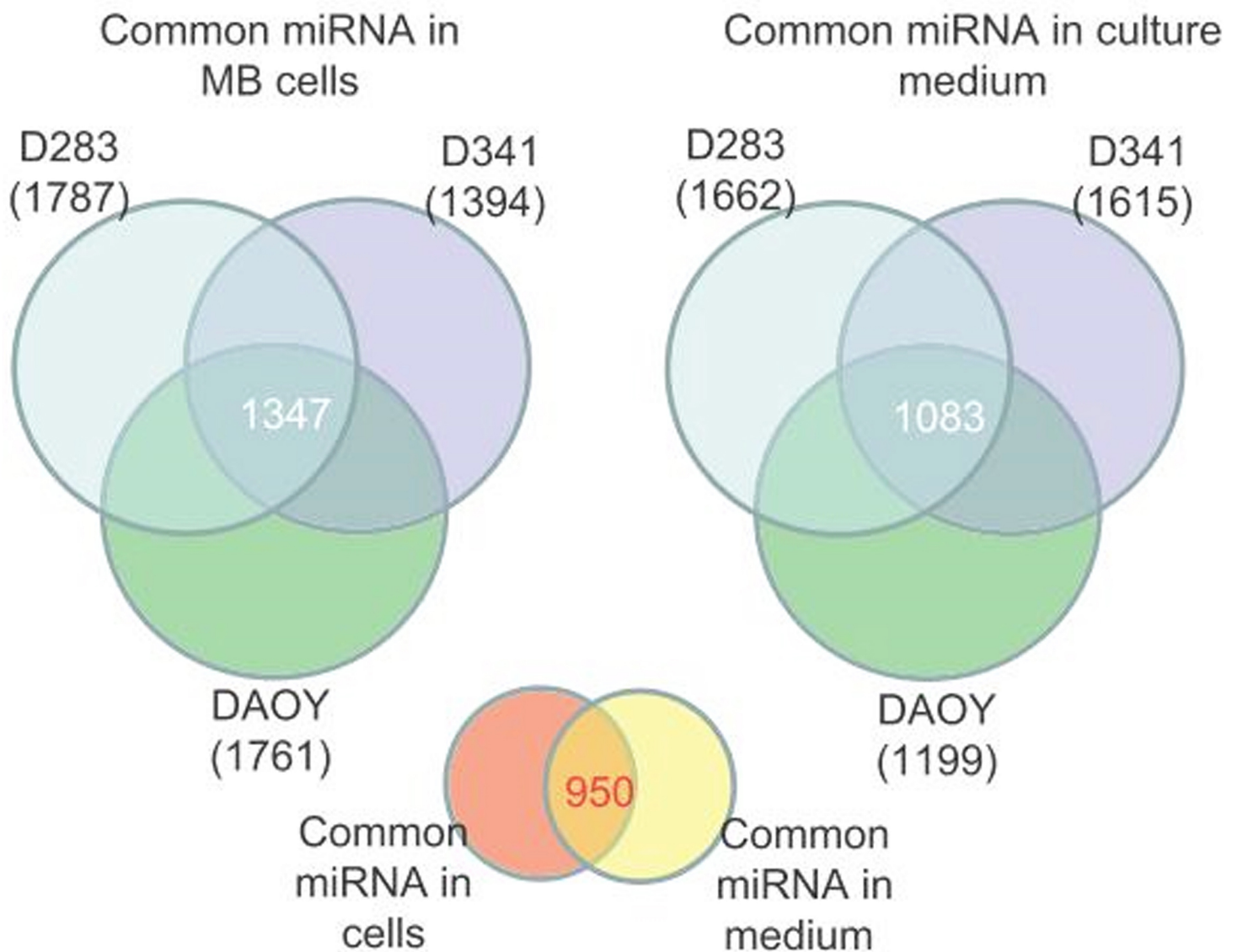


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Risk factors and molecular mechanisms of esophageal cancer: differences between the histologic subtypes

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ABSTRACT

The two major histologic subtypes of esophageal cancer have different risk factors as well as different molecular mechanisms. In this review, the differences in risk factors and genetic/epigenetic alterations between esophageal squamous cell carcinoma (ESCC) and esophageal adenocarcinoma (EAC) will be discussed. Cigarette smoking and alcohol consumption are risk factors for ESCC, while gastroesophageal reflux, cigarette smoking, and obesity are the main EAC risk factors. Commonly mutated genes of both subtypes are *TP53* and *PIK3CA*. Recent genome-wide analysis revealed that the activation of the RAC1 pathway may contribute to EAC tumorigenesis. Clustered abnormality in copy number was observed in several genes in ESCC, whereas a few genes were specifically altered at high frequency in EAC. Epigenetic changes, such as DNA methylation, histone modifications, and altered expression of microRNAs, have been revealed to influence carcinogenesis and progression of both ESCC and EAC.

Key words: Epigenetic alterations, esophageal cancer, genetic alterations, risk factors

Introduction

Esophageal cancer affects more than 450,000 people every year worldwide^[1] and is the 6th leading cause of cancer-related mortality.^[2] The two major histologic subtypes of esophageal cancer are esophageal squamous cell carcinoma (ESCC) and esophageal adenocarcinoma (EAC). ESCCs are by far more common in South East and Central Asia (79% of the total global ESCC cases), while the highest number of EAC is found in Northern and Western Europe, North America and Oceania (46% of the total global AC cases).^[3] The remarkable variations in geographic distribution indicate that different environmental risk factors likely affect the occurrence of esophageal cancer.

Recent progress in molecular biology has revealed that several genetic and epigenetic alterations are implicated in both carcinogenesis and progression of esophageal cancer. Genetic alterations include a chromosomal loss or gain, loss of heterozygosity (LOH), and amplification or mutations of genes. Epigenetic changes, such as DNA methylation, histone modifications, and altered expression of microRNAs regulate gene expression through mechanisms other than changes in DNA

sequence. It has become evident that molecular mechanisms also differ greatly between the two histologic subtypes.

In this review, the differences in both risk factors and molecular mechanisms between ESCC and EAC will be summarized.

Risk Factors

There are different risk factors between ESCC and EAC. Demonstrated in Table 1 are the major risk factors for each histologic subtype.

Both cigarette smoking and alcohol consumption are well-established risk factors for ESCC,^[4,5] with the risk in heavy smokers/drinkers being 50 times greater than those who neither drank nor smoked.^[6] Recently, deficiency in the enzyme aldehyde dehydrogenase 2 (ALDH2), which causes so-called alcohol flushing response, has been revealed to increase the risk of alcohol-related ESCC.^[7] In East Asian populations, there is a variant of ALDH2, resulting from the replacement of glutamate at position 487 with lysine, with the lysine allele encoding an inactive protein.^[8] Drinking hot beverages may also increase the risk of ESCC.^[9] In addition, patients with achalasia are at markedly increased risk of developing ESCC,^[10] while both ESCC and EAC may develop as a late complication of caustic injury.^[11] Oncogenic human papillomaviruses may increase the risk of ESCC, but the evidence is inconclusive.^[12]

Gastroesophageal reflux disease (GERD), cigarette smoking, and obesity are the main EAC risk factors.^[13] At least weekly symptoms of GERD increases the odds

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of EAC five-fold, while daily symptoms increased the odds seven-fold, when compared with those with less frequent episodes.^[14] The relative risk of esophageal and gastric cardia AC was 2.32 for current smokers and 1.62 for ex-smokers, as compared with never-smokers.^[15] However, a meta-analysis provided definite evidence of an absence of association between alcohol drinking and esophageal and gastric cardia AC risk.^[16] A systematic review and meta-analysis revealed a high body mass index (BMI) to be associated with a summary odds ratio for gastroesophageal AC of 1.5.^[17] A recent prospective cohort study in the United States found that a BMI ≥ 35 was associated with a hazard ratio of 3.67 compared with those with a normal-range BMI.^[18] Obesity may predispose to reflux through mechanical means, while adipokines and cytokines secreted from adipocytes and inflammatory cells are known to influence tumor development.^[19] *Helicobacter pylori* infection has been reported to actually decrease the risk of EAC by 41%^[20] through gastric atrophy, which leads to acid reduction.

Radiotherapy for thoracic diseases, such as breast cancer and Hodgkin's lymphoma, increases the risk of both ESCC and EAC.^[21,22] The incidence of both ESCC and EAC increases with age. There is a strong male predominance with up to eight men/one woman for EAC and three men/one woman for ESCC.^[23,24] Fat distribution in obese men is predominantly abdominal, and increasing abdominal diameter has been associated with an increased EAC risk.^[25] However, the male predominance of ESCC can be explained by the prevalence of smoking and alcohol drinking among males.^[26] Although an inhibitory effect of estrogen in the growth of esophageal cancer cells has been reported, there is no firm conclusion on the role of estrogen in human esophageal cancer etiology.^[27] The familial form of ESCC is rare, although familial aggregation has been reported in a high incidence area in China.^[28] In contrast, familial clustering of Barrett's esophagus and EAC has been observed. In a European cohort study, 7% of cases of Barrett's esophagus and EAC were familial.^[29]

The efficacy of endoscopic surveillance for high-risk individuals is controversial. Both lugol chromoendoscopy and an innovative optical image-enhanced technology such as the narrow band imaging have been reported to be useful in detecting early ESCC.^[30,31] In addition, endoscopic esophageal surveillance has been recommended for newly-diagnosed head and neck cancer patients.^[32] However, there is no study evaluating the efficacy of endoscopic surveillance or screening among people heavily exposed to ESCC risk factors. In contrast, endoscopic screening is recommended for patients with multiple risk factors in Barrett's esophagus, although there is no randomized clinical trial that has shown efficacy in preventing deaths due to esophageal cancer.^[33] For patients with Barrett's esophagus without dysplasia, endoscopic surveillance at intervals of

3-5 years has been recommended, and endoscopic eradication therapy is the treatment of choice for those with high-grade dysplasia (HGD).^[33] Recently, however, lengthening surveillance or discontinuing surveillance of patients with persistent non-dysplastic Barrett's esophagus (NDBE) has been discussed because of an annual cancer incidence of only 0.1-0.3% in such patients.^[34]

Molecular Mechanisms

Mutations

Recently, the results of whole-exome or whole-genome sequencing to identify somatic mutations in ESCC^[35] and EAC^[36] have been reported. The frequently mutated genes in esophageal cancers are shown in Table 2. The commonly mutated genes of both subtypes are *TP53* and *PIK3CA*. *TP53* is a major tumor-suppressor gene, its primary function being maintenance of genetic stability and DNA repair capacity.^[37] *PIK3CA* is a kinase activator of the phosphoinositide 3-kinase (PI3K)/AKT pathway and is frequently mutated in many types of human cancers,^[38] including ESCC.^[39] *NOTCH1*, *FAT1*, *FAT2*, *KMT2D* and *ZNF750* are also significantly mutated in ESCC. *NOTCH1* encodes one of the notch family receptors, and the notch signaling is a key pathway of the stem cell signaling network.^[40] There are other recently identified mutated genes^[35] and the much about the functions remains to be researched.

Table 1: Risk factors of esophageal cancer

Squamous cell carcinoma	Adenocarcinoma
Cigarette smoking	Gastro-esophageal reflux disease
Alcohol drinking	Barrett's esophagus
ALDH2 deficiency	Reflux symptoms
Drinking very hot liquids	Obesity
Achalasia	Cigarette smoking
Caustic injury	Diet (high in processed meat, low in fruits, vegetables)
History of thoracic radiation	History of thoracic radiation
Tylosis	Anticholinergic agents
Human papilloma virus infection	Family history
N-nitrosamines	<i>Helicobacter pylori</i> infection (decreased risk)

Table 2: Representative mutated genes in esophageal cancer

Squamous cell carcinoma	Adenocarcinoma
<i>TP53</i>	<i>TP53</i>
<i>KMT2D</i>	<i>CDKN2A</i>
<i>FAT1</i>	<i>SMAD4</i>
<i>FAT2</i>	<i>ARID1A</i>
<i>NOTCH1</i>	<i>PIK3CA</i>
<i>ZNF750</i>	<i>SPG20</i>
<i>PIK3CA</i>	<i>TLR4</i>
	<i>ELMO1</i>
	<i>DOCK2</i>

Bold: Genes commonly mutated in both subtypes

CDKN2A, *SMAD4*, *ARID1A*, *SPG20*, *TLR4*, *ELMO1* and *DOCK2* are significantly mutated in EAC. p16^{INK4a}, encoded by *CDKN2A*, inhibits CDK4 and 6 that bind to cyclin D1 and blocks abnormal cell growth and proliferation.^[41] *SMAD4* is a key intracellular mediator of transforming growth factor-beta signaling and is known to act as a tumor suppressor.^[42] *ARID1A*, which is one of the chromatin remodeling genes, is frequently mutated in a variety of human cancers.^[43] Among the remaining four newly identified genes, *ELMO1* and *DOCK2* are upstream modulators of RAC1 GTPase, suggesting the potential activation of the RAC1 pathway as a contributor to EAC tumorigenesis.^[36]

Recently, comparison of mutated genes among NDBE, HGD, and EAC revealed the majority of recurrently mutated genes in EAC, except *TP53* and *SMAD4*, were also mutated in NDBE.^[44] Mutations of *TP53* and *SMAD4* were stage-specific, confined to HGD and EAC, respectively.^[44]

DNA copy number alterations

Clustered abnormality in copy number was observed in several genes in ESCC [Table 3], whereas a few genes were specifically altered at high frequency in EAC.^[45] Instead, EAC samples demonstrated more widespread genomic instability and the total DNA copy number alterations were an independent prognostic factor.^[45]

Amplification and LOH observed in ESCC are summarized in Table 3. Amplification and overexpression of *CCND1*, which positively regulates G1/S transition, are frequently observed.^[46] The PI3K/AKT pathway is activated by amplification and overexpression of receptor tyrosine kinases (fibroblast growth factor receptor 1 and epidermal growth factor receptor), *KRAS*, and *PIK3CA*.^[35] The transcriptional genes *MYC* and *SOX2* are occasionally amplified. Deletion of several tumor suppressor genes, including *TP53*, *adenomatous polyposis coli* (*APC*), *CDKN2A*, and *FHIT*, is observed in ESCC. *APC* suppresses canonical Wnt signaling through inhibition of β -catenin, while it plays roles in several other fundamental cellular processes such as cell adhesion, migration, and chromosome segregation.^[47] Loss of *FHIT* transcripts affects development and progression of various types of cancers.^[48] Loss of *FHIT* expression was reported to be associated with exposure to environmental carcinogens.^[49,50]

Amplification/overexpression of *ERBB2* (also known as human epidermal growth-factor receptor 2/*neu*) gene has been observed in 24-32% of esophagogastric junction AC.^[51] The positive rate in EAC has been reported to be higher than that observed in gastric cancer.^[51] Trastuzumab, an antibody to *ERBB2*, added to chemotherapy, improved survival in patients with HER-2 positive advanced gastric or gastroesophageal junction AC compared with chemotherapy alone.^[52]

Comparison of cancer-associated genetic abnormalities in the columnar-lined esophagus, with and without goblet cells, has revealed frequent copy number abnormalities in intestinal metaplasia, whereas no such changes were observed in nongoblet cell metaplasia.^[53]

Epigenetic alterations

The promoter hypermethylation of several tumor suppressor genes, such as *APC*, *CDKN2A*, *CDH1*, *FHIT*, *RARB*, *Ras-association domain family 1* (*RASSF1*), *MGMT*, *MLH1*, and *MSH2*, causes decreased expression of these genes and has been known to affect carcinogenesis of ESCC^[54] [Table 4]. E-cadherin, encoded by *CDH1*, is a calcium-dependent adhesion molecule that plays a crucial role in the maintenance of intercellular junctions in normal epithelial cells.^[55] The *RARB* gene encodes retinoic acid receptor beta, a central regulator to normal growth and differentiation of a variety of epithelial cells.^[56] The *RASSF1* encodes a protein similar to RAS effector proteins. RASSF1A protein modulates a broad range of cellular functions essential for normal growth control.^[57] The *MGMT* gene encodes O⁶-methyl-guanine-DNA methyltransferase, a DNA repair

Table 3: Representative amplified or deleted genes in squamous cell carcinoma of the esophagus

Genes	Location	Function
Amplification		
<i>CCND1</i>	11q13	Cell cycle progression
<i>FGFR1</i>	8p11	Mitogenesis, differentiation
<i>EGFR</i>	7p12	Proliferation
<i>PIK3CA</i>	3q26	Cell growth, survival, proliferation
<i>MYC</i>	8q24	Cell cycle progression, transformation
<i>SOX2</i>	3q26	Stemness
<i>KRAS</i>	12p12	Proliferation
Loss of heterozygosity		
<i>TP53</i>	17q13	Cell cycle arrest, DNA repair, apoptosis
<i>APC</i>	5q21	Antagonist of Wnt signaling pathway
<i>CDKN2A</i>	9p21	Cell cycle arrest
<i>FHIT</i>	3p14	Purine metabolism

Table 4: Representative hypermethylated genes in esophageal cancer

Squamous cell carcinoma	Adenocarcinoma
<i>APC</i>	<i>APC</i>
<i>CDKN2A</i>	<i>TIMP3</i>
<i>CDH1</i>	<i>CDKN2A</i>
<i>FHIT</i>	<i>CDH1</i>
<i>RARB</i>	<i>MGMT</i>
<i>RASSF1</i>	<i>DAPK</i>
<i>MGMT</i>	<i>FHIT</i>
<i>MLH1</i>	<i>AKAP12</i>
<i>MSH2</i>	<i>SOCS-3</i>

Bold: Genes commonly hypermethylated in both subtypes

enzyme, which removes methyl- or alkyl-groups from guanidine after chemical modulation, therefore protecting cells from G to A mutations.^[58] *MLH1* and *MSH2* are two key DNA mismatch repair genes and epigenetic silencing of these genes may lead to microsatellite instability.^[59]

Promoters of *APC*, *tissue inhibitor of metalloproteinases 3 (TIMP3)*,^[60] *CDKN2A*, *CDH1*, *MGMT*, *DAPK*, *FHIT*,^[61] *AKAP12*,^[62] and *suppressors of cytokine signaling (SOCS)*^[63] have been reported to be frequently hypermethylated in EAC [Table 4]. *TIMP3* belongs to a family of genes that inhibit matrix metalloproteinases, a group of peptides involved in degeneration of extracellular matrix.^[64] Death-associated protein kinase 1 is a positive mediator of gamma-interferon-induced programmed cell death.^[65] A-kinase anchoring protein 12 is a multivalent anchoring protein and an important regulator of the beta2-adrenergic receptor complex.^[62] SOCS proteins act as negative regulators of JAK/STAT pathways and may represent tumor suppressors.^[66] Promotor methylation and subsequent transcript down-regulation of *SOCS-3* and to a much lesser extent, *SOCS-1* were involved in the multistep carcinogenesis of Barrett's AC.^[63]

Genome-wide DNA hypomethylation may also contribute to tumorigenesis. Long interspersed element 1 (LINE-1) is a retrotransposon comprising about 17% of the human genome, and the levels of LINE-1 methylation can be a surrogate marker of genome-wide DNA methylation.^[54] Hypomethylation levels of LINE-1 are frequently observed in ESCC and correlate with a poor prognosis.^[67] On the other hand, genome-wide methylation analysis also revealed that overall methylation of CpG islands was higher, but outside of CpG islands was lower, in Barrett's esophagus and EAC tissues than in normal esophageal tissues.^[68]

Histone modifications, including acetylation, methylation, phosphorylation, and ubiquitination, regulate gene expression and are implicated in carcinogenesis. Levels of acetylation/deacetylation of histone proteins are determined by two opposing groups of enzymes, histone acetyltransferases, and histone deacetylases (HDACs).^[69] HDAC inhibitors have demonstrated antitumor effects in various cancers.^[70] Of interest, high HDAC2 expression has been associated with aggressive EAC behavior.^[71]

MicroRNAs (miRs), small, noncoding RNA molecules consisting of 19-25 nucleotides, also regulate gene expression epigenetically.^[72] MicroRNAs can act as tumor promoters (onco-miR) through targeting expression of tumor suppressor genes or as tumor suppressors (ts-miR) through targeting expression of oncogenes. miR-21 functions as an onco-miR because it is overexpressed in many types of cancers, including ESCC^[73,74] and EAC.^[75] Targets of miR-21 have been shown to be PDCD4 (programmed cell death 4)^[73] and phosphatase and tensin homolog.^[76] Serum or serum exosomal miR-21 has been reported to be a biomarker

in ESCC.^[77,78] miR-375 is considered as ts-miR in several cancers, including both histologic subtypes of esophageal cancer.^[79,80] Reduced levels of miR-375 in cancerous tissue of EAC patients with Barrett's were strongly associated with a worse prognosis.^[80] miR-205 was down-regulated in both ESCC and EAC.^[81,82] Knockdown of miR-205 expression enhanced expression of zinc finger E-box homeobox 2, accompanied by a reduction of E-cadherin, leading to epithelial-mesenchymal transition.^[82] miR-223 expression was significantly higher in ESCC with an inverse relationship with F-box and WD repeat domain-containing 7, a cell cycle regulatory gene whose protein product ubiquitinates cell cycle regulators such as c-Myc, cyclin E and c-jun.^[83]

Recently, changes in expression of several miRs have been reported in Barrett's esophagus.^[84] miR expressions were compared between 2 groups of patients with Barrett's esophagus who either developed or did not develop EAC over a course of 5 years.^[85] As a result, 4 miRs (miR-192, miR-194, miR-196a, and miR-196b) were found to show significantly higher expression in patients with progression to EAC than in those without.

