sites<sup>[2]</sup>. Only small retrospective series have evaluated the behavior of hard palate and upper alveolus, and suggest that they have a low rate of regional node metastases<sup>[3-8]</sup>. However, recent studies find higher rates of both neck lymph node involvement and neck recurrence in these malignancies, and, there is a need to identify those aggressive cases that would benefit from more aggressive treatment<sup>[9,10]</sup>.

Several clinicopathological features have been implicated in recurrence risk and prognosis in HNSCC. These include tumor size, nodal involvement, tobacco and alcohol consumption, and presence of human papillomavirus (HPV) infection<sup>[11,12]</sup>.

The prevalence of HPV infection is higher in oropharyngeal squamous cell carcinoma (OPSCC) (35.6%) and has been associated with both better prognosis and higher response rate to chemoradiation<sup>[12,13]</sup>. P16 staining is highly correlated with HPV infection in OPSCC and has also been associated with good prognosis<sup>[14-17]</sup>. There is no information about the rate of p16 expression in rare locations like hard palate and upper alveolar ridge.

The aim of the present study was to evaluate predictive factors associated with node involvement, prognostic factors, and prevalence of p16 staining in hard palate and upper alveolar ridge SCC.

## METHODS

### Study population

All patients treated at Department of Head and Neck at Instituto Nacional de Enfermedades Neoplásicas with maxillary SCC between January 1997 and December 2011 were screened for the study. Inclusion criteria included having a primary tumor located in the upper alveolar ridge or hard palate, having a squamous histology, and having history of resection of primary tumor. Patients with primary tumor of nasal cavity and paranasal sinuses were excluded. The procedure of neck management was selected by the Institute surgeon. It included neck dissection in cases of clinically involved lymph nodes and in cases of metastasis risk factors like greater depth of primary tumor deep invasion. Selection of ipsilateral or bilateral dissection was also determined by the Institute surgeon and took into account clinical factors like proximity to midline.

Information about clinicopathological variables was taken from patient files and pathology report. Data included age, gender, tobacco use, alcohol use, tumor subsite, depth of invasion, histologic grade, margin status, perineural invasion (PNI), lymphovascular invasion (LVI), clinical and pathological stage (TNM classification), surgical procedure, radiation or chemotherapy administration, and date of last follow-up or death. Some standard pathological features that were not reported in patient file were prospectively completed by a pathologist (LC). The institutional review board approval was obtained from The Instituto Nacional de Enfermedades Neoplásicas (Lima, Peru). Since the study was based on a secondary source and there was no contact with the patients, no informed consent was applied; however, the identity and personal data of patients' medical records were protected at all times.

### P16 immunohistochemistry assay

Pathologists evaluated H&E slides under light microscopy and the most representative tissue were selected. A 0.6-cm punch was taken from each formalin-fixed paraffin-embedded (FFPE) sample selected and was

transferred to an empty paraffin recipient block in order to construct tissue microarrays (TMA). FFPEs samples were fixed for 6 to 8 h in 10% neutral buffered formalin and routinely processed with standard methodologies.

In total, 41 tissue cores were distributed into ten TMA blocks. Tissue sections were cut at 3 mm and float-mounted on adhesive (silanized) glass slides. Immunohistochemistry (IHC) for p16 status was performed using the DAKO EnVision™ FLEX+ detection system together with the Autostainer Link instrument (DAKO Corp, Carpinteria, California) on FFPE tissue. Antigen was retrieved using EnVision™ FLEX Target Retrieval Solution, High pH, and p16 was detected using p16 mouse monoclonal antibody (clone 16p04, JC2, BSB 5828, prediluted, Bio SB, Santa Barbara, California). The EnVision™ FLEX+, Mouse, High pH, (LINK) Kit was used to perform the assay according to the manufacturer's instructions. It contains the substrate chromogen 3-3'-diaminobenzidine (DAB), which, on staining, results in a brown-colored precipitate at the antigen site.

Positive p16 expression was defined as a strong and diffuse nuclear and cytoplasmic staining in at least 70% of the tumor cells.

Immunohistochemical evaluation was carried out by three pathologists in independent readings (LC, HG, and SC). Reports that varied among readers were reevaluated to determine a consensus.

### Statistical analysis

The log-rank statistic was used for univariate analysis, and Cox proportional hazards regression was used for multivariable analysis. Categorical comparisons were carried out using the chi-square statistic or Fisher exact test. In all cases, the level of alpha was set at 0.05 a priori. Survival analysis was calculated using the Kaplan-Meier method. All analyses were performed in SPSS version 17.0 (SPSS, Chicago, IL).

## RESULTS

Tumor primary location for this cohort was distributed in 5 patients for hard palate and 73 for upper alveolar ridge. Mean age was 64.47 years old and 55.1% were female. There were tobacco use and alcoholism history in 10.3% and 6.4% cases, respectively. Two (40%) hard palate and 52 (71.2%) upper alveolar ridge tumors were clinically classified cT4, and 3 (60%) hard palate and 21 (28.8%) upper alveolar ridge tumors were clinically node-positive at presentation. Clinical stages I-IV of upper alveolar ridge SCC were found in 1 (1.4%), 13 (17.8%), 5 (6.8%) and 54 (74%) of cases, respectively. Clinical stages II-IV of hard palate SCC were found in 1, 1 and 3 cases, respectively [Table 1].

The primary tumor was resected in all cases ( $n = 78$ ). Neck dissections were initially performed in 24 cases (21 in clinically node-positive and 3 in node-negative). Nineteen (79.16%) of cases who went to neck dissections had confirmed nodal metastases on pathological examination (including the 3 clinically node-negative cases). Cervical metastases in these 19 node-positive cases were distributed between levels I (94.7%), II (73.7%), and III (26.3%). Extracapsular extension at presentation was noted in 7 specimens of upper alveolar ridge tumors.

In no instances were age ( $P = 0.329$ ), location ( $P = 0.590$ ), cT ( $P = 0.629$ ), histological grade ( $P = 0.361$ ), PNI ( $P = 0.825$ ), or LVI ( $P = 0.080$ ) associated with cervical metastases [Table 2].

### Neck recurrences

Altogether, 18 patients (75%) developed cervical recurrences, and 8 (44.4%) of them went to neck dissection rescue (3 of them with additional radiation). Altogether, 18 patients (75%) developed cervical recurrences and 8 (44.4%) of them went to neck dissection rescue (3 of them with additional radiation). Ten patients did not go to surgery and treatment for them were: radiation alone ( $n = 2$ ), radiation and chemotherapy



**Table 1. General features**

	Cases	%
Age (years), mean (range)	64.47 (21-89)	
Gender		
Female	43	55.1
Male	35	44.9
Tobacco		
Yes	8	10.3
No	70	89.7
Alcohol		
Yes	5	6.4
No	73	93.6
Location		
Hard palate	5	6.4
Upper alveolus	73	93.6
cT		
T2-T3	22	28.2
T4	54	69.2
cN		
cN0	54	69.2
cN-positive	24	30.8
Clinical stage		
I	1	1.3
II	14	17.9
III	6	7.7
IV	57	73.1
Histological grade		
Poor/moderate differentiation	34	43.6
Well differentiated	44	56.4
Perineural infiltration		
No	31	39.7
Yes	21	26.9
Lymphovascular invasion		
No	40	51.3
Yes	13	16.7
p16		
Negative	38	48.7
Positive	3	3.8
Neck dissection		
No	54	69.2
Yes	24	30.8
Time from initial surgery to neck recurrence (months), mean (range)	8.6 (2-29)	
pN		
N0	9	11.5
N1	3	3.8
N2	15	19.2
N3	1	1.3
NX	50	64.1
Lymph node levels		
I	9	11.5
II	4	5.1
I, II	5	6.4
I, III	1	1.3
II, III	1	1.3
I, II, III	1	1.3
II, V, contralateral	1	1.3
I, II, III, contralateral	1	1.3
I, II, III, IV, contralateral	1	1.3
Without registration data	54	69.2
Extracapsular extension	7	29.2
N+ and N- with neck recurrence	33	42.3

**Table 2. Relationship between clinicopathological features and lymph node involvement**

	Neck lymph node (%)		<i>P</i>
	Negative ( <i>n</i> = 59)	Positive ( <i>n</i> = 19)	
Age (years), mean (range)	65.41 (31-89)	61.58 (21-88)	0.329
Location			
Upper alveolus	56 (94.9)	17 (89.5)	0.590
Hard palate	3 (5.1)	2 (10.5)	
cT			
T2-T3	19 (32.2)	5 (26.3)	0.629
T4	40 (67.8)	14 (73.7)	
Grade			
Poor/moderate differentiation	24 (40.7)	10 (52.6)	0.361
Well differentiated	35 (59.3)	9 (47.4)	
PNI			
No	23 (60.5)	8 (57.1)	0.825
Yes	15 (39.5)	6 (42.9)	
LVI			
No	32 (82.1)	8 (57.1)	0.080
Yes	7 (17.9)	6 (42.9)	
Tobacco			
No	53 (89.8)	17 (89.5)	1.000
Yes	6 (10.2)	2 (10.5)	
Alcohol			
No	55 (93.2)	18 (94.7)	1.000
Yes	4 (6.8)	1 (5.3)	
p16			
Negative	30 (90.9)	8 (100.0)	-
Positive	3 (9.1)	0 (0.0)	

PNI: perineural invasion; LVI: lymphovascular infiltration

(*n* = 2), chemotherapy alone (*n* = 2) or no-treatment (*n* = 4). Cervical metastases in these 18 cases of cervical recurrences were distributed between levels I (61.1%), II (100%) and III (27.8%).

Fourteen of the patients who were clinically node-negative (25.9%) and 4 of the patients who were pathologically confirmed node-positive at presentation (16.7%) had recurrences in the neck. The mean time to neck recurrence was 8.6 months (2 to 29 months). A factor associated with neck failure was high histological grade (*P* = 0.037). Recurrences were not associated with age (*P* = 0.725), cT (*P* = 0.754), N (*P* = 0.536), or PNI (*P* = 0.624) [Table 3].

### Expression of p16

A total of 41 (52.5%) lesions were tested for p16 expression. Overall, 7.3% (3 of 41) were p16 positive: 1 of 2 in hard palate (50%) and 2 of 39 in alveolar ridge (5.1%) [Figure 1]. The p16 positive tumors were not associated with age (*P* = 0.067), tumor location (*P* = 0.143), cT (*P* = 1.000), or histological grade (*P* = 0.560) [Table 4].

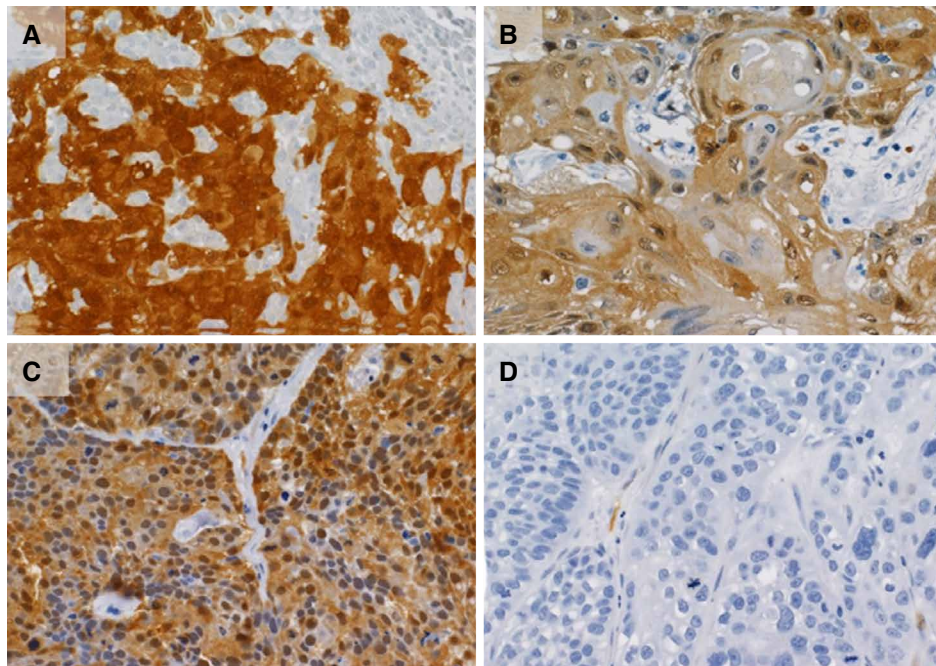
### Survival analysis

Median overall survival (OS) was 40 months. Neither smoking nor alcohol consumption was associated with shorter disease-free survival (DFS) (*P* = 0.815 and 0.507) nor OS (*P* = 0.597 and 0.634). LVI (*P* = 0.026) was associated with shorter DFS in univariate analysis. Larger cT (*P* = 0.019), presence of PNI (*P* = 0.039), LVI (*P* = 0.021), and neck dissection (*P* = 0.010) were associated with shorter OS in univariate analysis [Figure 2]. Neck involvement had a trend both for shorter DFS (31% vs. 48.7%, *P* = 0.278) and shorter OS (25.1% vs. 48.5%, *P* = 0.340). There was also a trend to shorter OS (9.3% vs. 52.3%, *P* = 0.064) in the presence of neck recurrence [Table 5].

**Table 3. Relationship between clinicopathological features and neck recurrence (*n* = 78)**

	Neck recurrence (%)		<i>P</i>
	No ( <i>n</i> = 60)	Yes ( <i>n</i> = 18)	
Age (years), mean (range)	64.8 (21-89)	63.4 (31-80)	0.725
cT			
T1-T2-T3	19 (31.7)	5 (27.8)	0.754
T4	41 (68.3)	13 (72.2)	
N			
N(-)	44 (73.3)	15 (83.3)	0.536
N(+)	16 (26.7)	3 (16.7)	
Location			
Upper alveolus	55 (91.7)	18 (100.0)	-
Hard palate	5 (8.3)	0 (0.0)	
Grade			
Poor/moderate differentiation	30 (50.0)	4 (22.2)	0.037
Well differentiated	30 (50.0)	14 (77.8)	
PNI			
No	24 (61.5)	7 (53.8)	0.624
Yes	15 (38.5)	6 (46.2)	
LVI			
No	30 (75.0)	10 (76.9)	1.000
Yes	10 (25.0)	3 (23.1)	
Tobacco			
No	52 (86.7)	18 (100.0)	-
Yes	8 (13.3)	0 (0.0)	
Alcohol			
No	56 (93.3)	17 (94.4)	1.000
Yes	4 (6.7)	1 (5.6)	
P16			
Negative	25 (89.3)	13 (100.0)	-
Positive	3 (10.7)	0 (0.0)	

PNI: perineural invasion; LVI: lymphovascular infiltration

**Figure 1.** P16 by immunohistochemistry in upper maxilla. (A, B, C) Positive status of p16 staining indicated by brown staining of nuclear and cytoplasmic membrane in three cases; (D) negative status for p16 staining. (Magnification 40x)

**Table 4. Relationship between p16 staining and clinicopathological features (n = 41)**

	p16 staining (%)		P
	Negative (n = 38)	Positive (n = 3)	
Age (years), mean (range)	63.71 (21-89)	48.67 (44-55)	0.067
cT			
T1-3	10 (26.3)	1 (33.3)	1.000
T4	28 (73.7)	2 (66.7)	
cN			
N (-)	30 (78.9)	3 (100.0)	-
N (+)	8 (21.1)	0 (0.0)	
Location			
Upper alveolus	37 (97.4)	2 (66.7)	0.143
Hard palate	1 (2.6)	1 (33.3)	
Grade			
Poor/moderate differentiation	15 (39.5)	2 (66.7)	0.560
Well differentiated	23 (60.5)	1 (33.3)	
PNI			
No	12 (40.0)	1 (50.0)	1.000
Yes	18 (60.0)	1 (50.0)	
LVI			
No	22 (73.3)	2 (100.0)	-
Yes	8 (26.7)	0 (0.0)	
Tobacco			
No	35 (92.1)	2 (66.7)	0.271
Yes	3 (7.9)	1 (33.3)	
Alcohol			
No	35 (92.1)	3 (100.0)	-
Yes	3 (7.9)	0 (0.0)	

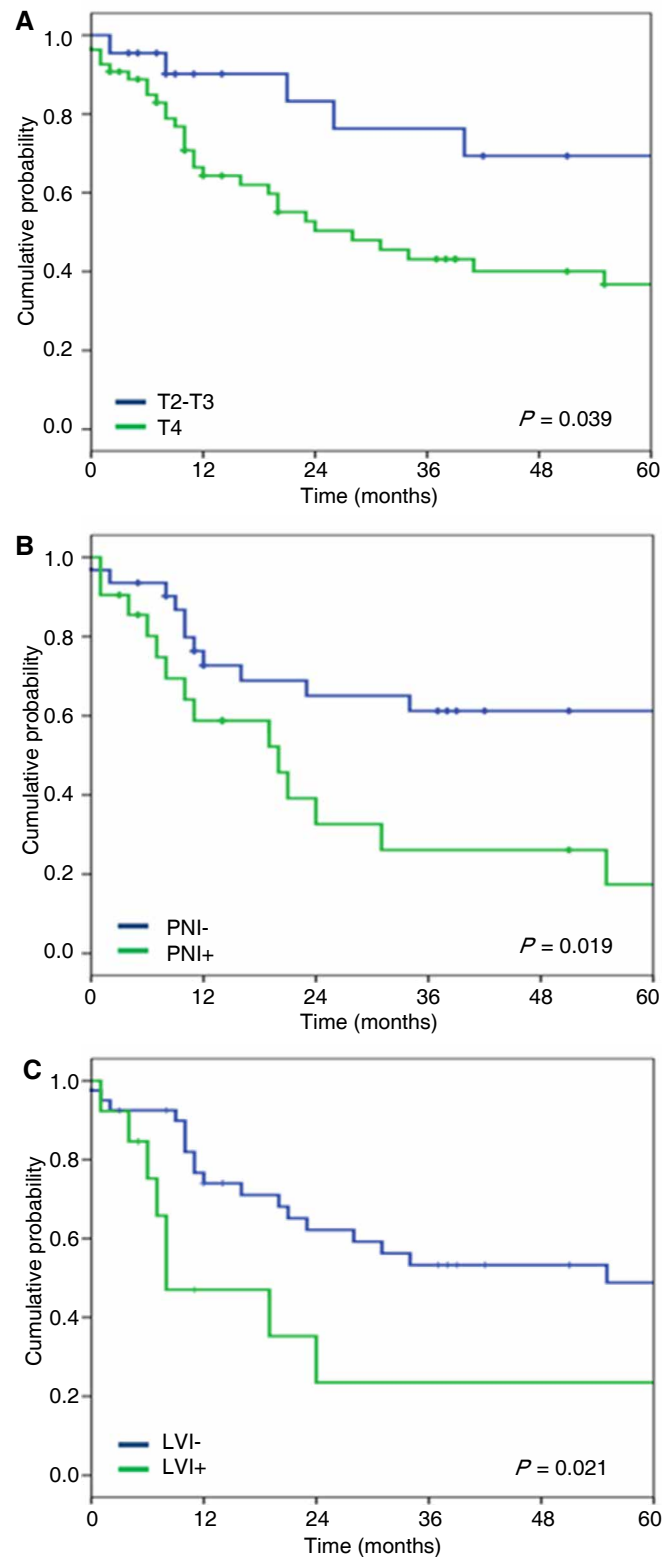
PNI: perineural invasion; LVI: lymphovascular infiltration

## DISCUSSION

Our rate of survival at 5 years was 44.5% and is similar to those reported by other studies (21% to 76%)<sup>[18,19]</sup>. This poor prognosis could reflect higher prevalence of neck node involvement at diagnosis or higher prevalence of poor prognostic factors like p16-negative status<sup>[1]</sup>.

The incidence of neck metastasis has been extensively described in cancer of the tongue and floor of the mouth (20% to 30%) and has been assigned a significant prognostic role in patients with clinically node-negative disease<sup>[20,21]</sup>. Clinicopathologic factors like large tumor size, tumor depth, higher grade, and microvascular invasion have been associated with the development of cervical lymph node metastasis in oral SCC<sup>[21]</sup>. Elective treatment of the neck with staging neck dissection is generally carried out in patients with SCC of the oral cavity when the risk of clinically occult metastases exceeds 15% to 20%, and treatment of the clinically N0 neck is now accepted for certain oral cavity subsites, such as the tongue and floor of mouth, where elective neck dissection produces a survival advantage<sup>[22-26]</sup>.

The understanding of the behavior of hard palate and upper alveolar cancers is poor due to their low incidence and because some of these studies indistinctly included different both other head and neck malignancy locations and special pathological entities like salivary gland tumors<sup>[27,28]</sup>. A series of 606 upper and lower alveolar SCCs reported 37% of cervical metastasis and 19% of harbored occult disease in elective neck dissections. Lymph node involvement at level II to V carried shorter survival than negative lymph node involvement<sup>[19]</sup>. A series of 347 cancers of the upper and lower gums that had an elective neck dissection rate of 58% found occult disease in 5.6%. Neck recurrence was found in 9% of the whole group. Ipsilateral and contralateral neck node involvement predicted cervical recurrence. Positive neck lymph nodes, tumor stage, and involved soft-tissue margins were significant covariates in survival prediction; clinical stage remained significant in multivariate analysis<sup>[29]</sup>. A series of 252 cases of palate SCC including 62 in the specific region



**Figure 2.** Estimated curves of OS regarding clinical tumor (A), perineural infiltration (B) and lymphovascular infiltration (C). OS: overall survival; PNI: perineural invasion; LVI: lymphovascular infiltration

of the hard palate found node involvement in more than 29% of the hard palate tumors. Neck recurrence was predicted by the presence of fixed or contralateral node metastases, but not by the presence of nodal metastasis itself. Size of the primary tumor and histological grade was significantly associated with survival,



**Table 5. Survival analysis**

	5-year-OS	P	5-year-DFS	P
All group	43.9%		44.5%	
Age				
≤ 60 years	56.1%		41.8%	
> 60 years	38.9%	0.667	45.8%	0.643
Gender				
Female	53.4%		51.0%	
Male	31.2%	0.539	37.8%	0.323
Tobacco				
Yes	21.9%		52.5	
No	46.2%	0.597	43.7	0.815
Alcohol				
Yes	0.0%*		40.0%**	
No	46.6%	0.634	44.8%	0.507
Location				
Hard palate	66.7%		53.3%	
Upper alveolus	42.7%	0.707	43.6%	0.851
cT				
T2-T3	69.3%		44.2%	
T4	36.7%	0.019	44.0%	0.743
Grade				
Poor/moderate differentiation	47.0%		51.7%	
Well differentiated	42.1%	0.715	39.9%	0.289
PNI				
No	61.2%		52.4%	
Yes	26.1%	0.039	36.3%	0.334
LVI				
No	48.8%		50.9%	
Yes	23.5%	0.021	24.7%	0.026
P16				
Negative	27.9%		35.0%	
Positive	100.0%	-	100.0%	-
Neck dissection				
No	53.4%		50.1%	
Yes	18.8%	0.010	29.6%	0.129
pN				
N-	48.5%		48.7%	
N+	25.1%	0.340	31.0%	0.278
Neck recurrence				
No	52.3%			
Yes	9.3%	0.064		
N+ and N- with neck recurrence				
No	55.5%			
Yes	21.6%	0.192		

\*Estimated at 41 months; \*\*estimated at 9 months. PNI: perineural invasion; LVI: lymphovascular infiltration

and clinical stage was the most important prognostic indicator<sup>[30]</sup>. Recent retrospective series with 26 to 146 upper alveolar ridge and hard palate cases reported a neck lymph node involvement between 11% and 36.6%, and regional recurrence in N0 neck from 14% to 27%<sup>[1,5-8]</sup>. These studies had several findings: cases with neck node involvement had higher grade; clinical stage but not margin status was associated with prognosis; and T3 (55%) and T4 (52%) tumors exhibited higher rates of neck lymph node involvement than smaller tumors (T1 = 15%; T2 = 28%). An analysis of the Surveillance, Epidemiology, and End Results (SEER) database evaluated 314 hard palate SCC and 411 upper alveolar ridge cases. They found a 13.65% prevalence of cervical metastasis and its correlation with larger tumor (4.1% for T1 to 24.7% for T4 tumors,  $P < 0.001$ ). Extension of lymph node involvement was correlated to survival ( $P < 0.001$ ).

We found a neck lymph node involvement rate of 24.4% and it has a trend associated with shorter survival ( $P = 0.340$ ). The traditional concept has been that SCC of the hard palate and maxillary alveolus exhibits a low rate of occult metastasis<sup>[7,31,32]</sup>. However, our results suggest that regional lymph node involvement is also frequent and relevant, and an elective treatment of the neck should be performed.

Regional recurrence rates in oral cancer have been described as between 30% and 47% in T1-2 carcinoma with untreated N0 neck, and they produce a significant decrease in patient survival. Some studies, including two prospective randomized trials, describe that neck recurrence rates decrease with the use of elective neck dissection<sup>[22,33-35]</sup>. Regional recurrences in oral malignancies were associated with poor differentiation, larger tumor size, positive lymph node, and extracapsular involvement<sup>[33,35]</sup>. A series of 114 cases with SCC of the maxillary alveolus and hard palate report regional recurrence rates of 26% in the N0 cohort ( $n = 100$ ), and 35% of the patients had either initially N-positive neck or a later conversion from N0 to N-positive neck. Neck recurrence was associated with diminished overall survival but not with larger tumor size or postoperative radiation to the neck. Patients with initial diagnoses of N-positive and those who later developed neck recurrences had similar OS<sup>[36]</sup>.

Neck node recurrences occurred in 18 cases (23.1%) of our series and appeared at a mean time of 8.6 months; this likely represents occult metastases at presentation. Therefore, we had 42.3% of neck node involvement if we consider initial patients with positive nodes and N0 patients who developed neck recurrence. We also found that 25.9% of cases without clinical evidence of neck involvement developed recurrences at the neck. Neck recurrence had a trend to poor prognosis but did not achieve significance, probably because these cases received effective treatment including surgery (44.4%) or chemoradiation (11.1%).

Large tumor size, PNI, and LVI have been extensively associated with nodal metastasis and with shorter survival in HNSCC<sup>[37,38]</sup>. Evaluation of classical prognostic factors in our series confirmed that larger tumors ( $P = 0.019$ ), presence of PNI ( $P = 0.039$ ), and LVI ( $P = 0.021$ ) were associated with shorter OS, and LVI ( $P = 0.026$ ) was associated with shorter DFS.

HNSCCs associated with smoking or drinking alcohol has been associated with a poor prognosis and are frequently located in laryngeal and hypopharyngeal cancer, respectively<sup>[39]</sup>. Our analysis did not indicate higher prevalence rates of these carcinogen agents and did not find an association with prognosis in the upper maxilla.

Expression of p16 is a confident biomarker of HPV infection in OPSCC and both are associated with better outcome<sup>[12,13,40-44]</sup>. In contrast to OPSCC, the rates of positive HPV in oral cancer are low, and recent studies suggest a disparity between the detection of HPV DNA and p16 expression when the prevalence of HPV is low<sup>[45]</sup>. Evaluation of p16 staining in our series found that only 3 (7.4%) of upper maxilla SCC cases were considered positive for p16 staining. The p16-positive cases had a trend to be younger (48.7 vs. 63.7 years,  $P = 0.067$ ), and all 3 cases were free of neck recurrence and alive at 5 years. This is the first time to our knowledge that p16 staining has been evaluated in upper maxilla SCC and could identify a group of patients with specific behavior. However, our analysis has the weakness of its small sample size and it needs to be confirmed by larger series (required size of series increases because of the low rates of p16-positive status in non-OPSCC).

The results of this retrospective analysis reveal that tumors of the hard palate and upper alveolus are associated with a high rate of neck node involvement and regional failure, which had a tendency to result in poor survival. Expression of p16 has a low rate in this pathology and could be associated with specific features.

## DECLARATIONS

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### Authors' contributions

Conception and design of the study and performed data analysis and interpretation: Salas E, Castaneda CA, Sanchez P, Postigo J

Performed data acquisition, as well as providing administrative, technical, and material support: Castillo M, Villegas V, Postigo J, Cano L, Casavilca S, Bernabe LA, Villa-Robles MR, Mantilla R, Belmar C, Guerra H

Drafted the article and made critical revisions related to the intellectual content of the manuscript, and approved the final version of the article to be published: all authors

### Data source and availability

No additional data are available.

### Financial support and sponsorship

None.

### Conflicts of interest

There are no conflicts of interest.

### Patient consent

Not applicable.

### Ethics approval

The institutional review board approval was obtained from The Instituto Nacional de Enfermedades Neoplasias (Lima, Peru).

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Meta-Analysis

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# The potential prognostic and predictive roles of programmed cell death protein 1 expressed by tumor-infiltrating lymphocytes in solid tumors: a meta-analysis

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## Abstract

**Aim:** Several previous studies have evaluated the potential role of programmed cell death protein 1 (PD-1) expressed by tumor-infiltrating lymphocytes (TILs) in various solid tumors and performed its prognosis role in patients' survival with inconsistent results. This study aims to further systematically evaluate the association of PD-1 by TILs with clinicopathological parameters and clinical outcomes in solid tumor patients.

**Methods:** A comprehensive search was conducted in PubMed, Embase, Web of Science, CNKI and Wanfang databases for relevant studies. The potential prognostic and predictive roles of PD-1 were assessed by pooled hazard ratio (HR), odds ratio (OR) and 95% confidence intervals (CI). A total of 1863 patients were selected for in-depth analysis.

**Results:** The results demonstrated that PD-1 by TILs was correlated to overall survival for ovarian cancer (HR = 0.40, 95% CI: 0.26-0.61,  $P < 0.00001$ ). Higher PD-1 expression was associated with lymph node metastasis (OR = 2.55, 95% CI: 1.22-5.29,  $P = 0.01$ ) and tumor grade (OR = 3.08, 95% CI: 2.07-4.57,  $P < 0.00001$ ).

**Conclusion:** The prognostic role of PD-1 by TILs is variant in different tumor types, which highlights the role of PD-1 by TILs as a potential predictive and prognostic biomarker and the development of strategies against the PD-L1/PD-1 axis would be a promising therapeutic target for some solid tumors.



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**Keywords:** Programmed cell death protein 1, meta-analysis, solid tumors, prognosis

## INTRODUCTION

Programmed cell death protein 1 (PD-1), a member of the CD28 receptor family, is expressed by activated lymphocytes and inhibits their proliferation functions after binding to PD-1 ligands such as PD-L1<sup>[1]</sup>. The interactions with PD-1/PD-L1 signaling has been shown to improve clinical outcome and restore functional T-cell responses in several cancers<sup>[2]</sup>.

Although PD-1 has generated increasing interest as a target for immune modulation in cancers, the prognostic values of PD-1 expressed by tumor-infiltrating lymphocytes (TILs) in solid tumors were still unclear<sup>[3]</sup>. Several previous studies have reported the PD-1 by TILs is more than a predictive biomarker but as a worse prognosis marker in multiple solid tumors such as gastric cancer<sup>[4]</sup>, non-small cell lung cancer (NSCLC)<sup>[5]</sup>, renal cell cancer<sup>[6]</sup> and nasopharyngeal cancer<sup>[7]</sup>. Another studies showed that PD-1 expression is associated with favorable survival in breast cancer<sup>[8]</sup>, glioblastoma<sup>[9]</sup>, metastatic melanoma<sup>[10]</sup>, ovarian cancer<sup>[11]</sup> and primary human papillomavirus-positive head and neck cancers<sup>[12]</sup>. Furthermore, one study displayed that the positive expression of PD-1 expression is not correlated with overall survival (OS) for esophageal squamous cell carcinoma (ESCC)<sup>[13]</sup>. The different of tissue samples, detection methods and evaluation criterions might be partly responsible for the inconsistent results.

And with the development of PD-L1/PD-1 targeted therapy, some predictive and prognostic biomarkers are crucial to be identified for the option of individualized anti-PD-1 targeted treatment<sup>[14]</sup>. Therefore, we conducted this meta-analysis to comprehensively evaluate the prognostic value of PD-1 by TILs in solid tumors, which will further facilitate the development of PD-L1/PD-1 immune check-point targeted therapy and identify novel strategies targeting PD-1.

## METHODS

### Publication searching

The eligible studies published in PubMed, Embase, Web of Science, CNKI and Wanfang databases were searched using the following keywords: “programmed cell death 1 receptor” or “PD-1” or “programmed death 1” or “CD279 antigen” and “cancer” or “tumor” or “neoplasm” or “carcinoma” and “prognosis” or “outcome” or “survival”. In addition, we also manually screened the reference lists derived from randomized controlled trials and systematic review to avoid omitting related publications. The search language was limited to English and Chinese.

### Inclusion and exclusion criteria

Inclusion criteria for this meta-analysis are: (1) full text available; (2) study focus on the association of PD-1 with clinicopathological parameters and OS; (3) cohort study, cross-sectional study or case-control study; (4) sufficient data or higher dots per inch of K-M survival curves. In addition, the exclusion criteria are as follows: (1) cell or animal studies; (2) case reports or review; (3) conference abstracts or comments; (4) repeated articles.

### Data extraction and quality assessment

Two investigators (Liu RZ and Ku JW) independently extracted the data from the relevant studies. The disagreements were resolved by consensus. The extracted data are as follows: first author name, publication year, patient source, cancer type, number of patients, detection method, clinicopathological parameters, effect size, hazard ratio (HR) and 95% confidence intervals (CI). The quality of eligible studies were assessed through the Newcastle-Ottawa scale (NOS) method<sup>[15]</sup>. Study with NOS scores above to 6 point were usually considered to be higher quality.

**Table 1. Features of included studies**

Authors	Year	Country	Cancer type	No. of patients	PD-1(+) patients	Clinicopathological parameters	Effect size	HR, 95% CI	NOS score
Badoual <i>et al.</i> <sup>[12]</sup>	2013	France	HNSCC	64	31/33(++/+)	NR	OS	Yes	7
Feng <i>et al.</i> <sup>[13]</sup>	2016	China	ESCC	88	45	B, C, D, E, G	OS	Yes	6
Zheng <i>et al.</i> <sup>[22]</sup>	2016	China	NSCLC	42	15/27(++/+)	B, H, I	OS	NR	7
Shen <i>et al.</i> <sup>[23]</sup>	2017	China	Pancreatic cancer	94	47/47(++/+)	A, B, C, D, E, G	OS	Yes	7
Harter <i>et al.</i> <sup>[24]</sup>	2015	Germany	NSCLC	62	18/44(++/+)	NR	OS	NR	6
Webb <i>et al.</i> <sup>[25]</sup>	2015	Canada	Ovarian cancer	195	75	NR	OS	Yes	6
Duchnowska <i>et al.</i> <sup>[27]</sup>	2016	Poland	Breast cancer	84	17	NR	OS	Yes	6
Chen <i>et al.</i> <sup>[28]</sup>	2016	China	ESCC	349	117	A, B, C, D, E, F	OS	Yes	7
Muenst <i>et al.</i> <sup>[29]</sup>	2013	USA	Breast cancer	660	104	C, D, E, G	OS	Yes	6
Sun <i>et al.</i> <sup>[30]</sup>	2015	China	ESCC	225	69	A, B, C, D	OS	Yes	6

HNSCC: head and neck squamous cell carcinoma; NSCLC: non-small cell lung cancer; ESCC: esophageal squamous cell carcinoma; NR: not reported; A: age; B: gender; C: tumor invasion depth; D: lymph node metastasis; E: tumor stage; F: tumor location; G: tumor grade; H: histology type; I: treatment method; OS: overall survival; HR: hazard ratios; CI: confidence interval; NOS: Newcastle-Ottawa scale

## Statistical analysis

All statistical analysis were conducted using the RevMan5.2 and STATA version 12.0 (STATA Corporation, College Station, TX, USA). HR and 95% CI were combined to assess the survival impact of PD-1 in solid tumors. For studies that offered only Kaplan-Meier curves, Engauge Digitizer (version 4.1) was performed to extract the survival data and calculate the estimated HRs and 95% CIs according to Tierney's method<sup>[16]</sup>. Additionally, pooled odds ratio (OR) and 95% CI were used to determine the association of PD-1 and clinicopathological features.

Heterogeneity is assessed using Cochrane's *Q* test and *I*<sup>2</sup> measurement (no heterogeneity, *I*<sup>2</sup> = 0%-25%; low heterogeneity, 25%-50%; moderate heterogeneity, 50%-75%; high heterogeneity, 75%-100%)<sup>[17]</sup>. *P* < 0.1 or *I*<sup>2</sup> > 50% indicate a significant heterogeneity. Random effects model was initially applied to combine the estimates of effect<sup>[18]</sup>. Otherwise, a fixed effects model was utilized<sup>[19]</sup>. Sensitivity analysis was used to illustrate any significant heterogeneity among studies. Begg's<sup>[20]</sup> and Egger's test<sup>[21]</sup> were deemed to explain publication bias with *P* value of less than 0.05.

## RESULTS

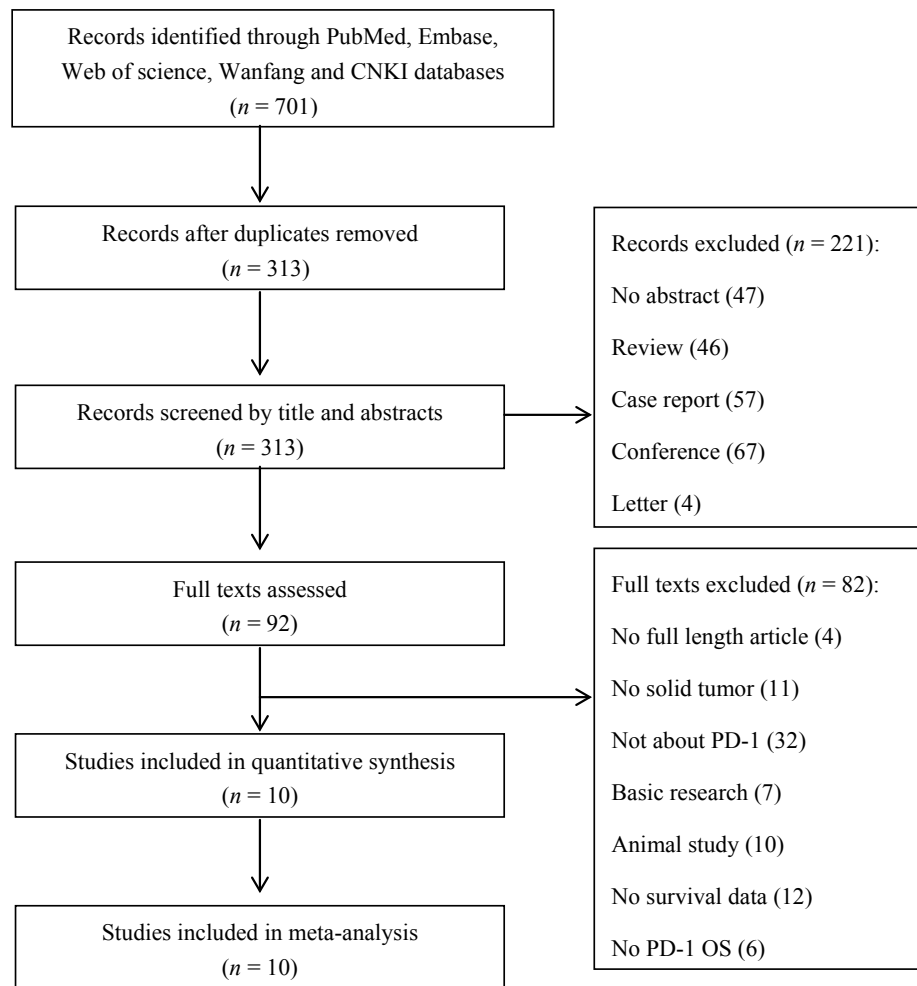
### Characteristics of included studies

A total of 701 studies were identified by electronic search and 388 studies were excluded because of duplication. After reading the titles and abstracts, 221 studies were excluded and 92 possible full text studies were carefully reviewed. Finally, 10 manuscripts containing 12 retrospective cohort studies were included for quantitative analysis in the meta-analysis [Figure 1]. The patients were diagnosed with various solid cancers including: ESCC, NSCLC, hepatocellular carcinoma, pancreatic cancer, breast cancer and ovarian cancer. The features of included studies were presented in Table 1.

To detect the expression of PD-1 by TILs, all studies used immunohistochemistry, except for 2 studies<sup>[22,23]</sup>, which used quantitative immunofluorescence, but the proportion of PD-1 expression was consistent with the others in that study. The detailed methodologies used to detect PD-1 are summarized in Table 2. Furthermore, 2 cohorts of patients were reported by Harter *et al.*<sup>[24]</sup> and Webb *et al.*<sup>[25]</sup>, respectively. PD-1 by TILs was assessed and the survival curves were reported independently, so they have been statistically analyzed as 4 individual studies.

### PD-1 by TILs and overall analysis

A total of 12 studies with 1863 patients were enrolled in survival analysis. Seven studies with data on PD-1 positive expression and OS in solid tumors. There are 2 studies provided OS for breast cancer (2 cohort



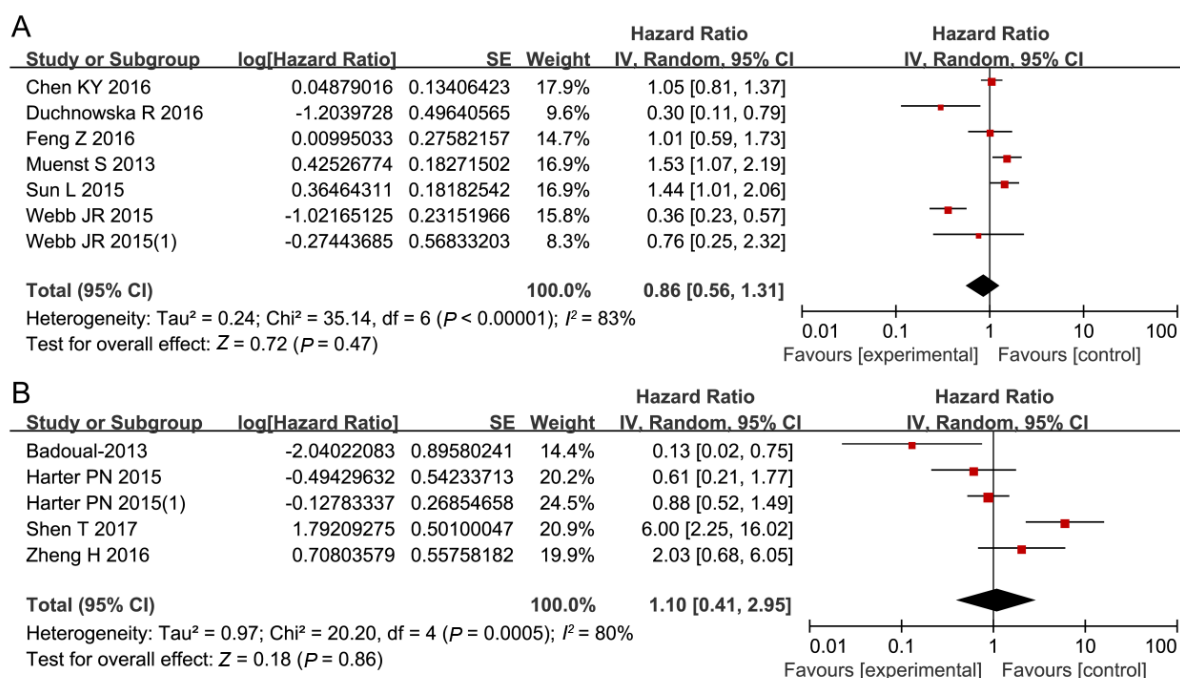
**Figure 1.** Flow diagram for selection of studies. PD-1: programmed cell death protein 1; OS: overall survival

**Table 2. Evaluation of human PD-1 by immunohistochemistry**

Authors	Detection method	Antibody clone	Antibody dilution	Antibody source	Cutoff value
Badoual <i>et al.</i> <sup>[12]</sup>	IHC	CT-011	1:100	CureTech LTD	NR
Feng <i>et al.</i> <sup>[13]</sup>	IHC	NR	NR	NR	NR
Zheng <i>et al.</i> <sup>[22]</sup>	IFC	NR	NR	BioLegend	> 12.27% of cells
Shen <i>et al.</i> <sup>[23]</sup>	IFC	AB52587	1:200	Abcam	NR
Harter <i>et al.</i> <sup>[24]</sup>	IHC	NAT-105	1:50	Abcam	Total score > 1 <sup>a</sup>
Webb <i>et al.</i> <sup>[25]</sup>	IHC	NAT-105	1:200	Biocare Medical	NR
Duchnowska <i>et al.</i> <sup>[27]</sup>	IHC	NBP1-88104	1:100	Novus	Total score > 1 <sup>b</sup>
Chen <i>et al.</i> <sup>[28]</sup>	IHC	NAT105	1:100	Abcam	Total score > 1 <sup>b</sup>
Muenst <i>et al.</i> <sup>[29]</sup>	IHC	MRQ-22	1:50	Rocklin	NR
Sun <i>et al.</i> <sup>[30]</sup>	IHC	MRQ-22	1:100	Abcam	Total score > 1 <sup>b</sup>

<sup>a</sup>All samples were scored according to the frequency of positive cells related to all cells (as percentage) on the stained TMA core: frequency 0-1% score 0; 1%-10% score 1; 10%-25% score 2; 25%-50% score 3; > 50% score 4; additionally we multiplied the frequency score with the intensity of staining (1 weak staining, 2 moderate staining, 3 strong staining). <sup>b</sup>Total score was calculated by adding a score of staining percentage to another score of staining intensity. The area of staining was scored as 0 (no tumor cells stained), 1 (< 25% of cells stained), 2 (≥ 25% of cells stained). Staining intensity was graded as 0 (no staining), 1 (weak staining), 2 (moderate staining), 3 (strong staining). PD-1: programmed cell death protein 1; IHC: immunohistochemistry; IFC: immunofluorescence; NR: not reported

studies in the same one paper), 3 studies for ESCC and 2 studies for ovarian cancer. A random effect model was used to calculate the pooled HR and 95% CI due to the high heterogeneity ( $P < 0.0001$ ,  $I^2 = 83\%$ ). The results showed that PD-1 expression was not associated with patients OS (HR = 0.86, 95% CI: 0.56-1.31,  $P = 0.47$ )



**Figure 2.** Forest plots of PD-1 expression and OS in solid tumor patients. The squares and horizontal lines correspond to the study-specific HR and 95% CI. The area of the square reflects the study-specific weight. The diamonds represents the pooled OR and 95% CI. The solid vertical line is at the null value (HR = 1). The associations between positive or negative expression of PD-1 (A) and strong or moderate positive expression of PD-1 (B) with OS are shown. PD-1: programmed cell death protein 1; OS: overall survival; HR: hazard ratio; OR: odds ratio; CI: confidence interval

[Figure 2A]. Another 5 studies provided data on PD-1 high or low expression and OS. There are 2 studies provided OS for NSCLC, 1 study for head and neck squamous cell carcinoma, 1 study for pancreatic cancer and 1 study for melanoma. The pooled HR was 1.10 (95% CI: 0.41-2.95,  $P = 0.65$ ) in solid tumor patients with high heterogeneity ( $I^2 = 80\%$ ,  $P = 0.0005$ ) [Figure 2B].

### PD-1 by TILs and subgroup analysis

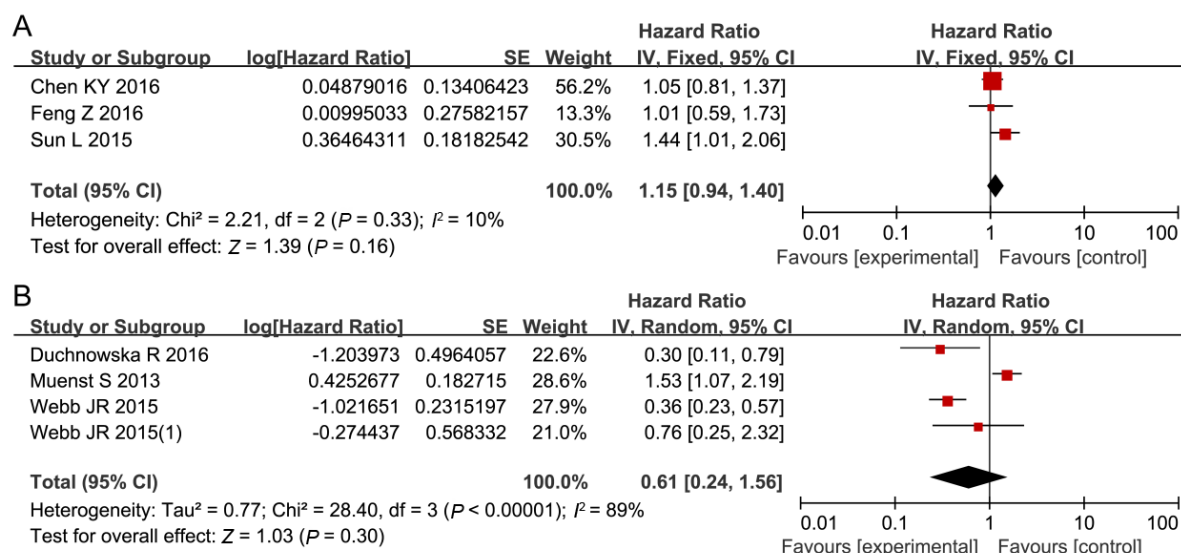
We also conducted subgroup meta-analysis to explore the possible source of heterogeneity. In the subgroup analysis stratified by patients source, pooled HR estimate for OS was 1.15 (95% CI: 0.94-1.40,  $P = 0.16$ ) for Asian patients with low heterogeneity ( $I^2 = 10\%$ ,  $P = 0.33$ ) [Figure 3A], and 0.61 (95% CI: 0.24-1.56,  $P = 0.30$ ) for non-Asian patients with high heterogeneity ( $I^2 = 89\%$ ,  $P < 0.0001$ ) [Figure 3B]. In the stratified analysis by cancer type, there are 2 studies provided OS for breast cancer, 3 studies for ESCC and 2 studies for ovarian cancer. There was no significant association between PD-1 expression and patients OS of breast cancer (HR = 0.72, 95% CI: 0.15-3.55,  $P = 0.69$ ) [Figure 4A] and ESCC (HR = 1.15, 95% CI: 0.94-1.40,  $P = 0.16$ ) [Figure 4B]. With no significant heterogeneity ( $P = 0.22$ ,  $I^2 = 33\%$ ), a fixed-effects model was conducted to evaluate their relationship for ovarian cancer. The results found that PD-1 expression was statistically significantly associated with patients OS (HR = 0.40, 95% CI: 0.26-0.61,  $P < 0.00001$ ) [Figure 4C].

### PD-1 by TILs and clinicopathological parameters

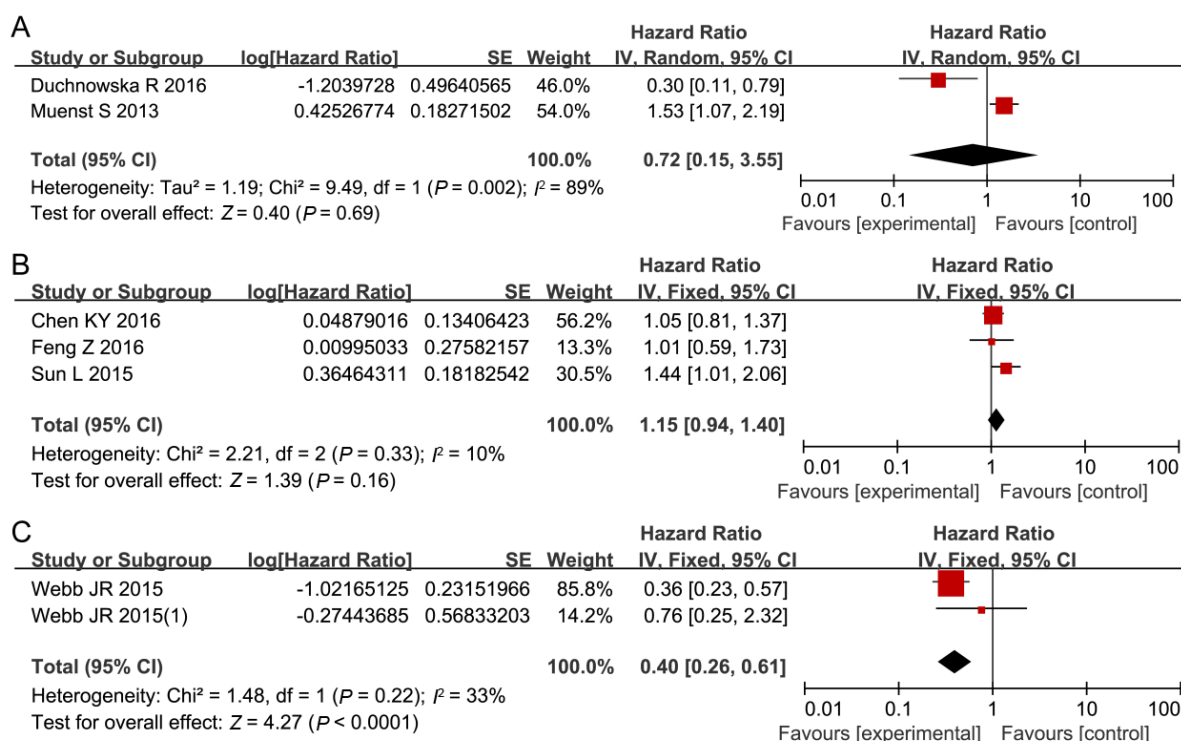
The average positive expression rates of PD-1 by TILs were 31.35% in all of the studies. There were the higher PD-1 overexpression in NSCLC, ESCC and pancreatic cancer, with accounting for 35.71%, 61.23% and 50.01%, respectively. And PD-1 expression levels in melanoma, breast cancer and ovarian cancer ranged from 8.59% to 22.97%.

Four studies including 1209 tissue samples investigated the association of PD-1 overexpression with status of lymph node. With significant heterogeneity ( $P = 0.0008$ ,  $I^2 = 82\%$ ), a random-effects model showed a



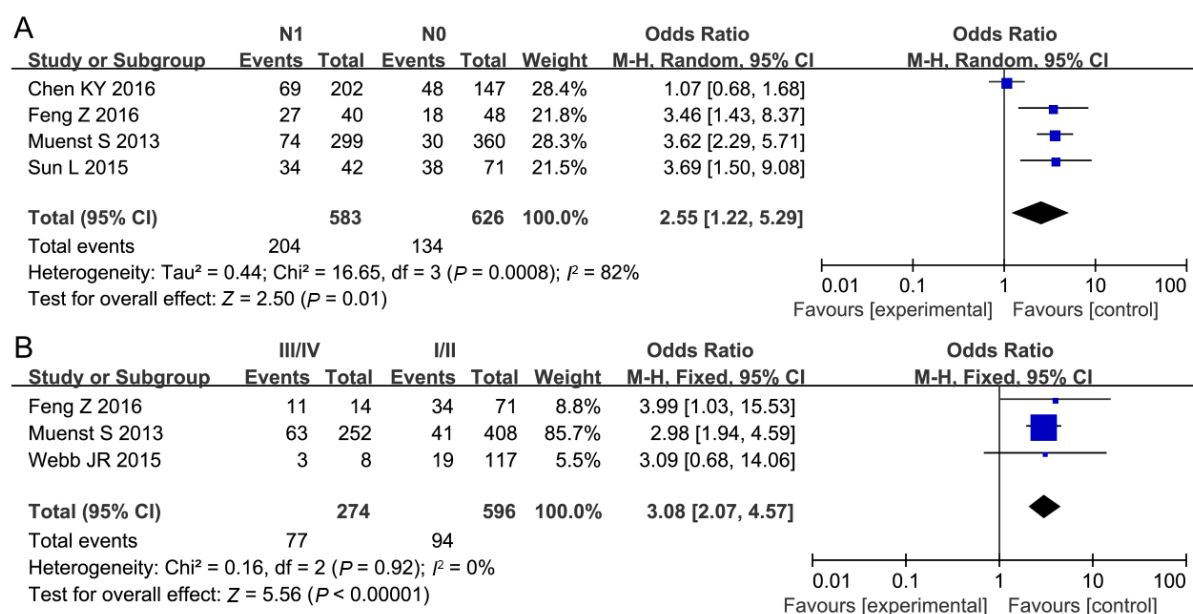


**Figure 3.** Forest plots for subgroup meta-analysis by patient source. The relationships between PD-1 overexpression and OS in Asia patients (A) and in non-Asia patients (B) are shown. PD-1: programmed cell death protein 1; OS: overall survival; CI: confidence interval



**Figure 4.** Forest plots for subgroup meta-analysis stratified by cancer type. The relationships between PD-1 expression and OS in breast cancer (A), ESCC (B) and ovarian cancer (C) are shown. PD-1: programmed cell death protein 1; OS: overall survival; ESCC: esophageal squamous cell carcinoma; CI: confidence interval

significant difference between lymph node metastasis group (35.0%) and lymph node non-metastasis group (21.4%) ( $OR = 2.55$ , 95% CI: 1.22-2.59,  $P = 0.01$ ) [Figure 5A]; 3 studies reported the relationship of PD-1 overexpression with tumor grade. With no significant heterogeneity ( $P = 0.92$ ,  $I^2 = 0\%$ ), a fixed-effects model was used in the study. The results revealed a significant difference between 274 grade 3/4 tissues (28.1%) and



**Figure 5.** Forest plots of PD-1 expression and the clinical pathological parameters of patients with solid tumors. The squares and horizontal lines correspond to the study-specific OR and 95% CI. The area of the square reflects the study-specific weight. The diamonds represents the pooled OR and 95% CI. The solid vertical line is at the null value (OR = 1). The associations of PD-1 expression with lymph node status (A) and tumor grade (B) are shown. PD-1: programmed cell death protein 1; OR: odds ratio; CI: confidence interval

**Table 3. Associations of PD-1 expression and clinical features**

Variables	No. of study	OR ( 95% CI)	Z, P (OR)	Heterogeneity ( $I^2$ , P bias)	Publication bias (Egger test) (t, P)	Pooling model
Age (years): $\leq 65$ vs. $> 65$	2	1.20 (0.48-3.02)	0.39, 0.70	73%, 0.06	-	Random
Gender: male vs. female	3	0.96 (0.78-1.18)	0.36, 0.72	0%, 0.81	0.42, 0.748	Fixed
T: T3/T4 vs. T1/T2	4	1.17 (0.61-2.24)	0.48, 0.64	91%, 0.000	0.57, 0.627	Random
Lymph node metastasis: yes vs. no	4	2.55 (1.22-2.59)	2.05, 0.01*	82%, 0.0008	1.09, 0.389	Random
Tumor grade: 3/4 vs. 1/2	3	3.08 (2.07-4.57)	5.56, $< 0.0001$ *	0%, 0.92	0.12, 0.923	Fixed
TNM stage: III/IV vs. I/II	4	1.04 (0.71-1.54)	0.21, 0.84	0%, 0.93	3.38, 0.077	Fixed

\*Statistical significance. PD-1: programmed cell death protein 1; OR: odds ratio; CI: confidence interval

596 grade 1/2 tissues (15.8%) (OR = 3.08, 95% CI: 2.07-4.57,  $P < 0.00001$ ) [Figure 5B]. We did not find the significant association of PD-1 with age, TNM stage or tumor invasion depth in solid tumor [Table 3].

### Publication bias

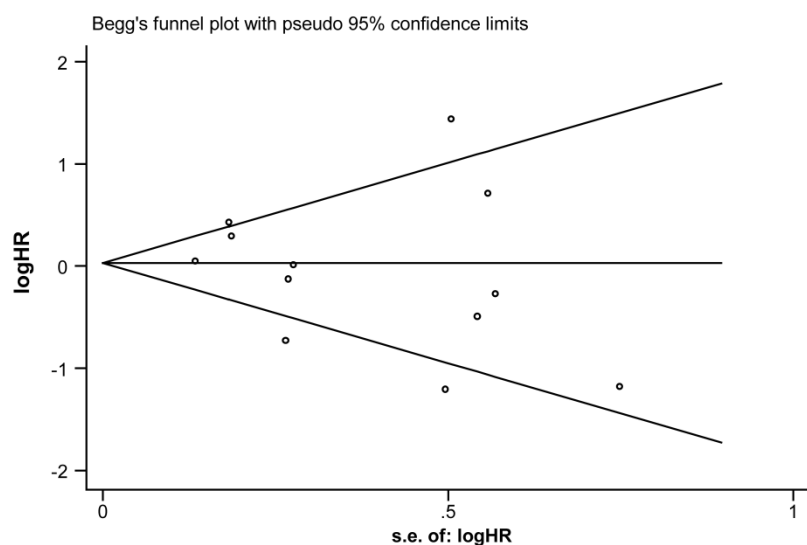
Begg's and Egger's test were applied to evaluate the publication bias of the included studies. No obvious asymmetry was presented through the visual assessment of the Begg's funnel plots [Figure 6]. Furthermore, the formal evaluation of Egger's test also failed to find the significant bias ( $P = 0.723$ ).

### Sensitivity analysis

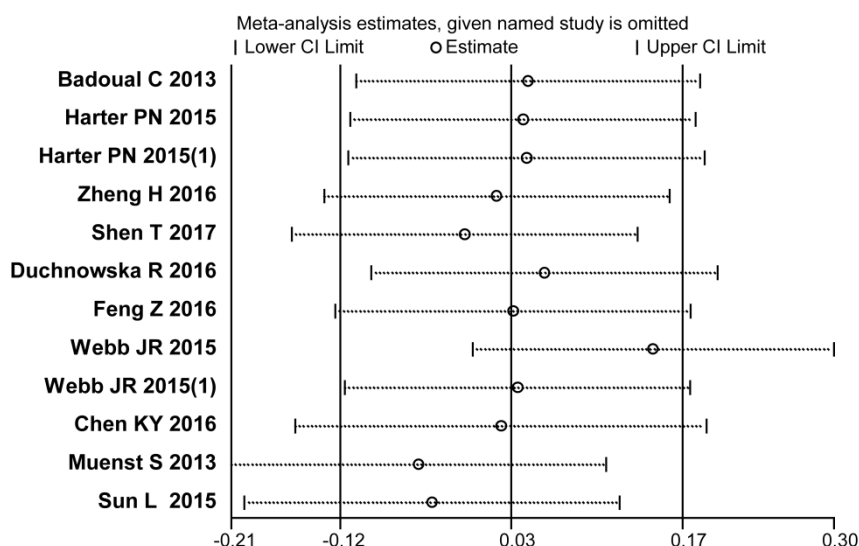
Sensitivity analysis was conducted to justify the influence of individual study on the synthetic results of OS. The pooled HR was not significantly influenced after omitting any singly study for the effect of PD-1 expression on OS in our study [Figure 7].

## DISCUSSION

PD-1, as one of the co-inhibitory receptors, plays an important role in cancer immunity equilibrium and immunity escape stages<sup>[26]</sup>. In the present study, we comprehensively assessed the association of PD-1



**Figure 6.** Begg's funnel plot for publication bias analysis. HR: hazard ratio



**Figure 7.** Sensitivity analysis of the meta-analysis. CI: confidence interval

expressed by TILs with OS in solid tumor and revealed that the prognostic role of PD-1 by TILs is variant in different solid tumor types. This study included 10 eligible publications with 12 cohort studies and a total of 1863 patients. To the best of our knowledge, this is the first systematic assessment of the association of PD-1 by TILs with OS in solid tumor.

With respect to the tumor type, when we performed the subgroup meta-analysis stratified by tumor types, ovarian cancer was correlated with better survival for patients with high PD-1 levels rather than other solid tumor. Although PD-1 by TILs was not associated with OS for all of included studies in the meta-analysis<sup>[12,13,22-30]</sup>. However, the results of studies using different clone to PD-1 antibodies were controversy in breast cancer<sup>[27,29]</sup> [Supplementary Figure 1] in our meta-analysis. One recent study reported the opposite results using variant PD-L1 antibodies in melanoma and lung cancer<sup>[14]</sup>. The difference of antibody clones, limited specificity, or distinct IHC protocols used may be partly explain the contradictory results<sup>[31]</sup>. Further studies are urgent to clarify the impact of antibodies on the results of studies.

Another important finding in the present study is that patients with lymph node metastasis and tumor grade 3/4 have higher PD-1 by TILs than patients with non-lymph node metastasis and 1/2 tumor grade. It is known that tumor grade and lymph node metastasis are usually major barriers to cancer treatment. And patients developed lymph node metastasis and tumor grade 3/4 have lower survival rates. To a certain extent, PD-1 by TILs may be contributed to the immunosuppression to aggravate the tumor growth and carcinogenesis, and further negatively affecting patients' survival. One study in clinical trials showed that PD-1-positive tumors tend to be more responsive to anti-PD-1 or anti-PD-L1 therapies<sup>[32]</sup>. It is reasonable to suggest that patients with lymph node metastasis and tumor grade 3/4 seem to be more sensitive to anti-PD-1 or anti-PD-L1 antibodies-based therapies.

Besides, PD-L1 expression state is another key point of PD-1/PD-L1-mediated tumor immune escape. In tumor tissues, PD-1 was mainly expressed by TILs, and PD-L1 was detected by both tumor cells and TILs<sup>[33]</sup>. PD-1 by TILs was significantly correlated with PD-L1 expressed by tumor cells<sup>[34,35]</sup>. Furthermore, the findings that PD-L1-positive TILs in cancer provides a suitable microenvironment for the development of tumor growth and treatment resistance, which was known to be mediated by the induction of activated IL-6 signaling<sup>[36,37]</sup>. Although immunotherapy using recombinant antibodies and vaccines, such as the therapies targeting PD-L1/PD-1, have been linked with prognosis and treatment response for a few solid tumors including a number of GI malignancies<sup>[38,39]</sup>, the expression of PD-L1 by CIK cells, TILs, and tumor cells within the tumor microenvironment remains to be elucidated.

Although the quality assessment of included studies is higher, there are still some limitations in the study. First of all, the quality of included studies is with selection bias due to the deletion of some unqualified literatures. Secondly, the screening of language is only English and Chinese and could not represent the whole population. Thirdly, the research objects are mainly cancerous tissues and the potential role of PD-1 in blood specimen remains unclear. Finally, the sample size in some of studies is small and further studies with larger sample size are still needed.

In conclusion, this meta-analysis demonstrates that PD-1 expressed by TILs is associated with lymph node metastasis and tumor grade in solid tumor. And more importantly, the prognostic role of PD-1 is variant in different solid tumors, which assumed that PD-1 by TILs seems to be a potential predictive biomarker and the development of strategies against the PD-L1/PD-1 axis would be a promising therapeutic target for some solid tumors.

## DECLARATIONS

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### Authors' contributions

Conception and design: Zhang DY, Liu RZ, Ku JW, Ma YH, Yi YJ

Manuscript writing: Zhang DY, Liu RZ, Ku JW, Ma YH

Manuscripts review and editing: Zhang DY

### Data source and availability

Data are searched in PubMed, Embase, Web of Science, CNKI and Wanfang databases.

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## Conflicts of interest

All authors declare no conflicts of interest.

## Patient consent

Not applicable.

## Ethics approval

Not applicable.

## Copyright

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Review

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# Prostate cancer cells at a therapeutic gunpoint of the autophagy process

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## Abstract

In a normal prostate, the process of controlling cell death is essential to maintain tissue homeostasis and its inhibition may lead to the development of cancer. Androgen receptor signaling plays pivotal roles in the prostate development and homeostasis as well as in the progression of prostate cancer. The main treatment for prostate cancer is a combination of androgen deprivation therapy (ADT) using anti-androgens and docetaxil administration. However, ADT eventually fails due to a pathological unbalance of cell death processes, in particular apoptosis and autophagy. As a result prostate tumors may re-grow and progress into the castration resistant stage. The role of autophagy in tumorigenesis is complex and it could be a double-edged sword process, as autophagy defects promote cancer progression in association with various dangerous cellular processes, while functional autophagy enables cancer cell survival under stress and likely contributes to the resistance of treatment. Autophagy is often impaired in prostate cancer, due to either activation of the Akt/mTOR pathway, which normally inhibits autophagy, or through allelic loss of Beclin-1 (*BECN1*), an essential autophagy gene. In particular, elucidating the interplay between autophagy and tumor cell metabolism will provide unique opportunities to identify new therapeutic targets and to develop synthetically lethal treatment strategies that preferentially target cancer cells, while sparing normal tissues.

**Keywords:** Prostate cancer, autophagy, androgen deprivation therapy, mTOR, autophagosome, LC3-II, Beclin-1

## PROSTATE CANCER INCIDENCE AND GENETICS

Prostate cancer is a tumor that develops in the prostate, a gland in the male reproductive system. Most prostate cancers are slow growing but there are cases of aggressive forms. Tumor cells may metastasize from the prostate to other parts of the body, particularly the bones and lymph nodes. Prostate cancer may cause pain, difficulty in urinating, problems during sexual intercourse, or erectile dysfunction. In particular,



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**Table 1. Main genes involved in prostate cancer**

Gene	Full name	Function/references
<i>BRCA1</i>	Breast cancer susceptibility protein type 1	DNA repair <sup>[7]</sup>
<i>BRCA2</i>	Breast cancer susceptibility protein type 2	DNA repair <sup>[7]</sup>
<i>HPC1</i>	Hereditary prostate cancer	Prostate cancer susceptibility ribonuclease L <sup>[8]</sup>
<i>VDR</i>	Vitamin D receptor	Inhibition of cell growth, metastasis and angiogenesis; apoptosis modulation and cell differentiation <sup>[8]</sup>
<i>CD82</i>	Cluster of differentiation 82	Metastasis suppressor attenuates the matrix adhesion <sup>[9]</sup>
<i>PTEN</i>	Phosphatase and tensin homolog	Tumor suppressor and cell cycle regulation <sup>[9]</sup>
<i>mTOR</i>	Mammalian target of rapamycin	Key signaling pathway linked to tumorigenesis and resistance to therapy <sup>[10]</sup>
<i>PSA</i>	Prostate specific antigen	Dissolver of cervical mucus, allowing the entry of sperm in the uterus <sup>[11]</sup>
<i>BCL2</i>	B-cell lymphoma 2	Pro-survival protein associated with the development of androgen-independent prostate cancer <sup>[12]</sup>
<i>MKI67</i>	Antigen Ki-67	Nuclear protein involved cell proliferation <sup>[13]</sup>
<i>ERK-5</i>	Mitogen-activated protein kinase 7	Signaling processes of various receptor molecules. In response to extracellular signals, this kinase translocate to the nucleus, where it regulates gene expression and activates different transcription factors <sup>[14]</sup>
<i>SP1</i>	Transcription factor Sp1	Involved in many cellular processes, including cell differentiation, growth, apoptosis, immune responses, DNA damage, and chromatin remodeling <sup>[15]</sup>
<i>TPD52</i>	Tumor protein 52	Unknown <sup>[16]</sup>

prostate cancer tends to develop in men over the age of fifty<sup>[1]</sup>. Rates of detection of prostate cancers vary widely across the world, with South and East Asia detecting less frequently than in Europe and in the United States. Globally, it is the sixth leading cause of cancer-related death in men<sup>[2]</sup>. More than 200,000 new cases are estimated in the United States in 2013, with a mortality rates over per 10 cases. Moreover, there are different ways of classifying patients with prostate cancer: the tumor-node-metastases (TNM) classification of malignant tumors evaluates the extension of the tumor, the involvement of lymph nodes and the metastatic dissemination. The Gleason Grading system is additionally used to evaluate the prognosis of men with prostate cancer. A Gleason score is given to prostate cancer based upon its microscopic appearance: cancers with a higher Gleason score are more aggressive and have a worse prognosis<sup>[3,4]</sup>.

Many factors, including genetics and diet, have been implicated in the development of this cancer. As suggested by association studies, genetic background can contribute to prostate cancer risk with family, race, and specific gene variants. Men who have a first-degree relative (brother or father) with prostate cancer have twice the risk of developing the cancer, and those with two first-degree relatives affected have a fivefold greater risk compared with men with no family history<sup>[5]</sup>. Studies of twins in Scandinavia suggest that 40% of prostate cancer risk can be explained by inherited factors<sup>[6]</sup>.

A summary of the different genes implicated in prostate cancer are highlighted in Table 1<sup>[7-16]</sup>.

## METABOLISM AS A PRIVILEGED TARGET IN PROSTATE CANCER CELLS

### Different metabolic targets and sub-targets in prostate cancer

The specific alterations in metabolic pathways observed in cancer cells confirm that tumors need unusual amounts of energy and biosynthetic precursors to survive and grow<sup>[17]</sup>.

However, the unique intermediary metabolism in prostate cancer cells is substantially different from that found in other cancer cell types<sup>[18]</sup>. In particular to satisfy the energy demand and to generate ATP, most cancer cells are mainly derive energy from aerobic glycolysis<sup>[19]</sup>. In androgen-dependent prostate cancer cells, Warburg<sup>[19]</sup> has demonstrated that glucose does not play a major metabolic role because LNCaP cells, and androgen-sensitive human prostate adenocarcinoma cells<sup>[20]</sup> widely employed in *in vitro* prostate cancer studies, can even grow in presence of low glucose concentrations<sup>[21]</sup>. Therefore, the metabolic state of prostate cancer cells is altered to satisfy the increased demand for energy that is required to support the

stimulated protein synthesis; moreover, these cells also have an inefficient autophagy process due to reduced catabolism<sup>[22]</sup>. The inhibition of glycolysis by the promoting some kind of metabolic stresses may be used to improve therapies. A novel therapeutic paradigm was the treatment introduced by DiPaola *et al.*<sup>[23]</sup>, using 2-Deoxy-D-glucose (2DG), an inhibitor of glycolysis and a glucose analogue that blocks the uptake of glucose and induced cytotoxicity and autophagy in different prostate cancer cells. It was, therefore, hypothesized that prostate cancer is metabolically fragile because of dependence on glycolysis and impaired autophagy.

Interestingly, altered lipid metabolism has also been demonstrated by multiple groups to play an important role in prostate cancer progression<sup>[24]</sup>. Fatty acid synthase (FAS), a rate-limiting enzyme in *de novo* lipogenesis, is frequently over-expressed in prostate cancers<sup>[25,26]</sup>. Correspondingly, pharmacological or molecular inhibition of either FAS or other lipogenic enzymes, like acetyl-coenzyme A carboxylase (ACC) and ATP citrate lyase (ACL), suppressed both *in vitro* and *in vivo* tumor growth<sup>[27,28]</sup>. FASs are also stimulated by androgen hormones as seen in LNCaP cell accumulation of lipid droplets (LDs) within the cytoplasm<sup>[29]</sup> containing both triacylglycerols (TGs) and cholesterol, which are enveloped by a monolayer of phospholipids and associated proteins<sup>[30]</sup>. LDs can be metabolized by hormone-regulated cytosolic lipases that break down the TGs into fatty acids which are then utilized for  $\beta$ -oxidation<sup>[31]</sup>, but there is a second pathway involving lipolysis mediated by autophagy. Recently, Singh *et al.*<sup>[32]</sup> reported that in rat hepatocytes, autophagosomes sequestered LDs and caused lysosomal lipolysis. An alternative pathway of lipolysis has been observed also in prostate cancer cells, further explaining how prostate cancer cells may adapt to survive in hostile environmental conditions<sup>[33]</sup>. Although androgens promote prostate cancer cell growth in part by increasing the expression of several of these lipogenic enzymes<sup>[24,34,35]</sup>, it is not known whether androgens may promote the formation of these lipid reservoirs by additional mechanisms that may also be critical for tumorigenesis<sup>[36]</sup>.

Statins (or HMG-CoA reductase inhibitors) are a class of drugs used to lower cholesterol levels by inhibiting the enzyme HMG-CoA reductase, which plays a central role in the production of cholesterol in the liver. Statins such as atorvastatin (ATO), in addition to their effects on cholesterol biosynthesis, have attracted considerable interest for their possible utility in cancer prevention and therapy<sup>[37]</sup>. It has been demonstrated by *in vitro* studies that autophagy and autophagy-associated cell death in PC3 prostate cancer cells can be induced by ATO<sup>[38]</sup>. Clinically, lowering of serum cholesterol is the first effect of statin treatment; even though the inhibition of prostate cancer cell growth could seem independent from the lowering of serum cholesterol, both can be mediated by effects on the mevalonate pathway. Recently, it has been discovered that ATO inhibited the synthesis of geranylgeranyl pyrophosphate (i.e. an intermediate in the HMG-CoA reductase pathway used by organisms in the biosynthesis of terpenes and terpenoid), played an important role in the induction of autophagy and suppressing prostate cancer cell growth<sup>[39]</sup>. Specifically, the authors found a stress-responsive miRNA, called miR-182, which mediates the activity of ATO in prostate cancer cells.

### **A landscape of ADT in prostate cancer**

In patients with advanced prostate cancer, ADT remains the most effective standard treatment, inducing programmed cell death and inhibiting cell proliferation<sup>[40]</sup>. Unfortunately, after short term remissions, cancer cells may escape from this treatment, survive and develop androgen-independent growing capabilities by several mechanisms<sup>[41]</sup>. Surviving tumor cells shows a phenotype known as Castration-resistant prostate cancer (CRPC) and death usually occurs within 3 years in the majority of patients<sup>[42]</sup>. The principal androgens produced in the testes are testosterone and the more active metabolite dihydrotestosterone. Androgens work after binding and trans-activating androgen receptor (AR), which regulates gene expression by interacting with different co-regulators during prostate cancer progression. The down-regulation of the levels of androgens, or preventing their entrance into prostate cancer cells, can reduce the tumor growth. To date, there are many hormone therapy protocols to achieve this goal. ADT is now performed with surgical castration (bilateral orchiectomy) or with luteinizing hormone-releasing hormone (LHRH) agonist therapy.

LHRH can improve a disease-free phase and a moderate survival (if it's combined with primary radiation), reducing circulating testosterone levels to so-called castrate levels ( $< 0.5$  ng/mL). Anti-androgen therapy is part of the common hormone therapy that is used with drugs which can stop the action of particular hormones. Presently, the anti-androgen therapy is always combined with orchiectomy or with LHRH agonists as a first-line hormone therapy, referred as combined androgen blockade (CAB). During the first days of treatment with LHRH analogues there could be an overload of testosterone: to counteract this event, specific LHRH's antagonists have been proposed<sup>[43]</sup>.

At the first symptoms of metastasis, in CRPC patients, the cytotoxic chemotherapy is usually initiated<sup>[44]</sup>. Although cancer cells still express ARs, at some point they no longer respond to ADT, and prostate cancer become recurrent<sup>[45]</sup>. It has been discovered that there are some AR mutations often expressed in hormone-refractory prostate cancer and these mutations cause a deregulation of transcriptional activity. These events are in contrast with the purpose of targeted therapies designed specifically to inhibit the receptor functions<sup>[46]</sup>. Eventually, it has been studied that prostate cancer cells can resist to ADT, surviving and developing an androgen independence in different ways, such as stimulating growth factor pathways, activating stress-dependent survival genes, increasing cytoprotective chaperone networks, and escaping from apoptosis processes<sup>[47-49]</sup>.

The regulatory effects of androgens on prostate cancer cells are still debated; in particular, the effects of modulation of the autophagy process during androgen deprivation have been investigated<sup>[50]</sup>. Previously, it was observed that autophagy was induced if androgen-sensitive LNCaP cells were cultured in the absence of serum; otherwise, if dihydrotestosterone was introduced, the autophagic process was reduced. This suggests that specific androgenic hormones produce a down regulation of autophagy process<sup>[51]</sup>. In addition, two independent studies have shown that cell death increases if LNCaP cells undergo androgen deprivation, suggesting that autophagy might exert a protection role toward prostate cancer cells<sup>[51,52]</sup>.

## THE DIFFERENT EFFECTS OF AUTOPHAGY MODULATION IN PRECLINICAL MODELS OF PROSTATE CANCER CELLS

### The autophagy process

Autophagy is a homeostatic process whereby cellular components are engulfed into vesicles known as autophagosomes, which then fuse with lysosomes and are consequently subjected to proteolytic degradation<sup>[53]</sup>.

In 1963 the Nobel Laureate, Christian de Duve, introduced the concept of autophagy<sup>[54]</sup>, now this definition has been assigned to several intracellular processes, including micro- and macro-autophagy, chaperone-mediated autophagy, and all of them eventually converge towards a common degradation phase mediated by lysosomes<sup>[55,56]</sup>.

Macro-autophagy, generally referred to as autophagy, has been experimentally proven to be involved in the pathogenesis of different diseases including cancer<sup>[57]</sup>.

At a molecular level, the kinase mTOR is a critical regulator of autophagy induction, with activated mTOR (MAPK and Akt signaling) it suppresses autophagy, whereas a negative regulation of mTOR, p53 and AMP-activated protein kinase (AMPK) signaling, promotes it. Three related serine/threonine kinases, UNC-51-like kinase -1, -2, and -3 (i.e. ULK1, ULK2 and ULK3) act downstream of the mTOR complex. ULK1 and ULK2 form a large complex with the mammalian homolog of an autophagy related gene product (mAtg13) and the scaffold protein FIP200. Class III phosphoinositide 3 kinase (PI3K) complex, containing hVps34, Beclin-1, p150, and Atg14-like protein or ultraviolet irradiation resistance-associated gene product (UVRAG), is required for the induction of autophagy. Autophagosome formation is controlled by Atg genes proteins through Atg12-Atg5 and LC3-II complexes. Atg12 is conjugated to Atg5 in an ubiquitin-like



reaction that requires Atg7 and Atg10. The Atg12-Atg5 complex then interacts non-covalently with Atg16 to form a larger complex. LC3/Atg8 is cleaved at its C-terminus by Atg4 protease to generate the cytosolic LC3-I. LC3-I is then conjugated to phosphatidylethanolamine following an ubiquitin like reaction that requires Atg7 and Atg3. Then, the lipidated form of LC3, known as LC3-II, is attached to the autophagosome membrane.

An extensive crosstalk and a dynamic balance exists between apoptosis and autophagy. Autophagy is a survival mechanism that typically is switched on during a nutrient deficiency; however, its excessive activation can lead to cell death, with morphological features different from apoptosis ones. Proteins typically placed at the cross roads of this processes are Beclin-1 and Bcl-2. In particular, Beclin-1-dependent autophagy is inhibited by Bcl-2, which works as an anti-autophagic regulator and as a pro-survival mechanism<sup>[58]</sup>. Autophagy, like other metabolic pathways, can be regulated by various inducers and inhibitors. For example, autophagy can be induced by deprivation of amino acids or serum, whereas it can be reduced by 3-methyladenine (3-MA), an inhibitor of class III PI3K, that blocks the generation of phosphatidylinositol 3-phosphate (PI3P), an essential docking molecule for the formation of phagophores at early stage of autophagy. In addition, to investigate the autophagic flux some antibiotics are used such as bafilomycin A1 and concanamycin A, because they inhibit specific ATPase activities and acidification of the lysosome, and therefore the final fusion event between the lysosomes and autophagic vesicles<sup>[58]</sup>.

The process of autophagy has been identified as an important mechanism of cellular resistance, or alternatively of cell death<sup>[59,60]</sup>. Autophagy is a response to the cell's energy demand, whereby the loose cytoplasm and the cellular organelles undergo lysosomal degradation to compensate for the demand for alternative energy during periods of nutritional limitation. Besides the recycling of nutrients, autophagy also plays a role for degrading damaged organelles by proteolysis to maintain a cellular quality control.

A combined inhibition of autophagy and proteasome degradation pathway induces an accumulation of intracellular protein aggregates reminiscent of neuronal inclusion bodies, causing a significant cancer cell death than blocking the proteasome degradation pathway alone. As a result, proteasome inhibition activates autophagy via a eukaryotic initiation 2 alpha-dependent mechanism to eliminate protein aggregates and alleviate proteotoxic stress<sup>[61]</sup>. On the other hand, sustained autophagy under conditions of protracted starvation has also been proposed to lead to cell death; thus, the survival or death consequences of autophagy are condition-dependent<sup>[62-65]</sup>. Therefore, in cancer, autophagy has a controversial role, it can protect cancer cells from adverse conditions or induce the death of cancer cells. In particular, in human prostate cancer, autophagy is often impaired due to allelic loss of Beclin-1 locus<sup>[66]</sup> or to the activation of the PI3K kinase/Akt/mTOR pathway that finally inhibits autophagy. It has been demonstrated, in particular in *in vitro* studies on epithelial prostate cancer cells, that autophagy can provide a survival mechanism for cells that are undergoing some kind of starvation, favoring tumor growth<sup>[67]</sup>.

### Autophagy in prostate cancer

As molecular events, cancer development is often associated with deletion or silencing of tumor suppressors genes such as PTEN, a negative regulator of the PI3K/Akt/mTOR pathway<sup>[68]</sup>, leading to resistance to various therapies in both preclinical and clinical trials<sup>[69]</sup>. Therefore, the PI3K/Akt/mTOR pathway plays a central role in various cellular processes, including protein cell survival, motility, synthesis, cell cycle, cell growth, and angiogenesis. The deregulation on this pathway may contribute to the malignant phenotype. Many small-molecule inhibitors targeting Akt, mTOR, and/or PI3K, and typically promotes growth arrest rather than cell death in solid tumors, and, therefore, use of small molecule inhibitors have been limited<sup>[70]</sup>. However, some of them have been successfully used in prostate cancer therapeutic schemes. In particular, some prostate cancer cell lines such as PC346-Flu1 and LNCaP were sensitive to monotherapy with the novel AKT inhibitor AZD5363, resulting in an increase in apoptosis at concentrations achievable in preclinical

models<sup>[71]</sup>; on the contrary, other prostate cancer cell lines, such as PC3 and DU-145, were quite resistant to the treatment. Recently, Lamoureux *et al.*<sup>[72]</sup> showed that AZD5363 induced cell death in the drug-resistant prostate cell lines by means of a chloroquine-mediated autophagy inactivation.

Chloroquine is known as a drug for the treatment of rheumatoid arthritis and malaria and to achieve an anti-HIV activity<sup>[73]</sup>. Chloroquine may be a clinically effective drug in prostate cancer, due to its ability to block lysosome acidification, preventing fusion with autophagosomes and, therefore, inhibiting the autophagy process<sup>[74]</sup>. Currently, many clinical trials used chloroquine to increase the effects of different targeted therapies such as bortezomib, temsirolimus, or gemcitabine in various cancers<sup>[75]</sup>. Early antitumor activities have been demonstrated in some of these trials. Furthermore, some studies evidenced that breast cancer cells could be sensitized to cisplatin by chloroquine, in an autophagy inhibition-independent manner, irrespective to Atg12 or Beclin-1 expression<sup>[76]</sup>. Previous studies reported that cell death in breast cancer<sup>[77]</sup> and in glioma cells<sup>[78]</sup> is increased by the combination of chloroquine (or other lysosomotropic agents) and PI3K pathway inhibitors. It was demonstrated that *in vitro* administration of D,L-sulforaphane (SNF), a synthetic racemic analogue of broccoli constituent L-sulforaphane, can inactivate histone deacetylase 6, therefore, interfering with the expression of androgen receptor genes in prostate cancer cells<sup>[79]</sup>. However, SNF also induced a cytoprotective autophagy in cultured human prostate cancer cells and it can be further enhanced with the pharmacologic inhibition of autophagy using chloroquine. The combined treatment was associated with decreased cell proliferation, increased apoptosis, alterations in protein levels of autophagy regulators Atg5 and phospho-mTOR, and suppression of biochemical features of epithelial-mesenchymal transition<sup>[80]</sup>.

Pyruvate kinase isoenzyme type M2 (PKM2), a modulator of glycolysis, also regulates the autophagy process by up-regulating LC3B or Beclin-1 in glioma cells or in cancer-associated fibroblasts<sup>[81,82]</sup>. Since Sp-1 directly regulates PKM2, Ling *et al.*<sup>[15]</sup> (2017) have recently found that a specific microRNA, miR-361-5p, inhibits CRPC cell proliferation, metabolism, and autophagy by directly targeting Sp1/PKM2 signaling, which is a potential target in PCa therapy.

Recently, it has been reported that the retinoic acid receptor responder (*RARRES1*)/tazarotene-induced gene-1 (*TIG-1*), a novel retinoid inducible gene first identified in skin raft cultures, modulates a series of signaling pathways inducing autophagy and inhibiting angiogenesis. The over-expression of *RARRES1* can lead to the block of MAPK, to the increase of Beclin-1, Atg3, LC3-II protein expression, and finally, the inhibition of mTOR expression<sup>[83]</sup>. These studies strongly indicated the attractive prospect of blocking autophagy processes combined with targeted therapy as a promising therapeutic approach for prostate cancer<sup>[72]</sup>.

Zeng *et al.*<sup>[84]</sup> (2018) have very recently investigated the role of a prostate leucine zipper protein (PrLZ) in combination with docetaxel-(the first-line standard approach in PCa) resistance in PCa, focusing on PrLZ-regulating autophagy pathway. PCa cells are protected from docetaxel induced-apoptosis by overexpression of PrLZ. The negative regulation of autophagy by PrLZ is mediated through LKB1/AMPK signaling pathway. The autophagy pathway and PrLZ can become a good therapeutic target for CRPC and, especially, docetaxel-resistant CRPC therapy<sup>[84]</sup>. Autophagy has, generally, a protective function on cancer cells so maybe, if autophagy is properly inhibited, it could be a clinical strategy to contrast therapeutic resistance in prostate cancer, when is associated with partial failure of radiation or chemotherapeutic schemes<sup>[85]</sup>. On the contrary, in androgen-independent prostate cancer cells, it has been shown that autophagy induction may sensitize cells to radiation<sup>[86]</sup>. Despite these contrasting results, a therapeutic benefit for prostate cancer patients can come from either induction or inhibition of autophagy, depending on the specific tumor environment, and ultimately, to the adopted therapeutic scheme<sup>[49]</sup>. Radiation therapy is a cytoprotective autophagy inducer in prostate cancer cells<sup>[87]</sup>, it was also reported that incubation of LNCaP cells in serum-free medium lead to a pro-death autophagy process<sup>[67]</sup>. Li *et al.*<sup>[51]</sup> found out that the inhibition of autophagy can lead LNCaP

cells to apoptosis in a serum-free medium, but not in cells in medium with serum or dihydrotestosterone. This suggests that autophagy process during androgen deprivation can protect LNCap cells from death. In other cancer cell lines it has been demonstrated that autophagy is modulated by growth factors contained in serum through activating the mTOR pathway<sup>[75]</sup>.

Cell death, in certain androgen deprivation situations under *in vitro* condition on epithelial prostate cancer cells, can arise by blocking autophagy processes via interfering with genetic or pharmacology means.

Furthermore, it has been observed that there is a parallelism between autophagy stimulation by androgen-ablation in prostate cancer cells and autophagy induction in some breast cancer cells during anti-hormone therapy. It was hypothesized that breast cancer cells tend to increase autophagy levels to develop a resistance to anti-estrogens<sup>[88]</sup>. To this regard, chloroquine induce cell death in LNCaP cells in a time and dose-dependent way, combined with an androgen deprivation<sup>[89]</sup>. At the same time, efficacy of androgen-ablation cell death can be enhanced by a combination of pharmacological inhibition of autophagy and chemotherapy<sup>[90]</sup>. Additional drugs that potentially are known to interfere with autophagy flux include bafilomycin A1, 3-methyladenine and pepstatin A<sup>[91]</sup>. However, these pharmacological molecules produces many off-target effects in different cellular pathways<sup>[91]</sup>.

At this moment there are not enough *in vivo* studies on the combination of androgen deprivation and autophagy inhibition, but the *in vitro* results obtained to date show the potentiality of the combination of conventional ADT and autophagy-modulation in prostate cancer patients<sup>[50]</sup>.

### Autophagy and androgen receptor interplay

In regulation of prostate development as well as in carcinogenesis, AR is a critical transcription factor, but in the autophagy process, the role of AR remains poorly understood<sup>[92]</sup>. In fact, in PC3 AR-negative cells, statin is an autophagy inducer, but not in LNCaP AR-positive cells<sup>[93]</sup>. In contrast, in LNCaP cells autophagy process is inhibited by dihydrotestosterone treatment, but this does not happen in PC3 cells<sup>[51]</sup>. In addition, other studies showed that cell death may increase, under androgen deprivation by inhibiting autophagy process in LNCaP cells and suggesting a role of autophagy as a protector of prostate cancer cells<sup>[93,94]</sup>. Due to these contrasting results, in prostate cancer cell, the role of androgen/AR signals in altering autophagy remains unclear<sup>[95]</sup>. Traditional androgen deprivation therapy to treat prostate cancer may not reverse the AR regulated autophagy pathway because this pathway was found under different conditions at different androgen concentrations. In particular, Jiang *et al.*<sup>[95]</sup> have used the compound ASC-J9 to specifically degrade AR in AR-positive cells.

Results revealed increased autophagy and decreased cell growth compared to those of sham-treated AR-positive cells. Therefore, targeting AR to promote autophagy may represent a new potential therapeutic approach to prostate cancer<sup>[39]</sup>.

It is emerging that different mechanisms regulate the autophagy process in androgen-ablation conditions<sup>[96]</sup>. In case of hypoxic conditions, autophagy can be induced by different independent pathways including the inhibition of mTOR kinase and hypoxia-inducible factors (HIF-1). Another mechanism that activate an autophagic response is controlled by energetic stress<sup>[94]</sup>. In particular, androgen deprivation may cause the genesis of autophagic vesicles which incorporate LD. The catabolism of lipids, known as lipophagy, represents a way to support energy demand and helps in the surviving of cells during ADT<sup>[25]</sup>. The loss of energy production leads to an activation of AMPK which, again, leads to suppression of mTOR signaling; this events cause fatty acid oxidation, glycolysis<sup>[97]</sup> and, lastly, autophagy<sup>[98]</sup>. It is very interesting that about 40% human prostate cancers have an over-expression of AMPK, which confirms its activation in different metabolic pathways<sup>[99]</sup>.

A recent work by Scherz-Shouval *et al.*<sup>[100]</sup> showed that elevated levels of reacting oxygen species (ROS) activate autophagy. In addition, it was highlighted that androgen-mediated ROS generation promoted prostate cancer cell growth<sup>[101]</sup>, which provided the rationale that androgenic regulation of autophagy required a specific ROS signal. This evidence was recently further confirmed, reporting that elevated ROS levels contributed to the androgen-induced autophagy, to intracellular lipid accumulation, and finally to tumor cell growth<sup>[36]</sup>. Overall, it is clear that the regulation of ROS levels within the cells is critical: although too much ROS can trigger apoptosis, moderate levels promote cell signaling activities that are needed for both proliferation and survival<sup>[102]</sup>.

### Autophagy and apoptosis crosstalk in prostate cancer

It is known that autophagy is particularly important as a survival mechanism in tumors with defects in the apoptotic pathway, supporting an already suggested therapeutic paradigm of a dual apoptotic and autophagic inhibition<sup>[103]</sup>. Prostate cancer cells could be sensitized to different apoptotic stimuli by inhibiting autophagy, which happens during ADT. In fact, appropriate stimuli can lead prostate cancer cells to apoptosis, even though these cells tend to evolve into an androgen-resistant phenotype<sup>[104]</sup>. To this regard, a recent investigation by Saleem *et al.*<sup>[105]</sup> demonstrated that employing the well-established Bcl-2 inhibitor, ABT-737, in combination with chloroquine resulted in enhanced cytotoxicity in prostate cancer *in vitro* and *in vivo*. These results also highlighted the importance in clinical studies for the evaluation of the crosstalk pathways between apoptosis and autophagy<sup>[100]</sup>. Tumor necrosis factor- $\alpha$  (TNF- $\alpha$ ) and TNF-related apoptosis-inducing ligand (TRAIL), members of the death receptor ligand superfamily, as apoptotic markers, have been suggested as potential anti-prostate cancer pharmacological targets<sup>[106,107]</sup>. In the LNCap cells the apoptotic response was enhanced by inhibiting pharmacological autophagy. Furthermore, the apoptotic cytotoxicity induced by TRAIL, in prostate cancer cell lines, was effectively increased by blocking autophagy by siRNAs targeting autophagic genes such as *BECN1* or *ATG7*<sup>[108]</sup>.

Shin *et al.*<sup>[109]</sup> reported that docosahexaenoic acid (DHA), an omega-3 fatty acid present in cold-water fatty fishes, leads to mitochondrial ROS generation and reduces phospho-mTOR and phospho-Akt expression levels in concentration-dependent manner in p53-mutant DU145 and PC3 cells. These results suggest that DHA may be beneficial for patients with p53-mutant prostate cancer and show its possible use in clinical therapies<sup>[109]</sup>.

Many natural compounds are studied for their antitumor features. Recently, the effects of Marchantin M (Mar), a naturally occurring macrocyclic bisbenzyls, have been tested, which resulted in a favorable apoptosis modulation<sup>[110]</sup>. Through this observation, it was hypothesized that caspase-independent mechanisms can also contribute to its cytotoxic effect on prostate cancer cells. Very recently, Jiang *et al.*<sup>[111]</sup> revealed that the Mar-induced cell death was additionally associated with the activation of autophagy, together with the induction of ER stress and the inhibition of proteasome activity. These results enforced the goal of the identification of chemotherapeutic compounds able to trigger apoptotic as well as autophagic cell death in prostate cancer cells, for a successful application in cancer therapy.

### Novel molecular actors for autophagy tuning in prostate cancer models

Recent contributions highlighted tyrosine kinases (TKs) and histone deacetylase (HDAC) inhibitors as promising modulators of autophagy activity in novel therapeutic schemes in prostate cancer models<sup>[112]</sup>. It was reported that TKs play a key role in tumor sensitivity to radiation and chemical-induced apoptosis<sup>[113]</sup>. Non-receptor tyrosine kinases (NRTK) are shown to participate in processes such as cell proliferation and migration in prostate cancer. There are several NRTK families, classified based on their structural similarities, that might potentially interfere with cell death balance in prostate cancer<sup>[23-25]</sup>. In particular, it has been shown that the administration of autophagy interfering molecules or drugs sensitized these cells toward Src tyrosine kinase inhibitor-based therapies<sup>[114]</sup>. Specifically, AR is phosphorylated by Src kinase complex, resulting in AR nuclear

translocation and activation; it was additionally reported that this kinase played an important role in the development of castration-resistant disease state<sup>[115]</sup>. Indeed, tyrosine kinase inhibitors targeting Src can inhibit androgen-independent growth of prostate cancer cells, but did not induce significant apoptosis. Therefore, an autophagy blocking strategy might significantly potentiate the effects of tyrosine kinase inhibitors as pro-apoptotic inducers<sup>[116]</sup>. In addition to cell migration, Src assists in tumor invasion through its regulation of matrix metalloproteinases (MMPs) via degradation of the extracellular matrix. Another interaction that involves Src in CaP is with steroid receptors. It has been demonstrated that in low androgen conditions, AR can activate Src in the cytoplasm, thereby triggering downstream signaling events independent of AR transcriptional and DNA-binding activity<sup>[38,48]</sup>. In fact, DNA synthesis can be inhibited by Src (as a dominant negative factor) after stimulation with low amount of androgens, but the Src pathway can be bypassed with higher concentrations of androgen coupled with AR over-expression. Src in addition to binding with AR, if stimulated with estradiol, can also interact with the estrogen receptor (ER) and thereby promote cell proliferation<sup>[38,49,50]</sup>; thus, it can be hypothesized that Src serves as a scaffolding protein for the AR-ER complex.

Focal kinase (FAK) adhesion, in addition to migration and proliferation, may also be involved in angiogenesis and apoptosis in CaP cells. There are evidence that FAK induces vascular endothelial growth factor (VEGF) transcription in an ERK1/2-dependent, Rap1-dependent, and Raf-dependent but Ras-independent manner<sup>[91-93]</sup>.

PTEN, a tumor suppressor gene with dual phosphatase activity, is part of the negative FAK regulators, and is deleted in the aggressive CaP<sup>[94]</sup>. The formation of the Lyn-PI3K-NEP complex can be regulated indirectly, in a negative way, by FAK<sup>[60]</sup>. ETK/BMX complex, discovered in 1994, belongs to the Tec family of NRTK<sup>[117]</sup>. In CaP, ETK is downstream of PI3K on the induction of the neuroendocrine differentiation following IL-6 stimulation in LNCaP cells<sup>[118]</sup>. It is also known that it works as an anti-apoptotic factor. Over-expression of ETK leads to a resistance to apoptosis in CaP cells due to its interaction with PI3K<sup>[118]</sup>. The activation of ETK does not require PI3K<sup>[27]</sup>. Rather, the interaction of ETK with p53 could be another mechanism of protection against apoptosis<sup>[119]</sup>. The introduction of ETK C-terminal fragment into PC-3 cells lead to apoptosis after proteolytic cleavage of ETK by caspases<sup>[120]</sup>. ETK is a signal transducer between SRC and AR downstream and FAK upstream. However, ETK alone is not enough efficient to activate AR, since it requires to interact with Pim1 protein<sup>[117,121]</sup>.

Several studies have suggested that inhibition of HDAC in the progression of autophagy could be a new way for treatments of prostate cancer<sup>[122]</sup>. It is known that HDAC inhibitors are among the most promising targeted anticancer agents and are potent inducers of growth arrest, differentiation, and autophagic cell death of prostate cells<sup>[123]</sup>. Very recently, Patra *et al.*<sup>[124]</sup> developed a novel HDAC inhibitor (MHY219) that induced cancer cell death and might be employed as a chemotherapy adjuvant in clinical studies. Similarly, other HDAC inhibitors have been tested in prostate cancer studies<sup>[125-127]</sup>. In another recent contribution, Vallo *et al.*<sup>[122]</sup> assayed PXD101, a potent pan HDAC inhibitor, to prevent the onset of castration-resistant phenotype and to potentiate hormonal therapy. A very interesting aspect is that there is a functional link between HDAC and liver X receptors (LXRs) members of the nuclear receptor family that regulates intracellular lipid homeostasis<sup>[128]</sup>. As already mentioned, lipids play a complex role in the progression and maintenance of prostate cancer. In fact, the increasing *de novo* synthesis of cholesterol and/or fatty acids is associated with the development of prostate cancer. Therefore, by inhibiting HDAC it was possible to reduce the levels of intracellular cholesterol and consequently it reduced the proliferation of tumor cells. Inhibitors HDCA and LXRs can, therefore, inhibit the proliferation of tumor cells<sup>[128]</sup>.

Currently, the only drug, approved to be applied in the chemotherapy of PCa, is docetaxel. Recently, a new drug was introduced, Salen-MN, a novel type of synthetic reagent bionic and exerts remarkable anticancer activities, but its effect is not been completely elucidated in PCa. In particular, treatment with Salen-Mn inhibited growth in PC-3 and DU145 cells. Moreover, Salen-Mn *in vitro* administration induced



an increase in the expression of apoptotic proteins such as Bcl-2-associated X protein (Bax), cleaved poly (ADP-ribose) polymerase (PARP), and cleaved caspase-3. Furthermore, it has been observed that Salen-Mn induced expression of LC3-I/II in both dose- and time-dependent manner. It was documented that Salen-Mn increased autophagy by means of AMPK phosphorylation. Therefore, Salen-Mn might represent a novel promising candidate for the treatment of prostate cancer<sup>[129]</sup>.

## CONCLUDING REMARKS

Basal autophagy helps to maintain homeostasis by contributing to organelle and protein turnover, but it is also a survival mechanism that is efficiently induced in stressed cells. Autophagy defects have been implicated in various health states and diseases, including infection, myopathy, Crohn's disease, neuro-degeneration and cancer. However, the role of autophagy in cancer is quite complicated and still somewhat controversial; it appears to be tumor suppressive during cancer development, but contributes to tumor cell survival during cancer progression. Furthermore, tumor cells can use autophagy to resist to various anti-cancer therapies. Cancer cells experience higher metabolic and energy demands and exposed to stresses than normal cells because of their rapid proliferation and altered glycolytic metabolism. These cells depend more heavily on autophagy for survival.

The therapeutic benefits of various cancer therapies have been improved because of the inhibition of autophagy, which allows a methodology to specifically target cells characterized by higher levels of autophagy. There is still much to be discovered about autophagy and its regulation, but the ongoing results are delineating a promising pharmacological target for cancer treatment. However, it is necessary to discover additional biomarkers to evaluate the complex dynamism of autophagy processes and to establish new methods to assess autophagy in clinical samples.

The data here reviewed from the current scientific literature generally indicated that the modulation of autophagy may be therapeutically beneficial in various tumors because of their ability to sensitize cancer cells to the different therapies, including DNA-damaging agents, anti-hormone therapies and radiation and chemotherapeutic combined strategies.

In particular, it is emerging that in prostate cancer, a promising combined treatment during androgen deprivation therapy is to target metabolic stress-induced signaling pathways. These complex pathways are intimately controlled by various molecular actors that play important roles in programmed cell death pathways including autophagy and apoptosis. In particular, autophagy is clearly becoming a central regulator of the main physiological and pathological processes, which through a precise and sensitive balancing determine pro-death or pro-survival fate of the cell. Therefore, the modulation of autophagy process in malignant cell types can be regarded as a potential strategy in cancer therapy.

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The authors declare that there is no conflict of interests regarding the publication of this paper.

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Review

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# Recent trend in gastric cancer treatment in the USA

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## Abstract

Gastric adenocarcinoma (GAC) is estimated as the fifteenth most common cancer in the USA. Incidence rate has been gradually decreasing, but prognosis remains dismal. For patients with locally advanced GAC (stage > T1B and < T4B), multimodality therapies, such as surgery, chemotherapy, and radiation therapy, are needed. Perioperative chemotherapy or postoperative chemoradiation/chemotherapy is recommended. For metastatic GAC patients, combination of two cytotoxics (platinum compound and fluoropyrimidine) has become a common place in the USA, and when HER2 is positive, trastuzumab is added. When GAC progresses after the first line therapy, additional biomarkers (microsatellite instability and programmed death ligand 1) should be tested so that checkpoint inhibitors can be used. Overall, the options for advanced GAC patients are limited and more research is needed.

**Keywords:** Gastric adenocarcinoma, chemotherapy, chemoradiation, preoperative treatment

## EPIDEMIOLOGY IN THE USA

Gastric adenocarcinoma (GAC) is estimated as the fifteenth most common cancer in the USA; 28,000 new cases are estimated in a year, which is 1.7% of all new cancer cases<sup>[1]</sup>. Incidence rate has been gradually decreasing; number of new cases per 100,000 people is 11.7 in 1975, 9.3 in 1990, 8.1 in 2000, and 6.6 in 2014<sup>[1]</sup>. In total 10,960 deaths are estimated in a year, which is 1.8% of all cancer death<sup>[1]</sup>. The 5-year survival rate of GAC in the USA is 30.6%; 53% GAC are localized at diagnosis, and the 5-year survival rate of localized GAC (no lymph node involvement) and regional GAC (regional lymph node involvement) is 67.2% and 30.7%, respectively<sup>[1]</sup>; 35% GAC are diagnosed as metastatic disease and have a poor outcome<sup>[1]</sup>.



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**Table 1. Summary of NCCN guideline for resectable gastric adenocarcinoma**

Stage	Treatment (recommendation category or comments)	Preferred regimen (recommendation category)
cT1a	Surgery Endoscopic resection	
cT1b	Surgery	
cT2 higher	Perioperative chemotherapy (1) (3 cycle preoperative and 3 cycle postoperative)	Fluorouracil and cisplatin (1) Fluoropyrimidine and oxaliplatin (1A) Epirubicin, cisplatin/oxaliplatin, and fluoropyrimidine (2B)
	Preoperative chemoradiation (2B)	Paclitaxel and carboplatin(1) Fluorouracil and cisplatin (1) Fluoropyrimidine and oxaliplatin (1)
	Postoperative chemoradiation (1) (for patients without preoperative treatment)	Fluoropyrimidine (1A) (before and after fluoropyrimidine-based chemoradiation)
	Postoperative chemotherapy (2A) (for patients after D2 lymph node dissection)	Capecitabine and oxaliplatin (1)

Location of GAC had dramatically changed in the USA. Most of GAC originate from the proximal lesser curvature, cardia, and the gastroesophageal junction<sup>[2]</sup>. This location trend is considered due to environmental risk factors, such as *Helicobacter pylori* infection, smoking, high salt intake, and obesity.

### STANDARD TREATMENT FOR RESECTABLE GAC IN THE USA

Resectable GAC patients with  $\geq$  cT1b can proceed to surgery (in the community setting) or receive preoperative therapy (in the university setting) [Table 1]. If GAC patients directly undergo surgery, postoperative chemoradiation is recommended based on the pathological stage or quality of surgery. Endoscopic resection is performed according to Japanese guideline<sup>[3]</sup>, but early stage (stage I) GAC is rare in the USA.

At our institution, we prefer the strategy of induction chemotherapy followed by chemoradiation and surgery<sup>[4,5]</sup>. This strategy originated at our institution (also, feasible in multi-institutional settings) and has been pursued based on excellent results recently reported<sup>[5]</sup>. Induction chemotherapy consists of 4 doses 5-fluorouracil (5-FU) and oxaliplatin administered every 2 weeks, and chemoradiotherapy consists of 45 Gy in 25 fractions with concurrent 5-FU/capecitabine with or without another cytotoxic like a platinum compound or taxane (when gastroesophageal junction is involved). After 6-8 weeks from the end of chemoradiation, a D2 dissection is attempted.

### Postoperative chemoradiation

SWOG 908/INT-0116, which started in 1991, is one of the most cited trials showing the survival benefit of postoperative chemoradiation for resected GAC in the USA<sup>[6,7]</sup>. In this trial, a total of 556 patients who underwent R0 resection were randomly assigned to surgery alone or surgery plus postoperative chemoradiotherapy (bolus 5-FU and leucovorin with 45 Gy radiotherapy). Compared with surgery alone group, postoperative chemoradiotherapy group showed better overall survival (OS) and relapse-free survival (RFS); the hazard ratio (HR) for OS is 1.32 [95% confidence interval (CI) 1.10-1.60;  $P = 0.0046$ ], and the HR for RFS is 1.51 (95% CI 1.25-1.83;  $P < 0.001$ ). Both overall relapse and locoregional relapse were decreased in postoperative chemoradiotherapy group<sup>[6,7]</sup>. According to these results, postoperative chemoradiation therapy became the standard treatment. It is appropriate only for those patients who undergo suboptimal surgery and do not received preoperative chemotherapy.

INT 0116 had some inherent drawbacks since surgical method was not part of the protocol. Thus, in the INT-0116 trial, D0, D1, and D2 lymph node dissections underwent in 54%, 36%, and 10% patients, respectively. Therefore, the efficacy of postoperative chemoradiation after D2 resection remains unclear. The ARTIST (Adjuvant Chemoradiation Therapy in Stomach Cancer) trial in Korea compared postoperative treatment with capecitabine plus cisplatin (XP) and XP plus radiation after curative resection with D2 lymph node dissection<sup>[8]</sup>. This trial showed that the estimated 3-year disease free survival rates were 78.2% in the chemoradiation



group and 74.2% in XP alone group ( $P = 0.862$ ), suggesting the addition of radiation to adjuvant XP did not significantly reduce recurrence after D2 dissection<sup>[8]</sup>. Additionally, the randomized phase III CRITICS-study assessed perioperative chemo vs. postoperative chemoradiation after preoperative chemotherapy. Patients had D1+ dissection with gastrectomy in this trial. In total 788 patients were randomized into chemotherapy group ( $n = 393$ ) and chemoradiation group ( $n = 395$ ), and the 5-year survival is 41.3% for chemotherapy group and 40.9% for chemoradiation group ( $P = 0.99$ )<sup>[9]</sup>. These results suggest that postoperative chemoradiation is not useful if optimal or near-optimal surgery is performed.

Several chemotherapy regimens before and after chemoradiation were evaluated<sup>[10-12]</sup>. For instance, Korean study evaluated 5-FU plus cisplatin (FP) before and after concurrent radiotherapy with capecitabine, and this regimen was well tolerated<sup>[10]</sup>. Epirubicin, cisplatin, and 5-FU (ECF) before and after concurrent radiotherapy was assessed, and this regimen was feasible, but did not improve survival<sup>[11,12]</sup>.

### Perioperative chemotherapy

Trials evaluating perioperative chemotherapy were held in Europe and its results have impacted NCCN Guideline as category 1 evidence. MAGIC trial showed an advantage in OS but control and experimental arms performed poorly<sup>[13]</sup>. The NCCN guidelines have not downgraded ECF based on toxicity issues and poor efficacy<sup>[13]</sup>. FNCLCC/FFCD trial randomly assigned 224 patients into the 2 groups: 113 to surgery plus perioperative chemotherapy (2 or 3 preoperative and 3 or 4 postoperative cycles of FP) and 111 to surgery alone<sup>[14]</sup>. Compared with the surgery alone group, the perioperative chemotherapy group had a favorable overall survival (5-year rate, 38% vs. 24%; HR 0.69; 95% CI 0.50-0.95;  $P = 0.02$ ) and significantly increased the R0 resection rate (84% vs. 73%;  $P = 0.04$ ), but 75% of patients in this trial had esophageal adenocarcinoma<sup>[14]</sup>. Recently, MRC-OEO5 trial compared two perioperative chemotherapy regimen, 2 cycles FP and 4 cycles ECF/ECX (epirubicin, cisplatin and capecitabine)<sup>[15]</sup>. This study showed no OS benefit for ECF/ECX compared with FP (3-year rate, 42% vs. 39%; HR 0.92; 95% CI 0.79-1.08;  $P = 0.30$ ), suggesting that addition of epirubicin and longer duration does not provide any advantage. However, this trial predominantly included patients with lower esophageal and junctional (types I and II) adenocarcinoma, not GAC.

The FLOT4 trial, which is multicenter, randomized, and phase 3 trial, compared perioperative chemotherapy with docetaxel, oxaliplatin, and fluorouracil/leucovorin (FLOT) and ECF/ECX<sup>[16,17]</sup>. Of 716 patients, 360 patients is assigned into ECF/ECX group and 356 patients assigned into FLOT group, and FLOT improved median progression-free survival (PFS) (30 months vs. 18 months; HR 0.75;  $P = 0.001$ ) and median OS (50 months vs. 35 months; HR 0.77;  $P = 0.012$ ) compared with ECF/ECX. Fifty percent of patients in FLOT group completed the planned postoperative treatments, while 37% of patients in ECF/ECX completed. Perioperative complications were similar across the 2 groups<sup>[16,17]</sup>. However, the FLOT regimen resulted in considerable toxicity and mortality. Some of the follow up is too early. FLOT could be recommended to only occasional fit patient for perioperative chemotherapy and we don't recommend it for regular use.

### Preoperative chemoradiation

Preoperative chemoradiation for GAC is not the standard of care in the USA but it is a developing strategy. The strategy has several advantages. Firstly, radiation field is planned more accurately because primary is in place. Postoperative radiation fields were redesigned in about 35% patients in the INT-0116 trial<sup>[6,7]</sup>. Secondly, preoperative chemoradiation increases R0 resection, resulting in low local relapses rate<sup>[5]</sup>. Finally, preoperative chemoradiation might reduce peritoneal dissemination during surgery, however this is debatable.

A multi-institutional trial, where patients received 2 cycles of FP followed by 45 Gy of radiation concurrent with 5-FU, demonstrated that R0 resection rate was 70% and pathologic complete response (pCR) rate was 30%<sup>[18]</sup>. Patients who achieved a good pathological response (< 10% residual carcinoma in the primary) had a significantly longer OS than those who did not (63.9 months vs. 12.6 months;  $P = 0.03$ )<sup>[18]</sup>. In another trial,

**Table 2. Summary of NCCN guideline for metastatic gastric adenocarcinoma**

Line	Preferred regimen (recommendation category)	
First-line therapy	HER2 overexpression	Trastuzumab combination with fluoropyrimidine and cisplatin (1)
		Trastuzumab combination with other chemotherapy agents (2b)
	HER2 negative	Fluoropyrimidine and cisplatin (1)
		Fluoropyrimidine and oxaliplatin (2A)
		Paclitaxel with cisplatin or carboplatin (2A)
		Docetaxel with cisplatin (2A)
		Fluoropyrimidine (2A)
		Docetaxel or paclitaxel (2A)
		Fluorouracil and irinotecan (2A)
		DCF modification (2A)
		ECF or ECF modification (2B)
Second-line therapy	Ramucirumab and paclitaxel (1)	
	Paclitaxel (1)	
	Docetaxel (1)	
	Irinotecan (1)	
	Ramucirumab (1)	
	Fluorouracil and irinotecan (2A)	
	Irinotecan and cisplatin (2A)	
	Docetaxel and irinotecan (2B)	

DCF: docetaxel, cisplatin, and intravenous 5-FU; ECF: epirubicin, cisplatin, and 5-FU; 5-FU: 5-fluorouracil

paclitaxel-based induction chemotherapy and chemoradiotherapy were also assessed. This trial demonstrated that pCR rate was 20%, and over 36 months median survival had been estimated<sup>[19]</sup>. In these trials, laparoscopic staging and endoscopic ultrasonography were used for initial staging. Moreover, surgery was a part of sequential treatment strategy and thus was required to be high quality, such as D2 dissection. Therefore, this strategy was considered to be limited in some specialized institutions. The RTOG 9904 assessed quality, survival, and safety of this strategy with 20 institutions and demonstrated its feasibility. In this trial, the pCR and R0 resection rates were 26% and 77%, respectively. A D2 dissection was performed in 50% of patients<sup>[20]</sup>.

Phase III trials to assess the value of preoperative chemoradiation in GAC, TOPGEAR trial, is currently evaluating the efficacy of adding preoperative chemoradiation to perioperative ECF (MAGIC trial regimen)<sup>[21]</sup>. The CRITICS-II trial started to assess the optimal preoperative regimen by comparing three arms; preoperative chemotherapy followed by surgery, preoperative chemotherapy and subsequent chemoradiation followed by surgery, and preoperative chemoradiation followed by surgery (NCT02931890). Results of these trials are forthcoming.

## STANDARD TREATMENT FOR METASTATIC GAC IN THE USA

### First line therapy

The recommended first-line therapy for patients with good performance status is a 2-drug combination of oxaliplatin plus 5-FU or capecitabine [Table 2]. Trastuzumab is added to the first line cytotoxic therapy in patients with HER2 positive GAC based on the ToGA trial<sup>[22]</sup>. Irinotecan in the first line setting did not produce OS advantage and used only for patients who are unable to tolerate platinum-based chemotherapy<sup>[23-25]</sup>. Three-drug combination of docetaxel, cisplatin, and intravenous 5-FU (DCF) or its modification is used by some but it is discouraged for two reasons: (1) it is toxic and provides marginal OS advantage and (2) it is better to avoid a taxane in the first line because one would not be able to take advantage of paclitaxel and ramucirumab in the second line. ECF is not recommended anymore in this situation<sup>[26]</sup>.

5-FU alone or in combination with various reagents used to be the key chemotherapeutic agent against metastatic GAC in the USA; FAM (5-FU, doxorubicin, and mitomycin), and FAMTX (methotrexate, 5-FU and adriamycin) used to be standard treatment<sup>[27,28]</sup>. EAP (etoposide, adriamycin, and cisplatin) was temporarily used in the 1990s, but was discontinued due to toxicity<sup>[29]</sup>. A randomized trial showed that ECF was better than FAMTX, however remained controversial<sup>[30,31]</sup>.

5-FU-based and cisplatin-based combinations were considered as an acceptable standard therapy according to trial in Asia<sup>[32]</sup>. Then, capecitabine, which is an oral fluoropyrimidine, and oxaliplatin, which is third-generation diaminocyclohexane platinum compound, were developed. A phase III in Germany showed that the combination of fluorouracil, leucovorin, and oxaliplatin improved median PFS compared with fluorouracil, leucovorin, and cisplatin (5.8 months vs. 3.9 months), but not significant<sup>[33]</sup>. The REAL-2 trial demonstrated possible replacement of 5-FU into capecitabine or cisplatin into oxaliplatin<sup>[34]</sup>. These results have led to trend toward preference of capecitabine plus cisplatin or capecitabine plus oxaliplatin in the USA.

S-1, which is oral fluoropyrimidine preferred in Japan, was reported to be similarly effective for survival with a better toxicity compared with infusional fluorouracil in West<sup>[35,36]</sup>. However, dose of S-1 administered each time in West (25 mg/m<sup>2</sup>) is lower than that in Asia (40-60 mg/body)<sup>[37]</sup>. Thus, more evidence is needed to get acceptance for S-1 in the USA.

DCF was evaluated in a randomized study, V-325 in 2006<sup>[38,39]</sup>. It showed that median OS of DCF was significantly longer than CF (9.2 months vs. 8.6 months;  $P = 0.02$ ), but DCF produced more toxicity<sup>[38,39]</sup>. Several modified DCF regimens demonstrated the efficacy and the safety<sup>[40-42]</sup>. Thus, the original DCF is not recommended, and modified DCF is still one of the option in specific cases.

### Second/third line therapy

For second line therapy, ramucirumab (an anti-VEGFR2 monoclonal antibody) is the only molecular-targeted drug with a confirmed minimal survival benefit in a global phase 3 trial. The REGARD trial compared ramucirumab and placebo, and showed that median OS in ramucirumab group was better than that in placebo group (5.2 months vs. 3.8 months)<sup>[43]</sup>. The RAINBOW trial compared paclitaxel with and without ramucirumab, and showed that OS in ramucirumab plus paclitaxel was significantly longer than in placebo plus paclitaxel (median 9.6 months vs. 7.4 months)<sup>[44]</sup>. Ramucirumab plus paclitaxel is the preferred regimen in the second line setting. Docetaxel, irinotecan and paclitaxel have significantly prolong OS compared to best supportive care, but all these trials were flawed<sup>[45-47]</sup>.

Immune checkpoint blockade has received global attention in recent years<sup>[48-50]</sup>. Keynote-059 assessed efficacy and safety of pembrolizumab, programmed death-1 (PD-1) inhibitor, monotherapy showed that overall response rate (ORR) was 11.2% and median duration of response (DOR) was 8.1 months in all cohort<sup>[51]</sup>. ORR was higher in PD-1 ligand (PD-L1) positive patients than PD-L1 negative patients (15.5% vs. 5.5%). Checkmate 032 assessed the combination of two checkpoint inhibitors, nivolumab (PD-1 inhibitor) and ipilimumab (cytotoxic T-lymphocyte-associated protein 4 inhibitor), and showed that ORR for combination therapy in PD-L1 positive patients was 40%, which was higher than nivolumab monotherapy<sup>[52]</sup>. Interestingly, among 7 patients with high microsatellite instability (MSI-H) tumors in Keynote-059, ORR was 57% and the CR rate was 14.3%. Given this result, the FDA has approved pembrolizumab for the treatment of patients with PD-L1 positive GAC who have received 2 or more lines of chemotherapy. Pembro is also approved for MSI-H tumor patients. Therefore, now we have to consider all 3 biomarkers for gastroesophageal adenocarcinoma patients (Her2, PD-L1, and MSI).

## PERSPECTIVE FOR TARGETED THERAPY AND IMMUNOTHERAPY

### Targeted therapies against stem cells

Cancer stem cells possess the capacity to self-renew and to cause the heterogeneous lineages of cancer cells. Several makers and pathways related to gastric cancer stemness have been identified<sup>[53]</sup>. Cancer stem cells are resistant to several chemotherapy, and thus targeting cancer stem cells is a potential therapy to overcome treatment resistance. Two stemness related pathways, Hedgehog and signal transducer and activator of transcription 3 (STAT3) pathway, were assessed in clinical trials so far. Vismodegib, which inhibit Hedgehog signals by binding smoothened (SMO), in combination with FOLFOX was assessed in phase 2, but did not benefit PFS (11.5 months vs. 9.3 months;  $P = 0.34$ )<sup>[54]</sup>. Moreover, BRIGHTER study assessed napabucasin,

**Table 3. Key trials for gastric or gastro-esophageal junction adenocarcinoma**

Study	Enrolled number	Treatment	Survival	HR (95% CI)	P value	Ref.
Pre or postoperative treatment						
INT-0116	281	Surgery → 5-FU/45 Gy	Median OS: 36 months	1.35 (1.09-1.66)	0.005	[6]
	275	Surgery	Median OS: 27 months			
ARTIST	228	Surgery → XP	3-year DFS: 74%	-	0.86	[8]
	230	Surgery → XP/45 Gy	3-year DFS : 78%			
CRITICS	393	ECC → surgery → ECC	5-year OS: 41%	-	0.99	[9]
	395	ECC → surgery → ECC/45 Gy	5-year OS: 41%			
FNCLCC/FFCD	113	CF → surgery (n = 113)	5-year rate: 38%	0.69 (0.50-0.95)	0.02	[14]
	111	Surgery (n = 111)	5-year rate: 24%			
MAGIC	250	ECF → surgery → ECF	5-year rate: 36%	0.75 (0.60-0.93)	0.009	[13]
	253	Surgery	5-year rate: 23%			
MRC	446	ECF → surgery	3-year rate: 39%	0.92 (0.79-1.08)	0.30	[15]
OEO-5	451	CF → surgery	3-year rate: 42%			
FLOT4	360	ECF → surgery → ECF	Median OS: 35 months	0.77 (0.63-0.94)	0.012	[17]
	356	FLOT → surgery → FLOT	Median OS: 50 months			
Targeted therapy						
ToGA	298	Trastuzumab + XP	Median OS: 13.8 months	0.74 (0.60-0.91)	0.0046	[22]
	296	Placebo + XP	Median OS: 11.1 months			
REGARD	238	Ramucirumab	Median OS: 5.2 months	0.78 (0.60-0.99)	0.047	[43]
	117	Placebo	Median OS: 3.8 months			
RAINBOW	330	Ramucirumab + paclitaxel	Median OS: 9.6 months	0.81 (0.68-0.96)	0.017	[44]
	335	Placebo + paclitaxel	Median OS: 7.4 months			

OS: overall survival; DFS: disease free survival; HR: hazard ratio; CI: confidence interval; XP: cisplatin and capecitabine; ECC: epirubicin, cisplatin and capecitabine; CF: cisplatin and 5-FU; ECF: epirubicin, cisplatin and 5-FU; FLOT: docetaxel, oxaliplatin, leucovorin, and 5-FU; 5-FU: 5 fluorouracil

STAT3 inhibitor, in combination with paclitaxel<sup>[55]</sup>. Although detail result of this trial is not available as of this date, napabucasin did not benefit OS<sup>[55]</sup>. However, these strategies might be effective for tumor with high expression of stem cell markers<sup>[56]</sup>. Further research is expected.

### Immunotherapy

To enhance immune checkpoint blockade therapy, combination with several agents have been assessed. Firstly, DNA methyltransferase inhibitor have been found to upregulate interferon signaling and tumor antigen presentation<sup>[57]</sup>. Therefore, a phase 1/2 study have been evaluating azacitidine in combination with pembrolizumab and epacadostat (NCT02959437). Secondly, because inducible CO-stimulator of T cells (ICOS) activate T cell and stimulate an anti-tumor immune response<sup>[58]</sup>, JTX-2011, an agonist of ICOS, in combination with nivolumab is being assessed (NCT02904226).

### TREATMENT FOR PERITONEAL METASTATIC GAC IN THE USA

Recommended therapy for peritoneal metastasis is systemic chemotherapy or best supportive care<sup>[59]</sup>. Hyperthermic intraperitoneal chemoperfusion (HIPEC) is a potential therapy for peritoneal metastases<sup>[60]</sup>. Our institution performed phase II study which evaluated neoadjuvant laparoscopic HIPEC (mitomycin C 30 mg and cisplatin 200 mg) for GAC patients with peritoneal metastasis<sup>[61]</sup>. Seven patients (37%) had negative peritoneal cytology after HIPEC, and the median OS from the date of diagnosis of metastatic disease was 30.2 months<sup>[61]</sup>. However, performing only HIPEC without systemic therapy might impair control of primary or distant disease. Therefore, further phase II trial of HIPEC (NCT02891447) is ongoing in our institution, and this result is expected.

### SUMMARY

In summary, perioperative chemotherapy or preoperative chemoradiation is recommended for localized advanced GAC. Postoperative chemoradiation is option for GAC patients who undergo surgery without preoperative treatment [Table 3]. Result of trials comparing preoperative chemotherapy to chemoradiation

is expected. Treatment strategies for metastatic GAC with HER2 negative is two-drug cytotoxic regimen; a platinum compound and a fluoropyrimidine. For GAC with HER2 positive, trastuzumab should be added. Metastatic GAC should be treated based on global trial [Table 3].

## DECLARATIONS

### Authors' contributions

Conception and design: Harada K, Ajani JA

Acquisition of data: Harada K

Manuscript writing: Harada K, Baba H, Ajani JA

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### Conflicts of interest

The authors declare that they have no conflicts of interest.

### Patient consent

Not applicable.

### Ethics approval

Not applicable.

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Review

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# The role of long noncoding RNAs in cancer metastasis

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## Abstract

Signaling pathways are tightly controlled systems that regulate the appropriate timing of gene expression required for the differentiation of cells down a particular lineage essential for proper tissue development. Proliferation, apoptosis and metabolic pathways are just a few examples of the signaling pathways that require fine-tuning, so as to control the proper development of a particular tissue type or organ system. An estimated 70% of the genome is actively transcribed, only 2% of which codes for known protein-coding genes. Long noncoding RNAs (lncRNAs) in particular, are a large and diverse class of RNAs > 200 nucleotides in length, and not translated into protein. lncRNAs are essential transcriptional and post-transcriptional regulators that control the expression of genes in a spatial, temporal, and cell context-dependent manner. The aberrant expression of lncRNAs is therefore linked with a number of chronic diseases including cardiac dysfunction, diabetes, and cancer. In this review, we highlight the specific role lncRNAs have in promoting the metastatic cascade across a number of epithelial cancer models.

**Keywords:** Long noncoding RNA, long intergenic noncoding RNA, microRNA, competitive endogenous RNA, breast cancer, brain cancer, lung cancer, prostate cancer, metastasis, therapeutics



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## INTRODUCTION

Metastasis is the leading cause of cancer-related deaths world-wide<sup>[1]</sup>. Understanding the biological processes that control the initiation and progression of metastasis is crucial in reducing tumor-related deaths associated with carcinomas<sup>[2,3]</sup>. Metastasis consists of the following phases: (1) escape of cells from the primary tumor and invasion into the surrounding mesenchyme<sup>[4]</sup>; (2) intravasation into adjacent vasculature and the lymphatic system<sup>[5]</sup>; (3) upregulation of cell survival mechanisms via resistance to apoptosis and anoikis<sup>[6]</sup>; (4) extravasation from the vasculature and subsequent infiltration into the parenchyma of a distant organ site<sup>[7]</sup>; and (5) the ability to undergo micro-metastatic colonization, and survival within a new tissue microenvironment<sup>[8,9]</sup>. The epithelial to mesenchymal transition (EMT) is a key developmental regulatory program describing the initiating processes of metastasis, and involves a linear series of events including tightly organized epithelial cells undergoing a loss of cellular polarity, and the ability for cells to survive under anchorage-independent conditions, both of which supports the propagation of migratory cells able to invade distant organ sites<sup>[10]</sup>. EMT essentially reactivates the embryonic morphogenesis and wound healing programs normally kept inactive within differentiated epithelial cells<sup>[11-13]</sup>. Therefore, investigating the series of cellular reprogramming events required for differentiated epithelial cells to acquire an invasive mesenchymal phenotype will aid in the development of therapeutics that specifically target metastatic cells.

While many zinc finger transcription factors (TFs) have been identified as regulators of EMT, including zinc-finger enhancer binding 1 (ZEB1), Snail, and Slug, little is known regarding the initiating steps that drive the transition of polar cells of an epithelial origin towards those with mesenchymal characteristics<sup>[14,15]</sup>. Furthermore, given invasive metastatic cells hone to various tissue sites depending upon the tissue of origin from which the primary tumor derives (i.e., the “seed and soil hypothesis”), one can hypothesize that ubiquitously expressed TFs such as Snail cannot be the sole contributor of a cell-context dependent regulatory process such as metastasis<sup>[16,17]</sup>. In fact, in a recent survey of the human genomic landscape, there is striking evidence that noncoding RNAs (ncRNAs) play an important and diverse role in regulating developmental transitions. Moreover, ncRNAs control the spatial and temporal tuning of cellular signaling pathways important for the proper execution of functional phenotypes such as enhanced cellular proliferation, migration, and/or survival<sup>[18-26]</sup>. Furthermore, in cancers that are dependent upon changes in the abundance and bioavailability of steroid hormones such as 17 $\beta$ -estradiol, ncRNAs have been identified to play a key role in the abrogating hormone-mediated metastasis<sup>[19,27-33]</sup>.

Therefore, ncRNAs are considered important epigenetic regulators of the transcriptome that modulate context-specific processes involved in promoting a metastatic phenotype. One class of ncRNA includes microRNAs (miRNAs), which are short 22-nucleotide (nt) ncRNAs that undergo biochemical processing from a longer primary miRNA (pri-miRNA) transcript via a series of interactions with RNase-III type proteins that include DROSHA and DICER. miRNAs operate via a distinct mechanism of action that relies upon imperfect complementarity or Watson-Crick base-pairing between a miRNA and the 3' untranslated region (3' UTR) of a target messenger RNA (mRNA)<sup>[34]</sup>. miRNAs therefore serve as guides that recruit RNA binding proteins (RBPs) such as AGO2 to specific mRNA targets resulting in reduced gene expression either via translational inhibition or via RNA degradation<sup>[25,35-37]</sup>.

Given this imperfect complementarity, miRNAs function as pleiotropic regulators of cell signaling pathways critically important in maintaining proper tissue development, as well as inhibiting the initiation and progression of tumorigenic cascades<sup>[38]</sup>. Given miRNAs operate by fine-tuning gene expression, and themselves function as either oncogenes or tumor suppressors when dysregulated, these ncRNAs subsequently present as potential targets for therapeutic development across a wide number of genetic disorders. miRNAs also modulate the expression of genes considered initiators of EMT, as well as mediators of downstream metastatic processes such as micro-metastatic colonization, anoikis, and interactions within the surrounding tumor microenvironment. For instance, miR-10b is a miRNA expressed at high levels in metastatic breast cancer



samples, and the use of anti-miRNA oligonucleotides significantly reduces metastatic lesions in mouse models<sup>[39,40]</sup>. Additionally, miRNAs such as miR-148a regulate the levels of E-cadherin and subsequently the progression of EMT via the modulation of DNMT1 activity<sup>[41]</sup>, while the miR-17 family of miRNAs controls metastatic phenotypes in lung cancer via dampening the expression of transforming growth factor (TGF)- $\beta$ <sup>[42]</sup>. Comprehensive reviews of miRNA in cancer are discussed in greater detail elsewhere<sup>[43,44]</sup>.

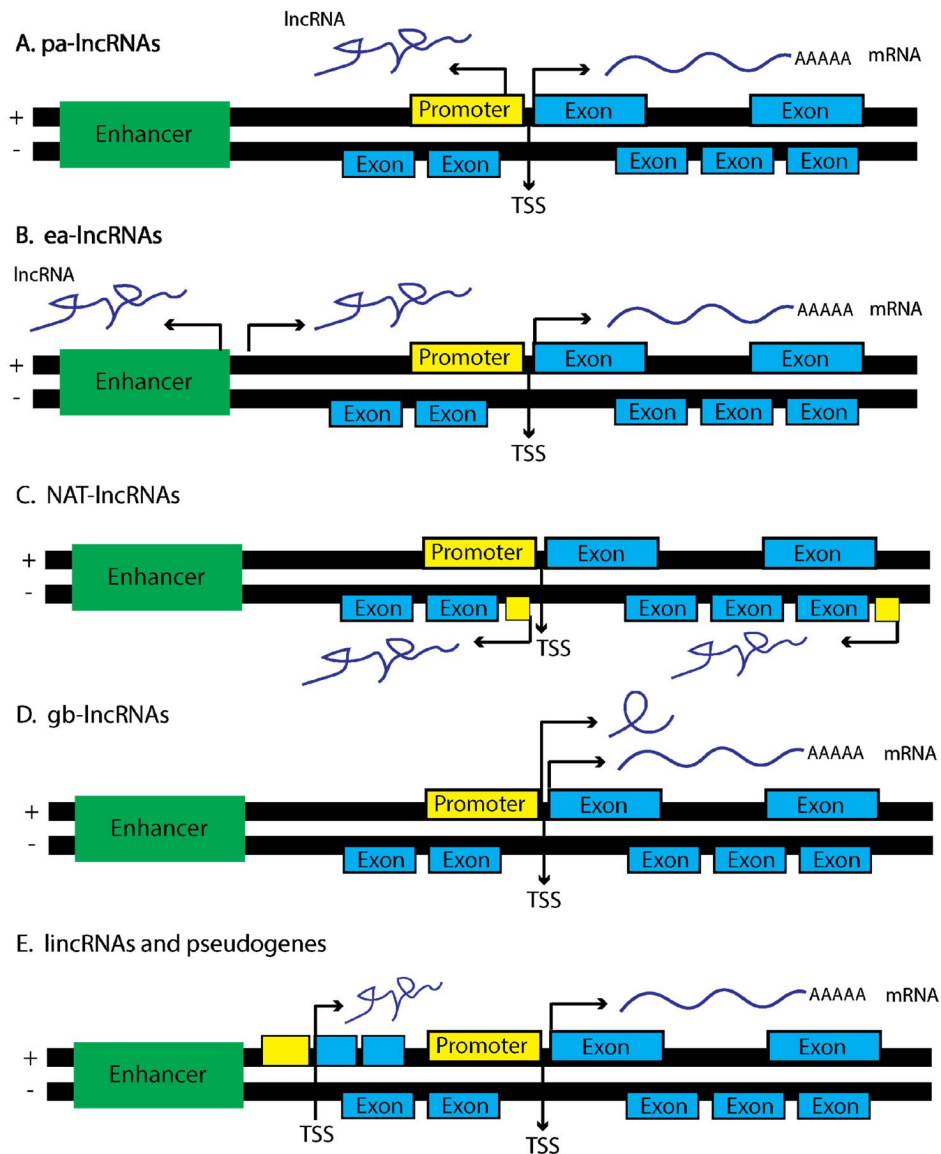
lncRNAs are a newly discovered class of ncRNA important in dampening stochastic gene expression by modulating the epigenetic landscape of the genome. lncRNAs are a divergent class of ncRNA molecule greater than 200 nt in length that lack protein-coding capacity, yet control a diverse array of biological processes via the recruitment of chromatin modifiers to specific genomic loci or by modulating post-transcriptional processes<sup>[45,46]</sup>. Currently there are over 118,000 high confidence lncRNA transcripts identified in *Homo sapiens* (<http://www.lncipedia.org>), many of which have no ascribed biological function. However, a number of studies have begun elucidating particular ncRNAs dysregulated across multiple cancer types<sup>[19,47-49]</sup>. The challenge in studying lncRNAs is their relatively low abundance and reduced conservation across species as compared to protein coding transcripts and other ncRNAs such as miRNAs<sup>[50]</sup>. This led many to believe that lncRNAs derive from leaky transcriptional processes and, therefore, have limited functionality in regulating cellular processes. However, there is considerable evidence that lncRNAs regulate the physiological pathways required for the initiation and maintenance of the metastatic process.

Broadly speaking, the diminished level of a lncRNA within a cell, results in the reduced bioavailability of a particular enzymatic substrate important in modulating chromatin structure as well as the transcriptional activity of neighboring protein coding genes. This occurs via a chaperone mechanism whereby a lncRNA brings into proximity RBPs, as well as components of the transcriptional machinery including RNA polII, to discrete genetic loci facilitating proper TF binding<sup>[35,45,51-58]</sup>. Therefore, the abundance of any particular lncRNA is important in providing the specificity necessary to promote certain phenotypic outcomes required during the metastatic cascade<sup>[59,60]</sup>. While investigators have identified specific roles for lncRNAs that control a number of cellular functions including differentiation, invasion, and metastasis, this review focuses on the role of lncRNAs within the metastatic process [Table 1].

## LNCRNA NOMENCLATURE

lncRNAs are a heterogeneous class of ncRNA transcribed from a number of regions within the genome, and in varying orientations that flank neighboring protein coding genes, promoting a diverse combination of functional phenotypes. The nomenclature of lncRNAs is still controversial; however, a concerted effort has been made to group lncRNAs into functional categories based on the genomic localization of these transcripts, as well as the regulatory functions they confer [Figure 1]. For instance, promoter-associated lncRNAs (pa-lncRNAs) are transcribed in an antisense orientation from a shared promoter of a neighboring protein coding gene<sup>[61]</sup>. A majority of pa-lncRNAs operate in cis and recruit chaperone proteins that modulate the transcription of the neighboring protein coding gene, though this is not always the case<sup>[62,63]</sup>. For instance, a pa-lncRNA was found to be transcribed from the cyclin D1 promoter and is important in mediating the inhibitory activity of certain histone acetyltransferases<sup>[64]</sup>.

Enhancer-associated lncRNAs (ea-lncRNAs) are similar to pa-lncRNAs, yet they originate from active enhancer regions within the genome that promote cis-activation of transcription via DNA looping at the proximal promoters of nearby protein coding genes<sup>[65]</sup>. ea-lncRNAs are also released from the site of transcription, and modulate the activity of distal gene promoters in trans through the recruitment of co-activators such as, p300/cAMP response element-binding protein (CREBP), as well as, demethylases such as lysine-specific demethylase 1 (LSD1)<sup>[66,67]</sup>. As an example, Braveheart (Bvht) is a lncRNA transcribed from an enhancer region marked by H3K27Ac, associates with cardiac specific transcriptional enhancers, and



**Figure 1.** lncRNAs derive from a number of genetic loci and associate with specific lncRNA function. (A) pa-lncRNAs originate from a bi-directional promoter from the sense strand of gene foci. These lncRNAs tend to operate in cis and regulate the neighboring protein coding gene; (B) ea-lncRNAs are similar to pa-lncRNAs yet are transcribed from enhancer regions within the genome; (C) NAT-lncRNAs are transcribed from the antisense strand and contain fully or partially complementary sequences to sense-strand transcripts, depending upon the surrounding genetic elements that regulate transcription of NATs; (D) gb-lncRNAs are transcribed in sense orientation, typically are one exon in length, and could share exons from protein coding transcripts; (E) lincRNAs are transcribed from genetic loci in either sense or antisense fashion and span regions considered transcriptionally active, coding or otherwise. Portions of this figure were adapted from Martens-Uzunova *et al.*<sup>[229]</sup>, with permission

when lost in mice perturbs the development of cardiomyocytes indicating Bvht is an important regulator of mammalian cardiac development<sup>[67,68]</sup>.

Natural antisense transcripts (NATs) are considered full length RNA transcripts initiated on the antisense strand of a respective protein coding gene<sup>[69,70]</sup>. Given this type of lncRNA has high complementarity to the mRNA transcript deriving from the sense strand, the formation of localized RNA duplexes results in enhanced RNA stability through HuR binding, or degradation via activation of RNA interference (RNAi) pathways. HIF1A-AS2, for instance, is transcribed from the HIF1A locus and operates as a scaffold, recruiting chromatin remodeling complexes, as well as RBPs such as IGF2BP2 to distinct genetic loci<sup>[71]</sup>.

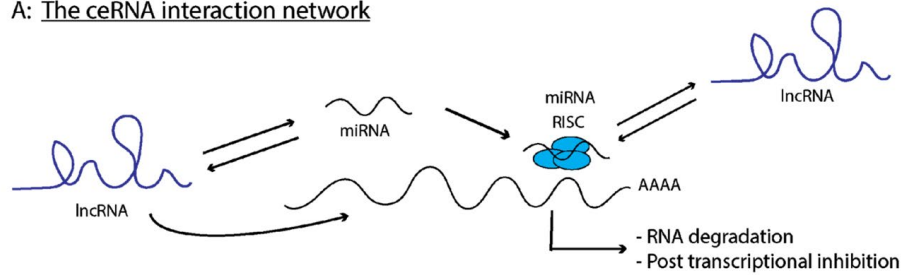
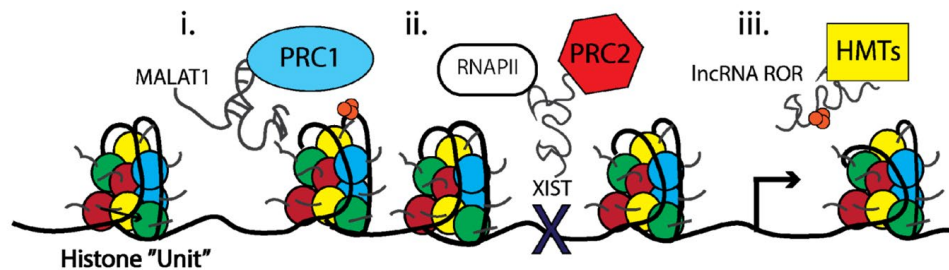
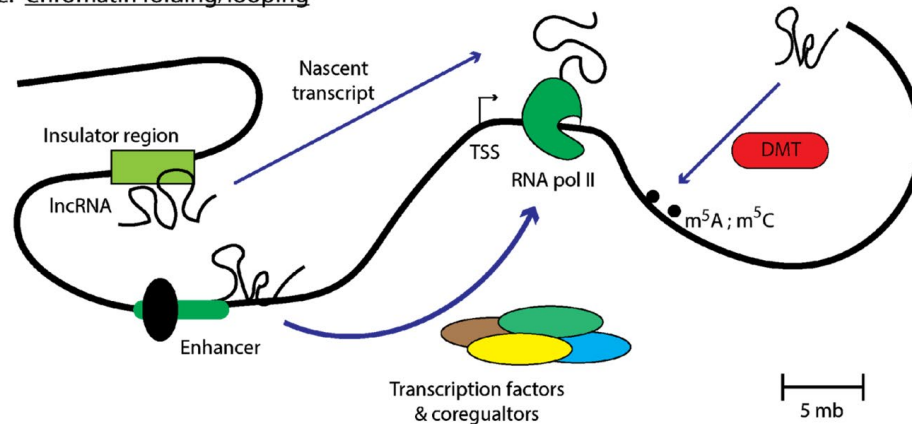
**Table 1. lncRNAs associated with and tumorigenesis and EMT pathways**

lncRNA	Cancer type	Expression in cancer	References	Role in metastasis
MALAT1	Lung	Upregulated	[190]	Suppression of E-cadherin <sup>[191]</sup>
	Bladder	Upregulated	[192]	
	Breast	Upregulated	[193]	
	Pancreatic	Upregulated	[194]	
MEG3	Meningioma	Downregulated	[195]	Regulation of autophagy <sup>[196]</sup> and DNA repair <sup>[197]</sup>
	Lung	Downregulated	[198]	
	Gastric	Downregulated	[199]	
HOTAIR	Liver	Upregulated	[200]	Reprogramming of chromatin state <sup>[201,202]</sup>
	Breast	Upregulated	[203]	
	Pancreatic	Upregulated	[204]	
GAS5	Liver	Downregulated	[205]	Controls invasion by control of miRNAs <sup>[205,206]</sup>
	Gastric	Downregulated	[207]	
	Breast	Downregulated	[208]	
H19	Liver	Upregulated	[209]	Chromatin remodeling <sup>[210]</sup> and TGF- $\beta$ regulation <sup>[211]</sup>
	Pancreatic	Upregulated	[137,212]	
	Gastric	Upregulated	[213]	
HULC	Liver	Upregulated	[147]	Regulates tumor microenvironment interactions <sup>[214]</sup>
	Gastric	Upregulated	[148]	
	Breast	Upregulated	[92,146]	
SPRY4-IT1	Melanoma	Upregulated	[139]	Proliferation and invasion via regulation of EZH2 <sup>[143]</sup>
	Lung	Upregulated	[215]	
	SCC	Upregulated	[142]	

HIF1A-AS2 is also important in regulating hypoxic responses in A549 lung cancer cells<sup>[72]</sup>. In fact, hypoxia induces HIF1A-AS2 expression, which in turn binds and represses HIF1 $\alpha$  levels under hypoxic conditions presumably through a process of RNA-mediated decay.

Gene body associated lncRNAs (gba-lncRNAs) differ from NATs, and originate instead from the sense strand of a respective protein coding gene loci<sup>[73-75]</sup>. An example of a gba-lncRNA is the pseudogene transcribed from the CCAAT/enhancer binding protein alpha (C/EBP $\alpha$ ) locus, termed ecCEBPA, which utilizes a separate open reading frame (ORF) and transcriptional start site (TSS) neighboring the C/EBP $\alpha$  gene locus<sup>[76]</sup>. ecCEBPA interacts with DNMT1, resulting in decreased methylation of the CEBPA gene. Mutagenesis studies further indicated that ecCEBPA contains hairpin structures that favor DNMT1 binding suggesting lncRNAs are important in modulating not only transcriptome wide DNA methylation, but are also present at sites of active transcription. Other gba-lncRNAs can operate as sponges for ncRNAs, thereby modulating the bioavailability of mRNA transcripts within a cell<sup>[73,74]</sup>. This competitive endogenous RNA (ceRNA) code or hypothesis is discussed in greater detail later in the review.

Finally, long intergenic noncoding RNAs (lincRNA) span extensive regions of the genome and are found within intronic regions of a coding gene, rather than as discrete genetic elements<sup>[71]</sup>. Examples include HOTAIR and MALAT1 [Figure 1]. One of the first lincRNAs discovered, X inactive specific transcript (XIST), produces an approximately 20 kilobase (kb) noncoding lincRNA and functions to silence the expression of genes derived from the inactive X chromosome (Xi) through recruitment of the polycomb repressive complex (PRC1/2)<sup>[77]</sup>. The precise mechanism by which XIST recruits PRC1/2 to the X-chromosome is still unclear, as X-inactivation requires an evenly distributed presence of PRC1/2 across the Xi so as to ensure the proper silencing of both coding and noncoding transcripts [Figure 2B]. Some have indicated that the silencing of Xi is accompanied by phosphorylation events on p53, indicating XIST cooperates with the p53 DNA-repair machinery during X-inactivation<sup>[78]</sup>. Given other ncRNAs such as miR-34 are known regulators of TP53 expression in cancer cell lines as well as during development<sup>[79]</sup>, this raises the notion that lincRNAs cooperate with ncRNAs to carry out specific cellular programs. XIST also mediates epigenetic interactions between PRC1/2 and specific gene loci via interactions with chromatin modifiers such as SHARP, SAF-A,

**A: The ceRNA interaction network****B: Chromatin modifiers and transcriptional repressors****C: Chromatin folding/looping**

**Figure 2.** lncRNAs regulate transcriptional and post-transcriptional processes important in modulating gene expression. (A) Depicts the number of interactions lncRNAs have with other ncRNAs to modulate the ceRNA network. Specifically, lncRNAs can interact with miRNA recruiting these small RNAs from cognate mRNA targets. miRNAs can also compete for lncRNA or mRNA target binding depending upon the respective transcript abundance. Finally, lncRNAs can alter the stability of mRNAs either by recruiting RBPs such as HuR, or by preventing miRNA-mediated mRNA degradation; (B) represents a number of interactions that modulates the chromatin-architecture, such as MALAT1 regulation of the PRC1 complex that can modulate the euchromatin state. Additionally, XIST can recruit PRC2 to chromatin sites that preclude RNAPII chromatin binding. Finally, lncRNA ROR sponge histone methyltransferases away from heterochromatic regions, promoting transcription and (C) depicts a special chromatin modulation termed “chromosomal looping” which brings seemingly distance chromosomal regions into proximity for transcriptional control under cis-regulatory interactions. Chromosomal looping also favors additional chromatin modifications to occur at specific genomic locations. Parts of figure are adapted from Long *et al.*<sup>[66]</sup>, with permission

and LBR to initiate transcriptional silencing<sup>[77]</sup>. Taken together, it is crucial that these biochemistry-focused studies continue, such that, novel therapies can be developed to modulate a specific biological activity mediated by a particular lncRNA of interest.

**LNCRNA MECHANISM OF ACTION****lncRNAs communicate with other ncRNAs via the “ceRNA code”**

The ceRNA hypothesis, specifically the notion that RNA-RNA interactions operate in a complex regulatory pattern through competitive Watson-Crick base-pairing interactions, formed after the discovery that

**Table 2. lncRNA-RNA associations involved in metastatic signaling cascades**

lncRNA	Interacting ncRNA	Mechanism of action	Cancer type	Phenotype	References
<i>MALAT1</i>	miR-9	miR-9 downregulation of <i>MALAT1</i>	Osteosarcoma	Reduced proliferation and colony formation	[216]
	miR-1	competitive binding between miR-1, <i>MALAT1</i> , and <i>Cdc42</i>	Breast cancer	Enhanced migration and invasion	[217]
	miR-22-3p	competitive binding between miR-22-3p, <i>CXCR2</i> , and <i>MALAT1</i>	Sarcomas	Regulates angiogenesis	[218]
<i>HOTAIR</i>	miR-545	Feedback mechanism between <i>HOTAIR</i> , miR-545, and EGFR	Gastric cancer	Promotes EGFR-induced proliferation	[219]
	miR-148a	<i>HOTAIR</i> is a miR-148a sponge regulates Snail2	Esophageal cancer	Promotes EMT expression	[220]
	miR-568	<i>HOTAIR</i> epigenetically represses miR-568	Breast cancer	Promotes metastasis via enhanced angiogenesis	[221]
<i>lncRNA-ATB</i>	miR-200	<i>lncRNA-ATB</i> operates as a sponge for <i>let-7</i>	Liver and gastric cancer	Regulation of ZEB and EMT	[91,115]
	miR-372	<i>lncRNA-ATB</i> competes with miR-372 and <i>LATS2</i>	Liver cancer	Modulates PKA signaling and energy metabolism	[92]
	miR-141-3p	<i>lncRNA-ATB</i> competes with miR-141-3p and TGF- $\beta$	Gastric cancer	Alters cell-cycle arrest and tumor growth	[222]
<i>H19</i>	let-7	<i>H19</i> operates as a sponge for <i>let-7</i>	Pancreatic cancer	Increases HMGA2-mediated EMT	[215]
	miR-141	<i>H19</i> operates as a sponge for miR-141	Gastric cancer	Induces EMT through regulation of ZEB	[223]
<i>HULC</i>	miR-675	<i>H19</i> and miR-675 compete with <i>Igfr</i> and <i>Tgfb1</i> binding HuR	Prostate cancer	Regulated development and angiogenesis pathways	[224,225]
	miR-372	<i>lncRNA-ATB</i> competes with miR-372 and <i>LATS2</i>	Liver cancer	Modulates PKA signaling and energy metabolism	[92]
	miR-200	<i>HULC</i> modulates Myc expression via miR-200a as sponge	CML	Inhibits tumor growth	[226,227]
<i>lincRNA-ROR</i>	miR-205	<i>lincRNA-ROR</i> sponges miR-205	Breast cancer	Induces EMT through regulation of ZEB	[228]

EMT: epithelial to mesenchymal transition; ZEB: zinc-finger enhancer binding 1; PKA: protein kinase A

PTENP1, a particular ncRNA with similar sequence homology to the protein coding gene PTEN, functioned as a sponge for ncRNA repressors of PTEN<sup>[80]</sup> [Figure 2A]. Specifically, PTENP1 binds a number of miRNAs, such as miR-21, causing disruption of cognate miR-21-PTEN base-pairing in cells<sup>[24,80,81]</sup>. When sufficient levels of PTENP1 are present, miR-21 is sequestered by the PTENP1 pseudogene which contains homologous miR-21 binding sites similar to PTEN. This results in the elevation of PTEN transcript levels, thereby promoting a tumor suppressive phenotype as PTEN inhibits the PI3K/AKT cell survival pathway<sup>[82-84]</sup>. However, one can imagine a ping-pong effect, whereby the ratio of PTENP1-PTEN abundance changes and levels of the PTEN transcript becomes more abundant. This results in miR-21 preferentially binding to PTEN causing a de-repression of the PTENP1 pseudogene. Under normal cellular conditions one can image a balanced scenario whereby miR-21 binds to either PTENP1 or PTEN in a 1:1 stoichiometric relationship. However, under certain chronic disorders, the over-abundance of any individual pseudogene can disrupt this balance, promoting inappropriate expression of transcripts that support either pro-proliferative or pro-survival signalling pathways (i.e., via the repression of PTEN transcripts)<sup>[80,85]</sup>. Therefore, further elucidating the mechanisms of ceRNA networks specific to metastasis are warranted and requires additional study.

Studies by Karreth *et al.*<sup>[86]</sup> have investigated these ncRNA interactions on a genome-wide level, and found that the ceRNA hypothesis can be applied to any number of ncRNAs that have the capacity for Watson-Crick base-pairing with another RNA molecule, either coding or non-coding. As an example, miRNAs can bind to and promote the decay of certain lncRNAs as these transcripts also contain a 3'UTR that in many cases harbor similar sequence motifs of the neighboring mRNA transcripts (i.e., lincRNA-p21 and CDKN1A) [Table 2]. Many of the mechanisms that facilitate miRNA-lncRNA interactions are similar to those that regulate miRNA-mRNA interactions. For instance, let-7 post-transcriptionally represses the RAS



and HMGA2 oncogenes in epithelial tumors, altering the metastatic potential of these cells<sup>[87-90]</sup>. However, let-7 also binds to well established oncogenic lncRNAs such as H19 and HOTAIR<sup>[91]</sup>, which promotes post-transcriptional repression of gene targets via AGO2-mediated lncRNA degradation. let-7 also reduces lncRNA levels through a separate mechanism of RNA decay by recruiting HuR binding proteins to AU-rich regions of the targeted ncRNA transcript. Other examples of lncRNAs that are regulated via the ceRNA hypothesis include interactions with lncRNA sponges. For instance, HULC and lncRNA-ATB can bind miR-372 and miR-200 respectively, but does not result in the degradation of the lncRNA<sup>[92,93]</sup>. Rather, miR-372 binding to HULC precludes miR-372 binding to bona fide mRNA targets such as LATS2<sup>[94]</sup>. This sponging phenotype of removing an inhibitor of LATS2 expression is relevant as LATS2 itself is a tumor suppressor. Therefore, HULC along with a number of other lncRNAs function as sponges or decoys that operate together to modulate the ncRNA network important for the manifestation of a particular cellular phenotype.

### **lncRNAs modulate cell signaling pathways**

In bacteria and yeast systems researchers observed that lncRNAs associate with protein modules localized to the cellular membrane<sup>[95]</sup>, indicating lncRNAs are not only present within the cytoplasm, but also operate within functionally discrete cytoplasmic compartments. The result of these lncRNA-chaperone protein interactions is the modulation of cell signaling networks through the activation or inhibition of a particular receptor tyrosine kinase (RTK) via the recruitment of cytoplasmic kinases or phosphatases. For instance, in eukaryotic systems, Uchl1 codes for an important enzyme specifically expressed within dopaminergic neurons, and the activity of this protein is regulated by an antisense SINEB2 element as well as an antisense transcript AS-Uchl1<sup>[96,97]</sup>. Under conditions of metabolic stress, such rapamycin treatment, AS-Uchl1 subcellular localization transitions from being primarily nuclear in abundance towards cytoplasmic enrichment, with discrete foci detectable by FISH at active polysomes due to the 5' cap-independent translation of Uchl1.

In another scenario, lnc-DC is a lncRNA expressed within dendritic cells, and is a crucial component for the activation of STAT3 signaling. This modulation of STAT3 activity occurs because lnc-DC binds to SHP1-containing protein foci, preventing SHP1-STAT3 interactions, and, in turn, allowing for phosphorylation of STAT3 at residue tyrosine-705 by a number of kinases<sup>[98]</sup>. This implies that lncRNAs function as scaffolds that recruit cytoplasmic enzymes (i.e., ubiquitinases, or kinases) essential in mediating post-translational modifications of cytoplasmic proteins. These observations also raise questions as to whether lncRNAs can recruit adaptor proteins such as GRB2 to the vicinity of the carboxy-termini of membrane-bound receptors containing SH2 domains, which are responsible for the direct modulation of RTK-mediated cell signaling cascades. These observations also support an earlier hypothesis from studies on RNA viruses, concerning RNA-lipid interactions, specifically those with charged moieties including phosphatidylcholine (PC) and phosphatidylserine (PS), are crucial in supporting life<sup>[99-102]</sup>. Further work is warranted to elucidate the complexities of these lncRNAs that operate as trans regulators of cell signaling pathways.

### **ROLE FOR NCRNAS IN CANCER METASTASIS**

The role of ncRNAs in the progression of the metastatic cascade has gained interest over the past decade. Small nucleolar RNAs (snoRNAs) for instance regulate the presence of ribosomal RNA (rRNA) modifications important in modulating a number of cellular phenotypes. snoRNAs are essential modulators of pre-rRNA processing through formation of a 10-21 nt RNA duplex around a specific base-pair modification<sup>[103-105]</sup>. These modifications direct the snoRNA complex to the location of enzymatic cleavage of A-sites on the pre-rRNA molecule resulting in liberation from the rRNA processing complex. snoRNAs are also important in the regulation of the spliceosome complex and the splicing of introns across a number of RNA molecules, including mRNAs, lncRNAs, and rRNAs<sup>[106,107]</sup>. snoRNAs can also regulate mRNA molecules at single nt resolution, mostly via methylation of adenosines (i.e., m6A) that alter the post-transcriptional processing of those modified mRNAs, or via 2'-O-ribose methylation of the spliceosomal machinery. Finally, snoRNAs are

expressed from independent transcripts indicating each snoRNA gene is potentially regulated in a spatial and temporal pattern, and implies the regulatory mechanisms controlling a process such as RNA splicing is highly cell-context specific<sup>[108]</sup>. For instance, snoRNAU50 is responsible for methylating residue C248 on the 28S rRNA subunit, and has been further implicated in supporting a tumor-suppressor phenotype in breast and prostate cancer<sup>[109]</sup>. However, SNORD26 and SNORD30 are important regulators of rRNA processing, and are expressed at higher abundance in metastatic prostate tumor samples, as compared to those having low Gleason scores, presumably to support the increased demand for protein synthesis required during tumorigenesis<sup>[17,110]</sup>. These studies support the notion that ncRNAs such as snoRNAs operate in a cell-context dependent manner, and therefore the continued investigation of specific ncRNAs responsible for modulating RNA splicing events, or the addition of RNA modifications that support a favorable cellular environment for processes such as metastasis to occur, are important. Additional ncRNAs such as miRNAs also have a well described role within the metastatic cascade yet are beyond the scope of this review. Herein, we highlight the role lncRNAs play in promoting the metastatic cascade across a variety of cancer models<sup>[45,111,112]</sup>.

lncRNAs have been reported to control one of the most well described processes within the metastatic cascade, namely the loss of E-cadherin expression on epithelial cells. Loss of E-cadherin expression is crucial in ensuring proper epithelial cell-cell adhesion is maintained, as cell-cell connections are present so as to support a state of quiescence within differentiated epithelial tissue<sup>[113]</sup>. Evidence supporting this notion includes studies assessing the mutational inactivation of E-cadherin or the elucidation of mechanisms underlying the post-transcriptional repression of E-cadherin mRNA levels<sup>[114]</sup>. Taken together, observations by numerous investigators support a widely accepted hypothesis that loss of cellular polarity through the disruption of cell-to-cell or cell-to-extracellular matrix (ECM) contacts is required for the initiation of metastasis.

Specific lncRNAs crucial in controlling E-cadherin abundance include FEZF1-AS1, which is found to be dysregulated in non-small cell lung cancer (NSCLC) samples, as compared to adjacent normal tissue samples<sup>[115]</sup>. FEZF1-AS1 is also highly expressed in poorly differentiated tumor tissues as well from as those of advanced tumor stage. FEZF1-AS1 abrogates the expression of E-cadherin by directly competing for LSD1 binding, which disrupts the required association between E-cadherin and the LSD1/EZH2 complex necessary for reducing turnover of the E-cadherin molecule itself. Therefore, lncRNAs operate not only as sponges or decoys that modulate the RNA network within a cell, but also as disruptors of cytoplasmic protein complexes essential in maintaining cellular polarity.

Another example of a lncRNA that regulates E-cadherin abundance includes lncRNA-ATB, which promotes the invasion of colorectal cancer cells after TGF- $\beta$  activation. This is a relevant mechanism to study, as lncRNA-ATB harbors clinicopathologic significance, and correlates with tumor stage, as well as the presence of metastatic foci within the sentinel lymph node and/or at distant organ sites. Furthermore, lncRNA-ATB associates with reduced overall- and disease-free survival within colon cancer patients<sup>[116]</sup>, and is elevated in the serum of patients post-surgery, indicating lncRNAs are present in circulating biofluids and function as biomarkers for tumor progression. Overall, with the advent of genome-wide transcriptomic studies, consortiums such as ENCODE<sup>[117]</sup> and TCGA<sup>[27]</sup> have amassed a vast array of information that investigators can utilize to elucidate how a particular lncRNA can modulate a series of RNA interaction networks involved in the attenuation of metastatic phenotypes within a cell. Given this effort, there are a number of newly identified lncRNAs strongly associated with metastasis that have the potential to be clinically relevant readouts for this biological process. Here, we report on several lncRNAs that play an important role in the metastatic process<sup>[118-120]</sup>.

### **The new linc's on the block**

Since 2012, the number of studies highlighting lncRNA involvement within the metastatic process has increased nearly 20-fold to approximately 200 manuscripts being reported in Pubmed.gov this year. Half of

these papers discuss how particular lncRNAs regulate the biochemical steps crucial for the initiation and maintenance of metastatic dissemination. These new lncRNAs are intriguing entities to study, as they have putative tumorigenic activity across a number of epithelial tumors, and are expressed at levels sufficient enough for investigators to perform both gain- and loss-of-function studies, and to assess the phenotypes that result upon lncRNA dysregulation.

Elucidating the role of these lncRNAs could further illuminate our understanding of the regulatory processes involved in the initiation of cellular depolarization and motility, as well as the crucial genetic factors required for metastatic dissemination. Below, we highlight examples of a few lncRNAs that regulate important cellular activities in epithelial tumors, which could be utilized for the development of new therapeutics for patients with metastatic disease.

### H19

H19 is a 2.3-kb oncofetal lncRNA gene derived from the IGF2 locus important in regulating cellular differentiation programs during development, including maternal imprinting<sup>[120]</sup>. While H19 is expressed from only one parental allele, robust levels of H19 are present during embryonic development, which is rapidly downregulated postnatally<sup>[121-125]</sup>. Improper H19 gene dosage compensation due to the lack of maternal imprinting results in embryonic lethality in mice, associates with certain clinical manifestations of those with Beckwith-Wiedemann syndrome, and correlates with an increased risk of developing Wilms tumor of the kidney<sup>[126-129]</sup>. H19 is also highly expressed in a number of tumors, and supports metastases by antagonizing ncRNAs and epigenetic regulators, such as chromatin modifiers crucial in maintaining epithelial polarity<sup>[130]</sup>. A recent study indicated that a single nucleotide polymorphism (SNP), rs2107425 located within an intron of the H19 gene, was associated with reduced metastatic free survival. This SNP does not affect the abundance of H19 in breast cancer patients, as compared to those not harboring the rs2107425 variant. Instead, this SNP alters the activity of H19 either by preventing binding to a cognate ncRNA or RBP responsible for modulating metastatic processes, or by promoting an alternative splicing event resulting in the modulation of the ceRNA network<sup>[131]</sup>.

H19 also has a direct role in regulating the cellular processes of invasion and angiogenesis crucial for the progression of metastatic disease. For instance, H19 associates with the TF enhancer of zeste homolog 2 (EZH2) in turn downregulating the expression of gatekeeper genes such as E-cadherin and adenoma polyposis coli (APC)<sup>[131,132]</sup>. H19 also supports constitutive WNT signaling by inhibiting the activity the WNT-antagonist Nkd1. Given Nkd1 inhibits WNT activity, it is plausible H19 is a crucial regulator of an autoregulatory feedback loop important in preventing the stochastic expression of WNT family members. The importance of WNT signaling as regulators of metastatic progression are discussed later in this review. However, Nkd1 itself is a specific regulator of clock and is regulated in an oscillatory manner by a number of factors. This also implies H19 synergizes with WNT/NKD1 signaling to regulate the circadian rhythm pathways essential for proper vertebrate embryogenesis, but also the molecular clock genes that provide important spatial information for the inappropriate re-activation of embryonic genes that induce tumorigenic processes such as proliferation, invasion, angiogenesis, as well as EMT<sup>[133,134]</sup>.

Understanding the regulation of H19 is important as certain types of cancer are dependent upon H19. In fact, BC-819 is an approach utilizing a plasmid expression system coding for diphtheria toxin under the control of an H19 regulatory sequence. Intratumoral injection of BC-819 *in vivo* as well as intraperitoneal (IP) injection of the compound in ovarian cancer patients is undergoing phase I/II clinical trials and shows promise at extending survival rates by reducing tumor burden<sup>[135-137]</sup>. Additional clinical trials include the ectopic expression of BC-819 via intravesical instillation in bladder cancer patients, and BC-819 vaccination in combination with gemcitabine for those with pancreatic adenocarcinoma. While both trials show promise as an effective approach to deliver lncRNAs in cancer patients<sup>[135-137]</sup>, it will be interesting to determine the

specificity of BC-819 in mitigating the number of metastatic foci detectable in these patients or if this therapy extends tumor latency.

### **SPRY4 intronic transcript 1**

Recently, SPRY4 intronic transcript 1 (SPRY4-IT1) was reported as a novel lncRNA crucial in regulating the initiation and progression of EMT across a number of epithelial carcinomas<sup>[138]</sup>. SPRY4-IT1 is transcribed from an intronic region within the SPRY4 gene and is approximately 708bp in length. SPRY4-IT1 is unique in that this lncRNA contains several known hairpin structures that associate with particular RBPs<sup>[139]</sup>. Therefore, investigators have some notion regarding the mechanisms by which SPRY4-IT1 controls cellular processes important in supporting a metastatic phenotype and includes altering the expression of regulatory genes such as MCM2, XIAP, LPIN2<sup>[138]</sup>. Functional studies indicate that aberrant expression of SPRY4-IT1 also modulates the migratory and invasive capacities across a number of *in vitro* cancer models and does this in part by regulating DNA repair genes, such as MDM2 and CDK1.

SPRY4-IT1 also controls the process of EMT through the modulation of intermediate filament proteins, such as fibronectin and vimentin, resulting in the fine-tuning of the molecular inputs initiated by Snail and TGF- $\beta$  localization and activity. Specifically, in esophageal squamous cell carcinoma (ESCC), the overexpression of SPRY4-IT1 disrupts the nuclear localization of Snail, and facilitates TGF- $\beta$ -induced EMT<sup>[140-142]</sup>. Mechanistically, it is still unclear as to how SPRY4-IT1 reduces the expression of epithelial cadherins (i.e., E-cadherin), while subsequently promoting the expression of neuronal or mesenchymal cadherins (i.e., N-cadherin). SPRY4-IT1 is known to modulate gene activity via dampening global H3K27me3 distribution, which is not in of itself not biologically informative; however, SPRY4-IT1 can induce H3K27me3 methylation of the EZH2 gene promoter, and subsequently promote the repression of E-cadherin gene expression<sup>[143]</sup>. This post-transcriptional chaperone activity of chromatin modifying complexes to particular gene loci is a typical feature of lncRNAs and is crucial in dampening unwarranted transcripts during cellular development.

It would also be interesting to determine if SPRY4-IT1 interacted with a selective subset of miRNAs that supported the development of angiogenesis. For instance, miR-126 inhibits tumor growth and results in decreased micro-vessel density in cervical cancer<sup>[144,145]</sup>. SPRTY-related proteins, such as SPRED1 have been reported to bind to miR-126 and control tumor neo-angiogenesis, as well. Therefore SPRY4-IT1 may be a crucial component of the ncRNA network responsible for tumor neo-angiogenesis.

### **Highly upregulated in liver cancer**

Highly upregulated in liver cancer (HULC) was first identified in hepatocellular cancer and is highly expressed in liver cancer, as well as in a number of carcinomas that metastasize to the liver, including colon and breast cancer<sup>[146,147]</sup>. Two recent studies suggest that HULC promotes angiogenesis as well as neo-angiogenesis, essential processes in the progression of metastasis as micro-metastatic lesions require an oxygen-rich environment to meet the demand of tumor growth within an hypoxic environment. For instance, Zhao *et al.*<sup>[148]</sup> found that overexpression of HULC in hepatocellular cancer cells results in an increase in the pro-angiogenic factor SPHK1. Specifically, HULC functions by sequestering miR-107, a bona fide target of which is the TF E2F1. The derepression of E2F1 results in the enhanced transcription of SPHK1, and by extension enhanced rates of vessel tube formation as well as increased tumor burden in a murine xenograft model. This HULC-specific upregulation of angiogenic processes was further tested in a chicken chorioallantoic membrane (CAM) assay, whereby condition medium from HULC overexpressing cells promoted increased growth of vessels within the chicken embryo.

Additional mechanisms by which HULC induces angiogenesis includes the sponging of miR-372 away from genes important in modulating the growth and survival of cancer cells. Additionally, HULC itself is transcriptionally upregulated by pro-metastatic growth factors, receptors, and RBPs including IGF2 mRNA-

binding protein 1 (IGF2BP1), as well as members of the protein kinase A (PKA) signalling pathway<sup>[146,149]</sup>. Going forward, HULC shows promise as a therapeutic target for patients with metastatic disease, and therefore further investigation is warranted.

### Estrogen receptor regulated lincRNA 01

The role for lncRNAs regulating hormone-signaling pathways related to the metastatic cascade are less well understood. In general, 17 $\beta$ -estradiol is known to regulate the activity and abundance of TGF $\beta$  signaling<sup>[150,151]</sup>, as well as modulate the levels of E-cadherin<sup>[152]</sup>. Together, 17 $\beta$ -estradiol, and moreover active ER $\alpha$  signaling are crucial in maintaining an epithelial phenotype by suppressing the pathways associated with EMT. 17 $\beta$ -estradiol/ER $\alpha$  signaling also controls the activity of certain ncRNAs, such as the miR-200 family of miRNAs, which regulates EMT promoting TF regulators such as ZEB1 and Smad interacting protein 1 (SIP1)<sup>[31]</sup>. This indicates steroid hormone signaling pathways can modulate ncRNA networks responsible for tumorigenesis and metastatic dissemination. For instance, our group identified lncRNA estrogen receptor regulated lincRNA 01 (ERRLR01) as a prognostic biomarker in breast cancer, which is regulated by ER $\alpha$  activity in breast cancer tumors. Specifically, ERRLR01 is highly expressed in triple-negative breast cancers, yet not in samples derived from patients with ER $\alpha$ + tumors. Follow up experiments indicated 17 $\beta$ -estradiol also altered the levels of ERRLR01 in ER $\alpha$ + cells lines (i.e., MCF-7 and T47D)<sup>[25,153]</sup>.

Given 17 $\beta$ -estradiol is a crucial regulator of EMT<sup>[28]</sup>, we surmise ERRLR01 and other lncRNAs are crucial mediators of the metastatic cascade. Another example of a hormone-sensitive lncRNA is linc00461, which modulates the activity of CREB, a known 17 $\beta$ -estradiol regulated TF. Interestingly, linc00461 interacts with miR-9 as a sponge releasing miR-9 from its cognate mRNA targets, thereby altering the activity of CREB, and subsequently modulating the proliferation and migration of glioma cells<sup>[154]</sup>. linc00461 also regulates tumorigenic and metastatic phenotypes in melanoma cells<sup>[155]</sup>, therefore further work elucidating the mechanism of action for linc00461 is warranted.

### Colon cancer associated transcript 2

The lncRNA colon cancer associated transcript 2 (CCAT2) was first discovered via genome-wide SNP-association studies whereby investigators determined if particular genomic variants associated with cancer incidence<sup>[156]</sup>. Previously many SNPs remain understudied because they occur within the ncRNA region of the genome. With our current understanding of the ncRNA landscape, new variants are being reassessed for functional significance in cancer. SNP, rs6983267, was of particular interest as this variant maps to the 8q24 region of the genome, which correlates with higher incidences of a number of epithelial tumors, including colorectal, prostate, ovarian, and inflammatory breast cancer<sup>[157]</sup>. Subsequent studies indicate CCAT2 levels are expressed at higher frequencies in tumor samples from colorectal cancer (CRC) patients with metastatic disease<sup>[111,156,158,159]</sup>. CCAT2 is also highly expressed in small cell lung cancer (SCLC) samples and is highly correlated with poor prognosis, as well as the presence of metastasis, signifying CCAT2 is an independent prognostic biomarker and/or therapeutic target for a disease with limited therapeutic options.

The regulatory mechanisms by which CCAT2 controls the progression of metastatic events is still unclear. Gain- or loss-of-function studies demonstrate that CCAT2 can modulate the proliferation and invasion potential of cancer cells *in vitro*, as well as the number of micro-metastatic lesions at distant organ sites utilizing murine xenograft models. However, the only reported cellular mechanism of action by which CCAT2 alters the metastatic potential of cells involves WNT signaling. The WNT gene family are crucial regulators of metastatic progression as WNT coupling to Frizzled receptors on the cell surface allows for the release of  $\beta$ -catenin from the GSK-3 $\beta$  ubiquitination complex<sup>[160,161]</sup>.  $\beta$ -catenin then enters the nucleus and operates as a transcriptional co-activator along with TCF/LEF, which together promotes the transcription of genes supporting metastatic progression. Here, CCAT2 overexpressing cell lines harbor increased WNT activity, while siRNAs directed towards CCAT2 reduced both the nuclear and cytoplasmic abundance of



$\beta$ -catenin, which in turn resulted in reduced CCND1 and MYC protein expression<sup>[162-168]</sup>. Moreover, the phenotypes observed under CCAT2 knockdown conditions operated synergistically with small molecule WNT inhibitor, FH-535. These studies indicate CCAT2 imparts a specific regulatory function through the augmentation of the WNT signaling pathway, and as such, contributes to a pro-metastatic phenotype.

### Myocardial infarction associated transcript

The lncRNA myocardial infarction associated transcript (MIAT) has been linked to several chronic disorders including myocardial infarction<sup>[169,170]</sup>, paranoid schizophrenia<sup>[171]</sup>, and neuroendocrine-derived prostate cancers<sup>[110]</sup>. MIAT interacts with a number of epigenetic modifiers in neuronal crest cells including PRC1/2 and ZEB1 that when altered results in the modified migratory capacities of these cell lines<sup>[172]</sup>. MIAT is also disrupted in a number of cancer cell lines, and is expressed at significantly lower levels in grade I-II breast tumors, as compared to those considered high grade III-IV tumors<sup>[110]</sup>. MIAT also modulates the invasive capacities of breast cancer cells by mediating the ncRNA interaction networks between ZEB1 and certain miRNAs, such as miR-150 and miR-29<sup>[173]</sup>. For instance, knockdown of MIAT promotes the expression of miR-150, yet also results in the reduction of miR-29 levels. This results in breast tumor cell lines transitioning from a pro-proliferative state towards a more quiescent yet migratory phenotype<sup>[72,174,175]</sup>. There are two mechanisms by which MIAT could alter these miRNA interaction networks. The first is that MIAT functions as a ceRNA and operates as a sponge for miR-150, reducing the bioavailability of miR-150 to interact with cognate mRNA transcripts such as ZEB1. The second is that MIAT operates as a chaperone or scaffold that recruits co-repressor complexes to the promoter of the MIR150 host gene, subsequently reducing the transcriptional output of miR-150. While both scenarios may not be mutually exclusive from one another, these regulatory interactions reinforce the notion that EMT is a highly controlled cellular program responsible for modulating the expression of E-cadherin, which is initiated by several key transcriptional repressors including ZEB1<sup>[176,177]</sup>. Interestingly, the resulting consequence of the MIAT-miR-150 interaction is the increased expression of ZEB1 and, in turn, a pro-metastatic phenotype, through the transcriptional upregulation of the MIR29 host gene. miR-29 promotes the invasive capabilities of cells by diverting the energy demand required for cellular proliferation and redirecting those energies towards signaling pathways that encourage motility and invasion<sup>[178,179]</sup>. This occurs in part by modulating the expression of cell-cycle checkpoint genes such as CDKN2A. Therefore, MIAT represents a characteristic example of how lncRNAs can modulate the activity of RNA-RNA interactions by controlling the bioavailability of other ncRNAs, in turn, reprogramming cellular signaling cascades to support a pro-metastatic phenotype.

### BMP/OP-responsive gene

Recently, the lncRNA BMP/OP-responsive gene (BORG) was identified to play a vital role in augmenting proliferation and survival cues within breast cancer cells<sup>[71,180]</sup>. Specifically, BORG interacts with the TRIM28 TF, which modulates the transcriptional co-repression of Cdkn1a and Gadd45a<sup>[181,182]</sup>. The presence of this BORG-TRIM28 binding complex is also linked with shorter tumor latency within breast cancer patients and correlates with a faster outgrowth of cancer cells in 3D culture systems. TRIM28 can also function as a transcriptional activator or repressor depending upon the chromatin architecture or extent of heterochromatinization within the nucleus. BORG localizes predominantly to the nucleus and has a unique function as it reinforces the repressive actions associated with TRIM28. As an example, repression of BORG in metastatic D2.A1 breast cancer cells prevents migratory outgrowths within 3D matrigel culture systems, as well as the abundance of micro-metastatic colonies in lung tissue utilizing an invasive breast cancer transplant model<sup>[180]</sup>. Furthermore, in aggressive metastatic breast cancer the expression of BORG is higher as compared to samples derived from non-malignant mammary tissues. Therefore, BORG clearly modulates the invasive capacities of breast cancer cells.

While RNA immunoprecipitation (RIP) experiments indicate that TRIM28 in fact requires BORG for binding to specific gene promoter regions, such as those neighboring Cdkn1a and Gadd45a<sup>[180]</sup>, it is still

unclear as to how BORG modulates TRIM28 binding to specific chromatin regions that in turn modulates a metastatic phenotype. One assumption is that specific sequence regions of BORG, outside the identified TRIM28 binding sequence, interacts with TRIM28 or additional TRIM domain-containing TFs through stacking interactions. These lncRNA structure-based interactions, mediate recruitment of the TRIM28 transcriptional protein complex to the proximal promoters of genes such as *Cdkn1a* and *Gadd45a*. BORG also confers a unique transcriptional signature that is enriched for KRAS signaling, as compared to non-metastatic D2.OR breast cancer cells. Further studies elucidating the role of BORG in human breast cancer cells, as well as the regulatory role within the metastatic process, are warranted.