Conclusion

In this review, the risk factors and molecular mechanisms of esophageal cancer, with special reference to the differences between two histologic subtypes, have been discussed. In spite of advances in the diagnostic tools and therapeutic strategies, esophageal cancer still remains one of the most lethal malignancies. In order to improve outcomes, early detection of tumors based on knowledge of risk factors is needed. In addition, efforts to identify novel therapeutic targets through molecular biological techniques are essential.

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Patient-derived xenograft models for oncology drug discovery

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ABSTRACT

The success of targeted therapies for cancer patients rests on three major components: the right target(s), the right drug and drug combination, and the right patient population. Although much progress has been made in understanding the mechanism of disease and in refining pharmaceutical properties of therapeutic agents, the attrition rates between target discovery and drug marketing approval have been high, especially in oncology. One of the main reasons underlying this undesirable statistics is believed to be the lack of predictive power of the model systems used in the preclinical setting. Several strategies have been employed with the aim of improving the predictive value of the preclinical studies, such as incorporating genomic profiling and molecular segmentation into model selection, and enhancing the development and application of patient-derived xenograft models even during early stage of drug discovery. This brief review will summarize some of the recent concept and practice in incorporating patient-derived models into all stages of drug discovery process, from target to clinical development.

Key words: Animal models, drug discovery, oncology, patient-derived xenograft, translational research

Introduction

The past decades have witnessed an explosive growth of scientific understanding of human diseases especially those of highly unmet medical needs. In the field of oncology, the significant progress in basic research coupled with technology advancement in drug discovery has resulted in a significant number of breakthrough therapies with improved efficacy and manageable toxicity. However, the overall track record of oncology drug research and development remains one of the worst in all therapeutic areas, with high attrition rate and prohibitive cost.^[1,2] Recent survey indicated that in oncology drug development, close to 95% of drugs tested in Phase I trials failed to reach marketing authorization stage.^[3] Significant efforts have been invested in scrutinizing every aspect of the drug discovery and development process and looking for ways to improve the success rate and efficiency. Among all, three pivotal areas have received much attention. First, it is commonly accepted that more refined, clinically relevant preclinical models are critical for accurately predicting patient response in clinical trials. Second, as we have fully embraced the concept and practice of personalized medicine and targeted therapy, tumor profiling and patient segmentation based on predictive biomarkers need to be an integral part of preclinical and clinical

research and drug development. Finally, there is a need for bi-directional flow of information between preclinical and clinical investigators, and for increased collaboration between industry, academia and regulatory agencies to ensure optimal alignment of interests and resources. This short review will only focus on patient-derived models as a promising approach for improving the successful rate of oncology programs.

Patient-derived Xenograft Models for Target Identification and Validation

In the past 4 decades, significant progress has been made in the understanding of cancer biology and emergency of new classes of targeted therapies that have significantly changed the landscape of cancer treatment and management. The key to these successes has been the identification and validation of cancer targets that distinguish cancer cells and tissues from normal ones, as elegantly summarized in the landmark articles by Hanahan and Weinberg.^[4,5] Although a dauntingly complex disease, cancer can be viewed as evolved around a number of rational commonalities, or hallmarks, necessary for tumor initiation, progression, metastasis, evasion of immune surveillance and resistance to therapeutic intervention. These processes involve not only genetic and epigenetic changes in the cancer cells themselves, but also recruitment and alterations in the tumor-associated stroma and micro-environmental factors. Therefore, it is conceivable that therapeutic approaches involving targeting multiple hallmark functions will continue to be the cornerstone for targeted cancer therapy and management.^[6]

Cancer target identification traditionally involves the search for differential expression and function between cancer

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and normal cells and tissues at the DNA, RNA, protein and microRNA levels. Multiple approaches of various through-put have been developed to identify differentially expressed genes and proteins.^[7,8] Recent advances in transcriptomics, proteomics, genomics, functional genomics, epigenomics and metabolomics have significantly expanded the scope and depth of novel targets as well as utility of existing targets.^[6,9-11] Although cell lines have been traditionally used due to their availability and accessibility, most recent efforts have been focused on patient samples, tumor biopsies and resections, for example, for their clinical relevance and heterogeneity. Once a potential candidate target is identified, the next key step is to functionally validate the target in the context of relevant patient population. The routinely employed approaches include tool compound, blocking antibody, dominant negative and RNA interference/short hairpin RNA. In addition, it is imperative to investigate whether the target identified in a small set of cells and tissues are reflected in a larger population ideally identifiable with selective biomarkers. To this end, a collection of large number of clinically collected tumor samples and patient-derived tumor models are critical to ensure translatability from target to drug and from laboratory to clinic.

Although cancer cell lines are the most widely used starting material as they are readily available and propagated to provide sufficient material for *in vitro* manipulation and *in vivo* tumor growth, most of them have been established long time ago and have been selected and cultured under nonphysiological conditions. In contrast, the least manipulated samples are those directly obtained from patients through surgical procedures or needle biopsies. However, one of the major challenges of using primary patient tumors is their limited “shelf-life” and very low quantity in most cases. Compared with cell line models and patient tissues, patient-derived xenografts (PDXs) provide a practical solution by both preserving the fidelity of clinical characteristics and providing tumor supply sufficient for most target identification and validation strategies.^[12,13] Another significant benefit of using PDX for target identification and validation is that the process from target identification to validation and then to efficacy screening can be streamlined around the same models, therefore, offering a complete circle from patient to mouse and then back to patient.

Patient-derived Xenograft Model Characterization

Typically, when patient samples are obtained for establishing PDX models, basic patient information (such as age, sex, ethnicity, clinical diagnosis) with the exception of patient identity will be provided. Once the tumors are established in immune-compromised mice, comprehensive characterization at DNA, RNA and protein levels will be carried out to gain detailed understanding

of the histological, biochemical, molecular and genomic characteristics of the models.^[14-16] As many of the technologies have become more efficient and affordable, whole-genome or transcriptome sequencing is increasingly being used to replace traditional microarray-based gene expression profiling and copy number variation studies. Next generation sequencing (NGS) approaches such as exome sequencing or whole genome sequencing also provide information on mutations and chromosomal aberrations such as duplication, deletion and translocation, many of which identify tumor suppressors or oncogenic drivers^[17] and potentially predict drugs likely to be efficacious in particular patient subgroups.^[18]

A number of studies were carried out to study the impact of successive passages on the gene expression, chromosomal stability and copy number variation. Although not definitive and most likely model-dependent, the general consensus in the field is that PDX models should be used at early passages.^[19] At relatively low passage, the histological features, gene expression profile, copy numbers and chromosomal stability remains very similar to the matching tumor directly harvested from patient.^[20-23] On the other hand, with each passage to a new mouse host, subsequent genetic changes may occur at different tendencies intrinsic to individual tumors, although the extent and impact of these alterations remain unclear.^[24]

In reality, each cancer patient’s tumor is heterogeneous and unique. And within each of the tumor indications mainly defined by anatomic locations of tumor incident (e.g. lung cancer, breast cancer), many subtypes can be identified by histopathology and immunohistochemistry (IHC) of an abbreviated panel of markers. Although these approaches have been widely used to describe and categorize tumors, they have largely failed to capture the variation of disease within indications. Recently, gene expression profiling and NGS have helped further refine the models via molecular subtyping within individual cancer indications.^[25-29] Such molecular subtyping can be particularly helpful in delineating subtypes that can be challenging to distinguish with routine histopathology or IHC. For example, traditionally, breast cancer subtyping is mainly based on histology findings of IHC staining of selected markers. Recent molecular profiling has identified six distinct subtypes (luminal A, luminal B, human epidermal growth factor receptor 2, basal-like, claudin-low, and a normal-like) with clinically significant differences in risk factors, incidence, prognosis, and treatment response.^[30-33] A similar approach has also been used in lung cancer to define clinically relevant subtypes to which targeted therapy can be applied to achieve optimal efficacy. In lung cancer, especially in non-small cell lung cancer (NSCLC), recurrent oncogenic drivers such as epidermal growth factor receptor, KRAS, anaplastic lymphoma kinase, as well as their related pathways can

be successfully employed to select responsive patients and predict response and resistance.^[34-36]

Patient-derived Xenograft Models More Accurately Reflect Human Cancer

Accumulating evidence has indicated PDX models are superior to traditional cell line xenograft models because they maintain more similarities to the tumors found in actual patients.^[14] For example, a detailed cytogenetic analysis of PDX models revealed strong preservation of the chromosomal architecture observed in patients.^[23] Furthermore, other studies have shown strong fidelity in histology,^[37,38] transcriptome,^[39] polymorphism^[40] and copy number variations.^[41] In some cases, certain oncogenic gene amplification can be found in cell lines at levels that are several-fold higher than in patient tumors, a cell culture-derived artifact that may lead to over-predict drug response in the clinic (unpublished data). On the other hand, emerging data started to show that PDX models may be more accurately reflect clinical response when treated with therapeutic agents at clinically relevant doses (CRDs).^[21]

Modeling Drug Resistance

Despite the continuously growing arsenal of new and improved anti-cancer drugs, for most cancer patients with advanced diseases, treatment failure remains an inevitable outcome. To a given treatment, only a fraction of the patients would respond the regimen favorably (responders), which stresses the importance of selecting patients with the appropriate molecular and pathological characteristics for maximal therapeutic benefit. On the other hand, even when a particular treatment is initially efficacious in selected patients, drug resistance will develop overtime. Therefore, drug resistance is a fundamental cause of therapeutic failure in cancer therapy. Numerous studies have attempted to unravel the mechanisms of drug resistance to traditional chemotherapeutic agents and to recently developed targeted, small molecule and antibody based drugs. Briefly, the mechanisms of resistance can be roughly mapped to four categories: (1) Multi-drug resistance (MDR). MDR is caused by expression and/or induction of efflux proteins, which are members of the ABC transporter superfamily involved in the transport of both hydrophobic and hydrophilic compounds.^[42] This mechanism is relatively more common for cytotoxic drugs and payload of antibody-drug conjugates^[42] than targeted agents; (2) Tumor initiating cells/cancer stem cells (TICs/CSCs). As discussed earlier, these cells have the capability of self-renewal and differentiation, remain relatively quiescent, and can tolerate higher level of DNA damaging agents and oxidative stress. These characteristics are important for TICs to survive chemotherapy and radiation and ignite tumor re-growth when the condition permits;^[43-46] (3) Tumor genetic and

epigenetic alterations. These alterations can take place at multiple points during tumor initiation, progression and treatment, and they can be preexisting mutations, acquired mutations, or changes in downstream genes and pathways. For example, resistance to EGFR tyrosine kinase inhibitors can be attributed to multiple mechanisms, such as gatekeeper mutation (T790M),^[47-49] c-Met amplification,^[50] activation of alternative pathways such as insulin-like growth factor receptor and AXL,^[48,51] trans-differentiation to mesenchymal cells^[52] or small cell features;^[53] and (4) Tumor microenvironment. Emerging data has indicated tumor microenvironment as a key mediator of drug resistance.^[54] For example, several potential mechanisms of resistance to anti-angiogenic drugs are microenvironment-derived, including up regulation of alternative pro-angiogenic signals,^[55,56] recruitment of bone marrow progenitors,^[57] and increased pericyte coverage.^[58] Another example can be found in pancreatic ductal adenocarcinoma, in which gemcitabine resistance has been attributed to inefficient drug delivery due to poorly perfused tumors.^[59]

There are obvious advantages of using PDX models to study drug resistance mechanism and to characterize therapeutic agents for efficacy. As discussed earlier, PDX models are heterogeneous in nature, and more closely reflective of tumors in actual patients,^[60] and a more appropriate system for understanding acquired and de novo drug resistance through enrichment of preexisting changes in subsets of cells.^[61,62] A large collection of PDX models can best represent a broad patient population with various preexisting mutations and susceptibility to generate additional mutations, which cannot be achieved by other models including cell line xenografts. In addition, PDX models contain TICs/CSCs, and proper tumor stroma (albeit controversial) that can potentially contribute to resistance as well. Furthermore, it has become possible to establish PDX models with tumors that had already been treated and later became refractory. This is an important point because in clinic, most patients entering clinical trials have been treated with standard of cares previously and have relapsed with refractory disease. Compared to cell line xenografts, PDX models should better recapitulate patients with refractory and metastatic cancer.^[63]

A number of studies have taken the advantages of PDX models to study drug resistance. Krumbach *et al.*^[60] investigated response to cetuximab in 79 PDX models generated from colon, gastric, head and neck, lung and mammary cancer. After an in-depth analysis of different molecular characteristics of the tumors, they identified c-MET activation as a key mechanism for drug resistance, especially in NSCLC adenocarcinomas. In another study: using PDX models of NSCLC, Dong *et al.*^[64] identified foci of resistance cells after cisplatin treatment as a single agent or in combination with vinorelbine, docetaxel, or gemcitabine. The authors

suggested that these drug-resistant cells were TICs-like and could be responsible for tumor recurrence.

Patient-derived Xenograft Models for Pharmacology and Biomarker Studies

Traditionally, pharmacology, biomarker and pharmacokinetics/pharmacodynamics studies for oncology programs almost exclusively relied on tumor xenograft and to a much lesser degree, syngeneic models. With the significant increase in the availability and affordability of PDX models offered by both academic institutions and contract research organizations, PDX models have seen increasingly their utility in routine research activities. A quick survey of oncology discovery programs published in the past 3 years shows that increasing number of programs use PDX models at some point during the preclinical discovery and translational research stages.^[14,65-67] In addition, there is an industry-wide trend to include PDX model readout as a key component of the required data package for both internal use as well as regulatory submission. The history of using incorporating PDX models in drug discovery can be traced back to several decades ago. For example, one of the earliest reports involving cancer drugs and PDX models by Fiebig *et al.*^[68] studied a number of chemotherapy drugs at their respective maximal tolerated doses (MTDs) in PDX models derived from 34 patients, and demonstrated 92% accuracy in predicting efficacy and 97% in predicting no-response. Similar predictive value was seen in a later study by the same group.^[69] However, additional studies suggest that the predictive value can fluctuate due to factors such as tumor histology and location, stage of disease from which the models are derived, the quality of PDX models, sample size and dosing regimen.^[64,70,71] In addition to selecting models that are histologically, molecularly and genetically relevant to the patients in clinical, another important factor for improving translatability of preclinical findings is the drug exposure. Not surprisingly, preclinical model species, in most cases immunocompromised mice, can exhibit different tolerability and adsorption, distribution, metabolism and excretion property than those in human. It is commonly seen that drug exposure levels at MTD dose in mice are higher than clinically achievable levels in human.^[72] Therefore, a compound given at mouse MTD to xenograft, allograft or syngeneic models may generate exaggerated efficacy that over-predicts human response in the clinic. This phenomenon has been seen for both chemotherapy agents^[12,73,74] as well as targeted agents such as vascular endothelial growth factor receptor inhibitors and PI3K inhibitors.^[75] A key concept and practice to avoid the pitfalls of using mouse MTD dose and exposure as the sole basis for efficacy prediction is to use CRD or clinically relevant exposure (CRE) whenever a CRD or CRE can be determined.

Patient-derived Xenograft Models for Mouse Clinical Trial

An evolving concept and practice, PDX mouse clinical trial, has started to yield positive results that had real-life impact on selected patients.^[76] In this setting,

PDX models established from the very same patients on trial are being treated ahead of patient therapy or concurrently, and results from the mouse trial is provided in real-time to help guide clinical management of the patient's tumor. Further powered by the molecular characterization of the tumors, this highly personalized approach has the potential to revolutionize the drug development and patient care.^[77] For example, a recent study by Stebbing *et al.*^[78] reported 22 sarcoma PDX models were successfully established from 29 patients (76% take rate) and screened for drug sensitivity to a panel of therapeutic agents. The entire process typically took 3-6 months depending on individual tumor growth characteristics and treatment regimen. Of the 22 patients, 6 died before data became available. Of the 16 remaining patients, 13 (81%) demonstrated a correlation between the results from their PDX mouse trial and clinical outcome. Similar approach has also been reported in advanced adenoid cystic carcinoma,^[79] ovarian,^[80] and other cancer types.^[81] The current data, although limited, appears to support the use of PDX models to prioritize therapeutic agents against individual tumors. However, some key challenges remain before this strategy can be broadly implemented in clinical practice. For example, establishment of PDX models is still a technically challenging and time-consuming process, even after much progress has been made to improve the take rate and optimize the expansion scheme. In addition, the algorithm for the selection of agents to be tested needs to be further developed and refined. Lastly, to effectively demonstrate the feasibility and clinical benefit of the PDX-guided treatment prioritization in the patient care setting, properly controlled clinical trials are needed.

Limitations of Patient-derived Xenograft Models

Although PDX models present an exciting opportunity for improving predictive value of preclinical and translational studies, and offer a number of advantages over conventional cell line xenograft models, just like any other preclinical model platforms, there are several limitations that one needs to be aware of. First, the utilization of severely immune-compromised host mouse strains, particularly the nonobese diabetic severe combined immune deficiency gamma mice, while allowing higher take rate and more consistent growth of xenografted human tumors, is inherently inadequate in modeling immune responses. Although human stroma components including immune cells originally present in the tumor biopsy can be grafted together with the tumor tissue,^[82] they normally cannot survive beyond the first passage, and will be completely lost in the subsequent expansion.^[83] The other stroma components including fibroblasts and vasculature are quickly replaced by murine counterparts.^[83] The lack of functional immune system limits the utility of these models in studies where immune responses are required. For example, immunotherapy cannot be readily studied in the PDX

models established in immune-compromised mice. It is well documented and accepted that immune system is an important part of tumor stroma and significantly contributes to tumor initiation, progression, metastasis and therapeutic response.^[84,85] The introduction of mice with partially or completely humanized immune systems can potentially ameliorate this issue, but significant technical challenges still exist.^[86,87]

Second, although technical advances have gradually improved the tumor take, different tumor types, and different subtypes within the same tumor type, have varying rates of success. This has led to imbalanced representation of tumor types/subtypes that is more determined by take rate rather than clinical incidence rate. Although PDX models can avoid artificial selection in extended culture on plastic, the *in vivo* selection process exists as soon as the tumors are implanted. For example, high-grade, fast proliferating tumors tend to be easier to establish as PDX models than low-grade, slowly growing but progressive tumors.^[88,89]

Additionally, compared to cell lines, PDX models are difficult to manipulate genetically. Most PDX models are established from and passaged as tumor fragments, and conventional transfection or transduction are not efficient to genetically modify the tumors or introduce detection markers (such as luciferase or fluorescent proteins). Therefore, PDX tumors are rarely established as orthotopic models, unless there is a surrogate biomarker that be readily used to measure tumor burden noninvasively.^[90]

Conclusion

Although hardly a new concept, PDX models have gained much attention and premium status in the past few years as they are becoming increasingly available and affordable, and are believed to offer a superior predictive value over conventional cell line xenograft models. Ample data indicated that PDX models maintain heterogeneity and tumor initiation ability, as well as molecular and genetic characteristics reflective of human tumors. Emerging data indicated an improved predictive value of the PDX models; however, it is still early to conclude whether the advantage in translatability is applicable to large sample size and to various therapeutic mechanisms and modalities. The mouse clinical trial has the potential to accelerate and de-risk human clinical trials and hopefully reduce clinical attrition rates for novel compounds, and to prioritize therapies by allowing parallel testing of multiple treatment schemes for an individual patient. However, there are still much to be done to address technical challenges to make this approach feasible and affordable and to convince the medical and insurance community of the value this approach can offer. At the same time, one cannot overlook the limitations of PDX models and should take into consideration of their shortcomings when design and

interpret studies. Collectively, these new developments emphasize the importance of employing PDX models in key areas of oncology drug discovery and development.

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Association between the cytotoxic T-lymphocyte antigen-4 polymorphisms and breast cancer risk and prognosis

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ABSTRACT

Aim: The aim was to evaluate the potential influences of cytotoxic T-lymphocyte antigen-4 (*CTLA-4*) gene polymorphisms on breast cancer risk, the distribution of *CTLA-4* single nucleotide polymorphisms (1661AG) in breast cancer patients and control subjects was investigated. **Methods:** In this case-control study, 100 patients with breast cancer as case group and 100 healthy participants as a control group were compared. Genotypes were determined by the polymerase chain reaction-restriction fragment length polymorphism method. Demographic characteristics of the study population, as well as tumor size, tumor grade and stage were collected in a questionnaire designed for this study. The collected data were statistically analyzed by SPSS-16.0 (SPSS Inc., Chicago, USA) predictive analytic software using the Chi-square test. **Results:** The mean age of women was 43.42 ± 13.1 years. The AA genotype was frequent in case group (43%) whereas the AG genotype was found more in the control group (69%). There was no significant relationship between the studied polymorphisms and the grade, stage and size of the tumor, nor between the studied polymorphisms and estrogen receptor, progesterone receptor and lymph node involvement ($P > 0.05$). Significant association between the studied polymorphisms and breast cancer metastases was found ($P = 0.02$). **Conclusion:** According to the results of the study, the AA genotype is associated with breast cancer, but none of the studied gene polymorphisms is associated with prognostic factors such as tumor stage, grade or size.

Key words: Breast cancer, cytotoxic T-lymphocyte antigen-4, polymorphism, prognosis

Introduction

Breast cancer is the most common cancer in women (30% of all cancers among women in developed countries), but is treatable if early diagnosis and treatment occur.^[1] The incidence of breast cancer has constantly increased since 1940^[2] and based on WHO reports, there is a 2% annual increase in breast cancer prevalence.^[3] There are no exact statistics on the prevalence of breast cancer among the Iranian population (the source of our study); estimates show that Iran has moderate, but increasing, prevalence.^[4] Both genetic and environmental susceptibilities are included in breast cancer etiology,^[5-8] but the exact etiology has not been definitively identified. Current studies confirm the role of the immune system on the etiology of breast cancer.

It was shown that cancer cells provoke immune recognition, but the biologic importance of antitumor

innate and adaptive responses, which are frequently detected in cancer-bearing hosts, remains incompletely understood.^[9] The most considerable antitumor response is made by human cellular immunity mediated by T-lymphocyte and natural killer (NK) cells. It follows that variants of genes included in the regulation, and proliferation of T-lymphocyte and NK cells would be effective in predicting the risk of breast cancer. Cytotoxic T-lymphocyte antigen-4 (*CTLA-4*) is coded by a gene on chromosome 2q33. It is a member of the immunoglobulin super family, which transmits an inhibitory signal to T cells. *CTLA-4* binds to B-7 on antigen-presenting cells, and polymorphism of *CTLA-4* gene interferes with surface activity of B-7, preventing T-lymphocyte from activating.^[10,11] In fact, *CTLA-4* prevents immune response^[12] and its tumor-killing activity.^[13] *CTLA-4* gene is composed of 4 exons and possibly plays a significant role in diseases related to T cells. More than 100 single-nucleotide polymorphisms are recognized on the *CTLA-4* gene. Among them, AG dysmorphisms, located on +49 of exon 1, could make amino acid (threonine into alanine) on *CTLA-4* protein.^[14]

Current studies show that this polymorphism affects the ability of *CTLA-4* to bind to B-7 cells and to activate T cells.^[15,16] These surveys show that translocation of A allele to G allele on +49 zone decreases the role of

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CTLA-4 on T-cell responses,^[15-18] although there are contradictory reports on the relationship between +49 A to G polymorphisms and cancer development.

Higher expression of CTLA-4 is seen in persons with thiamine on zone -318 of *CTLA-4* gene promoter or homogenous adenine on exon 1 of codon +49.^[14] There have been several studies on the relationship between polymorphisms on *CTLA-4* gene and autoimmune diseases such as graves, diabetes mellitus type one, lupus and Hashimoto thyroiditis^[19-22] and the tendency to develop cancer.^[23-27] Results of some studies show an inverse relation between polymorphisms of autoimmune diseases and malignancies on *CTLA-4* gene. Alleles discovered in autoimmune diseases are not seen in malignancies or are related to a good prognosis of cancers. In a study in Iran, results suggested higher risk of breast cancer among AA and AG genotypes on +49 zone, but there was no difference between -318 CT and -1666 AG among case and control groups.^[23,24]

Considering the high prevalence of breast cancer and also some confirmed evidence of a relationship between *CTLA-4* gene polymorphisms and breast cancer, we conducted this study to assess the relationship between *CTLA-4* gene polymorphisms and both incidence and clinic pathologic features of breast cancer. The results of this study would help physicians to recognize the prognosis and risk ratio of patients with a high risk of breast cancer.

Methods

Study subjects

The study group consisted of a total of 100 Iranian women with breast cancer and 100 healthy cancer-free control individuals. Informed consent was obtained from each subject, and each participant was then interviewed to collect detailed information on demographic characteristics such as sex and age. Some clinic pathologic features of breast cancer patients, such as tumor size, lymph node involvement, tumor type, and estrogen receptor (ER), were also obtained from their medical files [Table 1].

Patients were recruited between February 2013 and October 2014 at the Shahid Sadoughi Hospital and Cancer Hospital, Yazd, Iran. Control subjects were cancer-free individuals, and they were randomly selected from the same regions and the same time period as the patients were collected. The selection criteria included no individual history of breast or other cancers.

This study was approved by the Ethics Committee of Shahid Sadoughi University of Medical Sciences, Yazd, Iran. A written informed consent was taken from all patients.

Table 1: Relation between *CTLA-4* gene polymorphisms and tumor grade, stage, size, metastasis, estrogen receptor, progesterone receptor and age

	AA (%)	AG (%)	GG (%)	P
Tumor grade				
1	20 (80)	23 (71.9)	1 (33.3)	0.21
2	3 (12)	5 (15.6)	1 (33.3)	
3	2 (8)	4 (12.5)	1 (33.3)	
Tumor stage				
≤ 2	15 (75)	18 (72)	3 (100)	0.50
> 2	5 (25)	7 (28)	0 (0)	
Tumor size				
≤ 2 cm	11 (68.8)	14 (58.3)	0 (0)	0.50
2-5 cm	3 (18.8)	8 (33.3)	1 (100)	
> 5 cm	2 (12.5)	2 (8.3)	0 (0)	
Metastasis				
No	27 (64.3)	43 (87.7)	5 (83.3)	0.02
Yes	15 (35.7)	6 (12.2)	1 (16.7)	
Estrogen receptor				
Negative	10 (37)	9 (23.7)	2 (33.3)	0.49
Positive	17 (63)	29 (76.3)	4 (66.7)	
Progesterone receptor				
Negative	12 (44.4)	8 (21.6)	2 (33.3)	0.15
Positive	15 (55.6)	29 (78.4)	4 (66.7)	
Lymph node involvement				
No	36 (83.7)	38 (74.5)	5 (83.3)	0.53
Yes	7 (16.3)	13 (25.5)	1 (16.7)	
Age				
Under 40 years	24 (29.3)	54 (65.9)	4 (4.9)	0.30
Over 40 years	47 (29.8)	66 (55.9)	5 (4.2)	

Data are presented as n (%). *CTLA-4*: Cytotoxic T-lymphocyte antigen-4

Polymorphism genotyping

Peripheral blood (5 mL) was collected from subjects after informed consent was obtained. Genomic DNA was extracted from peripheral blood using the DNA extraction kit (BioFlux, cat: BSC 06M1, Hangzhou, Bioer Technology Co., Ltd, China).

Genotyping was performed by polymerase chain reaction (PCR)-restriction fragment length polymorphism method. The polymorphic region was amplified by PCR using the following primers: (forward) 5'-CTAAGAGCATCCGCTTGCACCT-3' and (reverse) 5'-TTGGTGTGATGCACAGAAGCCTTT-3' in a 25 µL reaction solution containing 0.3 µg of genomic DNA, ×1 PCR buffer, 0.3 mmol/L MgCl₂, 0.2 mmol/L dNTPs, 2 U tag DNA polymerase and 0.1 µmol/L of each primer.

The following PCR program was run: 94 °C for 4 min, 30 cycles of 94 °C for 30 s, 58 °C for 30 s and 7 °C for 45 s. Final extension was carried out at 72 °C for 5 min. The lengths of the PCR products were 486 bp (1661AG).

The PCR products were digested with restriction enzymes TruI (MseI) according to the manufacturer's instructions (Thermo Scientific Fermentas, USA) and

analyzed by 2% agarose gel electrophoresis. The cut site for TruI (MseI) was 5'-TTAA-3'.

The digested fragments in 1661AG were 139 and 347 bp. Presence of the A allele was recognized by detecting digested 347 and 139 bp fragments on gel, and the G allele by detecting intact primary 486 bp band [Figure 1]. Comparisons of genotype and allele frequencies in cases and controls were assessed by Chi-square and *t*-test using SPSS-16.0 (SPSS Inc., Chicago, USA). Statistical software and statistical significance were set at $P \leq 0.05$. The odds ratio and 95% confidence interval were also calculated.

Results

A total of 100 women with breast cancer and 100 healthy controls were enrolled in this study. Mean age of the case group was 48.92 ± 9.85 years and of the control group was 37.92 ± 13.67 years ($P < 0.001$; *t*-test). This study was done in patients who presented at Shahid Sadoughi Hospital and Cancer Hospital, Yazd, Iran. The grade, stage and size of the tumor in the case group are shown in Table 1. About 73.3% of patients had grade one tumors, and about 75% had stages 1 and 2 tumors. Tumor size in more than 60.9% of patients was ≤ 2 mm.

Polymorphisms of *CTLA-4* gene are shown in Figure 2. There is a significant relationship between groups according to gene polymorphisms ($P = 0.03$). Frequency of AA polymorphisms in the case group is higher than in controls whereas AG polymorphisms are more frequent in the control group.

Table 1 shows polymorphisms according to tumor stage. There is no significant difference among study groups according to tumor grade ($P = 0.21$). Also there was no relationship between polymorphism and tumor stage ($P = 0.50$) and tumor size ($P = 0.50$).

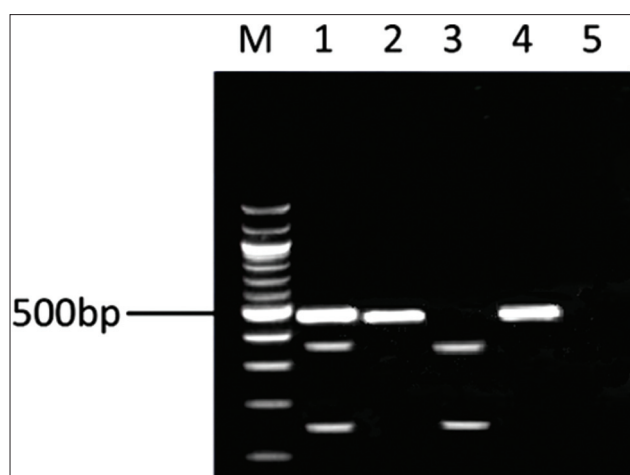


Figure 1: A gel image for the polymerase chain reaction-restriction fragment length polymorphism experiment which illustrates the band patterns of AA, AG and GG genotypes. Line M: molecular weight marker; Line 1: AG genotype; Lines 2 and 4: GG genotype; Line 3: AA genotype; Line 5: negative control

Considering the difference in mean age between the two study groups, we analyzed polymorphisms of *CTLA-4* gene in all participants according to age groups of under or over 40 years, but found no significant difference ($P = 0.30$) [Table 1]. We determined that the age difference between the two groups had no confounding effect on the study results.

Analysis of metastasis showed that there is a significant relationship between *CTLA-4* gene polymorphism and metastasis. Patients with AA genotype had higher rates of metastasis ($P = 0.02$) [Table 1].

Analysis also showed no relationship between *CTLA-4* gene polymorphisms and ER ($P = 0.49$), progesterone receptor ($P = 0.15$) and lymph node involvement ($P = 0.53$) [Table 1].

Discussion

There is increasing attention to the relationship between several genes' polymorphisms and polygenetic diseases such as hypertension, diabetes, and various malignancies.^[28] T cells and NK cells have a substantial role in working against tumors.^[29] T-lymphocyte, especially T killer cell, is the most important in defending cells against tumors. CTLA-4 molecule expresses on T-lymphocyte as an inhibitor and plays different roles in T-cell activity. It could inhibit amplification of T cells or even induce apoptosis of activated T cells.^[30] CTLA-4-mediated suppression of tumor immunity has been previously reported.^[13] Several studies have demonstrated the effect of CTLA-4 blockade in enhancing immunity to tumors.^[14-16] There are some studies on the relationship between *CTLA-4* gene polymorphisms and breast cancer, but results were contradictory.^[31] In order to clarify the role of genetic variants of *CTLA-4* gene in immune suppression of patients with cancer, the distribution of *CTLA-4* gene single nucleotide polymorphisms (1661AG), in breast cancer patients and

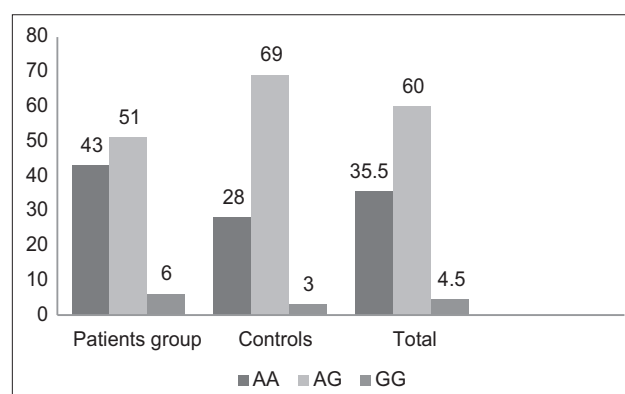


Figure 2: Frequency of AA, AG and GG polymorphisms of cytotoxic T-lymphocyte antigen-4 gene in patients, controls and total study group, showing that AA genotype in patients is more than this genotype in controls while AG polymorphism is more frequent in the control group. The Y-axis is showing the number of the cases and the X-axis is showing different types of genotypes

control subjects, were investigated and their associations were assessed with prognostic factors. The present case control study was done to determine possible relationships between AA, AG and GG polymorphisms of *CTLA-4* gene and breast cancer-related factors. Results revealed that the frequency of AA and GG genotypes in breast cancer patients is higher than in controls, while AG genotype is more frequent in healthy controls. These results confirm the findings of two other studies in Iran^[23] and China,^[32] which found GG genotype is more frequent in breast cancer patients. However, Erfani *et al.*^[24] did not find any difference between their study groups in terms of AG genotype. Another study in China suggested that AG genotype is more prevalent in breast cancer patients.^[33] Furthermore, Sun *et al.*^[15] reported that T cells with AA genotype are less active than those with GG genotype, and AG is related to different cancer incidence in humans.

The results of our study did not find any relation between AA, AG and GG genotypes with respect to tumor stage, grade, receptors or lymph node involvement. These results are in agreement with results of Wang *et al.*,^[16] who found no significant relationship between AG genotype and tumor size and lymph node involvement. Erfani *et al.*^[24] found a relationship between AA genotype and lower lymph node involvement and higher ER expression, but Ghaderi *et al.*^[23] found that AA genotype is related to higher rates of lymph node involvement and tumor size; these findings are different from our findings. Bi *et al.*^[34] in his study reported that *CTLA-4* expression is higher in stage 2 than stage 3 patients. There are also some other studies, which revealed that *CTLA-4* gene polymorphisms are related to higher stages and lymph node metastasis,^[6,32] which is not consistent with our study. According to age, in our study and also in the Bi *et al.*,^[34] there was no relationship between *CTLA-4* gene polymorphisms and age.

Li *et al.*^[32] found a relationship between all *CTLA-4* gene polymorphisms with estrogen and progesterone receptors. Also, Erfani *et al.*^[24] detected a relationship between AG genotype and ER expression, which was not consistent with our findings.

One of the limitations of our study is the failure to take into account risk factors such as age at menarche, menopausal status, and environmental factors. It is important to investigate the interaction between single-nucleotide polymorphisms and these factors on the risk of breast cancer in a larger sample size in further studies.

Based on our study, there is a relationship between *CTLA-4* gene 1661AG polymorphisms and incidence of breast cancer, but these polymorphisms are not effective for prognosis. Considering the controversial reports on this issue, more studies are needed with larger sample size. Also, a critical review and possible meta-analysis of

present studies are needed to make an exact estimation of the results of current studies.

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Changes in human epidermal growth factor receptor 2 status between primary breast/gastric carcinomas and synchronous metastatic lymph nodes: how can we explain them?

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ABSTRACT

Aim: Previous studies demonstrated discordant expression of human epidermal growth-factor receptor 2 (HER2) between primary cancer and their recurrence/metastasis. This study further evaluated HER2 status between primary gastric and breast invasive carcinomas and paired metastatic disease to lymph nodes. **Methods:** This study collected formalin-fixed paraffin-embedded representative tissue blocks from 62 gastric and 65 breast primary carcinomas as well as synchronous metastatic lymph nodes (male:female = 39:88; age ranged between 44 and 95 years with mean age of 69.32 years) for immunohistochemical staining of HER2 expression (DAKO HercepTest™ kit). If immunohistochemical HER2 score reached to 2+, HER2 amplification was then assessed using fluorescence *in situ* hybridization (PharmDx™ kit DAKO). **Results:** The discordant HER2 pooled rate, regardless either negative or positive conversion, was 9.67% in primary gastric carcinoma and corresponding nodal metastasis, while the changes in HER2 expression were revealed in 4.61% of mammary and lymph node neoplastic samples. A high-level concordance in HER2 expression between primary carcinoma and synchronous metastatic lymph nodes was confirmed in both types of cancer; the observed event of discordant HER2 status should be ascribed to intra-tumor heterogeneity, mostly appreciable in gastric cancer. **Conclusion:** In any case, the shift from positive to negative HER2 expression suggests that trastuzumab could be the targeted treatment choice whereas the opposite shift should be evaluated by a simultaneous HER2 determination in both primary and metastatic lymph nodes.

Key words: Breast cancer, epidermal growth-factor receptor 2, gastric cancer, lymph node, metastasis

Introduction

Expression or amplification of human epidermal growth-factor receptor 2 (HER2) frequently occur in primitive neoplastic tissues from patients with breast carcinoma (BC).^[1-4] However, in recent years, several studies have demonstrated that HER2 status may vary in the metastatic lesions compared to the primary tumor,^[5-8] and this discrepancy is more frequently found in distant metastases than in loco-regional ones.^[9-13] Discordance in HER2 status was not only found between primary BC and its metastases, but also among the consecutive relapses of the same tumor, with similar proportions of cases turning from negative to positive or vice versa and the changes mainly appeared in the second or following progressions.^[13-16]

HER2 amplification may also be detected in gastric carcinomas (GCs), with a prevalence ranging between

7.7% and 25% depending on localization and histology of the cancer,^[17-19] a higher rate of HER2 amplification occurs in unusual aggressive histology types, such as the hepatoid variant.^[20,21] However, until date, there were only a few studies reporting HER2 heterogeneity in paired primary and metastatic GC samples,^[22-24] and demonstrating a low rate of discordance in HER2 amplification with either positive and negative conversion.^[23,24]

The potential divergence in the HER2 status between the primitive BC/GC and their metastasized diseases, or among the successive metastases of the same tumor, has a significant clinical relevance since it may modify the patient's sensitivity to targeted therapies,^[8] which might be appropriate for the primitive tumor, but not for the metastases or vice versa.^[12-15] For this reason, some investigators proposed that detection of HER2 status should be re-assessed in the neoplastic tissues from metastatic BC to establish whether the therapy is actually appropriate.^[1,2,16,17]

Thus, in this study, we evaluated HER2 status in paired samples of BC/GC and synchronous metastatic lymph nodes that were collected during the same surgical and tissue processing procedures, thus limiting and avoiding any potential technical bias due to external factors. Our aim was to explore the eventual HER2 discordance rate between primary

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BC and GC samples and corresponding lymph node metastases. The study was approved by review board of University of Messina.

Methods

This cohort contained 127 surgical BC and GC specimens, together with the corresponding regional synchronous metastatic lymph nodes. In brief, 65 primary BC and 62 primary GC (male:female = 39:88; age ranged between 44 and 95 years with mean age of 69.32 years) were retrospectively collected from the archive of the Department of Human Pathology at the University of Messina. No patients had received neo-adjuvant chemotherapy or other therapies before surgery.

The primary GC was classified for localization and histology type according to WHO 2010, Lauren's classification and HER2 status of the tumor were available for all cases. Similarly, histology, grade, hormone receptor status, Ki-67, and HER2 status were recorded for all BC cases. Patient identification was not disclosed in this publication, and all patients had provided written consent to their medical information being used for research purposes, according with the Helsinki declaration.