### Prostate cancer associated transcript 1

Through genome wide RNA sequencing experiments, Prensner *et al.*<sup>[183]</sup> identified prostate cancer associated transcript 1 (PCAT1) as a lncRNA highly upregulated in metastatic prostate cancer samples, as well as those considered high grade (i.e., stage II-IV). Upon knockdown of PCAT1 in prostate cancer cell lines, Prensner *et al.*<sup>[183]</sup> identified 370 genes expressed differentially, many of which were associated with cell-cycle progression and mitosis, as well as cytoskeleton and microtubule regulation. Knockdown studies indicated that loss of PCAT1 resulted in an approximate 25% reduction in cellular proliferation, though the mechanism by which PCAT1 promotes an invasive phenotype is still unclear. One possibility may be due to the involvement of PCAT1 in the homologous recombination (HR) repair pathway. One can surmise, for instance, that as epithelial progenitor cells proliferate, the acquisition of successive mutations within the genome across daughter generations provides an opportunity for such cells to undergo a process such as EMT<sup>[12]</sup>. Interestingly, PCAT1 is inversely correlated with BRCA2 expression in LNCaP cells, while the knockdown of PCAT1 resulted in the upregulation of BRCA2, a crucial component of the DNA repair pathway<sup>[184,185]</sup>. Moreover, PCAT1 overexpression alters the formation of RAD51 and  $\gamma$ -H2aX foci after radiation-induced DNA damage, while naturally occurring polymorphisms within the genome, such as rs7463708, can promote an enhanced proliferative and migratory state within prostate cancer cell lines<sup>[186]</sup>. Therefore, it is entirely plausible that the reduction in chromosome stability via enhanced PCAT1 activity supports not only a pro-tumorigenic state, but a pro-metastatic phenotype as well. Separating these two distinct yet equally important mechanisms will be crucial in developing novel therapeutics to treat those with advanced prostate cancer.

### CONCLUSION

Overall, lncRNAs play a multifaceted role in controlling the ncRNA network, which is vitally important throughout embryogenesis and vertebrate development. Here we discussed the ways in which lncRNAs can function as metastatic regulators, primarily by controlling epigenetic mechanisms, such as the recruitment of co-repressor complexes including PRC1/2, as well as co-transcriptional complexes such as CREB/REST to specific chromatin regions. Therefore, lncRNAs represent a unique class of ncRNA that operate as scaffolds to bring specific chromosomal foci into proximity with epigenetic regulators and chromatin modifiers. lncRNAs also control the appropriate expression of the DNA methylation machinery such as DNMT1, and function as competitive binding partners for other ncRNAs with complementary sequences. As such, lncRNAs serve as potent disruptors of conserved RNA-RNA regulatory networks.

Interestingly, lncRNA sequences are not highly conserved across species, however lncRNAs harbor a conserved positional synteny that is linked with the regulatory function of that specific lncRNA. This presents a unique challenge for the lncRNA field in that determining the importance of a lncRNA molecule found to be differently expressed under certain experimental conditions cannot be further studied by assessing the conservation of the sequence. Investigators will require a more nuanced approach in studying the landscape of the surrounding genomic architecture, the proximity of certain DNA response elements, and if specific protein coding genes flank the lncRNA, while also keeping in mind the state of the surrounding chromatin architecture and determining if the DNA region is highly hetero- or eu-chromatinized [Figure 2C].

This also means that the function of a particular lncRNA using a mouse model of metastasis for instance, does not always imply that the mechanism of action of those lncRNAs function similarly in human cells. For instance, the use of genetically engineered mouse models (GEMMs) that have a propensity to develop metastasis may provide some useful information yet may not provide a complete picture regarding the mechanisms by which a specific lncRNA promotes metastasis in human systems. Therefore, additional technologies will be required to assess the functionality of metastatic-specific lncRNAs. For instance, the use of humanized mouse models, 3D culture systems, and use of conditionally reprogrammed cells from human tissue, all will aid investigators in determining the bona fide relevance of functionally conserved lncRNAs.

In many cases lncRNAs are expressed at lower abundance than cytoplasmic mRNA, thereby making it difficult to assess whether lncRNAs are functionally relevant, or present as a result of leaky transcriptional activity. As an example, lncRNAs can regulate the processing of nascent transcripts generated from RNA polII-based transcription. These lncRNAs may only number a few copies in the cell at any given time yet can bind in a 1:1 stoichiometric relationship with the nascent mRNA altering the stability of the newly synthesized RNA molecule. Technologies such as global run on sequencing<sup>[187]</sup>, which is a sensitive and high throughput type of nuclear-run on assay, have been developed to specifically determine the relevant abundance of a particular lncRNA binding these nascent transcripts. Additionally, techniques such as high-throughput sequencing of RNAs isolated by crosslinking immunoprecipitation (CLIP)<sup>[188]</sup> and cross-linking, ligation and sequencing of hybrids (CLASH)<sup>[189]</sup> have been utilized to gauge the abundance, enrichment, and/or composition of ncRNAs within particular RNA-RBP cytoplasmic complexes. As sequencing technology develops and the cost to perform these analyses decrease, the utilization of these biochemical approaches coupled with these high-throughput sequencing methods will pave the way for new discoveries regarding lncRNA function.

Despite these challenges, it is clear that lncRNAs play a crucial role in driving a metastatic phenotype, and in particular regulate the initiating steps of metastasis such as EMT. Given EMT is the process of cell fate switching, or reactivation of embryogenic programs that convert epithelia cells to those harboring a mesenchymal phenotype, the continued approach of utilizing reductionist-based investigations within well-defined model systems will help in elucidating the mechanisms by which individual lncRNAs regulate the underlying biology of metastasis. Given the advances in sequencing technology as well as a renewed scientific interest in lncRNA biology, the number of publication discussing the role of lncRNAs in metastasis will most likely double in the next year. The continued demand for reliable biomarkers of metastasis will also fuel research towards the development of prognostic and predictive indicators for patients with high grade tumors harboring metastatic dissemination. In conclusion, the lncRNA field is certainly in its infancy, yet is considered to be the wild-west of the post-genomic era and has the potential to unlock the key to some of the most prevalent challenges associated with treating patients with metastatic disease.

## DECLARATIONS

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Brian D. Adams is President/CEO of The Brain Institute of America and holds patent interests with AUM LifeTech. Authors have no other conflicts of interest to disclose. The authors declare that the research was conducted in the absence of any commercial or financial activities.

### Patient consent

Not applicable.

### Ethics approval

Not applicable.

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Letter

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## Hypoxia in prostate cancer

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Dear Editor,

I have read with great interest the review “Current challenges and opportunities in treating hypoxic prostate tumors” by McKenna *et al.*<sup>[1]</sup>. In this review, the authors present, as a key information in Table 1 of their article, values of oxygen partial pressures ( $pO_2$ ) in human tumors and the respective normal tissues, published earlier by our group<sup>[2,3]</sup> and “adapted” by McKeown<sup>[4]</sup> later.

In their article, McKenna *et al.*<sup>[1]</sup> have reviewed current knowledge about the impact of the “hallmark feature” hypoxia on pathways promoting cancer growth, malignant progression, therapeutic resistance and tumor immune escape<sup>[5-7]</sup>. Certainly, this information is of utmost interest to experimental and clinical oncologists. However, since this review contains some misleading/inappropriate oxygenation data, some additional information that may be of interest for the distinguished readership of this highly reputed journal, may serve for clarification.

In Table 1 of their review, McKenna *et al.*<sup>[1]</sup> present oxygen partial pressure ( $pO_2$ ) values together with oxygen concentration ( $cO_2$ ) data. When reviewing the biological role of hypoxia in malignant tumors, authors lacking an expertise in respiratory physiology often convert - without any need - the *in vivo*  $pO_2$  values, originally measured in tumors (and in normal tissues) using  $pO_2$  histography<sup>[2]</sup>, into  $O_2$  concentrations using either Dalton's law (only valid for gas mixtures within the airways) or Henry's law for gases dissolved in solutions, which cannot describe the relationship between partial pressures and concentrations of gases in heterogeneous media (e.g., tissues with lipid-rich membranes, the cytosol and the extracellular space, the latter with a high content of free water in cancers). Therefore, it is strongly suggested to avoid any conversion of measured  $pO_2$  values into  $cO_2$  data since the  $O_2$  solubility coefficient is: (1) highly dependent on the tissue water content; and (2) usually not known for heterogeneous cancer tissues in patients. In this context, it has



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to be mentioned that authors not familiar with respiratory physiology often use “local O<sub>2</sub> concentrations” by mistake, although pO<sub>2</sub> values have been measured in the original studies<sup>[2,3]</sup> (for typical examples see Table 1 in the review by McKenna *et al.*<sup>[1]</sup>).

Considering Henry’s law ( $cO_2 = \alpha \times pO_2$ ;  $\alpha$ : oxygen solubility coefficient), McKenna *et al.*<sup>[1]</sup> have communicated questionable oxygenation data grounded on wrong/doubtful O<sub>2</sub> solubility values for malignant and normal tissues, which originally have been communicated for blood plasma, i.e., irrelevant data when heterogeneous tissues such as prostate cancer are considered<sup>[8]</sup>.

Oxygen solubility coefficients for heterogeneous tissues (e.g., for experimental tumors<sup>[9]</sup>) are significantly lower than those for blood or blood plasma<sup>[10]</sup>. Due to this misconception, the O<sub>2</sub> concentration data of Table 1 in the review by McKenna *et al.*<sup>[1]</sup> are misleading/not correct and should, therefore, be removed from the table. There is no need to present concentration data in this comprehensive review.

## DECLARATIONS

### Authors’ contributions

Vaupel P contributed solely to the paper.

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### Conflicts of interest

There are no conflicts of interest.

### Patient consent

Not applicable.

### Ethics approval

Not applicable.

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Original Article

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# The combined analysis of solid and liquid biopsies provides additional clinical information to improve patient care

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## Abstract

**Aim:** To investigate if the genetic information provided by sequencing of both solid and liquid biopsies can shed light on tumor heterogeneity, and to understand the clinical usefulness of adding blood profiling to standard tissue analysis in cancer care.

**Methods:** Data from 351 patients with stage IV solid tumors for whom molecular profiling of their solid and liquid biopsies was available were studied, with a focus on the discrepant molecular information found between tissue and blood samples.

**Results:** In 86% of patients, solid and liquid biopsies provided different molecular information. Discrepant gene mutations with a functional impact on the corresponding protein were studied in detail. In 97% of cases, these additional mutations provided clinical value, mainly predicting sensitivity or resistance to targeted therapies. Specifically, 42% of the mutations found only in the liquid biopsy were directly predictive of approved therapies (80% targeted therapies), while 54% were inclusion criteria for molecularly-matched trials.

**Conclusion:** This study suggests that the addition of blood profiling should be considered in routine clinical oncology, especially for patients with metastatic disease where integrated analysis of solid and liquid biopsies provides a more complete characterization of tumor heterogeneity and provides valuable clinical information for patient treatment.

**Keywords:** Molecular profiling, solid tumor, liquid biopsy, solid biopsy, tumor heterogeneity, next-generation sequencing, precision medicine, targeted therapies



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## INTRODUCTION

In recent years, the field of cancer therapy has evolved from a “one-size-fits-all” approach towards precision medicine, where therapeutic options are tailored specifically to each patient. This patient-tailored strategy is based on the molecular characterization of the tumor through biomarker analysis using tumor biopsy samples<sup>[1]</sup>. It is becoming clear that genetically different tumor subtypes need to be treated with distinct targeted approaches<sup>[2]</sup>, for example the monoclonal antibody trastuzumab in HER2-positive breast cancers or vemurafenib in BRAF (V600E)-positive melanoma. Nonetheless, the use of targeted therapies is limited by either the presence of primary resistance or the development of acquired treatment resistance<sup>[2]</sup>, and tumor heterogeneity has been clearly associated with such resistance<sup>[3]</sup>. The advent of deep sequencing studies has demonstrated that human cancers display both temporal (different genetic events taking place during the disease course) and spatial intratumor heterogeneity, harbouring subclones with both shared and unique genomic aberrations<sup>[4]</sup> that respond differently to targeted therapy. Spatial discrepancy can be explained by clonal heterogeneity within the primary tumor and by the presence of metastasis. Driven by the Darwinian model, during the metastatic process a selection of the “most efficient” clones occurs, due to external forces such as the treatment given to the patient or the tumor environment, for example the presence of hypoxia<sup>[5]</sup>. It has been reported that tumors with high levels of clonal heterogeneity may show poor prognosis<sup>[6]</sup>.

As mentioned above, heterogeneity in cancer contributes to primary and acquired resistance<sup>[3]</sup>, and that is why approaches providing a global vision of the genomic landscape of the tumor are important for selecting the most appropriate targeted therapy for each patient. Although solid biopsies are the standard way of tumor characterization and will continue to play a central role in cancer management<sup>[7]</sup>, they show some limitations. One of them is that they may not capture tumor heterogeneity, as the aberrations found in a single solid biopsy can be different depending on the area where the sampling was performed, and this could lead to a biased characterization of the tumor that would influence therapy decision<sup>[4]</sup>. Fortunately, this limitation can be partially overcome by the use of liquid biopsies, such as the free circulating tumor DNA (ctDNA) in blood. ctDNA belongs to the pool of the total cell-free DNA (cfDNA) molecules; in individuals without cancer, the concentration of cfDNA is low, but tumor patients generally have significantly higher levels of cfDNA because of the high turnover of cancer cells. The ctDNA contains DNA mutations of both primary and metastatic lesions<sup>[7]</sup>, since it is released from multiple tumor regions. Therefore, one potential advantage of ctDNA over tissue biopsies is the detection of molecular heterogeneity<sup>[8]</sup>; as such, ctDNA can harbour mutations that are undetected in the corresponding solid biopsy<sup>[9]</sup>.

In this study we set out to determine the different genetic information revealed by solid and liquid biopsies, and examine the clinical utility of adding ctDNA profiling to the information obtained through tissue biopsies. To this end, we analysed data from 351 patients who had been previously characterized through sequencing of tissue and ctDNA samples.

## METHODS

### Patient population

This work is a retrospective study evaluating 351 patients with stage IV solid tumors whose tissue and blood samples were tested from May 2016 to November 2017 using the OncoSTRAT&GO™ profiling solution (OncoDNA SA, Gosselies, Belgium), and who had failed at least one line of therapy before undergoing molecular profiling. In all cases the oncologist suggested this solution to the patient, who gave informed consent for the tumor analysis data to be published. For objectivity, all samples were included in our analysis without prior selection for age, cancer type, treatment, profiling results or follow-up data.

### Samples

Matched tissue and blood samples from different tumor types were included in the analysis. The cancer types studied comprise breast (19.9%), colorectal (11.7%), lung cancer (11.4%), sarcoma (7.7%), ovarian (6.3%),



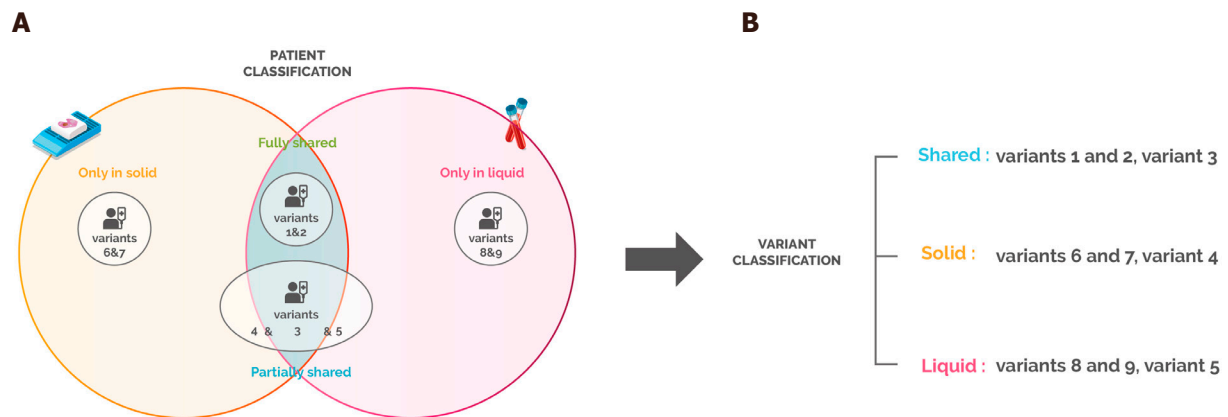
prostate (6.3%), hepatobiliary (5.7%), brain (5.1%), pancreatic (5.1%), gastric (4.6%), carcinoma of unknown primary (3.4%), head and neck cancer (1.7%) and other cancers (11.1%). Solid biopsies were obtained as a formalin-fixed paraffin-embedded (FFPE) block from either the primary or the metastatic tumor and underwent review by a pathologist to determine if the criteria for sample acceptance were met: tumor tissue > 10% of the whole sample and tumor size > 5 mm<sup>2</sup> in order to have enough tumor material, and lymphocyte invasion < 20% in the region where the tumor cells were located to avoid lymphocyte DNA contamination. In addition, tissue samples were macro-dissected to remove contaminating normal tissue. Blood was collected in two Cell-Free DNA BCT® CE tubes (Streck, La Vista, USA) and underwent visual inspection to determine that no hemolysis had occurred.

### **Sample preparation and next-generation sequencing**

DNA was extracted from FFPE tissue and cfDNA was extracted from blood using the Qiagen DNA FFPE Tissue Kit or Qiagen DNA Blood Mini Kit (Qiagen, Valencia, USA) respectively. DNA quantity was measured using the Qubit 2.0 Fluorometer (Thermo Fisher Scientific, Waltham, USA). To identify somatic alterations in tumor samples, we used our proprietary solution OncoSTRAT&GO™ (OncoDNA, Gosselies, Belgium). This solution is built based on the Oncomine Comprehensive Panel v2 designed by ThermoFisher, which we updated in order to analyse genome regions or genes not included in that version but shown to be important in cancer. OncoSTRAT&GO™ is based on AmpliSeq technology, amplifying for next-generation sequencing (NGS) whole exons and hotspot mutations of 192 genes (gene panel for the solid biopsy part of OncoSTRAT&GO™) or hotspot mutations of 27 genes (gene panel for the liquid biopsy part of OncoSTRAT&GO™). Briefly, the targeted sequencing libraries were generated using the Ion AmpliSeq Library kit 2.0 according to the manufacturer's instructions (Thermo Fisher Scientific, Waltham, USA). The starting material consisted of 10 ng DNA from FFPE or blood samples per pool of amplification (4 for the solid part and 2 for the liquid part, respectively). The primers used for amplification were partially digested by Pfu enzyme. The product of digestion was then ligated with corresponding barcoded adapters and purified using Ampure Beads (Beckman Coulter Inc., Indianapolis, USA). The product of purification was amplified for 5 more cycles and subsequently re-purified using Ampure Beads to generate the library sample. The quality of the libraries was assessed using the Qubit dsDNA HS Assay kit (Thermo Fisher Scientific, Waltham, USA). Ten pmol/L of each library was loaded into the IonChef system (Thermo Fisher Scientific, Waltham, USA) for the emulsion polymerase chain reaction. Libraries were then loaded into the sequencing chip that was placed in either the Personal Genome Machine, the Proton or the 5XL device (Thermo Fisher Scientific, Waltham, USA) depending on the required throughput. An average coverage of 1000× was generated in order to detect single-base substitutions down to 5% for the FFPE, and of 10000× to detect base substitutions down to 0.1% for blood samples.

### **Primary processing of next-generation sequencing data and identification of putative somatic mutations**

The data generated from the FFPE and blood samples were first aligned to the human reference sequence and annotated using the Consensus Coding DNA Sequences, RefSeq, and Ensembl databases. NGS data were then analysed using the Torrent Suite Software (Thermo Fisher Scientific, Waltham, USA). Next, somatic mutations were identified with the Variant Caller 4.0 software (Thermo Fisher Scientific, Waltham, USA) using the somatic high stringency parameters to ensure sufficient coverage of the analysed bases and to exclude mapping and sequencing errors [Supplementary Tables 1 and 2]. Genetic aberration analysis was focussed on single-base substitutions, small insertions and deletions. Candidate somatic alterations were further filtered based on: coverage of > 100 in solid biopsy analysis; a forward-reverse ratio of 10%, 90%; the exclusion of intronic and silent changes; and the retention of mutations resulting in missense mutations, nonsense mutations, frameshifts, or splice site alterations in the protein coding region. A manual visual inspection step was used to further remove artefactual changes.



**Figure 1.** Patient and variant categories. (A) Patient categories. Fully shared: patient having all the variants (1&2) detected in both the solid and liquid biopsies; partially shared: patient having different variants detected in the solid and/or liquid biopsies (4&5), and some variants that are shared (3); only in solid: patient having variants (6&7) only detected in the solid biopsy; only in liquid: patient having variants (8&9) detected only in the liquid biopsy; (B) Variant categories. Shared: variants that are detected in both the solid and liquid biopsy. These variants can be from “fully shared” patients or common variants from “partially shared” patients; solid: variants that are detected only in the solid biopsy. These can be from “only solid” patients or variants present only in tissue biopsy in “partially shared” patients; liquid: variants that are detected only in the liquid biopsy. These can be from “only liquid” patients or variants present only in blood in “partially shared” patients

### Clinical value of the detected aberrations

To evaluate the impact of the variants on the function of the proteins and on clinical benefit, a literature search was performed to identify *in vitro* studies, Food and Drug Administration (FDA) labels, guidelines and published retrospective and prospective clinical studies pertaining to genomic alterations in each gene and their association with functional impact on the protein and outcomes in cancer patients. Based on this research, variants were classified into four categories.

For the purposes of this study, the data shown refer only to the 27 genes of the panel comprising the liquid biopsy part of OncoSTRAT&GO™ [Supplementary Table 3], which are also included in the solid biopsy panel.

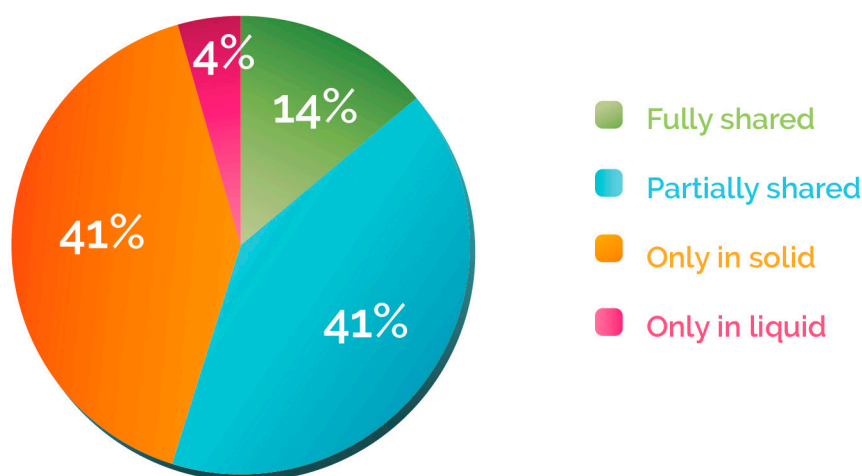
## RESULTS

We analysed data from 351 patients who were molecularly characterized from May 2016 to November 2017 using the OncoSTRAT&GO™ profiling solution, which combines the analysis by NGS of genetic variants in the solid and liquid (blood) biopsies.

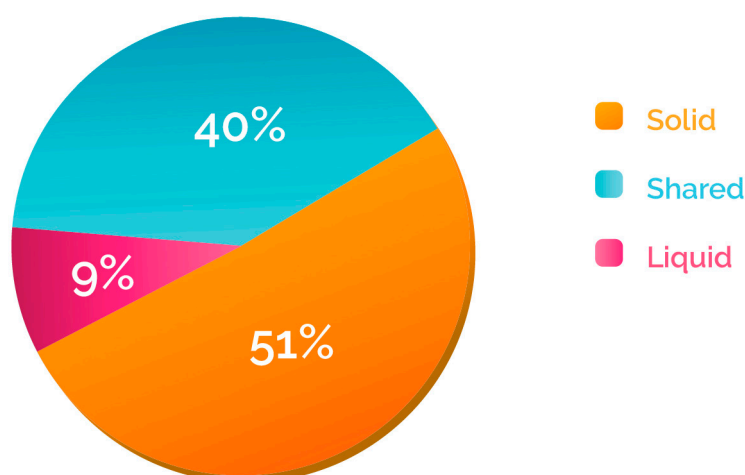
### Different genetic information revealed in the solid vs. the liquid biopsy

Patients with no variant detected in the tissue or the blood biopsy represented 11% of the total population and were excluded from further analysis. We classified the remaining patients ( $n = 313$ ) into four categories according to the mutation discrepancies found between their solid and liquid biopsies [Figure 1A]. This analysis showed that 41% of the patients carried mutations that were detected only in the solid biopsy, while for 4% of the cases, variants were found only in the blood. On the other hand, 41% of samples had partially shared mutations, and only 14% of the samples fully shared the gene variants in both biopsies [Figure 2]. These results suggest that in 86% of the patients, solid and liquid biopsies provide different information regarding genetic alterations.

Since the category “partially shared” includes patients who share variants in the two biopsies, we further performed a classification at the variant level by grouping the mutations in three categories: shared, solid and liquid [Figure 1B]. This analysis indicated that in the majority of the cases (60%), tissue and blood biopsies analysis showed discrepant patterns: 51% of the variants were detected only in the solid biopsy, while



**Figure 2.** Patient distribution according to discrepancy between solid and liquid biopsies. Patients were classified according to Figure 1A. The percentage of “fully shared”, “partially shared”, “only in solid” and “only in liquid” patients was then calculated



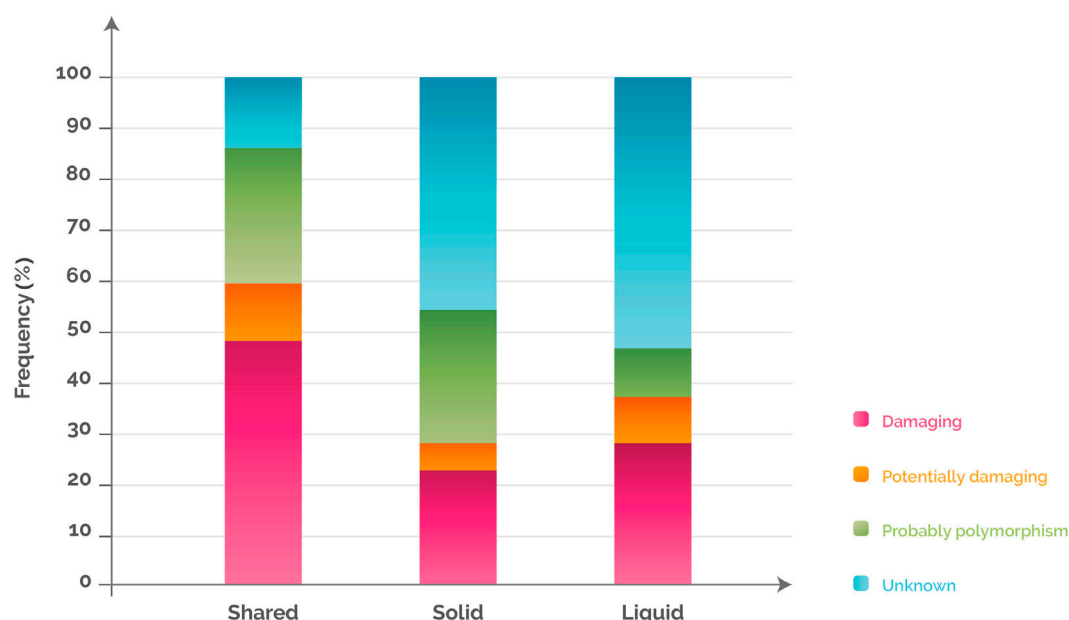
**Figure 3.** Variant distribution according to discrepancy between solid and liquid biopsy. Variants detected in the samples across different cancer types were classified according to Figure 1B. The percentage of “shared”, “solid” and “liquid” was then calculated

mutations detected only in the blood, which can provide information about spatial tumor heterogeneity, represented 9% of the total [Figure 3].

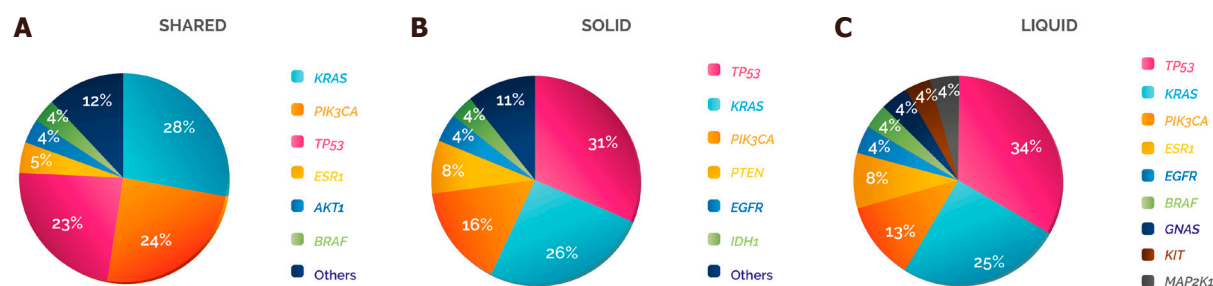
Next, we studied how the distribution of these three variant categories could be influenced by the time elapsed between the collection of the solid biopsy and the blood biopsy. As expected, the percentage of “shared” variants decreased progressively as the time elapsed between sampling increased. The opposite trend was observed for the percentage of “solid” and “liquid” [Supplementary Table 4]. In fact, when the solid biopsy was collected more than one year before the blood sample, the number of shared variants was only one third that of recent biopsies (less than 30 days), while the number of “solid” or “liquid” variants increased by around one-third and double, respectively. These data confirm that the mutations in a tumor change over time, and that this temporal heterogeneity can be demonstrated by comparing the solid and liquid biopsies at different collection times.

### Therapeutic implication of addressing tumor heterogeneity

In order to understand the biological and clinical implications of the variants identified in the tissue and blood analyses, we grouped the mutations into four different categories based on their impact on the function



**Figure 4.** Variant distribution according to functional classification and discrepancy between solid and liquid biopsy. Variants detected only in solid, only in liquid or in both were analysed for their functional impact on the corresponding protein, and were classified into 4 categories as follows: (1) damaging: a variant for which several published studies demonstrated a functional impact on the protein (activating or inhibiting) and where clinical information is also available confirming the impact; (2) potentially damaging: a variant for which only one publication has shown a functional impact based on an *in vitro* model and for which no clinical information is available; (3) unknown: a variant for which there are no publications associated with a functional impact and that is not known as a single nucleotide polymorphism in the NCBI dbSNP database; (4) polymorphism: a variant identified in the NCBI dbSNP database as a polymorphic variant with a minor allele frequency of at least 1%. NCBI: National Center for Biotechnology Information; dbSNP: Single Nucleotide Polymorphism Database



**Figure 5.** Damaging variant distribution per gene and type of biopsy. The distribution of the damaging variants identified in Figure 4 was analysed according to whether they were detected in (A) shared, (B) solid or (C) liquid

of the protein, and then sorted them according to the discrepancy between solid and liquid biopsy. 48%, 23% and 28% of the variants detected in “shared”, “solid” and “liquid”, were damaging [Figure 4; see legend for explanation of functional classification]. These percentages were cancer dependent. For example, when analysing the colorectal cancer samples separately, the percentage of damaging variants present in “shared”, “solid” and “liquid” rose to 59%, 31% and 44%, respectively [Supplementary Figure 1].

The genes showing the highest frequencies of damaging variants in the three categories were *TP53*, *KRAS* and *PIK3CA*, although the order varied: in “shared”, *KRAS* was the gene with the most damaging mutations (28%), followed by *PIK3CA* (24%) and *TP53* (23%), while in “solid” and “liquid” the gene with a higher frequency of damaging variants was *TP53* (31% and 34%), followed by *KRAS* (26% and 25%) and *PIK3CA* (16% and 13%, respectively) [Figure 5].

**Table 1. Damaging variants detected in the “liquid” category and their clinical implication according to cancer type**

Cancer type analysed	Gene	No. of variants	Therapeutic value
Breast cancer HR+	<i>ESR1</i>	2	Resistance to aromatase inhibitors <sup>[10]</sup>
	<i>KRAS</i>	1	Phase 2 trial (NCT02576444)
	<i>TP53</i>	3	Phase 2 trial (NCT02576444)
Cholangiocarcinoma	<i>PIK3CA</i>	1	Phase 2 trial (NCT02465060)
Colorectal cancer	<i>KRAS</i>	4	Resistance to anti-EGFR antibodies <sup>[11]</sup>
	<i>PIK3CA</i>	1	Resistance to anti-EGFR antibodies <sup>[12]</sup>
	<i>TP53</i>	2	Phase 2 trial (NCT02576444)
Endometrial carcinoma	<i>TP53</i>	1	Phase 2 trial (NCT02576444)
Gastric cancer	<i>PIK3CA</i>	1	Phase 2 trial (NCT02465060)
GBM	<i>BRAF</i>	1	Phase 2 trial (NCT02465060)
GIST	<i>KIT</i>	1	Resistance to KIT/PDGFRA-tyrosine kinase inhibitors <sup>[13]</sup>
NSCLC	<i>EGFR</i>	1	Resistance to EGFR-tyrosine kinase inhibitors <sup>[14]</sup>
	<i>GNAS</i>	1	Unknown
	<i>MAP2K1</i>	1	Sensitivity to MEK inhibitors <sup>[15]</sup>
Pancreatic cancer	<i>KRAS</i>	1	Phase 2 trial (NCT02576444)
Prostate cancer	<i>TP53</i>	2	Phase 2 trial (NCT02576444)

HR: hormone receptor; GBM: glioblastoma multiforme; GIST: gastrointestinal stromal tumor; NSCLC: non-small cell lung cancer

To address whether these mutations could have a clinical impact on the patients, we analysed the potential clinical benefit associated with each damaging variant detected in the samples. In 97% of the cases, the damaging mutations found solely in the solid or in the liquid biopsy were predictive of either sensitivity (82%) or resistance (15%) to specific cancer treatments - mainly targeted therapies (98%). When we studied in more detail the damaging variants detected in “liquid”, we observed that 96% were clinically actionable: 42% were directly predictive of approved therapies in the indicated cancer type, either targeted therapies (80%) or hormone therapies (20%, aromatase inhibitors), whereas 54% were inclusion criteria for trials using targeted therapies in molecularly selected patients (which were actively recruiting at the time of submission) [Table 1]. These data highlight that the integrated analysis of both biopsy samples provides valuable clinical information that could guide the use of cancer therapy.

## DISCUSSION

This study compared the discrepant distribution of mutations found in the molecular profiling of solid vs. liquid biopsies of metastatic cancer patients in order to better understand tumor heterogeneity. This analysis highlighted that the addition of ctDNA testing to tissue profiling might increase therapeutic value and could better guide oncologists in precision medicine.

The results show that in the majority of the cases, the information obtained by sequencing tumor tissue DNA and ctDNA is complimentary. We observed a higher percentage of mutations detected only in the solid biopsy (51%) compared to only in the liquid or to shared variants. One factor that can explain this is the tumor location, as it has been previously demonstrated that different cancer types shed different amounts of DNA into the blood<sup>[16]</sup>. Bettgowda *et al.*<sup>[16]</sup> showed that ctDNA was detectable in 100% of patients with stage IV colorectal cancer, while less than 10% of patients with advanced gliomas harboured detectable ctDNA (as the blood-brain barrier could prevent the entry of ctDNA into the circulation). In accordance with this, in our analysis we found that 56% of the variants were shared between solid and liquid biopsies in colorectal cancer patients, while in glioblastoma multiforme patients, 89% of the mutations were found in “solid”, 11% only in liquid biopsies and none in “shared” (data not shown). This confirms that the location of the tumor has an impact on the utility of ctDNA. A second factor that can explain why a high percentage of variants were only present in tissue DNA is the temporal heterogeneity, which is influenced by patient-specific selective pressures<sup>[17]</sup> such as the prescribed treatments and fluctuations in tumor microenvironment. In fact, the percentage of “shared” mutations markedly decreased when the time space of the collection dates increased.



This temporal heterogeneity provides interesting information about how the subclonal heterogeneity of a tumor evolves over the course of treatment, with some clones persisting or disappearing while new clones appear<sup>[18]</sup>. Note that this temporal analysis might not always provide useful information about the current treatments that would be of clinical benefit to the patient, as the current molecular status of the tumor is the critical factor in therapy personalization.

On the other hand, 9% of the variants were detected only in the blood, which are a reflection of spatial heterogeneity. This intratumor heterogeneity occurs either within different regions of the same tumor mass, or between the primary tumor and its metastases<sup>[4]</sup>. Moreover, different metastatic sites may also harbour different molecular features<sup>[19]</sup>. Previous studies have reported the existence of mutations that were only found in ctDNA but not in the corresponding tissue<sup>[7]</sup>. Since ctDNA can be shed into the blood from the primary tumor and/or the metastases, it can potentially provide tumor information from all cancer sites<sup>[4]</sup>. On the other side, spatial heterogeneity cannot be fully determined with a single-site solid biopsy.

We observed that on average across cancer types, “solid” and “liquid” variants with a demonstrated functional impact on the protein (damaging) were around 25% of all the mutations found, although the frequency was different depending on the cancer type. The genes that harboured the highest number of damaging variants were *TP53*, *KRAS* and *PIK3CA*. *TP53* is a key tumor suppressor that responds to several cellular stress signals by promoting different responses, such as cell cycle arrest, senescence or apoptosis<sup>[20]</sup>. *TP53* mutations have been found in almost every cancer type, and its inactivation is a common event in the tumorigenesis process<sup>[21]</sup>. *KRAS* is also one of the most frequently mutated genes in many cancers. It plays an important role in the regulation of cell division and activating mutations can lead to cell transformation because they impair the ability of the *KRAS* protein to switch between the “on” and the “off” state. Finally, *PIK3CA*, another commonly mutated oncogene in cancer<sup>[22]</sup>, is involved in many cellular processes, such as cell growth and proliferation and, when mutated, the increased kinase activity of *PIK3CA* protein contributes to cellular transformation<sup>[23]</sup>.

What are the clinical implications of analysing the discrepancy between the damaging variants found solely in the solid and solely in the liquid biopsy? In almost all the cases, these mutations were clinically actionable, meaning that they provided information about tumor sensitivity or resistance to approved or investigational targeted therapies. When we took into consideration only the spatial heterogeneity (“liquid” category), we again found a very high frequency of clinically actionable variants (96%), most of them either related to approved targeted therapies - for example *KRAS* mutations in colorectal cancer are associated with resistance to anti-EGFR antibodies<sup>[11]</sup>, and *MAP2K1* mutations in non-small cell lung cancer are related to sensitivity to MEK inhibitors<sup>[15]</sup> - or to actively recruiting trials of targeted therapies, such as NCI-MATCH (NCT02465060). This emphasizes that the addition of ctDNA profiling to the analysis of solid biopsies, the current gold standard for tumor molecular characterization, can provide valuable extra information for oncologists, either for the prescription of an approved treatment or for enrolling them in relevant clinical trials. In fact, the utility of ctDNA analysis for tumor characterization and for guiding treatment choice is being increasingly recognized, as reflected by the first FDA approval of a blood-based companion diagnostic to guide targeted therapy in non-small cell lung cancer (cobas EGFR Mutation Test v2).

Limitations of this study include the lack of information about patient follow-up regarding oncologists’ treatment decisions and patient outcomes. Strengths include the number of patients and the cancer types analysed, as well as having addressed the importance of understanding tumor heterogeneity in the clinical setting.

In conclusion, this study suggests that the combination of recent solid and liquid biopsies provides the most comprehensive and therapeutically valuable characterization of the heterogeneity of the patient’s tumor,

which cannot be achieved by performing only one type of biopsy, and that the inclusion of ctDNA profiling should be considered in routine oncology care especially for cancers where targeted therapies are approved or in development.

## DECLARATIONS

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### Authors' contributions

Conceived and designed the study: Laes JF

Worked on the acquisition of data, the writing of the article and the analysis and interpretation of data: all authors

Reviewed and agreed on the manuscript before submission: all authors

### Data source and availability

Due to privacy reasons, the data of this study cannot be open for readers. Nonetheless, if a researcher asks for data, they could be shared upon the signature of a confidentiality agreement.

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This study was sponsored by OncoDNA SA. The costs associated with the development and publishing of the manuscript were provided by OncoDNA SA.

### Conflicts of interest

All authors are employees of OncoDNA. Ghitti G and Laes JF report ownership of OncoDNA shares.

### Patient consent

Patients gave informed consent for their tumor analysis data to be published.

### Ethics approval

This study is not approved by IRB; however, all patients signed an informed consent before their samples underwent molecular profiling according to Belgian law.

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Review

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# Conversion surgery for stage IV gastric cancer

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## Abstract

Gastric cancer with distant metastases, such as para-aortic lymph node metastases, hepatic metastases, and peritoneal dissemination, is classified as stage IV. In this situation, cancer cells have formed micrometastases throughout the body; therefore, according to the algorithm of the Japanese guidelines, stage IV cancer is outside the indication for curative resection. Recent advances in some chemical agents have been remarkable, and some patients have survived for long periods even with stage IV gastric cancer. Thus, even in patients with stage IV gastric cancer, there is a possibility that gastrectomy as conversion surgery could play an important role in the treatment strategy. Gastrectomy as conversion therapy can be safely conducted without perioperative mortality and is considered a sufficiently acceptable treatment strategy. However, the significance of conversion surgery for stage IV gastric cancer remains controversial. In this review, we summarize the treatment strategies and outcomes of conversion surgery for stage IV gastric cancer.

**Keywords:** Gastric cancer, stage IV, gastrectomy, conversion surgery, outcome

## INTRODUCTION

Gastric cancer is a highly malignant tumor that can metastasize at high rates by lymphogenous spread, hematogenous spread, and dissemination. In stage IV advanced gastric cancer, which is characterized by distant metastasis to sites other than regional lymph nodes, cancer cells are considered to have formed micrometastases throughout the body. Such cancer is outside the indication for curative resection. As stated in the Japanese treatment guidelines, chemotherapy remains the main therapeutic approach for stage IV gastric cancer, and surgery for these patients is usually confined to palliative resection or a bypass operation to relieve symptoms<sup>[1]</sup>. The European Society for Medical Oncology (ESMO)<sup>[2]</sup> and the National Comprehensive



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Cancer Network (NCCN)<sup>[3]</sup> guidelines for gastric cancer also recommended the doublet or triplet platinum/fluoropyrimidine combinations for metastatic gastric cancer as a palliative chemotherapy.

Recent advances in chemotherapy and molecular targeted therapy have been remarkable, and some patients have survived for long periods. Some of these patients include those who have successfully undergone curative resection after chemotherapy. However, the significance of surgical resection after chemotherapy, termed conversion surgery, remains controversial for patients with gastric cancer.

Factors that make curative resection impossible include tumor invasion to adjacent structures (T4b), extensive nodal disease (para-aortic and/or bulky lymphnode metastasis located on supra-pancreatic area), hepatic metastases, peritoneal dissemination, peritoneal cytology positive for cancer cells, and other metastatic disease. The treatment strategies and outcomes differ according to each noncurative factor. In this chapter, we review the treatment outcome of conversion surgery for each type of unresectable advanced gastric cancer.

## TREATMENT STRATEGIES AND OUTCOMES FOR CONVERSION SURGERY

### Lymph node metastases

Para-aortic lymph node metastases from gastric cancer are classified as M1, and surgery with curative intent is not indicated according to the treatment algorithm of the current guidelines<sup>[1]</sup>. In addition, a standard treatment strategy including a role for para-aortic lymph node dissection (PAND) in patients with more advanced nodal disease has not yet been established. Systemic PAND was attempted in clinical studies in Japan until its survival benefit was denied in a randomized trial in which only patients without lymph node swelling in the para-aortic region were eligible<sup>[4]</sup>. Based on the results of that study, prophylactic PAND for patients with no signs of para-aortic lymph node metastasis was discontinued. However, no prospective study has either supported or opposed PAND in patients with surgically resectable para-aortic lymph node metastases at station numbers 16a2-b1.

Tokunaga *et al.*<sup>[5]</sup> retrospectively analyzed 178 patients who underwent R0 resection and were found to have metastasis to the para-aortic lymph nodes after examination of the resected specimens. Of these patients, 50 were treated by D2 gastrectomy plus PAND and 128 were treated by D2 with sampling of para-aortic nodes that were suspected to have cancer involvement. The 3-year survival rate was 21%. Perioperative chemotherapy was administered at the physicians' discretion but was not consistently delivered throughout the series. The authors concluded that D2 gastrectomy + PAND could be beneficial for carefully selected patients with metastasis to the para-aortic lymph nodes.

The effectiveness of PAND for patients with para-aortic lymph node metastases was shown in phase II trial by the Japan Clinical Oncology Group (JCOG) (JCOG0405). The treatment strategy was as follows. Two courses of neoadjuvant chemotherapy with S-1 plus cisplatin followed by gastrectomy with D2 plus PAND were performed. Patients with bulky nodal disease with or without lymphadenopathy restricted to the station No. 16a2-b1 region were eligible. Peritoneal metastasis was ruled out and the CY1 status was determined by staging laparoscopy prior to registration. The trial showed favorable results: a curative resection rate of 82% and 3- and 5-year overall survival (OS) rates of 59% and 53%, respectively<sup>[6]</sup>. Therefore, this treatment strategy could be recommended for institutions with sufficient expertise in PAND.

Another phase II trial exploring multimodal treatment for patients with para-aortic lymph node metastases limited to stations No. 16a2-b1 was performed in China. This study employed a combination of capecitabine and oxaliplatin (XELOX) as induction chemotherapy. In total, 48 patients were enrolled. After a median of 4 cycles of chemotherapy, 28 of the 48 patients (58.3%) underwent conversion surgery. The median OS of



all patients was 29.8 months, although these premature data were calculated after a median follow-up time of only 12.4 months. However, only D2 lymph node dissection was performed in that study; the fact that PAND was not performed should be considered<sup>[7]</sup>. The authors' strategy was to convert chemotherapy to surgical therapy for selected responders in the hope that up to six cycles of chemotherapy might cure the cancers outside the confines of standard surgical dissection. In contrast, the Japanese investigators treated patients by neoadjuvant chemotherapy to eliminate micrometastases that may or may not have been present, followed by surgery with curative intent to dissect all cancerous tissues that had been detected prior to the treatment. Therefore, the philosophy behind the two strategies is quite different.

Whether the preoperative diagnosis of para-aortic lymph node metastasis is reliable must be considered when discussing these treatment options. Lymph node metastasis is currently diagnosed when the lymph node diameter shows either a minor axis of  $\geq 8$  mm or major axis of  $\geq 10$  mm on abdominal computed tomography (CT). The JCOG 1302A trial, which evaluated the accuracy of clinical diagnosis and pathological stage III gastric cancer, showed that the sensitivity and specificity of the CT criteria for nodal metastasis were 62.5% (505/808) and 65.7% (278/423), respectively<sup>[8]</sup>. A recent prospective study indicated that multidetector-row CT achieved relatively high overall accuracy (76%) in preoperative detection of nodal metastasis<sup>[9]</sup>. Furthermore, Marrelli *et al.*<sup>[10]</sup> reported that the sensitivity and specificity of multidetector-row CT in detecting para-aortic lymph node metastasis were encouragingly high at 85% and 95%, respectively. Improvements in diagnostic accuracy also contribute to improvements in diagnostic modality.

### Liver metastases

Colorectal liver metastases are widely considered targets of surgery with curative intent because they often present as liver-only diseases, and R0 resection showed favorable survival in a recent clinical study<sup>[11]</sup>. However, the necessity of surgical resection of liver metastases of gastric cancer is still controversial.

The guidelines do not recommend surgery for stage IV gastric cancer; therefore, most patients with liver metastases of gastric cancer receive systemic therapy<sup>[1]</sup>. In contrast, several studies have shown that long-term survival can be obtained by performing hepatectomy for liver metastases of gastric cancer. However, only retrospective analyses of small cohorts collected over several decades have been performed, and most were single-institution studies. No prospective trial exploring the benefits of hepatectomy has been conducted.

We reviewed the 7 largest studies reported from 2012 to 2017, each with  $\geq 50$  patients who underwent hepatectomy for liver metastases from gastric cancer<sup>[12-17]</sup> [Table 1]. In these series, the 3- and 5-year OS rates were 14.0% to 51.4% and 9.3% to 42.3%, respectively, with a median survival time (MST) of 13.0 to 40.8 months<sup>[12-18]</sup>. Solitary metastasis or a small number of metastatic nodules was highlighted as a favorable prognosis in most of the studies. After multivariate analysis, Oki *et al.*<sup>[16]</sup> reported that more than two liver metastases [hazard ratio (HR), 2.14; 95% confidence interval (CI), 1.16-3.97] and Kinoshita *et al.*<sup>[13]</sup> reported that three or more liver metastases are independent factors that is associated with worse prognosis (HR, 2.33; 95% CI, 1.62-3.36). Oki *et al.*<sup>[16]</sup> also reported that the presence of three or more lymph node metastases was a factor that is associated with worse prognosis. Moreover, a size of  $\geq 3$  cm<sup>[15]</sup> or  $\geq 5$  cm<sup>[12,13]</sup> or serosal invasion<sup>[12,13,18]</sup> have been reported as an independent risk factors for the primary gastric cancer itself.

However, these reports were the results of accumulation of cases over a long period of 10 to 20 years. Therefore, with the given the recent advances in imaging studies, it is possible that the diagnosis of the number of liver metastasis might not be reliable. Thus, hepatectomy may be considered for patients with a small number of metastatic nodules and not restricted to a solitary tumor, provided that no other noncurative factor is present. At present, it may be reasonable to keep the indication for hepatectomy when a patient has three or fewer metastases.

**Table 1. Literature overview of outcomes following hepatectomy for gastric cancer liver metastases**

Ref.	Year	Country	Study interval	No. of patients	3-year OS	5-year OS	MST (months)
Takemura <i>et al.</i> <sup>[12]</sup>	2012	Japan	1993-2011	64	50.0	37.0	34.0
Kinoshita <i>et al.</i> <sup>[13]</sup>	2015	Japan	1990-2010	256	41.9	31.1	31.1
Tiberio <i>et al.</i> <sup>[14]</sup>	2015	Italy	1997-2011	53	14.0	9.3	13.0
Oki <i>et al.</i> <sup>[16]</sup>	2016	Japan	2000-2010	94	51.4	42.3	40.8
Tiberio <i>et al.</i> <sup>[18]</sup>	2016	Italy	1990-2013	105	20.3	13.1	14.6
Guner <i>et al.</i> <sup>[15]</sup>	2016	South Korea	1998-2013	68	40.6	30.0	24.0
Song <i>et al.</i> <sup>[17]</sup>	2017	China	2001-2012	96	47.6	21.7	34.0

OS: overall survival; MST: median survival time

Although chemotherapy has been successful and surgical cases are increasing, there is no evidence for the recommended chemotherapy regimen in this particular situation. Therefore, systemic chemotherapy is performed with reference to the treatment recommended by the guidelines<sup>[1]</sup>. However, Tiberio *et al.*<sup>[18]</sup> reported that adjuvant chemotherapy was a prognostic factor. Therefore, adjuvant chemotherapy after hepatectomy will be discussed as increasingly more cases are accumulated.

### Peritoneal dissemination

The peritoneum is a frequent site for metastases in patients with advanced gastric cancer, and peritoneal dissemination is one of the most important life-threatening factors in such patients. Systemic chemotherapy is administered to patients with peritoneal dissemination as well as other patients with stage IV gastric cancer. Systemic chemotherapy for gastric cancer has steadily progressed in recent years, and 5-fluorouracil-based or cisplatin-based regimens are generally accepted as possible standard chemotherapy. However, an adequate therapeutic effect has not been obtained. Otherwise, the treatment strategy for patients with only positive peritoneal cytology remains controversial. The Japanese Gastric Cancer Association advocates classification of free cancer cells in the peritoneal cavity as M1, and surgery with curative intent is not indicated according to the treatment algorithm of the current guidelines. However, the guidelines suggest that a cytology-positive status in the absence of other noncurative factors (i.e., macroscopic disease) can be managed with D2 gastrectomy and perioperative chemotherapy<sup>[1]</sup>.

Intraperitoneal (i.p.) chemotherapy has recently been conducted to improve the treatment outcomes for peritoneal dissemination. Ishigami *et al.*<sup>[19]</sup> developed a regimen involving the addition of weekly i.p. paclitaxel (PTX) to an established systemic chemotherapy regimen of S-1 and intravenous PTX for the treatment of peritoneal metastasis of gastric cancer. The i.p. PTX was administered to enhance antitumor activity against peritoneal metastasis by maintaining a high concentration of the drug in the peritoneal cavity over a long period, and its clinical effects have been verified by several convincing clinical trials involving patients with ovarian cancer with peritoneal metastasis<sup>[20]</sup>. In a phase II trial conducted by Ishigami *et al.*<sup>[21]</sup>, 40 patients with gastric cancer that was positive for peritoneal metastases and/or peritoneal cytology were enrolled. The authors reported a 1-year OS rate of 78%. In addition, malignant ascites disappeared or decreased in 13 of 21 (62%) patients, and cancer cells detected by peritoneal cytology diminished in 24 of 28 (86%) patients. In a phase III trial comparing this i.p. chemotherapy to S-1 plus cisplatin (PHOENIX-GC trial), the primary analysis did not show the statistical superiority of the i.p. regimen ( $P = 0.08$ ; HR, 0.72; 95% CI, 0.49-1.04), however, prolongation of the MST by 2.5 months was recognized in the i.p. group, and the i.p. chemotherapy could thus be considered a promising treatment option<sup>[22]</sup>. Furthermore, Ishigami *et al.*<sup>[23]</sup> performed a retrospective study of 100 cases of P1 and/or CY1 gastric cancer and found that conversion surgery was performed in 64 patients, among whom R0 resection was performed in 44 (69%).

Table 2 shows the promising results of several phase II clinical trials of i.p. taxanes after 2010. In these series, the 1-year OS rates were 69% to 78%, with an MST of 16.2 to 24.6 months<sup>[21,24-27]</sup>. Notably, the possibility of negative peritoneal cytology was very high at 81.8% to 97.0%.

**Table 2. Phase II clinical trials with intraperitoneal taxanes for gastric cancer with peritoneal disease**

Ref.	Year	No. of patients	1-year OS	MST (months)	Turned negative for cytology (%)
Ishigami <i>et al.</i> <sup>[21]</sup>	2010	40	78.0	22.6	24/28 (86)
Fujiwara <i>et al.</i> <sup>[24]</sup>	2012	18	76.0	24.6	–
Imano <i>et al.</i> <sup>[25]</sup>	2012	35	66.7	21.3	–
Fushida <i>et al.</i> <sup>[26]</sup>	2013	27	70.4	16.2	18/22 (81.8)
Yamaguchi <i>et al.</i> <sup>[27]</sup>	2013	35	77.1	17.6	28/29 (97)

OS: overall survival; MST: median survival time

Staging laparoscopy may be useful for the evaluation of resectability after chemotherapy. Several societies have provided recommendations for staging laparoscopy in patients with advanced gastric cancer<sup>[1,2]</sup>. If information on the CY status is available prior to surgery, a chemotherapy-first strategy can be taken, whereby only patients whose cytology status turns negative are indicated for surgery. To verify the effect of preoperative chemotherapy on positive cytology, Jamel *et al.*<sup>[28]</sup> reviewed studies in which staging laparoscopy was performed. Pooled analysis demonstrated that positive cytology was associated with significantly reduced OS (HR, 3.46; 95% CI, 2.77-4.31;  $P < 0.0001$ ). Interestingly, negative cytology following neoadjuvant chemotherapy was associated with significantly improved OS (HR, 0.42; 95% CI, 0.31-0.57;  $P < 0.0001$ ). The absence of macroscopic peritoneal disease with positive cytology was associated with significantly improved OS (HR, 0.64; 95% CI, 0.56-0.73;  $P < 0.0001$ ). This study suggests that patients with initial positive cytology may have a good prognosis following neoadjuvant treatment if the cytology results become negative after treatment.

Yoshida *et al.*<sup>[29]</sup> proposed new categories for the classification of stage IV gastric cancer that focused on the biology and heterogeneous characteristics of stage IV gastric cancer. They divided cancers based on the absence (categories 1 and 2) or presence (categories 3 and 4) of macroscopically detectable peritoneal dissemination, the biological outcome of which differs from that of hematological metastasis. Using this classification, Yamaguchi *et al.*<sup>[30]</sup> performed a retrospective study to clarify the role of conversion surgery in the treatment of stage IV cancer. Even in patients with macroscopic peritoneal dissemination without other organ metastasis (category 3), the survival of those who underwent conversion surgery was prolonged (31.0 months), and even the MST of those who failed to undergo conversion surgery was relatively good (18.5 months). However, patients with involvement of other organs in addition to peritoneal disease (classified as category 4; noncurable metastasis) understandably had fewer chances for surgical intervention, and their MST was 10 months.

### Postoperative complications

Kubota *et al.*<sup>[31]</sup> reported that postoperative complications that cause prolonged inflammation have an obvious impact on not only OS but also disease-specific mortality of patients with gastric cancer, even if the tumor is curatively resected. Thus, when performing conversion surgery, it is necessary to perform safe gastrectomy that does not cause complications.

Gastrectomy as conversion therapy can be safely conducted without perioperative mortality. The reported incidence of postoperative complications after gastrectomy is 24% to 29%<sup>[30,32]</sup>, which is similar to that in patients undergoing conventional radical surgery for gastric cancer (20.9% in patients with D2 lymph node dissection and 28.1% in patients undergoing an extended operation with aortic lymph node dissection) (JCOG9501)<sup>[33]</sup>.

### Predictive factors for long-term outcome

Several reports have described the long-term outcomes of conversion surgery for stage IV gastric cancer. In various studies, the prognosis of patients who underwent conversion surgery was significantly better than that

of patients who did not undergo conversion surgery<sup>[30,32,34,35]</sup>. Furthermore, whether R0 resection is performed may greatly affect the prognosis. Yamaguchi *et al.*<sup>[30]</sup> analyzed the treatment outcomes of 259 patients with stage IV gastric cancer and found that the MST of those who underwent R0 resection (41.3 months) was significantly better than that of patients who underwent R1 and R2 resection (21.2 months). Sato *et al.*<sup>[32]</sup> evaluated the treatment outcomes of initially unresectable gastric cancer treated with docetaxel, cisplatin, and S-1 (DCS) chemotherapy in a clinical trial. Conversion therapy was achieved in 33 of 100 patients (33%), and R0 resection was performed in 28 (84.8%) patients. The authors focused on the pathological response of the primary tumor, and the pathological response rate was 78.8%. Furthermore, multivariate analysis showed that pathological response was the only independent prognostic factor for conversion therapy ( $P = 0.009$ ). These findings suggest the clinical significance of performing conversion surgery for stage IV gastric cancer.

## VOLUME REDUCTION SURGERY

The JCOG and Korea Gastric Cancer Association conducted an open-label, randomized phase III trial (JCOG0705/KGCA01) comparing gastrectomy plus chemotherapy vs. chemotherapy alone in patients with advanced gastric cancer with a single noncurative factor. The patients were randomly assigned to gastrectomy followed by chemotherapy or chemotherapy alone. The chemotherapy regimen was S-1 plus cisplatin, which is a standard treatment for advanced gastric cancer. The 2-year OS rate was 31.7% (95% CI, 21.7-42.2) for patients assigned to chemotherapy alone compared with 25.1% (95% CI, 16.2-34.9) for those assigned to gastrectomy plus chemotherapy. The median OS was 16.6 months (95% CI, 13.7-19.8) for patients assigned to chemotherapy alone and 14.3 months (95% CI, 11.8-16.3) for those assigned to gastrectomy plus chemotherapy (HR, 1.09; 95% CI, 0.78-1.52;  $P = 0.70$ ). Thus, no evidence in support of volume reduction surgery was found for patients with advanced gastric cancer, even those with a single noncurative factor<sup>[36]</sup>.

The German AIO study group conducted the RENAISSANCE (AIO-FLOT5) trial: effect of chemotherapy alone vs. chemotherapy followed by surgical resection on survival and quality of life in patients with limited metastatic adenocarcinoma of the stomach or esophagogastric junction. This trial is a prospective, multicenter, randomized, investigator-initiated phase III trial aimed to evaluate the effects of perioperative chemotherapy with FLOT (5-fluorouracil, leucovorin, oxaliplatin, and docetaxel) in chemo naive patients with limited metastatic disease<sup>[37]</sup>. If the RENAISSANCE concept proves to be effective, this could potentially lead to a new standard therapy for metastatic gastric cancer.

## CONCLUSION

Long-term survivors exist among patients who have undergone conversion surgery with R0 resection for stage IV gastric cancer. Adequate selection of patients with stage IV gastric cancer for conversion therapy is very important to increase the likelihood of long-term survival. Furthermore, even with surgery, the prognosis of patients with other involvement of other organs in addition to peritoneal dissemination is poor. Therefore, surgical intervention in such patients should be performed cautiously. Further cooperation of specialists, such as surgeons and physicians, is necessary to allow for the establishment of diagnostic methods, surgery with fewer complications, and development of more effective agents. In the future, an approach applying the concept of conversion surgery might expand the eligibility for surgery with curative intent to include even patients with currently considered unresectable for metastases.

## DECLARATIONS

### Authors' contributions

Concept, design, literature search and manuscript preparation: Ida S  
Manuscript editing and review: Watanabe M

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Authors declare that they have no conflicts of interest.

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Review

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# Treatment strategy for metastatic gastric cancer in Japan

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## Abstract

Despite recent progress in diagnostic imaging, gastric cancer (GC) is occasionally found at an advanced stage with distant metastasis. As metastatic GC is difficult to cure, the treatment strategy should be considered individually based on the physical and socioeconomic status of patients as well as on the GC symptoms. The first choice of treatment for metastatic GC is chemotherapy, and several chemotherapeutic regimens for metastatic or recurrent GC have been developed through randomized controlled trials. Ongoing clinical trials will provide novel therapeutic options for patients with metastatic GC in the near future, while individualization of treatment based on detailed molecular information, so-called precision medicine, is eagerly anticipated. In this article, we review recent publications and guidelines focusing on recent progress in the treatment of metastatic GC in Japan.

**Keywords:** Gastric cancer, chemotherapy, molecularly targeted drug, para-aortic lymph node metastasis, liver metastasis

## INTRODUCTION

Gastric cancer (GC) is the fourth most commonly diagnosed cancer and the second leading cause of cancer mortality worldwide<sup>[1,2]</sup>. A large-scale database analysis in the United States revealed that distant metastases were present in 34% of GC patients at the time of their GC diagnosis<sup>[3]</sup>. Although systematic screening programs have been developed in Japan to enable detection of early stage GC<sup>[4]</sup>, GC is occasionally found at an advanced stage with distant metastasis. The first choice of treatment for patients with metastatic GC is chemotherapy<sup>[5]</sup>. Although recent advances in chemotherapy, including immune checkpoint inhibitors



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**Table 1. Results of trials with chemotherapy for metastatic gastric cancer in Japan**

Authors, year	Regimen	Patients (n)	OS (months)	HR
Koizumi <i>et al.</i> <sup>[8]</sup> , 2008	S-1 + cisplatin	148	13.0	0.77 (0.61-0.98)
	S-1	150	11.0	1
Yamada <i>et al.</i> <sup>[33]</sup> , 2015	S-1	234	11.4	0.83 (0.68-1.00)
	Cisplatin + irinotecan	236	12.3	0.82 (0.68-0.99)
	5-FU continuous infusion	234	10.8	1
Koizumi <i>et al.</i> <sup>[34]</sup> , 2014	S-1 + docetaxel	314	12.5	0.84 (0.71-0.99)
	S-1	321	10.8	1
Boku <i>et al.</i> <sup>[35]</sup> , 2009	S-1	234	11.4	0.83 (0.68-1.00)
	Cisplatin + irinotecan	236	12.3	0.82 (0.68-0.99)
	5-FU continuous infusion	234	10.8	1
Narahara <i>et al.</i> <sup>[36]</sup> , 2011	S-1 + irinotecan	155	12.8	0.93
	S-1	160	10.5	1
Hironaka <i>et al.</i> <sup>[44]</sup> , 2013	Weekly paclitaxel	108	9.5	1.13 (0.86-1.49)
	Weekly irinotecan	111	8.4	1
Shitara <i>et al.</i> <sup>[45]</sup> , 2017	Tri-weekly nab-paclitaxel	247	10.3	1.06 (0.87-1.31)
	Weekly nab-paclitaxel	246	11.1	0.97 (0.76-1.23)
	Weekly paclitaxel	248	1.06	1

OS: overall survival; HR: hazard ratio; 5-FU: 5-fluorouracil

and drugs targeting specific molecular pathways, have achieved an increase in the response rate, it is difficult to cure metastatic GC with chemotherapy alone. The current goals of treatment, therefore, are to relieve GC-related symptoms and to prolong survival. The median survival time achieved in clinical trials for metastatic or recurrent GC remains between 6 and 13 months<sup>[6-8]</sup>, although it has been proven that chemotherapy prolongs survival when compared with the best supportive care (BSC)<sup>[9,10]</sup>. Recently, it has been reported that curative resection may be performed for patients with liver metastasis, para-aortic lymph node metastasis, or positive peritoneal cytology, especially when chemotherapy has been effective<sup>[11-19]</sup>. In this review, we summarize the publications and guidelines that have focused on recent progress in the treatment of metastatic GC in Japan.

## TREATMENT STRATEGY FOR METASTATIC GC

The main treatment for metastatic GC is chemotherapy. Table 1 shows the representative trials for metastatic or recurrent GC in Japan. The first chemotherapeutic agent of choice against metastatic GC was 5-fluorouracil (5-FU), which was used either alone or in combination with various agents. In Japan, 5-FU as a key drug for GC was replaced by S-1 (tegafur-gimestat-otastat potassium), based on favorable results in trials involving Japanese patients<sup>[8,20]</sup>. Thereafter, trials focused on identifying the best combination regimen using S-1. Recently, many drugs designed to target the molecular pathways involved in the development or progression of cancer have been studied for metastatic GC<sup>[21-31]</sup> [Table 2]. In patients with GC overexpressing human epidermal growth factor receptor 2 (HER2), the addition of trastuzumab, an antibody targeting HER2, to the first-line cytotoxic drug regimens significantly prolonged the survival of patients<sup>[21]</sup>. Therefore, the presence or absence of HER2 overexpression is the first branch point when selecting the treatment regimen. The recommended treatment algorithm for patients of metastatic GC in the 5th edition of the Japanese Gastric Cancer Treatment Guideline is shown in Figure 1. Recommendation A indicates that the regimen is strongly recommended based on the certain evidence while recommendation B suggests that the regimen is weakly recommended because of insufficient evidence. Figure 2 demonstrates the alternative algorithm for patients who are unfit for the standard treatment due to comorbidities or social situations.

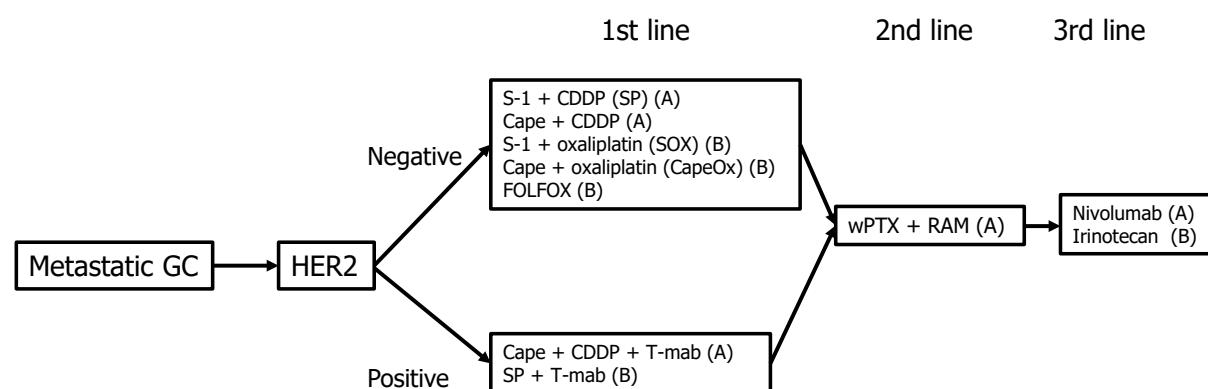
## HER2-negative advanced GC

In Japan, the first choice of chemotherapy for metastatic GC is S-1 and cisplatin (SP), according to the results of a phase III trial (SPIRITS trial<sup>[8]</sup>). This trial showed that patients treated with SP had significantly better overall survival (OS) than those treated with S-1 alone, with a median OS of 13 vs. 11 months ( $P = 0.04$ ). Progression-free survival (PFS) was also significantly longer in patients treated with SP than in those treated

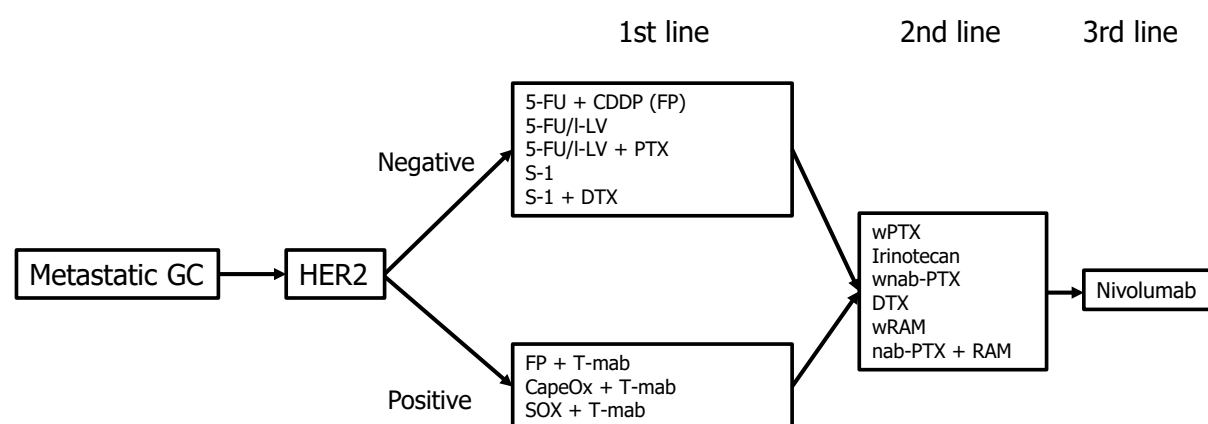
**Table 2. Results of completed phase III trials with molecular targeted therapy in advanced gastric cancer**

Target	Trial	Regimen	Patients (n)	OS (months)	HR
HER2	ToGA <sup>[21]</sup>	Cisplatin, capecitabine or 5-FU ± trastuzumab	584	13.8 vs. 11.1 (1st line)	0.74 (0.60-0.91)
HER2	LOGIC <sup>[48]</sup>	Capecitabine, oxaliplatin ± trastuzumab	545	12.2 vs. 10.5 (1st line)	0.74 (0.73-1.12)
HER2	TyTAN <sup>[49]</sup>	Paclitaxel ± lapatinib	261	11.0 vs. 8.9 (1st line)	0.84 (0.64-1.11)
EGFR	EXPAND <sup>[26]</sup>	Cisplatin, capecitabine ± cetuximab	679	9.4 vs. 10.7 (1st line)	1.09 (0.92-1.29)
EGFR	REAL3 <sup>[27]</sup>	Oxaliplatin, capecitabine, epirubicin ± panitumumab	553	8.8 vs. 11.3 (1st line)	1.37 (1.07-1.76)
VEGFR-2	REGARD <sup>[31]</sup>	BSC ± ramucirumab	355	5.2 vs. 3.8 (2nd line)	0.77 (0.60-0.99)
VEGFR-2	RAINBOW <sup>[30]</sup>	Paclitaxel ± ramucirumab	665	9.6 vs. 7.4 (2nd line)	0.80 (0.68-0.96)
VEGFR-A	AVAGAST <sup>[24]</sup>	Cisplatin, capecitabine or 5-FU ± bevacizumab	774	12.1 vs. 10.1 (1st line)	0.87 (0.73-1.03)
mTOR	GRANITE-1 <sup>[25]</sup>	BSC ± everolimus	633	5.4 vs. 4.3 (2nd or 3rd line)	0.90 (0.75-1.08)

OS: overall survival; HR: hazard ratio; 5-FU: 5-fluorouracil; BSC: best supportive care



**Figure 1.** The treatment algorithm for advanced gastric cancer in Japan



**Figure 2.** The treatment algorithm for patients who are unfit for the standard treatment in Japan

with S-1 alone, with a median PFS of 6 vs. 4 months ( $P < 0.0001$ ). The response rate of SP in this study was 54%; among 87 patients in the SP group, 46 (52.9%) achieved partial response and 1 (1.1%) had a complete response.



Capecitabine and cisplatin (Cape/CDDP) combination is one of the standard first-line regimens for patients with metastatic or recurrent GC worldwide. Cape/CDDP has been employed as a control regimen in global phase III trials, including the ToGA<sup>[21]</sup> and AVAGAST trials<sup>[24]</sup>. The subset analyses of the Japanese participants in these trials have shown safety and efficacy of this regimen; therefore, Cape/CDDP is a first-line treatment choice for Japanese patients.

The REAL-2 trial<sup>[32]</sup> evaluated whether fluorouracil could be replaced with capecitabine, and cisplatin replaced with oxaliplatin, in the epirubicin, 5-FU and cisplatin (ECF) regimen. This trial demonstrated that capecitabine and oxaliplatin are as effective as 5-FU and cisplatin, respectively, in patients with previously untreated esophagogastric cancer. Cisplatin causes renal toxicity and intravenous hydration is required to decrease the toxicity. Oxaliplatin does not require hydration and can be administered in an outpatient clinic. In Japan, the combination of S-1 plus oxaliplatin (SOX) appears to be as effective as SP for metastatic GC, with a favorable safety profile<sup>[33]</sup>.

The superiority of a combination of S-1 and docetaxel (DTX) to S-1 monotherapy as first-line treatment was evaluated in the START trial<sup>[34]</sup> which included Japanese and Korean patients with metastatic or recurrent GC. Although the initial survival analysis failed to demonstrate superiority after clarifying the outcomes of censored cases, a reanalysis demonstrated the efficacy of this regimen [OS 12.5 vs. 10.8 months, hazard ratio (HR) 0.84, 95% CI: 0.71-0.99,  $P = 0.032$ ]. Therefore, S-1/DTX can be selected as an alternative to SP, Cape/CDDP, or SOX. Both irinotecan (CPT-11) plus cisplatin and S-1 plus CPT-11 combinations failed to demonstrate survival benefit over 5-FU alone or S-1 alone, and are not recommended as a first-line regimen<sup>[35,36]</sup>.

Regarding triplet regimens, the V325 trial<sup>[37]</sup> demonstrated survival benefits of docetaxel, cisplatin, and 5-FU (DCF) over cisplatin and 5-FU (CF), although grade 3 or 4 toxicities were more frequent with DCF than CF. In Japan, a triplet regimen consisting of S-1, cisplatin and docetaxel is currently being evaluated in a phase III trial, JCOG1013, based on the favorable results of a phase II trial in Japan<sup>[38-40]</sup>.

Based on these findings, the Japanese guidelines recommend SP or Cape/CDDP as first-line treatment of HER2-negative metastatic GC, and SOX, CapeOX, FOLFOX, FP and S-1/DTX are recommended as alternatives.

### HER2-positive advanced GC

The ToGA trial showed that trastuzumab combined with conventional chemotherapy provided a significant survival advantage compared with chemotherapy alone in patients with HER2 positive metastatic or recurrent GC<sup>[21]</sup>. A total of 584 patients who had HER2-positive advanced GC or gastroesophageal junction cancer were randomly assigned to chemotherapy (consisting of CF or Cape/CDDP) with or without trastuzumab. The addition of trastuzumab significantly improved OS from 11.1 to 13.8 months ( $P = 0.0046$ ), as compared with chemotherapy alone. In addition, PFS increased from 5.5 to 6.7 months (HR 0.7, 95% CI: 0.59-0.85,  $P = 0.0002$ ). In the subgroup analysis, the survival benefit was more evident in the group of patients who had immunohistochemistry (IHC) 3+ or IHC 2+/fluorescent in-situ hybridization (FISH)-positive tumors than in the others. The addition of trastuzumab increased survival from 11.8 to 16.0 months (HR 0.65, 95% CI: 0.51-0.83,  $P = 0.036$ ) among this cohort. Therefore, trastuzumab is recommended for patients with IHC 3+ or IHC 2+/FISH positive tumors. A phase II trial to explore the efficacy and toxicity of trastuzumab combined with triweekly SP enrolled a total of 56 patients<sup>[41]</sup>. The response rate and the disease control rate were 68% (95% CI: 54%-80%) and 94% (95% CI: 84%-99%), respectively. The median OS and PFS were 16.0 and 7.8 months, respectively. Major grade 3 or 4 adverse events included neutropenia (36%), anorexia (23%), and anemia (15%). Although the study was not a randomized controlled trial, SP plus trastuzumab is considered to be a first-line chemotherapy choice for HER2-positive metastatic GC in Japan.

Accordingly, the recommended first-line treatment of HER2-positive metastatic GC in Japan is a combination of trastuzumab and Cape/CDDP or a combination of trastuzumab and SP.

### Second-line treatment

Second-line chemotherapy is known to prolong the survival of metastatic GC patients, and is recommended for patients with acceptable performance status. Among cytotoxic agents, monotherapy with DTX, CPT-11 or paclitaxel (weekly administration, wPTX) are available options. Randomized trials conducted in Germany<sup>[42]</sup> and Korea<sup>[43]</sup> have indicated survival benefits of DTX or CPT-11 over BSC. The German study<sup>[42]</sup> compared CPT-11 as a second-line chemotherapy with BSC but was ended prematurely due to poor accrual. The median OS was 4.0 vs. 2.4 months in the CPT-11 and placebo arms, respectively ( $P = 0.012$ ). The Korean study<sup>[43]</sup> compared either CPT-11 or DTX as salvage chemotherapy (SLC) with BSC. The median OS of the SLC and the BSC arms were 5.1 and 3.8 months ( $P = 0.004$ ), respectively. The WJOG4007<sup>[44]</sup> compared wPTX with CPT-11 in Japanese patients with advanced GC, after failure of primary combination chemotherapy using fluoropyrimidine plus cisplatin. The median OS of wPTX and CPT-11 groups was 9.5 and 8.4 months, respectively ( $P = 0.38$ ). In addition, third-line chemotherapy was administered in 89.8% of the wPTX group and in 72.1% of the CPT-11 group. Based on these findings, both wPTX and CPT-11 are considered reasonable second-line treatment options for advanced GC.

More recently, two large international phase III trials (REGARD and RAINBOW) have revealed the survival benefits of ramucirumab, a fully human monoclonal antibody against the vascular endothelial growth factor receptor (VEGFR)-2<sup>[30,31]</sup> for previously treated advanced gastric or gastroesophageal junction (GEJ) adenocarcinoma. In the REGARD trial<sup>[31]</sup>, patients were randomly assigned (2:1) to receive BSC plus either intravenous ramucirumab 8 mg/kg or placebo once every 2 weeks. The median OS was 5.2 months in the ramucirumab group and 3.8 months in the placebo group (HR: 0.77, 95% CI: 0.60-0.99;  $P = 0.047$ ), while the median PFS was 2.1 and 1.3 months, respectively. Ramucirumab appeared to be well tolerated, although rates of hypertension were higher in the ramucirumab group than in the placebo group. The RAINBOW trial<sup>[30]</sup> compared ramucirumab plus PTX vs. placebo plus PTX in patients with previously treated advanced GC. Patients were randomly assigned in a 1:1 ratio to receive either intravenous ramucirumab 8 mg/kg or placebo on days 1 and 15, plus intravenous PTX 80 mg/m<sup>2</sup> on days 1, 8, and 15 of a 28-day cycle. OS was significantly longer in ramucirumab plus PTX group than in the placebo plus PTX group (the median OS of 9.6 and 7.4 months,  $P = 0.017$ ). The toxicity of ramucirumab plus PTX was tolerable.

Nab-paclitaxel (nab-PTX) is a nanoparticle-albumin-bound paclitaxel and it does not contain the solvent cremophor EL and ethanol. Therefore, nab-paclitaxel can reduce the risk of a hypersensitivity reaction and can be administered to patients who are intolerant of alcohol. The ABSOLUTE trial<sup>[45]</sup> is a phase III study to evaluate the efficacy and safety of nab-PTX vs. wPTX in Japanese patients with advanced GC refractory to first-line chemotherapy. The median OS was 10.3 months in the nab-PTX every 3 weeks group, 11.1 months in the weekly nab-PTX group and 10.9 months in the wPTX group. Weekly nab-PTX was non-inferior to wPTX in terms of OS.

In summary, the recommended second-line treatment for metastatic GC in Japan is ramucirumab plus wPTX, and the alternative choice is monotherapy of either DTX, CPT-11, nab-PTX or ramucirumab.

### Immune checkpoint inhibitors in GC treatment

The ATTRACTION-2 (ONO-4358-12) trial evaluated the efficacy and safety of nivolumab, a fully human IgG4 monoclonal antibody inhibitor of programmed death-1, in patients with advanced GC or GEJ cancer who had been treated with two or more chemotherapy regimens<sup>[46]</sup>. The median OS was 5.26 months in the nivolumab group and 4.14 months in the placebo group (HR 0.63, 95% CI: 0.51-0.78;  $P < 0.0001$ ). The

safety profile of nivolumab in patients with advanced GC or GEJ cancer was manageable and similar to that reported in patients with other advanced solid tumors. Based on these results, the Japanese Ministry of Health, Labor and Welfare approved nivolumab for the treatment of unresectable advanced or recurrent GC which has progressed after chemotherapy. Currently, trials are ongoing to evaluate the efficacy of immune checkpoint inhibitors in earlier lines of GC treatment.

### **Ongoing trials in Japan**

The RAINFALL trial is ongoing to evaluate the effectiveness of ramucirumab in combination with Cape/CDDP compared to Cape/CDDP alone as first-line treatment of metastatic GC or GEJ adenocarcinoma (NCT02314117). The SOLAR trial, a phase III trial comparing TAS-118 (S-1 plus leucovorin) and oxaliplatin vs. SP as first-line treatment, is recruiting patients with advanced GC in Japan and Korea (NCT02322593).

### **Precision medicine for GC**

Treatment of cancer is likely to shift and be tailored towards personalized therapy based on detailed molecular information, known as precision medicine. The Cancer Genome Atlas Research Network reported the results of molecular classification of GC through integrative genomic analysis, which suggested that GC could be divided into four subtypes<sup>[47]</sup>: (1) Epstein-Barr virus-related tumors; (2) microsatellite instability represented as elevated mutation rates and MLH1 silencing; (3) genomically stable tumors that are strongly related with diffuse histology, RHOA mutations, and CLDN18-ARHGAP fusion; and (4) chromosomal instability that mainly comprises intestinal histology, TP53 mutation, and focal amplification of the receptor tyrosine kinase. Another study reported that GC can be classified into four subtypes<sup>[48]</sup>: (1) microsatellite unstable; (2) microsatellite stable (MSS) with TP53 mutation; (3) MSS without TP53 mutation; and (4) MSS with epithelial-to-mesenchymal transition (EMT). This study found that the MSS/EMT subtype was related to poor prognosis. Further analysis is needed to establish genome-based precision medicine.

### **CONCLUSION**

The main goal of treatment for metastatic GC patients is to prolong patient survival while preserving quality of life. In addition to the combination of conventional cytotoxic drugs, several newly developed agents, including targeted molecules and immune checkpoint inhibitors, have shown favorable results in the treatment of metastatic GC. Efforts should be focused on achieving precision medicine based on the molecular information of GC.

### **DECLARATIONS**

#### **Authors' contributions**

Concept, design, literature search, and manuscript preparation: Eto K

Manuscript editing: Ida S

Design, manuscript editing, and manuscript review: Watanabe M

Manuscript review: Baba H

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None.

#### **Conflicts of interest**

Authors declare that they have no conflicts of interest.

#### **Patient consent**

Not applicable.

## Ethics approval

Not applicable.

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Review

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# Management of metastatic esophagogastric junction adenocarcinoma

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## Abstract

The prognosis of metastatic disease of esophagogastric junction adenocarcinoma remains poor, despite using a variety of regimens using cytotoxic agents. Recent understanding of molecular characteristic and tumor microenvironment of this cancer is currently instigating new therapeutic options. In this review, we summarized previous evidences of cytotoxic agents widely used worldwide, and updated recent developments of molecular targeted drugs, and immune checkpoint inhibitors.

**Keywords:** Esophagogastric junction, adenocarcinoma, advanced, molecular targeted drug, immune checkpoint inhibitor, immunotherapy

## INTRODUCTION

The esophagogastric junction (EGJ) adenocarcinoma is defined as tumors which have their center within 5 cm proximal or distal to the anatomical esophagogastric junction<sup>[1-3]</sup>. In Western countries, the incidence of EGJ adenocarcinoma has been increasing rapidly over the last few decades, in the background of decreasing rate of *Helicobacter pylori* infection, and increasing trends of obesity and gastroesophageal reflux disease (GERD). EGJ adenocarcinoma is usually diagnosed with unresectable disease because of difficulty in early detection. Even after curative resection, many cases experience recurrent disease, resulting in lower survival rates of this tumor<sup>[4,5]</sup>. In spite of multidisciplinary treatments, median overall survival (OS) is around 12 months in advanced EGJ or gastric adenocarcinoma<sup>[6,7]</sup>. Therefore, the treatment goal for metastatic



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disease of this tumor should include survival benefit with symptom relief, and systemic chemotherapy is a major treatment option for those cases<sup>[8]</sup>. Treatments for advanced EGJ adenocarcinoma has been developed as a type of advanced gastric cancer, and many clinical trials were conducted targeting both EGJ and gastric adenocarcinoma. Recent comprehensive molecular analysis for upper GI cancers reveals molecular differences between EGJ and gastric adenocarcinoma<sup>[9,10]</sup>. Here, we update recent evidences of treatments for advanced EGJ adenocarcinoma, and discuss future perspective.

### CYTOTOXIC AGENTS (FOR HER2-NEGATIVE TUMORS)

Fluoropyrimidine (ftorafur, S-1, or capecitabine), platinum (cisplatin, or oxaliplatin), irinotecan, and taxanes (paclitaxel, or docetaxel) are globally used for metastatic disease of EGJ adenocarcinoma. In addition, trastuzumab is a humanized monoclonal antibody that selectively binds with high affinity to the extracellular domain of the human epidermal growth factor receptor, and approved for tumors with HER2+ [protein overexpression by immunohistochemistry (IHC) or gene amplification by *in situ* hybridization (FISH)] EGJ adenocarcinoma. Considering chemotherapeutic managements, tumor HER2 status is a valuable information for adding trastuzumab to cytotoxic agents. As a first-line therapy, there is no widely accepted first-line standard regimen for advanced EGJ adenocarcinoma.

In the USA and Europe, fluorouracil and platinum-based agents (CF) or docetaxel, fluorouracil, and cisplatin (DCF) is widely used regimen based on the clinical trial. In 2006, the V-325 study group showed no superiority between DCF and DC (docetaxel and cisplatin) in OS. Median OS was 9.6 months for DCF, and 10.5 months for DC. The incidence of hematologic toxicities was high, but it was comparable between DCF and DC. Grade 3 or 4 neutropenia was the most common in hematologic toxicity; it occurred in 86% in the patients with DCF, and 87% in DC cases, although non-hematologic toxicities of DCF had a higher incidence than that of DC<sup>[11]</sup>.

In Europe, epirubicin, cisplatin, and fluorouracil (ECF), epirubicin, cisplatin, and capecitabine (ECX), epirubicin, oxaliplatin, and fluorouracil (EOF), or epirubicin, oxaliplatin, and capecitabine (EOX) is a major regimen for advanced EGJ or stomach adenocarcinoma. The REAL-2 trial assessed above-mentioned four regimens with different three-drug combination, and showed median OS of 9.9 months with ECF, 9.9 months with ECX, 9.3 months with EOF, and 11.2 months with EOX, respectively. One-year-survival rates were 37.7%, 40.8%, 40.4%, and 46.8%. The trial showed capecitabine and oxaliplatin were as effective as fluorouracil and cisplatin<sup>[12]</sup>.

In Asia, the recommended first-line treatment is S-1 plus cisplatin (SP) or capecitabine plus cisplatin (XP). In the SPIRITS trial [phase III, including advanced gastric adenocarcinoma ( $n = 298$ )], OS was better in patients treated with SP than with S-1 alone. Median OS was 13.0 months [interquartile range (IQR) 7.6-21.9] in those assigned to SP compared with 11.0 months (IQR 5.6-19.8) in those assigned to S-1 alone [hazard ratio (HR) 0.77; 95% CI 0.61-0.98;  $P = 0.04$ ]. Progression-free survival (PFS) was significantly longer in those assigned to SP than S-1 alone [median PFS 6.0 months (3.3-12.9) for SP vs. 4.0 months (2.1-6.8) for S-1 alone;  $P < 0.0001$ ]. The trial showed more grade 3 or 4 adverse events including leucopenia, neutropenia, anemia, nausea, and anorexia, in patients assigned to SP than in patients assigned to S-1 alone<sup>[13]</sup>. However, the incidence of EGJ cancer remains low in Japan, and this clinical trial included only gastric cancer patients. Therefore, the standard treatment for EGJ cancer has not yet been established in Japan and patients with EGJ cancer are usually treated based on the evidence for gastric cancer.

### MOLECULARLY TARGETED DRUG

In the first decade of this century, molecularly targeted drugs have been developed for advanced EGJ adenocarcinoma [Table 1]. To date, trastuzumab and ramucirumab are the only molecularly targeted drugs

**Table 1. Clinical trials testing targeted therapies for esophagogastric junction and gastric adenocarcinoma**

Trial	Target	Patients (EGJ)	Treatment	Outcome (EGJ + gastric cases)	Outcome (EGJ)	Outcome (gastric)	Primary endpoint	Refs
1st line								
ToGA	HER2	594 (106)	XP <i>vs.</i> XP + trastuzumab	Positive	Negative	Positive	OS	[6]
LOGiC	HER2	545 (49)	CapeOx <i>vs.</i> CapeOx + lapatinib	Negative	Negative	Negative	OS	[18]
EXPAND	EGFR	904 (144)	XP <i>vs.</i> XP + cetuximab	Negative	Negative	Negative	OS	[24]
REAL3	EGFR	553 (169)	EOC <i>vs.</i> EOC + panitumumab	Negative	Negative	Positive	OS	[26]
RILOMET-1	MET	609 (124)	ECX <i>vs.</i> ECX + rilotumumab	Negative	Negative	Positive	OS	[27]
METGastric	MET/HGF	562 (130)	mFOLFOX6 <i>vs.</i> mFOLFOX6+onartuzumab	Negative	Negative	Negative	OS	[29]
AVAGAST	VEGFR-A	774 (130)	XP <i>i.</i> XP + bevacizumab	Negative	Negative	Negative	OS	[17,49]
2nd line								
RAINBOW	VEGFR2	665 (137)	Paclitaxel <i>vs.</i> paclitaxel + ramucirumab	Positive	Positive	Positive	OS	[7]
REGARD	VEGFR2	355 (90)	Placebo <i>vs.</i> paclitaxel + ramucirumab	Positive	Negative	Negative	OS	[14]
TyTAN	HER2	261 (2)	Paclitaxel or docetaxel <i>vs.</i> trastuzumab-emtansine	Negative	Negative	Negative	OS	[19]
GATSBY	HER2	345 (110)	Paclitaxel <i>vs.</i> paclitaxel + lapatinib	Negative	Negative	Negative	OS	[31]
GRANITE-1	mTOR	656 (187)	Placebo <i>vs.</i> everolimus	Negative	Negative	Negative	OS	[50,51]

CapeOx: capecitabine and oxaliplatin; EGFR: epidermal growth factor receptor; ECX: epirubicin, cisplatin and capecitabine; EOC: epirubicin, oxaliplatin and capecitabine; HER2: human epidermal growth factor 2; HGF: hepatocyte growth factor; mFOLFOX6: leucovorin, fluorouracil and oxaliplatin; mTOR: mechanistic target of rapamycin; OS: overall survival; PFS: progression-free survival; VEGF-A: vascular endothelial growth factor A; VEGFR2: vascular endothelial growth factor receptor 2; XP: capecitabine and cisplatin

with confirmed survival benefit in phase III trials. In this section, we focus on the results of phase III clinical trials.

### Trastuzumab (anti-HER2 antibody)

Trastuzumab is a monoclonal antibody targeting HER2. In 2010, ToGA trial [phase III, including EGJ ( $n = 106$ ) and advanced gastric adenocarcinoma ( $n = 478$ )] was to assess the efficacy and safety of trastuzumab plus first-line chemotherapy (XP or FP) of advanced HER2 positive 106 EGJ and 478 gastric adenocarcinoma. HER2 status was tested by IHC and FISH. HER2 positivity was defined as samples with 3+ by IHC, or those with both 2+ IHC and FISH positive. HER2 positivity was frequently observed in tumors located at EGJ, compared to those in stomach (33.2% for EGJ *vs.* 20.9% for stomach;  $P < 0.001$ ). Median OS was significantly longer in trastuzumab plus chemotherapy groups than in chemotherapy alone [median 13.8 months (95% CI 12-16) *vs.* median 11.1 months (95% CI 10-13), HR 0.74; 95% CI 0.60-0.91;  $P = 0.0046$ ]. However, in a subgroup analysis of EGJ adenocarcinoma, there were no survival benefit of trastuzumab (trastuzumab plus chemotherapy groups *vs.* chemotherapy alone, HR 0.67; 95% CI 0.42-1.08). The most common adverse events in both groups were nausea, vomiting and neutropenia. Rate of overall grade 3-4 adverse events (68% in trastuzumab plus chemotherapy groups *vs.* 68% in chemotherapy alone) and cardiac adverse events (6% in trastuzumab plus chemotherapy groups *vs.* 6% in chemotherapy alone) did not differ between the groups<sup>[6]</sup>. NCCN guideline recommends the addition of trastuzumab to any chemotherapy combination for patients with HER2-positive tumors.

### Ramucirumab (VEGFR-2 inhibitor)

Ramucirumab is a human IgG1 monoclonal antibody VEGFR-2 antagonist. The REGARD trial and the

RAINBOW trial showed a significant benefit of ramucirumab for advanced EGJ and gastric adenocarcinoma, as the second-line chemotherapy.

The REGARD trial [phase III, including advanced EGJ ( $n = 90$ ) and gastric adenocarcinoma ( $n = 265$ )] exhibited a significant benefit of ramucirumab (OS 5.2 months for ramucirumab vs. OS 3.8 months for placebo; HR 0.776, 95% CI 0.603-0.998;  $P = 0.047$ ). However, in a subgroup analysis of EGJ adenocarcinoma, the trial did not exhibit any significant benefit of ramucirumab (ramucirumab groups vs. placebo groups, HR 0.76; 95% CI 0.47-1.21). The incidence of hypertension was higher in the ramucirumab group than in the placebo group (16% vs. 8%)<sup>[14]</sup>. In the RAINBOW trial [phase III, including advanced EGJ ( $n = 137$ ) and gastric adenocarcinoma ( $n = 528$ )], the ramucirumab plus paclitaxel conferred a significantly prolonged OS, compared to the placebo plus paclitaxel group (9.6 vs. 7.4 months, HR 0.807; 95% CI 0.678-0.962;  $P = 0.017$ ). In a subgroup analysis of EGJ adenocarcinoma, the trial revealed survival benefit of adding ramucirumab, either (ramucirumab plus paclitaxel groups vs. placebo plus paclitaxel groups, HR 0.39; 95% CI 0.26-0.59). Grade 3 or 4 adverse events that occurred in more than 5% of patients in the ramucirumab and paclitaxel group vs. placebo and paclitaxel group were as follow; neutropenia (41% vs. 19%), leucopenia (17% vs. 7%), hypertension (14% vs. 2%), fatigue (12% vs. 5%), anemia (9% vs. 10%), and abdominal pain (6% vs. 3%)<sup>[7]</sup>.

### Bevacizumab (anti-VEGF antibody)

Bevacizumab, which is a monoclonal antibody that targets vascular endothelial growth factor A (VEGF-A), inhibiting tumor growth in preclinical studies<sup>[15,16]</sup>. In AVAGAST trial [phase III, including advanced EGJ ( $n = 103$ ) and gastric adenocarcinoma ( $n = 671$ )], bevacizumab did not confer any survival benefit (median OS 12.1 months in bevacizumab plus XP; vs. median OS 10.1 months in XP alone). In a subgroup analysis of EGJ adenocarcinoma, there was no survival benefit (data not available)<sup>[17]</sup>.

### Lapatinib

Lapatinib is the dual inhibitor of epidermal growth factor receptor (EGFR) and human epidermal growth factor receptor 2 (HER2) tyrosine kinases. In the TRIO-013/LOGiC trial [phase III, including advanced EGJ ( $n = 46$ ), esophageal ( $n = 20$ ) and gastric adenocarcinoma ( $n = 424$ )], lapatinib plus capecitabine and oxaliplatin (CapeOX) showed no additional efficacy as the first-line treatment for HER2 positive patients [median OS 12.2 months in CapeOX + lapatinib groups (95% CI 10.6-14.2) vs. median OS 10.5 months in CapeOX groups (95% CI 9.0-11.3), HR 0.91; 95% CI 0.73-1.12;  $P = 0.35$ ]. In a subgroup analysis of EGJ adenocarcinoma, there was no survival benefit (CapeOX + lapatinib groups vs. CapeOX groups, HR 0.90; 95% CI 0.44-1.85;  $P = 0.77$ )<sup>[18]</sup>.

TyTan study [phase III, including advanced EGJ ( $n = 2$ ) and gastric adenocarcinoma ( $n = 259$ )] demonstrated that lapatinib plus paclitaxel did not improve OS in HER2-positive patients compared to paclitaxel alone [median OS 11.0 months in lapatinib plus paclitaxel group (95% CI 9.5-14.5) vs. median OS 8.9 months in paclitaxel alone group (95% CI 7.4-11.1), HR 0.84; 95% CI 0.64-1.11;  $P = 0.1044$ ]<sup>[19]</sup>.

### Cetuximab, or panitumumab (anti-EGFR antibody)

Cetuximab is an EGFR antibody, widely used for patients with *KRAS* wild-type metastatic colorectal cancer<sup>[20,21]</sup>, recurrence or metastatic squamous-cell carcinoma of the head and neck<sup>[22]</sup>, and advanced non-small-cell lung cancer<sup>[23]</sup>. In the EXPAND trial [phase III, including advanced EGJ ( $n = 144$ ) and gastric adenocarcinoma ( $n = 747$ )], the efficacy of adding cetuximab to capecitabine plus cisplatin was examined. However, there was no benefit to adding of cetuximab to chemotherapy compared to chemotherapy alone in the first-line treatment [median PFS 4.4 months in cetuximab plus capecitabine and cisplatin groups (95% CI 4.2-5.5); vs. median PFS 5.6 months in capecitabine and cisplatin alone groups (95% CI 5.1-5.7); HR 1.09; 95% CI 0.92-1.29;  $P = 0.32$ ]. In a subgroup analysis of EGJ adenocarcinoma, there was no benefit to add cetuximab, either [median PFS 5.6 months in cetuximab plus capecitabine and cisplatin groups vs. median PFS 5.6 months in capecitabine and cisplatin alone groups; HR 1.12; 95% CI 0.73-1.71]<sup>[24]</sup>.



Panitumumab is a fully human immunoglobulin G2 monoclonal antibody targeting EGFR. In advanced colorectal adenocarcinoma, panitumumab significantly improved PFS<sup>[25]</sup>. The REAL3 trial [phase III, including advanced EGJ ( $n = 169$ ), esophageal ( $n = 220$ ) and gastric adenocarcinoma ( $n = 167$ )] revealed no survival benefit of adding panitumumab to epirubicin, oxaliplatin and capecitabine (EOC) chemotherapy [median OS 11.3 months in EOC alone groups (95% CI 9.6-13.0) vs. median OS 8.8 months in panitumumab plus EOC groups (95% CI 7.7-9.8), HR 1.37; 95% CI 1.07-1.76;  $P = 0.013$ ]. In a subgroup analysis of EGJ adenocarcinoma, the trial revealed no survival benefit of adding panitumumab, either (EOC alone groups vs. panitumumab plus EOC groups, HR 1.27; 95% CI 0.78-2.07)<sup>[26]</sup>.

### **Rilotumumab, and onartuzumab (MET/HGF inhibitor)**

Rilotumumab is a fully human monoclonal antibody that selectively targets the ligand of the MET receptor, hepatocyte growth factor (HGF). In the RILOMET-1 trial [phase III, including advanced EGJ ( $n = 124$ ), distal esophageal ( $n = 67$ ) and gastric adenocarcinoma ( $n = 63$ )], median OS was 8.8 months (95% CI 7.7-10.2) in the rilotumumab group, compared with 10.7 months (95% CI 9.6-12.4) in the placebo group (HR 1.34, 95% CI 1.10-1.63;  $P = 0.003$ ), demonstrating that rilotumumab conferred no survival benefit. In a subgroup analysis of EGJ adenocarcinoma, rilotumumab conferred no survival benefit (the rilotumumab group vs. the placebo group, HR 1.28; 95% CI 0.83-1.98)<sup>[27]</sup>.

Onartuzumab is a recombinant, fully humanized, monovalent monoclonal antibody that binds the extracellular domain of MET, blocking interaction with HGF<sup>[28]</sup>. In METGastric trial [phase III, HER2-negative and MET-positive tumors, including advanced EGJ ( $n = 130$ ) and gastric adenocarcinoma ( $n = 432$ )], no survival benefit was observed in onartuzumab plus mFOLFOX group, compared to placebo plus mFOLFOX (median OS 11.3 months in placebo plus mFOLFOX group vs. median OS 11.0 months in onartuzumab plus mFOLFOX group, HR 0.82; 95% CI 0.59-1.15;  $P = 0.24$ ). In a subgroup analysis of EGJ adenocarcinoma, no survival benefit was observed in onartuzumab plus mFOLFOX group (median OS not estimable in placebo plus mFOLFOX group vs. median OS 11.0 months in onartuzumab plus mFOLFOX group, HR 1.12; 95% CI 0.58-2.19)<sup>[29]</sup>.

### **Everolimus (mTOR inhibitor)**

Everolimus is an oral mTOR inhibitor. In GRANITE-1 [phase III, including advanced EGJ ( $n = 187$ ) and gastric adenocarcinoma ( $n = 656$ )], everolimus did not significantly improve OS, compared to placebo alone (median OS, 5.4 months in everolimus vs. median OS, 4.3 months in placebo, HR 0.90; 95% CI 0.75-1.08;  $P = 0.124$ ). In a subgroup analysis of EGJ adenocarcinoma, everolimus did not significantly improve OS, either (everolimus vs. placebo, HR 0.84; 95% CI 0.61-1.16)<sup>[30]</sup>.

### **Trastuzumab emtansine (anti-HER2 antibody)**

Trastuzumab emtansine (T-DM1) is anti-HER2 monoclonal antibody consisting of trastuzumab linked to emtansine (DM1), which is a microtubule inhibitor. In GATSBY [phase II/III, including HER2-positive advanced EGJ ( $n = 110$ ) and gastric adenocarcinoma ( $n = 235$ )], there was no superiority of T-DM1 to taxane [median OS 7.9 months with T-DM1 (95% CI 6.7-9.5) vs. median OS 8.6 months with taxane (95% CI 7.1-11.2), HR 1.15; 95% CI 0.87-1.51;  $P = 0.86$ ]. In a subgroup analysis of EGJ adenocarcinoma, similarly to the above, there was no superiority of T-DM1 to taxane (median OS 7.1 months with T-DM1 vs. median OS 8.5 months with taxane, HR 1.18; 95% CI 0.70-2.01)<sup>[31]</sup>.

### **Future prospect of molecularly targeted drugs**

Although precision medicine still remains developing for the upper gastrointestinal malignancies, there are some new approaches such as VIKTORY, and PANGAEA trials. PANGAEA is a phase II trial that gastroesophageal tumors are classified into the following six categories (HER2+, MET+, FGFR2+, VEGFR2+, MSI-H, and EGFR+), and then paired specific targeted therapies (trastuzumab, TBD, anti-EGFR antibody

ABT-806, TBD2, ramucirumab, and nivolumab) are assigned according to the biomarkers, along with standard chemotherapy<sup>[32]</sup>. VIKTORY is a screening trial without drug intervention for metastatic GC patients who failed or progressed on first-line chemotherapy, using cancer panel/nanostring CNV and immunohistochemistry<sup>[33]</sup>. These efforts may create new algorithms in upper gastrointestinal cancers.

## IMMUNOTHERAPY

The most advanced of the emerging development in EGJ and gastric adenocarcinoma is immunotherapy. Programmed death protein 1 (PD1), programmed cell death 1 ligand 1 (PD-L1) and cytotoxic T lymphocyte protein 4 (CTLA4) are the key drugs to regulate cellular immune functions. Pembrolizumab and nivolumab, which are being developed as anti-PD1 antibodies, have been examined in clinical trials.

### Pembrolizumab

Pembrolizumab is a selective, humanized, high-affinity IgG4-κ monoclonal antibody. By binding to PD1, pembrolizumab block the interaction between PD-1 and its ligands. In the USA, pembrolizumab was approved by the FDA for the treatment of melanoma, non-small-cell lung cancer and head and neck cancer. In a phase Ib trial (KEYNOTE-012), the safety and activity of pembrolizumab was assessed in patients with PD-L1 positive advanced EGJ and gastric adenocarcinoma. The median PFS and the median OS were 1.9 months (95% CI 1.8-3.5) and 11.4 months (95% CI 5.7) respectively<sup>[34]</sup>. The KEYNOTE-061 is a phase III trial as a second-line therapy for PD-L1-positive patients, comparing pembrolizumab with paclitaxel. The KEYNOTE-062 is phase III trial of pembrolizumab alone or combination with FP or capecitabine vs. FP or capecitabine alone as a first-line therapy for PD-L1-positive patients. Both of these trials are still in progress.

### Nivolumab

Nivolumab is a fully human IgG4 monoclonal antibody inhibitor of PD-1. In the ATTRACTION-2 study, which was a randomized phase III trial, investigating the efficacy and safety of nivolumab as a third-line for unresectable advanced and recurrent EGJ and gastric adenocarcinoma regardless of PD-L1 expression. Median OS was 5.26 months (95% CI 4.60-6.37) in the nivolumab group and 4.14 months (95% CI 3.42-4.86) in the placebo group (HR 0.63, 95% CI 0.51-0.78;  $P < 0.0001$ ), resulting in a new treatment option for these cancers<sup>[35]</sup>. The other anti PD-L1 antibody, such as avelumab, durvalumab and atezolizumab have been expected to advanced EGJ and gastric adenocarcinoma. Two randomized phase III trials of avelumab in EGJ and gastric adenocarcinoma are undergoing [Table 2].

CheckMate-032 is an ongoing trial, evaluating nivolumab alone, and nivolumab in combination with ipilimumab, for various solid tumors including previously treated advanced EGJ and gastric adenocarcinoma, regardless of PD-L1 expression status. Patients were randomly assigned in to the following three groups, NIVO3 group (nivolumab: 3 mg/kg, once every 2 weeks), NIVO1 plus IPI3 group (nivolumab: 1 mg/kg plus ipilimumab: 3 mg/kg, once every 3 weeks) and NIVO3 plus IPI1 group (nivolumab: 3 mg/kg plus ipilimumab: 1 mg/kg, once every 3 weeks). The median OS were 6.2 months (95% CI 3.4-12.4) in NIVO3 group, 6.9 months (95% CI 3.7-11.5) in NIVO1 plus IPI3 group and 4.8 months (95% CI 3.0-8.4) in NIVO3 plus IPI1 group<sup>[36,37]</sup>. In addition, CheckMate 649 examining nivolumab plus ipilimumab or nivolumab plus chemotherapy compared with patients receiving chemotherapy alone are also in progress<sup>[38]</sup>. Utilizing nivolumab in combination with the other agents may be a major option for EGJ and gastric adenocarcinoma.

### Future prospect of immunotherapy

Many study reported that PD-L1 expression has been related with poor prognosis and associated with response to immunotherapy<sup>[39-42]</sup>. On the other hands, only a few studies reported that PD-L1 blockade was effective without PD-L1 expression<sup>[35]</sup>. These results indicated that PD-L1 is not yet established as a biomarker for PD-L1 inhibitors. Recent reports suggested that host microbiome and tumor and stromal genomic profiles may be related with response to immune checkpoint blockade<sup>[9,10]</sup>. The diversity and

**Table 2. The phase III clinical trials of immunotherapy for esophagogastric junction and gastric adenocarcinoma**

<b>Trial</b>	<b>Drug</b>	<b>Target</b>	<b>Patients (EGJ)</b>	<b>Treatment</b>	<b>Primary endpoint</b>
CheckMate 649 (NCT02872116)	Nivolumab	PD1	594 (106)	Nivolumab and ipilimumab vs. 5-FU and oxaliplatin	OS
KEYNOTE-062 (NCT02494583)	Pembrolizumab	PD1	545 (49)	Pembrolizumab vs. pembrolizumab, 5-FU and cisplatin or capecitabine vs. 5-FU and cisplatin	PFS and OS
KEYNOTE-061 (NCT02370498)	Pembrolizumab	PD1	665 (137)	Pembrolizumab vs. paclitaxel	PFS and OS
ONO-4538-12 (NCT02267343)	Nivolumab	PD1	261 (0)	Nivolumab vs. placebo	OS

CTLA-4: cytotoxic T-lymphocyte-associated protein 4; OS: overall survival; PD1: programmed death protein 1; 5-FU: 5-fluorouracil; PFS: progression-free survival

abundance of specific bacterial species in the oral and fecal microbiome enhanced systemic and antitumor immunity<sup>[43,44]</sup>. For example, in the patients with advanced tumor who received immunotherapy, the use of antibiotics caused poor prognosis. In addition, oral administration of bacteria improved anti-tumor effect<sup>[45]</sup>. Some immune checkpoints, such as lymphocyte activation gene 3 protein (LAG3)<sup>[46]</sup>, T-cell immunoglobulin and mucin domain 3 (TIM3)<sup>[47]</sup>, T-cell immune-receptor with Ig and ITIM domains (TIGIT)<sup>[48]</sup> are being currently investigated in clinical trials, in order to develop new drugs in the near future.

## CONCLUSION

Global standard treatment for metastatic EGJ and gastric adenocarcinoma is the combination of platinum-agents and fluoropyrimidine. The availability of targeted agents such as trastuzumab or ramucirumab, have become a new hope to the patients with this aggressive tumor. Immune checkpoint inhibitors have emerged as a novel therapeutic option. Discovering the best combination of these drugs may lead a dramatic improvement of the prognosis of these aggressive tumors.

## DECLARATIONS

### Authors' contributions

Concept, design, literature search and manuscript preparation: Toihata T

Concept, design, and manuscript editing: Imamura Y

Manuscript review: Watanabe M, Baba H

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None.

### Conflicts of interest

There are no conflicts of interest.

### Patient consent

Not applicable.

### Ethics approval

Not applicable.

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Original Article

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# Anti-oxidation properties of leaves, skin, pulp, and seeds extracts from green papaya and their anti-cancer activities in breast cancer cells

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## Abstract

**Aim:** Breast cancer is typically detected either during a screening examination or after a woman notices a lump. Breast cancers have different phenotypes depending on the presence/absence of an estrogen receptor (ER) and/or an epidermal growth factor (Her-2) receptor. The objective of the present investigation was to investigate growth inhibitory activity of methanol-, ethanol-, and water-extracts from papaya fruit and leaves on MDA-MB-231 (ER<sup>-</sup>/Her-2<sup>-</sup>), MCF-7 (ER<sup>+</sup>/Her-2<sup>-</sup>), SK-BR-3 (ER<sup>-</sup>/Her-2<sup>+</sup>) and MDA-MB-361, AU565 (ER<sup>+</sup>/Her-2<sup>+</sup>) breast cancer cells.

**Methods:** The anti-oxidation potential of papaya extracts was determined by assessing their total polyphenol content, total flavonoid content and by assaying their anti-oxidation capacity. The effects on breast cancer cells proliferation were determined using a WST-1 assay.

**Results:** The seeds and leaves contained higher anti-oxidation potential than that of the skin and pulp fractions. Our data indicate that methanol- and ethanol-extracts of papaya leaves, skin, pulp, and seeds have no effect on any of the breast cancer cell lines, whereas water-extract of leaves and seeds caused low to modest cytotoxic effects only on ER-negative breast cancer cell lines.

**Conclusion:** Our data suggest that bioactive compound in papaya leaves can be potentially used to develop anti-cancer agents for ER-negative breast cancer.



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**Keywords:** Breast cancer, papaya leaves, antioxidants, polyphenols

## INTRODUCTION

Cancer is the second most common cause of death in the US<sup>[1]</sup>. About 1,685,210 people were estimated to have been diagnosed with cancer, and an estimated 595,690 were expected to die from it in 2016<sup>[2]</sup>. According to a published report, an estimated 14 million cases of cancer reported worldwide and nearly half (about 13% of the total worldwide deaths) died from cancer<sup>[3]</sup>. According to National Cancer Institute (2016), the most common cancer in the world is breast cancer in females and prostate cancer in males, followed by lung cancer. However, lung cancer causes more deaths than breast or prostate cancer<sup>[4]</sup>. Breast cancer is characterized in different molecular phenotypes based on three cellular receptors: estrogen receptor (ER), progesterone receptor (PR), and the epidermal growth factor receptor family member (Her-2/Neu). According to this classification a breast cancer can be ER<sup>+</sup>/PR<sup>+</sup>/Her-2<sup>+</sup>, ER<sup>+</sup>/PR<sup>+</sup>/Her-2<sup>-</sup>, ER<sup>-</sup>/PR<sup>-</sup>/Her-2<sup>+</sup>, or ER<sup>-</sup>/PR<sup>-</sup>/Her-2<sup>-</sup><sup>[5]</sup>. These subtypes have different tumor biology and treatment strategies.

The early and advanced hormone positive breast cancers can be effectively treated with endocrine therapy which blocks the estrogen production and/or inhibits the effect of estrogen at the receptor level<sup>[6]</sup>. Tamoxifen is a selective modulator of ER which is used as a gold-standard adjuvant treatment since 1995 for pre- and postmenopausal patients at low-risk of recurrence<sup>[7]</sup>. More recently, aromatase inhibitors (AIs) including drugs letrozole, anastrozole, and exemestane are developed which inhibit enzyme aromatase and reduce estrogens formation from androgens<sup>[8,9]</sup>. Other recent developments in breast cancer therapy include the development of strategies to inhibit Her-2 activity, especially in Her-2 positive breast cancer by monoclonal antibodies and by antibody fragments<sup>[10,11]</sup>. The recently developed humanized monoclonal antibody, trastuzumab (Tra), specifically targets the extracellular domain of Her-2, which is approved by the Food and Drug Administration (FDA) for the treatment of Her-2<sup>+</sup> breast cancer<sup>[12,13]</sup>. Another strategy to treat breast cancer is to develop inhibitors for angiogenesis. Angiogenesis is a process where new blood vessels are formed from existing vessels<sup>[14]</sup>. The strategies to reduce angiogenesis are (1) the development of antibodies or small molecules against vascular endothelial growth factor, basic fibroblast growth factor or platelet-derived growth factor to inhibit action of these proangiogenic factors and (2) the use of endogenous angiogenesis inhibitors including thrombospondin-1, endostatin, angiostatin, arresten, canstatin and tumstatin<sup>[15,16]</sup>. Although successful, these treatments for breast cancer have considerable side effects and often patients develop resistance to these drugs. There is a growing interest to use natural products for as an alternative or adjunct strategy to treat and prevent breast cancer.

Several epidemiological studies have shown that consumption of fruits, and vegetables (especially soy and cruciferous vegetables) are linked to reduced risk of breast cancer<sup>[17,18]</sup>, and some dietary natural products consumption might increase the survival rate of breast cancer by reducing the recurrence<sup>[19,20]</sup>. Several experimental studies have also shown that dietary natural products and their bioactive compounds can be very effective in reducing breast cancer growth because they are able to downregulate ER- $\alpha$  expression and activity; inhibit tumor proliferation, metastasis and angiogenesis of breast tumor cells; induce apoptosis and cell cycle arrest; and sensitize breast tumor cells to radiotherapy and chemotherapy<sup>[21,22]</sup>. It has been shown that the breast carcinogenesis occurs due to oxidative damage of mitochondrial DNA by reactive oxygen species (ROS)<sup>[23,24]</sup>. In a review article, studies were discussed indicating that the effects on cancer cells by fruits and vegetables, which are rich in flavonoids and other phenolic compounds, have been associated with their abilities to reduce or inhibit free radical-mediated damage to cellular macromolecules, such as proteins, lipids, and DNA<sup>[25]</sup>. These observations suggest that there may be an inverse association between anti-oxidation properties and cancer cell growth. The consumption of natural-dietary substances is, therefore, suggested as a useable approach for the prevention and/or treatment of breast cancer<sup>[26]</sup>.

The papaya (*Carica papaya* Linn) tree that belongs to a family *Caricaceae*, is originated in southern Mexico and Costa Rica. Now it is grown all over the world including Australia, Hawaii, Philippines, Sri Lanka, South Africa, India, and in all tropical and subtropical regions. Some counties produce papaya on a commercial scale for export; however, in most of the tropical regions it can be grown in home gardens<sup>[27]</sup>. Traditionally, all parts of papaya including roots, seeds, flowers, fruit, latex, barks, and leaves have been used to treat a number of diseases in various regions in the world. Papaya has also been studied for its anticancer activities for colorectal<sup>[28]</sup>, prostate<sup>[29,30]</sup>, cervical<sup>[31]</sup> and breast cancers<sup>[32]</sup>. The fruit, seeds, or leaves extracts of papaya have been shown to possess cytotoxic and anti-proliferative activities for a number of cancer cells lines including breast (MCF-7), liver (HepG2) and cervical carcinoma (Hela), lung adenocarcinoma (PC14), oral squamous cell carcinoma (SCC25), pancreatic epithelioid carcinoma (Panc-1), mesothelioma (H2452), and cancer of haematopoietic cell lines, including T cell lymphoma (Jurkat), plasma cell leukemia (ARH77), Burkitt's lymphoma (Raji), and anaplastic large cell lymphoma (Karpas-299) and human promyelocytic leukaemia (HL-60)<sup>[33-35]</sup>. The lipophilic extracts of papaya pulp inhibited cell proliferation of ER<sup>+</sup> breast cancer MCF-7 cells but did not inhibit ER<sup>-</sup> breast cancer MDA-MB-231 cells<sup>[36]</sup>. During present investigation, the effect of papaya extracts from leaves, skin, pulp and seeds were assessed on estrogen and Her-2-dependent and -independent breast cancer using representative cells lines.

## METHODS

### Materials

MDA-MB-231 (ER<sup>-</sup>/PR<sup>-</sup>/Her-2<sup>-</sup>; triple negative), MCF-7 (ER<sup>+</sup>/PR<sup>+</sup>/Her-2<sup>-</sup>), SK-Br-3 (ER<sup>-</sup>/PR<sup>-</sup>/Her-2<sup>+</sup>), AU565 (ER<sup>-</sup>/PR<sup>-</sup>/Her-2<sup>+</sup>), and MDA-MB-361 (ER<sup>+</sup>/PR<sup>+</sup>/Her-2<sup>+</sup>) breast cancer cell were purchased from ATCC (Manassas, VA 20110). F-12K (21127-022) media was purchased from Gibco (Grand Island, NY14072). Fetal bovine serum was purchased from RAMBIO (Missoula, Montana). Antibiotics: penicillin and streptomycin, and phosphate buffered saline was purchased from Fisher (Fair lawn, New Jersey). Folin-Ciocalteu, aluminum chloride, Diphenyl-1-picrylhydrazyl (DPPH), quercetin, gallic acid, and Trolox were purchased from Sigma Chemical Co (St Louis, MO). Green papaya was obtained from Randolph Farm at Virginia State University. WST-1 (MK400) was purchased from Talkara (Kusatsu, Shiga, Japan).

### Isolation of papaya leaves, skin, pulp, and seeds

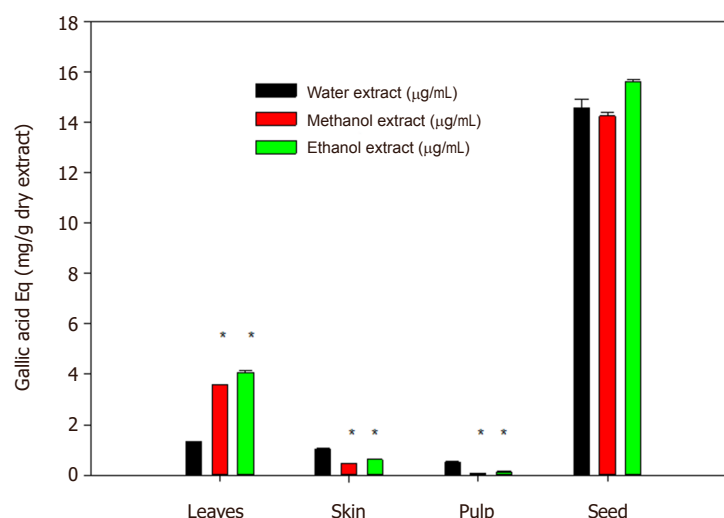
Unripe green papaya (2-3 kg) was obtained from Randolph Farm at Virginia State University, Petersburg VA. The papaya was washed with distilled water, then blotted dry with paper towel. The skin was peeled off using a kitchen peeler. The unskinned papaya was cut into half to remove seeds and then the pulp was cut into small pieces. The leaves and seeds were washed with distilled water. All fractions (leaves, skin, pulp and seeds) were spread on a plastic trays and left for drying in a chemical hood until a constant weight was obtained. The dried leaves, skin, pulp and seeds were ground to a fine powder using a mortar and pestle. The dried powder was flash frozen with nitrogen and stored at -80 °C until used.

### Preparation of leaves, skin, pulp and seeds extract

A known quantity (5 g) of dried papaya powder was mixed with 200 mL of 80% methanol, 60% ethanol, or 100% distilled water. The mixtures were placed on a shaker at room temperature overnight. The next day, the mixture was centrifuged at 1500 g for 20 min using a Thermo Scientific centrifuge (Waltham, MA). The supernatant was collected and the residues were washed 2 times by suspending them again in the respective solutions, mixing, and placing on shaker overnight. The collected supernatant was pooled together and the residues were discarded. The ethanol and methanol extracts were dried in a nitrogen evaporator (Organomation Associates, Inc, Berlin, MA) whereas the water extract was freeze dried. The dried extract was stored in a -20 °C freezer.

### Determination of total phenolic content

The total phenolic content (TPC) of papaya extract was determined by using Folin-Ciocalteu method as



**Figure 1.** Total polyphenols analysis from various papaya fractions. The total phenolic content (TPC) of papaya extract was determined by using Folin-Ciocalteu method. Results are mean  $\pm$  SD for at least 3 experiments as gallic acid equivalents. The TPC in methanol and ethanol extracts were compared to that in the water extracts. The significant differences, as marked “\*”, are reported at  $P < 0.05$

described<sup>[37]</sup>. The TPC content of the papaya extract was calculated as gallic acid equivalents.

#### Determination of total flavonoid content

An aluminum-chloride based assay was used to determine the total flavonoid content (TFC) of the extracts<sup>[38]</sup>. Quercetin was used as standard and flavonoid content was determined as quercetin equivalent.

#### Anti-oxidation capacity assay

The anti-oxidation activity in papaya extracts was assayed by using 2, 2-Diphenyl-1-picrylhydrazyl (DPPH) method<sup>[39]</sup>. The data is reported as % inhibition of DPPH oxidation.

#### Cell culturing and anti-proliferation assay

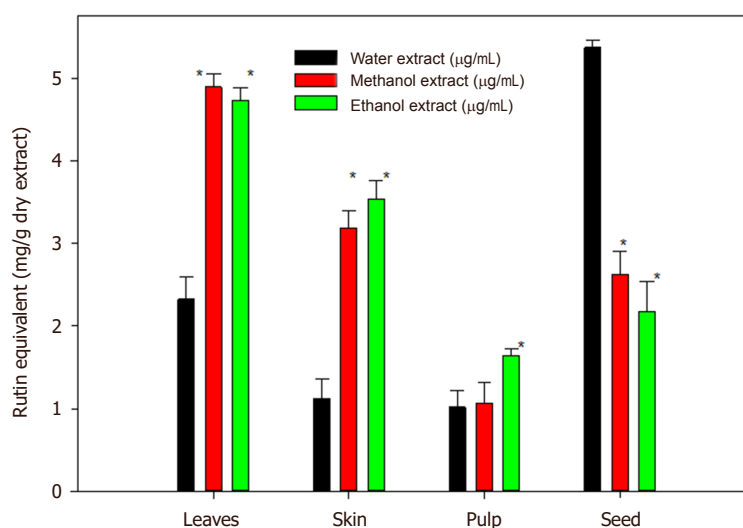
MDA-MB-231, MCF7, MDA-MB-361, and AU565 cells were maintained in Dulbecco's modified eagle medium (DMEM; Invitrogen; Carlsbad, CA) supplemented with penicillin (100 units/mL), streptomycin (100 μg/mL) and 10% FBS. SK-Br-3 cells were maintained in McCoy's 5A medium (ATCC) supplemented with penicillin (100 units/mL), streptomycin (100 μg/mL) and 10% FBS. All cell cultures were incubated in a humidified incubator at 37 °C and 5% CO<sub>2</sub>. Media was changed every 3 days and cells were subculture when they became confluent. Effect of papaya leaves, skin, pulp and seeds extract on cell proliferation was determined using a WST-1 assay as per manufacturer instructions. The results are expressed as % change from control.

## RESULTS

#### Characterization of papaya extract for anti-oxidation activity

The anti-oxidation potential of papaya extracts was determined by assessing their total polyphenol content, total flavonoid content and by assaying their anti-oxidation capacity. The data showing the TPC is presented in Figure 1. The highest amount of TPC was found in the seeds extract that ranged from 14-16 mg/g dry weight of the extracts. There was no significant difference in TPC content between water, ethanol and methanol extracts. The leaves were second highest in TPC content but had a considerably lower amount of TPC than that of seeds. The leaves contained TPC in 1-4 mg/g of dry weights. Water extract contained a lower amount of TPC (~1 mg/g dry weight) than that of ethanol or methanol extract. The amounts of TPC between ethanol and methanol extracts from papaya leaves were not significantly different. The skin





**Figure 2.** Total flavonoid content in various papaya fractions. Aluminum chloride complex forming assay was used to determine the total flavonoid content (TFC) of the extracts. Results are mean  $\pm$  SD for at least 3 experiments as quercetin equivalent. The TFC in methanol and ethanol extracts were compared to that in the water extracts. The significant differences, as marked "\*\*", are reported at  $P < 0.05$

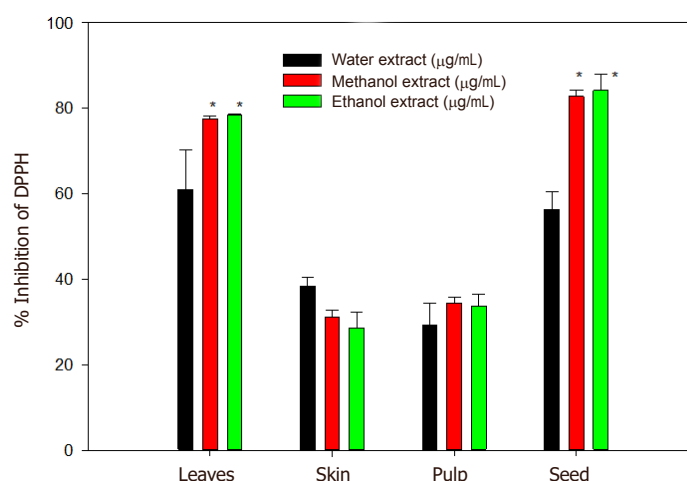
extracts contained TPC under 1 mg/g dry weight whereas the pulp extracts have very small amounts of TPC (0.05-0.5 mg/g dry weight).

The data for the TFC content in various papaya fraction are presented in Figure 2. The seeds and leaves extracts contained the highest amounts of TFC ranging 2-5.5 mg/g dry weight. The ethanol or methanol extract of leaves contained more TFC (~5 mg/g dry weight) than that of water extract (~2 mg/g dry weight). However, water extracts of seeds contained more TFC (~5.5 mg/g dry weight) than that of ethanol or methanol extracts (~2-2.5 mg/g dry weight). The amount of TFC in pulp and skin were less than that of seeds and leaves. In skin, higher amounts of TFC were present in the ethanol and methanol extracts (~3.2-3.5 mg/g dry weight) than that of water extract (> 1 mg/g dry weight). The pulp contained a lower amount of TFC than that of other fractions. The total amount of TFC in pulp ranged 1-1.5 mg/g dry weight.

The anti-oxidation capacity of papaya fractions was measured by assaying the inhibition of DPPH oxidation and is shown in Figure 3. The seeds and leaves contained the most anti-oxidation capacity than that of the skin and pulp fractions. The ethanol and methanol fractions of seeds and leaves contained more anti-oxidation activity than that of water extracts. The ethanol and methanol fractions of seeds and leaves inhibited DPPH oxidation by 75%-85% whereas the water extracts of these fractions inhibited DPPH oxidation by 50%-70%. The skin and pulp inhibited DPPH oxidation from 25% to 35%. There was no significant difference between water, ethanol or methanol extracts of skin or pulp.

#### Effect of papaya leaves, skin, pulp and seeds extract on MDA-MB-231 breast cancer cells

This experiment was carried out to investigate effect of papaya extracts on ER<sup>-</sup>/Her-2<sup>-</sup> breast cancer cell line using MDA-MB-231. The effect of water extract from leaves, skin, pulp and seeds is shown in Figure 4A. When the cells were treated with water extract of papaya leaves, the cells viability is reduced in a dose-dependent manner reaching a significant reduction of 20% ( $P < 0.05$ ) at 150  $\mu$ g/mL. On further increasing the concentration of extract, the cell viability was further reduced to 30% ( $P < 0.05$ ). Water extract of skin has no significant effect except at the highest concentration (250  $\mu$ g/mL) where cell viability is reduced by a marginal 10% ( $P < 0.05$ ). The pulp extract has no significant effect at any concentration. The water extract of seeds exhibited an effect similar to the water extract of the leaves causing a significant reduction in cell viability by 20% ( $P < 0.05$ ).



**Figure 3.** Anti-oxidation capacity of papaya fractions. The anti-oxidation activity in papaya extracts was determined by using 2, 2-Diphenyl-1-picrylhydrazyl (DPPH) method. The data is reported as % inhibition of DPPH oxidation. Results are mean  $\pm$  SD for at least 3 experiments. The results of methanol and ethanol extracts were compared to that of the water extracts. The significant differences, as marked “\*”, are reported at  $P < 0.05$

The effect of methanol extract from leaves, skin, pulp and seeds is shown in Figure 4B. The data indicate that none of the papaya fractions has any significant effect on cell viability of MDA-MB-231 breast cancer cells.

The effect of ethanol extract from leaves, skin, pulp and seeds is shown in Figure 4C. The extract from leaves, skin, and pulp has no significant effect; however, seed extract reduced cell viability starting at 75  $\mu\text{g/mL}$ . The cell viability was reduced significantly by 20% ( $P < 0.05$ ) at the highest concentration of 250  $\mu\text{g/mL}$ .

### Effect of papaya leaves and seeds extract on MCF-7 breast cancer cells

As we found from our previous experiment, neither the water, methanol nor ethanol extracts from pulp and skin had any effect on breast cancer cells; however, leaves and seeds showed effects on cell proliferation. We, therefore, carried out subsequent experiments on the extracts from leaves and seeds only. The effect of water extract from leaves and seeds is shown in Figure 5A. The extract from seeds, has no significant effect; however, leaves extract reduced cell viability significantly by 30% ( $P < 0.05$ ) at the highest concentration of 250  $\mu\text{g/mL}$ .

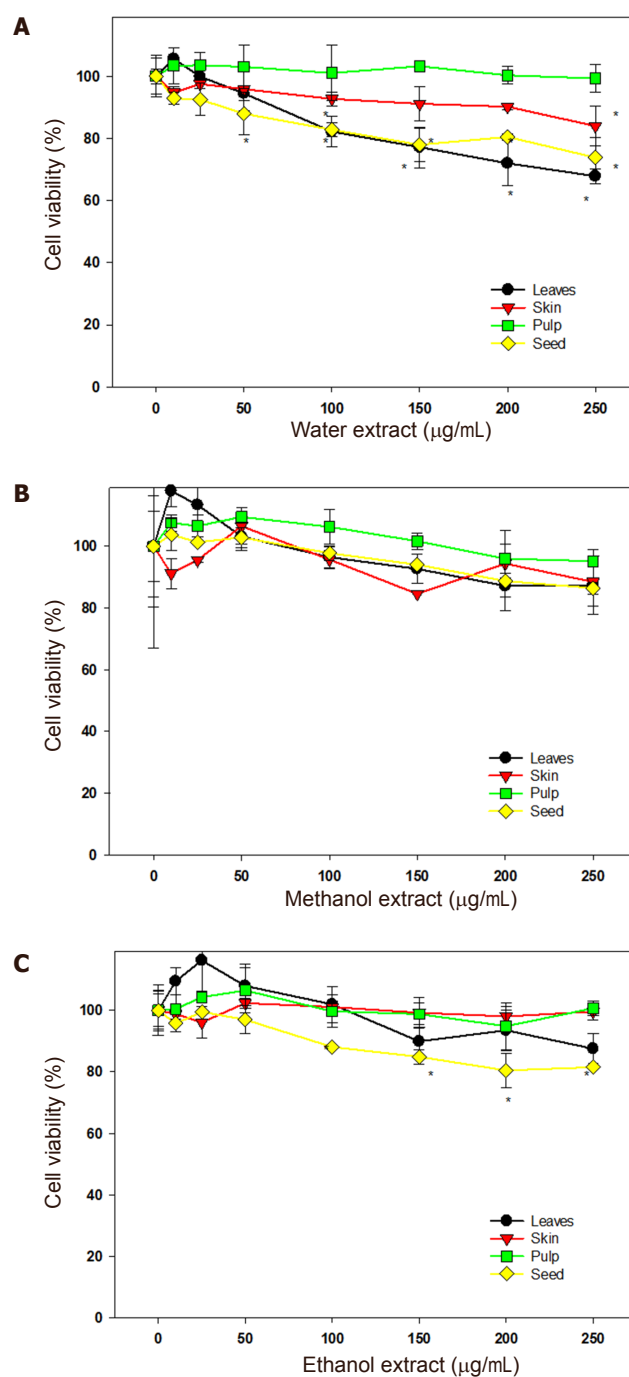
The effect of methanol extract from leaves and seeds is shown in Figure 5B. The data indicate that none of the papaya fractions has any significant effect on cell viability of MCF-7 breast cancer cells.

The effect of ethanol extract from leaves and seeds is shown in Figure 5C. Similarly, our data indicate that none of the papaya fractions has any significant effect on cell viability of MCF-7 breast cancer cells.

### Effect of papaya leaves and seeds extract on SK-Br-3 breast cancer cells

We performed further experiments to test the effect of papaya extracts in ER/Her-2<sup>+</sup> breast cancer using SK-Br-3 cells. As explained above, we only tested extracts from leaves and seeds on these cells.

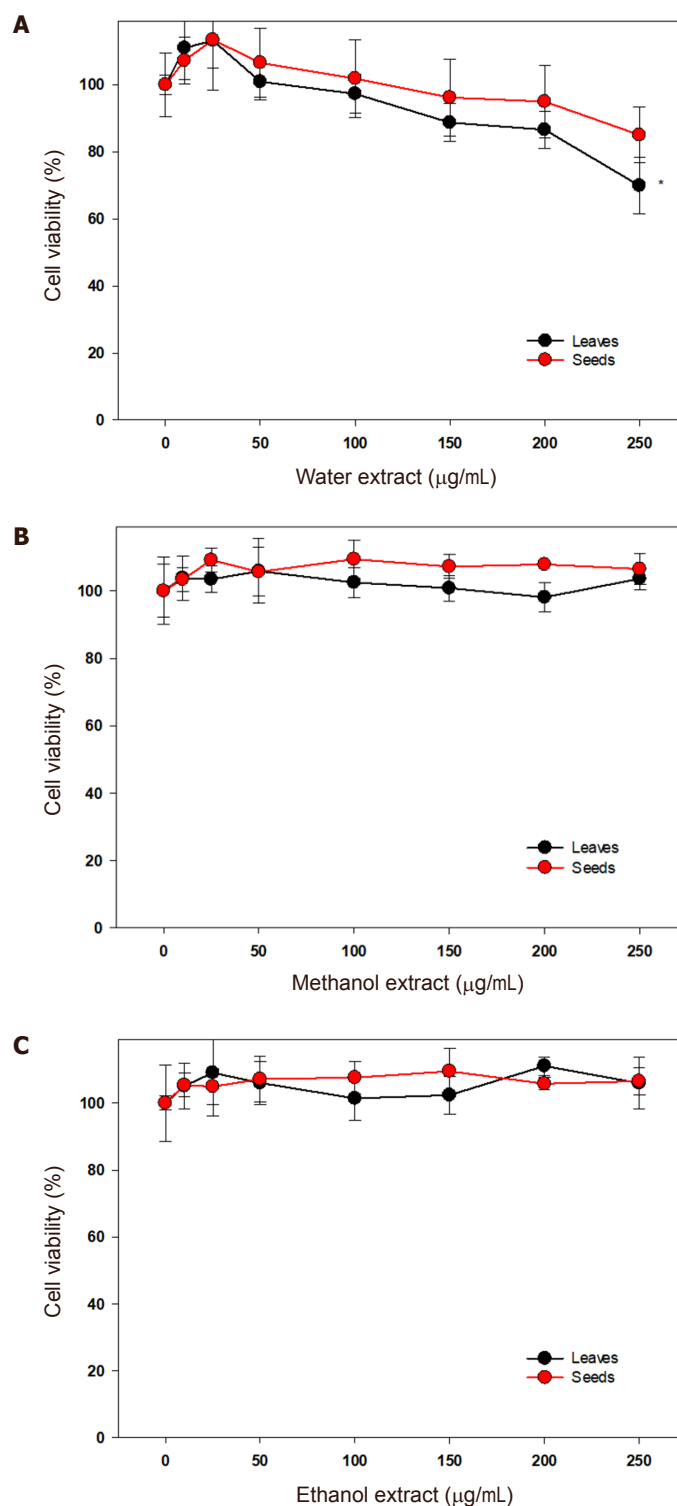
The effect of water extract from leaves and seeds is shown in Figure 6A. The extract from seeds has no significant effect except at the highest concentration (250  $\mu\text{g/mL}$ ) where cell viability is reduced by 25 % ( $P < 0.05$ ). However, leaf extract reduced cell viability in a dose-dependent manner starting at 10  $\mu\text{g/mL}$ . The cell variability was reduced significantly by 50% ( $P < 0.05$ ) at the highest concentration of 250  $\mu\text{g/mL}$ .



**Figure 4.** Effect of papaya extracts on MDA-MB-231 breast cancer cells. The effect of water (A), methanol (B), or ethanol (C) extracts on viability of MDA-MB-231 (ER<sup>+</sup>/Her-2<sup>+</sup>) breast cancer cells was measured as described in the "METHODS". Data is calculated as % inhibition of cell growth. All significant differences between control and treated cells are indicated by "\*" and are reported at  $P < 0.05$

The effect of methanol extract from leaves and seeds is shown in Figure 6B. The data indicate that none of the papaya fractions has any significant effect on cell viability of SK-Br-3 breast cancer cells.

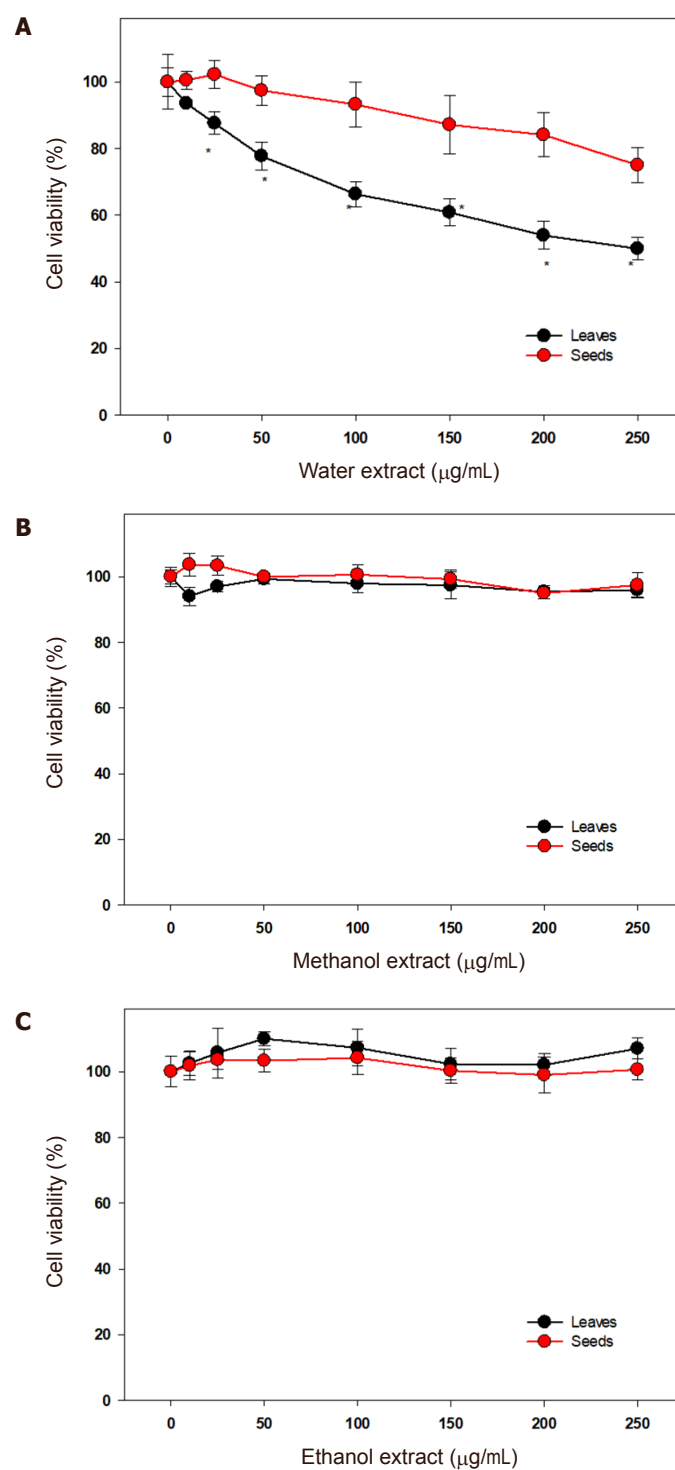
The effect of ethanol extract from leaves and seeds is shown in Figure 6C. Similar to methanol extracts, the data indicate that none of the ethanol extract from papaya leaves or seeds has any significant effect on cell viability of SK-Br-3 breast cancer cells.



**Figure 5.** Effect of papaya extracts on MCF-7 breast cancer cells. The effect of water (A), methanol (B), or ethanol (C) extracts on viability of MCF-7 (ER<sup>+</sup>/Her-2<sup>+</sup>) breast cancer cells was measured as described in the "METHODS". All significant differences between control and treated cells are indicated by "\*" and are reported at  $P < 0.05$

### Effect of papaya leaves and seeds extract on MDA-MB-361 breast cancer cells

The effect of papaya extract on ER<sup>+</sup>/Her-2<sup>+</sup> breast cancer cell lines were investigated using MDA-MB-361 cell lines. We again tested only extracts from leaves and seeds as explained earlier. The effect of water extract

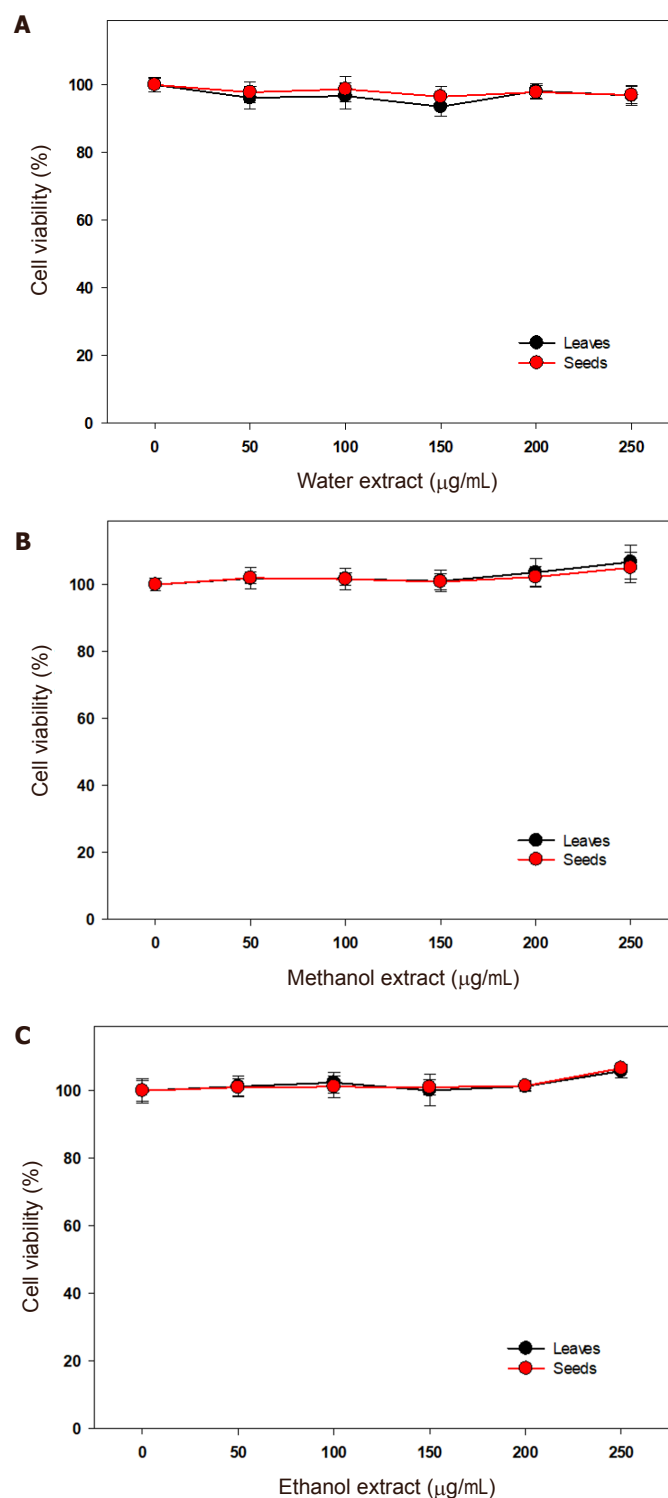


**Figure 6.** Effect of papaya extracts on SK-Br-3 breast cancer cells. The effect of water (A), methanol (B), or ethanol (C) extracts on viability of SK-Br-3 (ER<sup>+</sup>/Her-2<sup>+</sup>) breast cancer cells was measured described in the "METHODS". All significant differences between control and treated cells are indicated by "\*" and are reported at  $P < 0.05$

from leaves and seeds is shown in [Figure 7A](#). The extract from seeds and leaves have no significant effect on cell viability of MDA-MB-361 breast cancer cells.

The effect of methanol extract from leaves and seeds is shown in [Figure 7B](#). The data indicate that none of the papaya fractions has any significant effect on cell viability of MDA-MB-361 breast cancer cells.



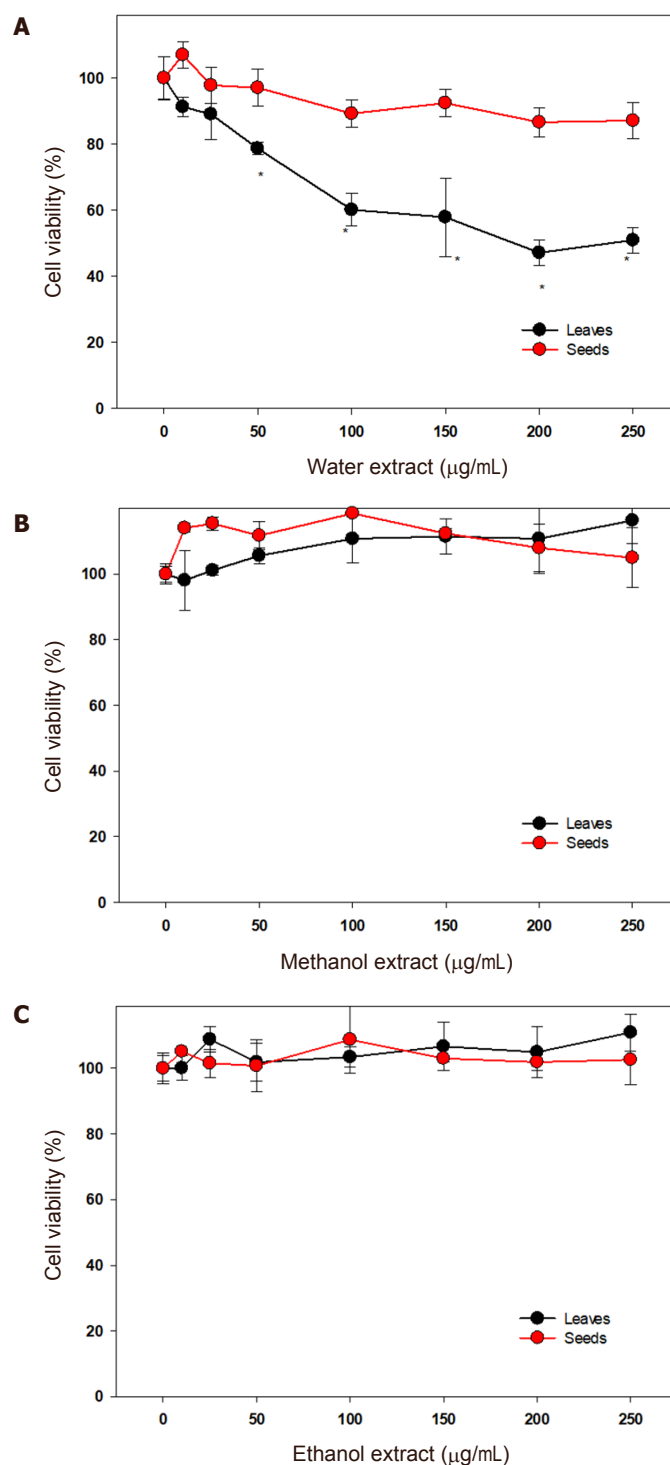


**Figure 7.** Effect of papaya extracts on MDA-MB-361 breast cancer cells. The effect of water (A), methanol (B), or ethanol (C) extracts on viability of MDA-MB-361 (ER<sup>+</sup>/Her-2<sup>+</sup>) breast cancer cells was measured as described in the "METHODS". All significant differences between control and treated cells are indicated by "\*\*\*" and are reported at  $P < 0.05$

The effect of ethanol extract from leaves and seeds is shown in Figure 7C. The data indicate that none of the papaya fractions also has any significant effect on cell viability of MDA-MB-361 breast cancer cells.

#### Effect of papaya leaves and seeds extract on AU565 breast cancer cells

From data shown above, we found that papaya extract from leaves and seeds were effective against ER<sup>-</sup> cell



**Figure 8.** Effect of papaya extracts on AU565 breast cancer cells. The effect of water (A), methanol (B), or ethanol (C) extracts on viability of AU565 (ER<sup>-</sup>/Her-2<sup>-</sup>) breast cancer cells was measured as described in the "METHODS". All significant differences between control and treated cells are indicated by "\*" and are reported at  $P < 0.05$

lines (MDA-MB-231 & SK-Br-3) irrespective to their Her-2 receptors expression whereas papaya extract from seeds and leaves were not effective on ER<sup>+</sup> cell lines (MCF-7 & AU565). To further validate the effects on ER<sup>+</sup> breast cancer cells, we used another ER<sup>-</sup> breast cancer cell lines, AU565 cell line (ER<sup>-</sup>/Her-2<sup>+</sup>).

The effect of water extract from leaves and seeds is shown in Figure 8A. The extract from seeds has no significant effect; however, leaves extract reduced cell viability starting at 50 μg/mL. The cell viability

was reduced significantly by 50% ( $P < 0.05$ ) at 200-250  $\mu\text{g/mL}$ . The effect of methanol extract from leaves and seeds is shown in Figure 8B. The data indicate that methanol extracts of the papaya leaves and seeds fractions had no significant effect on cell viability of AU565 breast cancer cells. The effect of ethanol extract from leaves and seeds is shown in Figure 8C. The data indicate that ethanol extracts of the papaya leaves and seeds fractions also had no significant effect on cell viability of AU565 breast cancer cells.

## DISCUSSION

The present study was carried out to investigate the effect of water, methanol, and ethanol fractions of papaya's leaf, skin, pulp, and seeds on breast cancer cells. We initially tested these fractions for their anti-oxidation activity. Most the anti-oxidation activity in fruits and vegetables is due to their total polyphenolic content<sup>[40]</sup>. Our data indicates that seeds have the highest amount of polyphenols than that of other fractions. The leaves also contained substantial amounts of TPC which was 1/3 to that of seeds. Other fractions have very small amounts of TPC. In seeds the amount of TPC was similar in all three extracts but in leaves water extract has less TPC than that methanol or ethanol. Different solvents including water, methanol and ethanol were used during present investigation because the phenolic compounds have different chemical characteristics and polarities and their solubility varies in polar and non-polar solvents<sup>[41]</sup>. Polar solvents are often used for extracting polyphenols from plant samples. Methanol is a very efficient solvent for extracting polyphenols of lower molecular weight, whereas aqueous acetone is generally used for extraction/isolation of higher molecular weight flavanols<sup>[42]</sup>. Ethanol is also a good solvent for polyphenol extraction which is also safe for human consumption. In addition, aqueous mixtures containing methanol, ethanol, ethyl acetate, or acetone have also been used by several investigators. Currently, over 8000 phenolic structures have been identified in fruits and vegetables<sup>[43]</sup>, and flavonoids are one of the major phenolic class comprising of almost 4000 compounds present in different edible plants<sup>[43]</sup>. We also determined the total flavonoids in the papaya extracts. Our data show that water extract of seeds and methanol and ethanol extracts of leaves contained the highest amount of TFC, which represented about 30%-40% of total phenolic compounds in seeds and leaves. These data suggest that seeds may contain water soluble small molecular weight polyphenols whereas leaves may contain less water-soluble high molecular weight polyphenols.

Next the anti-oxidation activities were determined in these fractions. The leaves and seeds possess the highest anti-oxidation activities. Although leaves and seeds have different phenolic and flavonoids contents, they have similar profile for their anti-oxidation activity. It was interesting to note that methanol and ethanol extracts exhibited higher anti-oxidation activity than the water extract in all papaya fractions. This difference could be due to the differences in the chemical structure of polyphenols and perhaps polyphenols are more soluble in an aqueous mixture of methanol or ethanol than water alone.

The different extracts from various fractions of papaya were used to determine their effects in breast cancer cells. As explained in the introduction section, breast cancer is characterized by different molecular phenotypes. In our initial studies, not much anti-cancer activity was found in pulp and skin extracts. These fractions were also very low in their polyphenolic contents. On the other hand, leaves and seeds showed anti-cancer activity and these fractions have significant amounts of polyphenols. The subsequent experiments were, therefore, performed only using leaves and seeds extracts. MDA-MB-231 was a representative of ER<sup>-</sup>/Her-2<sup>-</sup> breast cancer subtype. The water extracts of leaves and seeds were effective on these cells. The methanol and ethanol extract showed no significant effect. MCF-7 cells line was used as a representative of ER<sup>+</sup>/Her-2 breast cancer. None of the extracts from seeds or leaves exhibited any effect on these cell lines. Sk-Br-3 cells were used as a representative of ER<sup>-</sup>/Her-2<sup>+</sup> breast cancer subtype. It is clear from the data that only water extract of leaves exhibited effect on these cells line. MDA-MB-361 cell line was used as a representative of ER<sup>+</sup>/Her-2<sup>+</sup> breast cancer subtype. Again, no effect was found by any of the leaves or seeds extract on these cell lines. From this data it is clear that only ER<sup>-</sup> breast cancer cells irrespective to their Her-2 expression were significantly affected by water extracts of leaves. Whereas ER<sup>+</sup>

cells were largely unaffected. To further validate this finding, AU565, another ER<sup>-</sup> breast cancer cell line, was used. Similar to Sk-Br-3, water extract of papaya leaves also inhibited growth of this cell lines. These data, therefore, confirm the effect of water extracts from leaves on ER subtypes of breast cancer, which are difficult to treat. This observation indicates that water-soluble, small molecular weight compounds present in papaya leaves may be responsible for the anticancer activity in ER<sup>-</sup> breast cancer cell lines.

It is also interesting to note that both seeds and leaves extracts were similar in their anti-oxidation activity; however, leaf extracts inhibited ER breast cancer cell growth more potently than that of seeds extracts. It is clear from this observation that the antioxidation activity may not have attributed to their anticancer activity. Our results are consistent to other studies where growth inhibition of MCF-7 cells by pomegranate extract was not attributed to its high antioxidant potential<sup>[44]</sup>. It appears that the inhibitory compounds in the water extract of papaya leaves acted on cellular mechanism that are involved in regulating cell growth. During the present investigation, the phenolic composition of water extracts of leaves was not determined. However, it has been shown that papaya leaves extract contained proanthocyanidins and saponins classes of phenolic compounds. Water extract of papaya is commonly consumed to prevent/treat other diseases in traditional medicine<sup>[45]</sup>. This observation suggests that consumption of papaya leaf extract is probably safe. Furthermore, our data are consistent with previous studies, suggesting that papaya leaves can be beneficial for a number of cancers including breast cancer<sup>[35]</sup>.

Our data is interesting because ER<sup>-</sup> or triple negative tumors have limited options for the treatment. Triple-negative breast cancers do not have estrogen, progesterone, or Her-2 receptors and treatment with drugs designed to interfere hormone activities is not effective in these receptor-negative cancer cells. These cancers often grow faster than receptor-positive breast cancers. In most cases pre-menopausal women develop hormone receptor-negative cancers and appears to be common in younger women and in women with African-American or Hispanic/Latina ancestry<sup>[46]</sup>. Some of the ER<sup>-</sup> breast cancers are Her-2 positive that cancer are now effectively targeted with Herceptin, which is an antibody for Her-2 proteins<sup>[47]</sup>. The ER/Her-2<sup>-</sup> breast cancers do not benefit from anti-estrogen and/or anti-Her-2 based therapy. The options for treating such cancer are limited and involved surgery or chemotherapy, or both<sup>[48]</sup>. The drugs that are used to target such tumors are based on inhibition of cell proliferation pathways. Drugs including taxanes, anthracyclin, acts on DNA repair complex like, anti-oncogene P53, and stabilizing microtubules<sup>[49]</sup>. The effect of papaya leaves water extracts on ER<sup>-</sup> breast cancer cells in our study appears to be at a moderate level. However, the extract can be concentrated for more aggressive effects. In addition, papaya leaves extracts can be used as an adjunct therapy with the generally prescribed anti-cancer drugs, which may improve the efficacy of the drugs and also reduce the side effects and drug resistance in breast cancer patients. Furthermore, our *in vitro* data needs to be validated in an *in vivo* animal model.

In conclusion, water extract of papaya leaves containing water soluble polyphenols may have a potential to inhibit ER<sup>-</sup> breast cancer. However, further studies are required to determine the actual chemical nature of these phenolic compounds and their mechanism of action.

## DECLARATIONS

### Authors' contributions

Concept and design: Siddiqui RA, Rafei R

Manuscript preparation, editing, and review: Siddiqui RA, Kaseloo P, Witiak SM

Experimental studies, data acquisition, data analysis, statistical analysis: Li H, Hadadi SA

### Data source and availability

Data were obtained in Food Chemistry and Nutrition Science Laboratory, Agricultural Research Station, Virginia State University, Petersburg, VA 23806. The data are available in electronic format as Excel and Sigma plot files upon request.

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### Conflicts of interest

There are no conflicts of interest.

### Patient consent

Not applicable.

### Ethics approval

Animal or human tissues were not used; no ethical approval was required.

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Review

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# Pancreaticoduodenectomy for gastric cancer

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## Abstract

Pancreaticoduodenectomy (PD) is performed to achieve an R0 resection for gastric cancer with pancreatic and/or duodenal invasion. Several retrospective case series have been published, but the sample cohorts in each study were heterogeneous and small. Moreover, the absence of prospective studies results in a lack of solid evidence that will help determine who can benefit from this procedure. Although the morbidity and mortality of PD have been reported by most studies to be acceptable and that the procedure is feasible, these remained to be much higher than those of standard gastrectomy. Therefore, careful selection of patients should be considered. Based on a review of previous case series and our own experience, PD appears to be beneficial to patients with gastric cancer with pancreatic invasion when R0 resection is possible. In addition, multidisciplinary treatment such as neoadjuvant chemotherapy, is anticipated to improve survival. Nevertheless, considering that prospective randomized studies are difficult to perform, a large-scale multicenter retrospective cohort study is required to evaluate this highly invasive procedure.

**Keywords:** Gastric cancer, pancreaticoduodenectomy, multivisceral resection

## INTRODUCTION

Gastric cancer is the fifth most common cancer and is the third leading cause of cancer deaths worldwide<sup>[1]</sup>. Its incidence is higher in Eastern Asia, including Japan, Korea, and China, than in Western countries. Although approximately 50% of the patients in Japan are diagnosed during the early stages of gastric cancer, several patients are diagnosed in the advanced stages<sup>[2]</sup>. For gastric cancer treatment, radical surgical resection with lymph node dissection is the established standard and complete surgical resection without residual disease (R0 resection) is the cornerstone. For tumors that invade adjacent organs, combined resection is necessary for achieving complete tumor clearance.



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The pancreas is the organ most frequently invaded by gastric cancer<sup>[3-6]</sup>. When a tumor and/or lymphadenopathy invades the pancreatic head or infiltrates the duodenum, pancreaticoduodenectomy (PD) is the only possible treatment for achieving R0 resection. However, PD is a highly invasive procedure that cannot be performed on all patients. Since the first reported case of a patient who underwent PD for gastric cancer in 1978<sup>[7]</sup>, all case series published<sup>[8-17]</sup> were retrospective and single-center studies and no prospective study has been done. Because of the limited number of patients and heterogeneous data of the studies, definite indications for PD have not been established. Here we reviewed the literature on PD for gastric cancer and our own experience to clarify short- and long-term outcomes and the role of PD in gastric cancer.

## METHODS OF LITERATURE SEARCH

We conducted a literature search on PubMed using keywords “gastric cancer”, “pancreaticoduodenectomy”, and “multivisceral resection” considering articles published until November 2017. We excluded inaccessible abstracts or articles not written in English. In addition, we reviewed patients who underwent distal or total gastrectomy with PD at Shizuoka Cancer Center (Shizuoka, Japan) between September 2002 and December 2015. We collected patients’ characteristics and pathological and surgical findings from our database and individual patients’ electronic medical records. In addition, we statistically analyzed our data using R Statistics version 3.4.0 (R Foundation for Statistical Computing, Vienna, Austria). Furthermore, we calculated 5-year survival rates using the Kaplan-Meier method and compared them between the groups using the log-rank test. The statistical significance of data was defined as  $P < 0.05$ .

## SHORT-TERM SURGICAL OUTCOMES

PD is a highly invasive procedure that requires high surgical skills. When Buchholtz *et al.*<sup>[7]</sup> first reported PD for gastric cancer in 1978, they concluded that this treatment should not be performed because of the unacceptable risk without an additional and greater degree of palliation or likelihood of cure; however, they did not discuss their reasons in detail. Several studies have demonstrated short-term surgical outcomes of PD, including intraoperative blood loss, operation time, morbidity, and mortality [Table 1]<sup>[8-17]</sup>. The median amount of blood loss was reported to be  $> 1000$  mL and the median operation time was as long as 7 h.

Although several studies have concluded that PD for gastric cancer is feasible in terms of safety, the incidence of postoperative complications ranged widely from 22% to 74%, probably because of discrepancies in the definitions of complication. No study defined the exact criteria for postoperative complications because many of these reports were published before the definitive criteria for postoperative complications, the Clavien–Dindo classification<sup>[18]</sup>, were established. The mortality rate of PD was reported to be from 0% to 13%; however, the definition of the period of operative death differed among the studies; some defined mortality as death from any cause within 30 days after surgery, whereas the others did not mention the period. The study by Nunobe *et al.*<sup>[14]</sup>, who defined mortality as death from any cause before discharge, reported the highest mortality of 13%.

Although Min *et al.*<sup>[16]</sup> reported the lowest complication rate of 22% among the reported rates of the previous studies, they also demonstrated one of the highest mortality rates, which was 11%. These results meant that half of the patients who suffered from postoperative complications died; this 50% mortality rate among patients who suffered postoperative morbidity seemed to be a bit high, which was possibly due to the variable definitions of all the complications. At the same time, Yonemura *et al.*<sup>[8]</sup> reported a 23% incidence of pancreatic fistula, but did not report the incidence of all complications.

Saka *et al.*<sup>[11]</sup> reported the highest complication rate of 74%, with pancreatic fistula being the most frequent in 44% of patients; all patients recovered with conservative management and none reported operation-related

**Table 1. Summary of studies on pancreaticoduodenectomy for gastric cancer**

Authors	Patients (n)	Morbidity	Mortality	Blood loss (mL)	Operation time (min)	Overall survival	P value	Subset analysis		Overall survival by subset analysis	P value by subset analysis
Yonemura <i>et al.</i> <sup>[8]</sup>	PD = 26	23%*	0%	1600	288	NR	NR	Duodenal inv. cases only	PD vs. non-PD	NR	NS
	Non-PD = 63	3%*	3%	1200	216	NR		pN3 cases only	PD vs. non-PD	33% vs. 17% (5-year)	< 0.05
								Pancreatic inv. cases only	PD vs. non-PD	55% vs. 0% (5-year)	< 0.01
Hirose <i>et al.</i> <sup>[9]</sup>	PD = 10	70%	0%	1402	580	40% (5-year)	NS	Stage IV cases only	PD vs. non-PD	44% vs. 20% (5-year)	< 0.05
								pSI cases only	PD vs. non-PD	19 vs. 9 months (MST)	0.0478**
	Non-PD = 69	32%	0%	563	330	45% (5-year)		pN3 cases only	PD vs. non-PD	19 vs. 20 months (MST)	NS
Ajisaka <i>et al.</i> <sup>[10]</sup>	PD = 22	NR	NR	NR	NR	35% (5-year)	NS	Length of duodenal inv.	< 30 mm vs. ≥ 30 mm	21.2% vs. 26% (5-year)	NS
	Non-PD = 47	NR	NR	NR	NR	16% (5-year)		Duodenal inv. type	Mucosal type vs. submucosal type vs. nodal type	28% vs. 9.2% vs. 0% (5-year)	0.058 <sup>a</sup> , < 0.001 <sup>b</sup> , 0.304 <sup>c</sup>
								R0 cases only	PD vs. non-PD	37.3% vs. 33.8% (5-year)	NS
Saka <i>et al.</i> <sup>[11]</sup>	PD = 23	74%	0%	1600	480	34% (5-year)		R0 vs. R1/2	R0 vs. R1/2	47.4% vs. 0% (5-year)	0.035
	Non-PD = 45	NR	NR	NR	NR	28% (5-year)					
Lee <i>et al.</i> <sup>[12]</sup>	PD = 25	32%	0%	NR	349.5	15.8% (5-year)	NR	NR			
Chan <i>et al.</i> <sup>[13]</sup>	PD = 7	43%	0%	600	480	60% (5-year)	NR	NR			
Nunobe <i>et al.</i> <sup>[14]</sup>	PD with U7 LN = 23	13%*	13%	1700	535	7.7% (5-year)	0.014	Pancreatic inv. pattern	Tumor inv. vs. lymph node inv.	NR	0.324
	PD with ≤ 6 LN = 8	12.5%*	12.50%	1731	499	50% (5-year)		Tumor inv. cases only	U 7 LN vs. M 6 LN	NR	0.692
								Lymph inv. cases only	U 7 LN vs. M 6 LN	NR	< 0.001
Wang <i>et al.</i> <sup>[15]</sup>	PD = 17	71%	0%	NR	NR	34% (3-year)	0.0064	NR			
	Non-PD = 36	NR	NR	NR	NR	6% (3-year)					
Min <i>et al.</i> <sup>[16]</sup>	PD = 9	22%	11.10%	NR	420	0% (5-year)	0.013	NR			
	Non-PD = 58	31%	0%	NR	254	27.4% (5-year)					
Ryu <i>et al.</i> <sup>[17]</sup>	PD = 16	31.3%	6.30%	NR	NR	12.5% (5-year)	NR	R0 vs. R1/2	R0 vs. R1/2	15.4% vs. 0% (5-year)	0.458
								Postoperative chemo	Chemo vs. no-chemo	22.2% vs. 0% (5-year)	< 0.01
Present study	PD = 24	87.5% <sup>‡</sup>	8.3%	1218	449	27.5% (5-year)	NR	R0 vs. R1	R0 vs. R1	38.8% vs. 0% (5-year)	0.078
								R0 cases only	Pancreatic inv. vs. duodenal inv.	54.5% vs. 0% (5-year)	0.048
									diff. vs. undiff.	68.6% vs. 0% (5-year)	0.004

\*Pancreatic fistula only; \*\*by Wilcoxon test; <sup>a</sup>mucosal vs. submucosal type; <sup>b</sup>mucosal vs. nodal type; <sup>c</sup>submucosal vs. nodal type; <sup>‡</sup>Clavien-Dindo Grade II or more. PD: pancreaticoduodenectomy; NR: not reported; LN: lymph node metastasis; NS: not significant; MST: median survival time; OS: overall survival; inv.: invasion; chemo: chemotherapy; diff.: differentiated adenocarcinoma; undiff.: undifferentiated adenocarcinoma

death. Nunobe *et al.*<sup>[14]</sup> featured the largest number of patients, including 31 patients with gastric cancer who underwent PD. Although their center is one of the largest high-volume centers in Japan, with > 300 cases of gastrectomy performed during one year, the mortality rate of PD was as high as 13%. The most frequently observed complication was pancreatic leakage (13%), followed by intraabdominal abscess (6%) and colitis (6%); however, they did not report the rates of the other postoperative complications.

In our center, 24 gastric cancer patients underwent PD from 2002 to 2016; 19 patients underwent distal gastrectomy and 5 patients underwent total gastrectomy. Differentiated adenocarcinoma was noted in 15 patients and undifferentiated adenocarcinoma was noted in nine. The median blood loss was 1218 mL and the median operative time was 449 min. R0 resection was performed on 17 patients (70.8%) and R1 was performed on 7 patients (29.2%) owing to positive lavage cytology (CY1). There were no patients with tumor-positive resection margins. Four patients had a small number of peritoneal deposits adjacent to the stomach, which were completely resected during operation.

### SURVIVAL BENEFITS OF PD FOR PATIENTS WITH GASTRIC CANCER

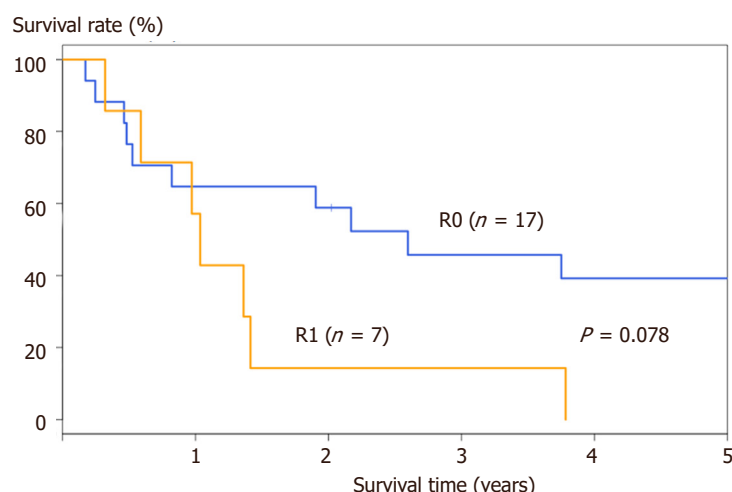
Several studies have evaluated the survival outcomes of patients undergoing PD for gastric cancer [Table 1]. However, conflicting results were reported, mainly because of heterogeneous data and small sample size in each study.

According to studies that evaluated multivisceral resection for gastric cancer clinically invading the adjacent organs (T4b) or for pathologic T4b gastric cancer, R0 resection and lymph node status were the independent prognostic factors<sup>[3,4,6,19]</sup>; however, few studies have shown poor survival outcomes for patients who underwent combined resection of the pancreas or a tumor invading the pancreas<sup>[16,20]</sup>. It is important to note that, in these studies, the number of patients who underwent PD was few or unknown. Among these, the retrospective study on the prognostic factors in patients with T4b gastric cancer by Min *et al.*<sup>[16]</sup> reported the highest number of patients who underwent PD; there were a total of 243 T4b gastric cancer patients, including 67 patients that had tumor invasion to the pancreas. In that study, pancreatic invasion was identified as an independent unfavorable prognostic factor by multivariate analysis. Moreover, among the operation methods used for pancreatectomy in the pancreatic invasion group, the PD group ( $n = 9$ ) had a significantly lower 5-year survival rate, compared with that of the other pancreatectomies group ( $n = 58$ ) (0% vs. 27.4%,  $P = 0.013$ ). Therefore, they did not recommend PD for T4b gastric cancer invading the pancreatic head.

In contrast, studies that compared PD and gastrectomy alone for T4b gastric cancer have found a therapeutic benefit of PD. Wang *et al.*<sup>[15]</sup> evaluated 53 patients with gastric cancer and pancreaticoduodenal region involvement and found that PD improved the 3-year survival rate, compared with that of palliative gastrectomy (34% vs. 5.6%,  $P = 0.0064$ ). Hirose *et al.*<sup>[9]</sup> showed that among patients with gastric cancer invading the pancreatic head, the median survival time (MST) was better in the PD group than in the palliative gastrectomy group (19 months vs. 9 months,  $P = 0.0478$ ). Yonemura *et al.*<sup>[8]</sup> also demonstrated that, compared with gastrectomy alone, PD with right hemicolectomy improved the 5-year survival rate of patients with pancreatic invasion (55% vs. 0%,  $P < 0.01$ ). Saka *et al.*<sup>[11]</sup> investigated 23 patients who underwent R0 resection with PD for gastric cancer macroscopically infiltrating the pancreatic head and showed that the 5-year survival rate was significantly better in patients without incurable factors, such as para-aortic lymph node metastasis, positive lavage cytology (CY1), and peritoneal dissemination, than in those with incurable factors (47.4% vs. 0%,  $P = 0.035$ ). It should be noted that in that study, CY1 cases were treated as R0 resection, which is considered an R1 resection according to the 7th edition UICC TNM classification.

In patients undergoing PD, there are two patterns of invasion to the pancreatic head, including direct invasion of the primary tumor and invasion via metastatic lymph nodes. Although most studies have not investigated survival according to the pattern of pancreatic invasion, the study by Nunobe *et al.*<sup>[14]</sup> showed





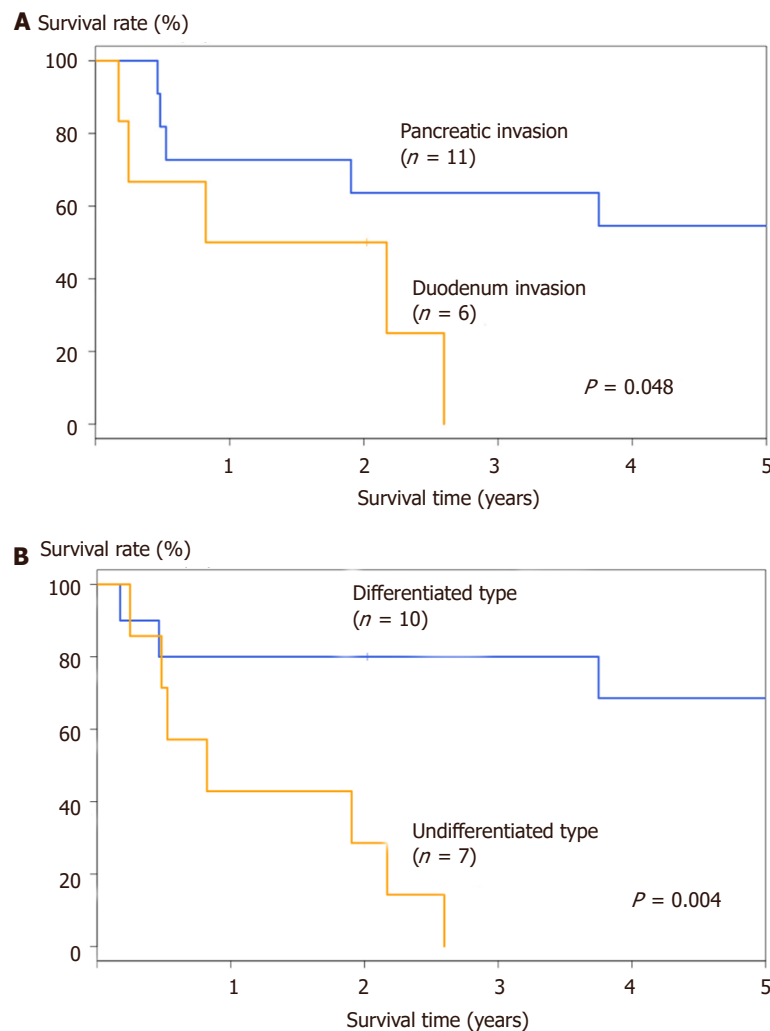
**Figure 1.** OS curve of 24 patients. There were 17 patients who underwent R0 resection and 7 patients who underwent R1 resection. The 5-year OS was better in patients who underwent R0 resection (38.8%) than in those who underwent R1 resection (0%), although the difference was not statistically significant ( $P = 0.078$ ). OS: overall survival

no difference in survival between these two patterns of invasion ( $P = 0.324$ ). According to these studies, if R0 resection is considered possible, PD should be performed for patients with either primary tumor or metastatic lymph node invasion to the pancreatic head.

Regarding the therapeutic benefit of PD for patients with tumors infiltrating the duodenum, no unified view has been obtained so far. Yonemura *et al.*<sup>[8]</sup> reported a survival benefit of PD over gastrectomy for T4b tumors, but not for tumors with duodenal invasion. Ajisaka *et al.*<sup>[10]</sup> evaluated 69 gastric cancer patients with duodenal invasion; among them, 22 patients underwent PD and 47 patients underwent gastrectomy alone. When a negative resection margin was achieved (i.e., R0 resection), the 5-year survival rates were almost the same (37.3% for PD vs. 33.8% for gastrectomy alone), although patients who underwent PD had more frequent adjacent tissue infiltration and significantly longer extent of duodenal invasion. They also found that survival was worse when duodenal invasion was from lymph node metastasis than from the primary tumor. Therefore, they concluded that curative PD for gastric cancer improved the survival of patients with duodenal invasion, except when duodenal invasion was of the nodal type.

Two studies have investigated the survival benefit of PD for patients with extensive lymph node metastases. Yonemura *et al.*<sup>[8]</sup> reported that PD improved the 5-year survival rate of patients with N3 lymph node metastasis (33% vs. 17%,  $P < 0.05$ ). They used the first English edition of the Japanese Classification of Gastric Carcinoma<sup>[21]</sup>, in which there were five N stages, with N3 referring to metastases in the hepatoduodenal, pre- and retropancreatic, and superior mesenteric nodes. In contrast, Hirose *et al.*<sup>[9]</sup> demonstrated that compared with palliative gastrectomy, PD had a tendency to not improve MST for patients with N3 lymph node metastases (19 months vs. 20 months, the differences were not significant). Therefore, it is difficult to reach a conclusion from these opposing results.

The other reported factors associated with better survival in patients who underwent PD included well-differentiated histologic type<sup>[15]</sup>, adjuvant intravenous chemotherapy<sup>[17]</sup>, and metastatic lymph nodes less than seven<sup>[14]</sup>. Based on our experience of patients who underwent PD for gastric cancer, the 5-year overall survival (OS) rate was 27.5% and the MST was 17.2 months. The 5-year OS rate was 38.8% in patients who underwent R0 resection ( $n = 17$ ) and 0% in those who underwent R1 resection ( $n = 7$ ), although this difference was not statistically significant ( $P = 0.078$ ), possibly due to the small sample size [Figure 1]. The OS curves of patients who underwent R0 resection are shown in Figure 2. The 5-year survival rate was significantly higher in patients with predominantly pancreatic invasion than in those with duodenal



**Figure 2.** OS curves of 17 patients who underwent R0 resection. The 5-year OS rate was significantly better (A) in patients with pancreatic invasion than in those with duodenal invasion (54.5% vs. 0%;  $P = 0.048$ ) and (B) in patients with differentiated tumors than in those with undifferentiated tumors (68.6% vs. 0%;  $P = 0.004$ ). OS: overall survival

invasion ( $n = 11$ , 54.5% vs.  $n = 6$ , 0%;  $P = 0.048$ ) [Figure 2A]. Likewise, the 5-year OS rate was significantly higher in patients with differentiated tumors than in those with undifferentiated tumors ( $n = 10$ , 68.6% vs.  $n = 7$ , 0%;  $P = 0.004$ ) [Figure 2B]. The univariate analysis of patients who underwent R0 resection is shown in Table 2.

Although conclusive results are difficult to obtain from previous studies, which had limited number of patients and heterogeneous data, it appeared that R0 resection is the minimum requirement for cure and that PD should not be performed in cases of CY1. In addition, tumors with duodenal invasion have little chance for cure; therefore, in cases with a positive resection margin, palliative surgery followed by chemotherapy or radiotherapy may be an alternative to PD. However, evidence proving this hypothesis is lacking.