For each case, 3 µm thick tissue sections from two different formalin-fixed paraffin-embedded representative tissue blocks of the primary tumor and metastatic lymph nodes (at least four for each case) were prepared and immunohistochemical stained for HER2 expression. In brief, the immunohistochemistry was carried out by using a DAKO HercepTest™ kit (Dako, Glostrup, Denmark) with an automated procedure (DAKO Autostainer Link 48) according to manufacturer's instructions. Antigen retrieval was performed by 3 cycles in 0.01 mol/L citrate buffer pH 6.0 in a microwave oven at 750 W. For HER2 score was used to semiquantitatively assess HER2 expression level, that is, for the primary GC, 0, absent staining; 1+, faint and discontinuous membranous staining in < 10% of neoplastic elements; 2+, light to moderate lateral, baso-lateral or complete membranous staining in > 10% of neoplastic elements; 3+, strong, intense lateral, baso-lateral or complete staining in > 10% of neoplastic elements and for BC, 3+ score was defined when strong membranous staining was noted in at least 30% cells, 2+ when weak to moderate complete membranous staining was evidenced in 10-30% of tumors cells, 1+ when a faint or weak and incomplete membrane staining was observed and 0 when no staining was observed or when staining was present in < 10% of neoplastic cells.

Furthermore, fluorescence *in situ* hybridization (FISH) was performed using a HER2 FISH PharmDx™ kit (Dako) in those cases with HER2 immunostaining score for 2+ or more. HER2 amplification was recorded when HER2 to CEP17 signal ratio was > 2.0.

Fleiss-Cohen weighted K statistics was used to assess the concordance rate between HER2 status of the primary carcinomas and metastatic synchronous lesions. K values between 0 and 0.2 were regarded as no agreement, between 0.21 and 0.4 as fair agreement, between 0.41 and 0.6 as moderate agreement, between 0.61 and 0.8 as substantial agreement, and between 0.81 and 1 as almost perfect agreement. The statistical association between HER2 status and the other histopathological parameters was assessed using Chi-squared test. $P < 0.05$ was considered statistically significant. All statistical analyses were performed using the SPSS package version 6.1.3 (SPSS, Chicago, IL, USA).

Results

Thirty GC cases (48.40%) were localized in the lower third of the stomach, 22 (35.48%) in the middle third and 10 (16.12%) in the upper-third (four of which were localized at gastro-esophageal junction). Thirty-five GC cases (56.45%) were diagnosed histopathologically according to the WHO criteria as adenocarcinoma (tubular, papillary, tubulo-papillary, and mucinous), 20 cases (32.25%) as poorly cohesive carcinoma, and 7 cases (11.30%) were mixed both. According to Lauren's classification, 35 cases (56.45%) were classified as intestinal type, 20 cases (32.25%) as diffuse and 7 cases (11.30%) as mixed. Thirty-two of these 62 primary GC (51.61%) were recorded as low-grade tumors, while 30 cases were high grade (48.39%). HER2 immunohistochemical staining showed that 11 primary GCs (17.74%) were scored for 3+ HER2 expression, while 4 cases were 2+ (6.42%), 5 cases 1+ (8.10%), and 42 cases (67.74%) were not expressed HER2 at all. FISH analysis revealed no amplification in all of these cases with HER2 scores of 2+ or more. Taken together, in primary GC, HER2 was overexpressed in 11 cases (17.74%) but there was no HER2 amplification in 51 cases (82.26%). The overall concordance rate of HER2 status in primary GC between corresponding synchronous metastases was 90.32%, whereas a change in HER2 status was observed in 6 (9.68%) [Table 1], e.g. 4 cases with HER2 amplification in the primary GC but no amplification in the metastasized tumors [negative conversion; Figure 1a and b], two of these discordant cases did not show HER2 amplifications in the primitive tumor but amplified in the lymph node metastases [positive conversion; Figure 1c and d and Table 2].

In the primary BC, the most frequent histology type was ductal invasive carcinomas with the following grading: 4 G1 (6.25%), 28 G2 (43%), and 33 G3 (50.75%). HER2 overexpression occurred in 14 (21.53%) of primary BC, 4 (6.15%) of which exhibited a score 2+, 2 (3.09%) a score 1+, while 45 (69.23%) cases didn't

express HER2 at all. FISH analysis was conducted in those cases with the HER2 score of 2+ or more and

Table 1: Clinicopathological and HER2 concordance in 62 GC patients

	Discordant GC	Concordant GC	P
Gender			
Male	4	36	0.739
Female	2	20	
Site			
Lower	3	27	0.389
Middle	1	21	
Upper	2	8	
Lauren histotype			
Intestinal	3	31	0.369
Diffuse	1	19	
Mixed	2	6	
WHO histotype			
Tubular	4	31	0.672
Poorly cohesive	1	19	
Mixed	1	6	
Grade			
Low	4	28	0.728
High	2	28	
Stage			
I-II	3	21	0.875
III-IV	3	35	
T			
1-2	2	18	0.689
3-4	4	38	
N			
1	3	24	0.922
2-3	3	32	

GC: Gastric carcinoma; HER2: Human epidermal growth factor receptor 2

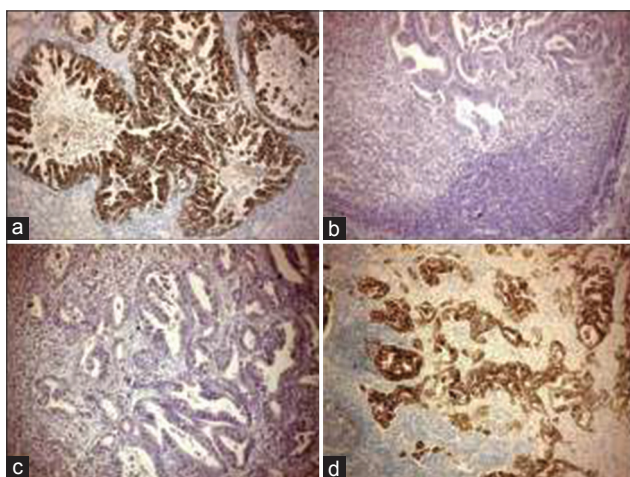


Figure 1: Expression of HER2 protein. A score of 3+ HER2 expression was encountered in neoplastic elements in a primary GC (a, $\times 200$) but vanished in the corresponding metastatic lymph node (negative conversion) (b, $\times 160$) (IHC, Mayer's hematoxylin counterstain). HER2 immunohistochemical negative staining in primary GC (c, $\times 200$), demonstrated a positive reactivity in the metastatic synchronous lymph node (positive conversion) (d, $\times 200$) (IHC, Mayer's hematoxylin counterstain). HER2: Human epidermal growth factor receptor 2; GC: Gastric carcinomas; IHC: Immunohistochemistry

the data revealed no HER2 amplification in these cases. Among 1+ cases, FISH was carried out in only two selected carcinomas showing high grade, high Ki-67 value, N+ status, and the absence of endocrine receptors expression, but no HER2 amplification was identified. HER2 was amplified in 14 BC cases (21.54%) but there was no HER2 amplification in these 51 cases (78.46%). The overall concordance rate was 95.39%, whereas changes in HER2 status between primary carcinoma and corresponding synchronous metastases were evidenced in 3 (4.61%) cases [Table 3]. Two of the discordant cases were HER2 negative in the primitive tumor but positive in the metastasized tumors [Figure 2a and b], whereas one case was HER2 positive in the primary BC and turned to negative in the metastatic tumor [Figure 2c and d and Table 4].

After that, we performed statistical analyses and found that the K value for the concordance rate in the HER2 status between primitive tumors and metastases was 0.651 (substantial agreement). HER2 amplification was significantly more frequent in the intestinal-type GC than that of diffuse-type while no significant differences in HER2 expression were noted among BC histology types. No statistical significant correlation emerged between HER2 and clinicopathological parameters (hormone receptors, growth fraction, pT, pN, and grade) either in GC as well as BC.

Discussion

In the current study, we retrospectively analyzed HER2 expression in surgical GC and BC specimens versus the corresponding metastatic lymph nodes. Our results firstly confirmed the presence of a high level of concordance in HER2 status between the primary GC/BC and their corresponding lymph node

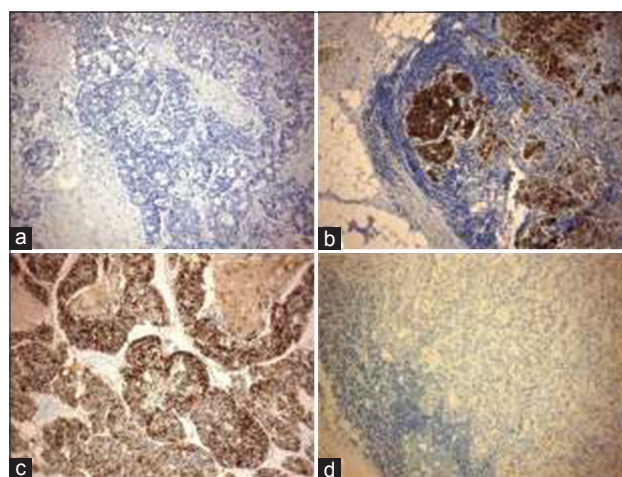


Figure 2: Expression of HER2 protein. A negative HER2 immunostaining in a primary infiltrative ductal carcinoma (a, $\times 160$) became positive in the lymph node metastasis (positive conversion) (b, $\times 120$). The strong and complete HER2 immunoreactivity in a case of primary BC (c, $\times 200$) was not present in the synchronous lymph nodal metastasis (negative conversion) (d, $\times 200$) (IHC, Mayer's hematoxylin counterstain). HER2: Human epidermal growth factor receptor 2; BC: Breast carcinoma; IHC: Immunohistochemistry

Table 2: HER2 discordant status in primary GC and corresponding synchronous nodal metastases

Sex	Stage	pT	pN	Histotype	Grade	Primary GC	Metastatic LN
Male	III	3	3	Mixed	High	3+	0
Female	III	2	3	Intestinal	Low	3+	0
Male	II	2	1	Intestinal	Low	3+	1+
Male	III	2	3	Intestinal	Low	3+	0
Male	II	3	1	Mixed	Low	0	3+
Male	II	3	1	Diffuse	High	2+*	3+

*Not amplified by FISH. LN: Lymph node; GC: Gastric carcinoma; HER2: Human epidermal growth factor receptor 2; FISH: Fluorescence *in situ* hybridization

Table 3: Clinicopathological and HER2 concordance in 65 BC patients

	Discordant BC	Concordant BC	P
ER status			
ER+	2	47	0.743
ER-	1	15	
PR status			
PR+	2	45	0.662
PR-	1	17	
Ki-67			
KI ≤ 14%	1	16	0.701
KI > 14%	2	46	
Grade			
G1	1	3	0.07
G2	0	28	
G3	2	31	
T			
1-2	2	39	0.630
3-4	1	23	
N			
1	2	22	0.630
2-3	1	40	

BC: Breast carcinoma; HER2: Human epidermal growth factor receptor 2; ER: Estrogen receptors; PR: Progesterone receptor

Table 4: HER2 discordant status in primary BC and corresponding synchronous nodal metastases

pT	pN	Grading	ER %	PR %	Ki-67 %	Primary BC	Metastatic LN
T2	N3	G3	90	15	20	3+	0
T1c	N1	G1	80	80	10	3+	1+*
T3	N1	G3	0	0	20	0	3+

*Not amplified by FISH. HER2: Human epidermal growth factor receptor 2; ER: Estrogen receptors; PR: Progesterone receptor; BC: Breast carcinoma; LN: Lymph node; FISH: Fluorescence *in situ* hybridization

metastases (90.32% and 95.39% respectively), which is consistent with previous observations of metachronous metastases (87.5-94.9%).^[19,23,25] Moreover, we also found evidence of HER2 differences between primary

carcinomas and their nodal metastases, that is, 9.68% GC cases and 4.61% BC cases did have the discordance between the primary and secondary tumors. Specifically, four cases had HER2 amplifications in the primary GC but there were no HER2 amplifications in the metastatic tumors. In contrast, two of the gastric discordant cases showed no HER2 amplifications in the primitive tumor but amplified in the lymph node metastatic tumors. Similarly, there were two of the discordant BC cases showed negative HER2 in the primitive tumor but became positive in the metastatic tumors, whereas one case was from positive HER2 in the primary BC to negative in the metastases. Therefore, a positive or negative conversion was encountered in either GC or BC cases, although with a different discordance rate. A possible explanation for the discordance observed in GC than in BC cases could be attributed to the most frequent occurrence of a heterogeneity in GC cases, compared to BC.^[18,21,26] Hence, the biopsies or tissue microarray assays do not seem adequate for assessment of HER2 expression, in contrast to that elsewhere reported.^[27,28] In addition, the multisampling method performed in this study using at least two tissue blocks of primary tumors and four of metastatic lymph nodes could identify more discordant cases and compensate a potential heterogeneous HER2 expression. The possible explanation of HER2 positive conversion may be related to the selection of a new HER2 positive clone in metastatic lymph nodes as a result of disease progression.^[29] Loss of HER2 amplification (negative conversion) in metastatic tumors could not be only attributed to the development of resistance to trastuzumab therapy since our patients had not been subjected to any neo-adjuvant treatment.^[29]

Changes in HER2 status between primary GC/BC and synchronous lymph node metastases may have relevant clinical impact. For example, only HER2 positive GC and BC currently support the use of trastuzumab in these patients; thus, our present finding suggests a need to reassess HER2 status before trastuzumab treatment. As a matter of fact, assessment of HER2 expression in the primary GC and BC may exclude from the targeted treatment a significant percentage of patients with a negative primary tumor, but positive metastases. Finally, the influence of discordant HER2 status in the therapeutic management as well as in the prognostic impact of patients affected by GC and BC should be greatly considered in order to correctly identify possible eligible candidates for trastuzumab-based therapy, even among patients with HER2 negative primary carcinomas.

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Incidence of bone metastasis in squamous cell carcinoma of the buccal mucosa

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ABSTRACT

Aim: This retrospective study was performed to show the incidence of bone metastasis from carcinoma of the buccal mucosa. Head and neck cancer is a leading health problem in India due to an increased incidence of tobacco use and poor oral hygiene. Squamous cell carcinoma of the buccal mucosa is common and roughly 2.5% of all malignancies that present to our center. Moreover, most patients present at late stages (III/IV) and consequently, survival rates are low. Bone metastasis in advanced cases of such carcinomas is rarely reported worldwide but is more prominent in parts of India. **Methods:** Here, we present a series of patients diagnosed with buccal mucosa carcinomas within the past 5 years that also demonstrated bone metastases. **Results:** These patients were young, with a history of tobacco chewing with locally advanced disease and bone metastases that developed within one year of diagnosis. Flat bones and vertebrae were mainly involved and the survival was short after diagnosis of metastasis despite treatment with local radiotherapy and chemotherapy. The cause of such frequent metastases cannot be proved but subclinical seeding of malignant cells before the eradication of the primary tumor is probable contributory with advanced local and nodal disease with high grade tumor. **Conclusion:** A pretreatment bone scan should be performed in locoregionally advanced buccal mucosa carcinomas at the time of diagnosis to define the treatment plan.

Key words: Bone metastases, buccal mucosa, squamous cell carcinoma

Introduction

Carcinoma of the buccal mucosa is the most common oral cavity cancer diagnosed in India. The National Cancer Registry Programme of the Indian Council of Medical Research estimates that head and neck cancer forms 20% of all new cancers in India. Males of the Ahmedabad urban area showed the highest age adjusted rate (AAR) for mouth cancer (12.9), followed by Bhopal (9.9). For females, however, Bengaluru showed^[1] the highest AAR (6.5) followed by the Kamrup urban district (5.8). In hospital, based cancer registry reports, cancer of the mouth is also ranked as the leading site in Mumbai in males and within the first five leading sites in all registries in males. In developed countries, carcinoma of the buccal mucosa is relatively uncommon compared to the Indian subcontinent. The high incidence of carcinoma of the buccal mucosa in our country is attributable to the oral consumption of tobacco, betel leaves, and nuts with lime. Alcohol, smoking habits, and poor socio-economic conditions also are contributing factors. Here, two-thirds of head and neck cancers

present in an advanced local and nodal stage, leading to poor results, with chances of distant metastasis also increasing. Importantly, up to 70% of patients diagnosed with advanced solid tumors develop bone metastases primarily from breast and prostate carcinoma. Bone metastasis is rarely seen in head and neck cancers and primary buccal mucosa malignancies rarely metastasize to distant sites. They usually metastasize to lymph nodes or spread locally. The development of newer radiotherapy techniques and availability of better chemotherapy drugs used concurrently have led to better control of such cancers. In fact, better control of local disease may lead to an increased incidence of distant metastasis,^[2] affecting survival. Bone metastases depend on the primary site of involvement, T and N stage and control of the nodal disease. It has been shown that patients presenting with advanced nodal disease show a higher incidence of distant metastasis, especially when there is extensive soft tissue or jugular vein involvement in the neck.^[3] In this study, we found a surprisingly high incidence of bone metastasis in carcinoma of the buccal mucosa patients, mostly in those who underwent surgery. Thus, we present the incidence and discuss possible causes of such metastasis and provide treatment recommendations. This study was approved by review board of SAIMS.

Methods

From January 2008 to October 2014, a total of 5791 cases of cancer were registered at the Sri Aurobindo Hospital in Central India. Head and neck cancer represented 25.8% of all malignancies and carcinoma of the buccal

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mucosa was 9.9% of all head and neck cancers and 2.6% of all malignancies. It was more common in males than females, with a ratio of 4:1. Tobacco chewing and poor oral hygiene were common factors associated with the patient characteristics. Carcinoma of the buccal mucosa was more common in younger individuals, with median age at diagnosis of 45.87% patients presented in stage III and IV and 75% of patients reported after surgery for adjuvant treatment. Four patients all developed bone metastases. All 4 patients had locally advanced disease [Table 1] and all underwent hemi-mandibulectomy with ipsilateral neck node dissection. Patients then received postoperative concurrent chemotherapy and radiotherapy, 60 Gy in 6 weeks with 6 MeV photons by linear accelerator. Upon completion of treatment, all 4 patients had local control but within 2-8 months, all 4 patients developed bone metastases. At the time of diagnosis of bone metastasis, patients received local radiotherapy, 30 Gy in 10 fractions to the involved bone, followed by chemotherapy with cisplatin and paclitaxel. None completed the planned six cycles of chemotherapy and had died within 6-9 months.

Results

Carcinoma buccal mucosa was reported in 9.9% of all head and neck cancers, although most patients did not complete the planned treatment. On follow-up, of 148 patients, 24 (16.2%) had local recurrence within one year, one patient developed second primary after 8 years, and 2 patients had lung metastasis and 4 patients developed bone metastasis. The incidence of bone metastasis was 0.2% of all head and neck cancer as compared to 0.1% reported in the literature worldwide.^[1] Sacrum, pelvis, vertebrae and index finger were commonly involved. Bone metastases developed 6-9 months upon completion of primary treatment, with all 4 patients presenting with locally advanced disease and nodal metastasis. All 4 patients underwent surgery as the primary treatment, followed by adjuvant concurrent chemotherapy and radiotherapy. All patients had grade II to III squamous cell carcinoma. Our incidence of bone metastasis of all carcinomas of buccal mucosa was 2.71%. We could not find the reported incidence in the literature worldwide. All 4 patients had advanced local (T4) lesions and 3-8 nodes were involved. None had extra nodal spread but one had perineural spread. All had deep muscle infiltration, with 2 patients also having mandibular bone involvement. After postoperative radiotherapy, all had local control but within 6-9 months, patients complained of severe local bone pain and an X-ray/computed tomography scan showed lytic bone lesion at the site of involvement in all 4 cases [Figures 1-3]. Fine needle aspiration cytology from the bone metastatic sites of all four cases indicated pathology consistent with metastatic disease [Figure 4]. All patients received palliative local radiotherapy to the involved bone to relieve pain, followed by chemotherapy

Table 1: Four patients developing bone metastases all had locally advanced disease

No.	pT stage	pN stage	Invasion	Tumor grade
1	2	2b	Deep muscle	I
2	4	2b	Mandible	II
3	4	2b	Deep muscle	II
4	4	2b	Muscle and mandible	II

with cisplatin and paclitaxel. After radiotherapy, all patients had complete bone pain relief. Despite treatment, the disease progressed and all patients died within 6-9 months of development of bone metastasis.