## DIAGNOSIS OF PANCREATIC INVASION BEFORE OR DURING OPERATION

Intraoperative diagnosis of tumor invasion to the pancreas has been reported to be difficult, with an accuracy rate ranging from 39% to 56.7%<sup>[5,6,22]</sup>. Adhesions secondary to desmoplastic reaction or tumor inflammation can be mistaken for local invasion<sup>[23]</sup>, which could lead to patients being subjected to unnecessary multivisceral resection and result in increased morbidity and mortality without oncological

**Table 2. Univariate analysis of the factors affecting the survival of patients who underwent R0 resection**

Covariates	n	5-year OS (%)	MST (months)	P value
Reason for PD				
Pancreatic invasion	11	54.5	-	0.048
Duodenal invasion	6	0	26.4	
Macroscopic type				
Non-type 4	15	40	31.6	0.551
Type 4	2	0	2.1	
Histological type				
Differentiated	10	68.6	-	0.004
Undifferentiated	7	0	10	
Type of gastrectomy				
DG	14	35.7	31.6	0.68
TG	3	66.7	-	
pT stage				
T1-3	7	57.1	-	0.339
T4	10	25	23.1	
pN stage				
N0/1/2	10	40	26.4	0.813
N3	7	38.1	45.6	
pStage				
Stage II-III	13	35.2	31.6	0.652
Stage IV	4	50	23.1	

OS: overall survival; MST: median survival time; PD: pancreaticoduodenectomy; DG: distal gastrectomy; TG: total gastrectomy

benefit. In our experience, pancreatic invasion from a tumor was suspected intraoperatively in 11 patients, but it was confirmed pathologically in only 8 patients (72.7%). In patients who were suspected to have pancreatic invasion of the tumor, the 5-year survival rate tended to be poor in patients with pathologically positive invasion than in those with pathologically negative invasion (66.7% vs. 12.5%,  $P = 0.150$ ).

Preoperative imaging, including multidetector computed tomography (MDCT)<sup>[24]</sup> and endoscopic ultrasound (EUS)<sup>[25]</sup>, may facilitate identification of pathological invasion. However, the accuracy of MDCT and EUS for the assessment of pathological tumor depth was low and varied between 77.1%–88.9% and 65%–92.1%, respectively<sup>[26]</sup>.

## PREOPERATIVE CHEMOTHERAPY

Neoadjuvant chemotherapy had been described by only one study; Chan *et al.*<sup>[13]</sup> reviewed nine patients with locally advanced gastric cancer involving the duodenum and/or pancreatic head. All patients underwent diagnostic laparoscopy or exploratory laparotomy prior to the surgery to exclude peritoneal metastases. Two patients did not undergo PD because of disease progression with liver metastasis and patient refusal. Of the seven remaining patients who underwent PD, three did not receive neoadjuvant chemotherapy due to patient refusal and bleeding from the tumor. Although the study involved quite a small number of patients and its follow-up was short, it showed a significantly better survival in patients who received neoadjuvant chemotherapy than in those who did not receive neoadjuvant chemotherapy (log-rank test;  $P = 0.039$ ).

In our experience, the benefit of neoadjuvant chemotherapy was difficult to assess because only 2 of the 24 patients received the treatment. Nevertheless, one of those patients survived longer than 5 years after surgery without recurrence and the other one remained alive at the end of this study period. Therefore, neoadjuvant chemotherapy seems to be a promising treatment to improve the survival of patients with gastric cancer who undergo PD.

Another therapeutic option for patients with initially incurable or unresectable gastric cancer is conversion

therapy, which is defined as surgical resection intending to achieve radical cure following chemotherapy and/or radiotherapy<sup>[27]</sup>. Several studies have reported positive outcomes from this treatment<sup>[28-32]</sup>, although none of them evaluated conversion therapy for patients who underwent PD. As we previously demonstrated, PD has a high morbidity and mortality, and its survival benefit appears to be limited. Therefore, neoadjuvant chemotherapy and conversion therapy should be considered as an alternative treatment strategy for patients requiring PD for curative resection.

## CONCLUSIONS

Although there is currently no solid evidence that PD may be recommended for advanced gastric cancer with pancreatic invasion when R0 resection is possible, but the high morbidity and mortality should be considered. In addition, multidisciplinary treatment, such as neoadjuvant chemotherapy, is anticipated to improve survival. Nevertheless, a large-scale multicenter cohort study is required to evaluate this highly invasive procedure.

## DECLARATIONS

### Authors' contributions

Designed the study, reviewed the literature, and wrote the manuscript: Makuuchi R

Contributed to writing the manuscript, drafting, critical revision, editing, and final approval of the final version: Terashima M

Contributed to critical revision of the manuscript and final approval of the final version: Irino T, Tanizawa Y, Bando E, Kawamura T

### Availability of data and materials

Not applicable.

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### Conflicts of interest

All authors declared that there are no conflicts of interest.

### Ethical approval and consent to participate

Not applicable.

### Consent for publication

Not applicable.

### Copyright

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Commentary

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# Cancer, circulating tumor cells, and metastasis: could protein-derived peptide fragments impede brain metastases?

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## Abstract

The majority of cancer deaths can be attributed to cancer cell metastases that migrate to distant target organs. Brain metastases constitute one of the leading causes of morbidity and mortality among cancer patients, occurring in about 40% of patients with metastatic disease. Thus, there exists an unmet need for early detection, diagnosis, and treatment directed against early stage cancer cell metastasis. Previous studies have reported the development of methods to detect and identify early circulating tumor cells (CTCs) in the bloodstream prior to their seeding into distant organs. Using a comprehensive analysis of total CTCs mRNA content, investigators have developed a mRNA "transcriptome signature" of 126 genes involved in CTC metastatic events. The genes were parsed into various metastatic-related activities indicating that CTCs sustained a semi-dormancy state bent on: (1) stress survival; (2) metabolic maintenance; (3) DNA and translational stability; and (4) chemotactic pro-inflammatory capabilities. These activities suggested that CTCs might be susceptible to interactions with protein-derived peptide segments whose actions are involved with metastatic activities such as cell invasiveness, contact, adhesion, motility, spreading, and migration. The use of protein-derived (encrypted) peptides to impede CTC metabolic activities and disrupt signaling pathways could have therapeutic potential in patients with early metastatic disease.

**Keywords:** Breast cancer, brain, metastasis, peptides, plasma proteins, tumor cells, circulation, transcriptome

## INTRODUCTION

Cancer metastases to the brain have been reported to occur in 10% to 20% of adult patients with malignant



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disease<sup>[1,2]</sup>. As such, brain metastases are one of the leading causes of morbidity and mortality among cancer patients with metastatic disease in the United States<sup>[3]</sup>. Indeed, the vast majority of cancer deaths can be attributed to tumor cell metastasis to distant sites rather than demise from the primary tumor mass itself. Several past reports have demonstrated that the presence of early circulating tumor cells (CTCs) provide a “feeder source” of break-away cells from the tumor mass cells that intravasate into the bloodstream to circulate, aggregate, then eventually migrate to metastatic sites of chemo-attracted target organs<sup>[4-6]</sup>. It may take extended time periods for the circulating cancer cells to develop aggregates detectable by radio-imaging as metastatic cell masses<sup>[7]</sup>. The early stages of disseminated tumor cells are able to aggregate to form micro-metastatic cell islets which further progress into macro-metastatic cell clusters. By the time the metastatic cells have migrated and “nested” into target tissues such as bone marrow and brain, the cells are already proliferating at exponential rates<sup>[8,9]</sup>. This advanced growth state of the tumor cell greatly reduces therapeutic options for the cancer patient. Therefore, it becomes crucial to detect and identify CTCs that represent very early stages of cells which disseminate from the primary tumor mass.

A recent report has, in fact, described use of a cutting-edge mRNA technology to characterize such CTCs. Hence, the first objective of the present commentary was to discuss the development of a biomarker mRNA signature that could screen and identify CTCs utilizing human breast cancer as the model. A second objective was to propose use of a potential therapeutic tool which could be directed against CTCs. These novel agents are referred to as “protein-derived peptide fragments” as discussed below.

## BACKGROUND STUDIES

It may be deduced from the above discussion that early screening and identification of CTCs associated with metastasis could be beneficial for evaluation of treatment options and their responses to brain metastasis. In a recent study using breast cancer (BC) cells, Boral *et al.*<sup>[10]</sup> reasoned that mRNA characterization of CTCs from BC derived cells could provide a means for early diagnosis of brain metastases; this knowledge could aid in planning therapeutic strategies and determining the effectiveness of targeting metastatic cells. Boral’s studies confirmed that patients with BC produce CTCs that express high levels of biomarkers that are associated with regulation of cell growth, proliferation, and signal transduction pathways directly involved with metastasis. Using a comprehensive analysis of CTC total mRNA transcriptomes, these investigators derived a unique “transcriptome signature” (TS) that distinguished circulating BC cells from those of the primary tumor mass. Even more intriguing, the derived TS revealed distinct signaling pathways inherent to BC-derived circulating cells that could provide the means of metastases to the brain. The Boral’s report concluded that the CTC biomarker profile and knowledge of their signaling pathways could be valuable as screening tools for: (1) micro- and macro-metastatic cell characterization; (2) decision-making in treatment modalities; and (3) monitoring post-treatment responses in patients with metastatic disease.

Previous literature reports have emerged which enumerated epithelial cell adhesion molecules (EpCAM) present on CTCs, thus providing an estimate of the overall metastatic cell burden present in BC patients<sup>[11-13]</sup>. Using previous EpCAM-related studies as a starting point, Boral *et al.*<sup>[10]</sup> devised a computer-based genomic and mRNA workflow strategy which revealed a higher cell surface frequency and expression of metastatic-associated biomarkers in BC patient’s cells versus cells from healthy blood donors<sup>[10]</sup>. Finally, these investigators utilized parametric flow cytometry and MRI-proven metastasis brain scans to analyze their BC patient’s cell populations.

## COMPONENTS OF THE CTC SIGNATURE

The CTC signature derived from circulating BC cells was parsed down to 126 genes involved in metastatic cell activities and signaling cascades<sup>[10]</sup>. Overall results from the 126 genes demonstrated that mRNA from 73 genes in the BC-CTC group were up-regulated while 53 genes were down-regulated. All of the CTC genes

detected were distinct and none clustered with their corresponding cells and tissues from the primary BC mass. Some of the biomarker constituents of the CTC mRNA signature included cell activities involving growth regulation, cell adhesion, cell-to-cell contact, spreading, migration, and motility. Such biomarkers included CD86, PARP6, ER $\alpha$ , GBP2, Adam-17, DDIT4, SLC2A3, SRGN, and NOTCH-1. Additional biomarkers were involved with chemotaxis, pro-inflammatory factors and immunomodulatory networks which included CD44, CD45, CD24, TNF, IL-1B, NFkB, CXCL8, CXCR4, and PDGF-BB. Brain-related biomarkers encompassed NCAM, Serpin I1, plasmin, neuroserpin B2, and UPAR which are required for stealth transpassage through the blood brain barrier [Supplementary Table 1]. It is of interest that proteins such as plasmin, serpins, and UPAR are especially crucial to CTCs for brain entry<sup>[14]</sup>.

A circumspect examination of some of the gene constituents of the CTC signature revealed that proteins related to various cellular activities and pathways could be parsed into several functional sub-groups. These groups displayed mRNA transcripts that were either enhanced (up-regulated) or reduced (down-regulated) in the blood circulating cells. The regulated gene transcripts encompassed cell activities such as: (1) growth and proliferation; (2) DNA transcription and translation; (3) signal transduction; (4) cell invasiveness and migration; and (5) mitotic and metabolic events. In summation, one could deduce from the above listing that CTC's appeared to be groomed for maintaining a metabolic "status quo" semi-dormancy state in order to survive in the blood circulation while preparing for migration to a distant organ site<sup>[4,15]</sup>. While so doing, the CTC have to retain their functional cell maintenance in order to detach from cell-to-cell contacts, adhere to blood platelets, and migrate to target organs (i.e., bone marrow or brain) with the aid of inflammatory chemokine molecules.

## PROTEIN-ENCRYPTED PEPTIDES, CTCs, AND METASTASIS

The containment of a class of growth factor, extra-cellular matrix, and angiogenic peptide fragments encrypted within the polypeptide chain of a full-length protein is known but is not widely recognized. However, some of the most potent growth inhibitors are derived from short peptide fragments (segments) already existent in naturally-occurring mammalian full length proteins that themselves affect cell growth and proliferation in an opposite function from the mother proteins. This less-recognized concept of a protein-derived reserve containing peptide growth Inhibitor fragments is becoming a recurring theme in the field of growth regulation, intracellular signaling, and cross-talk between signal transduction pathways. Classical examples of such occult (cryptic) peptides include the following examples; (1) tenascin binding peptide derived from fibronectin<sup>[16]</sup>; (2) angiostatin from plasmin<sup>[17]</sup>; (3) endostatin from type XVIII collagen<sup>[18]</sup>; (4) vasostatin from calreticulin<sup>[19]</sup>; and (5) constatin from type-IV collagen<sup>[20]</sup>. Such cryptic peptide sites can be exposed following a conformational change on a protein or can be released following proteolytic cleavage from a larger protein. These peptides can also be chemically synthesized as single fragments of 20-45 amino acids. A well-published example of a peptide site revealed following a conformational transition change on a full-length protein is an encrypted "growth inhibitory" site on alpha-fetoprotein (AFP), normally a growth promoting molecule<sup>[21-23]</sup>. The encrypted peptide segment, termed the growth inhibitory peptide (GIP), is a 34 amino acid segment concealed in a hydrophobic cleft of the completely-folded AFP molecule. The GIP site is revealed following protein unfolding in chemical environments containing high ligand concentrations of estrogens, fatty acids, and growth factors. This transitory GIP form converts the usually growth-enhancing AFP molecule into a growth-inhibiting polypeptide. This conversion occurs via protein un-folding into a conformational change resembling the denatured intermediate state of a molten globular form (MGF) of protein<sup>[22]</sup>. Since the MGF of AFP is a transitory intermediate form, AFP can refold back to its native tertiary fold following excess ligand removal. Because the AFP-MGF form is unstable, the GIP segment itself has now been synthesized, purified, and characterized as a distinct 34-mer synthetic peptide segment<sup>[23]</sup>. The 34-mer GIP fragment can inhibit both growth factor and estrogen-induced growth in a concentration-dependent fashion in addition to blocking metastatic-associated activities<sup>[24,25]</sup>.

## AFP-DERIVED PEPTIDES, CTCs, AND METASTASIS

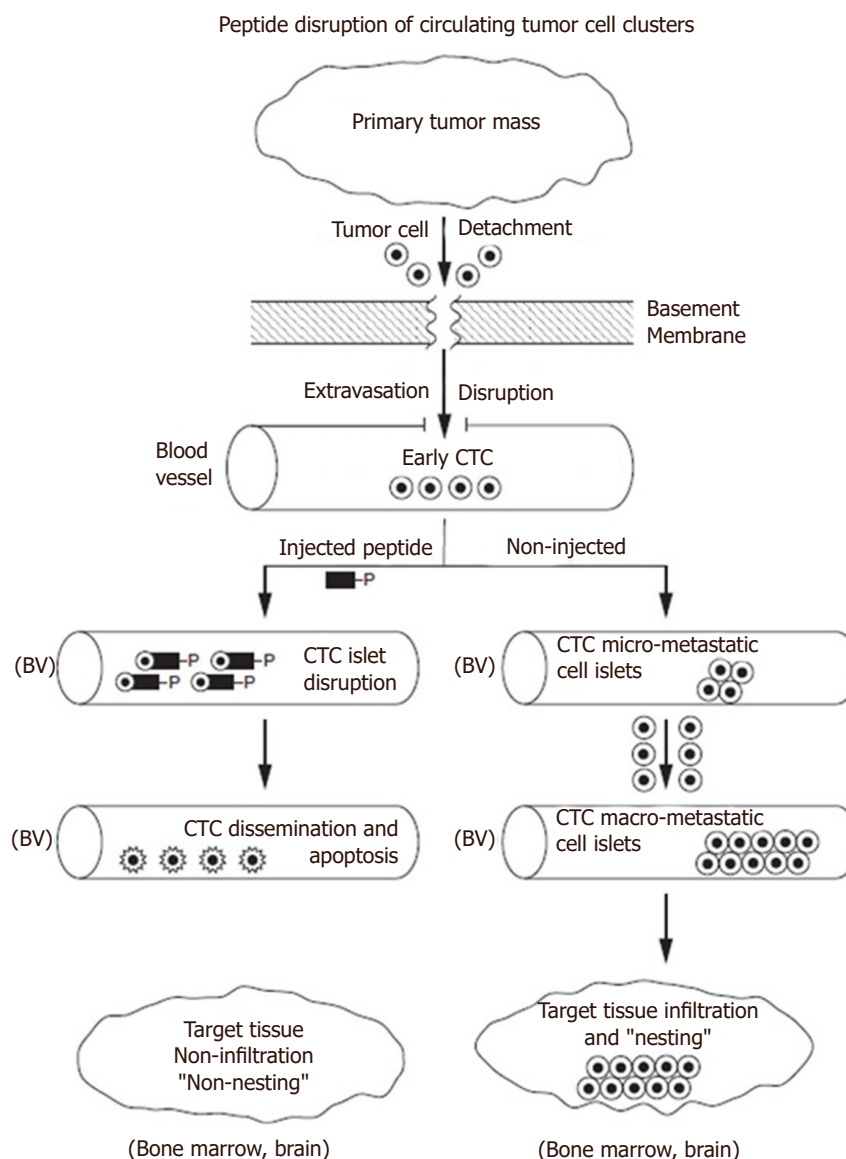
It is germane to the present commentary that full-length AFP mRNA detected in CTCs from hepatocellular carcinoma patients has been reported to serve as a predictive marker for metastasis<sup>[26]</sup>. Furthermore, a computer bioinformatics study of multiple metastatic protein interactions with AFP (and its derived peptides) has recently been reported<sup>[27]</sup>. In that study, many of the “*in silico*” AFP interaction with metastatic associated proteins were experimentally confirmed. Both *in vitro* and *in vivo* BC studies have been performed using GIP which demonstrated both anti-growth and anti-metastatic activities. For example, in a microarray study, GIP was found to down-regulate the mRNA (1.5 to 8 fold) of many proteins detected in the “CTC signature” of the BC-derived circulating cells described by Boral *et al.*<sup>[10]</sup>. Such proteins included CD44, CD40, TNF, NFkB, IL-1 receptor, Serpin I-1, and p53 AIP1 among others. Many of these metastasis-associated proteins were reported to interact with AFP in protein-to-protein interactions; such metastases-related proteins included the laminin receptor, collagen-IV, Integrin B-1, IL-1B, and the neural cell adhesion molecule (NCAM). It is of interest that, Serpin-I1 and plasminogen activator are known to promote cancer cell survival in brain metastasis by means of brain plasmin inhibition<sup>[28]</sup>. In the pro-inflammatory arena, AFP itself was found to interact and block CCR5 and CXCR4 chemokine receptors which are required for metastatic BC cell migration<sup>[29-31]</sup>.

## BIOLOGICAL ACTIVITIES OF AFP-DERIVED PEPTIDES

Other sets of data involving AFP-derived peptides have been generated *in vitro* involving cancer cell adhesion, cell-to-cell interactions, cell spreading, motility, migration, and growth<sup>[32,33]</sup>. Regarding cancer cell proliferation, GIP was found to inhibit growth in multiple BC cell lines *in vitro* and to inhibit cell-to-cell contact inhibition overgrowth in cultured MCF-7 cells<sup>[33-35]</sup>. In addition, both full length AFP and GIP were both found capable of inhibiting platelet aggregation<sup>[36]</sup>, a process necessary for CTC survival in the bloodstream; this activity involved integrins  $\alpha 2\beta 1$ ,  $\alpha 5\beta 1$ , and  $\alpha 2\beta 3$ . CTCs are known to adhere to blood vessel inner walls and to platelets, thereby cloaking themselves from circulating cytotoxic lymphocyte destruction<sup>[13,35,36]</sup>. Furthermore, GIP was found to block both adhesion of extra-cellular matrix (ECM) proteins to substrata as well as tumor cell adhesion to ECM-coated wells of microtiter plates<sup>[35]</sup>. The ECM proteins included laminin, fibrinogen, collagen-IV, fibronectin, thrombospondin, and vitronectin. In addition, both collagen-IV and NCAM have been reported to bind to the third domain of AFP at amino acid segments #433 to 545<sup>[34]</sup>. It has been further demonstrated *in vitro* that GIP notably interrupted the migration and invasion of follicular thyroid cancer cells<sup>[37]</sup>. It has also been reported that GIP could inhibit 60% of the cell spreading and migration of MCF-7 tumor cells in culture assays<sup>[34]</sup>. Because integrins and ECM proteins are both involved in cell migration by modulating the fine balance between cell-to-contact, adhesion, and cell detachment, it was noteworthy that GIP was found capable of disrupting the interaction between receptors and binding proteins in such activities. The final involvement of GIP with cancer cell activities was demonstrated using *in vivo* models of human BC xenografts in mice. GIP was reported to suppress cancer growth/proliferation in both xenograft and homograft models of MCF-7, GI-101, MDA-MB-231, and 6WI-1 BC tumors in host mice<sup>[25,32]</sup>. In the human MDA-MB-231 BC *in vivo* mouse model, GIP injections resulted in a 3-fold reduction in BC metastasis to the lungs as compared to controls. In the 6WI-1 *in vivo* mouse homograft model, GIP suppressed BC cell migration, invasiveness, and adherence to surrounding cells and tissues. Thus, GIP injections not only demonstrated BC growth suppression but also reduced metastatic-associated events in BC cells such as cell adherence, invasiveness, and migration in addition to decreased metastatic cell accumulation in distant organs. Finally, GIP administered *in vitro* produced an inhibition of cell membrane-induced agglutination, and induced cell shape changes via enhanced microtubule polymerization<sup>[24,34,35]</sup>.

## CONCLUDING STATEMENTS

It can be concluded from the above discourse that peptide fragments derived from plasma, ECM, and angiogenic-associated proteins are capable of tumor growth inhibition and suppression of metastatic spread.



**Figure 1.** An injected peptide disruption of blood circulating tumor cells (CTCs) is depicted in the flow diagram. The primary mass of malignant tumors are known to shed cells which can migrate through the intercellular spaces en route to metastasis. The detached migratory tumor cells can extravasate through the blood vessel basement membrane and endothelial cell lining into the lumen of blood vessels (BV). Early CTCs soon form micro-metastatic clusters which further aggregate to form macro-metastatic islets (right side of diagram). CTCs eventually infiltrate into distal target tissues (bone marrow, brain) and “nest” there. However, if designer peptides home toward and bind to tumor cell membrane proteins/receptors as decoy ligands (see text for mRNA expressed proteins), tumor cell clusters could be disrupted and disseminated (left side of diagram). Single circulating cells including CTCs demonstrating cell membrane ruffling and disruption can become susceptible to apoptosis and/or immune surveillance destruction

Such physiological events include cell-to-contact, cell migration, adhesion, detachment, spreading, and chemokine and receptor interactions. Following cell detachment from the BC tumor mass, the disseminated tumor cells extravasate through the tissue extracellular compartments, pass through disrupted (proteolysis) basement membranes, and emerge into the bloodstream. Once in the blood circulation, tumor cells can adhere and cluster into micro- and macro-metastatic islets that attach to blood platelets cloaking them from detection by cytotoxic lymphocytes. It is just prior to the stage of islet cluster formation that the metastatic cells are most vulnerable to blockade of signal transduction pathways [Figure 1]. Discovery of the CTC mRNA signature of CTCs en route to “nesting” in distant target organs, such as the brain, might allow investigators to design therapeutic strategies to impede metastatic invasion to the distant tissues and organs.



One such group of potential metastatic disruptive agents could include plasma-, ECM-, and angiogenic protein-encrypted peptide fragments as discussed above. Such metastatic (migration) interfering peptides might be therapeutically beneficial to BC patients in early stages of micro-metastasis. Small peptides are known to have short half-lives (hours), little or no side effects, and could be intravenously administered. The screening of CTCs using the “signature” identification methodology, followed by peptide therapy, could potentially provide a novel 2-step detection/therapy strategy for select cancer patients with early metastatic disease.

## DECLARATIONS

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Mizejewski GJ contributed solely to the commentary.

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The author declared that there are no conflicts of interest.

### Ethical approval and consent to participate

Not applicable.

### Consent for publication

Not applicable.

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Review

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# Immunotherapy in the treatment of colorectal cancer: a new kid on the block

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## Abstract

In the last few years, the success of anti-PD1 and anti-PDL1 drugs in solid cancers treatment and the advances in molecular biology have provided new potential treatment strategies for patients with metastatic colorectal cancer. Unfortunately, only patients with mismatch repair deficiency seem to benefit from immunotherapy and they represent a small subset of the metastatic population. New ongoing studies focus on converting an immune ignorant tumour into an inflamed one by combination therapies and on introducing an immunotherapeutic approach in earlier stages of disease (neoadjuvant and adjuvant setting). In this review we summarize the current knowledge about the molecular and immune landscape of colorectal cancer and propose new potential combination strategies to enhance the efficacy of immunotherapy.

**Keywords:** Colorectal cancer, immunotherapy, microsatellite instability, pembrolizumab, nivolumab, atezolizumab

## INTRODUCTION

Colorectal cancer (CRC) is the third most common cancer in males and the second in females, representing the fourth leading cause of cancer-related deaths worldwide in older adults<sup>[1,2]</sup>.

However, CRC-related mortality has declined progressively in the past decades, due to cancer screening programs, standardization of preoperative and postoperative care, improved surgical techniques and more-effective systemic therapies for early and advanced-stage disease<sup>[3]</sup>.



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Nevertheless, about 50% of patients develops metastases during the course of their disease. In these patients, chemotherapy (fluoropyrimidines, oxaliplatin and irinotecan) with biological agent [anti-vascular endothelial growth factor (anti-VEGF), anti-epithelial growth factor receptor (anti-EGFR) and multikinase inhibitor such as regorafenib] remains the standard of care, with median overall survival approaching 30 months<sup>[4]</sup>.

The molecular characterization of colorectal cancer has led to the identification of favorable and unfavorable immunological features linked to clinical outcome<sup>[5]</sup>.

Currently, CRC is classified into four consensus molecular subtypes (CMS), with unique clonal, stromal and immune dependences<sup>[6]</sup>.

The immune system has a substantial effect on cancer, especially as a suppressor of tumour initiation and progression. Additionally, it influences the response to immunotherapeutic and conventional treatment options (e.g., chemotherapy, radiotherapy and targeted therapies). However, the tumor can establish several mechanisms to escape immune surveillance. Therefore, different strategies may be pursued to restore the immune response against cancer cells, both as an active immunotherapy (cytokines, immune checkpoint inhibitors, co-stimulatory pathways and cancer vaccines) and as a passive immunotherapy (adoptive cellular therapy and monoclonal antibodies) approach<sup>[7]</sup>. FDA has recently approved checkpoint inhibitors (nivolumab and pembrolizumab), for the treatment of patients with microsatellite instability (MSI) metastatic CRC. However, the most unsolved problem is the lack of efficacy of these antibodies in microsatellites stable (MSS) tumours, which represent the majority of CRC.

In this review we summarize the biological bases and the recent clinical evidences related to the use of immunotherapy in metastatic colorectal cancer (mCRC) to suggest different treatment strategies according to different CMS, transcriptomic pathways and stroma-immune microenvironment.

## RATIONALE FOR IMMUNOTHERAPY IN CRC

The immune system has a major role in cancer: immune cells can act both as suppressors of tumor initiation and progression and as promoters of proliferation, infiltration and metastasis.

In 1970 Burnet<sup>[8]</sup> proposed the concept of immune-surveillance, that was updated by Dunn *et al.*<sup>[9]</sup> and Schreiber *et al.*<sup>[10]</sup> with the identification of the process of immunoediting. This process consists of three well-defined phases: elimination, equilibrium and escape. The elimination phase refers to active surveillance, and includes innate and adaptive immune responses to tumour cells. First of all, cells of the innate immune system (NK cells, NK T cells, macrophages and dendritic cells) recognize the presence of a growing tumor after its stromal remodeling, a local tissue damage and the release of inflammatory signals, which recruit these cells to the tumor site. They produce IFN-gamma and IL-12, and destroy most of cancer cells, even if some of them survive and reach the “equilibrium” phase. Therefore, in the elimination phase, the release of IFN-gamma and production of chemokines as CXCL10, CXCL9 and CXCL11 determine the inhibition of angiogenesis. Meanwhile dendritic cells migrate into the draining lymph nodes and promote the differentiation of Th1 cells into cytotoxic CD8+ T cells. In the equilibrium phase tumor cells that have escaped the elimination phase and have a non-immunogenic phenotype are selected for growth. Progressively these cells become unstable and acquire various mutations, so they will be able to grow despite immune attack and reach the escape phase. In this third phase, tumor cells continue to grow and may lead to malignancies.

In this process we can identify three main characters<sup>[11]</sup>:

1. Tumour cells have several mechanisms that block the activity of effector antitumor CD4+ and

CD8<sup>+</sup> T cells, in order to reduce local tumour-infiltrating immune responses. Frequent mechanisms include loss of MHC class I expression, dysregulation of antigen processing machinery, production of immunosuppressive factors (TGF- $\beta$ ; IL-10; VEGF; indoleamine 2,3 dehydrogenase), recruitment of immunosuppressive cells (i.e T-reg)<sup>[12]</sup> and activation of negative costimulatory signals in tumour microenvironment like PD-L1<sup>[13]</sup>.

2. Tumour microenvironment consists of regulatory immune cells, extracellular matrix proteins, and fibroblasts (cancer-associated fibroblasts, CAFs). They secrete tumour-promoting factors that contribute to tumor invasion and neoangiogenesis<sup>[14]</sup>. CAFs play a critical role in CRC immunosuppression, particularly in CRC RAS mutated; in fact they lead to tumour progression by activating epithelial mesenchymal transition and TGF- $\beta$ /SMAD signalling<sup>[15]</sup>: high levels of CAFs markers are correlated with poor prognosis in CRC<sup>[16]</sup>.
3. The immune system includes innate and adaptive immune cells. The innate cells consist of macrophages, mast cells, neutrophils, dendritic cells, and myeloid-derived suppressor cells (MDSCs) and natural killer (NK) cells. The adaptive T cells include CD8<sup>+</sup> cytotoxic T lymphocytes (CTLs), CD4<sup>+</sup> Th1 cells, CD4<sup>+</sup> Th17 cells and regulatory T cells (Tregs)<sup>[7]</sup>. A strong lymphocytic infiltration is associated with better clinical outcome in many tumors, including colorectal cancer. Particularly, high densities of CD3<sup>+</sup> T cells, CD8<sup>+</sup> cytotoxic T cells and CD45RO<sup>+</sup> memory T cells are associated with a longer disease free survival (DFS) and improved overall survival (OS)<sup>[17]</sup>. Therefore in CRC also the infiltration of M1 macrophages, DCs and NK cells is associated with good prognosis, while the presence of M2 macrophages, MDSCs, Th17 and B cells is related with poor outcome<sup>[18]</sup>.

All these findings have been translated into the elaboration of the so called “immunoscore”. It is based on the quantification of two lymphocytes populations (CD3/CD45RO, CD3/CD8 or CD8/CD45RO) in both tumor core and invasive margin. It ranges from 0 (low density of both cells in both cancer regions) to 4 (high density) and may predict DFS and OS in CRC<sup>[19]</sup>. It may help identify patients with early stage disease who might benefit from adjuvant chemotherapy or immunotherapies according to T-cell densities<sup>[20]</sup>.

## FROM MOLECULAR SUBTYPES TO STROMAL CLASSIFICATION

The development of CRC is supported by the accumulation of genetic and epigenetic alterations that transform colonic epithelial cells into colon adenocarcinoma cells. The genomic instability occurs early in carcinogenesis and it facilitates the acquisition of alterations in tumour suppressor genes and oncogenes in a clone of cells, resulting in cancer<sup>[21,22]</sup>. Three fundamental pathways are implicated in this process:

1. Chromosomal instability (CIN): CIN is observed in the majority of sporadic CRCs (65%-75%) and is characterized by a variety of chromosomal alterations leading to defects in chromosomal segregation, telomere stability, and the DNA damage response as well as a loss of heterozygosity. CIN-high tumours typically accumulate mutations in specific tumor-suppressor genes and oncogenes that activate critical pathways for CRC pathogenesis, including KRAS, CTNNB1, PIK3CA, APC, TP53 and SMAD4.
2. CpG-island methylator phenotype (CIMP): it is defined by hypermethylation of the symmetrical dinucleotide CpG and a global DNA hypomethylation. In many human genes there's a CpG island in their promoter region, and the methylation of the cysteines of the CpG-island implicates the transcriptional silencing of the gene<sup>[23]</sup>. When this happens in promoters of tumour suppressor genes, it supports the possible development of cancer<sup>[24]</sup>.
3. Microsatellite instability (MSI): it occurs in 15% of early-stage colorectal tumors. It is caused by a lack of expression in the DNA mismatch repair (dMMR) proteins, which normally are involved in the correction of DNA replication errors. This defect results in accumulation of mistakes in microsatellite regions, which are short repetitive sequences of DNA, with unit length ranging from one to six bases. They are scattered throughout the coding and noncoding regions of the genome. MSI can be due



to germline mutations of MMR enzymes, as MLH1, MSH2, MSH6, and PMS2, which causes the so called Lynch syndrome<sup>[25-27]</sup>. There are also sporadic dMMR CRCs, which arise mainly from epigenetic silencing of MLH1 promoter, and they are associated with CIMP phenotype and BRAF V600E mutations.

To define MSI, five microsatellites are evaluated through PCR based assay: if  $\geq 2/5$  are unstable, the sample is defined as MSI-high (MSI-H), while 1/5 or 0/5 are MSI-low (MSI-L) and MSS, respectively, which have a similar behavior<sup>[28]</sup>.

The progressive findings in the molecular characterization of CRC along with the identification of specific gene alterations as prognostic and predictive factors in this cancer, led to the elaboration of various CRC classifications, essentially based on gene expression<sup>[29-33]</sup>. However, there were many differences among these classifications, so in 2015 the CRC Subtyping Consortium (CRCSC) developed a new classification, identifying four consensus molecular subtypes (CMS), analyzing the results of six CRC subtyping algorithms<sup>[34]</sup>. Each CMS group had a specific pattern:

- CMS1 (MSI Immune, 14%): CMS1 samples were hypermutated, with low prevalence of somatic copy number alterations (SCNAs), enriched of MSI and CIMP tumours with hypermethylation status. A particular characteristic of this group was a more frequent presence of BRAF mutations, compared to the other CMS. This subtype was defined as immune, because of the rich immune infiltrate (especially Th1, cytotoxic T cells and NK cells) and the strong activation of immune evasion pathways, as we typically see in MSI CRC<sup>[35]</sup>.
- CMS2 (Canonical, 37%): this group exhibited the typical CIN pattern; it also showed more frequent copy number gains in oncogenes and losses in tumour suppressor genes. It was characterized by epithelial differentiation, with WNT and MYC activation, higher expression of the oncogenes *EGFR*, *ERBB2* (also known as *HER2*), insulin-like growth factor 2 (*IGF2*), insulin receptor substrate 2 (*IRS2*) and transcription factor hepatocyte nuclear factor 4 $\alpha$  (*HNF4A*), as well as cyclins2.
- CMS3 (Metabolic, 13%): CMS3 samples were characterized by few SCNAs, a 30% significant hypermutation with a mixed MSI status, a higher prevalence of CIMP low cluster and an intermediate hypermethylation status. This subtype was defined as “metabolic” according to the common metabolic alterations and the higher expression of KRAS mutations, which made this group of cancers similar to a recently identified gastric cancer subtype<sup>[12]</sup>.
- CMS4 (Mesenchymal, 23%): similarly to CMS2, this group had a high prevalence of SCNAs. It showed the typical mesenchymal pattern, as the upregulation of genes involved in epithelial mesenchymal transition, the TGF $\beta$  activation, angiogenesis, matrix remodeling, with a consequent stromal infiltration, particularly CAFs.

This classification reflects also significant clinical and prognostic differences among the various subtypes: CMS1 cancers are frequent in females with right-sided tumours and have a higher histopathological grade, while CMS2 cancers are more frequently left-sided. Moreover, CMS4 cancers are often diagnosed at advanced stages and they show worse overall survival (OS) and relapse-free survival (RFS). Patients with the typical CMS1 pattern have poor survival after relapse, consistently with the known bad prognosis of patients with MSI and BRAF mutated CRC after relapse<sup>[36]</sup>, differently from CMS2 population, which has the best survival after relapse of these CMS groups<sup>[37,38]</sup>.

## IMMUNOTHERAPY IN MSI mCRC

Mismatch repair deficient CRCs represent 15% to 20% of stage II and III CRCs and are associated with better prognosis than proficient (pMMR) tumors. In the metastatic setting, dMMR CRCs represent only around 5% and are associated with a poor prognosis<sup>[39]</sup>, as confirmed in the recent results presented at the 2017 ASCO

meeting (OS at 17.9 months and PFS at 3.9 months), whatever the chemotherapy regimen or targeted therapy used (bevacizumab or anti-EGFR)<sup>[40]</sup>.

Actually, immunotherapy is a prominent therapeutic approach in many cancers, such as melanoma, non-small cell lung cancer, kidney and bladder cancer. However, significant advances have been made also in CRC. A first study utilizing a CTLA-4 antagonist monoclonal antibody, tremelimumab, showed a possible usefulness of immune checkpoint inhibitors in CRC, obtaining one 6-month durable response<sup>[41]</sup>.

Then, in the phase II trial conducted by Le *et al.*<sup>[42]</sup>, the clinical activity of pembrolizumab was evaluated in three cohorts of patients: MSI-H CRC, MSI-H non CRC, and MSS CRC. The immune-related objective response rate (ORR) and the immune-related 6-month PFS rate were 40% and 78%, respectively, in the dMMR CRC patients, 0% and 11% in the pMMR CRC patients. These findings currently are being evaluated in the KEYNOTE-177 phase III trial in patients with dMMR metastatic CRC who have been randomized to treatment with pembrolizumab vs. standard therapy.

In Checkmate 142, nivolumab alone and the combination of nivolumab + ipilimumab were evaluated in patients with metastatic CRC, with or without MSI. Seventy patients with MSI-H CRC were enrolled and treated with nivolumab monotherapy (3 mg/kg every 2 weeks). At the preliminar presentation of the trial results, of the 47 patients which had at least 12 weeks of follow-up, 26% had an objective response while 30% had stable disease, with disease control rate of 55%. In the update published on *Lancet*, 23 of 74 patients achieved an objective response (ORR 31%) and 51 of 74 patients had disease control for 12 weeks or longer (DCR 69%)<sup>[43]</sup>. By the use of combination therapy (nivolumab 3 mg/kg q2 week plus ipilimumab 1 mg/kg q3 week × 4 doses, followed by nivolumab monotherapy), investigator-assessed ORR was 55%, and disease control rate for ≥ 12 weeks was 80%<sup>[44]</sup>. In this heavily pre-treated population, 12 months overall survival was 73% and 85% with monotherapy and combination therapy respectively. Grade 3 and 4 drug related adverse events (AEs) were reported in 25 patients treated with nivolumab (20%) mainly asymptomatic increasing of amylases and lipase: only 5 patients (7%) stopped the treatment due to toxicities. In the combination group, grade 3 and 4 AEs were reported in 32% of patients: 15 patients (13%) discontinued treatment because of study drug-relates AEs.

All these data supported the benefit of immunotherapy in MSI-H CRC, and for this reason FDA approved the use of nivolumab and pembrolizumab in patients with unresectable or metastatic MSI-H and dMMR CRC, that have progressed after previous treatment.

Similar results were not reached in MSS CRC, in fact in the pivotal pembrolizumab study, no response was achieved, with very poor PFS and OS, as subsequently confirmed in other trials<sup>[45,46]</sup> [Table 1].

There are several combination clinical trials and novel immunotherapeutic strategies under active investigation for metastatic CRC [Table 2].

## STRATEGIES TO CONVERT AN IMMUNE IGNORANT TUMOR INTO AN INFLAMED ONE

There are mainly 3 different tumour immune phenotypes [Figure 1]:

1. Highly immune-infiltrated tumours with favourable immune microenvironment, enriched of Th1-type functional TILs;
2. Highly immune-infiltrated tumours with unfavourable tumour microenvironment with active angiogenic and immunosuppressive pathways;
3. Poorly immunogenic tumours with minimal immune cell infiltration<sup>[47]</sup>.

**Table 1. Immunotherapy trials in metastatic colorectal cancer**

Population	Drugs	Target	Patients	Response rate
Refractory MSI-H CRC	Pembrolizumab (42)	PD-1	25	57%
				Sporadic cases: 100% ( $n = 6/6$ ) LS cases: 27% ( $n = 3/11$ )
	Nivolumab (43)	PD-1	74	31%
				Sporadic cases: 36% ( $n = 10/36$ ) LS cases: 30% ( $n = 8/27$ )
	Nivolumab + ipilimumab (44)	PD-1 + CTLA-4	119	55%
Refractory MSS CRC	Pembrolizumab (42)	PD-1	28	0%
	Nivolumab + ipilimumab (44)	PD-1 + CTLA-4	20	5%
Refractory CRC	Tremelimumab (41)	CTLA-4	49	2%
	Nivolumab (45)	PD-1	19	0%
	BMS-936559 (46)	PD-L1	18	0%
	Atezolizumab + bevacizumab (49)	PD-L1	14	7%
	Atezolizumab + FOLFOX/bevacizumab, 70% first line (49)	PD-L1	30	40% (total) 48% (first-line)
	Atezolizumab + cobimetinib (53)	PD-L1 MEK	23	17%
				(3 MSS, 1 unknown)

CRC: colorectal cancer; CTLA-4: cytotoxic T lymphocyte associated antigen 4; PD-L1: programmed cell death ligand-1; PD-1: programmed cell death protein 1; MSS: stable microsatellite; MSI-H: high microsatellite instability

**Table 2. Ongoing studies in colorectal cancer**

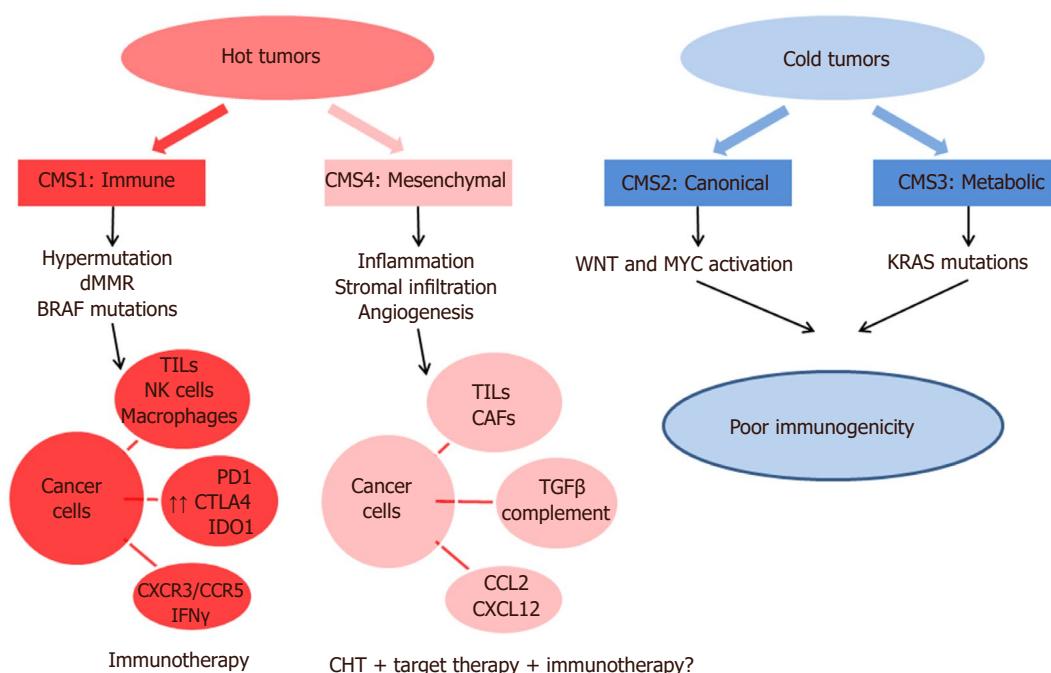
Trial	Treatment	Patient population	Endpoints
Keynote-177 NCT02563002	Pembrolizumab monotherapy vs. standard care chemotherapy	MSI-H CRC, 1st line metastatic	PFS
Keynote-164 NCT02460198	Pembrolizumab monotherapy	MSI-H CRC, metastatic refractory (Cohort A) or $\geq 1$ prior therapy (Cohort B)	ORR
NRG-GI004/S1610 NCT02997228	Atezolizumab vs. atezolizumab + FOLFOX + bevacizumab vs. FOLFOX + bevacizumab	MSI-H CRC, 1st line metastatic	PFS
Alliance A021502 NCT02912559	Atezolizumab + FOLFOX vs. FOLFOX alone	MSI-H CRC, stage III	DFS
NCT02870920	BSC + durvalumab + tremelimumab vs. BSC	MSS, chemorefractory mCRC	OS
NCT02788279	Atezolizumab vs. atezolizumab + cobimetinib vs. regorafenib	Chemorefractory mCRC	OS
NCT02873195 BACCI study	Atezolizumab + capecitabine + bevacizumab	Chemorefractory mCRC	PFS
NCT02948348	Nivolumab + chemoradiotherapy	Locally advanced CRC	Pathological complete response
NCT02888743	Durvalumab + tremelimumab +/- radiation	Chemorefractory mCRC	ORR

CRC: colorectal cancer; MSS: stable microsatellite ; MSI-H: high microsatellite instability; BSC: best supportive care; PFS: progression free survival; OS: overall survival; DFS: disease free survival; ORR: overall response rate

CMS1 tumours are characterized by upregulation of PD-1, PDL-1, CTLA-4 and IDO as described above and so they are the best candidates for immunotherapy.

A major challenge is to render pMMR mCRC (second and third group described above) sensitive to immune checkpoint inhibitors. In the pivotal pembrolizumab trial by Le *et al.*<sup>[42]</sup>, no responses in the MSS CRC pretreated cohort were observed with median overall survival of 5 months. Furthermore in the “combo” experience Checkmate 142 with nivolumab and ipilimumab, only 1 response was observed among 20 patients with pMMR CRC<sup>[44]</sup>.

CMS4 tumours should be considered “hot tumours” with immunosuppressive signalling ongoing:



**Figure 1.** Immune subtypes classification. CMS: Consensus molecular subtype; dMMR: deficit mismatch repair; TILs: tumor-infiltrating lymphocytes; PD1: programmed death protein 1; CTLA4: cytotoxic T-lymphocyte-associated protein 4; IDO1: indoleamine-pyrrole 2,3-dioxygenase; IFN $\gamma$ : interferon gamma; CXCR3-CCR5: chemokine receptor type 3-5; CAFs: cancer associated fibroblasts; TGF $\beta$ : transforming growth factor beta; CCL2-CXCL12: chemokine ligand 2-12

TGF- $\beta$  signalling and an angiogenic microenvironment should be targeted to restore a “CMS-1-like” immune microenvironment. Preclinical data suggested a synergic effect of TGF- $\beta$  and PD-1 inhibition in mouse model of mesenchymal CRC<sup>[48]</sup>. The role of chemotherapy alone in CMS4 tumours is ambiguous: chemotherapy has a detrimental effect on the immune system but the cell-necrosis induced and the subsequent release of neo antigens should be immunogenic, promoting the activity of APC and thus an immune-response. Based on previous clinical data suggesting an adjuvant immune effect of anti-VEGF antibody combined with standard chemotherapy, the combination of FOLFOX, bevacizumab and atezolizumab has been evaluated in a cohort of 23 naïve patients. Almost all patients demonstrated clinical benefit with 11 (48%) achieving partial response and 9 (40%) stable disease: how these results should be interpreted is still unclear but the clinical benefit reached in almost 90% of patients deserves further investigations<sup>[49,50]</sup>. Several other clinical trials investigating the combination of chemotherapy, bevacizumab and check-point inhibitors are ongoing.

CMS2 and CMS3 tumours are typically “cold tumours” with downregulation of MHC class I and lack immune cell infiltration<sup>[47]</sup>. A lot of strategies are still under evaluation to convert these immune-ignorant tumours into “hot tumours”.

In preclinical experiences, MEK and PD-1 co-inhibition showed a synergistic effect in colorectal, melanoma and breast cancer models<sup>[51]</sup>. Cobimetinib, a MAPK inhibitor, upregulates IFN- $\gamma$ , HLA molecules and PDL-1 expression stimulating CD8+ CTLs activity in the tumour microenvironment<sup>[52]</sup>.

Based upon these preclinical data, a phase I study that combined MEK and PDL-1 inhibition (cobimetinib and atezolizumab) was designed in which 4 of the 23 patients enrolled achieved partial response(17%): 3 of these 4 patients were pMMR, 1 had an unknown MSI/MMR status<sup>[53]</sup>. Actually, a three arms phase III study in which patients with pMMR chemo-refractory CRC are randomized to receive atezolizumab, or a

combination of atezolizumab and cobimetinib or regorafenib has completed the accrual and its results are eagerly awaited.

Another strategy to inflame these “cold cancers” could be enhancing T cell infiltration, typically poor in these tumors. Histone deacetylase (HDAC) inhibitors like romidepsin (preclinically tested for this capacity) have been actually combined with anti-PD1 therapy in a phase I/II trial currently ongoing in CRC<sup>[54]</sup>.

Another option could be the use of BITEs (Bispecific T cell engager) that bind the CD3 subunit of the T cell receptor and a tumor specific antigen.

Interesting results from preclinical experiences<sup>[55]</sup> lead to a phase I trial with CEA-CD3 TCB (RG7802, RO6958688). CEA-CD3 TCB is a novel T-cell bispecific antibody targeting CEA on tumour cells and CD3 on T cells increasing intratumoral T cell infiltration and activation and enhancing the PD-L1/PD-1 pathway<sup>[56]</sup>.

The phase I trial results, presented at ASCO 2017 by Tabernero *et al.*<sup>[57]</sup>, suggested antitumor activity in monotherapy and enhanced efficacy in combination with atezolizumab in patients with advanced CEA+ solid cancers with manageable safety profile.

## COMBINATIONS WITH RADIOTHERAPY

Radiotherapy determines cell death in targeted lesions inducing local and systemic immune-mediated anti-tumour effects. In 1953, Mole<sup>[58]</sup> proposed the term “abscopal effect” referring to the effects of ionizing radiation at a distance from the irradiated volume but within the same organism. Almost 50 years later, the role of the immune system in this “off target” effect has been settled. RT may affect antitumor immunity by enhancing antigen presentation by upregulation of major histocompatibility complex class I (MHC-1) expression of malignant cells and upregulation of tumor-associated antigens<sup>[59]</sup>. The clinical use of immune checkpoint inhibitors has greatly increased the number of abscopally responding patients. In a preclinical trial, Park *et al.*<sup>[60]</sup> achieved complete regression of primary tumour and partial response in distant metastases via abscopal responses with combination of radiotherapy and anti-PD1. At ASCO 2016, preliminary results of a phase II trial evaluating the abscopal effects of pembrolizumab after liver radiofrequency ablation or external beam radiotherapy had been presented. Tolerable safety profile and a partial response in non-irradiated lesions over 23 patients treated have been demonstrated<sup>[61]</sup>. A phase II trial investigating the efficacy of durvalumab-tremelimumab in combination with radiotherapy in patients with liver limited disease is underway (NCT02888743).

Trials with long-course chemoradiation in combination with PD-1 inhibition in locally advanced rectal cancer are still ongoing and so answers about this approach should be available in the next few years (NCT02948348, NCT03038477).

## IMMUNOTHERAPY COMBINATIONS

The clinical activity of epacadostat (IDO-inhibitor) alone appears limited but combination with pembrolizumab in melanoma patients reported ORR of 58%<sup>[62,63]</sup>. Actually epacadostat has been investigated in combination with pembrolizumab and azacitidine in refractory MSS CRC.

Also cetuximab, an anti-EGFR antibody actually approved for treatment of pan-RAS wt colorectal cancer, demonstrated a T-cell response and antigen liberation in HNSCC; in mCRC patients treated with cetuximab a relevant intratumoral T-cell infiltrates has been shown<sup>[64]</sup>. For these reasons, an ongoing phase I-II trial is examining the role of cetuximab-pembrolizumab combination in mCRC.



## OTHER STRATEGIES TO ENHANCE THE IMMUNOTHERAPY EFFECT

Other strategies are actually ongoing to enhance the response to immunotherapy<sup>[65]</sup>.

MGN1703 is a DNA-based Toll-like receptor that acts as an immunomodulator with immune activation in heavily pre-treated patients with mCRC in the phase II IMPACT trial in maintenance setting<sup>[66]</sup>. Patients who had completed first line standard chemotherapy + bevacizumab were randomly allocated to lefitolimod or placebo. There was a statistically significant better PFS in the experimental arm from start of induction therapy with the greatest benefit for patients with relatively low tumour burden<sup>[67]</sup>. Data on the use of MGN1703 (lefitolimod) as switch maintenance in patients with mCRC responding to first line chemotherapy are awaiting (Impala phase III trial).

In the perioperative liver-limited disease setting, the role of immunotherapy in association of chemotherapy was evaluated for the first time in 1996: a preoperative injection of IL-2 in patients with DUKES D tumours neutralized surgery-induced immunosuppression with improved overall survival due to postoperative mean numbers of T lymphocytes, natural killer cells and activated lymphocytes significantly higher in IL-2-treated patients than in controls<sup>[68]</sup>. Results from ongoing trials with check-point inhibitors also in this setting are awaited.

In the CRC prevention setting, the role of immunotherapy alone or in combination with chemotherapy is under evaluation. Vaccination is by far the first approach evaluated. Based on a MUC-1 vaccination clinical trial that enrolled 39 patients and suggested the presence of immunosuppressive mediators in premalignant stages<sup>[69]</sup>, a multicenter randomized phase II trial for testing the efficacy of this vaccine is actually ongoing (NCT02134925). Therapeutic KRAS mutated vaccines have been tested in preclinical trials for advanced tumors<sup>[70]</sup>.

Combinations of immune-modulating agents and chemopreventive drugs have been tested in preclinical studies<sup>[71]</sup>. The synergic effect of combining a non-steroidal anti-inflammatory drug (NSAID) with an immune checkpoint inhibitor is supported by preclinical data: aspirin induced upregulation of PD-L1 and PD-L2 expression<sup>[72]</sup>.

In a trial by Zelenay *et al.*<sup>[73]</sup>, the combination of COX-inhibitor and PD-1 inhibitor, was effective in eradicating BRAF-mutated melanoma neoplastic cells in mice with a significant increase in IFN, CXCL10, IL12 expression as immune-stimulating factors.

There are a lot of exogenous and endogenous factors (collectively called exposome) which are able to influence the development of CRC. However, for a comprehensive evaluation of tumor immunity, both the neoplastic cells and the immune system need to be deeply analyzed. Immune cells analyses in the tumor microenvironment have not been integrated into experimental immunological studies. In this regard, molecular pathological epidemiology (MPE) offers the opportunity to a multilevel research using bioinformatics and omics technologies to integrate immunology into population health sciences, providing a deeper understanding of the interaction between tumor, exposome and immune system and offering new insights for the development of intervention strategies, thus moving towards the era of precision medicine<sup>[74]</sup>. For example, the relation between microbiota and efficacy of chemotherapy and immunotherapy has been extensively evaluated in experimental studies across various malignancies<sup>[75-78]</sup>. Analysis of microbiota can be easily conducted by using oral swab or stool and integrated into immunology-MPE research<sup>[79]</sup>.

Routy *et al.*<sup>[80]</sup> observed the negative impact of antibiotics assumption during immunotherapy in terms of ORR, PFS, OS in patients with NSCLC, renal cancer, urothelial cancer. To confirm the hypothesis

**Table 3. Transcriptomic pathways involved and potential treatment strategies for each molecular subtypes**

Molecular subtypes	Transcriptomic pathways	Potential treatment strategies	Stroma-immune microenvironment	Strategies for immunotherapy
CMS1 (14%)	Immune activation JAK-STAT activation	(1) Immune checkpoint inhibition (2) Anti PD-1 + anti CTLA-4/anti-IDO	Highly immunogenic	Immune checkpoint inhibition (anti PD1/PDL-1/ anti-CTLA-4/anti-IDO)
CMS2 (37%)	WNT targets MYC activation EGFR activation VEGF or VEGFR activation Integrins activation TGFβ activation	(1) Pan-RAS + BRAF + PI3K wt: polichemotherapy + anti-EGFR (2) BRAF mutated: BRAF inhibitor + anti-EGFR + MEK-inhibitor (3) HER-2 amplified: anti-HER2 + anti-EGFR	Poorly immunogenic	(1) Combined EGFR pathway inhibition and immune checkpoint inhibition (2) Combined HDAC inhibitors and immune checkpoint inhibition (3) Immuno-chemotherapy
CMS3 (13%)	DNA damage repair Glutaminolysis Lipidogenesis Cell cycle	(4) KRAS or NRAS mutated: polichemotherapy + anti-VEGF	Poorly immunogenic	(1) Combined MEK-inhibitor and immune checkpoint inhibition (2) Combined HDAC inhibitors and immune checkpoint inhibition (3) Immuno-chemotherapy
CMS4 (23%)	Mesenchymal transition Complement activation Immunosuppression	(1) Polichemotherapy + anti-VEGF (2) Chemotherapy + anti-TGFR	Inflamed (immune tollerant)	(1) Combined TGF pathway inhibition and immune checkpoint inhibition (2) Combined anti-VEGF and immune checkpoint inhibitors (3) Anti-T-reg and/or anti-MDSCs treatment

CMS: consensus molecular subtypes; EGFR: epidermal growth factor receptor; JAK: Janus kinase; STAT: signal transducer and activator of transcription; TGFβ: transforming growth factor-β; VEGF: vascular endothelial growth factor; VEGFR: VEGF receptor; PD1: programmed death protein 1; CTLA4: cytotoxic T-lymphocyte-associated protein 4; IDO1: indoleamine-pyrrole 2,3-dioxygenase; HDAC: histone deacetylase; MEK: mitogen-activated protein kinase (MAPK) kinase; MDSCs: myeloid derived suppressor cells

that dysbiosis might affect the therapeutic efficacy of immune check-point inhibitors, they explored the composition of gut microbioma of these patients and observed that *Akkermansia muciniphila* was overrepresented in the faeces of patients who later benefited from PD-1 inhibition.

Furthermore, they observed improving CPIs efficacy and increasing CCR9+CXCR3+CD4+ TILs levels when they transplanted faecal microbiota from cancer patients who responded to immunotherapy into antibiotic-free mice<sup>[80]</sup>.

## MOLECULAR DRIVEN THERAPEUTIC HYPOTESIS

With the CMS classification system, approximately 85% of colorectal cancers could be molecularly classified. The evolution of precision medicine should be based on association of molecular information (mutations, methylation status, gene regulation), biological and clinical characteristics of the tumour [Table 3].

Early-stage patients with CMS1 tumours and in particular MSI tumours (most CMS1 cancers) have good prognosis with low recurrence rate. No adjuvant therapy should be considered for stage II tumours, while for stage III MSI-H CRC it is plausible that the addition of oxaliplatin could overcome the potential detrimental effect of fluoropyrimidine monotherapy<sup>[81]</sup>. For these subgroups of patients with MSI, hypermutated, hypermethylated cancers characterized by strong infiltration of immune cells, the usefulness of immune check-point inhibitors as the main treatment of advanced disease should be considered. Recently Shin *et al.*<sup>[82]</sup> identified acquired mutations in 4 patients treated with pembrolizumab with previous clinical benefit: these mutations caused mistakes in antigen presentation and immune escape of cancer cells. New efforts should

be done in investigating mechanisms of innate or acquired resistance to immunotherapy in these subgroups of tumours.

CMS2 cancers have low mutation rate compared to CMS1 and in most cases no mutations of BRAF and RAS are detected. Typically MYC and WNT pathways are activated so multiple efforts to interact with these signal transduction cascades should be considered<sup>[6]</sup>. For patients with KRAS, NRAS, BRAF and PIK3CA wild-type tumours (almost 30% of all cases), anti-EGFR antibody in combination with chemotherapy should remain the standard of care though retrospective analysis from the main phase III trials suggested differential benefit of anti-EGFR treatment according to primary tumour sidedness<sup>[83,84]</sup>. Anti-EGFR benefit seems to be restricted to patients with distal primary tumor with overexpression of EGFR ligands and amplifications of EGFR and IRS2<sup>[85]</sup>.

CMS3 cancers are characterized by KRAS mutations (almost 68%) and enriched for multiple metabolism signatures including glutamine, fatty acid and lysophospholipid metabolism<sup>[6]</sup>. Most CMS3 cancers don't have an easily identifiable therapeutic target but trials ongoing are evaluating the potential inhibition of these metabolic processes with glycolysis inhibitors such as inhibitors of glycogen synthase kinase or inhibitors of pyruvate dehydrogenase kinases. For tumours with HER-2 overexpression (3%-5% of this group), anti-HER2 and pan-ERBB TKI combination should overcome primary resistance to anti-EGFR treatment. Furthermore, in KRAS mutated cell lines, preclinical trials suggested that combination of pan-RAF and MEK inhibitors may be considered<sup>[47]</sup>.

As mentioned above, CMS2 and CMS3 tumours are so called “cold” or immune-ignorant tumours. Multiple clinical trials are evaluating combinations of chemo-immunotherapy and targeted agents and immunotherapy (anti MEK + anti PDL-1, CEA-CD3 TCB + anti PDL-1, anti-EGFR + anti-PD1) after negative results of previous trials with anti-PD1 and anti-PDL1 as monotherapy. Some interesting suggestions with COX-inhibitors, HDAC inhibitors should be confirmed by future phase III trials.

For CMS4 group, the identification of actionable targets is of major interest considering the worse relapse-free and overall survival. These tumours, characterized by mesenchymal stem-cell features, seems to have no benefit from standard adjuvant therapy with 5-FU and oxaliplatin because of EMT activation<sup>[86]</sup>.

For BRAF mutated tumours, target combination therapies with BRAF-inhibitors and MEK-inhibitors have shown lower clinical benefit than in melanoma<sup>[87]</sup>. In a recent clinical experience by Kopetz *et al.*<sup>[88]</sup>, the addition of anti-EGFR to the previous combination seems to be more effective.

Combinations of TGFR inhibitors and chemotherapy are under evaluation in ongoing clinical trials for tumours with “TGF-B activated” signature. The pro-cancerogenic effect of TGF-B develops through a direct effect on cancer cells but also on immune cells with inhibition of CTLs and NK cells associated with an expansion of Treg cells and MDSCs. In preclinical trials, combination of TGFR-B inhibitor with an OX40 agonistic mAb or with anti-PD1, showed a potential synergistic effect with high tumour-specific IFN $\gamma$  response<sup>[89]</sup>.

Another possible target could be angiogenesis: the subgroup analysis of CALGB 80404 trial confirmed clinical benefit with bevacizumab especially in CMS4 group<sup>[40]</sup>. Furthermore retrospective analysis from Correct trial with Regorafenib (multiple TKIs that targets VEGFR1-2-3) highlighted CMS4 group as the one who benefits the most from this treatment<sup>[90]</sup>.

The efficacy of check-point inhibitors in this subgroup of patients is limited by the intense immunosuppressive response in the tumour microenvironment.

Alternative approaches to enhance the immunotherapy efficacy in this subgroup of patients that are under evaluation, include blockade of immunosuppressive chemokine signalling circuits and pathways or elimination of MDSCs as observed in other neoplastic setting with immunosuppressive microenvironment<sup>[90,91]</sup>.

## CONCLUSIONS AND FUTURE PERSPECTIVES

The development of immunotherapeutic agents has opened the way to a new era in the treatment of many solid tumors, such as renal cell carcinoma, melanoma, bladder or non-small cell lung cancer. However, despite tangible improvements in the prognosis of these malignancies, in most cases acquired resistance finally develops and leads to significant clinical progression and death. Therefore, researchers have focused primarily on the identification of the molecular bases that underlie the development of resistance in patients treated with immunotherapy. Immune checkpoint inhibitors, such as anti-PD1 and anti-PD-L1, are the most utilized among immunotherapeutic agents in the treatment of a broad spectrum of malignancies. In the near future, uncovering the molecular mechanisms responsible for primary and acquired resistance to these agents will certainly be of paramount importance. Firstly, it might allow a more accurate selection of patients who are less suitable candidate to receive immunotherapy, thus leading to a more rational allocation of the economic resources. As of today, basic research has focused primarily on PD-L1 expression as a potential predictive biomarker of response to anti-PD1 and anti-PD-L1, but, in most of the cases, it is far away from being defined a reliable biomarker. Secondly, by identifying the mechanisms underlying acquired resistance, it might be possible to develop potential strategies to overcome them.

In the era of precision medicine, the recent consensus on molecular classification of CRC has paved the way to a more personalized approach in the treatment of this disease, especially in the metastatic setting. In particular, it is now established that patients with CMS1 subtypes CRC (mainly MSI-H) are the best candidate for immunotherapy, with clinical trials demonstrating unprecedented results that lead to regulatory approval of pembrolizumab and nivolumab. Nevertheless, some clinical challenges need to be addressed in the near future in the treatment of MSI-H CRC. Firstly, as mentioned above, we need to understand why some patients are primarily resistant to these drugs and the molecular mechanisms of the development of secondary resistance. Secondly, it might be crucial to explore the role of immunotherapy in other settings, such as in the prevention of CRC, in the conversion therapy of potentially resectable liver metastases, in the adjuvant treatment of early-stage disease or in the neoadjuvant treatment of locally advanced rectal cancer.

However, MSI represents unfortunately an hallmark of a small fraction of patients with metastatic CRC. Therefore, one of the major challenges that researchers need to face in the next few years is to define strategies to convert immune-ignorant tumors (like CMS-2 and CMS-3 subgroups) into inflamed ones and to restore a “CMS-1 like” immune microenvironment in CMS-4 tumors. Many clinical trials are ongoing with new combination therapies. The results of these trials will hopefully help clinicians to abandon the therapeutic paradigm of ‘one size fits all’ and allow a more selective biomarkers-driven approach.

Therefore, now that immunotherapy revolution has begun with a “new kid on the block” in the therapeutic armamentarium of patients with CRC, enrollment in these clinical trials is largely encouraged.

## DECLARATIONS

### Authors' contributions

Conceived and designed the manuscript: Spallanzani A, Gelsomino F, Caputo F, Santini C

Analysed and interpreted the data, drafted the article, revised it critically for important intellectual content and finally approved the version to be submitted: all authors

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**Conflicts of interest**

All authors declare that there are no conflicts of interest.

**Ethical approval and consent to participate**

Not applicable.

**Consent for publication**

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Editorial

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# Bioconjugate structures vs. composite nanoparticulate carriers: the battle for the future of smart, effective and safe cancer management

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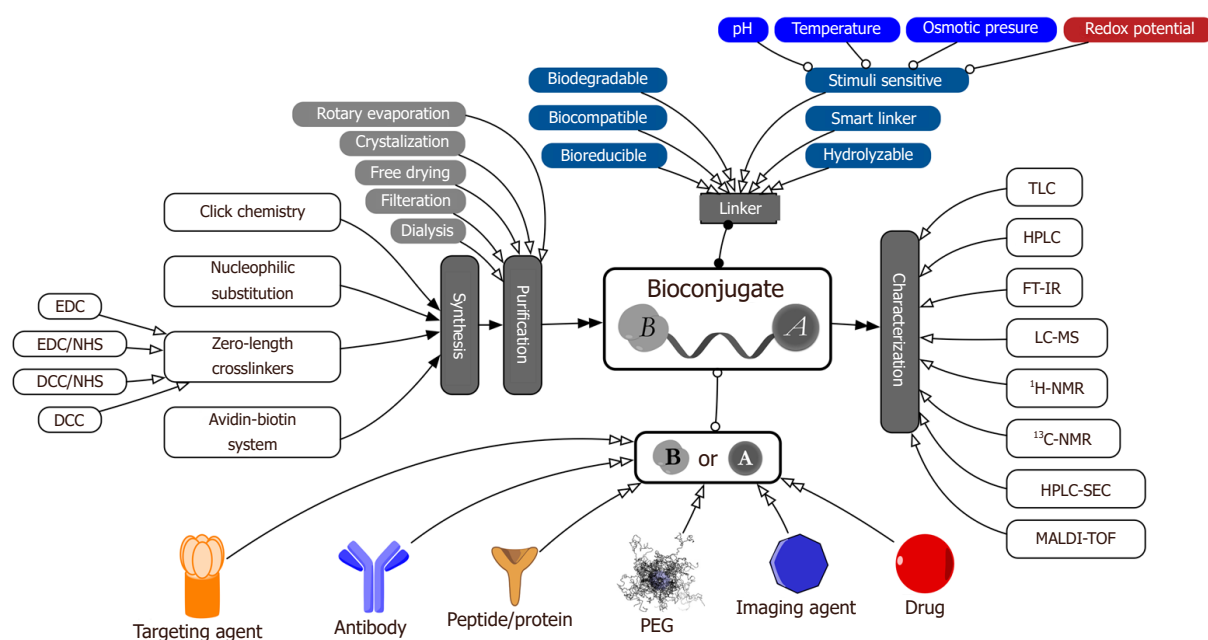
For the past couple of decades, academic research has been mainly focusing on novel carrier systems and nanoparticulate colloidal technologies for drug delivery, such as nanoparticles, nanospheres, vesicular systems, liposomes, nanocapsules, *etc.* Such efforts aided in the creation of newly marketed products such as Doxil® in the market<sup>[1,2]</sup>. Such systems provide the tools to customize a superior drug delivery system, impart novel functions to old drugs such as longer half-life and stealth properties (as in the case of Doxil®), and provide them with either passive or active targeting properties via grafting the carrier system with targeting moieties and/or imaging agents or another drug within the same carrier system<sup>[3]</sup>. Such technologies opened the gate towards more sophisticated and effective multi-acting platform(s) which can offer site-targeting, imaging, and treatment using a single multi-functional system<sup>[4]</sup>. Unfortunately, such technologies are faced with major problems including low stability profile, short shelf-life, and poor reproducibility across and within production batches leading to harsh bench-to-bedside transformation. The commercial scale-up processes of composite nanoparticulate carriers are challenging, time-consuming and costly. Such scale-up processes from the bench to pilot small-scale production, and subsequently to the full-scale process involve significant major pre-formulation and formulation developmental steps along with the design of rugged and robust *in vitro* characterization techniques to ensure safety and efficacy of the final formulation along with quantitative determination of intra/inter-batch variability to comply with pharmacopeial standards and regulations. Additionally, the majority of such novel therapeutic systems' inactive adjuvants and reagents used in the pre-formulation and formulation steps are not yet approved by the FDA and not listed in their approved inactive ingredient database (IID).



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**Figure 1.** Schematic diagram of bioconjugates' structure, design, synthesis, purification and characterization

Initiatives to overcome such setbacks either involved the design and development of novel new chemotherapeutic agents, chemical or physical modifications of currently used chemotherapeutic agents or novel smart bioconjugates<sup>[5]</sup>. Currently, pharmaceutical industry along with academic research is investing heavily in bioconjugate structures. The major purpose of bioconjugation is to create a stable conjugate between two molecules via a covalent link, at least one of which is a biomolecule<sup>[6]</sup>. By design, the covalent linkage should be easily biologically-cleavable to enable the release of the bioactive molecule at the desired target site. The main advantages of bioconjugation and the generated biomolecules include enhanced physical and chemical stability in the active pharmaceutical ingredient (API) journey to the target site, providing better safety and efficacy profiles, delivering enhanced API protection against proteolysis and immune responses and enhancement of the targeting powers of such novel bioconjugates nanoparticulate systems<sup>[6]</sup>.

Bioconjugate technologies offer an appealing and advantageous alternative to nanoparticulate delivery systems with all its flexible benefits when it comes to customized design and tailored grafting along with avoiding most of its shortcomings. Bioconjugates offer the flexibility in customized designing of personalized products. Bioconjugates facilitate simple and easy drug (active pharmaceutical ingredient) conjugation, using various smart biocompatible, bioreducible, or biodegradable linkers, to targeting agents, PEG layer or another drug [Figure 1]. Such technology enables the formation of smart multi-functional platform(s) offered by nanoparticulate carriers and bioconjugates structures. Furthermore, conjugates are still considered chemical compounds. This fact simply allows the use of traditional analytical and manufacturing technologies in the characterization and manufacturing of traditional active pharmaceutical ingredients offering high probability for their successful transition from bench to bedside. Moreover, the final formulation could be a simple injectable or solid formulation, which offers long shelf-life and enhanced stability profile.

Subsequently, bioconjugation technologies can aid in creating safer, cheaper, stable, and effective novel therapeutics. It can also be a rate-limiting step in reinventing old drugs and imparting new functions to them that would enhance their targetability, pharmacokinetic and pharmacodynamic parameters, and their overall formulation patient compliance, easing their transition to market<sup>[7]</sup>. A major focus should be the transformation of such novel bioconjugates' technologies from bench to bedside. The use of click chemistry, bioconjugation technologies, ligand post-insertion and labeling techniques need to be extensively

researched for ease of scale-up and proper bench-to-bedside transformation. Consequently, a current focus is on simple bioconjugate structures, which can be easily synthesized with high yield, reduced cost and high stability profile of the final formulation. This could provide a practical direction for the development of novel management tools and therapeutics, paving the road to affordable, scalable, stable, efficient and safe disease-management strategies.

## **DECLARATIONS**

### **Authors' contributions**

Nounou MI conceived the presented idea, developed the theoretical formalism and fully contributed to the writing of the manuscript.

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Not applicable.

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The author declares that there are no conflicts of interest.

### **Ethical approval and consent to participate**

Not applicable.

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Review

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# Pancreatic cancer: treatment approaches and trends

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## Abstract

Pancreatic cancer is one of the most challenging diseases due to its often late diagnose which results in limited therapeutic options and poor prognosis. To date, the only curative treatment is complete tumor removal surgery but only a few patients are eligible to do it. The median survival period after surgery followed by chemotherapy adjuvant treatment is about 2 years. Since its approval by the FDA, Gemcitabine has become the first-line chemotherapy agent for treatment of advanced pancreatic cancer. The FOLFIRINOX regimen is also used as a treatment scheme for pancreatic cancer; however, this regimen has resulted in small improvements in overall patient's survival. It is appropriated to clarify that the FOLFIRINOX regimen can only be administered in patients with good performance status. Due to the absence of outstanding result after patient's treatment with diverse chemotherapeutic agents combinations or unsuccessful administration of single-agent drugs to treat pancreatic cancer, the immunotherapy has become a new hope. A more comprehensive understanding of cancer microenvironment and the chemical communication between cancer cells and immune cells can result in new therapeutic approaches that will improve the elimination of pancreatic cancer cells, enhancing life quality for these patients and increasing the overall survival.

**Keywords:** Pancreatic cancer, chemotherapy, gemcitabine, immunotherapy

## INTRODUCTION

In recent decades the worldwide incidence of cancer has increased substantially. It has been estimated that 609,640 Americans will die from cancer this year<sup>[1]</sup> and pancreatic cancer is ranked in the fourth position among cancer-related deaths in the United States<sup>[2-5]</sup>. This cancer type is responsible for 331,000 deaths per year<sup>[6]</sup>, and according to GLOBOCAN, 2016 almost 340,000 new cases of pancreatic cancer are diagnosed each year worldwide.



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Pancreatic cancer is more common in elderly persons (between 60 and 80 years) and some studies have shown an increased incidence among diabetes<sup>[7,8]</sup> or chronic pancreatitis patients<sup>[2,9,10]</sup>. Both environmental and inherited factors<sup>[11]</sup> can contribute to the development of this disease and the most common risk factors associated to this type of cancer are smoking<sup>[12,13]</sup> and overweight obesity<sup>[14]</sup>.

The adenocarcinoma is the most common pancreatic cancer, representing 85% of all cases<sup>[15]</sup>. Furthermore, the pancreatic adenocarcinoma remains one of the most challenging malignancies with limited therapeutic options and poor prognosis<sup>[3]</sup> because it is usually diagnosed at an advanced stage<sup>[16]</sup>. This aspect partially can be explained by the fact that early stages of pancreatic cancer often present none or nonspecific symptoms, which can be translated in diagnosis challenges<sup>[12]</sup>.

Normally, advanced pancreatic cancer patients can present symptoms like nausea, vomiting, bloating, unexplained weight loss, jaundice, abdominal pain, dyspepsia and sometimes pancreatitis<sup>[9]</sup>. Moreover, 70% of patients present diabetes mellitus, usually with a diabetes history of less than 2 years<sup>[17]</sup>. The poor prognosis is also attributed to the high incidence of metastasis, leading to an aggressive disease course combined with the limited efficacy of systemic treatments<sup>[5]</sup>.

Surgery procedures are considered the most effective treatment and the only curative intervention but only 20% of patients are fit for it based on disease staging<sup>[4]</sup> and up to 80% of these patients relapse. When compared to other resected solid tumors, the poorest outcomes are observed in patients with resected pancreatic cancer. After surgery, those resected patients are selected for adjuvant therapy with chemoradiation or chemotherapy alone and they present a median survival post-surgery combined with adjuvant therapy averaging 2 years<sup>[14]</sup>, with only 20% of patients reaching 5-year survival rate<sup>[18]</sup>. Regarding that, there are some studies with neoadjuvant chemotherapy administered in patients with resectable, borderline resectable or locally advanced disease aiming to increase resectability by achieving higher margin-negative resections and conversion rates<sup>[19]</sup>.

According to the American Cancer Society, the 5-year relative survival of pancreatic cancer patients is 29% for localized stage at diagnose period, 11% for regional stage and only 3% for distant stage<sup>[20,21]</sup>. These statistical data indicate that there is an increased need for development of efficient and well-tolerated treatment options. This work intends to summarize the approved adjuvant chemotherapy approaches [Table 1] for advanced pancreatic cancer and some immunotherapy treatment trends for this aggressive and devastating disease.

## TREATMENT OPTIONS

Treatment of pancreatic cancer is multimodal, and most patients will receive more than one type. The primary and only curative intervention is surgery. In sequence, it includes adjuvant (treatment given after primary treatment) chemotherapy and/or radiation therapy, or palliative care depending on the stage of cancer, according to the staging system developed by American Joint Committee on Cancer, which is now in the 8th edition. Based on the cancer stage the patient will be directed to a kind of treatment. This staging system takes into account the TNM status which means: T - primary tumor size; N - lymph node involvement; M - distant metastasis [Table 2]<sup>[18]</sup>.

As mentioned, different treatment guidelines are used for each stage. Frequently, stage II (resected lesions) is treated by surgery and adjuvant chemotherapy, sometimes including chemoradiation; Stage III (locally advanced) chemotherapy with or without chemoradiation and stage IV (metastatic) with chemotherapy<sup>[22]</sup>.

## SURGERY

Pancreatic cancer patients are subdivided into four groups: resectable, borderline resectable, locally advanced nonresectable, and metastatic. Cancer that is confined to the pancreas without significant involvement of nearby

**Table 1. Summary of chemotherapy approaches**

Comercial name	Composition	FDA approval	Indication	Survival rate at 12 months	Median progression free survival	Median overall survival
Gemzar	Gemcitabine	1996	Advanced pancreatic cancer	18% compared to 2% 5-FU	-	5.65 months
Abraxane	Paclitaxel albumine-stabilized nanoparticle	2013 in combination with gemcitabine	Metastatic pancreatic cancer	35% compared to 22% of gemcitabine alone	5.5 months	8.5 months
FOLFIRINOX	5-FU, leucovorin, Irinotecan and oxaliplatin	-	Metastatic pancreatic cancer of good performance status patients	48% compared to 20% of gemcitabine	6.4 months	11.1 months
Onyvide	Nanoliposomal irinotecan	2015 in combination with + 5-FU + leucovorin	Gemcitabine resistant Advanced metastatic pancreatic cancer	26% compared to 16% in 5-FU + folinic acid	3.1 months	6.1 months

5-FU: fluorouracil

**Table 2. American Joint Committee on Cancer 8th edition staging system for pancreatic cancer**

Primary tumor (T)	Regional lymph node (N)		Distant metastase (M)
T1 Maximum tumor diameter $\leq$ 2 cm	N0	No regional lymph node metastasis	M0 No distant metastasis
T2 Maximum tumor diameter $>$ 2 cm but $\leq$ 4 cm	N1	Metastasis in 1-3 regional lymph nodes	M1 Distant metastasis
T3 Maximum tumor diameter $>$ 4 cm	N2	Metastasis in $\geq$ 4 regional lymph nodes	
T4 Tumor involves the celiac axis or the superior mesenteric artery (unresectable primary tumor)			
Stage			
Stage 1A	T1	N0	M0
Stage 1B	T2	N0	M0
Stage 2A	T3	N0	M0
Stage 2B	T1-3	N1	M0
Stage 3	Any T	N2	M0
	T4	Any N	
Stage 4	Any T	Any N	M1

blood vessels is called resectable. Cancer that is confined to the pancreas but involves nearby blood vessels or structures to a greater extent is called borderline resectable<sup>[23]</sup>. Cancer that involves nearby blood vessels or other structures to such a significant extent that it cannot be successfully removed by surgery is called locally advanced nonresectable<sup>[24]</sup>. Cancer that has spread outside the pancreas to other organs and tissues in the body is called metastatic. Patients with metastatic disease are not indicated to have surgical resection<sup>[25]</sup>.

All patients must undergo preoperative exams such as contrast-enhanced abdominal computed tomography or magnetic resonance imaging with cholangiopancreatography so the surgeons can decide what kind of procedure to apply on each patient.

For those patients that are possible to undergo resection there are three types of surgery: Whipple procedure, distal pancreatectomy, and total pancreatectomy. Conventional Whipple operation or pylorus preserving, also known as pancreaticoduodenectomy, with lymphadenectomy is the choice for head or neck pancreatic cancers. Distal pancreatectomy with splenectomy is the choice for body/tail cancer. The Whipple procedure removes the head of the pancreas, the gallbladder, duodenum, part of the bile duct, and often part of the stomach. It also removes the nearest lymph nodes to biopsy. The distal pancreatectomy removes the body and tail of the pancreas, some nearby lymph nodes, and sometimes the spleen and its blood vessels. The total pancreatectomy removes the gallbladder, duodenum, part of the bile duct and stomach, nearby lymph nodes, and sometimes the spleen<sup>[26-28]</sup>. The prognosis for patients that go through resection depends on



margin status. The one associated with the best outcomes is a R0 resection which means a total gross excision and negative histological margins; R1 resection is a total gross excision however with positive histological margins; and, R2 is a resection with residual gross tumor and patients that undergo R2 resection have similar prognosis of the unresectable patients treated with non-operative therapy, on account of that, surgeries that will result in R2 margins should not be consider as resectable<sup>[23,29]</sup>.

To improve survival for locally advanced patients neoadjuvant therapy has been evaluated aiming to shrink tumor, enhance resectability and also to increase rates of microscopic complete tumor resection<sup>[30]</sup>.

## CHEMOTHERAPY GEMZAR - GEMCITABINE

Gemcitabine is a deoxycytidine (dCTP) analogue, which is converted by nucleoside kinases into two metabolites diphosphate (dFdCDP) and triphosphate (dFdCTP). Each of these metabolites have a specific mechanism of action: (1) the diphosphate metabolite (dFdCDP) inhibits ribonuclease reductase, an enzyme known for catalyzing the reaction that generates ribonucleotides necessary for DNA synthesis; (2) the triphosphate metabolite (dFdCTP) competes with the natural dCTP for its incorporation into DNA newly synthesized strands. Once dFdCTP is incorporated, only one additional nucleotide is added to the growing DNA strands, which stops the DNA synthesis and eventually results in activation of apoptosis pathway leading the cells to death<sup>[31]</sup>.

Gemcitabine, as single-agent, became the first line treatment (1996) for advanced pancreatic cancer since a randomized trial showing that 23.8% of patients had experienced a clinical benefit response compared with 4.8% of patients treated with fluorouracil (5-FU). Gemcitabine also confers a modest improvement in overall survival than those observed in patients group treated with 5-FU. The patients' overall survival rates at 12 months were 18% for gemcitabine and 2% for patients treated with 5-FU<sup>[32]</sup>.

In the following decade, gemcitabine has become the backbone of combination regimen for new experimental approaches with either other cytotoxic molecules or novel chemotherapy agents<sup>[33]</sup>. Many phase II trials have demonstrated the efficacy of gemcitabine-based combinations, which comprise other cytotoxic molecules such as capecitabine, 5-FU, cisplatin, irinotecan<sup>[34-37]</sup> or the targeted agents sorafenib and cetuximab<sup>[38-40]</sup>. However, in some randomized phase III trials of gemcitabine based chemotherapy combinations, these combinations failed to show statistically significant improvement in patient's overall survival when compared to gemcitabine used as a single-agent<sup>[41-46]</sup>.

Nowadays, gemcitabine is used in combination with taxol, a paclitaxel albumin-stabilized nanoparticle formulation (nab-paclitaxel) that is commercially known as abraxane. Taxol is a microtubule dynamics inhibitor that promotes the stabilization of microtubules by preventing the catastrophe process, which induces cell cycle arrest at the G2/M phase resulting in cell death<sup>[44]</sup>. In preclinical studies, nab-paclitaxel improved the intratumoral concentration of gemcitabine. The FDA approval for this approach was obtained after a phase III study that demonstrated the efficacy and safety of this combination compared to monotherapy with gemcitabine in patients with metastatic pancreatic cancer. Von Hoff *et al.*<sup>[47]</sup>, randomized assigned 861 patients: 431 received nab-paclitaxel plus gemcitabine and 430 gemcitabine alone. The median overall survival was 1.8 months superior in the combination group, and the survival rate was 35% in the nab-paclitaxel-gemcitabine group compared to 22% in the gemcitabine group in 1 year. Moreover, this combination approach increased the median progression-free survival in 1.8 months. However, despite those benefits rates, peripheral neuropathy and myelosuppression were increased in the group that received nab-paclitaxel-gemcitabine combination<sup>[47]</sup>. De vita *et al.*<sup>[48]</sup> also confirmed the effectiveness in overall survival and progression free survival from patients treated with the combination of gemcitabine plus nabpaclitaxel.

Although not yet approved by the FDA as a treatment approach for pancreatic cancer, the ESPAC-4 study developed a phase III randomized trial that could establish the gemcitabine plus capecitabine combination

as the treatment of choice for adjuvant setting after resection<sup>[49]</sup>. In this study, they aimed to demonstrate the safety and efficacy of the combination for resected pancreatic cancer since a phase III randomized comparison between gemcitabine plus capecitabine and gemcitabine alone showed a significant improvement in objective response rate ( $P = 0.03$ ) and progression-free survival ( $P = 0.004$ ) and was associated with a trend toward improved overall survival ( $P = 0.08$ ) in patients with advanced pancreatic cancer that underwent the combination approach<sup>[50]</sup>. The capecitabine is an oral prodrug of 5-FU, a fluoropyrimidine carbamate, that provides prolonged fluorouracil tumor exposure at lower peak concentration. The conversion of capecitabine in the active drug needs an enzyme named thymidine phosphorylase which is present at higher levels in tumor cells compared to other tissues which improves tolerability and intratumor drug concentration<sup>[51]</sup>.

### **FOLFIRINOX REGIMEN - FLUOROURACIL, LEUCOVORIN, IRINOTECAN AND OXALIPLATIN**

5-FU is a fluoropyrimidine antimetabolite drug that exerts antitumoral effects inhibiting the enzyme thymidylate synthase, impairing the synthesis of the pyrimidine thymine, which is required for genetic material synthesis. The fluoronucleotides are misincorporated into RNA and DNA strands resulting in cell death<sup>[52]</sup>. Leucovorin is a metabolite of folinic acid, known as 5-formyltetrahydrofolic acid, which is the 5-formyl derivative of tetrahydrofolic acid<sup>[53]</sup>. Leucovorin is indicated for use as rescue therapy to reduce the toxicity associated of folinic acid antagonists that inhibits *de novo* synthesis of purines, pyrimidines and methionine. The combination of leucovorin and 5-FU can extend the survival in the palliative treatment of patients with advanced pancreatic cancer<sup>[54,55]</sup>. Irinotecan is a derivative of camptothecin that has a cytotoxic action via a potent and specific inhibition of DNA topoisomerase I, preventing the DNA strand ligation leading to double-strand DNA breakage and cell death<sup>[56]</sup>. Oxaliplatin is a platinum-based drug that belongs to the same family of cisplatin and carboplatin. In oxaliplatin the two amine groups were replaced by cyclohexyldiamine, which increases its antitumor effect. The chlorine ligands were replaced by the oxalato bidentate derived from oxalic acid that improves its water solubility<sup>[57,58]</sup>. Oxaliplatin is converted to active derivatives via displacement of the labile oxalate ligand. Its reactive species monoquo and diaquo daminocyclohexane platinum binds guanine and cytosine moieties of DNA and this association produces cross-linking of DNA inhibiting the DNA synthesis and transcription<sup>[59]</sup>.

A phase 1 study involving patients with advanced solid tumor was developed to determine the maximum-tolerated dose and the recommended dose of the triple combination (oxaliplatin, irinotecan, leucovorin/5-FU). A fair response in patients with advanced pancreatic cancer utilizing this combining regimen was observed<sup>[60]</sup>. Then, a phase 2 study of FOLFIRINOX regimen was conducted involving 46 advanced pancreatic cancer patients with good performance status. FOLFIRINOX showed a high efficacy against this malignant tumor, but it has produced severe neutropenia in half of the patients. It was prompted started the phase 2-3 trial in order to compare FOLFIRINOX regimen with gemcitabine as single antitumoral agent. In this trial, 342 patients were randomly assigned. The median overall survival and the median progression-free survival were significantly extended for the FOLFIRINOX regimen group (48% of patients submitted to FOLFIRINOX regimen were alive after 1 year compared to 20% treated with gemcitabine). Due to its high toxicity, the group treated with FOLFIRINOX showed more intense side effects such as grade 3 or 4 neutropenia, thrombocytopenia and grade 2 alopecia. However, despite the higher incidence of intense side effects, the FOLFIRINOX treated group showed a significant increase of time period that precedes the definitive deterioration of the quality of life compared to gemcitabine group. These results lead to the conclusion that FOLFIRINOX is an effective therapeutic option but only suitable for patients with metastatic pancreatic cancer that hold a good performance status<sup>[61]</sup>.

After the effectiveness of FOLFIRINOX regimen in the palliative setting has been established, Faris *et al.*<sup>[62]</sup> had performed a retrospective study in the Massachusetts General Hospital Cancer Center to answer two questions that remained unclear: will the benefit in response rate and overall survival in the metastatic setting translate to patients with locally advanced pancreatic cancer? And are curative-intent resections

possible in patients who respond to this treatment? They found that FOLFIRINOX regimen have substantial activity in locally advanced pancreatic cancer patients and also, that the use of FOLFIRINOX regimen could induce cancer conversion to resectability in more than 20% of patients. From those patients that could resect the cancer, 3 from 5 had recurrence and 1/3 of patients had experienced significant toxicity signals that required visits to emergency department or hospitalization. The most prevalent effects were anemia grade 1 or 2, thrombocytopenia (mostly grade 1), neutropenia, diarrhea/dehydration. Due to high toxicity of FOLFIRINOX regimen, further studies were suggested to reach an optimized treatment to patients with locally advanced pancreatic cancer.

In the other hand, FOLFIRINOX has been studied as neoadjuvant option for locally advanced and borderline resectable patients<sup>[63-65]</sup>. The neoadjuvant therapy can benefit by converting a few locally advanced tumors into resectable ones and increase R0 resectability in borderline tumors<sup>[66,67]</sup>. The FOLFIRINOX combination regime was associated with an increase in R0 resection rates when administered with or without radiotherapy before surgery in borderline resectable and locally advanced patients. The most important result is the down staging of the disease in locally advanced, thus making it possible for patients to undergo surgery and increasing the median progression free survival<sup>[19,68,69]</sup>. However, phase III studies should be prompted to confirm whether preoperative neoadjuvant vs. postoperative adjuvant treatment relates to better survival for those patients that can undergo surgery<sup>[70]</sup>.

### **ONYVIDE - NANOLIPOSOMAL IRINOTECAN, 5-FU AND FOLINIC ACID**

Nanoliposomal irinotecan has potential antineoplastic activity; its liposome encapsulation promotes better delivery of drugs into the cytosol from the endosome compartment of the cell. This encapsulation platform of drug delivery reduces the premature systemic drug release but maintains its intra tumoral release, enhancing antitumor activity<sup>[71]</sup>.

On October 22, 2015, the U.S. FDA has approved the onivyde (irinotecan liposome injection) in combination with 5-FU and leucovorin to treat patients with advanced metastatic pancreatic cancer who have been previously treated with gemcitabine-based chemotherapy. The approval was due to a phase III study, conducted after preceding trials showing promising activity of the nanoliposomal irinotecan in patients with metastatic pancreatic ductal adenocarcinoma previously treated with gemcitabine<sup>[72]</sup>.

In the phase III trial, nanoliposomal irinotecan was tested alone or in combination with 5-FU and folinic acid, compared with a common control (5-FU and folinic acid) in patients with metastatic pancreatic cancer progression after a regimen of gemcitabine. It was a global, randomized, open-label trial in 14 countries. Their results showed that nanoliposomal irinotecan plus 5-FU and folinic acid significantly improved the overall survival. Also, the results related with progression-free survival, objective tumor response, time to treatment failure and CA19-9 tumor marker response for those patients were significantly improved in contrast to the 5-FU and folinic acid control group. Neutropenia, fatigue, diarrhea and regurgitating were the main side effects observed in patients group (14.5%, 13.7%, 12.8%, 11.1% respectively) submitted to treatment with the combination of nanoliposomal irinotecan with 5-FU and folinic acid. With a manageable safety profile, this approach represents a new treatment option for many patients with metastatic pancreatic cancer that previously received an unsuccessful gemcitabine therapy<sup>[73]</sup>.

There is an ongoing trial, randomized, open-label, phase II study of onivyde vs. nab-paclitaxel + gemcitabine in patients with metastatic pancreatic adenocarcinoma (NCT02551991)<sup>[74]</sup>.

### **IMMUNOTHERAPY**

Despite all chemotherapy combinations and new trials with targeted therapies, overall survival of advanced pancreatic cancer patients remains poor. The establishments of new therapies that provide long-term

benefit are urgently needed. The spotlights are now on new immunotherapy approaches, since it is an unexplored and growing landscape and has been applied successfully in other types of cancer. There are many evidences showing that pancreatic cancer generates antitumor immune responses, suggesting that immunotherapies can be a promising alternative for those patients<sup>[75]</sup>. As already known, pancreatic cancer creates an immunosuppressive tumor microenvironment with mucin overexpression. To overcome this immunosuppressive microenvironment, Banerjee *et al.*<sup>[76]</sup> have developed a nanovaccine using recombinant fragments of MUC4, a highly expressed mucin which contributes to cancer aggressiveness, and immunized KPC mice. When compared to control group, the immunized mice exhibited a slower tumor growth kinetics and a greater accumulation of CD8+ and CD4+ T cells. The suppression of tumor progression caused by the immunization points the MUC4 nanovaccine to be a potential immunotherapy for pancreatic cancer.

Another potential immunotherapy approach resulted from the study in which they administered AMD3100 (plerixafor) in KPC mice. AMD3100 is an inhibitor of chemokine receptor CXCR4, a CXCL12 receptor. The inhibition of CXCR4 by the AMD3100 contributes to a fast T cell accumulation in regions of the tumor and acted together with the immunological checkpoint antagonist,  $\alpha$ -programmed cell death 1 ligand 1, to reduce cancer cells<sup>[77]</sup>.

Five main categories for immunotherapy applied to pancreatic cancer have been described<sup>[78]</sup>: (1) checkpoint inhibitors/immune modulators. This strategy aims to modulate immune system through inhibitory or stimulatory signals, such as inhibition of CD28 family receptors, which controls T cell responses, modulating the immune cytotoxic response, restoring or increasing the cytotoxic antitumor activities of T cell<sup>[79]</sup>; (2) therapeutic vaccines. In these cases, occurs a patient's active immunization with tumor specific antigen. This vaccine will trigger T cells and increase its activity against the tumor<sup>[80]</sup>; (3) adoptive T cell transfer. An adoptive T cell transfer is a kind of transfusion therapy that infuses mature T CD8+ specific cells in patients. These cells target surface proteins in tumor tissue, which are used to T CD8+ cells docking and eliminate cancer cells through granzyme and perforin secretion<sup>[81]</sup>; (4) monoclonal antibodies. This approach is a passive immunization using antibodies against the same cancer molecule epitope, created to target specific tumor antigens, which enhance the cancer cells recognition by phagocytes and T CD8+ cells improving its elimination; (5) cytokines use. The cytokines such as IL-10 and IL-17B are used to regulate tumor microenvironment, aiming to suppress the cancer cells property to express immunosuppressive cytokines that stop the immune activation against the cancer cells<sup>[78]</sup>.

Even though many encouraging results have been obtained for other types of cancer<sup>[82-84]</sup>, none of these treatments showed significant efficiency when applied as pancreatic cancer therapy<sup>[85,86]</sup>. Currently, although there are many ongoing trials for immunotherapy, therapeutic vaccines are the most cutting-edge clinical therapy applied as pancreatic cancer immunotherapy. Concerning to vaccines as immunotherapy category, the most advanced studies to date are those conducted with whole-cell vaccines and granulocyte-macrophage colony-stimulating factor (GM-CSF) vaccines.

### **THERAPEUTIC VACCINE IMMUNOTHERAPY WHOLE-CELL VACCINES**

Algenpantucel - L is an irradiated, live combination of two human allogeneic pancreatic cell lines that express the murine enzyme  $\alpha$ -1,3-galactosyl transferase. This enzyme performs the addition of  $\alpha$ -galactosyl epitopes on surface proteins and glycolipids of such cell lines. The human cells do not express murine  $\alpha$ -gal epitopes and these cells inoculation induce a hyperacute rejection of the vaccine pancreatic allograft cell. The hyperacute rejection results in the fast activation of antibody-dependent cell-mediate cytotoxicity. These processes will also stimulate the host immune system to eliminate endogenous pancreatic cancer cells<sup>[78,87]</sup>. Hardacre *et al.*<sup>[88]</sup> in 2013 performed a multi-institutional, open-label phase II trial to evaluate the use of algenpantucel-L in addition to standard adjuvant chemotherapy and chemoradiotherapy setting for resected pancreatic cancer patients (NCT00569387). In this study 70 patients were treated with gemcitabine

**Table 3. Therapeutic vaccines immunotherapy summary**

	Clinical trial	Biological	Intervention	Phase	Patient
Whole cell vaccines	NCT01072981 NCT01303172	Algenpantucel-L IMM-101	+ Gemcitabine + Gemcitabine	III ongoing II completed	Resected pancreatic cancer Advanced pancreatic cancer
GM-CSF vaccines		GVAX + CR207	Cy/GVAX + CRS207	II completed	Metastatic pancreatic cancer

GM-CSF: granulocyte-macrophage colony-stimulating factor; Cy: cyclophosphamide

and 5-FU based chemoradiotherapy as well as algenpantucel-L. The median follow-up was 21 months, and the one-year progression-free survival was 62% added to an 86% overall survival. Inoculation site pain and local tissue induration were the common side events; however, the allogenic cells administration was safe, and it proves to be a feasible combined approach. The results obtained from this phase II trial demonstrated that this immunotherapy component may improve survival, and due to such optimistical results a multi-institutional phase III study is ongoing (NCT01072981).

Another randomized phase II trial explored the safety and tolerability of an injectable immunomodulator from heat-killed mycobacterium obuense (IMM-101) used in combination with gemcitabine. This study showed that the administration of IMM-101 plus gemcitabine was safe and well tolerated as gemcitabine alone in patients with advanced pancreatic cancer, moreover the results from this phase II trial suggested a beneficial effect on overall survival which may support further evaluation of IMM-101 in a confirmatory study<sup>[89]</sup>.

### GM-CSF VACCINES

A recent phase II randomized multicenter study was conducted comparing cyclophosphamide (Cy)/GVAX followed by CRS-207 with Cy/GVAX alone in patients with metastatic pancreatic cancer. Cy/GVAX is composed of two irradiated GM-CSF-secreting allogeneic pancreatic cancer cell lines administered with low-dose of Cy to hinder regulatory T cells. GVAX induces T CD8+ cells activity against a tumor associated antigen named mesothelin that is over expressed in most pancreatic cancer cells. CRS-207 is a live-attenuated *Listeria monocytogene*-gene expressing mesothelin that induces innate and adaptative immunity response. The overall survival for the Cy-GVAX followed by CRS-207 was 6.1 months compared to 3.9 months of Cy-GVAX alone. Stable disease rate of 31% and 1-year survival rate of 24% are encouraging results. Furthermore, heterologous boost with Cy-GVAX and CRS-207 extended overall survival for pancreatic cancer patients with minimal related toxicities<sup>[90]</sup> [Table 3].

Worldwide efforts should be directed to identification and selection of specific antigens in order to induce immune response against pancreatic cancer cells aiming to eliminate the immunosuppressive microenvironment that this cancer produces. Appropriate selection of target antigens and combination of treatment protocols are critical to enhance treatment efficacy, lowering related toxicities and as already demonstrated improving the overall survival<sup>[91]</sup>.

Regardless of the advances in pancreatic tumor biology knowledge, mechanisms associated with the tumor microenvironment remain poorly understood, highlighting that the distinct composition of pancreatic tumor microenvironment could be a great barrier for immunotherapy success<sup>[92]</sup>. As a consequence of newly emerging information about tumor microenvironment, there was a shift in the cancer development concept from a tumor cell-centered view to a complex tumor ecosystem, which led to the acceptance that cancer cells interact with the extracellular matrix (ECM) and stromal cells<sup>[93,94]</sup>. A major component of the extracellular matrix is hyaluronic acid (HA), a hydrophilic glycosaminoglycan that is produced in bulk by many pancreatic cancer. Accumulation of HA in tumors is associated with malignancy and poor prognosis, because HA polymers bind and trap water molecules in the ECM as a fluid gel that increases interstitial fluid pressure and creates a physical barrier that restricts antibody and immune cells access the tumor. A pegylated recombinant human hyaluronidase (PEGPH20) is an agent that degrades the hyaluronic acid and normalizes interstitial



fluid pressure and has been applied to enhance the delivery of cytotoxic drugs<sup>[95]</sup>. Hingorani *et al.*<sup>[96]</sup> showed the results from a phase II comparison study between PEGPH20 [plus nab-paclitaxel/gemcitabine (AG)] (PAG) vs. AG in patients with untreated metastatic pancreatic ductal adenocarcinoma (NCT01839487). Because of an imbalance in thromboembolic events in PAG patients 40% patients were excluded from the study. In order to conclude this trial, the enoxaparin prophylaxis was applied in both arms and the phase II study comparison was successful. This randomized phase II met both primary endpoints (progression-free survival and thromboembolic event rate), with the greater improvement in the secondary endpoint which is the progression-free survival in HA-high patients. In the subset of 80 patients whose tumors had HA-high levels, the addition of PEGPH20 to chemotherapy resulted in an increase of 4 months of stable clinic conditions before disease progression when compared to chemotherapy alone. The results of the phase II trial suggested that HA has a potential predictive biomarker for patient's selection of PEGPH20, qualifying only patients with high levels of HA for the new phase III trial. The ongoing phase III trial (NCT02715804) intends to determine whether PEGPH20 actually increases patients' overall survival and not just their time to disease progression.

## RADIOTHERAPY

The effectiveness of radiotherapy has been continuously debated<sup>[97-99]</sup>. Recent studies have shown that the addition of radiotherapy to chemotherapy in the setting of locally advanced pancreatic cancer did not improve overall survival outcome<sup>[100,101]</sup>. A recent randomized phase III trial, LAP07 (NCT00634725) compared chemoradiotherapy in patients with locally advanced pancreatic cancer controlled after 4 months of gemcitabine-based chemotherapy with chemotherapy alone. No significant difference in overall survival was found. However, an increase in progression-free survival resulted in a longer period without treatment confirming association of chemoradiotherapy with decreased local progression<sup>[102]</sup>. Other studies have proposed that chemotherapy administered before simultaneous chemoradiotherapy could enhance survival<sup>[103,104]</sup>. Therefore, the benefits of radiation therapy in the management of locally advanced pancreatic cancer remain controversial.

## CONCLUSION

Although some studies had demonstrated a mild increase in survival rates, there are no available treatments to pancreatic cancer that are focused on preserving the patients' quality of life.

Considering this deadly disease, it is time to take into account the balance between overall survival and patient's life quality. Pancreatic cancer patients desperately need more specific drugs or drugs combinations capable of eliminating cancer cells without producing so many toxic effects. The real cost for one or two more months of life, is living in pain with severe diarrhea, vomits, neutropenia and immune deficiency.

The lack of an efficient therapy against pancreatic cancer has turned the spotlights to immunotherapy. Despite of many disappointments in several clinical trials, immunotherapy has become an established modality for treatment of other cancer types such as melanoma, breast and lung cancer. Clinical trials testing anticancer vaccines showed promising results to treat pancreatic cancer, however most of them have failed to demonstrate a significant efficacy in improving patient's overall survival and quality of life.

As already discussed, a more comprehensive understanding of cancer microenvironment and the chemical communication between cancer cells and immune cells can result in new molecules targets and pathways, which could be used to increase the immune responses against tumoral cells. These hypothetical targets may ultimately lead, alone or combined with a proper chemotherapy scheme, to a massive cancer cells elimination, improving quality of life and significantly extending overall survive of patients.

## DECLARATIONS

### Authors' contributions

Searched bibliographic references data: Pereira NP, Corrêa JR

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Both authors declare no conflicts of interest in association with this study.

### Ethical approval and consent to participate

Not applicable.

### Consent for publication

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Review

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# Update on targeted therapy and immune therapy for gastric cancer, 2018

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## Abstract

Gastric cancer (GC) remains one of the most common cancers and serious health problems worldwide. For unresectable or metastatic advanced gastric cancer, chemotherapy treatment is first selected. Although chemotherapy has improved survival in patients with advanced gastric cancer (AGC), the prognosis of these patients remains poor. In recent years, some therapies targeting biological molecules have been reported to prolong the survival of patients with AGC. Since trastuzumab, a monoclonal antibody that targets HER2, was established as standard therapy for unresectable GC in a HER2-positive patient, many other targets have been reported as new therapy targets. Many molecular targeted therapies, such as HER2, VEGFR or EGFR, have been verified as established standard treatments with or without chemotherapy in clinical trials. Furthermore, immunotherapy is expected to be an effective treatment with promising clinical trial data. Especially, immune checkpoint inhibitors, such as PD-1/PD-L1 or CTLA-4, have demonstrated innovative progression in GC therapy. Moreover, ongoing clinical trials including targeted therapy and immunotherapy have shown promising results in improving clinical outcomes, safety, and tolerability. In this article, we review targeting therapies and immunotherapies for GC and summarize future prospective treatments.

**Keywords:** Gastric cancer, targeted therapy, immunotherapy, immune checkpoint inhibitor

## INTRODUCTION

Gastric cancer (GC) is one of the most common cancers and the third-leading cause of cancer-associated deaths worldwide, especially in East Asia. Almost one million new cases (952,000 cases in 2012) have been estimated to occur annually<sup>[1]</sup>. In early GC, radical surgery with or without perioperative chemotherapy



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is the most recommended curative choice. For unresectable or metastatic advanced gastric cancer (AGC), chemotherapy treatment is first selected.

Conventionally, cytotoxic agents, such as 5-fluorouracil (5-FU), platinum agent, irinotecan, taxanes, and anthracyclines, are used for AGC. Among these, recommended as the first-line treatment, is a combination of 5-FU and platinum-based chemotherapy with or without docetaxel. This treatment results in a median overall survival (OS) of 10-15 months and a median progression-free survival (PFS) of 5-6 months<sup>[2]</sup>. For second-line treatment or after treatment, docetaxel, irinotecan, and paclitaxel are known to improve prognosis compared with best supportive care (BSC)<sup>[3]</sup>. Although chemotherapy has improved survival in patients with AGC, the prognosis of these patients remains poor.

However, some therapies targeting biological molecules have been reported to prolong the OS of patients with AGC. Trastuzumab, a monoclonal antibody for human epidermal growth factor receptor2 (HER2), has already been established with chemotherapy as first-line treatment for HER2-positive AGC patients<sup>[4]</sup>. In addition, ramucirumab, an anti-vascular endothelial growth factor receptor 2 (VEGFR2) antibody has also proven to be efficient for second-line treatment<sup>[5,6]</sup>. Therefore, this targeted therapy field is indeed currently evolving.

Recently, immunotherapy has also been expected to be an innovative therapy for several types of cancer. Cancer immunotherapy can reverse tumor immune escape associated with suppression of the immune checkpoint pathway. Immunotherapies targeting programmed death 1 (PD-1)/programmed death-ligand 1 (PD-L1) checkpoints have been identified to be an important scientific breakthrough and have already been approved in treatment of many types of cancer including melanoma, non-small cell lung cancer (NSCLC) and renal cell carcinoma. In addition, immunotherapy has begun to be approved for last-line treatment in GC patients based on the latest clinical trial data<sup>[7]</sup>. Immunotherapy has important clinical application with favorable outcomes, limitations, and acceptable adverse events, with it having been applied in many past and undergoing clinical trials.

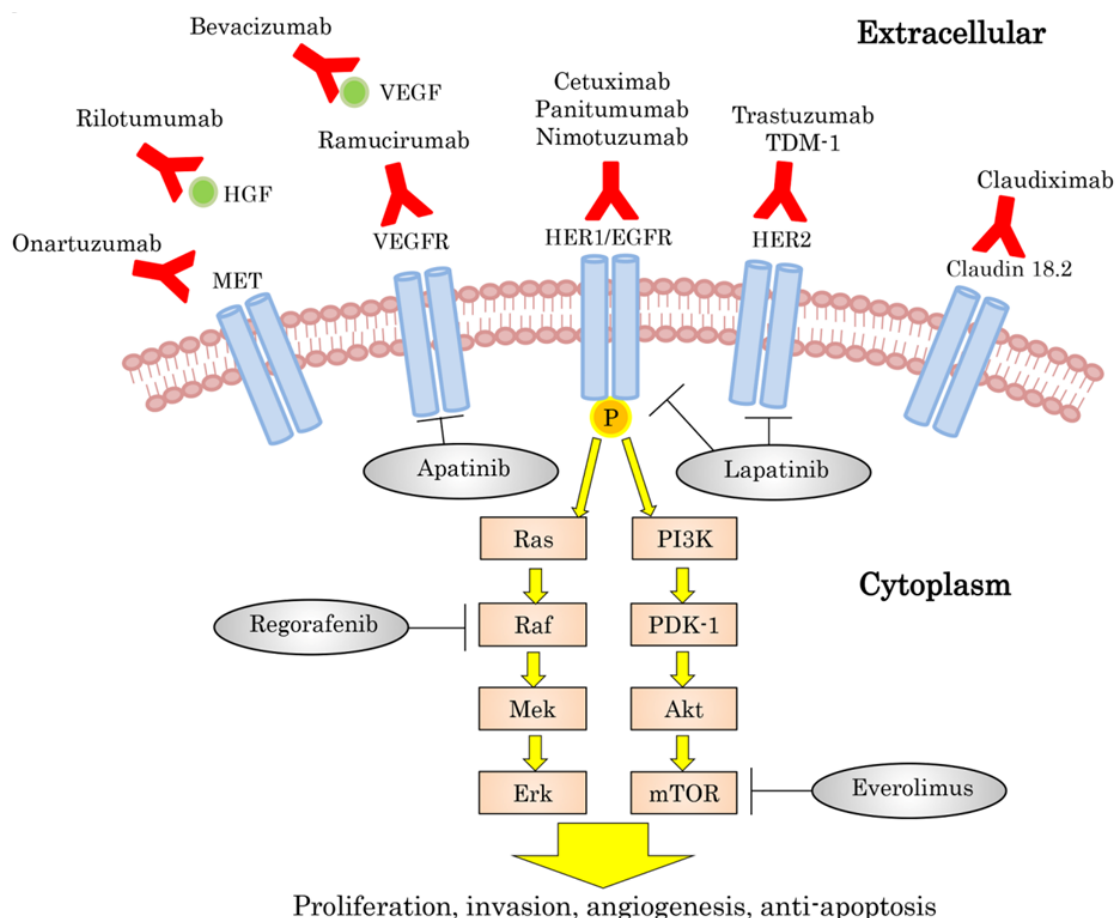
In this article, the latest knowledge of focused on common cancer targets, signaling pathways, targeting therapies, and immunotherapies for AGC are reviewed and future prospects for AGC treatment are described.

## TARGETED CHEMOTHERAPY

Since trastuzumab, a monoclonal antibody that targets HER2, was established as standard therapy for unresectable GC in a HER2-positive patient<sup>[4]</sup>, many other targets have been reported as new therapy targets. Furthermore, phase III trials that target HER2, epidermal growth factor receptor (EGFR), vascular endothelial growth factor (VEGF)/vascular endothelial growth factor receptor (VEGFR), MET, or the mechanistic target of rapamycin (mTOR) have been examined with new findings [Figure 1 and Table 1]. We introduce the current knowledge of targeting therapies for GC.

### Anti-HER2 monoclonal antibodies

HER2 is a proto-oncogene encoded by ErbB2 on chromosome 17. Trastuzumab was the first HER2-targeted drug to be developed and introduced for the treatment of HER2-positive metastatic breast cancer. Trastuzumab induces antibody-dependent cytotoxicity which causes the downregulation of cell cycle disorders. The ToGA trial, comprising randomized controlled trials recruiting patients with histology confirmed, inoperable, locally advanced, recurrent, or metastatic adenocarcinoma, was the first randomized phase III trial to show trastuzumab plus chemotherapy<sup>[4]</sup>. The result of this trial with trastuzumab plus chemotherapy is superior to chemotherapy alone for HER2-positive advanced or metastatic gastric cancer with regard to OS and DFS. Furthermore, up to 22.1% of patients were HER2-positive [Immunohistochemistry (IHC)2+/ fluorescence *in situ* hybridization (FISH)+ or IHC3+] in this trial and especially, the OS of the HER2 high-expression group was 16.0 months. This result precisely demonstrated the efficiency of trastuzumab for AGC.



**Figure 1.** Targeted therapy and oncogenic pathways in gastric cancer. EGFR: epidermal growth factor receptor; HER: human epidermal growth factor receptor; HGF: hepatocyte growth factor; mTOR: mammalian target of rapamycin; VEGF: vascular endothelial growth factor; VEGFR: vascular endothelial growth factor receptor

One novel antibody drug targeting HER2, trastuzumab-emtansine (TDM-1), was confirmed to be significantly effective in breast cancer. However, a phase III study for HER2-positive GC patients added with TDM-1 could not prolong OS and PFS during second-line treatment (GATSBY study)<sup>[8]</sup>. The reasons for this include the heterogeneity of HER2 expression in GC or the changed pattern of HER2 expression by first-line chemotherapy. The detailed analyses of those results are yet to be revealed.

### Tyrosine kinase inhibitors of EGFR/HER-2

Lapatinib, which is bound to the intracellular tyrosine kinase domains of epidermal growth factor receptor (ErbB1) and HER2 (ErbB2), blocks autophosphorylation and downstream signaling. In a phase III trial, for first-line treatment aimed at patients with HER2-positive GC, lapatinib with capecitabine + oxaliplatin (CapeOx) showed no significant difference in OS compared with placebo + CapeOx (LOGiC study)<sup>[9]</sup>. In the lapatinib arm, toxicities were increased, especially diarrhea. The effect of lapatinib was reportedly also dependent on region and age. Although the efficiency of lapatinib plus paclitaxel has also been evaluated in second-line treatment for a phase III trial, no increase in OS and PFS was observed (TyTAN trial)<sup>[10]</sup>. However, in patients with HER2-positive tumors or in China, clinical benefits have been shown. Therefore, the correlations in each condition need to be examined.

### Anti-EGFR monoclonal antibodies

Epidermal growth factor (EGF) is a protein that promotes cell proliferation, growth and differentiation by binding to EGFR<sup>[11]</sup>. Furthermore, EGFR is a transmembrane protein that activates by binding of ligands,

**Table 1. Phase II and phase III clinical trials of targeting therapy**

Target	Trial/ registry No./ authors	Regimen	Phase	Line	No. of patients	Median OS (months)		Median PFS (months)	
HER2	ToGA	FP/XP FP/XP + trastuzumab	3	1st	594	11.1 13.8	HR = 0.74 95%CI: 0.60-0.91 P = 0.0046	5.5 6.7	HR = 0.71 95%CI: 0.59-0.85 P = 0.0002
	LOGiC	CapeOx + placebo CapeOx + lapatinib	3	1st	545	10.5 12.2	HR = 0.91 95%CI: 0.73-1.12 P = 0.3492	5.4 6.0	HR = 0.82 95%CI: 0.68-1.00 P = 0.0381
	TyTAN	PTX PTX + lapatinib	3	2nd	261	8.9 11.0	HR = 0.84 95%CI: 0.64-1.11 P = 0.1044	4.4 5.4	HR = 0.85 95%CI: 0.63-1.13 P = 0.2241
	GATSBY	Docetaxel or paclitaxel TDM-1	3	2nd	345	8.6 7.9	HR = 1.15 95%CI: 0.87-1.51 P = 0.8589	2.9 2.7	HR = 1.13 95%CI: 0.89-1.43 P = 0.3080
EGFR	EXPAND	XP XP + cetuximab	3	1st	904	10.7 9.4	HR = 1.00 95%CI: 0.87-1.17 P = 0.95	5.6 4.4	HR = 1.09 95%CI: 0.92-1.29 P = 0.32
	REAL-3	EOC EOC + panitumumab	3	1st	553	11.3 8.8	HR = 1.37 95%CI: 1.07-1.76 P = 0.013	7.4 6.0	HR = 1.22 95%CI: 0.98-1.52 P = 0.068
	JapicCTI-090849	Irinotecan Irinotecan + nimotuzumab	2	2nd	83	7.7 8.4	HR = 0.994 95%CI: 0.618-1.599 P = 0.9778	2.9 2.4	HR = 0.860 95%CI: 0.516-1.435 P = 0.5668
	ENRICH	Irinotecan Irinotecan + nimotuzumab	3	2nd	Ongoing (primary endpoint: OS)				
VEGF	AVAGAST	XP + placebo XP + bevacizumab	3	1st	774	10.1 12.1	HR = 0.87 95%CI: 0.73-1.03 P = 0.1002	5.3 6.7	HR = 0.80 95%CI: 0.68-0.93 P = 0.0037
VEGFR2	REGARD	Placebo Ramucirumab	3	2nd	355	3.8 5.2	HR = 0.776 95%CI: 0.603-0.998 P = 0.047	1.3 2.1	HR = 0.483 95%CI: 0.376-0.620 P < 0.0001
	RAINBOW	Paclitaxel + placebo Paclitaxel + ramucirumab	3	2nd	665	7.36 9.63	HR = 0.807 95%CI: 0.678-0.962 P = 0.0169	2.86 4.4	HR = 0.635 95%CI: 0.536-0.752 P < 0.0001
	Li <i>et al.</i> <sup>[25]</sup>	Placebo Apatinib	3	3rd	267	4.7 6.5	HR = 0.709 95%CI: 0.537-0.937 P = 0.0149	1.8 2.6	HR = 0.444 95%CI: 0.331-0.595 P < 0.001
	RAINFALL	XP (or FP) XP (or FP) + ramucirumab	3	1st	Ongoing (primary endpoint: PFS)				
VEGFR, RET, RAF	INTEGRATE	Placebo Regorafenib	2	2nd or 3rd	147	4.5 5.3	HR = 0.74 95%CI: 0.51-1.08 P = 0.147	0.9 2.6	HR = 0.40 95%CI: 0.28-0.59 P < 0.001
HGF	RILOMET-1	ECX + placebo ECX + rilotumumab	3	1st	609	9.6 11.5	HR = 1.36 P = 0.021	2.86 4.4	HR = 1.27 P = 0.025
MET	METGastric	mFOLFOX + placebo mFOLFOX + onartuzumab	3	1st	562	11.3 11.0	HR = 0.82 95%CI: 0.59-1.15 P = 0.24	6.8 6.7	HR = 0.90 95%CI: 0.71-1.16 P = 0.43
mTOR	GRANITE-1	Placebo Everolimus	3	2nd or 3rd	656	4.34 5.39	HR = 0.90 95%CI: 0.75-1.08 P = 0.1244	1.41 1.68	HR = 0.66 95%CI: 0.56-0.78 P < 0.0001
Claudin 18.2	FAST	EOX EOX + claudiximab (extended by an arm3; EOX + high dose claudiximab)	2	1st	161 (+85)	8.4 13.4	HR = 0.51 95%CI: 0.36-0.73 P < 0.001	4.8 7.9	HR = 0.47 95%CI: 0.31-0.70 P = 0.0001
MMP-9	GAMMA-1	mFOLFOX + placebo mFOLFOX + andecaliximab	3	1st	Ongoing (primary endpoint: OS)				

GC: gastric cancer; OS: overall survival; PFS: progression free survival; HR: hazard ratio; CI: confidence interval; XP: capecitabine and cisplatin; FP: 5-fluorouracil and cisplatin; Capeox: capecitabine + oxaliplatin; EOC/EOX: epirubicin + oxaliplatin + capecitabine; ECX: epirubicin + cisplatin + capecitabine; FOLFOX: fluorouracil + leucovorin + oxaliplatin; TDM-1: trastuzumab-emtansine



including transforming growth factor  $\alpha$  (TGF $\alpha$ ) and ErbB. EGFR has been identified as an anticancer therapeutic target and many drugs that inhibit these bindings have been developed, including cetuximab, panitumumab, and nimotuzumab.

Cetuximab is an IgG1 monoclonal antibody that inhibits ligand binding to the EGFR<sup>[12]</sup> and stimulates cell-mediated cytotoxicity<sup>[13]</sup>. Addition of cetuximab to conventional chemotherapy has already been established as one of the first-line chemotherapy regimens in many types of cancer including patients with KRAS wild-type metastatic colorectal cancer (CRC)<sup>[14,15]</sup>. In a phase III trial of GC, addition of cetuximab to capecitabine + cisplatin did not improve OS and PFS (EXPAND study)<sup>[16]</sup>. These results were generally consistent between subgroups. Therefore, the critical factor of a negative result was unclear. In CRC, KRAS mutations are negative predictive biomarkers of cetuximab efficiency. However, KRAS mutations appear at low frequency in GC. Thus, this study could not have detected KRAS mutations as a predictive biomarker in GC patients. Other trials in advanced NSCLC have reported EGFR expression levels as a predictive biomarker of OS in patients treated with cetuximab. Searching the characteristics of molecular or patient groups is required for effective treatment with cetuximab.

Panitumumab is a recombinant, fully human, IgG2-monoclonal antibody that is highly selective for EGFR. Panitumumab in combination with chemotherapy has been established as the first-line and second-line treatment of KRAS wild-type metastatic colorectal cancer<sup>[17]</sup>. In a phase III trial of esophagogastric adenocarcinoma, addition of panitumumab to epirubicin + oxaliplatin + capecitabine (EOC), in interim analysis, median OS in patients allocated modified-dose EOC + panitumumab was inferior to that of patients allocated EOC and could not be recommended for use in populations with advanced esophagogastric adenocarcinoma (REAL3 study)<sup>[18]</sup>. Some factors associated with poor outcomes have been discussed. For one, combinations of EOC with full-dose panitumumab during the initial stages of the trial were associated with unacceptably high rates of grade 3 diarrhea. Therefore, oxaliplatin and capecitabine doses had to be reduced in this study. Another factor is that negative interaction might have occurred between panitumumab and EOC components or it may have been necessary to select patients by molecular characteristics. Therefore, identifying a subpopulation of patients benefiting from panitumumab or more details verifying the mechanism of molecular signaling is required.

Nimotuzumab is a recombinant humanized monoclonal immunoglobulin G1 antibody that acts against human EGFR and blocks the binding of EGF and transforming growth factor- $\alpha$  to EGFR<sup>[19,20]</sup>. This mechanism inhibits cancer-cell proliferation, angiogenesis, and induces apoptosis. Although a phase II trial, as second-line therapy, nimotuzumab plus irinotecan vs. irinotecan alone to AGC was performed, with no superiority of nimotuzumab plus irinotecan over irinotecan alone observed<sup>[21]</sup>. However, nimotuzumab plus irinotecan showed that potential improvement in a subgroup of patients with EGFR high expression subgroup was based on improved PFS, OS, and response rate. Therefore, in second-line treatment, a phase III study aimed at comparing the efficacy of nimotuzumab and irinotecan combination therapy on irinotecan alone in patients with EGFR overexpressed advanced GC or gastro-esophageal junction adenocarcinoma (GEJA) is ongoing (ENRICH study, NCT01813253). The results will be reported in 2017, with some efficiency expected.

### **Anti-VEGF monoclonal antibody**

VEGF is a signal protein produced by cells that stimulate the formation of blood vessels and mediate tumor angiogenesis<sup>[22]</sup>. Bevacizumab is a humanized monoclonal antibody that blocks angiogenesis by inhibiting VEGF-A, which stimulates angiogenesis in many types of cancer and was the first available angiogenesis inhibitor<sup>[23]</sup>. In a phase III trial, adding bevacizumab to capecitabine-cisplatin in first-line treatment of AGC did not increase OS compared with capecitabine-cisplatin (AVAGAST trial)<sup>[24]</sup>. However, adding bevacizumab to chemotherapy significantly increased PFS and overall response rate (ORR). Especially, in the European and Pan-American regions, the clinical benefit of the addition of bevacizumab increased the

most. The necessity to search a biomarker to detect patient groups who have responded to bevacizumab treatment in this trial has been discussed.

### **Anti-VEGFR monoclonal antibody**

Ramucirumab is directed against the VEGFR2 that mediated the majority of downstream effects of VEGF in angiogenesis by binding to VEGFR2 as a receptor antagonist blocking VEGF/VEGFR2. In a phase III study of AGC, ramucirumab monotherapy increased median survival time (MST) compared with placebo (REGARD study)<sup>[5]</sup>. Furthermore, in another phase III study of AGC, the combination of ramucirumab with paclitaxel significantly increased both OS and PFS compared with placebo with paclitaxel (RAINBOW study)<sup>[6]</sup>. Therefore, ramucirumab was established as one standard therapy for unresectable GC. The REGARD trial and RAINBOW trial both demonstrated the role of VEGFR-2 as an important therapeutic target in AGC. In the AVAGAST study, the efficiency of bevacizumab for Asian patients tended to be insufficient. In addition, second and further lines of therapy are more commonly received in Asia. In the REGARD trial, the control arm was designed in BSC. Therefore, other factors might experience difficulty in influencing the results. In the latest ongoing phase III study, the combination of ramucirumab with capecitabine and cisplatin is compared in PFS to capecitabine and cisplatin as first-line therapy in metastatic GC or GEJA (RAINFALL trial, NCT02314117).

### **TKIs**

Apatinib (also known as YN968D1) is a small-molecule tyrosine kinase inhibitor (TKI) that highly selectively binds to and strongly inhibits VEGFR2 and decreases the VEGF-mediated endothelial cell migration, proliferation, and tumor microvascular density. This agent also inhibits c-kit and c-SRC tyrosine kinases mildly. In a phase III trial, apatinib treatment significantly improved OS and PFS in patients who had at least two lines of prior chemotherapy fail compared with BSC<sup>[25]</sup>. Therefore, apatinib is focused on as a novel type of targeted treatment for AGC in several lines of therapy.

Regorafenib is an oral multikinase inhibitor, targeted angiogenic (VEGFR1, VEGFR2, and TIE2), stromal and oncogenic receptor tyrosine kinases. In a phase II trial, regorafenib significantly increased PFS compared with placebo as second-line or later-line therapy in AGC (INTEGRATE trial)<sup>[26]</sup>. Preliminary biomarker analysis from this trial suggested that the benefit of regorafenib was comparable in patients with VEGFA levels above and below the median. Especially, multitargeted tyrosine kinase inhibitors and similar have been required to define the subset of patients who could influence the clinical benefits. At the present time, a phase III trial is planned.

### **c-MET signaling pathway inhibitors**

c-MET is a transmembrane tyrosine kinase receptor for hepatocyte growth factor (HGF). c-MET activation promotes cell growth, invasion, and HGF/c-MET activation that occurs in several types of cancer including GC<sup>[27]</sup>. Furthermore, HGF/c-MET pathway has been related to tumor formation and metastasis.

Rilotumumab is a fully human IgG2 monoclonal antibody that acts against HGF that blocks the binding of HGF to its receptor and inhibits HGF/c-MET-mediated response. A phase III study (RILOMET-1) compared epirubicin + cisplatin + capecitabine (ECX) with or without rilotumumab in untreated patients with unresectable/advanced GC or GEJA who were c-MET positive and HER2 negative according to stained immunohistochemistry. The study was prematurely ended because of an imbalance of deaths and OS, PFS, ORR were worse in the rilotumumab arm<sup>[28]</sup>. Simultaneously, another phase III study (RILOMET-2) that compared capecitabine and cisplatin (XP) with or without rilotumumab was ceased for the same reason<sup>[29]</sup>. Considering these results, the influence of the aggression of cancer was mentioned. To clarify the cause of these results, clinical and biological analysis of the association between c-MET and GC is required.

Onartuzumab is a monovalent antibody that acts against c-MET and binds to the extracellular domain of c-MET preventing the ligand HGF. A phase III trial compared mFOLFOX6 with or without onartuzumab in MET-positive and HER2-negative gastroesophageal adenocarcinoma (GEC) (METGastric trial)<sup>[30]</sup>. However, addition of onartuzumab to first-line mFOLFOX6 did not significantly improve clinical benefits in OS, PFS, or ORR. Predictive biomarkers that identify patient groups who will most likely gain clinical benefit from onartuzumab require further investigation.

### **mTOR targeted therapies**

The mTOR is known to be the mammalian target of rapamycin and is encoded by the mTOR gene in humans. Phosphatidylinositol 3-kinase (PI3K)/Akt and mTOR activated in 30%-60% of gastric cancer PI3K/Akt/mTOR pathway dysregulations are associated with chemotherapy resistance<sup>[31]</sup>.

Everolimus is an oral mTOR inhibitor and was established as standard therapy in several types of cancer. Although everolimus in a phase II trial was demonstrated to be significantly beneficial clinically<sup>[32]</sup>, in a phase III trial, everolimus did not significantly improve OS for AGC after first- or second-line chemotherapy compared with BSC (GRANITE-1)<sup>[33]</sup>. The reason for these results was discussed to be partially attributable to the slightly higher percentage of placebo groups who initiated antineoplastic therapy after a study on drug discontinuation. In everolimus treatment, the predictive biomarker also needs to be investigated to determine its more effective use.

### **Anti-Claudin 18.2 monoclonal antibody**

The Claudin-18 splice variant 2 (CLDN18.2) belongs to a family of tight junction proteins. Claudin 18.2 is expressed in several types of cancer including GC. Claudiximab (IMAB362) is the chimeric monoclonal anti-CLDN18.2 antibody which activates antibody and component dependent cytotoxicity. In the FAST study, combinations of claudiximab with first line chemotherapy was evaluated in patients with advanced or recurrent GC or GEJA (NCT01630083). Claudiximab in combination with EOX (epirubicin + oxaliplatin + capecitabine) as first line have been showed clinically benefit in PFS and OS in this study<sup>[34]</sup>. Therefore, it will be expected to establish the evidence from a phase III trial in future.

### **Anti-matrix metalloproteinase-9 antibody**

Matrix metalloproteinase (MMPs) is a matrixin, a class of enzymes that belong to the zinc metalloproteinases family. MMP-9 is an extracellular enzyme which progress angiogenesis, tumor proliferation, and metastasis. GS-5745 is a monoclonal antibody that inhibits MMP-9 and has been combined with other chemotherapies<sup>[35]</sup>. Andecaliximab (GS-5745) is now being examined in a phase III trial in GC with mFOLFOX as 1st line (NCT02545504).

## **IMMUNOTHERAPY FOR GASTRIC CANCER**

Cancer immunotherapy is the use of the immune system in humans themselves to treat cancer. Conventionally, active immunotherapy, adoptive immunotherapy or antibody therapy have been developed as anticancer treatment. Active immunotherapy, which has been used in an attempt to stimulate the host's immune response to disease, such as a cancer vaccine, dendritic cell (DC) therapy, or cytokine therapy, was approved for some cancer types. Antibodies play a key role in adaptive immune response. Adoptive immunotherapy and antibody therapy use anti-tumor responses, monoclonal antibodies, lymphocytes and cytokines. However, the clinical effect of these therapies is limited and disparity exists for each evidence level for cancer treatment.

Most recently, immunotherapy has been acknowledged to be one of the most advanced therapies available in the treatment of cancer. We can experience the paradigmatic shift in the treatment of cancer including melanoma, NSCLC, renal cell carcinoma, and gastrointestinal cancer. Many clinical trials have been

**Table 2. Phase II and phase III clinical trial of immunotherapy in patients with GC**

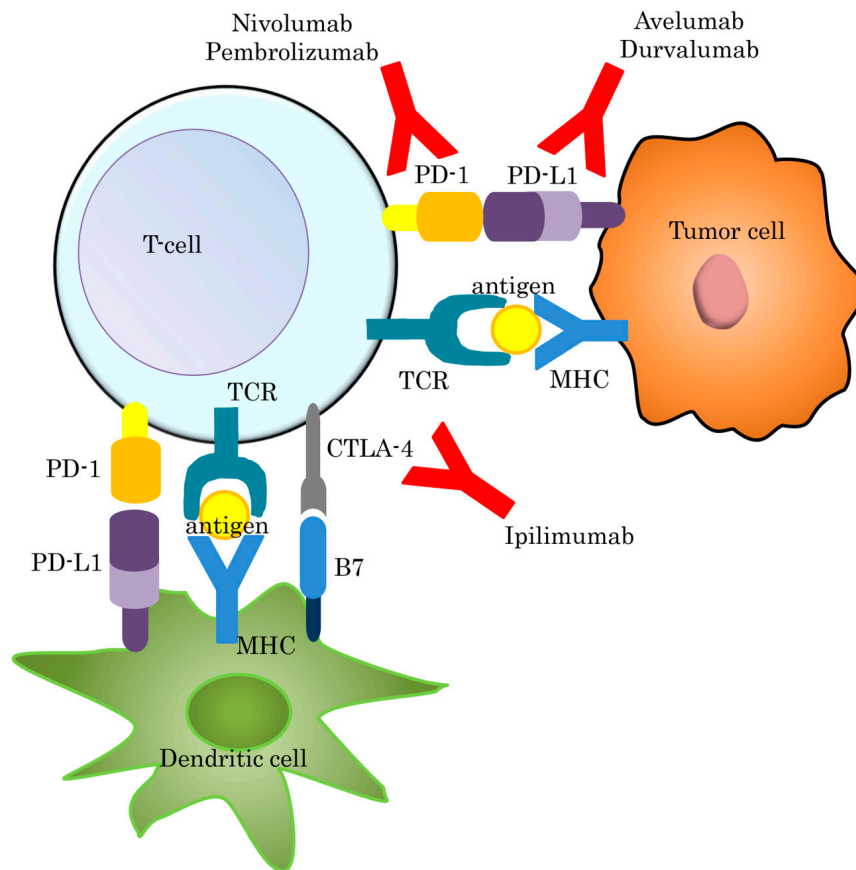
Target	Trial/ registry No./ authors	Regimen	Phase	Line	Result/primary endpoints				
PD-1	ONO-4538-12	Placebo Nivolumab alone	3	3rd or after	$n = 493$	Median OS (months) 4.14 5.32	HR = 0.63 95%CI: 0.50- 0.78 $P < 0.0001$	Median PFS (months) 1.45 1.61	HR = 0.60 95%CI: 0.49-0.75 $P < 0.0001$
	KEYNOTE-059	Pembrolizumab alone (previously treated patients ) Pembrolizumab alone (previously untreated patients ) Pembrolizumab + 5-FU + cisplatin or capecitabine	2	1st	Ongoing (adverse events, ORR)				
	KEYNOTE-061	Paclitaxel Pembrolizumab	3	2nd	Ongoing (PFS, OS)				
	KEYNOTE-062	Pembrolizumab alone Pembrolizumab + cisplatin + 5-FU or capecitabine Placebo + cisplatin + 5-FU or capecitabine	3	1st	Ongoing (PFS, OS)				
	CheckMate649	5-FU + oxaliplatin Nivolumab + 5-FU + oxaliplatin Nivolumab + ipilimumab	3	1st	Ongoing (OS)				
PD-L1 CTLA-4	JAVELIN Gastric 100	BSC after response or stability to oxaliplatin + fluoropyrimidine Avelumab	3	Maintenance after 1st-line	Ongoing (PFS, OS)				
	JAVELIN Gastric 300	BSC Paclitaxel or irinotecan + BSC Avelutinib + BSC	3	3rd	Ongoing (OS)				
PD-L1 CTLA-4	NCT02340975	Durvalumab Tremelimumab Durvalumab + tremelimumab	1b/2	2nd	Ongoing (adverse events, ORR, PFS)				
PD-L1 IDO1	ECHO-203	Durvalumab Epacadostat + durvalumab	1/2	2nd	Ongoing (adverse events, ORR)				
PD-1 LAG-3	NCT01968109	Relatlimab Relatlimab + nivolumab	1/2a	Last	Ongoing (adverse events, PFS)				

GC: gastric cancer; OS: overall survival; PFS: progression free survival; ORR: overall response rate; XP: capecitabine and cisplatin; FP: 5-fluorouracil and cisplatin

performed and have demonstrated clinical benefits in several types of cancer. Furthermore, the common advantages of immunotherapy compared with other chemotherapies have been reported to be safe and applicable to a large number of patients. In GC, many clinical trials or research studies have been promoted with promising evidence presented [Table 2]. This section will describe immune checkpoint inhibitors [Figure 2], peptide based inhibitors, and other immunotherapies in GC.

### Cancer vaccine

Cancer vaccines have been developed as therapeutic vaccines that activate tumor-associated antigen-specific T cells and reactivate existing tumor-specific T cells that are in a dormant or anergic state. This therapeutic mechanism depends on stimulating dendritic cells (DC) and activating natural killer (NK) cells, B cells, and naïve and memory T cells. Although cancer vaccines are verified depending on whether or not it sees an improvement in the prognosis of patients with solid tumor, they have not been shown to contribute to prolonging OS in phase III trials<sup>[36]</sup>. In recent years, in a phase I trial, vaccination with up-regulated lung cancer 10 and VEGFR epitope peptide was demonstrated to safely treat AGC<sup>[37]</sup>. Furthermore, a clinical trial of combined cancer vaccine with immune checkpoint inhibitors is planned in several types of cancer. Therefore, results of this trial about cancer vaccine in the future are expected.



**Figure 2.** Immune check point inhibitors in gastric cancer. CTLA-4: cytotoxic T-lymphocyte antigen-4; MHC: major histocompatibility complex; PD-1: programmed death 1; PD-L1: programmed death-ligand 1; TCR: T cell receptor

### Other immunotherapies

Other immunotherapies are being verified as potential therapies. Lymphocyte activation gene3 (LAG3), member of the immunoglobulin superfamily that exerts a wide variety of biological impacts on T cell function, is another vital checkpoint that is expected to have a synergistic interaction with PD-1/PD-L1. The combining anti-LAG3 “relatlimab” with nivolumab in patients with solid tumors have been assessed in clinical trial (NCT01968109). Glucocorticoid-induced TNFR family-related protein (GITR) is expressed at high levels on regulatory T cells (Treg). The agonist GITR antibody was reported to inhibit Treg-mediated suppression by eliminating GITR-expressing tumor-infiltrating Treg, or by causing them to become unstable, thereby attenuating their suppressive activity<sup>[38]</sup>. This anti-GITR-mAb (TRX518) is also examined in phase I trials to determine the safety of treatment in stage III or IV melanoma and other solid tumors including GC (NCT01239134). These trials are ongoing with reports detailing their results expected in the near future. Recently, TCR-inducible costimulatory receptor, marker of effector Treg, was a reportedly promising target for direct Treg-targeted therapeutic agents for GC<sup>[39]</sup>. From the progress of these research activities, Treg is also considered to be an attractive target of treatment for patients with AGC.

Polysaccharide-k (PSK) is a protein-bound polysaccharide isolated from *Trametes versicolor*. In the past, addition of PSK to chemotherapy was evaluated to be efficient and its use was attempted in GC treatment after curative gastrectomy as adjuvant treatment<sup>[40]</sup>. However, PSK’s clinical benefit was determined to be limited; therefore, few studies have included its use in recent years.



### Immune checkpoint inhibitors

Nivolumab is a human IgG4 monoclonal antibody that acts against PD-1 and has been approved for monotherapy and combination therapy for metastatic melanoma, NSCLC, renal cell carcinoma. Nivolumab works as a checkpoint inhibitor blocking signal that prevents activated T cells from attacking cancer. In a phase I/II trial, patients with nivolumab monotherapy received two or more prior regimens (CheckMate-032)<sup>[41]</sup>. The ORR was 14%, median PFS was 1.4 months, MST was 5.0 months and disease control rate was 32%. The 6-month survival rate was 49% and the 12-month survival rate was 36%. A phase III trial demonstrated that nivolumab significantly prolonged OS in patients with AGC or GEJA who had failed two or more standard chemotherapies (ONO-4538-12, ATTRACTION-2)<sup>[7]</sup>. In this trial, MST was 5.32 months with nivolumab *vs.* 4.14 months with placebo, and 12 months OS in the nivolumab group was 26.6% *vs.* 10.9% in the placebo group. Therefore, these results strongly support establishing treatment with nivolumab as a standard therapy for patients with GC. In practice, nivolumab was approved and started for treating AGC as a third-line treatment or after treatment. In addition, in a phase III trial, nivolumab was added to cytotoxic chemotherapy as first-line chemotherapy and evaluated (CheckMate649). This trial is expected to present new clinical benefits in the near future.

Pembrolizumab is a selective, humanized, high-affinity IgG4κ monoclonal antibody designed to bind to PD-1 and block interactions between PD-1 and its ligands. In patients with recurrent or metastatic PD-L1-positive GC enrolled in a phase Ib trial, pembrolizumab reportedly had a manageable toxicity profile and effective antitumor activity (KEYNOTE-012)<sup>[42]</sup>. In particular, 22% of patients with pembrolizumab had an overall response and 13% patients had grade3 or 4 treatment-related adverse events. In addition, that trial suggested a possible association between PD-L1 expression levels and pembrolizumab activity in GC. In a phase II trial, as a first-line treatment for AGC or GEJA, pembrolizumab as a monotherapy has been combined with cisplatin + 5-fluorouracil or capecitabine in subjects, with examinations ongoing (KEYNOTE-059, NCT02335411). Furthermore, in two ongoing phase III trials, pembrolizumab is compared with paclitaxel as second-line treatment for AGC or GEJA (KEYNOTE-061, NCT02370498), and pembrolizumab monotherapy is compared with a combination therapy of 5-FU (or capecitabine) plus cisplatin plus pembrolizumab or placebo as first-line treatment for patients who are PD-L1 positive and HER2 negative (KEYNOTE-062, NCT02494583).

Ipilimumab is a human IgG1 monoclonal antibody that acts against cytotoxic T-lymphocyte antigen 4 (CTLA-4)/B7 interaction to restore CD4 and CD8 effector activation. In a phase II trial, ipilimumab was compared with BSC for patients who had received first-line chemotherapy that was not significantly superior in efficiency as maintenance therapy<sup>[43]</sup>. Comparing the efficiency of nivolumab as a single agent or in combination with ipilimumab was performed in phase I/II trial (checkmate-032)<sup>[44]</sup>. The nivolumab + ipilimumab group showed a relatively higher ORR than the nivolumab monotherapy group (14% with nivolumab monotherapy and 26% with nivolumab + ipilimumab). A phase III trial of nivolumab + ipilimumab in patients with AGC is ongoing (NCT02872116).

Avelumab is an intravenously administered PD-L1 blocking human IgG1 lambda antibody for the treatment of various tumors. Avelumab has now been approved by the Food and Drug Administration (FDA) for the treatment of Merkel-cell carcinoma. In GC, the focus of a phase III study, avelumab is compared with best supportive care after response or stability to oxaliplatin and fluoropyrimidine, with examination ongoing (JAVELIN Gastric 100, NCT2625610). Avelumab is also now being verified to compare avelumab and BSC *vs.* paclitaxel or irinotecan and BSC in third-line treatment of AGC (JAVELIN Gastric 300, NCT02625623).

Durvalumab (MEDI4736) is a human IgG1κ monoclonal antibody that blocks the interaction of PD-L1 with PD-1 and CD80 molecules. This antibody has been approved for the treatment of patients with locally

advanced or metastatic urothelial carcinoma. Durvalumab has also been shown to be efficient in GC. Therefore, evaluating the safety, tolerability, antitumor activity, PK, pharmacodynamics and immunogenicity of durvalumab in combination with tremelimumab, which is a human IgG2 fully monoclonal antibody that acts against CTLA-4, and examining tremelimumab monotherapy in subjects with metastatic or recurrent GC or GEJA are ongoing in a phase Ib/II trial. Durvalumab has also been evaluated for efficiency with another medicine, epacadostat (INCB024360) (ECHO-203, NCT02318277)<sup>[45]</sup>. Epacadostat is a potent and novel indoleamine-2, 3 dioxygenase (IDO1) inhibitor. IDO1 is an enzyme responsible for oxidizing tryptophan into kynurenine and is implicated in immune modulation through its ability to limit T cell function and engage mechanisms of immune tolerance. IDO1 is focused upon as an immune subversion strategy and therapies targeting IDO1 are being evaluated in many types of cancer including GC.

## DISCUSSION AND CONCLUSION

Although several clinical trials have attempted to improve prognosis in GC patients<sup>[46]</sup> and their survival rate has been improving in recent years, unresectable or metastatic AGC has been untreatable, and median survival at this stage remains poor<sup>[47]</sup>. Therefore, research into more effective therapeutic targets, biological mechanisms, and treatments for AGC is essential.

Research that targeted therapy, including target genes, signaling pathways and drugs, is being developed day by day. Over the past few years, trastuzumab, as a first-line treatment for AGC<sup>[4]</sup> and ramucirumab, as a second-line treatment<sup>[5]</sup> has been recommended worldwide. Although many clinical trials have failed and have been unable to contribute new clinical benefits, further research has been conducted into establishing the next standard treatment for GC. In the present article, we summarized recent clinical trials. At this time, many useful basic research and preclinical trials are being performed and their progress is expected to provide us with better treatment for patients with GC in the near future.

In the field of immunotherapy, especially, more innovative treatments and combinations with other therapies are expected to be established. In addition to the clinical trials listed in this article, various other clinical trials and preclinical research are conducted in several types of cancer. Immunotherapeutic approaches with other agents, chemotherapies, targeting therapies, radiations, or different kinds of immunotherapies are currently being investigated to determine whether each clinical outcome is improved or not. In combined immunotherapy and cytotoxic chemotherapy, the synergism of these combinations is expected to lead to immunogenic cell death (ICD)<sup>[48]</sup>. ICD is a form of cell death induced by cytotoxic agents such as oxaliplatin. As another mechanism, gemcitabine or docetaxel reportedly inhibits the increasing myeloid-derived suppressor cells and B cell. Based on those mechanisms, a trial combination comparing nivolumab and conventional chemotherapy and other clinical trials is ongoing. In phase I or I/II trials, combinations of targeting therapies and immunotherapies, such as atezolizumab, which is a PD-L1 inhibitor, and bevacizumab, are examined in participants with solid tumors including GC (NCT02715531). In other types of cancer, the safety and tolerability of these therapies have been confirmed. If such trials demonstrate clinical benefit in GC, we can expect an increase of various combination patterns of therapies that have different anticancer mechanisms. Simultaneously, a start on evaluating adverse events and long-time clinical benefits should be made as soon as possible. This is particularly necessary considering that a diverse range of adverse events in immunotherapy have already been reported in many trials including GC themes; furthermore, whether or not exacerbation factors exist in some combination therapies must be confirmed. In preclinical research, the combination of multiple immune checkpoint therapy has been verified in mice. In light of this knowledge, more innovative treatment for GC is expected to be developed.

In another respect, combinations of multiple drug regimens that have been approved for other types of cancer are expected for adaptation expansion to GC. For example, in melanoma, the FDA has already approved many drugs and combinations, such as ipilimumab alone, combined nivolumab and ipilimumab, pembrolizumab

alone, the oncolytic virus therapy talimogene laherparepvec “T-VEC”, or other immunotherapies, targeting therapies, or chemotherapies. Investigation into whether or not these drugs with or without some combinations improve GC prognosis is the next possible step.

Although molecular targeted therapy and immunotherapy have been already accepted based on phase III trials, many clinical trials have resulted in negative results. Some possible specific problems in GC exist. First, GCs have heterogeneous characteristics with diverse histological types and genotypes<sup>[49]</sup>. A previous study revealed associations between histological subtypes and germline mutations. Furthermore, infection with *Helicobacter pylori* (*H. pylori*) and Epstein-Barr virus (EBV) has been shown to promote carcinogenesis in GC. The frequencies and association with *H. pylori*, EBV and GC or cancer control including prevention of these infections differed in each country and the conditions of these infections might affect the results of clinical trials. Based on this knowledge, development of a global consensus for gastritis with these infections has begun<sup>[50]</sup>. In recent years, The Cancer Genome Atlas (TCGA) analyzed many DNA alternations of GC and proposed four GC subtypes: EBV-infected tumors, microsatellite instability (MSI) tumors, genomically stable tumors, and chromosomally unstable tumors, at the molecular levels<sup>[51]</sup>. In addition, EBV-infected tumors are associated with high PD-L1 expression, and high MSI tumors are associated with high response rates to immunotherapies in other types of solid tumors<sup>[51,52]</sup>. Especially, MSI tumors have been reported to be possibly associated with sensitivity toward immune checkpoint blockade, regardless of the cancer tissue's origin<sup>[53]</sup>. Therefore, GC subtypes or tumor mutation must also be evaluated as a predictor of response to targeted therapy and immunotherapies.

Second, no established biomarkers exist to select optimum patient groups in GC treatment. Accordingly, discovery of useful biomarkers in GC treatment in previous clinical trials has not been reached. As aforementioned, in CRC, significant clinical benefits have been gained from selecting treatment methods based on genetic mutation as a predictive biomarker of response to targeted therapy, such as KRAS mutation. Therefore, identifying reliable biomarkers to accurately select patients would lead to selection of best treatment for each personalized tumor, implementing personalized medicine.

To overcome such problems, new technology such as miRNA, lncRNA which considered potential biomarker or to regulate GC progression at the transcript or transcript level has also been developed<sup>[54]</sup>. Although these researches are still in the preclinical stage, they have been expected as a solution for the recent problems.

In future, development of multidisciplinary treatment, including targeted therapy and immunotherapy, is expected to contribute to performing individualized therapy depending on the characteristics of the GC. Revealing each patient's genomic, biological, and immunological condition will contribute to selecting the most curate treatment. However, these approaches are unable to overcome our big health problem, GC.

## DECLARATIONS

### Authors' contributions

The conception and design of the study: Kiyozumi Y, Iwatsuki M, Baba H

Literature search: Kiyozumi Y

Data acquisition: Kiyozumi Y, Yamashita K, Koga Y

Manuscript preparation: Kiyozumi Y, Iwatsuki M

Manuscript editing: Iwatsuki M, Yoshida N

Manuscript review: Baba H

### Availability of data and materials

Not applicable.

## Financial support and sponsorship

None.

## Conflicts of interest

All authors declare that there are no conflicts of interest.

## Ethical approval and consent to participate

Not applicable.

## Consent for publication

Not applicable.

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Review

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# Circulating tumor cells in gastric cancer

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## Abstract

Circulating tumor cells (CTCs) have received a lot of attention as a novel biomarker for cancer research in past decades. CTCs infiltrate the bloodstream derived from the primary tumor, and are significantly involved in cancer metastasis and recurrence. Although clinical applications have been challenging owing to the difficulties of CTC identification, recent development of technology for specific enrichment and detection of CTCs contributes to diagnosis and treatment. Furthermore, CTC analyses will shed new light on the biological mechanisms of cancer progression and metastasis. A number of clinical studies have already been carried out on the basis of CTC technology. Nevertheless, the clinical utility of CTCs is still unknown in gastric cancer. In this review, we elaborate on the latest advances of CTC research in gastric cancer.

**Keywords:** Circulating tumor cells, gastric cancer, cancer progression and metastasis, tumor heterogeneity, epithelial mesenchymal transition, cancer stem cells, immune check point blockade

## INTRODUCTION

Gastric cancer is the sixth most common cancer and the third leading cause of cancer death worldwide<sup>[1]</sup>. Although diagnostic and therapeutic modalities for gastric cancer have been developed, it remains difficult to treat and manage patients with gastric cancer owing to the high frequency of metastasis and recurrence even after curative resection. Thus, to improve prognosis of gastric cancer, it is crucial to understand the process of metastasis and recurrence.



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Cancer metastasis and recurrence have been conventionally diagnosed by imaging test or serum tumor marker; however, these modalities cannot provide a precise and timely assessment of the process of metastasis and recurrence. This process has been interpreted as involving the circulating tumor cells (CTCs), which are infiltrated into the bloodstream. The detection of CTCs was first described in 1869<sup>[2]</sup>, and the “seed and soil” hypothesis was proposed in 1889<sup>[3]</sup>. This hypothesis suggested that the dissemination of metastatic tumor cells was organ-specific and not simply anatomic. Because the isolation and detection of CTCs in the blood was technically difficult, the critical role of CTCs has finally been demonstrated more than a century later<sup>[4]</sup>. At last, recent technology has contributed to the diagnosis and treatment of various cancers. The utility of CTCs for early diagnosis, prediction of prognosis, monitoring of the response to anticancer drugs, and early detection of recurrence has been demonstrated in several types of human cancer<sup>[5-8]</sup>. Moreover, it is expected that the research of CTCs elucidates the biological mechanisms of cancer metastasis and leads to better understanding of tumor heterogeneity. However, the clinical significance of CTCs and its biology in gastric cancer remain controversial. In this article, we review the latest progress of CTCs in gastric cancer.

## CANCER METASTASIS AND CTCS

Cancer metastasis is composed of several complex and interrelated steps, including transformation, migration, local invasion, intravasation into circulation, detachment, arrest at organs, extravasation, colonization, and proliferation. All steps are absolutely integral to the establishment of metastasis. In these processes, CTCs exhibit phenotypic diversity, such as epithelial mesenchymal transition (EMT) phenotype, and cancer stem cell (CSC) phenotype<sup>[9]</sup>, which facilitates metastasis.

EMT has been shown to play a critical role in metastatic spread by enhancing cancer cell mobility<sup>[10]</sup>. During EMT, epithelial cells change phenotype, such as reduction of cell-cell contacts, loss of polarity, development of cell mobility and invasiveness, repression of epithelial markers, and acquisition of mesenchymal phenotype. Epithelial markers [e.g., epithelial cells adhesion molecule (EpCAM), cytokeratin (CK), or E-cadherin] downregulate, while mesenchymal markers (e.g., vimentin, or N-cadherin) upregulate through EMT. Cancer cells undergoing EMT may intravasate as CTCs. Iwatsuki *et al.*<sup>[11]</sup> suggested that vimentin-positive tumor cells could survive in the peripheral circulation and the bone marrow and that vimentin-positive cancer cells invading intratumoral vessels must have undergone mesenchymal transition in gastric cancer. Furthermore, Wu *et al.*<sup>[12]</sup> reported that mesenchymal CTCs detected by using EMT markers were more commonly found in patients with metastatic sites of several types of human cancers.

In the EMT process, cancer cells can acquire stem cell-like properties, such as self-renewal, tumor initiation, undifferentiated status, and treatment resistance<sup>[13]</sup>. CD44 has been reported as a representative marker of CSCs in gastric cancer<sup>[14]</sup>. It has been demonstrated that CD44-positive CTCs were associated with cancer progression and recurrence in gastric cancer<sup>[15]</sup>. A recent study revealed that CD44-positive CTCs decreased after surgery or chemotherapy; therefore, they may be a predictive marker of treatment response in gastric cancer<sup>[16]</sup>.

## METHODOLOGY IN CTCS IDENTIFICATION

CTC identification typically undergoes two processes of “enrichment” and “detection”. The enrichment process is needed to detect CTCs efficiently, because CTCs are extremely rare, ranging between 1 to 10 cells per 10 mL in the peripheral blood<sup>[17]</sup>. CTCs can be enriched based on their biological and physical properties. Then, CTCs are detected using immunological, molecular, and functional assays [Table 1 and Figure 1]<sup>[18]</sup>.

### Enrichment techniques

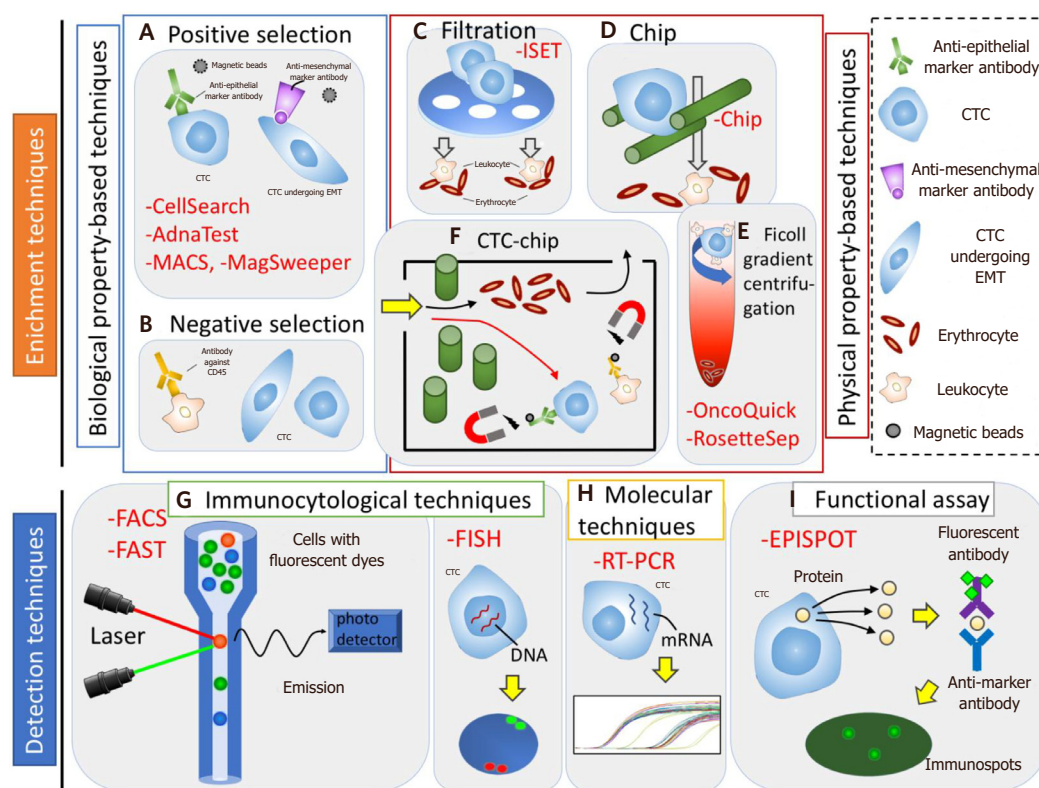
#### *Biological property-based techniques*

The biological enrichment techniques are based on specific surface makers detected by antibodies. Epithelial

**Table 1. CTC enrichment and detection technologies**

Method	System	Principle	Limitations
Enrichment technologies			
Biological	CellSearch	EpCAM antibodies coated with ferrofluid beads	Dependent on EpCAM
	AdnaTest	Immunomagnetic detection of EpCAM	High rate of false positive
	CTC-chip	EpCAM antibodies coated with microposts	Dependent on EpCAM
	MACS	Immunomagnetic beads coated with EpCAM antibodies	Dependent on EpCAM
	MagSweeper	Immunomagnetic beads coated with EpCAM antibodies	Dependent on EpCAM
Physical	ISET	Size	Variations in cell size
	OncoQuik	Density	Loss of CTCs
	RosetteSep	Density, negative selection	Loss of CTCs
Detection technologies			
Immunocytological	FACS	Antigen expression optical	Limited throughput
	FAST	Antigen expression optical	Loss of CTCs
	FISH	Detects chromosomal DNA sequence	Loss of viability
Molecular	RT-PCR	Measures nucleic acid	High rate of false positive
Functional assay	EPISPOT	Antigen expression	Enzymatic activity varies

CTC: circulating tumor cell; EpCAM: epithelial cells adhesion molecule; MACS: magnetic activated cell sorting; ISET: isolation by size of epithelial tumor; FACS: fluorescence-assisted cell sorting; FAST: fiber-optic array scanning technology; FISH: fluorescence *in situ* hybridization; RT-PCR: reverse transcription polymerase chain reaction; EPISPOT: epithelial immunospot



**Figure 1.** Circulating tumor cells (CTCs) enrichment (A-F) and detection (G-I) technologies. A and B: biological property-based techniques. A: positive selection - CTCs can be positively enriched using anti-epithelial or anti-mesenchymal marker antibody; B: negative selection - CTCs can be negatively enriched by depleting leukocyte using antibody against CD45. C-E: physical property-based techniques. C: filtration - CTCs are filtered using a membrane on the basis of the CTC size; D: chip - CTCs are trapped using microchip on the basis of CTC size and deformability; E: ficoll gradient centrifugation - CTCs are separated through a centrifugation on a ficoll density gradient on the basis of CTC density. F: physical and biological property-based techniques, CTC-chip - firstly, CTCs are selected on the basis of CTCs size, and then CTCs are isolated by magnetic bead-conjugated EpCAM antibodies, while normal hematopoietic cells are depleted by magnetic bead-conjugated antibodies against CD45. G: immunocytological techniques - CTCs can be detected by using a combination of anti-epithelial, anti-mesenchymal, anti-tissue-specific marker, or anti-tumor-associated antibodies. H: molecular techniques - CTCs can be detected by using RNA-based technologies. I: functional assay - viable CTCs can be isolated by detecting secretion of specific tumor proteins from CTCs. MACS: magnetic activated cell sorting; FACS: fluorescence-assisted cell sorting; FAST: fiber-optic array scanning technology; FISH: fluorescence *in situ* hybridization; RT-PCR: reverse transcription polymerase chain reaction; EPISPOT: epithelial immunospot; EMT: epithelial mesenchymal transition

cell markers are present on normal epithelial surface and epithelial tumors (i.e., carcinomas), but absent on normal blood cells; therefore, they can be used to identify the cancer cells in the bloodstream apart from normal blood cells. EpCAM and members of the CKs family (e.g., CK8, CK18, and CK19) are frequently used for positive selection of epithelial CTCs. However, epithelial cells can undergo EMT, resulting in downregulation of epithelial markers. To prevent false-negatives caused by EMT, N-cadherin and vimentin are used for identification of mesenchymal CTCs. In addition, to enrich CTCs specifically, negative selection is performed by using antibodies against CD45. CD45 is specifically expressed on the surface of leukocytes, whereas it is not expressed on carcinoma cells; thus, anti-CD45 antibody can deplete unnecessary leukocytes.

Tumor-specific makers, such as carcinoembryonic antigen (CEA), or  $\alpha$ -fetoprotein (AFP), are also used for biological CTC enrichment. In particular, human epidermal growth factor 2 (HER2) is suggested to be important biomarkers in the context of recent targeted therapies<sup>[19]</sup>.

On the basis of these techniques, there are various enrichment techniques. Magnetic activated cell sorting (MACS) uses magnetically labeled antibodies to enrich EpCAM-positive CTCs<sup>[20]</sup>. MagSweeper (Illumina, Hayward, CA, USA) is an automated immunomagnetic cell isolator for separation of rare endothelial cells<sup>[21]</sup>.

CellSearch System® (Veridex) captures CTCs using ferrofluid beads coated with anti-EpCAM antibody. Then, captured EpCAM-positive CTCs are stained with anti-CK and anti-CD45 fluorescently-conjugated dyes. Finally, enumeration of EpCAM-positive, CK-positive, and CD45-negative CTCs is completed by immunofluorescence<sup>[22]</sup>. The US Food and Drug Administration (FDA) has approved CellSearch for clinical use in breast, colorectal, and prostate cancer patients<sup>[6,23,24]</sup>. However, CTC detection by this system is not suitable for non-epithelial phenotype or EMT phenotype not expressing EpCAM and CK.

AdnaTest (AdnaGen AG, Langenhagen, Germany) is also an assay combining the enrichment and detection processes; that is, enriched by the magnetic procedure and detected by RT-PCR for identification of tumor-associated transcripts<sup>[25]</sup>.

CTC-chip is based on a microfluidic platform that contains an assortment of microposts coated with anti-EpCAM antibodies. Whole blood is pumped through this chip and EpCAM-positive cells are isolated and detected by cameras identifying their morphology, viability and the expression of tumor markers<sup>[26]</sup>.

#### *Physical property-based techniques*

Other enrichment techniques depend on physical properties of CTCs, such as size, diameter, density, deformability, and electric charge. The tumor cells were previously thought to be larger ( $> 8 \mu\text{m}$ ), and less deformable than blood cells. Isolation by size of epithelial tumor cells (ISET; RareCells, Paris, France) isolates epithelial cancer cells by using blood filtration with a membrane with  $8 \mu\text{m}$  pores; thus, larger cancer cells are filtered. ISET can detect a single CTC from 1 mL of peripheral blood<sup>[27]</sup>.

Density-dependent cell separation uses an inert polysucrose called Ficoll (GE Healthcare Bio-Science, Pittsburgh, PA; BD Bioscience, San Jose, CA). Ficoll was originally developed to isolate intact mononuclear blood cells from whole blood. Oncoquick™ (Greiner Bio One, Frickenhausen, Germany) based on Ficoll is a density gradient centrifugation system that can separate CTCs from whole blood samples<sup>[28]</sup>.

RosetteSep™ (StemCell Technologies, Vancouver, BC, Canada) is based on negative selection consisting of the depletion of the majority of the leukocytes and erythrocytes. This method employs a complex of antibody-targeted hematopoietic cells in human whole blood and crosslinks them to multiple erythrocytes, which leads to immunorosette formation. A centrifugation over a buoyant density medium such as Ficoll-Paque® allows for the precipitation of immunorosettes and unbound red blood cells, while CTC fractions can be



recovered from the medium.

Although cell filtration and centrifugation force have been investigated on the basis of these properties in past decades, it has been demonstrated that variations in CTC size have identified, and CTCs after undergoing EMT could be as deformable as leukocytes<sup>[29]</sup>. Therefore, new approaches have been developed to improve specificity of CTC enrichment.

### Detection techniques

After CTC enrichment, CTCs are detected by many different assays. Recent CTC identification assays combine enrichment and detection processes (e.g., CellSearch System, ISET, AdnaTest, CTC-chip, and EPISPOT). Other detection technologies include immunocytological techniques, molecular techniques, and functional assays.

#### *Immunocytological techniques*

Immunocytological techniques detect CTCs using antibodies against various antigens. These provide characteristics with high accuracy and subpopulation quantification with high specificity for simultaneous analysis with multiple parameters. However, the drawback of these techniques is lower sensitivity compared with molecular techniques.

Fluorescence-assisted cell sorting (FACS) is widely used to separate a specific cell population by using antibodies. Since FACS can analyze many parameters simultaneously, it is a versatile method with a wide range of applications. FACS sorts each cell individually, meaning that throughput of FACS is limited. Moreover, sorting conditions may be harmful to certain types of cells<sup>[30]</sup>.

Fiber-optic array scanning technology (FAST; SRI International, Menlo Park, CA) can more efficiently analyze large numbers of immunofluorescent-labeled cells in peripheral blood. FAST applies laser-based techniques to scan broad fields of view, and can detect and characterize CTCs extremely quickly and accurately. As FAST can analyze larger volumes of peripheral blood, it does not require an additional enrichment step and reduces the risk of cell loss<sup>[31]</sup>.

Fluorescence *in situ* hybridization (FISH) can precisely detect specific DNA sequences within chromosomes by using fluorescent probes. However, FISH requires high proficiency, and sometimes cannot provide clear results. To overcome these problems, a novel technology named Ikoniscope® (Ikonis, New Haven, CT) was developed for rare cell detection<sup>[32]</sup>. This system can detect one CTC per milliliter of peripheral blood. However, cells no longer have viability after FISH; therefore this technology has limited application for analyzing CTC.

#### *Molecular techniques*

Quantitative reverse transcription polymerase chain reaction (qRT-PCR) can analyze the expression of specific markers in CTCs. Specificity of qRT-PCR has been reported to be superior to that of immunohistochemistry<sup>[33]</sup>. Nowadays, a multiplex RT-PCR approach combined with liquid bead array detection has been developed to perform simultaneous amplification and detection of multiple biomarkers. However, there are several limitations, such as the contamination of non-malignant cells, the high rate of false positives, and amplification of cell-free nucleic acids<sup>[34]</sup>. In addition, once RNA has been collected from cells, the cells cannot undergo advanced analysis.

#### *Functional assay*

Epithelial immunospot (EPISPOT) detects specific tumor marker proteins secreted by CTCs<sup>[35]</sup>. Only viable CTCs are detected by EPISPOT because non-viable CTCs are not enough to detect secretion of proteins. EPISPOT is much more sensitive than ELISA when detecting secretion of CK19 from CTCs<sup>[36]</sup>. However,

because EPISPOT detects only CXCR4-positive CTCs, analysis of the heterogeneity of CTCs captured is limited.

While these developments can make CTC isolation accurate, further research on molecular characterization is necessary to confirm the significance of CTCs. Thus, the number of validation studies focusing on the characterization of CTCs has increased in recent years.

## CLINICAL UTILITY OF CTCs IN GASTRIC CANCER

There have been many previous studies of CTCs in gastric cancer, as summarized in Table 2. Although there are various methodologies of CTCs identification (e.g., RT-PCR, FACS, CellSearch System), determining the most appropriate detection method and marker of CTCs in gastric cancer remains controversial. Several meta-analyses have demonstrated that the presence of CTC is associated with advanced clinicopathological features and poor survival in gastric cancer<sup>[37,38]</sup>. Huang *et al.*<sup>[39]</sup> indicated that CTCs was associated with advanced stage, undifferentiated histological type, lymphatic invasion positive, and poorer survival.

Furthermore, CTC detection has been suggested to be a useful biomarker of diagnosis. Although previous meta-analysis showed that CTC cannot be recommended as a screening test of gastric cancer owing to lower and inconsistent sensitivity estimates for CTCs, a recent study demonstrated that CTC detection based on FAST technique, in contrast to previous studies mainly based on RT-PCR, can be an available biomarker for early diagnosis of gastric cancer with high sensitivity and specificity<sup>[40]</sup>. In addition to diagnosis and prediction of prognosis, recent studies reported that monitoring changes of CTCs during treatment may be a predictive marker of response to treatment. Li *et al.*<sup>[41]</sup> demonstrated that elevated CTCs ( $\geq 3$ ) during treatment were significantly associated with poor response rates and shorter survival. Notably, conversion to CTCs less than 3 after therapy improved the prognosis, while change to CTCs 3 or higher exhibited significantly worse prognosis. Shimazu *et al.*<sup>[42]</sup> reported that gastric cancer with diffuse bone metastases might have a very high CTC count ( $> 200$ ) in a small cohort. In cases with decrease of CTC count after treatment, tumor was sensitive to chemotherapy. They suggested that the change of CTC counts during treatment could be a predictive biomarker<sup>[42]</sup>.

HER2 has become a significant molecule for targeted therapy in gastric cancer. Trastuzumab (anti-HER2 monoclonal antibody) improved survival for patients with HER2 overexpressing gastric cancer. Although the assessment of HER2 status is usually performed on biopsy tissues from primary site, it has been reported that a discrepancy of HER2 status between the primary and the metastatic site was observed in some cases<sup>[43]</sup>. There has been an attempt to use CTCs for reassessment of HER2 status in recurrence or metastatic sites<sup>[44]</sup>. Mishima *et al.*<sup>[45]</sup> found a number of patients whose primary tumors were HER2 protein negative but who had *HER2* gene positive CTCs by using 3D-FISH in gastric cancer. Furthermore, those patients had a favorable response to trastuzumab, and the second stage of the phase 2 trial is ongoing.

## FUTURE PERSPECTIVES

### Heterogeneity of CTCs

Tumor heterogeneity has been well-known to show genetic and phenotypic diversities between different tumor types, and within the same tumor and the same patient. It has been reported that heterogeneity was associated with the response and resistance to treatment<sup>[46]</sup>. Since the tumor heterogeneity changes throughout treatment, the serial profiling of disease is needed. However, there have been no diagnostic modalities or biomarkers available for timely and accurate assessment of heterogeneity. Therefore, much attention has been paid to monitoring dynamic changes of tumor heterogeneity during treatment by detecting CTCs, which is a minimally invasive and repeatable procedure, and may allow for reassessing the biology even in recurrence or metastasis. Scher *et al.*<sup>[47]</sup> demonstrated that the degree of heterogeneity could

**Table 2. Clinical utilities of CTCs in gastric cancer**

Author	Year	Case	Method	Molecular marker	Clinical utility
Wu <i>et al.</i> <sup>[53]</sup>	2006	64	MAH	TERT, CK19, CEA, MUC	The expression of all 4 mRNA markers was an independent predictor for postoperative recurrence/metastasis
Uen <i>et al.</i> <sup>[54]</sup>	2006	52	RT-PCR	MUC1, c-Met	OS was shorter in patients with positive c-Met or MUC1 mRNA expression than in patients with negative c-Met or MUC1
Pituch-Noworolska <i>et al.</i> <sup>[55]</sup>	2007	57	ICC	CK8, 19, 20	There was no significant difference in the 5-year survival of patients, with or without CK in the blood
Koga <i>et al.</i> <sup>[56]</sup>	2008	101	RT-PCR	CK18, 19, 20	CK19 was a better marker than CK18 and CK20, and could be clinically useful to estimate prognosis
Yie <i>et al.</i> <sup>[57]</sup>	2008	55	RT-PCR, ELISA	Survivin	Survivin-expressing CTCs were statistically shown to be a significant and independent predictor for cancer recurrence
Mimori <i>et al.</i> <sup>[58]</sup>	2008	810	RT-PCR	CK7, 19, 20, VEGFR	Elevated expression of VEGFR-1 was associated with hematogenous metastases in gastric cancer
Bertazza <i>et al.</i> <sup>[59]</sup>	2009	70	RT-PCR	Survivin	Survivin mRNA levels were retained as an independent prognostic factor
Qiu <i>et al.</i> <sup>[60]</sup>	2010	123	RT-PCR	CEA	CEA mRNA positivity were independent factors for DFS
Arigami <i>et al.</i> <sup>[61]</sup>	2010	94	RT-PCR	B7-H3	The 5-year OS rate was significantly lower in patients with than without B7-H4 expression
Matsusaka <i>et al.</i> <sup>[62]</sup>	2010	52	CellSearch	EpCAM, CK8, 18, 19	Patients with U 4 CTCs at 2-week points and 4-week points after initiation of chemotherapy had a shorter median PFS
Cao <i>et al.</i> <sup>[63]</sup>	2011	98	RT-PCR, ELISA	Survivin	The detection of CTCs expressing survivin mRNA was an independent prognostic factors of DFS
Ito <i>et al.</i> <sup>[64]</sup>	2012	65	ICC	GFP, EpCAM	There was a significant relationship between the number of GFP-positive CTCs and overall survival
Arigami <i>et al.</i> <sup>[65]</sup>	2013	93	RT-PCR	STC2	The 5-year OS rate was significantly lower in patients with STC2 expression compared to patients without STC2 expression
Uenosono <i>et al.</i> <sup>[66]</sup>	2013	148	CellSearch	EpCAM, CK8, 18, 19	The detection of CTCs was an independent factor of shorter OS and PFS
Okabe <i>et al.</i> <sup>[67]</sup>	2015	136	CellSearch	EpCAM, CK8, 18, 19	The detection of CTCs was an independent factor of shorter PFS
Lee <i>et al.</i> <sup>[68]</sup>	2015	100	CellSearch	EpCAM, CK8, 18, 19	The detection of CTCs was associated with poor response to chemotherapy in metastatic gastric cancer
Kubisch <i>et al.</i> <sup>[69]</sup>	2015	62	Immune-magnetic	MUC1, EpCAM	The detection of CTCs was associated with shorter PFS and OS for patients undergoing chemotherapy
Li <i>et al.</i> <sup>[70]</sup>	2016	136	CellSearch	EpCAM, CK8, 18, 19	Conversion to a favourable CTC level (< 3 CTCs per 7.5 mL) following therapy improved the prognosis

MAH: membrane-array hybridization; ICC: immunocytochemistry; ELISA: enzyme-linked immunosorbent assay; DFS: disease free survival; OS: overall survival; PFS: progression free survival; CTC: circulating tumor cell; EpCAM: epithelial cells adhesion molecule; RT-PCR: reverse transcription polymerase chain reaction

serve as a biomarker of therapy option.

Furthermore, the advances in single-cell technologies have enabled individual CTC characterization, leading to improved understanding about tumor heterogeneity. Alix-Panabières and Pantel<sup>[48]</sup> reviewed genomic, transcriptomic, and proteomic characterization of single CTCs in different cancer types, and suggested that analysis of single CTCs may play a key role in understanding the mechanism of resistance to cancer therapy.

### PD-L1 expression on CTCs

Immune check point blockade with programmed cell-death protein 1 (PD-L1) inhibitor has recently attracted attention as a novel anticancer approach for treatment of advanced cancers. Overexpression of PD-L1 has been considered a potential mechanism of tumor escape immune elimination<sup>[49]</sup>. PD-L1 inhibitors are currently being most actively investigated for clinical use in various cancers. PD-L1 expression has been evaluated by mainly immunohistochemistry for primary tumor site as a predictive biomarker of response. However, recent studies reported tumor heterogeneity in both primary and distant metastatic site<sup>[50]</sup>.

CTCs survive in the bloodstream by exploiting immune escape mechanisms, including immune check point molecule. Therefore, it is crucial to understand the interaction of CTCs with the immune system to utilize more effective immunotherapies. Mazel *et al.*<sup>[51]</sup> demonstrated that PD-L1 frequently upregulated in CTCs of metastatic breast cancer patients. Furthermore, Strati *et al.*<sup>[52]</sup> showed that the detection of CTCs overexpressing *PD-L1* mRNA at the end of treatment was associated with poor survival, and the absence of *PD-L1* overexpression at the end of treatment was related with complete response in head and neck squamous cell carcinoma.

### CONCLUSION

Although there are many studies focusing on the utility of CTCs for diagnosis, prediction, monitoring, and choosing therapy, CTCs have not been used yet in clinical practice for gastric cancer. Therefore, further investigation and clinical studies are necessary to achieve clinical utility of CTC in gastric cancer.

### DECLARATIONS

#### Authors' contributions

Wrote the initial draft of the manuscript: Nakamura K

Contributed to interpretation of data, and assisted in the preparation of the manuscript: Iwatsuki M

Contributed to data collection and interpretation, and critically reviewed the manuscript: Kurashige J, Ishimoto T, Baba Y, Miyamoto Y, Yoshida N, Watanabe M, Baba H

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There are no conflicts of interest.

#### Ethical approval and consent to participate

Not applicable.

#### Consent for publication

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Systematic Review

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# Surgical treatment of stage IV gastric cancer: is it worthwhile?

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## Abstract

**Aim:** To analyze clinical features and survival outcomes of patients with surgically-treated stage IV gastric cancer, in order to evaluate the suitability of surgery in these patients.

**Methods:** We performed a systematic literature search using PubMed, MEDLINE, and Embase on October 9th, 2017. Survival outcomes data were collected.

**Results:** The original search returned 2434 papers. Thirty-nine studies were included in the final review, of which 26 evaluated liver metastasis resection, four pulmonary metastasis resections and nine palliative gastrectomies. In total 933 patients underwent hepatectomy for liver metastasis from gastric cancer and median survival rates were 73%, 37% and 27% at 1-, 3- and 5-year respectively, with a median overall survival of 22 months (9-52 months). Data regarding resection of lung metastases were scarce and extremely heterogeneous. In total 1115 patients underwent palliative gastrectomy and median overall survival of patients was 12 months (8-53 months). In the only randomized controlled trial, no survival benefit of additional gastrectomy over chemotherapy alone was found, in contrast with the retrospective studies.

**Conclusion:** Survival benefit of surgery in advanced gastric cancer is still unclear. Surgery may play an important role in highly selected patients. However, further randomized controlled trials are necessary to clarify the actual impact of surgery in these patients.

**Keywords:** Gastric cancer, metastasis, surgical treatment



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## INTRODUCTION

Gastric carcinoma is the second leading cause of cancer-related death worldwide<sup>[1]</sup>. The 5-year survival for patients with gastric cancer is 30.6%. This decreases to 5.2% in patients with distant metastases, who comprise 35% of total patients with a diagnosis of gastric cancer<sup>[2,3]</sup>. Liver metastases occur in 4%-14% of cases, while around 15% of patients develop pulmonary metastases<sup>[4,5]</sup>. Approximately 70% of patients are considered ineligible for surgical treatment with curative intent at the time of presentation, due to the presence of locally advanced disease or distant metastases<sup>[2]</sup>. In addition, recurrence occurs in 30%-50% of cases, even after curative R0 resection, mainly in the first two years after gastrectomy<sup>[6,7]</sup>.

In this setting, neoadjuvant chemotherapy offers new perspectives in controlling systemic disease and down-staging locally advanced gastric cancer prior to surgery<sup>[8]</sup>. Moreover, several studies have reported promising outcomes of surgical resection in patients with advanced gastric cancer with hepatic or pulmonary metastases. However, the current guidelines are not consistent regarding the most appropriate treatment strategy. The Japanese Gastric Cancer Association (JGCA) and the National Comprehensive Cancer Network (NCCN) guidelines<sup>[9,10]</sup> do not recommend surgery with curative intent in these patients, leading most patients with metastatic gastric cancer to receive palliative treatment. By contrast, the Guidelines Committee of the JGCA recently reconsidered the treatment of potentially resectable M1 disease in highly selected patients<sup>[9,11]</sup>. The definition of "stage IV" gastric cancer has varied greatly over the last few years; the 7th and 8th versions<sup>[12,13]</sup> of the American Joint Committee on Cancer (AJCC)/Union for International Cancer Control (UICC) 2010 tumor-node-metastasis (TNM) staging system clearly defined stage IV as any lesion with hematogenous metastases (M1), while previous versions<sup>[14]</sup> have also included "locally advanced" cases, such as lesions with massive (> 15) lymph node metastases (N3) or with direct invasion of adjacent structures (T4). The Japanese Classification of Gastric Cancer did not classify pancreatic head (station 13 and 17) and para-aortic (station 16) lymph node metastases as "distant" (M1) up until the 3rd English Edition in 2011<sup>[15]</sup>, whereas western staging systems had accepted this concept long before<sup>[14]</sup>. Which patients with stage IV gastric cancer (either locally advanced or with M1) should be offered a surgical resection and the exact survival benefit of this remain unclear.

This study sought to systematically review the literature in order to evaluate the outcomes of surgical treatment for stage IV gastric cancer and to provide an update on the surgical treatment strategies for this condition.

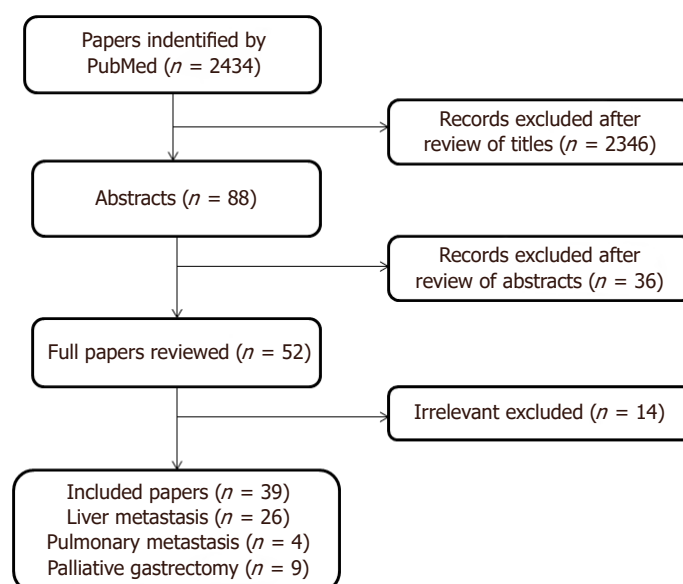
## METHODS

A systematic literature search was carried out on October 9th, 2017. All references from 2002 to 2017 were potentially eligible for inclusion in the study. The following search strategy was used in PubMed, MEDLINE and Embase: (((((((("gastric cancer") OR "gastric carcinoma") OR "gastric neoplasm") OR "stomach cancer") OR "stomach carcinoma") OR "stomach neoplasm"))) AND ((("metastatic") OR metastas\*)) AND (((("liver") OR "hepatic") OR "lung") OR "pulmonary"))) AND (((("surgery") OR "resection") OR "palliative surgery") OR "palliative gastrectomy") OR "surgical"))).

A title search was conducted with title review of all identified references. Studies deemed unrelated to study aims were excluded. Abstracts for the remaining studies were retrieved and screened for relevance. Full papers were retrieved for all abstracts deemed potentially eligible. Full papers underwent authors' review and assessment of inclusion/exclusion criteria. Any disagreement during the search and selection process was resolved by consensus.

## Inclusion criteria

- Papers presenting data regarding liver and pulmonary metastasis resection in patients with gastric cancer, without evidence of peritoneal metastases or metastases to other organs.



**Figure 1.** Diagram showing literature selection strategy

- Papers presenting data on patients undergoing palliative gastrectomy, defined as gastric resection without radical intent (microscopic or macroscopic residual disease) in patients with locally advanced disease or in patients with distant metastases.
- Original data (no review papers).
- Survival outcomes data available for at least 1 year following surgical resection. Papers relating to hepatic metastases must have reported at least the median survival time.
- Patient recruitment after 1980.

### Exclusion criteria

- Non-English language studies.
- Full manuscript not available (e.g., abstracts presented at conferences).
- Studies with less than ten patients.
- Malignancy other than epithelial carcinoma<sup>[16,17]</sup>.

The following data were collected: author details, country, recruitment period, study design, median follow-up, sample size, gender, positive and negative findings, and methodological quality. The primary outcome assessed was survival following surgical resection.

Considering the extreme heterogeneity of inclusion criteria of each paper, we aimed to review the literature descriptively without an intent of inference.

## RESULTS

The original search returned 2434 papers. [Figure 1](#) shows the study search strategy. Overall, 39 studies were included in the final review (26 for hepatic resection<sup>[18-43]</sup>, 4 for pulmonary resection<sup>[44-47]</sup> and 9 for palliative gastrectomy<sup>[48-55]</sup>).

### Liver metastasis surgical treatment

The 26 studies included provided data on 933 patients who underwent gastrectomy and synchronous or metachronous hepatectomy. The median sample size was 24 patients (range 11-256). Baseline characteristics



are described in [Table 1](#) and [Supplementary Table 1](#). The median age of patients undergoing hepatectomy was 64 years (range 57-72 years) and 78% of patients were males. There was a wide variety of disease burden in patients undergoing hepatectomy. Hepatic lesions were solitary and unilobar in 65% and 78% respectively. Fifty five percent of patients developed synchronous metastases, while 45% developed metachronous lesions. The majority of hepatectomies were minor resections for limited disease, although 42% of patients underwent major resections.

Details on chemotherapy used were also reported in 19 studies, including data on 775 patients. Of these, 15% received neoadjuvant chemotherapy and 46% received adjuvant chemotherapy. A wide variety of chemotherapy regimens were described, while seven studies did not state what chemotherapeutic agents their patients received<sup>[18,23,24,29,34,39,41]</sup>.

Survival outcomes are summarized in [Table 2](#). Median follow-up was 24 months (range 9-65 months). Twenty-one studies presented 1-year survival rates ranging from 36% to 96%, 19 presented 3-year survival rates ranging from 14% to 70% and 25 studies presented 5-year survival rates of between 9% to 42%. Median survival rates were 73%, 37% and 27% at 1-, 3- and 5-year respectively. Median overall survival was 22 months (range 9-52 months).

Seven of the 26 studies compared survival outcomes between resected patients and those who underwent chemotherapy alone. Surgery demonstrated a survival advantage in all of them.

### **Pulmonary metastasis surgical treatment**

Eighty-three patients provided by four studies underwent resection of pulmonary metastases from gastric cancers. Resection of gastric cancer lung metastases has rarely been reported and few data are available regarding short- and long-term outcomes of this procedure. The majority of patients with pulmonary metastases from gastric cancer present with carcinomatous lymphangitis or pleuritis, whereas nodular lesions are less common<sup>[56]</sup>.

Baseline characteristics are described in [Table 3](#) and [Supplementary Table 2](#). Median age was 66 years (range 56-68 years), and males represented the majority of resected patients (83% vs. 17%). All patients underwent gastrectomy and subsequent pulmonary metastasectomy. Hundred percent of included patients displayed metachronous metastases and 73% of these were solitary lesions. Overall 39% of patients underwent lobectomy, while wedge resection or segmentectomy was performed in 61%. In 3 studies<sup>[45-47]</sup>, indications for performing surgery were decided based on Thomford's criteria<sup>[57]</sup>. Shiono *et al.*<sup>[44]</sup> did not specify the criteria for surgical resection. Details on chemotherapy were reported in 3 studies. No patients underwent neoadjuvant chemotherapy, while adjuvant treatment was carried out in 42% of patients.

Median follow-up was 25 months (range 18-27 months). Overall survival outcomes are summarized in [Table 3](#). Iijima *et al.*<sup>[46]</sup> reported an overall 3-year survival rate of 30%. Kobayashi *et al.*<sup>[47]</sup> showed a median survival time following pulmonary resection of 67 months and an overall 5-year survival rate of 59%, while Shiono *et al.*<sup>[44]</sup> reported a value of 28%. By contrast, Yoshida *et al.*<sup>[45]</sup> followed patients for a median time of 27 months and the overall survival rates at 1, 3 and 4 years were 100%, 100%, and 75%, respectively. None of the included studies reported data regarding palliative treatment arms involving chemotherapy alone.

### **Palliative gastrectomy**

Nine studies providing data on 1115 patients who underwent palliative gastric resection were included [[Table 4](#)]. One of these was a randomized controlled trial (REGATTA)<sup>[58]</sup>. The median sample size was 137 patients (range 23-218), and 68% of patients were males. Except for the randomized controlled trial, inclusion criteria and study structure were very heterogeneous between series and, consequently a comparison of results between them was

**Table 1. Basic characteristics of studies regarding hepatectomy for hepatic metastasis from gastric cancer**

Study	Area	Year of publication	Recruitment period	Median follow-up (months)	Sample size (n)	Median age (years)	Mean age (years)	M/F (n)	Solitary/multiple (n)	Unilobular/bilobar (n)	Synchronous/metachronous (n)	Hepatectomy (minor/major) (n)	Hepatectomy (%) (minor/major)	Neo/adjunct therapy (n)	Neo/adjunct therapy (%)
Baek <i>et al.</i> <sup>[18]</sup>	South Korea	2013	2003-2010	13	12	NR	61	11/1	11/1	11/1	3/9	8/4	67/33	0/6	0/50
Chen <i>et al.</i> <sup>[19]</sup>	China	2013	2007-2012	NR	20	NR	57	12/8	8/12	11/9	20/0	6/14	30/70	20/20	100/100
Cheon <i>et al.</i> <sup>[20]</sup>	South Korea	2008	1995-2005	NR	41	60	NR	34/7	28/13	NR/NR	30/11	29/3	71/7	0/37	NR/88
Dittmar <i>et al.</i> <sup>[21]</sup>	Germany	2012	1995-2009	NR	15	57	NR	12/3	8/7	12/3	9/6	7/3	53/20	NR/NR	NR/NR
Garancini <i>et al.</i> <sup>[22]</sup>	Italy	2012	1998-2007	20	21	64	NR	14/7	12/9	16/5	12/9	17/4	81/19	NR/NR	NR/NR
Kinoshita <i>et al.</i> <sup>[23]</sup>	Japan	2014	1990-2010	65	256	64	NR	207/49	NR/NR	NR/NR	106/150	73/183	29/71	45/84	18/33
Koga <i>et al.</i> <sup>[24]</sup>	Japan	2007	1985-2005	NR	42	NR	NR	NR	29/13	NR/NR	20/22	35/7	83/16	0/14	NR/31
Komeda <i>et al.</i> <sup>[25]</sup>	Japan	2014	2000-2012	NR	24	69	NR	21/3	17/7	NR/NR	1/23	10/14	42/58	11/15	46/63
Li <i>et al.</i> <sup>[26]</sup>	Taiwan	2017	1996-2012	29	34	NR	62	23/11	NR/NR	NR/NR	0/34	NR/NR	NR/NR	NR/NR	NR
Liu <i>et al.</i> <sup>[27]</sup>	China	2012	1995-2010	38	35	NR	NR	29/6	12/23	12/23	35/0	NR/NR	NR/NR	NR/NR	NR/NR
Makino <i>et al.</i> <sup>[28]</sup>	Japan	2010	1992-2007	NR	16	NR	NR	13/3	9/7	11/5	9/7	16/0	100/0	5/13	19/88
Miki <i>et al.</i> <sup>[29]</sup>	Japan	2012	1995-2009	NR	25	72	NR	23/2	18/7	20/5	16/9	NR/NR	NR/NR	0/0	0/0
Morise <i>et al.</i> <sup>[30]</sup>	Japan	2008	1989-2004	NR	18	64	NR	16/2	14/4	15/3	11/7	14/4	78/22	NR/NR	NR/NR
Nomura <i>et al.</i> <sup>[31]</sup>	Japan	2009	1991-2005	20	17	NR	66	13/4	NR/NR	NR/NR	9/8	14/3	82/18	3/10	18/59
Qiu <i>et al.</i> <sup>[32]</sup>	China	2013	1998-2009	38	25	NR	NR	22/3	16/9	21/4	NR/NR	NR/NR	NR/NR	4/14	16/60
Roh <i>et al.</i> <sup>[33]</sup>	South Korea	2005	1988-1996	19	11	61	NR	10/1	11/0	11/0	8/3	10/1	91/9	NR/NR	NR/NR
Sakamoto <i>et al.</i> <sup>[34]</sup>	Japan	2003	1985-2001	17	22	63	NR	13/11	16/6	17/5	12/10	19/3	86/14	0/8	0/40
Sakamoto <i>et al.</i> <sup>[35]</sup>	Japan	2007	1990-2005	NR	37	64	NR	29/8	21/16	30/7	16/21	32/5	86/14	0/6	0/16
Takemura <i>et al.</i> <sup>[36]</sup>	Japan	2012	1993-2011	27	64	65	NR	49/15	37/27	NR/NR	34/30	50/14	78/22	18/26	28/41
Thelen <i>et al.</i> <sup>[37]</sup>	Germany	2008	1988-2002	9	24	64	NR	17/7	13/11	18/6	15/9	16/8	67/33	NR/NR	NR/NR
Tiberio <i>et al.</i> <sup>[38]</sup>	Italy	2015	1997-2011	NR	53	NR	NR	NR	NR/NR	NR/NR	53/0	38/14	72/26	0/22	0/42
Tsujiimoto <i>et al.</i> <sup>[39]</sup>	Japan	2010	1980-2007	29	17	NR	66	16/1	13/4	17/0	9/8	NR/NR	NR/NR	0/14	0/82

Viganò <i>et al.</i> <sup>[40]</sup>	Italy	2013	1997-2008	43	20	61	NR	12/8	15/5	18/2	9/11	11/9	55/45	8/NR	40/NR
Wang <i>et al.</i> <sup>[41]</sup>	China	2012	2003-2008	NR	30	60	NR	27/3	22/8	27/3	NR/NR	23/7	77/23	0/30	0/100
Wang <i>et al.</i> <sup>[42]</sup>	China	2014	1996-2008	14	39	NR	64	26/13	31/8	34/5	39/0	NR/NR	NR/NR	0/39	0/100
Zacherl <i>et al.</i> <sup>[43]</sup>	Austria	2002	1980-1999	NR	15	62	NR	10/5	8/7	NR/NR	10/5	12/3	73/20	0/2	NR/14

NR: not reported

challenging. In addition, in these retrospective studies, the indications for palliative gastrectomy were judged individually by surgeons based on patients' general health, performance status, symptoms, extent of disease, and feasibility of resection.

The minority of palliative resections were total gastrectomies, while 62% of patients underwent minor resection. Data regarding adjuvant treatment were reported in 7 of the 9 studies, and overall 71% of patients underwent post-operative chemotherapy; only one study reported data about neoadjuvant treatment<sup>[49]</sup>. Median overall survival was 12 months (range 8-53 months). In 6 of the 8 retrospective studies, a comparison with nonresected patients was carried out, and gastrectomy showed a significant survival advantage in 5 of them; however, these results had limitations related to the retrospective nature and the selection bias for surgery. In fact, in the REGATTA trial, the median overall survival was 16.6 months for patients assigned to chemotherapy alone and 14.3 months for those assigned to gastrectomy plus chemotherapy, in whom significantly higher rates of adverse events were also seen.

## DISCUSSION

The most appropriate treatment in cases of stage IV gastric cancer is still debated. Indications and advantages of a surgical approach to treat advanced gastric neoplasms in comparison to conservative therapy such as chemotherapy have not yet been established. Gastric cancers are mostly of advanced stage at diagnosis. However, location and number of metastases, as well as patient characteristics, influence the benefit of surgical treatment and overall survival outcomes. This systematic review showed that surgery seems to play an important role even in patients with incurable gastric cancer.

In our study, surgical resection of gastric cancer with hepatic metastasis in the absence of peritoneal disease is associated with 1-, 3- and 5-year survival rates of 73%, 37%, and 27% respectively. Median overall survival was 22 months (range 9-52 months). Compared to the results of randomized controlled trials based on chemotherapy, hepatectomy seems to offer in selected patients better survival outcomes<sup>[59,60]</sup>. Liao *et al.*<sup>[61]</sup>, consistent with previous reviews, described significantly improved overall survival in patients treated with hepatectomy compared to palliative chemotherapy. In light of these studies, the Japanese guidelines reconsidered the role of hepatectomy in the treatment of liver metastasis in gastric cancer, however, which patients may actually benefit from surgical treatment is still controversial. Medical comorbidities of patients undergoing hepatectomy were poorly described in the included studies, as were other confounders like metastatic features, use of neoadjuvant and adjuvant treatment, and surgical techniques. With regard to surgical approach, minor hepatectomy was performed more commonly than major resection (58% vs. 42%). Synchronous, multiple, or bilobar metastases were associated in some studies with poorer prognosis, but were not necessarily considered contraindications for surgery<sup>[24,28,35]</sup>. Chemotherapy was commonly used in the adjuvant and neoadjuvant setting. However, there was a wide variation in timing and regimens between studies. Therefore, from the current literature, the indications for a surgical approach to gastric cancer metastatic to the liver, in particular, the threshold for number of metastases and their location and the administration of chemotherapy in relation to surgery, remain undetermined.

**Table 2. Survival outcomes following hepatectomy for liver metastasis from gastric cancer, with a comparison with nonresected patients (chemotherapy alone)**

Study	1-year survival (%)	3-year survival (%)	5-year survival (%)	Median survival (months)	Nonresected patients, median OS (months)	P value
Baek <i>et al.</i> <sup>[18]</sup>	65	NR	39	31	-	-
Chen <i>et al.</i> <sup>[19]</sup>	NR	NR	15	22	5.5	0.000
Cheon <i>et al.</i> <sup>[20]</sup>	75	32	21	17	NR	0.0001
Dittmar <i>et al.</i> <sup>[21]</sup>	NR	NR	27	48	9	0.002
Garancini <i>et al.</i> <sup>[22]</sup>	68	31	19	11	-	-
Kinoshita <i>et al.</i> <sup>[23]</sup>	77	42	31	31	-	-
Koga <i>et al.</i> <sup>[24]</sup>	76	48	42	34	-	-
Komeda <i>et al.</i> <sup>[25]</sup>	78	40	40	22	-	-
Li <i>et al.</i> <sup>[26]</sup>	74	37	25	26	3.13	0.001
Liu <i>et al.</i> <sup>[27]</sup>	58	22	NR	15	-	-
Makino <i>et al.</i> <sup>[28]</sup>	88	56	42	38	15	0.001
Miki <i>et al.</i> <sup>[29]</sup>	74	43	37	33	NR	0.04
Morise <i>et al.</i> <sup>[30]</sup>	56	27	27	13	-	-
Nomura <i>et al.</i> <sup>[31]</sup>	NR	NR	31	21	-	-
Qiu <i>et al.</i> <sup>[32]</sup>	96	70	29	38	-	-
Roh <i>et al.</i> <sup>[33]</sup>	73	NR	27	19	-	-
Sakamoto <i>et al.</i> <sup>[34]</sup>	73	38	38	21	-	-
Sakamoto <i>et al.</i> <sup>[35]</sup>	NR	NR	11	31	-	-
Takemura <i>et al.</i> <sup>[36]</sup>	84	50	37	34	-	-
Thelen <i>et al.</i> <sup>[37]</sup>	38	16	10	9	-	-
Tiberio <i>et al.</i> <sup>[38]</sup>	50	14	9	13	-	-
Tsujimoto <i>et al.</i> <sup>[39]</sup>	NR	NR	32	34	-	-
Viganò <i>et al.</i> <sup>[40]</sup>	95	63	33	52	-	-
Wang <i>et al.</i> <sup>[41]</sup>	43	17	17	11	-	-
Wang <i>et al.</i> <sup>[42]</sup>	56	18	10	14	NR	NR (but referred < 0.05)
Zacherl <i>et al.</i> <sup>[43]</sup>	36	29	14	9	-	-

NR: not reported; OS: overall survival

Resection of lung metastases from gastric cancer has rarely been reported, and only small amounts of heterogeneous data are available regarding short- and long-term outcomes. The majority of articles present in the literature are case reports or small series. Only four studies were included in our evaluation. They reported favorable results in the surgical group; however, the series were small, and comparison between studies was difficult because of the heterogeneity of inclusion criteria for each study. Overall 3-year survival rates ranged from 30% to 100%. The frequent occurrence of extrapulmonary metastases before pulmonary metastasectomy restricts surgical treatment to highly selected patients<sup>[56]</sup>. In this setting, lung metastasectomy seems not to have a determined role in the standard management of these patients.

In the 1115 patients included in this review who underwent palliative gastrectomy, median overall post-resection survival was 12 months. The rationale for non-curative gastrectomy was the reduction of tumor burden and/or the palliation of symptoms, such as obstruction, perforation, bleeding or ascites. In 6 of the 8 retrospective studies included in this review, overall survival of resected patients was significantly better than the nonresected group. However, all studies highlighted as a limitation, the possible relationship between these positive results and the selection bias of patients. A previous meta-analysis was consistent with these results<sup>[62]</sup>. Moreover, they analyzed survival rates of patients that received palliative gastrectomy with or without chemotherapy, and it was shown that surgery combined with chemotherapy offered a survival benefit<sup>[62]</sup>. By contrast, the results of the REGATTA trial showed no survival benefit of additional gastrectomy over chemotherapy alone, not justifying gastric resection in patients with metastatic gastric cancer<sup>[58]</sup>. In light of this randomized controlled trial, chemotherapy alone remains the standard of care for

**Table 3. Demographics and survival data from the cited lung resection studies for metastatic gastric cancer**

Study	Country	Year of publication	Recruitment period	Median follow-up (months)	Sample (n)	Mean age (years)	M/F (n)	Neoadj/ adjuvant therapy (n)	1-year survival (%)	3-year survival (%)	5-year survival (%)	Median survival (months)	Nonresected patients, median OS (months)	P value
Iijima <i>et al.</i> <sup>[46]</sup>	Japan	2016	1985-2012	27	10	64	10/0	NR	NR	30	NR	27	-	-
Kobayashi <i>et al.</i> <sup>[47]</sup>	Japan	2012	1998-2012	23	12	56	10/2	0/10	NR	NR	58.40	67	-	-
Shiono <i>et al.</i> <sup>[44]</sup>	Japan	2013	1980-2011	18	51	68	40/11	NR/18	NR	NR	28	29	-	-
Yoshida <i>et al.</i> <sup>[45]</sup>	Japan	2014	2003-2012	27	10	67	9/1	0/3	100	100	NR	NR	-	-

None of the 4 papers reported a comparison with nonresected patients. M: male; F: female; NR: not reported; OS: overall survival

**Table 4. Demographics and survival data of patients undergoing palliative gastrectomy, with a comparison with nonresected patients**

Study	Country	Year of publication	Recruitment period	Median follow-up (months)	Sample (n)	Mean age (years)	M/F (n)	Gastrectomy total/subtotal	Neo/adjuvant therapy	1-year survival (%)	3-year survival (%)	5-year survival (%)	Median survival (months)	Nonresected patients, median OS (months)	P value
Li <i>et al.</i> <sup>[48]</sup>	China	2010	1992-2002	NR	137	58	76/61	21/116	NR/NR	NR	NR	NR	12	7	0.001
Ko <i>et al.</i> <sup>[49]</sup>	South Korea	2012	1992-2007	NR	178	58	122/56	63/115	0/120	NR	NR	NR	12	-	-
Hartgrink <i>et al.</i> <sup>[50]</sup>	The Netherlands	2002	1989-1993	NR	156	NR	NR	63/93	NR	NR	NR	NR	8	5	0.001
Saidi <i>et al.</i> <sup>[51]</sup>	USA	2006	1990-2000	NR	24	57	14/10	4/10	NR/12	NR	NR	5	13	6	0.006
Kokkola <i>et al.</i> <sup>[52]</sup>	Finland	2012	2000-2009	NR	23	61	NR	11/12	NR/15	NR	NR	NR	11	6	0.152
Samarasam <i>et al.</i> <sup>[53]</sup>	India	2006	1999-2003	NR	107	NR	NR	39/68	NR/107	NR	NR	NR	24	12	0.003
Lin <i>et al.</i> <sup>[54]</sup>	China	2008	1994-2001	NR	183	60	143/40	NR	NR/112	80.3	20.8	6	NR	-	-
Sougiultzis <i>et al.</i> <sup>[55]</sup>	Greece	2011	1997-2007	NR	218	NR	NR	NR	NR/218	NR	NR	NR	53	16	0.001
Fujitani <i>et al.</i> <sup>[58]</sup>	Japan/ South Korea/ Singapore	2016	2008-2013	NR	89	NR	61/28	57/32	0/89	NR	NR	NR	14	16	0.070 (one-side)

M: male; F: female; NR: not reported; OS: overall survival



these patients, even though the chemotherapy regimen used in the REGATTA trial was based on S-1, which shows reduced tolerability in Western patients<sup>[63]</sup>.

In summary, from the present literature, a surgical approach for stage IV gastric cancer shows uncertain survival benefits and is not justified in all patients. Further randomized controlled trials are necessary to clarify the actual impact of surgery in these patients. Probably, surgery may play an important role in highly selected patients. Criteria to select patients who can benefit more from surgical treatment have not yet been identified, and this needs further investigation.

## DECLARATIONS

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### Authors' contributions

Concept, design, definition of intellectual content: Pergolini I, Ciano P, Guercioni G, Catarci M

Literature search, data acquisition, data analysis, statistical analysis: Pergolini I, Ciano P

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Manuscript editing, manuscript review: Pergolini I, Ciano P, Guercioni G, Catarci M

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All authors declared that there are no conflicts of interest.

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Not applicable.

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Case Report

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# Necrotizing fasciitis as a complication of taxanes: a case report

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## Abstract

Necrotizing fasciitis is a rare complication of chemotherapy, however, few reports were published as a specific complication of taxanes. We are reporting this rare complication of a lady who was treated with taxanes as an adjuvant therapy for her breast cancer who was referred to us from the medical department and turned out to be necrotizing fasciitis in her right thigh. We are also presenting the literature review of this type of complication.

**Keywords:** Necrotizing fasciitis, breast cancer, taxanes, docetaxel, complication of chemotherapy

## INTRODUCTION

Necrotizing fasciitis is a rare complication of chemotherapy. It is characterized by necrosis of the soft tissue subcutaneous fat and fascia. Necrotizing fasciitis secondary to taxanes alone is very rare, and only 7 cases have been reported by the WHO adverse drug reactions<sup>[1]</sup>. Taxanes, however, as a chemotherapy, has been used as an adjuvant treatment in combination with other chemotherapy like fluorouracil and cyclophosphamide with reduction of the risk of recurrence by 25% and death by 17%. Many complications have been reported from the minor nausea, vomiting, alopecia, neuropathy to the development of secondary malignancy. Depression of the immune system is one of the most worrying side effects where patients become prone to develop all spectrums of infections, including necrotizing fasciitis as in our case. We are presenting this case in order to highlight the presence of this complication despite its rarity.



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**Figure 1.** (A) Post debridement; (B) post skin grafting

## CASE REPORT

A 52-year-old female presented to our emergency department complaining of painful right lower limb swelling, generalized weakness and fever for 2 days. There were no history of trauma, intervention or insect bites and no other chronic medical illness. The lady was diagnosed with breast cancer 6 months prior to this admission and she had breasts conserving wide local excision of the tumor, followed by 7 cycles of adriamycin and cyclophosphamide, then 3 cycles of docetaxel. The last cycle of the taxane was just 12 days prior to her admission. On examination, she looked unwell, tachycardia with generalized swelling and tenderness in the medial aspect of the right thigh. There was no distal neurovascular compromise and no palpable lymphadenopathy. Her complete blood picture and inflammatory markers were normal on admission.

Following aggressive resuscitation in the intensive care unit, she was taken to the operating theatre where extensive debridement of necrotic tissue was done from the right thigh. This was repeated four times on different occasions till nice granulation tissue was obtained, which was then covered by split skin graft [Figure 1]. The histopathology features showed focal ulceration, marked hemorrhage, congestion, full thickness necrosis, fibro purulent exudate and micro abscesses formation along with degenerating muscle fibers consistent with necrotizing fasciitis.

## DISCUSSION

Necrotizing fasciitis is an uncommon infection with high mortality rate<sup>[2]</sup> caused by wide spectrum of micro-organisms, of which two thirds are polymicrobial (type A) and one third is monomicrobial, mainly cocci (type B)<sup>[3]</sup>. It involves inflammation and necrosis of subcutaneous tissue, fascia, and muscles and later of skin. High index of suspicion is needed for early diagnosis. Appearance of swelling, tachycardia, tense oedema, ecchymosis, blister or bullae, crepitus and hypotension are late signs<sup>[4]</sup>.

Scoring system suggested by Wong *et al.*<sup>[5]</sup> based on the level of haemoglobin, leucocyte count, C reactive protein, creatinine, glucose, and sodium will aid with the diagnosis. Diagnosis, however, is usually confirmed intraoperatively when we find the classical foul smell “dish water” discharge, necrosis with positive “finger test”.

Aggressive and radical debridement is critical for improving the outcome and lowering mortality rate<sup>[6]</sup>.

In our patient, there was no other predisposing factor found for the development of necrotizing fasciitis apart from the fact that the patient was on docetaxel at the time of infection, where she received 3 cycles 12 days



prior to admission. It has been documented that chemotherapy leading to alterations on the mucosa, soft-tissue, and skin along with immunosuppression might be a triggering factor<sup>[7]</sup>. The skin toxicity (including dry skin, erythema, pigmentation, pruritus, rash/desquamation, urticaria, dermatitis, and other) has been reported in previous studies<sup>[8]</sup>. It was hypothesized that taxanes metabolites are excreted through the sweat glands which are abundant on the palms and soles.

The WHO however, reported in their adverse drug reaction database only 7 cases of necrotizing fasciitis associated with docetaxel.

To conclude, we should always have a low threshold point in suspecting necrotizing fasciitis when reviewing patients presenting with pain while on chemotherapy. Also, keeping in mind that patients can appear systemically well despite the presence of necrotizing fasciitis due to immune suppression - as these patients are not able to respond to infection adequately and skin manifestation may present different due to their blunted immunological response system.

## DECLARATIONS

### Authors' contributions

Assisted in collection of the data and the patients' notes from the file, refined the literature and wrote the paper: Amer NM

Did the primary collection of the patients' notes, lab results and the photos, collected the literature and assisted with the writing of the manuscript: Niaz R

### Availability of data and materials

All data and information are available from the corresponding author upon request.

### Financial support and sponsorship

None.

### Conflicts of interest

The authors declare that there are no conflicts of interest.

### Ethical approval and consent to participate

Not applicable since it is only a case report and not a study.

### Consent for publication

Patient gave full informed consent for writing and publishing this paper.

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Review

Open Access



# Current trends in gastric cancer treatment in Europe

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## Abstract

Gastric cancer is one of the major causes of cancer-related deaths, despite the gradual decrease of its incidence in the West. Minimally invasive procedures, such as endoscopic resection and laparoscopic gastrectomy, have been successfully introduced in European high-volume centres, in the treatment of early gastric cancer. Regarding advanced, localized gastric cancer a number of prospective trials have been completed in search of better therapeutic options, aiming to optimize the efficacy *vs.* adverse effect ratio. From the results of these prospective randomized trials, the therapeutic strategy has in the last decades shifted emphasis from adjuvant therapy to neoadjuvant or perioperative chemotherapy, in curatively intended treatment. Moreover, recent studies have shown promising results in the use of molecular targeted agents, both in perioperative and palliative settings. The introduction of molecularly targeted therapy will enable a personalized approach based on each patient's and tumor's characteristics, maximizing the benefits from chemotherapy. The present review article focuses on recent therapeutic trends, as well as future perspectives, of surgical and oncological gastric cancer treatment in the Western setting, mainly based on landmark clinical trials.

**Keywords:** Gastric cancer, surgery, neoadjuvant, adjuvant, perioperative, chemotherapy, Western

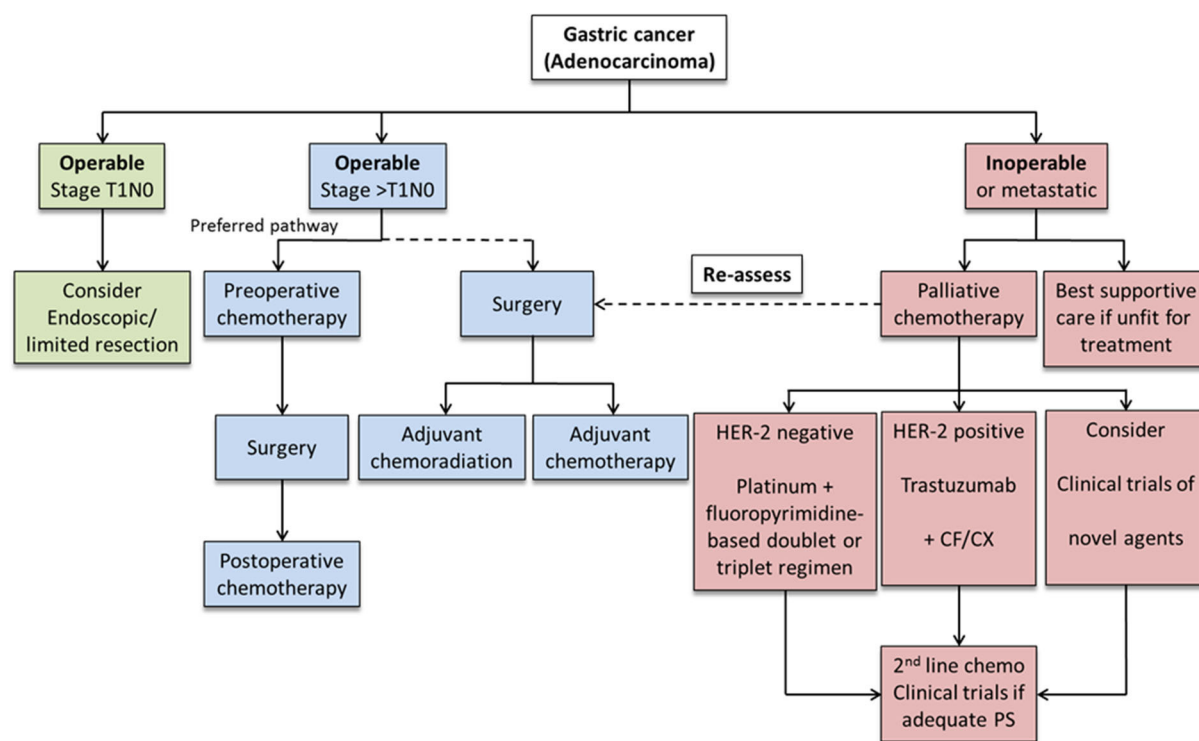
## INTRODUCTION

Recent evidence provided by clinical trials and modern technical developments have strongly facilitated the employment of a multimodal approach in gastric cancer treatment. Endoscopic resection is now accepted as a curative option for early gastric neoplastic lesions<sup>[1,2]</sup>. At the same time, laparoscopic gastrectomy has increased in popularity in recent years<sup>[3,4]</sup>. For locally advanced gastric cancer, radical gastrectomy with D2 lymph node dissection has become the standard surgery in most European high volume centers<sup>[5]</sup>. In addi-



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**Figure 1.** Algorithm for the management of gastric cancer in Europe<sup>[15]</sup>

tion, perioperative chemotherapy (CT) is the standard therapy in curatively intended disease in most European countries<sup>[6-10]</sup>, and also molecular targeted therapy has been implemented in human epidermal growth factor receptor (HER)-2 positive tumors in the palliative setting<sup>[11]</sup>. This review gives an overview of current surgical and perioperative management in curatively intended treatment for localized gastric cancer, as well as palliative management for metastatic disease, in Europe. Furthermore, we discuss recent therapeutic trends and future directions for gastric cancer management in a European setting.

## GASTRIC CANCER IN EUROPE

There were 140,000 new cases of gastric cancer diagnosed across all European countries in 2012<sup>[12]</sup>. Gastric cancer is the sixth most common cancer and the fourth most common cause of cancer related death in Europe, causing 107,000 death annually. The treatment policy in Europe has lately, in several respects, been influenced by the Japanese Guidelines<sup>[13,14]</sup> and this is reflected in most European professional organization guidelines such as those from ESMO/ESSO/ESTRO<sup>[15]</sup>. For planning treatment, ESMO/ESSO/ESTRO guidelines require multi-disciplinary team conferences including surgeons, medical oncologists, gastroenterologists, radiologists, pathologists, dieticians and nurse specialists. Figure 1 shows an algorithm for the management of gastric cancer in Europe.

Gastric cancers in the West tend to have a large proportion of the diffuse type histology, often located in the proximal stomach, compared to typical histology and tumor position in the East, which more commonly tend to be of the intestinal type and typically located in the distal stomach. Furthermore, gastric cancer in Europe is more likely to be diagnosed in advanced stages due to the low incidence and consequential lack of screening programs<sup>[16,17]</sup>. Consequently, due to the difference in disease characteristics, the proportion of total gastrectomies performed is substantially higher in Western treatment populations and studies.

**Table 1. Criteria for endoscopic submucosal dissection<sup>[19]</sup>**

Criteria	Absolute indication		Extended indication		Out of indication
European guidelines	T1a (m)	T1a (m)	T1a (m)	T1a (m)	T1b (sm, < 500 μm)
	< 2 cm	> 2 cm	< 2 cm	< 3 cm	< 3 cm
	Differentiated	Undifferentiated	Differentiated	Differentiated	Differentiated
Japanese guidelines	UI (-)	UI (-)	UI (-)	UI (+)	UI (-)
	T1a (m)	T1a (m)	T1a (m)	T1a (m)	Any submucosal invasion (> T1b)
	< 2 cm	> 2 cm	< 2 cm	< 3 cm	
	Differentiated	Differentiated	Undifferentiated	Differentiated	
	UI (-)	UI (-)	UI (-)	UI (+)	

m: intramucosal; sm: submucosal; UI: ulceration

## MANAGEMENT OF LOCAL/LOCOREGIONAL DISEASE

### Endoscopic treatment

Only around 10%-15% of gastric cancers in Europe are diagnosed as early gastric cancers. Although adoption of endoscopic submucosal dissection (ESD) in the West has been slow, due to a lower incidence of early gastric cancer, European Society of Gastrointestinal Endoscopy (ESGE) guidelines recommend ESD as the treatment of choice for most superficial neoplastic gastric lesions<sup>[18]</sup>. Guidelines from the National Cancer Center in Tokyo have expanded these criteria based on a large number of patients<sup>[2,19]</sup>. ESD should be considered for lesions with very low risk of lymph node metastasis, no matter if it meets the absolute or expanded indication criteria [Table 1]. Western studies have demonstrated an *en-bloc* and R0 resection rate of 98.4% and 90.2%, respectively, which are comparable to corresponding results from Eastern Asian institutions<sup>[20]</sup>. The delayed bleeding rate was 6% and perforation rate was 1% which are also equivalent to Eastern Asian rates<sup>[21-24]</sup>. The potential benefits of ESD are now acknowledged and ESD has become a promising treatment option, alongside conventional endoscopic mucosal resection (EMR), for early gastric cancer in Western countries.

### Surgical treatment

Surgical resection remains the only treatment modality that is potentially curative for locally advanced gastric cancer. However, the extent of surgical resection and lymph node dissection is still, to some degree, controversial. Most European guidelines, nevertheless, recommend D2 dissection for stage II and III disease<sup>[15]</sup>. At the same time, minimally invasive gastrectomy is becoming more and more common<sup>[25]</sup>.

#### *Extent of gastric resection*

The extent of resection is basically determined by the tumor location as well as the tumor stage, the type and extension of stomach resection has a direct impact on patient's postoperative quality of life (QOL)<sup>[26,27]</sup>. In Western, in contrast to Far Eastern countries, most gastric cancers are diagnosed in the proximal stomach as locally advanced tumors, which subsequently usually require total gastrectomy with D2 lymph node dissection for optimized prognosis. Therefore, the number of suitable cases for function preserving surgical techniques, such as proximal and pylorus-preserving gastrectomy, which have been popularized in Eastern Asia due to advantages of improved postoperative QOL, are very few in European populations<sup>[28]</sup>. The vast majority of diagnosed European gastric cancer cases are instead more suitable for subtotal or total gastrectomy. Several studies have shown some functional advantages and comparable overall survival (OS) rate in subtotal gastrectomy compared with total gastrectomy<sup>[26,27,29,30]</sup>. ESMO/ESSO/ESTRO guidelines recommend macroscopic proximal margins of 5 cm between the proximal tumor margin and esophagogastric junction (EGJ) for subtotal or distal gastrectomy, and of 8 cm for the diffuse histological type of gastric cancer<sup>[15]</sup>. Nonetheless, some studies reported equivalence regarding oncological outcome with shorter proximal margin<sup>[31,32]</sup>.



### *Lymph node dissection*

Lymph node dissection is an important part of achieving local tumor control in gastric cancer treatment, and there has been much debate over the years on the optimal extent of this dissection. Traditionally, D2 lymph node dissection has been performed in Japan as standard practice since the 1960s, on the basis of excellent long-term outcomes in Japanese case series<sup>[13]</sup>. In Japan, D2 is the norm, while many surgeons in the West still prefer to perform D1 dissection. One of the reasons is the results of the well-known Dutch randomized clinical trial<sup>[33]</sup>, which compared the survival advantage of D2 lymph node dissection with D1 resection, failing to demonstrate any benefits in D2 group in the main overall survival analysis. However, in this trial the postoperative mortality was very high in the D2 arm, which counterweighed any potential survival advantage of the extended lymph node dissection at 5 years follow-up. A stratified analysis showed that a large proportion of the morbidity and mortality in the D2 group was related to synchronous splenectomy and pancreatectomy while in the subgroup of patients without pancreaticosplenectomy the risk of relapse was significantly lower in the D2 compared to D1 group. However, in 10-year follow-up there was a significant advantage in overall survival for the D2 group<sup>[34]</sup>, despite the great losses in the early postoperative period. This and other publications showing excellent short term outcomes<sup>[35]</sup> after D2 gastrectomy in Western high volume centres has led to the current Western consensus that D2 dissection should be the standard procedure if carried out in specialized, high-volume centers<sup>[5]</sup>.

### *Laparoscopic gastrectomy*

Laparoscopic gastrectomy was launched in 1991 and the first laparoscopic total gastrectomy with D2 lymphadenectomy for advanced gastric cancer was reported in 2000 in Japan<sup>[36,37]</sup>. The clinical objective with this technique was to minimize the surgical access trauma while still providing the same oncological operation, in terms of T- and N-radicality, as open gastrectomy. Advantages suggested and to some extent proven with laparoscopic gastrectomy, compared to open surgery, are less postoperative pain, earlier recovery of bowel function, shorter hospital stay and better cosmetic result<sup>[37-39]</sup>. Furthermore, the concern from sceptics regarding the efficacy of the laparoscopic lymphadenectomy, has been relieved, as the number of harvested lymph nodes has been shown to be comparable to that of open surgery<sup>[40]</sup>. Although laparoscopic distal gastrectomy for early gastric cancer is gradually accepted as an oncologically safe alternative to open gastrectomy in Europe, laparoscopic total gastrectomy and laparoscopic D2 lymph node dissection for advanced cases are still considered challenging, due to their technical nature. With respect to surgical and oncological safety, these procedures should be carefully implemented in experienced hands at centres with high annual caseloads.

## **ADJUNCT THERAPY**

Many clinical phase III trials on adjunct therapy for gastric cancer have been conducted worldwide. Despite high-level evidence supporting the principle of adjuvant or neoadjuvant treatment, there is no standard of care for adjunct treatment in gastric cancer. Main landmark trials are summarized in Table 2. The two major studies of adjunctive therapy in western populations, the North American Intergroup INT0116 trial<sup>[41]</sup>, the MAGIC trial<sup>[42]</sup>, demonstrated two major directions, postoperative chemoradiotherapy (CRT) and perioperative CT. Through many clinical trials, new regimens such as FLOT<sup>[43]</sup>, enhancement of preoperative treatment, and application of molecular targeted therapeutics are attracting much attention.

### **Postoperative chemotherapy and chemoradiotherapy**

The INT0116 trial, the first randomized study evaluating the benefit of adjuvant CRT<sup>[41]</sup>, and a subsequent retrospective Dutch trial demonstrated that postoperative CRT improved OS and reduced local recurrence rates following D1 lymph node dissection or R1 resection<sup>[34]</sup>. Also the additional survival benefit of adjuvant CT has shown by Asian phase III ACTS-GC<sup>[44]</sup> and CLASSIC trial<sup>[45]</sup> in Asian patients. However, the ARTIST trial<sup>[46]</sup>, a phase III trial from Korea, and the recent Dutch CRITICS trial failed to show a survival advantage of postoperative additional radiation therapy to perioperative CT<sup>[47,48]</sup>. In the CRITICS trial, only 47%

**Table 2. Landmark trials of perioperative and palliative chemo/chemoradiotherapy in gastric cancer**

Study name (year)/region	Focus of trial	Treatment arms	Main results (95% CI)	
INT-0116 (2001) <sup>[41]</sup> North America	Adjuvant CRT	Surgery alone Surgery + 5-FU/LV/RT	m-OS: 27 months m-OS: 36 months	HR = 1.35 (1.09-1.66) <i>P</i> = 0.005
ACTS-GC (2007) <sup>[44]</sup> Japan	Adjuvant CT	Surgery alone Surgery + S-1	3-OS: 70.1% 3-OS: 80.1%	HR = 0.68 (0.52-0.87) <i>P</i> = 0.003
CLASSIC (2012) <sup>[45]</sup> South Korea	Adjuvant CT	Surgery alone Surgery + capecitabine/oxaliplatin	3-OS: 59% 3-OS: 74%	HR = 0.56 (0.44-0.72) <i>P</i> < 0.0001
ARTIST (2012) <sup>[46]</sup> South Korea	Adjuvant CRT	Surgery (D2 resection) + XP Surgery (D2 resection) + XP/RT	3-DFS: 74.2% 3-DFS: 78.2%	<i>P</i> = 0.086
ARTIST-II <sup>[49]</sup> South Korea	Adjuvant CRT	Surgery (D2 resection, node-positive) + XP Surgery (D2 resection, node-positive) + XP/RT	In progress	
MAGIC (2006) <sup>[42]</sup> Europe	Perioperative CT	Surgery alone ECF + surgery + ECF	5-OS: 23% 5-OS: 36%	HR = 0.75 (0.60-0.93) <i>P</i> = 0.009
FLOT (2017) <sup>[54]</sup> Germany	Perioperative CT	ECF or ECX + surgery + ECF or ECX FLOT + surgery + FLOT	m-OS: 35 months m-OS: 50 months	HR = 0.77 (0.63-0.94) <i>P</i> = 0.012
CRITICS (2011) <sup>[47,48]</sup> The Netherlands	Perioperative CT plus adjuvant RT	ECX or EOX + surgery + XP/RT ECX or EOX + surgery + ECX or EOX	5-OS: 40.9% 5-OS: 41.3%	<i>P</i> = 0.99
POET (2009) <sup>[55]</sup> Germany	Neoadjuvant CRT	PLF + surgery PLF/RT + surgery	3-OS: 27.7% 3-OS: 47.4%	HR = 0.67 (0.41-1.07) <i>P</i> = 0.07
TOPGEAR (2017) <sup>[57]</sup> Australia/New Zealand/ Europe/Canada	Perioperative CT plus neoadjuvant RT	ECF + surgery + ECF ECF/RT + surgery + ECF	In progress Equivalent in gastrointestinal (32% vs. 30%) and hematological (50% vs. 52%) toxicity	
MAGIC-B <sup>[59]</sup> UK	Perioperative CT plus molecular targeted	ECX + surgery + ECX ECX/lapatinib or bevacizumab + surgery + ECX/lapatinib or bevacizumab	In progress	
INNOVATION (2016) <sup>[58]</sup> Europe	Perioperative CT plus molecular targeted	FP or XP FP or XP/trastuzumab FP or XP/trastuzumab/pertuzumab	In progress	
V-325 (2006) <sup>[65]</sup> Europe	Palliative CT	FP DCF	2-OS: 9% 2-OS: 18%	Severe adverse event: 59% Severe adverse event: 69%
REAL-2 (2008) <sup>[51]</sup> UK	Palliative CT	ECF vs. ECX vs. EOF vs. EOX	m-OS: 9.9 vs. 9.9 vs. 9.3 vs. 11.2 months ( <i>P</i> = 0.02) 1-OS: 37.7% vs. 40.8% vs. 40.4% vs. 46.8%	
ML17032 (2009) <sup>[52]</sup> South Korea	Palliative CT	XP FP	m-OS: 10.5 months m-OS: 9.3 months	HR = 0.85 (0.64-1.13) <i>P</i> = 0.008
German AIO (2011) <sup>[69]</sup> Germany	Palliative CT	BSC Irinotecan	m-OS: 2.4 months m-OS: 4.0 months	Symptom improvement: 7% Symptom improvement: 50%
COUGAR-02 (2014) <sup>[70]</sup> UK	Palliative CT	BSC BSC/docetaxel	m-OS: 3.6 months m-OS: 5.2 months	HR = 0.67 (0.49-0.92) <i>P</i> = 0.01
ToGA (2010) <sup>[11]</sup> South Korea	Palliative CT plus molecular targeted	XP or FP XP or FP/trastuzumab	m-OS: 11.1 months m-OS: 13.8 months	HR = 0.74 (0.60-0.91) <i>P</i> = 0.005
RAINBOW (2014) <sup>[73]</sup> Germany	Palliative CT plus molecular targeted	Paclitaxel Paclitaxel/ramucirumab	m-OS: 7.4 months m-OS: 9.6 months	HR = 0.81 (0.68-0.96) <i>P</i> = 0.017
REGARD (2014) <sup>[74]</sup> USA	Palliative molecular targeted	BSC (placebo) Ramucirumab	m-OS: 3.8 months m-OS: 5.2 months	HR = 0.78 (0.60-1.00) <i>P</i> = 0.047
ATTRACTION (2017) <sup>[75]</sup> South Korea	Palliative molecular targeted	BSC (placebo) Nivolumab	m-OS: 4.1 months m-OS: 5.3 months	HR = 0.63 (0.51-0.78) <i>P</i> < 0.0001

5-FU: fluorouracil; BSC: best supportive care; CRT: chemoradiotherapy; CT: chemotherapy; DCF: docetaxel/cisplatin/fluorouracil; DFS: disease free survival; ECF: epirubicin/cisplatin/fluorouracil; ECX: epirubicin/cisplatin/capecitabine; EOF: epirubicin/oxaliplatin/fluorouracil; EOX: epirubicin/oxaliplatin/capecitabine; FLOT: fluorouracil/leucovorin/oxaliplatin/docetaxel; FP: fluorouracil/cisplatin; HR: hazard ratio; LV: leucovorin; m-OS: median overall survival; OS: overall survival; PLF: cisplatin/leucovorin/fluorouracil; RT: radiotherapy; XP: capecitabine/cisplatin

and 52% of patients completed postoperative CT and CRT therapy, to a large extent due to low postoperative treatment tolerance in Western patients. This study suggested that Western adjunct treatment should shift to

preoperative strategies, considering patients' tolerability for treatment. The subsequent ARTIST-II trial which focused on adjuvant CRT for node-positive patients<sup>[49]</sup> and CRIRTICS-II trial to evaluate the significance of preoperative CRT strategies for curative gastric cancer are now in progress.

### Perioperative chemotherapy

In general, prior to surgery, patients usually tolerate adjunct treatment rather well, perhaps due to an intact performance status. Neoadjuvant chemotherapy has not been shown to increase postoperative morbidity or mortality, while neoadjuvant chemoradiotherapy may be associated to increased morbidity, at least for junctional tumors<sup>[50]</sup>. On this basis, all guidelines recommend this type of down-staging treatment for patients with locally advanced gastric cancer and perioperative therapy has therefore been widely adopted as the standard of care throughout Europe. The MAGIC trial was the first to provide the perioperative therapeutic option for resectable gastric cancer with favorable results of perioperative CT compared with surgery alone<sup>[42]</sup>. However, the epirubicin, cisplatin and fluorouracil (ECF) protocol used in the MAGIC trial has requirements that limit its use in non-trial situations (e.g., the need for a central line and constant specialized handling) and poor postoperative completion rates (42%). The REAL-2 trial showed that the ECF and epirubicin, oxaliplatin, and capecitabine (EOX) regimens were equally effective for advanced tumors, whereas a meta-analysis of the data from the REAL-2 and ML17032 trials suggested better response rates and OS with capecitabine combinations<sup>[51-53]</sup>. EOX regimen is now widely accepted as adjunct treatment in the West.

The recent FLOT4-AIO trial offered a new option with favorable results for locally advanced gastric cancer<sup>[43]</sup>. In this trial, 716 patients who had clinical stage T2 or higher and/or nodal positive disease were randomly assigned to either three pre- and postoperative cycles of epirubicin, cisplatin and either infusion of fluorouracil (5-FU) or capecitabine (ECF/ECX group) or four pre-and postoperative cycles of 5-FU/leucovorin, docetaxel and oxaliplatin (FLOT group). Thirty-five percent of patients in the FLOT group had at least one serious adverse event involving a perioperative medical or surgical complication and 51% had grade 3-4 neutropenia, which was higher than 39% in ECF/ECX group. Overall 5-year survival was 45% in FLOT group, significantly better than the 36% in ECX/ECF group with a hazard ratio (HR) of 0.77 (95% confidence interval 0.63-0.94)<sup>[54]</sup>. FLOT type perioperative chemotherapy can now be considered the Western gold standard regimen in the treatment of locally advanced, non-metastatic gastric cancer.

A number of new clinical trials are in progress to investigate new neoadjuvant and adjuvant regimens to further improve outcomes. The reinforcement of preoperative treatment is one possible future direction. The German POET trial, which aimed to clarify the impact of additional preoperative radiotherapy to neoadjuvant CT for patients with EGJ adenocarcinoma, demonstrated a non-statistically significant improved median survival compared to the CT-alone group<sup>[55]</sup>. However, it showed a substantially higher rate of pathological complete response<sup>[56]</sup>, in the CRT group (15.6% vs. 2.0%). These results emphasized the importance of strengthening the preoperative therapy, and thus neoadjuvant CRT has been suggested to be effective and beneficial. The TOPGEAR trial is currently evaluating the impact of additional preoperative radiotherapy to perioperative CT<sup>[57]</sup>.

Another option for improving surgical outcomes is molecular targeted therapy, which has been demonstrated in the palliative setting. Some molecular targeted agents, such as trastuzumab and lapatinib, are being introduced into perioperative use. The INNOVATION trial, a 3-arm randomized phase II trial evaluating if neoadjuvant dual HER-2 blockade with CT, may lead to higher pathologic complete response rates than trastuzumab and CT, or CT alone, in resectable gastric cancer<sup>[58]</sup>. The MAGIC-B trial is also investigating the additional tyrosine kinase inhibitor lapatinib to perioperative ECX in the subset of the patients with HER2 overexpressing tumors<sup>[59]</sup>. These new studies are expected to provide new, molecularly tailored treatment options.

## EUROPEAN TRENDS IN THE MANAGEMENT OF ADVANCED/METASTATIC GASTRIC CANCER

The aim of palliative CT is to increase survival and palliate the clinical symptoms of the disease, with as little toxicity and negative impact on QOL as possible. Available data from randomized clinical trials clearly show a statistically significant advantage of palliative CT, compared with best supportive care (BSC)<sup>[60]</sup>. To improve the efficacy and to reduce the adverse effects of CT, optimal agents and combinations are currently being sought.

### First line

Historically, doublet regimens using platinum and fluoropyrimidine have been frequently used in palliative setting. Alternative to platinum/ fluoropyrimidine doublet regimen, taxane-based regimen and irinotecan plus 5-FU are suggested<sup>[61]</sup>. Irinotecan and oxaliplatin have shown better tolerability and equivalent time-to-progression in comparison to cisplatin, and guidelines suggest that these agents are promising substitutes for cisplatin in combination with fluoropyrimidines as well as capecitabine for 5-FU within doublet and triplet regimen<sup>[53,62,63]</sup>.

A meta-analysis demonstrated that adding anthracycline to platinum and fluoropyrimidine doublet significantly improved survival<sup>[64]</sup>. Additional docetaxel to 5-FU/CDDP (DCF) is another option to strengthen the CT but careful use is necessary in the palliative setting, due to low margins to toxicity. The V-325 trial and FLOT trial showed the advantage of additional therapeutic effects by taxane-based triplet regimen<sup>[65,66]</sup>. Although DCF in V-325 trial was superior to CF in response rate (RR) (37% *vs.* 25%), time-to-progression (5.6 *vs.* 3.7 months) and 2-year survival rate (18% *vs.* 9%), the absolute benefit in terms of survival was less than 4 weeks and was counterbalanced by a significant high 3-4 adverse events rate. Similarly, FLOT regimen showed improved response rate (49% *vs.* 28%), better progression free survival (PFS) (9.0 *vs.* 7.1 months, *P* = 0.79) and no significant benefit in median OS (17.3 *vs.* 14.5 months, *P* = 0.39). Although there were no differences in serious adverse events, QOL was worse in FLOT group.

These triplet regimens have not demonstrated convincing benefits in terms of survival, but instead increased toxicity rates. Therefore, these regimens are not generally accepted in the palliative setting, so far. The clinical question of which subgroups may be suitable for the stronger triplet regimens, for locally advanced or metastatic disease, is currently under investigation.

### Second line and more

ESMO/ESSO/ESTRO Guidelines recommend the use of irinotecan, docetaxel and paclitaxel as second line therapy, since these agents have shown to improve OS and QOL compared to BSC in patients with a good performance status<sup>[67]</sup>. The European guidelines also stress the fact that both paclitaxel and irinotecan have been directly compared in a Japanese Phase III trial showing similar efficacy in median OS for 8 to 9 months<sup>[68]</sup>. The German AIO phase III study demonstrated superiority of irinotecan compared to BSC in terms of improvement in tumor-related symptoms as second line therapy<sup>[69]</sup>. The COUGAR-02 trial confirmed that docetaxel achieved a significant benefit in OS (5.2 *vs.* 3.6 months, *P* = 0.01) in patients with a performance status of 0-2 after failure of fluoropyrimidine/platinum regimen<sup>[70]</sup>. In spite of the fact that 21% of patients treated with docetaxel experienced grade 4 toxicities, significantly less pain and a trend towards less dysphagia and nausea, were reported. Based on the results of these well conducted randomized trials, a benefit of irinotecan and docetaxel as second-line treatment was clearly established for patients with good performance status. These treatment options should be offered with close monitoring of potential adverse effects.

### Palliative radiation therapy

Guidelines mention that hypo-fractionated radiotherapy is an effective and well-tolerated treatment option for symptomatic locally advanced or recurrent disease<sup>[71]</sup>. In non-comparative observational studies, the

overall response rates for bleeding, pain and obstruction symptoms were 74%, 67% and 68% respectively, low biological equivalent dose of > 39 Gy regimens appear to be adequate for symptom palliation<sup>[72]</sup>.

## TARGETED THERAPIES

As in other solid organ tumors, the biological abnormalities triggering the development and progression of gastric cancer are increasingly elucidated through ongoing research. These findings have potentially important implications as investigators attempt to elucidate the key pathways driving the tumor in each individual patient.

Overexpression of the HER2 gene, which is present in approximately 10%-20% of gastric cancers, is more common in intestinal type than diffuse type gastric cancer and more common in EGJ cancer than distal gastric cancer. Following the phase III ToGA trial, which demonstrated statistically significant improvement in PFS and OS with the addition of trastuzumab to a cisplatin/5-FU doublet regimen, trastuzumab was licensed in Europe for use in HER-2 positive disease in combination with capecitabine or 5-FU and cisplatin doublet<sup>[11]</sup>. This regimen currently represents the standard of care for these palliative patients. Also, the large phase III RAINBOW trial and REGARD trial, have shown the survival benefit of ramucirumab, a monoclonal antibody VEGFR-2 antagonist, as second line in the palliative setting<sup>[73,74]</sup>. Moreover, nivolumab, a fully human IgG4 monoclonal antibody inhibitor of programmed death-1, significantly improved the median overall survival in patients with advanced gastric cancer, or gastro-esophageal junction cancer, who had been previously treated with two or more chemotherapy regimens (ATTRACTION-2 trial)<sup>[75]</sup>. Several studies targeting HER2, VEGF, EGFR, T-DM1 are currently ongoing with some potentially favorable results<sup>[76-80]</sup>. Further developments in molecular subtyping of gastric cancer are likely to offer new possibilities in personalized treatment of gastric cancer in the future<sup>[81-84]</sup>.

## CONCLUSION

It is clear that the multimodal therapy encompassing both radical surgical treatment and perioperative CT/CRT offers the best possibility to cure resectable gastric cancer. In Western countries, minimally invasive approaches and D2 dissection have been successfully implemented at some high-volume centers. However, these procedures are still not standardized in the whole population-based case-load of incident cases, due to the low incidence of gastric cancer in Europe and other Western populations. Although the prospective clinical trials performed have achieved clear improvements in the therapeutic outcomes and patients' prognosis in the last decades, an optimal treatment for advanced gastric cancer has not been established, given the still poor overall survival. Recent advances in molecular tumor biology of adenocarcinoma of the stomach offer us important clues about future tailoring of gastric cancer treatment. Furthermore the rapid developments in sequencing techniques are likely to revolutionize our understanding of disease biology in the next decades. It is very likely that a number of new biomarkers will provide completely new options for personalized therapy, which may realize substantial therapeutic improvements, with excellent efficacy and tolerable adverse effects.

## DECLARATIONS

### Authors' contributions

Designed the study, reviewed the literature, and wrote the manuscript: Kamiya S

Contributed to writing the manuscript, drafting, critical revision, editing, and final approval of the final version: Nilsson M

Contributed to critical revision of the manuscript and final approval of the final version: Rouvelas I, Lindblad M

### Availability of data and materials

Not applicable.



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## Conflicts of interest

All authors declare that there are no conflicts of interest.

## Ethical approval and consent to participate

Not applicable.

## Consent for publication

Not applicable.

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Review

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# Circulating microRNAs as a liquid biopsy: a next-generation clinical biomarker for diagnosis of gastric cancer

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## Abstract

Accumulating evidence has suggested the potential clinical utility of novel body fluid biomarkers, or “liquid biopsy”, using circulating tumor cells and cell-free nucleic acids from cancer patients. Noninvasive and reproducible, liquid biopsy could provide the basis for individualized therapeutic strategies by identifying genetic and epigenetic aberrations that are closely associated with cancer initiation and progression. MicroRNAs (miRNAs) are short noncoding RNAs that post-transcriptionally regulate gene expression. They also play important roles in various physiological and developmental processes as oncogenic or tumor-suppressive regulators. Specific miRNA expression signatures have been identified in a number of human cancers. Circulating miRNAs have been detected in plasma and serum, and this in blood has attracted the attention of researchers for their potential as noninvasive biomarkers. Circulating miRNAs have emerged as tumor-associated biomarkers that reflect not only the existence of cancer, but also the dynamics, malignant potential, and drug resistance of tumors. Herein, we review the recent biological and clinical research on the circulating miRNAs of gastric cancer and discuss future perspectives for their clinical applications as a liquid biopsy.

**Keywords:** Liquid biopsy, circulating nucleic acids, circulating microRNA, biomarker, gastric cancer

## INTRODUCTION

Gastric cancer is third-leading cause of death among all cancers worldwide<sup>[1]</sup>. While improved perioperative management and diagnostic techniques have boosted early detection and decreased mortality in recent years,



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gastric cancer continues to constitute a global health problem as a prevalent form of cancer<sup>[1]</sup>. Gastric cancer patients at advanced stages of the disease have a very poor prognosis<sup>[2]</sup>. Despite these continued difficulties, no biomarker molecule has been employed for the early diagnosis of gastric cancer in clinical settings, and researchers have validated only a scant number of molecules as therapeutic targets<sup>[3-7]</sup>. Therefore, for gastric cancer, identifying novel molecular targets and clinical biomarkers remain vital clinical challenges.

Recently, the concept of a “liquid biopsy” has become widely accepted in the clinical setting. Liquid biopsy is a less approaches for obtaining genetic and epigenetic aberrations that are closely associated with cancer initiation and progression<sup>[8]</sup>. Moreover, liquid approaches allows for repeated sampling and this makes it possible to evaluate the longitudinal evolution of a tumor and its heterogeneous characteristics, which single sampling may fail to capture<sup>[9-13]</sup>. Understanding circulating tumor cells and cell-free nucleic acids in cancer patients may bring new insights into prognostic and predictive value of liquid biopsy. In this article, we review recent research on the circulating miRNAs of gastric cancers, and discuss future perspectives on next-generation clinical biomarkers and treatment targets in gastric cancer.

## THE MOLECULAR FEATURES AND BIOLOGICAL SIGNIFICANCE OF MICRORNAS

Small noncoding RNAs known as microRNAs (miRNAs) regulate how specific protein-coding genes are translated. After miRNAs were discovered in 1993<sup>[14]</sup>, researchers have correlated changes in miRNA expression with diseases progression in multiple forms of cancer<sup>[15-18]</sup>. Numerous recent studies have detailed how miRNAs can be detected in plasma/serum while keeping their impressive stability<sup>[16,19-22]</sup>. Plasma/serum miRNAs resist endogenous ribonuclease activity through binding with plasma proteins such as Argonaute 2 and high-density lipoprotein (HDL)<sup>[23,24]</sup> or being surrounded by different secretory vesicles, including plasma/serum exosomes and apoptotic bodies<sup>[19,25-27]</sup>. Thus, miRNAs in peripheral blood are not digested by RNase or damaged by other conditions such as low or high pH, extended storage, boiling, and multiple freeze-thaw cycles. In addition, numerous extracellular miRNAs are made present by active secretion in addition to cell lysis<sup>[10,28,29]</sup>; such miRNAs are able to play a role as intercellular transmitters<sup>[22,28,30,31]</sup>. As one possible mechanism, the extracellular miRNAs involved in exosome vesicles has been reported to be released through ceramide-dependent secretory systems and function in recipient cells<sup>[29]</sup>.

## CIRCULATING MICRORNAS ARE A PROMISING SOURCE OF DIAGNOSTIC AND PROGNOSTIC INFORMATION IN SOLID TUMORS

Mitchell *et al.*<sup>[19]</sup> first reported that circulating miRNAs had potential utility as new biomarkers in patients with solid cancers. As noninvasive and reproducible biomarkers in cancer patients, circulating miRNAs have since attracted the attention of researchers. As indicated by the usefulness of cell-free DNA and circulating tumor cells, the concept of “liquid biopsy” through circulating miRNAs may also provide ideal individualized therapeutic strategies for cancer patients and contribute to the development of precision medicine. Indeed, previous studies, including our own, have identified various blood-based miRNA biomarker candidates, which are useful for cancer detection, monitoring tumor dynamics, and predicting malignant potential, prognosis, and chemoresistance in cancer patients<sup>[32-45]</sup>.

## HIGH LEVELS OF CIRCULATING MICRORNAS IN PLASMA/SERUM IN GASTRIC CANCER

Various studies have identified circulating miRNAs for use in the diagnosis and prognosis of gastric cancer patients [Table 1]. In 2010, we reported the usefulness of circulating miRNAs and demonstrated their feasibility as biomarkers in the plasma of patients with gastric cancer. We selected four miRNAs (miR-17-5p, 21, 106a, and 106b) that has been previously reported as upregulated in gastric cancer tissues, analyzed their levels in plasma using RT-qPCR, and confirmed their utility as diagnostic biomarkers<sup>[32]</sup>. We then identified plasma miR-451 and miR-486 as novel cancer screening markers using the Toray® 3D-Gene microRNA

**Table 1. High level of circulating microRNAs in plasma/serum in gastric cancer**

miR	Sample	Ethnicity	Gastric cancer patients	Controls	Value	Ref.
miR-16	Plasma	China	200	200	D	Zhu <i>et al.</i> 2014 <sup>[55]</sup>
		China	50	47	D	Wang <i>et al.</i> 2014 <sup>[69]</sup>
		China	155	111	D	Zhang <i>et al.</i> 2015 <sup>[58]</sup>
miR-17-5p	Plasma	Japan	69	30	D	Tsujiura <i>et al.</i> 2010 <sup>[32]</sup>
		China	65	NA	P, M	Wang <i>et al.</i> 2012 <sup>[70]</sup>
	All blood	China	90	27	D	Zhou <i>et al.</i> 2010 <sup>[71]</sup>
miR-18a	Plasma	Japan	104	65	D, M	Tsujiura <i>et al.</i> 2015 <sup>[40]</sup>
miR-19b	Plasma	China	155	111	D	Zhang <i>et al.</i> 2015 <sup>[58]</sup>
miR-20a	Plasma	China	65	NA	P, M	Wang <i>et al.</i> 2012 <sup>[70]</sup>
		China	60	60	D	Cai <i>et al.</i> 2013 <sup>[72]</sup>
		China	101	91	D	Zhou <i>et al.</i> 2015 <sup>[73]</sup>
	Serum	China	55	55 (post-operative)	P, M	Yang <i>et al.</i> 2017 <sup>[74]</sup>
miR-21	Plasma	Japan	69	30	D, P	Tsujiura <i>et al.</i> 2010 <sup>[32]</sup> Komatsu <i>et al.</i> 2013 <sup>[54]</sup>
		China	70	70	D	Li <i>et al.</i> 2012 <sup>[75]</sup>
		China	53	20	D	Zheng <i>et al.</i> 2012 <sup>[48]</sup>
		China	174	39	D	Wang <i>et al.</i> 2012 <sup>[76]</sup>
		Japan	87	114	D	Shiotani <i>et al.</i> 2013 <sup>[77]</sup>
		China	103	NA	M	Song <i>et al.</i> 2013 <sup>[78]</sup>
		China	50	50	D	Wu <i>et al.</i> 2015 <sup>[57]</sup>
		China	92	89	D	Huang <i>et al.</i> 2016 <sup>[79]</sup>
		Poland	20	20	D	Sierzega <i>et al.</i> 2017 <sup>[80]</sup>
		China	138	50	D, P	Zhuang <i>et al.</i> 2016 <sup>[81]</sup>
miR-23b	Plasma	China	138	50	D, P	Zhu <i>et al.</i> 2014 <sup>[55]</sup>
miR-25	Plasma	China	200	200	D	Zhu <i>et al.</i> 2014 <sup>[55]</sup>
		China	20	20	D, P	Zhang <i>et al.</i> 2014 <sup>[82]</sup>
		China	101	91	D	Zhou <i>et al.</i> 2015 <sup>[73]</sup>
		China	65	65	D	Li <i>et al.</i> 2017 <sup>[83]</sup>
miR-92a	Plasma	China	200	200	D	Zhu <i>et al.</i> 2014 <sup>[55]</sup>
miR-92b	Plasma	China	101	91	D	Zhou <i>et al.</i> 2015 <sup>[73]</sup>
miR-93	Plasma	China	65	65	D	Li <i>et al.</i> 2017 <sup>[83]</sup>
		China	20	20	D, P	Zhang <i>et al.</i> 2014 <sup>[82]</sup>
miR-100	Serum	China	50	47	D	Wang <i>et al.</i> 2014 <sup>[69]</sup>
miR-106	Serum	Japan	87	114	D	Shiotani <i>et al.</i> 2013 <sup>[77]</sup>
		China	118 (with chemotherapy)	20 (without chemotherapy)	P	Song <i>et al.</i> 2017 <sup>[84]</sup>
miR-106a	Plasma	Japan	69	30	D	Tsujiura <i>et al.</i> 2010 <sup>[32]</sup>
	All blood	China	90	27	D	Zhou <i>et al.</i> 2010 <sup>[71]</sup>
miR-106b	Plasma	Japan	69	30	D	Tsujiura <i>et al.</i> 2010 <sup>[32]</sup>
		China	60	60	D	Cai <i>et al.</i> 2013 <sup>[72]</sup>
		China	20	20	D, P	Zhang <i>et al.</i> 2014 <sup>[82]</sup>
		China	65	65	D	Li <i>et al.</i> 2017 <sup>[83]</sup>
		Iran	36	36	D	Ayremlo <i>et al.</i> 2015 <sup>[84]</sup>
miR-107	Serum	Iran	36	36	D	Ayremlo <i>et al.</i> 2015 <sup>[84]</sup>
miR-181c	Plasma	China	30	60 (30 gastric ulcer and 30 gastritis)	D	Cui <i>et al.</i> 2013 <sup>[85]</sup>
miR-185	Plasma	China	101	91	D	Zhou <i>et al.</i> 2015 <sup>[73]</sup>
miR-191	Serum	China	57	58	D	Peng <i>et al.</i> 2014 <sup>[86]</sup>
miR-192	Plasma	China	96	36	D	Chen <i>et al.</i> 2014 <sup>[52]</sup>
miR-199a-3p	Plasma	China	230	130	D	Li <i>et al.</i> 2013 <sup>[51,87]</sup>
miR-200c	All blood	Spain	52	15	D, P	Valladares-Ayerbes <i>et al.</i> 2012 <sup>[49]</sup>
	Serum	China	98	100	P	Zhang <i>et al.</i> 2015 <sup>[88]</sup>
miR-210	Plasma	China	101	91	D	Zhou <i>et al.</i> 2015 <sup>[73]</sup>
miR-221	Plasma	China	60	60	D	Cai <i>et al.</i> 2013 <sup>[72]</sup>
miR-222	Plasma	China	114	56	D, P	Fu <i>et al.</i> 2014 <sup>[53]</sup>
miR-223	Plasma	China	70	70	D	Li <i>et al.</i> 2012 <sup>[75]</sup>
	Serum	China	50	47	D	Wang <i>et al.</i> 2014 <sup>[69]</sup>
miR-331	Serum	Poland	20	20	D	Sierzega <i>et al.</i> 2017 <sup>[80]</sup>
miR-370	Plasma	Taiwan	40	12	D	Lo <i>et al.</i> 2012 <sup>[89]</sup>
miR-378	Serum	China	40	41	D	Liu <i>et al.</i> 2012 <sup>[47]</sup>

miR-421	Serum	China	90	90	D	Wu <i>et al.</i> 2015 <sup>[50]</sup>
miR-451	Plasma	Japan	56	30	D	Konishi <i>et al.</i> 2012 <sup>[46]</sup>
		China	200	200	D	Zhu <i>et al.</i> 2014 <sup>[55]</sup>
miR-486-5p	Plasma	Japan	56	30	D	Konishi <i>et al.</i> 2012 <sup>[46]</sup>
		China	200	200	D	Zhu <i>et al.</i> 2014 <sup>[55]</sup>
miR-664	Serum	China	118 (with chemotherapy)	20 (without chemotherapy)	P, M	Song <i>et al.</i> 2017 <sup>[84]</sup>

D: diagnostic value; P: prognostic value; M: monitoring value

array-based approach on pre- and postoperative samples<sup>[46]</sup>. The area under the curve (AUC) values for these markers were high, at 0.96 and 0.92, respectively for the diagnosis of gastric cancer<sup>[46]</sup>. Additionally, genome-wide miRNA expression profiles followed by RT-qPCR assays revealed that circulating miR-378 had an AUC of 0.861 with 87.5% sensitivity and 70.73% specificity<sup>[47]</sup>. As shown in Table 1, many circulating miRNAs have been previously identified (by our group and others) as promising blood biomarker candidates for the detection of gastric cancer: miR-16, miR-17-5p, miR-18a, miR-19b, miR-20a, miR-21, miR-23b, miR-25, miR-92a, miR-92b, miR-93, miR-100, miR-106, miR-106a, miR-106b, miR-107, miR181c, miR-185, miR-191, miR-192, miR-199a-3p, miR-200c, miR-210, miR-221, miR-222, miR-223, miR-331, miR-370, miR-378, miR-421, miR-451, miR-486-5p, and miR-664, all of which are up-regulated in plasma/serum. These are promising diagnostic biomarkers<sup>[32,40,46-55,57,58,69-89]</sup>.

### LOW LEVEL OF CIRCULATING MICRORNAS IN PLASMA/SERUM IN GASTRIC CANCER

Kosaka *et al.*<sup>[29,59,60]</sup> recently suggested that healthy cells secrete some tumor-suppressor miRNAs as a way of slowing aberrant cell growth. We have previously found that blood-borne tumor-suppressor miRNAs, such as let-7a<sup>[32]</sup> and miR-375<sup>[35,45]</sup> were significantly downregulated in comparison to those of normal volunteers. Circulating miRNAs are released from both normal and cancer tissues, and the majority of these tumor-suppressor miRNAs are thought to arise from normal tissues. We therefore hypothesize that the progression of cancer causes healthy cells to become depleted of some tumor-suppressor miRNAs. That hypothesis is supported by our previously data that shows that a decrease in the plasma level of the tumor-suppressor miR-375 in esophageal cancer patients<sup>[34]</sup> and this<sup>[61]</sup> is correlated with reduced survival. We have also proposed that tumor progression and the resultant poor prognostic outcomes are correlated with the downregulation of tumor-suppressor miRNAs in the bloodstream<sup>[34,35]</sup>. As shown in Table 2, various circulating tumor-suppressor miRNAs have previously been identified as promising blood biomarker candidates for the detection and diagnosis of gastric cancer. These include miR-15a, miR-17, miR-26a, miR-31, miR-92a, miR-93, miR-106b, miR-122, miR-181b, miR-195-5p, miR-203, miR-204, miR-206, miR-218, miR-375, miR-503, miR-940, and let-7a, which are downregulated in plasma/serum with a great degree of diagnostic ability<sup>[32,52,56,62,75,79,84,90-100]</sup>.

### CIRCULATING MICRORNAS RELATED TO MALIGNANT POTENTIAL, TUMOR RECURRENCE, AND PROGNOSIS BIOMARKERS IN PLASMA/SERUM IN GASTRIC CANCER

Wang *et al.*<sup>[70]</sup> have reported that high levels of plasma miR-17-5p and miR-20a were significantly correlated with poor overall survival in gastric cancer patients. Valladares-Ayerbes *et al.*<sup>[49]</sup> have also reported that higher expression levels of miR-200c in blood are associated with poor overall survival. We demonstrated that the postoperative cause-specific survival was significantly poorer in gastric cancer patients with high plasma miR-21 levels than in those with low levels<sup>[54]</sup>. Moreover, the incidence of vascular invasion was also slightly higher in gastric cancer patients with high miR-21 levels, and multivariate analysis revealed that the presence of high miR-21 plasma levels was an independent prognostic factor<sup>[54]</sup>. Therefore, various up-regulated circulating miRNAs have previously been identified as blood-based prognostic biomarkers for gastric cancer: miR-17-5p, miR-20a, miR-21, miR-23b, miR-25, miR-93, miR-106, miR-106b, miR-200c, miR-222, and miR-664 [Table 1]<sup>[49,53,54,70,74,81,82,84,88]</sup>.

**Table 2. Low level of circulating microRNAs in plasma/serum in gastric cancer**

miR	Sample	Ethnicity	Gastric cancer patients	Control	Value	Ref.
miR-15a	Serum	China	118 (with chemotherapy)	20 (without chemotherapy)	P	Song <i>et al.</i> 2017 <sup>[90]</sup>
miR-17	Serum	China	40	36	D	Zeng <i>et al.</i> 2014 <sup>[91]</sup>
miR-26a	Plasma	China	285	285	D	Qiu <i>et al.</i> 2016 <sup>[92]</sup>
miR-31	Serum	China	92	89	D	Huang <i>et al.</i> 2016 <sup>[79]</sup>
miR-92a	Serum	China	92	89	D	Huang <i>et al.</i> 2016 <sup>[79]</sup>
miR-93	Serum	China	118 (with chemotherapy)	20 (without chemotherapy)	P	Song <i>et al.</i> 2017 <sup>[84]</sup>
miR-106b	Serum	China	40	36	D	Zeng <i>et al.</i> 2014 <sup>[91]</sup>
miR-122	Plasma	China	96	36	D	Chen <i>et al.</i> 2014 <sup>[52]</sup>
miR-181b	Serum	China	92	89	D	Huang <i>et al.</i> 2016 <sup>[79]</sup>
miR-195-5p	Serum	China	62	36	D, P	Shen <i>et al.</i> 2016 <sup>[93]</sup>
	Plasma	Turkey	20	190	D	Gorur <i>et al.</i> 2013 <sup>[94]</sup>
miR-203	Serum	China	92	89	D	Huang <i>et al.</i> 2016 <sup>[79]</sup>
		Japan	130	22	P, M	Imaoka <i>et al.</i> 2016 <sup>[62]</sup>
miR-204	Serum	China	115	40	P, M	Chen <i>et al.</i> 2016 <sup>[95]</sup>
miR-206	Serum	China	150	150	D	Hou <i>et al.</i> 2016 <sup>[96]</sup>
miR-218	Plasma	China	70	70	D	Li <i>et al.</i> 2012 <sup>[75]</sup>
	Serum	China	68	56	P	Xin <i>et al.</i> 2014 <sup>[97]</sup>
miR-375	Serum	China	NA	NA	D	Zhang <i>et al.</i> 2012 <sup>[98]</sup>
miR-503	Serum	China	68	32	D, P	Wu <i>et al.</i> 2016 <sup>[99]</sup>
miR-940	Plasma	China	110	30	D	Liu <i>et al.</i> 2016 <sup>[56]</sup>
let-7a	Plasma	Japan	69	30	D	Tsujiura <i>et al.</i> 2010 <sup>[32]</sup>
	Serum	China	80	NA	D	Wang <i>et al.</i> 2013 <sup>[100]</sup>

D: diagnostic value; P: prognostic value; M: monitoring value

Regarding tumor-suppressor miRNAs, Imaoka *et al.*<sup>[62]</sup> reported that serum expression of miR-203 was significantly lower in stage IV than in stages I-III of gastric cancer patients. Serum miR-203 expression was significantly lower in gastric cancer patients with worse malignant potential, as indicated by higher T stage, vessel invasion, and nodal, peritoneal, and distant metastases. Low expression of serum miR-203 was correlated with poor disease-free survival and overall survival. This low expression was an independent predictive marker for metastases, including nodal, peritoneal, and distant metastases, and a poor prognosis in gastric cancer patients<sup>[62]</sup>. Therefore, various downregulated circulating miRNAs have been identified as blood-based prognostic biomarkers for gastric cancer: miR-15a, miR-93, miR-195-5p, miR-203, miR-204, miR-218 and miR-503 [Table 2]<sup>[62,84,90,93,95,97,99]</sup>.

## DIFFERENT EXPRESSION LEVELS OF SOME CIRCULATING MIRNAS BETWEEN PLASMA AND SERUM IN GASTRIC CANCER

From the viewpoint of liquid biopsy using blood miRNAs, many issues must still be addressed before novel findings can be translated into clinically useful and noninvasive screening strategies for gastric cancer patients. Because plasma includes more abundant proteins, such as coagulation factors, than does serum, miRNA profiles in the plasma of cancer patients differ considerably from those in the serum<sup>[63]</sup>, as has been shown in esophageal cancer<sup>[37,64]</sup> and pancreatic cancer<sup>[63]</sup>. In gastric cancer, the expression levels of some circulating miRNAs, such as miR-17, miR-92a, miR-93, and miR-106b, moved in opposite directions in the plasma and serum [Tables 1 and 2]. Although detailed mechanisms remain unknown, the data strongly suggest that these issues should be considered in future clinical applications of cancer treatments.

## FUTURE PERSPECTIVES ON CIRCULATING TUMOR-SUPPRESSOR MICRORNAS FOR TREATMENT TARGETS IN GASTRIC CANCER

Multiple researchers have recently examined therapeutic miRNA-based drugs by using synthetic miRNA

mimics<sup>[101]</sup>. Various efforts have been made to develop miRNA-based therapies in the past several years, and two studies have shown particular promise. The first study focused on the therapeutic silencing of disease-associated miRNAs using miRNA inhibitors. Miravirsen (Santaris Pharma) is one of several promising miRNA inhibitors; it can bind to miR-122 and inhibit its biogenesis. Miravirsen was developed for the treatment of hepatitis C and is currently under evaluation in clinical trials<sup>[65-67]</sup>. The second study examined therapeutic miRNA-based drugs through the use of synthetic miRNA mimics. Recently, a phase I clinical trial using the miRNA mimic MIRX34 (Mirna Therapeutics, Inc.) was performed<sup>[68]</sup>. MIRX34 is a synthetic miRNA mimic of the tumor suppressor miR-34 and was administered to patients with primary or metastatic liver cancer. This trial was ended because of serious adverse immune-related effects. The administration of tumor-suppressor miRNA mimics continues to bear undesirable risks and negative, unexpected physiological effects because multiple genes, regulating multiple biological functions, can be impacted by miRNAs. Restoring tumor-suppressor miRNAs, which are abundantly detected in the plasma/serum of healthy individuals but lowered in patients with cancer [Table 2], may minimize the physiological risks of systemic administration. We recently reported that restoring and maintaining the miR-107 plasma level significantly inhibited tumor progression in mice<sup>[61]</sup>. The systemic delivery of tumor-suppressor miRNAs in gastric cancer patients may thus provide significant advantages because effects can be repeatedly examined repeatedly using blood-based miRNA levels.

## CONCLUSION

The development of liquid biopsy-based analyses could improve diagnosis and therapy for patients with gastric cancer. As a liquid biopsy, circulating miRNAs have the potential to diagnose gastric cancer at an early stage, predict prognosis and recurrence, evaluate patient status and therapeutic efficacy, and provide optimal, individualized treatment strategies. It should be noted that the present review is limited by examining a relatively small number of retrospective cohort studies. Additional research with large cohorts or prospective clinical trials with longer follow-up periods are therefore necessary to confirm the usefulness of candidate miRNAs. Translation into clinically useful gastric cancer treatments also requires significant additional work. The physiological effects of tumor-suppressor miRNAs must be examined in greater detail before they can be safely administered systemically, and their tumor-suppressive functions must be validated *in vivo* before clinical use. Delivery systems for miRNAs must be further refined to surmount problems such as cellular uptake and bloodstream stability. Finally, more powerful anticancer tumor-suppressor miRNAs should be found by examining the plasma of patients with different cancers, through methods such as microarray analysis, next-generation sequencing, and digital PCR-based approaches. Currently under evaluation, these strategies will likely provide the future's next innovations.

## DECLARATIONS

### Authors' contributions

Designed the research and wrote the paper: Komatsu S

Collected the data and performed data analyses: Komatsu S, Kiuchi J, Imamura T

Reviewed the paper: Komatsu S, Ichikawa D, Otsuji E

### Availability of data and materials

Not applicable.

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None.

### Conflict of interest

All authors declared that there are no conflicts of interest.



**Ethical approval and consent to participate**

Not applicable.

**Consent for publication**

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Original Article

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# New insights into the role of intra-tumor genetic heterogeneity in carcinogenesis: identification of complex single gene variance within tumors

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## Abstract

**Aim:** Present cancer hypotheses are almost all based on the concept that accumulation of specific driver gene mutations cause carcinogenesis. The discovery of intra-tumor genetic heterogeneity (ITGH), has resulted in this hypothesis being modified by assuming that most of these ITGH mutations are in passenger genes. In addition, accumulating ITGH data on driver gene mutations have revealed considerable genotype/phenotype disconnects. This study proposes to investigate this disconnect by examining the nature and degree of ITGH in breast tumors.

**Methods:** ITGH was examined in tumors using next generation sequencing of up to 68,000 reads and analysis tools that allowed for identification of distinct minority variants within single genes, i.e., complex single gene variance (CSGV).

**Results:** CSGV was identified in the androgen receptor genes in all breast tumors examined.

**Conclusion:** Evidence of CSGV suggests that a selection - as opposed to a mutation - centric hypothesis could better explain carcinogenesis. Our hypothesis proposes that tumors develop by the selection of preexisting *de novo* mutations rather than just the accumulation of *de novo* mutations. Thus, the role of selection pressures, such as changes in tissue microenvironments will likely be critical to our understanding of tumor resistance as well as the development of more effective treatment protocols.



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**Keywords:** Intra-tumor genetic heterogeneity, breast cancer, complex single gene variance

## INTRODUCTION

### Current carcinogenesis hypotheses

The traditional understanding of carcinogenesis, that cancer cells accumulate somatic driver mutations that give them a growth advantage<sup>[1]</sup> is beginning to be questioned as data reveal the presence of driver gene mutations involved in carcinogenesis in normal tissues<sup>[2]</sup>. Further, a critical issue still to be elucidated is how these mutations create a gain-of-function in cells that results in them acquiring new oncogenic properties, rather than just the loss-of-function of factors that control cell growth and division. One indication as to why these properties might be more complicated than a simple case of excessive or distorted growth is that cancer genes are generally not over-expressed in the tissues from which the cancer develops<sup>[3]</sup>. For example, out of 130 highly specific-cancer genes only four are most highly expressed in the tissue from which the cancer originates<sup>[3]</sup>. Thus, other factors besides protein accumulation are likely to be involved. Compounding this conundrum is the observation that there are often different mutations in different cancer-associated genes in different cancer tissues<sup>[1]</sup>. Raising the question as to how these differences are related to the tissue specificity of certain cancer mutations.

Further, in a recent study looking for associations between specific cancer genes and specific cancer tissues some genes did not behave as expected<sup>[1]</sup>. The analyses suggested that both cell-intrinsic (i.e., genomic and epigenetic) and cell-extrinsic (i.e., environmental, both internal and external) factors could explain the differences in the cell type-specificity of cancer genes. For example, in breast cancer, specific external environmental factors have included estrogen receptor alpha (ER) activation by estradiol<sup>[4]</sup> and conversion of estrogen into genotoxic metabolites that can cause DNA double-strand breaks<sup>[5]</sup>. However, in most cases it has not been possible to associate any specific intrinsic or extrinsic factor with cancer tissue specificity. Underlying these fundamental questions is a growing awareness of substantial amounts of genetic heterogeneity not only within different types of cancer tissues<sup>[6]</sup>, but within single tumor cancer tissues as well. These latter observations have been labelled as intra-tumor genetic heterogeneity (ITGH)<sup>[7]</sup>.

### Intra-tumor genetic heterogeneity

ITGH identified within breast tumors, has revealed numerous alterations in different genes, with the assumption that most mutations are in “passenger” genes<sup>[8]</sup>, including studies using single cell sequencing techniques<sup>[9]</sup>. However, such studies have also not drawn many definitive conclusions as to precise roles of many of the “driver” genes in carcinogenesis. Genes being identified as drivers: (1) if they are either oncogenes or tumor suppressor genes; (2) if they function in some aspect of cell growth; (3) if their location are close to any of these types of genes<sup>[10]</sup>. Further, a recent paper noted that passenger genes can also have damaging effects on cancer progression<sup>[11]</sup>.

We believe this confusion is partly because of a failure to investigate the nature and degree of genetic heterogeneity within single genes, a condition that we have labelled, complex single gene variance (CSGV), as opposed to just identifying mutations in different cancer-associated genes. Why this is important is that as natural selection is being increasingly identified as a critical process in cancer biology<sup>[12]</sup>, there needs to be a better understanding of the nature of the genetic variation that is being subjected to selection.

### Identification of single gene genetic heterogeneity

The question as to why genetic heterogeneity within individual genes has not been studied before is partially because the approach to identifying gene variants is based on using sequence analysis algorithms and tools that make it inherently difficult to identify CSGV. Essentially, they are designed to ignore or minimize the

possibility that different mutations of an individual gene can exist in a single person's tissues. The assumption being that finding multiple variants of a single gene within an individual's tissues is highly unlikely and therefore if identified is likely the result of either PCR or sequencing errors. Indeed, almost all NGS analyses rely on the use of filters and other techniques such as sequence alignment tools to remove such variants<sup>[13]</sup>. These techniques further reduce the possibility of finding multiple mutations within an individual gene, as some are likely to be at very low frequencies, and will be present in only a small minority of cells within an individual tumor, as noted in a recent review of post-zygotic somatic mosaicism<sup>[14]</sup>. Therefore, one of the challenges of the study was to develop a sequencing analysis approach that allows for the identification of CSGV. Further, an important practical consideration for identifying CSGV is that it is increasingly becoming apparent that every driver gene mutation does not produce a cancer phenotype, with some driver mutations even being present in non-cancer tissues<sup>[15,16]</sup>. In the present study, we have used a sequencing approach that makes it easier to detect multiple mutations of the androgen receptor gene (*AR*) within individual breast tumors.

### Androgen receptor and breast cancer

In the case of breast cancer (BC), the *AR* is more widely expressed than either estrogen receptor (ER) alpha or progesterone receptor (PR) genes, and so it is not surprising that the *AR* has become a significant marker in defining BC subtypes<sup>[17]</sup>. The *AR* has therefore started to be singled out as a possible therapeutic target, particularly in triple-negative [ER-/PR-/herceptin receptor (HER) 2-] BC (TNBC)<sup>[18,19]</sup>. Indeed, a large cohort study reported *AR* expression in 32% of TNBC cases<sup>[20]</sup>. In another study examining cases of ER-positive breast carcinoma, tumor cells changed after treatment from ER-dependent to AR-dependent, possibly explaining why such cells become resistant to aromatase inhibitor treatment<sup>[21]</sup>. At present, most studies have focused on *AR* expression during different BC stages, and, indeed, *AR* expression has been identified as a possible critical marker in predicting BC survival<sup>[22]</sup>. While androgen-based therapeutics have been used for over 50 years to treat BC<sup>[23]</sup>. The authors believe that to truly exploit potential AR related mechanisms to provide clinical therapeutic benefits, a more detailed understanding of AR variant distribution and frequency in BC tissues, i.e., AR CSGV, both before and throughout carcinogenesis, will be required.

Further, examining CSGV occurrence in other critical driver genes may help resolve the genotype-phenotype disconnects between the mutational status of putative cancer-associated genes and the occurrence and progression of cancer. For, if it is assumed that somatic clonal evolution is the mechanism driving carcinogenesis, then tissue microenvironments need to be able to select from different variants of individual genes. As the presence of a single variant would not allow cells and tissues sufficient flexibility to adapt to different selection pressures produced by different tissue microenvironments. Further, the ability to collect such data about all potential driver genes may well provide new insights into resistance to treatment as well as to treatment failures.

## METHODS

### Laser capture microdissection and DNA extraction

Frozen tumors were obtained from a breast cancer tissue bank [Table 1] that had been set up with all the required experimental permissions and vetted by the Jewish General Hospital's ethics board. Histological slides of 5-7 µm thick were prepared and stained using a standard hematoxylin/eosin protocol. To ensure the maximum purity of the cancer samples, following histo-pathological characterization by an expert pathologist, cells from cancer tumor areas were dissected by LCM using an AutoPix 100 (Molecular Devices, Sunnyvale, CA). An average of 2500 cells was dissected from each different section. Genomic DNA was extracted from the cells using a QIAamp DNA Micro kit (QIAGEN, Germantown, MD) following the manufacturer's directions.

**Table 1. Clinical data**

Specimen No.	Age at diagnosis	Nuclear grade	Histology grade	Menopausal	T	N	M	TNM stage	ER	PR	HER2
T-44	55	III	III	+	pT2	pN2a	pM1	IV	+	+	+
T-102	78	III	III	+	pT2	pN3a	pM0	IIIC	+	+	+
T-106	64	II	II	+	pT1c	pN0	pMx	I	+	+	-
T-112	60	III	III	+	pT2	pN0(i+)	pM0	IIA	-	-	-
T-121	62	I	I	+	pT1c	pN1a	pM0	IIA	+	+	-
T-125	60	II	II	+	pT1c	pN0(i-)	pM0	I	+	+	-

T: tumor stage; N: lymph node stage; M: metastatic stage; TNM stage: overall breast cancer stage; ER: estrogen receptor  $\alpha$ ; PR: progesterone receptor; HER2: herceptin receptor

### PCR amplification

Amplification of *AR* exons was carried out using the Fast Start High Fidelity PCR kit (Roche, Indianapolis, IN). PCR products were generated using 36 different pairs of fused primers designed to flank the *AR* sequences of exons 4-8, which has been shown to be the region of the *AR* that contains a high proportion of mutations, including those associated with cancer<sup>[24]</sup>. The primers also included the sequence of introns 3-8 [Table 2]. Each primer consisted of a 5' overhang of 19 bp, a 3 bp patient-specific barcode, and a 20-27 bp *AR*-specific sequence. The 5' overhang was used to facilitate emulsion PCR (em-PCR) and sequencing. The 3 bp barcode facilitated sample identification post sequencing, by allowing the pooling of different DNA samples for em-PCR. To ensure consistency three separate PCR preparations were prepared for each of the samples.

### Ultra-deep pyrosequencing (next generation sequencing)

After conventional PCR amplification, the DNA from each sample was quantified by PicoGreen® dsDNA Assay (Invitrogen, Carlsbad, CA) and pooled equimolarly (em). For optimal em-PCR, the theoretical distribution ratio of beads and ssDNA is 1:1 for the clonal amplification. Based on this ratio, the initial eight em-PCR reactions were performed to determine the optimal ratio for em-PCR, based on bead recovery percentage (which was between 10%-15%). After the em-PCR reaction, the micro-reactors were broken and the beads captured by filtration. The biotin-labeled amplicon-positive beads were enriched using Streptavidin magnetic beads and then single stranded. The DNA beads were pre-incubated with DNA polymerase, sequencing primer and single strand binding protein (SSB), and then distributed into the wells on a PicoTiterPlate™ optical faceplate (454, Branford, CT), that contained 1.6 million wells. After adding the DNA beads and enzymatic beads (ATP sulfurylase and luciferase), the packing beads were layered onto the wells and the plate centrifuged for bead deposition. The signal processing and base-calls were performed using the software package from 454 (Branford, CT)<sup>[25]</sup>.

The sequence reads that passed quality control were aligned to the *AR* reference sequence (NM\_000044.2) mRNA sequence of *Homo sapiens* androgen receptor, transcript variant 1 using a BLAST-based approach to determine the direction of each read; exons 4-8 were examined. To determine the likelihood of identifying PCR and sequencing errors, which is known that the 454 sequencing technology can generate<sup>[26]</sup>, special care was taken in sequencing homopolymeric regions, which can generate spontaneous insertions/deletions. However, as the study only sequenced exons 4-8 of the *AR*, that do not contain any homopolymeric regions, such errors were unlikely be a problem.

### Sequence analysis

The sequencing data was aligned using MAFFT version 7.050, a multiple sequence alignment software. The data was then filtered by the length of each read, only reads that were the expected length were retained. The mode of the length of the total reads was used to imply expected length. Since sequencing errors are known to depend on position within the read, with more errors occurring near the end of each read, we further fil-

Table 2. Primers used for sequencing

Sample	Primers	Tag	Primer sequences
T-44	4A/882718B	AAC	GCCTCCCTCGGCCCATCAGAACATTCAAGTCTCTTCTCTTC / GCCTTGCCAGCCGCTCAGAACCAAGGAGTGGGCTGGTTGTT
T-102	4A/882718B	AAG	GCCTCCCTCGGCCCATCAGAACATTCAAGTCTCTTCTCTTC / GCCTTGCCAGCCGCTCAGAACCAAGGAGTGGGCTGGTTGTT
T-106	4A/882718B	ATG	GCCTCCCTCGGCCCATCAGAACATTCAAGTCTCTTCTCTTC / GCCTTGCCAGCCGCTCAGAACCAAGGAGTGGGCTGGTTGTT
T-112	4A/882718B	ATC	GCCTCCCTCGGCCCATCAGAACATTCAAGTCTCTTCTCTTC / GCCTTGCCAGCCGCTCAGAACCAAGGAGTGGGCTGGTTGTT
T-121	4A/882718B	ACA	GCCTCCCTCGGCCCATCAGAACATTCAAGTCTCTTCTCTTC / GCCTTGCCAGCCGCTCAGAACCAAGGAGTGGGCTGGTTGTT
T-125	4A/882718B	ACT	GCCTCCCTCGGCCCATCAGAACATTCAAGTCTCTTCTCTTC / GCCTTGCCAGCCGCTCAGAACCAAGGAGTGGGCTGGTTGTT
T-44	605A/4B	AAC	GCCTCCCTCGGCCCATCAGAACGACAGTGTACACATTGAAGGCTATG / GCCTTGCCAGCCGCTCAGAACGCTCATAGAGCGTTCACT
T-102	605A/4B	AAG	GCCTCCCTCGGCCCATCAGAACGACAGTGTACACATTGAAGGCTATG / GCCTTGCCAGCCGCTCAGAACGCTCATAGAGCGTTCACT
T-106	605A/4B	ATG	GCCTCCCTCGGCCCATCAGATGGACAGTGTACACATTGAAGGCTATG / GCCTTGCCAGCCGCTCAGATGGTCCATAGAGCGTTCACT
T-112	605A/4B	ATC	GCCTCCCTCGGCCCATCAGATGGACAGTGTACACATTGAAGGCTATG / GCCTTGCCAGCCGCTCAGATGGTCCATAGAGCGTTCACT
T-121	605A/4B	ACA	GCCTCCCTCGGCCCATCAGATGGACAGTGTACACATTGAAGGCTATG / GCCTTGCCAGCCGCTCAGATGGTCCATAGAGCGTTCACT
T-125	605A/4B	ACT	GCCTCCCTCGGCCCATCAGATGGACAGTGTACACATTGAAGGCTATG / GCCTTGCCAGCCGCTCAGATGGTCCATAGAGCGTTCACT
T-44	5A/5B	ACC	GCCTCCCTCGGCCCATCAGACCTCTCTGCCCCACAGGGACTC / GCCTTGCCAGCCGCTCAGACCAACCAACAGGCTCTG
T-102	5A/5B	ACG	GCCTCCCTCGGCCCATCAGACCTCTCTGCCCCACAGGGACTC / GCCTTGCCAGCCGCTCAGACCAACCAACAGGCTCTG
T-106	5A/5B	AGA	GCCTCCCTCGGCCCATCAGATGATCTCTGCCCCACAGGGACTC / GCCTTGCCAGCCGCTCAGATGAGTGGTCTCTCTGAATCTC
T-112	5A/5B	AGC	GCCTCCCTCGGCCCATCAGATGATCTCTGCCCCACAGGGACTC / GCCTTGCCAGCCGCTCAGATGAGTGGTCTCTCTGAATCTC
T-121	5A/5B	AGG	GCCTCCCTCGGCCCATCAGATGATCTCTGCCCCACAGGGACTC / GCCTTGCCAGCCGCTCAGATGAGTGGTCTCTCTGAATCTC
T-125	5A/5B	TAA	GCCTCCCTCGGCCCATCAGATGATCTCTGCCCCACAGGGACTC / GCCTTGCCAGCCGCTCAGATGAGTGGTCTCTCTGAATCTC
T-44	6A/6B	TAT	GCCTCCCTCGGCCCATCAGTATCAATCAGAGACATTCCTCTGG / GCCTTGCCAGCCGCTCAGTATAGTGGTCTCTCTGAATCTC
T-102	6A/6B	TAG	GCCTCCCTCGGCCCATCAGTATCAATCAGAGACATTCCTCTGG / GCCTTGCCAGCCGCTCAGTATAGTGGTCTCTCTGAATCTC
T-106	6A/6B	TTA	GCCTCCCTCGGCCCATCAGTATCAATCAGAGACATTCCTCTGG / GCCTTGCCAGCCGCTCAGTATAGTGGTCTCTCTGAATCTC
T-112	6A/6B	TTT	GCCTCCCTCGGCCCATCAGTATCAATCAGAGACATTCCTCTGG / GCCTTGCCAGCCGCTCAGTATAGTGGTCTCTCTGAATCTC
T-121	6A/6B	TTC	GCCTCCCTCGGCCCATCAGTATCAATCAGAGACATTCCTCTGG / GCCTTGCCAGCCGCTCAGTATAGTGGTCTCTCTGAATCTC
T-125	6A/6B	TTG	GCCTCCCTCGGCCCATCAGTATCAATCAGAGACATTCCTCTGG / GCCTTGCCAGCCGCTCAGTATAGTGGTCTCTCTGAATCTC
T-44	7A/7B	TCA	GCCTCCCTCGGCCCATCAGTATGTTGTCAGAAAACCTTGGTG / GCCTTGCCAGCCGCTCAGTATGGCTCTATCAGGCTGTTCTC
T-102	7A/7B	TCT	GCCTCCCTCGGCCCATCAGTATGTTGTCAGAAAACCTTGGTG / GCCTTGCCAGCCGCTCAGTATGGCTCTATCAGGCTGTTCTC
T-106	7A/7B	TCC	GCCTCCCTCGGCCCATCAGTATGTTGTCAGAAAACCTTGGTG / GCCTTGCCAGCCGCTCAGTATGGCTCTATCAGGCTGTTCTC
T-112	7A/7B	TCG	GCCTCCCTCGGCCCATCAGTATGTTGTCAGAAAACCTTGGTG / GCCTTGCCAGCCGCTCAGTATGGCTCTATCAGGCTGTTCTC
T-121	7A/7B	TGA	GCCTCCCTCGGCCCATCAGTATGTTGTCAGAAAACCTTGGTG / GCCTTGCCAGCCGCTCAGTATGGCTCTATCAGGCTGTTCTC
T-125	7A/7B	TGG	GCCTCCCTCGGCCCATCAGTATGTTGTCAGAAAACCTTGGTG / GCCTTGCCAGCCGCTCAGTATGGCTCTATCAGGCTGTTCTC
T-44	8A/8B	ATT	GCCTCCCTCGGCCCATCAGATTACCTCTTGTACACCTGTTT / GCCTTGCCAGCCGCTCAGATTAAAGGCACATGACAGAGGATAG
T-102	8A/8B	AGT	GCCTCCCTCGGCCCATCAGATTACCTCTTGTACACCTGTTT / GCCTTGCCAGCCGCTCAGATTAAAGGCACATGACAGAGGATAG
T-106	8A/8B	TGT	GCCTCCCTCGGCCCATCAGATTACCTCTTGTACACCTGTTT / GCCTTGCCAGCCGCTCAGATTAAAGGCACATGACAGAGGATAG
T-112	8A/8B	TGC	GCCTCCCTCGGCCCATCAGTGCACCTCTTGTACACCTGTTT / GCCTTGCCAGCCGCTCAGTGCAGGCACTGCAGAGGATAG
T-121	8A/8B	TGG	GCCTCCCTCGGCCCATCAGTGCACCTCTTGTACACCTGTTT / GCCTTGCCAGCCGCTCAGTGCAGGCACTGCAGAGGATAG
T-125	8A/8B	CCG	GCCTCCCTCGGCCCATCAGTGCACCTCTTGTACACCTGTTT / GCCTTGCCAGCCGCTCAGTGCAGGCACTGCAGAGGATAG

The primers sequencing coverage includes introns from 3 to 8

**Table 3. Number of sequencing reads**

Exon	Patient					
	T-44	T-102	T-106	T-112	T-121	T-125
4A	37,704	68,001	33,819	15,660	20,289	59,399
4B	2884	1317	3882	n/a	2862	6640
5	4206	3765	4488	3705	4460	3763
6	9612	2683	4198	3108	1434	2853
7	19,248	7104	3729	1260	6188	2993
8	3443	3836	4430	1569	1662	1795

tered the data by retaining only the sequence between the fifth and one hundred and fiftieth bp. All variants in the data sets were then identified.

## RESULTS

The samples were analyzed by ultra-deep sequencing at a depth of up to 68,000 reads for each sample [Table 3]. The analyses revealed 53 exonic mutations [Table 4]. These included 20 mutations in exon 4, 11 mutations in exon 5, 10 mutations in exon 6, 4 mutations in exon 7, and 8 mutations in exon 8. It was noted that a significant number of the mutations (18 out of 53) had previously been identified as either associated with androgen insensitivity syndrome (AIS) (11 mutations) or prostate cancer (7 mutations). Twenty-one mutations occurred in several of the tumor samples, with 4 of the mutations occurring in at least 4 of the tumor samples. The distribution of the mutations in each tumor was unique, resulting in a different set of AR variants being present in each of the tumors [Figure 1].

## DISCUSSION

### Do CSGVs really exist?

Before discussing the results, it seems reasonable to address the controversy with regards to whether intra-tissue genetic heterogeneity really exists, particularly as it has been identified not just within tumors, but within normal tissues as well<sup>[27,28]</sup>. Indeed, questions have been raised as to the possible role of methodological errors in generating genetic heterogeneity in both tumors<sup>[29]</sup> and tissues in general<sup>[30]</sup>. To address these questions, it is important to discuss the sequence analysis tools used in our NGS protocols. In traditional sequencing approaches, coverage is based on genome mapping approaches, which use a theoretical redundancy in coverage based on the expression  $LN/G$ , where  $L$  is the read length,  $N$  is the number of reads and  $G$  is the haploid genome length<sup>[31]</sup>. Unfortunately, many factors can result in unequal coverage that produces gaps or much lower coverage than expected<sup>[32]</sup>. Further, problems such as the choice of alignment algorithms means that even the best mapping algorithms cannot align all reads to a reference genome<sup>[33]</sup>. As the cost of sequencing has come down, so has the depth of sequencing increased, and this has had a profound effect on the sensitivity of sequencing and the ability to detect rare mutations accurately<sup>[34]</sup>. Experimental data has confirmed that the major factors that influence detection sensitivity are read depth and experimental precision<sup>[34]</sup>. Indeed, it would appear possible to accurately detect mutations at a frequency of as low as 0.1%, provided there is sufficient read depth<sup>[34]</sup>. Somewhat surprisingly, the use of filters used to eliminate false reads *etc.* does not necessarily prevent low frequency mutations from being detected<sup>[35]</sup>. Indeed, if used correctly they can in fact enhance the ability to detect low frequency mutations, and in cases of tumor genetic heterogeneity, such an ability is likely to be extremely important<sup>[35]</sup>. In the case of the present study we believe we have adopted a sufficiently precise sequencing technique that we can use a 0.1% cutoff value to identify the mutations present in our breast tumor samples.

### Importance of identifying changing frequencies of driver gene variants during carcinogenesis

At present, identification of ITGH has solely been based on whether specific driver gene variants have been

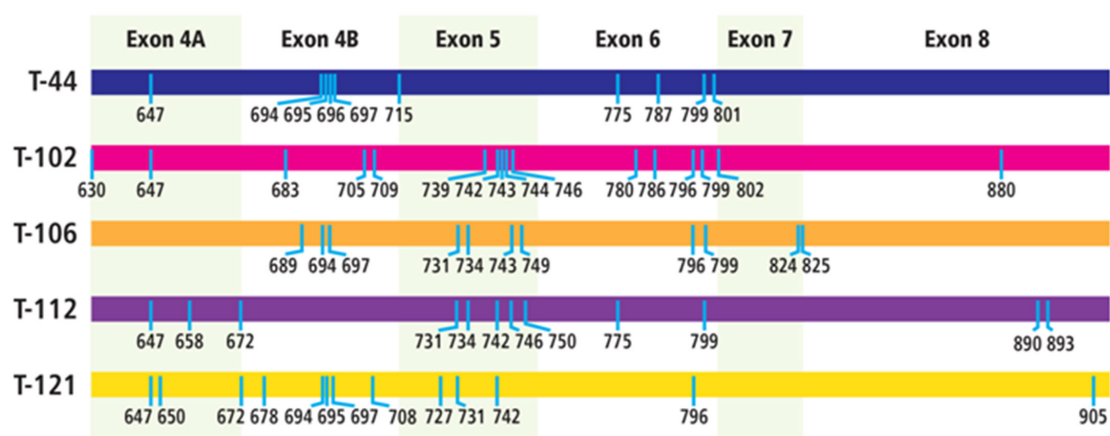


**Table 4. Summary of androgen receptor exonic mutations**

Codon	WT NT	Mutant	Context	WT AA	AA change	Patients tumor						Disease phenotype
						T-44	T-102	T-106	T-112	T-121	T-125	
Exon 4A						Number of reads						
						37,704	68,001	33,819	15,660	20,289	59,399	
						Number of mutants						
630	1888C	T	CC <u>C</u> GG	Arg	Trp		68				72	
647	1941C	T	T <u>C</u>	Ser	Ser	40	118		21	23	107	
649	1947C	T	AC <u>C</u>	Thr	Thr						66	
650	1950C	T	AC <u>C</u>	Thr	Thr					31	73	PCa
652	1955C	T	C <u>CCC</u>	Pro	Pro						67	
658	1972C	T	ACC <u>C</u> AG	Gln	Stop				20		63	CAIS
672	2015C	T	<u>CCC</u>	Pro	Pro				21		60	
Exon 4B						Number of reads						
						2884	1317	3882	n/a	2862	6640	
						Number of mutants						
672	2015C	T	<u>CCC</u>	Pro	Pro					4		
678	2021C	T	C <u>C</u> TG	Leu	Leu					3	9	
683	2047C	T	<u>C</u> CA	Pro	Ser		2					
689	2065G	A	<u>G</u> GA	Gly	Arg			4				
694	2080C	T	C <u>C</u> AG	Gln	Stop	4		4		4	7	CAIS
695	2084C	T	<u>CCC</u>	Pro	Leu							
695	2085C	T	<u>CCC</u>	Pro	Pro	7				3		
696	2086G	A	<u>G</u> AC	Asp	Asn	4						CAIS
697	2091C	T	T <u>C</u>	Ser	Ser	4		5		6		
705	2113C	T	C <u>C</u> TC	Leu	Ser		2					
708	2124G	A	CT <u>G</u> GGA	Leu	Leu					4		
709	2125G	A	CTG <u>G</u> GA	Gly	Arg		2					CAIS
715	2141A	AA (ins A)	GT <u>A</u> CAC	His	fs	6						
Exon 5						Number of reads						
						4206	3765	4488	3705	4460	3763	
						Number of mutants						
727	2180G	A	C <u>C</u> GC	Arg	His					5		
731	2191G	A	GT <u>G</u> GTA	Val	Ala			6	4	7		
734	2200C	T	GAC <u>C</u> AG	Gln	Stop			5	4			CAIS
739	2218T	TT (ins T)	AT <u>I</u>	Gln	fs	10	6		4			
742	2225G	A	T <u>G</u> G	Trp	Stop		7		9	7	6	PCa
743	2229G	A	AT <u>G</u> GGG	Met	Ile		5	6				PAIS
744	2231G	A	ATG <u>G</u> GG	Gly	Arg		5					CAIS
746	2238G	A	AT <u>G</u> GTG	Met	Ile		4		4			
749	2246C	T	<u>G</u> CC	Ala	Val			8				PCa
750	2250G	A	AT <u>G</u> GGC	Met	Ile				4		4	PCa
752	2255G	A	T <u>G</u> G	Trp	Stop						5	PCa
Exon 6						Number of reads						
						9612	2683	4198	3108	1434	2853	
						Number of mutants						
775	2323C	T	TAC <u>C</u> GC	Arg	Cys	11			4			CAIS
779	2337C	T	T <u>C</u> CGG	Ser	Ser						4	
780	2338C	CC (ins C)	TCC <u>C</u> GG	Arg	Pro fs		3					
786	2354T	C	G <u>T</u> C CGA	Val	Ala		3					
787	2359C	T	GTC <u>C</u> GA	Arg	Stop	21					3	PCa, CAIS
796	2390G	A	TTT <u>G</u> GA	Gly	Arg		3	5		3		
797	2391G	A	T <u>G</u> G CTC	Trp	Stop						3	CAIS
799	2395C	T	CTC <u>C</u> AA	Gln	Stop	20	6	6	5		5	CAIS
801	2403C	T	ACC <u>CCC</u> CAG	Thr	Thr	12						
802	2405C	T	ACC <u>CCC</u> CAG	Pro	Leu		3					

Exon 7						Number of reads					
						19,248	7104	3729	1260	6188	2993
						Number of mutants					
824	2471A	AA (ins A)	AAA AAT CAA AAA	Asn Gln	Lys fs			5			
825	2472T	TA (ins A)	AAA AAT CAA AAA	Gln	fs					21	
825	2473C	CA (ins A)	AAA AAT CAA AAA	Gln Lys	Gln Lys fs			4			
Exon 8						Number of reads					
						3443	3836	4430	1569	1662	1795
						Number of mutants					
880	2638T	TT (ins T)	ACT TTT GAC	Asp	Stop		4				
887	2661G	A	ATG GTG	Met	Ile					2	PCa
890	2670G	A	GTG CAC	Val	Val				2	2	
893	2678C	T	TTT CCG GAA	Pro	Leu				2		CAIS
893	2678C	CC (ins C)	TTT CCG GAA	Pro	Pro fs				2		
893	2679G	A	TTT CCG GAA	Pro	Pro					2	
905	2715C	CC (ins C)	GTG CCC AAG	Pro	Pro fs					3	

n/a: not available; PCa: prostate cancer; CAIS: complete androgen insensitivity syndrome



**Figure 1.** AR exonic mutations present in each of the tumor samples. T- refers to individual tumor samples. AR refers to codon within which mutations were found

present within cancer tissues, but their frequencies have generally not been assessed. This is because it has been assumed that such variants are present in most tumor cells and are therefore responsible for the cancer phenotype, so that ITGH just reflects the complex genetic makeup of individual tumors, but that the basic mutation-centric paradigm still applies. However, evidence that driver gene mutations can also be present in normal tissues has considerably confused the role of these driver genes in carcinogenesis. We believe that identifying cases of CSGV is likely to be helpful in resolving the phenotype/genotype disconnect, because the data will reveal the actual frequency of the variants and put them in context within a tumor. In a previous study examining an AR CAG repeat length polymorphism in breast tumors, changes in the frequency of these polymorphisms in normal and cancer tissues from individual tumors, as well as in matching blood samples were investigated. This revealed the distribution frequencies of different length AR CAG repeat variants associated with carcinogenesis<sup>[6]</sup>. A similar approach applied to analyzing driver gene CSGV is likely to give further information to help elucidate the significant genetic events of carcinogenesis.

### How can identifying CSGV in tumors contribute to our understanding of cancer genetics?

Clearly, the presence of CSGV within cancer tissues clashes with our present understanding that carcinogenesis is the result of “purifying” selection pressure on single gene variants in a tumor that eventually will lead to removal of all the non-selected variants of that gene<sup>[36]</sup>. This argument in turn justifies being satisfied with the identification of a single variant per gene, and therefore to ignore any other low frequency variants within the same gene, on the assumption that they must be artifacts, possibly due to PCR or sequencing errors. The recognition that a selection of different single gene variants can remain in individual tumors, is clearly not in line with our present understanding of the occurrence and distribution of cancer mutations. However, our present results would question the validity of this understanding as CSGV were identified in the AR within all 6 breast tumors examined and suggests that the role of mutations in carcinogenesis is more complex than previously thought.

### How can identifying CSGV help in understanding treatment resistance?

First, it suggests a mechanism to explain how some tumors can become rapidly resistant to treatment by proposing the existence of genetic variants that can be selected for in genes that have been targeted by chemotherapy. Indeed, the selection of such variants could be a response to ensure the survival of cells that contained the targeted gene as postulated by the atavistic model<sup>[37]</sup>, which considers resistance of cancer cells to treatment as one of their major characteristics. Second, it places much more emphasis on understanding the role of selection pressures generated by different tissue microenvironments on carcinogenesis<sup>[38,39]</sup>. It also suggests that analyzing the makeup of tissue microenvironments may facilitate the recognition of specific factors involved in the selection of cancer-associated variants.

### A different paradigm to explain carcinogenesis

The principle of “parsimony” has underwritten our understanding of science since the middle of the 19th century by telling us to choose the simplest scientific explanation that fits (all) the observed evidence. In studying the genetics of cancer this has been reflected in our belief that identifying common gene mutations present in tumor tissues is one of the keys to understanding the ontology of solid tumors. However, the validity of this concept is being challenged by accumulating evidence of genetic diversity within individual tumors, which this study has further expanded by revealing evidence of AR CSGV in breast tumors. As noted previously, current cancer hypotheses are almost all based on the concept that accumulation of specific *de novo* individual driver mutations within specific tissues can result in carcinogenesis. However, the lack of a consistent relationship between driver mutations and cancer types and the discovery of the presence of many different driver mutant genes within the same types of cancer tissues has resulted in complex genetic profiles. These have effectively meant that many of these driver gene mutations have been reduced to risk factors, albeit with significant clinical implications, rather than gene mutations that are directly responsible for carcinogenesis.

Interestingly, such phenotype/genotype lack of precision has been found not just in multifactorial diseases such as cancer, but in locus specific genetic disorders as well. For example, in certain locus specific diseases a significant number of individuals that exhibit the disease phenotype do not have a mutation in the putative disease-causing gene, such as in the case of androgen insensitivity syndrome<sup>[24]</sup> and PKU<sup>[40]</sup>. Further, a review of genotype-phenotype relationships in a wide range of genetic diseases has revealed many cases of reduced or even zero penetrance<sup>[41]</sup>. While whole genome sequencing studies have found individuals that can have well known disease-causing gene mutations but do not exhibit the disease phenotypes<sup>[42]</sup> including cancer-associated genes in healthy individuals<sup>[43]</sup>.

Other recent evidence has further complicated the genetics of cancer, by revealing the effect on cancer phenotypes of processes such as epigenetic regulation, DNA and RNA editing, cellular differentiation hier-

archies, gene expression stochasticity and protein-protein interactions<sup>[44]</sup>. However, their roles are not well defined at present, as in many cases these factors are analyzed as separate events, rather than studying their integrated effect on the selection pressures of the complete tissue microenvironment<sup>[45]</sup>.

One possible hypothesis we have previously proposed is that while intra-tissue genetic heterogeneity may provide the genetic underpinnings for carcinogenesis. It is tumor microenvironment selection pressure on preexisting *de novo* mutations that is the carcinogenic trigger, rather than just the accumulation of *de novo* mutations<sup>[46]</sup>. We have further postulated that these mutations occur early in human embryogenesis<sup>[45]</sup>, as has now been suggested in another recent study<sup>[47]</sup>.

We believe that this hypothesis is supported by the presence of genetic heterogeneity in both cancer and normal tissues, as well as by the evidence of non-genomic, often environmental factors as risk factors for cancer. Indeed, the complexity of post-zygotic variation<sup>[14]</sup> has only added to the importance of variant selection due to environmental factors within tissue microenvironments in determining cancer phenotypes<sup>[48]</sup>. A detailed examination of the arguments favoring a selection-centric paradigm has been given in a recent paper<sup>[49]</sup>, which the identification of AR CSGV in breast tumors has further strengthened.

### How the identification of CSGV could affect approaches to cancer treatment

Based on many cases of individual-gene genetic heterogeneity that have recently been identified in normal as well as cancer tissue, it seems reasonable to believe that CSGV is likely to also occur in normal tissue. The presence of multiple variants within single genes at low frequencies in normal tissue and cells prior to tissue becoming cancerous would further strengthen the selection-centric paradigm of carcinogenesis. This paradigm could also better explain many observations in which, environmental factors that are clearly non-mutagenic, i.e., diet, exercise, *etc.*, can somehow direct mutations in specific “driver” genes<sup>[50]</sup>. Thus, “healthy” lifestyle factors can result in the selection of environments that are “cancer resistant”, while other environments identified as “cancer causing”, that are often man-made, can lead to cancer<sup>[51]</sup>. CSGV could then simply explain a “cancer resistant” environment as one that selects for pre-existing wild-type gene variants and a “cancer causing” environment as one that selects for pre-existing oncogenic gene variants.

Based partially on the principle of parsimony discussed previously, success of species, tissues or cells, has always been considered to eventually result in a specific species, tissues or cells eliminating the competition. However, in the case of CSGV this clearly does not seem to be the case, as while gene variants may not be selected, they are not eliminated entirely either. Thus, in the case of cancer, just destroying the cancer cells and not changing the conditions that allow for them to be preferentially selected, is possibly going to allow other cancer cells with different gene variants to eventually be selected, as the environmental conditions that selected cells with oncogenic properties have not been altered. Our present approach to cancer treatment of removing cancer cells, does of course not preclude the possibility of cancer recurring. However, the presence of CSGV would suggest an approach to cancer treatment that in addition to removing the cancer would also seek to select the normal tissue and cells that are always present within cancer tissues, although normally only as a very small minority of cells. This new treatment approach would therefore require that cancer tissue microenvironments be returned to conditions that would once again select for normal cells, although this is clearly not a simple task.

Recently, more attention has started to be given to the carcinogenic role of the tumor microenvironment including in both tumorigenesis<sup>[52]</sup> and differential tissue responses to therapy<sup>[53]</sup>. These studies have begun to analyze and reveal some of the tumor micro-environmental factors that may play a critical role in carcinogenesis. Naturally, these data are also likely to help reveal the tissue micro-environmental properties

within normal, non-cancer tissues. However, our understanding of what constitutes tissue-specific micro-environment conditions is still very incomplete. Also, it is highly likely that individuals will have their own set of micro-environmental, chemical and biological conditions, so it will be necessary to analyze their tissue microenvironments in considerable detail. Clearly, cells and tissues exist in complex three-dimensional environments, which include both extra- and intracellular environments. To analyze these microenvironments new technologies are being developed, including atomic force microscopy<sup>[54]</sup>, quantitative extracellular matrix proteomics<sup>[55]</sup>, and single cell multiomics<sup>[56]</sup> that are being used to create complex databases of tissue micro-environmental factors that will hopefully facilitate the identification of those significant factors that allow for the selection of normal as opposed to cancer cells.

However, at first glance there appears to be the same underlying problem with this approach as the one that has characterized attempts to analyze the genomic and post-genomic events that cause cells to become oncogenic. Namely, the inability to identify the critical oncogenic events involved because we can only measure conditions before and after a cell becomes cancerous. However, the tissue micro-environmental conditions that result in normal cells being selected do not suffer from this drawback, as normal cells remain dominant in tissue over relatively long periods of time, presumably because they are subject to relatively consistent tissue micro-environmental conditions. Nevertheless, it is important to note that tissue microenvironments are likely to be highly individualized, so that even within an individual different tissue microenvironments might exist around different tissues.

## Conclusion

Before the discovery of ITGH and now CSGV, the novel approach to cancer treatment that we are suggesting would have never been considered. However, if it is proven that cancer-associated genes within tumors as well as normal tissue consistently exhibit CSGV. Then a treatment approach that includes the goal of reselecting normal tissues by adjusting the tissue microenvironment, would seem to be the logical way to ensure that cancer treatments finally result in the permanent elimination of cancer.

## DECARATIONS

### Authors' contributions

Design: Gottlieb B

Literature research: Gottlieb B

Sequencing: Babrzadeh F, Wang C, Gharizadeh B

Analysis of data: Oros KK, Greenwood CMT

Tissue and DNA preparation: Alvarado C

Tumor samples: Basik M

Manuscript writing: Gottlieb B

Manuscript editing: Beitel LK, Trifiro M

### Availability of data and materials

Data is available from Dr. Bruce Gottlieb. Materials are unavailable.

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### Conflicts of interest

All authors declare that there are no conflicts of interest.



## Ethical approval and consent to participate

This study was approved by the ethical review board of Jewish General Hospital.

## Consent for publication

Not applicable.

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Review

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# Laparoscopic personalized function-preserving gastrectomy with sentinel node mapping for early-stage gastric cancer

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## Abstract

Laparoscopic gastrectomy is considered as an indispensable option between endoscopic resection and standard gastrectomy with open laparotomy for patients with early-stage gastric cancer. However, the extent of gastrectomy and remnant gastric function may affect patients' quality of life (QOL) after surgery. Therefore, function-preserving gastrectomy in addition to laparoscopic surgery could be considered in patients with early-stage gastric cancer. A prospective multicenter trial and meta-analyses of sentinel node (SN) mapping and biopsy for early-stage gastric cancer have demonstrated favorable SN detection rates and accuracy of nodal metastatic status. Although a combination of radioactive colloids with blue dyes as tracers is currently considered as the promising procedure of SN mapping in early-stage gastric cancer, several new technologies, such as indocyanine green fluorescence imaging, may markedly improve its accuracy. For early-stage gastric cancer, the development of laparoscopic personalized minimized gastrectomy with SN mapping may help retain patients' QOL after surgery. A recently developed full-thickness partial gastrectomy with SN mapping and basin dissection would become a reliable minimally invasive gastrectomy for treating patients with cNO early-stage gastric cancer.

**Keywords:** Sentinel node, gastric cancer, laparoscopic, nonexposed endoscopic wall-inversion surgery

## INTRODUCTION

In East Asian countries, such as Japan and Korea, early-stage gastric cancer (cT1) is identified in many patients owing to endoscopic diagnosis and surveillance<sup>[1]</sup>. Nowadays endoscopic submucosal dissection



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(ESD) is accepted as a less invasive procedure without gastrectomy for the resection of cT1 gastric cancer<sup>[1]</sup>. Laparoscopic gastrectomy is considered as an indispensable option between ESD and distal or total gastrectomy with open laparotomy for early-stage gastric cancer<sup>[2]</sup>. Laparoscopic distal gastrectomy (LDG) is comparable with open distal gastrectomy for early gastric cancer, and can be performed in clinical practice<sup>[3,4]</sup>. Many patients with gastric cancer currently undergo LDG and laparoscopic total gastrectomy (LTG) with standard lymphadenectomy<sup>[1-4]</sup>. LDG and LTG contribute to better aesthetics and earlier postoperative recovery after surgery<sup>[5]</sup>. However, the extent of gastrectomy and remnant gastric function may affect patients' quality of life (QOL) after surgery, resulting in several complications such as dumping syndrome and loss of body weight due to the disturbance of oral food intake. Therefore, function-preserving gastrectomy in addition to laparoscopic surgery could be considered in patients with early-stage gastric cancer indicated for these procedures.

Function-preserving minimized gastrectomy procedures, including partial and segmental gastrectomy, with modified lymphadenectomy are thought to improve postoperative gastric function compared to the standard gastrectomy. However, certain incidences of nodal skip metastasis in the second compartment or unpredicted station remain to be solved in these procedures. The sentinel node (SN) mapping and biopsy could overcome these issues as a novel intraoperative examination for accurate diagnosis of nodal metastasis in early-stage gastric cancer.

The SN is considered as the first lymph node(s) receiving lymphatic drainage from the primary tumor site<sup>[6,7]</sup>, and are regarded to be the first possible node(s) of metastasis from the primary lesion. Theoretically if SNs are pathologically negative for cancer metastasis, unnecessary extended lymphadenectomy can be avoided. SN navigation surgery is defined as a less invasive surgical procedure with modified lymphadenectomy by the diagnosis of SN metastasis. SN navigation surgery can prevent unnecessary lymphadenectomy and the occurrence of associated postoperative complications, and result in improving the patients' QOL.

SN mapping and biopsy were firstly utilized in breast cancer and melanoma, and subsequently attempted to other solid tumors<sup>[7-9]</sup>. Several studies involving SN mapping and biopsy for early-stage gastric cancer showed favorable SN detection rates and accuracy to predict nodal metastatic status<sup>[10,11]</sup>. Based on the studies, we have been developing a novel approach which combines laparoscopic function-preserving gastrectomy with SN mapping.

## LAPAROSCOPIC SN MAPPING AND BIOPSY PROCEDURES FOR GASTRIC CANCER

Combination of radioactive colloids with blue or green dyes as a dual tracer method is currently thought to be the standard procedure for successful SN mapping in early-stage gastric cancer<sup>[10,11]</sup>. The accumulation of radioactive colloids in SN enables the detection of SN using hand-held gamma probes. In addition, blue dye is useful for real-time visualization of lymphatic flow even in laparoscopic surgery. Technetium<sup>99m</sup> tin colloids and technetium<sup>99m</sup> sulfur colloids are mainly utilized as radioactive tracers, and indocyanine green (ICG) is commonly used for dye tracer.

In our institutions, the indication to SN mapping and biopsy is currently limited to the patients with clinical T1 tumors over the ESD criteria, primary tumors of < 4 cm in tumor diameter, with clinical No gastric cancer<sup>[10,11]</sup>. In our institution, 2.0 mL (150 MBq) of technetium<sup>99m</sup> tin colloid is injected endoscopically a day before surgery into the submucosal layer surrounding the primary lesion. Injection of the tracer into the submucosal layer using an endoscopic puncture needle facilitates more accurate tracer administration than laparoscopic injections from the seromuscular site of the gastric wall. Technetium<sup>99m</sup> tin colloid which has relatively large particle size (approximately 200 nm in diameter) accumulates in the SNs after the endoscopic injection into the primary tumor site.



Blue or green dyes are also injected in the submucosal layer at primary lesion in a similar manner right after surgery began. Blue- or green-stained lymphatic vessels as well as lymph nodes are visualized during laparoscopic observation within 15 min after the injection. A hand-held gamma detector is also useful to locate the radioactive SN accurately. Moreover, laparoscopic gamma probing is feasible using a gamma detector which is available via trocar ports<sup>[10,11]</sup>.

For intraoperative SN biopsy, the pickup method is commonly employed in breast cancer and melanoma. However, the intraoperative SN sampling for gastric cancer should be accompanied with sentinel lymphatic basin dissection, which is a selected lymphatic basin dissection including identified SN<sup>[10,11]</sup>. The lymphatic basins around the stomach are currently divided into five basins along the main gastric arteries: basins along left gastric artery, right gastric artery, left gastroepiploic artery, right gastroepiploic artery, and posterior gastric artery<sup>[12]</sup>.

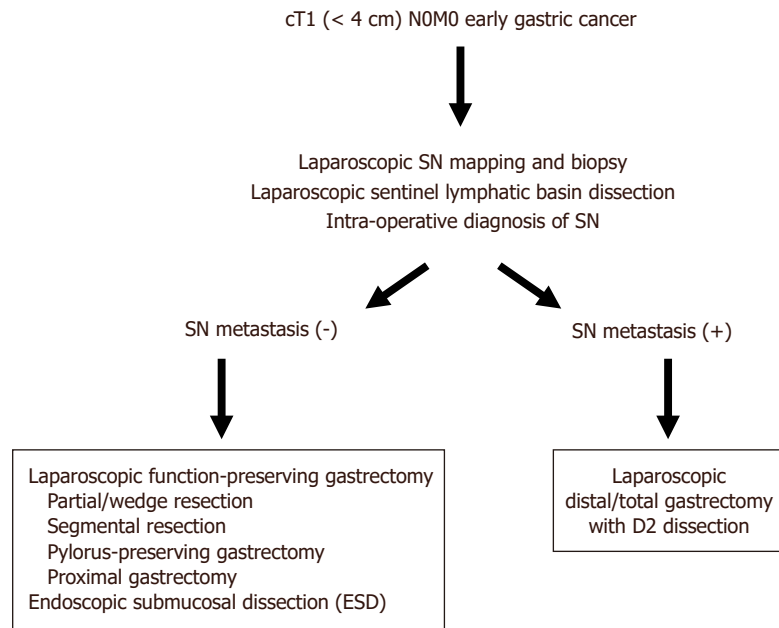
ICG has excitation and fluorescence wavelengths in the near-infrared range<sup>[13]</sup>. Many studies have clearly demonstrated the clinical utility of intraoperative ICG infrared imaging for laparoscopic SN mapping using infrared ray electronic endoscopy (IREE) to date<sup>[13,14]</sup>. IREE is useful to visualize ICG-stained lymphatic vessels and SN more clearly than normal laparoscopy. Subsequently, ICG fluorescence imaging was also developed as a reliable novel technique for SN mapping<sup>[15,16]</sup>. SN can be clearly visualized using laparoscopic ICG fluorescence imaging in comparison with conventional normal light imaging. Although the efficacy of ICG infrared or fluorescence imaging should be carefully evaluated by further prospective studies regarding SN detection rate and accuracy to predict the nodal metastasis, and compared with radio-guided methods, the new technologies may markedly improve the accuracy of laparoscopic SN mapping and biopsy in early-stage gastric cancer.

## FEASIBILITY OF SN MAPPING IN GASTRIC CANCER

Until now, approximately 100 single institutional studies of SN mapping have indicated favorable SN detection rate and accuracy to predict nodal metastasis for early-stage gastric cancer. These results are as good as those of SN mapping for breast cancer and melanoma<sup>[11]</sup>. A meta-analysis, which consisted of 38 SN mapping studies including 2128 patients with gastric cancer, showed that the SN detection rate and accuracy of nodal status determination were 94% and 92%, respectively<sup>[17]</sup>. The study also indicated that SN mapping for gastric cancer is reliable especially in patients with T1 tumor, use of dual tracers and submucosal injections of tracers.

A Japanese group previously conducted a prospective multicenter trial (UMIN ID: 000000476) to evaluate the feasibility of SN mapping for gastric cancer using the dual tracer method<sup>[10]</sup>. In this study, SN mapping and biopsy were performed for 397 patients with cT1NoMo or cT2NoMo single tumor with primary lesion diameter of < 4 cm and those without any previous treatment. To estimate the accuracy of the SN mapping, D2 or modified D2 gastrectomy was essentially performed for enrolled patients after SN mapping according to the guidelines for standard care by The Japan Gastric Cancer Association.

As the results of the study, the SN detection rate was 97.5% (387 of 397), and 14.7% of patients (57 of 387) showed lymph node metastasis. Fifty-three (93.0%) of the 57 patients with nodal metastasis showed positive SN for metastasis. False-negative rate was 7% (4 of 57), and the overall accuracy to determine nodal metastatic status based on SN mapping was 99.0% (383 of 387). Of the 53 patients with positive SN, 32 (60.4%) had nodal metastases limited to only SN. Of the 21 SN-positive/non-SN-positive patients, 15 (71.4%) had metastatic non-SN located within SN basins and 6 (28.6%) had metastatic non-SN located outside the SN basins but within the extent of the D2 lymphadenectomy. Of the 4 patients with false-negative SN biopsy, 3 patients had either primary tumors of more than 4-cm diameter or pT2 tumor or both, and only 1 patient who



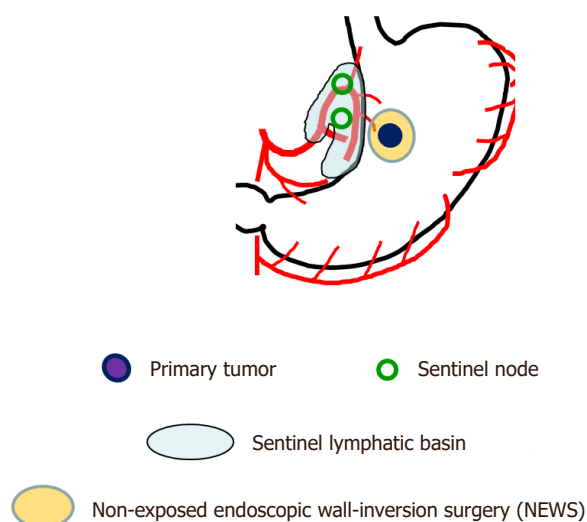
**Figure 1.** Laparoscopic function-preserving gastrectomy for cT1N0M0 gastric cancer with sentinel node mapping. ESD: endoscopic submucosal dissection

had a primary tumor more than 4-cm had a metastatic non-SN outside the SN basin<sup>[10]</sup>. The prospective multicenter trial verified that SN mapping for gastric cancer is technically feasible and reliable regarding SN detection rate and overall accuracy. The study would provide perspectives on the future of minimally invasive personalized gastrectomy based on SN mapping for early-stage gastric cancer.

## MINIMALLY INVASIVE GASTRECTOMY BASED ON SN MAPPING IN EARLY-STAGE GASTRIC CANCER

Pathological status of SN and distribution of SN basins would provide the information in minimizing the extent of gastric resection and avoiding distal or total gastrectomy with D2 lymphadenectomy. Laparoscopic function-preserving gastrectomy for cT1N0 gastric cancers, including partial/wedge resection, segmental gastrectomy, proximal gastrectomy, and pylorus-preserving gastrectomy would be determined based on the SN status for each patient [Figure 1]<sup>[18-20]</sup>. Retention of patients' QOL in addition to earlier postoperative recovery could be obtained using laparoscopic minimized gastrectomy with SN mapping.

Ichikura *et al.*<sup>[21]</sup> previously reported 35 patients with limited gastrectomy such as wedge resection and segmental gastrectomy with SN basin dissection for early gastric cancer with pathologically negative SN biopsy. As the results showed, all patients could survive without any recurrence of tumor in the study. Moreover the extent of the resected stomach in patients with limited gastrectomy was significantly less than that in patients with the standard gastrectomy. Based on these studies, our group in Japan has been conducting a multicenter prospective trial (UMIN ID: 000014401) which aims to elucidate laparoscopic function-preserving gastrectomy with SN mapping and SN basin dissection regarding long-term survival and postoperative patients' QOL for patients with clinical T1N0M0 gastric cancer with primary lesions of < 4 cm in tumor diameter. In the study, *en-bloc* SN basin dissection including SN even in patients with SN-negative for metastasis is thought to be essential to warrant the curability of the surgery because of certain possibility of false-negative SN. A Korean group has also been conducting a prospective multicenter randomized controlled trial to clarify the oncological safety, including long-term survival, of laparoscopic function-preserving gastrectomy with SN basin dissection compared with laparoscopic standard gastrectomy<sup>[22]</sup>.



**Figure 2.** Schema of nonexposed endoscopic wall-inversion surgery (NEWS) with laparoscopic sentinel node mapping and sentinel lymphatic basin dissection for early-stage gastric cancer

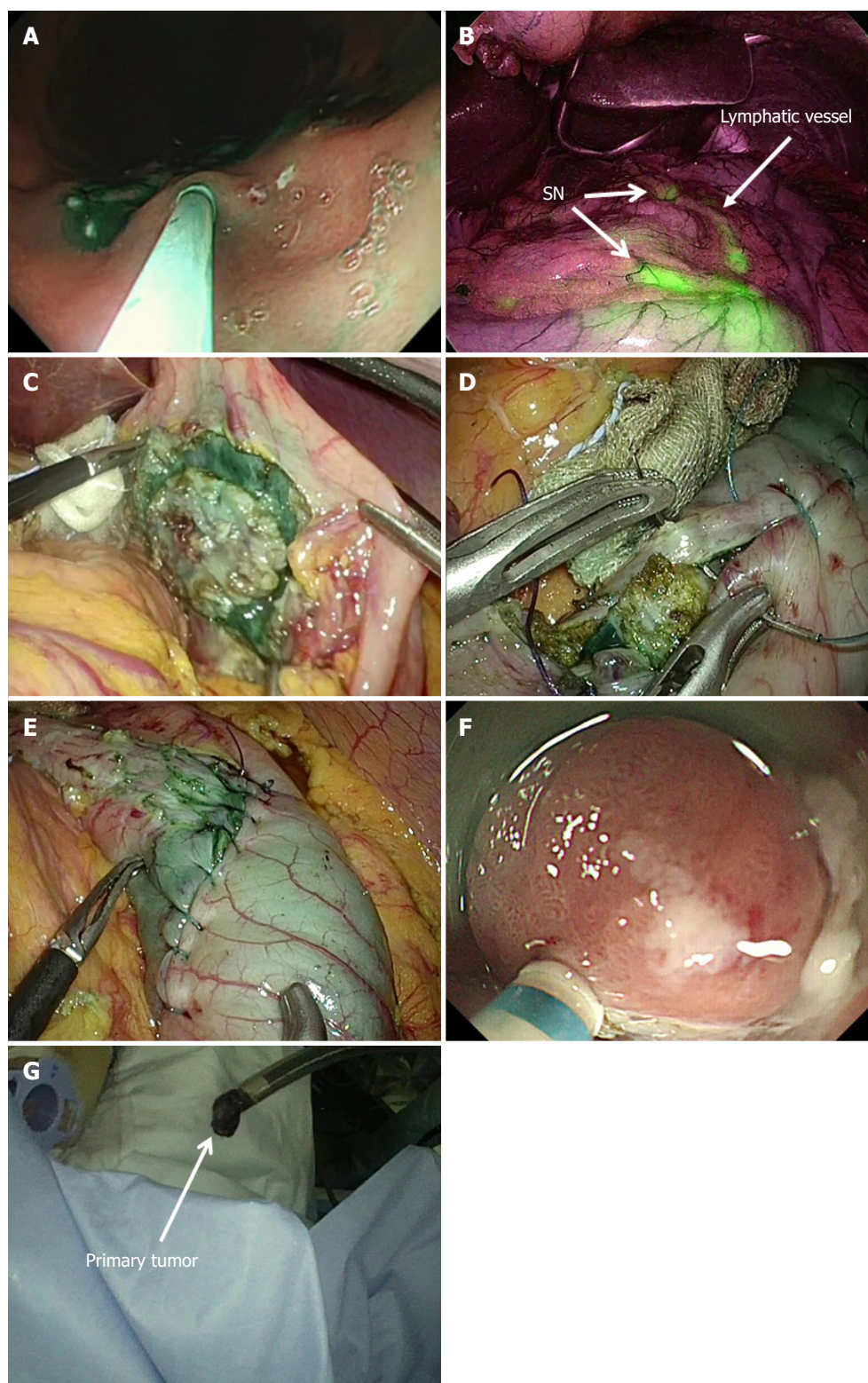
A combination of ESD with laparoscopic SN mapping for early-stage gastric cancer views another desirable option as a new minimally invasive stomach-preserving treatment. When all SNs are pathologically negative for metastasis in laparoscopic SN mapping and biopsy, then theoretically, ESD instead of gastrectomy might be sufficient for the curative resection of cT1 gastric cancer beyond the ESD criteria<sup>[20,23]</sup>. However, further studies are needed to certify the reliability of laparoscopic SN mapping with ESD.

Currently, LDG or LTG is frequently employed in patients with early-stage gastric cancer based on the pathological assessment of primary lesion obtained using ESD in the practice. Until now, whether SN mapping would be feasible or not even after ESD remains unknown. One of the most important concerns is that the lymphatic flow from the primary lesion to the original SN might be altered after ESD. However, a previous study reported that at least the SN basin was not markedly changed by ESD prior to surgery<sup>[20,23]</sup>. Laparoscopic limited gastrectomy based on SN mapping and biopsy could be feasible even after ESD.

## NONEXPOSED ENDOSCOPIC WALL-INVERSION SURGERY WITH MINIMALLY INVASIVE SN BIOPSY

In laparoscopic partial gastrectomy, the demarcation line of the primary tumor cannot be identified accurately because the approach of gastrectomy is usually from the outside of the stomach. Therefore, a wider resection of the stomach cannot be avoided to prevent a positive surgical margin of primary tumor site. Recently, a new technique called nonexposed endoscopic wall-inversion surgery (NEWS) has been developed. The procedure is a full-thickness partial resection of the stomach, which can minimize the extent of gastric resection using endoscopic and laparoscopic surgeries without transluminal access designed to resect early-stage gastric cancer. In our ongoing clinical trial, the cases of NEWS with laparoscopic SN mapping and sentinel basin dissection have been accumulated in cT1NoM0 early-stage gastric cancer [Figure 2]<sup>[24,25]</sup>.

In brief, after placing circumferential mucosal markings of primary tumor, ICG was endoscopically injected into the submucosal layer around the primary lesion to identify the SNs [Figure 3]<sup>[24]</sup>. The SN basin including SNs was dissected, and no metastasis in all SN was confirmed by intraoperative pathological examinations. After the SN mapping and biopsy, NEWS was performed for the resection



**Figure 3.** Nonexposed endoscopic wall-inversion surgery (NEWS) with laparoscopic sentinel node (SN) mapping and sentinel lymphatic basin dissection for early-stage gastric cancer. (A) Indocyanine green (ICG) was endoscopically injected into the submucosal layer around the primary lesion; (B) ICG fluorescence imaging results in clear visualization of SNs and lymphatics; (C) laparoscopic circumferential seromuscular incision surrounding the primary tumor; (D and E) laparoscopic seromuscular suturing and inversion of the primary tumor site to the inside of the stomach; (F) endoscopic circumferential mucosal and submucosal incision for primary tumor resection; (G) endoscopically retrieved primary tumor



of primary tumor site. After placing circumferential serosal markings laparoscopically, submucosal injection was endoscopically administered. Next, circumferential seromuscular incision of the primary tumor and suturing of outer edge of the seromuscular incision were laparoscopically performed, with the primary lesion inverted to the inside of the stomach. Subsequently, the circumferential mucosal and submucosal incision of the primary lesion was endoscopically added, and the primary lesion was perorally removed [Figure 3].

NEWS in combination with laparoscopic SN mapping enables us to minimize the area of gastric resection as full-thickness partial gastrectomy in patients with SN-negative for metastasis<sup>[25]</sup>. NEWS does not require intentional perforation of the gastric wall during the procedure. Therefore we can apply this technique for treating gastric cancers without the risk of iatrogenic dissemination of tumor cells into the peritoneum and abdominal cavity. The NEWS combined with laparoscopic SN mapping are expected to become a promising minimally invasive, function-preserving gastrectomy to cure cN0 early-stage gastric cancer.

## LIMITATION AND FUTURE PERSPECTIVE OF SN NAVIGATION SURGERY

Many single institutional studies and the prospective multicenter trial of SN mapping and biopsy for early-stage gastric cancer have demonstrated acceptable SN detection rates and accuracy to predict nodal metastatic status. However, SN mapping techniques in details such as the choice of dyes, dual or single tracer methods, and the timing of tracer injection need to be standardized for universal application of SN biopsy in clinical practice. In addition, proper indication of laparoscopic function-preserving gastrectomy such as laparoscopic local resection with SN navigation surgery based on the tumor location has not been verified yet. Also, further improvement in SN navigation techniques should be required for more accurate SN mapping. Results of ongoing Japanese and Korean prospective trials would be expected to verify the postoperative patients' QOL and long-term survival in patients undergoing laparoscopic function-preserving gastrectomy with SN mapping.

## CONCLUSION

For early-stage gastric cancer, good prognosis can currently be guaranteed by conventional standard gastrectomy. However, the personalized, minimally invasive treatments retaining the patients' QOL have to be developed as a next step. Although further studies are required for careful evaluation, laparoscopic function-preserving gastrectomy, such as full-thickness partial gastrectomy, in combination with laparoscopic SN mapping would become an ideal strategy to reach the goal.

## DECLARATIONS

### Authors' contributions

Writing the manuscript: Takeuchi H

Supervising as a primary investigator of the clinical trials in the manuscript: Kitagawa Y

### Availability of data and materials

Not applicable.

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None.

### Conflicts of interest

All authors declare that there are no conflicts of interest.



**Ethical approval and consent to participate**

Not applicable.

**Consent for publication**

Not applicable.

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Review

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# Molecular mechanism of peritoneal dissemination in gastric cancer

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## Abstract

Peritoneal dissemination (PD) is the most common cause of metastasis in gastric cancer (GC). Because there are no standard treatments for PD, it is associated with a poor prognosis. Although clinicians have performed intraperitoneal chemotherapy for GC with PD, the outcome remains unsatisfactory. Therefore, the development of novel treatments and diagnostic tools for PD is expected to improve the prognosis of GC patients with PD. Notably, it is essential to elucidate the molecular mechanisms involved in the development of PD in GC. In this review, the molecular mechanisms of PD (three steps: detachment from the primary tumor, adaptation to the microenvironment of the peritoneal cavity, and attachment to peritoneal mesothelial cells) and new topics in GC are highlighted.

**Keywords:** Gastric cancer, peritoneal dissemination, molecular mechanism

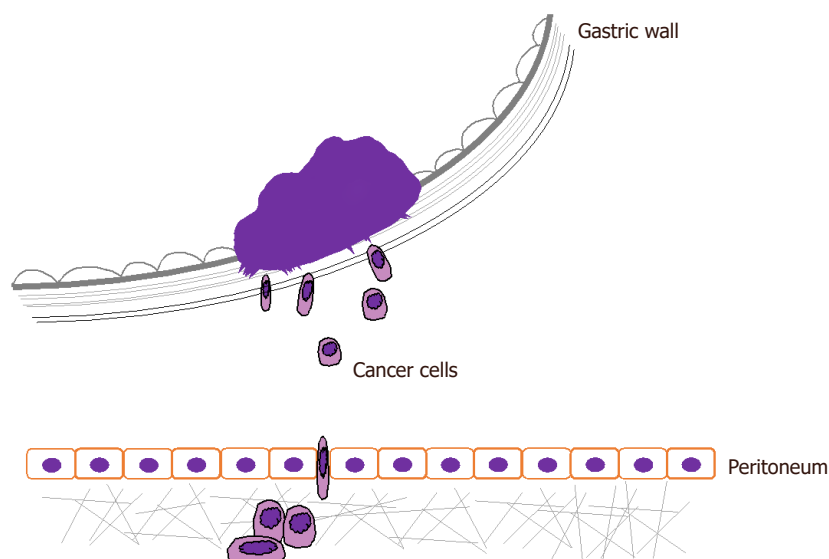
## INTRODUCTION

Gastric cancer (GC) is one of the most prevalent cancers worldwide and is associated with a high mortality rate<sup>[1]</sup>. The malignant potential of GC is characterized biologically by the dissemination of cancer cells from the primary site throughout the peritoneal cavity. Almost 50% of recurrence was peritoneal dissemination in GC, and GC patients with peritoneal dissemination (PD) had a poor prognosis<sup>[2]</sup>. Although molecularly-targeted therapy has improved the prognosis of advanced and recurrent GC, the outcome remains unsatisfactory particularly in GC patients with PD<sup>[3,4]</sup>. Therefore, clarification of the



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**Figure 1.** The metastatic cascade of peritoneal dissemination in gastric cancer

**Table 1.** The major molecules involved in development of peritoneal dissemination in gastric cancer

	Molecule	Biological function	Associated molecules/pathways	References
Detachment from the primary tumor	E-cadherin	Cell-cell adhesion	Wnt, Rho GTPase, NF- $\kappa$ B pathway, EMT	[14-19]
	ARL4C	GTP-binding protein	Rho GTPase, EGF, Wnt	[23,24]
Adaptation to the peritoneal cavity microenvironment	HIF1 $\alpha$	Regulation of cellular and systemic homeostatic responses to hypoxia	EMT, NF- $\kappa$ B pathway, Glucose metabolism	[39-42]
	LOX	Lysyl oxidase	EMT	[43]
	ANGPTL4	Resistance to anoikis	FAK/Src/PI3K/Akt/ERK	[46]
	CXCL12	Chemokine ligand	EMT, CXCL12/CXCR4	[55,56]
	Akt	Serine-threonine kinase	PI3K/Akt, PTEN/PI3K/NF- $\kappa$ B/FAK	[50-54]
	FAK	Tyrosine kinase	Fak/Src	[53,54]
Attachment to peritoneal mesothelial cells and tumor growth	Integrin $\alpha$ 3 $\beta$ 1	Cell adhesion	Lamine-5	[63]
	VEGF	Vascular endothelial growth factor	Angiogenesis	[61,65-67]

molecular mechanisms of PD is important for developing novel therapies and improving the clinical outcomes of GC patients.

The metastatic cascade of GC consists of lymphatic metastasis, hematogenous metastasis, and PD. Although the lymphatic metastasis and hematogenous metastasis are the major dissemination processes in solid cancers, PD is the most frequent metastatic type in GC patients, according to the annual report 2009 from Japanese Gastric Cancer Association. Unlike the lymphatic metastasis and the hematogenous metastasis, the peritoneal dissemination is initially driven by direct invasion from gastric wall to the peritoneal cavity.

Many metastasis-related factors, such as adhesion molecules, matrix proteases, and motility factors, are involved in the development of PD, which is a multistep process<sup>[5-9]</sup>. The first step involves detachment of cancer cells from the primary tumor, followed by survival of the cells in the microenvironment of the peritoneal cavity. The last step is attachment of circulating tumor cells to peritoneal mesothelial cells and tumor growth. In this review, we highlight the major molecular mechanisms of PD [Table 1 and Figure 1] and new topics in GC.

## THE DETACHMENT OF CANCER CELLS FROM THE PRIMARY TUMOR

The development of PD is initiated by penetration of cancer cells through the gastric wall. In this step, cancer cells must have the ability to migrate and invade for successful detachment from the primary tumor and for gaining access to the peritoneal cavity. E-cadherin is a calcium-dependent cell-cell adhesion molecule that plays a crucial role in establishing the epithelial architecture and maintaining cell polarity. Dysregulation of E-cadherin contributes to tumor invasion by promoting cell motility<sup>[10,11]</sup>, resulting in PD. Moreover, E-cadherin and the cadherin-catenin complex may promote invasion and migration by modulating various signaling pathways in epithelial cells, including Wnt signaling<sup>[12]</sup>, Rho GTPase<sup>[13,14]</sup>, and NF- $\kappa$ B pathways<sup>[15,16]</sup>, as well as epithelial-mesenchymal transition (EMT)<sup>[13,17]</sup>.

The activation of Rho GTPases (RhoA, cdc42, Rac) also drives cancer cell motility and invasion by promoting actin cytoskeleton reorganization<sup>[18-20]</sup>. The formation of lamellipodia and filopodia (resulting in actin cytoskeleton reorganization), which are regulated by Rac and cdc42, respectively, contributes to cancer cell motility<sup>[18]</sup>. In a previous study, ADP-ribosylation factor-like 4C (*ARL4C*), a downstream factor of EGF signaling and Wnt signaling, was reported to promote cell motility by activating Rho GTPases<sup>[21]</sup>. We recently found that *ARL4C* is associated with PD in GC, possibly by promoting the invasive capacity of cancer cells via activation of both EMT and actin cytoskeleton reorganization<sup>[22]</sup>. *ARL4C* is proposed to be a novel biomarker and potential therapeutic target for GC patients with PD.

In the process of cancer cell invasion, overexpression of matrix metalloproteinases (MMPs) is required for degradation of the extracellular matrix (ECM)<sup>[23,24]</sup>. High expression of MMP-7 is a reported risk factor for PD in GC<sup>[25]</sup>, and MMP-2 and MMP-9 are also associated with the invasive capacity of gastrointestinal cancer cells<sup>[24,26,27]</sup>. Furthermore, MMP-14 can activate MMP-2 in addition to degradation of ECM<sup>[28]</sup>.

EMT is an essential phenotypic conversion mechanism that has been implicated in the initiation of metastasis and tumor progression in many types of cancers<sup>[29]</sup>. During EMT, epithelial cells exhibit enhanced motility and invasiveness<sup>[30]</sup>, low expression of E-cadherin, high expression of vimentin, a spindle shape, and reduced adhesion. The major ligands involved in EMT are EGF, TGF $\beta$ , Wnt, Notch, and integrin. The major transcription factors that induce EMT via downregulation of E-cadherin expression<sup>[13]</sup> are Twist, Snail, Slug, Zeb1, and Zeb2<sup>[31-33]</sup>. We focused on the influence of EMT on PD and found that discoidin domain-containing receptor 2 promoted PD in GC via induction of EMT<sup>[34]</sup>.

## CELL SURVIVAL IN THE MICROENVIRONMENT OF THE PERITONEAL CAVITY

The microenvironment of the free abdominal space is hypoxic and deficient in glucose<sup>[35]</sup>. The cancer cells, which are seeded in the peritoneal cavity, must survive, proliferate, and migrate in this environment. Cell adhesion to appropriate ECM components with integrin and cadherin is essential for cell survival, and loss of this adhesion induces cell death, which has been termed “anoikis”. Therefore, anoikis resistance is required for cells surviving in the peritoneal cavity and anchorage-independent growth<sup>[36]</sup>.

HIF1 $\alpha$  is reportedly involved in PD in GC, colorectal cancer, and pancreatic cancer<sup>[35,37]</sup>. HIF1 $\alpha$  is induced by hypoxia and functions as a master regulator of cellular and systemic homeostatic responses to hypoxia by activating the transcription of many genes, including those involved in glucose metabolism and other adaptations to hypoxia<sup>[38-40]</sup>. Interestingly, HIF1 $\alpha$  induces EMT by activating the transcription of genes in the LOX family<sup>[41]</sup>. EMT contributes to not only migration and invasion but also anoikis resistance in cancer cells<sup>[42,43]</sup>. HIF1 $\alpha$  also induces angiopoietin-like-4 (*ANGPTL4*), a secreted protein essential for tumor growth and resistance to anoikis in GC cells<sup>[44]</sup>.

Cancer cells develop anoikis resistance via several mechanisms, including changes in integrin repertoire expression, induction of EMT, oncogene activation, and adaption of their metabolism<sup>[45-47]</sup>. In gastrointestinal



cancers, the PI3K/Akt<sup>[48-50]</sup> and PTEN/PI3K/NF- $\kappa$ B/FAK pathways<sup>[51,52]</sup> are involved in the formation of PD and anoikis resistance. FAK is a key integrin signaling molecule involved in cell survival pathways<sup>[51,52]</sup>. Moreover, the CXCL12/CXCR4 pathway can induce EMT<sup>[53,54]</sup> and is associated with PD and anoikis resistance<sup>[55,56]</sup> in multiple human cancers.

## THE ATTACHMENT OF FREE TUMOR CELLS TO PERITONEAL MESOTHELIAL CELLS AND TUMOR GROWTH

Cancer cells seeded in the peritoneal cavity attach directly to the peritoneal surface. However, the mesothelium, a membrane composed of simple squamous epithelium that forms the lining of peritoneum, prevents the cancer cells from penetrating into the submesothelial space. The connective tissue under the mesothelium contributes to the formation of a microenvironment (niche) for seeding cancer nodules in the process of PD<sup>[6,57,58]</sup>. The production of MMPs and integrin is important for the penetration into the submesothelial space<sup>[59]</sup>. Notably, MMP-7 functions as a key factor in the degradation of ECM, promoting the penetration of cancer cells into the submesothelial space and the formation of PD. Integrins, transmembrane receptors that facilitate cell-ECM adhesion, were found to be overexpressed in GC cell lines with high PD potential<sup>[60]</sup>. Takatsuki *et al.*<sup>[61]</sup> reported that inhibition of integrin  $\alpha 3 \beta 1$  reduced the number of disseminated nodules in GC cells. Laminin-5, a ligand with a high affinity for integrin  $\alpha 3 \beta 1$ , is a major ECM glycoprotein. Inhibition of laminin-5 reduced the adhesion of free cells to parietal peritoneum, suggesting that integrin  $\alpha 3 \beta 1$  plays a key role in cell penetration into the submesothelial space<sup>[61]</sup>. Recently, it was reported that mesothelial cells create a novel tissue niche that facilitates GC invasion, resulting in PD<sup>[62]</sup>.

Cancer cells that have attached to connective tissue underlying the mesothelium induce angiogenesis for tumor growth through high expression of vascular endothelial growth factor (VEGF)<sup>[59]</sup>. VEGF is a well-known signaling protein that stimulates formation of blood vessels. Previous studies suggest that VEGF is associated with PD in GC<sup>[63-65]</sup>. VEGF receptor antisense therapy inhibited angiogenesis and PD in GC<sup>[65]</sup>. Targeting VEGF is considered an attractive strategy to inhibit PD in GC.

## NEW TOPICS IN GC

Immune checkpoint inhibitors enhance antitumor T-cell activity through inhibition of immune checkpoints such as the programmed death-1 (PD-1) receptor. Recent trials showed that anti-PD-1 receptor antibodies (pembrolizumab evaluated in KEYNOTE-012 and nivolumab in ONO-4538-12) exert antitumor activity in patients with advanced GC or gastro-esophageal junction cancer<sup>[66,67]</sup>. In a subgroup analysis of the ONO-4538-12 trial, there are no interactions between PD and nivolumab treatment, indicating that nivolumab is effective for treatment of GC patients with or without PD. Immune checkpoint inhibitors are expected to improve the outcome of GC patients with PD.

With the accumulation of genomic/epigenomic data, many public data and online analysis tools are now available. The Cancer Genome Atlas (TCGA) is a large cancer genome project that has accumulated RNA sequencing, exome sequencing, SNP array, DNA methylation, reverse-phase protein lysate microarray, and clinical data across multiple cancers, and these data sets can be downloaded easily. Recently, TCGA reported a molecular classification that divides GC into four subtypes [Epstein-Barr virus (EBV)-positive, microsatellite instability (MSI), genomically stable (GS), chromosomal instability (CIN)] based on integrated genomic/epigenomic data (copy number analysis, whole exome sequencing, DNA methylation arrays, RNA sequencing, microRNA arrays, protein arrays)<sup>[68]</sup>. This classification provides a consistent and unified framework for further clinical and preclinical translational research. Elucidation of the molecular characterization of PD in GC is still needed but is expected to promote the development of novel treatments for GC patients with PD.

Recently, the perinuclear compartment (PNC), a complex nuclear structure associated with metastatic behaviors of cancer cells has drawn much attention<sup>[69,70]</sup>. Metarrestin, a PNC inhibitor, inhibits invasion in vitro, suppresses distant metastatic development in three mouse models of human cancer<sup>[71]</sup>. The invasion is required for the formation of PD, suggesting that metarrestin could also disturb the metastatic cascade of PD. Metarrestin will be submitted to the Food and Drug Administration for approval as an investigational drug in the near future.

## CONCLUSION

The formation of PD is a multistep process, in which cancer cells must detach from the primary tumor, adapt to the microenvironment of the peritoneal cavity, and develop disseminated nodules. GC is characterized by genome instability and intratumoral heterogeneity, which contribute to the development of cancer by enabling adaptation to any change in environment. The same genomic/epigenomic alterations across all clones maybe an attractive therapeutic target for GC patients with PD. Further elucidation of the molecular mechanism underlying PD is essential for developing novel treatments and improving the outcome of GC patients with PD.

## DECLARATIONS

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### Authors' contributions

Designed the study, and wrote the initial draft of the manuscript: Hu QJ

Modified the draft of the manuscript: Ito S

Collected and interpreted the data, and critically reviewed the manuscript: Yanagihara K, Mimori K

Approved the final version of the manuscript: All authors

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### Conflicts of interest

All authors declared that there are no conflicts of interest.

### Ethical approval and consent to participate

Not applicable.

### Consent for publication

Not applicable.

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Review

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# Robotic gastrectomy for gastric cancer

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## Abstract

Robotic gastrectomy (RG) is increasingly performed, particularly in East Asia. With articulated devices, surgeons are able to perform every procedure more comfortably and meticulously, which makes RG ideal from the surgeon's standpoint. However, it is still unclear whether it is a suitable treatment strategy from the patient's viewpoint, due to the lack of solid evidence obtained from randomized controlled trials. The feasibility of RG has been demonstrated in many retrospective comparative studies, which showed similar trends, including relatively less estimated blood loss and longer operation time with RG than laparoscopic gastrectomy (LG), equivalent number of harvested lymph nodes and similar length of postoperative hospital stay between RG and LG. However, considering the higher medical expenses associated with RG, its superiority in terms of long-term survival outcomes will need to be confirmed for it to be accepted more widely.

**Keywords:** da Vinci, robot, gastric cancer, robot assisted gastrectomy, laparoscopic gastrectomy

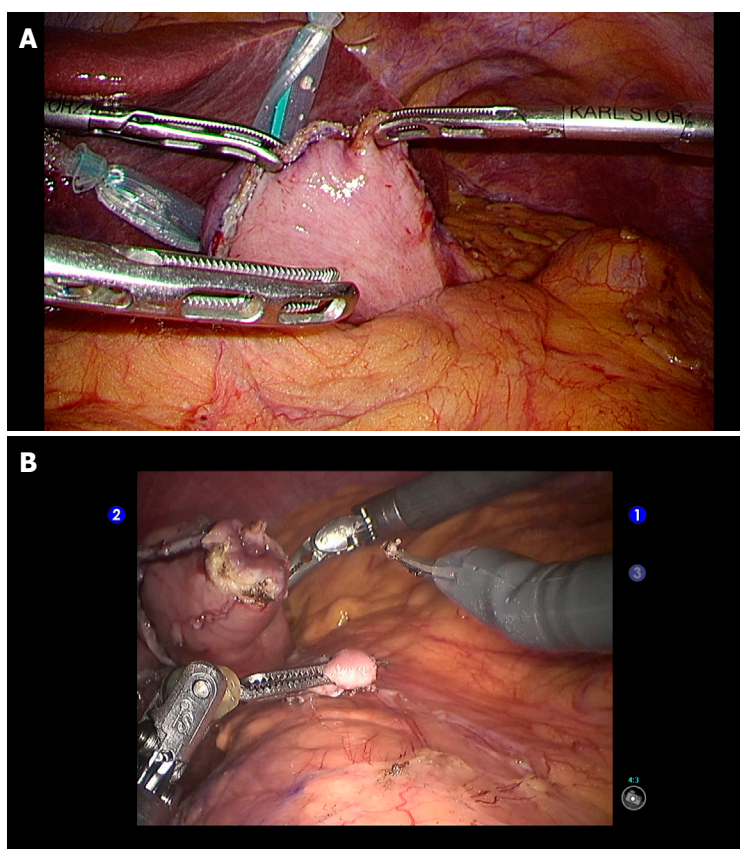
## INTRODUCTION

Minimally invasive surgery (MIS) for gastric cancer has been increasingly performed in the East, where incidence of the disease is high and approximately half of cases are diagnosed at an early stage<sup>[1-3]</sup>. The non-inferiority of laparoscopic gastrectomy (LG) for early gastric cancer comparing to open gastrectomy in terms of short- and/or long-term outcomes has been confirmed by randomized controlled trials, and that for advanced gastric cancer is under investigation and may be shown in the near future<sup>[4-7]</sup>. However, LG has several shortcomings which include limitation in the movement range of forceps and the two-dimensional surgical view available to operating surgeons, and it will be necessary to overcome these issues for MIS to be accepted more widely.



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**Figure 1.** (A) Surgical field during LG: straight devices without articulation are used; (B) surgical field during RG: articulated devices are used

Using the da Vinci® Surgical System (Intuitive Surgical, Sunnyvale, CA, USA), a system for robotic surgery, surgeons are able to attain a three-dimensional view, instrument flexibility, tremor suppression, and improved ergonomics, which are thought to be advantages of robotic gastrectomy (RG)<sup>[8-11]</sup>. With these advantages, theoretically, RG enables surgeons to perform more precise surgery with less trauma, which could result in superior outcomes over LG. However, the number of comparative prospective studies between RG and LG is quite limited, and therefore, solid evidence supporting RG does not yet exist<sup>[12-16]</sup>.

Herein, we would review the comparative retrospective and prospective studies which have investigated the differences in short- and long-term surgical outcomes between RG and LG.

### Clear advantages of RG over LG

There are several clear benefits of RG which contribute to reducing invasiveness and trauma compared with LG. Articulated devices, which are only available in RG, make each surgical technique more meticulous and precise, and are thought to be one definitive advantage of RG [Figure 1]<sup>[8-13]</sup>. Other apparent advantages include a tremor suppression function, which is helpful to keep a stable surgical field and effective to reduce organ injury, and a three-dimensional image, which has become available in LG although special equipment is necessary. With these clear advantages, RG is expected to have advantages over LG. Clear and possible advantages and disadvantages of both procedures are summarized in Table 1.

### Clear disadvantages of RG

Because RG requires expensive machines and devices, cost effectiveness is an intriguing issue for surgeons, and seems to be an absolute disadvantage of RG. In Korea and Japan, where more than half of reports have

**Table 1. Advantages and disadvantages of RG vs. LG are summarized**

Articulated devices	RG favor
3D image	RG favor
Tremor suppression	RG favor
ergonomics	RG favor
Intraoperative blood loss	Equivalent
Morbidity rate	Equivalent
Mortality rate	Equivalent
Medical expense	LG favor
Operation time	LG favor

RG: robotic gastrectomy; LG: laparoscopic gastrectomy

been published, the cost for RG is not yet reimbursed by government, and therefore patients or hospitals have to pay additional fees<sup>[17]</sup>. In contrast, medical expense for LG is partially covered by national insurance systems, and the cost burden on patients and hospitals is obviously less than for RG. The additional fee for RG differs between surgeries depending on how many disposable and re-usable instruments are used. Previously, some comparative studies investigated the difference in medical expense between RG and LG and reported that RG expenses were approximately twice as great<sup>[18-21]</sup>. In a prospective comparative study conducted in Korea, significantly higher total cost in the RG group (US\$13,432) than the LG group (US\$8090) was also reported<sup>[14]</sup>. However, if medical expenses associated with RG decrease in the future, they will no longer be an absolute disadvantage of RG.

## COMPARISON OF SHORT-TERM SURGICAL OUTCOMES BETWEEN RG AND LG

Short-term surgical outcomes between RG and LG have been compared in many retrospective and a few prospective studies<sup>[9,14-20,22-44]</sup>. Among short-term surgical outcomes, intraoperative blood loss, the duration of surgery, the number of retrieved lymph nodes, the incidence of postoperative complications, and the length of postoperative hospital stay are thought to reflect surgical quality, and were assessed in most studies.

Intraoperative blood loss was generally equivalent or less during RG than LG [Table 2]. The magnified fine three-dimensional view attained in RG enables surgeons to recognize even very small vessels, and with articulated devices, they can surely stanch bleeding. However, the reported statistically significant differences in intraoperative bleeding between LG and RG were generally less than 100 mL except for one report from Korea<sup>[38]</sup>, and it is unclear whether the difference is clinically significant or not. Statistically significant more blood loss in RG was also reported in two Japanese studies, but the differences were less than 20 mL<sup>[33,41]</sup>.

The duration of surgery is significantly longer in RG than in LG in all report, and the difference was statistically significant in most series [Table 3]. Although the difference ranged from 14 to 124 min, it took RG generally approximately 60 min more operation time than LG. There are several probable explanations for longer operation time in RG. Firstly, it takes 15 to 30 min, known as docking time, to prepare before an operator begins the surgery at a console. Secondly, during RG, a surgeon uses four robotic arms, which is less than the average number of five ports used during conventional LG. Although an additional port for an assistant can be used in RG, it is under the assistant's not the surgeon's control, and is sometimes useless due to collisions with robotic arms. As a result, it becomes difficult to make a fine surgical field, particularly in patients with high visceral fat volume or advanced disease, and therefore might cause longer operation time.

The number of retrieved lymph nodes was reported to be almost equal between RG and LG. The duration of postoperative hospital stay was also similar, although a few investigators reported that it was shorter following RG than LG.

**Table 2. Comparison of blood loss**

Author	Year	Country/area	Approach	Number of patients (n)	Blood loss (mL)
Kim <i>et al.</i> <sup>[30]</sup>	2010	Korea	ODG vs. LDG vs. RDG	12 vs. 11 vs. 16	<sup>a</sup> 79 vs. 45 vs. 30 <sup>**</sup>
Caruso <i>et al.</i> <sup>[22]</sup>	2011	Italy	OG vs. RG	120 vs. 29	<sup>a</sup> 386 vs. 198 <sup>**</sup>
Woo <i>et al.</i> <sup>[42]</sup>	2011	Korea	LG vs. RG	591 vs. 236	<sup>a</sup> 148 vs. 92 <sup>**</sup>
Huang <i>et al.</i> <sup>[25]</sup>	2012	Korea	OG vs. LG vs. RG	586 vs. 64 vs. 39	<sup>a</sup> 400 vs. 100 vs. 50 <sup>**</sup>
Kim <i>et al.</i> <sup>[29]</sup>	2012	Korea	OG vs. LG vs. RG	4542 vs. 861 vs. 436	<sup>a</sup> 192 vs. 112 vs. 85 <sup>**</sup>
Uyama <i>et al.</i> <sup>[41]</sup>	2012	Japan	LDG vs. RDG	25 vs. 225	<sup>a</sup> 81 vs. 52 <sup>**</sup>
Huang <i>et al.</i> <sup>[19]</sup>	2014	Taiwan	LG vs. RG	73 vs. 35	<sup>a</sup> 116 vs. 80 <sup>**</sup>
Junfeng <i>et al.</i> <sup>[27]</sup>	2014	America	LG vs. RG	394 vs. 120	<sup>a</sup> 138 vs. 118 <sup>**</sup>
Kim <i>et al.</i> <sup>[28]</sup>	2014	Korea	LDG vs. RDG	481 vs. 172	<sup>a</sup> 135 vs. 60 <sup>**</sup>
Lee <i>et al.</i> <sup>[32]</sup>	2015	Korea	LDG vs. RDG	267 vs. 133	<sup>a</sup> 87 vs. 47 <sup>**</sup>
Seo <i>et al.</i> <sup>[37]</sup>	2015	Korea	LDG vs. RDG	40 vs. 40	<sup>a</sup> 227 vs. 76 <sup>**</sup>
Suda <i>et al.</i> <sup>[40]</sup>	2015	Japan	LG vs. RG	438 vs. 88	<sup>a</sup> 34 vs. 48 <sup>*</sup>
Nakauchi <i>et al.</i> <sup>[17]</sup>	2016	Japan	LG vs. RG	437 vs. 84	<sup>a</sup> 33 vs. 44 <sup>*</sup>
Procopiuc <i>et al.</i> <sup>[36]</sup>	2016	Romania	OG vs. RG	29 vs. 18	<sup>a</sup> 564 vs. 208 <sup>**</sup>
Shen <i>et al.</i> <sup>[38]</sup>	2016	China	LG vs. RG	330 vs. 93	<sup>a</sup> 213 vs. 177 <sup>**</sup>
Yang <i>et al.</i> <sup>[43]</sup>	2017	Korea	OG vs. LG vs. RG	241 vs. 511 vs. 173	<sup>a</sup> 149 vs. 66 vs. 53 <sup>**</sup>
Song <i>et al.</i> <sup>[9]</sup>	2009	Korea	LDG (early) vs. RDG	20 vs. 20	-
			LDG (later) vs. RDG	20 vs. 20	40 vs. 94 <sup>**</sup>
Eom <i>et al.</i> <sup>[18]</sup>	2012	Korea	LDG vs. RDG	62 vs. 30	88 vs. 153 <sup>**</sup>
Park <i>et al.</i> <sup>[20]</sup>	2012	Korea	LDG vs. RDG	120 vs. 30	60 vs. 75 <sup>*</sup>
Hyun <i>et al.</i> <sup>[26]</sup>	2013	Korea	LG vs. RG	83 vs. 38	131 vs. 131 <sup>**</sup>
Noshiro <i>et al.</i> <sup>[33]</sup>	2014	Japan	LDG vs. RDG	460 vs. 21	115 vs. 96 <sup>**</sup>
Son <i>et al.</i> <sup>[39]</sup>	2014	Korea	LTG vs. RTG	58 vs. 51	211 vs. 153 <sup>**</sup>
Park <i>et al.</i> <sup>[35]</sup>	2015	Korea	LG vs. RG	622 vs. 148	146 vs. 171 <sup>**</sup>
Cianchi <i>et al.</i> <sup>[23]</sup>	2016	Italy	LDG vs. RDG	41 vs. 30	119 vs. 100 <sup>**</sup>
Okumura <i>et al.</i> <sup>[34]</sup>	2016	Korea	OG vs. RG	132 vs. 49	157 vs. 85 <sup>**</sup>

\*median; \*\*mean. <sup>a</sup> $P < 0.05$ . LDG: laparoscopic distal gastrectomy; LG: laparoscopic gastrectomy; LTG: laparoscopic total gastrectomy; RDG: robotic distal gastrectomy; RG: robotic gastrectomy; RTG: robotic total gastrectomy; ODG: open distal gastrectomy; OG: open gastrectomy

The incidence of postoperative complication was compared between the approaches [Table 4]. Many investigators have thought that RG could be safer than LG, because articulated devices, the three-dimensional image, and the tremor suppression function could make recognition of anatomical structures much easier and lymphadenectomy much safer. However, unexpectedly, significantly lower morbidity rate was reported only in two reports, and the difference, even if morbidity rate was lower in RG than LG, was not statistically significant in other reports<sup>[33,41]</sup>. Considering the current status of LG, which is already a well-established safe procedure, it seems to be very difficult to show that RG could further improve the safety. Mortality rate was not statistically significant between RG and LG in any of the studies, and therefore, both RG and LG seem to be safe procedures in terms of postoperative morbidities and mortality.

### Long-term outcomes between RG and LG

The number of reports focusing on long-term survival outcome is quite limited [Table 5]. Three Korean series, which were from a single institute with different study populations, and one Japanese series, reported long-term outcomes with a median follow up period of at least three years<sup>[32,33,35,40]</sup>. In the Korean series, Lee *et al.*<sup>[32]</sup> focused on patients undergoing D2 distal gastrectomy, Son *et al.*<sup>[39]</sup> included patients undergoing spleen-preserving total gastrectomy, and Okumura *et al.*<sup>[34]</sup> compared long-term survival outcomes of elderly (70 years old or older) patients between RG and LG. None of these studies showed significant survival differences. The Japanese series by Nakauchi *et al.*<sup>[17]</sup> compared three-year overall and recurrence free survival between RG and LG, and reported that no statistically significant difference was found even after stratification by pathological stage. However, the lack of the results of prospective comparative studies focusing on long-term survival makes it difficult to obtain any conclusive result in terms of long-term survival outcomes.