Discussion

Head and neck squamous cell carcinoma has a high propensity for loco-regional spread through lymphatic and/or hematogenous spread and occurs in about 10% of cases. Sites of metastases most commonly include the lungs, brain, bones, and skin.^[4] Newer diagnostic regimens and more thorough work-up at diagnosis have improved our understanding of squamous cell carcinoma and consequently loco-regional control of cancer above the clavicles has increased.^[5] However, the overall disease-free survival rate has not improved^[6] and the incidence of distant metastases and second primary tumors has increased.^[7] Risk factors for hematogenous spread include higher tumor stage, size of the primary lesion (T4), tumor grade, and the lesion site. The incidence of distant metastasis is hypopharynx 60%, base of tongue 53%, and anterior tongue cancer 50%.^[8]

Distant metastasis to bones from buccal mucosa is extremely rare and we could find only one report.^[9] In contrast, in the last 5 years, our center diagnosed 4 cases of squamous cell carcinoma of the buccal mucosa which had metastasized to bones. All patients were young, had T4 disease, and grade I-II squamous cell carcinoma and were using chewing tobacco.

Distant metastases were all seen within one year of completion of primary treatment. Thus, there is probably subclinical seeding of malignant cells before the eradication of the primary tumor. The average survival with distant metastasis ranged between 21 and 33 weeks.^[10,11] In this series, bone metastases occurred within an average of 9 months from diagnosis and survival was only 6-9 months after development of bone metastasis.

There was one study of patients with locally advanced head and neck cancers at presentation who developed metastases.^[12] The usual primary sites were base of tongue and tonsil, with solitary bone metastases and a postoperative buccal mucosa case where multiple osteolytic bone lesions were seen. The cause of distant metastases after local control is not known although all patients who developed bone metastasis had advanced

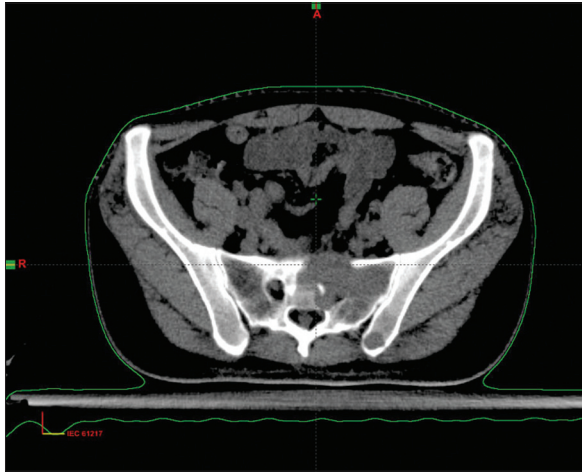


Figure 1: Osteolytic lesion with soft tissue involvement in sacrum

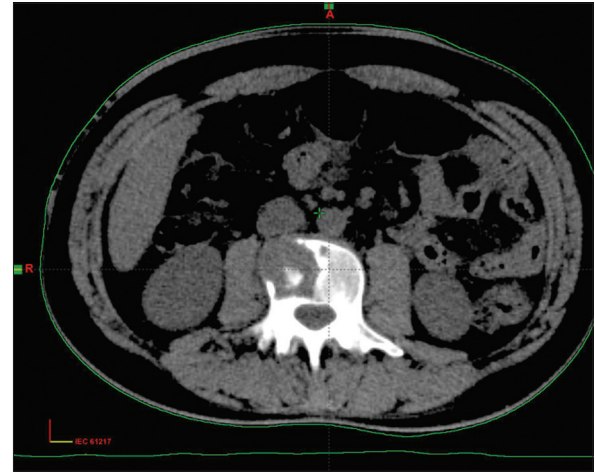


Figure 2: Osteolytic lesion in lumbar vertebra



Figure 3: Osteolytic lesion in proximal phalanx of index finger

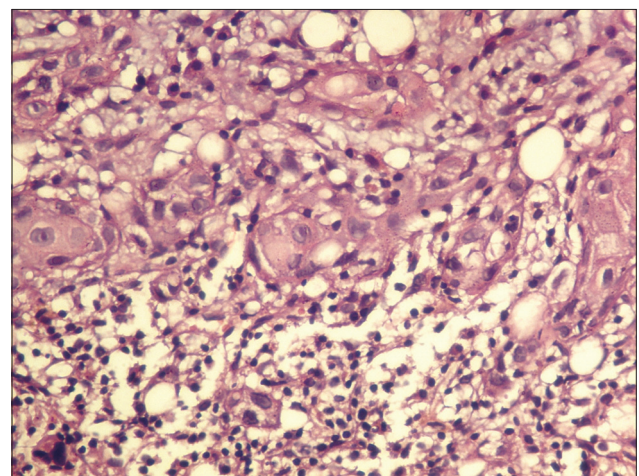


Figure 4: Squamous cell carcinoma metastasis within bone marrow (×40)

nodal disease and one patient also had extra capsular spread. All patients in our study had undergone surgery and had advanced local and nodal disease at presentation.

A strong correlation was seen between clinical nodal disease and pathologically involved lymph nodal status. Patients with clinically palpable lymph nodal (N1-N3) disease were operated and histologically had three or more lymph nodes showing metastases with extra capsular spread and/or lymphovascular invasion were more prone to develop distant metastasis. Also, in present study, the patients who developed bone metastasis had higher nodal disease [Table 1].

Axial skeleton is the most common site of bone metastasis in our cases, involving spine, pelvis, and ribs, with lumbar spine being the most common.^[13] In the appendicular skeleton, the proximal femur and humerus are mainly involved. Patients in this series have involvement of the flat and appendicular bones which are the usual sites involved. One study reviewed radiographs and nuclear medicine studies of 363 patients of head and neck cancers retrospectively.^[14] It was found that 1% developed bone metastasis, mainly involving pelvic bones, femur, humerus, ribs, and thoracic vertebra. These lesions were mainly osteolytic, with moth-eaten or permeated borders. In our series, we also found that the flat parietal bones of skull, ribs, and sacrum, and long bones such as shaft of femur and radius were involved. Osteolytic lesions usually appeared within 3-12 months

of completion of the primary treatment. The prognosis of carcinoma buccal mucosa patients who develop bone metastasis is usually poor with a median survival about 8 months.^[15] We also saw that bone metastases occurred an average of 9 months after the primary treatment.

A probability of subclinical seeding of malignant cells before the eradication of the primary tumor should be considered. In young patients with locally advanced disease distant metastases can affect different organ systems including the bones and almost invariably herald a poor prognosis. Treatment is always palliative and survival remains less than one year. In locoregionally advanced cases of all head and neck carcinoma cases, a bone scan should be done prior to definitive treatment in order to avoid unnecessary local treatment and start systemic treatment earlier to improve survival.

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Early stage squamous cell carcinoma of the tonsil presenting with multiple organ metastases including skin and brain after successful local treatment

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ABSTRACT

Early stage carcinoma of the tonsil is curable, and the incidence of systemic metastasis is very low and central nervous system involvement is very rare. A patient diagnosed with early stage tonsillar carcinoma treated with chemoradiation was followed by brachytherapy boost. One and half years after completion of treatment, the patient presented with disseminated metastasis to the skin, lung, liver, bone, and brain. He had all favorable prognostic parameters except being a young adult.

Key words: Brain metastasis, distant metastasis, early stage cancer tonsil, skin metastasis

Introduction

Early stage carcinoma of the tonsil is curable with a very low incidence of distant metastasis. Advanced stage carcinoma of the oropharynx has a probability of distant metastasis from 15% to 20%.^[1] Considering all stages and sub-sites of head and neck cancer, the incidence of distant metastasis is reported to be of about 10-15%.^[2] Evidence of distant metastasis in early stage disease is not available in the literature. We diagnosed a case of early stage carcinoma tonsil that had been treated effectively with concurrent chemoradiotherapy followed by brachytherapy boost. The patient had locally controlled disease but developed multiple visceral metastases through hematogeneous dissemination within a few months of treatment completion.

Case Report

A 34-year-old Indian male with a history of being a chronic smoker and alcoholic presented with complaints of dysphagia, foreign body sensation during deglutition of 3 months duration. On physical examination, the patient appeared to be in a good performance and adequate nutritional status. Clinical examination revealed a proliferative growth at left tonsil without evidence of any palpable neck nodes. Direct laryngoscope revealed

proliferative growth of 3 cm diameter at left tonsil; involving the anterior and posterior pillar and encroaching to the soft palate and lateral pharyngeal wall. The valleculae and epiglottis were free. The histopathological report of the tonsillar fossa biopsy indicated moderately differentiated, infiltrating squamous cell carcinoma. Contrast-enhanced computed tomography scan of the head and neck revealed 3.1 cm × 2.2 cm sized mass at the left tonsillar fossa. In the neck, there was no evidence of lymph node metastasis. Ultrasonography (USG) of the abdomen and pelvis was normal except for mild fatty changes in the liver. Chest skiagram was normal. Hence, the patient was diagnosed as a case of carcinoma tonsil cT₂N₀M₀ and was planned for radical therapy. He was treated with external beam radiotherapy (EBRT) of 6 MeV photon with conventional bilateral portal plan, 50 Gy in 25 fractions, along with 5 cycles of concurrent chemotherapy with cisplatin (65 mg). Thereafter, a boost radiation by interstitial high-dose-rate brachytherapy of 20 Gy/5 fractions to residual tumor and tonsillar fossa was given.

After completion of treatment, the patient was asked to visit the follow-up (FU) for clinical evaluation every 3 months. The 1st year of FU was unremarkable. At the FU visit at 18 months, he presented with a recent history of dry cough for more than 1 week. Chest skiagram showed only pneumonitic changes at the lung bases. He was managed conservatively and responded well. At the FU of 20 months, the patient was suffering from backache which has progressed over a short period in spite of taking analgesics. Digital X-ray of the lumbar sacral (LS) spine revealed no obvious findings. However, within a few weeks, the pain increased severely, and the patient was unable to walk or stand without support. Clinically he had a severe tenderness over the lumbar

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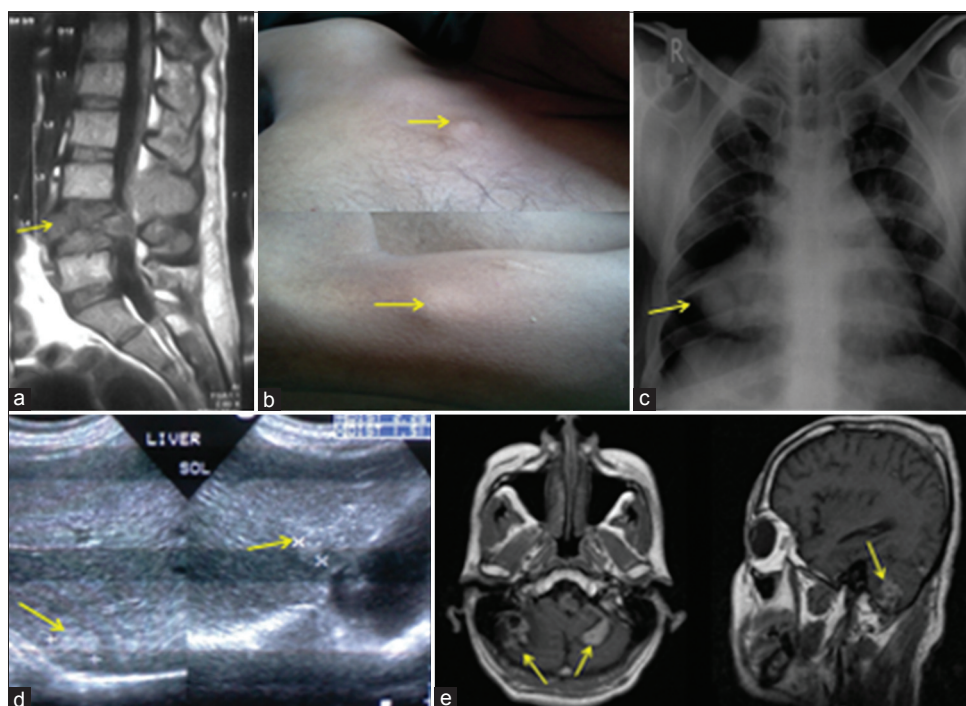


Figure 1: Multiple metastatic sites in a treated case of early stage cancer of tonsil: (a) spine; (b) skin nodules; (c) lungs; (d) liver; (e) brain. The yellow arrows in the respective sites point to the metastatic sites

spine. Magnetic resonance imaging of LS spine showed wedge compression fracture of L4 vertebral body and altered marrow signal intensity with associated periosteal component at L3 and L4 level; it's likely to be a metastasis [Figure 1a].

There was no evidence of loco-regional failure. During clinical examination of the patient multiple subcutaneous nodules were found, mainly in the upper part of the body (over chest, arms, nape of the neck, scalp) [Figure 1b]. Excision biopsy from the nodule over left arm showed histopathological features of metastatic deposit from squamous cell carcinoma. He underwent a metastatic workup. His chest skiagram showed bilateral, multiple nodular opacities suggestive of metastasis [Figure 1c]. USG whole abdomen also revealed multiple hypoechoic space occupying lesion in both lobes of the liver (largest being 2.25 cm × 1.5 cm) [Figure 1d].

Magnetic resonance imaging of brain central nervous system (CNS) showed multiple altered signal intensity in both cerebellar hemispheres and also similar deposits at right parietal, left temporal, parietal and occipital bony calvarium, and adjacent scalp resulting in bony destruction [Figure 1e]. He was treated by palliative radiotherapy (30 Gy in 10 fractions) to whole brain and to lumbar the spine along with other supportive care. He had 50-60% of pain relief and marginal improvement of neurological symptoms. After treatment, he was taken home and offered best supportive care; he died after 3 months of completion of radiotherapy.

Discussion

For head and neck squamous cell cancers, only 5% of cases with loco-regionally controlled disease may present with distant metastasis.^[2] Early stage cancer of head and neck is considered to have a good prognosis in general irrespective of sub-site or stage of the disease. Carcinoma tonsil with T₂ disease is considered to have 80% curability. Treatment with EBRT followed by brachytherapy boost is a standard practice.^[3] The case merits discussion for a number of reasons. In the first place, this is probably the first case reported in English literature with a head neck malignancy having such widespread metastasis involving lung, liver, bones, skin, and brain. The literature supports that the distant metastasis from carcinoma tonsil commonly spreads to lungs and rarely to bones or liver.^[4,5] However, involvement of so many organs is unknown. Second, there has been a very few reports of head and neck cancers metastasizing CNS by the hematogenous route.^[1,6] The natural history of the disease in this patient confirms surely that he had a hematogenous metastasis to the CNS along with other organs.

The current treatment options limit the survival of metastatic head and neck cancers patients to < 1 year^[7] and our patient with extensive multiorgan metastasis survived for only 3 months.

We attempted to identify the risk factors for distant metastasis in head and neck carcinoma by retrospective analyses.^[4,8] In univariate and multivariate analysis, the most significant factors were neck node

involvement (number, level of neck node involved) and site of the primary tumor ($P < 0.001$). Among other factors, T stage of the primary tumor, histopathological grade of tumor, response to treatment, and young age are also mentioned but with varying significance in different studies. In this context, this case had features of favorable prognostic group (stage cT₂N₀M₀, tonsil, moderately differentiated squamous cell carcinoma, excellent loco-regional control) but except for young age. Similar cases need to be reported and documented so that more aggressive FU can be advised in this group of patients.

The reasons for this unusual presentation of such widespread metastasis in a patient with apparently good prognostic factors is not known. However, we came across two very interesting reports while preparing this case report. In a study on prostate cancer patients treated by brachytherapy, the authors concern that the cells liberated at the time of brachytherapy increases the risk of metastatic deposits and may results in a systemic failure, as measured by serum prostate-specific antigen levels.^[9] Similar observation had been made in case of a glioblastoma multiforme is treated by brachytherapy.^[10] There are no further evidences in this regards, but those interesting incidences need to be reviewed in the context of our case to find out the rarest possibility of any such mechanism.

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Malignant ascites with omental metastasis: a rare event in prostate cancer

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ABSTRACT

Prostate cancer is the most common type of male malignancy in the world and approximately 10-20% of prostate cancer shows a metastatic disease at initial diagnosis commonly to the bones, vertebrae, ribs, long bones, and skull. However, prostate cancer metastasis to the omentum with malignant ascites is extremely uncommon. In this study, we report such a case, which also highlights a repeatedly negative ascitic fluid cytology even with multiple omental metastatic nodules. The purpose of this case report is to provide awareness to physicians for this rare occurrence.

Key words: Immunohistochemical staining, malignant ascites, omental metastasis, prostate cancer, prostatic specific antigen

Introduction

Prostate cancer is the most common male malignancy in the world with an estimated 1,100,000 new cases and 307,000 cancer-related deaths in 2012.^[1] Although most prostate cancer patients have localized disease with a favorable prognosis, advanced prostatic cancer metastasize frequently to the bone and regional lymph nodes, but prostate cancer metastasis to the omentum with malignant ascites is very rare.^[2] In this study, we report such a rare case.

Case Report

A 65-year-old man was initially diagnosed as prostate adenocarcinoma with Gleason score 7 (4 + 3 = 7 out of 10) in 2004. The level of prostatic specific antigen (PSA) was 233 ng/mL at cancer diagnosis. He then underwent bilateral orchiectomy and hormonal therapy with 50 mg dose of bicalutamide, but discontinued after 5 months treatment. In 2009, his PSA level raised to 90 ng/mL, but there was no evidence of metastasis detected by either computed tomography (CT) or bone scan. He was again on bicalutamide treatment, but his PSA response lasted for approximately 2 years. In December 2011, bicalutamide treatment was discontinued, and Fosfestrol was started. However, in November 2013, his PSA level was increasing to 27.4 ng/mL and therefore, fosfestrol was discontinued and the patients were treated with ketoconazole and prednisolone.

One month after this regime of treatment, patient presented with 10 days history of abdominal distension and found to have gross ascites. A diagnostic and therapeutic paracentesis was conducted and removed 1,500 mL straw colored fluid. Fluid analysis showed to be exudate and cytology was negative for malignant cells. Ascitic fluid adenodeaminase titer and polymerase chain reaction showed negative for tuberculosis. Ascitic fluid was taken and tested for multiple times, but all were negative. Moreover, esophageal-gastroduodenoscopy and colonoscopy were normal. Contrast-enhanced CT abdomen in March 2014 showed prostatic mass with gross ascites with thickened omentum [Figure 1]. Bone scan shows no evidence of skeletal metastasis. Serum and ascitic PSA were 316 ng/mL and 175 ng/mL, respectively. A ultrasound-guided biopsy of the thickened omentum and histology showed a metastatic adenocarcinoma [Figure 2], which was immunohistochemically positive for cytokeratin (CK) and PSA [Figure 3] and focally positive for CK7, whereas negative for CK2. Patient was then planned for Taxotere-based chemotherapy.

Discussion

Although prostate cancer can metastasize to nearly any organs in the body, metastasis without osseous involvement is extremely rare. Arnheim showed in 1948 that in 176 postmortem cases, the bone, lymph nodes, and lungs were the most common metastasis of prostate cancer,^[2] whereas the uncommon metastasis sites included the adrenal gland, kidney, brain, pancreas, genitalia, and breast. Malignant effusion, whether pleural or peritoneal, was an extremely rare.^[2] Moreover, Rapoport and Omenn^[3] reviewed the autopsy of 523 prostate cancer cases and found that 13 cases had peritoneal deposits, but with no other metastasis elsewhere in the body,

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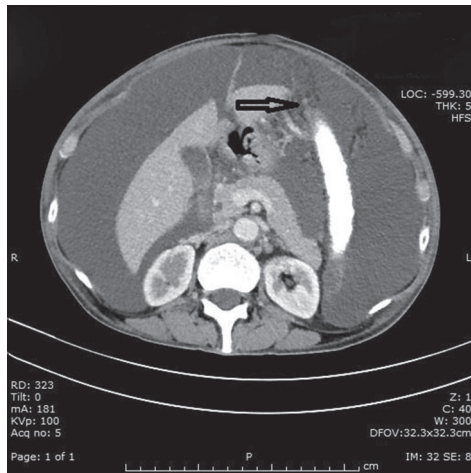


Figure 1: The computed tomography (CT) scan. The CT data show diffuse nodular thickening (black arrow) of the omentum and ascites

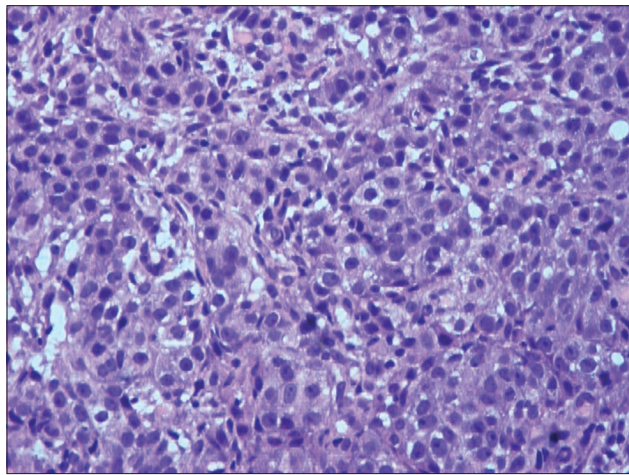


Figure 2: Hematoxylin and eosin staining of the omental biopsy. Tissue section shows omental tissue infiltrated by poorly differentiated adenocarcinoma

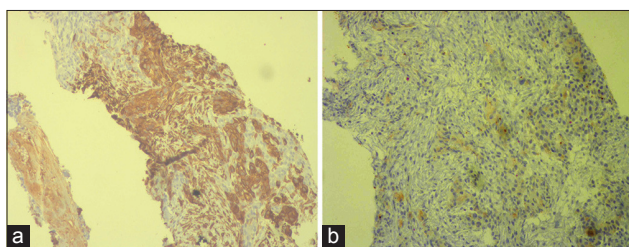


Figure 3: (a) Immunohistochemical staining of tissue biopsy for pan-cytokeratin; (b) prostatic specific antigen

indicating that there were effusions occurring in prostate cancer patients without any involvements of the more common metastatic sites. Thus, if other benign (like tuberculosis in India) and malignant (especially gastrointestinal)^[4] etiologies should be excluded, these

patients could reasonably be thought to be due to prostate cancer-induced ascites. Until date, there have been only 16 published cases of malignant ascites in prostate cancer^[5] and most cases presenting with malignant ascites were associated with other metastatic sites, including the bone, lymph nodes, omentum, rectal wall, liver, adrenal, and pleural effusions.^[6]

The mechanism of malignant ascites may include peritoneal seedlings or lymphatic obstruction. Tumor cells in an effusion may have exfoliated from the primary lesion as evidenced by the positive cytology after repeated cytological examinations of ascetic fluid. However, negative cytology could be very difficult to make a differential diagnosis between benign and malignant ascites, such as the current case. The immunohistochemical staining can provide a valuable adjunction. For example, immunostaining of prostatic acid phosphatase and/or prostate specific antigen could be useful in the diagnosis of prostate cancer with a malignant effusion.^[7] Usually, malignant effusions in prostate cancer patients are associated with very poorer prognosis.^[8]

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Complete response with fotemustine and bevacizumab after early progression following radiotherapy and temozolomide treatment in patient with glioblastoma multiforme

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ABSTRACT

Glioblastoma multiforme is the most common type of primary central nervous system tumor and is noted for its short survival and poor response to chemotherapeutic agents. Unfortunately, the relapse rate is very high, and there is no reference drug for second-line treatment. In this study, a patient was treated with the Soffietti regimen. The induction phase was fotemustine 75 mg/m² at day 1 and day 8 and bevacizumab 10 mg/kg at day 1 and day 15. The maintenance phase was fotemustine 75 mg/m² and bevacizumab 10 mg/kg every 3 weeks for two cycles. Follow-up magnetic resonance imaging showed post-surgical changes at the left occipital level, without contrast enhancement, and toxic left leuko-encephalopathy post-treatment without mass effect and with no evidence of tumor residue. The patient then was maintained with bevacizumab monotherapy until it was withdrawn when pulmonary thromboembolism occurred. Following tumor regrowth, fotemustine was started again as maintenance therapy. The patient achieved stabilization of his disease until his death due to thromboembolic and infectious complications.

Key words: Bevacizumab, brain tumor, fotemustine

Introduction

Glioblastoma multiforme (GBM) is the most common type of primary central nervous system (CNS) tumor and is noted for its short survival and poor response to chemotherapeutic agents.^[1] Adjuvant temozolomide and radiotherapy is the gold-standard treatment.^[2] Unfortunately, the relapse rate is very high, and there is no reference drug for second-line treatment.^[3-5]

Case Report

This report describes the case of a 58-year-old patient with a history of hypertriglyceridemia and psoriasis who was admitted to the emergency department after a 4-day episode of disorientation to time and place, speech disturbance, 2/5 lack of muscle strength, right hemi-temporal blindness, and motor dysphasia. Chest, abdominal, and pelvic computed tomography was unremarkable. A brain magnetic resonance imaging (MRI) showed an oval left parieto-occipital lesion with the anteroposterior diameter of 27 mm, nodular contrast medium enhancement and white matter edema. In February 2011, the lesion was resected and

was diagnosed as a WHO grade 4 GBM, with a 30% mind bomb E3 ubiquitin-ligase 1 proliferation index.

In March 2011, external radiotherapy (total dose 60 Gy, fractioned in 2 Gy/day) was started with concomitant temozolomide at 75 mg/m²/day, followed by temozolomide monotherapy (150 mg/m² for 5 days each 28 day cycle in the first cycle, and 200 mg/m² in the second cycle). Thereafter, an episode of gait imbalance with motor disturbance of the right upper limb occurred.

Three months after finishing radiotherapy, a brain MRI showed a cystic left parieto-occipital lesion measuring 40 mm × 40 mm × 30 mm, and edema. This MRI suggested tumor relapse [Figure 1a].

The patient rejected surgery and chemotherapy according to the Soffietti *et al.*^[6] regimen was started. The induction phase was fotemustine 75 mg/m² at day 1 and day 8 and bevacizumab 10 mg/kg at day 1 and day 15, followed by an interval of 3 weeks, and maintenance phase: fotemustine 75 mg/m² and bevacizumab 10 mg/kg, every 3 weeks for two cycles. Follow-up MRI showed post-surgical changes at the left occipital level, without contrast enhancement, toxic left leuko-encephalopathy post-treatment, without mass effect and with no evidence of tumor residue [Figure 1b]. There was a clinical response and from a radiological point of view, the mass had disappeared and there was no contrast enhancement (Response Assessment in Neuro-Oncology criteria were used to assess this).^[7] The patient was discharged on a physiological replacement dose of corticosteroids and maintenance bevacizumab monotherapy.

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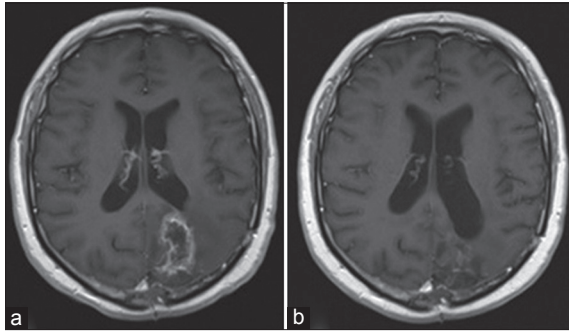


Figure 1: (a) Magnetic resonance imaging 3 months after finishing radiotherapy during temozolomide monotherapy; (b) response after two cycles of fotemustine and bevacizumab treatment

In April 2012, pulmonary thromboembolism occurred, and the bevacizumab was withdrawn. Low molecular weight heparin treatment was initiated. Two months later, repeat MRI showed a 2.5 cm enhancement area in the surgical site, suggesting tumor relapse. In June 2012, fotemustine was restarted as monotherapy at 75 mg/m² every 3 weeks (two cycles). In August 2012, MRI showed tumor stabilization. In November 2012, the patient suffered bronchoaspiration and unfortunately died.

Discussion

Fotemustine is a third-generation nitrosourea with alkylating cytotoxic activity and high lipophilicity that allows it to cross the blood-brain barrier. It achieves therapeutic levels in the CNS and has proven antitumor activity, either as monotherapy or in combination.^[5,8-10] Bevacizumab is a humanized monoclonal antibody that inhibits vascular endothelial growth factor and has activity in distinct tumors like GBM, either in monotherapy or combination with irinotecan.^[11,12] The combination of these two drugs has shown promising results. Soffietti *et al.*^[6] published the results of a phase II study in which fotemustine and bevacizumab were combined according to the following scheme: induction phase (fotemustine 75 mg/m² at day 1 and day 8, and bevacizumab 10 mg/kg at day 1 and day 15); followed by an interval of 3 weeks, and maintenance phase (fotemustine 75 mg/m² and bevacizumab 10 mg/kg, every 3 weeks) until tumor progression, unacceptable toxicity, or withdrawal of consent. The combination of these two drugs showed promising results: overall response rate was 52%, and a significant neurologic improvement was observed in 60% of symptomatic patients. Progression-free survival at 6 months was 42.6%, and overall survival at 6 months was 75.9%. Median progression-free survival was 5.2 months, and median overall survival was 9.1 months. Toxicity^[6] with this regimen was predictable and manageable; grade 1 or 2 appeared in the majority of patients. Neutropenia (13%), thrombocytopenia (9%), wound dehiscence (5.5%), and deep venous thrombosis (4%) are the main grade 3 toxicities. Pulmonary embolism appeared as grade 4 toxicity in 4% of patients. These results encouraged us to use this therapeutic regimen in our patient.

The early initial progression, occurring shortly after the second adjuvant temozolomide cycle, made us consider a scheme that could achieve a high rate of disease control. The outstanding response obtained at 4 months of treatment with fotemustine plus bevacizumab, without radiological evidence from the pre-existing tumor mass, prompted us to continue with bevacizumab maintenance.^[12] When this patient had received temozolomide monotherapy, he presented with instability and vertiginous symptoms. During bevacizumab and fotemustine therapy, the neurological symptoms disappeared. The withdrawal of bevacizumab after 7 months of treatment, due to pulmonary thromboembolism, caused a relapse of the disease. Nonetheless, the patient achieved stabilization of the disease from reintroduction of fotemustine until his death due to thromboembolic and infectious complications.

We consider this case interesting because treatment with bevacizumab plus fotemustine achieved rapid response, in 4 months, in a patient with rapid progression to first-line treatment. Furthermore, it is notable because the patient responded to treatment with fotemustine after the progression that occurred following withdrawal of bevacizumab maintenance.

We consider that this combination scheme should be tested in further clinical trials. Due to the promising results reported by Soffietti *et al.*, and confirmed by our own clinical experience, fotemustine should be considered as rescue treatment for relapsed GBM.

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Metachronous metastasis of renal cell carcinoma to bilateral testis

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ABSTRACT

Unusual site metastasis as a presenting complaint of renal cell carcinoma (RCC) has been reported previously in the literature. RCC is a tumor with notoriously unpredictable behavior. The authors report an unusual case of metachronous bilateral testicular metastasis in a patient who operated for RCC. The case highlights the unique behavior of RCC with an unusual site of metastasis. A 72-year-old patient presented with bilateral scrotal swelling of 1-month duration. There was a history of left radical nephrectomy for RCC 4 years prior. He underwent a bilateral high inguinal orchidectomy and diagnosis of chromophobe RCC was made on histopathological examination.

Key words: Metachronous metastasis, renal cell carcinoma, testis

Introduction

Renal cell carcinoma (RCC) is a relatively rare adult solid tumor accounting for 3.0% of malignancies worldwide. It is an unpredictable entity due to its atypical metastatic profile at presentation. Thirty percent of these tumors may be accompanied by synchronous metastatic disease at diagnosis.^[1] The organs most affected by metastatic spread are: lung, bone, liver, brain, and lymph nodes.^[2]

However, other structures can also be affected by RCC metastases: eyes, mouth, neck and thyroid, heart, breast, rectum abdominal muscle, intra-scrotal structures, and vagina.^[3] Although metastatic foci are present in about 30.0% of RCCs at the time of primary diagnosis (synchronous), metastatic disease can develop as part of the latency of the tumor, with delayed development of metastases, especially if the tumor is well-differentiated.

Case Report

A 72-year-old smoker presented to surgical outpatient department (OPD) with a complaint of a progressively increasing bilateral scrotal swelling of 1-month duration. There was a history of left radical nephrectomy for RCC 4 years prior. His general physical examination was unremarkable. Local examination showed bilateral hard testicular masses, 12 cm × 5 cm on the right side and

10 cm × 6 cm on the left side, extending to epididymis, with absent testicular sensation. Examination of the abdomen did not reveal any abnormality. Blood samples for serum lactate dehydrogenase, β human chorionic gonadotropin, and serum alpha fetal protein were sent, which were found to be within normal limits. Metastatic workup was done: contrast-enhanced computed tomography of whole abdomen and pelvis was within normal limits. Ultrasound of testis showed bilateral homogenous enlargement of testis size 10 cm × 5 cm × 3 cm on right side and 10 cm × 5 cm × 3 cm on left side extending to epididymis, with focal areas of necrosis suggestive of testicular malignancy. After all routine hematological and biochemical investigations, he was consented and undertook a bilateral high inguinal orchidectomy. Post-operative period was uneventful.

Biopsy finding

On gross examination: right testicular mass of 13 cm × 8 cm × 6 cm with cut surface showing a solid variegated appearance. Left testicular mass of 10 cm × 8 cm × 6 cm with cut surface showing a solid variegated appearance. Microscopy revealed malignant tumor cells arranged in large islands and nests in the interstitium. The tumor cells were large with clear cytoplasm in a fair number of cells, and eosinophilic cytoplasm in other cells with moderate nuclear pleomorphism along with some mitotic figures [Figure 1a]. The seminiferous tubules and epididymis were pushed to one side [Figure 1b], but all the margins were free and testicular vein was not involved on either side.

Immunohistochemistry was done epithelial membrane antigen positive and cytokeratin pan, S100, CD10 were negative. A diagnosis of chromophobe variety of RCC was made.

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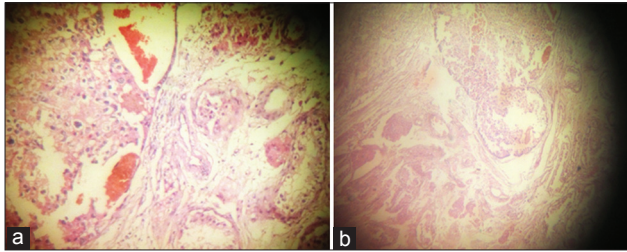


Figure 1: (a) Histopathology shows malignant tumor cells arranged in large islands and nests in the interstitium; (b) histopathology shows the seminiferous tubules and epididymis were pushed to one side

Post-operative follow-up

Patient was followed up every 3 months in surgical OPD. Clinical examination routine hematological investigations and abdominal ultrasound were undertaken at each visit. He was asymptomatic till 2 years post-operatively but is now lost to follow-up.

Discussion

An unpredictable clinical behavior is often characteristic of RCC. The occurrence of metastatic localizations in unusual sites is widely described. The interval between primary diagnosis and the occurrence of distant metastasis can vary from synchronous to very long.^[4] RCC metastasizing to testes is rare. Amongst the urinary tract malignancies, prostate is the most common primary site for testicular secondaries constituting 35% of all testicular malignancies.^[5] The left testis is more involved than right, and it is believed that metastasis from RCC to left testis occurs via left testicular vein.^[6] The pathogenesis of right testicular involvement is less clear and is probably of hematogenous nature by involvement of inferior vena cava by invasion.^[7] Tran *et al.*^[8] published a rare case of metastasis of RCC to ipsilateral spermatic cord in 2013.

With regard to metastatic testicular involvement, the incidence of secondary testicular tumors ranges from 0.3% to 3.6%.^[9] In this case, the patient had bilateral testicular metastasis following RCC similar to simultaneous bilateral testicular metastases from renal clear cell carcinoma.

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Cancer preventing spices

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Sir,

Cancer is a major burden of disease worldwide not only in developed countries, but also in developing countries.^[1] Jemal *et al.*^[2] have reported that about 12.7 million cancer cases and 7.6 million cancer deaths are estimated to have occurred in 2008 worldwide, in which 56% of the cases and 64% of the deaths were in the economically developing world. Moreover, the WHO has published that deaths from cancer worldwide are projected to continue to rise to over 12 million in 2030.^[3]

Whether we have a history of cancer in our family or are currently battling the disease, lifestyle factors, including our diet, can make a huge difference in helping fight off cancer. Ongoing research supports the hypothesis that some foods actually increase our risk of cancer, while others may reduce cancer risk by a variety of mechanisms. These include a number of traditional spices that contain compounds with chemopreventive properties. Some common Indian spices with cancer-fighting properties are turmeric (*Curcuma amada*), black pepper (*Piper nigrum*), cardamom (*Amomum aromaticum*), cinnamon (*Cinnamomum zeylanicum*), clove (*Syzygium aromaticum*), garlic (*Allium sativum*), ginger (*Zingiber officinale*), cumin (*Cuminum cyminum*), saffron (*Crocus sativus*), coriander (*Coriandrum sativum*), dill (*Anethum graveolens*), basil (*Ocimum basilicum*), caraway (*Carum carvi*).^[4] Active constituents are listed in Table 1.^[5]

To date, hundreds of compounds have been identified as potential cancer modifiers, several of which are active ingredients in above mentioned spices such as curcumin in turmeric, piperine in black pepper.^[6,7] Despite a rapidly growing body of experimental evidence supporting the cancer preventive properties of spices, minimal data exist regarding actual dietary intake levels of spices and the pharmacokinetics of active components. Today,

Table 1: Plants with anti-cancer activity

Plant name/family	Active constituent	Class
<i>Zingiber officinale</i> / Zingiberaceae	Gingerenone A, Gingerols, shogaols, zingerone	Curcuminoids
<i>Allium</i> <i>sativum</i> /Liliaceae	Alliin, allicin alliin, alliinase, S-allylcysteine, diallyl disulfide	Flavonoids
<i>Curcuma amada</i> Linn./Zinziberaceae	Tumerone, curcumine	Phenol
<i>Ocimum basilum</i> Linn./Laminaceae	Eugenol, orientin, and vicenin	Volatile oil, flavonoids, phenolic compounds

spices are increasingly appreciated not only for their culinary properties but also for their potential health benefits. For some spices, health attributes associated with spice use may arise from their antioxidant properties. In other cases, the biological effects of spices may arise from their ability to modulate a number of cellular processes, including those involved with drug metabolism, cell division, apoptosis, differentiation, and immunocompetence.^[6]

Spices can potentially inhibit the bioactivation of carcinogens, decrease free radical formation, suppress cell division and promote apoptosis in cancerous cells, regulate inflammation, and suppress microbial growth. The low toxicity may make them particularly useful as a subtle personal dietary change that may decrease the risk for several diseases. The addition of about 1 g/day of herbs to one's diet can significantly provide to total antioxidant intake (> 1 mmol).^[8] Because several spices are effective antioxidants, they may be particularly important in decreasing oxidative damage due to environmental stress, including excess calorie intake.^[9] Spices can be added directly to foods, as has been done historically, or used as dietary supplements.

To conclude, the potential chemopreventive properties of common spices deserve further, rigorous investigation in preclinical and clinical studies. There is considerable ongoing research on the pharmacological properties of individual compounds extracted from spices or herbal supplements (e.g. curcumin, resveratrol). However, basic and preclinical research on these compounds has been hampered

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by poor bioavailability and pleiotropic mechanisms of action that are difficult to study in traditional “one compound-one molecular target” experiments. It should be pointed out that natural products like spices contain complex mixtures of compounds that can affect each other’s pharmacokinetics, solubility and potentially, pharmacodynamics. Future studies should take the complexity of natural products into account and use “systems biology” approaches to dissect their pleiotropic pharmacological properties.

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Introduction to Volume 1 Issue 2 of *Journal of Cancer Metastasis and Treatment*

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It is my privilege to introduce the second issue of the *Journal of Cancer Metastasis and Treatment*. In keeping with the format we have chosen for our journal, this issue contains two reviews focusing on fields of great translational and clinical interest, five original articles, and three case reports. The first review discusses the rapidly evolving field of circulating tumor cells (CTC) as cancer biomarkers. Since the introduction of the first Food and Drug Administration approved CTC test to assess the progression of metastatic breast, colorectal and prostate cancer, CTC have generated tremendous interest among clinicians seeking sensitive progression biomarkers and basic scientists interested in isolating and studying these cells. A number of new sorting, capture, and enumeration technologies are being evaluated. Competing technologies have emerged, such as circulating tumor DNA. The review by Potdar and Lotey summarizes the current experience with CTC as diagnostic and prognostic biomarkers and the future of this technology. The review by Alfonso *et al.* describes recent progress in our understanding of urothelial cancer, a poorly understood malignancy that can be very difficult to treat, based on novel translational science. The original articles cover a variety of translational and clinical topics: the rapidly growing field of extracellular microRNA (miRNA) detection and quantification, as applied to medulloblastoma; the management of diffuse large B-cell non-Hodgkin lymphoma (NHL) in elderly Egyptian patients; the prognostic value of GATA3 and FOXA1 detection in breast cancer; current clinical criteria

for the management of squamous non-small cell lung cancer (NSCLC); and the pharmacological properties of *Withania somnifera* extract in a triple-negative breast cancer model. miRNAs and other non-coding RNAs in biological fluids promise to revolutionize the world of biomarkers. However, there remain significant technical issues surrounding the reproducible quantification of miRNAs for clinical purposes. The manuscript by Shalaby *et al.* is an example of progress in this highly promising field. The manuscript by Zeeneldin *et al.* describes the experience of treating diffuse large B-cell NHL in a geriatric setting where safety and efficacy considerations have to be balanced against each other and where monoclonal antibodies are not always available. The study by Chivukula *et al.* proposes the intriguing hypothesis that immunohistochemical detection of the transcription factor GATA3 and “pioneer” factor FOXA1 has prognostic value in breast cancer. The manuscript by Savini *et al.* describes the experience of this group treating squamous NSCLC, analyzing variables associated with improved survival in their patient population. The article by Ray *et al.* describes the effect of a promising natural product, an extract from well-known medicinal plant *Withania somnifera*, on the production of cytokines associated with metastasis in a standard triple-negative breast cancer model. Finally, the three case reports describe unusual presentations, including histologically different metastases from an unknown primary lesion, orbital metastasis from rectal carcinoma and a mature spinal teratoma presenting in an elderly patient.