**Table 3. Comparison of operation time**

Author	Year	Country/area	Approach	Number of patients (n)	Operation time (min)
Song <i>et al.</i> <sup>[9]</sup>	2009	Korea	LDG (early) <i>vs.</i> RDG	20 <i>vs.</i> 20	<sup>a</sup> 290 <i>vs.</i> 203 <sup>**</sup>
			LDG (later) <i>vs.</i> RDG	20 <i>vs.</i> 20	<sup>a</sup> 134 <i>vs.</i> 203 <sup>**</sup>
Kim <i>et al.</i> <sup>[30]</sup>	2010	Korea	ODG <i>vs.</i> LDG <i>vs.</i> RDG	12 <i>vs.</i> 11 <i>vs.</i> 16	<sup>a</sup> 127 <i>vs.</i> 204 <i>vs.</i> 259 <sup>**</sup>
Caruso <i>et al.</i> <sup>[22]</sup>	2011	Italy	OG <i>vs.</i> RG	120 <i>vs.</i> 29	<sup>a</sup> 222 <i>vs.</i> 290 <sup>**</sup>
Woo <i>et al.</i> <sup>[42]</sup>	2011	Korea	LG <i>vs.</i> RG	591 <i>vs.</i> 236	<sup>a</sup> 171 <i>vs.</i> 220 <sup>**</sup>
Eom <i>et al.</i> <sup>[18]</sup>	2012	Korea	LDG <i>vs.</i> RDG	62 <i>vs.</i> 30	<sup>a</sup> 189 <i>vs.</i> 229 <sup>**</sup>
Huang <i>et al.</i> <sup>[25]</sup>	2012	Korea	OG <i>vs.</i> LG <i>vs.</i> RG	586 <i>vs.</i> 64 <i>vs.</i> 39	<sup>a</sup> 320 <i>vs.</i> 350 <i>vs.</i> 430 <sup>**</sup>
Kim <i>et al.</i> <sup>[29]</sup>	2012	Korea	OG <i>vs.</i> LG <i>vs.</i> RG	4542 <i>vs.</i> 861 <i>vs.</i> 436	<sup>a</sup> 158 <i>vs.</i> 176 <i>vs.</i> 226 <sup>**</sup>
Park <i>et al.</i> <sup>[20]</sup>	2012	Korea	LDG <i>vs.</i> RDG	120 <i>vs.</i> 30	<sup>a</sup> 140 <i>vs.</i> 218 <sup>*</sup>
Yoon <i>et al.</i> <sup>[44]</sup>	2012	Korea	LTG <i>vs.</i> RTG	65 <i>vs.</i> 36	<sup>a</sup> 210 <i>vs.</i> 306 <sup>**</sup>
Huang <i>et al.</i> <sup>[19]</sup>	2014	Taiwan	LG <i>vs.</i> RG	73 <i>vs.</i> 35	<sup>a</sup> 330 <i>vs.</i> 358 <sup>**</sup>
Junfeng <i>et al.</i> <sup>[27]</sup>	2014	America	LG <i>vs.</i> RG	394 <i>vs.</i> 120	<sup>a</sup> 221 <i>vs.</i> 235 <sup>**</sup>
Kim <i>et al.</i> <sup>[28]</sup>	2014	Korea	LDG <i>vs.</i> RDG	481 <i>vs.</i> 172	<sup>a</sup> 167 <i>vs.</i> 206 <sup>**</sup>
Noshiro <i>et al.</i> <sup>[33]</sup>	2014	Japan	LDG <i>vs.</i> RDG	460 <i>vs.</i> 21	<sup>a</sup> 315 <i>vs.</i> 439 <sup>**</sup>
Son <i>et al.</i> <sup>[39]</sup>	2014	Korea	LTG <i>vs.</i> RTG	58 <i>vs.</i> 51	<sup>a</sup> 210 <i>vs.</i> 264 <sup>**</sup>
Han <i>et al.</i> <sup>[24]</sup>	2015	Korea	LPPG <i>vs.</i> RPPG	69 <i>vs.</i> 68	<sup>a</sup> 194 <i>vs.</i> 258 <sup>**</sup>
Lee <i>et al.</i> <sup>[32]</sup>	2015	Korea	LDG <i>vs.</i> RDG	267 <i>vs.</i> 133	<sup>a</sup> 171 <i>vs.</i> 218 <sup>**</sup>
Park <i>et al.</i> <sup>[35]</sup>	2015	Korea	LG <i>vs.</i> RG	622 <i>vs.</i> 148	<sup>a</sup> 189 <i>vs.</i> 255 <sup>**</sup>
Suda <i>et al.</i> <sup>[40]</sup>	2015	Japan	LG <i>vs.</i> RG	438 <i>vs.</i> 88	<sup>a</sup> 361 <i>vs.</i> 381 <sup>*</sup>
Cianchi <i>et al.</i> <sup>[23]</sup>	2016	Italy	LDG <i>vs.</i> RDG	41 <i>vs.</i> 30	<sup>a</sup> 262 <i>vs.</i> 323 <sup>**</sup>
Kim <i>et al.</i> <sup>[31]</sup>	2016	Korea	LDG <i>vs.</i> RDG	288 <i>vs.</i> 87	<sup>a</sup> 230 <i>vs.</i> 248 <sup>**</sup>
Nakauchi <i>et al.</i> <sup>[17]</sup>	2016	Japan	LG <i>vs.</i> RG	437 <i>vs.</i> 84	<sup>a</sup> 361 <i>vs.</i> 378 <sup>*</sup>
Okumura <i>et al.</i> <sup>[34]</sup>	2016	Korea	OG <i>vs.</i> RG	132 <i>vs.</i> 49	<sup>a</sup> 174 <i>vs.</i> 227 <sup>**</sup>
Procopiuc <i>et al.</i> <sup>[36]</sup>	2016	Romania	OG <i>vs.</i> RG	29 <i>vs.</i> 18	<sup>a</sup> 243 <i>vs.</i> 320 <sup>**</sup>
Shen <i>et al.</i> <sup>[38]</sup>	2016	China	LG <i>vs.</i> RG	330 <i>vs.</i> 93	<sup>a</sup> 226 <i>vs.</i> 257 <sup>**</sup>
Yang <i>et al.</i> <sup>[43]</sup>	2017	Korea	OG <i>vs.</i> LG <i>vs.</i> RG	241 <i>vs.</i> 511 <i>vs.</i> 173	<sup>a</sup> 193 <i>vs.</i> 174 <i>vs.</i> 202 <sup>**</sup>
Uyama <i>et al.</i> <sup>[41]</sup>	2012	Japan	LDG <i>vs.</i> RDG	25 <i>vs.</i> 225	345 <i>vs.</i> 361 <sup>**</sup>
Hyun <i>et al.</i> <sup>[26]</sup>	2013	Korea	LG <i>vs.</i> RG	83 <i>vs.</i> 38	220 <i>vs.</i> 234 <sup>**</sup>
Seo <i>et al.</i> <sup>[37]</sup>	2015	Korea	LDG <i>vs.</i> RDG	40 <i>vs.</i> 40	224 <i>vs.</i> 243 <sup>**</sup>

\*median; \*\*mean. <sup>a</sup> $P < 0.05$ . LDG: laparoscopic distal gastrectomy; LG: laparoscopic gastrectomy; LTG: laparoscopic total gastrectomy; LPPG: laparoscopic pylorus preserving gastrectomy; RDG: robotic distal gastrectomy; RG: robotic gastrectomy; RTG: robotic total gastrectomy; RPPG: robotic pylorus preserving gastrectomy; ODG: open distal gastrectomy; OG: open gastrectomy

Considering the total medical expense of RG, long-term outcomes of RG need to be better than those of LG, and should be confirmed by future prospective trials.

## PROSPECTIVE STUDIES

Although quite a few retrospective studies already exist, the number of prospective studies, particularly that of prospective comparative studies, is extremely limited so far<sup>[12-14,16]</sup>.

Kim *et al.*<sup>[14]</sup> reported the results of a prospective non-randomized comparative study. In their study, a total of 423 patients selected either RG or LG after they received a comprehensive explanation of each procedure, and were matched according to surgeon, extent of gastric resection, and sex. Similar early surgical outcomes including morbidity and mortality rate, except for longer operation time in the RG group were reported.

The results of a single-center prospective randomized trial, in which patients were allocated to either open ( $n = 153$ ) or robotic ( $n = 158$ ) gastrectomy groups, were reported by Wang *et al.*<sup>[16]</sup>. Similar complication rates between the groups, and less estimated blood loss, longer duration of surgery, and shorter postoperative hospital stay in the robotic group than the open group were reported.



**Table 4. Comparison of postoperative morbidity and mortality**

Author	Year	Country/area	Approach	Number of patients (n)	Morbidity rate	Mortality rate
Huang <i>et al.</i> <sup>[19]</sup>	2014	Taiwan	LG vs. RG	73 vs. 35	<sup>a</sup> 8% vs. 13%	1.4% vs. 1.4%
Suda <i>et al.</i> <sup>[40]</sup>	2015	Japan	LG vs. RG	438 vs. 88	<sup>a</sup> 11% vs. 2%	0.2% vs. 1.1%
Nakauchi <i>et al.</i> <sup>[17]</sup>	2016	Japan	LG vs. RG	437 vs. 84	<sup>a</sup> 12% vs. 2%	-
Yang <i>et al.</i> <sup>[43]</sup>	2017	Korea	OG vs. LG vs. RG	241 vs. 511 vs. 173	<sup>a</sup> 25% vs. 12% vs. 5%	0.8% vs. 0.4% vs. 0%
Song <i>et al.</i> <sup>[9]</sup>	2009	Korea	LDG (early) vs. RDG	20 vs. 20	5% vs. 5%	0% vs. 0%
			LDG (later) vs. RDG	20 vs. 20	10% vs. 5%	0% vs. 0%
Kim <i>et al.</i> <sup>[30]</sup>	2010	Korea	ODG vs. LDG vs. RDG	12 vs. 11 vs. 16	17% vs. 9% vs. 13%	0% vs. 0% vs. 0%
Caruso <i>et al.</i> <sup>[22]</sup>	2011	Italy	OG vs. RG	120 vs. 29	43% vs. 41%	3.3% vs. 0%
Woo <i>et al.</i> <sup>[42]</sup>	2011	Korea	LG vs. RG	591 vs. 236	14% vs. 11%	0.3% vs. 0.4%
Eom <i>et al.</i> <sup>[18]</sup>	2012	Korea	LDG vs. RDG	62 vs. 30	7% vs. 13%	0% vs. 0%
Huang <i>et al.</i> <sup>[25]</sup>	2012	Korea	OG vs. LG vs. RG	586 vs. 64 vs. 39	15% vs. 16% vs. 15%	1.4% vs. 1.6% vs. 2.6%
Kim <i>et al.</i> <sup>[29]</sup>	2012	Korea	OG vs. LG vs. RG	4542 vs. 861 vs. 436	11% vs. 9% vs. 10%	0.5% vs. 0.3% vs. 0.5%
Park <i>et al.</i> <sup>[20]</sup>	2012	Korea	LDG vs. RDG	120 vs. 30	8% vs. 17%	0% vs. 0%
Uyama <i>et al.</i> <sup>[41]</sup>	2012	Japan	LDG vs. RDG	25 vs. 225	11% vs. 17%	0% vs. 0%
Yoon <i>et al.</i> <sup>[44]</sup>	2012	Korea	LTG vs. RTG	65 vs. 36	15% vs. 17%	0% vs. 0%
Hyun <i>et al.</i> <sup>[26]</sup>	2013	Korea	LG vs. RG	83 vs. 38	39% vs. 47%	0% vs. 0%
Junfeng <i>et al.</i> <sup>[27]</sup>	2014	America	LG vs. RG	394 vs. 120	4% vs. 6%	-
Kim <i>et al.</i> <sup>[28]</sup>	2014	Korea	LDG vs. RDG	481 vs. 172	4% vs. 5%	0.6% vs. 0%
Noshiro <i>et al.</i> <sup>[33]</sup>	2014	Japan	LDG vs. RDG	460 vs. 21	10% vs. 10%	0% vs. 0%
Son <i>et al.</i> <sup>[39]</sup>	2014	Korea	LTG vs. RTG	58 vs. 51	22% vs. 16%	0% vs. 2.0%
Han <i>et al.</i> <sup>[24]</sup>	2015	Korea	LPPG vs. RPPG	69 vs. 68	22% vs. 19%	0% vs. 0%
Lee <i>et al.</i> <sup>[32]</sup>	2015	Korea	LDG vs. RDG	267 vs. 133	13% vs. 11%	-
Seo <i>et al.</i> <sup>[37]</sup>	2015	Korea	LDG vs. RDG	40 vs. 40	30% vs. 28%	-
Park <i>et al.</i> <sup>[35]</sup>	2015	Korea	LG vs. RG	622 vs. 148	8% vs. 8%	0.5% vs. 0%
Cianchi <i>et al.</i> <sup>[23]</sup>	2016	Italy	LDG vs. RDG	41 vs. 30	12% vs. 13%	4.9% vs. 3.3%
Kim <i>et al.</i> <sup>[31]</sup>	2016	Korea	LDG vs. RDG	288 vs. 87	9% vs. 6%	0.3% vs. 1.1%
Okumura <i>et al.</i> <sup>[34]</sup>	2016	Korea	OG vs. RG	132 vs. 49	18% vs. 14%	0% vs. 0%
Procopiuc <i>et al.</i> <sup>[36]</sup>	2016	Romania	OG vs. RG	29 vs. 18	28% vs. 22%	0% vs. 0%
Shen <i>et al.</i> <sup>[38]</sup>	2016	China	LG vs. RG	330 vs. 93	10% vs. 10%	-

<sup>a</sup>*P* < 0.05. LDG: laparoscopic distal gastrectomy; LG: laparoscopic gastrectomy; LTG: laparoscopic total gastrectomy; LPPG: laparoscopic pylorus preservingl gastrectomy; RDG: robotic distal gastrectomy; RG: robotic gastrectomy; RTG: robotic total gastrectomy; RPPG: robotic pylorus preservingl gastrectomy; ODG: open distal gastrectomy; OG: open gastrectomy

**Table 5. Studies which provided long-term survival outcomes**

Author	Year	Country/area	Approach	Number of patients (n)	Median Follow up period (months)	5y-OS (%)	5y-DFS (%)
Son <i>et al.</i> <sup>[39]</sup>	2014	Korea	LTG vs. RTG	58 vs. 51	<sup>a</sup> 70	<sup>a</sup> 91.1 vs. 89.5	<sup>a</sup> 90.2 vs. 91.2
Lee <i>et al.</i> <sup>[32]</sup>	2015	Korea	LDG vs. RDG	267 vs. 133	<sup>a</sup> 75	<sup>a</sup> N.S.	-
Okumura <i>et al.</i> <sup>[34]</sup>	2016	Korea	OG vs. RG	132 vs. 49	<sup>a</sup> 58	<sup>a</sup> N.S.	-
Junfeng <i>et al.</i> <sup>[27]</sup>	2014	America	LG vs. RG	394 vs. 120	19 vs. 15	69.9 vs. 67.8 (3y)	-
Han <i>et al.</i> <sup>[24]</sup>	2015	Korea	LPPG vs. RPPG	69 vs. 68	19 vs. 23	-	-
Nakauchi <i>et al.</i> <sup>[17]</sup>	2016	Japan	LG vs. RG	437 vs. 84	42 vs. 41	88.8 vs. 86.9 (3y)	86.3 vs. 86.9 (3y)
Procopiuc <i>et al.</i> <sup>[36]</sup>	2016	Romania	OG vs. RG	29 vs. 18	32 vs. 25	N.S.	-

<sup>a</sup>median follow up period longer than 3 years. N.S.: statistically not significant difference; LDG: laparoscopic distal gastrectomy; LG: laparoscopic gastrectomy; LTG: laparoscopic total gastrectomy; LPPG: laparoscopic pylorus preservingl gastrectomy; RDG: robotic distal gastrectomy; RG: robotic gastrectomy; RTG: robotic total gastrectomy; RPPG: robotic pylorus preservingl gastrectomy; OG: open gastrectomy

## DISCUSSION

RG has several absolute advantages, which include articulated devices, tremor suppression function, and a fine three-dimensional view, and surgeons can perform operations comfortably with these technologies. However, these advantages are from the surgeons' perspective, and it is unclear whether these technologies applied to RG are also advantageous from the patients' viewpoint. Theoretically, the more meticulous and precise surgeries are, the better the outcomes will be. However, for RG to be more widely accepted, advantages from the patients' side should be demonstrated in clinical trials, ideally in prospective randomized trials.

Short-term surgical outcomes such as intraoperative bleeding, surgical time, duration of postoperative hospital stay, and postoperative morbidity and mortality rate are thought to reflect surgical quality, and some of them directly affect patients' quality of life. Therefore, these factors are frequently compared between surgical procedures, when investigators need to show superiority or non-inferiority of a newly emergent procedure. Indeed, they have been compared in many studies of RG and LG. However, it seems difficult to conclude that RG is a superior procedure to LG in terms of short-term surgical outcomes, because RG is a more time-consuming procedure, but does not show any obvious benefits. Although some have reported that RG is associated with less bleeding, the differences, which were generally less than 100 mL, seem not to be clinically meaningful. It might be difficult to demonstrate that RG could further improve short-term surgical outcomes, because LG is already a well-established and satisfactorily safe procedure.

The number of studies focusing on long-term surgical outcomes is quite limited, due to insufficient follow-up period in each study. So far, similar long-term survival outcomes between RG and LG have been reported, and we need to wait for the results of currently ongoing studies to reach any conclusions about long-term survival outcomes.

Interpretation of the results of comparative studies should be done carefully because of possible selection bias. In most comparative studies, surgical approaches were selected by the patients themselves after thoughtful explanation of both procedures, but the possibility of selection bias should be taken into account. To overcome this issue, well designed prospective, hopefully randomized controlled, trials are necessary, and we have to at least wait for the results of prospective non-randomized comparative studies<sup>[14]</sup>.

To demonstrate the feasibility of RG, the surgical outcomes of RG are usually compared with those of LG. However, considering that both surgeries were developed on the concept of being minimally invasive, the differences between RG and LG might be marginal, even if RG is truly a superior procedure to LG. In addition RG is, so far, obviously the more expensive surgical procedure. Therefore, it seems unrealistic for RG to completely replace LG with all surgeries in the very near future. However, if the cost of RG decreases dramatically and high medical expense is no longer a problem, it may be a different story with RG becoming further widespread.

So far, RG seems to be as feasible as LG in terms of short- and long-term surgical outcomes. However, RG is an expensive procedure at present, and it is unclear whether RG is superior to LG from the patients' standpoint. The results of well designed prospective comparative studies are awaited to obtain conclusive results on this issue.

## **DECLARATIONS**

### **Authors' contributions**

Analysed and interpreted the data: Tokunaga M, Watanabe M, Sugita S, Tonouchi A, Kaito A, Kinoshita T  
Read and approved the final manuscript: Tokunaga M, Watanabe M, Sugita S, Tonouchi A, Kaito A, Kinoshita T

### **Availability of data and materials**

Not applicable.

### **Financial support and sponsorship**

None.

### **Conflicts of interest**

All authors declared that there are no conflicts of interest.

**Ethical approval and consent to participate**

Not applicable.

**Consent for publication**

Not applicable.

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Original Article

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# Extracellular control of chromosomal instability and maintenance of intra-tumoral heterogeneity

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## Abstract

**Aim:** Current cancer treatments are challenged by the plasticity of cancer cells, largely influenced by chromosomal instability (CIN) leading to variations in karyotype known as tumor-specific aneuploidy, which in turn, leads to intra-tumor cellular heterogeneity (TH). Cells with certain chromosomal defects often survive treatment and the growth-associated states of TH persist in recurrent tumors. Modulation of the CIN rate seems to reside within the tumor itself. In an attempt to develop a therapy targeting cancer plasticity, we studied the possible extracellular control of CIN rate in Chr7-defined TH in gliomas.

**Methods:** Chr7-fluorescence *in situ* hybridization was applied on various grades of gliomas, *in vitro* cultures and intracranial xenografts of two syngeneic glioma lines (U251 and U251-NS) derived from various cell-inoculating densities, with or without EFEMP1 overexpression.

**Results:** A grade-dependent increase of trisomy-7 population and Chr7-defined cell diversity was shown in gliomas. A negative association between Chr7-MS rate and initial cell-inoculating density was observed which was prevented by EFEMP1 overexpression.

**Conclusion:** Our data demonstrate that CIN is a major driver for cancer cell plasticity and suggest that CIN can be controlled by extracellular factors derived from normal and tumor cells, and EFEMP1 is one of these factors.

**Keywords:** Malignant glioma, intra-tumoral heterogeneity, functional tumor subpopulations, chromosome 7, chromosome mis-segregation, EFEMP1



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## INTRODUCTION

Cancer has long been known as a disease associated with genetic defects, largely represented by aneuploidy at both early and late stages, which are maintained in local recurrences and metastases of tumors and the derived cell lines<sup>[1-4]</sup>. Recent advances in high-throughput sequencing technology enabling analysis of single cancer cell's transcriptome and genomics by RNA and DNA sequencing, respectively<sup>[5,6]</sup>, have revealed shockingly large degrees of cancer cell phenotypic and genetic diversity<sup>[7,8]</sup>, which is consistent with the long-seen hallmark of cancer, namely intratumoral cellular heterogeneity (TH), cellular differences in morphology, transcriptome, metabolism, motility, and angiogenic, proliferative, immunogenic, and metastatic potential within a single neoplasm<sup>[9-12]</sup>.

There have been reports of TH for both non-heritable (non-transferable) and heritable (transferable) sources of diversity in tumor cell populations. Non-heritable sources are mainly described for phenotypes of cancer stem cells in re-initiation of cancer, and epithelial-mesenchymal transition and endothelial trans-differentiation that resemble those of embryonic cells by epigenetic re-programing. Heritable sources are cells with genetic mutations and karyotype and DNA copy number variations, and even epigenetic modifications. Tumor-specific aneuploid cells with different tumor-forming phenotypes and the stable states of TH are strongly influenced by chromosomal instability (CIN) and the tumor microenvironments. Studies by Hu *et al.*<sup>[13]</sup> demonstrated a connection between non-heritable and CIN-related heritable sources, and was supported by a further study by our lab<sup>[14]</sup>. These studies suggest that mis-segregation (MS) of tumor-specific chromosome or variable distribution of chromosomal fragments with oncogene amplification, known as double minute (DM), is one of the control mechanisms in maintaining diversity in tumor cell subpopulations that are functionally complementary in tumor formation, hence it underlies the recurrence of glioblastoma multiforme (GBM) after bulk tumor resection and chemo/radiation therapy.

The cancer-driving role of CIN is well supported by experimental data. As shown by Klein *et al.*<sup>[15]</sup>, activated oncogenes destabilize karyotypes and function indirectly, like carcinogens. Mitotic checkpoint defects are the major causes of aneuploid cells, and most turn out to be unviable<sup>[16-18]</sup>. The ability to produce aneuploid cells allows selection to take place which is essential to cancer evolution<sup>[19]</sup>. It is also a fast evolving mechanism employed by yeast<sup>[20]</sup>. The catalytic role of CIN on increasing tumor cell genetic clonal diversity in causing tumor progression has been suggested by a theoretical study of cancer progression<sup>[21]</sup>, and supported by a study on clinical samples of esophageal adenocarcinoma<sup>[22]</sup>. Cells differing in aneuploidy would differentially grow in different tumor microenvironments, e.g., hypoxia, low pH, providing a tumor survival benefit under changing environmental circumstances<sup>[23,24]</sup>. No doubt, CIN defined cancer plasticity has challenged cancer treatment thus far<sup>[25-31]</sup>.

As revealed by single-cell RNA and genomic sequencing, tumor cell subpopulations differ genetically (in number of genes and chromosomes and DNA methylation) and in transcriptome, which leads to phenotypic and functional subpopulation diversity and ultimately to cancer plasticity. The characteristics of tumor cell subpopulations and the dynamic steady state of tumor cell subpopulations are established through selection in favor of cancer persistence and growth. To understand the resistance of cancer to therapeutic interventions (bulk tumor resection, chemo/radiation therapy, targeted therapy, *etc.*), it is important to understand both the formation and maintenance of TH.

From CIN-empowered cell variables, the successful selection in favor of cancer development would simplify the tumor-ecology by streamlining subpopulation diversity down to only the essential subpopulations; to form a team of synergistically interactive functional tumor cell subpopulations that would drive the fast growth and invasive characteristics of cancer. In such stage of cancer evolution, CIN would work against cancer by de-stabilizing the optimal tumor-ecology. In this scenario, selection would be directed to suppress CIN. Thus both promotion and inhibition of CIN are important events favoring successful cancer evolution.

Understanding such “Yin” and “Yang” reciprocal aspects of CIN could facilitate development of therapeutic strategies, which could potentially prevent cancer recurrence.

This study attempts to explore this possibility, by studying a cell line model of GBM in which two tumor subpopulations have been functionally characterized as stem-like tumor initiating cell (STIC) and tumor mass-forming cell (TMC), defined by different copies of chromosome 7 (Chr7), and their inter-conversions via MS of Chr7<sup>[13]</sup>. We further studied our prior finding of changes in the steady state of Chr7-defined subpopulations in response to microenvironmental cues and an extracellular protein named fibulin-3, or EGF containing fibulin-like extracellular matrix protein 1 (EFEMP1)<sup>[32]</sup>.

## METHODS

### Ethics statement for human tissues

Tumors from Tissue Bank of UC Irvine and University of Arkansas for Medical Science were included in this study, with Institutional Review Board approval, as reported previously<sup>[33]</sup>.

### Cell cultures

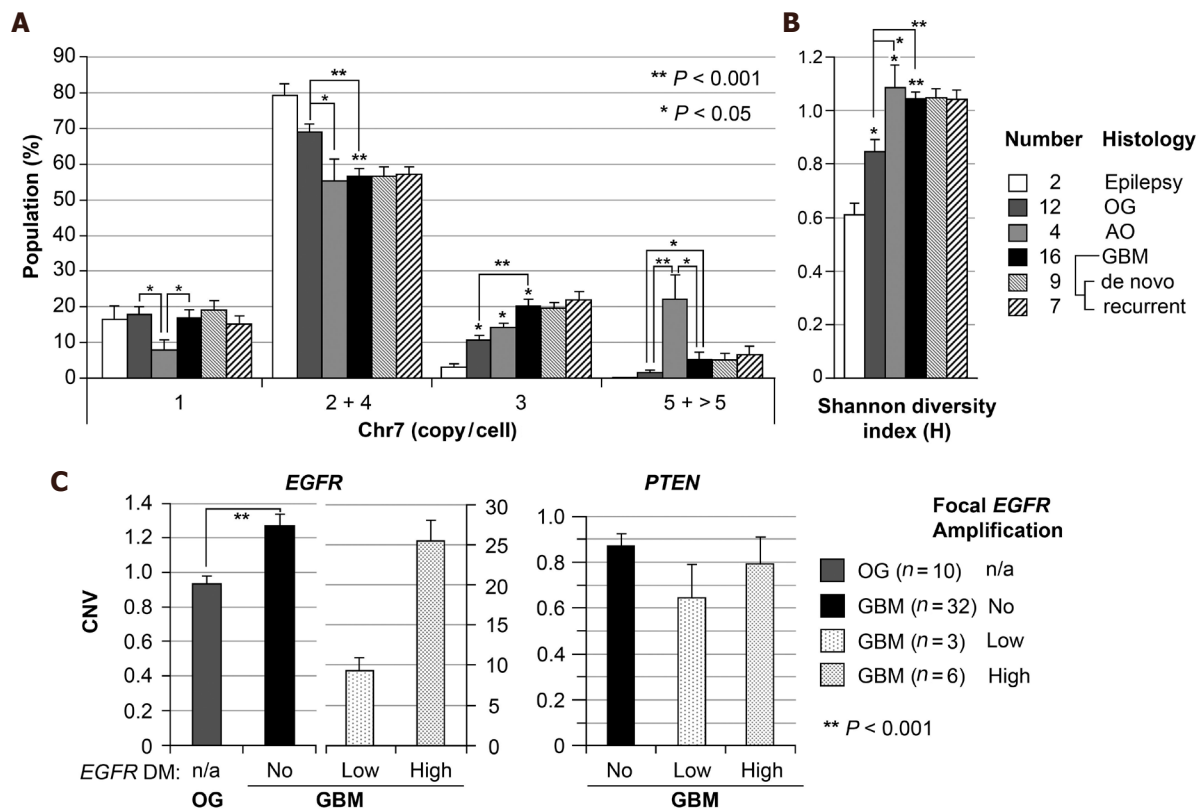
The human glioma cell line U251 (also known as U251HF) was obtained from M.D. Anderson Cancer Center, University of Texas. U251-NS is a single-cell line of neural sphere culture of U251 established at UC Irvine Brain Tumor Research Laboratory. Characterization of U251 with phenotypes defined as tumor mass-forming cells (TMC) and U251-NS as stem-like tumor initiating cells (STIC) and their Short Tandem Repeat (STR) profiles were reported previously<sup>[13]</sup>. EFEMP1 and Empty/pTRIPZ lentiviral vectors and their transduced glioma cells (U251 and U251-NS) were described by Hu *et al.*<sup>[32]</sup>.

U251 (including those infected by lentiviral vectors) was grown in monolayer cultures in DMEM/F12 supplemented with 5% bovine serum, respectively, while U251-NS (including those infected by lentiviral vectors) were grown in 1% agar-coated plates in DMEM/F12 supplemented with epidermal growth factor (EGF, 20 ng/mL), basic fibroblast growth factor (FGF, 10 ng/mL), and 1% B27 (Invitrogen, Carlsbad, CA). U251-NS was attached in fibronectin (10 µg/mL)-coated plates prior to FISH analysis.

### Fluorescence *in situ* hybridization

The methods for fluorescence *in situ* hybridization analyses on glioma specimens, glioma xenografts from intracranial models of mice, and cell cultures were reported previously<sup>[32]</sup>. Briefly, metaphase-spread slides were obtained by exposing exponentially growing cells to nocodazole solution (100 µg/mL final, Sigma) for 1 h. Then the cells were trypsinized (0.25% trypsin/EDTA, Invitrogen) to collect cell pellets, which were treated with a hypotonic solution (phosphate buffer) for 5 min at 37 °C. The cell pellets were fixed (methanol:glacial acetic acid = 3:1) for at least 30 min. Finally, the cell suspensions were dropped onto slides to get metaphase chromosome spreads. Cryosections (7 µm) of human glioma and epilepsy brain tissue frozen specimens, and mice brain with i.c. xenografts of glioma cells were fixed with 100% methanol for 5 min. The slides were further treated with 0.3% sodium citrate solution for 10 min in a pressure cooker, and rinsed with water briefly. FISH analyses on glioma cells and tissues were performed using Vysis LSI EGFR SpectrumOrange/CEP 7 SpectrumGreen Probes (Abbott Molecular Inc) following the manufacturer's instructions. Cells were counted on slides using a Nikon Eclipse TS100/TS100F fluorescent microscope with a 100× lens.

The numbers of Chr7 centromeres per nucleus, detected by the FISH CEP7 probe, were counted and the percentages of cells with different copies of Chr7 were determined based on counting of more than 250 nuclei per sample of tumors or cell cultures. These data were used to establish the level of tumor heterogeneity with regard to Chr7-defined cell subpopulations. The Shannon diversity index (H) was calculated to show the degree of diversity with regard to Chr7-tumor cell subpopulations as described previously<sup>[22]</sup>.



**Figure 1.** Increase of trisomy-7 cell percentage and Chr7-defined cell diversity in higher grade of glioma. (A) Chr7-subpopulations in gliomas of WHO grades II (Oligodendroglioma, OG), III (anaplastic oligodendroglioma, AO), and IV (glioblastoma multiforme, GBM), determined by FISH. Cells with 4 copies of Chr7 were considered as tetraploid cells with 2-Chr7; (B) Shannon diversity index (H) calculated based on the percentage of cells in four groups shown in A; (C) comparison of copy number variation (CNV) of *EGFR* and *PTEN* in gliomas by CQ-PCR analysis of DNA samples. Bar height and error bar are mean and SEM of individual tumors

### Real-time comparative quantitative polymerase chain reaction

DNA samples from frozen glioma specimens were isolated using a DNeasy kit (QIAGEN, Valencia, CA). Comparative quantitative polymerase chain reaction (CQ-PCR) standards (CQ101 for *EGFR* and CQ102 for *PTEN*) and PCR primers of *EGFR*, *PTEN*, and three reference genes on 2q34 (*SPAG16*), 3p14.3 (*ERC2*), and 5q31.2 (*SPOCK1*) were from Ziren Research LLC (Irvine, CA). It is a recombinant DNA containing PCR fragments of *EGFR* or *PTEN* and reference genes in one piece to determine copy number variation (CNV) as described previously<sup>[33]</sup>. Real-time PCR was carried out using FAST-START SYBR-Green I Master Mix (Roche).

### Statistical analysis

Two tailed *T*-tests with equal sample variation were performed to measure significance on pairwise comparisons, with  $P < 0.05$ , 0.01, and 0.001 are shown in presented figures.

## RESULTS

### Increase of trisomy-7 and Chr7-defined cell diversity in higher grades of glioma

Using FISH analysis with dual probes for centromere 7 and *EGFR*, our prior studies showed common alterations of Chr7, both in number and structure in established glioblastoma multiforme (GBM) derived cell lines of U251, A172, LN229, and T98G, and an increasing Chr7 score (average copy of Chr7 per cell) along with increase in the grade of human gliomas<sup>[13]</sup>. Here we re-analyzed the FISH data on these gliomas, and showed a significantly higher percentage of cells with 3 copies of Chr7 in GBM (grade 4,  $n = 16$ ) compared to oligodendroglioma (OG, grade 2,  $n = 12$ ) [Figure 1A]. The Shannon diversity index (H) was calculated

based on the percentages of four Chr7-defined cells shown in Figure 1A, to compare the degree of tumor subpopulation diversity between different grades of gliomas. As shown in Figure 1B, gliomas of all grades presented significantly higher value of H-index compared with non-tumoral brain tissues from patients with epilepsy. Furthermore, grade III AO and IV GBM both showed significantly higher values of H-index compared with grade II OG, due to significant increase of cells carrying 5 and 3 copies Chr7, respectively.

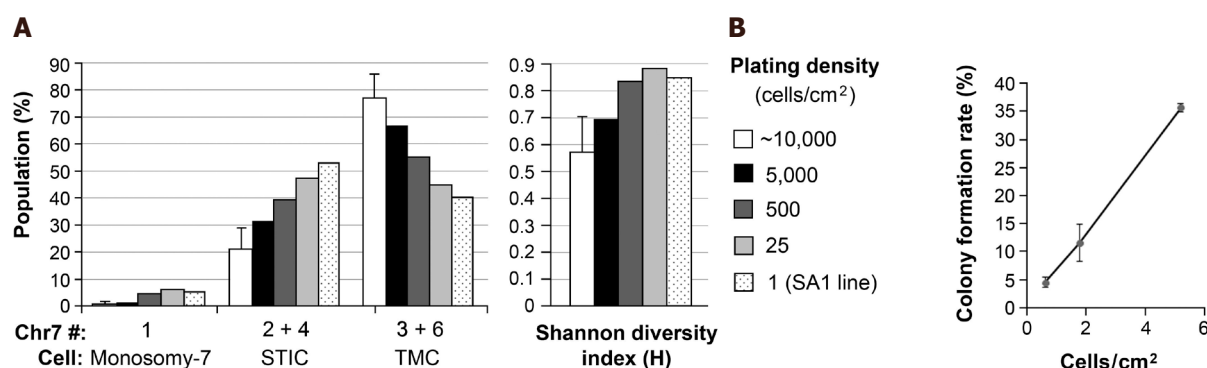
To determine if the observed increase in cells with 3 copies of Chr7 are trisomy-7 or triploid cells, we performed CQ-PCR for CNV of *EGFR* on 7p in reference to three single-copy genes (*ERC2*, *SPAG16* and *SPOCK1*) on three different chromosomes (3p, 2q and 5q, respectively) in a larger set of human glioma samples, including OG and GBM tissues used in FISH analyses. As shown in Figure 1C, most of GBMs (78%) showed significantly (on the average of 1.3-fold) higher copies of *EGFR* compared with that of OGs. About 22% of GBM showed very high copies of *EGFR*, with 7%, on the average 9-fold higher and 15%, on the average 26-fold higher, from focal *EGFR* amplification. As reported previously, this is due to extrachromosomal oncogene amplification or double minute chromosome (DM)<sup>[34]</sup>. In contrast to overall increase of *EGFR* CNV, CQ-PCR showed overall decrease of *PTEN* gene copy, with an average of less than 1 ratio of *PTEN* to one of the three reference genes. Taking together, data from FISH and CQ-PCR are consistent with increase of trisomy-7 population in GBM as compared with OG.

### Low cell-plating density caused increase of CIN rate

We have previously presented two GBM heterogeneity models where variations in Chr7 or DM status characterized tumor subpopulations functionally defined as TMC and STIC, which were enriched by certain *in vitro* culture conditions, known as serum-adherent (SA) and neurosphere (NS) conditions, respectively<sup>[13,14]</sup>. The dynamic state of tumor sub population diversity was stabilized with one dominant subpopulation over long-term passages at high cell-plating densities without changing culture conditions. However, under the same culture conditions, single-cell cultures, derived from single cell or soft agar colony, presented not only diverse cell populations, but also higher degrees of heterogeneity compared with their parental cultures. Examples are Chr7-defined subpopulations in single-cell SA and NS lines of four established GBM cell lines (U251, A172, LN229, and T98G)<sup>[13]</sup>, as well as DM-defined subpopulations in single-cell NS line of a GBM-derived primary culture 51A<sup>[14]</sup>. The explanation of this phenomenon would be an increase of CIN rate, shown by increase of MS rate of the subpopulation-defining chromosome or DM, due to loss, and dramatic weakening, of inhibitors of CIN (InCIN) in initial and subsequent cell divisions of single-cell lines.

To test the hypothesis that regulation of CIN rate is paracrine-mediated, Chr7-FISH was carried out in U251 derived from serial decrease of cell-plating density from that normally used in cell passages (~10,000 cells/cm<sup>2</sup>). U251 cells at above 90% and about 40% confluence from plating with 10,000 and 1000 cells in a 24-well plate were fixed 2 days later for FISH analysis. Cells from three selected wells, each containing 16 colonies one week after seeding 50 cells per well in a 24-well plate, were detached by trypsin-EDTA and passed into a 35-mm dish and cultured for two days prior to FISH analysis. The percentages of cells with Chr7 copy of 1, 2 & 4, and 3 & 6 of U251 derived from various cell-plating density were plotted in the left panel of Figure 2A, and the H-index calculated based on the percentages of these populations was shown in the right panel of Figure 2A.

We have shown previously that TMC in U251 carrying 3 copies of Chr7, 2 normal, 1 with q-arm deletion, denoted as 3-Chr7 (2n, 1d), and STIC carrying 2 copies of Chr7, 1 normal, 1 with q-arm deletion, denoted as 2-Chr7 (1n, 1d). Counting of whole chromosome number (WCN) for 145 metaphase nuclei of U251 and U251-NS showed that the majority (87%) have aneuploid karyotypes with a modal chromosome number of 50. Hence both cells with 2 or 3-copies of Chr7, which were differentially enriched in U251-NS and U251, respectively, had near diploid karyotypes. The small portion (4%-5%) of cells with 4 and 6-copies of Chr7 were therefore considered as transient tetraploid stages of STIC and TMC, respectively, as shown in Figure 2A. Monosomy-7 cells with 1 copy of Chr7 in U251 were also near diploid.



**Figure 2.** Increase in population diversity and decrease of cell viability both correlate with decrease in cell plating density. (A) Chr7-FISH was carried out in U251 cultures plated at various cell densities and in SA1, a clonal line of U251 established in serum-containing medium. Based on near diploid karyotypes of cells in U251, cells carrying 1, 2 & 4, and 3 & 6 copies of Chr7 were denoted as monosomy-7 cells, STIC and TMC, respectively, as described previously<sup>[13]</sup>. Shannon diversity index was calculated based on the percentages of these three cell subpopulations, as described previously<sup>[22]</sup>; (B) colony formation rate from one week culture of 50 cells of U251 in 35, 60, and 100 mm dishes in 3, 4, and 10 mL of medium, respectively, in 4-6 replicates

As shown in Figure 2A, decreasing cell plating density of U251 cultures in the same surface area and volume of culture medium caused a gradual decrease in percentage of TMC (67%, 55%, 45%, and 40%) along with a gradual increase in percentage of STIC (31%, 40%, 48%, 53%), leading to a gradual increase in population diversity, as shown by increase of H-index value. SA1 is a single-cell line of U251 formed and expanded in SA conditions. Its CGH profile confirmed its origin from a TMC in U251<sup>[13]</sup>. As shown in Figure 2A left panel, the percentage of STIC (53%) in SA1 was slightly higher than that of TMC (40%). While in its parental culture, TMC was the dominating population (average 77%), based on analyses of four different passages. Because there was no change in culture conditions from SA to NS, which is favorable or against the growth of STIC or TMC, respectively, the observed increase in percentages of STIC and corresponding decrease of TMC would mostly due to increase of Chr7-MS rate by TMC in responding to decrease of paracrine effect of InCIN from decrease of cell plating density.

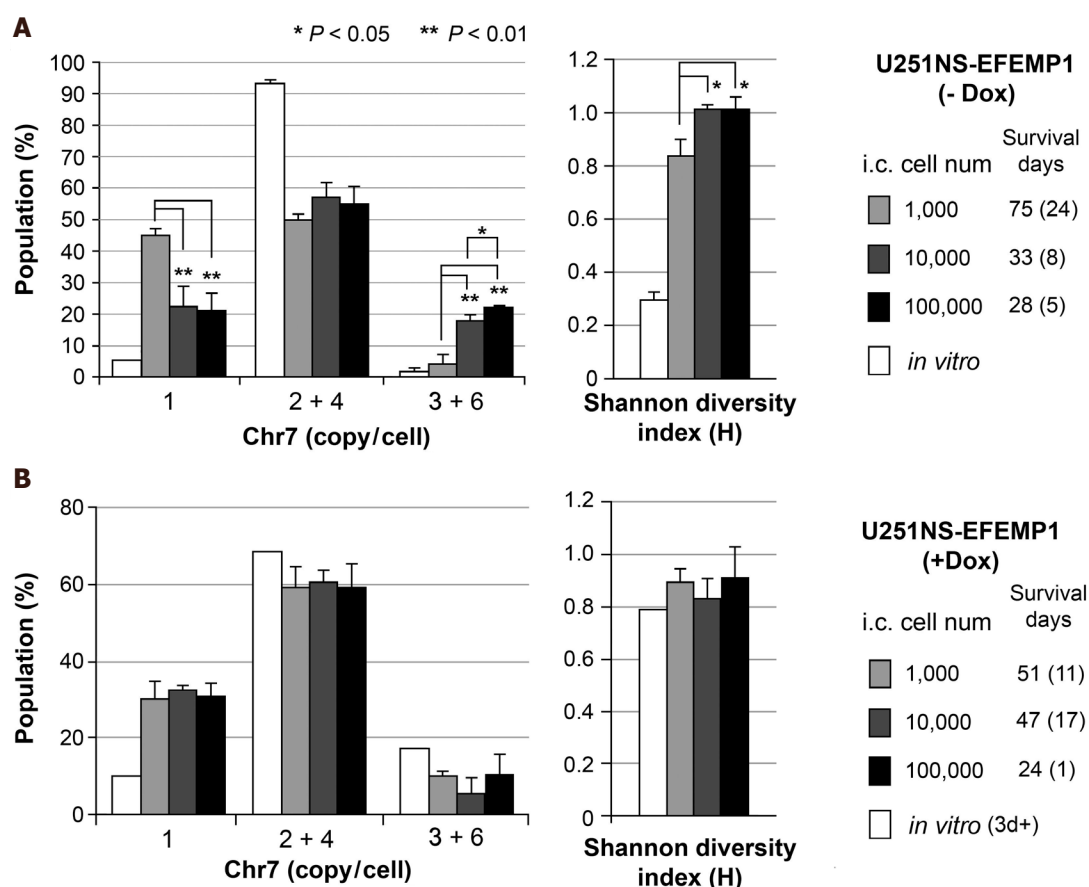
We then analyzed colony formation rate of U251 in SA conditions, by plating 50 cells of U251 in 35, 60 and 100 mm dishes with nearly 3-fold serial increase of surface areas from 10 to 28, and to 79 cm<sup>2</sup>. As shown in Figure 2B, there was a near 3-fold of serial decrease of colony formation rates, which is not related to changes in volume of culture medium (from 3 to 4, and to 10 mL), but to cell plating density. Clearly, it is more to the change of cell density that changed cell viability in colony formation assay. According to notion that most aneuploid cells from chromosomal MS are nonviable, decrease of cell survival would be consistent with increase of MS rate, in response to decrease of cell-plating density in above described colony formation assay.

Taken together, results of FISH analysis showed increase in population diversity, and colony formation assay showed decrease in colony formation rate due to increase of two dimensional cell density of U251. Overall our data are consistent with paracrine control of cancer cell CIN rate, by local concentration of extracellular factors secreted by its self as well as its neighboring cancer cells.

### Extracellular control of CIN in maintenance of TH

The above described increase of CIN rate in establishment of single-cell lines and a negative association between population diversity and cell-plating density suggest paracrine control of cancer cell CIN rate, with InCIN acting in the extracellular compartment. This conclusion was supported by FISH analyses of intracranial (i.c.) xenografts derived from U251-NS with different inoculum sizes. U251-NS is a single-cell line of U251 with 90% STIC under NS-conditions which did not support the growth of TMC<sup>[35]</sup>. The small (1%-2%) portion of TMC in U251-NS is likely from Chr7-MS of STIC as demonstrated in mathematical





**Figure 3.** Increase in TH *in vivo* due to decrease of cell inoculum size and inhibition of CIN from overexpression of EFEMP1. (A) comparison of Chr7-subpopulations in xenografts derived from intracranial (i.c.) implantation of U251-NS at various inoculum sizes (1000, 10,000, and 100,000 cells/3  $\mu$ L); (B) comparison of Chr7-subpopulations in xenografts of U251-NS from various inoculum sizes and with expression of ectopic EFEMP1 induced by treatment with Dox. See Figure 2 for Chr7-defined populations. Bar height and error bar are mean and SD of individual mice. Data from FISH analyses and mice survival were reported in Hu *et al.*<sup>[32]</sup>

modeling<sup>[13]</sup>. After changing the *in vitro* culture environment to orthotopic *in vivo* environment of glioma, the percentage of monosomy-7 cell and TMC markedly increased, which were found physically near each other in xenografts<sup>[13]</sup>, suggesting increased rate of Chr7-MS of STIC. The dramatic increase of monosomy-7 cell from 5% to more than 20% due to changing environments of *in vitro* to *in vivo* could be explained by increase of survivability or growth speed of monosomy-7 cells *in vivo*, as compared to *in vitro*.

We have previously reported FISH analyses of intracranial (i.c.) xenografts derived from intracranial implantation of U251-NS cells infected with lentiviral vector pTRIPZ to express EFEMP1 (named U251NS-EFEMP1) under promoter controlled by doxycycline (Dox)<sup>[32]</sup>. We observed similar cell subpopulations in i.c. xenografts of U251NS-EFEMP1 (-Dox) and U251-NS with inoculum size of 100,000, where 55% were STIC and 23% monosomy-7 cells. Here we compared Chr7-defined subpopulation proportion as the steady state of TH and Shannon diversity index value in xenografts derived from the same implantation of U251NS-EFEMP1 (-Dox) but variable inoculum sizes. As shown in Figure 3A, xenografts derived from a small inoculum (1000 cells) of U251NS-EFEMP1 (-Dox) were nearly equally (45%, 50%) composed of monosomy-7 cell and STIC, respectively, which was in striking contrast to xenografts of U251NS-EFEMP1 (-Dox) of 10- and 100-fold larger inoculum sizes. There were significantly higher percentage of TMC and lower percentage of monosomy-7 cells in xenografts of 10,000 and 100,000 inoculums leading to their higher H-index values and shorter survival of mice, compared with that of inoculum of 1000 cells.

The monosomy-7 cell remained slow-growing under both *in vivo* (as shown in [Figure 3A](#) by median 75 days survival of mice with i.c. xenografts containing 45% monosomy-7 cells vs. 33 days survival of mice with i.c. xenografts containing 20% monosomy-7 cells), and *in vitro* environments, and never became a population larger than 5% in both U251 and U251-NS *in vitro* cultures, as well as in single-cell or low-density cultures of U251. Thus, it would be the increase of Chr7-MS by STIC, not the increase of monosomy-7 cell growth speed that explains the dramatic difference in increase of monosomy-7 cell percentage in xenografts from a small number of cell implantation, as compared with that from 10 and 100-folds higher inoculum sizes. This demonstrates the negative association of cell density and Chr7-MS rate by STIC in initial and subsequent cell divisions following i.c. tumor cell implantation. The significantly higher percentage of TMC in xenografts of U251NS-EFEMP1 (-Dox) with inoculum size of 100,000 cells compared with that of 10,000 cells is functionally related to the shorter survival of mice from the fast growth features of TMC, although their differences on Shannon diversity index and survival are not significant, but both are significantly different from that of inoculum size of 1000 cells. Such cell density-related threshold of extracellular factors in control of Chr7-MS rate were also observed in TMC in U251 *in vitro* culture under SA-conditions [[Figure 2](#)], both demonstrating extracellular control of CIN in maintenance of TH.

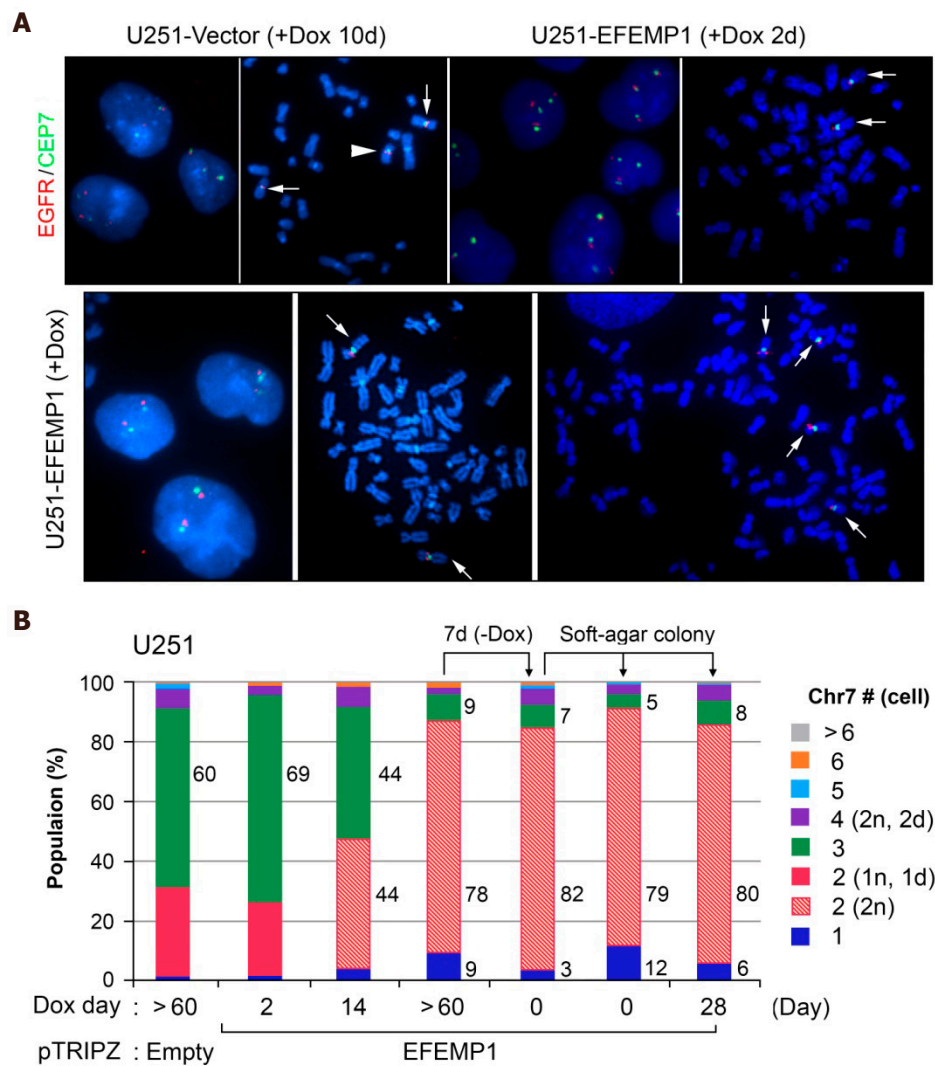
### EFEMP1 is an inhibitor of CIN

The cell density-dependent negative effect on CIN rate suggests paracrine-control of CIN. Below we present the CIN inhibition function of an extracellular matrix protein EFEMP1 (also known as fibulin-3) that was initially reported as a senescent protein<sup>[36]</sup>, and later widely reported in cancers<sup>[37]</sup>, with cell-context-dependent dual functions in TMC and STIC in U251 and U251-NS lines, respectively<sup>[32]</sup>.

Ectopic EFEMP1 was induced by adding Dox (1 µg/mL) to culture medium for about 1 day and maintaining EFEMP1 overexpression in xenografts was achieved by providing Dox (1 mg/mL) in drinking water of mice throughout the experiment. The tumor-promoting role of EFEMP1 in STIC, as suggested by its pro-invasive function in STIC shown in a matrigel invasion assay, could only be seen in small inoculum sizes of 1000 where the size of TMC number was too small to manifest EFEMP1's suppression role, as shown in xenografts from medium (10,000) and large (100,000) inoculum sizes<sup>[32]</sup>. FISH analyses showed lack of significant difference in both the steady state Chr7 subpopulations and H-index in xenografts of U251NS-EFEMP1 (+Dox) of various inoculum sizes [[Figure 3B](#)], which was in striking contrast to that of U251NS-EFEMP1 (-Dox) shown in [Figure 3A](#). Besides the dual functions of EFEMP1 in TMC and STIC, EFEMP1 was further demonstrated to carry a role as InCIN, to suppress the increase of Chr7-MS by STIC during formation of i.c. xenografts.

As reported previously in our studies of the tumor suppression function of EFEMP1 in glioma, long-time *in vitro* overexpression of EFEMP1 in U251 amplified a population carrying two normal copies, denoted as 2-Chr7 (2n), barely seen in parental culture, into the majority subpopulation (about 80%) in U251-EFEMP1 (+Dox). In contrast to high tumorigenicity of U251 where TMC (3-Chr7 (2n, 1d)) was the dominant subpopulation, U251-EFEMP1 (+Dox) with majority cells carrying 2-Chr7 (2n) showed significantly lower tumorigenicity even after withdrawal of Dox in subcutaneous xenograft models<sup>[13]</sup>. In this study, we examined the effect of EFEMP1 on control of Chr7-MS rate in 2-Chr7 (2n) cells enriched in U251-EFEMP1 (+Dox).

FISH analysis was carried out on *in vitro* cultures of U251 transduced with the empty vector of pTRIPZ and Dox-controlled transient- and stable-expression of ectopic EFEMP1. As shown in [Figure 4](#), Chr7-defined steady state of TH in U251 was similarly shown in U251-Vector after a 10-day Dox-treatment. The arrowhead marked one chromosome 7 with 7q deletion (1d), which was specifically found in both TMC (2n, 1d) and STIC (1n, 1d), as reported previously<sup>[13]</sup>. For studying the effect of EFEMP1, U251-Vector was used as control for the effect of vector and dox-treatment. As shown in [Figure 4](#), Chr7-defined steady state of TH in U251 was dramatically altered due to EFEMP1 overexpression, with 69%, 44%, 9% of TMC present in cultures



**Figure 4.** FISH analyses of *in vitro* cultures of U251 with transient and long-term expression of ectopic EFEMP1. (A) representative FISH interphase and metaphase nucleus images of U251 transduced by lentivirus of empty vector or doxycycline (Dox)-induction of ectopic EFEMP1. Normal Chr7 was shown by a white arrow, abnormal Chr7 (with amplification of p-arm and deletion of q-arm) by a white arrowhead; (B) comparison of Chr7-subpopulations in various U251 cultures with or without EFEMP1 overexpression

after 2, 14, and over 60 days of Dox-treatment. After lengthy induction of ectopic EFEMP1 by Dox, even after withdrawal of Dox for a week, nearly 80% of cells in U251-EFEMP1 (+Dox) and U251-EFEMP1 (withdrawal of Dox) carried similarly high percentages of 2-Chr7 (2n) cells. This demonstrated that the new steady state of tumor subpopulation induced by EFEMP1 persisted for some time, even after the extent of EFEMP1 overexpression was eliminated or minimized. Long-term expression of ectopic of EFEMP1 changed the steady state of U251 subpopulations with key subpopulation of 2-Chr7 (2n) of low tumorigenicity.

We then studied MS rate of this low tumorigenic 2-Chr7 (2n) subpopulation of U251, by analyzing two single-cell lines derived from soft-agar colonies of U251-EFEMP1 (withdrawal of Dox) formed and expanded with or without Dox-treatment. FISH analysis showed similarly high percentages of 2-Chr7 (2n) cells in U251-EFEMP1 (withdrawal of Dox) and its derived single-cell lines, regardless of Dox-treatment [Figure 4B]. The lack of increase in cell population diversity in single-cell lines of U251-EFEMP1 (withdrawal of Dox) suggests a lower CIN rate of cells with 2-Chr7 (2n) in U251, which is in striking contrast to that of single-cell lines of high tumorigenic glioma cell lines, as described above and shown in Figure 2A.

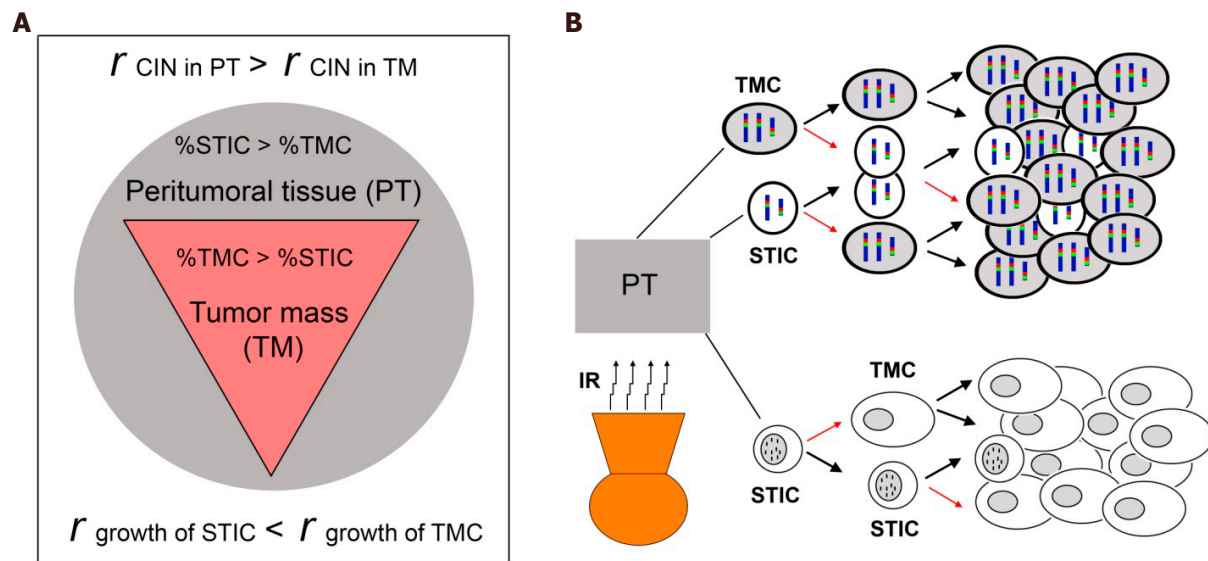
As in U251 and U251-NS cultures, monosomy-7 cell in U251-EFEMP1 (withdrawal of Dox) is a result of Chr7-MS following proliferation of 2-Chr7 (2n). Withdrawal of Dox-induced EFEMP1 from U251-EFEMP1 (+Dox) mainly caused a 3-fold decrease of monosomy-7 cell percentage from 9% to 3%, suggesting a pro-CIN effect of Dox or its induced ectopic EFEMP1. The latter has shown an InCIN effect in TMC and STIC populations as described above. FISH analyses showed that the percentage of monosomy-7 in single-cell lines of U251-EFEMP1 (withdrawal of Dox) without or with Dox-treatments were increased by four- and two-fold, respectively, compared with their parental line [Figure 4B]. Hence in 2-Chr7 (2n) cells of low tumorigenicity and low CIN rate, EFEMP1 has also played the role of InCIN.

## DISCUSSION

TH is a hallmark of the most malignant glioma, glioblastoma multiforme (GBM) where “M” stands for “multiforme” based on the degree of tumor cell diversity assessed solely with histopathology, both between different tumors and, within the overall cell population of any given individual tumor. If they do not succumb to their original tumor, most patients with GBM go on to experience tumor recurrence, despite surgical resection, post-operative radiation and chemotherapy. There is no histological or cytogenetic difference between primary and recurrent GBM (regardless of multiplicity of treatments and recurrences). Most GBMs (about 80%) show loss of chromosome 10 (monosomy 10)<sup>[38]</sup>, with activation of PI3K-mediated growth signaling as a result of loss of tumor suppressor PTEN leading to aggressive growth<sup>[39]</sup>. The other most commonly seen numerical chromosome aberration in GBM is gain of Chr7 (trisomy/polysomy 7)<sup>[40]</sup>. Chr7 copy number variation, including monosomy 7, occurs in both high- and low-grade gliomas, and appears to be associated with invasive and proliferative cell phenotypes<sup>[40-44]</sup>. Through FISH analysis of individual cells within glioma tissue and CQ-PCR analysis of whole tissue, we showed increased Chr7-defined cell diversity in comparison to non-tumoral tissues of brain, and the positive relation of this diversity to the malignant nature and behavior of these tumors. The grade-dependent increase of trisomy-7 cells may have functional implications, e.g., a high proliferative phenotype, as also suggested by other studies<sup>[40]</sup>. Comparing grade II and III gliomas with oligodendroglia components, the observation of high percentage of cells with 5 copies Chr7 and low percentage of cells with 1 copy of Chr7 in AO could be functionally significant with increase of malignant phenotype due to increase of CIN rate, which requires further study with larger sample sizes of AO.

From analyzing the distributions of Chr7-defined subpopulations in GBM-derived cell line U251 and its clonal subculture line U251-NS under both *in vitro* and *in vivo* conditions, overall our findings support the idea that MS rate increased by the dominating tumor cell subpopulation in U251 [Figure 2] and U251-NS [Figure 3A] in response to decrease of two and three dimensional cell densities, respectively, and in U251-EFEMP1 (+Dox) in forming soft agar colonies [Figure 4B]. Our conclusion of increasing MS rate is not from the direct measurement. The increase of MS rate of the dominating TMC subpopulation in U251 was concluded based on a serial reduction of its percentage along with increase of the minor STIC subpopulation [Figure 2A] and decrease of cell viability [Figure 2B] due to decrease of cell plating density. Given the same culture conditions that were unfavorable to monosomy-7, less supportive to STIC, and favorable to TMC, results from this experiment undermines the impact from cell plating density on each subpopulation's proliferation and/or death rate which may affect the state of TH. In contrast, it highlights the immediate impact from the dramatic decrease of local extracellular factors. In U251-NS, where STIC was the key cells, similar results was observed suggesting increase of MS rate due to decrease of cell density [Figure 3A]. Base on mouse survival that is negatively related to the speed of tumor growth, monosomy-7 cells remain slow-growing under *in vivo* conditions. The increase of monosomy-7 percentage in i.c. xenograft of U251-NS compared to that of *in vitro* culture suggests less apoptotic rate of monosomy-7 cells in conditions of *in vivo* vs. *in vitro*. The significant increase of monosomy-7 cell portion in xenografts from decrease of inoculum size could be mainly caused by an increase of MS rate of STIC in responding to dramatic decrease in concentration of local extracellular factors playing roles as InCIN, including EFEMP1.





**Figure 5.** A model of GBM with TH and CIN in control of tumor recurrence. (A) differential intra- and peri-tumoral distributions of slow-growing invasive STIC and fast growth TMC, and differential CIN rate in the bulk of tumor mass (TM) and parenchyma of peritumoral tissue (PT); (B) recurrent GBM models from Chr7- and DM-defined STIC and TMC based on published studies<sup>[13,14]</sup>. A thick black arrow shows the proliferation of cells to re-populate, and a thin red arrow shows the proliferation of cells with MS of Chr7- and DM, giving rise to other functional subpopulations

Relying on GBM's divergent “grow” or “go” cellular phenotypes of GBM cells, to study plasticity of GBM cells and the mechanisms of GBM recurrence after aggressive post-surgical therapies, we simplified our study by focusing on tumor cell subpopulations with these two diverse phenotypes. STIC subpopulation reflects the “go” phenotype and TMC subpopulation reflects the “grow” phenotype, with differing chromosomal markers defining these two functional subpopulations. Overall our published and new data presented here suggest that the plasticity of GBM cell is under paracrine-control of the CIN rate, represented by MS of a subpopulation-specific chromosome. Consistently, we showed that the more confluent the cells, the more the inhibition of CIN. A model for recurrence of GBM is presented, assuming differential intra- and peri-tumoral distributions of slow-growing invasive STIC and fast growth TMC, with a low CIN rate in the bulk of the tumor mass (TM) and a high CIN rate in invaded parenchyma of peritumoral tissue (PT) for both subpopulations [Figure 5A]. We propose that CIN rate is not only modulated by tumor microenvironment, but also by current cytotoxic therapeutic interventions, such as irradiation, which can assist in the re-establishment of TH optimized through evolutionary selection pressures leading to re-establishment of the steady state of subpopulations in prior established GBM [Figure 5B].

In these two GBM heterogeneity models, where Chr7 or DM-defined two key tumor subpopulations which function as STIC and TMC, we showed that the two subpopulations could be differentially enriched by SA and NS culture conditions. The steady state of TH with one subpopulation as majority remained stable over long-term passages under the same culture conditions (SA or NS). In a Chr7-defined heterogeneity model of GBM, the mathematical model revealed that it is Chr7-MS that prevents the phase out of the slow-growing subpopulations in either condition, even at a rate as low as ~0.01 or 0.001 for TMC or STIC, respectively, per cell division<sup>[13]</sup>. The calculated MS rates of TMC and STIC in Chr7-defined heterogeneity model of GBM are in the range of aneuploidy rates reported in human cancer cells<sup>[17]</sup> and yeast<sup>[20]</sup>. In a DM-defined heterogeneity model of GBM, we demonstrated regain of TH by STIC (with DM) giving rise to TMC without DM<sup>[14]</sup>. The MS rate of DM in stabilized status has not yet been determined. Overall, this model defines CIN, represented by MS of the subpopulation-defining chromosome (e.g., Chr7, DM), to cause TH with functionally diverse tumor subpopulations in *de novo* tumor and its restoration in recurrent tumors.



### The balance of between CIN and InCIN in cancer evolution

Aneuploidy in clinical specimens and their derived cell lines is a hallmark of cancer; thus CIN has been proposed to be a driving force of cancer evolution. CIN can readily and rapidly, in a time frame of one cell division, give rise to tumor cells with diverse genotypes that lead to dramatic changes in transcriptional profiles, and thus affect the behavior and survivability of the progeny cells. Based on CIN-created cell variables, cancer would start by successful selection of those cells with oncogenic functions and then progress by further successful selection of a team of synergistically interactive and mutually supportive functional tumor cell subpopulations that drive the fast growth and invasive characteristics of cancer. Paradoxically on occasion, CIN could also apparently interfere with cancer evolution by producing large number of cells lacking oncogenic function and viability as well as loosening the steady state of TH optimal for cancer's growth or de-stabilizing the optimal tumor-ecology. In these occasions, selection would be directed to suppress CIN, in maintaining the team of tumor cell subpopulations with diverse functions and symbiotic relationships. This leads to the ability to adjust the MS rate in proliferating tumor cells in accordance to their local extracellular cues from the dynamic tumor microenvironment.

The existence of inhibitor(s) of CIN made and secreted by cancer cells into extracellular compartments and their dose-dependent function on suppressing CIN was demonstrated by our data published and new experiments detailed above. The key evidence comes from findings that MS rate was uniformly increased in single-cell cultures of all examined GBM cell lines and primary cultures, and this increase of MS rate was associated with reduction of cell plating density *in vitro* and inoculum size *in vivo*. Results from both *in vitro* and *in vivo* models showed saturation effect on population diversity from a high cell density, such as 5000-10,000 cells/cm<sup>2</sup> for U251 and 10,000 and 100,000 inoculum size for U251-NS, suggesting a balance was reached between CIN and InCIN that benefit the overall growth of the culture or tumor under the described conditions.

Overall, the studies presented here suggest that both CIN and InCIN contribute to the establishment of steady state of TH optimal for tumor growth as well as survival and re-emergence after conventional therapy. The higher the grade of malignancy, the more efficient the component of tumor subpopulations and interactions are, for optimal growth and support from tumor microenvironment. Since gliomas can progress from lower grades after therapy to higher grades with increase of diversity in tumor subpopulations [Figure 1B], this proves increase of tumor cell diversity in cancer evolution. Selection in favor of tumor growth would lead optimal steady state of TH with specific tumor subpopulations and tumor ecology.

Tumor cells are further empowered with a sensing system to increase or decrease the rate of CIN in order to maintain the species of functional tumor subpopulations and the steady state of TH optimized in growth under a given environment, or to establish new species of functional tumor subpopulations and a new steady state of TH to cope with damages in their living environments, from over-growth or therapeutic interventions. If CIN is a primary driver in cancer evolution, InCIN would be a necessary component of that driver that empowers cancer development in a more effective and efficient way. This endows power of change and flexibility upon cancer evolution, which is an inherent mechanism of cancer recurrence, following surgical resection and therapeutic interventions currently practiced, such as chemo and radiation for GBM. Given the fact that at least one resistant subpopulation of tumor (e.g., STIC) has the ability to increase the MS rate by sensing InCIN dynamics, tumor recurrence in local (GBM) and distant (other types of cancers) places is guaranteed. Understanding the “Yin” and “Yang” reciprocal aspects of CIN and their control of TH dynamics would lead to an entirely new and exciting era towards improving cancer treatment involving directed perturbation of CIN and/or InCIN in ways that will not allow for establishment, or maintenance, of optimal synergistically interacting and mutually supporting tumor subpopulations and tumor-supporting micro-environment.

## DECLARATIONS

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### Authors' contributions

Conceived this study, designed and performed the experiments, analyzed the data, and wrote the manuscript: Zhou YH

Participated and edited the manuscript: Afrasiabi K, Linskey ME

### Availability of data and materials

Data and materials will be open to readers upon request.

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### Conflicts of interest

The authors declare that they have no personal circumstances or interest that may be perceived as inappropriately influencing the representation or interpretation of reported research results.

### Ethical approval and consent to participate

Tumors from Tissue Bank of UC Irvine and University of Arkansas for Medical Science were included in this study, with Institutional Review Board approval. No further consent is needed.

### Consent for publication

Not applicable.

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Review

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# Genomic heterogeneity meets cellular energetics: crosstalk between the mitochondria and the cell cycle

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## Abstract

Changes in cellular energetics and genomic instability are two characteristics of cancers that have been studied independently. Evidence of cross-talk between mitochondria function and nuclear function has started to emerge, suggesting that these pathways can influence one another. Here we review recent evidence that links the mitochondria and the cell cycle. This evidence indicates bidirectional cross-talk where mitochondria function can regulate the cell cycle and induce genomic instability, and conversely, the cell cycle machinery regulates mitochondria function. Implications for this cross-talk in the development of cancer are discussed.

**Keywords:** Mitochondria dynamics, cell cycle, mitochondria heterogeneity, genomic heterogeneity

## INTRODUCTION

Changes in metabolism and genomic instability were among the earliest characteristics of tumors to be identified. Boveri's hypothesis in the early 1900s that malignant tumors originated from cells with abnormal chromosome numbers<sup>[1]</sup>, initiated an era of research on the role of genomic instability in cancer development. Likewise, Otto Warburg's work on the metabolic changes in tumor cells<sup>[2,3]</sup> pioneered an era of research studying the role of changes in cell metabolism during cancer progression. These two fields, however, have mostly remained separate. Here we focus on emerging evidence of crosstalk between the processes occurring at the mitochondria and those in the nucleus, particularly as it relates to the cell cycle. These discoveries



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suggest an important connection between mitochondria heterogeneity and genomic heterogeneity that has key implications for our understanding of cancer development.

## GENOMIC INSTABILITY IN CANCERS

Genomic instability has long been associated with cancer development<sup>[4]</sup> and can range from single point mutations<sup>[5,6]</sup> to massive genomic rearrangements (e.g., chromothripsis)<sup>[7,8]</sup>. Parallel genome sequencing studies of cancer cells have revealed a wide variety of mutations and chromosomal abnormalities existent in cancer genomes: studies looking at mutations identified averages of 47-84 non-silent clonal mutations per tumor<sup>[9]</sup>. Studies focusing on clonal somatic chromosomal rearrangements have uncovered similar variation, from cancer cells containing a single chromosomal rearrangement per cell to cells with > 200 rearrangements, including deletions, duplications, and inversions<sup>[10,11]</sup>. Similarly, studies looking at gene copy number abnormalities identified a mean of 209 somatic copy number abnormalities per cancer genome<sup>[12]</sup>. A common finding throughout these studies is the great heterogeneity in the type of genomic instability as well as in the identity of the genes affected, with only a small number of genes found to be commonly affected in multiple cancers.

The complexity of genomic heterogeneity in cancer is further expanded when we consider that these differences are not limited to differences among clonal populations of cancer cells (inter-tumoral heterogeneity). Sequencing of different regions within a tumor reveals equally staggering intra-tumor genomic heterogeneity<sup>[13-17]</sup>, which is dynamic over time<sup>[18-22]</sup>. Taken together, these results are consistent with models of rapid genomic evolution within tumors and intra-tumoral genomic heterogeneity increasing over time, correlating with tumor aggressiveness and decreased patient survival<sup>[23,24]</sup>.

Genomic instability can be initiated by exogenous or endogenous agents. The role of external genotoxic agents (e.g., UV-light, x-rays, chemical mutagens) in inducing genomic changes has been extensively reviewed elsewhere<sup>[25-28]</sup>. Endogenous causes of genomic instability include errors in DNA replication<sup>[29]</sup>, transcription-induced stress<sup>[30]</sup>, spontaneous or activation-induced cytosine deamination<sup>[31]</sup>, transposon mobilization<sup>[32]</sup>, and defective or error-prone DNA repair<sup>[33]</sup>, among other factors. Another important and widely studied source of genomic instability is DNA damage induced by the reactive oxygen species (ROS) produced in the mitochondria during the respiration process. ROS function in the cell as signaling molecules that regulate multiple cellular pathways and are key for cell and organism homeostasis<sup>[34]</sup>. However, ROS can also generate direct DNA damage by oxidation of DNA bases<sup>[35,36]</sup>. Importantly, the complex relationship between mitochondria function and nuclear processes extends beyond the role of ROS.

## MITOCHONDRIA HETEROGENEITY IN CANCER

In addition to their role as the bioenergetics center of the cell, mitochondria are central to a myriad of cellular functions including iron<sup>[37]</sup> and calcium homeostasis<sup>[38]</sup>, metabolism of amino acids, lipids, nucleotides and carbohydrates, apoptosis, and a variety of signaling pathways<sup>[38-42]</sup>. Dysfunctions in many of these mitochondrial processes have been associated with cancer development<sup>[40,42]</sup> and chemoresistance<sup>[43]</sup>.

Similar to nuclear genomic heterogeneity, metabolic heterogeneity is also widespread in tumors. The initial findings by Otto Warburg of metabolic changes in cancer cells have been confirmed at multiple levels, from *in vitro* cancer cell models to *in situ* tumors in patients right before surgery<sup>[44,45]</sup>. Similar to the observations in genomic heterogeneity, these studies have revealed metabolic heterogeneity within different sections of the tumor<sup>[44]</sup> indicating that metabolic heterogeneity exists between tumors (inter-tumoral), within the tumor (intra-tumoral) and most likely also varies dynamically over time.

At the genetic level, comparisons using full genome sequencing in patient-derived pairs of cancer and normal tissues across multiple tumor types revealed the existence of somatic mtDNA mutations in a majority of

tumors<sup>[46,47]</sup>, with 31.1% of the tumors harboring multiple mtDNA mutations<sup>[47]</sup>. Unlike the nuclear genome, which contains two alleles of each gene, the mtDNA complement of a cell consists of hundreds to thousands of circular mtDNA molecules, allowing for different layers of mtDNA heterogeneity: alterations in mtDNA copy number, mutations in the mtDNA that occur in some but not all copies of the mtDNA genome within a cell (heteroplasmy), or mutations in the mtDNA that show dominance and accumulate until the mutant mtDNA becomes the only version present in the cell (homeoplasmy). Differences in mtDNA copy number, both increases and decreases of mtDNA relative to normal tissue, have been observed in many cancer types with some studies showing mtDNA copy number variation in up to 88% of tumors<sup>[48]</sup>. However, the role of mtDNA mutations or copy number variations as potential causative agents in cancer development have not been fully established due to the technological difficulties of manipulating the mtDNA genome. Studies in mice that have mtDNA from one strain and nuclear DNA from another strain (i.e., mice generated by mitochondrial-nuclear exchange) show effects in cancer progression models including changes in tumor size and metastatic burden<sup>[49]</sup>, suggesting that the mtDNA can affect cancer progression.

### INTERPLAY BETWEEN MITOCHONDRIA AND NUCLEAR FUNCTIONS

Genetic interconnections between the nucleus and the mitochondria are evident, since all but thirteen mitochondrial proteins are encoded by the nuclear genome. Associations between nuclear-encoded mitochondrial genes and tumorigenesis have been found, including mutations in several subunits of complex II, succinate dehydrogenase and isocitrate dehydrogenase, among other mitochondrial enzymes<sup>[50,51]</sup>. This nuclear control of the mitochondria by regulation of nuclear-encoded mitochondrial genes is termed anterograde signaling, and it is complemented by an equally important retrograde signaling system that allows the mitochondria to relay signals to the nucleus<sup>[52,53]</sup>. Retrograde signaling was first identified via changes observed in transcription of nuclear genes in response to respiration defects<sup>[54]</sup>. Later studies established that the retrograde signaling response is a mitochondria quality control mechanism in which the cell senses different mitochondrial functions (e.g., ROS production, the TCA cycle, calcium levels, the unfolded protein response), and communicates the status of these functions to the nucleus via signaling cascades<sup>[52,53]</sup>. These retrograde signals activate diverse nuclear responses, setting in motion multiple pathways that regulate energy homeostasis, oxidative stress, and mitophagy, among other functions<sup>[52,53,55]</sup>.

Importantly, mitochondria-dependent regulation of other nucleo-centric processes has started to emerge, including a role in regulation of the cell cycle.

### THE MITOCHONDRIA MEETS THE CELL CYCLE

The eukaryotic cell cycle consists of four phases G<sub>1</sub>, S-phase, G<sub>2</sub> and mitosis. These phases were historically defined by two genome-centric processes: DNA duplication (S-phase) and chromosome segregation (mitosis), interspersed with “gap” phases (G<sub>1</sub> and G<sub>2</sub>) to allow for cell growth<sup>[56]</sup>. It is now understood that the cell cycle involves more than duplication and segregation of DNA. During a cell cycle cells must also grow and segregate their organelles and other cellular structures<sup>[57-59]</sup>. This duplication of the genome and increase in cell biomass, followed by the complex division of all cell contents to form two fully functional daughter cells requires a large amount of energy and metabolites. Links between metabolism and the cell cycle were identified early in the history of cell cycle research via genetic screens in budding yeast that identified Cell Division Cycle (CDC) mutants<sup>[60]</sup>. Several of the original CDC alleles, which cause cell cycle defects when grown at the non-permissive temperature, were later discovered to also result in reduced carbon metabolism and lower ATP production<sup>[61]</sup>. Conversely, mutations in cell cycle genes, such as the cyclin-dependent kinase CDC28 were found to also affect mitochondria biogenesis<sup>[62]</sup>.

This metabolism-cell cycle connection has been studied in detail in budding yeast. Analysis of synchronously growing yeast populations uncovered cyclic changes of metabolism that associate closely with the phases

of the cell cycle<sup>[63]</sup>. These studies determined that in budding yeast the metabolic cycle consists of three phases<sup>[64]</sup>: (1) oxidative respiration, marked by increased oxidative phosphorylation, increased ATP and amino acid production. This phase is aligned with entry and progression into G<sub>1</sub> of the cell cycle; (2) reductive/building phase, characterized by an increase in glycolysis, increased production of nucleotides, nucleosides and ethanol. This phase occurs in synchrony with S-phase and mitosis; (3) reductive/charging characterized by production of complex carbohydrates for energy storage (e.g., glycogen, trehalose). This phase occurs during the end of mitosis and entry into quiescence (G<sub>0</sub>). The synchronicity of the cell cycle and metabolic cycle in budding yeast appears to be the result of a system of coupled oscillators, since the metabolic cycle can continue to oscillate in the absence of cell division<sup>[65,66]</sup>. Intriguingly, the expression of a number of cell cycle genes continues to oscillate with the metabolic cycle even in those cells that are not undergoing cell division, suggesting that the metabolic cycle can regulate cyclic expression of cell cycle genes independently of cell cycle controls<sup>[65]</sup>.

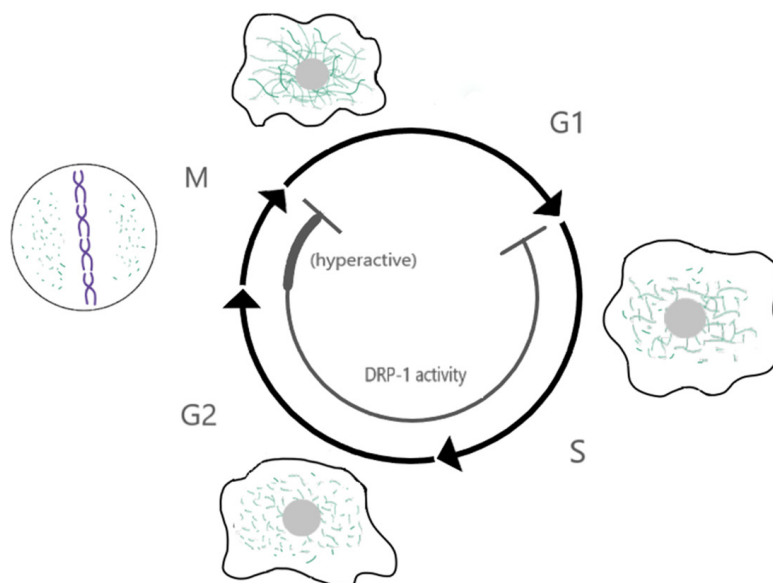
Overall the evidence in budding yeast reveals an interaction between mitochondrial metabolism and the cell cycle. Evidence of a similar interaction in other organisms has only recently started to emerge.

### MITOCHONDRIA DYNAMICS ARE REGULATED BY THE CELL CYCLE

Early studies on the connection between mitochondria processes and the cell cycle in human cells identified an increase in total mitochondria biomass that paralleled the increase in cell size during cell cycle progression<sup>[67]</sup>. The finding that mtDNA replication was not co-regulated with nuclear DNA replication<sup>[68]</sup>, led to the idea that mitochondria and cell cycle processes were mostly unlinked. This view has changed recently as mounting evidence has shown that mitochondria biogenesis, morphology, dynamics and function are regulated by the cell cycle.

Mitochondria are highly dynamic organelles undergoing constant fission and fusion. These dynamics depend largely on several members of the dynamin family of proteins: mitofusin 1 and 2 (Mfn1/2) drive fusion of the outer mitochondria membrane and optic atrophy protein 1 (Opa1) mediates inner mitochondria membrane fusion, while dynamin-related protein 1 (Drp1) is required for mitochondria fission<sup>[69,70]</sup>. Mitochondria fission is also facilitated by four receptors that cooperate to recruit Drp1 to the outer mitochondria membrane: Mff, MiD49/51 and Fis1<sup>[71,72]</sup>. Importantly, mitochondria morphology and dynamics change in a cell cycle-dependent manner<sup>[73]</sup>, with elongated mitochondria being dominant in G<sub>1</sub><sup>[74]</sup> and short mitochondria being dominant in mitosis<sup>[75]</sup>. These changes in mitochondria dynamics during the cell cycle are controlled via regulation of mitochondria-dynamics proteins by the cell cycle machinery [Figure 1].

Mitochondria fission during mitosis in human cells is driven by Drp1, whose activity is increased in mitosis via phosphorylation by the mitotic cyclin Cyclin B1/Cdk1<sup>[76]</sup>. Drp1 phosphorylation in mitosis is promoted by another mitotic kinase, Aurora A, via phosphorylation of the small GTPase RALA and its binding partner RALBP1, which in turn bind to and facilitate Drp1 phosphorylation by Cyclin B1/Cdk1<sup>[77]</sup>. Mitochondria fission in mitosis is important for mitochondria segregation. Depletion of RALA or RALBP1 result in asymmetric segregation of the mitochondria to the two daughter cells, presence of mitochondria bridges during cytokinesis, and in some cases cytokinesis failure due to interference of the indivisible mitochondria mass with the cytokinetic ring<sup>[77]</sup>. In turn, Drp1 promotes mitotic exit (adaptation) of cells arrested in mitosis with the microtubule-stabilizing drug taxol via regulation of Cyclin B1 levels<sup>[78]</sup>. Similarly, ATP depletion by addition of 2-deoxy-glucose (2-DG) and sodium azide promotes mitotic exit in cells arrested in mitosis with the microtubule depolymerizing drug nocodazole, and this adaptation is also due to reduction in Cyclin B levels<sup>[79]</sup>. These results indicate a bi-directional crosstalk where the mitotic machinery increases Drp1 activity and mitochondria dynamics in mitosis, which in turn feedbacks to regulate mitosis<sup>[80]</sup>. Once the cells exit mitosis, Drp1 is targeted for degradation by APC/C<sup>Cdh1</sup><sup>[81]</sup>, shifting the balance of mitochondria dynamics to favor mitochondria fusion.



**Figure 1.** Crosstalk between mitochondria dynamics and the cell cycle. Model showing the changes in mitochondria dynamics and Drp1 activity throughout the cell cycle. Mitochondria fusion is favored in G1 and mitochondria fission is dominant in mitosis. This leads to the formation of a highly elongated and interconnected network during late G1, and small disconnected mitochondria in mitosis. Changes in mitochondria dynamics are regulated by the cell cycle machinery, for example mitochondria fission is favored in mitosis by Drp1 phosphorylation by the mitotic kinase Cyclin B1/Cdk1. Conversely, mitochondria fusion is favored in G1 due, at least in part, to degradation of Drp1 by the ubiquitin ligase APC/C-Cdh1 in early G1. In turn, these changes in mitochondria morphology regulate the cell cycle. Hyperfused mitochondria promote the G1/S transition, while inhibition of Drp1 induces a G2 arrest and failure to fragment mitochondria in mitosis can interfere with cytokinesis. These phenotypes have started to reveal a profound level of cross-talk between these two processes

In addition to regulating mitochondria dynamics, cell cycle proteins also regulate respiration and other mitochondrial processes. Cyclin D1 represses mitochondria function by inhibiting nuclear respiratory factor 1 (NRF1), a transcription factor that induces expression of a set of nuclear-encoded mitochondrial genes<sup>[82]</sup>, and regulates gluconeogenesis<sup>[83]</sup>. A pool of Cyclin B1/Cdk1 localizes to the mitochondria, phosphorylates components of the OXPHOS machinery and increases their activity at the G2/M transition<sup>[84]</sup>. Some components of the spindle assembly checkpoint (e.g., Mad2, BubR1, p31-comet) have roles in insulin signaling<sup>[85]</sup>, while others (e.g., Mps1, Survivin) localize to the mitochondria and regulate apoptosis<sup>[86,87]</sup>. Together, these results indicate extensive regulation of mitochondria functions and/or cell metabolism by the cell cycle machinery.

### THE CELL CYCLE IS IN TURN REGULATED BY MITOCHONDRIA FUNCTION

Increased mitochondria fusion after mitotic exit leads to the formation of a hyperfused mitochondria network in late G1 which promotes the transition from G1 into S-phase<sup>[74]</sup>. The molecular mechanism by which mitochondria hyperfusion promotes S-phase entry has not been completely elucidated. However, it appears that mitochondria hyperfusion and the accompanying increase in mitochondria respiration in late G1 promotes accumulation of the S-phase cyclin, Cyclin E<sup>[74]</sup>. Conversely, inhibition of respiration in G1 using the uncouplers FCCP or CCCP results in decreased Cyclin E accumulation and delay in S-phase entry<sup>[74,88]</sup>. This model is supported by an analysis of mitochondrial potential ( $\Delta\Psi_m$ ) in a population of G1 cells which showed that G1 cells with low  $\Delta\Psi_m$  have a molecular profile corresponding to early G1 cells (e.g., low Cyclin E, high p27Kip1), while G1 cells with high  $\Delta\Psi_m$  have a late G1 molecular signature (e.g., high Cyclin E, low p27Kip1)<sup>[89]</sup>.

In addition to its role in promoting the G1/S transition, mitochondria dynamics also regulate the G2/M transition. Depletion of Drp1 results in a G2 arrest<sup>[90-92]</sup>, due to the presence of DNA damage and activation

of the DNA-damage kinases ATM and ATR<sup>[90]</sup>. A similar G2 delay accompanied by DNA damage is also observed after disruption of the Drp1 adaptor Fis1<sup>[93]</sup>. Disruption of other mitochondria functions, such as in a *Drosophila* knockout of the mitochondria-specific form of RNaseZ<sup>[94]</sup> and in human cells depleted of mtDNA (rho0 cells)<sup>[95]</sup> also cause a G2 delay. Furthermore the G2 delay after Fis1 depletion correlates with low expression of the cell cycle transcription factor FoxM1 and its downstream mitotic genes, including Cyclin B1, suggesting that defects in mitochondria dynamics/function can lead to transcriptional inhibition of the G2/M transition<sup>[93]</sup>. The link between mitochondria dynamics and cell cycle gene expression is further strengthened by observations of a correlation between Drp1 expression levels and expression of cell cycle genes in different cancers, particularly genes expressed in G2/M<sup>[96]</sup>. Other metabolic alterations such as starvation and the subsequent induction of autophagy, or hypoxia have also been shown to regulate cell cycle progression<sup>[97]</sup>.

Mitochondria dynamics/function have a role in the regulation of mitosis since Drp1 activity and ATP depletion promote mitotic exit in cells arrested in mitosis with microtubule-targeting drugs<sup>[78,79]</sup>. This exit from mitotic arrest when mitochondria function is compromised is due to premature degradation of Cyclin B1 by activation of the ubiquitin ligase APC/C<sup>Cdh1</sup><sup>[79]</sup>. These results indicate a complex cross-talk between mitochondria functions and the mitotic machinery, which has important implications for our understanding of the response of cancer cells to microtubule-targeting agents commonly used as cancer treatments (e.g., taxol, vinblastine). Additionally, other mitotic phenotypes are observed in cells with compromised mitochondria function, including amplification of centrosomes<sup>[90,95]</sup>, abnormal centrosome positioning<sup>[98]</sup>, chromosome misalignment<sup>[90]</sup> and multipolar spindles<sup>[95]</sup>. However, whether these phenotypes indicate a role for the mitochondria in the regulation of centrosome duplication or mitosis, or are merely consequences of the G2 delay and DNA damage observed in these cells has not been elucidated. Paradoxically, incubation with the Drp1 inhibitor Mdivi-1 seems to exert the opposite effect in cells damaged by x-rays. X-ray irradiation results in DNA damage, abnormal progression through mitosis (mitotic catastrophe), centrosome amplification and formation of micronuclei. In this scenario, incubation with the Drp1 inhibitor Mdivi-1 reduced the centrosome amplification and formation of micronuclei observed after irradiation<sup>[99]</sup>.

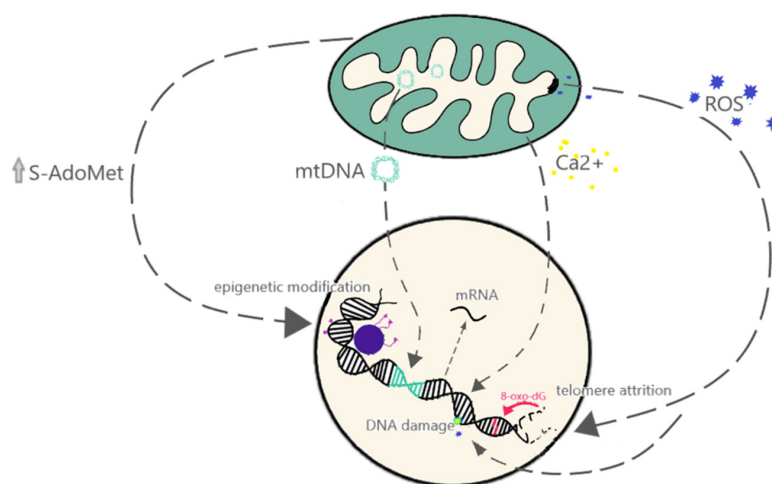
Taken together, these results provide clear evidence of a bidirectional link between mitochondria dynamics/function and cell cycle progression at multiple phases. However, more research is needed to fully understand the extent of interaction between these processes and to understand the molecular underpinnings of this crosstalk.

## OTHER LINKS BETWEEN THE MITOCHONDRIA AND NUCLEAR FUNCTION

As discussed previously, one of the best studied endogenous sources of genomic instability is the mutagenic potential of ROS, which can induce oxidative DNA damage<sup>[35,36]</sup>. Increased levels of ROS have also been shown to induce other types of damage such as telomere attrition and chromosome fusions<sup>[100]</sup>. However, other mechanisms by which mitochondrial dysfunction affects nuclear genome instability have started to emerge [Figure 2]. In budding yeast, loss of mtDNA leads to genomic instability and this was not correlated with defects in respiration, but rather with defects on the mitochondrial processing of iron-sulfur clusters<sup>[101]</sup>. In addition, mtDNA can affect nuclear DNA through direct transfer of genes. This process, termed numtogenesis<sup>[102]</sup>, was thought to be a rare event occurring at an evolutionary scale of millions of years. However, several reports have identified higher rates of numtogenesis in cancer cells. For example, a study identified mtDNA in the nuclei of up to 27.5% of cervical carcinoma cells compared to 0% of paired cells from the normal cervical epithelium<sup>[103]</sup>. Increased rates of numtogenesis were also observed via analysis of whole genome sequencing of adenocarcinoma samples<sup>[104]</sup>. Importantly, mtDNA integration into the nuclear genome can have important consequences such as activation of oncogenes<sup>[105]</sup>.

Another direct link between the mitochondria and genomic instability has been observed in cells that survive exposure to pro-apoptotic stimuli. Exposure of cells to a sub-lethal dose of the BH3-mimetic ABT737 results





**Figure 2.** Mitochondria functions impact nuclear functions. Model showing the different aspects of mitochondria function that have been shown to affect the nuclear genome at the level of gene expression (e.g., via calcium signaling pathways), genomic instability (e.g., DNA damage, telomere attrition) or epigenetic modifications (e.g., DNA methylation, histone modifications)

in partial mitochondria outer membrane permeabilization (MOMP), partial caspase activation and increased DNA damage<sup>[106]</sup>. One of the mitochondrial proteins, released from the mitochondria during MOMP is apoptosis inducing factor (AIF). AIF translocates to the nucleus and binds to the nuclear DNA, triggering condensation and fragmentation<sup>[107-109]</sup>. Importantly this nuclear translocation of AIF has also been observed in cells exposed to sublethal doses of oxidative stress<sup>[110]</sup>, suggesting that release of mitochondrial proteins like AIF can result in DNA damage without triggering apoptosis.

In addition to introducing genomic instability, the mitochondria also modulates nuclear functions via retrograde signaling that regulates nuclear gene expression. Mitochondria play a role in the epigenetic regulation of the nuclear genome<sup>[111,112]</sup>. DNA methylation patterns have been shown to change in cells depleted of mtDNA (rho0)<sup>[113]</sup> and in cells with different mtDNA haplotypes<sup>[114]</sup>. Mitochondria also have a role in calcium regulation, and mitochondrial stress can induce calcium release, activating signaling cascades that can lead to different nuclear gene expression responses and phenotypic changes, such as increases in invasive behavior<sup>[115]</sup>. Similarly, reduction in mtDNA content in breast cancer cells activates a calcineurin-dependent pathway that induces phenotypic changes similar to the epithelial-to-mesenchymal transition (EMT) associated with higher cancer aggressiveness<sup>[116]</sup>. Retrograde signaling, alteration of epigenetic regulation, direct transfer of genetic material, and ROS-mediated effects demonstrate the myriad of ways that mitochondrial dysfunction can play a role in nuclear genome instability and function.

## IMPLICATIONS OF MITOCHONDRIA-NUCLEAR INTERACTIONS FOR CANCER

Deregulation of cellular energetics is considered one of the emerging hallmarks of tumor development, while genomic instability has been established as an enabling characteristic of cancers<sup>[117]</sup>. The results discussed in this review provide evidence for a complex bidirectional cross-talk between mitochondria processes and nuclear processes involved in genomic maintenance, particularly regulation of the cell cycle. Identifying the molecular players involved in this cross-talk will not only open possibilities for the development of new cancer treatments, but it also reveals an unexpected complexity where genomic instability and defects in mitochondria function can synergize to accelerate cancer progression. That is, as cancer progresses and cell metabolism changes, these changes could lead to modifications in cell proliferation due to cell cycle dysregulation; while in turn modifications in the cell cycle or genomic instability can induce changes in mitochondria function, leading to a synergistic acceleration in the acquisition of cancer-associated traits.

This synergism also accelerates the acquisition of heterogeneity whereby increases in genomic heterogeneity will promote heterogeneity of mitochondria function, and vice versa.

## DECLARATIONS

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### Authors' contributions

All authors contributed to the writing of this review. In addition Herrera EL prepared the figures.

### Availability of data and materials

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### Conflicts of interest

All authors declared that there are no conflicts of interest.

### Ethical approval and consent to participate

Not applicable.

### Consent for publication

Not applicable.

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Review

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# Centrosome aberrations and chromosome instability contribute to tumorigenesis and intra-tumor heterogeneity

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## Abstract

Centrosomes serve as the major microtubule organizing centers in cells and thereby contribute to cell shape, polarity, and motility. Also, centrosomes ensure equal chromosome segregation during mitosis. Centrosome aberrations arise when the centrosome cycle is deregulated, or as a result of cytokinesis failure. A long-standing postulate is that centrosome aberrations are involved in the initiation and progression of cancer. However, this notion has been a subject of controversy because until recently the relationship has been correlative. Recently, it was shown that numerical or structural centrosome aberrations can initiate tumors in certain tissues in mice, as well as invasion. Particularly, we will focus on centrosome amplification and chromosome instability as drivers of intra-tumor heterogeneity and their consequences in cancer. We will also discuss briefly the controversies surrounding this theory to highlight the fact that the role of both centrosome amplification and chromosome instability in cancer is highly context-dependent. Further, we will discuss single-cell sequencing as a novel technique to understand intra-tumor heterogeneity and some therapeutic approaches to target chromosome instability.

**Keywords:** Centrosome, chromosome instability, intra-tumor heterogeneity, single-cell sequencing

## INTRODUCTION

Intra-tumor heterogeneity is a cancer hallmark that is characterized by the presence of different cell subpopulations within the same tumor<sup>[1,2]</sup>. These cell subpopulations foster tumor adaptation and evolution



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that hinders cancer treatment and leads to tumor recurrence and metastasis<sup>[3,4]</sup>. Therefore, despite the great conceptual and technological advancements in cancer research, recurrence and metastasis remain a key clinical challenge, making cancer the second leading cause of death in the United States. In this review, we discuss some classical experiments that have enlightened us as to our understanding toward cell cycle and centrosome regulation in order to understand how this modulates cancer initiation, maintenance, progression, and causes intra-tumor heterogeneity. We also discuss other causes of intra-tumor heterogeneity, such as the cancer stem cell theory. We also discuss the single-cell sequencing technique, as a novel technique to understand intra-tumor heterogeneity and relevant therapeutic targets that may aid our understanding of cancer and envision a more effective treatment.

## THE CAUSES AND CONSEQUENCES OF INTRA-TUMOR HETEROGENEITY IN CANCER

Intra-tumor heterogeneity describes the existence of different genetic subpopulations of cells in a given primary tumor<sup>[1]</sup>. Genetic heterogeneity is studied to determine the transcriptional expression, copy number or mutational/polymorphic status of genes within a tumor to provide an overall tumor genetic composition and determine the best treatment option for patients<sup>[5]</sup>, which is the basis for personalized medicine. Genetic, epigenetic, and metabolic changes are important contributors to tumor formation and progression<sup>[5]</sup>. Cancer stem cells, genetic and epigenetic alterations, copy number variation (CMV), single nucleotide variants (SNV), aneuploidy, genome duplication, and chromosome instability can initiate and sustain cancer progression and genetic heterogeneity. Intra-tumor heterogeneity supports the theory of clonal evolution that has been forced by selective pressures such as those exerted by chemotherapy or radiotherapy.

It is generally accepted that all cancer types display some degree of intratumoral heterogeneity, with thyroid and prostate cancers showing less heterogeneity, and cancers that include lung, stomach, glioblastomas and melanomas displaying a high degree of intratumoral heterogeneity<sup>[2]</sup>. In fact, transcriptomic and genomic profiling of multi-spatial biopsies of glioblastomas, medulloblastomas and renal cell carcinomas demonstrated that cells within a single tumor were rarely clonal, thus explaining single-agent therapy failure in cancers<sup>[6]</sup>. Genetic heterogeneity determines the fate of metastasis, with highly heterogeneous cancers such as colon displaying highly heterogeneous metastases within the same patient<sup>[7]</sup>. On the other hand, many high-grade serous ovarian cancers of patients with metastases are clonal, and most metastases originate from one clone<sup>[8]</sup>. Breast cancers are excellent examples of the role played by genetic heterogeneity in survival outcomes of affected patients<sup>[1]</sup>. Breast cancers are classified using mRNA expression microarrays and/or with several pathological markers, including the epidermal growth factor 2 (Her2), the estrogen receptor (ER), or the progesterone receptor (PR). The classification includes Luminal A (ER<sup>+</sup>PR<sup>+</sup>Her2<sup>-</sup>), Luminal B (ER<sup>+</sup>PR<sup>+</sup> and Her2<sup>+</sup> or Her2<sup>-</sup>), Her2<sup>+</sup> (ER<sup>-</sup>PR<sup>-</sup>Her2<sup>+</sup>) and basal (which includes 76% triple-negative breast tumors, ER<sup>-</sup>PR<sup>-</sup>Her2<sup>-</sup>)<sup>[9]</sup>. Luminal A breast cancer patients show the best survival of all breast cancer patients, followed by Luminal B, Her2<sup>+</sup> and basal<sup>[10,11]</sup>. More recent studies show that hormone receptor-negative breast tumors (Her2<sup>+</sup> and basal) display more chromosome instability and centrosome amplification (defined as the acquisition of three or more centrosomes that promote the formation of a bipolar mitotic spindle and equal segregation of chromosomes following mitosis) than luminal subtypes<sup>[12,13]</sup>. Also, Her2<sup>+</sup> and triple-negative basal breast cancer patients that initially respond to chemotherapy tend to relapse more readily than luminal breast cancer patients if residual disease remains<sup>[14]</sup>. Molecular subtypes also determine the preferred metastatic sites of breast cancer cells, since Luminal subtypes are more likely to invade the bone, and basal subtypes are more likely to invade into the lung<sup>[15]</sup>. The differences in survival outcomes between luminal and hormone receptor-negative breast cancers can be explained by the plethora of treatments available to treat luminal patients (including tamoxifen, Cdk4/Cdk6 and aromatase inhibitors). Nevertheless, the differences in survival can only be partly explained by differences in treatments available, since similar treatments are available for Luminal A and Luminal B breast cancers, and yet Luminal B breast cancers have poorer

survival<sup>[16]</sup>. We speculate that the higher relapse rates are due to the close relationship between aneuploidy, chromosome instability, and chemotherapy resistance<sup>[17,18]</sup>.

Intra-tumor heterogeneity origins can be explained by two theories: clonal evolution and stem cells origin. The first theory, clonal evolution, proposes that intra-tumor heterogeneity arises in response to tumor cell adaptation<sup>[1]</sup>. In this model, the existence of different genetic subpopulations of cells can be due to external pressures that drive the evolution of a tumor following the Darwinian evolutionary principles<sup>[19]</sup>. This theory was first described in 1976 by Peter Nowell, who described cancer progression as an evolutionary process driven by multiple somatic mutations, giving rise to uncontrolled growth and adaptation to the environment<sup>[19,20]</sup>. Then, Loeb proposed that this evolutionary process could be accelerated by a mutator phenotype initially caused by a mutation in genes that control genetic stability<sup>[21]</sup>. Many mouse models have given support to the evidence of such mechanism in mouse models, including experiments done by Fukasawa *et al.*<sup>[22]</sup>, who demonstrated using young mice harboring a genetic knockout of p53 frequent chromosome instability, aneuploidy, and centrosome amplification that preceded tumorigenesis. Other altered tumor suppressors that allow genomic instability include Brca1 and Brca2<sup>[23,24]</sup>. Oncogenes that can cause genetic instability include K-Ras<sup>G12D</sup>, v-Ras, H-Ras<sup>G12V</sup> and c-Myc<sup>[25-29]</sup>. More recent data by the Pellman group has shown that evolution can also occur from single, catastrophic events<sup>[30,31]</sup>. One of such mechanisms is known as chromothripsis, which is caused by the fragmentation and rearrangements of whole chromosomes contained in micronuclei (defined as missegregated whole chromosomes)<sup>[31]</sup>. Interestingly, centrosome amplification and failure of the spindle assembly checkpoint frequently cause whole chromosome losses<sup>[26,27,32-35]</sup>, implying that they may represent primary causes of these catastrophic events. Genetic mutations not only drive cancer initiation and progression but can sustain cancer cell survival by modulating the metabolism that supplies the high demand of building blocks required by cancer cells. For example, it has been reported that the transcription factors p53, c-Myc, and HIF can induce the expression and activity of glucose transporters involved in glycolysis and the hexose monophosphate shunt to fuel the TCA cycle<sup>[36]</sup>. Moreover, fatty acid  $\beta$ -oxidation is expressed differently in glioblastoma subtypes; this generates a different response to drug treatment and leads to lipid mobilization to generate more energetic compounds and building block for cancer development and progression<sup>[37]</sup>. This adaptation to the environment does not only create an effect in the microenvironment surroundings but also alters the response to therapy by creating cells resistant to chemotherapy.

The second theory, the cancer stem cell (CSC), states that the self-renewal capacity of a stem cell leads to intra-tumor heterogeneity<sup>[1]</sup>. This theory does not take in consideration aberrant genetic errors that may confer genetic advantages to the tumor as the clonal evolution theory does. The presence of CSCs was first observed in chronic myeloid leukemia and mouse models<sup>[19]</sup>. Furthermore, a study done in mice that were injected with breast cancer cells demonstrated the presence of a small subset of cells that displayed the cell surface marker of stem cells, CD44<sup>+</sup>CD24<sup>-/low</sup><sup>[38]</sup>. Another tenet of the CSC theory is that tissue-specific stem cells may arise from the accumulation of mutations over time that can initiate tumorigenesis (local or distant), and then become CSC<sup>[39]</sup>. For metastasis to occur, the cancer cells from a primary tumor need to detach, invade the vascular or lymphatic tissue, extravasate, and then proliferate by recruiting surrounding vasculature to grow at a distant site. CSC has been implicated in metastasis through epithelial to mesenchymal transition (EMT), a precursor of metastasis<sup>[40]</sup>. CSC gives origin to the generation of circulating tumor cells (CTCs), defined as rare (1 to 10<sup>6</sup>) cancer cells that circulate in the peripheral blood<sup>[39,41]</sup> and colonize adjacent tissues; thus contributing to tumor progression. External pressures create a microenvironment that changes the phenotypic and behavioral development of a tumor. This reasoning provides an initial explanation of drug resistance and metastasis initiation between patients with the same type of cancer<sup>[5,39]</sup>. The external pressures can be inflammatory responses, radiotherapy, or cytotoxic chemotherapy<sup>[19,42,43]</sup>. The microenvironment surrounding a tumor can also influence tumor fate. In a recent example, the genetic ablation of the E2F3 transcription factor in macrophages suppresses mammary tumor metastasis into the lungs, but not mammary tumor growth, suggesting that proper macrophage functions and specific microenvironments maintain specific cancer cell functions<sup>[44]</sup>.

**Table 1. Single-cell genomic sequencing methods**

Technique	Description	References
DOP-PCR	Allows the amplification of the nucleus genome using primers with ACTG combinations	[52]
MDA	No PCR phase; instead denaturalized DNA is amplified	[53-55]
MALBAC	Detects Copy Number Variants by amplifying the original DNA strand	[53]

### SINGLE-CELL SEQUENCING: A PROMISING TOOL FOR DECIPHERING TUMOR HETEROGENEITY

We discussed in the previous section that cancer stem cells, and changes in genetic and metabolic pathways in whole populations and single cells triggered by chromosome instability generate heterogeneity in cancer cell subpopulations. Even then, these cancer-cell subpopulations are limited in their functionality by distinct microenvironments or physical barriers, and tumor cells adapt to overcome these barriers. This confers adaptive tumor features and generates CTCs. Due to their critical role in intra-tumor heterogeneity, CTCs are well studied by single-cell sequencing. CTCs are found as clusters that reflect the intra-tumor heterogeneity and the potential capacity to initiate metastasis. Alternatively, CTCs can differentiate into different single cells from the initial tumor, thus increasing intra-tumor heterogeneity. Therefore, CTCs can serve as a diagnostic and evolutionary component to a better-targeted therapy<sup>[45-48]</sup>.

The most recent technique to study intra-tumor heterogeneity is single-cell sequencing (SCS). SCS is based on the principles that govern the next generation sequencing (NGS) technique. However, SCS is more informative than NGS because it reveals information from a single cell instead of making a pool of several cells that may have a heterogeneous genome and thus affect the results. The SCS procedure can be divided into two stages: single cell isolation and cell genomic profiling. Single cells can be obtained by the use of fluorescence-activated cell sorting (FACS)<sup>[49]</sup>, laser-capture microdissection (LCM)<sup>[47]</sup>, and micromanipulation<sup>[49]</sup>. Out of these, FACS appears to be the most efficient and easier to perform. After obtaining the single cell, single-cell genomic sequencing or single-cell transcriptomic sequencing can be done.

Single-cell genomic sequencing or single nuclear genome sequencing is useful to study mutations, single nucleotide variations, and indels (insertion and deletions)<sup>[50]</sup>. Multiple methods of SCS for single nuclear genome have been designed [Table 1]. One of such variants is the DOP-PCR, in which the amplification of the sequences is started with primers that in the 5'-3' ends have six possible ACTG combinations, which allow the hybridization of the template with the single cell DNA. This amplification of the sequences generates a database that is used to assess copy number assessment<sup>[39,41,51]</sup>. Another type of DNA sequencing of single cells is the multiple displacement amplification (MDA). This technique is characterized by not having a PCR phase amplification; instead denaturalized DNA from single cells are exposed to anneal with hexamer primers, synthesizing new DNA strands<sup>[52]</sup>. This type of sequencing is a better tool to detect mutations in the DNA strands. Another is the multiple annealing and looping-based amplification cycles (MALBAC) that amplify the original single cell DNA strand<sup>[51]</sup>. Creating a database that is useful for the detection of copy number variants (CNV)<sup>[53]</sup>. An aspect that differentiates all of these types of SCS is the generation of artifacts, false positive and false negative results that can affect the application of the proper algorithm to determine if the changes are significant of the population heterogeneity at the level of single nucleotide variants (SNV).

On the other hand, single-cell transcriptomic sequencing or whole transcriptome sequencing can be used to study the genetic network regulation in a certain cell subpopulation. Also, it can be useful to detect alternative splice sites, novel exons, retained introns, coding RNAs, and non-coding RNAs, among others<sup>[39,41,50]</sup>. Most of the sequencing protocols in cancer research use the whole transcriptome amplification (WTA). WTA uses reverse transcriptase to transform mRNA to cDNA via PCR amplification. This method was first used by Tang and colleagues<sup>[56]</sup>, and they used an oligo-dT primer at 5' and in the 3' they added a poly-A tail in the cDNA, generating data to detect alternative splice sites in the mRNA, generation of novel exons in the CTCs and genetic variants in the strand. Two main variants have been developed, Smart-Seq and Smart-Seq2, which differ in the 5' end primer of the strand<sup>[57,58]</sup>. Later, Quartz-seq was developed to detect the



**Table 2. Single-cell RNA sequencing methods**

Methods	Description	References
scRNA-Seq	Single cell transcriptome analysis	[56]
STRT-Seq	Provides adaptation of the template by switching oligonucleotide to barcode the 5' of the transcripts; allows for unbiased amplification among samples	[62]
Smart-Seq	Allows the evaluation of single nucleotide polymorphisms in a full length of cDNA to barcode 96 samples	[58]
Cel-Seq	Single cell <i>in vitro</i> technique that amplified mRNA linear that was multiplexed in a barcode manner	[60,61]
Smart-Seq2	Improved the sensitivity, coverage, and accuracy using an inaccessible RNA nucleotide (locked nucleic acid)	[57]
RCA	Whole transcriptome amplification from a small quantity of DNA	[64]
FISSEQ	<i>In situ</i> whole transcriptome amplification from a small quantity of DNA	[65]
UMI	Unique molecule identifiers that are tagged to cDNA allows for adjusted amplification bias, sensitivity, and background noise of samples	[66]
Microfluidics	96-single cell Smart-Seq2 that uses a microfluidic system	[67]
inDrop-Seq	Droplet-based; allows the sampling of thousands of cells to be sequenced with a barcode wrapped droplet	[68]
Drop-Seq		[69]
Cyto-Seq	Uses magnetic beads in combination with capture and poly(A) selection to analyze 100,000 cells	[70]
SUPeR-Seq	Uses a universal poly(A) independent RNA sequencing	[71]
G&T-Seq	Simultaneous genome and transcriptome sequencing	[72]
FRISCR-Seq	Uses intracellular staining; contains a low degree of bias	[73]
scMT-Seq	Simultaneously analyzes the methylome and the transcriptome of single cells	[74]
scTrio-Seq	Simultaneously sequence the genomic, transcriptomic, and methylome of single cells	[75]
Div-Seq	Scalable single nucleus RNA sequencing (sNuc-Seq), based that tracks dynamics of cells with high sensitivity	[76]
LCM-Seq	Laser capture microdissection <i>in situ</i> RNA sequencing	[77]
Small RNA-Seq	Analysis of micro, small, and transference RNAs	[78]

heterogeneity of gene expression between groups of SCS methods. This method reduces the amplification to detect expression of genes in different single cells types<sup>[59]</sup>. Cell expression by linear amplification and sequencing (Cel-Seq and Cel-Seq2) uses the method of molecular barcoding to identify different single cells in a pool of cells<sup>[60,61]</sup>. Despite the cost-effectiveness of the technique, it remains under- development. Single cell tagged reverse transcription (STRT) is a type of sequencing that quantifies the 5' mRNA gene expression in single cells, that is capable of locating promoters and enhancers. One of the latest is the Drop-Seq and Indrop-Seq by Islam *et al.*<sup>[62]</sup> in which thousands of cells in a droplet are sequenced by using a wrapped unique barcode. Another method has been developed from fixed cells, and additional transcriptome and methylome analyses have been studied to determine changes in expression of RNA in single cells<sup>[47,63]</sup>. Several other variants are exemplified in Table 2 and reviewed in more details elsewhere.

Despite being a time-consuming technique that requires multiple sampling and cannot be used to make generalizations, SCS can be used to diagnose rare tumor cells, detect earlier metastatic malignancies in CTCs, and study intra-tumor heterogeneity<sup>[50]</sup>. Even though this technique provides high replicability can have a high generation of false-positive or negatives or sequencing bias, affecting the applicability of the technique to drug treatment and diagnosis. Understanding intra-tumor heterogeneity can help improve current cancer treatments through precision medicine. Take for example breast cancer, which has been classified as at least 18-21 subtypes with unique histological and molecular characteristics; yet therapy is delimited to the ER, PR, Her2 criteria<sup>[79]</sup>. Since intra-tumor heterogeneity leads to chemotherapy resistance<sup>[79]</sup>, SCS can help detect rare genotypes that may be an aid in this process. Intra-tumor heterogeneity may also confer some adaptive features to the tumor through distinctive biomarkers, so SCS can also help identify such biomarkers to improve current treatment selection and move forward into precise medicine.

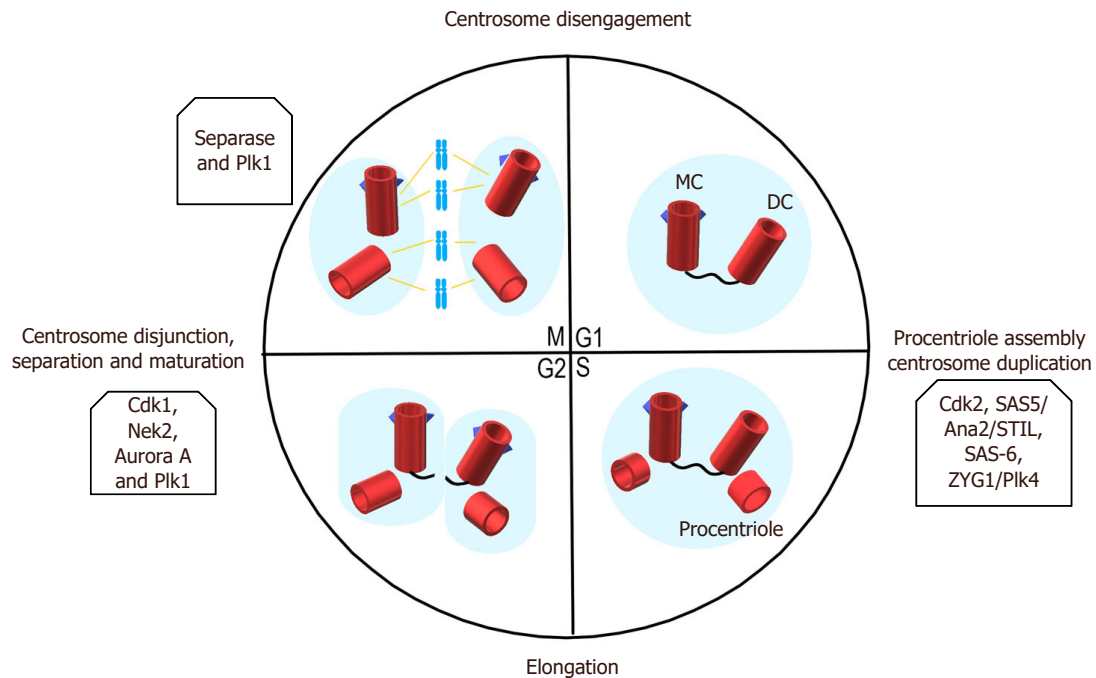
## CENTROSOME ABERRATIONS, CHROMOSOME INSTABILITY AND TUMORIGENESIS

Over 100 years ago, Theodor Boveri coined the term centrosome (independently and simultaneously discovered and called corpuscle central by van Beneden) and hypothesized that centrosome aberrations

leading to abnormal mitosis and abnormal chromosome constitutions may contribute to malignant tumors<sup>[80]</sup>. Since then, our laboratory and those of others have worked towards the elucidation of the mechanisms and consequences of centrosome aberrations in tumor initiation and progression. The centrosome is a small organelle composed of a pair of centrioles surrounded by pericentriolar material (PCM) that serves as the principal microtubule organizing center of vertebrate cells<sup>[81]</sup>. The centrosome duplicates only once to ensure proper spindle formation and equal chromosomal segregation during mitosis<sup>[82,83]</sup>. In order to maintain chromosome stability, the centrosome duplication cycle and the cell cycle must be tightly coordinated<sup>[84-88]</sup>. Laser ablation and microsurgical removal demonstrated that some immortalized mammalian cells (hTERT-RPE and -HMECs) can cycle without centrioles/centrosomes; however, some epithelial cells like BSC-1 African green monkey kidney cells go through G1 much more slowly or not at all if centrosomes are removed<sup>[89,90]</sup>. Centrosome removal sensitizes cells to various external stimuli such as blue light, which results in p53-dependent G1 arrest<sup>[89]</sup>. Similarly, silencing of 14 (out of 15) centrosome components arrests cells in G1 by activating p53, p21, p38, and inactivation of cyclin A-Cdk2 activity<sup>[91]</sup>.

Failure in the control of the centrosome cycle or of cytokinesis leads to numerical and structural centrosome aberrations, which have been identified in most cancer types<sup>[92-94]</sup>. A common centrosome aberration in many cancers is centrosome amplification<sup>[94]</sup>, which culminates in different degrees of aneuploidy (including single chromosome gains/losses all the way to whole genome doublings) and chromosome instability, thus contributing to intra-tumor heterogeneity. In order to maintain genomic stability, the cell cycle machinery also regulates the centrosome cycle<sup>[84,88,95-99]</sup>. One model states that the centrosome duplication cycle starts in G1-S when the pair of centrioles dissociates<sup>[88,100,101]</sup>. In a model proposed by Fukasawa<sup>[88]</sup>, centrosome disengagement in late G1 is licensed by the phosphorylation of nucleophosmin (NPM) by cyclin E/Cdk2 complexes<sup>[97,102-107]</sup>. Another model, evidenced by data from the Stearn group suggests that centriole disengagement occurs during anaphase, that it involves separase, and that this event licenses centriole duplication in the next cell cycle; in this model Cdk2 is required for centriole duplication, but not for licensing<sup>[108]</sup>. Our studies added additional complexity to these models, since the centrosomes from *cdk4*<sup>-/-</sup> mouse embryonic fibroblast did not achieve centrosome separation at G1/S, while these with a *cdk2*<sup>-/-</sup> genotype achieved premature separation, and the premature separation defect was exacerbated in *cdk2*<sup>-/-</sup>*cdk4*<sup>-/-</sup> mouse embryonic fibroblasts<sup>[104]</sup>. Early studies from the Nigg's group demonstrated that centriole duplication requires the activation of E2F transcription factors and the activity of the Cdk2-cyclin A complex<sup>[107]</sup>, and the Leone laboratory demonstrated that repression by E2F3 played a major role in preventing premature centriole duplication, centrosome amplification, and chromosome instability by controlling cyclin E levels and cyclin E-dependent kinase activity<sup>[109]</sup>. Although it is not entirely clear how the E2F activators (E2F1, E2F2 and E2F3a) control centrosome duplication, our laboratory has shown that the E2F activators control the transcription, protein stability, and protein levels of many targets that regulate the centrosome cycle and mitosis, including cyclin E, Rb, Plk4, Nek2, Mps1, SgoL1, and cyclin B<sup>[35,109,110]</sup>.

Albeit elucidating the entire centrosome duplication cycle is still a work in progress, much is now known about the cellular events controlling it, recently reviewed by Nigg and Holland<sup>[111]</sup>. Centriole assembly is controlled by phosphorylation of Ana2/STIL by Plk4; this event recruits Ana2 and Sas6 to initiate procentriole formation<sup>[112,113]</sup>. Centriole biogenesis is controlled by interactions between Cdk2 and the SKP1-Cullin-F-box E3 ligase  $\beta$ TrCP, where Cdk2 protects STIL from degradation by  $\beta$ TrCP<sup>[114]</sup>; STIL then interacts with CPAP to complete centriole duplication<sup>[115]</sup>. Cdk2 also controls the degradation of Mps1 in centrosomes to control centriole duplication<sup>[116]</sup>. Aurora kinase A (AURKA) is essential to the formation of a bipolar mitotic spindle by regulating centrosome separation<sup>[117]</sup>. The AURKA phosphorylation of Cdk1-cyclin B at G2 recruits the former to centrosomes, where it is activated to initiate mitotic entry<sup>[118]</sup>. Centrosome localization of Cdk1 and inhibition of Chk1 is present in mitosis to prevent premature activation of the Cdk1-cyclin B complex<sup>[119]</sup>. Accordingly, PLK1 regulates centrosome maturation<sup>[120]</sup>, centrosome disjunction through NEK2<sup>[121]</sup>, and centrosome microtubule-attachments<sup>[122]</sup>. Also, NEK2 regulates centrosome separation by



**Figure 1.** The centrosome duplication cycle. The mother centriole (MC) is depicted with blue triangles that represent the distal and sub-distal appendages to differentiate it from the daughter centriole (DC). In the G1 phase, the two centrioles are connected by a proteinaceous linker. The G1/S transition phase is characterized by the procentriole assembly, and some of the key proteins involved in this process are mentioned. In this stage, the DC starts to acquire the appendages that the MC has. During the S phase, the microtubules are synthesized, and rearrangement will occur to fully generate the procentriole. Till the G2 phase, the proteinaceous linker is broken, and the DC already has the distal and sub-distal appendages. This will convert DC into MC, and two pairs of centrioles will be formed. In the G2/M transition phase centrosome disjunction, separation, and maturation take place. Some key regulators have been listed above. During the M phase, the separated centrioles participate in bipolar spindle mitosis, and the centrosome cycle is completed when each daughter cell inherits two centrioles

phosphorylating and inactivating c-Nap1 and  $\beta$ -catenin<sup>[123,124]</sup>. Lastly, from metaphase to anaphase, the two centrosomes migrate to opposite cellular poles and form the mitotic spindle to which the kinetochore will attach<sup>[82]</sup>. Faithful segregation of chromosomes is ensured by the spindle assembly checkpoint (SAC) and associated proteins such as BUB1B<sup>[125]</sup>, MPS1<sup>[126]</sup>, among others. Other proteins that play important functions in chromosome integrity include Bub1, which maintains sister chromatid cohesion through the phosphorylation of Sgo1<sup>[127]</sup>; another protein that plays a key role in this activity is PP2A, which ensures localization of Sgo1 to centromeres<sup>[128]</sup>. Aurora kinase B, survivin, and ICENP play important roles in cytokinesis<sup>[129]</sup> [Figure 1].

Deregulation of the centrosome duplication cycle results in centrosome aberrations and chromosome instability that ultimately have an effect on tumorigenesis<sup>[87,88,130]</sup>. While centrosome aberrations are traditionally associated with cancer, mutations in genes that codify for centrosome proteins are also known to cause human diseases such as ciliopathies (e.g., autosomal recessive primary microcephaly, Bardet-Biedl disease, polycystic kidney disease, and primary ciliary dyskinesia)<sup>[131]</sup>. Centrosome aberrations are classified as numerical and structural<sup>[132]</sup>. Both aberrations co-occur in tumors<sup>[133,134]</sup>. Centrosome aberrations have been identified in most cancer types<sup>[94]</sup>. For example, pioneering studies from the Doxsey laboratory demonstrated structural abnormalities in number, position, shape, and size of centrosomes in primary solid tumors, including brain, breast, colon, lung, and prostate<sup>[92]</sup>. Likewise, studies from the Salisbury laboratory showed that breast cancer tissue displayed abnormal structural and numerical centrosome aberrations, abnormal mitoses and chromosome instability relative to normal breast tissue<sup>[133,135,136]</sup> and that centrosome amplification in breast cancers is indicative of tumor aggressiveness<sup>[137]</sup>.

Centrosome amplification is defined as an excess of normal components, specifically more than two centrosomes and more than four centrioles<sup>[138]</sup>. Centrosome amplification results in multipolar or pseudobipolar mitotic spindles that may culminate in aneuploidy and chromosome instability<sup>[101]</sup>. Also, centrosome amplification may lead to defects in cytokinesis that lead to tetraploidy<sup>[139]</sup>. Because tetraploidy and excess chromosome instability are associated with decreased cellular fitness<sup>[140,141]</sup>, cells with amplified centrosomes avoid cell death by clustering centrosomes in order to avoid the generation of multipolar mitosis, and excessive aneuploidy and chromosome instability<sup>[142,143]</sup>. However, cells with pseudobipolar spindles form merotelic attachments that lead to single chromosome gains and losses<sup>[144]</sup>. Either tetraploidy or single chromosome losses have been shown to be tumorigenic in mouse models of cancer<sup>[145,146]</sup>. In a more recent study, Sabino *et al.*<sup>[147]</sup> demonstrated that *Drosophila melanogaster* epithelial wing disc cells overexpressing Sak display extra centrosomes and exhibited mechanisms of clustering, but also inactivation of extra centrosomes. Inactivation of extra centrosomes is defined as the gradual loss of microtubule-nucleating capacity. Although inactivation culminates in normal spindle bipolarization, neither clustering nor inactivation was efficient and abnormal segregation was observed. Furthermore, epithelial cells with extra centrosomes generated tumors when transplanted into the wild-type host.

Although the role of numerical aberrations (i.e., centrosome amplification) in cancer has been extensively studied, its role in tumor initiation, progression, and metastasis remains controversial, and may be context-dependent. For example, centrosome amplification in hepatobiliary cancer is not associated with tumor stage, size or proliferative activity<sup>[94]</sup>. Likewise, there is no significant relationship between centrosome amplification and tumor size, stage or patient survival in lung cancer<sup>[94]</sup>. Moreover, studies from the Cleveland group in mice - with centrosome amplification induced by Cre-recombinase-mediated Plk4 expression - did not result in spontaneous tumorigenesis regardless of p53 status<sup>[148]</sup>. Concordantly, studies from the Basto's laboratory demonstrated that induction of centrosome amplification in mouse brains caused microcephaly due to increased apoptosis caused by multipolar divisions of neuronal stem cells<sup>[149]</sup>. Our own studies using an orthotopic model of breast cancer showed that rescuing back centrosome amplification in Her2<sup>+</sup> breast cancer cells silenced for E2F3 through the overexpression of GFP-Nek2 did not influence tumor growth or tumor burden<sup>[150]</sup>. In contrast, other models suggest that centrosome amplification can influence tumor initiation and progression. For example, centrosome amplification correlates with poor prognostic factors such as nodal status and hormone receptor-negative status in 103 primary invasive breast cancers<sup>[151]</sup>. Likewise, centrosome amplification is associated with triple-negative breast cancers, higher stage, and higher grade, correlating with decreased overall survival and relapse-free survival in a cohort of 362 breast cancer patients<sup>[152]</sup>. Another study confirmed the above results and correlated centrosome amplification with markers of aggressiveness in triple-negative breast cancer patients, including increased stage and the mesenchymal marker vimentin<sup>[153]</sup>. Several transgenic models suggest that centrosome amplification might have causal, rather than consequential effects on cancer. For example, centrosome amplification causes tumors in flies independently of chromosome instability<sup>[154,155]</sup>. Other studies using transgenic mouse models involved the temporal expression of the prolyl isomerase Pin1<sup>[156]</sup>, Aurora A<sup>[157]</sup>, or K-Ras<sup>G12D</sup><sup>[25]</sup> in mammary epithelial cells, which resulted in pre-malignant mammary epithelial lesions with centrosome amplification that preceded mammary tumors. In mice, centrosome amplification induced by Plk4 accelerates the time of onset of lymphomas and sarcomas associated with loss of p53<sup>[158]</sup>, and of skin tumors in p53-deficient epidermis<sup>[159]</sup>. More recently, Levine *et al.*<sup>[160]</sup> used a mouse model of intestinal neoplasia with a single truncated allele of the adenomatous polyposis coli (APC<sup>Min</sup>) tumor suppressor and generated a doxycycline-inducible mouse model exhibited increased levels of PLK4 (APC<sup>Min/+</sup>; Plk4<sup>Dox</sup>), which resulted in centrosome amplification and aneuploidy. Notably, the APC<sup>Min/+</sup>; Plk4<sup>Dox</sup> exhibited higher intestinal tumor incidence compared to the APC<sup>Min</sup> but no greater tumor burden. Therefore, these results demonstrate that centrosome amplification has a role in tumor initiation but not in tumor progression. To investigate if centrosome amplification can drive spontaneous tumorigenesis, Levine *et al.*<sup>[160]</sup> also developed a ROSA26-rtTA; tetO-Plk4 mouse model that expressed Plk4 in multiple mouse tissue upon doxycycline treatment. These mice developed lymphomas,

squamous cell carcinomas, and sarcomas that exhibited aneuploidy. However, it is still unknown why some tissue efficiently develop tumors, where others do not. Perhaps this is due to the high levels of centrosome amplification induced in these models, since high-level chromosome instability and aneuploidy affect the fitness of tumor cells, since they die, or stop proliferating after a few cell cycles<sup>[140,141]</sup>.

Moreover, studies from the Pellman group demonstrated that centrosome amplification also plays a role in tumor progression by promoting invasion<sup>[161]</sup>. In this particular study, invasion was measured using a 3D culture model after inducing centrosome amplification in untransformed human mammary epithelial MCF10A cells either by a genetic approach (through the overexpression of PLK4 in the cells by a doxycycline-inducible system) or by a pharmacological approach (through the inhibition of cytokinesis by the addition of 1,4-Dichlorobenzene, DBC, which also resulted in tetraploidy)<sup>[161]</sup>. The advantages of using such approaches are that this model allows the visualization of invasive protrusions and breast glandular structure formation, which cannot be achieved by conventional cell culture. The major findings were that centrosome amplification induced invasion in breast cells through an increase in the activity of Rac1 that disrupted cell to cell adhesions, and the invasion was independent of the induction of tetraploidy<sup>[161]</sup>. Likewise, our laboratory showed that rescuing back centrosome amplification in Her2<sup>+</sup> breast cancer cells downregulated for E2F3 by overexpressing GFP-Nek2 induced invasive protrusions in 3D culture<sup>[162]</sup>. The Aneja's laboratory also showed that induction of centrosome amplification by overexpression of Plk4 in MCF10A cells induced higher migration that correlated with vimentin expression<sup>[153]</sup>. Experiments done by Denu expressing Plk4 in non-transformed MCF10A mammary epithelial cells demonstrated that acute acquisition of centrosome amplification resulted in de-differentiation of cells, where CD24 levels were reduced, and CD44 increased, suggesting that these cells were acquiring stem-cell features<sup>[13]</sup>.

While the role of centrosome amplification in cancer is more clearly defined, the role of structural aberrations has been unclear until recently. Structural centrosome aberrations are defined as changes in size and composition of the pericentriolar matrix without changes in the number of centrioles<sup>[163]</sup>. Overexpression of Ninein-like protein (Nlp), a protein that is involved in microtubule nucleation<sup>[164]</sup> causes structural centrosome aberrations leading to spontaneous tumors in mice, including breast, ovary, and testicle<sup>[165]</sup>. The latest result from the Zhan laboratory is highly relevant to human disease since Nlp is overexpressed in breast, lung, ovarian, and squamous head and neck cancers<sup>[165-167]</sup>. Interestingly, structural centrosome aberrations lead to similar phenotypes as centrosome amplification, albeit by a non-cell autonomous mechanism, since overexpression of Nlp contributes to invasion by causing stiffness in epithelial cells that culminate in budding out of the acinar structures mitotic cells that do not contain centrosome aberrations<sup>[168]</sup>.

Together, these experiments suggest that centrosome amplification and structural aberrations can contribute to aggressive features of tumors by inducing invasion, increased grade/stage, and more stem-like features of cells. The studies above suggest that the effects of centrosome amplification in tumor cells appear to be context dependent.

## MECHANISMS DRIVING CENTROSOME AMPLIFICATION AND CHROMOSOME INSTABILITY

The Vande Woude group first identified the mechanism by which centrosome amplification is generated in tumors by showing that mouse embryonic fibroblasts lacking p53 displayed centrosome amplification<sup>[169]</sup>. Later on, other groups demonstrated that centrosome amplification was triggered by the loss of tumor suppressors that include APC<sup>[170]</sup>, BRCA1<sup>[24]</sup>, and BRCA2<sup>[171]</sup>. Regarding the mechanism, in p53-null mouse embryonic fibroblasts, silencing or genetic ablation of Cdk2 and Cdk4 suppressed centrosome amplification<sup>[104]</sup>. Also, centrosome amplification in Brca1- or GADD45- deficient cells was associated with the downregulation of Nek2<sup>[172]</sup>. Several studies revealed oncogenes could also drive centrosome amplification. For example, v-RAS drives centrosome amplification through the MAPK pathway<sup>[26,173]</sup>. Further, H-Ras<sup>G12V</sup> and H-Ras<sup>G12V</sup>, and c-Myc drive centrosome amplification through cyclin D1, Cdk4, and Nek2 in the non-transformed mammary



epithelial cells MCF10A<sup>[25]</sup>. Likewise, Her2<sup>+</sup> breast cancer cells require Cdk4 and Nek2 to signal centrosome amplification and chromosome instability<sup>[174]</sup>. Further, the inhibition of Cdk2 suppressed Aurora A-induced centrosome amplification in MCF7 breast cancer cells with inactive p53 by preventing the localization of Aurora kinase A to centrosomes<sup>[175]</sup>. However, not all oncogenes induce centrosome amplification as means to initiate tumors, despite the induction of proliferation and apoptosis in pre-malignant mammary epithelial lesions by c-Myc; the pre-malignant lesions were devoid of centrosome amplification<sup>[25]</sup>. Nevertheless, c-Myc eventually induced centrosome amplification in mammary tumors, suggesting that c-Myc requires other genetic or epigenetic alterations to induce this abnormal process in mammary tumors.

There has been vast evidence demonstrating the essential role of the RB/E2F pathway in cell cycle regulation and centrosome duplication, a pathway that is unregulated by oncogenes such as Ras and Myc<sup>[176]</sup>. For example, acute loss of Rb causes centrosome amplification<sup>[177]</sup>. Although the E2F transcriptional factors have redundant functions, each member of the family also has unique functions<sup>[178]</sup>. Take for example E2F3, whose loss in mouse embryonic fibroblasts results in unregulated cyclin E-dependent kinase activity, defects in nucleophosmin B association with centrosomes, and premature centriole separation and duplication that result in centrosome amplification, mitotic spindle defects, and aneuploidy<sup>[109]</sup>. On the other hand, genetic ablation of E2F1, E2F2, E2F4 or E2F5 does not cause centrosome amplification. Also, silencing E2F1 or E2F3 in Her2<sup>+</sup> breast cancer cells suppresses centrosome amplification, while overexpression of E2F1, E2F2, or E2F3a in MCF10A cells is sufficient to trigger centrosome amplification and chromosome instability<sup>[110]</sup>.

Chromosome instability is a broad term that refers to chromosome segregation errors, which results in chromosome losses or rearrangements. As reviewed elsewhere, chromosome instability can occur as a consequence of mitotic checkpoint defects, aberrations in centrosome duplication cycle, altered kinetochore function, microtubule attachment defects, chromosome cohesion defects, and mutations causing or allowing genomic instability<sup>[17]</sup>. Although it has been shown that centrosome amplification leads to chromosome instability<sup>[101]</sup>, a recent study from Kuznetsova *et al.*<sup>[179]</sup> showed that chromosome instability, tolerance of mitotic errors, and multidrug resistance can be promoted by tetraploidization in human cells without centrosome amplification. This study demonstrated that chromosome instability was tolerated by mutations in p53 and the downregulation of the pro-apoptotic factors iASPP and cIAP2. Even though it remains a question whether centrosome amplification is a cause or an effect of chromosome instability, both have been shown to occur exclusively in malignant tumors that display aneuploidy<sup>[138]</sup> and are associated with tumor recurrence<sup>[180]</sup>, metastasis<sup>[181,182]</sup>, and drug resistance<sup>[18,183,184]</sup>. Aneuploidy is defined by gains or losses of whole chromosomes that play a role in tumor initiation, maintenance, and progression<sup>[138]</sup>. Aneuploidy, as a consequence of chromosomal instability, along with genomic instability (defects in DNA damage detection and repair) lead to intra-tumor heterogeneity.

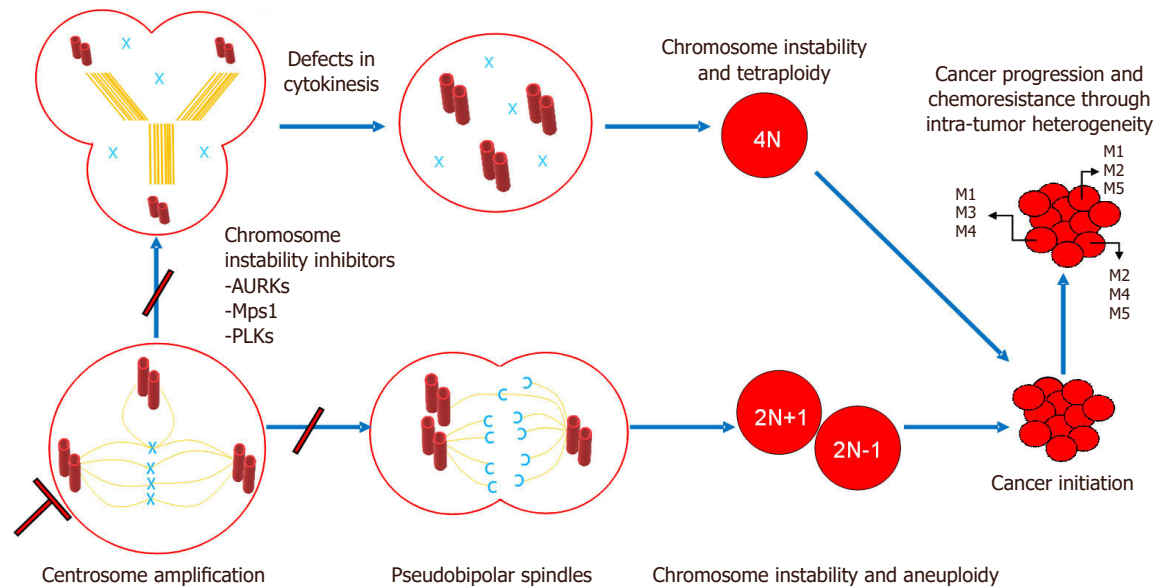
Chromosome instability occurs exclusively in malignant tumors that display aneuploidy; chromosome instability affects tumor progression by generating intra-tumor heterogeneity<sup>[181,182]</sup>. For example, chromosome instability has been shown to maintain intra-tumor heterogeneity in glioma cells<sup>[185]</sup>. A more recent study showed that chromosome missegregation drives intra-tumor heterogeneity in glioma cells; cells with double minute chromosomes were more radio-resistant than those without them<sup>[186]</sup>. Upon irradiation, the double minute chromosomes allowed glioma cells to invade and become angiogenic. Thus, in that setting, intra-tumor heterogeneity generated by the loss and gains of double minute chromosomes may hinder cancer treatment by increasing cell invasiveness and radio-resistant cells. Several studies have shown that chromosome instability also contributes to chemotherapy resistance<sup>[18,183,184,187]</sup>, making chromosome instability a good therapeutic target. However, it is noteworthy that there is a complex relationship between chromosome instability and therapeutic response that depends not only on the chromosome instability level, but also in the genetic context and tissue type<sup>[188]</sup>. As an example, a study conducted by Heerema *et al.*<sup>[188]</sup>

found that trisomies of chromosome 4 and 6 did not affect prognosis in patients with high hyperdiploid acute lymphoblastic leukemia, while concurrent trisomies of chromosomes 10 and 17 were associated with a better prognosis and trisomies of chromosome 5 was correlated with a worse prognosis. Later on, in this manuscript, we describe two approaches to target chromosome instability clinically. The first is by targeting some key proteins involved in the centrosome duplication cycle to decrease chromosome instability. The second approach aligns more with the notion that the cell will tolerate a certain level of chromosome instability and beyond that the cell will not be viable. Therefore, this approach aims to elevate chromosome instability levels to induce cell cycle arrest or apoptosis.

## INHIBITORS OF CHROMOSOME INSTABILITY IN CANCER TREATMENT

Given the numerous mechanisms attributed to chromosomal instability, several approaches have been proposed to target chromosome instability in cancer. One approach is to target centrosome-associated proteins that regulate microtubule dynamics and the SAC to prevent centrosome amplification, thus preventing chromosome instability<sup>[87,189,190]</sup>. The Cdk4/Cdk6 inhibitor Palbociclib (PD-0332991) in combination with the aromatase inhibitor letrozole has greatly improved the outcomes of ER<sup>+</sup>, Her2<sup>-</sup> advanced breast cancer patients<sup>[191,192]</sup>. Albeit that study did not measure centrosome amplification and chromosome instability, it is tempting to propose this as an approach to suppress active generation of these processes in cancer cells, since we have shown that silencing or genetic ablation of Cdk4 in p53-null fibroblasts, in mammary epithelial cells expressing H-Ras<sup>G12V</sup> or H-Ras<sup>G12V</sup> and c-Myc, or in Her2<sup>+</sup> breast cancer cells suppress these processes<sup>[25,104,174]</sup>. However, this approach neglects the fact that chromosome instability may occur by multiple mechanisms and multiple dysregulated proteins. In fact, Palbociclib is ineffective in basal breast cancer cells (the subtype with a higher degree of chromosome instability), and patients are harboring alterations in the Rb/E2F pathway<sup>[193,194]</sup>. Nevertheless, several inhibitors targeting polo-like kinases (Plks) and Aurora kinases (AURKs) have been tested in pre-clinical and clinical trials with mixed outcomes, and this has been extensively discussed elsewhere<sup>[189]</sup>. Notably, the inhibitor MLN8237 (Asertib) that targets AURKs exhibited efficacy for several solid tumors and T-cell lymphoma, but not acute myeloid leukemia<sup>[195,196]</sup>. The opposite was observed for the selective inhibitor of AURKB, AZD1152 (Barasertib)<sup>[189]</sup>.

Another strategy to kill tumor cells is to elevate chromosome mis-segregation. It has been proposed that there is an optimal level of chromosome instability for tumor maintenance and progression; beyond that level chromosome instability becomes detrimental for cancer cells<sup>[12,184]</sup>. For example, elegant experiments from the Sluder laboratory demonstrated that the acquisition of tetraploidy in most immortalized or cancer cells they investigated resulted in cell cycle arrest within a few cell cycles<sup>[140]</sup>. Also, the Cleveland group demonstrated that while low-level aneuploidy triggered by the loss of one copy of Cenp-E was tumor promoting in mice, aneuploidy can also be tumor-suppressive<sup>[197]</sup>. A recent pan-cancer analysis of genetic heterogeneity in cancer done by the Malley group showed that in general, cancers with intermediate levels of chromosome instability (measured by copy number variation analysis) had worst prognosis than cancers with low or high levels of instability<sup>[2]</sup>. However, their relationship varied depending of the adjuvant treatment given, suggesting that radiotherapy and adjuvant chemotherapy may be effective in treating cancers with intermediate chromosome instability by pushing the limits of tolerable chromosome instability. The Swanton's group also provided clinical evidence to support this hypothesis with their retrospective study conducted in a cohort of 246 primary breast cancer patients<sup>[12]</sup>. The study showed that extreme chromosome instability (measured with chromosome-specific markers and aCGH and correlated to the CIN70 score, MammaPrint, and GGI) correlated with improved long-term survival in ER-negative breast cancer patients; exhibiting a non-monotonic correlation<sup>[12]</sup>. This observation was confirmed in a study involving a larger cohort of ER<sup>-</sup> patients<sup>[198]</sup>. However, a linear correlation was observed in ER-positive breast cancer patients and extreme chromosome instability<sup>[12]</sup>; the same relationship was found with glioblastomas<sup>[2]</sup>. Thus, we have



**Figure 2.** Centrosome amplification leads to tumor initiation and cancer progression through intra-tumor heterogeneity. Two models are described above. First, centrosome amplification leads to pseudobipolar spindles that culminate in chromosome instability and aneuploidy. Second, centrosome amplification leads to defects in cytokinesis that culminates in chromosome instability and tetraploidy. Both mechanisms converge to initiate cancer. Cancer progression and chemoresistance occurs and is maintained as a consequence of intra-tumor heterogeneity. Chromosome instability inhibitors (e.g., AURKs, Mps1, and PLKs) are therapeutic targets that may prevent this chain of events by targeting early steps of this process

to be careful with proposing increasing chromosome instability as a strategy against cancer, since it is tumor suppressive in some cancers, and tumor promoting in others.

Mitotic kinases contribute to chemotherapy resistance, as illustrated by Janssen *et al.*<sup>[199]</sup>, who demonstrated that the reduction of essential levels of Mps1 and BubR1 sensitized several tumor cells to clinically relevant doses of paclitaxel (an anti-mitotic drug commonly used in cancer treatment). On the other hand, inhibition of these kinases did not induce cell death in normal cells. Currently, a Mps1 inhibitor is being tested in clinical trial Phase 1 (BAY1161909) in triple negative breast cancer patients<sup>[200]</sup>. In this clinical trial, the Mps1 inhibitor is administered along with paclitaxel (a microtubule-interfering agent) to induce tumor death by increased chromosome mis-segregation<sup>[200]</sup>. A similar approach can be tested with the combination of paclitaxel and BubR1, Hec1, Nek2, or Sgol1 inhibitors because all of these proteins play an important role in proper SAC functioning and our studies have demonstrated their role in centrosome amplification and chromosome instability downstream of the E2F activators<sup>[35,162,201]</sup>. Additionally, a study by Lee *et al.*<sup>[201]</sup> ranked 62 different anticancer drugs for their capacity to induce chromosome instability. The drugs evaluated in this study have several mechanisms of action (e.g., antimicrotubule activity, DNA replication and damage response, mitotic checkpoint inhibition, *etc.*) and can be evaluated in combination with inhibitors of centrosome-associated proteins to see if the effect of increase chromosome instability is potentiated. Thus, these findings present us with multiple possibilities that together with advances in precise medicine and technologies such as SCS can be explored in cancer patients with specific tumor genotype/phenotype (intra-tumor heterogeneity) to develop better treatment.

## CONCLUSION

Failure to properly regulate the cell cycle and the centrosome cycle leads to centrosome aberrations. One of such centrosome aberrations is centrosome amplification, which occurs in various cancer types. In our model depicted in Figure 2, we summarize two known mechanisms that denote the role of centrosome

amplification in tumor initiation, maintenance, progression, and chemo/radio-resistance through intra-tumor heterogeneity. One mechanism shows that centrosome amplification results in multipolar or pseudobipolar mitotic spindles that may culminate in aneuploidy and chromosome instability, thus contributing to intra-tumor heterogeneity. The other mechanism shows how defects in cytokinesis lead to tetraploidy and chromosome instability. This mechanism also promotes tumor initiation, maintenance, progression, and chemoresistance through intra-tumor heterogeneity. The reader should also keep in mind that centrosome aberrations may contribute to malignant phenotypes in cancer such as invasion through changes in polarity, and such phenotypes occur independently of chromosome instability.

However, the role of centrosome amplification in tumorigenesis needs to be further elucidated in human tumors because it has been shown that centrosome aberrations are highly context-dependent and several other mechanisms may apply<sup>[202]</sup>. Another aspect that is worth studying in the future is the effect of functional centrosome aberrations (microtubule nucleation, disorganized mitotic spindle, *etc.*) and other structural centrosome aberrations such as changes in shape, size position, and composition in cancer. Also, clustering mechanisms and normal spindle bipolarization through extra chromosome inactivation and how these vary in cancer. Nevertheless, proper classification of centrosome aberrations in human tumors might have a diagnostic or prognostic value. Therefore, it would be beneficial to explore the therapeutic applications of chromosome instability in cancer. As reviewed here, chromosome instability inhibitors such as AURKs, Mps1, and PLKs inhibitors can help improve cancer treatment by preventing centrosome amplification and chromosome instability. Another strategy will be to increase chromosome instability levels to promote cancer cell death, but this will be context dependent. For example, this strategy can be used for ER<sup>-</sup> breast cancers, since extreme chromosome instability correlates with better prognosis in patients with this molecular phenotypes. On the other hand, increasing chromosome instability in ER<sup>+</sup> breast tumors is a poor strategy, since there is a direct relationship between increases in chromosome instability and poor survival. In addition, increasing chromosome instability may increase chemotherapy resistance in some patients. SCS can help to address specific genotype that confers cancer cell subpopulations adaptive advantages and impede complete tumor clearance. The advances in both SCS and the identification of putative therapeutic targets are promising toward a complete understanding of cancer and how effective treatment can be achieved.

## DECLARATIONS

### Authors' contributions

Conceived the general idea of the review and made up the structure: Jusino S, Saavedra HI

Searched the literature and drafted the manuscript: Jusino S, Fernández-Padín FM

Read and approved the final manuscript: all authors

### Availability of data and materials

Not applicable.

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### Conflicts of interest

All authors declare that there are no conflicts of interest.

**Ethical approval and consent to participate**

Not applicable.

**Consent for publication**

Not applicable.

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Topic: How does the prostate cancer microenvironment affect the metastatic process and/or treatment outcome?

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# Expression and regulation of aldehyde dehydrogenases in prostate cancer

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## ABSTRACT

The functional role of aldehyde dehydrogenases (ALDHs) in prostate cancer remains an area of some controversy. Many studies have used high ALDH functional activity to isolate putative cancer stem cells with tumour-initiating and propagating properties, while evidence is also emerging about the involvement of specific isoforms in migration, invasiveness and metastasis. Identification of specific ALDH isoforms, which contribute to both drug resistance and aggressiveness of the disease remains a challenge within the complex heterogeneity of prostate cancer. The purpose of this perspective is to dissect functional roles for ALDH in the tumour microenvironment and to evaluate the potential of the ALDH gene family as biomarkers and/or targets for therapeutic intervention.

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## INTRODUCTION

Prostate cancer (PCa) is the most common cancer affecting men in the developed world. In the UK alone, over 47,000 new cases are diagnosed and more than 11,000 cancer related deaths are registered every year (Prostate Cancer UK, 2016). PCa is often present in the absence of apparent symptoms for many years, and so is considered to be slow-growing,

however this is not true for all PCa's. Whilst the underlying cause of PCa is not fully understood, the initial stages of PCa frequently depend on androgens for cellular proliferation. If radiotherapy or radical prostatectomy cannot be used to eradicate or remove the tumour, then it is effectively treated by androgen deprivation therapy (ADT)<sup>[1,2]</sup>, especially if the tumour has escaped the capsule. However, the tumour invariably relapses in most patients after ADT, leading to an aggressive form of PCa known as castration-



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**Table 1: Tissue distribution, subcellular distribution and substrates of human aldehyde dehydrogenases**

ALDH	Tissue distribution (main organs)	Subcellular localisation*	Substrate*
1A1	Most tissues <sup>[225]</sup>	Cytosol	Retinal
1A2	Testis <sup>[226]</sup>	Cytosol	Retinal
1A3	Retina, salivary gland and stomach <sup>[87]</sup>	Cytosol	Retinal
1B1	Small intestine, liver and pancreas <sup>[227]</sup>	Mitochondria	Retinal & acetaldehyde
1L1	Liver, kidneys and muscles <sup>[228]</sup>	Cytosol	10-formyltetrahydrofolate
1L2	Pancreas, heart and brain <sup>[229]</sup>	Mitochondria	10-formyltetrahydrofolate
2	Most tissues <sup>[230]</sup>	Mitochondria	Acetaldehyde
3A1	Stomach, lung and cornea <sup>[231]</sup>	Cytosol, partially in nucleus	Aromatic & aliphatic aldehydes
3A2	Liver <sup>[231]</sup>	Endoplasmic reticulum	Fatty aldehydes
3B1	Lung, prostate and kidneys <sup>[231]</sup>	Endoplasmic reticulum	Octanal
3B2	Salivary gland and placenta <sup>[232]</sup>	Endoplasmic reticulum	Unknown
4A1	Liver, kidney and placenta <sup>[26]</sup>	Mitochondria	Glutamate- $\gamma$ -semialdehyde
5A1	CNS, brain and blood <sup>[233]</sup>	Mitochondria	Succinate semialdehyde
6A1	Liver, kidney and heart <sup>[26]</sup>	Mitochondria	Malonate semialdehyde
7A1	Liver, kidney and heart <sup>[231]</sup>	Cytosol	$\alpha$ -amino adipic semialdehyde
8A1	Liver, kidney and brain <sup>[26]</sup>	Cytosol	Retinal
9A1	Liver, kidney and muscle <sup>[231]</sup>	Cytosol	$\gamma$ -aminobutyraldehyde
16A1	Bone, heart, kidney and lung <sup>[231]</sup>	Transmembrane protein	Unknown
18A1	Pancreas, ovary, testis and kidney <sup>[231]</sup>	Mitochondria	Glutamic- $\gamma$ -semialdehyde

\*Adapted from references<sup>[26,32]</sup>

resistant prostate cancer (CRPC), which remains an untreatable disease<sup>[1,3]</sup>.

The tumour microenvironment (TME) exerts a strong hold on tumour initiation, progression and metastasis<sup>[4]</sup>. TME is a general term encompassing a complex heterogeneous environment which includes inflammatory cells, blood vessels, extracellular matrix<sup>[5]</sup> and fibroblasts (stroma). In normal prostate homeostasis, a controlled interaction between non-epithelial components such as stroma and epithelial cells contributes to normal epithelial proliferation, differentiation and migration<sup>[5,6]</sup>. When prostate epithelial cells have acquired a malignant phenotype, this crosstalk between prostate epithelium and stromal cells is perturbed<sup>[6]</sup>. As a consequence, stromal cells play a critical role in activating cellular events within the TME that sustain and support cancer proliferation and metastasis<sup>[4,7]</sup>. Multiple studies of cell signalling associated with androgen<sup>[8]</sup>, Hedgehog<sup>[9]</sup>, fibroblast growth factor (FGF)<sup>[10]</sup>, Src family kinase<sup>[11]</sup>, transforming growth factor- $\beta$  (TGF- $\beta$ )<sup>[12]</sup>, Integrin<sup>[13]</sup> and Notch<sup>[14]</sup> pathways, implicate the TME, however many such observations are derived using a mixture of both human and mouse models in which the TMEs are radically different. Accordingly more careful attention is required to evaluate the impact of the TME.

Within a tumour, the entire population of replicating cancer cells has been hypothesised to be derived from a small subpopulation of cancer stem cells (CSCs) or tumour initiating cells (TICs)<sup>[15]</sup>. CSCs

have the ability to both self-renew and to produce progenitor and differentiated cells, generating phenotypically diverse tumour cell populations<sup>[16]</sup>. The stem cell microenvironment (SCME) is a specific anatomic location (or “niche”) where stem cells (SCs) are located, and the interplay between SCs and these niches can regulate the dynamic process of SCs’ role in tissue generation, maintenance and repair<sup>[17]</sup>. Several factors affect SC regulation within the SCME, including the interactions of SC with each other, with differentiated cells, and with extracellular matrix components<sup>[18]</sup>. Dysfunction of a cellular process or signalling pathway within the SC niche could contribute to the evolution of a CSC<sup>[19]</sup>. Although the presence of this CSC niche could pose obstacles for the treatment of PCa, it has also been proposed that the CSC niche also provides a potential target for biomarker and drug discovery<sup>[20-22]</sup>.

Aldehyde dehydrogenases (ALDH) have been exploited as selective markers for CSCs and have been assigned potential functional roles in differentiation, self-protection and expansion<sup>[23]</sup>. The ALDH superfamily consists of 19 genes with distinct chromosomal locations, which are found across 11 families and 4 subfamilies<sup>[23-25]</sup>. These enzymes have a varied tissue and organ distribution<sup>[26-28]</sup> and are localised in the cytoplasm, mitochondria, nucleus, and endoplasmic reticulum<sup>[23,24]</sup>. ALDH isoforms show distinct substrate specificity<sup>[26,29,30]</sup>, and are NAD(P)+ dependent [Table 1]. Their major role is the detoxification of endogenous and exogenous molecules, via oxidation of aldehyde substrates to

their corresponding acids. This catalytic oxidation is a fine-tuned reaction evolved to protect cells from the harmful effects of highly reactive aldehydes and maintains cellular homeostasis<sup>[24,25,31]</sup>. Vital functions include protection of cells from oxidative stress (e.g., reactive oxygen species, ROS) and promotion of retinoic acid (RA) metabolism and signalling<sup>[32]</sup>.

Mutations and polymorphisms in ALDH genes lead to a loss-of-function that are associated with various human pathologies<sup>[33-39]</sup>, which supports their important biological function. Plentiful studies have described the expression of ALDHs in human tissues, however their expression profile and functional activity is poorly understood within the TME. As a consequence of high and abundant expression, ALDHs have been considered to be biomarkers of specific tumour types<sup>[40-45]</sup>. Human ALDHs are among the regulatory proteins that catalyse the retinoic acid (RA) pathway, which has been linked with “stemness” characteristics<sup>[45]</sup>. The ALDH1A subfamily members have also been identified in a wide-range of human CSCs, and their expression has been associated with poor prognosis in patients with several cancer types including PCa<sup>[46-54]</sup>.

## ALDH EXPRESSION AND REGULATION IN PROSTATE CANCER

The rate and frequency of PCa progression varies considerably between individuals, ranging from relatively slow (indolent, non-invasive) in some patients whilst in other cases the disease is more aggressive and results in rapid metastasis<sup>[55]</sup>. At present, PCa is diagnosed at first by monitoring levels of serum prostate-specific antigen (PSA) and digital rectal examination<sup>[55]</sup>. However there is a substantial overlap in PSA levels between patients with benign prostatic hyperplasia (BPH) and patients with PCa<sup>[55]</sup>. About 25% of cases with PCa display no increase in serum PSA levels and thus must be detected by other methods<sup>[55]</sup>, such as diagnostic needle biopsies and MRI scans. Furthermore, it is crucial to determine indolent from aggressive forms of PCa, to offer patients earlier diagnosis and better treatment options. This is neither currently possible nor routine. In this regard, more detailed, in-depth understanding of the correlation between ALDHs and PCa progression may provide alternative biomarkers for disease diagnosis and treatment.

As indicated above, a complex interplay of PCa with the surrounding stroma, androgen receptor (AR) signalling, epithelial-to-mesenchymal transition

(EMT) and other signalling pathways within the TME support progression of the disease. Stromal cells such as fibroblasts and myofibroblasts are involved in hormone signalling, contributing to stromal-epithelial interactions in the primary tumour setting<sup>[56-58]</sup>. For example both stromal and epithelial ALDH1 expression, measured using IHC, have been shown to be a potential biomarker for breast cancer<sup>[59]</sup>. The epithelial and stromal ALDH1 expression (detected in 43% and 69% of benign breast biopsies, respectively) was associated with a predicted increase in the risk of breast cancer. However, as with many earlier studies<sup>[45]</sup> on profiling ALDHs in clinical specimens, no information is available to ascertain which ALDH was overexpressed from the subfamily (ALDH1A1, 1A2, 1A3, 1B1, 1L1, 1L2).

In PCa, several ALDH isoforms (1A1, 1A3, 3A1, 3A2, 4A1, 7A1, 9A1 and 18A1) have been found to be overexpressed<sup>[15,60-68]</sup>, but only a few isoforms appear to play critical roles in PCa. In a recent proteomic study<sup>[69]</sup>, ALDH1A3 expression was in part controlled via miR-187, as downregulation of this microRNA led to induction of ALDH1A3, while re-introduction decreased ALDH1A3 expression in PC-3, DU145 and LNCAP prostate cancer cells. Some ALDHs may also contribute to regulation of AR pathways, with implications for normal prostate development, prostate carcinogenesis and progression to androgen-independent disease<sup>[70-73]</sup>. AR is expressed in almost all primary PCas<sup>[74-76]</sup> and the transition from a localised hormone-naïve to a castration-resistant phenotype is based on a complex interplay of signalling molecules attributed to aberrant AR signalling<sup>[73,77-79]</sup>. Raised PSA suggests that AR function is still active but abnormal in CRPC<sup>[80]</sup>, due to a number of different mechanisms including AR amplification<sup>[81]</sup>, AR gain-of-function mutations<sup>[82]</sup>, intracrine androgen production<sup>[83]</sup>, elevated levels of AR cofactor that sensitises cancer cells to low levels of androgens<sup>[84]</sup>, ligand-independent activation of AR by growth factors and cytokines<sup>[85]</sup> and constitutively active messenger ribonucleic acid (mRNA) spliced variants of AR<sup>[86]</sup>. Consequently, AR remains a critical factor in the progression of early-stage PCa to CRPC.

ALDH1A3 is androgen responsive in human epithelial LNCaP cells since its expression was 4-fold higher after treatment with dihydrotestosterone (DHT), which indirectly affects both AR regulation and cell differentiation<sup>[59]</sup>. ALDH1A3 has also been correlated with AR signalling pathway in primary PCa tissue where expression was consistent with luminal layer localisation<sup>[65]</sup>. Significantly, the study also showed that knockdown of ALDH1A3 led to substantial



reductions in proliferation rate and the invasive ability of PC-3 cells. However, the regulation of ALDH1A3 expression is likely to be multifactorial<sup>[87]</sup>.

Outside the ALDH1 family, strong association of ALDH3A1 with PCa progression has also been demonstrated in both immortalised cancer cell lines and tumour xenografts<sup>[61]</sup>. In clinical tissues ALDH3A1 was detected in intra-epithelial neoplasia, with elevated levels in carcinomas in the absence of expression in normal prostate glands. Finally, in comparison with the paired local carcinomas, ALDH3A1 was upregulated in both lymph node metastatic tumours and was detectable in bone metastatic PCa.

ALDH7A1, which has also been related to the stemness of CSCs<sup>[88]</sup>, is mainly localised in the cytosol, but it has also been found expressed to a lesser degree in the mitochondria and nucleus<sup>[32,45]</sup>. In addition to catalysing aldehyde metabolism, ALDH7A1 also plays a role in protecting tissues from the damaging effects of osmotic stress<sup>[89]</sup> while mutation of the ALDH7A1 gene has been related to pyridoxine-dependent epilepsy<sup>[90,91]</sup>. In cancer, ALDH7A1 is expressed in nodular melanoma (NM)<sup>[92]</sup>, ovarian<sup>[93]</sup> and lung cancers<sup>[94]</sup> while in PCa the isoform has been shown to be involved in intra-bone growth and induced bone metastasis<sup>[64]</sup> as well as zoledronic acid resistance<sup>[95]</sup>. Gene expression profiling supports the involvement of ALDH7A1 in multiple molecular pathways related to the metastatic process in PCa<sup>[96]</sup>.

## EVIDENCE FOR EPIGENETIC CONTROL OF ALDHs

PCa can be initiated by genetic or epigenetic alterations, including DNA methylation in the promoter region of genes, normally linked to transcriptional silencing<sup>[55]</sup>. Epigenetic changes including DNA methylation and histone modifications of tumour suppressor genes (TSGs) preferentially occur in the early stages of cancer progression<sup>[55]</sup>. The promoter region of ALDH1A2 in primary PCa specimens has been shown to be densely hypermethylated in comparison to normal prostate tissues<sup>[97]</sup>. This observation is supported by another study that showed a low/absent expression of ALDH1A2 in PCa in formalin-fixed paraffin embedded sections compared to elevated levels of expression in normal prostate tissue<sup>[98]</sup>. On this basis it was suggested that ALDH1A2 act as a TSG in PCa, and that its epigenetic regulation could differentiate normal prostate cells from malignancy. In contrast,

ALDH1A3 has been demonstrated to be an androgen responsive gene<sup>[67]</sup> whose induction contributes to the conversion of retinol to RA with potential for supporting cellular proliferation<sup>[55]</sup>. Hypermethylation of the ALDH1A3 promoter region in clinical tissues has also been detected<sup>[99]</sup>, but this study used a relatively small sample size ( $n = 24$ ) and did not distinguish between methylation of basal and luminal PCa cells. Although larger studies are required, it is possible that methylation of the promoter regions of ALDH1A2 and ALDH1A3 could be used as a marker for PCa detection<sup>[55]</sup>.

## ALDH EXPRESSION IN CSC MICROENVIRONMENT

Growing evidence strongly supports initiation of PCa from CSCs residing within a basal niche<sup>[100-105]</sup>. In xenotransplantation experiments, less than 100 TICs are needed to generate a new tumour in mice and these cells exhibit a basal phenotype<sup>[106]</sup>. Furthermore, using human tissue biopsies the prostate SC markers CD44+,  $\alpha 2\beta 1$ -integrinhigh and CD133+ have been used to identify and isolate prostate CSCs with self-renewal capacity *in vitro*<sup>[100]</sup>. Additionally, there are other important markers that have been used to identify and isolate PCa SCs. ATP binding cassette (ABC) transporters which are proteins that play a vital role in the efflux of drugs have also been used to enrich CSCs. However, CD44+,  $\alpha 2\beta 1$ -integrin high and CD133+ ABC transporters are also expressed in normal SCs<sup>[107,108]</sup> which emphasises the need to employ at least two markers to avoid cross reacting populations of cells<sup>[107]</sup>. A growing body of evidence suggests that the functional activity of ALDHs can be used to identify and purify CSCs from e.g. breast<sup>[109]</sup>, ovary<sup>[110]</sup>, lung<sup>[111]</sup>, colon<sup>[112]</sup>, pancreas<sup>[113]</sup> and prostate cancer<sup>[114]</sup>. At present it is unclear if ALDH expression is significantly different between normal SCs and CSCs, hence more research is required to understand if any isoforms could be more predictive than e.g. CD44+,  $\alpha 2\beta 1$ -integrin high and CD133+ used as a PCa SC gene-expression signature<sup>[115]</sup>.

ALDHs expressed in SCs are members of the ALDH1 family (1A1, 1A2, 1A3, 1L1, 1L2), ALDH2, ALDH3A1, ALDH4A1 and ALDH7A1, which have all been linked with various critical roles including chemo-protection, DNA damage and regulation of the cell cycle<sup>[24]</sup>. The Aldefluor assay has frequently been used to identify and isolate CSCs, but as this assay does not distinguish between different isoforms many studies suffer from a lack of knowledge of the contributing ALDHs to the stemness of the isolated subpopulations with tumourigenic properties. However, some



studies have shown that e.g. the ALDH1A1 isoform positively correlates with the expression of CSC surface markers CD133<sup>[116]</sup> and CD34<sup>[117]</sup> with utility in characterising liver CSCs and leukaemia SCs, respectively. The association of ALDH3A1 has also been reported in PCa progression<sup>[61]</sup>. Stem cell-like cells from DU145 cells have elevated expression of ALDH3A1 compared to non-stem counterparts, and the stem cell-like population generated xenograft tumours with aggressive features<sup>[118]</sup>.

## ALDHs AND THE RETINOID SIGNALLING PATHWAY

Retinoic acid (RA, all-trans retinoic acid (ATRA), tretinoin) the physiologically active metabolite of vitamin A (retinol) is a potent regulator of signalling pathways during embryonic development<sup>[119]</sup>. RA is necessary for adult tissue homeostasis and acts through nuclear retinoic acid receptors (RARs)<sup>[120]</sup>, with diverse immune modulatory roles<sup>[121,122]</sup>, role in spermatogonial differentiation<sup>[123]</sup>, and cancer<sup>[124-126]</sup>. RA is endogenously produced from retinol (vitamin A) in two subsequent metabolic steps [Figure 1]: the first step is the retinol oxidation to retinaldehyde, which is catalysed by several alcohol dehydrogenases (also known as retinol dehydrogenases)<sup>[127,128]</sup>. The second step is the oxidation of retinaldehyde to retinoic acid, which is an irreversible step carried out by ALDHs (also known as retinal dehydrogenases)<sup>[129]</sup>. At least four ALDH isoforms, ALDH1A1, 1A2, 1A3 and 8A1, have been shown to be responsible for the oxidative formation of retinol to RA<sup>[128,130-132]</sup>. ALDH1A3 appears to be the most catalytically efficient enzyme for RA oxidation and has no apparent capacity to metabolise cis-retinal substrates<sup>[133]</sup>. The involvement of ALDHs in RA synthesis underpins their vital function in pathways associated with cell proliferation, differentiation and survival<sup>[87]</sup>.

The synthesised RA binds to nuclear RAR and retinoid X receptor (RXR) forming a heterodimeric complex, which binds to RA response elements (RAREs), leading to downstream regulation of gene expression and cell differentiation events<sup>[134-137]</sup>. RA and 9-cis-RA (isotretinoin) bind to RARs, whereas only 9-cis-RA can bind to RXRs<sup>[23]</sup>. In response to RA synthesis, cellular retinoic acid binding protein (CRABP) shuttles RA to the nucleus where it binds to the RAR/RXR heterodimer<sup>[138,139]</sup>. This subsequently results in the dissociation of co-repressors NCoR, SMRT and HDAC complex<sup>[140]</sup> and allows co-activators such as SRC/p160 family, p300/CBP and CARM-1 to bind<sup>[141,142]</sup>. The chromatin structure is

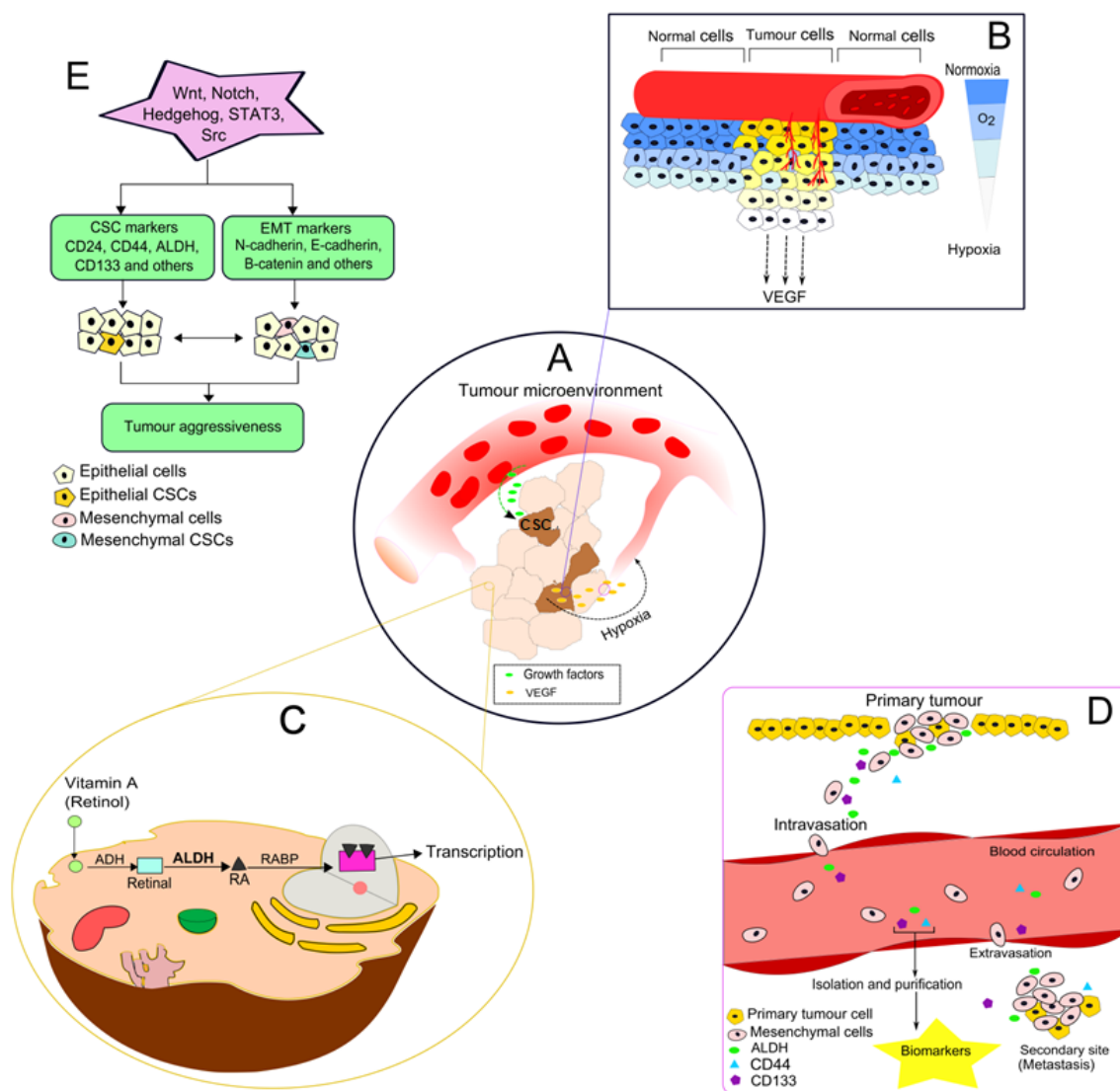
relaxed by the action of histone acetyltransferase (HAT) or methyltransferase activity<sup>[143]</sup>, facilitating the recruitment of transcriptional machinery which stimulates RA responsive gene transcription<sup>[144,145]</sup>.

The RA biosynthetic pathway is likely to be suppressed or activated depending on the local prostate microenvironment<sup>[146-148]</sup>. The effect of RA has been investigated in normal and malignant prostate tissues<sup>[129,149]</sup>. Differential expression of RA was demonstrated in normal prostate, BPH, and prostate carcinoma tissues<sup>[129]</sup>. For example it was found that endogenous retinol levels were 2-fold elevated in BPH compared to normal and PCa tissue while RA levels were found 5-8 times lower in PCa tissue compared with the other two tissues. The authors speculated that the reason for this elevated level of retinol in BPH could reflect (1) a reduced activity of the dehydrogenase that metabolises retinol to retinal or (2) uptake from serum that metabolises retinol to retinal. A possible cause for the reduced level of RA in PCa could be a more rapid degradation of RA by cytochrome P450 enzymes<sup>[150]</sup>.

In addition, RA also has variable effects on PCa signalling pathways, either directly or indirectly by regulating certain transcriptional factors such as NR2F1<sup>[151]</sup> and RA receptor responder 1 (RARR1)<sup>[152]</sup> since RA represses invasion and SC phenotype by induction of metastasis suppressors RARR1 and latexin (LXN) in PCa<sup>[153]</sup>.

Retinoids are used as cancer treatment, in part due to their ability to induce differentiation and arrest proliferation. In the clinic, RA has been clinically investigated in PCa as single treatment<sup>[154]</sup>, or with other agents in attempts to produce synergistic effects<sup>[155-157]</sup>. However, delivery of retinoids presents a challenge because of the rapid metabolism and the epigenetic alterations that can render cells retinoid resistant<sup>[158]</sup>. This poses new challenges rather than solutions. ALDH1A3 expression is regulated by many factors and is linked to many metabolic pathways including glycolysis and retinoid signalling, which has been recently reviewed<sup>[87]</sup> and hence not further discussed here.

The relationship between AR and ALDH1A3 has been studied in both normal and tumour tissues, to understand the exact mechanism of their interaction, and its relationship to the role of ALDH1A3 as a marker of CSCs in several tumour types. In breast cancer, a potential link between ALDH1A3 expression and RA signalling contributed to an increase in the rate of cancer progression<sup>[159]</sup>. In human epidermal



**Figure 1:** Aldehyde dehydrogenases (ALDHs) expression and function in the tumour microenvironment. ALDH expression in cancer stem cells (CSCs) and differentiated cells have been linked with several cellular processes including glycolysis/glucogenesis and amino acid metabolism, which are likely to be affected by the local microenvironment including impact by hypoxia (A, B). Various ALDH isoforms have been shown to be regulated by e.g. tumour suppressor genes, oncogenes and microRNAs, however a well-documented functional role is linked with the retinoic acid (RA) pathway resulting in transcriptional activation of a number of genes important in cell differentiation (C). High ALDH expression is frequently used as an endogenous marker that in combination with cell surface markers can be used to isolate CSCs (D). More research is required to understand how ALDH activity may contribute to signaling pathways, maintenance of CSCs and contribute to tumour aggressiveness (D, E)

keratinocytes, it has been shown that regulation of RA metabolism involved the transcriptional activation of only ALDH1A3 amongst a panel of ALDH genes<sup>[160]</sup>. ALDH1A3 activity induced by RA-regulated genes has been proposed to play a role in establishing a unique transcriptional profile that favours the CSC phenotype<sup>[161,162]</sup>. Conversely, a recent study revealed that ALDH1A1, 1A2 and 1A3, were downregulated in the undifferentiated embryonal cancer Wilms' tumour 1 (WT1) resulting in inhibition of RA synthesis<sup>[163]</sup>.

Blum *et al.*<sup>[164]</sup> investigated the regulation of both RA and ALDH1A3 in the urogenital sinus epithelium (UGE), which contains primitive foetal prostate cells. A number of the major regulators of the RA receptor, including ALDH1A3 were up-regulated in both primitive populations of adult and foetal prostate SCs, with 10-fold increased ALDH activity in adult prostate SCs compared to cell population (Sca-1Neg) with no regenerative potential. In addition, expression of CRABP, which transports RA into the nucleus to bind

RA receptors was 47-fold up-regulated in the UGE, as confirmed by qPCR analysis, and may indicate the potential of these cells to differentiate. In the context of PCa, ALDH1A3 might play a significant role in the CSC niche of the TME, thereby contributing to a survival mechanism.

## THE CSC NICHE, SIGNALLING PATHWAYS AND POTENTIAL FOR THERAPEUTIC INTERVENTION

Cancer cells acquire a more invasive and migratory phenotype through EMT<sup>[165-168]</sup>. Cell adhesion is reduced in early metastatic PCa by downregulation of expression of E-cadherin and  $\beta$ -catenin (characteristically expressed in normal epithelial cells)<sup>[169]</sup>. In contrast, the expression of N-cadherin (characteristically expressed in mesenchymal cells) is upregulated<sup>[170]</sup>. In clinical specimens there is lower E-cadherin and  $\beta$ -catenin expression and higher N-cadherin expression in higher grade PCa compared to lower grade PCa<sup>[171-174]</sup>. However restoration of elevated E-cadherin expression and  $\beta$ -catenin was seen in metastatic cells deposited in the bone<sup>[175]</sup>, implicating expression control rather than total E-cadherin gene loss.

The Wnt/ $\beta$ -catenin signalling pathway plays a significant role in maintaining the stemness of PCa<sup>[176,177]</sup>. In radioresistant ALDH+ (identified by Aldefluor assay) prostate progenitor cells, activation of EMT and the Wnt/ $\beta$ -catenin signalling pathways has been demonstrated. In this study, ALDH1A1 gene expression was regulated by the Wnt signalling pathway and correlated with simultaneous expression of  $\beta$ -catenin in whole prostate tumour specimens<sup>[178]</sup>. Encouragingly, inhibition of the Wnt pathway (by siRNA knockdown or the tankyrase inhibitor XAV939) resulted in reduced ALDH+ tumour progenitor population and radio-sensitisation of cancer cells<sup>[178]</sup>. The link between ALDH1A1 and  $\beta$ -catenin has also been demonstrated using spheroidal aggregates in a xenograft model comprised of ovarian cancer cells with stem cell characteristics<sup>[179]</sup>. In this study,  $\beta$ -catenin knockdown decreased ALDH1A1 expression, which subsequently led to inhibition of tumour growth and metastasis.

As described above, ALDH7A1 is highly expressed in primary PCa tissue<sup>[15,88]</sup>. ALDH7A1 knockdown decreased the stem/progenitor cell subpopulation in the human PCa cells and tumour migration ability *in vitro*<sup>[88]</sup>. The activity was correlated with increased TGF- $\beta$  signalling, which strongly induced ALDH7A1

activity while the activity could be inhibited with a TGF- $\beta$  signalling antagonist<sup>[88]</sup>. Overexpression of the TGF- $\beta$  signalling pathway correlates with poor clinical outcomes in PCa. TGF- $\beta$  promotes tumour progression by stimulating the metastasis and angiogenesis<sup>[180]</sup>.

As with many other studies, investigation of ALDH+ cells isolated from both PCa cell lines and primary cells have shown self-renewal, colony forming capacity and tumorigenicity. ALDH expression correlated with CD44 and  $\alpha\beta$ 1-integrin expression as well as phosphorylation of the transcription factor STAT3. Galiellalactone, a potent and specific inhibitor of STAT3 signalling, reduced ALDH1A1 expression and subpopulation of ALDH+ cells following treatment of DU145 PCa xenografts. This study highlighted the role of the STAT3 signalling pathway in putative prostate CSCs and further supports STAT3 as a potential therapeutic target<sup>[181]</sup>. In a separate study using primary tumour cells, STAT3 inhibition resulted in both cell death and CSC differentiation, resulting in a loss of both colony forming and tumour initiating capacity<sup>[182]</sup>.

## ALDH ASSOCIATED DRUG RESISTANCE IN THE TME

A number of studies have linked ALDH expression with chemotherapy resistance, although the underlying mechanisms are not well understood. Whilst chemotherapy reduces the bulk of a tumour, it also enriches the previously described CSC population<sup>[183-185]</sup> which are not susceptible to anti-mitotic drugs currently approved for clinical use. Although evidence is not available in PCa, CSCs have been shown to be highly resistant to both radiotherapy and chemotherapies including temozolomide, gemcitabine, etoposide, carboplatin, paclitaxel, fluorouracil, mitoxantrone, daunorubicin and cyclophosphamide (CPA)<sup>[186-200]</sup>, contributing to tumour recurrence and metastasis. There are several possible mechanisms for CSC resistance to cancer therapy. Firstly, CSCs are slow-proliferating cells in a quiescent state and thus resist drugs primarily designed to target rapidly dividing cells<sup>[201]</sup>. Secondly, CSCs resist irradiation because of increased activation of the DNA damage checkpoint response, as exemplified in a recent study of glioblastoma CSCs<sup>[202]</sup>. Thirdly, increased expression of ABC transporters protects CSCs from high concentrations of drugs<sup>[203]</sup>, as demonstrated by removal of Hoechst stain in analysis of side populations<sup>[204,205]</sup>. Lastly, high ALDH expression is likely linked to metabolic and detoxifying mechanisms, supporting a role as chemo-protecting enzymes<sup>[201]</sup>.

Early studies first demonstrated a chemo-resistant role for ALDHs in a CPA resistant L1210 leukaemia cell line<sup>[206]</sup>. This study showed that high levels of ALDH activity were found in L1210 cells and that treatment with disulfiram (ALDH inhibitor) reversed the resistance phenotype of the cells to CPA. A subsequent study confirmed the role of ALDH-mediated CPA resistance in medulloblastoma<sup>[207]</sup>. Similar studies demonstrated that high ALDH activity indicates CPA resistance in cancer and CSCs<sup>[208]</sup>. Accordingly, inhibition of ALDH activity can in principle serve to sensitise CSCs to drugs such as CPA<sup>[209]</sup>. More specifically, ALDH1A1 and ALDH3A1 were both shown to inactivate CPA analogues<sup>[210,211]</sup>.

The sphere forming cells (a common property of CSCs), from the sarcoma cell line MG63 were significantly insensitive to doxorubicin and cisplatin treatment compared with monolayer adherent counterparts. The sarcosphere cells with high ALDH1 activity were proposed as candidate sarcoma SCs, in which efficient drug detoxification is likely to have contributed to generation of a chemo-resistant CSC phenotype<sup>[191]</sup>. Furthermore, high ALDH expression in CSCs has shown chemo-resistance in both breast CSCs<sup>[190,212]</sup> and head and neck squamous cell carcinoma (HNSCC) SCs<sup>[213]</sup>, where ALDH expression was associated with high Snail expression, a marker of EMT. Knockdown of Snail expression significantly decreased the expression of ALDH1 whilst blocking the tumorigenic abilities of CD44+ CD24- ALDH1+ cells<sup>[213]</sup>. Although many chemotherapeutic drugs are less effective in ALDH-expressing cancer cells, the underlying mechanisms are poorly understood. None of the drugs contain aldehyde functional groups that are direct substrates for biochemical reactions with ALDHs, but esterase activity has been shown for some of these enzymes, which potentially provides an ALDH mediated resistance mechanism for drugs such as the taxanes. Phase 1 metabolism resulting in short lived aldehydes as illustrated for CPA are direct substrates for ALDH detoxification, providing a potential resistance mechanism in ALDH+ expression cells including CSC population within the TME [Figure 2]. Drug resistance can be reversed by co-treatment with an ALDH inhibitor such as DEAB. For example, doxorubicin, paclitaxel and radiotherapy resistance in breast cancer cell lines has been reversed following treatment with DEAB or RA<sup>[190]</sup>.

## ALDH, HYPOXIA AND TME

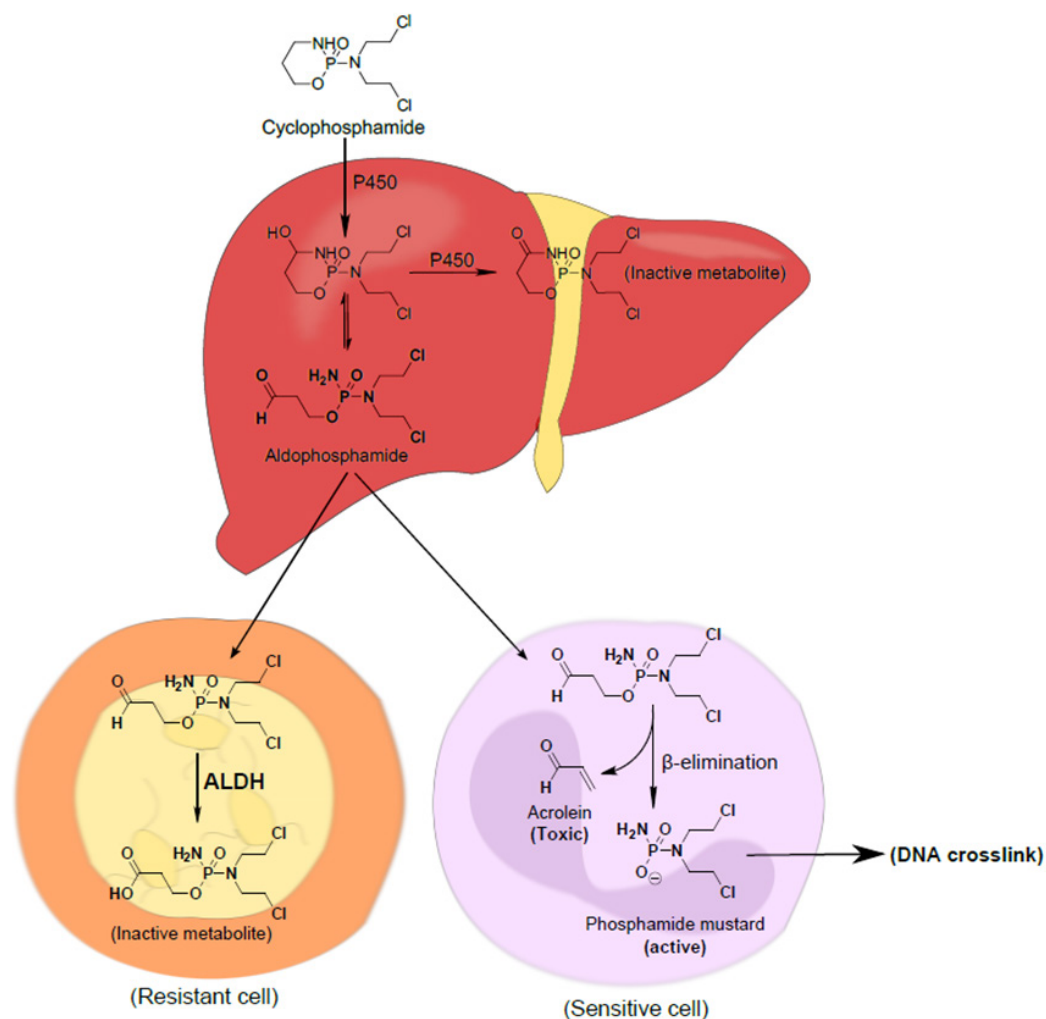
Hypoxia is not only a major feature of the tumour microenvironment but is also a potential contributor

to the multidrug resistance (MDR) and enhanced tumourigenicity of CSCs<sup>[214]</sup>. Within the proposed hypoxic CSC niche, the cells are surrounded by an acidic microenvironment that activates a subset of metastasis promoting proteases such as MMPs and cathepsins<sup>[215]</sup>. As a consequence of poor angiogenesis and the inaccessible location, hypoxic cells are exposed to insufficient drug concentrations, which promote the survival of a drug-resistant sub-population of cells. The lower oxygen tension increases resistance to radiotherapy and as discussed above, also enriches CSC niche within the TME. Hypoxia-activated prodrugs (HAPs) have been investigated for several decades and have shown considerable promise in combination with chemotherapy or radiotherapy, but no HAPs have yet been approved for clinical use. Unravelling the PCa microenvironment is likely to offer new insight and opportunities to molecularly stratify patients for treatment, based on their tumours' hypoxic signature, including analysis of enzymes with oxidase and/or reductase functionality. Prostate tumours are considerably hypoxic as discussed in this thematic issue<sup>[216]</sup> and enzymes such as ALDHs are likely to be expressed differentially within the TME due to different pressures including hypoxic stress and types of cells such as MDR and CSCs.

The limited sensitivity of hypoxic tumours to radiotherapy may in part be related to CSCs residing in the hypoxic niche. Primary human PCa samples express both elevated levels of ALDH1A1+ and hypoxia inducible factor 1 alpha (HIF-1α), which have been linked to radioresistance<sup>[217,218]</sup>. A recent study<sup>[219]</sup> demonstrated that irradiation enriched the CSC population of DU145 and PC-3 cells. The irradiated cells were shown to possess elevated ALDH functional activity as well as DNA damage response activity, and *in vivo* the irradiated ALDH+ cells were shown to maintain their tumorigenic properties, suggesting these might be radioresistant *in vivo*. Furthermore, in primary human prostate tumours, IHC analysis revealed co-localisation of ALDH1A1 and HIF-1α expression, implying that a subset of ALDH+ cells resides in the hypoxic niche and emphasising the need to target these to effectively eradicate heterogeneous prostate tumours.

In other tumours, for example radiation resistant mesenchymal glioma, the SCs (MGSCs) possess elevated glycolytic activity and ALDH activity, in contrast to benign proneural SCs. Expression of ALDH1A3 was increased in clinical high-grade glioma compared with low-grade glioma or normal brain tissue<sup>[220]</sup>. Encouragingly, although the MGSCs were very aggressive *in vitro* and *in vivo*, the pan-





**Figure 2:** Cytochrome P450 (CYP) activation of cyclophosphamide (CPA). Initial hydroxylation of CPA in the liver by CYP isoforms leads to generation of aldophosphamide, an intermediate which is a substrate for aldehyde dehydrogenases (ALDHs) metabolism. If aldophosphamide enters circulation it is very likely to be detoxified in ALDH-expressing cells including cancer stem cells (CSCs), but not in cancer cells with low or absent ALDH expression

ALDH inhibitor DEAB significantly reduced cellular proliferation *in vitro*. This investigation suggested that two subtypes of MGSCs, harbouring distinct metabolic signaling pathways, constitute intratumoural glioma heterogeneity. ALDH1A3 was proposed to play an important role in the glycolysis pathway, via catalytic metabolism of acetaldehyde to acetate that is in turn linked to the tricarboxylic acid (TCA) cycle<sup>[220]</sup>. The glycolysis pathway is interesting because of the link to the TME and what is defined as the “Warburg effect”. A recent study<sup>[221]</sup> reported on the mitochondrial pyruvate carrier 1 (MPC1) gene in knockout studies using CRISPR/Cas9 technology in RM-1 murine PCA cells. The MPC1 gene knockout cells revealed a metabolism reprogramming to aerobic glycolysis with reduced ATP production, increase in cell migration and resistance to both chemo- and radiotherapy. In addition, the MPC1 knockout cells expressed

significantly higher levels of the stemness markers Nanog, HIF-1 $\alpha$ , Notch1, CD44 and ALDH.

The latter study provides an alternative route for therapeutic intervention, focussed on reprogramming glycolytic pathways. ALDHs such as the 1A3 isoform could be a key player in such therapeutic intervention. However, as we<sup>[45]</sup> and others<sup>[46,87,222]</sup> have discussed previously, the expression of ALDHs in normal tissue expression remain a stumbling block towards a credible clinical therapy. However, advances in drug delivery technologies could in the future enable administration of an ALDH inhibitor, which is potentially selective for a specific isoform. For example, a recent report<sup>[223]</sup> indicate that the latter might be achieved in combination with radiotherapy, or as an option to sensitise heterogeneous prostate tumour responses to docetaxel.



## CONCLUDING REMARKS

The number of papers that report ALDH expression in the context of cancer is largely attributable to the use of the Aldefluor assay as a means to identify and isolate subpopulations with particularly stemness characteristics. However, selected ALDH isoforms are also emerging as critical players in chemo- and radioresistance and a signature of tumour aggressiveness in conjunction with cells capable of migration, invasion and metastasis. Still, as is clear from this review of ALDH expression and function in PCa and other recent reviews<sup>[45,46,87,222]</sup>, the ever increasing number of publications that reveal inconsistent and sometimes contradictory information is not helpful in clarifying ALDHs as potential biomarkers of specific cancer types or CSC population; e.g., many early studies that reported on ALDHs, utilised antibodies that only stained for e.g. ALDH1 but were not selective for 1A1, 1A2, 1A3, 1B1, 1L1 or 1L2. Equally the Aldefluor assay is not isoform-selective and has contributed to inefficient validation of these enzymes. Furthermore, previous studies were carried out when the understanding of cancer cell subtypes, and the involvement of TME was limited, resulting in incomplete ALDH profiling. Bearing this in mind, currently emerging evidence in PCa suggests the dominant isoforms are ALDH1A1, 1A2, 1A3, 3A1 and 7A1. The expression and function have been demonstrated using a number of different 2D and 3D cancer models as well as clinical samples. Further investigations of these isoforms are required in order to fully validate their potential as biomarkers or targets for therapeutic intervention. Such investigations should take better account on our choices of models as argued by Maitland in accompanying review<sup>[224]</sup> in this thematic issue. As discussed in this review, ALDH enzymes also play a functional role in CSC populations, in the context of the TME. This synergy will be important in future studies to dissect whether ALDH expression leads to drug resistance via direct or indirect mechanisms. Underpinning the role of the RA signalling pathways, and the glycolytic biochemical pathways associated with the Warburg effect form part of both a regulatory network and a vicious cycle of tumour aggressiveness. The TME no doubt plays a critical role in exerting this selective pressure on ALDH expression and function, and hence should be more carefully considered in unravelling the cellular roles for specific ALDH isoforms. In this regard, use of siRNA, CRISPR and the development of highly specific small molecules to probe ALDH function will enable us more quickly ascertain the importance of specific ALDHs.

## DECLARATIONS

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### Author's contributions

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The authors declare there are no conflicts of interest.

### Ethical approval and consent to participate

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Topic: How does the prostate cancer microenvironment affect the metastatic process and/or treatment outcome?

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# Introduction of the special issue “How does the prostate cancer microenvironment affect the metastatic process and/or treatment outcome?”

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It is with great pleasure that we introduce this special issue titled “How does the prostate cancer microenvironment affect the metastatic process and/or treatment outcome?”. Within this issue we, and our fellow authors, explore the role of the prostate cancer microenvironment in tumour metastasis and treatment outcome. Global statistics reveal that prostate cancer is the second most common form of cancer and is attributable to fifth of all cancer-related deaths to affect men worldwide. Whilst rates of disease incidence appear to be increasing a high proportion of men diagnosed with disease will survive for ten or more years. Yet, for some men, the disease is far more aggressive, resulting in tumour metastasis and failed response to treatment. Improving our knowledge of the prostate cancer microenvironment will undoubtedly lead to opportunities for providing better treatment options for patients with aggressive forms of this disease. To address this gap in knowledge, we here

present nine articles detailing the current state of art regarding development of aggressive prostate cancer.

Current methods of prostate cancer diagnosis lack both sensitivity and specificity. This can result in failed diagnosis of men likely to develop aggressive disease and over-treatment of men who do not require treatment who consequently suffer as a result. It is clear that if we are to improve the current paradigm clinicians and scientists must work together. The first and the last articles in this special issue, written by Mason<sup>[1]</sup> and Maitland<sup>[2]</sup>, explores how to improve on preclinical models and collaboration between scientists and clinicians in combined efforts towards improving the quality of lives of patients living with prostate cancer.

With our improved understanding of the prostate cancer development, it is clear that our attitudes towards cancer diagnosis and therapeutic intervention need to evolve



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also. Researchers require better models that more accurately represent the tumour microenvironment, but this requires collaboration between basic scientists and clinicians. Only by working together can we truly hope to develop personalised, effective, cancer therapies and therefore improve the survival of men with prostate cancer. The two commentaries by Mason<sup>[1]</sup> and Maitland<sup>[2]</sup> encapsulate seven in-depth reviews, which are focussed on key aspects that contribute to prostate cancer aggressiveness.

Perhaps one of the most studied aspects of prostate cancer is the role of androgens. The prostate requires androgens, a family of hormones including testosterone, which interact with the androgen receptor (AR) to regulate normal physiological function. Aberrant androgen signalling, however, can drive the formation and growth of prostatic tumours. Androgen deprivation therapy therefore remains a pivotal treatment for prostate cancer. Abiraterone and enzalutamide are two such therapeutic agents used to treat prostate cancer. Regrettably, tumours can become resistant to such therapies, and there is urgent need for new therapies to improve patient survival. Pippione *et al.*<sup>[3]</sup> review the androgen-AR axis and the potential targeting of steroidogenic enzymes as a means of overcoming resistance to existing prostate cancer treatments.

Cancer progression is associated with a dysregulated balance between cellular growth, division, and cell death. Such processes are regulated by transcription factors that work alone, or in combination with other proteins, to regulate genetic expression. One family of transcription factors, the HOX proteins, have been shown to contribute to interactions between prostate tumours and the surrounding microenvironment. Morgan and Pandha<sup>[4]</sup> review the numerous roles of HOX proteins in prostate cancer, ranging from regulation of androgen-receptor sensitivity to angiogenesis and tumour metastasis.

Tumour growth within the prostate, and other sites, requires remodelling of the surrounding environment. Matrix metalloproteinases (MMPs), a family of proteolytic enzymes, have long been associated with regulation of the extracellular matrix and tissue remodelling. They have also been implicated in the initiation, progression and metastasis of multiple cancer types, including prostate cancer. The MMP family is composed of two broad sub-groups, the soluble or secreted MMPs and the membrane-type MMPs (MT-MMPs). Whilst many studies have focussed on the soluble MMPs, the expression and roles of MT-MMPs remain less clear and form the subject of

review by Falconer and Loadman<sup>[5]</sup>. Furthermore, the authors consider MT-MMP expression and proteolytic capacity in the design of potential new therapies against metastatic prostate cancer.

Cell to cell communication plays a key role in the development of prostate cancers. As is evident from numerous articles within this special issue, such communication occurs not only between cancer cells but also between all cells within the tumour microenvironment, resulting in changes to the local environment that favour tumour growth and metastasis. Historically, many studies have focussed on soluble growth factors and cytokines, but there is emerging evidence highlighting the role of extracellular vesicles. Such vesicles can be divided into two broad sub-categories, microvesicles and exosomes. Whilst there have been many studies exploring the role of extracellular vesicles in cancer, relatively few of these studies have focussed on prostate cancer. Shephard *et al.*<sup>[6]</sup> review the reported functions of extracellular vesicles from diverse malignancies to identify those with potential relevance to prostate cancer. It is clear that extracellular vesicles represent a means of delivering a complex assortment of factors from one cell to another, actively contributing to the disease progress. Such vesicles also represent an attractive source of biomarkers for both diagnostic and prognostic purposes.

Despite the wealth of knowledge on prostate cancer and potential therapeutic interventions there remain many unanswered questions and challenges to address. One such area is that of tumour heterogeneity. Heterogeneity within prostatic tumours is a complex topic and forms the basis of an original research article by Frame *et al.*<sup>[7]</sup>. When we think of heterogeneity we should not only consider patient variability, but also differences between individual tumours and sub-populations of cells within a tumour. Not only do the authors consider how to tackle tumour heterogeneity, but they introduce a new model for assessing drug response that takes into account this heterogeneity. As an extension of tumour heterogeneity, McKenna *et al.*<sup>[8]</sup> discusses how the presence of hypoxia, which is well-known to contribute to cancer aggressiveness and resistance to chemo- and radiotherapy, can be turned into an opportunity for rationalised drug discovery and combination therapy while Ibrahim *et al.*<sup>[9]</sup> discusses how tumour-initiating cells with high expression of aldehyde dehydrogenase contribute to treatment resistance and tumour recurrence.

We would like to thank all of the contributing authors for their hard work in producing the enclosed articles.



We hope you enjoy this special issue of the Journal of Cancer Metastasis and Treatment.

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The authors declare there are no conflicts of interest.

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Original Article

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# Increased ARF6 activation correlates with HGF stimulation in non-invasive prostate cancer cells

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## Abstract

**Aim:** The effects of hepatocyte growth factor (HGF) on a non-invasive prostate cancer cell line (CAHPV-10), expressing cMET were studied, to mimic the possible effects neo-adjuvant androgen deprivation therapy may have in promoting tumour progression.

**Methods:** Prostate epithelial cells and prostate cancer cells derived from cancer metastatic sites were analysed using cell culture assays, immunofluorescence, quantitative real-time polymerase chain reaction and western blotting, with or without HGF stimulation.

**Results:** HGF significantly enhanced cell proliferation and induced cell scattering and invasion in CAHPV-10 cells compared to untreated controls. Active adenosine diphosphate-ribosylation factor 6 (ARF6) was found to be present in all metastatic prostate cancer cells, with levels highest in the most aggressive cell line, PC-3. Following stimulation with HGF, active ARF6 expression was substantially elevated in CAHPV-10 cells.

**Conclusion:** These findings provide further molecular insight into the progression of prostate cancer and highlights potential issues for early prostate cancer therapeutic strategies.

**Keywords:** Prostate cancer, adenosine diphosphate-ribosylation factor 6, hepatocyte growth factor stimulation, androgen deprivation therapy



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## INTRODUCTION

Prostate cancer (PCa) is the most common cancer in men in the UK. Every year more than 35,000 cases of PCa are diagnosed, which equates to one man every 15 min and accounts for approximately 12% of all male deaths from cancer in the UK<sup>[1]</sup>. Androgens play a significant role in the growth and progression of PCa. The current therapy for locally advanced and metastatic disease is androgen deprivation therapy (ADT) either through orchidectomy, luteinising hormone releasing hormone or blockade through the androgen receptor (AR). For men with localised PCa, the two major treatment options are surgery by radical prostatectomy or radiotherapy. However, ADT is becoming increasingly important in the early stages<sup>[2]</sup>. ADT can be given in the neoadjuvant setting to reduce tumour size for improved excision during surgery or increase the effectiveness of radiotherapy<sup>[3]</sup>. ADT can also be given for men with localised PCa who are unfit for curative treatment (surgery or radiotherapy) or whose cancer has begun to progress and become symptomatic<sup>[4]</sup>.

However, several studies have shown that following androgen suppression, expression of hepatocyte growth factor (HGF) or its receptor c-Met are elevated in prostate tissues<sup>[5,6]</sup>. Additional studies have also shown that suppression of the AR increases cMet expression in PCa cell lines<sup>[7,8]</sup> while increased c-Met expression is induced by removal of androgens in PCa cells<sup>[8]</sup>. HGF is a multifunctional cytokine, which in the prostate is secreted by prostate stromal cells and activates c-Met in a paracrine manner<sup>[9]</sup>. It is widely accepted that HGF dependent c-Met activation is involved in tumour development, including PCa, by inducing cell proliferation<sup>[10]</sup> and activating stages of the metastatic cascade by stimulating migration<sup>[10,11]</sup>, cell scattering<sup>[12]</sup>, invasion<sup>[13]</sup> and angiogenesis<sup>[14]</sup>.

It has been reported that serum levels of HGF correlate with stage of prostate malignancy. Thus, HGF serum levels are higher in men with localised PCa compared to healthy controls and further elevated in men with metastatic PCa compared to localised disease<sup>[15-17]</sup>. Plasma levels of HGF have also been documented as predicting PCa metastasis to the lymph nodes as well as recurrence following surgery<sup>[15]</sup>. Additionally, while c-Met expression is linked to disease progression<sup>[5,18,19]</sup> elevated c-Met has been documented in localised PCa tissue when compared to healthy controls<sup>[20]</sup>. With the importance of HGF and c-Met in PCa well documented, it has been hypothesised that while current ADT potentially inhibits AR mediated cell proliferation and survival, it could also abolish its suppressive role on the HGF/c-Met pathway<sup>[7]</sup> and therefore may unintentionally drive tumour progression. In addition, while there are a plethora of studies evaluating the effects of HGF in PCa<sup>[21-24]</sup>, the majority focus on metastatic cell lines and hence advanced cancer. We, however, have sought to investigate the effect of HGF on a non-invasive cell line called CAHPV-10, known to express cMET<sup>[25]</sup> with the aim of ascertaining the molecular effects that may result due to ADT therapy on early state PCa and its role in promoting tumour progression and metastasis.

Furthermore, HGF has been shown to activate adenosine diphosphate-ribosylation factor 6 (ARF6), which is a member of the Ras superfamily of GTPases<sup>[26,27]</sup>. Increased levels of activated ARF6 [ARF6-guanosine triphosphate (ARF6-GTP)] have been found to increase the invasive capacity of melanoma cells both *in vitro*<sup>[28]</sup> and *in vivo*<sup>[29]</sup>, while silencing ARF6, by small-interfering RNA, has been shown to inhibit the ability of breast cancer cells to invade through an artificial basement membrane<sup>[30]</sup>. While we have recently shown that ARF proteins are over-expressed in PCa tissue compared to normal control tissue<sup>[31]</sup>, published studies on the presence of ARF6 in PCa are scant. The aims of the present investigation were to determine 1) if ARF6 is up-regulated in invasive PCa cells; 2) to investigate whether HGF stimulation correlates with active ARF6 expression in cells derived from non-invasive PCa.

## METHODS

### Cell culture

Prostate epithelial cells (PNT2) and PCa cells derived from cancer metastasis to the lymph nodes (LNCaP) and bone (PC-3) were obtained from the European Collection of Cell Cultures. PCa cells derived from non-

invasive prostate cancer (CAHPV-10) and metastatic cancer to the brain (DU145) were purchased from the American Type Culture Collection. The cell lines were routinely cultured as described in Morgan *et al.*<sup>[32]</sup>.

### Gene expression analysis for ARF6

Gene expression analysis was carried out as described in Morgan *et al.*<sup>[32]</sup> and the following ARF6 primers were used: 5'-TGTGGGTTTCAACGTGGAGAC-3' and 5'-CAGTGTAGTAATGCCGCCAGAG-3'.  $\beta$ -actin and HPRT were used as reference genes, therefore the  $\beta$ -actin primers used were 5'-GATGGCCACGGCTGCTTC-3' and 5'-TGCCTCAGGGCAGCGGAA-3'. HPRT primers used were 5'-GACTGTAGATTTTATCAGACTGA-3' and 5'-TGGATTATACTGCCTGACCAA-3'.

### Western blot analysis

RIPA buffer (Sigma Aldrich, Dorset, UK) was used to extract the total protein, of which 30  $\mu$ g was run a 12% tris-glycine PAGE gels (Bio-Rad Laboratories, Hemel Hempstead, UK). The western blot was carried out as described in Morgan *et al.*<sup>[32]</sup>.

Primary mouse antibody to ARF6 (1:1000), as well as horseradish peroxidase conjugated secondary anti-mouse were used (1:1000) (supplied in the Active ARF6 pull down and detection.  $\beta$ -actin (1:1000) (New England Biolabs, Hertfordshire, UK) was a control for protein loading.

### Immunofluorescence for localisation of ARF6

All prostate cells were seeded at  $1 \times 10^5$  cells/mL onto sterile microscope slides. Slide preparation was carried out as previously described by Morgan *et al.*<sup>[32]</sup>. Immunofluorescence of ARF6 expression was performed by first incubating the slides with rabbit anti-ARF6 (1:1000 in 6% BSA/PBS) (abcam, Cambridge Science Park, Cambridge, UK) for 2 h at 37 °C. Following the washing steps to remove the primary antibody, secondary antibody anti-rabbit (Qdot525 Invitrogen, Paisley UK) (1:100 in 6% BSA/PBS) was added and the sample incubated for 1 h at room temperature. The slides were again washed in water and fixed in ethanol (70%, 85% and 95%) for 2 min each. The AxioCam fluorescent microscope (Carl Zeiss Ltd, Hertfordshire, UK) was then used to analyse the slides. Negative controls (to rule out autofluorescence) were carried out by substituting the primary antibody for 1% BSA/PBS.

### GST pull down assay for activated ARF6

All prostate cells were seeded in a T-175 cm<sup>3</sup> flask and grown to 80% confluence. Total protein was extracted and ARF6-GTP isolated using the Active ARF6 pull down and detection kit (Thermo Scientific Langensfeld, Germany) according to manufacturer's instructions. Briefly, cells were lysed using 1 mL lysis buffer and agitated on ice for 5 min before being scraped into a 2 mL tube and sheared through a 20 gauge needle 5 times and centrifuged at 16,000 g at 4 °C for 15 min.

The supernatant containing the total lysate was transferred to a new tube. An aliquot of the cell lysate was reserved for protein quantification using the Pierce BCA (Thermo Scientific, Langensfeld, Germany) and for detection of total ARF6 by western blot analysis.

For detection of activated ARF6, the remaining lysate was added to a spin column containing GST-GGA3-beads and incubated for 1 h at 4 °C with gentle rocking. The column was then centrifuged at 6000 g for 10-30 s and washed with 400  $\mu$ L of Lysis/Binding/Wash Buffer before centrifugation for a second time at 6000 g for 10-30 s. Twenty five microlitres of the elute (ARF6-GTP) and 25  $\mu$ L of total protein lysate (total ARF6) were used for western blot analysis, as described above and experiments were performed in triplicate.

### HGF induced cell proliferation

CAHPV-10 cells were seeded at 2000 cells/100  $\mu$ L in 96 well plates and incubated at 37 °C for 24 h to allow for cell adherence and growth. Cells were then washed twice in phosphate buffered saline (PBS) (Invitrogen,

Paisley UK) and the cells grown for a further 24 h in serum starved medium (0.5% FCS). Hepatocyte growth factor (R&D systems, Minneapolis, USA) was then added to the cells at 10, 25, 50 and 100 ng/mL for 24 and 48 h. PBS/0.1 BSA (Bovine Serum Albumen, Sigma Aldrich, Dorset, UK) was used as a negative control. Cell proliferation was assessed using the Cell 96® AQueous One Solution Cell Proliferation Assay (Promega, Southampton, UK) according to the manufacturers' instructions. Four replicates for each HGF concentration were performed and the experiment repeated in triplicate.

### HGF induced cell invasion

The invasive potential of CAHPV-10 cells following HGF treatment were assessed using a commercial cell invasion assay kit (Innocyte Cell Invasion Assay kit, Calbiochem, Merck, Middlesex, UK) utilising 8 µmol/L pore transwell inserts pre-coated with basement membrane extract. Briefly, the basement membrane extract was rehydrated by adding 300-400 µL of warm, serum-free medium for 30-60 min at room temperature. Following incubation the serum free medium was removed and 350 µL of a  $1 \times 10^6$  cell suspension was added to each insert. Five hundred microlitres of medium containing either 10-100 ng/mL HGF or 0.1% BSA/PBS vehicle control were added to both the upper and lower and the chambers of the transwell plate and incubated for 48 h. Following incubation, the inserts were removed and placed into unused 24-well plates containing 500 µL of cell staining solution. Cells that had migrated through the basement membrane to the underside of the insert were then dislodged by tapping the insert against the bottom of the well before incubating for a further 30 min. Following incubation the inserts were removed and the wells containing the dislodged cells was incubated for an additional 30 min. Finally, 200 µL of the dislodged cell suspension were added to triplicate wells of a black 96-well plate (Thermo Scientific Langenselbold, Germany) and the fluorescence measured using an excitation wavelength of 485 nm and an emission wavelength of 520 nm. This experiment was performed in triplicate.

### HGF induced cell scattering

CAHPV-10 and DU145 (positive control) cells were seeded at a density of  $1 \times 10^4$  cells/mL of culture medium in 6-well plates. Cells were incubated until small colonies formed. Cells were then serum starved (0.5% FCS) for 24 h prior to stimulation with 10-100 ng/mL HGF for 24 and 48 h. Changes in cell morphology were observed using a light microscope (Carl Zeiss Ltd, Hertfordshire, UK) and representative colonies were photographed using a Canon Powershot A640. Negative controls using 0.1% BSA/PBS were also performed. The experiment was performed in triplicate.

### HGF stimulation of CAHPV-10 cells for activation of ARF6

It was observed that CAHPV-10 cells, having very low levels of activated ARF6, were used to ascertain whether HGF stimulation could activate ARF6 in a non-invasive cell line. CAHPV10 cells were seeded as above and grown to 80% confluence. Cells were then treated with either 50 ng/mL HGF or 0.1% BSA/PBS vehicle control for 48 h. GST pull down assay was performed as described above.

### Statistical analysis

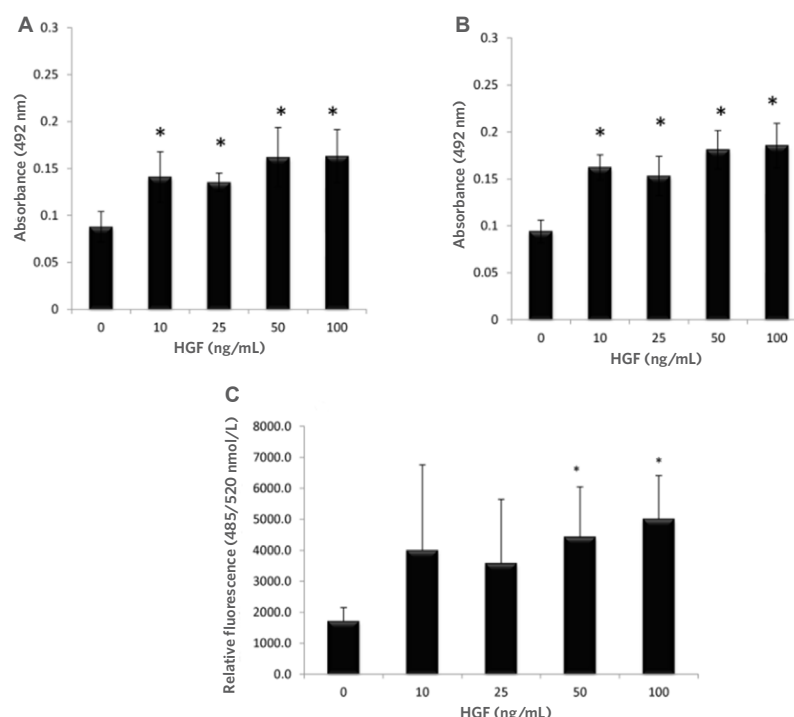
To ascertain if our data was normally distributed a one sample Kolmogorov-Smirnov statistical test was performed. The resulting  $P > 0.05$ , indicated the data had a normal distribution. Parametric analysis was carried out including ANOVA test, Dunnett's post hoc analysis to compare HGF treated with control cells and Tukey post hoc analysis was performed for multiple group comparisons. Statistical significance was considered for  $P < 0.05$ . For the invasion assay, Student T-test was performed and a  $P < 0.05$  was considered significant.

## RESULTS

### HGF stimulation induced cell proliferation and invasion

We wished to assess whether HGF could increase cell proliferation and invasion in a cell line derived from a non-invasive tumour. The results [Figure 1A and B] show PCa cell proliferation was significantly enhanced





**Figure 1.** HGF significantly increased the proliferation of CAHPV-10 non-invasive prostate cancer cells over (A) 24 h and (B) 48 h. CAHPV-10 cells were serum starved for 24 h before exposure to HGF. The data are expressed as mean  $\pm$  standard deviation (SD) (from 4 replicates repeated in triplicate). \*Denotes significant difference between treated and control cells ( $P < 0.05$ ); C: HGF significantly increased the invasive capacity of CAHPV-10 cells. Cells were seeded onto an artificial basement membrane extract in transwell inserts and serum starved for 24 h before treatment with HGF for 48 h. The data are expressed as mean (from triplicate experiments)  $\pm$  SD. \*Denotes significant difference between treated and control cells ( $P < 0.05$ ). HGF: hepatocyte growth factor

by HGF at 24 ( $P < 0.001$ ) and 48 h ( $P < 0.001$ ) when compared to untreated control cells, yet interestingly there was no significant difference between concentrations of HGF or time points, suggesting that the c-Met receptor or downstream signaling pathways have reached saturation point at 10 ng/mL.

It can also be seen from Figure 1C that 48 h HGF stimulation at 50 and 100 ng/mL significantly increased CAHPV-10 invasion through an artificial basement membrane ( $P < 0.042$  and  $P < 0.019$  respectively) with an approximate 2-fold increase in cell numbers crossing the artificial membrane compared to untreated controls. These results show that exposing PCa cells, derived from a localised tumour, to HGF increases not only their proliferation but also their invasive capacity.

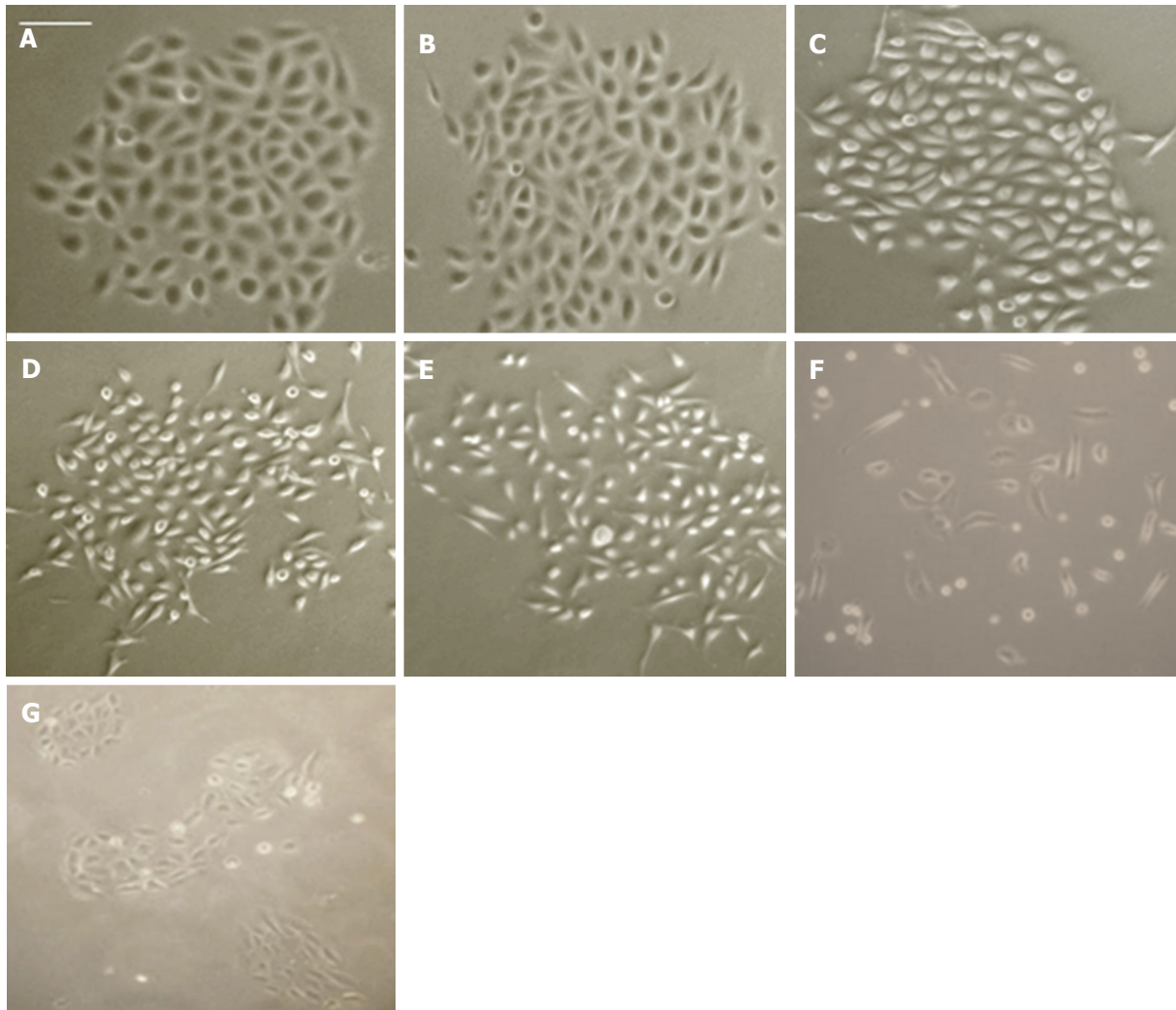
### HGF induced cell scattering in non-invasive cancer cells

To determine whether HGF stimulation could induce cell scattering in a non-invasive cell line, we exposed CAHPV-10 cells (grown in small colonies) to various concentrations of HGF over 24 and 48 h time period. As many studies have shown HGF induce cell scattering in DU145 PCa cells, we also used DU145 cells with and without HGF exposure as a positive and negative controls.

Concentrations of HGF ranged from 0-100 ng/mL. CAHPV-10 cells treated over a 24 h period did not show any significant signs of cell dissociation, spreading or motility. In contrast, following 48 h exposure to HGF, cell dissociation, spreading and motility became evident at 50 and 100 ng/mL HGF [Figure 2].

### ARF6 is expressed in PCa cells

A role for the GTPase ARF6 has been implicated in several cancers but information pertaining to its role in PCa is scant. Gene expression analysis on prostate cells derived from normal prostate epithelium through to aggressive, metastatic disease revealed ARF6 to be present in prostate cells [Figure 3A]. There was no signifi-



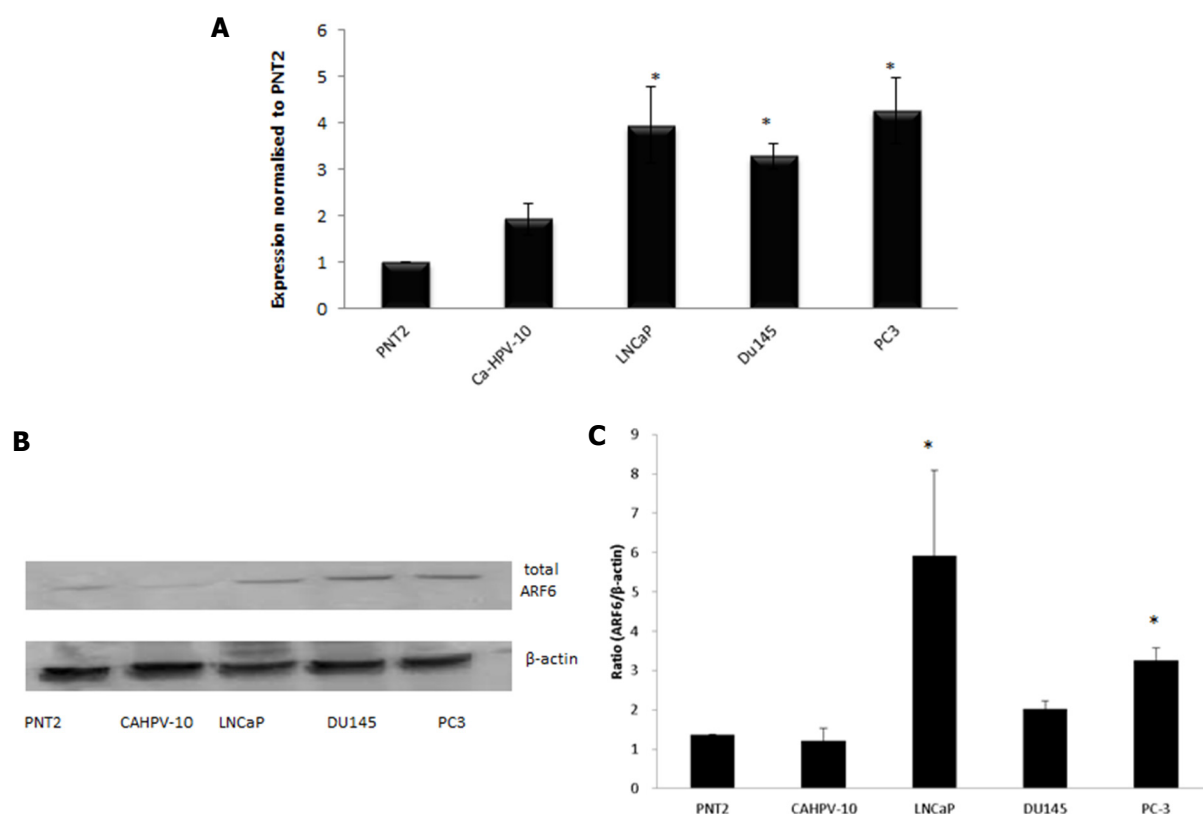
**Figure 2.** CAHPV-10 non-invasive prostate cancer cells treated with (A) control (PBS + medium); B: 10 ng/mL HGF; C: 25 ng/mL HGF; D: 50 ng/mL HGF; E: 100 ng/mL HGF for 48 h; F: DU145 cells treated with 10 ng/mL for 48 h as a positive control; G: DU145 cells without HGF for 48 h as a negative control. Scale bar (shown in A) represents 25× mol/L for (A-C) and 50× mol/L for D-G

cant difference in ARF6 levels when CAHPV-10 were compared to the normal prostate cell line PNT2 but there was a significant difference between non-invasive and invasive cancer cells. ARF6 levels were significantly higher in LNCaP (3.9-fold), DU145 (3.3-fold) and PC-3 (4.3-fold) cells compared to CAHPV-10 ( $P < 0.04$ , 0.032 and 0.009 respectively). There was no significant difference between the invasive cell lines regardless of their differing invasive capacities.

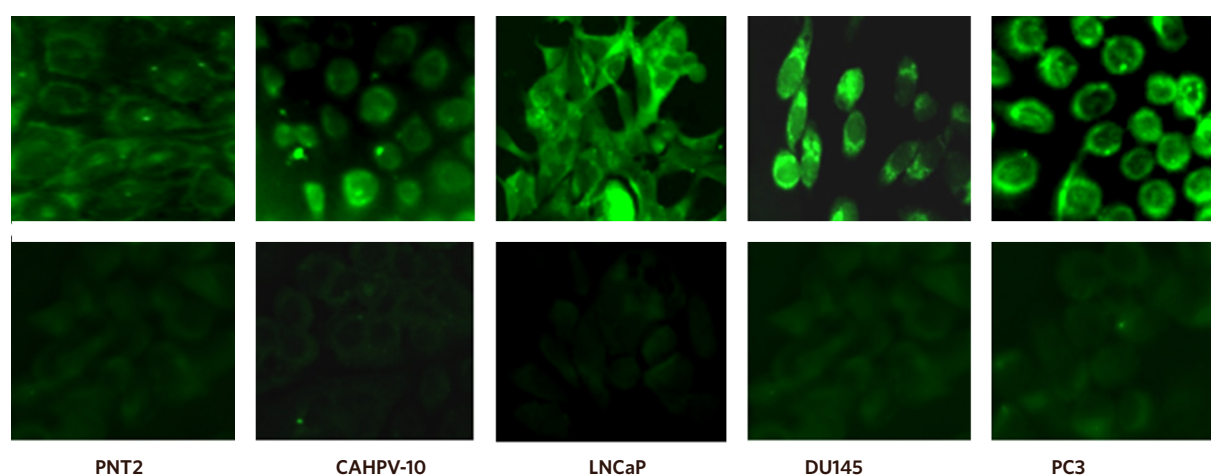
To determine whether mRNA levels were translated through to the protein level, we then performed western blot analysis. Protein bands correlating to ARF6 were evident in all samples [Figure 3A] but densitometry [Figure 3C] revealed total levels of ARF6 were not significantly different between PNT2 (1.37) and CAHPV-10 cells (1.21) yet were significantly increased in the metastatic cell lines LNCaP, 5.92 and PC-3, 3.26 ( $P < 0.001$  and  $P < 0.034$  respectively).

#### *Immunofluorescence shows ARF6 localises to the plasma membrane in aggressive cancer cells*

Inactive ARF6 (ARF-GDP) localises to the cytosol and endosomes and when activated (ARF-GTP) translocates to the plasma membrane. Therefore, we carried out immunofluorescence to ascertain the localisation of ARF6 in all our PCa cells. Figure 4 shows that staining for ARF6 was predominantly localised to the cell

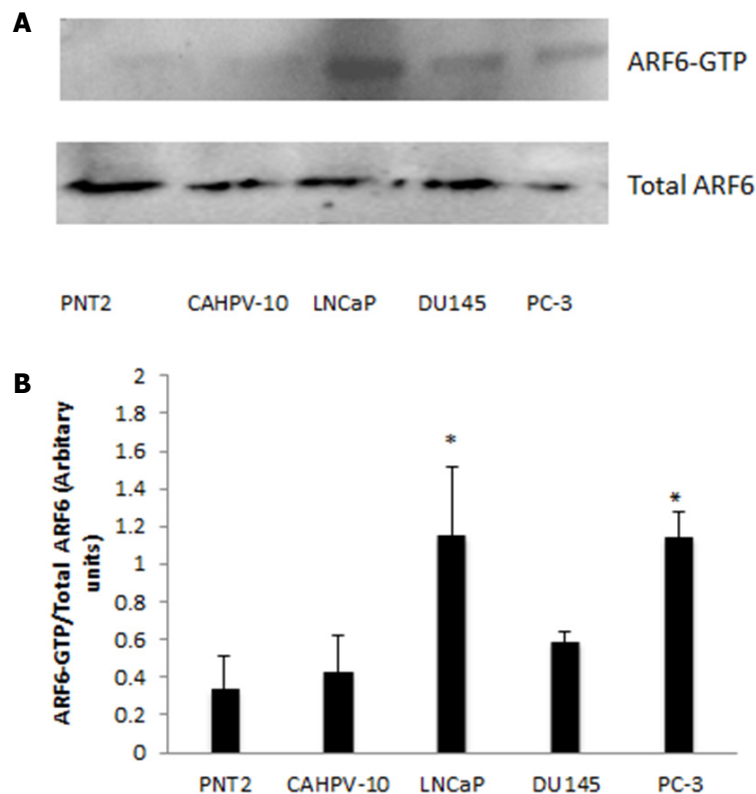


**Figure 3.** A: Gene expression analysis shows ARF6 is expressed in prostate cells derived from normal tissue (PNT2), localised prostate cancer (CAHPV-10), weakly metastatic (LNCAp), moderately (DU145) and aggressively (PC-3) metastatic prostate cancer tissue. ARF6 expression was similar between PNT2 and CAHPV-10. A significant increase in expression was observed in LNCAp, DU145 and PC-3 compared to CAHPV-10. The data are expressed as mean (from triplicate experiments), relative to PNT2,  $\pm$  SD. \*Denotes significant difference compared to PNT2 ( $P < 0.05$ ); B: western blot analysis for total ARF6 protein; C: densitometry data is presented relative to the level of  $\beta$ -actin. The data are expressed as mean (from triplicate experiments)  $\pm$  SD



**Figure 4.** Inactive ARF6 localises to the cytosol and endosomes and when activated translocates to the plasma membrane. Representative immunofluorescence images show cells stained with rabbit anti-ARF6 exhibit a stronger signal intensity at the plasma membrane in the metastatic cell lines (LNCAp, DU145, PC3) than those derived from normal tissue (PNT2) and localised prostate cancer (CAHPV-10). Corresponding negative controls are shown in the bottom row

periphery in all cell types. In PNT2 cells, ARF6 staining intensity was very weak but intensity increased with progression to an aggressive phenotype suggesting higher levels of the activated form of ARF6 is present at



**Figure 5.** A: GST pull down assay for ARF6-GTP was performed on prostate cells from normal prostate (PNT2), localised prostate cancer (CAHPV-10), weakly metastatic (LNCaP), moderately (DU145) and aggressively (PC-3) metastatic prostate cancer. Twenty five microlitres of GST assay elute was used in western blot analysis for ARF6-GTP detection. A: corresponding 25  $\mu$ L aliquot from total protein lysis was used for detection of total ARF6 protein; B: band density was measured by densitometry and the ratios of ARF6-GTP to total ARF6 are shown. The data are expressed as mean (from triplicate experiments)  $\pm$  SD. \*Denotes significant difference compared to PNT2 ( $P < 0.05$ )

the cell membrane in cells with an invasive phenotype compared to cells which are derived from normal prostate epithelium or non-invasive cancer tissue.

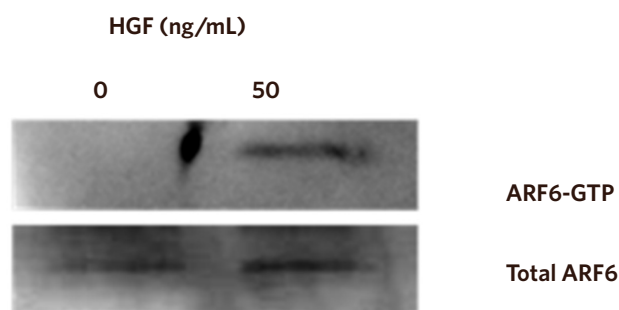
#### *Active ARF6 can be detected in aggressive PCa cells*

To quantify levels of ARF-GTP, we performed a GTP pull down assay, which utilises a fusion protein GST-GGA3 that specifically interacts with the GTP bound form of ARF6. This was performed on all prostate cells representing normal through to aggressive metastatic disease. Western blot analysis of ARF6-GTP and total ARF6 can be seen in [Figure 5A](#), which shows ARF6-GTP to be evident in the invasive cell lines whilst very low levels were observed in the non-invasive cells. Densitometry was performed and a ratio between ARF6-GTP and total levels of ARF6 were calculated. [Figure 5B](#) shows levels of ARF6-GTP were significantly higher in LNCaP ( $P < 0.033$ ) and PC3 ( $P < 0.034$ ) compared to non-cancerous prostate cells (PNT2). Furthermore, ARF6-GTP levels in LNCaP and PC-3 were significantly higher ( $> 2.5$  fold) than in the localised prostate cells CAHPV10 ( $P < 0.044$  and  $0.001$  respectively).

#### **HGF induces the activation of ARF6 in non-invasive cells**

HGF is known to activate ARF6, therefore we wished to determine whether HGF stimulation could activate ARF6 in the non-invasive CAHPV-10 cells. Fifty nanograms per millilitre was the lowest concentration that produced a significant effect on cell invasion and a discernable effect on cell scattering, thus, we chose these parameters to stimulate the CAHPV10 cells.

Western blot analysis revealed that the active form of ARF6 was substantially elevated in HGF treated cells compared to BSA/PBS treated control cells so much so that when not stimulated with HGF no discernible



**Figure 6.** HGF activates ARF6. CAHPV-10 cells were serum starved for 24 h before being exposed to 50 ng/mL HGF for 48 h. Active ARF6 pull down assay and western blot analysis shows activated ARF6 to be present in HGF treated cells but not in untreated control cells

band could be detected but after HGF stimulation ARF6 detection was abundantly expressed [Figure 6].

## DISCUSSION

While not standard of care, the use of ADT as an early stage treatment option is becoming increasingly important<sup>[2]</sup>. To mimic the possible effect of neo-adjuvant ADT in early PCa we stimulated a noninvasive cell line, known to express c-Met, with HGF. Our results revealed that HGF stimulation induced cell proliferation, scattering and cell invasion as well as activating ARF6. All of these processes are associated in one way or another with enhancing the aggressive nature of cancer cells and/or are essential components of the metastatic cascade. Cell proliferation was significantly increased in HGF stimulated cells when compared to untreated cells following both 24 and 48 h exposure. This finding is not surprising given that HGF plays an essential role in embryonic development and wound healing<sup>[33]</sup>. However, HGF stimulation did not produce a time or concentration dependent effect. We hypothesise that this effect is due to the c-MET receptor, or downstream pathways, reaching saturation point at 10 ng/mL and thus, any increases in concentration or length of time has no additional effect on cell proliferation. This is supported by other studies that have shown that changes in HGF and c-MET levels do not always invoke a concentration-dependent response either due to variants in HGF or c-Met or that down-stream signalling pathways become saturated and can no longer be phosphorylated<sup>[34]</sup>.

Interestingly HGF also caused these non-invasive cells to scatter when compared to untreated control cells. HGF has been shown to cause the disruption of a variety of normal epithelial cells resulting in cell migration<sup>[35]</sup> necessary for wound healing but it is also an essential attribute in the metastatic phenotype. HGF has been shown to induce cell scattering by inhibiting E-cadherin function resulting in cell-cell dissociation<sup>[36]</sup>, the disassembly of cell-cell adhesion complexes<sup>[37]</sup> and activation of the Ras/MAPK<sup>[38]</sup> and PI-3 kinase<sup>[39]</sup> pathways. Our results showed that HGF only induced cell dissociation, spreading and motility at the higher concentrations (50 and 100 ng/mL at 48 h). HGF is typically a paracrine factor, expressed by mesenchyme to activate c-MET in the neighbouring epithelia. Studies have shown that stromal cells secrete HGF in the range of 14-24 ng/mL<sup>[34]</sup>. Studies have also reported that serum levels increase as PCa progresses with one study showing that serum HGF levels in metastatic cancer patients were 2 times that of localised PCa patients<sup>[17]</sup>. Thus, *in vitro* HGF stimulation at high concentrations could possibly mimic the higher levels seen in the tumour microenvironment as a result of ADT and account for the observed phenotypic effects on cell dissociation.

It has been well documented that HGF stimulation enhances prostate tumour cell invasion *in vitro*<sup>[9,19,40,41]</sup> while blocking the expression of c-Met reverses the invasive properties of PCa cells<sup>[40,42]</sup>. Using 50 ng/mL HGF for 48 h we observed a significant increase in the invasive capacity of the CAHPV-10 cells compared to un-stimulated control cells. It may be suggested that an increase in cell proliferation may account for



the increased number of cells invading through an artificial basement membrane matrix. However, in our cell proliferation studies, significant increases in cell proliferation were seen at 10 ng/mL of HGF while the number of cells invading through the artificial membrane only significantly increased at 50 ng/mL. Thus, we believe that if the results of the invasion assay could be influenced by an increase in cell proliferation then we would have observed a significant number of cells invading the basement membrane at 10 ng/mL. Thus, unlike in previous studies that use cell lines that already have metastatic capabilities, we have shown, for the first time that treating non-invasive PCa cells with HGF can induce an invasive phenotype. Building on from this finding future work would need to expand the range of cell types used to include more non-invasive PCa cell lines to achieve a more rounded representation of “PCa”<sup>[43]</sup>.

ARF6 is a member of the Ras superfamily and can be activated by various growth factors, in particular, by HGF<sup>[28]</sup>. It functions in a range of biological activities and has been shown to play roles in adherens junction disassembly<sup>[26]</sup>, cell migration<sup>[44]</sup> and cell proliferation<sup>[45]</sup>. While there is very little information on the presence or activation of ARF6 in PCa we have recently shown that ARF proteins are over-expressed in PCa tissue compared to normal control tissue<sup>[31]</sup>. In this study, gene expression analysis revealed that ARF6 is expressed in all the prostate cells. Analysis showed that there was no significant difference in expression between cells derived from normal prostate epithelium and localised, non-invasive PCa but a significant difference in expression between non-invasive and invasive cancer cells was evident. ARF6 levels were significantly higher in LNCaP, DU145 and PC-3 cells compared to CAHPV-10 but there was no significant difference between the invasive cell lines regardless of their differing invasive capacities. Protein analysis confirmed the gene expression data and showed that ARF6 protein was detectable in all prostate cells. Like the majority of GTPases, ARF6 cycles between an inactive GDP-bound form and an active GTP-bound form<sup>[28]</sup>. Inactive ARF6 localises to the cytosol and endosomes and when activated it translocates to the plasma membrane<sup>[46]</sup>. Immunofluorescence revealed a strong defined signal at the periphery of the cells in both DU145 and PC-3 indicating a close association of ARF6 and the cell membrane while staining intensity appeared to be markedly reduced in both CAHPV-10, and PNT2. This data would appear to suggest that activated ARF6 increases with the aggressiveness of the cancer. To validate the immunofluorescence data, Western blotting and densitometry analysis of ARF6-GTP and total ARF6 were performed. ARF6-GTP was shown to be evident in the invasive cell lines but very low levels in the non-invasive cell lines. The ratio between ARF6-GTP and total levels of ARF6 were also calculated and highlighted the fact that levels of ARF6-GTP were higher in all the cells derived from invasive tumours. In particular, in the cell line PC-3, which is a cell line derived from a highly aggressive PCa that has metastasised to the bone and is representative of the main metastatic site for PCa, ARF6-GTP levels were found to be 3.5 and 2.7 times higher than the levels found in normal and non-invasive cells respectively.

It has been documented that HGF can activate ARF6 in epithelial cells<sup>[26]</sup>. As neo-adjuvant ADT can increase the levels of HGF, we wanted to determine whether HGF stimulation could activate ARF6 in the non-invasive cell line CAHPV-10. Using 50 ng/mL for 48 h we found that activated ARF6 levels increased compared to untreated CAHPV-10 cells. ARF6 plays an essential role in epithelial and endothelial cell migration and elevated levels of activated ARF6 have been found to increase the invasive capacity of melanoma cells both *in vitro*<sup>[28]</sup> and *in vivo*<sup>[29]</sup>, while silencing ARF6 has been shown to inhibit the ability of breast cancer cells to invade through an artificial basement membrane<sup>[30]</sup>. Whether HGF stimulated activation of ARF6 is responsible for the increased proliferation, motility and invasiveness observed following HGF stimulation of CAHPV-10 cells now needs to be determined.

In conclusion, ADT is increasingly being used in the neo-adjuvant setting. With the importance of HGF and cMet in PCa documented, it has been hypothesised that while current ADT may inhibit AR mediated cell proliferation and survival, it may abolish its suppressive role on the HGF/c-Met pathway, unintentionally driving tumour progression. We have shown that HGF enhanced cell proliferation, induced scattering and

cell invasion in a non-invasive cell line. We have shown that ARF6 is expressed in non-invasive PCa cells and that HGF stimulation correlates with increased levels of activated ARF6. All of these processes are associated with enhancing the aggressive nature of cancer cells. While further work is now needed to determine whether HGF activation of ARF6 is responsible for driving the cellular changes observed in this study, and on additional non-invasive cell lines, these findings provide further molecular insight into the progression of PCa and would suggest that ADT is unintentionally driving PCa through increasing levels of active ARF6.

## DECLARATIONS

### Authors' contributions

Performed the experiments: Morgan C, Whiteland H

Helped plan the experiments: Doak SH

Drafted the manuscript: Morgan C, Swithenbank LJ

Reviewed the manuscript: all authors

### Availability of data and materials

Data can be individually requested and information on materials is provided in the methods section.

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### Conflicts of interest

All authors declare that there are no conflicts of interest.

### Ethical approval and consent to participate

Not applicable.

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Not applicable.

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Review

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# Unmasking tumor heterogeneity and clonal evolution by single-cell analysis

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## Abstract

The intratumoral heterogeneity orchestrated by the tumor intrinsic and extrinsic mechanisms enable cancers to persist and spread notwithstanding the use of aggressive interventional therapies. The heterogeneity is revealed at multiple levels - at the level of individual tumor cells, in the cellular composition of tumor infiltrates and in the chemical microenvironment in which the cells reside. Deconvoluting the complex nature of the cell types present in the tumor, along with the homo and heterotypic interactions between different cell types can produce novel insights of biological and clinical relevance. However, most techniques analyze tumors at a gross level missing key inter-cell-type genotypic and phenotypic differences. The advent of single-cell sequencing has given an unprecedented opportunity to analyze the tumor at a resolution that not only captures the diversity of the cellular composition of a tumor but also provides information on the genetic, epigenetic and functional states of different cell types. In this review, we summarize the genesis of tumor heterogeneity, its impact on tumor growth and progression and their clinical consequences. We present an overview of the currently available platforms for isolation and sequencing of single tumor cells and provide evidence of its utility in precision medicine and personalized therapy.

**Keywords:** Intratumoral heterogeneity, single-cell sequencing, clonal evolution, circulating tumor cells, drug resistance

## INTRODUCTION

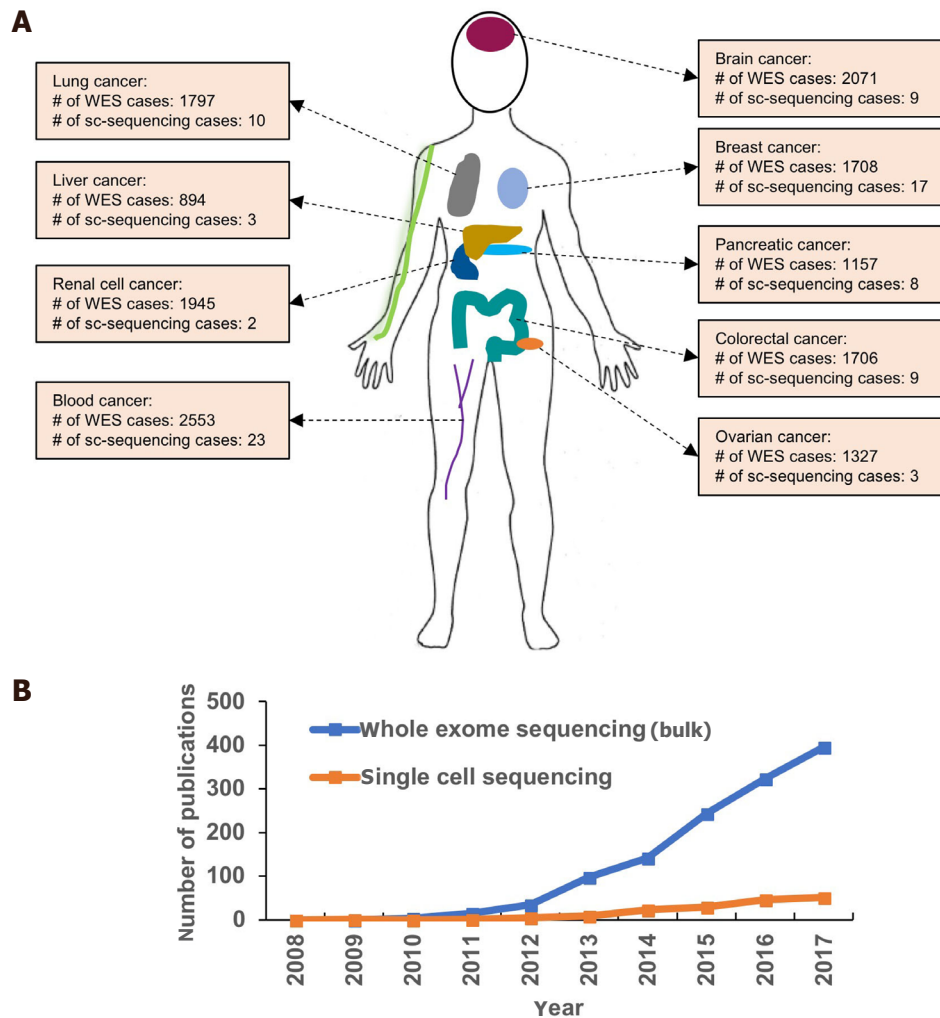
A single cell is the ultimate denominator of a multicellular organism. In the progression of cancer, a single cell begins its journey to evolve into a malignant tumor cell and forms distinct subpopulations leading to intratumoral heterogeneity (ITH). Clonal diversity, the source of ITH, is the characteristics of all cancers and plays a critical role in cancer invasion, metastasis and development of resistance to targeted and non-



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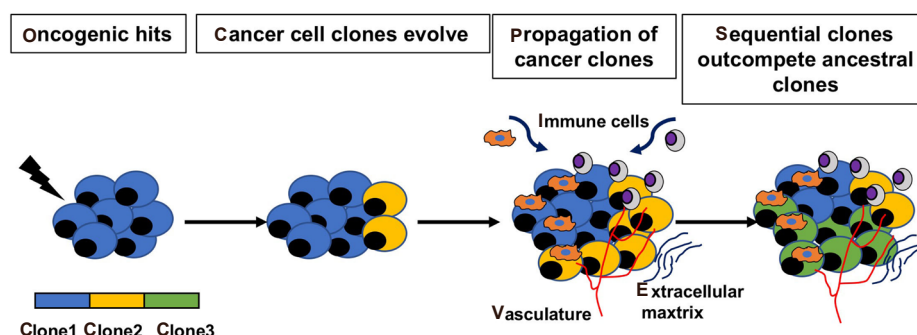






**Figure 1.** Application of whole exome sequencing (WES) and single-cell sequencing (sc-sequencing) to cancer research. A: Overview of patient cases to which WES and sc-sequencing were applied to characterize different types of human cancers to understand ITH and tumor microenvironment. The various types of cancers include liver cancer, lung cancer, renal cell cancer, blood cancer, brain cancer, breast cancer, pancreatic cancer, colorectal cancer and ovarian cancer compiled from public databases; B: the number of publications reporting applications of either whole exome or single-cell sequencing to cancer patients within the recent ten years. The key words "exome/single-cell sequencing" and "cancer patients" were used for searching articles from NCBI

targeted therapies<sup>[1-4]</sup>. Next-generation sequencing of bulk tumor tissues from many cancers has generated an unprecedented amount of multidimensional data bringing in novel insights into mechanisms of tumor initiation, progression and metastasis [Figure 1]. It has also unmasked the underlying deeper genotypic and phenotypic heterogeneity that exists between tumors belonging to the same cancer type. The ITH originating in the cancer genome can be revealed by deep exome and whole genome sequencing. However, transcriptome data from a complex mixture of cells derived from bulk tumor tissues fail to accurately elucidate the ITH, requiring technologies to study tumors at a single-cell resolution. Over the past ten years, there has been extraordinary progress in the development and application of single-cell analysis in cancer research as evidenced by the rise in publications describing different aspects of single-cell sequencing to characterize tumors at a deeper level [Figure 1]. In this review, we first introduce the concept of ITH and its clinical implications. Next, we outline new technologies enabling single-cell analysis with high sensitivity and finally provide examples of their applications in uncovering new perspectives in cancer diagnostics and treatment.



**Figure 2.** Origin of ITH. Upon certain oncogenic hits, some cells in the normal tissues undergo genetic alterations to generate cancer cells. ITH arises through clonal evolution in which cells are dictated by transcriptomic and epigenetic factors and the tumor microenvironment. Cancer clones (yellow) propagate and generate successive clones (green) which outcompete the ancestral ones

## ORIGIN OF ITH

ITH was first described by Fidler *et al.*<sup>[5]</sup> more than 30 years ago in murine models as a single tumor consisting of many cell subpopulations. However, this concept of heterogeneity in the composition of a tumor has now been expanded to include the genetic and molecular heterogeneity present within individual tumor cells and cells comprising the tumor microenvironment<sup>[6-9]</sup>.

### Genetic and epigenetic alterations

ITH arises as a result of both genetic and non-genetic changes in the tumor cells and the surrounding environment respectively [Figure 2]<sup>[10]</sup>. Increased genetic instability as a result of mutations in DNA damage checkpoint control genes and DNA repair genes is one of the hallmarks of cancer and generates divergent clonal population of cells as the tumor grows over time<sup>[11,12]</sup>. With the significantly high rate of cancer cell divisions, events of random mutagenesis increase, leading to local and global genetic alterations, that influence the future course of tumor development and progression<sup>[13]</sup>. In addition, these genetic alterations create a hotbed for competition between clones driven by selection processes imposed by changes in the tumor microenvironment and by the use of therapies<sup>[14,15]</sup>.

A vast majority of established driver mutations are clonal and arise early in the development of the tumor, however, subclonal *de novo* driver mutations may also arise in the later stages of tumorigenesis - to escape drug sensitivity and successful metastasis, for example<sup>[16]</sup>. In a recent UK-wide multi-center prospective longitudinal cohort study, "Tracking Renal Cell Cancer Evolution through therapy (TRACERx Renal)", clonal phylogeny and evolutionary subtypes were elucidated by multi-region sampling on matched primary and metastasis biopsies from 100 renal cell carcinoma patients<sup>[17]</sup>. Subclonal driver mutations in the *VHL* and *PBRM1* genes that were identified in the original tumor were absent in the widely disseminated metastatic tumor sites. Instead, these metastatic sites acquired loss of 9p and 14q mutations, suggesting that metastatic competence may not be driven by the founder driver mutations that established the primary tumor<sup>[17]</sup>.

Tumor heterogeneity can also arise from epigenetic variations through DNA methylation that can profoundly modulate the open and closed conformation of chromatin in tumor cells, leading to gene expression alterations and phenotypic changes<sup>[18]</sup>. For example, the methylation status of the tumor suppressor gene *CDKN2B* can be used as a biomarker of response to treatment in multiple diseases<sup>[19]</sup>. However, heterogeneous methylation was observed in individual patients with acute myeloid leukemia, posing a challenge in using *CDKN2B* methylation as a biomarker<sup>[20]</sup>. Similarly, differential microRNA expression is known to affect the diversity of cellular phenotype within a single tumor by modulating the expression of target genes<sup>[21]</sup>. Subclonal expression of microRNAs (miRNA-21, miRNA-34a, miRNA-125, and miRNA-126) in prostate cancer is associated with diverse patient outcomes<sup>[22]</sup>.

### Cellular composition of tumors

Cell types present in the tumor stroma, such as immune cells, fibroblasts, vascular cells play a critical role in shaping the composition of tumors by secreting cytokines growth factors and extracellular matrix that changes the stiffness of the tumor tissue<sup>[23]</sup>. In a tumor microenvironment infiltrated by CD8 T cells at the tumor site is associated with increased overall survival, whereas myeloid-derived suppressor cells (MDSCs) possessing strong immune suppressive activity decreases overall survival<sup>[24]</sup>. The diversity of these functionally different immune cell types creates a heterogeneous tumor microenvironment and regulate tumor growth, metastasis and treatment response<sup>[25]</sup>. In addition, the distribution and density of the vasculature impact the supply of nutrients and oxygen selecting for tumor cells with specific metabolic phenotypes further contributing to tumor heterogeneity<sup>[26,27]</sup>. Tumor heterogeneity has a significant bearing on the management of disease as summarized in the next section.

## CLINICAL IMPACT OF THE ITH

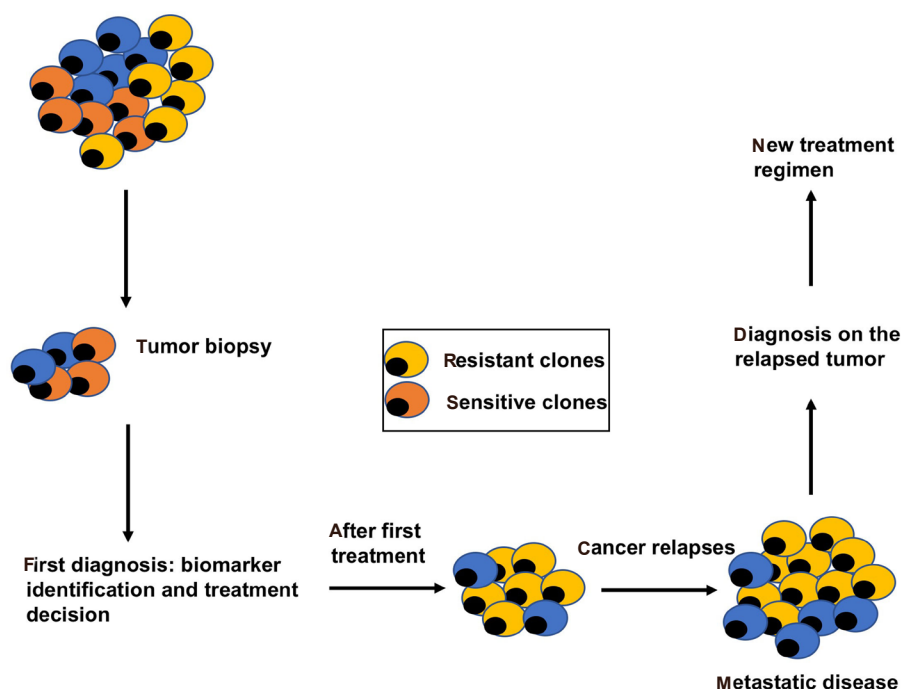
### Resistance to therapy

The resistance of tumors to therapies is often attributed to the presence of rare drug-resistant clones in the tumor before therapy or appears after treatment. An example of clonal resistance was observed in patients with anaplastic lymphoma kinase (*ALK* gene) rearranged non-small cell lung cancer (NSCLC) post treatment with *ALK* inhibitors<sup>[28]</sup>. Patients that developed drug resistance displayed a distinct spectrum of *ALK* resistance mutations in response to different generations of *ALK* inhibitors<sup>[28]</sup>. Particularly, *ALK*<sup>G1202R</sup> mutation is highly enriched in resistant tumors after treatment with second-generation *ALK* inhibitors, highlighting the significance of repeat biopsies and genotyping during the course of targeted therapy treatment<sup>[28]</sup>. In addition, studies investigating the mechanism of resistance of NSCLC tumors to EGFR tyrosine kinase inhibitors have revealed a variety of drug resistance mechanisms, including gatekeeper mutation T790M detected in > 50% of the EGFR TKI resistant tumors<sup>[29]</sup>, amplification of MET receptor tyrosine kinase<sup>[30]</sup>, activating mutation in PI3K pathway<sup>[31]</sup>, and other uncharacterized mechanisms involving changes in the cellular phenotype. The appearance of a rare clonal population of tumor cells harboring drug resistance mutations or drug resistance phenotype can be captured by single-cell sequencing of the tumor and may not be discernible from whole tumor analysis, especially when present at a very low frequency. In an alternative model of drug resistance, resistant clones can be pre-existing in the tumor as a rare cell population and emerge post clearance of the drug-susceptible clones. In fact, in a study involving a cohort of 20 breast cancer patients, 8 out of 10 patients that did not show complete clearance of the tumor displayed unique somatic mutations in chemoresistant clones by single-cell sequencing. These mutations were pre-existing and were adaptively selected by the chemotherapy treatment<sup>[32]</sup>. It is possible to detect *de novo* or drug-induced resistant clones present at low frequency by ultra-deep exome sequencing, however, two critical pieces of information - number of cells harboring the mutation and the zygosity of the mutation - cannot be accurately assessed from the bulk sequencing.

### Challenges in diagnostic and prognostic biomarker identification

Identifying clinically relevant diagnostic biomarkers are challenging given that the tumor is heterogeneous and diagnostic or prognostic biomarkers are not expressed uniformly in all cells and across longitudinal assessment periods [Figure 3]. For example, the divergent genetic landscape of metastatic cells can render biomarkers identified from primary tumors irrelevant [Figure 3]<sup>[33]</sup>.

In prostate cancer, ITH represents a major challenge for diagnostic and prognostic biomarker identification. Enhanced DNA ploidy and loss of *PTEN*, a tumor-suppressor gene, are critical prognostic markers of prostate cancer<sup>[34]</sup>. In a clinical study of 304 patients who underwent radical prostatectomy, a significant difference in DNA ploidy classification and loss of *PTEN* expression was observed by analyzing all tumor areas in comparison to a single biopsy sample, suggesting that the heterogeneous chromosomal alterations com-

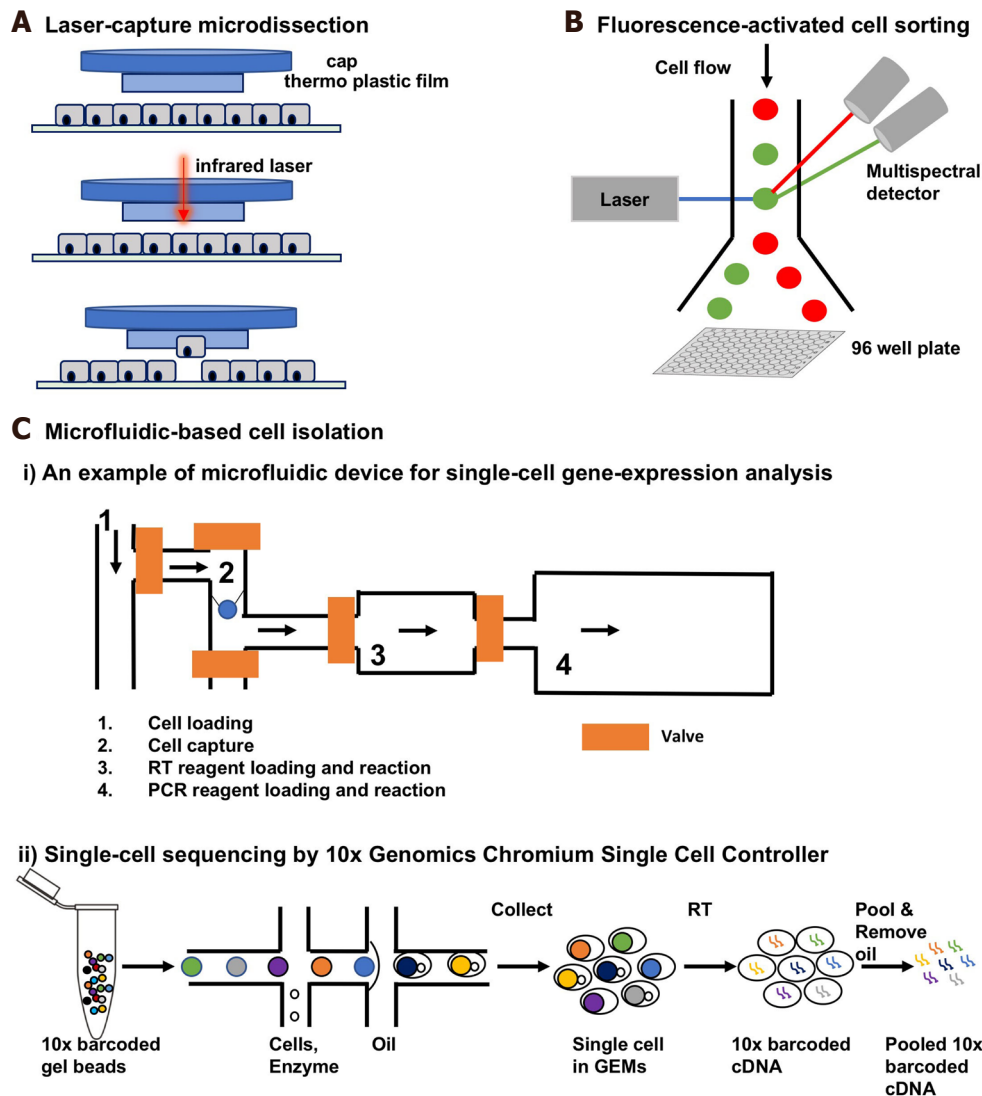


**Figure 3.** The clinical implications of tumor heterogeneity. Cancer diagnosis is commonly based on tumor biopsy, which is usually a small fraction of the total tumor mass and does not represent all subclones inside the tumor. Initial diagnosis is made based on the tumor biopsy. After the first-line treatment, dominant clones can be killed successfully whereas resistant clones persist and drive tumor progression. Metastasis may develop from the resistant clones that survive the initial treatment. New diagnosis needs to be made in order to apply the second-line treatment

promise the accuracy of histopathology analysis and confound disease prognosis<sup>[35]</sup>. Prognostic markers in ovarian cancer such as unique CpG methylation patterns have been suggested for progression-free survival as well as early disease recurrence following chemotherapy<sup>[36,37]</sup>. However, DNA methylation patterns are heterogeneous and occurs in both large and poorly defined genomic regions<sup>[20]</sup>, posing a challenge in using CpG methylation as a biomarker. In a recent study by Rajaram *et al.*<sup>[38]</sup>, a data-driven framework based on single-cell analysis has been reported that provides an estimate of the depth of sampling that may be minimally required to cover the full range of phenotypic heterogeneity for accurate biomarker discovery. Based on the analysis of 215 single-cell features, three replicates were sufficient to capture the heterogeneity for many features if they were defined by clear biomarkers without background noise<sup>[38]</sup>. For example, nuclear staining (the number of nuclei staining by DAPI: an easily detectable feature) requires 1-2 cores to capture the heterogeneity in > 90% of the patients, while 10 cores or more are needed to assess the heterogeneity of YAP transcription factor expression (a sparsely detectable feature)<sup>[38]</sup>. Therefore, both the complexity of the feature and the biomarkers that define the feature determine the number of samples required for studying heterogeneity<sup>[38]</sup>.

## UNCOVERING ITH BY SINGLE-CELL ANALYSIS

Single-cell analysis is a powerful tool to resolve ITH of solid tumors and to detect the genetic makeup of rare cancer cells such as circulating tumor cells (CTCs) to ultimately guide personalized treatment strategies. The sensitivity of detecting somatic variants or changes in gene expression at a single-cell level has improved dramatically over the years through the introduction of new technologies. Single-cell analysis workflow includes isolation of single cells, either from the tumor site or circulating tumor cells from the blood. Following tumor dissociation, single-cells can be obtained by serial dilution, flow cytometry or microfluidics technology and then sequenced at sufficient depth to capture the genetic changes.



**Figure 4.** Different ways of single-cell isolation. A: Laser capture microdissection. A thermolabile polymer is placed on a tissue section on a glass slide. An infrared laser fires through the cap over the cells of interest to melt the film. The cell of interest adheres to the film, leaving the unwanted cells behind; B: fluorescence-activated cell sorting. A stream of single cells passes through an excitation laser beam and the fluorescent signal is analyzed by a multispectral detector. Single cells can be sorted into a 96 well plate; C: microfluidic-based single-cell isolation: i) An example showing a microfluidic device for single cell gene expression analysis (figure is adapted from White et al.[94,95], 2011): (1) loading of single cells; (2) capturing single cells; (3) reverse transcription; (4) PCR; ii) Gel Bead-in-EMulsions (GEMs) formation and barcoding of 10× Genomics single-cell sequencing platform (figure is adapted from 10× Genomics Inc). Single cell GEMs are generated by passing cells with enzyme mix, partitioning oil and 10× barcoded gel beads. After GEM formation, the gel bead is dissolved and the co-partitioned cell is lysed. Reverse transcription occurs inside GEMs and barcoded full-length cDNA is generated. After RT, the GEMs are broken and the cDNA is pooled prior to library preparation for sequencing

### Single-cell isolation methods from solid tumors

A major challenge in single-cell analysis is obtaining a viable cell sample from complex tumor tissues. Current methods include mechanical or enzymatic dissociation of tissues followed by isolation of single cells. Once the tissue is processed, multiple techniques to isolate single cells can be implemented [Figure 4]. A more labor-intensive technique of laser capture microdissection (LCM) is also a viable approach for single-cell isolation from sectioned tumor samples. One challenge for single-cell transcriptomics is the poor RNA quality extracted from archival tumor samples such as formalin-fixed paraffin-embedded (FFPE) samples<sup>[39]</sup>. However, with the Smart-3SEQ method, it is now feasible to perform single-cell RNA-seq on FFPE samples<sup>[39]</sup>. Additionally, recent advances using the SMART seq technology and cDNA synthesis methods using



random priming (SMART-Seq Stranded Kit, Takara Inc.) have been beneficial in extracting reliable gene expression information from poor quality RNA from FFPE samples.

#### *Single-cell isolation by mechanical or enzymatic dissociation*

Conventionally, tumor tissues are dissociated into single cells by mechanical dissociation (e.g., meshing, trituration with a pipette/tip)<sup>[40-42]</sup> or by enzymatic dissociation<sup>[43-45]</sup> or a combination of both. Enzymes such as collagenase<sup>[41]</sup>, DNase<sup>[46]</sup>, trypsin<sup>[47]</sup> are commonly used for dissociating the cell-cell contacts and the extracellular matrix to generate single cell suspensions. The various dissociation methods may largely differ in their yield of viable cells<sup>[48,49]</sup>, limiting their downstream applications. Therefore, tumor dissociation protocols optimized for different tumor types is a key gap that needs to be addressed for high-throughput single-cell analysis.

#### *Single-cell isolation by LCM*

To preserve the native properties of tumor cells shaped by the complex tumor microenvironment, LCM can be used to isolate tumor cells directly from sectioned tissues. It is a method to procure subpopulations of tissue cells under direct microscopic visualization by cutting away unwanted cells and obtain histologically pure cell population [Figure 4A]<sup>[50]</sup>. A variety of downstream applications exist for microdissected cells such as DNA genotyping, RNA transcript profiling or cDNA library generation. Even though the majority of the studies take advantage of approximately 100-1000 dissected cells, LCM can also be used for single-cell isolation directly<sup>[51-53]</sup>.

### **Isolation of rare CTCs**

Currently, tumor biopsies are obtained to establish the diagnosis and determine whether the predictive biomarkers are consistent between the primary and the metastatic tumors. However, getting biopsies is invasive, expensive and not always feasible. Additionally, it is difficult to get biopsies of metastatic lesions or get repeat biopsies for difficult to access tumors. Analysis of disseminated tumor cells (DTCs) is a useful alternative to tumor biopsy in clinical setting for patient stratification, therapy selection and monitoring drug resistance during the course of treatment<sup>[54]</sup>. DTCs originate from the primary or metastatic tumors, extravasate into the bloodstream or lymphatics and carry genomic profiles of tumors from which they originate<sup>[55,56]</sup>. Disseminated cancer cells are usually detectable as CTCs in the circulation<sup>[54]</sup>. A small fraction of them that have reached to a secondary organ such as the bone marrow and lymph nodes is termed as DTCs<sup>[54]</sup>. Though for certain cancers, the presence of DTCs in distant organs is a strong predictive marker for cancer metastasis, the challenge with DTC isolation due to the invasive procedure is a deterrent in studying this population by single-cell sequencing. On the contrary, CTCs circulating in patient blood has proved to be a valuable resource for diagnostic and prognostic biomarker discovery<sup>[57]</sup>, although distinguishing a DTC from a pool of CTCs is challenging.

CTCs contain signatures of tumor heterogeneity and carry the spectrum of somatic mutations present in both the primary and metastatic lesions in different cancers<sup>[55,56,58]</sup>. Because conventional molecular analysis of whole tumors provides genotype/phenotype information of the dominant clones or aggregated information of all clones, single-cell analysis of the CTCs is a potential solution to investigate heterogeneity. By isolating and sequencing single CTCs in the blood, it is possible to measure somatic mutations that are present at both the primary and metastatic tumor sites without performing an invasive core biopsy<sup>[59,60]</sup>. Two types of isolation methods - microfluidic-based and immunoaffinity-based are used for capturing CTCs.

#### *Microfluidic-based cell isolation*

The microfluidic platform can be used for single-step isolation of CTCs from unprocessed blood specimens<sup>[61,62]</sup>. As whole blood flows through the CTC-chip, individual CTCs are captured onto the microposts coated with anti-EpCAM antibody. This type of microfluidic processing enables high yield of pure CTCs<sup>[63]</sup>. Subsequent studies demonstrated the ability and reliability to isolate CTCs from patients with metastatic

lung cancer using this CTC-chip to perform an EGFR mutational analysis<sup>[63]</sup>. An improved microfluidic CTC isolation platform, the herringbone (HB)-chip, is also developed by the same group<sup>[64]</sup>. The HB-chip uses calibrated microfluidic flow patterns to drive cells to come in contact with the antibody-coated walls of the device, thereby reducing cell collisions and improving target cell capture efficiency. A commercial microfluidic circuitry chip DEPArray System (Menarini Silicon Biosystems, Inc.) containing an array of individually controllable electrodes to create a dielectrophoretic (DEP) cage around each cell for single CTC isolation is also available<sup>[65]</sup>. Besides isolation of CTCs from blood, the microfluidic platform can also be used for single-cell isolation from other tissues<sup>[66,67]</sup>. For example, an innovative workflow using DEPArray system was established to examine tumor heterogeneity using FFPE samples, providing a solution for genetic analysis using minute archival clinical samples<sup>[68]</sup>.

#### *Immunoaffinity-based cell isolation*

The CellSearch Circulating Tumor Cell Kit (Menarini Silicon Biosystems, Inc.) is based on ferrofluid- and fluorochrome-couple antibodies with high binding affinities for the EpCAM antigen of CTCs. After immunomagnetic capture and enrichment, CTCs in peripheral blood are detected and enumerated as measured by fluorescence intensity. ITH has been reported for *PIK3CA* and *TP53* mutations in metastatic breast cancer using a combination of CellSearch and DEPArray technologies<sup>[69,70]</sup>. CTCs can also be purified and enriched using an immunomagnetic enrichment device termed MagSweeper<sup>[71]</sup>. Using this technique, high level of heterogeneity among individual CTCs was detected in the blood of metastatic breast cancer patients<sup>[72]</sup>.

#### **Isolation of single cells using Fluorescence-activated cell sorting**

Flow cytometry using fluorescence-activated cell sorting is a powerful method of isolating single cells that share the same marker from liquid suspensions. Cells passing through the lasers emit optical signals enabling their separation and capture from other cells that lack the signal<sup>[73,74]</sup>. Single cells can be sorted individually onto a 96 well plate format [Figure 4B]. Alternatively, a serial dilution can be performed using the sorted cell suspensions into a 96 well plate such that each well contains a single cell. Downstream sequencing can be performed using a 96 well plate format.

Isolated single cells can be interrogated by a variety of genomic technologies for deeper genotype-phenotype characterization. Significant technological advancement summarized in the next section is producing novel insights into the biology of the disease and applications in the clinic.

#### **Downstream analysis of single cells**

##### *Single-cell genomics*

The work-flow of single-cell sequencing involves amplification of genomic DNA or RNA transcripts to produce enough material for library construction. The earliest method of sequencing DNA from single-cells combined flow-sorting cells by DNA ploidy followed by single-nucleus sequencing by degenerative-oligonucleotide-PCR technique<sup>[74,75]</sup>. However, this method failed to generate genome-wide single nucleotide variants due to low coverage of ~6%<sup>[74,75]</sup>. A non-PCR-based multiple-displacement DNA amplification method using Phi29 enzyme and random hexamers [Table 1] produced good genome coverage with high sequence fidelity in multiple single-cell studies<sup>[58,76-79]</sup>. Another amplification method - multiple annealing and looping-based amplification cycles (MALBAC) reduced whole-genome amplification bias and improved genome coverage [Table 1]. In the MALBAC method, limited isothermal amplification using degenerate primers, followed by PCR amplification produced 93% genome coverage for a single cell and both copy-number variations and single nucleotide variations were detected<sup>[80]</sup>. Amplification bias is a serious limitation in single-cell sequencing, which can reduce the accuracy of genomic information from single-cell genomes<sup>[81]</sup>. Statistical models have been developed to calibrate allelic bias in single-cell whole-genome amplification to reduce the sequencing artifacts<sup>[81]</sup>.

**Table 1. Techniques for single-cell analysis**

	Methods	Example	Advantage	Disadvantage	Ref.
Genome	DOP-PCR		High-throughput, high coverage	Amplification bias, allelic dropout	[74,75]
	MDA		High-throughput, even coverage	Amplification bias, allelic dropout	[58,76-79]
	MALBAC		High-throughput, even coverage	Amplification bias, allelic dropout	[80]
Transcriptome	MMLV	Smart-seq	Full-length transcript, amplify quickly	Weak 3' bias	[83,84]
	IVT	CEL-Seq	Full-length transcript, specificity, ratio fidelity	3' bias, low efficiency	[87,88]
	Phi29 DNA polymerase		Full-length transcript, high efficiency, low bias	No strand specificity	[89,90]

MDA: multiple-displacement DNA amplification; DOP-PCR: degenerative-oligonucleotide-PCR; IVT: *In vitro* transcription

### Single-cell transcriptomics

The first study of single-cell RNA transcriptome of mouse blastomere detected novel splice junctions and expression of more genes than previous microarray studies<sup>[82]</sup>. However, this method was found to have a strong 3' bias due to the inefficiency of first-strand cDNA synthesis by reverse transcriptase. To overcome this problem, Smart-seq technique was developed using MMLV reverse transcriptase with template switching activity [Table 1]<sup>[83,84]</sup>. This Smart-seq method utilizes an intrinsic property of MMLV to add three to four cytosines specifically to the 3' end of the first cDNA strand, which is subsequently used to anchor a universal PCR primer for amplification<sup>[85]</sup>. In a single-cell RNA-seq of CTCs from melanoma patients, Smart-seq has improved read coverage across transcripts despite increased noise in gene expression estimates<sup>[83]</sup>. Moreover, distinct gene expression patterns including candidate biomarkers for melanoma CTCs were reported in this study<sup>[83]</sup>.

*In vitro* transcription (IVT) -based linear RNA amplification uses T7 RNA polymerase to produce transcripts with high specificity and low error rate [Table 1], it has the drawback of lower efficiency and is biased towards the 3' end of input transcripts<sup>[86]</sup>. CEL-Seq method of pooling cells and libraries reduced some of the limitations of IVT and was used to capture differential gene expression in two-cell stage embryo of *C. elegans*<sup>[87,88]</sup>.

The third strategy used Phi29 DNA polymerase for cDNA library generation from single cells [Table 1]<sup>[89,90]</sup>. RNA is reverse transcribed, circularized and then amplified using Phi29 polymerase which preserves full-length transcript coverage. Additionally, random primers can be incorporated to generate cDNA, making this method suitable for prokaryotes<sup>[89]</sup>.

### A combined method of single-cell isolation and single-cell sequencing

Microfluidic devices for single-cell isolation coupled with single-cell RT-qPCR or whole transcriptome has been developed by multiple groups<sup>[91-93]</sup>. A good example is a microfluidic device developed by White *et al.*<sup>[94,95]</sup> capable of performing high precision RT-qPCR measurements of gene expression from hundreds of single cells per run. This device combines cell loading, cell lysis, reverse transcription and quantitative PCR in one cell processing unit [Figure 4Ci]<sup>[94,95]</sup>. Once cells are loaded, a single cell is trapped in a cell capture chamber [Figure 4Ci]<sup>[94,95]</sup>. After cell lysis, the transcript target is reverse transcribed before being injected into the PCR chamber<sup>[94]</sup>. Master mixes for RT and qPCR are loaded onto the common feed channel sequentially to enable each reaction step. A similar device, featuring additional cell processing chambers and sample elution capabilities has been released as a commercial product (Fluidigm C1) in 2012. Since then, an increasing number of studies investigated ITH using Fluidigm's microfluidic device<sup>[96-98]</sup>.

Efforts to reduce amplification bias by incorporating unique molecular identifiers before transcriptome am-

**Table 2. Overview of single-cell studies on analyzing ITH**

Tumor type	Sample type	Method	Description	Ref.
Colorectal cancer	CTC	DNA-seq	Mutation profiling, clonal evolution	[55]
Prostate cancer	CTC	DNA-seq	Genetic lineage	[58]
Breast cancer	CTC	RNA-seq	Transcriptome profiling	[72]
Breast cancer	Primary tumor	DNA-seq	Clonal diversity	[75]
Melanoma	CTC	RNA-seq	Transcriptome profiling	[83]
Leukemia	Primary tumor	DNA-seq	Mutation profiling, clonal evolution	[97]
Glioblastoma multiforme	Primary tumor	RNA-seq	Clonal evolution	[106]
Acute myeloid leukemia	Primary tumor	DNA-seq	Mutation profiling, clonal evolution	[105]
Breast cancer	Primary tumor	DNA-seq	Copy number evolution, clonal evolution	[74]
Breast cancer	Primary tumor	DNA-seq	Copy number evolution, clonal evolution	[77]
Acute myeloid leukemia	Primary tumor	DNA-seq	Clonal evolution	[109]
Kidney cancer	Primary tumor	DNA-seq	Mutation profiling	[76]
Bladder cancer	Primary tumor	DNA-seq	Mutation profiling, clonal evolution	[110]
Colon cancer	Primary tumor	DNA-seq	Clonal evolution	[111]
Acute myeloid leukemia	Primary tumor	DNA-seq	Clonal evolution	[112]
Chronic lymphocytic leukemia	Primary tumor	DNA-seq, RNA-seq	Genotype-phenotype relationship clonal evolution, mutation profiling	[113]
Lung cancer	CTC	DNA-seq	Copy number evolution	[56]
Pancreatic ductal adenocarcinoma	CTC	RNA-seq	Phenotype characterization	[115]
Glioblastoma	Primary tumor	RNA-seq	Transcriptional profiling, phenotype characterization	[43]
Glioblastoma	Primary tumor	DNA-seq	EGFR evolution	[116]
B cell leukemia	Primary tumor	DNA-seq	Karyotype heterogeneity	[117]
Myeloproliferative neoplasm	Primary tumor	DNA-seq	Mutation profiling, clonal evolution	[78]
Melanoma	CTC	DNA-seq	Mutation profiling, copy number evolution	[118]
Breast cancer	CTC	RNA-seq	Transcriptome profiling	[120]
Various cancers	Primary tumor	RNA-seq	TCR repertoire analysis	[124,126]
Liver cancer	Primary tumor	RNA-seq	Characterization of T cell functional states	[130]
Breast cancer	Primary tumor	RNA-seq	Tumor microenvironment characterization	[132]
Prostate cancer	CTC	RNA-seq	Heterogeneity in signaling pathways	[136]
Prostate cancer	CTC	DNA-seq	Copy number evolution	[137]
Breast cancer	Primary tumor	DNA-seq, RNA-seq	Clonal evolution, transcriptome profiling	[32]

ITH: intratumoral heterogeneity; CTC: circulating tumor cell

plification are ongoing<sup>[99]</sup>. A novel technique termed Drop-seq uses the microfluidic chamber to isolate single cells followed by labeling RNA of individual cells with a different barcode, allowing pooling of cDNA during sequencing thereby greatly improving the multiplexing efficiency<sup>[100]</sup>. Applying Drop-seq to mouse retinal bipolar cells resulted in the identification of different types of neurons by matching molecular expression to cell morphology<sup>[101]</sup>. A similar technique was commercialized by 10× Genomics Inc [Figure 4Cii] in 2016. The 10x platform applies unique barcodes to separately index each cell by partitioning thousands of cells into Gel Bead-in-Emulsions. Libraries are generated and sequenced and the 10x barcodes are used to associate individual reads back to the individual cells. The platform can profile up to 10,000 cells from a complex mixture of different cell types.

## APPLICATIONS OF SINGLE-CELL SEQUENCING

Recent technical advances have enabled generation of unprecedented amount of information on genomics and transcriptomics at the single-cell level [Table 2]. Compared to bulk transcriptomics data obtained from tumor tissues, single-cell RNA-seq allows capturing of the gene expression profile from individual cells of heterogenous origin, which is a significant advantage over bulk sequencing that captures the average gene expression of a sample. Secondly, for the samples with limited amount of material, single-cell analysis is a good alternative to characterize the genotype. Taking CTCs for an example, mutations identified in CTCs

are also present in the primary tumor and may be found in the metastatic lesions<sup>[55]</sup>, suggesting that single-cell analysis on CTCs is an effective option to non-invasively monitor cancer progression and predict metastatic risk. Last but not the least, single-cell analysis facilitates researchers to dissect tumor heterogeneity at a much higher resolution than before. For example, the degree of karyotypic anomalies in human cancer is associated with tumor progression and therapeutic response to cancer treatment<sup>[102]</sup>. However, current karyotypic analysis methods rely on a small fraction of dividing mitotic subpopulations in the sample and do not provide in-depth information on copy number variations (CNV)<sup>[102,103]</sup>. Single-cell whole genome sequencing offers a significant advantage over traditional methods in analyzing karyotypic anomalies and CNVs at a much higher resolution.

### Understanding tumor evolution

Tumor evolution is a dynamic process and describes the emergence of cancer cell subpopulations under environmental pressure. As the tumor grows, each generation of cells acquire novel somatic mutations that provide cells with survival advantages thereby determining the overall fitness of the clonal population<sup>[104]</sup>. Waves of clonal expansion and contraction driven by changes in the tumor microenvironment govern the life cycle of a tumor. Single-cell sequencing can potentially identify low abundance clones carrying driver mutations, which can be further leveraged to refine therapeutic strategies. Although low abundance driver mutations are possible to detect by deep exome sequencing, the fraction of cells carrying the mutation, or the zygosity of the change (relevant for loss of function mutations in tumor suppressor genes) are hard to estimate without single cell sequencing. A computational approach to map single-cell mutational profile from exome sequencing was successfully used to chart the chronological acquisition of mutations and create a phylogenetic map of tumor evolution in both glioblastoma multiforme and secondary acute myeloid leukemia (AML)<sup>[105,106]</sup>. A similar analysis in breast cancer identified three clonal populations in the primary tumor of which only one clone was present in the metastatic lesion<sup>[74]</sup>. This observation supports the hypothesis that rare clones present in the primary tumor harbor genetic signatures of metastasis even before they have spread and colonized distant sites<sup>[74,107,108]</sup>. In a follow-up breast cancer study, aneuploidy rearrangements were shown to occur early in tumor evolution, which remained highly stable as the tumor grew, whereas, point mutations generated clonal diversity<sup>[77]</sup>. A similar pattern is observed in lymphoblastic leukemia patients where recurrent translocations appear earlier than structural nucleotide variants<sup>[109]</sup>. This suggests that large structural alterations offer selective advantage early during tumor growth followed by accumulation of mutations producing clonal diversity. This is supported by the finding that subclonal populations arise more frequently in tumors with high mutational burdens such as bladder and colon cancer, but not in tumors with low mutational burden such as renal cell carcinoma<sup>[76,110,111]</sup>. A clonal progression of multiple mutations was mapped in hematopoietic stem cells of AML patients, suggesting the clonal evolution of AML genomes from founder mutations<sup>[112]</sup>. An interesting finding from single-cell analysis is that phenotypic diversity fails to recapitulate genotypic diversity detected in subclones strongly implicating that a large proportion of genotypic variation may lack functional consequences, appearing and disappearing without contributing to tumor evolution<sup>[113]</sup>.

### Disease diagnosis and therapeutic stratification of patients

Modern cancer treatment relies heavily on accurate molecular and immuno/histopathological tissue analysis of needle biopsies or surgically resected tissues for diagnosis. Tumor heterogeneity often confounds accuracy of disease diagnostics by subsampling a subset of tumor cells that may not represent the whole tumor. This calls for obtaining multiregional and longitudinal samples to guide therapeutic intervention, which is often not routine. High-resolution single-cell analysis of tumor samples or CTCs can aid in refining diagnostic parameters and patient stratification.

In a single-cell sequencing study of CTCs from metastatic lung cancer, patients who share the same subtype of lung cancer displayed similar patterns of copy number variations in their CTCs, providing a potential



biomarker of CTC-based cancer diagnostics<sup>[56]</sup>. In pancreatic cancer, pancreas epithelial cells can be present in the blood at pre-cancerous stages in pancreatic ductal adenocarcinoma patients<sup>[114]</sup>. In another study, single-cell sequencing analysis on CTCs obtained from pancreatic ductal adenocarcinoma patients identified a macrophage-pancreatic tumor cell fusion product with high proliferative and metastatic potential<sup>[115]</sup>. These studies suggest that early detection of these pancreatic epithelial cells in the blood stream can serve as an important diagnostic tool for pancreatic cancer detection<sup>[114,115]</sup>.

The treatment of glioblastomas, an aggressive type of brain tumor has benefited from single-cell sequencing because of a high degree of tumor heterogeneity harboring a diverse population of cells with a large spectrum of stemness, differentiation states, and variable proliferative capacity<sup>[43]</sup>. By applying single-cell sequencing to EGFR-amplified glioblastomas, novel EGFR truncation variants were identified<sup>[116]</sup>. *In vitro* and *in vivo* functional studies revealed that a specific EGFR variant (EGFRvII, deletion of exons 14 and 15) was sensitive to EGFR inhibitors, which are currently in clinical trials<sup>[116]</sup>. In chromosomally unstable B cell leukemia patients, different degrees of karyotypic abnormalities were detected by single-cell whole genome sequencing, which bulk sequencing failed to detect. Because karyotypic abnormalities associate with poor clinical outcome in multiple cancers<sup>[102]</sup>, the degree of karyotypic anomalies assessed by single-cell sequencing can be utilized as an important readout for stratifying patient risk<sup>[117]</sup>. Single-cell analysis has identified novel mutations in JAK2-negative myeloproliferative neoplasm such as *SESN2* and *NTRK1*, chronic lymphocytic leukemia such as *LCP1* and *WNK1* and chromosomal abnormalities in melanoma such as chromosomal 12 amplification<sup>[78,113,118]</sup>, opening up opportunities to target these neoplasms. For example, *NTRK1* encodes a tyrosine kinase receptor and inhibitors are available to target its *NTRK1* gene fusions that results in constitutive activation of the kinase<sup>[119]</sup>. For patients who are *JAK2* mutation negative but harbor *NTRK1* mutation, it is tempting to speculate that *NTRK1* can be a target for the treatment of myeloproliferative neoplasm.

### Disease monitoring and prognostic biomarkers

Cancer heterogeneity in part is driven by selection pressure that arises during drug treatment. Capturing this dynamic heterogeneity at the genetic and cellular composition level prior to, during, or post-treatment is crucial in assessing drug efficacy and predicting patient survival. Single-cell analysis is an extremely powerful tool to capture the dynamic events at a molecular level for disease monitoring and in predicting prognostic biomarkers. Below are few examples of the application of single-cell sequencing in developing prognostic and predictive biomarkers.

#### CTC analysis

Single-cell analysis of CTCs can provide prognostic markers in several cancers. Microfluidics-based RNA sequencing has aided identification of CTC clusters held together by the cell junction component plakoglobin that mediate intercellular adhesion. Presence of high levels of CTC clusters over single CTCs correlated with poor prognosis indicating their role in the metastatic spread of cancer<sup>[120]</sup>. Indeed, heterogenous expression of plakoglobin in the primary tumor supports the evidence that tightly adhered groups of cells from the primary tumors serve as the precursors to CTC clusters in circulation. Thus, single-cell identification of plakoglobin-positive clonal cell populations of tumor cells in conjunction with the presence of CTC clusters in the patient blood is a potent prognostic marker of breast cancer metastasis<sup>[120]</sup>.

#### TCR repertoire analysis

Anti-tumor immunity is largely driven by antigen-specific CD8 T cells, which recognize tumor-derived neoantigenic peptides complexed with human leukocyte antigen also referred to as major histocompatibility complex (MHC) in mouse, to mount an anti-tumor immune response<sup>[121]</sup>. Adoptive cell therapy using autologous tumor infiltrating lymphocytes (TILs) has been shown to be effective for the treatment of multiple cancers<sup>[122,123]</sup>. The anti-tumor effects observed post T cell therapy are associated with the activation of

neoantigen reactive T cells<sup>[122]</sup>. To improve the efficacy of the T cell therapy, engineering TILs to express the neoantigen-specific TCR can be a promising next-generation immunotherapy drug<sup>[124]</sup>. However, to develop these engineered T cells, identifying paired sequences of both TCR  $\alpha$  and  $\beta$  chains from the vast repertoire of TCRs is a challenge. One way to overcome this challenge is to perform, single-cell TCR profiling to obtain paired TCR  $\alpha/\beta$  sequence information<sup>[125]</sup>. Using patient samples, neoantigen specific CD8 T cells were clonally expanded *in vitro* and multiple paired TCR sequences were identified by single-cell analysis<sup>[124]</sup>. Importantly, the transduced T cells expressing TCRs recognized the neoantigen presented by autologous antigen-presenting cells<sup>[124]</sup>. Another study using single-cell TCR repertoire analysis revealed that clonally expanded CD8 T cells were antigen-specific and showed cytotoxic activity against tumors in mouse models<sup>[126]</sup>. Intriguingly, the combination of 10x Genomics' single cell TCR sequencing platform coupled to gene expression holds enormous potential for assessing and monitoring patient response to cancer vaccines and immunotherapy drugs.

#### *Monitoring the functional state of CD8 T cells*

In the tumor microenvironment, the ability of CD8 T cells to secrete pro-inflammatory cytokines and exert cytotoxic function can be compromised during persistent immune activation<sup>[127]</sup>. Such exhausted CD8 T cells differ profoundly from memory CD8 T cells and co-express multiple co-inhibitory immune checkpoint regulators such as PD-1, LAG-3, and TIM-3 and lack successful anti-tumor immune response<sup>[127,128]</sup>. Even though various checkpoint inhibitors show clinical efficacy by unleashing cytotoxic T cells activity, a large fraction of patients fails to respond to these immunotherapies<sup>[129]</sup>. Therefore, a detailed understanding of the mechanisms of CD8 T cell exhaustion is required. Further, since the transcriptional signatures of T cell exhaustion are closely intertwined with their activated T cell state, single-cell analysis is an optimal approach to identify biomarkers specific to T cell dysfunction. In a single-cell RNA-seq analysis of T cells from hepatocellular carcinoma patients, 11 unique T cell subsets were identified based on their molecular and functional properties<sup>[130]</sup>. Exhaustion signature gene *LAYN* was identified and associated with inhibition of IFN- $\gamma$  production<sup>[130]</sup>. A single-cell RNA-seq of CD8 tumor-infiltrating lymphocytes from murine tumor models has also aided identification of novel molecular pathways of T cell exhaustion that is uncoupled from T cell activation<sup>[131]</sup>.

#### *Profiling of immune suppressive cell types present in the tumor microenvironment*

Single-cell transcriptome profiling enables characterization of the complex tumor microenvironment with its heterogeneous mixture of tumor cells along with stromal and immune cells<sup>[132]</sup>. Targeting of immunosuppressive cell types in the tumor microenvironment can sometimes be key to the efficacy of checkpoint inhibitors such as anti-CTLA-4 therapy. A variety of cell types including T regulatory cells (Tregs), tumor-associated macrophages, type 2 NKT cells, M2 macrophages and MDSCs enforce immune suppression in the tumor helping tumor cells to survive anti-tumor immune attack<sup>[133]</sup>. Identifying MDSCs has been challenging from bulk sequencing data due to the absence of unique MDSC markers. In addition, the presence of over 10 different myeloid subsets further complicates bioinformatics analysis<sup>[134]</sup>. Tregs are potent immune modulators and assessing their frequency, phenotype, and function at tissue sites has been profoundly challenging due to the fact that majority of the defining markers like CD25, FOXP3 and CTLA4 are also present in effector T cells<sup>[135]</sup>. Single-cell analysis of tumor infiltrated immune cells can help circumvent some of these hurdles in tumor characterization. In a recent single-cell analysis study tumor cells from 11 breast cancer patients, cancer cells were separated from immune cells based on their copy number variations<sup>[132]</sup>. Analysis of the immune cell fraction revealed the presence of immunosuppressive macrophages of M2 phenotype and activated T effector cells. Interestingly, the T cells also expressed markers of T cell exhaustion such LAG3 and TIGIT suggesting that they could be targeted by immune checkpoint inhibitors<sup>[132]</sup>.

#### **Understanding mechanisms of disease resistance**

Resistance to chemotherapy and molecularly targeted therapies is a major barrier to achieving long-term

benefit to treatment. ITH arising from diverse cell subpopulations with distinct molecular features produce varying levels of drug sensitivity and resistance<sup>[16]</sup>. Retrospective analysis of CTCs from patients who had developed resistance to inhibitors of the androgen receptor (AR) showed higher activation of non-canonical Wnt pathway beside altered expression and mutations in AR compared to untreated patients<sup>[136,137]</sup>. In castrate-resistant prostate cancers high content single-cell longitudinal profiling of CTCs from a patient undergoing chemotherapy and targeted therapy revealed a selective clonal expansion of cells with AR amplification supporting the adaptive model of therapy resistance evolution<sup>[137]</sup>. Similar observation of selective clonal persistence was seen in breast cancer patients treated with chemotherapy. In this study, single-cell sequencing post-chemotherapy revealed transcriptional reprogramming of resistant signatures, elucidating the mechanism of therapy resistance<sup>[32]</sup>.

Based on aforementioned studies, an accurate assessment of ITH by single-cell sequencing using multi-regional, longitudinal sampling is essential to understand the mechanism of drug resistance and facilitate the development of more effective therapies.

## **FUTURE DIRECTIONS**

With the development of precision microfluidic devices and sequencing technologies, single-cell analysis has transformed our understanding of ITH and clonal evolution. Single-cell genomics promises to deconvolute complex biological processes in cancer, reveal epigenetic alterations and monitor the evolution of metastatic and treatment resistance clones. By applying single-cell sequencing to different experimental systems, such as cells in culture, patient-derived xenografts, murine models and analysis of human tumors, novel diagnostics and therapies can be developed. A major hurdle in single-cell sequencing is the high cost of the technology. Moreover, the volume and complexity of single-cell sequencing datasets exceed that of the traditional bulk sequencing, calling for better statistical algorithms to deconvolute the data. Additional caution should be given on the transcriptome coverage and number of cells taken for single-cell analysis to ensure the accuracy of gene expression distribution estimates. Future breakthroughs in developing cost-effective sequencing methods and powerful data analysis pipeline for single-cell sequencing are likely to expand the scope of this technology beyond cancer to other diseases.

## **DECLARATIONS**

### **Authors' contributions**

Manuscript drafting: Shi X, Chakraborty P, Chaudhuri A

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Not applicable.

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All authors are full-time employees of MedGenome Inc.

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Not applicable

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Editorial

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# Introduction to the special issue on reviews of gastric cancer metastasis and treatment

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It is a great honor for me to introduce the special issue entitled reviews of Gastric Cancer Metastasis and Treatment. I've been focusing on 6 topics including tumor microenvironment (TME), biomarker research, regional variation in gastric cancer treatment, diagnosis and treatment for peritoneal carcinomatosis (PC), surgical treatment for advanced or metastatic gastric cancer, and novel treatment modalities. This special issue includes 18 review articles concerning these topics.

TME has been proven to be deeply implicated in tumor progression and metastasis in gastric cancer. Sawayama *et al.*<sup>[1]</sup> gave a comprehensive overview of the functions of each component of TME and reviewed the clinical impact of the alteration of TME. Cancer stem cells (CSCs) are known to be the main reason for resistance to anticancer agents as well as for the development of distant metastasis. Uchihara *et al.*<sup>[2]</sup> reviewed the impact of the TME on gastric CSCs.

Biomarkers play an increasingly important role in the clinical management of cancer patients. Nakamura *et al.*<sup>[3]</sup> reviewed recent progress in technology for specific enrichment and detection of circulating tumor cells (CTCs) that contribute to the diagnosis and treatment of gastric cancer. Liquid biopsy using CTCs and cell-free nucleic acids are considered as a tool that enables individualized or precision medicine. MicroRNAs (miRNAs) are short noncoding RNAs that post-transcriptionally regulate gene expression. Komatsu *et al.*<sup>[4]</sup> reviewed the recent biological and clinical research on the circulating miRNAs of gastric cancer and discussed the future perspectives.

There are regional differences in recommended treatment for gastric cancer. Kamiya *et al.*<sup>[5]</sup>, Karolinska



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University in Sweden, reviewed current trend in gastric cancer treatment in Europe. In Europe, perioperative chemotherapy is the standard care for locally advanced gastric cancer. The regimen for the perioperative chemotherapy has shift from the Medical Research Council Adjuvant Gastric Infusional Chemotherapy (MAGIC) regimen (Epirubicin, cisplatin, 5-fluorouracil/Epirubicin, Cisplatin, Capecitabine) to the fluorouracil, folinic acid, oxaliplatin, taxotere (FLOT) triplet. Harada *et al.*<sup>[6]</sup>, University of Texas, M.D., Anderson Cancer Center, summarized recent trend in gastric cancer in the USA. In the USA, postoperative chemoradiation is one of the standard care for locally advanced tumors. When cancer progresses after the first line therapy, additional biomarkers, including microsatellite instability (MSI) and programmed death-ligand 1 (PD-L1) should be tested for the screening of candidates for the checkpoint inhibitors. Eto *et al.*<sup>[7]</sup>, Cancer Institute Hospital in Japan, reviewed recent publications and guidelines focusing on the progress in treatment of metastatic gastric cancer in Japan. The incidence of adenocarcinoma in the esophagogastric junction (EGJ) has been increasing rapidly, especially in Western countries. Although treatment for EGJ adenocarcinoma has been developed as a type of gastric cancer, recent comprehensive molecular analysis revealed differences in molecular mechanisms between EGJ and gastric adenocarcinomas. Toihata *et al.*<sup>[8]</sup> reviewed recent evidence of treatment for advanced EGJ adenocarcinoma.

PC is frequently observed in patients with advanced gastric cancer and is considered to be an incurable disease. Hu *et al.*<sup>[9]</sup> reviewed the molecular mechanisms of three steps in the development of PC, including detachment from the primary tumor, adaptation to the microenvironment of the peritoneal cavity, and attachment to peritoneal mesothelial cells. Peritoneal lavage cytology (PLC) has been shown to be an independent predictor of cancer relapse after curative gastrectomy and poor prognosis. Matsuoka and Yashiro<sup>[10]</sup> reviewed the clinical roles and attributes of PLC in gastric cancer. Sugarbaker<sup>[11]</sup> summarized the role and efficacy of neoadjuvant systemic chemotherapy, neoadjuvant intraperitoneal and systemic chemotherapy, cytoreductive surgery, and perioperative chemotherapy including hyperthermic intraperitoneal chemotherapy and/or early postoperative intraperitoneal chemotherapy as prevention or treatment for PC. Macedo *et al.*<sup>[12]</sup> introduced pressurized intraperitoneal aerosol chemotherapy as a treatment option for PC.

Pergolini *et al.*<sup>[13]</sup> performed a systematic review of literature on surgical resection for metastatic gastric cancer. Survival benefit of surgery in advanced gastric cancer is still unclear. Surgery may play an important role in highly selected patients. However, further randomized controlled trials are necessary to clarify the actual impact of surgery in these patients. Recent advances in chemotherapy enabled conversion surgery for patients with initially unresectable gastric cancer. Ida and Watanabe<sup>[14]</sup> reviewed the treatment strategies for stage IV gastric cancer and discussed the potential efficacy of conversion surgery. Pancreaticoduodenectomy (PD) is the only possible treatment for achieving R0 resection when a tumor and/or lymph node metastasis directly invades the pancreatic head or infiltrates the duodenum. However, the efficacy and safety of PD for advanced gastric cancer remain unclear. Makuuchi *et al.*<sup>[15]</sup> reviewed the literatures on PD for gastric cancer and their own experience.

Recently, targeting therapies and immune checkpoint blockade have been introduced into gastric cancer treatment. Kiyozumi *et al.*<sup>[16]</sup> summarized the latest knowledge of focused common cancer targets, signaling pathways, targeting therapies, and immunotherapies for gastric cancer. The late-phase complication of the large-extent of gastric resection negatively influences patients' quality of life. Takeuchi and Kitagawa<sup>[17]</sup> introduced current status of sentinel lymph node (SN) biopsy and function-preserving gastrectomy based on the SN biopsy. Robotic assisted surgery is increasingly performed for many types of cancers. Tokunaga *et al.*<sup>[18]</sup> reviewed the comparative retrospective and prospective studies which have investigated the difference in short- and long-term outcomes between robotic gastrectomy and laparoscopic gastrectomy.

I would like to express my sincere gratitude to Professor Lucio Miele, Editor-in-Chief, Journal of Cancer Metastasis and Treatment, for giving me this opportunity. I would like to thank all of the contributing au-



thors for their hard work in producing the articles. I also thank Professor Hideo Baba, Department of Gastroenterological Surgery, Kumamoto University, for his guidance and support for this project. I am very happy if you enjoyed this special issue.

## DECLARATIONS

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Review

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# NSAID celecoxib: a potent mitochondrial pro-oxidant cytotoxic agent sensitizing metastatic cancers and cancer stem cells to chemotherapy

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## ABSTRACT

Intermittent hypoxia within tumor microenvironments causes pro-oxidative stress impairing oxidative phosphorylation (OxPhos) and increases mitochondrial production of reactive oxygen species (ROS). In primary tumors this provokes metabolic reprogramming of both tumor cells and cancer stem cells and emergence of highly metastatic cancer cells. Tumor reprogramming is initiated by activating nuclear respiratory factors and hypoxia-inducible factors in response to changes in oxygen and ROS levels. Hence, hypoxia-induced pro-oxidative stress drives invasion and metastasis. However, it is also the Achilles' heel of metastatic cancer cells because pro-oxidative agents further overload the mitochondria and intracellular milieu with excessive ROS to trigger apoptosis, whereas antioxidant agents promote their survival and tumor progression. Herein lies the metastatic tumor cell sensitivity to non-steroidal anti-inflammatory drugs (NSAIDs) and we and others have shown that the NSAID celecoxib exerts powerful pro-oxidative anticancer effects by directly targeting mitochondria to increase ROS production and trigger cancer cell death, including metastatic cancer cells and cancer stem cells. This review highlights the considerable benefits from appropriate NSAID use in humans against post-diagnosis metastatic tumors and the need to further develop their use as adjuvant therapy for advanced stage metastatic disease where they are already showing significantly improved clinical outcomes.

**Keywords:** Non-steroidal anti-inflammatory drug, celecoxib, metastasis, anticancer, mitocans, chemosensitizing, cancer stem cells, therapy



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## INTRODUCTION

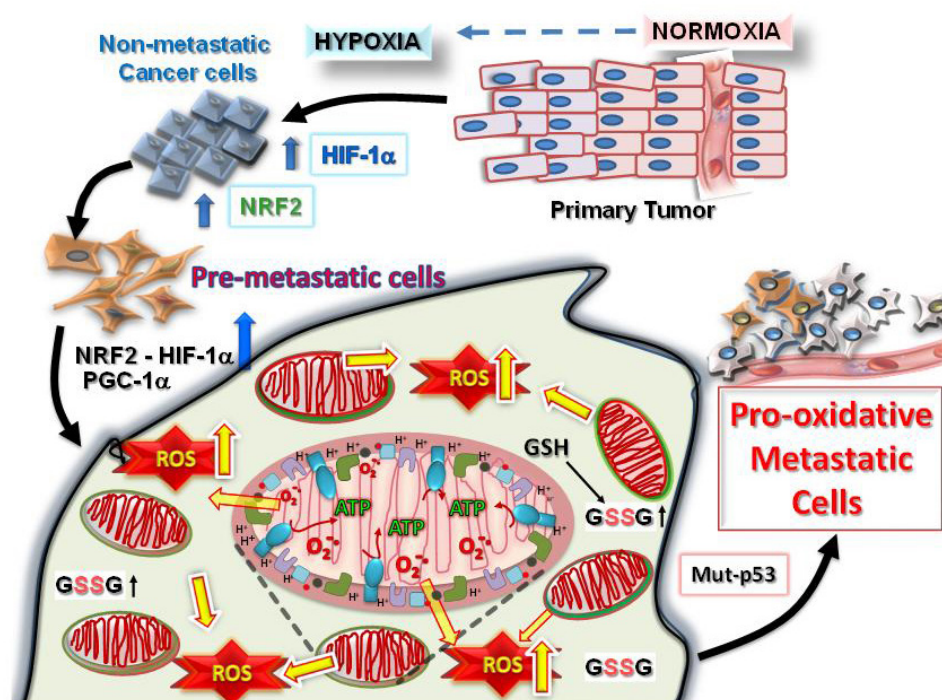
Cancer cells and their mitochondria adapt to higher levels of oxidative stress as they emerge from the primary tumor to become circulating tumor cells and migrate into the metastatic distant tissues<sup>[1-5]</sup>. It is clear that emerging metastatic cancer cells have undergone not only significant genetic but also metabolic changes including activation of their antioxidant systems which promote their survival by helping to detoxify heightened reactive oxygen species (ROS) levels to enable eventual metastatic outgrowth into diverse sites. In the first part of this review, the evidence for these changes in redox homeostatic mechanisms identified for reprogramming into highly metastatic cells and cancer stem cells are discussed. The second part of the review is focused on how drugs such as non-steroidal anti-inflammatory drugs (NSAIDs) like celecoxib are able to take advantage of and target the pro-oxidative state of highly metastatic cancer cells and cancer stem cells to force them into terminal states of ROS excess thereby activating cell death.

### **The metastatic potential of cancer cells is regulated by their redox status and ROS levels**

Several lines of independent evidence have established that the metastatic potential of tumor cells and cancer stem cells are directly related to their heightened redox status and greater intrinsic capacity for ROS production<sup>[1,5,6]</sup> which becomes particularly important for metastasis<sup>[2,3]</sup> [Figure 1]. The conditions that cause this development are the culmination of hypoxia generated in expanding primary tumors and resulting oxidative stress upregulating the expression and activation levels of two essential transcription factor families which allow cancer cells to cope with heightened ROS levels. These are the nuclear E2-related factors [a.k.a. nuclear respiratory factors (NRFs) as key regulators of the antioxidant and cytoprotective genes]<sup>[7]</sup>, that in turn increase expression of hypoxia-inducible factors (HIF's)<sup>[8]</sup>. Both the NRF and HIF families of factors act as crucial rheostat regulators of the redox state, affected by ROS levels in cells, and have both been shown to combine together and perform key roles in tumor survival and progression under hypoxia<sup>[8]</sup>. The question is whether it is best to increase or decrease ROS as an anticancer therapeutic strategy<sup>[6]</sup>. However, before addressing the question of anticancer therapeutic strategy, the next sections of the review focus on how increased ROS levels reprogram to sustain a heightened state of ROS production and greater metastatic potential.

These events are the consequence of major changes occurring at the level of gene expression during this adaptation process and reprogramming which results when the master transcriptional regulator, nuclear respiratory factor 2 (NRF2) becomes activated and is released from the mitochondrial outer membrane to the cytosol [Figure 2]. Upon its release NRF2 transits to the nucleus to form heterodimers with other basic leucine zipper (BZIP) family members (such as Maf), binding to antioxidant response elements (AREs) in the promoter regions activating the NRF2 target genes<sup>[8]</sup>. Amongst the over 500 NRF2 target genes are many encoding proteins that collectively promote malignant cancer cell survival, such as detoxifying enzymes, antioxidant enzymes [including several key proteins of both the reduced glutathione (GSH) and thioredoxin (Trx) systems], receptors, transcription factors, metabolic enzymes, p-Akt, proteases, and many more (reviewed in<sup>[7]</sup>). NRF2 activation can cause increased mitochondrial mass<sup>[4,9,10]</sup> and induction of peroxisome proliferator-activated receptor gamma coactivator 1-alpha (PGC1 $\alpha$ ) and PGC1 $\beta$ ; PGC1 $\alpha$  together with NRF2 stimulate the expression of the related gene NRF1 (reviewed in<sup>[11]</sup>). PGC1 $\alpha$  has been shown to form complexes with NRF2 as a transcriptional coactivator and promotes NRF2 and NRF1 binding to the manganese superoxide dismutase, SOD2 gene promoter<sup>[12]</sup>. Consequently, NRF1 activates nuclear genes that encode mitochondrial proteins, including mitochondrial transcription factor A (TFAM), promoting mitochondrial biogenesis<sup>[4,13]</sup> such that the tumor cells are modified to adopt increased pro-oxidative states with greater malignant potential<sup>[2,14]</sup> [Figure 3].

Early studies showed PGC1 $\alpha$  to be a potent stimulator of mitochondrial respiration and gene transcription in liver, heart, and skeletal muscle, activated under oxidative stress<sup>[15]</sup>. It is proposed that mitochondria,

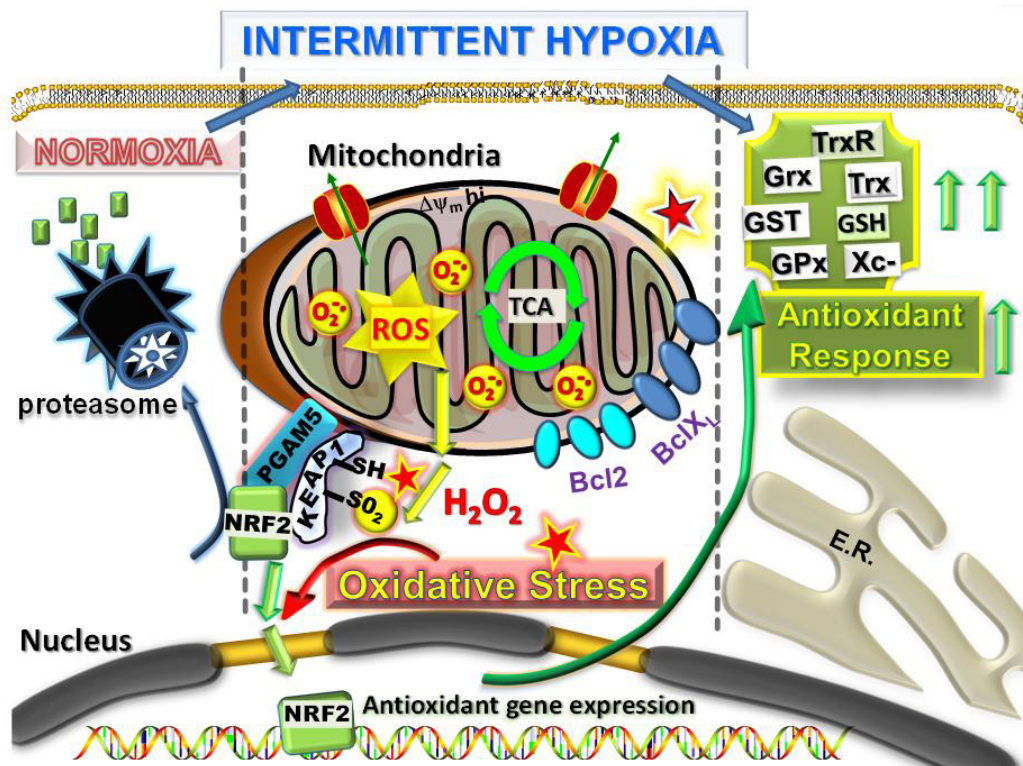


**Figure 1.** Tumorigenesis requires hypoxic driven metabolic reprogramming for metastatic progression. The progression of malignant states during carcinogenesis involves changes occurring within the tumor microenvironment, including regions of hypoxia, hypoglycemia and acidosis. These regions are where cancer cells evolve into pre-metastatic states after selection by the harmful conditions, resulting in the altered capacity for increased ROS production and protection from the greater oxidative stress. Eventually, other mutations (such as in cell cycle regulatory proteins, p53 or ARF) occur which allow the cells to adapt by further metabolic reprogramming to emerge as highly pro-oxidative metastatic cells. HIF: hypoxia-induced factor; PGC1α: peroxisome proliferator-activated receptor gamma coactivator 1-alpha; NRF: nuclear respiratory factor; ROS: reactive oxygen species

as an energy center important for cellular homeostasis, undergo biogenesis as an endogenous protective response mechanism designed to cope with ischemic/hypoxic insults and to counteract their detrimental effects. In either normal cells, SH-SY5Y neuroblastoma or immortalized mouse myoblast C2C12 cells undergoing oxidative stress, wild type p53 levels were shown to increase within several hours to form a complex with coactivator PGC1α and activate genes such as NRF1 and NRF2 but without affecting proliferation<sup>[16]</sup> [Figure 3]. Ischemia in the brain has been shown to increase mitochondrial DNA, total mitochondrial number and expression of the mitochondrial transcription factors downstream of PGC1α (including NRF1 and TFAM), whereas the ensuing reperfusion increases oxidative stress and mitochondrial biogenesis<sup>[17]</sup>. PGC1α is a powerful controller of cell metabolism and maintains a balance between production and scavenging of pro-oxidant molecules by coordinating mitochondrial biogenesis, promoting oxidative phosphorylation [OxPhos, i.e., mitochondrial adenosine triphosphate (ATP) synthesis] and the expression of antioxidants like GSH, although the exact role of PGC1α in cancer is unclear with no consistent relationship<sup>[18]</sup>.

In a study of breast cancer cells, PGC1α expression and activation were shown to significantly increase mitochondrial biogenesis and OxPhos to promote metastasis<sup>[19]</sup>, and increased PGC1α levels were detected in the circulating tumor cells and metastases from a range of different murine cancer models (4T1, B16F10 and MDA-MB-231) compared to levels in the corresponding primary tumors. PGC1α was linked with greater levels of migratory/invasive cancer cells, increased mitochondrial copy number, respiration and OxPhos [Figure 3]. Silencing PGC1α in the breast cancer cells severely decreased copy number of mitochondrial DNA and visible mitochondria within the cells, suspended their invasive potential and attenuated metastasis without affecting proliferation, primary tumor growth or the epithelial-to-

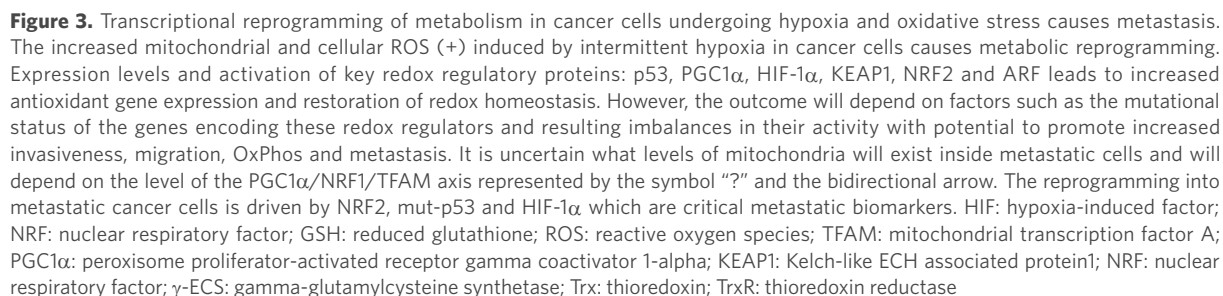




**Figure 2.** Intermittent hypoxia within the primary tumor microenvironment as a driver of mitochondrial ROS production and metastatic reprogramming. Mitochondrial ROS is produced extensively in cells undergoing rounds of intermittent hypoxia. In a similar manner to ischaemia/reperfusion in normal cells, the intermittent hypoxia of early primary cancer cells causes readjustments in redox homeostasis by increasing ROS activated NRF2 release from the outer redox hub (KEAP1/PGAM5) on the mitochondria, NRF2 transport to the nucleus and transcriptional activation of a large number of antioxidant defense response, including the GSH and Trx systems to counteract and detoxify the ROS. In addition, Bcl-2 and Bcl-XL are stabilized to promote cell survival under the conditions of pro-oxidative stress. NRF: nuclear respiratory factor; ROS: reactive oxygen species; Trx: thioredoxin; GSH: reduced glutathione

mesenchymal transition<sup>[19]</sup>. Unfortunately, this study did not compare mitochondrial ROS production or oxidative stress between primary and metastatic tumors. However, in another study of renal carcinoma, overexpressing PGC1 $\alpha$  in Von Hippel-Lindau (VHL) gene defective, constitutive HIF expressing clear cell renal cell carcinoma (ccRCC) impaired cancer cell growth and upregulated expression of antioxidant enzymes, but also showed greater ROS levels and oxidative stress<sup>[20]</sup>. The HIFs were shown to directly inhibit PGC1 $\alpha$  activity or its expression, reducing oxygen consumption and increased stabilization of HIF1 $\alpha$  protein caused a switch in metabolism away from PGC1 $\alpha$  driven OxPhos to increased glycolysis<sup>[20]</sup>.

It would appear that the reasons for differences in the PGC1 $\alpha$  relationship amongst different cancers may depend upon their levels of other factors such as expression of the HIF's as inhibitors vs. other PGC1 $\alpha$  coactivators such as p53, a transcriptional activator and interactive binding partner of PGC1 $\alpha$  [Figure 3]. For example, PGC1 $\alpha$  mRNA levels were substantially higher in wild-type p53 lung cancer cell lines compared to cell lines with p53 loss or missense mutations and siRNA knockdown of PGC1 $\alpha$  inhibited cell proliferation in wild-type p53 lung cancer cell lines<sup>[21]</sup>. These results are consistent with p53 binding the PGC1 $\alpha$  gene promoter, increasing expression<sup>[16]</sup>, thereby protecting the cells after promoting ROS detoxification capacities to enable cancer cell survival under states of oxidative stress<sup>[22]</sup>, particularly stress from mitochondrial ROS<sup>[23]</sup>. The increased PGC1 $\alpha$  complexes with p53, modifying transactivating function [Figure 3] to cause cancer cell cycle arrest and activation of metabolic target genes, promoting ROS clearance in response to metabolic stress, such as from low glucose<sup>[24]</sup>. However, loss of PGC1 $\alpha$  expression prevents the p53-mediated ROS clearance, instead enhancing p53-dependent cancer cell apoptosis<sup>[24]</sup>. Hence, when GSH levels are



depleted by the gamma-glutamylcysteine synthetase ( $\gamma$ -ECS) inhibitor, buthionine sulfoximine (BSO) or other metabolic oxidative stress, increased p53 binds to the PGC1 $\alpha$  gene promoter to increase its expression and together the complex then promotes cellular antioxidant defenses *via* NRF2-mediated expression of antioxidant enzymes such as SOD2 and c-GlutamylCysteine Ligase [a.k.a.  $\gamma$ -ECS, Catalytic subunit of the  $\gamma$ -ECS (a.k.a.  $\gamma$ -GCL) enzyme required in the first step of GSH synthesis], increasing GSH synthesis to restore redox homeostasis<sup>[16]</sup>. Knocking down either p53 or PGC1 $\alpha$  prevented induction of SOD2 or  $\gamma$ -ECS<sup>[16]</sup>. Hence, loss of p53 function would restrict the ability of cells to defend against oxidative stress. In the latter study, it should be noted that under conditions where NRF2 was activated by pro-oxidative stress using BSO to scavenge GSH, no change was detected in mitochondrial biogenesis and neither NRF1 nor TFAM was altered at the protein or mRNA levels<sup>[16]</sup>. Based on the results of this study, it follows that cancer cells with lower GSH/GSSG ratios and a more pro-oxidative status such as that commonly found in metastatic tumor cells<sup>[1-3,5,6]</sup>, particularly where p53 is either lost or mutated, will not show a strong PGC1 $\alpha$  response. This is supported by studies of vascular smooth muscle cell responses to oxidative stress (1 mmol/L diethylenetriamine/nitric oxide adduct (DETA/NO) as a nitric oxide donor for 24 h) comparing p53 wild type

to p53 null cell responses where higher ROS (2.7 fold greater increase) was produced in cells from the wild type p53 mice<sup>[25]</sup>. The question then is how does the metastatic cancer cell cope with the heightened level of oxidative stress, and whether this is related to changes in p53 function.

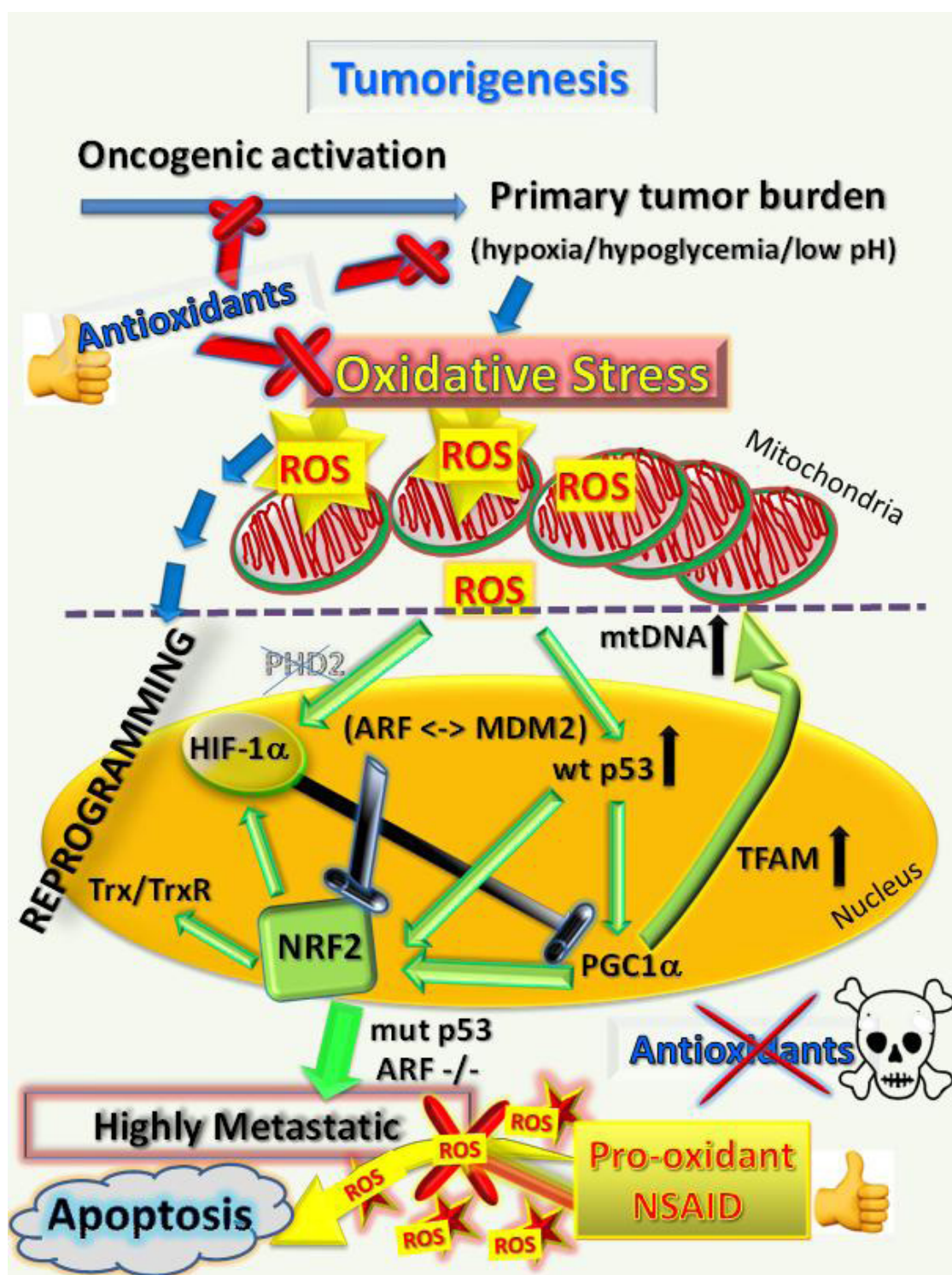
Recently, mutant p53 (mut-p53) was shown to interact with NRF2, increasing p53/NRF2 complexes on select antioxidant response element (ARE) containing gene promoters to activate transcription of a specific set of genes, whilst repressing most others<sup>[26-28]</sup>. In particular, the Trx gene (*TXN*) is unusual along with the thioredoxin reductase (*TXNRD1*) as mut-p53 activated NRF2 target genes enhancing the Trx system with pro-survival and pro-migratory functions in breast cancer cells under oxidative stress, while heme oxygenase 1 (*HMOX1*) is a mut-p53 repressed target displaying opposite effects<sup>[26]</sup>. Mut-p53 appears to sequester NRF2 preventing its activity on most of the NRF2 regulated genes impairing its canonical antioxidant activities, directly promoting greater ROS accumulation in cancer cells by inhibiting expression of the glutamate/cystine antiporter solute carrier family 7 member 11 (SLC7A11, also called xCT), a component of the cystine/glutamate antiporter as part of the Xc- system, diminishing cytosolic production of GSH<sup>[27,28]</sup>. Analysis across a cancer cell panel for accumulation of mut-p53 protein showed a significant association with increased basal mitochondrial and cytosolic ROS levels, and decreased endogenous GSH reserves. Also, consistent with this observation, by overexpressing mut-p53 in cancer cells, system Xc- activity and GSH levels were diminished resulting in a heightened level of ROS stress. In contrast, knockout of mut-p53 decreased basal ROS levels and conferred protection against H<sub>2</sub>O<sub>2</sub><sup>[27,28]</sup>. Hence, highly metastatic cancer cells with a mutant form of p53 or as p53 null cells which commonly occurs in advanced stages of malignancy would account for many of the cell phenotypes with greater mitochondrial ROS production [Figures 3 and 4], reflected by their lower or higher PGC1 $\alpha$  levels and related changes in mitochondrial mass.

ARF, the regulator of p53 protein levels in cells by binding the mouse double minute 2 (MDM2) homolog to prevent p53 turnover, also exerts its influence in regulating the NRF2 antioxidant system, also binding NRF2 to inhibit its activation, wherein ARF/NRF2 association prevents cAMP-response-element-binding protein (CREB) dependent acetylation of NRF2 and binding to target DNA<sup>[29]</sup>. Hence, ARF/NRF2 significantly represses NRF2 transcriptional activity and expression of SLC7A11 component of the cystine/glutamate antiporter Xc-system is dramatically suppressed when ARF is activated, again affecting GSH production and causing increased cellular ROS levels in cancer cells, much as mut-p53 does as outlined above. Hence, depending on the status of ARF and p53 mutations or deletions in cancer cells, NRF2 activation would be expected to be heightened in their absence, particularly in cellular states of elevated pro-oxidative stress *via* Kelch-like ECH associated protein1 (KEAP1) inactivation, potentially setting up a self-perpetuating scenario maintaining higher endemic intracellular ROS levels. Even in cells with wild type p53, activated NRF2 binds to an ARE in the MDM2 gene increasing expression<sup>[30]</sup> that in turn, promotes p53 ubiquitination and proteasomal degradation. Transcription of the SQSTM1 gene encoding the p62 autophagy regulatory protein is also stimulated by NRF2<sup>[31]</sup>. In turn, p62 sequesters KEAP1, thereby increasing NRF2 abundance<sup>[31]</sup> in another self-promoting cycle. Moreover, depending on the levels of oxidative stress, NRF2 together with mechanistic target of rapamycin (mTOR) serine/threonine kinase and adenosine-monophosphate-activated-protein-kinase (AMPK) coordinate alternative autophagy dependent pathways, with lower stress levels promoting survival whereas higher stress results in cell death<sup>[32]</sup>.

### **Chemoprevention of carcinogenesis mediated *via* the KEAP1-NRF2 redox sensory hub**

When cells undergo hypoxia, mitochondrial ROS production is promoted in the short term as a by-product from the respiratory chain<sup>[33,34]</sup>. Consequently, a number of events ensue ultimately resulting in greater activation of NRFs and HIFs. One of the main cell sensors of the oxidative stress resides on the outer mitochondrial membrane, comprising a ternary protein complex anchored *via* PGAM5





**Figure 4.** Antioxidants are effective cancer chemopreventatives until cancer becomes established, when they promote metastatic progression, whereas NSAIDs as pro-oxidants are effective by overloading cancer cells with excessive ROS to eliminate metastases. Once tumors are established, antioxidants will support and promote the further progression of cancer cells to metastasize. However, the opposing pro-oxidants such as the NSAID celecoxib cause excessive ROS overload and induce mass cell death in metastases or sensitize metastatic cells to enhanced chemotherapeutic killing. ROS: reactive oxygen species; HIF hypoxia-induced factor; NSAID: non-steroidal anti-inflammatory drug; Trx: thioredoxin; TrxR: thioredoxin reductase

(histidine mediated serine/threonine phosphatase)<sup>[35]</sup>. This complex act as a critical redox sensory hub consisting of the bound protein, KEAP1 with many redox regulated thiol-cysteine residues in its structure available for modification by electrophilic agents, ROS mediated oxidation or other xenobiotic compounds. The PGAM5-KEAP1 master redox controller on mitochondria is a pivotal regulatory complex involved in the actions of chemopreventive agents that inhibit the development of chemically-induced carcinogenesis [Figure 2]. The role of the KEAP1 complex in cancer has already been previously extensively reviewed<sup>[36-40]</sup> and it will not be reviewed further except in relation to mechanisms of action by anticancer chemopreventative agents.

The KEAP1 cysteine thiol residues with low pKa values are especially reactive with chemopreventatives such as the isothiocyanates<sup>[41]</sup>. At physiological pH, these cysteines exist as thiolate anions that are primed for nucleophilic attack by such electrophilic agents (termed inducers) (reviewed in<sup>[42]</sup>). These thiol modifications disrupt the function of KEAP1 as an anchor or tether holding KEAP1 binding proteins such as NRF2, Bcl2 or Bcl-X<sub>L</sub> in a complex on the outer membrane with PGAM5, as a histidine phosphatase involved in regulating these interactions [Figure 2]. KEAP1 is a redox-regulated substrate adaptor protein for the Cul3 E3 ubiquitin ligase and together this complex responds to oxidative stress by controlling availability of NRF2, as well as Bcl-2 and Bcl-X<sub>L</sub> pro-survival proteins. Hence, under normal redox conditions, KEAP1 targets NRF2, Bcl-2 and Bcl-X<sub>L</sub> for ubiquitination and proteasomal degradation (reviewed in<sup>[43]</sup>). Upon cellular/mitochondrial oxidative stress, KEAP1 thiols are oxidized and inactivated preventing substrate ubiquitination, allowing NRF2, Bcl2 and Bcl-X<sub>L</sub> stabilization and release [Figure 2]. The pro-survival proteins Bcl-2 and Bcl-X<sub>L</sub> then protect the mitochondria by preventing apoptosis while NRF2 migrates to the nucleus where it activates cellular antioxidant defense genes to restore redox homeostasis<sup>[29]</sup>. Therefore, the KEAP1 hub is a major regulator of the normal host cell defense responses against oxidative stress involved in maintaining the cellular redox balance.

In this regard, KEAP1 functions as a tumor suppressor protein to prevent tumor progression by negatively regulating substrates NRF2, IKK $\beta$  and Bcl-2/Bcl-X<sub>L</sub>, consistent with KEAP1 function as a guardian against cancer<sup>[40,44]</sup>. However, when subjected to increased levels of pro-oxidative stress and enhanced ROS levels, irreversible modification of the KEAP1 master regulator either *via* successively more severe chemical oxidation reactions (such as the irreversible transformation of the thiol-cysteine derived sulfenic acid into sulfinic or sulfonic acids, Figure 2) or genetic mutations or other chemical modification will occur. The end result is blocking of KEAP1 function, constitutive NRF2 activity and increased Bcl-2/Bcl-X<sub>L</sub> availability which together with adaption to heightened ROS levels is commonly found inside highly metastatic tumor cells<sup>[1-3,5,6]</sup>. At this point, use of chemopreventive agents targeting the KEAP1-NRF2 master complex will be either ineffectual or could even promote more rapid tumor progression to increase metastatic disease, as outlined below.

### **Constitutive NRF2 mediated HIF activation and reprogrammed state of emerging metastatic cancer cells**

Mutation and dysregulation of the NRF2-KEAP1 pathway are common events in cancer (reviewed in<sup>[40,44]</sup>) and KEAP1 inactivating somatic mutations have been detected in numerous cancer cells (reviewed in<sup>[40]</sup>). A study by the Cancer Genome Atlas Research Network reported that > 30% of squamous lung carcinomas have alterations in the NRF2-KEAP1-CUL3 pathway that result in high, constitutive expression of NRF2 and that somatic mutations in NRF2 will disrupt its interaction with KEAP1<sup>[45]</sup>. Epigenetic silencing of KEAP1 by hypermethylation of its promoter is found in 53% of colorectal cancers<sup>[46]</sup>. Thus, the constitutive activation of NRF2 common to metastatic cancer cells will result in many major modifications including altering the mitochondrial and cytosolic metabolism critical for tumor survival and metastasis<sup>[7]</sup>. NRF2 activation prevents cancer initiation<sup>[47,48]</sup> but in tumor cells promotes cancer progression as invasion,



migration<sup>[49-51]</sup> and metastasis<sup>[52-54]</sup>, and also induces multidrug resistance to chemotherapy by upregulating expression of the multidrug resistance proteins (MDR)/P-glycoprotein/ABC drug transporters<sup>[55,56]</sup>. In this regard, NRF2 functions in a similar manner during ischemia-reperfusion of normal tissues in the body<sup>[57]</sup> as well as in the metabolic shift that occurs during induced pluripotent stem cell colony formation with reprogramming after an initial burst of OxPhos and increased ROS production which increases NRF2 before a temporal peak in HIF-1 mediated glycolysis and shuttling *via* the pentose phosphate pathway<sup>[58]</sup>. In a related fashion a recent study showed that NRF2 activation also promotes the Warburg effect and stemness-associated properties of cancer-initiating cells<sup>[59]</sup>.

Although it is well known that NRF2 and HIF-1 signaling are both regulated in response to intermittent hypoxia and ROS accumulation [Figures 2 and 3], the evidence suggests that these two signaling pathways are not simply linked by cellular context but interact to promote metastasis and play complementary roles. For example, in the state of chemoresistance induced by hypoxia they both increase expression of the multidrug resistance MDR/P-glycoproteins (reviewed in<sup>[8]</sup>). The HIF-1 promoter contains two AREs and has been shown to be negatively regulated by NRF1 but is probably also activated by NRF2 during intermittent hypoxia<sup>[60]</sup>. However, during constant hypoxia, only HIF-1 is increased but not NRF2<sup>[60]</sup>. Hence, when limiting O<sub>2</sub> levels required for OxPhos are available, a build-up in tricarboxylic acid cycle intermediate metabolites (such as fumarate, succinate or oxaloacetate) can occur, which inhibits prolyl hydroxylase (PHD) activity (reviewed in<sup>[61]</sup>). The ensuing still relatively low ROS level (i.e., moderate oxidative stress) produced by the dysfunctional mitochondria in cells under moderate hypoxia also helps inactivate the PHDs<sup>[62]</sup>. The net effect is to prevent the enzymes such as PHD2 from targeting HIF for ubiquitination and proteasomal degradation [Figure 4]. Thus, hypoxia results in stabilization and activation of HIF-1 as part of the homeostatic mechanism to mediate adaptive responses *via* altered gene expression (reviewed in<sup>[63]</sup>).

One critical cell-autonomous adaptive response to hypoxia controlled by HIF-1 is to act as a feedback regulator to lower mitochondrial mass (by inhibiting PGC1 $\alpha$ , as outlined above) and alter mitochondrial and cytosolic metabolism (reviewed in<sup>[61,64]</sup>). Thus, HIF-1 is one of the main factors that mediates the adaptive metabolic responses to hypoxia, increasing glycolytic pathway flux and decreasing flux through the tricarboxylic acid cycle, in order to return mitochondrial ROS production to more normal low levels<sup>[65-67]</sup>. HIF-1 and NRF2 also help mediate increased flux through the serine synthesis pathway and mitochondrial one-carbon (folate cycle) metabolism to increase mitochondrial production of antioxidants (nicotinamide adenine dinucleotide phosphate (NADPH) and GSH). In this manner, HIF and NRF mediated reprogramming functions to protect cells from excessive oxidative stress and levels of mitochondrial ROS production, *albeit* promoting survival of cancer cells with heightened redox and greater oxidative status.

The evidence above provides clear support for mut-p53/NRF2 and HIF-1 in reprogramming metastatic cancer cell metabolism by blocking GSH production while increasing the pentose phosphate shunt in order to provide the NADPH required for Trx production to compensate for GSH loss and to buffer the resulting increased ROS levels in these cells to within a range that is beneficial for tumor progression. In 2015, studies were undertaken that specifically analyzed the importance of these antioxidant pathways and their role in cancer initiation *vs.* progression<sup>[68]</sup>. A range of murine cancer models deficient in *Gclm* (encoding the modifier or regulatory subunit of  $\gamma$ -ECS) showed that the inherent decreased GSH production caused delayed tumor initiation, invasiveness and progression, consistent with observations across a range of human cancers<sup>[68]</sup>. These studies also examined several drugs such as BSO to inhibit  $\gamma$ -ECS either used alone or combined with sulfasalazine (SSA) to block the Xc-glutamate/cystine antiporter and reduce cystine uptake or auranofin (AUR) to inhibit the enzyme TrxR. Early BSO (20 mmol/L) treatment in the drinking water of young animals dramatically reduced breast cancer burdens in the *Gclm*<sup>-/-</sup> mouse models, increasing oxi-

ductive stress damage that was proposed to hinder tumor growth. However, if BSO treatment was delayed until after the onset of tumors, then no differences in tumor development were noted. Primary breast epithelial cells isolated from the *Gclm*<sup>-/-</sup> mice were resistant to BSO because of a compensatory increase in the NRF2-mediated Trx antioxidant pathway, higher CD44, cystine and glutamate levels, as well as increased NADPH, but decreased GSH levels<sup>[68]</sup>. BSO (150 µmol/L) treating human MDA-MB-231 metastatic breast cancer cells or a range of other cancer lines in culture similarly increased cystine uptake whereas combining BSO with SSA (250 µmol/L) or AUR (250 nmol/L) to simultaneously diminish both GSH and Trx induced striking levels of ROS production [detected with 2',7'-dichlorodihydrofluorescein diacetate (DCFDA)] and apoptotic cell death. However, antioxidants N-acetyl-L-cysteine (NAC; 1 mmol/L) or Trolox (250 µmol/L) prevented the cell death<sup>[68]</sup>.

In summary, the results above are consistent with the NRF2-Trx mediated reprogramming of tumor cells into cancer stem cells and the emergence of highly metastatic cancer cells, inhibiting the GSH antioxidant pathway but increasing the Trx/TrxR antioxidant pathway, ROS production and oxidative stress.

### **The use of antioxidants in post-diagnosis cancer therapy will be harmful by promoting activation of the NRF2-HIF-1 axis, providing insight into the importance of redox status in cancer metastasis**

In 2015, an elegant study showed that human patient derived melanoma cell lines transplanted subcutaneously, intravenously or into the spleen of NSG immunodeficient mice produced circulating melanoma cells and metastases with high levels of mitochondrial ROS production, transmembrane electrical potential and NADPH levels *via* the folate pathway but also lower mitochondrial mass and GSH/GSSG ratios when compared to that of the primary tumors<sup>[69]</sup>. As outlined in the previous section, highly metastatic tumors are reprogrammed by NRF2-HIF-1 to undergo metabolic changes allowing them to increase mitochondrial NADPH levels to help detoxify and thus attenuate or buffer against higher ROS and oxidative stress. In the 2015 study, NAC was applied *in vivo* with the aim of inhibiting ROS in these cells and lowering metastasis in their melanoma model, but it failed. To the contrary, systemic treatment with NAC enhanced the levels of circulating tumor cells and significantly increased their numbers of metastases<sup>[69]</sup>. Unfortunately, these investigators did not examine the metastases for their levels of NRF2 expression or ROS level. It should be noted here that several studies have reported that NAC can activate NRF2 expression in treated cells<sup>[70,71]</sup>, possibly acting *via* miR141 to lower KEAP1 expression levels<sup>[72]</sup>.

Although antioxidant therapy was predicted early on to be a potentially effective means for treating cancer patients, it became a highly controversial area of debate<sup>[3,73]</sup>, similar to the role of ROS in cancer<sup>[74]</sup>. However, more recent studies like the one above have repeatedly shown that the use of antioxidants can be counter-productive and instead accelerate more malignant tumor phenotypes, particularly those associated with metastasis<sup>[52,69,75,76]</sup>. These findings are consistent with the intracellular redox status as playing a crucial role in tumor survival, progression and development of the metastatic malignant phenotype<sup>[77]</sup>. Alternative, more natural interventions to treat cancers with antioxidants like NAC or soluble vitamin E (Trolox) were originally aimed at decreasing ROS levels as the driver of malignancy. However, such interventions were only successful if applied during the early stages of carcinogenesis, as outlined above<sup>[68]</sup>. In another study of mouse models with B-RAF- and K-RAS-induced lung cancers, antioxidants were again shown to significantly increase the metastatic potential of pre-existing cancer cells by stimulating tumor progression and lowering survival rates<sup>[78]</sup>. Transcriptome analysis revealed that the structurally unrelated NAC and vitamin E produced similar changes, lowering expression of cellular antioxidant genes and increasing tumor proliferation by decreasing levels of ROS, oxidative DNA damage and p53 expression in murine and human lung tumor cells. Knockdown or inactivation of p53 similarly increased tumor growth and obviated the effects of antioxidants. This evidence implies that the use of antioxidants promotes oncogenic cancer cell growth once established by inhibiting the ROS-activated wild type p53 axis, which would otherwise act to suppress tumor initiation and development by causing cell death. However, once p53 is mutated or

deleted then the cell death from greater ROS levels and oxidative stress will be avoided.

In 2015, another study<sup>[76]</sup> administering NAC also showed increased lymph node and lung metastases in the *Braf<sup>C.A/+</sup>Pten<sup>fl/fl</sup>Tyr-Cre<sup>+/-</sup>* mouse model of spontaneous malignant melanoma, but had little impact on the number or size of the primary tumors (NAC dose 1 g/liter, ~6 mmol/L changed weekly, corresponding to 114 to 229 mg/kg body weight for an adult male mouse). Similarly, NAC or vitamin E at 200 and 20  $\mu$ mol/L respectively increased the migration and invasiveness of human malignant melanoma cells *in vitro* but did not affect their proliferation. Either of these two antioxidants greatly increased the GSH/GSSG ratios in the melanoma cells and in lymph node metastases from the mouse model. The effects of increased tumor migration *in vitro* were inhibited by BSO (1 mmol/L) showing a dependency on nascent GSH synthesis. Furthermore, NAC or vitamin E did not alter the ROS levels detected in treated cells but increased the activation of the small guanosine triphosphatase (GTPase) Ras homolog gene family, member A (RHOA) involved in tumor cell migration and invasion and blocking downstream RHOA signaling abolished the antioxidant-induced migration. These results confirmed that antioxidants and the GSH system are important in enhancing metastatic cancer progression.

It is noteworthy<sup>[79]</sup> that NAC treatment has been shown to increase activation and protein levels of HIF-1 $\alpha$  in rat brain after ischemia/reperfusion. In another study of fetal lung alveolar epithelial cells, NAC over the range 1-50 mmol/L given as a pretreatment for 24 h dose-dependently enhanced and stabilized the subsequent levels of hypoxia-induced (3% O<sub>2</sub> for 4 h) activation of HIF-1 $\alpha$  protein, but decreased nuclear p65 NF- $\kappa$ B and DNA binding activity<sup>[80]</sup>. Analyses of changes in GSH homeostasis with increasing O<sub>2</sub> + NAC levels revealed correspondingly increased GSH/GSSG ratios in cultured cells. These results indicate that the effects of NAC as an antioxidant will depend on the amount of the agent applied and available levels of oxygen and can activate HIF-1 in cancer cells<sup>[81]</sup>.

It should be noted that NAC reacts differently with the various oxyradicals found in cells undergoing pro-oxidative stress. For example, NAC rapidly reacts with hypochlorous acid or hydroxyl radicals with a rate constant of  $1.36 \times 10^{10} \text{ M}^{-1} \text{ s}^{-1}$  whereas reaction with superoxide (O<sub>2</sub><sup>-</sup>,  $65 \text{ M}^{-1} \text{ s}^{-1}$ ) and H<sub>2</sub>O<sub>2</sub> ( $0.16\text{-}0.85 \text{ M}^{-1} \text{ s}^{-1}$ ) is much slower<sup>[82,83]</sup>. In mitochondria, NAC becomes desulfurated to form H<sub>2</sub>S, which is subsequently oxidized to sulfane sulfur (protein-SH + H<sub>2</sub>S (from NAC) + 1/2 O<sub>2</sub>  $\rightarrow$  protein-SSH + H<sub>2</sub>O) as a key mediator of the antioxidative and cytoprotective effects of NAC<sup>[84]</sup>. However, NAC also undergoes direct interactions with proteins containing reactive cysteine thiol groups, such as Raf-1, MEK and ERK *via* thiol-disulfide exchange<sup>[85]</sup> and hence, could react with KEAP1 to allow NRF2 activation. Furthermore, NAC directly reacts with many other small molecules (isothiocyanates, diallyl sulfides or triterpenoids), which themselves have the ability to interact with thiol-containing proteins. Thus, great caution must be exercised when interpreting results where NAC has been used primarily for its antioxidant effect or to demonstrate the involvement of ROS in drug-induced cancer cell death, as NAC effects may vary depending on the concentration applied, the oxygen level and other reactive drugs in the system. Furthermore, agents like NAC could work independently of KEAP1 to directly activate NRF2 by acetylating critical lysine residues<sup>[86]</sup> enabling NRF2 to go to the nucleus of cells<sup>[87]</sup>.

The NAC mediated activation of HIF-1 protein stabilization is more likely to occur under long-term NAC treatment due to modifying redox homeostasis *via* PHD inhibition and NRF2 activation, whereas over the short-term, high levels of NAC directly inhibit HIF-1 activity and the hypoxic responses taking place inside cancer cells *in vitro* and *in vivo*<sup>[88-90]</sup>. Thus, treating either PyMT or EO771 breast cancer cell lines with 25 mmol/L NAC prevented HIF-1 $\alpha$  stabilization (over 2 h) under either hypoxia or normoxia *in vitro*<sup>[88]</sup>. NAC treatment had no effect on HIF-2 expression. Over 8 h, NAC treatment (10-25 mmol/L) prevented stabilization of HIF-1 $\alpha$  and decreased vascular endothelial growth factor (VEGF) secretion in response to hypoxia in breast tumor cells *in vitro*, but did not alter the hypoxia-induced increase in mRNA expression for

VEGF and lysyl oxidase (LOX). *In vivo*, mice supplemented with NAC (40 mmol/L fresh daily) in drinking water showed significantly increased GSH levels in their blood within 48 h and maintained these elevated levels for ensuing weeks of continued treatment. NAC (40 mmol/L/daily in drinking water) did not translate to a difference in the primary tumor growth or the hypoxic state of primary tumors (by either HIF-1 expression or hypoxia level detected with pimonidazole) which remained similar to that seen in primary tumors in the untreated control mice<sup>[88]</sup>. However, NAC treatment given *in vivo* did significantly increase the lung metastatic burden in the EO771 experimental breast cancer metastasis model, consistent with NAC antioxidant as not advisable for post-diagnosis cancer therapy. Again, it would have been of interest to examine the NRF2-HIF-1 levels inside the metastatic tumor cell population.

In a related study using several different models of tumorigenesis including human P493 B lymphoma cells with conditional *MYC* or PC3 prostate carcinoma cells with 10 mmol/L NAC diminished HIF-1 $\alpha$  protein stabilization and activity over 8-24 h and VEGF secretion under hypoxic conditions (1% O<sub>2</sub>)<sup>[89]</sup>, it was identified that NAC treatment also lowered the *MYC* induced ROS production and  $\gamma$ -H2AX level in the cancer cells but there were no other signs of genomic instability. In an inducible *MYC* transgenic murine hepatocarcinoma model, providing NAC (40 mmol/L/daily in drinking water) to pregnant females prevented offspring from subsequently developing liver cancers compared to untreated mice. Hence, NAC or vitamin C used at very high levels to remove cellular ROS caused PHD2 reactivation and HIF-1 $\alpha$  degradation in a VHL-dependent manner with loss of HIF-1 over the short term, even in hypoxia<sup>[89]</sup>. A study of epithelial to mesenchymal transition 6 (EMT6) triple negative breast cancer cells in mice undergoing metastatic colonization over several days during extravasation into the lungs showed during this time that metastasizing cells increased their HIF-1 activity in a manner that was hypoxia-independent but ROS-dependent<sup>[90]</sup>. This activation of HIF-1 most likely correlates with ROS mediated inactivation of KEAP1 and/or stabilization of active NRF2 to upregulate HIF-1 expression. The increased HIF-1 level was confirmed by correlating with induced expression of lactate dehydrogenase A and phosphorylation of the E1 $\alpha$  subunit of pyruvate dehydrogenase, consistent with HIF-1 reprogramming of energy metabolism from a predominant oxidative (OxPhos-dependent) state to a non-oxidative anaerobic glycolysis-dependent state.

The bolus administration of very high doses of NAC (1 g/kg/administration, 2 injections/day from 1 to 6 days after *i.v.* transplantation of tumor cells) or the use of the HIF-1 inhibitor, YC-1, impeded the metabolic reprogramming of cancer cells, eventually suppressing the formation of metastatic lung tumors<sup>[90]</sup>. These results are consistent with an earlier study of B16F10 metastasis to the lung after subcutaneous injection where increasing NAC to very high dosage (up to 4 g/kg dose, with the latter having no metastases at all) showed dose related inhibition of both primary tumor size and corresponding numbers of lung metastases after 4 weeks<sup>[91]</sup>. This situation would be consistent with NAC at very high levels inhibiting the ROS-NRF2-HIF-1-mediated metabolic reprogramming responsible for migration, invasion and survival of metastatic cancers during their metastatic colonization in the lungs. Very high NAC levels will help adapt the redox homeostasis in cancer cells back to a lower level by lowering ROS and pro-oxidative stress, increasing GSH/GSSG ratios and preventing tumor growth.

Further evidence for the role of HIF-1 in tumor metastasis was shown by treating B16F10 cells with intermittent hypoxia, in which case significantly increased levels of ROS generation and HIF-1 protein levels were obtained<sup>[92]</sup>. Mice were subjected to whole body intermittent hypoxia after implantation of B16F10 melanoma cells, which increased the number and weight of metastatic colonies growing in their lungs<sup>[92]</sup>. Examining the lungs containing tumor metastases showed greater oxidative stress assessed by increased p22phox, SOD mRNA levels and NRF2 protein levels, as well as increased inflammatory markers, TNF- $\alpha$  and IL-6 mRNA levels and NF- $\kappa$ B p65 protein levels. In these studies, mice were treated with Tempol (4-hydroxy-2,2,6,6-tetramethylpiperidine-N-oxyl), a broadly effective agent for detoxifying ROS as a cell membrane-permeable nitroxide that dismutates superoxide, facilitates hydrogen peroxide

metabolism by catalase-like actions, and limits formation of hydroxyl radicals when it is reduced to the amine derivative. Thus, Tempol treatment counteracted the hypoxia/ROS-induced melanoma lung metastasis in mice by decreasing the levels of oxidative stress and inflammatory responses.

A related study using intermittent hypoxia to treat breast cancer cell lines in culture showed similar findings when cells adapted after successive rounds of hypoxia were then injected intravenously into syngeneic mice<sup>[93]</sup>. Again, as in the studies above, intermittent hypoxia treatment of breast cancer cell cultures subsequently enhanced their metastatic seeding and outgrowth into the lungs when transplanted *in vivo*. Furthermore, exposing these mammary tumor cells to intermittent hypoxia promoted clonal diversity, upregulated metastasis-associated gene expression, induced a pro-tumorigenic secretory profile, increased stem-like cell marker expression, and gave rise to tumor-initiating cells at a relatively higher frequency<sup>[93]</sup>. Thus, the evidence from many studies is consistent that intermittent hypoxia reprograms cancer cells by inducing a number of genetic, molecular, biochemical, and cellular changes to support tumor cell survival, colonization, and the creation of a permissive microenvironment to enhance metastatic growth.

In a study repurposing common drugs used to treat human type 2 diabetes mellitus, including the hypoglycemic dipeptidyl peptidase-4 inhibitors (DPP-4i) saxagliptin and sitagliptin, as well as  $\alpha$ -lipoic acid, it was shown that their use did not increase the frequency of primary tumor incidence<sup>[52]</sup>. However, these drugs did increase the risk of metastasis from existing tumors<sup>[52]</sup>. Specifically, the drugs induced prolonged activation of NRF2 and a cellular antioxidant response by inhibiting KEAP1-dependent ubiquitination mediated NRF2 degradation. Therefore, it was proposed that these drugs were acting as antioxidants, which is doubtful. Rather they are more likely to be reactive drugs capable of NRF2 activation. Thus, in cellular states with heightened oxidative stress, the KEAP1 cysteine sulphydryl groups may be modified by electrophilic reactive species that disrupt KEAP1-NRF2 interactions [Figures 2 and 3]. This would cause NRF2 release and activation to upregulate expression of metastasis-associated proteins, increase cancer cell migration, promoting metastasis, as seen in xenograft mouse models<sup>[52]</sup>. Accordingly, knockdown of NRF2 expression attenuated naturally occurring or DPP-4i-induced tumor metastasis, whereas NRF2 activation accelerated metastasis. In human liver cancer tissue samples, higher NRF2 expression correlated with metastasis<sup>[52]</sup>. Hence, this is a further mechanism whereby agents that first appear to be antioxidants, when used during cancer therapy could activate greater NRF2 signaling to promote cancer metastatic progression in patients.

Another aspect to NRF2's role in tumorigenesis relates to the host immune response. It was shown that *Nrf2* deficiency in the host animal but not of the cancer cells led to increased local tumor growth in the *Nrf2* null mice after subcutaneous injection of wild type B16F10 melanoma cells, as indicated by an increased proportion of animals with locally palpable tumor mass and time-dependent increases in tumor volume at the primary site of injection<sup>[94]</sup>. Further, the *Nrf2* null host mice showed a remarkable increase in lung metastasis by B16F10 melanoma cells as compared with wild-type mice<sup>[94]</sup>. Thus, factors such as a hypoxic tumor microenvironment would normally promote an anticancer immune response, but not in the absence of any capacity for expression of NRF2 in the host stroma and immune cells. Again, the results are consistent with the proposal that the usage of systemic antioxidant therapy which will act to suppress NRF2 protein levels in host cells could also be highly counterproductive due to their inhibitory potential for host immune responses.

In summary, although dietary antioxidants may be beneficial in helping prevent carcinogenesis in the initiation stages, they appear to be ill advised in the period post-cancer diagnosis where these agents promote greater malignancy and metastatic progression by helping activate the NRF2-HIF-1 axis. Hence, a different approach will be required to enhance anticancer responses post-diagnosis which will target the specific reprogrammed differences existing in the more highly advanced/metastatic tumor cells.



### NSAIDs as chemopreventatives and effective anticancer agents

The NSAIDs are the most commonly used medicine for inflammatory diseases, providing effective management of pain, fever, flushing and edema. This therapeutic benefit is ascribed to their designated (purported) function as inhibitors of the cyclooxygenases (prostaglandin-endoperoxide synthases as enzymes involved in producing pro-inflammatory prostanoids, including thromboxane and prostaglandins)<sup>[95,96]</sup>. However, extensive support for NSAIDs having other functions as anticancer drugs is emerging and this evidence is reviewed here.

#### First evidence for anticancer activity from chemoprevention with the use of NSAIDs in familial adenomatous polyposis

Celecoxib was originally developed as a selective cyclooxygenase-2 (COX-2) NSAID used to treat the pain and inflammation of osteoarthritis, rheumatoid arthritis, ankylosing spondylitis and other acute forms of pain. Celecoxib was designed to provide analgesia similar to the earlier NSAIDs such as ibuprofen and naproxen but offering much lower gastrointestinal side effects by not targeting COX-1. Early on, NSAIDs were recognized for lowering the risk of colorectal cancers (for review, see<sup>[97]</sup>) and in 2004, *Celebrex* (celecoxib) was the first to gain United States Federal Drug Administration (USFDA) approval for the purpose of decreasing polyp formation in familial adenomatous polyposis (FAP) [Table 1]. In this situation, celecoxib acts as a chemopreventative agent, lowering the incidence of polyp formation by about 30%, thereby impeding patients' progression to developing advanced colorectal cancer<sup>[98]</sup>. In patients post-diagnosis after treatment for sporadic polyposis, taking celecoxib (400 mg daily) was also shown to decrease by 41% the incidence of adenoma recurrence or onset of advanced adenoma detected after 5 years<sup>[99,100]</sup>. More recently the USFDA has given the combination of another NSAID, sulindac with the ornithine decarboxylase inhibitor, eflornithine, [difluoromethylornithine (DFMO)] fast-track status for use in FAP, although it has yet to be approved. When the polyp burden was assessed for the entire colorectum by endoscopy, the DFMO/sulindac treated FAP patient group showed a lower 3-year incidence of subsequent high-risk adenomas by > 90 % vs. only a 36 % decrease ( $P=0.01$ ) in the sulindac monotherapy group. However, more clinical trials are required to complete the supportive evidence before approval can be granted<sup>[101]</sup>. A similar international randomized trial comparing combined celecoxib + DFMO to celecoxib alone showed a synergy with the combination providing an average decrease for video based assessment of global polyps by 80% vs. 33% for celecoxib alone ( $P = 0.03$ )<sup>[102]</sup>. From the above outcomes (see Table 1 for summary), it is clear that chemoprevention with NSAIDs works very successfully for colorectal cancer.

#### Evidence for NSAID based chemoprevention against colorectal cancer in general

Several more recent studies have indicated that low, non-toxic doses of NSAIDs (including the low cost drug, aspirin) should be considered for approval or at least recommended for extended use across the entire population for the chemoprevention of colorectal cancers<sup>[103]</sup>. For example, it has been shown that aspirin use was more effective than either fecal occult blood testing (RR = 0.36; 95% CI: 0.22-0.59) or flexible sigmoidoscopy (RR = 0.37; CI: 0.22-0.62) in preventing death from or cancer development in the proximal colon and was equally effective to the other screening methods for lowering the colorectal cancer incidence and mortality<sup>[104]</sup>. One biomarker for responsiveness to aspirin under consideration is the tumor phosphatidylinositol-4,5-bisphosphate 3-kinase, catalytic subunit alpha (PIK3CA) mutation linked with greater effectiveness from regular aspirin use post-diagnosis by lowering total mortality from colorectal cancer by 29%-46% (RR = 0.71; CI: 0.51-0.99,  $P = 0.04$ )<sup>[105]</sup> and HR = 0.54; CI: 0.31-0.94,  $P = 0.01$ )<sup>[106]</sup>. Aspirin (N-acetylsalicylic acid) extensively acetylates proteins *in vivo* and may also react to put salicylate groups on proteins<sup>[107]</sup>. As such, aspirin related drugs can modulate KEAP1 function<sup>[108]</sup> inducing NRF2 signaling as an antioxidant chemopreventative drug<sup>[109]</sup> or as an alternative mechanism similar to other NSAIDs by increasing ROS levels in cancer cells as outlined further below. The exact effects exerted by drugs such as

**Table 1. Summary of NSAID prevention against metastatic cancers in clinical trials.**

Type of cancer	NSAID	Treatment	Combination with	Clinical outcome	Ref.
FAP	Celecoxib	400 mg bi-daily	None	Lowering polyp formation (30%)	[97,98]
CRC	Celecoxib	400 mg daily	None	Lowering 5-year risk of advanced adenoma by 41%	[99,100]
CRC	Sulindac	150 mg daily	+DFMO (750 mg daily)	3-year high risk adenomas lower by > 90% vs. sulindac monotherapy (30%)	[101]
CRC	Celecoxib	400 mg bi-daily	+DFMO (250-1250 mg daily)	Decrease global polyp assessment by 80% vs. celecoxib monotherapy (33%)	[102]
Proximal colon	Aspirin	75, 100, 300 or 600 mg/day	None	50% reduction in deaths from CRC with metastasis free at diagnosis; 50%-70% reduction in distant metastases	[103,104]
CRC	Aspirin vs. other NSAIDS	Any dose	None	Long term low dose decreased CRC mortality by 56% over 10-year follow-up & by 40% post-diagnosis mortality in KRAS wild type CRC	[111]
Distant metastasis by Br or Pr Ca	Several NSAIDS	pre- vs. post-operative NSAID use vs. non-users	None	NSAIDS decreased incidence of metastatic cancer post-cancer diagnosis by ~ 50%	[120]
Unresectable metastatic CRC	Celecoxib	200 mg bi-daily	FOLFOX4	45% survival at 3 years, 4 CR's	[168-172]
REACT Her2-, resected Br Ca.	Celecoxib	400 mg bi-daily	Capecitabine	93/195 complete response rate	[173]
NSCLC meta-analysis	COXIBs		Chemotherapy	48% decreased recurrence after 2 years	[174]
STAMPEDE prostate cancer	Celecoxib	400 mg bi-daily	Zoledronic acid (4 mg)	40% increase in response rates	[175]
				22% increased overall survival at median follow-up of 5 years	

NSAID: non steroidal anti-inflammatory drug; CRC: colorectal cancer; Pr Ca: prostate cancer; NSCLC: non small cell lung cancer; DFMO: alpha-difluoromethylornithine; CR: complete response

NAC or aspirin on cellular redox will depend on their relative concentrations, reaction rates and affinity for GSH (pro-oxidative effect) or the Cys-thiol groups on redox regulatory proteins such as the KEAP1/NRF2 hub (antioxidant effect) vs. TrXR (antioxidant effect)<sup>[110]</sup>.

### NSAIDs as chemopreventatives post-cancer diagnosis lower the incidence of recurrence or metastasis

In a comprehensive study of 2,419 patients with invasive colorectal cancer during 1997-2008 from registries in the USA, Canada and Australia, with a median follow-up period of 10.8 years since diagnosis, survival in the post-diagnostic non-users was compared with NSAID users<sup>[111]</sup>. The results showed significant decrease in all-cause mortality [hazard ratio (HR) = 0.75; CI: 0.59-0.95] and marked reduction in colorectal cancer specific mortality (HR = 0.44; CI: 0.47-0.86), notably with aspirin use. By comparison, the decreased mortality from any NSAID use post-diagnosis was only significantly improved in the Kirsten Rat Sarcoma (KRAS) wild-type protein expressing tumors (HR = 0.60; CI: 0.46-0.80), but not for the more malignant KRAS-mutant tumors (HR = 1.24; CI: 0.78-1.96).