I hope you enjoy reading this issue, and the ones that will follow.

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Role of circulating tumor cells in future diagnosis and therapy of cancer

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ABSTRACT

Circulating tumor cells (CTCs) have become a blistering topic of discussion for oncologists because of their tremendous potential in the diagnosis and treatment of cancer. Over the past few years, they have been doled with quite an amount of research in this area understanding that CTCs are shed from tumors and circulate in the bloodstream. This process can also occur at an early stage of cancer. The major limitation in isolation of CTCs is their availability in limited numbers. Hence, many techniques have been developed and are under continuous improvement to enhance their efficacy of CTC isolation and enumeration. They have shown their potentiality to not just indicate the presence of a tumor but also to provide us with its core information. They have also proven to be useful in detecting minor subgroups of cells present in the primary tissue which might eventually be the cause of treatment resistance or relapse of the disease. Hence, detecting and characterizing CTCs can definitely become an inevitable step in treating solid tumor malignancies. In this review, we have tried to comprehend the basics of CTCs including isolation, detection, characterization, and molecular mechanism of their circulation in the blood stream. We have mostly focused on the significance of CTCs in diagnosis and therapies of four most common types of cancers, namely, breast, prostate, lung, and colorectal. This review provides the coverage of most of the advancements with regards to different tumor malignancies and their probable use in predicting outcomes of the disease to realize the concept of personalized medicine.

Key words: Cancer stem cells, circulating tumor cells, epithelial to mesenchymal transition, metastasis, molecular markers, personalized medicine

Introduction

Cancer is a collective term for uncontrolled malignant tumor growth taking place in any tissue of the body. More than 100 types of cancers are known till date, some of them being more common in specific genders such as in case of women; breast cancer is of the most common whereas in men, prostate cancer is quite common.^[1] Other types of cancer like lung, colon, blood, lymph are found in both men and women. Surgery, radiotherapy, chemotherapy are the established treatments for cancer which also constitute significant side effects. However, there is still a long way to go to constitute 100% efficacious results because of heterogeneity and resistant of tumor cells to available therapies of cancer.^[2] Each of the subtypes responds differently to the treatment and makes it difficult to attain a replete cytogenic response. Cancers are also known for attaining complex diversity which makes it difficult for clinicians to choose the treatment procedure.^[3] Some prevalent mutations or the ones attained during the course of treatment may also result resistance to the therapy. In such cases,

continuation of the same treatment only worsens the condition, therefore, there is a need of extremely specific and targeted therapy which can help the survival of patients in such situations.^[4] It is increasingly becoming a prerequisite to take a “fingerprint” of a given tumor and then proceed with a “tailor-made” treatment. Circulating tumor cells (CTCs) can provide us with the required information and pave a new avenue in future cancer therapies.

Mechanism of Cancer Development

Most cancer remains asymptomatic at early stages and start showing up signs only in later stages of development. It is difficult to treat the patient in advanced stages of cancer, because the tumor spreads itself in various tissues of the body which is referred as invasion and metastasis of cancer.^[5] The actual trigger which initiates this process remains obscure. However, CTC-based technologies may predict the pathway of metastasis. A malignant tumor cell has many cell cycle pathways abnormally regulated. Initially, the epithelial cells of a primary tumor infest nearby blood or lymphatic vessels and circulate in them as shown in Figure 1. Of the many altered pathways in these cells, one of them is the production of a protein called matrix metalloproteinase (MMP).^[6] Upon metastasis of a tumor cell, it breaks from the main tumor and enters the extracellular space which is mainly made up of collagenous fibers. The tumor cells secrete MMP, which breaks collagen fibers as well as the basement membrane surrounding the blood and lymph vessels.

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The tumor cells now gain direct access to the epithelial membrane of the vessels and squeeze into them through the tight junctions.^[7] Once into the bloodstream, they can easily transport to other tissues of the body and invade them. An aggressive tumor cell can attach itself to the endothelial membrane of the vessel and create a “pore” through which it escapes out and invade the nearby tissue.^[8] Other less aggressive tumor cells can use this pore to enter the same tissue and establish a new tumor. Malignant tumors also initiate angiogenesis to enhance blood supply around the tumor and support its growth and development.^[9] All this time, when several changes are taking place during the course of tumor growth, an important phenomenon is the shedding of cells from the primary tumor in the bloodstream as CTCs.^[10] These cells carry tremendous information about the presence of tumor, its growth stage and mutations that it harbors. Due to this vital data, they have enormous applications in the detection, staging and treatment guidance of solid tumor malignancies. In this review, we have discussed about their significance, isolation, enrichment techniques and the advancements in the field of molecular biology of CTCs in major types of cancers including breast, prostate, colorectal, and lung cancer.

Circulating Tumor Cells

CTCs are described as cells shed by a primary tumor into vasculature and they keep circulating in the blood stream of cancer patients.^[10] Scientists have tried to decipher their nature and significance. CTCs are known to be circulating in the body fluids before they metastasize to various parts of the body even in primary stages of the disease.^[10] However, they are not easily identified, as they are present in a very small numbers. It is estimated that a teaspoon of blood might contain just about 5-50 CTCs. CTCs first exuviate from the primary tumor and remain in the blood stream for a while till the time it wedges itself in a new tissue as shown in Figure 1. Some CTCs can adhere to the wall of capillaries and bunk to enter a new tissue. While in the blood stream, they might

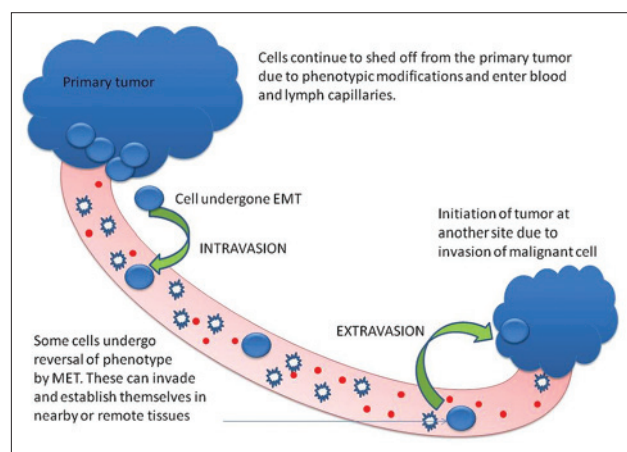


Figure 1: Cells migrating from primary tumor into blood stream and to a site of invading another tissue

even clog capillaries due to their big size.^[11] Many CTCs can be shed from a given tumor in different locations. A given tumor may vary in nature at different locations, that is, it may display heterogeneity. CTCs released from different locations of a tumor may exhibit discrepancies of a given tumor. Thus, CTCs can contribute to a potpourri of heterogeneous cells disgorged from the same tumor.^[11]

Despite consistent efforts, researchers are yet to gather its caboodle. Of the known properties, one of them is that they undergo epithelial to mesenchymal transition (EMT) as shown in Figure 2. This results in change with respect to epithelial markers and other cellular properties.^[12] An epithelial cell starts behaving like a mesenchymal cell and can detach itself from the parent tissue and become a free flowing entity. CTCs use this property to invade blood and lymph capillaries and swim freely in them. Not all CTCs undergo complete EMT; some of them undergo just partial changes or partial EMT. CTCs undergone complete EMT can revert their phenotype by undergoing mesenchymal to epithelial transition (MET) out of which some can contribute to micro or macro-metastasis leading to cancer progression.^[13] When a tumor cell undergoes reversion by MET, they regain properties of cell adhesion. These cells first adhere to the wall of capillaries and then evade from them to nearby tissues. Since they can now behave as epithelial cells again; they adhere to the target site and start dividing and re-dividing giving rise to a new tumor. However, EMT transition can also lead to a perplexed situation as there is a lot of diversity in the morphological transformations.

Significance of CTCs

One of the most axiomatic implications of CTCs is that they are minimally invasive indicators.^[12,13] Detection of CTCs can reveal mint of information rather than just the presence of a tumor. They can help us to realize the concept of tailor-made medicine. Analysis of CTCs

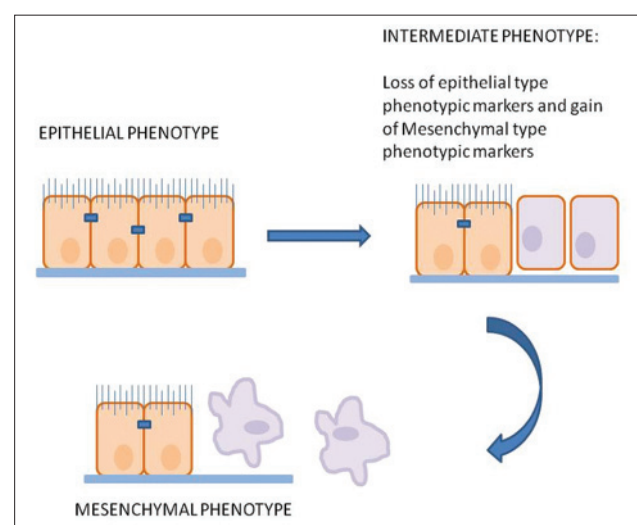


Figure 2: Transition of cells from epithelial to mesenchymal phenotype

can save a patient from worsening the condition with unsuitable medications. Furthermore, the earlier they are detected, faster and better treatment options can be made available to the patient. It provides the basis of understanding mutations and genotypic changes of malignant cells and hence provides the best suitable targeted therapy. CTCs are multifunctional biomarkers and enable us to assess the patient serially along the treatment journey. They are potentially an alternative to invasive biopsies for detection, characterization and monitoring of non-hematological cancers.^[14] Although as of now it is not clear whether CTCs are the cause of metastasis, they still hold the potential for being a cause for disease progression. Metastasis is better known to be caused by cancer stem cells (CSCs), which are highly motile, self-renewing cancer initiators. They also have increased resistance to apoptosis as well as to certain treatment drugs. CTCs with such properties can be metastatic in nature. CTCs after undergoing EMT can also make non-CSC type cells to behave like CSCs. In addition, it is yet to be clarified whether cells with metastatic potential have increased motility and aggressive nature of CTCs as compared to non-metastatic tumor cells. On the whole, CTCs give us biological insights of the disease condition, progression, and treatment prediction. Reports indicate that patients with fewer numbers of CTCs survive longer than the patients which have more number of CTCs.^[15] Another important implication of CTCs is that they can form the constitutional basis of tumor staging.^[16] The types and quantity of CTCs can form *prima facie* of the degree and type of cancer. They can be periodically used to keep a check on disease progression. In some cases, they have even been able to identify the drug targets by analyzing the enumerated CTCs and its phenotype. They can even help in the selection of secondary treatment options while the patient has failed to respond to first line treatments.^[17] One such example is the detection of human epidermal growth factor receptor 2 (HER2)-positive CTCs in HER2-negative breast cancers.^[18] Thus, it gives us hints and specks about quiescent population that may be present in the tumor and be the cause of drug resistance or relapse of the disease.^[19] Since, CTCs hold such critical information about a tumor and its characteristics; they can definitely form the pedestal of patient-specific treatments. The great enigma about cancer can adjudicate with the help of information retrieved from CTCs analysis.

Isolation and Analysis of CTCs

In the recent years, CTCs have gained increasing importance because of their multi potential uses. Despite their long known discovery and spates in clinical oncology, no method has been devised to isolate or enumerate CTCs efficiently. Primarily, their quantity in blood circulation is the biggest hurdle in isolation of CTCs. Out of the several CTCs shed by the primary tumor only about 0.1% survives in the circulation and only about 0.01% is responsible for metastasis.^[17] It has been reported that CTCs are not

continuously shed in the circulation. They are discontinuous and might not be present in homogenous condition. Thus, while isolating CTCs a single blood sample might fall insufficient or may give inaccurate results.^[20] This is accompanied by further reduction in their numbers when they get clogged in capillaries due to their large size. They can also form clusters while flowing and some of them may even adhere to the walls of the capillaries, or some might be cloaked by the platelets. Further reduction in CTCs number takes place during batch processes which are followed for their enrichment. Simpler methods involve size based separation, collagen adhesion method or density-based separation. Other sophisticated ones rely on epithelial markers, immunomagnetic techniques, microchips, and nanotech approaches.^[21]

Density-based Ficoll-Hypaque method

Gertler *et al.*^[22] 2003 have used Ficoll-Hypaque density-based separation method to separate tumor cells from bone marrow and peripheral blood aspirations. It is based on differential migration of cells which takes places during centrifugation and gives a layered separation of cells types. The porous barrier is permeable to the red blood cells and other smaller components of blood. The buffy coat above this layer is of concern, as it contains the tumor cells along with leukocytes. This layer can be easily aspirated and analyzed further to determine the presence and quantification of CTCs.^[21]

Immunomagnetic (antibody based) method

This method exploits the presence of surface markers on tumor cells or hematopoietic cells. In this method, antibodies are coupled with magnetic particles and then used for positive or negative selection of CTCs. In positive selection, surface markers of CTCs are targeted, whereas, in negative selection, depletion of blood cells other than CTCs is achieved by targeting their surface markers^[23] as shown in Figure 3. Epithelial cell adhesion molecule (EpCAM) is one of the most widely tapped markers on tumor cells. CD45 in case of lymphocytes and glycophorin for erythrocytes are two commonly used markers in case of negative selection. MACS® has introduced microbeads which can be used in such negative selection.^[24]

Food and Drug Administration (FDA) has approved CellSearch® (by Janssen Diagnostics) which is by far the most efficacious system for extraction and enumeration of CTCs.^[25] The CTCs according to this system are defined by a characteristic round oval shape cells with nucleus which is stained by 4',6-diamidino-2-phenylindole stain. This procedure may be laborious and intensive but gives the best enrichment results as a comparison to other existing techniques. It makes use of antibodies like EpCAM attached to magnetic beads for binding to specific tumor cell surface receptors. These cells can be pulled out from the rest of cells under the influence of a magnetic field.^[26] Some tumor cells might escape the

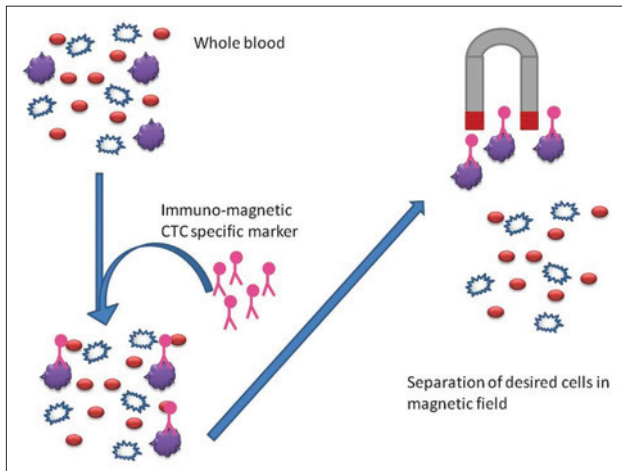


Figure 3: Immunomagnetic separation of circulating tumor cells

antibodies as they undergo EMT transitions while some other tumor cells belonging to a smaller sub-population might also be ignored. Some CTCs remain undetected throughout this process.^[27] Hence, although this method is being used currently for experimental purposes, there is yet lot of scope for improvisation in the quantitative as well as qualitative aspects of tumor cell detection.

Microfluidics method

As antibody-dependent cell sorting is not a completely reliable source. There are a lot of hurdles in accomplishing higher percent enrichment of cells from the whole blood. Hence, it is important to take into consideration other methods which rely on antibody-free systems. In this method, cell size-based sorting is accomplished using microfluidic technology.^[28] The microfluidic chamber is made up of special materials and is usually spiral or curvilinear. When whole blood is allowed to pass through this micro-chamber, inertial lift forces and drag forces help in sorting of the cells. These forces rely on differential sizes of cell in the sample. In case of CTCs, whole blood or leukocyte along with CTCs fraction can be used as a feed in input. As they pass through the microfluidic chamber, the forces will act on the cells and start separating them based on size. The CTCs incline more towards the inner wall (larger size) while other cells such as white blood cells and red blood cells will incline towards the outer side of the wall (smaller size).^[29] They can be collected in separate fragments at the end of the tube, where it bifurcates into collecting chambers as shown in Figure 4. Recent advances have allowed the procedure to be carried out with minimal loss of cell types.^[30,31]

Size based separation method

As CTCs are usually bigger in size compared to other components, this characteristic is put to use. This method can even be used to detect the presence of a single tumor cell in a quantity of blood as little as 1 mL (shown in Figure 5). ISET[®] is one such established method which

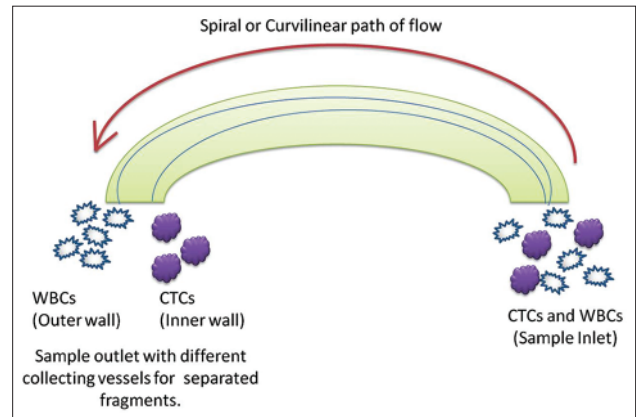


Figure 4: Microfluidic-based separation of circulating tumor cells

is used for such type of cell size based sorting. Specially designed filter are employed to allow blood components to percolate through them. CTCs being bigger in size will not be able to pass through the membrane and hence remain over it. They can be then collected from over the membrane filter and subjected to analysis.^[32]

Other techniques

The FDA approved cell detection method has quite some limitations. Hence, a lot of attempts are being made to invent better techniques which are highly efficient low on cost, less labor intensive, and time savers too. Metacell[®] is another cell size-based sorting method which has been introduced lately.^[32,33] Microchips and micro slides are being designed to exploit various differential properties of cells. Lu *et al.*^[34] have introduced a device which they refer to as Nan Velcro CTCs Chip. They claim that this device is much more efficient and reproducible as compared to CellSearch[®] kit. This kit is composed of a patterned silicon nanowire substrate which is overlaid with polydimethylsiloxane mixture.^[34] While another cell surface marker-based systems is a flow cytometry fluorescence-activated cell sorting.^[35] Another emerging technique is making use of dielectric constants of cells such as the DEPArray system.^[26] Ju *et al.*^[36] have described a method where they make use telomerase activity to isolate melanoma cells in peripheral blood. As telomerase activity is elevated in cancerous cells rather than normal cells, they made use of an adenoviral vector human telomerase reverse transcriptase to drive the expression of green fluorescent protein which can be used to isolate CTCs in this method. An interesting device called VeriFAST is an integrated system which can isolate cells as well as perform down streaming processes including staining with EpCAM and other antibodies to isolate CTCs.^[37] Many more such technological advancements have been reported by scientists all over the world. There are several newer assays are being introduced which are focused on marker free isolation such as chromatography, filtration, and dielectrophoresis for capturing CTCs from cancer patients.^[38] Few of them have been mentioned under specific cancer categories discussed ahead in this review.

Characterization and Molecular Profiling of CTCs

We have discussed various CTCs enrichment techniques which are being used for isolation of CTCs from metastatic cancer patients. However, none of them has achieved much of quantitative success. The results have shown a great amount of variation from 10% to 90% of isolated CTCs and hence, it is crucial to analyze the collected cells for their quantity as well as their exact phenotype. A numerical indication of collected CTCs may not be able to reveal the true picture of the type of cells isolated from cancer patients. Similarly, tumor cells can undergo a variety of changes and be present in heterogeneous subpopulations. Hence, a mere number of CTCs can lead to faulty conclusions. Therefore, there is a need for true characterization of these isolated CTCs cells to come to logical conclusions. Molecular profiling of these isolated cells will crystallize the picture, as it reveals the true nature of the isolated CTCs cells.