Beyond FAP and general colorectal cancer, the evidence is now sufficiently substantial for recommendations that the population consider taking NSAIDs regularly over the long term in low doses as a chemoprevention against all types of cancer<sup>[112-114]</sup>. Historically, many population-based longitudinal studies with other cancer types and patients prescribed NSAIDs have been reported, including several recent meta-analyses summarizing the findings<sup>[104,111,114,115]</sup>. The outcomes from many studies have consistently outlined the benefits accrued from using NSAIDs either in the setting of pre- or postoperative use to treat cancer<sup>[116]</sup>, and particularly in a manner similar to that with FAP, by lowering risks of recurrence or progression to metastatic cancer post-diagnosis<sup>[113,117-119]</sup>. Given the abundance of recent meta-analyses, such studies will not be reviewed here except for those having a direct bearing on the main point of this review - that the NSAIDs preferentially work when used as a therapy for advanced stage metastatic disease (for a summary of the clinical evidence, in Table 1).

In particular, in this regard, a recent very large retrospective meta-analysis of the decrease in cancer metastasis with NSAID use is noteworthy and reported on data from 16 previous studies of various cancer types and a total of 202,780 participants<sup>[120]</sup>. The common observation from their analysis was the significantly lower risk ratios for distant metastasis found across the majority of cancer types comparing pre- vs. postoperative NSAID use relative to non-users [overall response rate (ORR) = 0.708; CI: 0.586-0.856, and RR = 0.484; CI: 0.393-0.595, respectively]. This included prostate cancer (pre-diagnostic use: RR = 0.874; CI: 0.787-0.97; post-diagnostic use: RR = 0.482; CI: 0.359-0.647), and breast cancer (pre-diagnostic use: RR = 0.644; CI: 0.565-0.735; post-diagnostic use: RR = 0.485; CI: 0.362-0.651). These results are typical and show that the NSAIDs in general will decrease the incidence of metastatic cancer post-cancer diagnosis by about 50%.

### **Enhanced clinical outcomes from using NSAIDs combined with chemotherapy for advanced stage metastatic cancers**

The reasons for the consistent differences observed between pre- and post-diagnostic use or pre- vs. post-operative use, with post-use showing a much lower relative risk of cancer related mortality have yet to be conclusively identified. However, we propose that one essential basis for these differences relates to the effectiveness of the drugs with the timing of treatment (post being more important and NSAIDs are much more effective in this situation) together with the extent of metastatic burden of the disease (with the NSAIDs showing activity predominantly greater effective benefit in the context of metastatic disease for the reasons outlined below). Importantly, overall in the above large scale study, comparing to the reference non-user group, those cancer patients prescribed the NSAIDs showed a significant and marked reduction in their subsequent risk from developing metastatic tumors (RR = 0.623; CI: 0.515-0.753,  $P < 0.001$ ). From these studies and many others, it can be concluded that in a majority of cases the outcomes clearly demonstrate the benefits from NSAID prescriptions after cancer diagnosis, which are commonly associated with lower all-cause mortality amongst cancer patients [Table 1]. The lowering of post-diagnostic cancer with NSAID use applies not only to FAP and colorectal cancer but also to breast<sup>[121]</sup>, prostate<sup>[122,123]</sup>, melanoma<sup>[124]</sup>, oesophageal<sup>[125]</sup>, gastrointestinal<sup>[126]</sup> and endometrial<sup>[127,128]</sup> cancers. Clearly, if the NSAIDs are utilized and administered with the correct timing and for the appropriate stages of advanced disease, they should work across all types of cancers and lower the burden caused by metastatic disease.

At this point, it should be noted that a few studies have been reported which did not find associations between aspirin or NSAID use and lower cancer mortality<sup>[121,129,130]</sup> and in some cases, they have been associated with even greater mortality<sup>[131]</sup>. Importantly, considerable caution and care must be taken with such studies where patients may be using the NSAIDs to offset pain in the terminal phases of cancers. For example, when NSAID use during the last three years of patient follow-up before death was excluded, it completely reversed the findings from one of higher mortality to a much lower mortality shown for the NSAID users relative to non-users, in line with the majority of studies. Hence, including the time period up until death (i.e., overall survival) can greatly and grossly adversely affect the observations<sup>[122]</sup>. In addition, the importance of comprehensive exposure definitions (duration of use, timing, consistency and intensity/dose) and evaluation of potential effect modification, co-morbidities or other user characteristics such as gastrointestinal and cardiovascular status, blood pressure, body mass index or obesity should also be evaluated<sup>[132]</sup>.

### **The importance of cancer staging in the clinical benefit from the NSAIDs and why their use enhances outcomes as chemopreventatives or chemosensitizing agents that induce greater pro-oxidative stress**

To summarize, different cancer cell types and stages alter the efficacy of the NSAIDs considerably when tested as anticancer agents, particularly when the bulk of supportive evidence in the clinical setting of metastatic malignant disease post-cancer diagnosis is assessed. This situation has, until now, been further

compounded by a lack of a precise understanding of how the NSAIDs act to kill cancer cells (for review see<sup>[133]</sup>). Current understanding of NSAID function as anticancer agents and our recent elucidation of their mitochondrial targeting (as mitocans) is reviewed below, as well as clinical data from human trials in advanced cancer. The mounting evidence is now clear that NSAIDs, and particularly celecoxib, significantly enhance advanced cancer patient responses to the existing commonly used chemotherapies and lower the burden of metastatic disease. One of the main aims of this review is then to promote increased understanding and extended clinical usage of celecoxib when treating advanced stage metastatic disease, for example in drug unresponsive tumors like triple negative breast cancers.

Based on the deregulated redox homeostasis in cancer cells and increased ROS levels promoting tumor growth and malignant progression by metabolic reprogramming in tumors associated with enhanced antioxidant ability as a common feature, it has been proposed that tumors can be sensitized to chemotherapy and other canonical antitumor treatments by disabling antioxidant defenses (NADPH and GSH) through metabolic inhibition<sup>[134,135]</sup>. Overloading cancer cells by exacerbating oxidative stress potentiates chemotherapeutic responses and can also improve responses to radiation therapy<sup>[135,136]</sup>. Such studies underscore the importance of understanding the regulatory systems operating in cancer cells to then be able to use agents like the NSAIDs appropriately for therapeutic benefit in treating disease [Figure 4]. Whereas it has been commonly reported that the mechanism of cancer therapy obtained with NSAIDs can be ascribed to their activity as potent drugs capable of inhibiting the COX's, attempts to link RRs in cancer patients with tumor levels of COX expression have been largely unsuccessful<sup>[137,138]</sup>. While these actions may account for a fraction of the events in response to NSAID treatment occurring *in vivo*, the bulk of recent evidence shows that targeting such enzyme systems is inadequate and does not explain the majority of their anticancer functions, but rather, indicates that other more important off-target activities in cancer cells do exist. We propose that one of the key targets of NSAIDs is the mitochondria in cancer cells and that NSAIDs should be repurposed for post-diagnostic therapy of cancer by exploiting pro-oxidative ROS production to kill metastatic cancer cells.

### **NSAIDs function as pro-oxidative anticancer drugs independent of COX or other enzymatic inhibition**

Several lines of evidence have convincingly shown that COX inhibition is not the main mode of action for the anticancer effectiveness of NSAIDs. First, comparing the relative anticancer activities and structure/function of the different NSAIDs revealed that their actions as anticancer agents usually involve higher or lower drug concentrations than the inhibition constant ( $K_i$  or  $K_d$ ) required to inhibit the COX activities<sup>[139-141]</sup>, with many working independently of their COX inhibitory potential. For instance, doses of acetylsalicylic acid used to decrease inflammation are much higher than those required to inhibit COX activity. Second, several studies have established that NSAID derivatives and homologs that do not inhibit COX function, nevertheless exhibit undiminished anticancer responses<sup>[142-145]</sup>. Third, the evidence shows COX inhibitors to be equally effective against COX-null cancer cell lines<sup>[146,147]</sup>. Thus, on multiple bases, it can be concluded that COX inhibition is not the predominant driver of the anticancer effect exhibited by this class of drugs.

Similar to the other NSAIDs, structurally related homologs of celecoxib exist with even greater potency as anticancer agents, but they do not bind or inhibit COX's. For example, dimethylcelecoxib is a COX-null celecoxib derivative containing an additional methyl group compared to the prototypic 3-methylcelecoxib. Zhu *et al.*<sup>[148]</sup> (2002) showed that modifying the side groups and enlargement of the hydrophobic aryl moiety by adding the second methyl group (as 2,5-dimethylcelecoxib or more specifically 4-(5-(2,5-Dimethylphenyl)-3-(trifluoromethyl)-1H-pyrazol-1-yl)benzenesulfonamide) promoted apoptosis more effectively than celecoxib. This mechanism is in contrast to the action of celecoxib in COX-2 inhibition, which has stringent requirements in regard to the stereo-specific arrangement of the 3' methyl

group on celecoxib and the benzenesulphonamide moiety. Another COX-null homolog of celecoxib, E7123 or 4-(5-(2,5-dimethylphenyl)-3-(trifluoromethyl)-4,5-dihydro-1H-pyrazol-1-yl)benzenesulfonamide was also much more potent than celecoxib in killing cancer cells<sup>[149-151]</sup>.

### **NSAIDs target mitochondrial ROS production to trigger apoptosis of metastatic cancer cells and cancer stem cells and celecoxib is a potent exemplar**

Previously, we reviewed the role of NSAIDs as “hitting the bulls-eye” in cancer cells by targeting mitochondrial function to trigger cell death *via* the intrinsic mitochondrial pathway of apoptosis<sup>[152]</sup>. Our more recent studies examined five different NSAIDs and showed that adding them to metastatic cancer cell lines in culture resulted in a progressive increase in ROS production from mitochondria to trigger ensuing cytotoxicity, by activating the intrinsic apoptotic signaling pathway. Celecoxib showed much greater potency than the other NSAIDs tested. Similar observations applied to isolated and purified preparations of mitochondria where upon addition of celecoxib in low micromolar concentrations abruptly induced production of superoxide by disrupting the respiratory chain electron transfer and mitochondrial metabolism thereby inducing ROS production directly from the mitochondria<sup>[153]</sup>. These results applied to mitochondria isolated from both normal tissues and hepatoma cells. Thus, our data indicate that when the mitochondria are removed from their normal intracellular milieu with the cytosol full of antioxidant systems, they become very sensitive to the direct action of celecoxib on ROS production. Furthermore, we showed<sup>[153,154]</sup> that one aspect of celecoxib’s activities important for cancer cell death is that it can, at sufficiently high levels, directly inhibit mitochondrial respiration, the transmembrane electrical potential and ATP production and induces excess superoxide as a by-product from the electron transport chain, which in turn, triggers caspase activation and apoptosis of cancer cells. Thus, even at low levels, celecoxib interferes with the mitochondrial respiratory pathway of cancer cells to promote excessive ROS production<sup>[153]</sup>. Moreover, celecoxib at doses assayed to block OxPhos and cellular growth (10 µmol/L) severely decreased triple negative breast cancer cell (MDA-MB-231 and MDA-MB-468) migration (60%) and invasiveness (25%-55%) potential<sup>[154]</sup>. Celecoxib was recently shown to inhibit breast cancer stem cell self-renewal, sensitize against chemoresistance, inhibit EMT, and attenuate metastasis and tumorigenesis<sup>[155]</sup>. Although a similar report on bladder cancer suggested that the mechanism for the actions of celecoxib on cancer stem cells is mediated by inhibiting COX-2 and prostaglandin synthesis<sup>[156]</sup>, this is unlikely to be the cause given the evidence cited above for mitochondrial pro-oxidant activity.

Celecoxib (1-10 µmol/L) treatment of J774 myelomonocytic leukemia cells, vascular smooth muscle cells or human umbilical vein and aortic endothelial cells has been shown to increase mitochondrial ROS and NRF2 nuclear activation *via* PI3K/Akt, p-38 and p-ERK signaling<sup>[157,158]</sup> or AMPK/CREB<sup>[159]</sup>. Celecoxib together with hypoxia produced greater expression levels of heme oxygenase HO-1<sup>[157]</sup>. This activation was inhibited by pretreating cells for 30 min with 10 mmol/L NAC and was COX-independent but was not seen with rofecoxib, ibuprofen, naproxen or indomethacin<sup>[157-159]</sup>. It follows that celecoxib should uniquely activate NRF2 inside metastatic cancer cells or cancer stem cells, but this is unlikely to be sufficient to protect against the excessive mitochondrial ROS overloading the antioxidant system with ensuing cytotoxicity [Figure 4].

### **Celecoxib in combination with chemotherapy synergistically improves responses against advanced stage metastatic disease in pre-clinical animal models of cancer**

Although many reports of pre-clinical studies with animal or human xenografted cancer cell lines treated with NSAIDs have been published, only celecoxib will be reviewed here where the focus has been on celecoxib and its exceptional ability to target metastatic cancer cells and synergize with chemotherapy. Thus, synergistic anticancer effects have been attained by combining celecoxib in murine models of colorectal cancer with either 5-fluorouracil (5-FU)<sup>[160]</sup> or with oxaliplatin<sup>[161]</sup>; in melanoma models with dacarbazine<sup>[162]</sup> or with doxorubicin for metastatic murine breast cancer<sup>[163]</sup>. Hence, the pre-clinical findings are consistent with the ability of celecoxib to chemosensitize cancer cells rendering them more susceptible



to other anticancer drugs. Drugs like celecoxib have proven to offer further advantages in that they have been shown to kill cancer cells independently of MDR<sup>[164,165]</sup> or p53 or DNA mismatch repair enzymes (reviewed in<sup>[152,166]</sup>), because as we have shown, they kill by targeting mitochondrial metabolism<sup>[152-154,167]</sup>.

### **Celecoxib in combination with chemotherapy has shown curative efficacy in clinical trials of advanced stage metastatic human cancers**

An extensive analysis of NSAIDs and their use to treat human cancer is beyond the scope of this review. A basic Pubmed search restricted to celecoxib with the key words “clinical trial”, “cancer” and “celecoxib” provided about 424 studies. In the site <https://clinicaltrials.gov>, 359 listed studies include celecoxib and cancer for either the prevention, treatment and decreased symptoms or cancer recurrence for a wide range of cancers including: breast, bladder, pancreatic, colorectal, lung, head and neck, prostate, ovarian, uterine, liver and bile duct, cervical and renal. The main message from these studies is that where the NSAIDs have been combined with the standard of care treatments in the clinical setting for advanced stage metastatic disease, they have often shown significant improvements in outcomes. The successful results of clinical trials where celecoxib has been combined with commonly used chemotherapies are summarized in Table 1 and as follows.

A “curative” efficacy was reported following combination standard of care chemotherapy (FOLFOX4) with celecoxib for advanced unresectable metastatic adenocarcinoma of the colorectal area<sup>[168]</sup>. The Activate tumor from Dormancy And Potentiate its Targeting (ADAPT) phase II trial examined capecitabine and celecoxib ± radiation following first-line chemotherapy and showed a higher complete response (CR) rate and prolonged survival with the celecoxib combination in stage IV unresectable metastatic colorectal cancer patients at the 10-year follow-up<sup>[169-172]</sup>. The Randomised EuropeAn celecoxib trial (REACT) of primary breast cancer subgroup analysis after 5-year follow-up showed that the 655 breast cancer patients who did not have subsequent adjuvant chemotherapy, nevertheless greatly benefited from being prescribed celecoxib, with a decreased recurrence (HR = 0.62; CI: 0.38-1.00)<sup>[173]</sup>. In non small cell lung cancer (NSCLC), a statistically significant improved response with COX-2 inhibitors added to first-line treatment was reported for advanced stage disease (RR = 1.39; CI: 1.19-1.63). Increased ORRs were also observed with COX-2 inhibitors added to chemotherapy (RR = 1.40; CI: 1.20-1.63)<sup>[174]</sup>. In the large Systemic Therapy in Advancing or Metastatic Prostate Cancer: Evaluation of Drug Efficacy (STAMPEDE) trial, similar to the REACT breast cancer study above, subgroup analysis of patients with metastases at baseline showed a significant improvement in both the overall survival (HR = 0.78; CI, 0.62-0.99,  $P = 0.04$ ) and failure-free survival (HR = 0.77; CI: 0.63-0.93,  $P = 0.008$ ) for the celecoxib/zoledronic acid group compared with the control group<sup>[175]</sup>.

### **CONCLUSION**

It is becoming clear from the greater understanding of differences occurring in tumor cells during the progression to advanced stages of metastatic disease that a precise basis exists for specifically targeting such tumors and eliminating them. Thus, with reversible or irreversible changes in the NRF2-HIF-1 axis, redox mediated reprogramming of gene expression occurs associated with metabolic change and greater endemic mitochondrial ROS/pro-oxidative states. Hence, we have now identified a specific cancer drug target, the mitochondria. Based on the evidence, we can conclude that use of antioxidant strategies is ill advisable after cancer diagnosis, as it is too late to prevent tumors from arising, but instead will promote their further metastatic progression. However, pro-oxidative agents like celecoxib which target mitochondrial ROS production to further tip the redox balance over and beyond the limits of cell survival by overwhelming the antioxidant defense systems in these tumor cells, will cause their mass destruction. This pro-oxidative overkill synergizes when combined with standard chemotherapeutic treatments targeting other aspects of cancer cell replication and survival, significantly improving patient responses and survival with post-

diagnosis treatment, lowering recurrence rates. Such combination therapies have even shown significant curative benefits for hitherto refractory tumors. The implications from these findings are that repurposing drugs such as NSAIDs like celecoxib or other agents that work in similar fashion should be highly encouraged, as should their use in more clinical trials of metastatic disease and where biomarkers such as constitutive NRF2-HIF protein expression are well defined.

## DECLARATIONS

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### Conflicts of interest

All authors declared that there are no conflicts of interest.

### Ethical approval and consent to participate

Not applicable.

### Consent for publication

Not applicable.

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Case Report

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# Cryptic NUP214-ABL1 fusion with complex karyotype, episomes and intra-tumor genetic heterogeneity in a T-cell lymphoblastic lymphoma

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## Abstract

T-lymphoblastic lymphoma (T-LBL) is a rare and aggressive form of non-Hodgkin's lymphoma and little is known about their molecular background. However, complex karyotypes were already related to this group of malignancy and associated with poor outcome. Here, we describe a 17-year-old female being diagnosed with T-LBL and a normal karyotype after standard G-banding with trypsin-Giemsa (GTG)-banding. However, further analyses including high-resolution molecular approaches, array-comparative genomic hybridization (aCGH), multiplex ligation-dependent probe amplification, fluorescence *in situ* hybridization and multicolor chromosome banding revealed a cryptic complex karyotype, *NUP214-ABL1* gene fusion, episomes and intra-tumor genetic heterogeneity. In addition, homozygous loss of *CDKN2A*, as well as amplification of oncogene *TLX1* (*HOX11*) were detected. Actually, *NUP214-ABL1* fusion gene replicated autonomously in this case as episomes. Overall, highly amplification of *NUP214-ABL1* fusion gene defines possibly a new subgroup of T-LBL patients which accordingly could benefit from treatment with tyrosine kinase inhibitors. As episomes are missed in standard karyotyping aCGH should be performed routinely in T-LBL to possibly detect more of such cases.

**Keywords:** T-cell lymphoblastic lymphoma, *NUP214-ABL1* fusion, complex karyotype, episomes, intra-tumor genetic heterogeneity, molecular cytogenetics, array comparative genomic hybridization



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## INTRODUCTION

Lymphoblastic lymphoma (LBL) is a rare and aggressive form of non-Hodgkin's lymphoma (NHL). LBL develops from immature B cells committed to the B- (B-LBL) or T-cell lineage (T-LBL). LBL is morphologically indistinguishable from acute lymphoblastic leukemia (ALL) and 90% of it have a T-cell phenotype. LBL also accounts for approximately 2% of all NHL cases and occur in adult, children and adolescent, with a male predominance (three time more male are affected)<sup>[1-2]</sup>.

Chromosomal abnormalities in T-LBL are not well defined and cytogenetic data in T-LBL is limited. However, a few published cytogenetic studies revealed that typical chromosomal aberrations identified in T-cell ALL (T-ALL) are also present in T-LBL. These include translocations of T-cell receptor (*TCR*) gene to genes encoding transcription factors such as *TAL1*, *TLX1*, *LMO2*, and *LYL1*. In particular, the translocation t(9;17)(q34;q22~23) is typically found in T-LBL<sup>[1-4]</sup>. However, no single recurrent and typical genetic alteration for T-LBL could be identified. This is in contrast to other malignancies like translocation of *ALK* gene in anaplastic large cell lymphoma, *MYC* gene in Burkitt lymphoma or *BCL2* gene in follicular lymphoma.

Here we present the comprehensive analysis of a T-LBL case with a normal karyotype, according to standard G-banding with trypsin-Giemsa (GTG)-banding, using high resolution molecular methods, identifying also some intra-tumor genetic heterogeneity besides unusual acquired genetic alterations. Also here we report *NUP214-ABL1* gene fusion in this patient, which appears cryptic due to its localization in episomes.

## CASE REPORT

A seventeen-year-old female patient, who was initially diagnosed in South Africa with T-ALL, presented in the clinic in Poland with abdominal pain, accompanied by diarrhea and vomiting; she was here initially treated only symptomatically. A few days before, a blood test already revealed hyperleukocytosis ( $589 \times 10^9/l$ ) with presence of 94% lymphoblasts in blood smear, hemoglobin 8.5 g/dl, and platelet count  $53 \times 10^9/l$ . Bone marrow findings were: hypercellularity with 95% lymphoblasts, lack of megakaryocytes and Periodic-Acid-Schiff (PAS) staining identified in 70% of the blasts thick grains (data not shown). Ultrasound of abdomen showed enlargement of the spleen to 152 mm, and presence of fluid in the lower pelvis. Cervical lymph nodes were bilaterally enlarged with diameters of 3-4 cm, and small submandibular nodes were bilaterally enlarged to 2 cm in diameter.

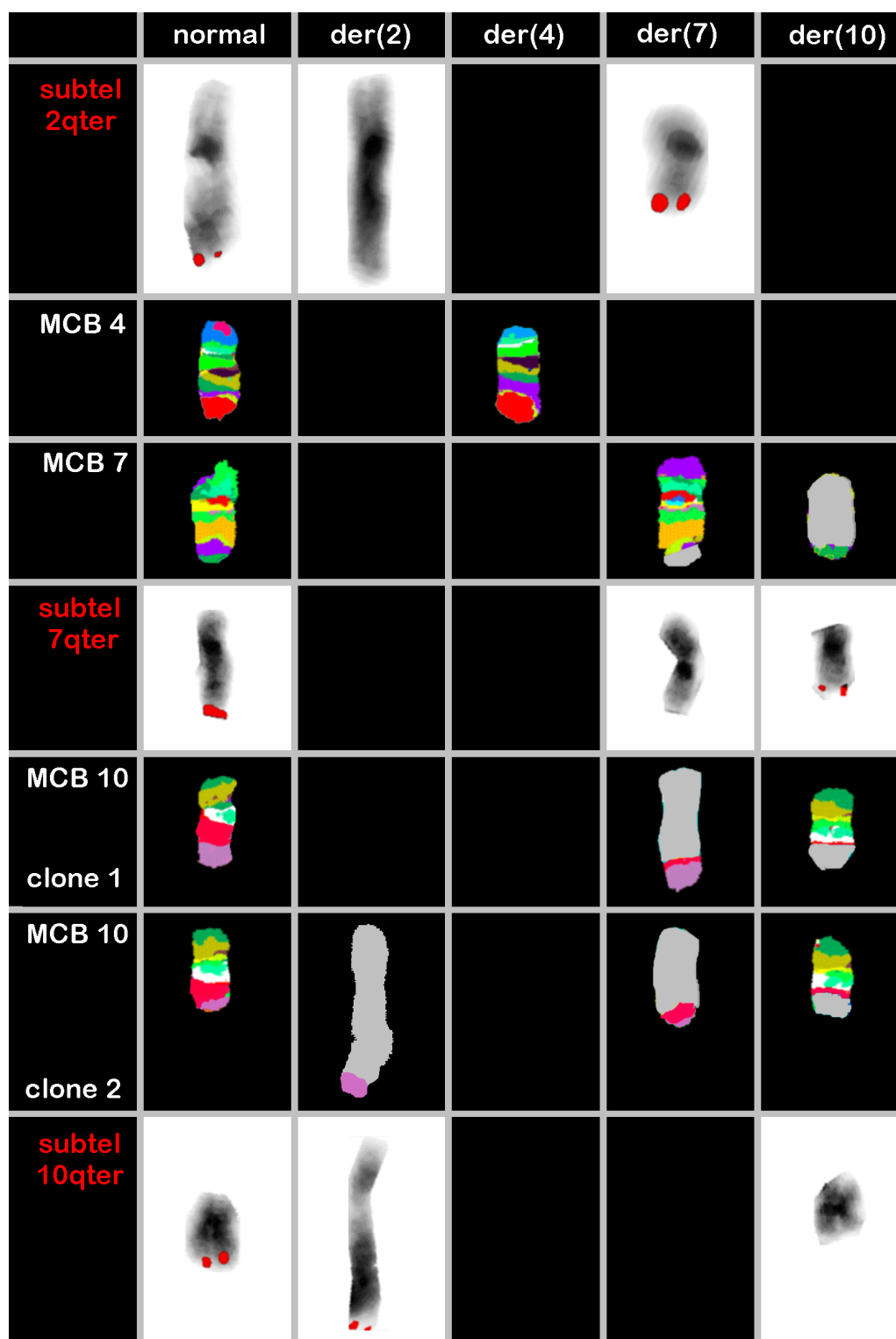
Cytogenetic and immunophenotypic analyses were done. The latter characterized a T-LBL due to high expression of CD45 (100%), CD2 (96.6%), CD4 (97.3%), CD8 (90%), CD7 (77.1%), CD5 (76.0%), sCD3 (71.2%), CD1a (70.0%) and the lack of TdT, CD19, CD34 and CD38.

Banding cytogenetic analyses were done in unstimulated bone marrow cells according to standard procedures<sup>[5]</sup> from the material taken at initial diagnoses. A total of 20 metaphases were available and analyzed on a banding resolution of 300 bands per haploid karyotype, revealing a normal female karyotype. Molecular diagnostic polymerase chain reaction (PCR)-based tests for presence of gene fusions *BCR/ABL* (p190 and p210), *TCF3/PBX1*, *MLL/AF4* and *SIL/TAL1* were negative (results not shown).

Also genomic DNA isolated from cells fixed in acetic acid-methanol (1:3) was subjected to array-comparative genomic hybridization (aCGH) as well as the multiplex ligation probe amplification (MLPA) studies, as previously reported<sup>[6]</sup>. Finally, fluorescence in situ hybridization (FISH) was done<sup>[6-8]</sup>, revealing a highly complex karyotype [Figure 1 and Table 1] with gene-amplification due to episomes (abbreviated here as epi), which can be reported as:

46,XX,der(2)t(2;7)(q37.3;q25.1),del(4)(p14p16),t(7;10)(q34;q24),del(9)(p21.3p21.3),epi(6;9)(q23.3;q34.12)x20~30[20%]/46,XX,der(2)t(2;7)(q37.3;q25.1),del(4)(p14p16),der(7)(7pter->7q34::10q24.1->10q25.1::2q37.3->2qter),del(9)(p21.3p21.3),der(10)t(10;7)(q23;q34),epi(6;9)(q23.3;q34.12)x20~30[40%]/46,XX[40%].





**Figure 1.** Result of multicolor banding (MCB) probesets for chromosomes 4, 7 and 10 are shown. MCB 10 showed the founder clone and subclone. Locus-specific probes (LSPs) for chromosomes 2, 7 and 10 characterized the breakpoints in 2q37.3, 7p34, 10q24.3 and 10q25.1 [Table 1]. The final karyotype after application of all approaches is summarized in the text. der = derivative chromosome

In the FISH-studies done here, between 15 and 25 metaphases were evaluated per applied probe-set, thus in the final karyotype overall percentages are given for the observed clones.

**Table 1. Locus specific probes used for FISH together with their location according to genome browser version NCBI/hg18; this version was used here as some here applied FISH-probes are no longer available in newer genome browser versions. Results obtained are presented using standard (gene) abbreviations and such used according to the international system of cytogenomic nomenclature**

Cytoband	Position [NCBI36/hg18]	Genes/locus	Probe	Result (signals on...)
2q37.1	chr2:234,552,641-234,701,765	n.d.	RP11-263G22	der(2)
2q37.2	chr2:236,163,266-236,349,539	n.d.	RP11-473L20	der(2)
2q37.3	chr2:238,251,662-238,463,936	n.d.	RP11-497D24	der(7)
2q37.3	chr2:242,433,475-242,633,697	D2S447	2qTEL (Vysis)	der(7)
6q23.3	chr6:135,544,146-135,582,003	MYB	SPEC MYB DCBAP (Zytovision)	amp(6)(q23.3q23.3)
7q31.2	chr7:116,099,695-116,225,676	MET	SPEC MET/CEN7 (Zytovision)	der(7)
7q33	chr7:133,287,726-133,474,337	n.d.	RP11-639H21	der(7)
7q33	chr7:134,684,542-134,842,811	n.d.	RP11-371N6	der(7)
7q33	chr7:136,263,935-136,416,924	n.d.	RP11-88K4	der(7)
7q33	chr7:137,919,273-138,093,873	n.d.	RP11-269N18	der(7)
7q34	chr7:141,674,679-141,819,906	TCRB	n.a.	n.a.
7q34	chr7:142,124,883-142,316,809	n.d.	RP11-39H3	der(10)
7q34-q35	chr7:142,787,852-142,859,896	n.d.	RP11-811J9	der(10)
7q35	chr7:143,536,879-143,690,749	n.d.	RP11-45N9	der(10)
7q35	chr7:145,715,880-145,867,471	n.d.	RP11-97H18	der(10)
7q35	chr7:147,084,270-147,259,380	n.d.	RP11-302C22	der(10)
7q36.3	chr7:158,400,001-158,600,424	VIJyRM2000	7qTEL (Vysis)	der(10)
9p21.3	chr9:21,792,635-21,984,490	MTAP CDKN2A/B	SPEC CDKN2A/CEN9 (Zytovision)	del(9)(p21.3p21.3)
9q34.13	chr9:132,579,089-132,752,883	ABL1	LSI BCR, ABL (Vysis)	amp(9)(q34.12q34.12)
10q23.31	chr10:89,613,175-89,718,512	PTEN	SPEC PTEN/CEN10 (Zytovision)	der(10)
10q24.31	chr10:102,880,252-102,887,526	TLX1	n.a.	n.a.
10q24.31-q32	chr10:102,895,115-103,074,760	n.d.	RP11-324L3	der(7)
10q24.32	chr10:104,652,453-104,813,482	n.d.	RP11-724N1	der(7)
10q25.1	chr10:106,748,189-106,912,787	n.d.	RP11-165P9	der(7)
10q25.1	chr10:107,741,530-107,812,754	n.d.	RP11-596L14	der(7)
10q25.2	chr10:112,350,581-112,499,609	n.d.	RP11-364E8	der(2)
10q25.2	chr10:116,774,286-116,971,219	n.d.	RP11-338L11	der(2)
10q26.13	chr10:123,227,834-123,347,962	FGFR2	SPEC FGFR2/CEN10 (Zytovision)	der(2)
10q26.3	chr10:134,925,980-135,126,361	D10S2290	10qTEL (Vysis)	der(2)

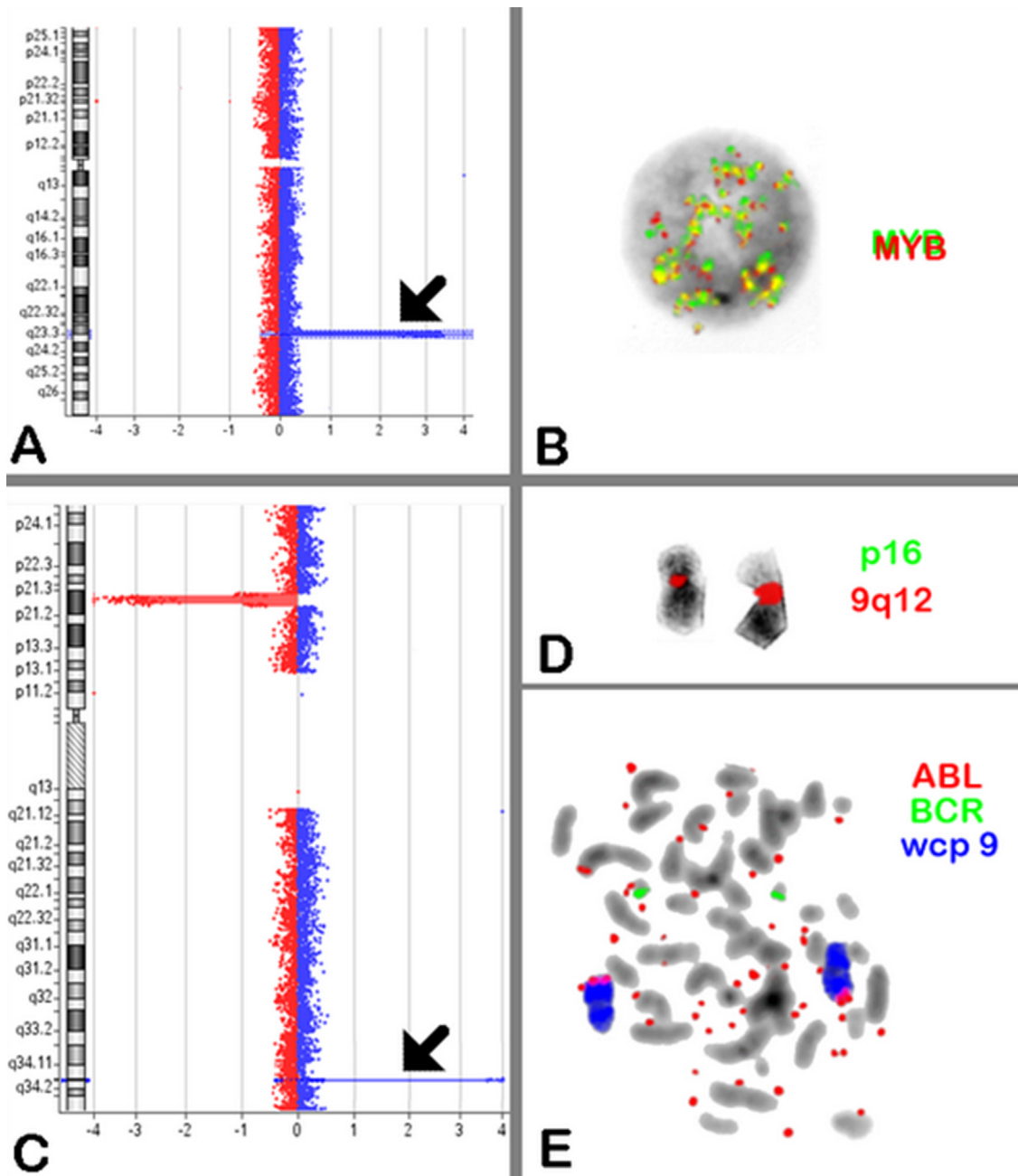
FISH: fluorescence *in situ* hybridization

*NUP214-ABL1* fusion could be deduced from aCGH data - the region being amplified ends on one side at *NUP214*- and on other side at *ABL1*-gene - as the amplified region is present as episomes, which are circular, there must be *NUP214-ABL1* fusion.

The patient was treated according to the Polish Adult Leukemia Group (PALG) protocol, with induction therapy consisting of prednisone, daunorubicin, vincristine and PEG-L-asparaginase. No remission was achieved and the patient was re-treated according to fludarabine, cytarabine, and mitoxantrone (FLAM) with consolidation course (metrotrexate, cyclophosphamide and PEG-L-asparaginase) and maintenance treatment. After ten months, the patient relapsed and was now treated according to Hyper-CVAD protocol (cyclophosphamide, vincristine, doxorubicin/Adriamycin and dexamethasone). Still, one month later the patient unfortunately died.

## DISCUSSION

Recurrent acquired genetic lesions play a key role in predicting and assessing risks, so are the treatment protocols to be applied. Still, little is known about the copy number alterations (CNAs) accompanying structural abnormalities in T-LBL, such as the *NUP214-ABL1* fusion gene. *ABL1* fusion proteins are sensitive to tyrosine kinase inhibitors, which potentially can be included in future treatment strategy and *NUP214* is a component of the nuclear pore complex, which mediates nucleocytoplasmic transport. *NUP214* is widely



**Figure 2.** A: Array comparative genomic hybridization (aCGH) analysis of chromosome 6 revealed high level of 6q23.3 amplification containing *MYB* gene (arrow); B: *MYB* Dual Color Break Apart Probe was applied and showed high level of amplification more than 20 copies/per cell; C: aCGH analysis of chromosome 9 revealed biallelic deletion of *CDKN2A* at 9p21.3 and high level of 9q34 amplification contains *ABL1* and *NUP214* (arrow); D: FISH confirmed the homozygous deletion of *CDKN2A* in metaphase; E: BCR, ABL Dual Color Probe was applied and showed variable number of episomes (20-30) in spread metaphases. aCGH: array-comparative genomic hybridization; FISH: fluorescence in situ hybridization; wcp: whole chromosome paint

expressed and is involved in the pathogenesis of acute myeloid leukaemia associated with the translocation t(6;9)(p23;q34) as *DEK-NUP214* fusion<sup>[9-11]</sup>.

To the best of our knowledge, a cryptic *NUP214-ABL1* fusion yet has only been identified in 6% of individuals with T-ALL and is the second most prevalent fusion gene involving *ABL1*<sup>[12-15]</sup>. Here we report this for the first time in a T-LBL case, and even detected it as a high level amplification; most probably after inversion, duplication or translocation, gene fusion, circularization and amplification happened. As *ABL1* is one of the best targetable tyrosine kinases, identification of *ABL1* gene fusion is clinically important, as patients may

**Table 2. Summary of CNAs detected by aCGH. Recurrent (R) and unique (U) acquired CNAs are correspondingly highlighted in first column. Results obtained are presented using standard (gene) abbreviations and such used according to the international system of cytogenomic nomenclature**

Chromosome (alteration U or R)	Cytobands	GRCH37/hg19	Size of imbalance [Mb]	Genes
1 (U)	del(1)(p36.31p36.23)	chr1:5,958,728-7,238,618	1.27	<i>NPHP4, KCNAB2, CHD5, RPL22, RNF207, ICM1, HES3, GPR153, ACOT7, HES2, ESPN, MIR4252, TNFRSF25, PLEKHG5, NOL9, TAS1R1, ZBTB48, KLHL21, PHF13, THAP3, DNAJC11, CAMTA1</i>
	del(1)(q22.2q22.2)	chr1:91,620,826-91,739,326	0.2	<i>HFM1</i>
4 (U)	del(4)(p16.3p14)	chr4:3,072,509-38,882,925	35.8	<i>HTT, C4orf44, RGS12, HGFAC, DOK7, LRPAP1, LOC100133461, ADRA2C, LOC348926, OTOP1, TMEM128, LYAR, ZBTB49, D4S234E, STX18, LOC100507266, MSX1, CYTL1, STK32B, C4orf6, EVC2, EVC, CRMP1, JAKMIP1, LOC285484, WFS1, PPP2R2C, MAN2B2, MRFAP1, LOC93622, S100P, MRFAP1L1, CNO, KIAA0232, TBC1D14, LOC100129931, CCDC96, TADA2B, GRPEL1, FLJ36777, SORCS2, PSAPL1, MIR4274, AFAP1, AS1, AFAP1, ABLIM2, SH3TC1, HTRA3, ACOX3, METTL19, GPR78, CPZ, HMX1, LOC650293, USP17, USP17L6P, DEFB131, MIR54812, DRD5, SL-C2A9, WDR1, MIR3138, ZNF518B, CLNK, MIR572, HS3ST1, HSP90AB2P, RAB28, LOC285547, NKX3-2, LOC285548, BOD1L, LOC152742, CPEB2, C1QTNF7, CC2D2A, FBXL5, FAM200B, BST1, CD38, FGFBP1, FGFBP2, PROM1, TAPT1, FLJ39653, LDB2, QDPR, CLRN2, LAP3, MED28, FAM184B, DCAF16, NCAPG, LCORL, SLIT2, LOC100505893, MIR218-1, PACRGL, KCNIP4, NCRNA00099, LOC100505912, GPR125, GBA3, PPARGC1A, MIR573, DHX15, SOD3, CCDC149, LGI2, SEPSECS, LOC285540, PI4K2B, ZCCHC4, ANAPC4, SLC34A2, SEL1L3, C4orf52, RBPJ, CCKAR, TBC1D19, STIM2, MIR4275, PCDH7, ARAP2, DTHD1, KIAA1239, C4orf19, RELL1, PGM2, TBC1D1, PTTG2, FLJ13197, KLF3, TLR10, TLR1, TLR6, FAM114A1, MIR574</i>
6 (R)	amp(6)(q23.3q23.3)	chr6:134,245,761-136,118,354	1.87	<i>TBPL1, SLC2A12, HMGA1P7, SGK1, ALDH8A1, HBS1L, MIR3662, MYB, AH11, NCRNA00271</i>
9 (R)	del(9)(p21.3p21.3)	chr9:20,605,923-21,218,606	0.61	<i>MLLT3, KIAA1797, MIR491, PTPLAD2, IFNB1, IFNW1, IFNA21, IFNA4, IFNA7, IFNA10, IFNA16</i>
	del(9)(p21.3p21.3)	chr9:21,252,517-23,002,377	1.75	<i>IFNA22P, IFNA5, KLHL9, IFNA6, IFNA13, IFNA2, IFNA8, IFNA1, LOC554202, IFNE, MIR31, MTAP, C9orf53, CDKN2A, CDKN2B-AS1, CDKN2B, DMRTA1</i>
	amp(9)(q34.1q34.1)	chr9:133,658,293-134,092,544	0.43	<i>ABL1, QRFP, FIBCD1, LAMC3, AIF1L, NUP214</i>
10 (U)	del(10)(q25.1q25.2)	chr10:111,634,169-112,348,580	0.71	<i>XPNPEP1, ADD3, MXI1, SMNDC1, DUSP5, SMC3</i>
15 (U)	amp(15)(q13.3q13.3)	chr15:32,098,670-32,539,666	0.44	<i>CHRNA7</i>

CNAs: copy number alterations; aCGH: array-comparative genomic hybridization

potentially benefit from tyrosine kinase inhibitors<sup>[16-17]</sup>.

Episomes are submicroscopic, circular and large acentric DNA fragments that can replicate autonomously. One of the common formation-mechanisms for extrachromosomal elements in cancer cells is episome-replication and unequal segregation during cell division, resulting finally in an increase of copy numbers. Still they that are invisible in banding cytogenetics; this is because episomes are composed of only several hundred kilobases of amplified oncogenes and/or drug-resistance genes, and thus are too small to be visualized by light-microscopy<sup>[18-20]</sup>. Of interest, we detected a variable number of episomes (20-30) in different cells. However, we suggest that c-MYB is also present on the same episomes [Figure 2]; due to lack of material we could not confirm this by FISH.

Additionally, recurrent acquired CNAs in different chromosomal regions were also identified besides unique ones for this case [Table 2]. Taken together, the genomic abnormalities in T-ALL and T-LBL are so similar

that they could be considered as identical diseases in the future<sup>[1,4,12,14,15,21-24]</sup>.

As shown in our case, *NUP214-ABL1* is accompanied with loss of cyclin-dependent kinase inhibitor 2A (*CDKN2A*), which encodes the tumorsuppressors p16INK4A and p14ARF, and affects cell cycle progression. *CDKN2A* gene deletion can be detected at initial diagnosis or acquired at relapse, suggesting that *CDKN2A* gene deletion is a secondary genetic event and associated with chromosomal rearrangements. This may as a result lead to the aberrant expression of a diverse group of T-cell-specific transcription factors, which again can function as oncogenes, such as *TLX1* and *TLX3*<sup>[2,21,25]</sup>. The translocation t(7;10)(q34;q24), resulting from the *TRB/TLX1* fusion gene, has been reported in several studies, and is present in 5% of pediatric and 30% of adult with T-cell ALL<sup>[25-28]</sup>.

Overall, in the present T-LBL case we identified substantial intra-tumor genetic heterogeneity and complexity. The founder clone has *TRB/TLX1* fusion gene and the subclone has *TRB/TLX1* fusion gene plus complex karyotype involving three-way translocation t(2;7;10)(2q37.3;7q34;10q25.1), further developing into a more complex subclone. Interestingly, the breakpoints at 2q37.3, 7q34, 10q24.3 and 10q25.1 were not previously reported in T-LBL<sup>[29]</sup>. Thus, this data provides genetic support for a multi-step pathogenesis: deletion of a tumor-suppressor gene (*CDKN2A*), deregulated expression of a transcription factor *TLX1* and most likely overexpression of a constitutively activated tyrosine kinase (*NUP214-ABL1*) and oncogene *c-MYB* due to epigenome amplification and the unique phenotypes of the T-LBL case mentioned above.

To conclude, this study demonstrates the power of high resolution molecular approaches. It may be considered that the use of such approaches is the most efficient and future standard method for screening *ABL1* alteration. Particularly in T-LBL patients this may be advantageous, as *ABL1* modulates T-cell development and plays a role in cytoskeletal remodeling processes in T-cells. Besides, the intra-tumor genetic heterogeneity in cancer has important implications for reservoirs of cells involved in progression of disease and drug resistance therapy. As *NUP214-ABL1* fusion is sensitive to the tyrosine kinase inhibitor, this suggests that new therapeutic approaches in T-LBL may improve outcome and/or decrease treatment-related morbidity.

## DECLARATIONS

### Authors' contributions

Did the FISH-studies and drafted the paper: Othman MAK

Performed array comparative genomic hybridization (aCGH) analyses and interpretation: Melo JB, Carreira IM, Othman MAK

Provided T-LBL-case including clinical and banding cytogenetic data: Grygalewicz B, Kołkowska-Leśniak A

Planned and organized the study and did final drafting of the paper: Liehr T

All authors read and approved the paper.

### Availability of data and materials

All data is provided in this article. Also the patient was mentioned previously in<sup>[30]</sup> as P61.

### Financial support and sponsorship

None.

### Conflicts of interest

All authors declared that there are no conflicts of interest.

### Ethical approval and consent to participate

The present study was approved by the Ethical Board at the Friedrich Schiller University (Jena, Germany; approval No., 1105-04/03). Consent to participate of the parent of the patient studied here is available on request from the authors of this paper.



**Consent for publication**

Not applicable.

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Original Article

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# Two sides to colon cancer: mice mimic human anatomical region disparity in colon cancer development and progression

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## Abstract

**Aim:** Strong evidence reveals important differences between cancers in the proximal *vs.* distal colon. Animal models of metastatic colon cancer are available but with varying degrees of reproducibility and several important limitations. We explored whether there were regional differences in the location of murine colon cancers and assessed the utility of murine models to explore the biological basis for such differences.

**Methods:** We re-analyzed data from our previous studies to assess the regional distribution of murine colon cancer. In survival surgery experiments, we injected HT-29 human colon cancer cells into the wall of the cecum or distal colon of Nu(NCr)-Foxn1<sup>nu</sup> or NOD.Cg-Prkdc<sup>scid</sup>Il2rg<sup>Tim1Wji</sup>/SzJ mice and compared the development of primary tumors and metastases.

**Results:** Within 7-17 weeks after intramural cecal injection of HT-29 cells, eight mice failed to develop solid primary tumors or metastases. In contrast, within four weeks after cell injection into the distal colon, 13 mice developed metastases - 12 mice developed subcutaneous metastases; of these, four developed liver metastases and one developed both liver and lung metastases. One mouse developed liver metastases only. Histological examination confirmed these lesions were adenocarcinomas.

**Conclusion:** Our findings reveal the preferential growth of murine colon neoplasia and invasive human orthotopic



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xenografts in the distal mouse colon. The new approach of injecting cells into the distal colon wall results in a pattern of colon cancer development that closely mimics the progression of metastatic colon cancer in humans. This novel model of colon neoplasia has great potential for exploring anatomical differences in colon cancer and testing novel therapeutics.

**Keywords:** Colorectal cancer, orthotopic tumor model, mouse model, HT-29 cells, colon

## INTRODUCTION

In developed countries, colorectal cancer (CRC) is the second most common cause of cancer-related death in men and the third in women<sup>[1]</sup>. Metastatic cancer is the chief reason for CRC-related death; primary tumors without metastases are readily cured by endoscopic or surgical therapy<sup>[1]</sup>. Intriguingly, strong evidence reveals important differences between cancers in the proximal vs. distal colon<sup>[2-6]</sup>.

Compared to cancers of the distal colon, proximal colon cancers are more common in women, are associated with microsatellite instability and the serrated pathway, and are more likely to be at advanced stages when first diagnosed. Distal colon tumors are more likely to be associated with chromosomal instability and arise from the pathway involving dysregulated *APC*, *K-ras*, *DCC*, and *p53*<sup>[6]</sup>. Previous studies reported conflicting findings with regards to whether mortality was significantly different in those with primary right- vs. left-sided colon cancer<sup>[7-9]</sup>. A meta-analysis found higher mortality in patients with right-sided compared to left-sided colon cancer<sup>[7]</sup>. A recent database study found that right-sided colon cancer was associated with lower cancer-specific mortality at the localization stage, equivalent mortality at the regional stage, and higher mortality at the metastatic stage<sup>[7]</sup>. Another recent retrospective study found those with left-sided colon cancer had better survival outcomes, especially with stage III cancers<sup>[10]</sup>. From 1998 to 2013, the SEER (Surveillance, Epidemiology, and End Results) database identified 90,635 and 112,679 persons diagnosed with left- and right-sided colon cancer, respectively<sup>[7]</sup>.

Few therapeutics are either effective or available to treat persons with metastases to the liver and other organs. To improve therapeutic outcomes, there is great urgency to gain a better understanding of the mechanisms underlying colon cancer dissemination as a basis to develop targeted therapies. For investigators to test such new therapeutics with some degree of reliability there is also a great need to conceive and develop novel models that more closely mimic human disease.

Several animal models of metastatic colon cancer are available, with varying degrees of reproducibility, limitations, and imperfect fidelity to the biology of human cancer. Current murine models are limited by location, depending on what model is used, and cancers in different locations have different genetic profiles. A case in point is *ApcMin* mouse models that were meant to recapitulate defective Wnt/ $\beta$ -catenin signaling present in ~90% of human colon cancer<sup>[11]</sup>; in the most commonly used *ApcMin* mouse strains, tumors are almost uniformly adenomas, not adenocarcinomas, and are located predominantly in the small intestine, not colon. Also, murine models using injection of human colon cancer cells are limited by the need to use immune-deficient mice to allow tumors to develop, thus excluding the testing of immunotherapies<sup>[12]</sup>. Nonetheless, using syngeneic models with murine colon cancer cells is also imperfect because these cell lines are less well-studied and their biology may not mimic that of human cancers<sup>[12]</sup>.

Despite their limitations, murine models have long served as the most reliable platform for preclinical evaluation of new drugs and technologies<sup>[12]</sup>. These include models employing chemical carcinogenesis, genetic engineering, and animal- or patient-derived xenografts<sup>[1,13]</sup>; the latter have been particularly helpful to study the mechanisms underlying the metastatic spread of human colon cancer and identify susceptible therapeutic targets<sup>[1,12]</sup>. Colorectal cancer xenografts grown subcutaneously in immunodeficient mice are limited by the lack of metastasis; instead, orthotopic tumor models involving injection of CRC tumor cells or implanta-

tion of tumor tissue directly into the wall of the colon have the potential to be more representative of human metastasis<sup>[14]</sup>.

We sought to determine if mice could serve as a model to explore regional differences in the location of cancers within the colon. To seek such differences in the growth and progression of colon neoplasia in mouse models, we first determined the location of colon tumors in mice treated with a colon-selective carcinogen or with a genetic predisposition to intestinal neoplasia. Next, based on our initial findings, we compared the proclivity of human colon cancer cells to grow and invade the proximal vs. distal colon of immune-deficient mice.

## METHODS

### Analysis of the distribution of colon neoplasia in our published studies of chemically-induced carcinogenesis in mice

To assess the regional distribution of murine colon cancer, we re-analyzed data from our published and unpublished murine colon cancer studies conducted from 2006 through 2018<sup>[15-19]</sup>. During this interval, we had treated 10- to 23-week-old male mice on a variety of genetic backgrounds with weekly intraperitoneal injections of 7.5 mg azoxymethane (AOM)/kg body weight for 4 weeks. In C57BL/6 mice that are resistant to AOM treatment alone<sup>[20]</sup>, we supplemented the drinking water with 2.5% dextran sodium sulfate (DSS) for 5 days. We euthanized mice 20 weeks after the first AOM injection. An investigator masked to mouse genotype and treatments measured tumor number and size, and tumors were characterized as adenomas or adenocarcinomas based on size, contour, and color. A senior pathologist classified colon tumors as adenomas or adenocarcinoma based on consensus recommendations<sup>[21]</sup>.

### Surgical induction of colon neoplasia

#### Cell culture

We purchased authenticated HT-29 cells from American type culture collection (ATCC). HT-29 cells were grown in McCoy's 5A medium (Life Technologies) supplemented with 10% FBS. We grew cells in a humidified incubator at 37 °C with 5% CO<sub>2</sub> and passaged weekly at subconfluence after trypsinization. We suspended cells in DPBS (50 × 10<sup>6</sup> cell/mL) containing 10 μmol/L Y27632 and 50% Matrigel.

#### Animals

All animal studies were conducted at the Baltimore VA Hospital Animal Facility and our laboratory in the Bressler Research Building at the University of Maryland School of Medicine. All surgical procedures were approved by the University of Maryland School of Medicine Institutional Animal Care and Use Committee under the Office of Animal Welfare Assurance. The Research and Development Committee at the Baltimore VA also approved animal studies. We used 11- to 14-week old male Nu(NCr)-Foxn1<sup>nu</sup> (nude) mice and NOD.Cg-Prkdc<sup>scid</sup>Il2rg<sup>Tim1Wji</sup>/SzJ (NSG) mice, obtained from both the University of Maryland Veterinary Resources and Jackson Laboratories (Bar Harbor, ME).

#### Surgical technique - laparotomy

In a biosafety cabinet (BSL2) we anesthetized mice with continuous vaporized isoflurane for general anesthesia and performed laparotomy and cell injections with the mice on a warming pad. After confirming a sufficient level of anesthesia by a toe pinch, we positioned mice prone. For corneal protection, we applied lubricant (Major Pharmaceuticals LubriFresh P.M Ophthalmic Ointment, Livonia, MI) to each eye. We disinfected the mouse's upper back with an alcohol swab and administered buprenorphine SR (concentration 0.3 mg/mL, dose 0.05-0.1 mg/kg body weight diluted 1:9 with sterile 0.9% saline) or carprofen (concentration 50 mg/mL, dose 5 mg/kg body weight diluted 1:9 with sterile 0.9% saline) subcutaneously for analgesia. We then placed mice supine and, if necessary, clipped the anterior abdominal hair. After skin preparation with alcohol, to provide local anesthesia we injected mice subcutaneously with 0.25% bupivacaine (concentration 2.5 mg/mL, dose 0.1 mL diluted 1:2 with sterile 0.9% saline) along the planned midline laparotomy site. Next,



we cleansed the abdomen with povidine-iodine solution and alcohol and applied sterile drapes. We made a small midline laparotomy and inserted a self-retaining retractor in the upper abdomen.

Following injection of human colon cancer cells and replacing the intestines, we approximated fascial edges with 5-0 vicryl running sutures and closed the skin primarily with 4-0 nylon interrupted sutures. After applying skin glue (3M Vetbond tissue adhesive, St. Paul, MN) to the suture line, we awakened mice slowly from anesthesia and placed them in a clean cage for recovery with close monitoring. After completion of the operation, the mice were administered analgesia for at least 72 h post-operatively and monitored closely.

#### *Surgical technique - cecal injection*

To explore the predilection of human colon cancer cells to form tumors in different regions of the mouse, we first injected  $2$  to  $5 \times 10^6$  (40-100  $\mu$ L) HT-29 human colon cancer cells into the cecum of nude or NSG mice. We chose these cell numbers based on previous reports describing successful metastatic models of colon cancer in mice<sup>[1,12,22,23]</sup>. We described pre-operative steps above. The cecum was located using moist sterile cotton tip applicators and brought outside the abdomen onto a moist  $2 \times 2$  sterile gauze. In all mice, we injected  $2$ - $5 \times 10^6$  cells (40-100  $\mu$ L) into the wall of the cecum using a 27-gauge needle. After injection, we applied light pressure at the injection site for approximately 30 s with a moist sterile tip applicator and inspected the area for leakage. We irrigated the cecum and abdominal cavity with warm DPBS, and then returned the cecum to its normal anatomic position within the abdomen. Closure of the abdomen was performed as describe above.

#### *Surgical technique - flank injection*

In mice failing to form cecal tumors, we confirmed the ability of the HT-29 cells to form xenografts and metastases following subcutaneous and splenic injection, respectively. For subcutaneous injections, we briefly anesthetized mice with vaporized isoflurane, disinfected their flanks with alcohol, and injected  $2 \times 10^6$  cells (40  $\mu$ L) in each flank. We recovered mice from anesthesia in their cages.

#### *Surgical technique - splenic injection*

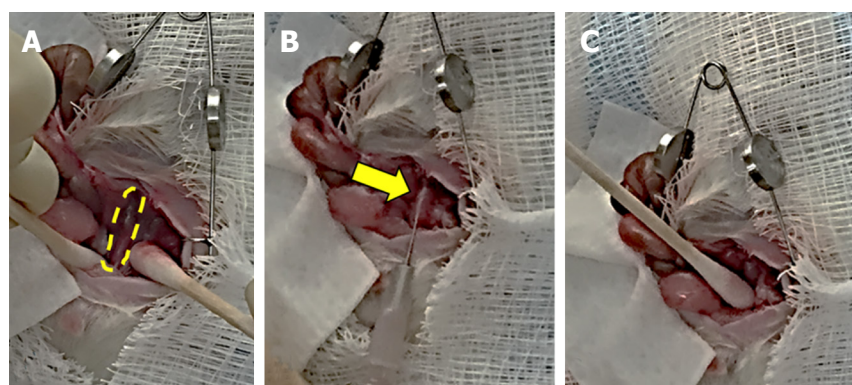
We described pre-operative steps above. The spleen was located using moist sterile cotton tip applicators and brought forward within the abdomen. In all mice, we injected  $5 \times 10^6$  cells (100  $\mu$ L) into the wall of the spleen using a 27-gauge needle. After injection, we applied light pressure at the injection site for approximately 30 s with a moist sterile tip applicator and inspected the area for leakage and bleeding. We irrigated the spleen and abdominal cavity with warm DPBS, and then returned the spleen to its normal anatomic position within the abdomen. After 1 h, we removed the spleen and irrigated the abdomen again with DPBS. We closed the abdomen as described above.

#### *Surgical technique - distal colon injection*

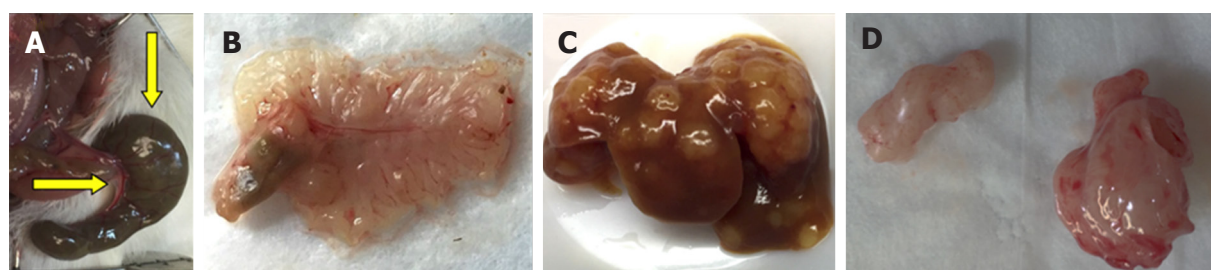
To induce colon cancer growth and metastasis, we injected  $5 \times 10^6$  (100  $\mu$ L) HT-29 human colon cancer cells into the wall of the distal colon of nude or NSG mice. We described pre-operative steps above. The distal colon was located using moist sterile cotton tip applicators [Figure 1A]. In all mice, we injected  $5 \times 10^6$  cells (100  $\mu$ L) into the wall of the distal colon using a 27-gauge needle [Figure 1B]. After injection, we applied light pressure at the injection site for approximately 30 s with a moist sterile tip applicator [Figure 1C] and inspected the area for leakage. We irrigated the distal colon and abdominal cavity with warm DPBS. We closed the abdomen as described above.

### **Statistical analysis**

We used the unpaired Student's *t* test (assuming unequal variance) to compare continuous variables between two independent groups. For multi-group comparisons, we applied two-way ANOVA with one between-subject factor (WT vs. FGF15-deficient) and one within-subject factor (normal tissue vs. tumor tissue) followed by post hoc tests with Tukey-Kramer's adjustment for *P* values. We used Fisher's exact test to compare



**Figure 1.** Main steps in the surgical approach to injecting colon cancer cells in the murine distal colon. A: Isolation of the distal colon (outlined) using moist sterile cotton tip applicators with retraction of the abdominal wall and evisceration of abdominal organs; B: injection of  $5 \times 10^6$  HT-29 human colon cancer cells into the wall of the distal colon (arrow) using a 27-gauge needle; C: applying pressure with a moist sterile cotton tip applicator at the injection site to prevent leakage and hemorrhage



**Figure 2.** Results of cecal, splenic, and subcutaneous injections of HT-29 human colon cancer cells. A, B: Serosal and mucosal images of normal cecum 15 weeks after injecting HT-29 cells; C: numerous liver metastases 4 weeks after splenic injection; D: representative xenografts harvested from mouse flanks 4 weeks after subcutaneous flank injection

proportions. We considered differences significant when  $P$  was less than 0.05.

## RESULTS

### Chemical induction of colon cancer

During a 12-year period, we induced colon neoplasia by treating 182 mice with AOM alone and 94 mice with AOM plus DSS. Strikingly, in all AOM- and AOM/DSS-treated mice that developed adenomas and adenocarcinomas [265 of 276 mice (96%)], all tumors were limited to the distal half of the colon; no proximal lesions were present ( $P < 0.001$ ). None of the 276 mice that developed primary colon tumors had metastases.

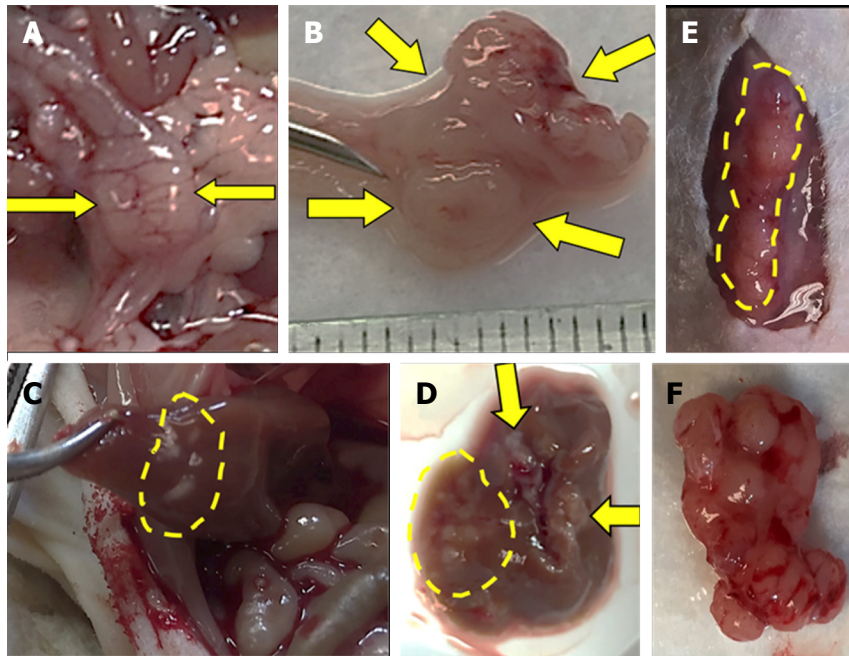
### Surgical induction of colon cancer

#### Cecal/flank/splenic injection

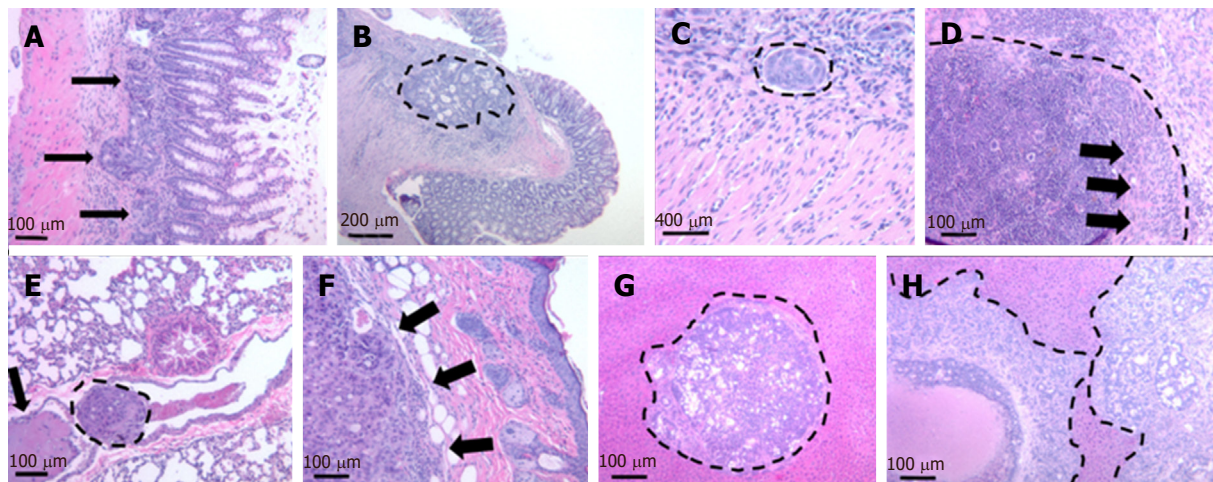
Seven to 17 weeks after cecal injection of HT-29 cells, none of 8 mice (5 nude, 3 NSG) developed a cecal lesion [Figure 2A and B]. Yet, within 4 weeks, injecting HT-29 cells into the spleens and flanks of nude mice (3 mice each) uniformly yielded liver metastases [Figure 2C] and xenografts [Figure 2D], respectively, confirming the cells were capable of developing solid tumors that grew and metastasized. No metastases developed after flank injection and xenograft formation.

#### Distal colon injection

Within four weeks after cell injection, 12 mice developed primary colon tumor at the injection site in the distal colon and 13 mice (4 NSG, 9 nude) developed metastases [Table 1]. Based on our preliminary mouse experiments as well as the results of previously published studies<sup>[1,23,24]</sup>, we euthanized mice four weeks after cell injection. Twelve mice developed subcutaneous anterior abdominal metastases; of these, four developed



**Figure 3.** Injected human colon cancer cells form solid tumors in the distal colon with liver, lung, and anterior abdominal wall metastases. Serosal (A) and mucosal (B) images show invasive solid tumor in the distal colon (arrows). Metastases in the liver, *in situ* (C) and *ex vivo* (D) (arrows and dashed lines). Subcutaneous metastases in the anterior abdominal wall, *in situ* (E) and *ex vivo* (F).



**Figure 4.** Representative histological images of local colon tumor as well as metastases to the lung, liver, and anterior abdominal wall. (A) and (B) Primary tumor invading the intestinal wall (arrows and dashed lines); C: tumor emboli in intramural and subserosal lymphatics (dashed lines); D: lymph node infiltration. Dashed lines delineate lymph node capsule, arrows indicate tumor cells; E: metastasis to the lung: intravascular tumor embolus (dashed lines) and thrombus (arrow); F: subcutaneous metastasis to anterior abdominal wall (arrows) with epidermis to the right; G and H: multiple metastatic tumor deposits within the liver (dashed lines).

liver metastases and one developed both liver and lung metastases [Figure 3]. One mouse developed liver metastases only. Histological examination confirmed the presence of adenocarcinoma within the wall of the mouse colon [Figures 4A and B], in the lymphatic spaces [Figures 4C and D], in the lung parenchyma [Figure 4E], in the anterior abdominal wall [Figure 4F], and multiple metastatic deposits within the liver [Figures 4G and H].

## DISCUSSION

Increasing evidence supports the presence of major differences in right- and left-sided colon cancers with regard to the host's clinical characteristics, microbiome, response to treatment and outcome. Although mo-



**Table 1. Distribution of primary colon tumors and metastases after injection of human colon cancer cells into the distal colon wall of 13 mice**

Mouse	Strain	Primary colon tumor	Liver metastases	Abdominal wall metastases	Lung metastases
1	NSG	√	-	√	-
2	NSG	√	-	√	-
3	NSG	√	-	√	-
4	NSG	√	-	√	-
5	Nude	√	-	√	-
6	Nude	√	√	√	-
7	Nude	√	√	√	√
8	Nude	-	-	√	-
9	Nude	√	-	√	-
10	Nude	√	√	√	-
11	Nude	√	√	√	-
12	Nude	√	√	√	-
13	Nude	√	√	-	-

NSG: NOD.Cg-Prkdc<sup>scid</sup>Il2rg<sup>Tim1Wji</sup>/SzJ.

lecular and genetic profiling of cancer cells has revealed some important differences, the reasons for this anatomical disparity remain unclear<sup>[25,26]</sup>. Whether the primary tumor is located in the right or left colon has been reported to play a prognostic role in metastatic colorectal cancer<sup>[25]</sup>, although new findings suggest primary tumor 'sidedness' may be a less important determinant of overall and disease-specific survival than patient characteristics and other pathological features<sup>[26]</sup>.

Our studies reveal that mice appear to be a suitable model to explore the predilection of cancer for different anatomical regions of the colon. We found that mice treated with a colon-selective carcinogen or with a genetic predisposition to intestinal neoplasia developed tumors only in the distal half of the colon; there were no proximal lesions. Likewise, immune-deficient mice preferentially developed colon cancer and metastases when we injected HT-29 human colon cancer cells into the distal, rather than proximal, colon.

In the course of these studies, we developed a novel method for inducing metastatic colon cancer in mice. We chose HT-29 cells for our studies because they are commonly used *in vitro* model of human colon cancer and express M3 type muscarinic receptors (M3R), a focus of our research program<sup>[27-29]</sup>. Injecting HT-29 human colon cancer cells between the mucosa and the muscularis external layers of the distal colon wall of immunodeficient mice resulted in a pattern of tumor dissemination that mimicked human disease. Nonetheless, while it is uncommon for colon cancer in humans to spread to the skin and subcutaneous tissues, we found that when injected in mice, HT-29 cells are capable of diffuse metastasis, including to the skin. At present, we cannot explain why these colon cancer cells had a predilection for the anterior abdominal subcutaneous tissue. The location of these skin metastases near the surgical incision site leads us to speculate that features of the inflammatory response to the skin incision (e.g., release of cytokines) may attract migrating colon cancer cells to this location, a testable hypothesis that may expand our understanding of the biology underlying tumor metastasis. We will test this hypothesis in future studies.

We believe this novel animal model will be an important adjunct to our *in vitro* studies and useful to study and test novel therapies that target M3R and its downstream signaling pathways to attenuate cancer cell dissemination. This model is relatively straightforward and the procedures easy to learn and perform by an investigator experienced in animal surgery, with reasonably rapid development of primary solid tumors and metastases. Unlike xenograft models, this method requires only one mouse and one operation to generate both colon cancer and metastasis. Our approach appears more biologically relevant than models in which investigators inject cells into the tail vein or footpad.

An important limitation of our new approach is that, as discussed in the Introduction, colon cancer immunotherapy cannot be studied in models using immune-deficient mice. However, humanizing the mouse immune system may achieve this goal. Next-generation models, including “immunoavatar mice” could offer the ability to study the effects of immunotherapy in colon cancer. Hemato-lymphoid humanized mouse models may allow the development of a complete human immune system in a human tumor-bearing mouse<sup>[30]</sup>. Yet, even these humanized models are likely to present important obstacles with regard to mimicking the physiological maturation of human immune cells and the progression of human colon cancer.

In conclusion, our findings identify preferential growth of murine colon neoplasia and invasive human orthotopic xenografts in the distal mouse colon. These data support the utility of mouse models to study anatomical variance in the development and progression of colon neoplasia. We describe a useful model for inducing metastatic colon cancer in mice that is neither laborious nor time-consuming. This novel approach furnishes animals that closely mimic the progression of metastatic colon cancer in humans. This approach shows promise for studying novel therapeutics targeting colon cancer dissemination and metastasis.

## DECLARATIONS

### Authors' contributions

Study Design: Felton J, Cheng K, Raufman JP

Experimental Methods: Felton J, Cheng K, Shang AC, Hu S, Drachenberg CB, Raufman JP

Manuscript Preparation: Felton J

Final Review: Felton J, Raufman JP

Manuscript Review: Cheng K, Shang AC, Hu S, Larabee SM, Drachenberg CB, Raufman JP

### Availability of data and materials

Not applicable.

### Financial support and sponsorship

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### Conflicts of interest

All authors declared that there are no conflicts of interest.

### Ethical approval and consent to participate

Not applicable.

### Consent for publication

Not applicable.

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Perspective

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# Pharmacogenetics and cancer management

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## Abstract

The science of one's genetic background and its impact on disease susceptibility and drug response has come of age and firmly established its proper place in the clinic. Its impact is felt more in the treatment of cancer than any other disease area several reasons: critical time, narrow therapeutic index and overlapping toxicity window. We realize that the true potential of pharmacogenetics will be realized when we have been able to integrate other variants like insertion-deletion, copy number variation, *etc.*, in addition to single nucleotide polymorphism for their collective influence on drug response and toxicity. Technology has rapidly evolved and has become affordable to be used in the clinic once it gets standardized and validated not only in one population but in several major world population -particularly those which are under-represented in human variant database.

**Keywords:** Pharmacogenetics, drug response, DNA variants, insertion-deletion, copy number variation, therapeutic efficacy, toxicity

At the announcement of the first draft of the Human Genome Project in 2000, US President Bill Clinton proclaimed, "future generation will know cancer only as a zodiac sign" hoping that deciphering the human genome will lead to the eradication of cancer. This announcement, although ambitious, paved the way to look at the human genome and attribute the regions of genome contributing to the formation of cancer cells and dictating the response to treatment. The human genome changed the rules of the game. This is also reflected in US President Obama's Precision Medicine Initiative launched in January 2015, "Precision medicine gives clinicians tools to better understand the complex mechanisms underlying a patient's health, disease, or condition, and to better predict which treatments will be most effective"



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(<https://obamawhitehouse.archives.gov/the-press-office/2015/01/30/fact-sheet-president-obama-s-precision-medicine-initiative>).

Earlier a very elaborate mix of population genetics, molecular genetics, and very complex statistical approach was used to identify the gene(s) attributed to the development of cancer<sup>[1]</sup>. *BRCA1* and *BRCA2* genes were localized on chromosomes 17 and 13 respectively, through this approach. The technique, known as positional cloning, was very elaborate and yet proved evasive in the case of cancer and many other polygenic diseases that are influenced by the environment as well. Subsequently, in the post-genomic era, a whole genome scan from case-control studies revealed several “probable” genes likely to influence cancer development, metastasis, and the treatment outcome<sup>[2,3]</sup>. The genes and their variants associated with cancer are found in all the major races and populations of the world, although their frequency occurrence may slightly differ among various populations. Therefore, what really matters is more about one’s own genetic background, than about which race or population one belongs to.

The human genome and its resultant tools and techniques have been tremendously useful in the cancer management. Although the hope expressed by Bill Clinton is beyond the horizon at the moment, we have learned many things about the origin of cancer cells, its spread and metastasis, and treatment. Perhaps the most important lessons of the past two decades are: (1) cancer etiology is very complex and heterogeneous, implying that the formation of cancer in two patients might have different molecular etiology. This is an important consideration as the same treatment may not be equally effective for both the patients; and (2) there is also heterogeneity of patient population implying that two patients, based on their genotypic and phenotypic make-up may respond differently to the identical treatment protocol<sup>[4]</sup>. The understanding that there is tremendous variability in drug response which is emanating from the individual’s genetic and metabolic variants gave rise to the science of pharmacogenetics.

Soon after the Human Genome Project, we saw a few individual genome analyses followed by massive genome-wide exon scans that collectively have enormously enriched the data of the human genome. As the scale of technologies expanded, the cost came down, and it became desirable as well as affordable to use genetic testing for determining individual’s genetic susceptibility to develop cancer and thereby its prevention, treatment regimen and its prognosis<sup>[5]</sup>. In 2001, the cost of one genome sequence was about 100 million USD, which has now come down to about 1000 USD. With this price tag, a scan of one’s individual genome has become a reality and may become a necessary tool in the health management<sup>[6]</sup>.

Pharmacogenetics can play an important role in identifying responders and non-responders to medications, avoiding adverse events, and optimizing drug dose. For example, Ciccolini *et al.*<sup>[7]</sup> have summarized utility of pharmacokinetic and pharmacogenetics in chemotherapy with gemcitabine. Realizing the importance of genetic biomarkers, US FDA maintains the list of Pharmacogenomic Biomarkers for drug labeling purpose (<https://www.fda.gov/downloads/Drugs/ScienceResearch/UCM578588.pdf>). Similarly, Genetic Testing Registry from NCBI/NIH has 241 tests listed for cancer of which 64 are pharmacogenetic tests for 18 genetically influenced drug responses (e.g., tamoxifen, irinotecan, thioguanine, fluorouracil) (<http://www.ncbi.nlm.nih.gov/gtr/>). As of now, there are dozens of companies which will test one’s DNA for the susceptibility to a variety of cancers for about 200 USD. Their panel of genes may have from about 20 to 100 genes associated with a variety of cancers. Such pre-symptomatic testing which has tremendous value in cancer prevention is slowly becoming acceptable and even desirable, especially if one of the family members has been afflicted by cancer. One can debate about the number of gene variants on the commercial tests, the fact is that the list can’t be exhaustive as it evolves with each new study, and the complexity of “system biology” where gene products may exhibit compensatory functions *in vivo*. I anticipate that for some genotyping tests, we may need it coupled with respective phenotyping parameter in the future.

While we welcome the trend, we should be wary of the limitations of such testing: (1) the science of association between a given gene variant and cancer is tenuous and still evolving; (2) the clinical studies indicating gene-cancer association may have been done in a given population and there are strong reasons that the studies need to be validated in other major populations<sup>[8]</sup>; (3) the human genome data is not really that representative of the world's population. It has been largely collected from the Caucasian population, and may not have adequate representation from African, Asians including Indian and Chinese populations<sup>[6]</sup>; (4) the statistical increased or reduced risk assessment may vary from one study to another, and based on it, it is tricky to counsel the general population about the risk; and (5) the population at large may not be prepared to understand the associated risk, and may not be prepared to deal with such predictive risk assessment.

Benefits of pharmacogenetics are two-fold: (1) with a certain probability, we can predict the risk from cancer for a given individual. This type of pre-symptomatic diagnosis can to a great extent prevent cancer mortality by adopting frequent screening for the suspected cancer risk and catching it at the very early stage. BRCA1 and BRCA2 are excellent examples where women with positive cancer biomarker can be vigilant and catch cancer before it had a chance to spread; and (2) in many cases, pharmacogenetic tests can help the oncologist to a better treatment regimen with minimum toxicity. Both of these benefits have become part of the cancer management. In American Society of Clinical Oncology (ASCO) post of May 2016, Dr. Stephen T. Sonis has summarized the role pharmacogenetics can play in cancer patient care (<https://am.asco.org/daily-news/personalizing-supportive-care-pharmacogenomics-and-risk-prediction>).

Pharmacogenetics can help us reduce the toxicity of chemotherapy by selecting the right drug and its dose for a given patient based on his drug response profile<sup>[9,10]</sup>. Good examples of such an approach are Herceptin and Xeloda, where predicted non-responders are spared from the respective treatment and the undue toxicity is minimized. I am reminded of my conversation about a decade ago with an oncologist who considered the optimum dose is the maximum dose a patient can tolerate, who subsequently agreed that it would be nice if we can know the effective dose and the toxic dose before treatment for each patient. For cancer where the time is very critical, the cost and the toxicity of chemotherapy are high, and where the therapeutic window overlaps with the toxicity window, the pharmacogenetics offers a valuable tool to select an appropriate drug and its dose for a particular patient, and achieve an optimized therapeutic outcome. I take liberty to quote Dr. Howard L. McLeod in October 2016 ASCO post, "The somatic genome can assist oncologists in predicting a patient's tumor behavior if left untreated (prognosis) or treated (efficacy prediction), and the germ-line genome can influence prognosis as well as help assess the level of drug-related toxicity the patient will likely experience." He further added, "As our data become richer, we will get to the point where we can predict all severe drug toxicities."

There are four fundamental limitations in our approach in taking the science of pharmacogenetics to the clinic: (1) the first and the foremost is the over-emphasis on single nucleotide polymorphism (SNP) in considering it synonym with genetic variants. The fact is that there are other forms of variants, like insertion, deletion, copy number variation (CNV) which are abundant in the human genome and may cover a larger part of the genome than that covered by SNP and may have more influence in cancer development and drug response<sup>[11,12]</sup>. Since these variants are relatively new and technologically not as convenient to type, their impact is undervalued. As the science of pharmacogenetics develops further, it will be hard to ignore them; (2) the second and equally important limitation is that most of our studies have tried to link a given gene or its SNP(s) to a very complex biology of cancer. We have realized, but yet not put to test, that ultimately a disease like cancer may not be associated with a single SNP or a gene. It has to be a complex combination of several genetic loci and their variants which can collectively lead a normal cell to become transformed. Since there are many possible permutations and combinations of variants and genes to be studied for their association with cancer, practically it has remained a daunting

task and perhaps awaits better technologies or illuminating algorithm to reveal such consortium of variants responsible for the transformation of a cell to become cancerous; (3) the third limitation is for us to realize that genotype is not everything. The manifestation of a genome continuously changes with age, physiological and environmental conditions. Hence over emphasis on genotype and underplaying phenotype may not help us understand cancer or lead us to its meaningful cure; and (4) since genotyping results are likely to influence medical decisions, it is imperative that the technology of genotyping is standardized and validated. This is of pivotal importance to eliminate lab to lab variation. Similarly standardized format should be used in reporting results. These issues have been rightly pointed out by Morvan *et al.*<sup>[13]</sup>. These simple but critical technical improvements will help in making pharmacogenetics an important tool in cancer management.

In conclusion, we have come a long way in our understanding of the role the genetic background of an individual plays in the susceptibility to cancer, in the treatment outcome and prognosis. We have a long way to go to utilize the new knowledge in the integration of our overall understanding of cancer biology and the ways to conquer it. The hope President Bill Clinton expressed, may become reality one day!

## DECLARATIONS

### Authors' contributions

The author contributed solely to the article.

### Availability of data and materials

Not applicable.

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None.

### Conflicts of interest

The author declared that there are no conflicts of interest.

### Ethical approval and consent to participate

Not applicable.

### Consent for publication

Not applicable.

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Original Article

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# Secondary malignancy estimation in patients after mastectomy and adjuvant therapy

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## ABSTRACT

**Aim:** Secondary malignancy estimation after radiotherapy of post mastectomy patients is becoming an important subject for comparative treatment planning. The data from modern treatment planning systems provide accurate three-dimensional dose distributions for each individual patients, thereby opening up new possibilities for more precise estimates of secondary cancer incidence rates in the irradiated organs.

**Methods:** This study estimates the probability of secondary malignancy using radiobiological model for post mastectomy patients in a low-resource center, Nigeria. The secondary cancer complication probability (SCCP) was computed for linear, linear-exponent and linear-plateau models.

**Results:** The result shows that comparing the three models the mean SCCP for the contralateral breast ranged between 0.41%-0.93%; for the lung (0.34%-5.93%); while for the chest wall is between 0.65%-31.95%. Also, the result showed that based on the differential dose volume histogram, the SCCP in the chest wall is highest compared to the lung and contralateral breast; while the linear model overestimate the risk of secondary malignancy, the linear-exponent and the linear plateaus gave values not outrageously high.

**Conclusion:** The models in this study have shown that the risk of secondary malignancy in these post mastectomy patients is low.

**Keywords:** Probability, radiobiological model, complication, malignancy



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## INTRODUCTION

The most common malignancy reported among women worldwide is breast cancer<sup>[1]</sup>. In Nigeria, majority of patients that are diagnosed with breast cancer each year are firstly treated with surgery followed by radiation therapy<sup>[2]</sup>. Recent technological developments in both diagnosis and treatment of breast cancer as well as awareness campaign of this disease have led to early detection and better treatment management. Subsequently, the increase in the population of long-term survivors of breast cancer patients<sup>[3]</sup>.

The early breast cancer trialists' collaborative group meta-analysis has shown an overall survival benefit in favour of adjuvant radiotherapy (RT) after breast cancer surgery<sup>[4]</sup>. Although, the risk for radiotherapy treated patients regarding the induction of secondary cancer is small, it remains a relevant consideration among post mastectomy patients<sup>[5]</sup>. Quite a number of population-based studies have shown the association between primary breast tumour irradiation and the risk of second cancer within or outside the treatment field<sup>[6-8]</sup>.

In most cases, the treatment of breast cancers are with surgery, radiotherapy and chemotherapy, or most often with a blend of all the above. A significant proportion of patients diagnosed with breast cancer usually undergo radiotherapy<sup>[9]</sup>. Although, following radiotherapy, the cure often a times comes at a price of developing the risk of a second cancer among breast cancer survivors, it is however higher than that for the general population<sup>[6,8,10-12]</sup>.

In particular, irradiation of surrounding tissues during breast RT can cause secondary malignancies to develop within these tissues<sup>[13]</sup>. Secondary malignancy refers to a new histologically proven primary cancer in a person who has survived an earlier cancer event. While the benefits of RT outweigh the risks of developing subsequent cancers, it is imperative to evaluate the long-term consequences of breast cancer therapy. Modelling secondary cancer risk is not very new, and has been applied for many cancer diseases, also for breast cancer patients<sup>[14,15]</sup>, however developing countries with low resource RT centers are yet to adopt this approach. Applying this modelling approach, will go a long way to give quality assurance as to the nature of treatment plan patients are exposed to. The aim of this study is to estimate the risk of secondary cancer after radiotherapy of post mastectomy patients using radiobiological model.

## METHODS

Forty-six patients treated in the Radiotherapy Unit, University of Benin Teaching Hospital, Benin city, Nigeria, between January 2012 and March 2014 for breast cancer after radical mastectomy were included in this study. All patients underwent computed tomography (CT) simulation in supine position on an angled board, with both arms placed above their head, which was rotated to the contralateral side (GE Brightspeed CT-scanner, GE Medical Systems). Patients received 50 Gy in 25 fractions for 5 weeks. The Elekta PrecisePlan was used for the computerised planning process. The organs at risk were the heart and lungs. The Elekta radiotherapy machine was used in treating the patients.

After the patients information have been anonymized the imported dose volume histograms (DVHs) from the computerised treatment planning system will now be used to calculate the equivalent uniform dose (EUD) and the secondary cancer complication probability (SCCP).

## EUD

This is defined as the uniform dose that, if delivered over the same number of fractions as the non-uniform dose distribution of interest, yields the same radiobiological effect<sup>[16]</sup>.

The phenomenological formula for the generalised EUD (i.e., normal and tumor cells) as proposed by

**Table 1. Parameters used to calculate the secondary cancer complication probability**

Organs	$\alpha$ (Gy <sup>-1</sup> )	$\delta$ (Gy <sup>-1</sup> )	$In_{org}$ (%/Gy)	Source
Breast	0.085	0.139	0.78 [0.6-1.0]	[21]
Lungs	0.085	0.150	1.68 [1.1-2.3]	[22]

Niemierko (1997)<sup>[17]</sup> is

$$gEUD = \left( \sum_i v_i D_i^a \right)^{\frac{1}{a}} \quad [18]$$

Where  $v_i$  is fractional organ volume receiving a dose of  $D_i$  and  $a$  is tissue-specific parameter that describes the volume effect.

## SCCP

The theory of SCCP adopted for this study is based on the Schneider model<sup>[19]</sup>:

$$(1) \quad SCCP = In_{org} OED_{org}$$

where  $In_{org}$  is the organ specific absolute cancer incidence rate for a low dose in percent per gray. These values represent lifetime risk, and assume a residual life expectancy of 50 years. Therefore, any effect of radiation-induced breast cancer associated with age was ignored in this study. Data from atomic bomb survivor was used to estimate the  $in_{org}$  for the breast and thereafter applied to whole-body irradiation.  $OED_{org}$  is the organ equivalent dose and represents the corresponding dose in gray for an inhomogeneous dose distribution, which if it was distributed evenly throughout the organ, would cause similar radiation-induced cancer incidence<sup>[19]</sup>.

Three different dose-response models: linear, linear-exponential, and linear-plateau based on the differential DVHs was used in this study to compute the organ equivalent dose (OED)<sup>[20]</sup>.

$$(2) \quad OED_{lin} = \left( \frac{1}{V_T} \right) \sum_i (v_i \cdot D_i)$$

$$(3) \quad OED_{lin-exp} = \left( \frac{1}{V_T} \right) \sum_i (v_i \cdot D_i e^{-\alpha D_i})$$

$$(4) \quad OED_{lin-plateau} = \left( \frac{1}{V_T} \right) \sum_i \left( v_i \cdot \left( \frac{1 - e^{-\delta_{org} D_i}}{\delta_{org}} \right) \right)$$

The parameters  $\alpha$  and  $\delta$  are the organ specific model parameters for their respective dose-response models. The parameters used to calculate SCCP is given in Table 1.

## Data analysis

The study employed descriptive and inferential statistics to analyse the data. Descriptive statistics used are mean, standard error of mean, percentage frequency distribution; while inferential statistics used include correlation analysis and one way analysis of variance; Scheffe post hoc was used to separate means where significant difference is observed in the SCCP of the different groups of mean dose and EUD. The level of significance was set at 0.05. The analysis was carried out using STATA version 12.

## RESULTS

Using SCCP to evaluate the plans for risk of secondary cancer complication in the contralateral and chest walls and the paired lungs, there was observed difference between the linear, linear-exponent and linear-plateau dose risk models for SCCP due to the fact that the linear model deviates from the other two models for dose larger than 5 Gy. This was very noticeable in the organs exposed with higher doses (paired lungs and planning target volume). This is given in Table 2.

**Table 2. The secondary cancer complication probability (linear, linear-exponent, plateau) indices for different organs**

Models	Contralateral breast (%)	Lung (%)	Chest wall (%)
Linear	0.93 ± 0.24	5.93 ± 0.54	31.96 ± 2.08
Linear exponent	0.41 ± 0.05	0.34 ± 0.03	0.65 ± 0.06
Plateau	0.48 ± 0.07	1.81 ± 0.12	4.83 ± 0.26

**Table 3. Correlation of dose volume histogram parameters of breasts, chest walls and lungs with the secondary cancer complication probability**

	Linear	Linear-exponent	Linear-plateau
Contralateral breast			
Max dose	0.437	0.179	0.546 <sup>*</sup>
Min dose	0.387	0.124	0.487 <sup>*</sup>
Mean dose	0.418	0.170	0.606 <sup>**</sup>
Volume	-0.113	-0.293	-0.139
EUD	-	-	-
Lung			
Max dose	0.318	0.096	0.283
Min dose	0.711 <sup>**</sup>	0.390	0.803 <sup>**</sup>
Mean dose	0.912 <sup>**</sup>	-0.125	0.870 <sup>**</sup>
Volume	-0.217	-0.059	-0.179
EUD	0.759 <sup>**</sup>	-0.079	0.732 <sup>**</sup>
Chest wall			
Max dose	0.040	0.085	0.059
Min dose	0.936 <sup>**</sup>	0.217	0.830 <sup>**</sup>
Mean dose	0.989 <sup>**</sup>	0.361	0.870 <sup>**</sup>
Volume	-0.373	-0.869 <sup>**</sup>	-0.469 <sup>*</sup>
EUD	-	-	-

\* $P < 0.05$ ; \*\* $P < 0.01$ . EUD: equivalent uniform dose

The relationship between DVH parameters and SCCP for the breasts, chest walls and lungs is presented in Table 3. It shows that the DVH parameters of the contralateral breasts did not show any significant relationship with the linear and linear-exponent models, while for the linear-plateau model a positive significant positive relationship exist between the max, min and mean doses. This shows that the max, min and mean doses on the DVH plan is predicative of secondary cancer. The DVH parameters of the lungs did not show any significant relationship with Linear-exponent SCCP; while the min, mean and EUD showed very strong positive relationship with the linear and linear-plateau SCCP. In the chest walls, the min and mean dose showed significant positive relationship with linear model SCCP, volume showed significant negative relationship with linear-exponent SCCP; while min and mean doses and volume showed significant positive and negative relationship respectively with linear-plateau model SCCP. It is interesting to note that in all the three organs, the minimum and mean doses are very strong positive parameters to be considered when planning a patient to reduce the risk of secondary cancer.

Table 4 shows the mean comparison of SCCP at different mean dose to the lung. From the table, it is evidence that for the linear model as the dose increases the SCCP value also increases significantly, but the linear-exponent model did not show any significance as increase dose did not affect the SCCP. The linear-plateau model also showed significance in the mean comparison. The different treatment groups (mean dose) had significantly different SCCP and it follows an increasing order with mean dose.

Table 5 shows the mean comparison of SCCP at different EUD to the lung. From the table, it is clear that for the linear and linear-plateaus models showed significant differences on comparing the EUD groups; while the linear-exponent model did not show any significant difference ( $P > 0.05$ ).



**Table 4. Mean comparison of the secondary cancer complication probability at different mean doses to the lung**

	< 5 Gy	5-10 Gy	Above 10 Gy	P
Linear	3.01 ± 0.91	5.87 ± 0.31	9.78 ± 0.60	0.000
Linear-exponent	0.39 ± 0.10	0.32 ± 0.02	0.32 ± 0.02	0.558
Linear-plateau	1.13 ± 0.28	1.86 ± 0.05	2.49 ± 0.12	0.000

Means with different superscripts are statistically significant at  $P < 0.05$

**Table 5. Mean comparison of the secondary cancer complication probability at different equivalent uniform dose to the lung**

	< 5 Gy	5-10 Gy	Above 10 Gy	P
Linear	4.13 ± 1.00	5.92 ± 0.35	10.16 ± 0.65	0.000
Linear-exponent	0.33 ± 0.05	0.35 ± 0.04	0.30 ± 0.02	0.851
Linear-plateau	1.45 ± 0.24	1.83 ± 0.09	2.59 ± 0.11	0.004

Means with different superscripts are statistically significant at  $P < 0.05$

## DISCUSSION

The risk of secondary malignancy in this study is 4.83% for the chest wall. This statistics is quite higher than the reported epidemiological result of Burt *et al.*<sup>[23]</sup> of approximately 3.4% of secondary malignancies were attributed to radiation therapy. This shows that to a great extent, radiobiological model agrees with epidemiological results; and can thus be incorporated into clinical evaluation of treatment plans during quality check by the medical physics. This statistics is lower than other studies where 6%-9% of the second cancers among irradiated breast cancer patients were estimated to be associated with radiotherapy<sup>[24,25]</sup>. This increase in the estimated risk could be as a result of initial treatment with chemotherapy<sup>[26-29]</sup>. This probability associated with the use of chemotherapy alone is lower than that of patients that underwent chemotherapy and radiotherapy<sup>[30]</sup>.

The finding from this study does not corroborate the findings of Corradini *et al.*<sup>[31]</sup> who reported a secondary cancer risk to the lungs as 0.65% and 2.49% using the linear exponent model at 50 years and 70 years respectively for free breathing technique; while 0.63% and 2.42% was reported for the plateau model at 50 years and 70 years respectively. These values are however lower than the reported values in this study, but may be smaller if the deep-inspiration breath-hold radiotherapy technique is employed. Although no study has ascertained any significant difference in the risk of secondary cancer to the lungs using this technique, they however reported higher values of secondary cancer risk as well as radiation induced lung cancer<sup>[32-36]</sup>. In a meta-analysis, including over 700,000 women treated for early breast cancer, it was demonstrated that radiation therapy is significantly associated with an excess risk of second cancers in organs with fairly close proximity to the former treatment fields<sup>[37]</sup>.

The average SCCP values for the lungs is  $0.34\% \pm 0.03\%$  using the linear-exponential model. In a previous study, average SCCP values using the linear-exponential model gave a prediction of  $5.3\% \pm 0.1\%$  for post mastectomy radiation therapy (PMRT)<sup>[38]</sup>; which is higher than the computed value in this study. It is however close to the value of  $5.93\% \pm 0.54\%$  obtained using the linear model. It is worthy of note here that the results from SCCP estimations are indicative of lifetime risk, with a mean residual lifetime of 50 years. It has been reported that smoking during radiation therapy or earlier caused an increase of the 15 years risk of developing a lung cancer after radiation therapy and breast conserving surgery by 4.7% and 6%, respectively when it was compared to 0.26% among non-smokers<sup>[39]</sup>. Apart from the inherent increased risk in cancer survivors due to lifestyle, chemotherapy and radiation therapy are both known to further boost the risk of second solid cancers<sup>[20]</sup>.

The risk of developing cancer on the contralateral breast cancer after radiotherapy appears to be common

among women who are in their premenopausal age (younger than age 40 to 45 years) when exposed to radiation therapy, however higher risk is observed for PMRT patients<sup>[40]</sup>. The mean age of patients in this study is  $57.8 \pm 8.7$  years (46-83 years). The mean SCCP of the patients in this study using the linear exponential dose-risk model was  $0.41\% \pm 0.05\%$ . This value is lower than the average SCCP value of 1.0% for volumetric modulated arc therapy reported by Nichols *et al.*<sup>[38]</sup> using the linear-exponential dose-response model. The result of this study is very important for younger patients (below 50 years) who are at greater risk for radiogenic second malignancies. Hernandez *et al.*<sup>[41]</sup> reported that no excess breast cancer risk has been found among women irradiated at age 40 years or older, while Boice *et al.*<sup>[42]</sup> showed that after the age of 45 years radiation exposure with mean radiation dose of 2.51 Gy entails very little, if any at all or no risk (relative risk, 1.01) of radiation-induced breast cancer for a female population with an average age of 51.7 years.

As much as several studies have reported second cancers attributed to the treatment of the primary, were identified in several anatomical sites<sup>[40-42]</sup>, several others have not shown any appreciable risk in developing second primary cancer after breast radiotherapy, outside the treatment field<sup>[43,44]</sup>.

There was significant increase in the risk of secondary malignancy as dose to the different organs increases. This agrees with the finding of Deutch *et al.*<sup>[45]</sup> who reported that higher dose of radiotherapy to lung in breast cancer patients was associated with increased incidence of subsequent radiation induced malignancies in both ipsilateral and contralateral lungs.

## DECLARATIONS

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### Authors' contributions

Saw and contoured the patients: Adeyemi OF

Literatures collection: Osahon OD

Designed the study and carried out the data analysis: Okungbowa EG

### Availability of data and materials

Data will be made available on request through the corresponding author.

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None.

### Conflicts of interest

All authors declared that there are no conflicts of interest.

### Ethical approval and consent to participate

We declare that the article does not require a Statement of Ethics, since all the clinical material was anonymised. Absolutely no information concerning the patients, themselves, were used, so no consent were necessary.

## Consent for publication

The study didn't make use of patients directly but through secondary data collection method. The patients already gave their consent before they went through radiotherapy.

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Review

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# Circulating tumor cells and the metastatic process: the complexity of malignancy

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## Abstract

Despite improvements achieved in terms of early detection and therapeutic approach, metastatic breast cancer remains one of the principal worldwide causes of death. In recent years, due to the heterogeneous response of each patient to chemotherapy, clinical research highlights the need of a personalized approach. Circulating tumor cells (CTCs) represents a promising tool for this purpose. Unfortunately, even if their correlation with severity, outcome and metastatic nature of the tumor has been established, several issues, mainly concerning their characterization and isolation, need to be solved. In this review, latest knowledge on CTCs and metastatic process in breast cancer were analyzed, aiming to understand their clinical utility and validity for a prospective therapeutic scenario.

**Keywords:** Breast cancer, metastasis, circulating tumor cells, personalized therapy

## INTRODUCTION

Breast cancer (BC) represents the second leading cause of death among women not only in Western countries but also, as proved by new evidences, in developing countries<sup>[1-5]</sup>. BC has been defined as a heterogeneous disease with multiple intrinsic tumor subtypes and the possibility to develop one of them is directly related to many factors, such as aging, genetics and lifestyle (obesity, lack of physical activity, sedentary behavior and frequent alcohol consumption)<sup>[6-8]</sup>. Furthermore, each BC subtype, with distinctive histopath-



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ological and biological characteristics, reflects different clinical outcomes and therapeutic strategies<sup>[6,9]</sup>. Estrogen and progesterone receptors (ER and PR) in addition to the human epidermal growth factor receptor 2 (HER-2) and the proliferation index (Ki-67) represent the most clinically used predictive biological markers<sup>[10,11]</sup>. Nowadays, it has been amply demonstrated how their expression is correlated with both BC intrinsic subtypes classification and the relative prognosis<sup>[6,12]</sup>. Concisely, the canonical molecular classification, firstly established by Perou in 2000, divided breast cancers in two principal subfamilies, ER- positive and ER-negative<sup>[6,12,13]</sup>. In the first subfamily are included the LUMINAL A (ER<sup>+</sup>PR<sup>+</sup>HER2<sup>-</sup>Ki67<sup>-</sup>) and LUMINAL B (ER<sup>+</sup>PR<sup>+</sup>Her2<sup>+/+</sup>Ki67<sup>+</sup>) subgroups that represent the most common subtypes among BC. Despite the highest incidence, luminal A has the best survival rate and is recurrence-free, while luminal B, due to their heterogeneity, presents a worse outcome together with an high risk of relapse, thus additional chemotherapy and anti-HER2 drugs treatment are needed<sup>[14,15]</sup>. The ER- subfamily includes two principal subgroups. The first subtype, called HER2 OVER-EXPRESSED (ER-PR-Her2<sup>+</sup>Ki67<sup>+</sup>), is correlated with poor prognosis and a higher risk of early relapse. Hopefully, it has been demonstrated that anti-HER2 drugs treatment brings an increment on survival and patients respond positively to chemo and neoadjuvant therapy<sup>[6]</sup>. The second ER- subgroup, the so-called BASAL LIKE, that represents 15% of BC, is characterized by an expression patterns including lack or low expression of ER, PR and HER2 in addition to a high expression of basal markers and Ki67. In the 60%-90% of cases, basal-like BC is TRIPLE NEGATIVE BC (TNBC), due to the absence of the principal three biological marker expressions<sup>[16]</sup>. TNBC represents a very heterogeneous subgroup comprised of further six subclasses, such as basal-like BL1 and BL2, mesenchymal-like, mesenchymal stem-like, luminal-androgen receptor expression, immunomodulatory and an unstable type subclasses<sup>[17]</sup>. In general, the TNBC subgroup exhibits, in addition to a high proliferation rate, an increase in basal/myoepithelial cells-related cytokeratins (CKs) and epidermal growth factor receptor (EGFR) expression<sup>[14]</sup>. Furthermore, even if its heterogeneity is correlated with different prognosis and severity levels, the high percentage of TNBC patients present the worse clinical outcome, a shorter relapse-free period and a strong possibility to develop bone, lung, brain and liver metastasis<sup>[18,19]</sup>. Actually, it is clearly demonstrated that there is a strict correlation between the survival of women with BC and the incidence of distant metastases<sup>[20,21]</sup>. The migration of tumor cells from the primary tumor into the blood stream and their subsequent dissemination to secondary locations throughout the body represents the *sine qua non* condition that acts as a trigger for the entire metastatic process<sup>[22]</sup>. Nowadays, circulating tumor cells (CTCs) represent an important prognostic biomarker in early BC disease and their presence is directly correlated with the patient's response to therapy and with poor prognosis in case of recurrence in radically resected BC or in metastatic disease<sup>[23-26]</sup>. Nevertheless, determination and utility of CTCs, in the common clinical practice, are still object of discussion<sup>[27]</sup>. Therefore, after a little excursion on CTCs characteristics and behavior during the metastatic process, the aim of this review is to make a point on clinical utility and validity of CTCs for a prospective therapeutic scenario.

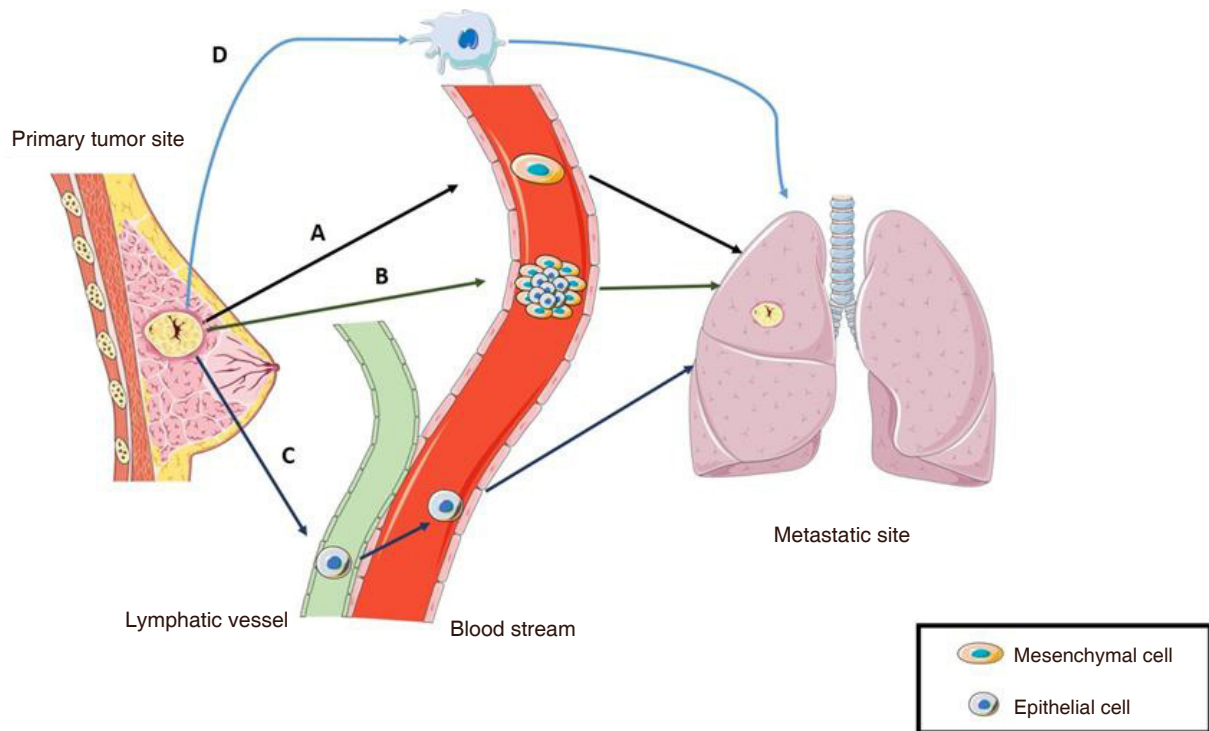
## CTCs AND THEIR PLASTICITY IN THE METASTATIC PROCESS

It is estimated that, at least in 90% of cases, metastases in distant organs represent an obstacle to the therapy and the primary cause of death in BC patients<sup>[23,28]</sup>. In the presence of metastatic cancers, chemotherapy is less effective on tumor cells and, as estimated by the American Cancer Society, only 22% of patients present a 5-year survival rate ([www.cancer.org](http://www.cancer.org)). Metastasis can be described as a complex dynamic multi-step process that begins with the intravasation of primary tumor-derived cells into blood or lymphatic vessels and goes on with the arrest, adhesion and extravasation of CTCs bringing to the colonization of distant organs<sup>[22,29,30]</sup>. Whenever these cells penetrate into the bone marrow, acquiring a status of dormancy, they are defined as "Disseminated Tumor Cells" (DTCs)<sup>[31,32]</sup>. Since their first detection in 1869 by Ashworth, several studies and clinical trials have demonstrated and confirmed, over the years, the strict correlation between detection and monitoring of CTCs in peripheral blood and metastatic BC (MBC), in terms of disease progression, prediction of treatment efficacy and overall-survival<sup>[33-43]</sup>. This concept has also been ratified in the eighth edition of the AJCC Cancer Staging Manual, in which circulating CTCs and bone mar-

row DTCs detection and enumeration have been included as important prognostic tools in both M0 and M1 BC classes<sup>[44]</sup>. The ability of CTCs to perform several functional and morphologic changes, conferring them a high degree of heterogeneity and plasticity, lie behind their clinical and therapeutic attractiveness. It has been deeply highlighted the important role of epithelial to mesenchymal transition (EMT) as an essential trans-differentiation process in many physio/pathologic mechanisms, such as mesoderm formation in embryonic development, tissue repair or fibrosis<sup>[45-47]</sup>. Generally, epithelial cells are defined as adherent cells, expressing E-cadherin, a transmembrane glycoprotein involved in tight junctions' formation between adjacent cells, and CKs, such as CK8, CK18 and CK19, that exhibit an apicobasal polarity and a dense network of intercellular adhesion complexes that prevent them from migrating. In contrast, mesenchymal cells are single spindle-shaped cells that do not present intercellular junctions and, consequently, are able to migrate. In addition, mesenchymal cells generally exhibit a specific proteins profile such as vimentin, fibronectin and alpha-smooth muscle actin ( $\alpha$ -SMA)<sup>[48]</sup>. Therefore, considering the first part of the metastatic process, in which cells loss their epithelial nature, acquire a mesenchymal-like expression profile and the detachment from the primary tumor site occurs, CTCs undergo EMT<sup>[49,50]</sup>. This multiple complex signaling system is triggered by the transforming growth factor- $\beta$  (TGF- $\beta$ ) that enhanced cell migration, invasiveness and increased ability to counteract apoptosis<sup>[51]</sup>. In fact, it has been demonstrated that TGF- $\beta$  is able to induce, in normal mammary epithelial cells, the phosphorylation of Smad2 and Smad3 and the activation of other EMT-related pathways, such as Notch, PI3K/AKT and Wnt<sup>[52,53]</sup>. This signal cascade activates EMT transcriptional factors, such as ZEB1, ZEB2, Twist, Snail and Slug, that downregulate the expression of E-cadherin<sup>[54-57]</sup>. Consequently, cell-cell adhesions are disintegrated, cytoskeleton fibers and extracellular matrix (ECM) component undergo remodeling bringing a loss of cell basal-apical polarity and a strong motile and invasive properties acquisition<sup>[58,59]</sup>. Together with E-cadherin, another epithelial-specific transmembrane protein, involved in EMT process, is the epithelial cell adhesion molecule (EpCAM). In normal conditions, this protein is localized in the intercellular space, where it is able to promote tight junctions formation and interact with E-cadherin, to maintain the epithelial integrity. On the contrary, in cancer tissue, after EMT-related cell-cell adhesion disintegration, EpCAM becomes ubiquitously distributed on the entire cancer cell surface and, for this reason, more easy to be detected with antibody-based assay. In view of this, CTCs have long been traditionally defined positive for EpCAM and CK markers expression and negative for the hematopoietic marker CD45 (EpCAM<sup>+</sup>/CK<sup>+</sup>/CD45<sup>-</sup>). However, in 2014, Lustberg *et al.*<sup>[60]</sup> identified different circulating cell populations in MBC patients composed of EpCAM/CK<sup>+</sup> cells expressing mesenchymal markers, with few or no epithelial markers, and cells with both hematopoietic and epithelial markers profile. This heterogenic nature of CTCs was also confirmed through several gene expression profiling. In fact, whilst they supported the correlation between CTCs, metastatic process and patient's overall-survival, to date no consensus has been established regarding biological markers to be used to identify these cells<sup>[61-63]</sup>. Currently, putting together different studies, among all the analyzed genes related to cell survival (IGFR1, FOXO3), the EMT process (TWIST1, SNAIL, SLUG, VIM) or tumor progression and invasion (HER2, CXCR4, uPAR, VEGFA, VEGFR, Cathepsin D) only CK19, mucin 1 (MUC1) and EpCAM result as the most accepted genes<sup>[61,64-68]</sup>. In addition, it has been demonstrated that metastasis exhibit, as primary tumors, an epithelial phenotype instead of a mesenchymal one, and that, using mice models, mammary tumors can promote an apparent EMT-independent lung metastatic process<sup>[69,70]</sup>. Considering all these evidences, an epithelial-mesenchymal plasticity (EMP) model has been proposed as a hallmark of CTCs in the metastatic process, in which circulating cells, during their migration to distant organs, are able to switch between a hybrid phenotype along the epithelial to mesenchymal spectrum conferring them the ability to adapt in different microenvironments<sup>[71-73]</sup>.

### CTCs migration models

In support of the EMP model, several histopathological, intravital microscopy and *in vitro* studies demonstrated that CTCs exhibit different invasion strategies (collective or individual) and are able to exchange toward them according to the surrounding microenvironment<sup>[74-81]</sup> [Figure 1]. The classical migration model



**Figure 1.** Cancer cells migration models. A schematic panel of tumor cells migration models discussed in the text: A: epithelial to mesenchymal transition/mesenchymal-epithelial transition process; B: collective migration model; C: lymphatic vessel pathway; D: mesenchymal to amoeboid transition process [Image created with Servier Medical Art (<https://smart.servier.com/>)]

depends on a reversible EMT process, known as mesenchymal to epithelial transition (MET). Primary tumor-derived CTCs, with a mesenchymal expression profile and an elongated cell shape that allows them to adhere on ECM substrate and direct their migration, are able to penetrate in the blood stream<sup>[82]</sup>. Once reached a desirable metastatic niche, CTCs promote disruption of cell adhesion and polarity, remodeling of the cytoskeleton and changes in cell-ECM adhesion<sup>[83,84]</sup>. This tissue remodeling process leads to the generation of crossing points relevant for migration and tissue invasion<sup>[77]</sup>. Subsequently, mesenchymal CTCs are able to promote MET in order to restore their epithelial profile as well as their proliferative ability. As a result, secondary tumor growth<sup>[78]</sup> is promoted. Instead of moving through the complex EMT/MET process, another proposed mechanism suggests that epithelial and mesenchymal cells could cooperate to migrate and promote the subsequent metastatic process. In the so-called “collective migration model”, it is assumed that hybrid phenotypes create and coexist in a multicellular cluster, called tumor micro-emboli or CTC cluster<sup>[85]</sup>. By comparing both collective and individual invasion mechanisms, it is clear that the cluster migration, instead of the individual one, provides several advantages to the metastatic process<sup>[77,82]</sup>. Functionally, this structure is able to guide migration and to invade the secondary organ thanks to the mesenchymal “leader cells” that create a protective microenvironment to the poorly mobile but highly proliferate epithelial “follower” cells, inserted in the core, to accomplish the metastatic process<sup>[76,78,86]</sup>. A third mechanism, called mesenchymal to amoeboid transition, refers to a single dissociated primary tumor-derived cell that lost its attachment to the ECM adopting a distinctive spherical and highly deformable morphology with bubble-like protrusions, able to infiltrate tissues<sup>[77,87,88]</sup>. In contrast with the previous models, amoeboid migration, because it is a protease-independent process in which cells mechanically displace ECM fibrils instead of degrading them, represents at the same time a simple and efficient strategy to move through tissues and between tissue barriers<sup>[89,90]</sup>. The Met receptor tyrosine kinase (Met-RTK), a growth factor receptor, is able to promote tumor growth and metastasis by enhancing motility, survival, proliferation of cancer cells and stimulating angiogenesis<sup>[91]</sup>. In 2014, Laser-Azogui *et al.*<sup>[87]</sup> demonstrated that BC cells ex-

press high levels of activated Met-RTK which are able to induce membrane blebbing and, as a consequence, cell dissociation, amoeboid motility and invasion. Furthermore, they highlighted a Met-induced protection from apoptosis and the ability of these Met-expressing cancer cells to promote the metastatic process. The lymphatic vessel pathway, due to its discontinuous structure, the high concentration of hyaluronic acid and the lymph fluid composition, which is able to improve cell survival and to reduced shear stress, represents a better and safer dissemination vehicle for cancer cells than the blood stream. Thus, it could be reasonable to consider the possibility that both epithelial and mesenchymal cancer cells migrate, preferably, through the lymphatic system, spread first to lymph nodes and then drain into the blood<sup>[92-94]</sup>. Accordingly, another mechanism of tumor EMT-independent metastasis, namely tumor-induced lymphangiogenesis, has been proposed<sup>[95]</sup>. Briefly, mesenchymal cancer cells, which are able to produce and release lymphangiogenic factors, such as vascular endothelial growth factor C and D (VEGF-C and VEGF-D), promote an increase of lymphatic vessel density in the peri- and intratumoral area, so that epithelial cells are able to colonize lymphatic system and lymph nodes can facilitate their entry into the systemic circulation<sup>[96-100]</sup>. It has been demonstrated that an increase in lymph vessel density, due to tumor-induced lymphangiogenesis, is correlated with a high amount of lymph node metastasis, VEGF-C expression and worse disease-free/overall survival in BC patients<sup>[101]</sup>.

### Immune escape

An important issue related to the EMP of CTCs and their metastatic potential is the immune-escape, which is the ability of tumor cells, during their migration, to counteract the elimination by the immune system and to increase their possibility to survive and to colonize distant organs<sup>[102-104]</sup>. One of the most studied immune evasion mechanism is the programmed death-ligand 1 (PD-L1)/programmed death receptor (PD-1) axis. In normal conditions, the PD-L1 and its PD-1 represent a physiological checkpoint of the immune system. Antigen-presenting cells express PD-L1 while PD-1 is detectable on the surface of activated T-cells. Once ligand/receptor interaction occurred, a strong inhibitory signal promotes apoptosis and functional exhaustion in T-cells<sup>[105]</sup>. In 2014, Chen *et al.*<sup>[106]</sup> have identified, in lung tumor, a molecular link between the overexpression of the EMT-effector ZEB1 and a more abundant presence of PDL1, able to promote the exhaustion of intratumoral T lymphocytes and the development of metastasis<sup>[106-108]</sup>. Similarly, in breast cancer, it has been demonstrated that PD-L1 expression is heterogeneous and it is generally associated with the presence of poor-prognosis factors, high proliferative index and aggressive molecular subtypes<sup>[109,110]</sup>. In 2015, for the first time, Mazel *et al.*<sup>[111]</sup> provided evidence that CTCs, isolated from the blood of BC patients, frequently express PD-L1 on their surface. The Fas/FasL axis represents another EMP-dependent immune escape mechanism based on the ligand/receptor interaction with a negative impact on the clinical outcome of BC patients<sup>[112]</sup>. Briefly, when the factor-associated suicide (Fas), a transmembrane receptor belonging to the tumor necrosis factor (TNF) family, interacts with its ligand (FasL), expressed on the surface of activated T lymphocytes, Fas-expressing cells go through apoptosis. During BC progression, Fas was found to be repressed in association with an increase of FasL level and TGF- $\beta$  secretion in tumor cells, conferring to CTCs the ability to induce cell death and escape immune recognition<sup>[113]</sup>.

### Metastatic niche

Despite the migration mechanism and the above-mentioned immune evasion systems adopted by cancer cells, only a few percentage of cells that extravasate are able to survive in the unsuitable secondary organ environment and promote metastatic growth. Thus, the microenvironment in the metastatic site represents a major challenge for invading cancer cells. Starting from the “seed and soil” hypothesis, postulated by Paget, up to date, it is well known that cancer cells (the seed) require a specific and compatible “soil” microenvironment, the pre-metastatic niche, which is able to evolve and to promote both cell engraftment, creating the metastatic niche, and cell proliferation, leading to the micro- to macro- metastatic transition<sup>[114-119]</sup>. Many evidences demonstrate how primary tumor site is able to modify, before cancer cells’ arrival, the secondary organ microenvironment, stimulating the creation of the pre-metastatic niche<sup>[120]</sup>.



Tumor-secreted factors, such as VEGF-A, TNF- $\alpha$  and TGF- $\beta$ , are able to promote bone marrow-derived hematopoietic progenitor cells (BMDCs) recruitment in the secondary organ. Accordingly, BMDCs recruitment results in an ECM remodeling, upregulating fibronectin (FN) and other molecules, such as MMPs, and stimulate angiogenesis<sup>[121]</sup>. Hypoxia-inducible factor (HIF) represents a major effector and adaptor in BC cells that, due to a massive and unregulated proliferation in association with vasculature dysfunctions, are exposed to a hypoxic microenvironment<sup>[122-124]</sup>. Lysyl oxidase (LOX), one of the principal HIF-dependent BC secreted factor, is strictly correlated with tumor invasiveness and lung and bone metastasis formation. In the pre-metastatic organ, LOX is able to co-localize with fibronectin and to modulate cell-ECM interactions<sup>[125]</sup>. Furthermore, through the interaction with type IV collagen, LOX recruits BMDCs and, in a second attempt, promotes the colonization of metastatic tumor cells<sup>[126-128]</sup>. In the matrix remodeling scenario, it has been demonstrated that the secretion of lysyl oxidase-like 2 (LOX-2) is also able to induce  $\alpha$ SMA expression in pre-metastatic fibroblasts, inducing their activation and the secretion of FN and LOX, generating a fibrotic microenvironment capable of supporting tumor cell persistence and survival<sup>[129,130]</sup>. Finally, the primary cancer secretion of VEGF, TGF- $\beta$  and TNF- $\alpha$  stimulates Angiopoietin-2 expression in the pre-metastatic niche increasing vascular permeability and, consequently, promoting the extravasation of CTCs so that metastatic process can move forward<sup>[131-133]</sup>.

## STATE OF THE ART IN CTCs ANALYSES

The intrinsic mark of rarity of CTCs, in addition to their highly heterogeneous nature, represents an obstacle to study their biology<sup>[134,135]</sup>. Nevertheless, several technologies are being developed for CTCs detection in patients' peripheral blood sample based on their knowing biological properties<sup>[136]</sup>. The most commonly used techniques are based on a combination of enrichment/isolation and detection procedures. In the first phase, CTCs are separated from hematologic cells, especially leukocytes that, due to their similar physiochemical and biological properties, could contaminate tumor cell pool<sup>[134]</sup>. The enrichment procedures exploit physical (size, deformability, density and electrical charge) or biological characteristics (cell surface protein expression, viability and invasive capacity) of CTCs<sup>[137,138]</sup>. The detection step consists of immunostaining methods ranging from classic immunocytochemistry (ICC) or immunofluorescence to flow cytometry<sup>[138]</sup>. Furthermore, RT-PCR approach represents another option to detect tumor related mRNA transcripts in patients' blood. Although this method does not require a prior CTCs enrichment, the inability to provide CTCs enumeration deeply restricts its utilisation<sup>[138]</sup>. Regarding CTCs isolation from blood components, density gradient centrifugation, such as Ficoll-Hypaque, Percoll (GEHealthcare Life sciences), OncoQuick (Greiner Bio-One), Cytotrack, Accucyte-cytfinder, represents the most commonly physical properties-based technique<sup>[139-141]</sup>. Other exploited approaches are based on cell-size separation, such as microfiltration (Screen Cell, CellSieve, ISET, Parylene filter, Filtration/Sequential ICC) or microfluidic test that combines size and deformability properties of CTCs (Ephesia, HB-CTC-chip, Iso-Flux, OncoCEE, Parsortix system, the ClearCell FX or Vortex)<sup>[135,142-151]</sup>. Nevertheless, even if all the described isolation methods represent rapid and less expensive alternatives, they are generally hampered by blood cells-related false-positive results, thus making necessary the combination with other enrichment methods and the loss of large CTCs and CTC clusters due to the high heterogeneity of CTC size<sup>[136,152]</sup>. Immunological assays, based on the extremely specific reaction between antibodies and the target antigens on the cell surface, provide a high purity rate of isolated CTCs<sup>[145,153-160]</sup>. Several of these techniques are based on EpCAM positive selection and, actually, the most standardized method is the CellSearch® system (Janssen Diagnostics), the only one approved by the U.S. Food and Drug Administration for CTCs enumeration in BC and other type of cancer<sup>[25,27,157,161]</sup>. Nevertheless, as reported by several clinical trials, in patients in which EMT occurring with the downregulation of EpCAM and other epithelial markers, this system may fail to capture the entire pool of CTCs and may result in false negative findings<sup>[74,134,162-165]</sup>. Furthermore, it has been demonstrated that the lack of EpCAM<sup>+</sup> CTCs detection does not reflect a status of benign prognosis. In fact, it could be directly related with negative hormone receptors, high tumor grade, triple-negative disease, inflammatory BC and brain metastasis (OR = 6.17, 95%CI: 2.14-17.79;  $P = 0.001$ ) or conversely with bone



metastasis (OR = 0.47; 95%CI: 0.27-0.80;  $P = 0.01$ )<sup>[166]</sup>. Hence, it is important to understand, using different epithelial and/or mesenchymal markers, how defined other clinically relevant sub-populations of CTCs. Accordingly, taking into account the attested probability of false-negative results, cell-surface vimentin and EGFR were suggested as alternative markers for detecting mesenchymal transitioned CTCs<sup>[136,167,168]</sup>. To recapitulate, the common issue underlined with positive selection procedures is to fail the capture of cells with low expression of EpCAM and non-epithelial phenotypes such as those that have undergone EMT. In addition, the isolated CTCs have reduced viability and this aspect represents an important obstacle to CTCs' biological characteristics understanding<sup>[137]</sup>. Otherwise, immunological methods based on negative selection are also available. The latter are commonly used to deplete cells that do not express CD45 leucocyte antigen or a cocktail of antibodies direct against red and white blood cells, such as RosetteSep, Easy-Sep, Dynabeads, mojoSort<sup>[137]</sup>. Cells isolated with this approach are relatively more viable but, at the same time, are highly impure. In fact, the purified cells pool contains epithelial and non-epithelial phenotypes together with normal blood vessel, stromal cells or other cells normally present in the circulation<sup>[137]</sup>. These evidences, as reported by a huge number of studies, confirm that the main challenge of CTCs isolation and characterization are the lack of specific standardized procedures that strongly restrict their use in clinical practice<sup>[134,169,170]</sup>.

## CLINICAL RELEVANCE OF CTCs

Despite progress achieved in terms of prevention, diagnosis and treatment, drug resistance and tumor relapse, whose severity and probability are specific for each patient, remain one of the principal issue in breast cancer. Therefore, as a good clinical practice, it has been established that a patient's 5-year follow up, since primary tumor, could lead to an early detection of recurrence or metastasis and to a more specific and efficient therapy<sup>[169]</sup>. Canonical tissue biopsy represent on one side a costly, painful and hard to repeat procedure. In addition, it is not able to provide a complete genetic or epigenetic tumor characterization in order to identify possible tumor phenotypical alteration<sup>[171]</sup>. In this optic, non-invasive liquid biopsies and the measurement of specific blood-based biomarkers represent an effective alternative parameter to monitored invasive BC patients. Cancer Antigen 15-3 (CA 15-3), carcinoembryonic antigen, tissue polypeptide antigen, tissue polypeptide-specific antigen and the soluble form of HER2 represent the most detected serum BC biomarkers<sup>[172-174]</sup>. Nevertheless, even if it has been demonstrated a correlation between single or combined circulating biomarker levels and recurrence incidence, many issues need to be solved<sup>[175-178]</sup>. For instance, there are still problems associated with the lack of a validated clinically relevant level to establish, for each biomarker, a cut-off parameter<sup>[169]</sup>. Furthermore, it has been demonstrated that biomarker prognostic efficacy depends on the recurrence site. In fact, higher levels of biomarkers were detected in BC distant metastases, such as bone or liver, than in loco-regional or lung recurrence<sup>[179]</sup>. Additionally, these biomarkers are inappropriate to figure out mechanisms of therapy resistance<sup>[169]</sup>. For these reasons, nowadays, the detection of CTCs from patient blood samples appears as a powerful tool in the management of early and advanced BC patients<sup>[138]</sup>. CTC-based liquid biopsy represents a more informative tool, able to improve patients' selection and monitoring for target treatments, than conventional tumor tissue based- biomarkers that focused only on the primary tumor or metastases. Indeed, in the last few years, several studies highlighted the prognostic relevance of CTCs in MBC. In particular, it has been demonstrated that patients with a persistent CTCs count > 5 cell per 7.5 mL blood had a worse patient free survival (PFS) and overall survival (OS) compared to those that have CTCs < 5 at baseline and during follow-up<sup>[25,27,180-182]</sup>. Furthermore, due to their characteristics and minimally invasive procedures, the use of CTCs permits to evaluate the dynamic change of tumor over time for each patient that may impair the response to specific targeted treatments<sup>[138,183]</sup>. From this point of view, CTC detection appears to hold promise of a better patients' management but up to date they are not still routinely used in clinical practice. In fact, CTC enumeration and variation during treatment were independent from any other baseline clinical or pathological characteristics and were not associated with pathological complete response<sup>[26,27]</sup>. Furthermore, as highlight by the SWOG S0500 randomize trial in advanced breast cancer, there is no evidence that changing or discontinuing therapy based on CTC level could

improve patients' health outcomes, quality of life or cost effectiveness. In addition, PFS and OS showed no difference in outcome when patients were switched to an alternate regimen<sup>[180]</sup>. Thus, the American Society of Clinical Oncology guidelines affirm that the use of CTC count alone may be prognostic but not predictive for monitoring response to treatment for metastatic breast cancer<sup>[184]</sup>. Nevertheless, several clinical trials based on the comparison in HER2/ER/PR expression profiles between patient's biopsy, from primary tumor or metastatic site, and CTCs, demonstrated a discrepancy between biopsies and circulating cells that could have important therapeutic implications<sup>[185]</sup>. In fact, it has been revealed in HER2<sup>-</sup> and ER<sup>+</sup> BC patients the presence, respectively, of HER2<sup>+</sup> and ER<sup>-</sup> CTCs associated with an increased mortality risk, poor PFS and low OS<sup>[186-188]</sup>. Therefore, knowing that the switch between HER2<sup>-/+</sup> or ER<sup>+/-</sup> can occur after multiple courses in patients under HER2<sup>-</sup> targeted or ER-endocrine therapies, the monitoring of CTCs becomes crucial<sup>[189,190]</sup>. Obviously, these evidences suggest a potential mechanism of a patient's specific therapy-resistance, which is still unknown and under investigation in ongoing clinical trials<sup>[191]</sup>. In conclusion, despite several issues needing to be overcome, CTCs could be considered as a "real-time" liquid biopsy, able to provide important molecular information about patient's current disease and, hopefully, to suggest the suitable personalized treatment regimen<sup>[138]</sup>.

## CONCLUSION

At present, personalized medicine represents one of the principal aims of medical research. For this reason, even the improvement achieved in treatment options and the better clinical outcomes for BC patients, conventional tissue biopsies are considered, up to date, a poor diagnostic procedure. The growing interest in CTCs and their in progress validation as diagnostic and prognostic biomarker, could represent the tool for achieving this wishes of "personalization". In fact, despite the still outstanding issues already covered in this review, CTCs could be crucial to the understanding of the complex BC heterogeneity, at the same time, they could be considered as a screening tool. Furthermore, their proved implication in the metastatic process and, most important, in chemoresistance, is stimulating the rapid development of new CTC isolation and single cell analysis platform. In the future, it is expected that the improvement in CTCs knowledge may pave the way to the discovery of new targets and to therapies that are more efficient.

## DECLARATIONS

### Authors' contributions

Conception and elaboration of the work: Di Raimo T, Angelini F

Provided administrative, technical, and material support: Di Raimo T, Angelini F

Revisiting the work critically for important intellectual content: Di Raimo T, D'Andrea MR, Angelini F

Final approval of the version: Di Raimo T, De Santis E, Coppola L, D'Andrea MR, Angelini F

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All authors declared that there are no conflicts of interest.

### Ethical approval and consent to participate

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Review

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# Immunotherapy in colorectal cancer treatment: actual landscape and future perspectives

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## Abstract

Colorectal cancer (CRC) represents the second most common cancer in Europe with marked differences in prognosis and response to treatments. In the past years research showed emerging interest in genomic and immunologic fields. The clinical heterogeneity, that occurs during the pathogenesis of CRC, is driven by chromosomal alterations and defective function of DNA mismatch repair genes. CRC is classified in four consensus molecular subtypes (CMS) with different immunogenic characteristics and prognosis. CMS1 microsatellite instable (MSI)-like and CMS4, both characterized by high levels of immune infiltration, are recognized as the most immunogenic subtypes, even though functional characteristic leading to different prognosis are reported. In particular, MSI tumors have been identified as the best candidates for immunotherapy treatment and a number of studies have evaluated the efficacy of anti-programmed cell death ligand-1 (PDL-1) and anti-cytotoxic T-lymphocyte-associated protein 4 (CTLA4) in this setting. However, literature data show that the majority of patients with CRC have microsatellite stable (MSS) tumors and this status seems related to lower response to PDL-1/programmed cell death-1 or CTLA4 blockade. The aim of this paper is to investigate the role of immunotherapy in MSI and MSS CRC.

**Keywords:** Colorectal cancer, immunotherapy, microsatellite instable, microsatellite stable mismatch repair, prognosis

## INTRODUCTION

Colorectal cancer (CRC) represents the second most common cancer in Europe with significant heterogeneity in prognosis and response to treatment. Prognostic factors include stage of disease, site of



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metastasis, and type of treatment given. Tumor genetic mutations gained a pivotal role as the prognostic factor. To date, the median overall survival (OS) for patients with metastatic CRC is about 30 months<sup>[1]</sup>. In 2015, 70% of new cases underwent potentially curative resection thanks to screening programs<sup>[2]</sup>.

In the past years, research on CRC has shown an emerging interest in genomic and immunologic fields. The clinical heterogeneity that occurs during the pathogenesis of CRC is driven by chromosomal alterations and defective function of DNA mismatch repair (MMR) systems<sup>[3]</sup>. In particular, about 15%-20% of CRC show deficient mismatch repair (dMMR) systems, while chromosomal instability with functioning DNA MMR, a status defined as microsatellite stable (pMMR), is found in 80%-85% of CRC<sup>[4]</sup>.

Microsatellites are defined as areas within the DNA sequence where a single nucleotide (mononucleotide) or units of two or more nucleotides are repeated in genome. They are usually located in the introns of genes and the number of repeats contained in every microsatellite is usually preserved in every single cell of the body<sup>[5]</sup>. Microsatellite instability (MSI) is defined as a clonal change in the number of repeated DNA nucleotide units in microsatellites and it arises in tumors with dMMR due to the inactivation of one of the four *MMR* genes: *MSH2*, *MLH1*, *MSH6*, and *PMS2*. Considering that a minority of tumors display instability in fewer than 20% of the markers studied, a classification has been proposed that identifies MSI-low (with just one unstable marker in out of the five-marker Bethesda panel) and MSI-high (with two or more unstable marker)<sup>[6]</sup>.

Clinical and biological differences between dMMR and pMMR are well established. Specifically, dMMR causes genetic instability (aneuploidy, allelic losses, amplifications, translocations, and chromosomal gains) that influences the expression of genes leading to CRC carcinogenesis<sup>[7,8]</sup>. On the other hand, dMMR CRCs have shown better prognosis compared to pMMR tumors<sup>[9-11]</sup>. The increased mutation rate of dMMR tumors triggers an increased production of potentially immunogenic peptides or epitopes establishing a rationale for immunotherapy in this CRC subtype while few data regarding immunotherapy efficacy in pMMR tumors are available in literature<sup>[12]</sup>. In this review we analyzed the role of immunotherapy and target agents in dMMR and pMMR.

### Role of the immune system in CRC

Conventionally, clinical and pathological features, along with tumor characteristics, are known to define cancer aggressiveness. Nevertheless, in the past years, tumor microenvironment (TME) has shown to play an important role in tumor growth and metastatic potential. TME is composed of epithelial cells, blood and lymphatic vessels, stromal cells, and infiltrating immune cells, including T lymphocytes, B cells, natural killer (NK) cells, dendritic cells (DCs), macrophages, and granulocytes. Each tumor displays a specific composition of TME and CRC shows a high degree of immune cell infiltration and high presence of mesenchymal stromal cells<sup>[13]</sup>.

Studies in this field highlighted that different constituents of TME may influence tumor proliferation, infiltration and metastatic spread in different ways. Cancer growth or inhibition represents the result of the interplay between tumor cells and TME. Immune system has been demonstrated to be a key-mechanism of tumor regulation.

Immune system recruits, in cancer surveillance, the coordinated and balanced activation of both innate immune cells [such as macrophages, neutrophils, myeloid derived suppressor cells (MDSC), mast cells, eosinophils, and antigen-presenting cells (APCs)] and adaptive immune cells (NK cells, T and B lymphocytes cells)<sup>[14]</sup>.

At first, innate immune system is recruited by abnormal cells without specific antigen recognition and



inflammatory response is activated promoting angiogenesis and tumor cells proliferation. Later, adaptive immune response is triggered by interaction and recognition between non-self-antigens and peptides presented by the major histocompatibility complexes (MHC) of APCs and T cells<sup>[15]</sup>.

Immune system cells play different roles during tumor immune response. CD4<sup>+</sup> cells sustain inflammatory response by secreting a variety of cytokines such as interferon  $\gamma$ , tumor necrosis factor  $\alpha$ , interleukin-2 (IL-2), and IL-17. CD4<sup>+</sup> cell activation promotes proliferation and function of a specific subgroup of CD8<sup>+</sup> cells called cytotoxic T lymphocytes, that are capable of direct lysis of tumor cells. CD8<sup>+</sup> cells can also secrete cytokines causing cytotoxic response. NK cells are involved in antibody-dependent cell-mediated cytotoxicity and natural cytolytic activity against tumor cells. Macrophages destroy cancer cells through phagocytosis and release matrix-degrading substances (metalloproteinases and cysteine cathepsin proteases). Consequently, high levels of metalloproteinase represent an important factor to predict CRC prognosis and metastasis<sup>[16]</sup>.

Part of the cells described above make up tumor-infiltrating lymphocytes (TILs) that showed to have a prognostic role in cancer treatment and appeared often to be associated with better clinical outcomes<sup>[17]</sup>.

Mesenchymal stem cells (MSC) are non-hematopoietic stromal cells with proliferative potential, immunosuppressive properties, and ability to differentiate into several cell types. Their immunosuppressive function is releasing of proinflammatory factors, inhibiting lymphocyte proliferation and DCs maturation, promoting the production of macrophages, and regulating T cells (Treg). MSC are also involved in tumor initiation, angiogenesis, resistance to chemotherapy, invasion and metastatic process.

Criteria such as composition, density and location of TILs have shown to correlate with different prognosis indicators. Notably, in CRC the number and location of cytotoxic and memory T lymphocytes can predict tumor recurrence and prognosis in early-stage CRC<sup>[18]</sup>. Mlecnik *et al.*<sup>[19]</sup> observed that CRC presenting low CD8<sup>+</sup> cytotoxic T-lymphocyte (CTL) infiltration were associated with higher tumor growth and metastatic spread. Conversely, patients whose tumor showed high density CD8<sup>+</sup> CTL were more likely to have early-stage exordium. Moreover, among patients relapsed, CD8<sup>+</sup> CTL infiltrate appeared to be low independently to TNM stage. These findings support the hypothesis that lymphocyte infiltration represents a strong and independent prognostic factor in CRC.

Tumors cells are well known to develop strategies of immune escape. Indeed, they may show genetic alterations that enhance the expression of mesenchimal transition or immunosuppressive genes along with chemokines responsible for immune suppressive cells recruitment, conferring to cancer cells innate resistance to anti-programmed cell death-1 (PD-1) drugs. Different mutations might be responsible for resistance acquired after an initial benefit out of immunotherapy; during clonal expansion a resistant clone develops high proliferation potential and drives resistance advance.

For example, loss-of-function mutations in Janus Kinases 1/2 (JAK 1/2) might be responsible both for primary and adaptive resistance to immunotherapy. These inactivating mutations affect interferon gamma signaling rendering cancer cells unable to respond to interferon gamma by expressing programmed cell death ligand-1 (PDL-1) and other interferon-stimulated genes, and patients with such tumors became unlikely to respond to PD-1 blockade therapy. This mechanism has already been described in melanoma patients. Zaretsky *et al.*<sup>[20]</sup> analyzed biopsy samples from paired baseline and relapsing lesions in four metastatic melanoma patients who experienced disease progression after an initial objective tumor regression and found resistance-associated loss-of-function mutations in the genes encoding JAK1 or JAK2, concurrent with deletion of the wild-type allele. Shin *et al.*<sup>[21]</sup> described the case of one patient with dMMR colon cancer who did not respond to anti-PD-1 therapy despite a high mutational load, thus identifying JAK1/2 mutations also as potential mediators of primary resistance to PD-1 blockade.

Another mechanism that has been accounted for acquired resistance to immunotherapy in melanoma is inactivation of beta-2-microglobulin (B2M), a fundamental component of the antigen-presenting MHC I. Le *et al.*<sup>[22]</sup> (which included in their study 40 patients with CRC and 46 patients with 11 other histologies) identified mutations of the *B2M* gene in four of five tumors with acquired resistance to anti-PD-1 therapy. However, no *B2M* mutations were identified in primarily resistant tumors. The recognition that the above-mentioned mutations would lead to primary or acquired resistance to PD-1 therapy might be useful to building oncogenic sequencing panels used to select patients for treatments.

The tight interaction between tumor and immune system has driven to the hypothesis of cancer immunoediting. This concept reinvented tumor immunosurveillance taking into account the dual role played by immune responses as host-protective and tumor-promoting. According to immunoediting cancer growth is structured in three different phases: elimination, equilibrium and escape. In the elimination phase immune system engages both innate and adaptive response to eliminate developing tumors before they become clinically evident. If this phase is satisfactorily fulfilled and the tumor results fully eradicated, the whole process might be considered completed. However if a single cancer cell variant escapes the elimination phase it proceeds to the equilibrium phase. During the second phase clonal growth of selected cell variant is prevented by immune system, but those cells still survive in a state of dormancy. Notably, adaptive responses are engaged in the equilibrium phase which is also the time of cancer immunoediting. Also equilibrium might be the end of the entire process whether the immune system keeps under control the “survivor cells” for the lifetime of the host. Nevertheless, the continuous immune pressure on tumor cells may lead them to enter the escape phase. In this third phase tumor variants elude immune system with different mechanisms and they outgrow to clinically apparent cancer<sup>[23,24]</sup>.

#### CMS 1-4 and immune classification

As previously reported CRC clinical pathological characteristics and tumor TMN stage largely affect CRC prognosis and drive treatment choices along with mutation in *RAS* and *BRAF* genes. Nevertheless, patients sharing same TNM stage and therapies end up with different outcomes suggesting that key factors are still missing to our knowledge and approach. To attempt a more inclusive classification, different criteria were proposed that take into account also composition, density and location of tumor immune infiltrate<sup>[25]</sup>. An example of these efforts is the “Immunoscore” for tumor immune classification promoted by Galon’s group. This immune-based classification demonstrated to have a good and independent prognostic value<sup>[26]</sup>.

Furthermore, an international consortium of experts has introduced a gene expression and immune -based classification system: the “consensus molecular subtypes” of CRC, providing new prognostic and predictive tools<sup>[27]</sup>. CRC is classified in four CMS and a fifth unclassified group. CMS1 group, also called MSI-like, includes tumors with instability of microsatellite due to mutations in MMR proteins and *BRAF* oncogene mutations. This subtype is also characterized by a diffuse immune infiltrate, composed of T helper cells and cytotoxic T cells. CMS2 subtype, also called canonical, encompasses tumors with chromosomal instability and upregulation of *c-MYC* and *Wnt* proto-oncogene pathways. CMS3 subtype, also known as metabolic, encompasses tumors with mutated *KRAS* and tumors presenting metabolic dysregulation. CMS4, also known as mesenchymal subtypes includes tumors with mesenchymal phenotype, high expression of mesenchymal genes, stromal infiltration, angiogenesis and transforming growth factor beta (TGF- $\beta$ ) activation. The four subtypes have different frequency, immunogenic characteristics and prognosis with CMS1 and CMS4 recognized as the most immunogenic subtypes, both characterized by high levels of immune infiltration<sup>[13,17]</sup>.

Becht *et al.*<sup>[28]</sup> in a retrospective analyses demonstrated that high levels of TILs reported in CMS1 and CMS4 have different functional characteristics leading to different prognoses. Although both subtypes have high CD8+ T lymphocytes and macrophage infiltrate, CMS1 patients show a Th1 polarization, with

favorable prognosis, while CMS4 subgroup have high density of endothelial, myeloid cells and fibroblasts with higher production chemokines and cytokines that support tumor-associated inflammation, stromal invasion and, angiogenesis, resulting in worse prognosis. These findings stress the role of TME functional orientation beyond TILs composition.

Regarding the others subgroups, CMS2 and CMS3 that occur approximately in 50% of CRC, have low immune and inflammatory infiltration and, intermediate prognosis<sup>[27,28]</sup>.

Also tumor genetic signature has a strong prognostic value. It is reported that stromal composition might strongly affect tumor transcriptional profile hiding tumor cell intrinsic transcriptional traits, especially in those tumors whose gene expression is largely sustained by stromal cells. Using patient-derived xenografts, Isella *et al.*<sup>[29]</sup> developed an approach to unmask CRC cell specific transcriptional features. Based on these findings, five CRC intrinsic subtypes (CRIS) were identified. CRIS-A includes MSI-like, *BRAF*- or *KRAS*-mutated tumors with mucinous expression and glycolytic, pro-inflammatory features. CRIS-B encompasses poorly differentiated tumors characterized by high TGF- $\beta$  driven activation and stressed epithelial-mesenchymal transition traits. CRIS-C groups *KRAS* wild-type tumor with chromosomal instability expressing elevated levels of epidermal growth factor receptor (EGFR). CRIS-D clusters stem phenotype tumors with active Wnt pathway and insulin-like growth factor-2 amplification and overexpression. In CRIS-E subtype Wnt signaling is again observed but it is associated with Paneth-like phenotype and mutations in TP53.

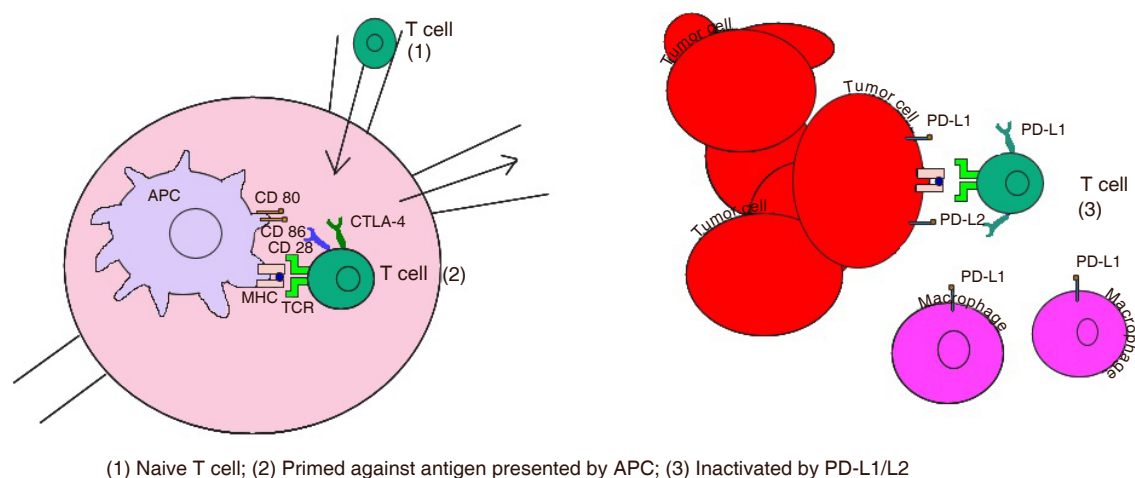
Many of these traits differ from those reported in other transcriptional classification, confirming the strong influence of stromal contexture. CRIS grouping may be applied both to primary and metastatic CRC with low overlap on previous transcriptional classifications. Interestingly, CRIS subtypes were demonstrated to have new prognostic and predictive potentials<sup>[29]</sup>.

### Immunotherapy in MSI CRCs

In the past years, research on immunology and molecular biology fields has clarified the role of the immune system in cancer growing and metastatic potential of tumors. Interestingly, MSI tumors show a marked predisposition to express a wide variety of neoantigens reflecting a significantly high mutational burden [20 fold higher compared to microsatellite stable (MSS)], due to dMMR. The load of neoantigens and the pronounced expression of T-cell recruiting chemokines cooperate to sustain an active immune TME characterized by diffuse immune infiltrate. This explains why CMS1 subtype is recognized as highly immunogenic. This consideration builds up a strong rationale for the use of immunotherapy in MSI CRC. Furthermore, Llosa *et al.*<sup>[30]</sup> proposed an interaction between tumor gene expression and immune microenvironment in CRC. Not only did they report an association between MSI tumors and Th1/CTL rich infiltrate, but they also observed that MSI tumors showed enhanced expression of several immune checkpoints, as to balance such an active immune microenvironment. This might explain both the natural development and growth of tumors that should be easily eliminated by the immune system and the possible efficacy of checkpoint inhibitors in this setting.

Immune system defends our bodies from non-self antigens activating immune response. However, it is pivotal that immune defenses arise at the appropriate time and are limited when they are no more requested in order to prevent chronic inflammation and autoimmune disease. A variety of co-inhibitory checkpoints are engaged to balance activation signals.

One of the most important immune checkpoints is represented by PD-1 and PDL-1. PD-1 is expressed on activated T-cells while PDL-1 is usually expressed on APCs' surface and their interaction mediates a co-inhibitory stimulus that limits excessive immune responses in peripheral tissues ensuring the maintenance



**Figure 1.** Interactions between cancer cells and T-cells and the role of PD-1/PDL-1 and CTLA4. PD-1: programmed cell death-1; PDL-1: programmed cell death ligand-1; CTLA4: cytotoxic T-lymphocyte-associated protein 4; APC: antigen-presenting cell; MHC: major histocompatibility complexes

of peripheral tolerance [Figure 1]. Another immune checkpoint is a cytotoxic T-lymphocyte-associated protein 4 (CTLA4), expressed on T-cell surface, which counteracts CD28 T-cell activation signal thus downregulating the amplitude of early stages T-cell activation<sup>[31,32]</sup>.

The biological significance of PD-1/PDL-1 and CTLA4 suggests a therapeutic role of blockade of these pathways in different types of cancer, including CRC<sup>[33,34]</sup>. Monoclonal antibodies that block PD-1/PDL-1 or CTLA4 are currently approved in melanoma, kidney, and lung cancer treatment and are under study in other neoplastic diseases including CRC cancer. In particular, the efficacy of anti-PD-1 treatment in metastatic CRC was evaluated in a phase 2 clinical trial where pembrolizumab was administered in patients with pMMR and dMMR. Pembrolizumab was administered at the dose of 10 mg/kg every 14 days. The results of this study showed that MMR status predicted clinical benefit of immune checkpoint blockade with pembrolizumab.

In particular, the immune-related objective response rate (ORR) and immune-related 6-month progression-free survival (PFS) were 40% and 78% respectively, for dMMR CRC patients (cohort A) and 0% and 11% for pMMR CRC patients (cohort B). The median PFS was 2.2 months (95% CI, 1.4-2.8) and OS was 5 months (95% CI, 3.0 to not estimable) in the cohort with pMMR CRC. The median PFS and OS were not reached in the cohort with dMMR CRC. Indeed, the authors revealed 1782 somatic mutations per tumor in dMMR compared with 73 in pMMR tumors ( $P = 0.007$ ), and high somatic mutation loads were associated with prolonged PFS ( $P = 0.02$ ). In conclusion Le *et al.*<sup>[35]</sup> underlined that dMMR CRC is more responsive to PD-1 blockade than pMMR [Table 1].

CheckMate142 investigated efficacy of both nivolumab monotherapy and nivolumab plus ipilimumab combination therapy in MSI CRC. In the monotherapy cohort, seventy-four pretreated dMMR/MSI-H metastatic CRC patients were treated with nivolumab 3 mg/kg every 14 days. Nivolumab provided evidence of benefit in previously treated patients with dMMR CRC, with an ORR of 34% (95% CI, 23.2-45.7) with a disease control rate (DCR) of 62% (95% CI, 50.1-73.2). Interestingly, durable responses were observed and 64% of patients had response lasting more than 12 months. Median PFS was 6.6 months and 12 months OS was 72% (95% CI, 60.0-80.9) with a median follow up of 21 months<sup>[36]</sup>.

CheckMate 142 combination cohort evaluated nivolumab plus ipilimumab, an anti-CTLA4 antibody. Indeed, nivolumab and ipilimumab can act synergistically to promote T cell antitumor activity. In this

**Table 1. Clinical trials of immunotherapy in colorectal cancer**

Trial	Phase	Drugs	n (%)	Setting	PFS months	OS months	ORR	Number of identifier
PD-1 Blockade in Tumors with Mismatch-Repair Deficiency dMMR CRC	II	Pembrolizumab 10 mg/kg each 14 days	21		NR		40	
pMMR CRC	II	Pembrolizumab 10 mg/kg each 14 days	11		2.2 (95% CI, 1.4 to 2.8)	5.0 (95% CI, 3.0 to not estimable)	0	
Other dMMR non-CRC	II	Pembrolizumab 10 mg/kg each 14 days	9		5.4 (95% CI, 3 to not estimable)	NR	71	
CheckMate 142 Ongoing (recruiting) Estimated primary completion date December 3, 2018	II	Nivolumab 3mg/Kg with Ipilimumab 1 mg/Kg every 3 weeks for 4 doses followed by Nivolumab 3mg/Kg every 2wk until progression		Pre-treated		NE* 9-mo rate 87%*	55%*°	NCT02060188
A Phase 2 Study of Pembrolizumab (MK-3475) in Combination With Azacitidine in Subjects With Chemo-refractory Metastatic Colorectal Cancer AAM 2017 n 3054 Ongoing (not recruiting) Actual primary completion date March 2016 Estimated study completion date November 2020	II	Pembrolizumab 200 mg on day 1 of every 21 day cycle PLUS Azacitidine 100 mg daily on days 1-5 every 21 days every 21 days Treatment continued for 9 cycles or until evidence of progression of disease or unacceptable toxicity Subjects with chemo-rfractorym CRC without any further standard treatment option	31	Pre-treated	2.1 m (1.8 to 2.8)	6.2 m (3.5 to 8.7)	3% CI (1-17)	NCT02260440

\*Preliminary data *BRAF* mutation and wild type; MSI: microsatellite instable; PFS: progression-free survival; OS: overall survival; ORR: objective response rate; PD-1: programmed cell death-1; MMR: mismatch repair; CRC: colorectal cancer; NR: not reached; AAM: American Society of Clinical Oncology Annual Meeting

cohort, one hundred ninety-nine previously treated patients with metastatic or recurrent dMMR CRC were treated with 4 doses of combination immunotherapy with nivolumab and ipilimumab followed by nivolumab. At median follow-up of 13.4 months, primary endpoint ORR was 55% (95% CI, 45.2-63.8) and DCR for 12 weeks or more was 80%. PFS rates were 76% at 9 months and 71% at 12 months while OS rates were 87% and 85%, respectively. Responses were observed irrespective of PDL-1 expression, *BRAF* or *KRAS* mutational status or history of Lynch syndrome. Regarding toxicity, no new safety signals were reported and no treatment related deaths were reported. Incidence of treatment related adverse events (73%) was comparable to monotherapy while grade 3-4 adverse events were 32% compared to 20% for monotherapy cohort. Common adverse events included fatigue, diarrhea, pruritus, fever, increase of aspartate aminotransferase and hypothyroidism.

Although the comparison is only indirect, these results suggest that a double-blockade might improve clinical outcomes, thus becoming a promising treatment option for MSI CRC<sup>[37]</sup>. Nevertheless, data from melanoma clinical trials have shown that combination of anti-PD-1 and anti-CTLA4 treatment may result in significant toxicity with 55% grade 3-4 adverse events<sup>[38]</sup>. In particular, diarrhea and colitis represented adverse events leading to discontinuation of treatment in a significant proportion of patients. On this basis, more studies about safety of this combination in treatment of CRC patients are warranted. Future investigations may further clarify the role of immunotherapy in pMMR CRC, in particular regarding the role of combination therapy compared to single agent anti-PD-1 treatment and the predictive value of PDL-1 expression [Table 2].

### Immunotherapy in other CRCs subtypes

Albeit dMMR tumors proved to be responsive to immune-checkpoint inhibition, the majority of patients with CRC have pMMR tumors and this status was related to lower response to PDL-1/PD-1 or CTLA4 blockade. Hence, other molecular subtypes require different strategies. Theoretically, immunotherapy



**Table 2. Ongoing clinical trials of immunotherapy in colorectal cancer**

<b>Trial</b>	<b>Phase</b>	<b>Drugs</b>	<b>Setting</b>	<b>Number of identifier</b>
MK-3475-177/KEYNOTE-177 Ongoing (not recruiting) Estimated primary completion date August 15, 2019 A Study to Investigate Efficacy and Safety of Cobimetinib Plus Atezolizumab and Atezolizumab Monotherapy vs. Regorafenib in Participants With Metastatic Colorectal Adenocarcinoma (COTEO IMblaze370) Ongoing, (not recruiting) Estimated primary completion date February 2019	III	Pembrolizumab 200 mg each 21 days for up to 35 treatments vs. chemotherapy	1st line	NCT02563002
A Phase 2 Study With Safety Lead-in, Evaluating TAS-102 Plus Nivolumab in Patients With Microsatellite Stable Refractory Metastatic Colorectal Cancer Ongoing (not recruit) Estimated completion date March 2018	III	Regorafenib (160 mg days 1-21 every 28 days) vs. Cobimetinib plus atezolizumab (cobimetinib 60 mg days 1 to 21 plus atezolizumab 840 mg IV on day 1 and day 15 in a 28-day cycle) and atezolizumab monotherapy (atezolizumab monotherapy 1,200 milligrams (mg) on day 1 in a 21-day cycle)	3rd line	NCT 02788279
MK-3475-158/KEYNOTE-158 Ongoing (recruiting) Estimated primary completion date August 28, 2023	II	TAS102 plus Nivolumab	3rd line	NCT02860546
Phase 2 Study of MK-3475 in Patients With Microsatellite Unstable (MSI) Tumors Ongoing (recruiting) Estimated primary completion date June 2021	II	Pembrolizumab 200 mg every 3 weeks for up to 35 administrations	Pre-treated	NCT02628067
A Phase I, Open-Label, Multi-Centre Study to Assess the Safety, Tolerability and Preliminary Anti-tumour Activity of Ascending Doses of Selumetinib (AZD6244 Hyd-sulfate) in Combination With MEDI4736 and Selumetinib in Combination With MEDI4736 and Tremelimumab in Patients With Advanced Solid Tumours Ongoing (recruiting) Estimated primary completion date July 10, 2018	II	-MSI Negative Colorectal Cancer: Pembroluzumab 10 mg/kg every 14 days -MSI Negative with Mutator Phenotype: Pembrolizumab 200 mg flat dose every 21 days	Pre-treated	NCT01876511
An Open-label, Phase II Basket Study of a hypoMETHylating Agent Oral Azacitidine and DURvalumab (MEDI4736) (Anti-PDL1) in Advanced Solid Tumors (METADUR) Ongoing (recruiting) Estimated primary completion date July 2021	I	Selumetinib + MEDI4736 Patients with known MSI-high status will be excluded; patients with MSS, MSI-low, or unknown MSI status may be enrolled	Pre-treated	NCT02586987
Evaluate the Efficacy of MEDI4736 in Immunological Subsets of Advanced Colorectal Cancer Ongoing (recruiting) Estimated primary completion date July 2019	II	Azacitidine 300 mg daily for 14 consecutive days of every 28 days cycle for 3 cycles. PLUS Durvalumab 1,500 mg on Day 1 of every 28 days cycle for 12 months or until disease progression Only Microsatellite Stable Colorectal Carcinoma	Pre-treated	NCT02811497
	II	subjects will receive MEDI4736 for 12 months, or until PD, initiation of alternative cancer therapy, unacceptable toxicity. Following the 12-month treatment period, subjects without evidence for PD or other reason to discontinue treatment will be monitored without further treatment. Upon evidence of PD during the monitoring period, administration of MEDI4736 may resume at the Q2W schedule, for up to another 12 months Locally advanced or metastatic MSI-H CRC	3rd line	NCT02227667

\*Preliminary data; MSI: microsatellite instable; MSS: microsatellite stable; TAS: Trifluridine/Tipiracil; PD: progressive disease; PDL-1: programmed cell death ligand-1; CRC: colorectal cancer

could be useful for all CRC if it was possible to convert the tumor towards a “CMS1-like” immune phenotype. CMS4 tumors (which showed the worse prognosis in terms of overall and relapse-free survival), for example, are characterized by an unfavorable, inflamed immune phenotype. They revealed high expression of mesenchymal genes, stromal cell infiltration and an angiogenic microenvironment.

Vascular endothelial growth factor-A (VEGF-A), a proangiogenic molecule produced by the tumors, has a crucial role in the development of the immunosuppressive microenvironment<sup>[39]</sup>. Given the immune-adjuvant effect that has been suggested for metastatic CRC patients treated with the anti-VEGF antibody

bevacizumab<sup>[40]</sup> when combined with conventional chemotherapy, researchers are trying to further enhance the effect on the immune system by coupling anti-angiogenic treatment with immunotherapy and this strategy might be particularly relevant for CMS4 tumors. Several clinical trials are investing whether combination of bevacizumab with either immunotherapy alone or combined with targeted therapies and conventional chemotherapy might show activity in this setting (NCT02873195, NCT02291289, NCT02876224).

Another key aspect of the TME of CMS4 tumors is represented by activation of TGF- $\beta$  signaling. Using a preclinical model of CT26 colon carcinoma cells, Triplett *et al.*<sup>[41]</sup> showed that combining aOX40 antibodies with an inhibitor of the TGF- $\beta$  receptor (SM16) had a synergic action and elicited complete regression of tumors. Targeting the TGF- $\beta$  pathway with galunisertinib as monotherapy and in combination with anti-PD-1 agents, induced anti-tumor immunity and tumor shrinkage also in a mouse model of mesenchymal CRC<sup>[42]</sup>. Based on these evidences, multiple TGF- $\beta$  targeted therapies are currently in clinical trials.

CMS2 and CMS3 are considered as “cold” tumors, meaning that they lack immune cell infiltration. The level of expression of immunosuppressive genes is low, thus suggesting different mechanisms of immune escape. For example, the downregulation of MHC class I observed in these tumors, results in reduced presentation of tumor-associated antigens<sup>[43]</sup>. CMS3 tumors are frequently *RAS* mutated. In a recent study by Lal *et al.*<sup>[44]</sup> who used The Cancer Genome Atlas RNA-seq, *KRAS*-mutant CMS2 samples had reduced infiltration of cytotoxic cells and neutrophils relative to CMS1 and CMS4 and to *KRAS* wild-type CMS2 samples. Deregulation of mitogen-activated extracellular signal-regulated kinase (MEK) pathway is involved in carcinogenesis and maintenance of cancers. This pathway is physiologically activated by growth factors, but in pathological conditions mutations of oncogenic proteins (such as *RAS* and *RAF*) can cause the systematic activation of the MEK cascade. MEK inhibition with cobimetinib upregulates tumor major histocompatibility complex-I expression, promotes intratumoral T-cell accumulation and enhances anti-PDL-1 responses<sup>[45]</sup>. In a recent phase Ib study presented at Gastrointestinal Cancer Symposium American Society of Clinical Oncology (ASCO) 2018, sixty-six patients were enrolled to receive atezolizumab in combination with Cobimetinib in metastatic or locally advanced CRC refractory to chemotherapy. Preliminary data showed interesting results: OS was 10 months with durable responses in patients with MSS or microsatellites instable-low tumors<sup>[46]</sup>. Conversely, CMS2 tumors are usually characterized by EGFR activation without mutations in downstream pathways (e.g., *KRAS* mutations). Cetuximab, an anti EGFR monoclonal antibody, revealed a potential synergistic effect with monoclonal antibodies targeted to CTLA4 and PD-1 antigens and *in vivo* studies, especially in patients with head and neck tumors and lung cancer, are promising<sup>[47]</sup>.

Other approaches that are being tested to improve immunotherapy response among CMS subtypes are represented by cytokine treatment, cancer vaccination and passive immunotherapy with adoptive T cell transfer or monoclonal antibody targeting tumor-associated antigens. Klein *et al.*<sup>[48]</sup> recently evaluated carcinoembryonic antigen (CEA)-IL2v (RG7813), an engineered IL-2 variant (IL-2v) with abolished IL-2 $\alpha$  (CD25) binding fused to an antibody targeting CEA to increase immune infiltration and activates NK and T cells both in the periphery and within tumors. In two ongoing dose-escalation phase I studies, Tabernero *et al.*<sup>[49]</sup> proved the antitumor activity of CEA CD3 TCB (RG7802, RO6958688), a novel T-cell bispecific antibody targeting CEA on tumor cells and CD3 on T cells, in 11% of adult patients with advanced CEA+ solid tumors who received RG7802 as monotherapy and in 50% of patients to whom the antibody was given in combination with atezolizumab 1200 mg Q3W.

Likewise, other malignancies, combining immunotherapy with conventional chemotherapeutic strategies or with radiotherapy (RT) might represent an useful and practical means to stimulate immune cell infiltration and elicit immune response. To this purpose, clinical trials testing the combination of anti-PDL-1/PD-1 treatment with RT or modified FOLFOX are ongoing (NCT02437071, NCT02375672). In the

first one is a phase II study to evaluate the safety and abscopal effect of pembrolizumab after palliative RT or ablation in pts with unresectable/recurrent pMMR metastatic colorectal cancer, who have received  $\geq 2$  standard therapies, with ORR in a non-targeted lesion as primary objective<sup>[50]</sup>. After enrolling 26 patients, pembrolizumab after RT or ablation resulted feasible with a tolerable safety profile, with one patient achieving a partial response (PR) in non-irradiated lesions after RT (abscopal effect). The second one is based on the hypothesis that combination of mFOLFOX6 and pembrolizumab may enhance immunogenic cell death and improve outcome in patients with untreated, advanced CRC irrespective of MMR status. After a median follow up of 24 weeks, clinical activity was seen in patients including those with proficient MMR, with a DCR rate of 100% at 8 weeks<sup>[51]</sup>.

A different strategy that is currently under evaluation to improve efficacy of immunotherapy in MSS/pMMR CRCs is combination of histone deacetylase inhibitor and PD-1 inhibitors. Entinostat, an oral, class I-selective histone deacetylase inhibitor is able to enhance anti-PD-1 activity by downregulation of immunosuppressive cell types in the TME<sup>[52]</sup> in models of renal and lung cancer.

Preliminary results of a phase II study of entinostat in combination with pembrolizumab have been recently presented at ASCO 2018 annual meeting. Sixteen pretreated MSS/pMMR CRC patients were enrolled and at data cut-off 6 patients remained on study (1 PR, 6 stable disease). The treatment showed acceptable safety with common adverse events including fatigue (37.5%), arthralgia (18.8%), and increased alkaline phosphatase (18.8%). These results can be viewed as promising, considering that have been obtained in a patient population in which objective responses have not been reported with anti-PD-1 monotherapy<sup>[53]</sup>.

In addition to immune strategies focused on PD-1/PDL-1 axis and CTLA4 and against cancer immunotolerance, a series of different approaches (albeit still on the side of immunotherapeutic approaches) are recently been investigated in CRC. T lymphocytes engineered to express chimeric antigen receptors (CAR-T cells) have been tested for their potential role as therapeutic agents in CRC. In a recent paper of Magee *et al.*<sup>[54]</sup>, CAR-T cells expressing the human specific GUC2YC antigen variable fragment were able to determine an increase of cytokine production and upregulation of markers of inflammation. The cells were also able to induce a somewhat specific killing of CRC cells who did express GUC2YC, whereas GUC2YC-deficient cells were spared. This was proven in *in-vitro* and in mice xenografts, suggesting further development of CAR-T cells engineered to express this antigen.

However, in another paper of Huang *et al.*<sup>[55]</sup>, it is also suggested that, albeit interesting, development of CAR-T cells therapy for CRC patients should first be complemented by the addition of some forms of treatment able to induce indoleamine 2,3-dioxygenase 1 (IDO1) downregulation. The authors have examined the effects of CAR-T cells targeting EGFR variant III on CRC cell lines and correlated the effectiveness of treatment on the basis of either IDO1 downregulation or normal expression on the basis of the expression, in cell lines, of mir-153. In particular, due to the inhibitory effect on the expression of IDO1 of mir-153, the authors were able to find a significant correlation between CAR-T cells mediated killing of CRC cells and high levels of expression of mir-153, thus suggesting that CAR-T cells treatment “per se” is not enough to induce some meaningful tumor response.

Albeit manipulation of the mutational load of CRC patients is a mere piece of science fiction, it is well-known that, for treatments that are focused on PD-1/PDL-1 axis, mutational load might represent the best way to identify those patients who could benefit from this kind of strategy (more than the simplistic way of assessment of patients as in microsatellite stable/unstable). In particular, in a recent paper of Fabrizio *et al.*<sup>[56]</sup>, authors tested 6004 cases of CRC by matching MSI assessment (MSS or MSI-L vs. MSI-H) and estimation of tumor mutational burden (TMB high or low). Authors found that the matching was not exactly perfect, with 302 cases (5% cases) having MSI-H status and 301/302 (99.7%) MSI-H cases having TMB high status

but also with 164/5702 (2.9%) MSS cases having also TMB high status. Authors were able to confirm the activity of an anti-PD1 inhibitor in patients having TMB high status, thus suggesting that screening patients on the basis of MSI-H status positivity is somehow restricting the number of patients that could ultimately benefit from anti-PD1/PDL-1 treatment.

These data suggest that, at least in the foreseeable future, more data are needed to further assess the clinical impact of these treatment approaches in everyday practice, as there are a few crucial topics still to be addressed (namely the fitness of T cells, how to increase sensitivity of the TME towards T cell mediated killing and the selection of patients that benefit best from these treatment approaches).

## CONCLUSION

In the past few years, introduction of new therapeutic approaches and better selection of patients have significantly changed treatment strategy of CRC and definitely improved patient outcome.

Immunotherapy has been the most important revolution in cancer treatment of recent years and it continues to show impressive results in lethal malignancies such as melanoma or lung cancer. Still, results observed in CRC with checkpoint inhibitors immunotherapy are modest if compared to other tumor entities and limited to a small subset of patients with MSI. In this context, a better knowledge of tumor immune microenvironment is essential to developing effective therapeutic strategies and overcoming resistance.

Interestingly, molecular characterization of CRC has shown that CMSs are associated with specific immune infiltration profiles corresponding with characteristic mechanisms of immune escape.

In particular, CMS1 subtype presents the most favourable situation for immunotherapy efficacy with high immune infiltration rich in Th1 cells and TILs, explaining the efficacy of checkpoint inhibitors in this subtype. CMS4 also presents high immune infiltrate but with an unfavourable, inflamed molecular orientation characterized by intratumoral MDSC, M2-macrophages and B-cells associated with pro-inflammatory gene expression, including myeloid chemokines, immune suppressive molecules and complement factors. In this situation, the combination of checkpoint inhibitors with TGF pathway inhibition represents a promising strategy as well as the use of angiogenesis inhibitors or anti-MDSCs treatment. On the contrary, CMS2 and CMS3 are poorly immunogenic tumors with scarce immune infiltrate. In this context, combination of checkpoint inhibitors with MEK-inhibition or anti-EGFR monoclonal antibodies could allow to overcome resistance. In addition, monoclonal antibodies targeting tumor-associated antigens, such as CEA, engineered with IL-2 may be able to increase immune infiltration and activates NK and T cells also in tumors with poor immune infiltration. Other strategies which may be effective in the setting of CMS2 and CMS3 are the combination of chemotherapy and immune checkpoint inhibitors or passive immunotherapy treatments as cancer vaccines with primed DCs.

In conclusion, the development of new effective immunotherapeutic strategies in CRC should be driven by a better knowledge of mechanisms of resistance to current treatments and take in account differences in immune microenvironment between different molecular subtypes to find the best treatment for each patient.

## DECLARATIONS

### Authors' contributions

Responsible for the paper: Berardi R

Concept, design, definition of intellectual content: Bittoni A

Literature search: Meletani T, Sotte V, Cantini L

Manuscript preparation: Meletani T, Sotte V, Cantini L, Giampieri R

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All authors declared that there are no conflicts of interest.

### Ethical approval and consent to participate

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### Consent for publication

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Original Article

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# Tumor cell invasion from the marginal sinus into extranodal veins during early-stage lymph node metastasis can be a starting point for hematogenous metastasis

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## Abstract

**Aim:** To investigate whether tumor cells in a lymph node (LN) can invade from the marginal sinus into extranodal veins via vessel branches that communicate with intranodal veins and whether this can be a starting point for hematogenous metastasis at the early stage of LN metastasis.

**Methods:** Vascular and lymphatic networks of LNs in MXH10/Mo-*lpr/lpr* mice were investigated using three-dimensional micro-computed tomography and histological methods. Flow in the blood vessel networks of LNs was investigated by fluorescence microscopy. Tumor cells were injected into the subiliac LNs of MXH10/Mo-*lpr/lpr* mice to induce metastasis to the proper axillary LNs. Tumor development in the proper axillary LN was detected using an *in vivo* bioluminescence imaging system. A two-dimensional image of the proper axillary LN microvasculature was reconstructed using a contrast-enhanced high-frequency ultrasound system.

**Results:** Extranodal veins communicated with intranodal veins via branches that penetrated the capsule, and blood flowed from intranodal veins to extranodal veins. Tumor cells that had metastasized to the marginal sinus invaded these communicating veins to develop hematogenous metastases.



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**Conclusion:** Metastatic LNs that would be considered by clinical imaging to be stage N0 can be a starting point for hematogenous metastasis. The study findings highlight the need for the development of novel techniques for the diagnosis and treatment of early-stage LN metastasis, i.e., when standard diagnostic imaging might incorrectly classify the LN as stage N0.

**Keywords:** Lymph node, metastasis, N0, lymph node-mediated hematogenous metastasis

## INTRODUCTION

Tumor cells reach the marginal sinus of a sentinel lymph node (LN) via afferent lymphatic vessels, after which they proliferate along the lymphoid sinus, invade the cortex and reach the medulla<sup>[1]</sup>. The abundant vascular network in a LN<sup>[2]</sup> allows tumor cells to grow without the induction of tumor neovasculature<sup>[3,4]</sup>. Since tumor cells growing in a LN can infiltrate both the lymphatic channel and the vascular network, a sentinel LN can be the origin of lymphatic metastasis to downstream LNs as well as hematogenous metastasis<sup>[5]</sup>. It has been suggested that high endothelial venules (HEVs) may be involved in the mechanisms underlying systemic metastasis<sup>[5,6]</sup>, but the details remain unknown. Clinically, a LN is judged as positive for metastasis (> N1) if tumor invasion is detected by diagnostic imaging or aspiration cytology. However, since a LN can be erroneously classified as stage N0 during the early stages of tumor invasion, a false-N0 LN can potentially be a source of systemic metastasis. For example, in the NSABP-32 trial<sup>[7]</sup>, patients with breast cancer judged incorrectly to be stage N0 had no difference in overall survival, disease-free survival and distant disease-free interval to patients judged to be stage N0. In other words, tumor cells may have undergone systemic metastasis at a stage when LNs were incorrectly classified as N0.

Recently, we demonstrated that a fluorescent dye injected locally into a LN flowed into both the efferent lymphatic vessel and extranodal veins and that intranodal and extranodal veins communicated via branches that passed through the capsule<sup>[8,9]</sup>, a feature not described in conventional textbooks of anatomy. Thus, tumor cells can undergo both lymphatic and hematogenous metastasis. Based on these results, we proposed a theory of LN-mediated hematogenous metastasis, whereby LNs can be the origin of systemic metastasis<sup>[8,9]</sup>.

In this study, we used a mouse model in which metastasis to the proper axillary LN (PALN) was induced by the inoculation of tumor cells into the subiliac LN (SiLN). We found that during early-stage PALN metastasis (confirmed by pathological imaging), the invasion of tumor cells from the marginal sinus into intranodal veins and then extranodal veins may be a first step in the mechanism of hematogenous metastasis from a LN.

## METHODS

Experiments were carried out in accordance with published guidelines and approved by the Institutional Animal Care and Use Committee of Tohoku University.

### Mice

MXH10/Mo-*lpr/lpr* (MXH10/Mo/*lpr*) mice (12-17 weeks old), which are a congenic strain of MRL/Mp-*lpr/lpr* and C3H/HeJ-*lpr/lpr* mice<sup>[10]</sup>, were bred under specific pathogen-free conditions in the Animal Research Institute, Graduate School of Medicine, Tohoku University, Sendai, Japan. The LNs enlarge to about 10 mm in diameter at 12 weeks of age due to invasion by *lpr*-T (CD4<sup>-</sup>CD8<sup>+</sup>B220<sup>+</sup>Thy<sup>+</sup>) cells<sup>[11]</sup>. The anatomical locations and nomenclatures of murine LNs have often been ignored or assigned incorrectly; in this study, we used the term “subiliac LN” instead of “inguinal LN”<sup>[12]</sup>.

### Micro-computed tomography imaging

Specimens were analyzed using high-resolution micro-computed tomography (micro-CT) scanning (scanXmate/E090, Comscan Tecno). Barium contrast agent (mean size, 935.7 nm) was prepared as

previously described<sup>[13]</sup>. With the mouse under deep general anesthesia, 0.1 mL heparin (Novo-Heparin, 1000 units/mL, Mochida Pharmaceutical) was administered intravenously and 0.05 mL papaverine hydrochloride (40 mg/mL, Nichi-Iko) was given subcutaneously. Ten min later, a syringe pump (Legato100, KD Scientific) was used to infuse 4 mL of saline (18 mL/h) into the left ventricle through a thoracotomy. After cutting of the caudal vena cava and draining of the blood from the body, 4 mL of contrast medium was injected into the left ventricle (18 mL/h) via a T-shaped stopcock. Following perfusion with contrast medium, the LNs were dissected and kept at 4 °C for > 2 h to allow fixation to occur. The samples were placed on the stage of a micro-CT scanner (the gutta percha was used as a landmark for positioning) and scanned (angiography) at resolutions of 5-30 µm and a slice thickness of 100 µm. Acquired slice data were rendered as 3D images using a 3D analysis suite (Amira, Maxnet).

### Cell culture

KM-Luc/GFP cells (mouse malignant fibrous histiocytoma-like cells derived from an MRL/Mp-*gld/gld* mouse expressing a fusion of the luciferase and enhanced-green fluorescent protein genes) were cultured as previously described<sup>[14]</sup>. FM3A-Luc cells (C3H/He mouse mammary carcinoma cells expressing the luciferase gene)<sup>[10]</sup> were maintained in RPMI-1640 medium supplemented with 10% fetal bovine serum, 1% L-glutamine-penicillin-streptomycin and 1 mg/mL G418 (Sigma-Aldrich). Both cell types had an H-2<sup>k</sup> haplotype, which is the same as that of MXH10/Mo/*lpr* mice, and expressed vascular endothelial growth factor (VEGF)-A and VEGF-B but not VEGF-C; KM-Luc/GFP but not FM3A-Luc cells showed slight VEGF-D expression<sup>[15]</sup>. The relative growth rates of KM-Luc/GFP and FM3A-Luc cells were 3.8/day and 1.1/day, respectively<sup>[8]</sup>. Cell lines were incubated (37 °C, 5% CO<sub>2</sub>/95% O<sub>2</sub>) until 80% confluence was achieved. Lack of *Mycoplasma* contamination was confirmed on the inoculation day (MycoAlert *Mycoplasma* Detection Kit; Lonza Rockland).

### NBD-liposomes

NBD-liposomes were synthesized from 1,2-distearoyl-sn-glycero-3-phosphatidylcholine (DSPC; MC8080, NOF Co.), 1,2-distearoyl-sn-glycerol-3-phosphatidylethanolamine-methoxy- polyethyleneglycol (DSPE-PEG[2000-OMe]; DSPE-020CN, NOF Co.), and 1,2-dipalmitoyl-sn-glycero- 3-phosphoethanolamine-N-(7-nitro-2-1,3-benzoxadiazol-4-yl) (NBD-DPPE; FE6060, NOF Co.)<sup>[9,16]</sup>. The size and zeta potential of the NBD-liposomes was 642 nm and -1.5 mV, respectively, as measured using a particle size and zeta potential analyzer (ELSZ-2; Otsuka Electronics).

### Visualization of venous flows internal and external to a LN

Under deep general anesthesia, an arc-shaped incision was made in the abdominal skin of a mouse (*n* = 1, 12 weeks old) from the subiliac region to the proper axillary region, and 100 µL of 0.01 µmol/L NBD-liposomes was injected into the tail vein (100 µL/min, 60 s). Fluid flow in the veins was visualized using a fluorescence stereomicroscope (M165-FC; fluorescence filter: GFP2; excitation: 460-500 nm; emission: > 510 nm; Leica) connected to a high-speed camera (10 frames/s; CoolSNAP HQ2; Photometrics)<sup>[8]</sup>.

### Induction of metastasis to the PALN by injection of tumor cells into the SiLN

KM-Luc/GFP ( $1.5 \times 10^6$  cells/mL) or FM3A-Luc ( $3.3 \times 10^5$  cells/mL) cells (passaged three times) were suspended in a mixture of 20 µL phosphate-buffered saline (PBS) and 40 µL of 400 mg/mL Matrigel (Collaborative Biomedical Products). The prepared cells were injected into the center of the SiLN of a mouse (aged 14-16 weeks) using a 27G needle, which was maintained in the same position for 5 min to allow solidification of the Matrigel. Tumor development in the SiLN and metastasis to the PALN (the rates of which depended on the tumor cell type) were detected using an *in vivo* bioluminescence imaging system (IVIS; Xenogen) at 4 and 7 days post-inoculation of KM-Luc/GFP cells and at 6, 13, 20 and 27 days post-inoculation of FM3A-Luc cells. Injection of tumor cells into the SiLN induces metastasis to the PALN via lymphatic vessels as well as hematogenous metastasis via the thoracoepigastric vein (TEV)<sup>[9,17]</sup>; the cell number is highest in the SiLN and second highest in the PALN<sup>[14]</sup>.



### Contrast-enhanced ultrasound imaging and spatiotemporal analysis of pixel intensity variations

A 2D image of the PALN microvasculature was reconstructed on days 6 and 9 post-inoculation of KM-Luc/GFP cells and on days 8, 15, 22 and 29 post-inoculation of FM3A-Luc cells using a contrast-enhanced high-frequency ultrasound (CE-HFUS) system with a 37.5-MHz transducer (RMV-710B, VisualSonics). Each transducer was fixed to a 3D stage control system (Mark-204-MS; Sigma Koki). Contrast images (slice thickness, 100  $\mu$ m) were captured before and 60 s after an intravenous bolus injection of 100  $\mu$ L microbubbles (Sonazoid, Daiichi Sankyo) into the tail vein. During imaging, the mouse was positioned on a heated stage and anesthetized with 2% isoflurane in oxygen<sup>[18]</sup>. Acquired images were analyzed using ultrasound contrast agent-detecting software<sup>[19]</sup> to measure temporal changes in the PALN microvessel density.

### Histological analysis

PALNs and SiLNs were harvested at the indicated time points, fixed in 10% formaldehyde in PBS for 3 days, placed on a shaker for 1 day at room temperature and then stored at 4 °C for 2 days. Next, the samples were dehydrated in 100% ethanol, placed into a tissue processor, embedded in paraffin and cut into 3- $\mu$ m serial sections using a fully-automated tissue-sectioning device (AS-400, Kurano). Samples were stained with hematoxylin and eosin (HE) or immunostained for detection of CD31-positive vascular endothelial cells.

### Statistical analysis

Data are presented as mean  $\pm$  SD or mean  $\pm$  SEM. Differences between groups were determined by one-way analysis of variance followed by Tukey's test or an unpaired *t*-test (GraphPad Prism 6J). *P* < 0.05 was considered to be statistically significant.

## RESULTS

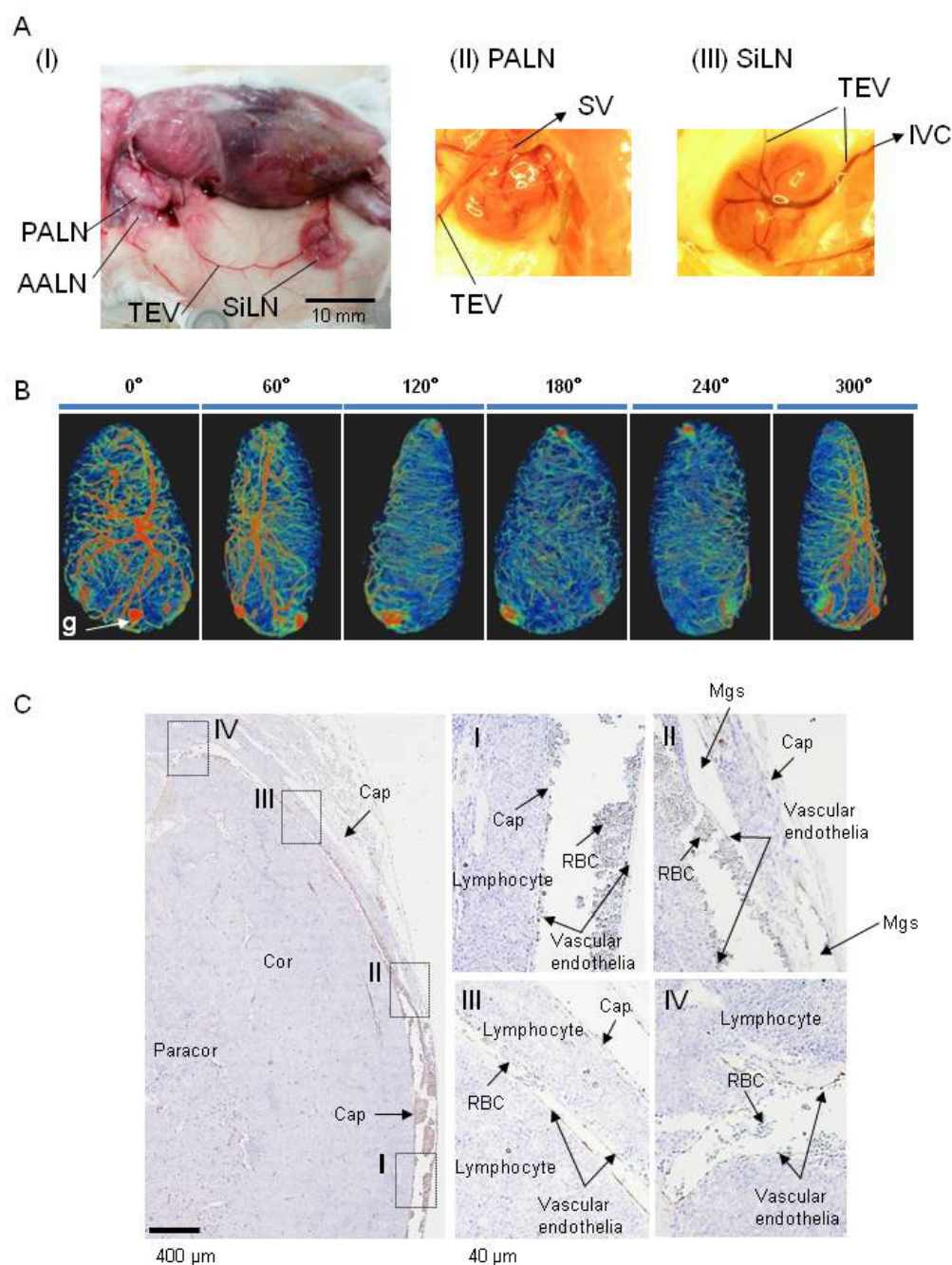
### Connection of the TEV to SiLN blood vessels

First, we examined the anatomical positions of the PALN, SiLN, accessory axillary LN (AALN) and TEV. An arc-shaped incision was made in the abdominal skin of a mouse from the subiliac region to the proper axillary region [Figure 1AI]. The axillary area contains two LNs, the PALN and AALN<sup>[16]</sup>. The SiLN and AALN lie upstream of the PALN in the lymphatic network. The TEV, which connects the subclavian vein and inferior vena cava, runs adjacent to the PALN [Figure 1AII] and SiLN [Figure 1AIII] and along the lymphatic vessels (not visualized in Figure 1AI) between these LNs. The TEV receives venous blood from the PALN and SiLN via small branches [Figure 1AII and AIII]<sup>[9,16]</sup>. There were many vessels on the LN that connected to intranodal vessels [Figure 1AII and AIII]. The hilum of the PALN [Figure 1AII] and of the SiLN [Figure 1AIII] was located behind the image.

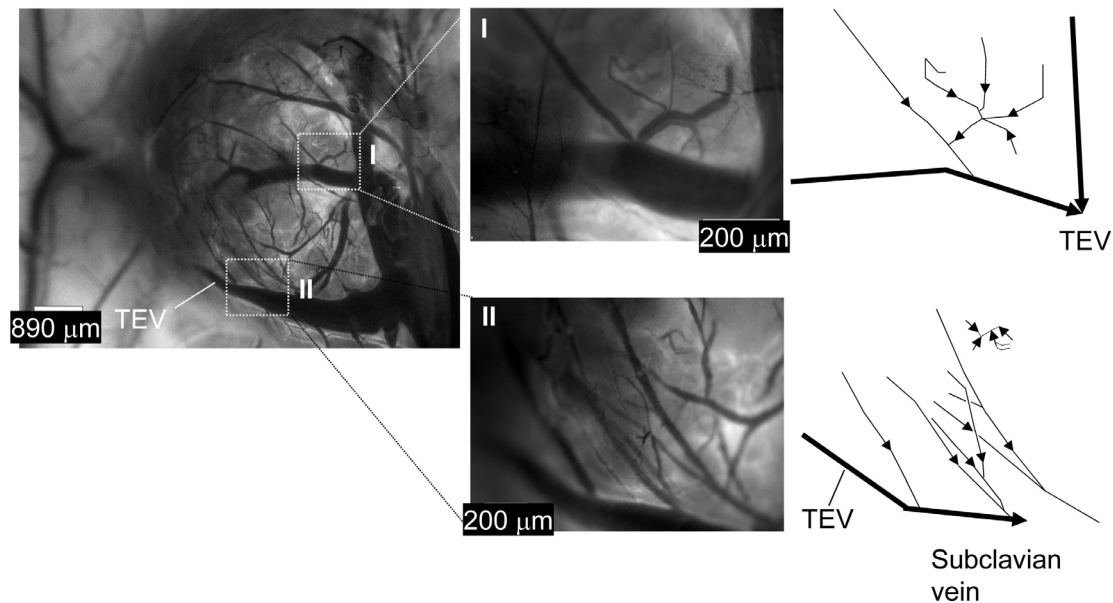
A series of 3D micro-CT images, rotated every 60°, revealed a complex vascular network in the PALN [Figure 1B], with many small branches penetrating the LN capsule and connecting the TEV to intranodal blood vessels. There were no similar networks on the reverse side [Figure 1B]. Immunolabeling of CD31 [Figure 1C] showed that the TEV ran along and penetrated the capsule of the SiLN [Figure 1CI-IV]. The TEV ran along the capsule (Cap; image I) and connected with intranodal veins penetrating the marginal sinus (Mgs; image II). The branches of TEV ran in the cortex (Cor) under the marginal sinus (image III) and branched into two vessels in the cortex (image IV).

### Flow dynamics between the PALN blood vessel network and TEV

The flow dynamics between the PALN blood vessel network and TEV were visualized under a fluorescence stereomicroscope after intravenous injection of NBD-liposomes [Figure 2]. Consistent with the results shown in Figure 1, the TEV communicated with intranodal blood vessels via many small branches [Figure 2]. In the region excluding the TEV [Figure 2I and Video 1], a large vein running along the PALN made connections with the intranodal veins, and the combined blood flow drained into the TEV. In the region including the TEV [Figure 2II and Video 2], the venous network inside the LN connected to the TEV. The blood flow



**Figure 1.** Vascular and lymphatic networks of lymph nodes (LNs). A: Vascular networks in LNs: (I) a macroscopic view of a 14-week-old mouse (right side) indicating the anatomical positions of the proper axillary LN (PALN), subiliac LN (SiLN), accessory axillary LN (AALN) and thoracoepigastric vein (TEV). The axillary area contains two LNs, the PALN and AALN<sup>[16]</sup>. The SiLN and AALN are upstream of the PALN in the lymphatic network. The TEV, which connects the subclavian vein (SV) and inferior vena cava (IVC), runs adjacent to the SiLN and PALN and along the lymphatic vessels between them (not visualized). The TEV receives venous blood from the SiLN and PALN via small branches; (II) blood vessels running on the PALN in a 17-week-old mouse. There were many vessels on the LN surface. The hilum was behind the image; (III) blood vessels running on the SiLN in a 17-week-old mouse. There were many vessels on the LN surface. The hilum was behind the image; B: three-dimensional micro-computed tomography (micro-CT) images showing the surface and internal vascular structure of the PALN in a 14-week-old mouse. The series of images shows the LN rotated by 60° each time. The TEV communicated with intranodal blood vessels via many small branches that penetrated the LN capsule. There were no similar networks on the reverse side. Scale: 2 mm. g: gutta-percha; C: images immunostained for CD31 showing the connections between the TEV and SiLN blood vessels in a 16-week-old mouse. Images I-IV (middle and right) are magnified views of the corresponding regions highlighted in the left-most image. The TEV ran along the capsule (image I) and connected with intranodal veins penetrating through the marginal sinus (image II). It is notable that the marginal sinus was extremely close to the intranodal vein. The branches of TEV ran in the cortex under the marginal sinus (image III) and branched into two vessels in the cortex (image IV). RBC: red blood cell; Cap: capsule; Mgs: marginal sinus; Cor: cortex; Paracor: paracortex



**Figure 2.** Flow in the blood vessel network of the proper axillary lymph node (PALN;  $n = 1$  mouse, 12 weeks old). Images I and II are magnified views of the corresponding regions highlighted in the left-most image. Image I shows a region that did not include the thoracoepigastric vein (TEV) and was a frame obtained from Video 1. Image II shows a region that includes the TEV and was a frame obtained from Video 2. The directions of flow in images I and II are shown schematically to the right of each image

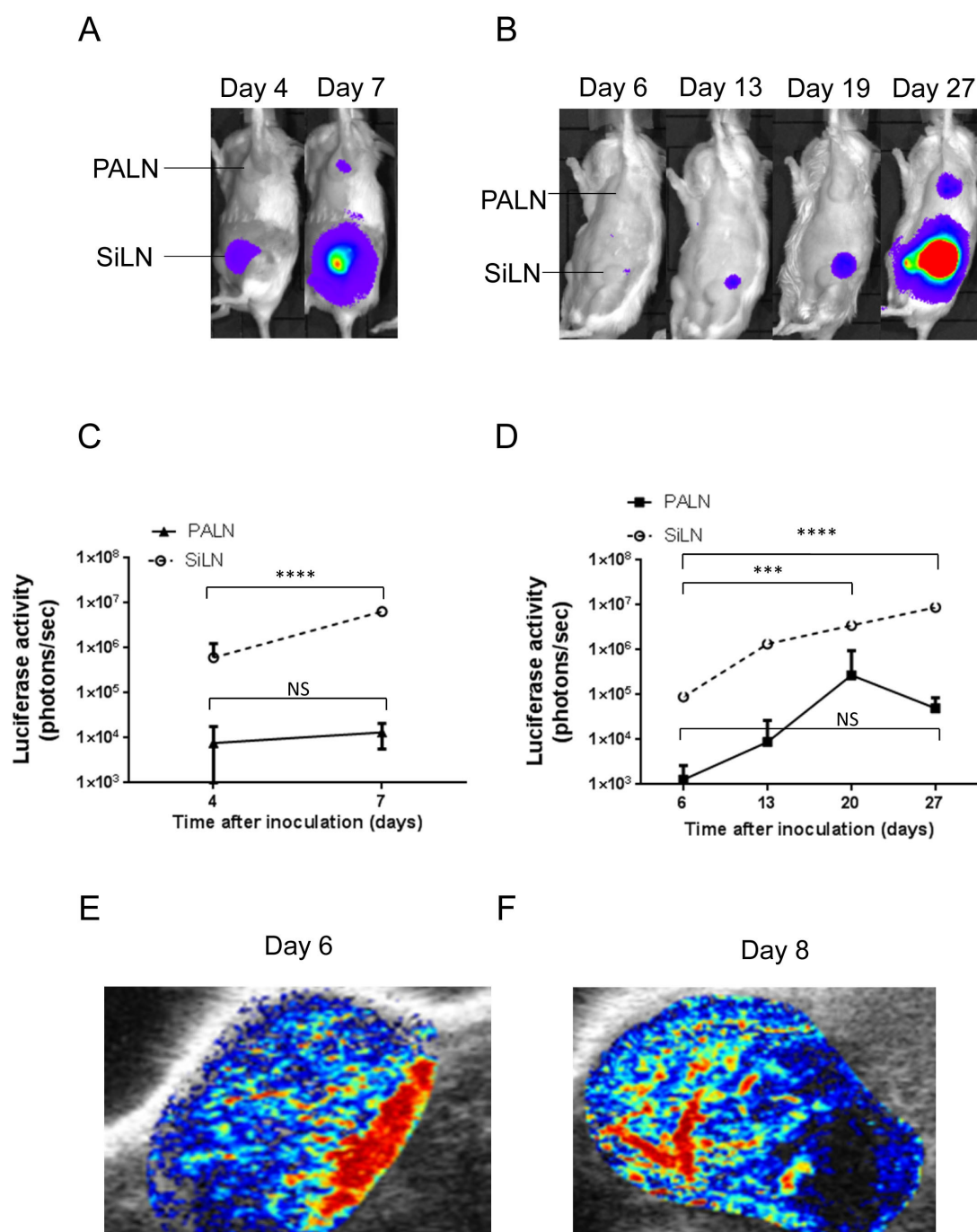
from the TEV enters the subclavian vein<sup>[16]</sup>; since the venous branches from the intranodal veins connect to the TEV, their blood is returned to the venous circulation.

### Metastatic SiLN at the false N0 stage

Next, we explored the interaction of tumor cells with intranodal vessels during the early stage of metastasis. Two different tumor cell types were used: KM-Luc/GFP [Figure 3A, C, E] and FM3A-Luc [Figure 3B, D, F]. The injection of tumor cells into the SiLN induced metastasis in the PALN. Luciferase activity in the SiLN and PALN increased with tumor progression for both cell lines (Figure 3A and C for KM-Luc/GFP, Figure 3B and D for FM3A-Luc). Metastasis was detected on day 7 after inoculation of KM-Luc/GFP cells [Figure 3A] and day 27 after inoculation of FM3A-Luc cells [Figure 3B]. Subsequently, we investigated flow dynamics in and around the metastatic PALN using CE-HFUS [Figure 3E and F]. Microbubbles flowing in the TEV were visualized in experiments using KM-Luc/GFP cells [Figure 3E and Video 3], while confluence of the TEV with other vessels was visualized in experiments using FM3A-Luc cells [Figure 3F and Video 4]. Our previous studies demonstrated no significant change in the volume of the metastatic PALN up to day 7 for KM-Luc cells<sup>[20]</sup> and day 22 for FM3A-Luc cells<sup>[21]</sup>. Thus, in a clinical setting, the metastatic PALN in these experiments would have been classified as stage No by diagnostic ultrasound.

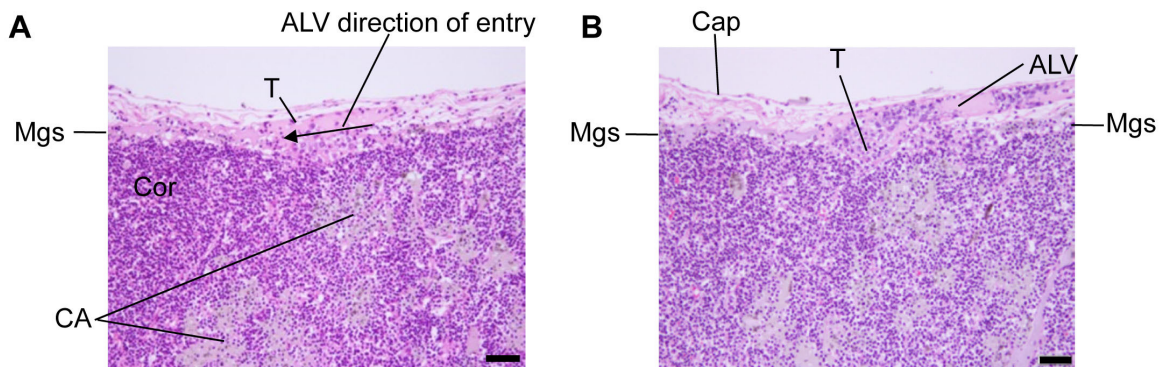
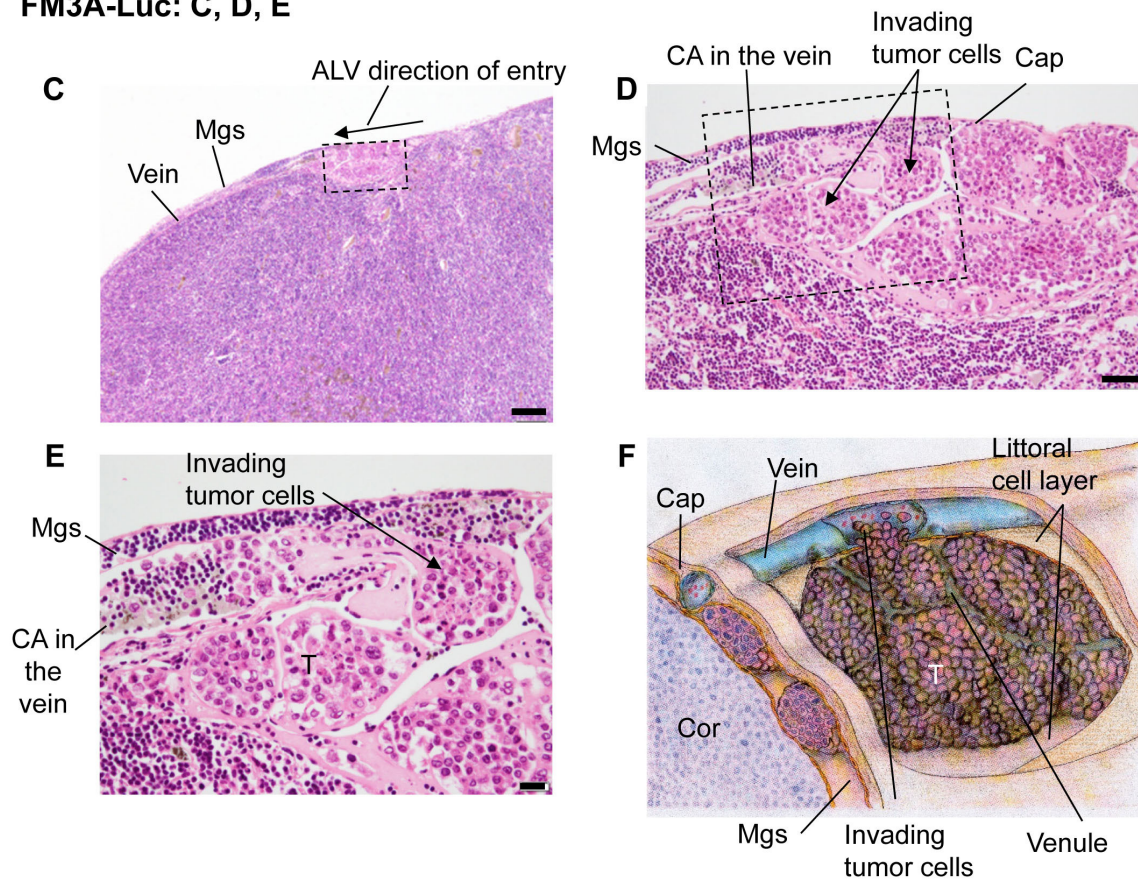
### Interaction of tumor cells with surrounding blood vessels in a LN at the false N0 stage

Histological techniques were used to investigate the interaction of tumor cells in the PALN (at the false No stage) with the surrounding blood vessels at day 6 for KM-Luc/GFP cells [Figure 4A and B] and day 8 for FM3A-Luc cells [Figure 4C-E]. The PALNs were removed after the micro-CT imaging experiments had been completed so that the blood vessels were filled with contrast agent. In experiments using KM-Luc/GFP cells, serial sections stained with HE [Figure 4A and B, Supplementary Figure 1] or immunostained for CD31 [Supplementary Figure 1] showed an afferent lymphatic vessel entering the LN. Tumor cells from the afferent lymphatic vessel had invaded the marginal sinus as well as vessels that were filled with contrast agent. In experiments using FM3A-Luc cells [Figure 4C and D], metastasized tumor cells were detected in the marginal sinus of the PALN. Importantly, tumor cells that had developed in the marginal sinus had



**Figure 3.** Induction of metastasis of KM-Luc/GFP cells or FM3A-Luc cells to the proper axillary lymph node (PALN) after inoculation of the cells into the subiliac lymph node (SiLN). KM-Luc/GFP cells (A, C, E); FM3A-Luc cells (B, D, F). A: Progression of KM-Luc/GFP cell metastasis from the SiLN to the PALN, as detected from measurements of luciferase activity; B: progression of FM3A-Luc cell metastasis from the SiLN to the PALN, as detected from measurements of luciferase activity; C: quantification of luciferase activity in the SiLN and PALN after inoculation of KM-Luc/GFP cells into the SiLN (SiLN,  $n = 5$  at day 4 and  $n = 3$  at day 7; PALN,  $n = 4$  at day 4 and  $n = 4$  at day 7). \*\*\*\* $P < 0.0001$ , unpaired  $t$ -test. The bars show mean  $\pm$  SEM values; D: quantification of luciferase activity in the SiLN and PALN after inoculation of FM3A-Luc cells into the SiLN (SiLN,  $n = 14$  at day 6,  $n = 10$  at day 13,  $n = 7$  at day 20 and  $n = 3$  at day 27; PALN,  $n = 14$  at day 6,  $n = 10$  at day 13,  $n = 7$  at day 20 and  $n = 3$  at day 27). \*\*\* $P = 0.0001$ , \*\*\*\* $P < 0.0001$ , one-way ANOVA and Tukey's test. The bars show mean  $\pm$  SEM values; E: representative images of the PALN at day 6, obtained using contrast-enhanced high-frequency ultrasound (CE-HFUS;  $n = 5$ ; Video 3), in a mouse inoculated with KM-Luc/GFP cells. The CE-HFUS image reveals microbubbles flowing in the vessel in the PALN; F: representative images of the PALN at day 8, obtained using CE-HFUS ( $n = 5$ ; Video 4), in a mouse inoculated with FM3A-Luc cells. The CE-HFUS image shows microbubbles flowing in the vessel in the PALN. NS: not significant



**KM-Luc/GFP: A, B****FM3A-Luc: C, D, E**

**Figure 4.** Invasion of tumor cells into a subcapsular vein in the proper axillary lymph node (PALN). A, B: Sections of the PALN at day 6 after inoculation of KM-Luc/GFP cells into the subiliac lymph node (SiLN). The sections were stained with hematoxylin-eosin (HE); the interval between each section was 12  $\mu$ m. Tumor cells (T) that had entered from the afferent lymphatic vessel (ALV) had invaded into the vein in the marginal sinus (Mgs) of the PALN. Scale bar: 100  $\mu$ m. Cor: cortex; CA: contrast agent; C-F: PALN at day 8 after inoculation of FM3A-Luc cells into the SiLN [C: tumor cells localized in the marginal sinus of the PALN. HE staining. Scale bar: 200  $\mu$ m; D: higher magnification of the rectangular region in (C). The marginal sinus was filled with tumor cells in a botryoidal configuration that projected toward the vascular wall. HE staining. Scale bar: 50  $\mu$ m; E: higher magnification of the rectangular region in (D). The tumor cells had disrupted the littoral cell layer of the marginal sinus and the vascular wall and had invaded into the vascular lumen (arrow). HE staining. Scale bar: 20  $\mu$ m; F: illustration of (D) showing the invasion of metastatic tumor cells from the marginal sinus into the subcapsular vein. Cap: capsule; Cor: cortex; Mgs: marginal sinus (subcapsular lymphatic sinus)]



entered the region close to the subcapsular vein and invaded the vessel [Figure 4E]. The schematic diagram in Figure 4F illustrates the internal features of the marginal sinus with metastatic tumor cells invading the subcapsular vein.

## DISCUSSION

The present study demonstrates that tumor cells in the marginal sinus of a LN can invade extranodal veins via branches that communicate with intranodal veins. We suggest that this may be the first step in hematogenous metastasis from LNs. Importantly, this form of metastasis occurs before tumor cells have interfered with well-developed vascular networks and HEVs within the LN<sup>[5,6]</sup>. Veins are ubiquitously present on the surface layer of the LN [Figure 1]<sup>[22]</sup> and communicate with intranodal veins, allowing blood to flow to the systemic circulation from intranodal vessels. This anatomical arrangement and flow characteristics have not been described previously. Kelch *et al.*<sup>[2]</sup> surgically excised individual mesenteric LNs from the mouse and clarified the topology of the entire intranodal vascular network using an automated confocal imaging system and custom-written software. However, unlike the present study, their analysis was limited to the vascular network within the LN. This study utilized malignant fibrous histiocytoma-like KM-Luc/GFP cells and breast cancer FM3A-Luc cells, both of which are syngeneic to the recipient mice. The invasion of tumor cells from the afferent lymphatic vessel into the intranodal veins was confirmed for both cell types [Figure 4, Supplementary Figure 1]. This implies that LN-mediated hematogenous metastasis may be a mechanism relevant to a wide variety of cancer types.

Lymphadenopathy in MXH10/Mo/*lpr* mice is due to the *lpr* gene and is characterized by the accumulation of *lpr*-T cells in the paracortical region. The metastatic lesions shown in Figure 4 are located in the marginal sinuses of the LNs. Thus, it is unlikely that there is a direct relationship between abnormal lymphocyte proliferation in the LN parenchyma and tumor cell proliferation and vascular invasion.

MXH10/Mo/*lpr* mice do not express the *fas* gene involved in apoptosis, since the *lpr* gene is a *fas*-deletion mutant gene. Thus, the immune system in MXH10/Mo/*lpr* mice is functional except for the signaling pathway related to *fas*. This precludes us from using human cell lines for our metastasis experiments, which would require immune-deficient mice such as SCID or nude mice. We selected MXH10/Mo/*lpr* mice for the present experiments because their characteristics make them better suited for use as a model of metastasis than immune-deficient mice implanted with human cell lines.

Fisher *et al.*<sup>[23]</sup> reported that LNs were not an effective barrier against tumor cell progression along the lymphatic system based on the observation that tumor cells were confirmed in the efferent lymphatic vessels after their injection into the lymphatic vessels of the rabbit. This means that tumor cells can flow through the lymphatic system via the marginal sinuses of LNs<sup>[17,24]</sup>, suggesting that lymphatic flow based on the anatomical structure of LNs should be distinguished from the mechanism of tumor metastasis.

Our findings suggest the possibility that tumor cells may undergo hematogenous metastasis at an early stage of LN metastasis, i.e., even before the stage when the infiltration of tumor into the LN is detectable by imaging or aspiration cytology. In other words, false-No metastatic LNs can be a source of hematogenous metastasis. The importance of false-No LNs in distant metastasis is supported by the results of clinical trials suggesting that LN dissection<sup>[25,26]</sup> and sentinel LN biopsy<sup>[27]</sup> do not always improve survival in patients with cancer.

The development of new methods to detect and treat metastasis in false-No LNs will be extremely important. In previous studies, we have shown that a lymphatic drug delivery system has great potential in the therapeutic and prophylactic treatment of false-No LNs<sup>[25,26]</sup>, and we expect this technique to be applied in the clinical setting in future.

## DECLARATIONS

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### Authors' contributions

Designed and performed the study, drafted the manuscript and prepared the figures: Kodama T  
Interpreted the data, reviewed the manuscript: All authors

### Availability of data and materials

Not applicable.

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### Conflicts of interest

All authors declared that there are no conflicts of interest.

### Ethical approval and consent to participate

Not applicable.

### Consent for publication

Not applicable.

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Review

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# Molecular biomarkers in current management of metastatic colorectal cancer

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## Abstract

Over the past two decades, the treatment outcomes in metastatic colorectal cancer (mCRC) have been remarkably improved, largely from the evolution of systemic therapy. Also, the molecular biomarkers have played a major role in this improvement by their predictive value in current treatment paradigm in mCRC. Currently, extended *RAS* mutation analysis is required for consideration of anti-epidermal growth factor receptor therapy in patients with mCRC. Several uncommon gene alterations have emerged as the potential targets for their matched molecular targeted therapy. Although, most patients with mCRC do not derive benefit from immunotherapy. By using microsatellite instability or mismatch repair test, we are now able to identify a small subgroup of patients with mCRC who have a very good response to immune checkpoint inhibitors. With the increasing number of required biomarkers in mCRC management, multiplex gene panel testing is now replacing single gene testing strategy. In patients accessible to matched molecular targeted therapy, especially for clinical trials, the comprehensive genomic profiling might be the preferred testing method. Although, it is potentially benefit in mCRC treatment, the liquid biopsy is not yet clinically applicable. The optimal utilization of molecular biomarker testing is required for best treatment outcomes in individual patients.

**Keywords:** Molecular biomarkers, metastatic colorectal cancer, treatment

## INTRODUCTION

Over the past two decades, the treatment outcomes in metastatic colorectal cancer (mCRC) have been significantly improved, largely because of the evolution of systemic therapy. Although chemotherapy is



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still the mainstay treatment in mCRC, its efficacy could be significantly enhanced by the biologic therapies including anti-angiogenesis and anti-epidermal growth factor receptor (anti-EGFR) agents. In a selected subgroup, the median overall survival is now up to 40 months with current treatment paradigm<sup>[1]</sup>. Recently, the molecular targeted therapy and immunotherapy have been demonstrated as the emerging effective therapeutic options for some patients with mCRC. The molecular biomarker plays a critical role as a tool for personalized therapy in current and upcoming treatment paradigm in patients with mCRC. The optimal utilization of molecular biomarker testing is required for best treatment outcomes in individual patients. This article reviews clinical application and limitation of current and emerging biomarkers in management of mCRC.

## BIOMARKERS FOR ANTI- EGFR THERAPY

EGFR is a transmembrane receptor tyrosine kinase playing a major role in carcinogenesis of several cancers including CRC. Although the EGFR expression was required for patients to be eligible in the initial anti-EGFR trials in mCRC<sup>[2,3]</sup>. The later reports demonstrated poor correlation between EGFR expression and treatment response<sup>[4,5]</sup>. Instead, *KRAS* mutation is a robust negative predictor for benefit of anti-EGFR in patients with mCRC. However, not all patients with wild-type *KRAS* mCRC will have benefit from first-line chemotherapy and anti-EGFR combination therapy, patient selection for anti-EGFR therapy has been evolved through biomarker analysis in subsequent clinical trials.

## RAS

RAS protein is a critical regulator of growth factor-induced cell proliferation and survival in both cancer and normal cells. There are three *RAS* family genes including *KRAS*, *NRAS* and *HRAS*. *KRAS* mutation is found in 30%-40% of CRC. *NRAS* mutation has been demonstrated in up to 3% of CRC while *HRAS* mutation was very rare in CRC<sup>[6]</sup>. In mCRC, *KRAS* exon 2 (codon 12 and 13) is the most frequent location for *RAS* mutation, with prevalence of 40%. Other *RAS* mutations were found at *KRAS* exon 3 and 4, and *NRAS* exon 2, 3 and 4, with prevalence of 15%-20%. Totally, the prevalence of all *RAS* mutations was around 55%-60% in patients with mCRC<sup>[7,8]</sup>. The mutations promote constitutive activation of GTP-bound RAS, resulting in activation of downstream signaling pathways especially the RAF/MEK/ERK pathway and PI3K pathway.

As a key downstream regulator of EGFR pathway, the activated mutation of *KRAS* might be able to abrogate the anti-EGFR treatment effects. In 2008, a retrospective analysis of *KRAS* exon2 mutation of a phase III trial, CO.17, demonstrated that cetuximab improved overall survival (OS) and progression free survival (PFS) only in patients with wild-type *KRAS* tumors, not in patients with mutant *KRAS* tumors<sup>[9]</sup>. This finding was subsequently confirmed in several cohorts of phase II and III trials of both available anti-EGFR agents including cetuximab and panitumumab<sup>[3,10,11]</sup>. In PRIME study, a prospective analysis of *KRAS* exon 2 mutation revealed a detrimental effect of additional panitumumab to chemotherapy for untreated patients with mutant *KRAS* mCRC<sup>[12]</sup>. In this cohort, a subsequent report demonstrated the extended analysis of *RAS* mutation, including *KRAS* and *NRAS* exon 2, 3 and 4, as the better predictive factor for panitumumab in patients with mCRC<sup>[13]</sup>. There was a detrimental effect of panitumumab in patients with wild-type *KRAS* exon2 with mutant other *RAS* mCRC. Similarly, this predictive effect of extended *RAS* mutation was subsequently confirmed in several phase II/III cetuximab and panitumumab trials. Therefore, the extended analysis of *RAS* mutation is required in selection of patients with mCRC for anti-EGFR therapy.

In contrast, *KRAS* mutation did not predict benefit of bevacizumab in patients with mCRC. In the analysis of phase III trial cohorts, additional bevacizumab to chemotherapy provides clinical benefit in both patients with wild-type and mutant *KRAS* mCRC<sup>[14,15]</sup>. Although patients with mutant *KRAS* mCRC seemed to live shorter than patients with wild-type *KRAS* mCRC in several anti-EGFR trials, prognostic



value of *KRAS* mutation was confounded by the effectiveness of anti-EGFR therapy in patients with wild-type *KRAS*<sup>[3,9-12]</sup>. There were conflicting results among the analysis in other RCT cohorts regarding the prognostic value of *RAS* mutation in mCRC<sup>[14,16]</sup>.

### **BRAF**

As *BRAF* is a key regulator in MAPK pathway, anti-EGFR therapy might not be effective in tumors with activated *BRAF* mutation. However, given the small number of patients with mutant *BRAF* tumors, the analysis of individual anti-EGFR randomized controlled trials did not consistently showed predictive effect of *BRAF* mutation for anti-EGFR therapy in patients with mCRC<sup>[13,17-20]</sup>. Recently, there were two metaanalyses regarding predictive effect of *BRAF* mutation for anti-EGFR treatment in patients with mCRC, although they showed no significant benefit of anti-EGFR therapy in patients with mutant *BRAF* mCRC. Pietrantonio *et al.*<sup>[21]</sup> suggested *BRAF* mutation as a negative predictive biomarker for anti-EGFR therapy, while Rowland *et al.*<sup>[22]</sup> concluded that there was insufficient evidence to exclude the benefit of anti-EGFR therapy in patients with mutant *BRAF* mCRC<sup>[21,22]</sup>. Although, there has been no definitive conclusion, the patients with mutant *BRAF* tumor are unlikely to derive treatment benefit from anti-EGFR therapy.

### **Other biomarkers**

As one third of patients with wild-type *RAS* mCRC will not have objective response to first-line chemotherapy and anti-EGFR combined therapy. The additional potential biomarkers would help optimizing anti-EGFR therapy in mCRC.

PI3K-AKT-mTOR pathway is another key downstream signaling pathway of EGFR. Alterations of PIK3CA and PTEN were explored for its predictive value for anti-EGFR therapy in mCRC. In contrast to other *RAS* and *BRAF*, PIK3CA mutation and PTEN alterations are not mutually exclusive with *KRAS* exon 2 mutation. The prevalence of PIK3CA mutation was 4%-11% in patients with wild-type *KRAS* mCRC<sup>[19,23-27]</sup>. The prevalence of PTEN loss and mutation was 19%-58% and 7%-9%, respectively, in patients with wild-type *KRAS* mCRC<sup>[19,24-27]</sup>. For those patients with wild-type *KRAS* exon 2 mCRC, PIK3CA mutation and PTEN alterations were associated with poorer objective response rate and OS for anti-EGFR therapy in two metaanalyses<sup>[28,29]</sup>. However, there might be different predictive effects between different PIK3CA mutations and different techniques detecting PTEN alterations.

EGFR ligands, epiregulin (EREG) and amphiregulin (AREG) are overexpressed in CRC<sup>[30,31]</sup>. EREG and AREG expressions in mRNA, tumor protein and plasma protein levels are associated with poor prognosis in CRC<sup>[31-33]</sup>. *In vitro* studies demonstrated their autocrine activation and reduction of cetuximab effect in AREG and EREG gene silencing CRC cells<sup>[34,35]</sup>. These preclinical studies led to several retrospective analyses demonstrating the correlation of EREG and AREG with anti-EGFR benefit in mCRC<sup>[25,36,37]</sup>. Most studies demonstrated association between high AREG and EREG mRNA expression and better survival in patients with CRC receiving anti-EGFR. In a metaanalysis, these associations were still statistically significant only in patients with wild-type *KRAS* mCRC<sup>[38]</sup>. In a tumor analysis of CO.17 trial, the benefit of cetuximab was found only in high expression but not low expression of EREG mRNA in patients with wild-type *KRAS* mCRC<sup>[39]</sup>. This predictive effect was not shown in patients with mutant *KRAS* mCRC. Recently, a retrospective analysis of PICCOLO trial demonstrated similar predictive effect of AREG/EREG mRNA expression for benefits of the additional panitumumab to irinotecan in patients with wild-type *KRAS* mCRC<sup>[40]</sup>. However, there are limitations for utility of AREG/EREG expression as a predictive biomarker for anti-EGFR including no standard cut off for these continuous variables and modest concordance between their expression in primary and metastatic sites. Although, the plasma levels of AREG and EREG might overcome these limitations, there have been limited data of their predictive value in patients with mCRC.

## BIOMARKERS FOR IMMUNOTHERAPY

Recently, immune checkpoint inhibitors including anti-programmed cell death-1 (PD-1)/PD-ligand 1 (PD-L1) and anti-cytotoxic T lymphocyte associate protein-4 (anti-CTLA-4), have emerged as a new treatment paradigm in several cancers especially non-small cell lung cancer and melanoma. Given the low tumor mutational burden, colorectal cancer was considered as a “cold” tumor for immune response. Correspondingly, the early anti-PD-1 trial revealed almost no response in mCRC. However, subsequent studies demonstrated high response rate in patients with mCRC with high microsatellite instability (MSI-H).

### MSI

MSI-H or DNA mismatch repair (MMR) deficiency is found in 12%-15% of CRC. It is a hallmark phenotype of Lynch syndrome caused by germline mutation of MMR genes including *MLH1*, *MSH2*, *MSH6* and *PMS2*. However, 80% of MSI-H/ deficient MMR (dMMR) CRC are sporadic and caused by epigenetic inactivation of *MLH1*. The prevalences of MSI-H/MMR deficiency are 20%, 12% and 5% in CRC stage II, III and IV, respectively<sup>[41,42]</sup>. MSI-H/dMMR CRCs have distinct clinicopathologic features including right-sided location, poor differentiation, mucinous type and lymphocyte infiltration. To detect dMMR tumor, there are two diagnostic methods including MSI test and MMR protein immunohistochemical staining. MSI-H/dMMR is a good prognostic factor in early stage CRC, but patients with MSI-H/dMMR mCRC had poorer prognosis than patients with microsatellite stable (MSS) or proficient MMR (pMMR) mCRC<sup>[41,43,44]</sup>. Although MSI-H/dMMR may negatively predict the benefit of adjuvant fluorouracil in stage II CRC, the metanalysis showed no significant difference in chemotherapy response rates between MSI-H/dMMR and MSS/pMMR mCRC<sup>[45]</sup>.

Currently, MSI-H/dMMR is the only predictive biomarker for immunotherapy in patients with mCRC. In early reports of anti-PD1 in human tumors, it seemed to be inactive in mCRC. However, 1 out of 33 patients with mCRC obtained complete response<sup>[46,47]</sup>. Given the hypermutated state and lymphocytic infiltration features of MSI-H/dMMR tumors, this particular subgroup has been explored for anti-PD-1 in mCRC. In a phase II trial, the response rates were 40% and 0% in patients with dMMR and pMMR mCRC, respectively<sup>[48]</sup>. Recently, the combination of anti-PD-1 and anti-CTLA-4, nivolumab and ipilimumab, had shown more robust treatment outcomes including response rate of 55% and 1-yr PFS of 71% in previously treated patients with dMMR mCRC<sup>[49]</sup>. Anti-PD-1 is now a standard treatment option in patients with MSI-H/dMMR mCRC.

### PD-L1

PD-1 is expressed in activated mature T cells, while PD-L1 is constitutively expressed in tissue including tumor cells. Ligation of PD-1 and PD-L1 results in co-inhibitory signal repressing the T cell response. PD-L1 expression is currently the predictive and companion biomarker for anti-PD1 especially pembrolizumab in several cancers. In CRC, PD-L1 expression rate varied among several reports<sup>[50-52]</sup>. Although some reports showed higher PD-L1 expression in MSI-H CRC than MSS CRC<sup>[50-52]</sup>. This correlation was not consistent as reported by Drieser and colleagues in the largest study with 1,491 tumor samples<sup>[53]</sup>. These inconsistent findings in CRC might be largely caused by the variation of PD-L1 expression assessment including staining techniques, antibodies and scoring systems. Also there are some evidenced of temporal and spatial heterogeneity of PD-L1 expression in mCRC<sup>[54,55]</sup>. With these limitations and no evidence of its predictive effect for anti-PD1 therapy, there is still no clinical application of PD-L1 expression in patient with mCRC.

### Tumor mutational burden

Recently, tumor mutational burden has become the potential predictive factor for anti-PD1 therapy in several cancers. Generally, CRC is considered low tumor mutational burden (TMB) cancer, but MSI-H/dMMR CRC is constitutively high TMB tumor. As mentioned, MSI is very robust in predictive effect for anti-PD1 in mCRC. Moreover, MSI and MMR test is simple and less expensive than TMB assessment.

Therefore, the clinical application of TMB is quite limited in patients with mCRC. Although, early report of TMB assessment by comprehensive genomic profiling (CGP) demonstrated 20% high TMB in MSS CRC, there was only 1% high TMB in MSS CRC in the subsequent study<sup>[56,57]</sup>. However, there were different cut off levels for high TMB among these studies, clinical validation of these cut points in association with benefit of anti-PD1 needed to be defined.

## BIOMARKERS FOR EMERGING TARGETED THERAPY

Over the past decade, there have been several emerging molecular targeted therapies playing key role in cancer personalized therapy. In CRC, the most common genomic alterations including APC, RAS and TP53 are still undruggable. However, the current genomic profiling landscape leads to the discovery of uncommon targetable genomic alterations such as BRAF mutation, human epidermal growth factor receptor 2 (HER2) amplification and receptor tyrosine kinase (RTK) rearrangements in CRC.

### BRAF

RAF protein is a key protein kinase transducing signal from RAS to MEK in MAPK pathway. *BRAF* mutation was found in 10% of mCRC<sup>[16,58,59]</sup>. *BRAF* V600E is the most common mutation resulting in an increased activity of *BRAF*<sup>[60]</sup>. In patients with mutant *BRAF* CRC, there are distinct clinicopathological features including proximal tumor location, T4 tumor, poor differentiation and older age<sup>[61]</sup>. However, recently, there was a report of patients with mutant *BRAF* 594 or 596 with different features including rectal location, non-mucinous and no peritoneal metastasis. *BRAF* mutation is mutually exclusive with *KRAS* mutation but associated with MSI<sup>[59]</sup>.

*BRAF* mutation is a poor prognostic factor. The analysis of phase III first-line chemotherapy studies in patients with mCRC demonstrated significantly shorter PFS and OS in patients with mutant *BRAF* tumors compared to wild-type *BRAF* tumors<sup>[16,41]</sup>. However, this prognostic effect was demonstrated only in patients with proficient MMR tumors<sup>[41]</sup>.

In contrast to melanoma, *BRAF* targeted therapy did not have meaningful activity in patients with mutant *BRAF* mCRC<sup>[62-65]</sup>. The preclinical study showed feedback activation of EGFR as a resistance mechanism to BRAF inhibitor in mutant *BRAF* mCRC<sup>[66]</sup>. Recently, a phase II trial showed significant improvement of PFS from 2.0 to 4.4 months and a response rate from 4% to 16% by additional vemurafenib to cetuximab and irinotecan in patients with mutant *BRAF* V600 mCRC<sup>[67]</sup>. The addition of MEK inhibitor or PI3K inhibitor to the dual therapy seemed to show better response rates and PFS<sup>[68,69]</sup>. Therefore, *BRAF* mutation is now an emerging target for combined BRAF inhibitor therapy in patients with mCRC.

### HER2

HER2 is an EGFR family member activating MAPK and PI3K pathways. HER2 amplification/ overexpression is a prognostic and predictive marker for breast and gastric cancers. In CRC, it accounts for 2%-3% of mCRC, but up to 5% in wild-type *KRAS* tumors<sup>[70]</sup>. It is very rare in patients with mutant *RAS/BRAF* mCRC<sup>[70]</sup>. HER2 amplification/overexpression could be conventionally detected by in situ hybridization or immunohistochemical staining in tumor samples. The HER2 testing recommendation has been a consensus in breast and gastric cancers, but not in CRC. In a matched sample analysis, Lee and colleagues showed high discordance in positive results of FISH test between primary and metastatic sites<sup>[71]</sup>. There was also the possibility of changes in HER2 status after anti-EGFR therapy in patients with mCRC as shown in an analysis of plasma samples<sup>[72]</sup>. Moreover, there has been no consensus in diagnostic criteria for HER2 overexpression in CRC. The more stringent criteria including an intensely positive > 50% of cancer cells required for positivity by immunohistochemical staining was validated in HERACLES study, an anti-HER2 targeted study<sup>[73]</sup>. In this study, there was 30% response rate to lapatinib and trastuzumab in patients with HER2 overexpressed mCRC. Another trial evaluating efficacy of combination of pertuzumab and trastu-

zumab demonstrated 37.5% response rate in patients with HER2 overexpressed mCRC<sup>[74]</sup>. Though, HER2 is currently a predictive marker for the emerging anti-HER2 therapy in patients with mCRC. The optimal HER2 testing still needs to be defined in patients with mCRC.

### RTK rearrangement

RTK rearrangements play a critical role in carcinogenesis of several cancers. These uncommon alterations are the emerging targets for novel effective therapies as demonstrated in ALK positive non-small lung cancer. Based on a few reports, RTK rearrangements are rare with prevalence of 0.2%-2.4% in CRC. Pietrantonio *et al.*<sup>[75]</sup> had reported the clinicopathological analysis of 27 patients with *ALK*, *ROS1* and *NTRK* gene rearrangement mCRC. As compared with 319 patients with no rearrangements, *ALK*, *ROS1* and *NTRK* gene rearrangements were significantly more frequent in elderly patients with right sided, MSI-H and *RAS/RAF* wild type tumor. The study also demonstrated significantly shorter survival and poorer response to anti-EGFR in these patients with *ALK*, *ROS1* and *NTRK* gene rearrangement<sup>[75]</sup>. By detection of these alterations, the patients could have benefit from the corresponding targeted therapy such as entrectinib in patients with *CAD-ALK* gene and LMNA-ETK1 rearrangement<sup>[76-78]</sup>. However, given its rarity, the optimal diagnostic approach for these subgroups should be defined.

### CLINICAL SAMPLE FOR BIOMARKER ANALYSIS

With the advancement of genomic analysis techniques, tumor genomic profiling is currently feasible in plasma samples. Although, tumor sample is still the gold standard for tumor genomic profiling, plasma sample or “liquid biopsy” addresses some limitations of tumor biomarkers.

### Tumor biomarkers

Genomic profiling on tumor sample is the mainstay strategy for biomarker analysis in mCRC. However, there might be various available tumor sample sites, including primary tumor and metastatic sites. Primary tumor sample is more likely available in most patients with mCRC. The high concordance rates of genomic profiling of 90%-100% especially for *RAS* and *BRAF* mutations between primary and metastatic CRC samples were demonstrated in many studies<sup>[79-81]</sup>. Though, these high concordance rates have not been shown for uncommon genomic alteration, either primary or metastatic tumor was acceptable for genomic profiling in mCRC. For MSI/MMR, Haraldsdottir and colleagues showed perfect concordance of MMR status between primary tumor and metastasis, but a couple of reports showed up to 20% discordance rates<sup>[82-84]</sup>. Although, the spatial heterogeneity seems to be small in mCRC, the temporal heterogeneity, especially after treatment is potentially an issue for management in mCRC<sup>[85]</sup>. Therefore, the appropriate tumor samples for biomarker testing should be defined for the emerging genetic alterations and MSI/MMR tests, in order to maximize the benefit of molecular targeted agents and immune checkpoint inhibitors in mCRC.

### Liquid biopsy

Not only a non-invasive and reproducible technique, but also a “liquid biopsy” would be able to overcome the limitation of tumor analysis including spatial and temporal heterogeneity. Currently, it is based on detection of circulating tumor DNA (ctDNA) by advanced technologies such as BEAMing method, droplet digital PCR or next generation sequencing (NGS). Several studies confirmed high concordance rate, 90%-100%, in *BRAF* and *KRAS* mutations between tumor and liquid biopsy<sup>[86,87]</sup>. Two prospective studies demonstrated that early reduction in ctDNA during chemotherapy treatment could predict good responder in patients with mCRC<sup>[88,89]</sup>. Also, the emergence of *KRAS* mutation could be detected before radiographic disease progression during anti-EGFR therapy in patients with wild-type *KRAS* mCRC<sup>[85,90]</sup>. So, the liquid biopsy for disease monitoring during anti-EGFR therapy is potentially useful for clinical management of mCRC. However, the comprehensive gene analysis of ctDNA in mCRC is still not ready for clinical application, given the rarity of other than *RAS* targetable gene mutation and test sensitivity in mCRC.

**Table 1. Current application of biomarker in metastatic colorectal cancer**

Biomarker	Frequency	Clinical features	Predictive value	Current status	Site of tumor sample	Detection method*
<i>RAS</i> mutation	55%-60%	None	Resistance to anti-EGFR therapy	Standard biomarker	Primary tumor or metastasis	Single gene assay <b>Multiplex gene panel assay</b> CGP
<i>BRAF</i> mutation	10%	Right-sided location, poorly differentiation, elderly, Wild-type <i>RAS</i> , <i>MSI-H</i>	Resistance to anti-EGFR therapy Benefit of combination <i>BRAF</i> inhibitors	Standard biomarker	Primary tumor or metastasis	Single gene assay <b>Multiplex gene panel assay</b> CGP
MSI/MMR	5%	Right-sided location, poor differentiation, mucinous type, lymphocyte infiltration	Benefit of immune checkpoint inhibitors	Standard biomarker	No recommendation	<b>MSI test</b> <b>IHC</b> CGP
Other rare genetic alterations						
HER2 amplification	2%-3%	None	Benefit of anti-HER2 therapy	Optional	No recommendation	<b>IHC</b> <b>FISH</b> CGP
RTK rearrangement	0.2%-2.4%	Right-sided location, elderly, <i>MSI-H</i> , wild-type <i>RAS</i> / <i>RAF</i>	Benefit of RTK inhibitors	Optional	No data	<b>FISH</b> <b>CGP</b>

\*Bold typing(s) is/are the preferred method(s). IHC: immunohistochemical staining; MSI: microsatellite instability; MSI-H: MSI-high; MMR: mismatch repair; EGFR: epidermal growth factor receptor; CGP: comprehensive genomic profiling; FISH: fluorescent in situ hybridization; HER2: human epidermal growth factor receptor 2; RTK: receptor tyrosine kinase

## MOLECULAR SUBTYPES OF COLORECTAL CANCER

The genomic profiling has been widely performed in several types of cancers including CRC. In 2015, the CRC Subtyping Consortium analyzed and coalesced six independent classification systems into four consensus molecular subtypes, CMS 1-4, based on 3,962 patient samples<sup>[91]</sup>. However, less than 10% of these samples were mCRC, resulting in limitation of its clinical application in mCRC. Although, patients with CMS 4 had the worst overall survival and relapse free survival, patients with CMS1 had worst survival after relapse, corresponding to the findings of *BRAF* mutation and *MSI-H* as the poor prognostic factors in mCRC. In contrast to CMS1, patients with CMS2 had better survival after relapse than other subtypes, reflecting the good prognosis of wild-type *RAS*/*BRAF* mCRC. As current strategy in governance of systemic therapy is largely dependent on driver gene alterations, the molecular subtype is still not yet applicable in management of patients with mCRC.

## COMPREHENSIVE GENOMIC PROFILING

With the advancement of molecular techniques such as NGS, the CGP is now available for clinical utility in management of patients with advanced cancer. Tumor CGP can provide insight into clinical relevant genetic alterations (CRGAs) guiding matched treatment selection for an individual patient. As described earlier, the current CRGAs in mCRC are *RAS* mutation, *BRAF* mutation and *MSI-H*, accounting for 75% of all mCRC. All these alterations might be already known in the majority of patients with mCRC by sequential testing. Currently, CGP is used for detecting other rare CRGAs such as *HER2* amplification, RTK rearrangement or other potential targets, and TMB assessment. However, this advantage of CGP is quite limited due to the rarity of these CRGAs and uncertain benefit of those matched therapeutic agents.

## CLINICAL APPLICATIONS OF BIOMARKERS IN MCRC

As described above, most molecular biomarkers are currently used for treatment selection. For untreated patients with mCRC, *RAS* and *BRAF* mutations are required as the initial test for consideration of anti-EGFR therapy. Although immune checkpoint inhibitors are not currently first-line therapy, *MSI/MMR* should also be included in those initial tests for a comprehensive treatment plan for each particular patient. Extended *RAS* mutation analysis including *KRAS* exon 2, 3, 4 and *NRAS* exon 2, 3, 4 is the standard test



for evaluation *RAS* status. In addition to anti-EGFR therapy consideration and prognostification, *BRAF* mutation is now the target for combination *BRAF* inhibitors. Other biomarkers such as *HER2*, *RTK* rearrangement or rare potential emerging targets were considered as beyond standard biomarkers. With the increasing number of required biomarkers in mCRC management, multiplex gene panel testing is now replacing single gene testing strategy. Of those patients accessible to matched molecular targeted therapy, especially for clinical trials, CGP might be the preferred testing method. Liquid biopsy is not yet clinical applicable in mCRC, but there is potential benefit of the detection of drug resistance and dynamic change of biomarker status. The current application of biomarkers in mCRC was summarized in [Table 1](#).

## DECLARATIONS

### Authors' contributions

Responsible for the paper, concept, design, literature search, and manuscript preparation, manuscript editing, manuscript revision: Tanasanvimon S

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The author declared that there are no conflicts of interest.

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Original Article

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# Altered energy metabolism and metabolic gene expression associated with increased metastatic capacity identified in MDA-MB-231 cell line variants

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## Abstract

**Aim:** Despite current advances in therapies and the gradual decline in breast cancer-related mortality, metastasis remains a major therapeutic challenge for treatment. Energy reprogramming is now recognized to be an important part of tumorigenic processes, but its relevance in metastatic dissemination has yet to be elucidated.

**Methods:** Using the MDA-MB-231HM.LNm5 cell line, a novel, highly metastatic variant line derived from TN human breast adenocarcinoma MDA-MB-231 line, alteration in growth and energy metabolisms associated with enhanced metastatic potential were described. Glycolysis and oxidative phosphorylation (OXPHOS) was characterized using the seahorse XF analyzer. Whole transcriptome sequencing (RNA-seq) and quantitative real-time PCR was used to ascertain expression differences in metabolic genes.

**Results:** We observed reduced proliferation, and an elevation of both glycolytic and OXPHOS metabolism in the



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highly metastatic daughter line. The elevated metabolic rate is only partially reflected by transcript levels of relevant metabolic regulators. Heightened mitochondrial respiration is potentially underpinned by increased expression mitochondrial electron transport chain components. However, increased glycolysis was not underpinned by up-regulation of metabolic genes encoding enzymes participating in glycolysis.

**Conclusion:** Our results indicate breast tumour cells with elevated metastatic propensity are more metabolic active. We also identified differentially expressed metabolic genes, such as IDH2, that may play a part in the metastatic process beyond energy reprogramming.

**Keywords:** Breast cancer, energy reprogramming, cancer metabolism, metastasis, RNA-seq

## INTRODUCTION

The majority of breast cancer-related deaths are not caused by the primary tumor itself, but are due to the results of metastasis to vital organs<sup>[1]</sup>. Although only a small percentage of patients are initially diagnosed with late stage or metastatic breast cancer, the 5-year survival for these patients is 25% compared with 99% for patients diagnosed with localized disease<sup>[2]</sup>. In addition, current prognostic markers are unable to accurately predict the risk of metastasis development and approximately 30% of patients first diagnosed with earlier-stage breast cancer will eventually develop recurrent metastatic disease<sup>[3]</sup>. Therefore, despite current advances in therapies and the gradual decline in breast cancer-related mortality<sup>[4]</sup>, the diagnosis and management of metastatic disease remains a major therapeutic challenge for breast cancer treatment.

The dysregulation of cellular energetics is now regarded as one of the hallmarks of cancer<sup>[5]</sup>. The metabolic phenomenon describing increased glycolytic capacity in cancer cells, known as “the Warburg effect”<sup>[6]</sup>, stimulated decades of research directed towards the characterization of the reprogramming of energy metabolism during cellular transformation and its role in tumor development. The Warburg effect emerged as just one component of global changes in energy metabolism occurring in both cancer cells and the tumor microenvironment<sup>[7,8]</sup>. Additionally, an increasing number of studies suggest that metabolic reprogramming plays an important role not only in the process of malignant transformation, but also in the growth and survival of tumor cells within a hostile environment, such as the often limited nutrient and oxygen supply in solid tumours<sup>[9-11]</sup>. However, despite the significant number of studies that investigated the metabolic programming of primary cancer cells, less is known about metabolic alterations in the context of metastatic disease, especially in breast cancer.

Comparison of breast cancer cell lines panel reveals that cell lines with molecular subtypes associated with more aggressive disease progression exhibit an overall increase in energy metabolic processes, including glycolysis and oxidative phosphorylation (OXPHOS)<sup>[12-15]</sup>. Studies using metastases derived from the same primary tumour reported more puzzling metabolic changes. In a xenograft model using circulating tumor cells from a breast cancer patient, a proteomic comparison between parental cells and cells that metastasized to the brain demonstrated up-regulation in enzymes involved in both glycolysis and mitochondrial respiration pathways<sup>[16]</sup>. Moreover, compared to primary tumour cells, circulating tumour cells derived from 4T1 mouse mammary tumors exhibited elevated expression of mitochondrial respiration pathway genes, but not glycolytic genes, while lung metastasis from the same primary tumour revealed modest metabolic change<sup>[17]</sup>. Consistent with this finding, increased OXPHOS, were observed with increased metastatic potential in several metastatic cell line variants derived from the same primary breast cancer<sup>[13,18]</sup>. These findings provide evidence that energy reprogramming may be an important feature of the complex process of breast cancer metastasis, but also raise the question of whether the metabolic profiles of metastatic cells vary depending on the stage of metastasis and site of distant metastasis.

To gain a better understanding of the metabolic changes underlying the process of breast cancer metastasis, we characterized a highly metastatic variant line of the commonly used triple-negative human breast ad-

enocarcinoma cell line MDA-MB-231<sup>[19]</sup>, and compared cellular and metabolic alterations. The MDA-MB-231HM.LNm5 cell line is a highly angiogenic and metastatic variant of the MDA-MB-231 cell line derived by *in vivo* passaging whereby spontaneous secondary lesions were isolated and expanded *ex vivo*<sup>[20-23]</sup>. We recently demonstrated that the metastatic ability of MDA-MB-231HM.LNm5 line is highly elevated compared to the parental MDA-MB-231 cells. In a metastasis model involving surgical resection of the primary tumour, NSG immune-deficient mice bearing the HM.LNm5 line exhibited primary tumour recurrence, as well as significant lung, liver, spleen, lymph and spine metastasis. By comparison, no metastatic lesions were detected in secondary organs of MDA-MB-231-innoculated mice<sup>[23]</sup>.

In this study, the metabolic profiles of MDA-MB-231HM.LNm5 were compared to the parental MDA-MB-231 cell line using the Extracellular Flux (XF) Analyzer thus enabling simultaneous measurement of the two major cellular energy-producing pathways, glycolysis and OXPHOS. We then used whole transcriptome sequencing (RNA-seq) and quantitative real-time PCR (RT-qPCR) to ascertain expression differences in metabolic genes that were associated with enhanced breast cancer metastatic ability.

## METHODS

### Cell culture

The MDA-MB-231 human breast adenocarcinoma cell line was purchased from ATCC<sup>[19]</sup>. MDA-MB-231HM cells<sup>[20,21]</sup> were kindly provided by ZM Shao and ZL Ou (Breast Cancer Institute, Fudan University, Shanghai, China). MDA-MB-231HM.LNm5 cells were derived as described below. All lines were maintained in phenol red - containing RPMI1640 (Invitrogen) supplemented with 10% (v/v) heat-inactivated fetal calf serum (FCS), 2 mmol/L L-glutamine, 1% (v/v) non-essential amino acids, 5% (v/v) sodium pyruvate, 100 U/mL penicillin, 15 mmol/L HEPES buffer, and 0.2% (v/v) sodium bicarbonate (Sigma-Aldrich). Cells were maintained at 37 °C in 5% CO<sub>2</sub> and passaged every 4-5 days.

### Generation of reporter gene tagged MDA-MB-231 variants

To facilitate optical imaging of tumors *in vivo*, both parental MDA-MB-231 and MDA-MB-231HM cells were transduced with retrovirus encoding tdTomato fluorescent protein, selected with Blasticidin S and bulk sorted for tdTomato expression by flow cytometry (FACS Aria, Beckton Dickinson), as previously described<sup>[24]</sup>. Both populations were also transduced with retrovirus encoding Firefly luciferase and selected using puromycin<sup>[24]</sup>. Parental MDA-MB-231 cells were additionally transduced with retrovirus encoding enhanced GFP (encoded by the pFBneoGFP plasmid, a kind gift from Hiroshi Nakagawa, University of Pennsylvania), selected using G418, and bulk sorted for GFP expression using flow cytometry (FACS Aria, Beckton Dickinson). MDA-MB-231HM.LNm5 cells were isolated from a spontaneous axillary lymph node metastasis that developed from a reporter gene tagged MDA-MB-231HM primary inguinal mammary tumour in a BALB/c-SCID mouse.

### Cellular proliferation

Cells seeded at 10<sup>5</sup> cells/cm<sup>2</sup> into 24-well plastic plates were allowed to adhere overnight and were then rendered quiescent by incubation in serum-free medium containing 0.25% (v/v) bovine serum albumin for 24 h before re-exposing to FCS (5%, 10%) for 48 h. Viable cells were identified by the trypan blue (0.06% w/v) exclusion<sup>[25]</sup> and enumerated (blinded) manually using a haemocytometer chamber.

### Cellular proliferation using Resazurin (Alamar Blue)

Cell proliferation was also assessed using the Resazurin dye method by measuring reduction of the redox dye resazurin to resorufin<sup>[26]</sup>. Cells were seeded and treated as described in previous section, and were then incubated with Resazurin reagent containing 1.5% (w/v) Resazurin, 0.25 (w/v) methylene blue, 2.9% (w/v) potassium hexacyanoferrate (III) and 4.22% (w/v) potassium hexacyanoferrate (II) trihydrate for 2 h at 37 °C in 5% CO<sub>2</sub>. Resorufin formation was measured fluorometrically (excitation 570 nm; emission 620 nm) using a FlexStationII (Molecular Devices, Sunnyvale, CA, USA). Results are expressed in relative fluorescent units.

### Extracellular flux assay

The extracellular acidification rate (ECAR) and oxygen consumption rate (OCR) were measured in real-time using the XF24 extracellular flux bioanalyzer (Seahorse Bioscience, Agilent Technologies Australia, Mulgrave, Vic, Australia), as described previously<sup>[27]</sup>. Briefly, MDA-MB-231 or MDA-MB-231HM.LNm5 cells were seeded at a concentration of 100,000 cells/well in RPMI medium the day before the assay. One hour before the start of the metabolic profiling assay the medium was changed to XF Base medium (Seahorse Bioscience) supplemented with sodium pyruvate (1 mmol/L), D-glucose (25 mmol/L) and adjusted to pH7.4. To determine glycolytic parameters, ECAR was measured at baseline and after injection of oligomycin (5 µmol/L) [Supplementary Figure 1A]. To determine respiration parameters, OCR was measured at baseline and after injection of Oligomycin (5 µmol/L), [carbonyl cyanide-4 (trifluoromethoxy) phenylhydrazine (FCCP), 1 µmol/L] and a combination of antimycin A and rotenone (2.5 µmol/L each). Parameters of mitochondrial respiration were measured according to the XF cell Mito Stress test user manual [Supplementary Figure 2A].

### RT-qPCR

RNA samples were isolated from 3 or more independent experiments. Total RNA was isolated using TRIzol® reagent (Life technologies). RNA (100 ng) was reverse-transcribed using the High Capacity cDNA Reverse Transcription Kit (Invitrogen, Mulgrave, Vic, Australia). Reactions of 5 µL total volume were performed using a Mastercycler® Pro (Eppendorf, Hamburg, Germany). cDNA (1 ng) was used for real-time PCR using iTaq™ universal SYBR® Green Supermix (Bio-Rad, Gladesville, NSW, Australia) and an ABI Prism 7900HT sequence detection system (Applied Biosystems), as described previously<sup>[22]</sup>. Gene expression was normalized to 18S ribosomal RNA using the  $2^{-\Delta Ct}$  method<sup>[28]</sup>. Specificity of the primer sets was confirmed by dissociation curve analysis. Primer sequences are listed in Supplementary Table 1.

### RNA-seq library preparation and sequencing

RNA was pooled from 9 individual cultures of independent culture passage of MDA-MB-231HM.LNm5 cell line and the parental MDA-MB-231 cell line. Total RNA was isolated using TRIzol® (as above). RNA-seq libraries were constructed from 500 ng total RNA using NEBNext Ultra RNA library prep kit for Illumina (#E7530) with NEBNext Poly(A)mRNA Magnetic Isolation Module (#E7490). Prior to library preparation, RNA was confirmed to be of high quality (RNA integrity number > 8) by Agilent Bioanalyzer 2100 analysis. Paired end 2 × 50 bp rapid sequencing was performed on an Illumina HiSeq 2500 (Melbourne Translational Genomic Platform, University of Melbourne). Raw data was filtered by removing reads with adaptor sequences, reads where the percentage of unknown bases is greater than 10%, and reads considered to be of low quality (where bases with quality ≤ 5 constitute greater than 50% of base reads) to obtain “clean reads”. All subsequent analyses are based on “clean reads” only.

### RNA-Seq data analysis

FASTQ files were first analysed using FASTQC software (<http://www.bioinformatics.babraham.ac.uk/projects/download.html>) before proceeding with an integrated sequence trimming and alignment step against the UCSC hg19 human reference genome downloaded from Illumina's iGenomes ([https://support.illumina.com/sequencing/sequencing\\_software/igenome.html](https://support.illumina.com/sequencing/sequencing_software/igenome.html)) using Rsubread package (v 1.20)<sup>[29]</sup>. Reads that were aligned to annotated coding regions of the genome were counted using the “featureCounts” feature from Rsubread<sup>[30]</sup>. These counts were subsequently normalized using the trimmed mean of M-value method<sup>[31]</sup> and transformed into counts per million (CPM) to describe gene expression level. As a single replicate per condition was used, we assigned a biological coefficient of variation of 0.3 to proceed with the pairwise comparison analyses for the detection of differentially expressed genes using EdgeR software<sup>[32]</sup>.

### Gene Ontology analysis and gene list extraction

A list of all 1,158 nuclear-encoded mitochondrial genes was obtained from the MitoCarta2.0 human inventory<sup>[33]</sup>. Genes associated with the processes of glycolysis (canonical glycolysis GO:0061621; glycolytic process GO:0006096; positive regulation of glycolytic process GO:0045821; negative regulation of glycolytic process

GO:0045820; regulation of glycolytic process GO:0006110) and tricarboxylic acid (TCA) cycle (GO:0006099) were extracted from the gene ontology consortium website using the AmiGO Gene Ontology browser (<http://amigo.geneontology.org/amigo>)<sup>[34]</sup>. Genes contained within the mitochondrial respiratory chain complexes [The Hugo Gene Nomenclature Committee (HGNC) family ID: 639; Complex I GO:006120; complex II GO:006121; complex III GO:006122; complex IV GO:006123; complex V GO:006124] as well as mitochondrial respiratory chain complex assembly factors (HGNC family ID:645) were extracted from HGNC data base (<http://www.genenames.org>) under the “gene family” browser<sup>[35]</sup>. Genes with CPM < 1 were excluded from analysis.

### Statistical analyses

All statistical analyses were conducted using Prism v6.0 software (Graph Pad, San Diego, CA, USA). Results are expressed at mean  $\pm$  SEM from  $n$  independent experiments (performed on separate days on cells from a different passage) and analysed as grouped data. Cell proliferation data are expressed as a percentage of unstimulated control cell number. Two-way ANOVA with repeated measures with Bonferroni's *post hoc* test was performed to ascertain statistical significance. For XF analyser profiles and qRT-PCR analysis, significance was determined by paired two-tailed Student's *t*-test.  $P < 0.05$  was considered a significant difference for all analyses.

## RESULTS

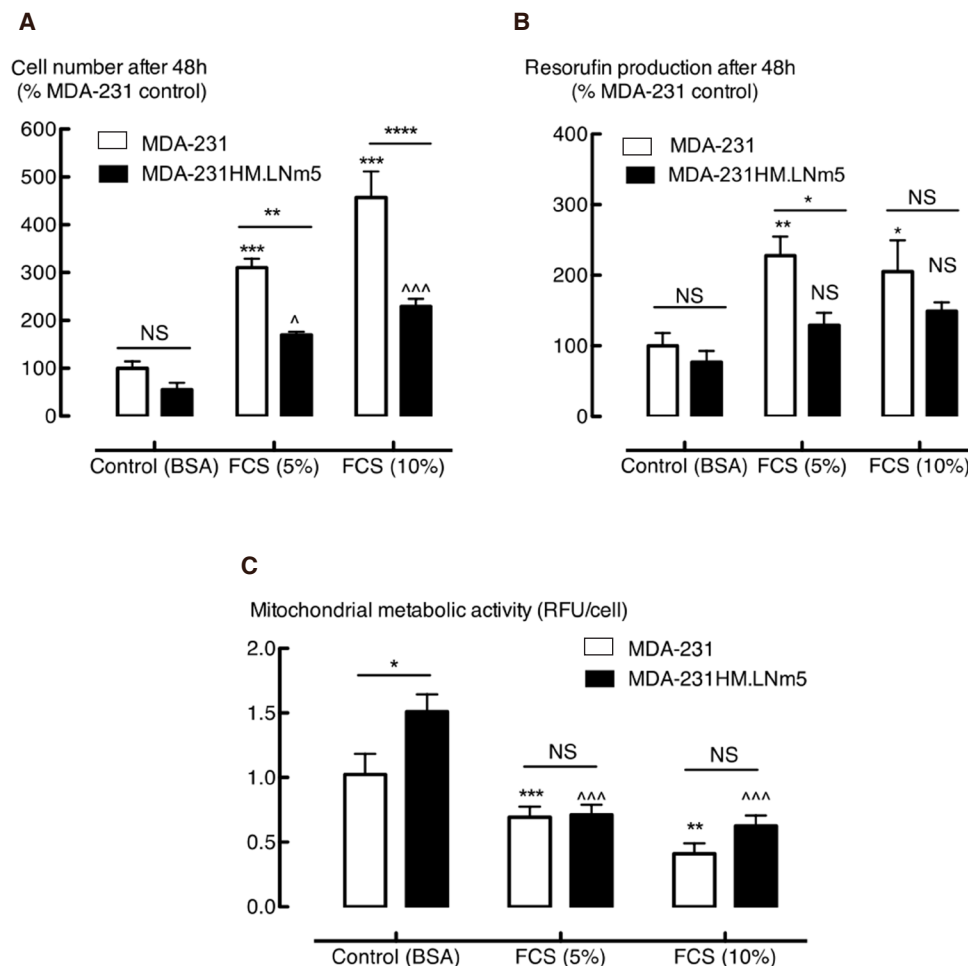
### The MDA-MB-231HM.LNm5 cell line shows slower serum-induced proliferation *in vitro* than the parental MDA-MB-231 cell line

MDA-MB-231HM.LNm5 cells exhibit lower migratory and invasive capabilities compared to the parental MDA-MB-231 cells, despite enhanced metastatic potential<sup>[22]</sup>. Here we see decreased MDA-MB-231HM.LNm5 cell proliferation compared to the parental cells, as measured by either cell enumeration or resazurin dye reduction. FCS (5% or 10%) increased the number of both parental MDA-MB-231 and MDA-MB-231HM.LNm5 cells [Figure 1A]. However, the number of cells resulting from 48 h of proliferation was significantly reduced in MDA-MB-231HM.LNm5 cells compared to parental cells [Figure 1A]. The commonly used Resazurin “proliferation” assay demonstrated a similar percentage increase in MDA-MB-231 cell number at 5% and 10% FCS [Figure 1B], whereas the FCS response of MDA-MB-231HM.LNm5 cell was barely detectable. The differences in the outcomes of experiments using the two different methodologies not only illustrate the limitation of Resazurin use for assessment of cell proliferation, but also emphasize that the indirect measurement of cell number using metabolically converted substrates without independent verification is prone to generate incorrect conclusions.

### The metastatic line MDA-MB-231HM.LNm5 is more metabolically active than the parental MDA-MB-231 cells line

Although the conversion of resazurin to resorufin is widely used as a “proliferation” assay, an estimate of mitochondrial metabolic activity could be extracted by calculating resorufin production per cell, as resazurin undergoes enzymatic reduction in the mitochondria to generate the fluorescent resorufin product<sup>[36]</sup>. Unstimulated MDA-MB-231HM.LNm5 cells have significantly higher basal mitochondrial activity compared to the parental cells, a difference that was not observed in the presence of serum [Figure 1C]. However, as both cytosolic and microsomal enzymes have the ability to reduce resazurin<sup>[37]</sup>, we sought a more precise method for quantification of the metabolic changes potentially associated with enhanced metastatic phenotype.

The XF bioanalyser facilitates real time measurement of the two major energy-producing pathways in the cell, namely glycolysis and OXPHOS. The ECAR, is a measure of glycolysis, and is determined by the net production and extrusion of protons into the culture medium as a result of the conversion of glucose to pyruvate and subsequently to lactate plus  $H^+$ . Simultaneously, OXPHOS is measured by calculating the OCR.

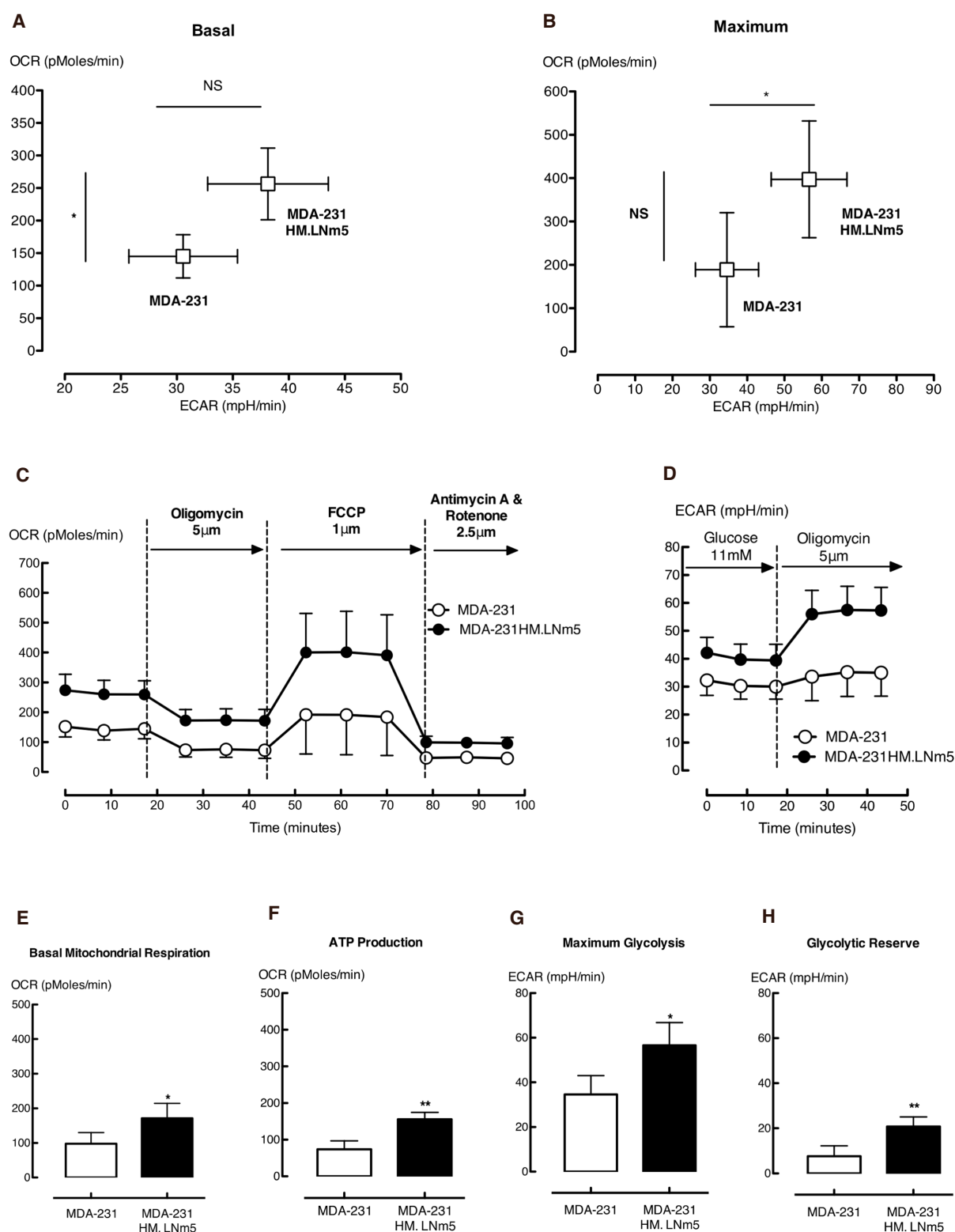


**Figure 1.** Proliferation of the highly metastatic MDA-MB-231HM.LNm5 (MDA-231HM.LNm5) and parental MDA-MB-231\_ATCC (MDA-231) cell lines. Cell growth in the presence and absence of fetal calf serum (FCS) (5% or 10%) after 24 h serum starvation was measured by enumeration of viable cells (trypan blue exclusion) (A) or the resazurin fluorometric method (B). Mitochondrial metabolic activity (C) was also determined [total relative fluorescent units (RFU) divided by the total number of cells]. Data is presented as mean  $\pm$  SEM,  $n = 4$ . Two-way ANOVA with Bonferroni's post hoc test was used to test for statistical significance \* $P < 0.05$ ; \*\* $P < 0.01$ ; \*\*\* $P < 0.001$  vs. MDA-231 control; ^ $P < 0.05$ ; ^^ $P < 0.001$  vs. MDA-231HM.LNm5 control. BSA: bovine serum albumin; NS: not significant

Measurement of OCR and ECAR baseline conditions in the absence of glutamine and lipids showed a near doubling of OXPHOS (OCR) and an approximately 25% increase in glycolysis (ECAR) in the MDA-MB-231HM.LNm5 cells compared to the parental MDA-MB-231 cells [Figure 2A]. Both cell lines were challenged to their maximum glycolytic and respiratory capacity by treatment with oligomycin and FCCP, respectively. Oligomycin inhibits ATP production by inhibiting the mitochondrial ATP synthase (complex 5). This subsequently triggers any cellular energy production that was occurring by respiration to shift to glycolysis, thus revealing the maximum glycolytic rate [Supplementary Figure 1A]. FCCP, on the other hand, is an uncoupling agent that disrupts the mitochondrial membrane potential and stimulates the respiratory chain to operate at maximum capacity [Supplementary Figure 2A]. Compared to the parental MDA-MB-231 cell line, MDA-MB-231HM.LNm5 cells showed higher maximum glycolytic and marginally higher respiratory capacity [Figure 2B].

To ensure that the metabolic alteration observed was independent of the exogenous fluorescent proteins and luciferase in the cells the assay was repeated in reporter gene-free MDA-MB-231 and MDA-MB-231HM.LNm5 lines. Basal OCR and ECAR were increased in the MDA-MB-231HM.LNm5 lines compared to the parent, albeit the differences were less striking. Maximum OCR was also higher in the MDA-MB-231HM.LNm5 lines, while maximum ECAR remained similar [Supplementary Figure 3].





**Figure 2.** The metabolic phenotype of the highly metastatic MDA-MB-231HM.LN5 (MDA-231HM.LN5) and parental MDA-MB-231 (MDA-231) cell lines. Baseline oxygen consumption rate (OCR) and extracellular acidification rate (ECAR) calculated in the presence of glucose were combined to generate the basal phenogram (A), while maximum ECAR (Oligomycin treated) and maximum OCR [carbonyl cyanide-4 (trifluoromethoxy) phenylhydrazine (FCCP) treated] were combined to generate the maximum phenogram (B). The glycolytic profile shows ECAR readings in the presence of glucose (11 mmol/L) from which the basal glycolytic rate was calculated (C) [Supplementary Figure 1]. Following injection of oligomycin (5  $\mu$ mol/L), both maximum glycolytic rate (G) and glycolytic reserve (H) could be determined. The respiration profiles of both cell lines (D) show OCR readings in the presence of glucose and following subsequent addition of oligomycin (5  $\mu$ mol/L), FCCP (1  $\mu$ mol/L) and finally antimycin plus rotenone (2.5  $\mu$ mol/L of each). This procedure allows the quantification of basal mitochondrial respiration (E) and ATP production (F), as well as maximal mitochondrial respiration, spare respiration capacity, proton leak and non-mitochondrial respiration [Supplementary Figure 2]. Data are presented as mean  $\pm$  SEM,  $n = 5-7$ . The student's  $t$ -test was used to test for statistical significance. NS: not significant; \* $P < 0.05$ , \*\* $P < 0.01$

### MDA-MB-231HM.LNm5 cells display enhanced glycolytic reserve, mitochondrial respiration and ATP synthesis

The increase in ECAR in the presence of oligomycin not only demonstrates the maximum glycolytic rate, but also shows the glycolytic reserve [Supplementary Figure 1A]. MDA-MB-231HM.LNm5 cells showed a larger increase in ECAR compared to the MDA-MB-231 parental line following oligomycin treatment [Figure 2D], revealing higher maximum glycolytic rates and reserves [Figure 2G and H]. Similarly, the difference between maximum OCR and basal OCR allows calculation of the spare respiratory capacity, which did not differ between the two cell lines [Supplementary Figure 2B].

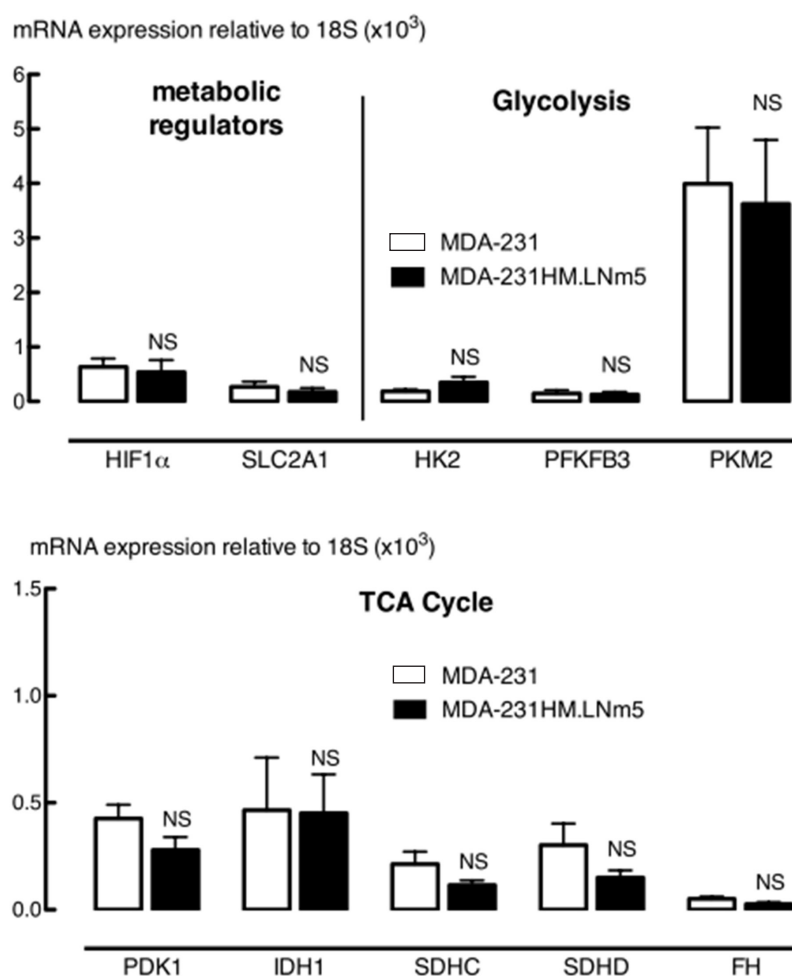
Both mitochondrial and non-mitochondrial respiration contributed to the basal and maximum OCR. The combination of rotenone, a complex I inhibitor, and antimycin A, a complex III inhibitor, shut down mitochondrial respiration completely, leaving respiration driven by processes outside the mitochondria only. MDA-MB-231HM.LNm5 cells showed significantly higher mitochondrial-dependent basal respiration [Figure 2E] and similar mitochondrial and non-mitochondrial -dependent maximum respiration rates compared to the parental cells [Supplementary Figure 2C and D].

The two processes that control basal mitochondrial respiration, ATP production and proton leak, can be probed with the blockade of ATP synthase using oligomycin. Measuring the reduction in OCR upon addition of oligomycin revealed significantly higher mitochondrial ATP synthesis in MDA-MB-231HM.LNm5 cells compared to parental MDA-MB-231 cells [Figure 2F], but unchanged proton leak-driven respiration [Supplementary Figure 2E].

### Gene expression analysis of energy metabolism pathways

In order to associate the observed metabolic changes with specific genetic or epigenetic alterations, we first selected several genes encoding enzymes that participate in glycolysis and the TCA cycle that were documented to contribute to altered metabolism in cancer cells<sup>[38]</sup>. RT-qPCR analysis of glucose transporter type 1 [solute carrier family 2 member 1 (*SLC2A1*)], hexokinase 2, fructose-2,6-bisphosphatase 3, muscle pyruvate kinase 2, pyruvate dehydrogenase kinase 1, cytosolic isocitrate dehydrogenase-1 (*IDH1*), succinate dehydrogenase complex subunits C and D and fumarate hydratase mRNA showed similar expression levels between the MDA-MB-231HM.LNm5 cells and parental cells [Figure 3].

To produce an unbiased analysis, the whole transcriptome of each cell line was then deep-sequenced using RNAseq, and the expression of genes involved in key pathways of energy metabolism was compared, including those influencing glycolysis and mitochondrial respiration. Gene expression level was expressed as CPM and the expression level of gene sets was compared by calculating the ratio between two cell lines using the MDA-MB-231 parental line as the denominator. Transcript per million was also compared and yields similar ratio (data not shown). The comparison of all mitochondrial genes showed a symmetrical distribution of expression around a log-fold change of 0, indicating no predominant direction of effect, although some genes were dysregulated between the two cell lines [Figure 4A]. Genes encoding enzymes directly involved in glycolysis were expressed at lower levels in MDA-MB-231HM.LNm5 cells compared to the parental cells [Figure 4B, Supplementary Table 2]. In particular, hexokinase domain containing 1 (*HKDC1*), encoding the hexokinase isoform HKDC1 which catalyzes the rate-limiting and obligatory first step of glucose metabolism<sup>[39]</sup>, was significantly down-regulated (Table 1  $\log_2FC = -6.64$ ). However, the majority of genes involved in regulating glycolytic processes showed unaltered expression between the two cell lines. The most differentially expressed genes were those that were down-regulated in metastatic cells [Figure 4B, Table 1, Supplementary Table 3], including MLX interacting protein-like (*MLXIPL*,  $\log_2FC = -6.73$ ), encoding a leucine zipper transcription factor of the Myc/Max/Mad superfamily, and *FBP1* ( $\log_2FC = -5.36$ ), encoding the gluconeogenesis regulatory enzyme fructose-1,6-bisphosphatase-1. Reduced expression of these genes in MDA-MB-231HM.LNm5 was confirmed by RT-qPCR [Figure 5].



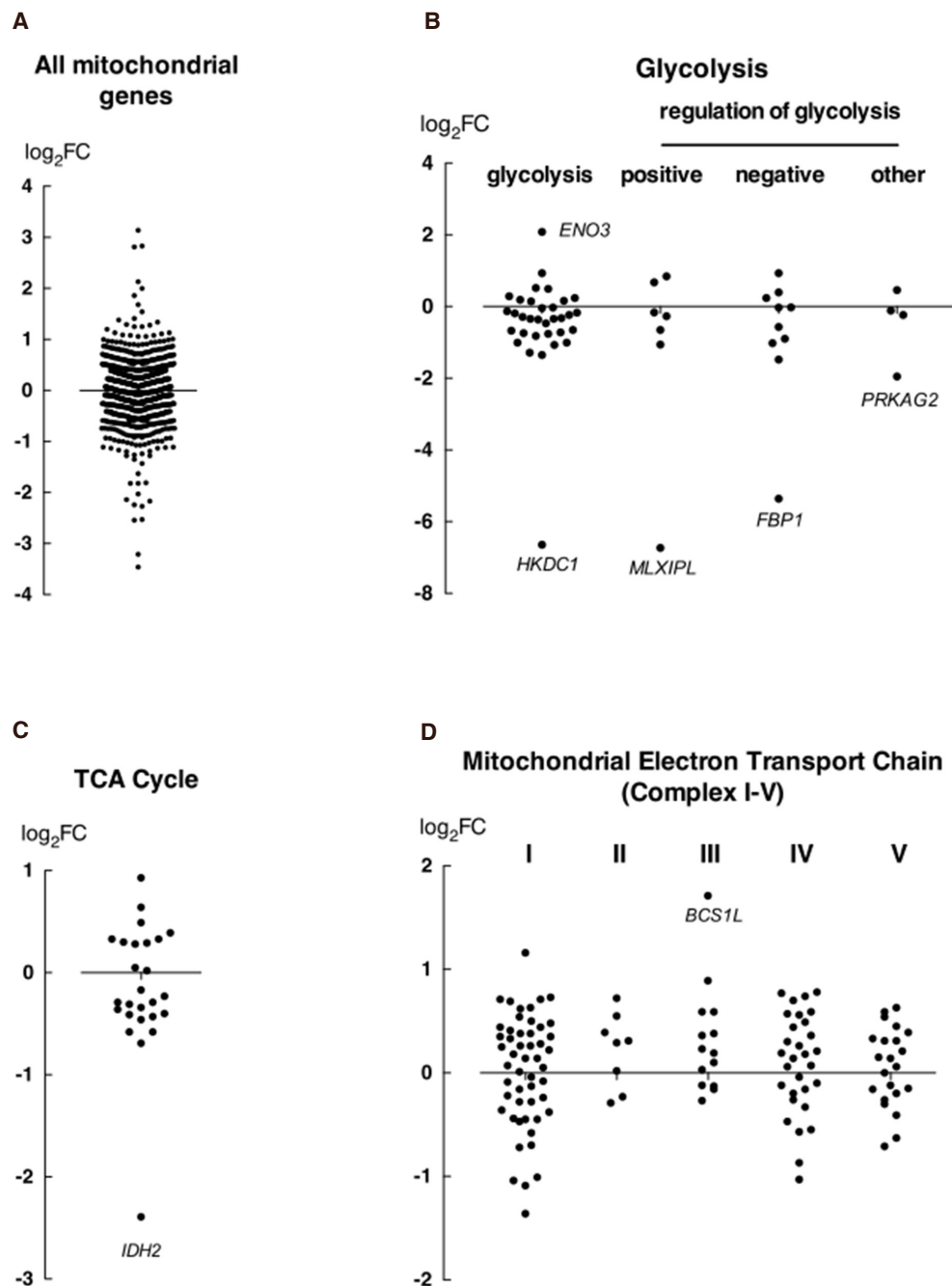
**Figure 3.** qRT-PCR analysis of metabolic regulatory genes, glycolysis and tricarboxylic acid (TCA) cycle genes in MDA-MB-231HM.LNm5 (MDA-231HM.LNm5) and parental MDA-MB-231 (MDA-231) cell lines. Expression of hypoxia inducible factor 1 alpha subunit (*HIF1α*), solute carrier family 2 member 1 (*SLC2A1*)/GLUT1, hexokinase 2 (*HK2*), fructose-2,6-biphosphatase 3 (*PFKFB3*), pyruvate kinase, muscle (*PKM2*), pyruvate dehydrogenase kinase 1 (*PDK1*), cytosolic isocitrate dehydrogenase-1 (*IDH1*), succinate dehydrogenase complex subunits C and D (*SDHC*, *SDHD*), and fumarate hydratase (*FH*) were measured by SYBR-green qRT-PCR relative to 18S rRNA levels. Results are presented as mean  $\pm$  SEM,  $n = 7-8$ . The student's *t*-test was used to test for statistical significance. Not significant (NS), compared to MDA-MB-231 cells

Expression of TCA cycle genes was similar between two cell lines with the exception of *IDH2* (mitochondrial isocitrate dehydrogenase), which was expressed at one-fifth the levels of the parental MDA-MB-231 cells (Figure 4C,  $\log_2 FC = -2.39$ , Supplementary Table 4). This down-regulation was confirmed by RT-qPCR [Figure 5].

The electron transport chain (ETC) in mitochondria is a key site for oxidative phosphorylation and is the major energy source used to produce ATP. The aforementioned XF mitochondrial stress test quantitatively probes this process. Expression levels of all five complexes were higher in metastatic daughter line compared to parental line, while genes belonging to complex II and III showed the greatest up-regulation. The expression of ubiquinol-cytochrome C reductase complex III chaperone (*BCS1L*), encoding a ubiquinol-cytochrome C reductase complex III chaperone, was the most strikingly elevated of all the ETC genes (Figure 4D,  $\log_2 FC = 1.71$ , Supplementary Table 5).

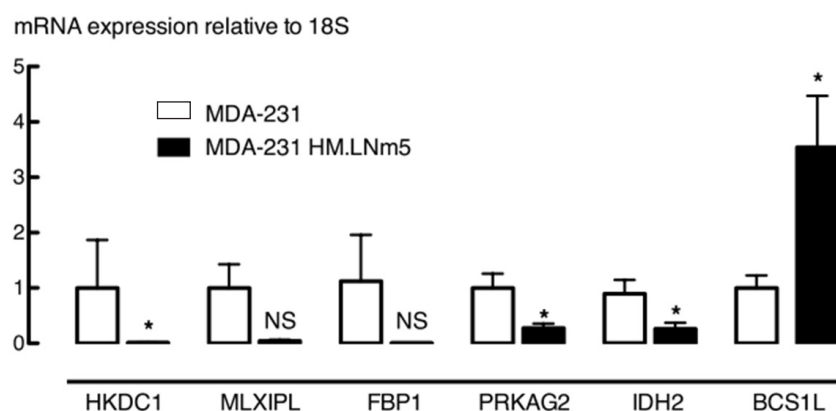
## DISCUSSION

MDA-MB-231 human breast cancer cells, originally derived from the pleural effusion of a patient with metastatic dissemination<sup>[19]</sup>, exhibit a gene expression signature predicting poor-prognosis<sup>[40]</sup>. Although this line



**Figure 4.** RNA-seq analysis of metabolic gene expression alteration between the MDA-MB-231HM.LNm5 and parental MDA-MB-231 cell lines. Expression level of all mitochondrial genes (MitoCarta 2.0) were compared (A), as well as genes involved in key processes of energy metabolism, including glycolysis (B) (glycolytic process: GO: 0006096; positive regulator of glycolytic process: GO: 0045821; negative regulator of glycolytic process: GO: 0045820; regulation of glycolytic process: GO: 0006110), tricarboxylic acid (TCA) cycle (C) (GO: 0006099), and the electron transport chain (D) (mitochondrial respiratory chain complexes: HGNC family ID: 639 & mitochondrial respiratory chain complex assembly factors HGNC family ID: 645). The  $\log_2FC$  (y-axis) is derived from counts per million (CPM) values for MDA-MB-231HM.LNm5 divided by CPM values for MDA-MB-231, where a positive FC value represents up-regulation in the MDA-MB-231HM.LNm5 cells and a negative value represents down-regulation. Genes with a CPM value of < 1 across both samples were not included. FC: fold change; *ENO3*: enolase 3; *HKDC1*: hexokinase domain containing 1; *MLXIPL*: MLX interacting protein-like; *FBP1*: fructose-1,6-bisphosphatase-1; *PRKAG2*: protein kinase, AMP-activated, gamma 2 non-catalytic subunit; *IDH2*: isocitrate dehydrogenase-2; *BCS1L*: ubiquinol-cytochrome C reductase complex III chaperone

has been used to study breast cancer metastasis, and despite deriving from tumors with metastatic capability in the original patient, the MDA-MB-231 cell line often displays poor spontaneous metastatic ability when used in immuno-compromised mice, including BALB/c nude and NOD.SCID strains<sup>[41]</sup>. The MDA-MB-



**Figure 5.** qRT-PCR analysis of candidate differentially expressed metabolic genes between MDA-MB-231HM.LNm5 (MDA-231HM.LNm5) and parental MDA-MB-231 (MDA-231) cell lines. Results are presented as mean  $\pm$  SEM,  $n = 9$ . Expression in MDA-231 was set to 1. The student's *t*-test was used to test for statistical significance. NS: not significant; \* $P < 0.05$  compared to MDA-MB-231 parental cells

231HM.LNm5 cell line, on the other hand, provides a much better model for the study of breast cancer metastasis *in vivo*. Compared to the parental MDA-MB-231 cells, this lymph node metastasis-derived sub-line not only shows aggressive spontaneous metastasis, but also mimics the organ tropism of metastatic human breast cancer, with spontaneous metastasis to lung, liver, spleen and sentinel lymph node<sup>[22,23]</sup>.

Real time bioenergetics assessment revealed an elevated glycolytic rate and oxidative phosphorylation in MDA-MB-231HM.LNm5 cells compared to the parental line, suggesting that the more metastatic line offers greater energy plasticity. This increased metabolic capacity reflects a composite of both energy demands for energy production used in macromolecule biosynthesis and metabolism and could be a result of an increased energy requirement accompanying the acquisition of metastatic potential. Interestingly, we showed that this enhanced metastatic ability was associated with reduced *in vitro* migratory and proliferative phenotype<sup>[22,23]</sup>.

Enhanced proliferative rate has long been considered as a hallmark of tumor cells, which is the basis for conventional chemotherapy<sup>[5]</sup>. Early molecular profiling studies of human breast tumors revealed that increases in proliferative gene signatures (for example genes directly associated with cell cycle progression) were associated with worse clinical outcome<sup>[42,43]</sup>. However, evidence also shows migratory, and thus invasive phenotype and proliferative phenotype are not expressed simultaneously in breast cancer. Indeed, breast cancer subpopulations with elevated metastatic activity are not more proliferative than their parental population<sup>[44]</sup>. Recent finding revealed MDA-MB-468 cells with reduced E-cadherin (inducing EMT) were more migratory, invasive and less proliferative<sup>[45]</sup>. Others showed positive correlation between bone marrow metastasis and the levels of circulating but non-proliferating breast cancer cells<sup>[46]</sup>. Furthermore, the correlation between breast cancer cell lines extracted from tumours of various disease stages and their growth rate indicate that proliferation decreases with disease progression<sup>[47]</sup>. These observations, together with our own, support the phenomenon known as the “migration/proliferation dichotomy”<sup>[48]</sup> or a “go or grow” mechanism<sup>[49]</sup>, where cell motion and proliferation appear to be mutually exclusive phenotypes.

The inverse relationship observed between cell proliferation and metastatic ability may be explained by the cancer stem cells theory, where quiescent/slowly dividing cells exhibit increased tumorigenic potential<sup>[50-52]</sup>. In addition to slow growth rate, these quiescent stem cells are also relatively resistant to current chemotherapy and radiotherapy treatments<sup>[53]</sup>, show increased metastatic ability through the epithelial-to-mesenchymal transition<sup>[54]</sup> and potentially explain the inter-tumoural heterogeneity and therapeutic failure seen in metastatic breast cancer<sup>[55]</sup>.



**Table 1. Differentially expressed metabolic genes in MDA-MB-231HM.LNm5 (MDA-231HM.LNm5) and parental MDA-MB-231 (MDA-231) cell lines, as detected by RNA-seq**

Metabolic process	Gene symbol	Gene name	Entrez gene ID	CPM		Log <sub>2</sub> FC
				MDA-231	MDA-231 HM.LNm5	
Canonical glycolysis (GO:0061621); glycolytic process (GO: 0006096)	<i>ENO3</i>	Enolase 3 (beta, muscle)	2027	12.8	31.9	2.08
	<i>BPGM</i>	2,3-bisphosphoglycerate mutase	669	18.5	9.5	-1.01
	<i>ENO2</i>	Enolase 2 (gamma, neuronal)	2026	204.2	115.1	-1.01
	<i>PGK1</i>	Phosphoglycerate kinase 1	5230	804.8	408.4	-1.07
	<i>PFKL</i>	Phosphofructokinase, liver	5211	255.6	117.1	-1.29
	<i>PPP2R5D</i>	Protein phosphatase 2, regulatory subunit B', delta	5528	214.5	88.7	-1.35
Positive regulator of glycolytic process (GO: 0045821)	<i>HKDC1</i>	Hexokinase domain containing 1	80201	19.5	3.2	-6.64
	<i>INSR</i>	Insulin receptor	3643	65.5	34.9	-1.06
	<i>MLXIPL</i>	MLX interacting protein-like	51085	3.8	0.5	-6.73
Negative regulator of glycolytic process (GO: 0045820)	<i>PPARA</i>	Peroxisome proliferator-activated receptor alpha	5465	45.3	17.7	-1.02
Regulation of glycolytic process (GO: 0006110)	<i>IER3</i>	Immediate early response 3	8870	590.1	278.6	-1.48
	<i>FBP1</i>	Fructose-1,6-bisphosphatase 1	2203	29.1	2.4	-5.36
	<i>PRKAG2</i>	Protein kinase, AMP-activated, gamma 2 non-catalytic subunit	51422	71.4	32.7	-1.95
Tricarboxylic acid cycle (TCA) (GO:0006099)	<i>IDH2</i>	Isocitrate dehydrogenase 2 (NADP+), mitochondrial	3418	30.7	6.5	-2.39
Mitochondrial Complex I (GO:006120) (HGNC family ID: 640, 645)	<i>NDUFA5</i>	NADH dehydrogenase (ubiquinone) complex I, assembly factor 5	79133	10.1	18.0	1.16
	<i>NDUFB1</i>	NADH dehydrogenase (ubiquinone) 1 beta subcomplex, 11, 17.3 kDa	54539	44.0	26.2	-1.01
	<i>NDUFB2</i>	NADH dehydrogenase (ubiquinone) 1 beta subcomplex, 2, 8 kDa	4708	73.4	36.7	-1.04
	<i>NDUFV3</i>	NADH dehydrogenase (ubiquinone) flavoprotein 3, 10 kDa	4731	37.8	19.9	-1.09
	<i>NDUFV2</i>	NADH dehydrogenase (ubiquinone) flavoprotein 2, 24 kDa	4729	18.2	11.1	-1.36
	<i>BCSL1</i>	BC1 (ubiquinol-cytochrome c reductase) synthesis-like	617	23.3	55.2	1.71
Mitochondrial Complex IV (GO:0006123) (HGNC family ID: 643, 645)	<i>COA6</i>	Cytochrome c oxidase assembly factor 6	388753	16.8	9.2	-1.03

The parental is used as the denominator when calculating fold change (log<sub>2</sub>FC). Genes with log<sub>2</sub>FC absolute value of 1 or more (2 FC) were considered differentially expressed. CPM: count per million; FC: fold change

Speculation can be made on other biological capabilities requiring higher cellular energy that contribute to increased metastatic potential, including the ability to resist cell death (especially in the circulation), induce angiogenesis, and evade immune destruction<sup>[5]</sup>. Emerging evidence suggests that some key cellular energetics regulators and processes can also be linked to the induction of angiogenesis<sup>[56]</sup>, the triggering of cancer cell death<sup>[57]</sup>, and shaping the immune micro-environment in the tumor stroma<sup>[58]</sup>. However, the nature of the relationship between these biological processes and cancer metabolism phenotype has been largely unexplored and warrants further study.

Our results show that the increased glycolysis in the MDA-MB-231HM.LNm5 cells was not underpinned by up-regulation of metabolic genes encoding enzymes participating in glycolysis. On the contrary, glycolytic genes were expressed at a comparatively lower level in the metastatic daughter line. Interestingly, reductions in HKDC1 and MLXIPL expression have been reported to be associated with reduced glucose uptake<sup>[59,60]</sup>, although we did not observe any change in expression levels of any of the major glucose transporters such as GLUT1 (SLC2A1). Protein post-translational modification (PTM) is a key mechanism of regulation in signal

transduction pathways. Studies have shown that up-regulated glycolysis can be influenced through diverse PTMs including phosphorylation, acetylation, glycosylation and oxidation of glycolytic enzymes as well as other signaling mediators (reviewed<sup>[61]</sup>). It is not unlikely that the observed elevation of glycolytic activity in the metastatic cells was the result of PTMs and gene expression were lowered as compensating mechanism. Further studies would need to be carried out to investigate whether proteomic changes are correlated with transcriptomic observations.

The XF mitochondrial stress test revealed that the elevated oxidative phosphorylation observed in the metastatic cells is independent of leaky mitochondria and is mainly explained by the enhanced production of ATP. The result further suggests a higher energy demand in the metastatic MDA-MB-231HM.LNm5 line compared to the parental line. Additionally, we found increased expression of all five complexes of the mitochondrial electron transport chain, which are the mediators of oxidative phosphorylation. Although this elevation was modest in magnitude, it may be sufficient to shift the entire metabolic profile of the cells.

In addition to the XF analyzer, metabolic status could also be measured by a variety of assays such as direct measurements of various metabolic enzymes, substrates, or ATP as surrogates of total energy metabolism. Although these metabolic assays each have their limitations and are mostly single-point measurements, it would have added valuable verification of our XF observation.

IDH2 expression was significantly reduced in MDA-MB-231HM.LNm5 cells while IDH1 levels remain unchanged. Interest in this family of enzymes in relation to cancer biology arose from reports of recurring mutations in *IDH1* and *IDH2* genes in several cancers including colorectal cancer and gliomas<sup>[62]</sup>. The functionality of these mutants and their impact on cancer progression has been the focus of many studies. Currently, inhibitors of mutant IDH1 and IDH2 are in Phase I/II clinical trials for both solid and myeloid tumors. In breast cancer, *IDH* gene mutations are detected at a frequency of less than 5%<sup>[63]</sup>. Compared to the substantial focus on mutant forms of IDH, little is known about the role of wild-type IDH1, and even less of wild-type IDH2, in cancer progression and metastasis. Hepatocellular carcinoma patients with reduced levels of IDH2 in tumors were at increased risk of metastatic progression and showed worse prognosis<sup>[64]</sup>. Similarly, in osteosarcoma, IDH2 levels were inversely correlated with pathological grade and metastasis<sup>[65]</sup>. The suggestion from these correlative observations, that wild-type IDH2 suppresses metastatic processes, is further supported by our data. In addition, our findings suggest that the mechanism by which IDH2 may inhibit metastasis is independent of cellular energy pathways.

Our transcriptomic findings warrant further studies that directly investigate the role of the abovementioned DEGs in metastatic behaviors of breast cancer cells. Knockdown and/or ectopic overexpression of genes of interest found in our study, such as BCS1L or IDH2, in the metastatic MDA-MB-231HM.LNm5 and/or non-metastatic MDA-MB-231 cells may reveal the relationship between these genes and metastatic phenotypes including metabolic reprogramming. Moreover, related animal experiments involving the manipulation of the expression of these genes of interest would further characterize their contribution in breast cancer growth and progression.

We acknowledge the limitation of having carried out the metabolic and transcriptomic studies in cultured cells. The clinical relevance of human cell line models has been questioned. Indeed, there is not always a linear correlation between *in vitro* proliferation or motility and spontaneous metastatic capacity *in vivo*, as other cellular phenotypes, influencing intravasation, extravasation and survival in the circulation (among others) also play a role. However, to determine precise ECAR and OCR measurement *in vivo* would be technically challenging. Future studies involving metabolic and transcriptomics analysis of tumour cells isolated *in situ* are required.

In conclusion, until recently, metabolic reprogramming in the context of metastatic dissemination has been largely unexplored in breast cancer. In the present study, a model of spontaneous metastatic breast cancer was used to identify metabolic alterations involved in breast cancer progression. The highly metastatic MDA-MB-231HM.LNm5 line displayed higher glycolytic activity and elevated oxidative phosphorylation compared to the parental MDA-MB-231 line, despite reduced proliferative ability. We also showed that this enhanced metabolic rate is only partially reflected by transcript levels of relevant metabolic regulators. Consideration of protein translation, and post-translational modifications, may provide further insight into the molecular alterations underlying the elevated glycolysis and oxidative phosphorylation in cells with higher metastatic capacity. Characterization of the metabolic changes correlated to enhance metastatic potential would deepen knowledge of metastatic mechanisms, and could facilitate the development of new strategies for therapeutic interventions and clinical management of patients with metastatic breast cancer.

## DECLARATIONS

### Authors' contributions

Derived the MDA-MB-231HM.LNm5 line, generated the reporter gene tagged MDA-MB-231 and MDA-MB-231HMLNm5 lines: Johnstone CN

Conducted the Seahorse XF assays and contributed to the analysis and interpretation of the data: Ryall JG

Conducted the RNA-seq library preparation: Keenan CR

Analysed the FASTQ files from RNA sequencing: López-Campos GH

Conducted the majority of the experiments in the study, performed data analysis and interpretation, and drafted the article: Tu Y

Contribution to conception and design of the study: Tu Y, Johnstone CN, Stewart AG

Major contributor to the conception and design of described work, contributed to writing and editing of the manuscript, and will be the guarantor for this article: Stewart AG

### Availability of data and materials

The datasets generated during and/or analysed during the current study are available from the corresponding author on reasonable request.

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### Conflicts of interest

All authors declared that there are no conflicts of interest.

### Ethical approval and consent to participate

Not applicable.

### Consent for publication

Not applicable.

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Review

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# Are memantine, methylphenidate and donepezil effective in sparing cognitive functioning after brain irradiation?

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## Abstract

One strategy to reduce neurocognitive deterioration in patients after brain irradiation is the use of neuroprotective medication. To generate up-to date knowledge regarding neuroprotective agents we performed a systematic review on the clinical effectiveness of three agents that were reported to have neuroprotective characteristics: memantine, methylphenidate and donepezil. The use of memantine after brain irradiation showed a delay in cognitive deterioration, although at 24 weeks this did not reach significance ( $P = 0.059$ ). Lack of significance is likely to be the result of the limited statistical power of 35% and memantine did show significant differences in secondary outcomes. The study on methylphenidate was not conclusive. Donepezil revealed significant differences in a few cognitive tests however no difference in global cognition was found. In addition, larger effects were observed in individuals with greater cognitive dysfunction prior to treatment.

**Keywords:** Memantine, donepezil, methylphenidate, brain irradiation, neuroprotection, whole brain irradiation, neuroprotective agent, lung cancer

## INTRODUCTION

Radiotherapy is an important treatment modality for patients who suffer from primary brain tumours or brain metastases. Adverse effects of brain irradiation include fatigue, nausea, cognitive decline, ataxia



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and alopecia. These side effects may be mild and transient, but can also be progressive and even persistent with structural brain damage on MRI scanning. Neurocognitive decline, especially memory dysfunction, is a major complaint following brain radiotherapy. In the United States, approximately 200,000 patients receive brain irradiation each year<sup>[1]</sup>. Due to both tumour progression and treatment, up to ninety percent of these patients experience cognitive dysfunction<sup>[1]</sup>. The treatment-related neurocognitive decline is poorly understood<sup>[2]</sup>, but causes a severe decline in the quality of life of these patients. Radiation injury is a multifactorial and complex event, characterized by vascular modification, inflammation, gliogenesis abnormalities and, when high-dose radiation is administered, even necrosis.

The incidence of radiation necrosis generally rises with an escalating radiation dose, fraction size and the administration of chemotherapy<sup>[3]</sup>. The precise mechanism of the neurotoxicity remains to be answered. However, two hypotheses (the vascular hypothesis and the glial hypothesis) explaining this neurocognitive decline have arisen<sup>[4]</sup>. The vascular hypothesis suggests that radiation induces vascular injury which leads to vascular inadequacy and so contributes to neurotoxicity. This neurotoxicity will eventually lead to neurocognitive decline. The degree of vascular inadequacy seems correlated to the extent of cognitive impairment<sup>[3]</sup>. The second hypothesis, the glial hypothesis, states that radiation therapy leads to a hold of gliogenesis because of a microglial inflammatory response induced by IL-6, inducing demyelinating necrosis. White matter networks are essential for cognitive function. By damaging these networks, as caused by demyelinating necrosis, cognitive impairment may occur<sup>[3]</sup>. However, in experimental animal studies, gliogenesis occurred to be fairly spared following radiation therapy, making this hypothesis less plausible. In contrast to this sparing of astrocytes and oligodendrocytes, a 97% reduction in “newborn” neurons was found in neurogenesis after brain irradiation<sup>[5]</sup>.

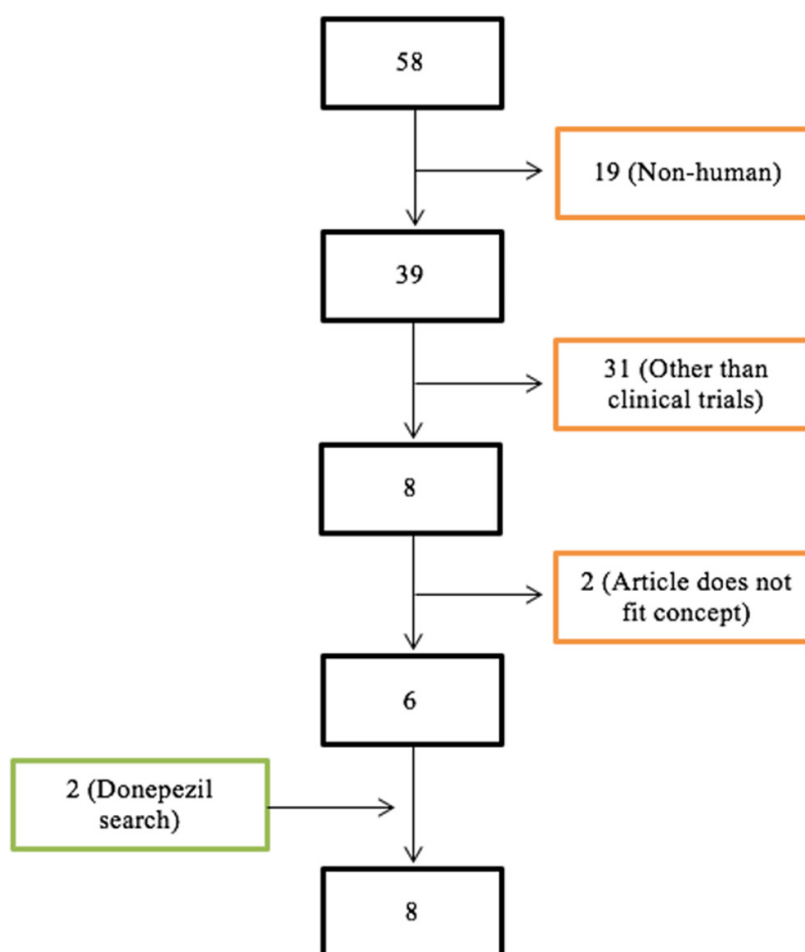
Whole brain radiotherapy (WBRT) or prophylactic cranial irradiation (PCI) exposes the whole cerebrum to a modest dose of radiation. Since the influence of brain irradiation on the long-term cognitive performance is a concern, several strategies, such as partial brain irradiation, hippocampal avoidance irradiation and the use of neuroprotective agents, aim to prevent or reduce radiation-induced cognitive deterioration<sup>[6]</sup>.

Memantine<sup>[7]</sup>, donepezil<sup>[8]</sup> and methylphenidate<sup>[9]</sup> have been widely studied in Alzheimer’s disease and influence cognition. Memantine was reported to be effective in the treatment of moderate to severe Alzheimer’s disease, whereas donepezil reduces the likelihood of progression of cognitive impairment at 12 months significantly ( $P = 0.004$ ). In addition, these agents are suggested to be neuroprotective<sup>[6,10,11]</sup>, thereby possibly limiting cognitive deterioration after brain radiotherapy. This led to the following research question: are memantine, methylphenidate and donepezil successful in sparing cognitive functioning after cerebral radiotherapy-treatment?

A literature study was performed to determine the effect of memantine, methylphenidate and donepezil on the neurocognitive function of patients after partial or whole-brain radiotherapy.

## LITERATURE SEARCH STRATEGY

A search in PubMed was conducted to evaluate the effect of memantine, methylphenidate and donepezil on cognition after brain-radiation therapy. In the [Supplementary Table 1](#) you will find the details on the search strategy. The search date was January 2018. The articles had to be in English language. This search provided 58 articles. After applying the filters “human subjects” and “clinical trials”, only eight articles were selected [[Figure 1](#)]. Each publication was carefully examined and identified to fit the research question based on the eligibility criteria. Including criteria for studies consisted of “human beings”, “cranial irradiation therapy”, “brain tumours or -metastases”, the use of “memantine”, “methylphenidate” or “donepezil” and “cognitive assessment”. In addition, the trials had to be clinical trials, written in the English language.



**Figure 1.** Flowchart of the PubMed search results

After the initial search, three additional searches were performed focussing on memantine, methylphenidate and donepezil. The search on donepezil revealed nine articles of which two were fitting the criteria. A total of eight articles were identified. The level of evidence of the individual studies was determined using Levels of Evidence by the Oxford Centre for Evidence-based Medicine<sup>[12]</sup>.

Data extraction was performed by studying the identified articles and interpreting the results focusing on the effect of memantine, methylphenidate and donepezil on sparing cognitive function after the cranial radiation therapy.

## MEMANTINE

Memantine is a non-competitive NMDA-receptor antagonist which blocks the effects of excessive levels of glutamate that could cause neuronal dysfunction, which is currently used for the treatment of Alzheimer's disease and vascular dementia. For memantine, one large phase III clinical trial has been performed in patients with brain metastases [Table 1]. Brown *et al.*<sup>[6]</sup> conducted a large placebo-controlled, double-blind, randomized trial of 508 subjects, to evaluate the potential beneficial effects of memantine on cognition in patients receiving WBRT. Memantine was administered in a daily dose of 20 mg, within three days after the start of WBRT, and appeared to be well tolerated. The primary endpoint of the study, delayed recall Hopkins Verbal Learning Test - Revised (HVLTR) at 24 weeks, showed less decline however, this lacked statistical significance ( $P = 0.059$ ). At 8 weeks the memantine arm indicated benefit; the median decline

**Table 1. Detailed information on study- and tumour type, neurocognitive tests performed, radiation treatment and level of evidence of the eight articles identified**

Author + Year	Agent + dose	Study type + n	Tumour type	Neurocognitive tests	Radiation therapy + dose	Level of evidence
Brown <i>et al.</i> <sup>[6]</sup> , 2013	Memantine 20 mg/d	Phase III n = 508	Brain metastases	HVLT-R, COWA	WBRT 37.5 Gy (15 × 2.5 Gy)	1b
Rapp <i>et al.</i> <sup>[11]</sup> , 2015	Donepezil 5 mg/d & 10 mg/d	Phase III n = 198	Brain tumours	HVLT-R, mROCF, TMT, COWA, DST, GP-D	P/WBRT U 30 Gy	2b
Shaw <i>et al.</i> <sup>[15]</sup> , 2006	Donepezil 5 mg/d & 10 mg/d	Phase II n = 35	Brain tumours	MMSE, TMT, DST, mROCF, COWA, CVLT-2	P/WBRT (dose not specified)	4
Correa <i>et al.</i> <sup>[16]</sup> , 2016	Donepezil 5 mg/d & 10 mg/d	Pilot n = 24	Childhood brain tumours	DST, BTA, DST (WMS-III), TMT, HVLT-R, BVMT-L	RT/chemotherapy (dose not specified)	4
Castellino <i>et al.</i> <sup>[17]</sup> , 2012	Donepezil 5 or 10 mg/d	Pilot n = 13	Brain tumours	D-KEFS, WRAML-2, CPT, WISC-IV, Woodcock Reading Mastery Test	P/WBRT > 23.5 Gy	4
Jatoi <i>et al.</i> <sup>[18]</sup> , 2005	Donepezil 5 mg/d & 10 mg/d	Phase III n = 9	SCLC	MMSE, BDS	PCI (dose not specified)	4
Butler <i>et al.</i> <sup>[13]</sup> , 2007	Methylphenidate 5 mg/d & 10 mg/d & 15 mg/d	Phase III n = 68	Brain tumours and/or brain metastases	MMSE	P/WBRT U 25 Gy (10 × 1.8-3.0 Gy)	2b
Meyers <i>et al.</i> <sup>[14]</sup> , 1998	Methylphenidate 10 mg/d & 20 mg/d & 30 mg/d	Cohort n = 30	Brain tumours	DST, HVLT, COWA, TMT, grooved pegboard	RT (not specified)	4

SCLC: small cell lung cancer; WBRT: whole brain radiotherapy; P/WBRT: partial or whole brain radiotherapy; RT: radiotherapy; PCI: prophylactic cranial irradiation; HVLT-R: Hopkins Verbal Learning Test - Revised; TMT: trail making test; MMSE: Mini Mental Status Examination; BVMT: Brief Visuospatial Memory Test; WRAML-2: Wide Range Assessment of Memory and Learning scale 2; COWA: Controlled Oral Word Association Test; mROCF: modified Rey-Osterrieth complex figure; DST: digit span test; GP-D: grooved pegboard-dexterity; CVLT-2: California Verbal Learning Test-2; BTA: brief test of attention; D-KEFS: Delis-Kaplan Executive Function System; CPT: Conners Continuous Performance Test; WISC-IV: Wechsler Intelligence Scale for Children-Fourth Edition; BDS: Blessed Dementia Scale

was -0.36 in the memantine arm and -0.72 in the placebo arm ( $P = 0.069$ ). The time to cognitive decline, the rate of decline in memory using HVLT-R as well as executive function trail making test (TMT) part B and processing speed (TMT part A) were delayed favouring the memantine arm (HR 0.78, 95% CI: 0.62-0.99,  $P = 0.01$ ) as compared to the placebo. A 21% relative reduction was found in the probabilities of cognitive function failure at 24 weeks; the probability of cognitive function failure in the memantine arm was 53.8% whereas 64.9% was found in the placebo arm. Superior results were seen in the memantine arm for executive function at 8 ( $P = 0.008$ ) and 16 weeks ( $P = 0.0041$ ) and for processing speed ( $P = 0.0137$ ) and delayed recognition ( $P = 0.0149$ ) at 24 weeks. Moreover, time to cognitive decline was found to significantly favour the memantine arm. Lack of significance is likely to be the result of the limited statistical power of 35%, because of a high dropout rate due to tumour progression and/or death. However, the almost significant finding could be beneficial in the long term for patients. The authors stated that the potential beneficial effects of memantine on cognitive function after WBRT may be more likely in patients with better prognostic factors or in the patients that respond well to radiation therapy.

## METHYLPHENIDATE

Methylphenidate, mainly known as ritalin, was studied in clinical trials by both Butler *et al.*<sup>[13]</sup> and Meyers *et al.*<sup>[14]</sup>. Butler *et al.*<sup>[13]</sup> performed a double-blind, placebo-controlled randomized trial to determine the effects of methylphenidate (5-15 mg daily) on cognitive function in brain tumour patients receiving partial or WBRT to a dose of > 23.5 Gray (Gy). The investigators did not find an advantage for the use of methylphenidate before WBRT in patients with primary brain tumours or metastatic brain tumours using the Mini Mental Status Examination (MMSE). Meyers *et al.*<sup>[14]</sup> conducted a phase III trial on the effect of methylphenidate on

cognition using an extensive test battery including memory recall and recognition and the trail making test (TMT) after radiotherapy treatment. The delivered radiation dose was not specified and only 30 patients with primary brain tumours were included. Each receiving 10-30 mg of methylphenidate twice daily for as long as the duration of the study which was not specified. Unlike Butler *et al.*<sup>[13]</sup>, this study indicated a significant improved function in psychomotor speed, memory, visual-motor function, executive function, motor speed and dexterity.

## DONEPEZIL

Donepezil is an acetylcholinesterase inhibitor and is widely studied. Rapp *et al.*<sup>[11]</sup> performed a phase III placebo-controlled trial in 198 subjects to determine whether donepezil improves cognitive function in primary brain tumour patients and patients with brain metastases treated with partial brain irradiation or WBRT receiving  $\geq 30$  Gy. Patients received 5 mg of donepezil for six weeks and 10 mg of donepezil for 18 weeks after completing their course of radiation therapy (WBRT as well as partial brain irradiation). They found no difference in global cognition at 24 weeks. However, significant differences favouring donepezil were observed for recognition memory and motor speed and dexterity. The authors reported that the benefit from donepezil increased as the pre-treatment level of cognitive impairment increased. Shaw *et al.*<sup>[15]</sup> performed a phase II clinical trial to evaluate cognitive functioning in partial or WBRT for patients with brain tumour after a 24-week donepezil treatment. Like Rapp *et al.*<sup>[11]</sup> doses of 5 and 10 mg daily were used. This study showed significant improvement in the following cognitive domains; attention/concentration, verbal memory and figural memory with a favourable trend for donepezil for verbal fluency. However, no change in global cognitive function was found, which is in line with the findings of Rapp *et al.*<sup>[11]</sup>, Correa *et al.*<sup>[16]</sup> performed a pilot study including only 24 patients with brain tumours. Fifteen of these 24 patients received donepezil, after completion of therapy (80% RT with or without chemotherapy, 20% received chemotherapy only), in the same quantities as Rapp *et al.*<sup>[11]</sup> and Shaw *et al.*<sup>[15]</sup>. This pilot study showed a significant post-baseline improvement in some aspects of attention; longest digit span forward, graphomotor speed, digit symbol subtest and Brief Visuospatial Memory Test - Revised for delayed recall. Improvements in other measurements were not conclusive or significant. Another pilot study was carried out by Castellino *et al.*<sup>[17]</sup> to assess the toxicity and efficacy of donepezil in childhood brain tumour survivors. Thirteen children were enrolled into the study receiving a daily dose of 5 to 10 mg of donepezil (depending on the child's weight). The median time from radiation therapy to study enrolment was extremely long: 4.7 years. This long interval possibly influences the effect of the donepezil treatment on cognitive sparing after cranial irradiation. This study showed improved as well as non-improved outcomes. Memory measured with the Wide Range Assessment of Memory and Learning scale was improved and a small effect in number/letter memory was found. Attention and concentration showed only non-significant effects. Other outcome measures like letter fluency and sorting tasks did not show significant improvement. Lastly, Jatoi *et al.*<sup>[18]</sup> conducted a double blind, placebo-controlled trial to test how donepezil 5 mg/day (with dose escalation to 10 mg/day after one month), and vitamin E, would affect the cognitive function of small-cell lung cancer patients after completing PCI. However, this study only accrued nine out of the calculated 104 patients and no results were available.

## DISCUSSION

In this literature search, the neuroprotective effect of memantine, methylphenidate and donepezil was studied in patients with primary brain tumours, brain metastases or PCI treated with partial irradiation or WBRT. Memantine appeared to benefit cognitive outcomes after partial or WBRT, however the benefit did not reach significance at 24 weeks. Donepezil revealed significant differences in a few cognitive tests however the global cognition was not influenced. Methylphenidate showed indistinct results in the performed trials. Leaving the benefits of its use during brain irradiation unanswered. Overall, it is hard to conclude whether a possible neuroprotective agent we studied is effective in preserving cognitive function in patients receiving brain irradiation because of three reasons: in the reported studies, patient populations differ as



well as the radiation treatment they have received and the neurocognitive tests varied which makes the exact difference between the three agents hard to determine. Another difficulty is that disease regression or progression interferes with neurocognitive improvement or deterioration. Our literature study identified eight papers of three studied agents. For memantine the evidence for delaying neurocognitive decline found in a single randomized trial that examined the effect of memantine on cognition in patients with brain metastases treated with WBRT was not statistically significant, although there was a trend that approached significance ( $P = 0.059$ ; 35% statistical power). However, the secondary endpoints showed that memantine deferred the time to cognitive decline and also reduced the rate of this decline significantly. So on the long term, memantine could be beneficial for patients with brain tumours or brain metastases. Methylphenidate showed positive results for cognitive preservation in a small group of brain tumour patients undergoing brain irradiation. However, no advantage on MMSE was found in a double blind randomized trial between patients receiving methylphenidate and patients receiving placebo. Methylphenidate was studied in two clinical trials by Butler *et al.*<sup>[13]</sup> and Meyers *et al.*<sup>[14]</sup>. The results were conflicting and the endpoints of the trials were different. Butler *et al.*<sup>[13]</sup> used the MMSE whereas Meyers *et al.*<sup>[14]</sup> used multiple, more sensitive cognitive tests. Butler *et al.*<sup>[13]</sup> found no advantage for prophylactic use of methylphenidate using the MMSE. The study was prematurely closed because of slow accrual, a high dropout rate and an interim analysis which did not show an effect for methylphenidate. Meyers *et al.*<sup>[14]</sup> studied the use of methylphenidate on 30 brain tumour patients and found a significant improvement in the following cognitive domains; psychomotor speed, memory, visual-motor function, executive function, motor speed and dexterity. Besides the small sample size an important limitation of this study is the lack of long-term follow up. As a result, the observed differences could have been no more than just the result of chance findings. Rapp *et al.*<sup>[11]</sup> conducted a placebo-controlled clinical trial in 198 brain tumour patients on cognition after the use of donepezil at 24 weeks. The improvement in cognitive function using multiple well-validated cognitive test-batteries occurred in both the donepezil-group and in the placebo-group. This means that there is an anti-tumour effect due to the irradiation and therefore improvement in cognitive function in both treatment arms. This trial emphasises the importance of a placebo-controlled trial to answer the question of neuroprotection. Without a placebo control group, an effect of the anti-cancer treatment on neurocognition could not be distinguished from an improvement due to the neuroprotective effects of the studied drug. Lastly, since the study was carried out in two academic medical centres (Wake Forest University Baptist Medical Centre and MD Anderson Cancer Centre), geographic diversity of the study population was achieved.

In accordance to the study by Rapp *et al.*<sup>[11]</sup>, the study by Shaw *et al.*<sup>[15]</sup> showed significant improvement in attention/concentration, verbal memory and figural memory and a trend toward significance for verbal fluency. However, their study population was small and only MMSE was used. Unlike Rapp *et al.*<sup>[11]</sup>, the study by Shaw *et al.*<sup>[15]</sup> clearly lacked a control-group.

The pilot studies carried out by Correa *et al.*<sup>[16]</sup> and Castellino *et al.*<sup>[17]</sup> lacked a placebo-control group and both included extremely small study populations of respectively 24 and 13 subjects. The study by Castellino *et al.*<sup>[17]</sup> included only childhood subjects, aged 8-17 years. Jatoi *et al.*<sup>[18]</sup> reported on a prematurely stopped trial after only nine patients were included and results are not available.

There are several limitations to our study. There has been heterogeneity in the selected patients: patients with primary as well as secondary brain tumours have been included. Besides, the type of radiation therapy differed: four out of eight studies included patients receiving partial and WBRT whereas one study included patients receiving WBRT and one study included patients only treated with PCI. Correa *et al.*<sup>[16]</sup> even included patients receiving concurrent chemotherapy. Meyers *et al.*<sup>[14]</sup> did not specify the type of irradiation used. In addition, the delivered radiation dose was not specified in four trials. Furthermore, there is no consensus on the optimal neurocognitive test battery to be used. In some trials only the MMSE was used. The HVLT-test was most commonly used, but no standard test was applied to determine the

level of cognitive functioning. Also, neurocognitive decline may be due to tumour progression or based on neurotoxicity caused by the irradiation.

Besides, the drug administration started at different points in time. Most of the identified studies administered the study drugs after completion of the radiation therapy, but some studies administered the drug during the irradiation. In the trial by Castellino *et al.*<sup>[17]</sup> the median time from radiotherapy to enrolment of the study was 4.7 years (range 1.9-11.9 years) making it hard to determine whether donepezil would have any preventive effect on neurocognitive function. Another issue is the fact that a neuroprotective drug could interfere with the cytotoxic tumour effect of the irradiation if given concurrently.

A previously published review in 2014 by Attia *et al.*<sup>[19]</sup> analysed different treatment options for radiation-induced cognitive decline. We included two more recent articles on donepezil; Rapp *et al.*<sup>[11]</sup> (2015) and Correa *et al.*<sup>[16]</sup> (2016). Attia *et al.*<sup>[19]</sup> reported a statistically significant improvement after administration of donepezil in several cognitive domains as based on the trial by Shaw *et al.*<sup>[15]</sup>. These domains include verbal memory, working memory, visual-motor and psychomotor performance and executive functioning. Importantly, in the trial by Shaw *et al.*<sup>[15]</sup> (2006) no significant change was reported in global cognitive function or executive function.

The article on donepezil by Rapp *et al.*<sup>[11]</sup> is a randomized placebo-controlled clinical trial in 198 subjects. This study did not show a global improvement in cognitive function, but differences in a few cognitive tests were shown.

Several trials are ongoing at the time of this literature study. One of these trials (NCT03342443) is a large ( $n = 240$ ) randomized, double-blind, placebo-controlled trial carried out by the Sun Yat-Sen Memorial Hospital of Sun Yat-Sen University. This trial aims to determine the effect of memantine on cognitive function in patients with radiotherapy-related cognitive impairment due to head- and neck cancer. Another large ( $n = 510$ ) ongoing trial is the randomized phase III trial by NRG Oncology (NCT02360215). This trial aims to evaluate whether memantine and WBRT with or without hippocampal avoidance in patients with brain metastases can reduce neurocognitive decline.

## CONCLUSION

In conclusion, the results of this systematic review on neurocognitive preservation in patients undergoing brain irradiation with memantine, methylphenidate or donepezil showed heterogeneity in the selected patients, the neurocognitive test used and the radiation treatment. Valuable clinical placebo controlled trials on neurocognitive preservation in patients undergoing brain irradiation are sparse. The results of this systematic review showed some evidence for the use of memantine to delay cognitive decline in patients undergoing brain irradiation. The results for methylphenidate remain inconclusive. Donepezil did show benefit in some domains although the global cognition was not influenced. Results from two ongoing trials on memantine (NCT03342443 and NCT02360215) are to be awaited.

## DECLARATIONS

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All authors declared that there are no conflicts of interest.

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Not applicable.

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Not applicable.

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Original Article

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# Levels of chemical element contents in thyroid as potential biomarkers for cancer diagnosis (a preliminary study)

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## Abstract

**Aim:** Thyroid cancer is an internationally important health problem. The aim of this exploratory study was to evaluate whether significant changes in the thyroid tissue levels of Al, B, Ba, Br, Ca, Cl, Cu, Fe, I, K, Li, Mg, Mn, Na, P, S, Si, Sr, V, and Zn exist in the malignantly transformed thyroid.

**Methods:** Thyroid tissue levels of twenty chemical elements were prospectively evaluated in 41 patients with thyroid malignant tumors and 105 healthy inhabitants. Measurements were performed using a combination of non-destructive and destructive methods: instrumental neutron activation analysis and inductively coupled plasma atomic emission spectrometry, respectively. Tissue samples were divided into two portions. One was used for morphological study while the other was intended for trace element analysis.

**Results:** It was found that contents of Al, B, Br, Ca, Cl, Cu, K, Mg, Mn, Na, P, S, and Si were significantly higher (approximately 3.2, 4.6, 9.3, 1.8, 2.3, 3.6, 1.6, 1.6, 1.6, 1.2, 2.5, 1.1, and 2.8 times, respectively) while content of I lower (nearly 26 times) in cancerous tissues than in normal tissues.

**Conclusion:** There are considerable changes in chemical element contents in the malignantly transformed tissue of thyroid.

**Keywords:** Thyroid malignant tumors, intact thyroid, chemical elements, biomarkers for cancer diagnosis, instrumental neutron activation analysis, inductively coupled plasma atomic emission spectrometry



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## INTRODUCTION

Thyroid cancer (TC) is the most common endocrine malignancy. TC incidence has dramatically increased in the recent decades<sup>[1]</sup>. During the same period no other cancer has increased as much as TC. With the worldwide increase in the incidence of TC, it has become the fifth most common cancer in women<sup>[2-4]</sup>. In some countries, the incidence of TC has increased extremely fast, and it has been the most common cancer over the last years<sup>[5]</sup>.

Although the etiology of TC is unknown, several risk factors including deficiency or excess of such micronutrient as I have been well identified<sup>[6-17]</sup>. It was also reported that the incidence of TC and mortality from this disease increases progressively with advancing age<sup>[18,19]</sup>. For example, a 37-fold increase in hazard ratio from age < 40 years to age > 70 years was shown in the study of 3,664 TC patients that received surgery and adjuvant treatment at Memorial Sloan Kettering Cancer Center from the years 1985 to 2010<sup>[19]</sup>.

Besides I involved in thyroid function, other trace elements have also essential physiological functions such as maintenance and regulation of cell function, gene regulation, activation or inhibition of enzymatic reactions, and regulation of membrane function. Essential or toxic (mutagenic, carcinogenic) properties of trace elements depend on tissue-specific need or tolerance, respectively<sup>[20]</sup>. Excessive accumulation or an imbalance of the trace elements may disturb the cell functions and may result in cellular degeneration, death or malignant transformation<sup>[20-22]</sup>.

In our previous study a significant positive correlation between age and some chemical element contents in the thyroid was observed<sup>[23-28]</sup>. It was concluded that an age-dependent excess of intra-thyroidal I and Zn concentration is probably one of the factors acting in both initiation and promotion stages of thyroid carcinogenesis<sup>[9,24,25]</sup>, as it was earlier shown by us for I in thyroid and for Zn in prostate gland<sup>[29-34]</sup>. Moreover, it seems fair to suppose that besides I and Zn, many other chemical elements also play a role in the pathophysiology of the thyroid.

This work had two aims. The first was to assess the Al, B, Ba, Br, Ca, Cl, Cu, Fe, I, K, Li, Mg, Mn, Na, P, S, Si, Sr, V, and Zn mass fraction contents in TC tissue using a combination of non-destructive and destructive methods: instrumental neutron activation analysis with high resolution spectrometry of short-lived radionuclides (INAA-SLR) and inductively coupled plasma atomic emission spectrometry, respectively. The second aim was to compare the levels of chemical elements in the malignant thyroid with those in intact (normal) gland of apparently healthy persons.

## METHODS

All patients suffering from TC ( $n = 41$ , mean age Mean  $\pm$  SD was  $46 \pm 15$  years, range 16-75) were hospitalized in the Head and Neck Department of the Medical Radiological Research Centre. Thick-needle puncture biopsy of suspicious nodules of the thyroid was performed for every patient, to permit morphological study of thyroid tissue at these sites and to estimate their chemical element contents. In cases of surgically operated patients with TC the specimens of resected materials were also used for morphological and chemical investigation. In all cases the diagnosis has been confirmed by clinical and morphological results obtained during studies of biopsy and resected materials. Histological conclusions for malignant tumors were: 25 papillary adenocarcinomas, 8 follicular adenocarcinomas, 7 solid carcinomas, and 1 reticulosarcoma.

Normal thyroids for the control group samples were removed at necropsy from 105 deceased (mean age  $44 \pm 21$  years, range 2-87), who had died suddenly. Samples were obtained within 48 h after a sudden death. The majority of deaths were due to trauma. A histological examination in the control group was used to control the age norm conformity, as well as to confirm the absence of micro-nodules and latent cancer.



All tissue samples were divided into two portions using a titanium scalpel<sup>[35]</sup>. One was used for morphological study while the other was intended for chemical element analysis. After the samples intended for chemical element analysis were weighed, they were freeze-dried and homogenized<sup>[36]</sup>.

The pounded samples weighing about 5-10 mg (for biopsy) and 100 mg (for resected materials) were used for chemical element measurement by INAA-SLR. The samples for INAA-SLR were sealed separately in thin polyethylene films washed beforehand with acetone and rectified alcohol. The sealed samples were placed in labeled polyethylene ampoules. The content of Br, Ca, Cl, I, K, Mg, Mn, and Na were determined by INAA-SLR using a horizontal channel equipped with the pneumatic rabbit system of the water-water-reactor-special research nuclear reactor (Branch of Karpov Institute, Obninsk). Thyroid samples irradiated by neutrons were measured using a gamma spectrometer. The gamma spectrometer included the 98 cm<sup>3</sup> Ge(Li) detector with on-line computer-based multichannel analyzer system (NUC 8100, Hungary) and provided a resolution of 1.9 keV on the <sup>60</sup>Co 1332 keV line.

After INAA-SLR investigation the thyroid samples were taken out from the polyethylene ampoules and used for inductively coupled plasma-atomic emission spectrometry (ICP-AES). The samples were decomposed in autoclaves. For this 1.5 mL of concentrated HNO<sub>3</sub> (nitric acid at 65%, maximum of 0.0000005% Hg; GR, ISO, Merck, Darmstadt, Germany) and 0.3 mL of H<sub>2</sub>O<sub>2</sub> (pure for analysis) were added to each thyroid samples, which were placed in one-chamber autoclaves (Ancon-AT2, Ltd., Moscow, Russia) and then heated for 3 h at 160-200 °C. After autoclaving, they were cooled to room temperature and solutions from the decomposed samples were diluted with deionized water (up to 20 mL) and transferred to plastic measuring bottles. Simultaneously, the same procedure was performed in autoclaves without tissue samples (containing only HNO<sub>3</sub> + H<sub>2</sub>O<sub>2</sub> + deionized water), and the resultant solutions were used as control samples. Sample aliquots were used to determine the Al, B, Ba, Ca, Cu, Fe, K, Li, Mg, Mn, Na, P, S, Si, Sr, V, and Zn mass fractions by ICP-AES using the spectrometer ICAP-61 (Thermo Jarrell Ash, USA). The determination of the ChE content in aqueous solutions was made by the quantitative method using calibration solutions (High Purity Standards, USA) of 0.5 and 10 mg/L of each element. The calculations of the ChE content in the probe were carried out using software of a spectrometer (ThermoSPEC, version 4.1).

Information detailing the NAA-SLR and ICP-AES methods used and other details of the analysis were presented in our earlier publications concerning chemical element contents in human thyroid, scalp hair, and prostate<sup>[7,23,27,37-42]</sup>.

To determine contents of the elements by comparison with a known standard, biological synthetic standards (BSS) prepared from phenol-formaldehyde resins were used<sup>[43]</sup>. In addition to BSS, aliquots of commercial, chemically pure compounds were also used as standards. Ten sub-samples of certified reference material (CRM) International Atomic Energy Agency (IAEA) H-4 (animal muscle) and five sub-samples of CRM of the Institute of Nuclear Chemistry and Technology (INCT, Warszawa, Poland), INCT-SBF-4 Soya Bean Flour, INCT-TL-1 Tea Leaves, and INCT-MPH-2Mixed Polish Herbs were treated and analyzed in the same conditions as those for thyroid samples to estimate the precision and accuracy of results.

A dedicated computer program for INAA mode optimization was used<sup>[44]</sup>. All thyroid samples were prepared in duplicate, and mean values of chemical element contents were used. Mean values of chemical elements contents were used in final calculation for the Br, Fe, Rb, and Zn mass fractions measured by two methods. Using Microsoft Office Excel, a summary of the statistics, including, arithmetic mean, standard deviation, standard error of mean, minimum and maximum values, median, percentiles with 0.025 and 0.975 levels was calculated for chemical element contents. The difference in the results between two age groups was evaluated by the parametric Student's *t*-test and non-parametric Wilcoxon-Mann-Whitney *U*-test.

**Table 1. Instrumental neutron activation analysis with high resolution spectrometry of short-lived radionuclides data of chemical element contents in the IAEA H-4 (animal muscle) reference material compared to certified values (mg/kg on dry mass basis)**

Element	Certified values			This work results M ± SD
	Mean	95% confidence interval	Type	
Br	4.1	3.5-4.7	C	5.0 ± 0.9
Ca	188	163-213	C	238 ± 59
Cl	1890	1810-1970	C	1950 ± 230
K	15800	15300-16400	C	16200 ± 3800
Mg	1050	990-1110	C	1100 ± 190
Mn	0.52	0.48-0.55	N	0.55 ± 0.11
Na	2060	1930-2180	C	2190 ± 140

M: arithmetic mean; SD: standard deviation; C: certified values; N: non-certified values

**Table 2. Inductively coupled plasma-atomic emission spectrometry data of chemical element contents in certified reference materials (M ± SD, mg/kg on dry mass basis)**

Element	Soya Bean Flour (INCT-SBF-4)		Tea Leaves (INCT-TL-1)		Mixed Polish Herbs (INCT-MPH-2)	
	Certificate	This work result	Certificate	This work result	Certificate	This work result
Al	45.5 ± 3.7	37.1 ± 1.4	2290 ± 280	2248 ± 61	670 ± 111	485 ± 79
B	39.9 ± 4.0	34.5 ± 1.4	26 <sup>a</sup>	24.8 ± 1.2	-	28.8 ± 8.1
Ba	7.30 ± 0.23	7.38 ± 0.23	43.2 ± 3.9	44.7 ± 2.6	32.5 ± 2.5	32.2 ± 0.6
Ca	2467 ± 170	2737 ± 190	5820 ± 520	6296 ± 360	10800 ± 700	10250 ± 294
Cu	14.3 ± 0.5	14.2 ± 0.8	20.4 ± 1.5	19.7 ± 1.1	7.77 ± 0.53	8.28 ± 0.47
Fe	90.8 ± 4.0	80.5 ± 6.9	432 <sup>a</sup>	493 ± 39	460 <sup>a</sup>	459 ± 33
K	24230 ± 830	25230 ± 1090	17000 ± 1200	17810 ± 1320	19100 ± 1200	20280 ± 870
Li	-	0.0047 ± 0.0018	-	0.217 ± 0.034	-	0.574 ± 0.044
Mg	3005 ± 82	2983 ± 340	2240 ± 170	2415 ± 115	2920 ± 180	2955 ± 159
Mn	32.3 ± 1.1	30.0 ± 1.0	1570 ± 110	1628 ± 145	191 ± 12	197 ± 5
Na	-	10.2 ± 3.4	24.7 ± 3.2	24.2 ± 3.5	350 <sup>a</sup>	338 ± 17
P	6555 ± 355	6782 ± 248	1800 <sup>a</sup>	2457 ± 150	2500 <sup>a</sup>	3022 ± 481
S	4245 ± 471	4468 ± 529	2470 ± 250	2500 ± 230	2410 ± 140	2409 ± 159
Si	-	26.7 ± 4.8	-	325 ± 34	-	268 ± 64
Sr	9.32 ± 0.46	8.76 ± 0.21	20.8 ± 1.7	19.8 ± 1.0	37.6 ± 2.7	37.4 ± 2.1
V	-	≤ 0.22	2.0 ± 0.4	1.8 ± 0.2	0.95 ± 0.16	0.90 ± 0.04
Zn	52.3 ± 1.3	54.8 ± 6.6	34.7 ± 2.7	36.0 ± 3.7	33.5 ± 2.1	32.0 ± 6.1

M: arithmetic mean; SD: standard deviation; a: informative values

## RESULTS

[Table 1](#) depicts our data for Br, Ca, Cl, K, Mg, Mn, and Na mass fractions in ten sub-samples of CRM IAEA H-4 (animal muscle) and the certified values of this material.

[Table 2](#) presents our data for Al, B, Ba, Ca, Cu, Fe, K, Li, Mg, Mn, Na, P, S, Si, Sr, V, and Zn mass fractions in five sub-samples of INCT-SBF-4 Soya Bean Flour, INCT-TL-1 Tea Leaves and INCT-MPH-2 Mixed Polish Herbs CRMs and the certified (or informative) values of this material.

The comparison of our results for the Ca, K, Mg, Mn, and Na mass fractions (mg/kg, dry mass basis) in the normal human thyroid obtained by both INAA-SLR and ICP-AES methods is shown in [Table 3](#).

[Table 4](#) presents certain statistical parameters (arithmetic mean, standard deviation, standard error of mean, minimal and maximal values, median, percentiles with 0.025 and 0.975 levels) of the Al, B, Ba, Br, Ca, Cl, Cu, Fe, I, K, Li, Mg, Mn, Na, P, S, Si, Sr, V, and Zn mass fraction in normal and cancerous thyroid tissue.

**Table 3. Comparison of the mean values ( $M \pm \text{SEM}$ ) of the chemical element mass fractions (mg/kg, on drymass basis) in the normal human thyroid (males and females combined) obtained by both instrumental neutron activation analysis with high resolution spectrometry of short-lived radionuclides and inductively coupled plasma-atomic emission spectrometry methods**

Element	INAA-SLR ( $M_1$ )	ICP-AES ( $M_2$ )	$\Delta$ , %
Ca	1692 $\pm$ 109	1633 $\pm$ 108	3.5
K	6071 $\pm$ 306	6764 $\pm$ 298	-11.4
Mg	285 $\pm$ 17	308 $\pm$ 17	-8.1
Mn	1.35 $\pm$ 0.07	1.21 $\pm$ 0.07	10.4
Na	6702 $\pm$ 178	7154 $\pm$ 201	-6.7

ICP-AES: inductively coupled plasma-atomic emission spectrometry; M: arithmetic mean; SEM: standard error of mean;  $\Delta = [(M_1 - M_2)/M_1] \times 100\%$

The comparison of our results with published data for Al, B, Ba, Br, Ca, Cl, Cu, Fe, I, K, Li, Mg, Mn, Na, P, S, Si, Sr, V, and Zn mass fraction in normal and cancerous thyroid<sup>[45-74]</sup> is shown in Table 5.

The ratios of means and the difference between mean values of Al, B, Ba, Br, Ca, Cl, Cu, Fe, I, K, Li, Mg, Mn, Na, P, S, Si, Sr, V, and Zn mass fractions in normal and cancerous thyroid are presented in Table 6.

## DISCUSSION

### Precision and accuracy of results

A good agreement of our results for the Al, B, Ba, Br, Ca, Cl, Cu, Fe, I, K, Mg, Mn, Na, P, S, Sr, V, and Zn mass fractions with the certified values of CRM IAEA H-4, INCT-SBF-4, INCT-TL-1, and INCT-MPH-2 [Tables 1 and 2] as well as the similarity of the means of the Ca, K, Mg, Mn, and Na mass fractions in the normal human thyroid determined by both INAA-SLR and ICP-AES methods [Table 3] demonstrates an acceptable precision and accuracy of the results obtained in the study and presented in Tables 4-6.

The mean values and all selected statistical parameters were calculated for twenty chemical elements (Al, B, Ba, Br, Ca, Cl, Cu, Fe, I, K, Li, Mg, Mn, Na, P, S, Si, Sr, V, and Zn) mass fractions [Table 4]. The mass fraction of Al, B, Ba, Br, Ca, Cl, Cu, Fe, I, K, Li, Mg, Mn, Na, P, S, Si, Sr, V, and Zn were measured in all, or a major portion of normal and cancerous tissue samples.

### Comparison with published data

The means obtained for Al, B, Ba, Br, Ca, Cl, Cu, Fe, I, K, Mg, Mn, Na, P, S, Si, Sr, V, and Zn mass fraction, as shown in Table 5, agree well with the medians of mean values reported by other research for the human thyroid, including samples received from persons who died from different non-thyroid diseases<sup>[45-65]</sup>. The mean obtained for Li is two orders of magnitude lower than the median of previously reported data. Moreover, it is outside the range of previously reported means. The mean obtained for V is one order of magnitude higher than the median of previously reported data, but it is inside the previously reported range of means. A number of values for chemical element mass fractions were not expressed on a dry mass basis by the authors of the cited references. Hence we calculated these values using published data for water 75%<sup>[75]</sup> and ash 4.16% on dry mass basis<sup>[76]</sup> contents in thyroid of adults.

In cancerous tissues [Table 3] our results were within the range of means published for Br, Ca, Cu, Fe, I, Mg, Mn, and Zn contents. The obtained means for V was approximately three orders of magnitude lower median of previously reported mean [Table 5]. The obtained mean for Cl was almost one order of magnitude higher than the only reported result and the mean for K was some higher than the median of previously reported means and also higher than the upper level of the range of these means [Table 5]. No published data referring Al, B, Ba, Li, Na, P, S, Si, and Sr contents of cancerous thyroid tissue were found.

The ranges of means of Al, B, Ba, Br, Ca, Cl, Cu, Fe, I, K, Li, Mg, Mn, Na, P, S, Si, Sr, V, and Zn levels reported in the literature for normal and for untreated cancerous thyroid vary widely [Table 5]. This can be

**Table 4. Some statistical parameters of Al, B, Ba, Br, Ca, Cl, Cu, Fe, I, K, Li, Mg, Mn, Na, P, S, Si, Sr, V, and Zn mass fraction (mg/kg, dry mass basis) in normal and cancerous thyroid**

Tissue	Element	M	SD	SEM	Min	Max	Median	P 0.025	P 0.975
Normal <i>n</i> = 105	Al	10.5	13.4	1.8	0.800	69.3	6.35	1.19	52.9
	B	0.476	0.434	0.058	0.200	2.30	0.300	0.200	1.73
	Ba	1.12	1.15	0.15	0.0480	5.00	0.680	0.0838	4.48
	Br	14.9	11.0	1.2	1.90	54.1	11.6	2.56	49.3
	Ca	1682	999	106	373	5582	1454	444	4183
	Cl	3400	1452	174	1030	6000	3470	1244	5869
	Cu	4.08	1.22	0.14	0.500	7.15	4.10	1.57	6.41
	Fe	223	95	10	52.0	489	210	72.8	432
	I	1841	1027	107	114	5061	1695	230	4232
	K	6418	2625	290	1914	15293	5948	2947	13285
	Li	0.0208	0.0155	0.0022	0.0015	0.0977	0.0178	0.0041	0.0487
	Mg	296	134	16	66.0	930	284	95.8	541
	Mn	1.28	0.56	0.07	0.470	4.04	1.15	0.537	2.23
	Na	6928	1730	175	3686	13453	6835	3974	10709
	P	4290	1578	207	496	8996	4221	1360	7323
	S	8259	2002	263	644	11377	8399	3662	11208
	Si	50.8	46.9	6.2	5.70	180	36.0	7.11	174
	Sr	3.81	2.93	0.34	0.100	12.6	2.90	0.365	11.3
	V	0.102	0.039	0.005	0.0200	0.250	0.100	0.0440	0.192
	Zn	94.8	39.6	4.2	7.10	215	88.5	34.9	196
Cancer <i>n</i> = 41	Al	33.0	25.5	7.1	4.50	96.5	21.3	5.70	85.6
	B	2.21	1.89	0.52	1.00	5.60	1.00	1.00	5.42
	Ba	1.42	1.30	0.35	0.220	4.09	0.945	0.259	3.93
	Br	139	203	36	6.20	802	50.2	7.75	802
	Ca	3013	2966	699	452	9768	1578	467	8938
	Cl	7699	2900	703	4214	14761	7216	4240	13619
	Cu	14.5	9.4	2.6	4.00	32.6	10.9	4.21	31.4
	Fe	255	168	27	60.6	880	217	74.6	673
	I	71.8	62.0	10.1	2.00	261	62.1	2.93	192
	K	10054	4018	877	1660	18814	9204	4073	17559
	Li	0.0314	0.0307	0.0090	0.0078	0.111	0.0182	0.0088	0.0995
	Mg	478	194	42	130	933	467	166	881
	Mn	2.01	1.34	0.29	0.100	5.95	1.61	0.250	5.23
	Na	8576	2433	531	4083	14048	8107	4901	12925
	P	10493	3238	866	5382	15403	9694	5767	15391
	S	9448	1605	429	7139	12591	9422	7211	12204
	Si	143	156	42	18.6	523	64.2	19.8	472
	Sr	6.26	7.61	1.59	0.93	30.8	3.00	0.985	25.0
	V	0.0904	0.0308	0.0100	0.0580	0.170	0.0870	0.0600	0.154
	Zn	96.9	80.0	12.6	28.7	375	69.8	36.3	374

M: arithmetic mean; SD: standard deviation; SEM: standard error of mean; Min: minimum value; Max: maximum value; P 0.025: percentile with 0.025 level; P 0.975: percentile with 0.975 level

explained by a dependence of element content on many factors, including the region of the thyroid, from which the sample was taken, age, gender, ethnicity, mass of the gland, and the cancer stage. Not all these factors were strictly controlled in cited studies. Another leading cause, in our opinion, of inter-observer variability can be attributed to the accuracy of the analytical techniques, sample preparation methods, and inability of taking uniform samples from the affected tissues. It was insufficient quality control of results in these studies. In many reported papers tissue samples were ashed or dried at high temperature for many hours. In other cases, thyroid samples were treated with solvents (distilled water, ethanol, formalin *etc.*). There is evidence that by using these methods some quantities of certain trace elements are lost as a result of this treatment, which concerns not only such volatile halogen as Br, but also other trace elements investigated in the study<sup>[36,77,78]</sup>.

**Table 5. Median, minimum and maximum value of means Al, B, Ba, Br, Ca, Cl, Cu, Fe, I, K, Li, Mg, Mn, Na, P, S, Si, Sr, V, and Zn contents in the normal and cancerous thyroid according to data from the literature in comparison with our results (mg/kg, dry mass basis)**

Tissue		Published data [Reference]		This work
Element	Median of means (n)*	Min of means M or M ± SD, (n)**	Max of means M or M ± SD, (n)**	Males and females M ± SD
Normal				
Al	33.6 (12)	0.33 (-) <sup>[45]</sup>	420 (25) <sup>[46]</sup>	10.5 ± 13.4
B	0.151 (2)	0.084 (3) <sup>[47]</sup>	0.46 (3) <sup>[47]</sup>	0.476 ± 0.434
Ba	0.67 (7)	0.0084 (83) <sup>[48]</sup>	≤ 5.0 (16) <sup>[49]</sup>	1.12 ± 1.15
Br	18.1 (11)	5.12 (44) <sup>[50]</sup>	284 ± 44 (14) <sup>[51]</sup>	16.3 ± 11.6
Ca	1600 (17)	840 ± 240 (10) <sup>[52]</sup>	3800 ± 320 (29) <sup>[52]</sup>	1663 ± 999
Cl	6800 (5)	804 ± 80 (4) <sup>[53]</sup>	8000 (-) <sup>[54]</sup>	3400 ± 1452
Cu	6.1 (57)	1.42 (120) <sup>[55]</sup>	220 ± 22 (10) <sup>[53]</sup>	3.93 ± 1.43
Fe	252 (21)	56 (120) <sup>[55]</sup>	2444 ± 700 (14) <sup>[51]</sup>	223 ± 95
I	1888 (95)	159 ± 8 (23) <sup>[56]</sup>	5772 ± 2708 (50) <sup>[57]</sup>	1841 ± 1027
K	4400 (17)	46.4 ± 4.8 (4) <sup>[53]</sup>	6090 (17) <sup>[49]</sup>	6418 ± 2625
Li	6.3 (2)	0.092 (-) <sup>[58]</sup>	12.6 (180) <sup>[59]</sup>	0.0208 ± 0.0154
Mg	390 (16)	3.5 (-) <sup>[45]</sup>	840 ± 400 (14) <sup>[60]</sup>	296 ± 134
Mn	1.82 (36)	0.44 ± 11 (12) <sup>[61]</sup>	69.2 ± 7.2 (4) <sup>[53]</sup>	1.28 ± 0.56
Na	8000 (9)	438 (-) <sup>[62]</sup>	10000 ± 5000 (11) <sup>[60]</sup>	6928 ± 1730
P	3200 (10)	16 (7) <sup>[63]</sup>	7520 (60) <sup>[50]</sup>	4290 ± 1578
S	11000 (3)	4000 (-) <sup>[54]</sup>	11800 (44) <sup>[50]</sup>	8259 ± 2002
Si	16.0 (3)	0.97 (-) <sup>[45]</sup>	143 ± 6 (40) <sup>[64]</sup>	50.8 ± 46.9
Sr	0.73 (9)	0.55 ± 0.26 (21) <sup>[47]</sup>	46.8 ± 4.8(4) <sup>[53]</sup>	3.81 ± 2.93
V	0.042 (6)	0.012 (2) <sup>[65]</sup>	18 ± 2 (4) <sup>[53]</sup>	0.102 ± 0.039
Zn	118 (51)	32 (120) <sup>[55]</sup>	820 ± 204 (14) <sup>[51]</sup>	94.8 ± 39.7
Cancerous				
Al	-	-	-	33.0 ± 25.5
B	-	-	-	2.21 ± 1.89
Ba	-	-	-	1.42 ± 1.30
Br	15.7 (4)	9.6 (1) <sup>[66]</sup>	160 ± 112 (3) <sup>[67]</sup>	139 ± 203
Ca	1572 (6)	390 (1) <sup>[68]</sup>	3544 (1) <sup>[66]</sup>	3013 ± 2966
Cl	940 (1)	940 ± 92 (4) <sup>[53]</sup>	940 ± 92 (4) <sup>[53]</sup>	7699 ± 2900
Cu	6.8 (11)	4.7 ± 1.8 (22) <sup>[69]</sup>	51.6 ± 5.2 (4) <sup>[53]</sup>	14.5 ± 9.4
Fe	316 (8)	69 ± 51 (3) <sup>[68]</sup>	5588 ± 556 (4) <sup>[53]</sup>	255 ± 168
I	78.8 (12)	< 23 ± 10 (8) <sup>[70]</sup>	800 (1) <sup>[71]</sup>	71.8 ± 62.0
K	6878 (4)	636 ± 64 (4) <sup>[54]</sup>	7900 (1) <sup>[72]</sup>	10054 ± 4018
Li	-	-	-	0.0314 ± 0.0307
Mg	320 (2)	316 ± 84 (45) <sup>[69]</sup>	544 ± 272 (6) <sup>[73]</sup>	478 ± 194
Mn	1.83 (4)	1.6 ± 0.8 (22) <sup>[69]</sup>	186 ± 18 (4) <sup>[53]</sup>	2.01 ± 1.34
Na	-	-	-	8576 ± 2433
P	-	-	-	10493 ± 3238
S	-	-	-	9448 ± 1605
Si	-	-	-	143 ± 156
Sr	-	-	-	6.26 ± 7.61
V	81.2 (1)	81.2 ± 8.4 (4) <sup>[53]</sup>	81.2 ± 8.4 (4) <sup>[53]</sup>	0.0904 ± 0.0308
Zn	112 (13)	48 ± 8 (5) <sup>[74]</sup>	494 ± 37 (2) <sup>[72]</sup>	96.9 ± 80.0

M; arithmetic mean; SD: standard deviation; (n)\*: number of all references; (n)\*\*: number of samples

### Effect of malignant transformation on chemical element contents

From Table 6, it is observed that in cancerous tissue the mass fractions of Al, B, Br, Ca, Cl, Cu, P, and Si are approximately 3, 5, 9, 2, 2, 4, 2, and 3 times, respectively, higher than the mass fractions of K, Mg, Mn, Na, and S, which are almost 57%, 61%, 57%, 24%, and 14%, respectively, higher than in normal tissues of the thyroid. In contrast, the mass fraction of I is almost 26 times lower. Thus, if we accept the chemical element contents in thyroid glands in the control group as a norm, we have to conclude that with a malignant transformation the levels of Al, B, Br, Ca, Cl, Cu, K, Mg, Mn, Na, P, and S in thyroid tissue significantly increased whereas the levels of I drastically decreased.



**Table 6. Differences between mean values ( $M \pm SEM$ ) of Al, B, Ba, Br, Ca, Cl, Cu, Fe, I, K, Li, Mg, Mn, Na, P, S, Si, Sr, V, and Zn mass fraction (mg/kg, dry mass basis) in normal and cancerous thyroid**

Element	Thyroid tissue				Ratio
	Norm $n = 105$	Cancer $n = 41$	Student's <i>t</i> -test <i>P</i>	<i>U</i> -test <i>P</i>	Cancer to norm
Al	10.5 $\pm$ 1.8	33.0 $\pm$ 7.1	<b>0.0083</b>	$\leq 0.01$	3.14
B	0.476 $\pm$ 0.058	2.21 $\pm$ 0.52	<b>0.0062</b>	$\leq 0.01$	4.64
Ba	1.12 $\pm$ 0.15	1.42 $\pm$ 0.35	0.446	$> 0.05$	1.27
Br	14.9 $\pm$ 1.2	139 $\pm$ 36	<b>0.0016</b>	$\leq 0.01$	9.33
Ca	1682 $\pm$ 106	3013 $\pm$ 699	0.076	$\leq 0.05$	1.79
Cl	3400 $\pm$ 174	7699 $\pm$ 703	<b>0.000013</b>	$\leq 0.01$	2.26
Cu	4.08 $\pm$ 0.14	14.5 $\pm$ 2.6	<b>0.0017</b>	$\leq 0.01$	3.55
Fe	223 $\pm$ 10	255 $\pm$ 27	0.278	$> 0.05$	1.14
I	1841 $\pm$ 107	71.8 $\pm$ 10.1	<b>0.0000000001</b>	$\leq 0.01$	0.039
K	6418 $\pm$ 290	10054 $\pm$ 877	<b>0.00060</b>	$\leq 0.01$	1.57
Li	0.0208 $\pm$ 0.0022	0.0314 $\pm$ 0.0090	0.265	$> 0.05$	1.51
Mg	296 $\pm$ 16	478 $\pm$ 42	<b>0.00043</b>	$\leq 0.01$	1.61
Mn	1.28 $\pm$ 0.07	2.01 $\pm$ 0.29	<b>0.024</b>	$\leq 0.01$	1.57
Na	6928 $\pm$ 175	8576 $\pm$ 531	<b>0.0069</b>	$\leq 0.01$	1.24
P	4290 $\pm$ 207	10493 $\pm$ 866	<b>0.0000054</b>	$\leq 0.01$	2.45
S	8259 $\pm$ 263	9448 $\pm$ 429	<b>0.027</b>	$\leq 0.01$	1.14
Si	50.8 $\pm$ 6.2	143 $\pm$ 42	<b>0.047</b>	$\leq 0.01$	2.81
Sr	3.81 $\pm$ 0.34	6.26 $\pm$ 1.59	0.144	$> 0.05$	1.64
V	0.102 $\pm$ 0.005	0.0904 $\pm$ 0.0100	0.305	$> 0.05$	0.89
Zn	94.8 $\pm$ 4.2	96.9 $\pm$ 12.6	0.877	$> 0.05$	1.02

M: arithmetic mean; SEM: standard error of mean; statistically significant values are in bold

### Role of chemical elements in malignant transformation of the thyroid

Characteristically, elevated or reduced levels of chemical elements observed in cancerous tissues are discussed in terms of their potential role in the initiation and promotion of TC. In other words, using the low or high levels of the chemical element in cancerous tissues researchers try to determine the carcinogenic role of the deficiency or excess of each chemical element in investigated organ. In our opinion, abnormal levels of many chemical elements in tumor could be the cause and also the effect of malignant transformation. From the results of such kind of studies, it is not always possible to decide whether the measured decrease or increase in chemical element level in pathologically altered tissue is the reason for alterations or vice versa.

#### Al

The trace element Al is not described as essential, because no biochemical function has been directly connected to it. At this stage of our knowledge, there is no doubt that Al overload impacts negatively on human health, including the thyroid function<sup>[79]</sup>.

#### B

Trace element B is known to influence the activity of many enzymes<sup>[80]</sup>. Numerous studies have demonstrated beneficial effects of B on human health, including anti-inflammatory stimulus - which reduces levels of inflammatory biomarkers, such as high-sensitivity C-reactive protein and tumor necrosis factor  $\alpha$ ; as well as raises levels of antioxidant enzymes, such as superoxide dismutase (SOD), catalase, and glutathione peroxidase<sup>[81]</sup>. Why B content in cancerous thyroid is higher than normal level and how an excess of B acts on thyroid are still to be cleared.

#### Br

This is one of the most abundant and ubiquitous of the recognized trace elements in the biosphere. Inorganic bromide is the ionic form of bromine which exerts therapeutic as well as toxic effects. An enhanced intake of bromide could interfere with the metabolism of iodine at the whole-body level. In the thyroid gland the biological behavior of bromide is more similar to the biological behavior of iodide<sup>[82]</sup>. In our previous studies,

we found a significant age-related increase of Br content in human thyroid<sup>[23,26-28]</sup>. Therefore, a goitrogenic and, probably, carcinogenic effect of excessive Br levels in the thyroid of old females was assumed. On the one hand, elevated levels of Br in TC tissues, observed in the present study, support this conclusion. But, on the other hand, bromide compounds, especially KBr, NaBr, and NH<sub>4</sub>Br are frequently used as sedatives in Russia<sup>[83]</sup>. It may be the reason for elevated levels of Br in specimens of patients with TC. Nevertheless, the accumulation of Br in neoplastic thyroid tissues could possibly be explored for diagnosis of TC.

### Ca

In addition to the elevated Br level, an excess in Ca mass fractions in thyroid tissue may contribute to harmful effects on the gland. Many reviews and numerous papers raise the concern about role of Ca in the prostate, breast, lung and other organ malignant transformation<sup>[84-94]</sup>.

### Cl

Cl is a ubiquitous, extracellular electrolyte essential to more than one metabolic pathway. Cl exists in the form of chloride in the human body. In the body, it is mostly present as sodium chloride. Therefore, as usual, there is a correlation between Na and Cl contents in tissues and fluids of human body. It is well known that Cl mass fractions in samples depend mainly on the extracellular water volume, including the blood volumes, in tissues<sup>[95]</sup>. Cancerous tissues are predominantly highly vascularized lesions. Thus, it is possible to speculate that thyroid malignant tumors are characterized by an increase of the mean value of the Cl mass fraction because the level of tumor vascularization is higher than that in normal thyroid tissue. Overall, the elevated levels of Cl in neoplastic thyroids could possibly be explored for diagnosis of TC.

### Cu

Cu is a ubiquitous element in the human body which plays many roles at different levels. Various Cu-enzymes (such as amine oxidase, ceruloplasmin, cytochrome-c oxidase, dopamine-monooxygenase, extracellular SOD, lysyl oxidase, peptidylglycineamidating monooxygenase, Cu/Zn SOD, and tyrosinase) mediate the effects of Cu deficiency or excess. Cu excess can have severe negative impacts. Cu generates oxygen radicals and many investigators have hypothesized that excess copper might cause cellular injury via an oxidative pathway, giving rise to enhanced lipid peroxidation, thiol oxidation, and, ultimately, DNA damage<sup>[96-98]</sup>. Thus, Cu accumulation in thyroid parenchyma with age may be involved in oxidative stress, dwindling gland function, and increasing risk of goiter or cancer<sup>[26,28]</sup>. The significantly elevated level of Cu in thyroid malignant tumors, observed in the present study, supports this speculation. However, an overall comprehension of Cu homeostasis and physiology, which is not yet acquired, is mandatory to establish the exact role of Cu in the thyroid malignant tumors etiology and metabolism. Anyway, the accumulation of Cu in neoplastic thyroids could possibly be explored for diagnosis of TC.

### I

Compared to other soft tissues, the human thyroid gland has higher levels of I, because this element plays an important role in its normal functions, through the production of thyroid hormones (thyroxine and triiodothyronine) which are essential for cellular oxidation, growth, reproduction, and the activity of the central and autonomic nervous system. Malignant transformation is accompanied by a loss of tissue-specific functional features, which leads to a significant reduction in I content associated with functional characteristics of the human thyroid tissue. Drastically low level of I content in neoplastic thyroids could possibly be explored for diagnosis of TC.

### K

An uncontrollable cell proliferation characterizes the malignant tumors. Therefore, morphological structures of TC tissue differ from the structure of normal thyroid parenchyma. Because K is mainly an intracellular electrolyte, an elevated level of K content in the TC tissue might reflect the increase of ratio “mass of

transformed thyroid cell - mass of follicular colloid”. Nevertheless, the accumulation of K in neoplastic thyroids could possibly be explored for diagnosis of TC.

### Mg

Mg is abundant in the human body. This element is essential for the functions of more than 300 enzymes (e.g., alkaline phosphatases, ATP-ases, phosphokinases, the oxidative phosphorylation pathway). It plays a crucial role in many cell functions such as energy metabolism, protein and DNA syntheses, and cytoskeleton activation. Moreover, Mg plays a central role in determining the clinical picture associated with thyroid disease<sup>[99]</sup>. Experimental data have shown that high doses of magnesium increase the activity of the thyroid gland<sup>[100]</sup>. Magnesium deficiency can influence bioavailability and tissue distribution of selenium which then appears diminished<sup>[101]</sup>. From these data, one can conclude that Mg is involved in the thyroid function. If so, significant reduction in Mg content can be associated with TC, because malignant transformation is accompanied by a loss of thyroid-specific functional features. However, it is well known that malignant tumors usually have higher Mg levels than normal tissues<sup>[102-107]</sup>, possibly caused by the “retention” of Mg by the tumor<sup>[108]</sup>, as a result of the high Mg requirement of growing cells. In addition, cultured proliferating cells have long been known to contain more magnesium than quiescent cells, and experimental conditions that decreased magnesium availability affected cell proliferation rate<sup>[109]</sup>. Thus, the elevated levels of Mg in neoplastic thyroids could possibly be explored for the diagnosis of TC.

### Mn

The trace element Mn is a cofactor for numerous enzymes, playing many functional roles in living organisms. The Mn-containing enzyme, Mn-SOD, is the principal antioxidant enzyme which neutralizes the toxic effects of reactive oxygen species (ROS). It has been speculated that Mn interferes with thyroid hormone binding, transport, and activity at the tissue level<sup>[110]</sup>. There is the opinion that Mn deficiencies in humans are rare and humans maintain stable tissue levels of this trace element<sup>[111]</sup>. It was reported that intracellular Mn content was positively correlated with Mn-SOD, suggesting that the intracellular Mn level is associated with Mn-SOD activity<sup>[112]</sup>. However, an overall comprehension of Mn homeostasis and physiology, which is not yet acquired, is mandatory to establish Mn exact role in the thyroid malignant tumors etiology and metabolism. Anyway, the accumulation of Mn in neoplastic thyroids could possibly be explored for diagnosis of TC.

### Na

The knowledge concerning ion regulation in many normal and abnormal cell processes has had a rapid development. It was found, among other regulations, that sodium-calcium exchange is associated with the cytoskeleton and the cell membrane. A hypothesis was eventually established that a wide variety of pathological phenomena ranging from acute cell death to chronic processes, such as neoplasia, have a common series of cellular reactions<sup>[113]</sup>. In accordance with this hypothesis, concentrations of sodium were found to be enhanced in human and animal neoplastic tissues<sup>[114,115]</sup>. Moreover, the hypothesis that physiological and biochemical changes are associated with proliferating malignant tumors may cause an increase in total tissue sodium concentration was tested with non-invasive, quantitative <sup>23</sup>Na magnetic resonance imaging in patients with benign and malignant breast tumors. It was shown that elevated Na concentrations in breast lesions appear to be a cellular-level indicator associated with malignancy<sup>[116]</sup>. In addition, Na is mainly an extracellular electrolyte and its elevated level in malignant tumors might be linked with a high tumor vascularization (see Chlorine). Anyway, it seems that the accumulation of Na is a generic property of malignant tumors.

### P

P is necessary for several, various biological roles in the signal transduction of cells and energy exchange of human body. About 80%-90% of phosphorus is founded in teeth and bones in the form of hydroxyapatite.

Calcium phosphates are one of the main constituents of mineral deposits in aortic wall and tissues<sup>[117]</sup>. Thus, the high P level in TC can be intimately linked with tumor calcification<sup>[86-96]</sup>.

S

Proteins contain between 3% and 6% of sulfur amino acids. Sulfur amino acids contribute substantially to the maintenance and integrity of the cellular systems by influencing the cellular redox state and the capacity to detoxify toxic compounds, free radicals and ROS<sup>[118]</sup>. ROS are generated during normal cellular activity and may exist in excess in some pathophysiological conditions, such as inflammation. Therefore exploring fundamental aspects of sulfur metabolism such as the antioxidant effects of sulfur-containing amino acids<sup>[119]</sup> may help elucidate the mechanism by which the S content increases in TC. Thus, it might be assumed that the elevated S level in cancerous thyroid reflects an increase in concentration of ROS in malignant tissue.

Our findings show that mass fraction of Al, B, Br, Ca, Cl, Cu, I, K, Mg, Mn, Na, P, and S are significantly different in TC as compared to normal thyroid tissues [Table 6]. Thus, it is plausible to assume that levels of these chemical elements in thyroid tissue can be used as tumor markers. However, this subject needs in additional studies.

### Limitations

This study has several limitations. Firstly, analytical techniques employed in this study measure only twenty element (Al, B, Ba, Br, Ca, Cl, Cu, Fe, I, K, Li, Mg, Mn, Na, P, S, Si, Sr, V, and Zn) mass fractions. Future studies should be directed toward using other analytical methods which will extend the list of chemical elements investigated in normal and cancerous thyroid tissue. Secondly, the sample size of TC group was relatively small. It does not allow us to carry out the investigations of chemical element contents in TC group using differentials like gender, histological types of tumors, stage of disease, and dietary habits of healthy persons and patients with TC. Lastly, the generalization of our results may be limited to Russian population. Despite these limitations, this study provides evidence on cancer-specific tissue Al, B, Br, Ca, Cl, Cu, I, K, Mg, Mn, Na, P, and S level alteration and shows the necessity to continue chemical element research of malignant thyroid tumors.

### DECLARATIONS

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#### Authors' contributions

Collected thyroid samples, designed the INAA and ICP-AES of samples, carried out the statistical analysis of results: Zaichick V

Managed the literature searches, wrote the first draft of the manuscript, translated the manuscript into English: Zaichick S

Read and approved the final manuscript: Zaichick V, Zaichick S

#### Availability of data and materials

Data were obtained in Radionuclide Diagnostic Department, Medical Radiological Research Center, Obninsk 249036, Russia. The data are available in electronic format as Excel and Word files upon request.

### Financial support and sponsorship

None.

### Conflicts of interest

All authors declared that there are no conflicts of interest.

### Ethical approval and consent to participate

All studies were approved by the Ethical Committees of the Medical Radiological Research Centre, Obninsk. All procedures performed in studies involving human participants were in accordance with the ethical standards of the institutional and/or national research committee and with the 1964 Helsinki declaration and its later amendments or comparable ethical standards.

### Consent for publication

Not applicable.

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Both personal authors and organization as author	Vallancien G, Emberton M, Harving N, van Moorselaar RJ; Alf-One Study Group. Sexual dysfunction in 1,274 European men suffering from lower urinary tract symptoms. <i>J Urol</i> 2003;169:2257-61. [PMID: 12771764 DOI: 10.1097/01.ju.0000067940.76090.73]
Journal articles not in English	Zhang X, Xiong H, Ji TY, Zhang YH, Wang Y. Case report of anti-N-methyl-D-aspartate receptor encephalitis in child. <i>J Appl Clin Pediatr</i> 2012;27:1903-7. (in Chinese)
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Books	Sherlock S, Dooley J. Diseases of the liver and biliary system. 9th ed. Oxford: Blackwell Sci Pub; 1993. pp. 258-96.
Book chapters	Meltzer PS, Kallioniemi A, Trent JM. Chromosome alterations in human solid tumors. In: Vogelstein B, Kinzler KW, editors. <i>The genetic basis of human cancer</i> . New York: McGraw-Hill; 2002. pp. 93-113.
Online resource	FDA News Release. FDA approval brings first gene therapy to the United States. Available from: <a href="https://www.fda.gov/NewsEvents/Newsroom/PressAnnouncements/ucm574058.htm">https://www.fda.gov/NewsEvents/Newsroom/PressAnnouncements/ucm574058.htm</a> . [Last accessed on 30 Oct 2017]
Conference proceedings	Harnden P, Joffe JK, Jones WG, editors. Germ cell tumours V. Proceedings of the 5th Germ Cell Tumour Conference; 2001 Sep 13-15; Leeds, UK. New York: Springer; 2002.
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Unpublished material	Tian D, Araki H, Stahl E, Bergelson J, Kreitman M. Signature of balancing selection in Arabidopsis. <i>Proc Natl Acad Sci U S A</i> . Forthcoming 2002.

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