A fundamental process in EMT, down-regulates E-cadherin, which can be attained by many transcriptional factors.^[39] Most of the molecular markers that have been isolated for characterizing CTCs are EMT indicators. During EMT process, a metastatic cell goes through a lot of modifications at cellular and molecular levels and many genes undergo transcriptional alterations.^[39] Some of these genes play a role in initiating the effect of EMT while others play a role in regulating and maintaining its transited state. The other factors like inflammatory cytokines and physical changes in the tumor microenvironment also play a role in EMT promotion.^[39] *TWIST1* and *TWIST2* genes are most strongly expressed genes in EMT process which are responsible for inducing transformation alone or in co-operation with other factors such as TGF β , Wnt, Notch, *etc.*^[40] E-cadherin is one of the most important proteins for maintaining the epithelial nature of cells. Snail1 and Snail2 suppress the transcription of E-cadherin as well as *Zeb1* and *Zeb2* genes. This results into downregulation of E-cadherin, which leads to initiation of EMT process.^[41,42] Other gate keeper's genes of epithelial state, such as alpha and gamma catenins are also been down-regulated along with downregulation of E-cadherin in this process.^[43,44]

Induction of certain mesenchymal characters during EMT process requires upregulation of two extracellular matrix proteins, that is, vimentin and fibronectin in these cells which escape the barriers of local tissue and proceeded for invasion. Similarly, other genes such as N-cadherin, CD44, integrin $\beta 6$ are also implicated for proper migration of these cells.^[43-46] Even understanding the mutational changes, abnormal size, and characteristics of CTCs, scientists are still pondering over the fact that these cells are able to survive in an environment which is totally hostile for them. It is postulated that out of the several hundred CTCs shed by the tumor, only a few

remain in the circulation. There are reports suggesting that CTCs bearing mutations, such as upregulation of CD47, help them in escaping attack by natural killer cells and macrophages. Similarly, downregulation of chaperone protein-calreticulin again helps them to dodge the immune system.^[47,48] Schölch *et al.*^[49] in their studies have referred this state as an “immune-evasive” to the period between EMT and MET in circulation. Thus, overall it seems that CTCs have very evolved mechanisms to maintain and express their invasive aggressive nature by surpassing the body's natural immune system.

CTCs in Breast Cancer Diagnosis and Treatment

Breast cancer is one of the most common types of cancer detected in women. Last two decades, due to early diagnosis and advancement in treatment protocols, breast cancer mortality has been considerably reduced. However, there is no hope of survival when patient condition progresses to the metastatic stage. Recent studies have shown that CTCs which are shed from tumor are mediator of metastatic dissemination and form micrometastasis at distant organs.^[50] Due to advancement in technology, several methods have been established to isolate CTCs from metastatic breast cancer patients. CTCs derived from breast cancer patients are among the most extensively studied for diagnosis and treatment of breast cancer.^[50] There is a direct co-relation of CTCs with disease prognosis and survival has been reported in many cases. It has been shown that if there is more number of CTCs, there are less chances of survival.^[50] The progression of the disease and its response to treatment can be very well-monitored by characterizing CTCs which are disseminated from the primary tumor. It has shown that the presence of CTCs, despite of ongoing treatment, is an indicative of worse overall survival.^[51] Hence, it is very important to characterize CTCs for better understanding of this disease progression and cure.^[51]

Due to large size and few numbers of CTCs in blood circulation of metastatic breast cancer patients, the isolation and enumeration of captured CTCs have proven to be of prognostic value in breast cancer evaluation and treatment. One of technologies presently in use is the CellSearch[®] system, which works on a principle of selecting CTCs as per the positive expression for EpCAM and cytokeratin (CK) protein on the surface of these cells.^[52] Although it has proven greatly useful and reproductive, it may limit the selection due to EMT transition process. CTCs which have undergone EMT will show downregulation of epithelial markers including EpCAM.^[52] In some cases, it has been observed that HER2-positive metastatic breast cancer shows the presence of EpCAM negative CTCs.^[53] Hence, EpCAM independent methods could fetch an increase in number of capture of CTCs. Second, detecting CTCs on the

basis of HER2 expression has been suggested in many cases. CTCs vary in expression and frequency of this gene, and it can be directly correlated with the disease's progression and survival.^[54] Not only about correlating the primary tumor's characteristics, CTCs can reveal more vital information, which is at times not detected by mere analysis of primary tumor. In a particular group of HER2- breast cancer patients, HER2+ CTCs are identified.^[55] This leads to consideration of revision in the ongoing treatment of the disease. Trastuzumab-based therapy is applied to these patients with HER2+ CTCs and HER2- primary tumor. This study has shown that 1 out of 4 patients are treated completely while 2 patients have attained partial response to this treatment. Even though this study number of patients are few, it has given important facts about CTCs. It has helped in identifying the changing course of the disease well before time. Thus, CTCs hold the potential to represent the metastatic state of HER2- breast cancer.^[55]

CTCs have been reported to harbor many types of mutations and transformations. Obermayr *et al.*^[56] have shown that genes like EpCAM and secretoglobulin, family 2A and SCGB2A2, can be used as important markers in the detection of CTCs in breast cancer. CTCs have been reported to establish mutations after dissemination from the primary tumor and some of these mutations may help the circulating cells to attain enhanced survival and therefore molecular profiling of CTCs holds the importance in understanding the real state of disease. Monitoring the CTCs with respect to CK19 expression can reveal the nature of metastatic potential of the tumor. CK19 expression in CTCs has been prognostic for worse overall outcome of the disease. CK19 as well as TP53 mutations are mostly found in all of the CTCs derived from triple negative breast cancer patients.^[57] Some researchers believe that it can be one of the driving factors to the progression of the disease to triple negative stage.^[53] It has also been shown that breast cancer patients which expressed *KRT19*, *SCGB2A2*, and *ERBB2* genes showed poor survival rates.^[56] IGF-IR mutation has also been observed to be expressed in patients of breast cancer at a metastatic stage of disease. Furthermore, mutations in *PIK3CA* gene and *ERBB2* mutations are reported in CTCs of some patients whose primary tumor did not share this state of disease.^[57,58] Apart from these mutations, EMT changes have been one of the critical properties of CTCs. Most of the CTCs isolated from breast cancer patients show the presence of EMT markers such as *ETV5*, *NOTCH1*, *SNAIL*, *TGFB1*, *ZEB1*, and *ZEB2*.^[41] The mutational and transitional changes taking place in CTCs make them gain an aggressive behavior which in turn helps them to break apart from the basement membrane and disseminate from the tumor.^[59] EMT pathway and *PIK3CA* mutations have been related to progression of the disease to metastasis in many cases.^[60,61] Hence, molecular profiling of CTCs is becoming increasingly important both to understand the state of the disease and then select

an optimal treatment for a given patient.^[62] It has been reported that the presence of genes like *EpCAM*, *CCNE2*, *DKFZp762E1312*, *EMP2*, *MAL2*, *PPIC*, or *SLC6A8* can be related with the presence of CTCs in peripheral blood.^[56,62]

In breast cancer, chemotherapy is one of the standard modes of treatment. During the course of this therapy, CTCs values are determined before and after rounds of chemotherapy. In most of the cases with non-metastatic state of breast cancer, reduction in number of CTCs is observed after the first round of chemotherapy. However, it was also noted that CTCs had a tendency to attain resistance to the therapy. Hence, it is suggested that a regimen of increasing doses should be deployed in the progressing rounds of chemotherapy.^[63] Studies by Peeters *et al.*^[64] have revealed some statistics about CTCs count and disease state. In a small group under their study, about 80% of patients who had more than 80 CTCs in 7.5 mL of blood died within one year from diagnosis of metastasis of disease. In a similar study by Smerage *et al.*,^[65] it has been observed that CTCs continued to remain detected after first round of chemotherapy in some patients of breast cancer. It is observed that such patients are in rapid progression of the disease to metastasis. They have further suggested that in such cases, it can be ideal to opt for some alternative treatment with some of the novel therapeutic agents rather than continuing with the same chemotherapy.^[66]

CTCs in Prostate Cancer Diagnosis and Treatment

Prostate cancer originates in the prostate gland of male reproductive system. Fusion of *TMPRSS2* and *ERG* genes is identified as one of prime reasons leading to prostate cancer which is often accompanied by loss of *PTEN*.^[67] Biopsy remains the test for full confirmation of this disease. Less invasive processes are sometimes conducted to detect this disease as well as to understand its progression. Prostate-specific antigen (PSA) level detection is one of such tests which can be used to identify the presence of disease and monitor the treatment effect in these patients. However, PSA levels may not be always necessarily indicative of the disease progression as PSA level may raise due to reasons other than prostate cancer. Similarly, fall in levels of PSA during treatment may not be necessarily indicative for the eradication of the prostate tumor. It has been shown that drugs targeting androgen receptor (AR) may bring down levels of PSA but not necessarily cure the disease simultaneously. Hence, a better prognostic marker is greatly demanded. When PSA testing falls insufficient to validate the course of treatment, CTCs isolation enumeration and characterization can act as a reliable marker for diagnosis and therapy of prostate cancer.^[68,69]

In a study by Attard *et al.*^[70] have captured circulating, non-apoptotic nucleated, EpCAM+ CK+ CD45- cells

from prostate cancer patient's blood and confirmed for their malignant origin and hormone-regulated expression of ERG1. Thus, CTCs hold great potential to identify and stage the prostate cancer with minimal invasive procedures.^[70] Giesing *et al.*^[71] have identified overexpression of five genes, namely, *SOD2*, *GPX1*, *AR*, *cyclin B*, and *bFGF* which have predicted the clinical stage of metastasis and 3 of these genes are related to bone metastasis.^[72] CTCs are known for their heterogeneity acquired due to frequent transitions from epithelial to mesenchymal state. Some of these EMT mutations are more frequent in castration-resistant prostate cancer than compared to hormone-sensitive prostate cancer. They can be used to identify specific targets in variants of the same type of cancer. These mutations can be used as a checkpoint and also help to speed up this testing as well as validation of upcoming therapies. In prostate cancer, CTCs have been proposed to act as intermediate or surrogate endpoints for survival and to shorten timelines for drug approval.^[73,74]

Changes in levels of CTCs can be correlated with the disease status. Patients with lower levels of CTCs have shown slower disease progression in comparison to those having a higher amount of CTCs.^[75] CTCs are sure to provide a better overall picture of the state of disease as there are molecular variations in different sites of metastasis. Shaffer *et al.*^[76] demonstrate an example of variation in EGFR ranging from 0% to 100%. Hence, understanding the heterogeneity in the disease cannot be understood from the single site biopsy and profiling of these CTCs becomes a necessity. Leversha *et al.*^[77] have shown that molecular characterization of CTCs may be possible for reporting genomic amplification of AR and chromosomal instability in prostate cancer patients. There is very much high expression of *MYC* and *TMPRSS-ETV* genes and downregulation of *PTEN*.^[77] Such copy number alterations have been related to aggressive tumors.^[78] CTCs exome sequencing has proven its clinical significance. Major percentage of cancer mutations are detected in CTCs, which matched the primary tumor. Furthermore, a great percentage of mutations could be predicted and matched with the metastatic site of tumor. The presence of more than 5 CTCs in 7.5 mL of blood has been related to poor outcome of the disease treatment in metastasis.^[75] Hence, not only is it beneficial in providing prognostic information, but it can also act as a gateway to treat those patients in a better manner whose tumors do not shed CTCs.^[79]

Newer technologies continue to emerge with the growing research. Lu *et al.*^[34] have introduced NanoVelcro CTCs chip which claims to have better and reproducible results as compared to FDA approved CellSearch® kit. Olmos *et al.*^[80] have made use of reverse transcription polymerase chain reaction (PCR) to identify telomerase activity in CTCs for which they are very sensitive. However, individual CTCs can be identified with

this method. Galletti *et al.*^[81] made use of prostate cancer-specific antibodies i.e. prostate-specific membrane antigen, PSA, prostate specific stem cell antigen and EpCAM to evaluate isolation of CTCs in the metastatic stage of disease which might escape EpCAM specific selection. They have indicated isolation of specific CTCs including the one which undergoes EMT and escape EpCAM selection and organ-specific CTCs in the metastatic stage of prostate cancer.^[81]

CTCs in Colorectal Cancer Diagnosis and Treatment

Colorectal cancer (CRC) is one of the most dreaded diseases and has its poor prognosis. Although survival rates have drastically improved over time, timely prognosis would aid the treatment to a great extent using CTCs testing. The data available for earlier stages are yet bare and lacks good sample size for studies on CRC. Romiti *et al.*^[82] have analyzed the prognostic role of CTCs, highlighting the importance of CTCs count before and after chemotherapy. However, to avoid misleading CTCs counts after surgery, it has been suggested that there should be a time gap of at least 24 h prior to post-surgical sampling. This is because the procedure may contribute to a temporary rise in CTCs which are rapidly cleared within 24 h. CTCs follow-up for patients with the aggressive disease can form an inevitable tool and also help in selecting better emphatic treatments.^[82,83] Barbazán *et al.*^[84] have done molecular profiling of CTCs derived from metastatic CRC. They have studied various molecular markers, such as VCL, ITGB5, BMP6 for invasive phenotype, TLN1, APP, CD9, LIMS1, and RSU1 for adhesion and migration for deeper understanding of the behavior of these prostate cancer cells. These markers can be used to profile the type of tumor and to assist in selecting a suitable treatment. In some reports, researchers claim that a higher amount of CTCs is reported in mesenteric blood rather than peripheral blood. CTCs can be used to diagnose patients symptomatic for CRC in addition to fecal occult and lower gastrointestinal endoscopy.^[85] Like other malignancies, dormancy of CTCs in CRC is another aspect to discuss because even after significant exposure to treatment, some CTCs continue to be detected in the circulation. Molnar *et al.*^[86] mention about detection of CTCs as individual cells or as clusters by a CK-based, immunomagnetic cell separation method. Although the number of CTCs decreases with the progressing treatment methods, at least a few of these cells or clusters are observed to be circulating in the blood stream for a long time despite operation. This could be explained by assuming that some CTCs remain dormant in condition for long durations and that they still continue to be present in the circulation.

Mutational analyses of CRC derived CTCs carried out by Bork *et al.*^[87] have pointed out some clinically significant characteristics. In particular, KRAS mutant CTCs are

discovered in patients, whose primary tumor is KRAS wild type. An ultra-deep sequencing revealed the presence of KRAS mutated group of cells in the primary tumor. This is another example revealing the crucial importance of CTCs sequencing which helps us find out details of the heterogeneity in the tumor which is otherwise not possible by single biopsy. CTCs have been directly related to state to disease and predicting treatment outcome in CRCs just like other cancers. Individual markers such as KRT19, MUC1, EpCAM, CEACAM5, and BIRC5 are studied by de Albuquerque *et al.* showing positively ranging between 15% and 35%. They have observed a shorter progression-free survival in patients showing more of these CTCs compared to the group of patients with lesser or no CTCs.^[88] In an interesting study by Allen *et al.*,^[89] we come across the finding that CRC tumor-associated events such as apoptotic CTCs and CTCs debris are more indicative of liver metastasis in particular than just CTCs count. These events are more indicative of the site of metastasis rather than primary tumor and hence are clinically very significant.^[20] CRC has often been related to liver metastasis in particular. This is supported by detection of increased number of CTCs in mesenteries than peripheral blood. Though the prognostic value of these CTCs has not yet been validated. Denève *et al.*^[90] in their studies strongly support that liver is the filter site for CTCs and that viable CRC disseminated cells can be isolated from hepatic tissue. Reports are pointing out that the EpCAM+ CTCs are often detected in liver indicating a strong signal of association of liver metastasis in CRC. In another study by Antolovic *et al.*^[91] have noted worse overall survival in later stages of CRC patient-derived CTCs having CEA/CK/CD133 positive mRNA than those who are negative for these markers. They have discussed about use of additional markers like CD133 for detection of not only CK20+ and CEA+ subpopulation of CTCs but also for more aggressive type CD133+ disseminated cells.

Improvements in the detection of CTCs continue to evolve as the need does. In one study Antolovic *et al.*^[91] have suggested the use of Ficoll gradient isolation prior to use of EpCAM enrichment technique of CTCs for enhanced results. In many cases, it is easier for clinicians to treat a suffering patient if the malignancy is detected earlier. Hence, in cases such as that of CRC where early detection still awaits some efficient technique, CTCs can play a good enough role in not only detecting and personifying but also providing a real-time means of the disease status along the treatment journey.

CTCs in Lung Cancer Diagnosis and Treatment

Lung cancer is broadly classified as small cell lung cancer (SCLC) and non-SCLC (NSCLC). Early detection continues to remain a challenge in lung cancer, reducing the chances of survival. Dissemination is an early event in both these types of lung cancer and hence CTCs can be of great use in lung cancer as the available biopsies are not readily procurable.^[92-94] A study by

Casavant *et al.*^[93] provides significant data of study on animal models demonstrating use of CTCs in SCLC as “liquid biopsy” and paving way for personalized medicine. The most common methods used for isolating CTCs in lung cancer are CellSearch® and ISET kits. Both these methods indicate a higher number of isolation of CTCs in SCLC than NSCLC. Taenzer *et al.*^[92] have explained this by the possibility of EMT in NSCLC, which makes the disseminated cells escape EpCAM selection. Mutations on exon 19 and 21 of EGFR are the prime target of drug-based therapies. Other mutations such as T790M, EML4-ALK rearrangement, BRAF, KRAS, HER2, PIK3CA/AKT1, ROS, FGFR1, and MET are also of greater interest in lung cancer as clinical trials are now focused on mutation based therapies.^[95,96] Molecular characterization of CTCs holds great importance as it can provide a very plausible means of mutation detection. Furthermore, one can be easily monitored periodically for the development of any resistant mutations during the course of treatment.^[97-99] Higher number CTCs in lung cancer has been associated with larger tumor size and in particular in bone metastasis.^[100-102] CTCs have surely gathered lot of enthusiasm and effort towards their research with their staggering clinical potentials. But till date research on them has been limited by many factors, such as their small capture number being a major problem. Kolostova *et al.*^[35] have drawn an attractive protocol for isolation and culturing *in vitro* CTCs of human lung cancer. If CTCs can be cultured *in vitro* like other cells, it will be of great beneficial value as it will pace up the investigation on the nature of CTCs and its characterization. Furthermore, circulating tumor micro-emboli (CTMs) have been reported in many cases of lung cancer. CTMs are cluster of disseminated tumor cells in circulation. CTM are of particular interest in this case as they are considered to be markers of extreme metastatic potential.^[103] Treatment response is a major question in advanced lung cancer. CTCs count can potentially help approve the ongoing treatment and also help in suggesting any alterations if required.^[104,105] Ilie *et al.*^[105] in their discussion on CTCs in lung cancer have indicated possibilities of the presence of these cells even before angiogenesis. CTCs can be present in circulation long time before the disease can be actually detected. Therefore, they can become the core of research in regards of early detection of the disease for symptomatic patients.^[106-108]

CTCs in Other Cancers Diagnosis and Treatment

CTCs can be detected in almost all of the solid tumor malignancies and changes in the disease state can be predicted with the help of CTCs. Genes like *VIM*, *TGFBR2*, *TGFB*, and *SERPINE1* which are indicative of mesenchymal phenotype, are expressed in higher levels in the CTCs of glioblastoma cancer in comparison to the cells of the primary tumor or cell culture.^[109-111] TWIST and Vimentin are considered as diagnostic markers for hepatocellular carcinoma. TWIST is known to suppress expression of E-cadherin while overexpression

of Vimentin is strongly related with the mesenchymal phenotype of these CTCs. Overexpression of ZEB1 and ZEB2 is also reported in this cancer.^[112] So far, no specific marker has been reported for bladder cancer. However, it has been suggested that overexpression of H-RAS oncogene and mutations in *FGR-R* genes in CTCs could be considered as a diagnostic tool.^[113] Apart from genes, other cellular transformations like loss of cellular junctions which aid in cell to cell communications are indicative of mesenchymal phenotype. Markers such as CK20, UP II, and EGFR have been related to diagnosis of bladder cancer. CTCs can be assessed to detect the presence of these markers and aid in the diagnosis of the malignancy.^[114,115] Alonso-Alconada *et al.*^[41] have done molecular profiling of CTCs isolated from metastatic endometrial carcinoma (EC) patients. They have shown that there is an overexpression of stem cell related genes, i.e. ALDH and CD44, EC related genes such as *BRAF*, *PIK3CA*, *RELA*, *RUNX1*, and *EMT* related genes, that is, *ETV5*, *NOTCH1*, *SNAIL*, *TGFBI*, *ZEB1* in these patients.^[41] Of these genes, *ETV5*, in particular, is strongly related to increased metastasis and CTCs plasticity.^[41] Häfner *et al.*^[116] have shown that the evaluation of level of HPV16-E6 mRNA by real-time PCR is more sensitive molecular marker expressed in CTCs isolated from metastatic cervical cancer patients than that of commonly used CK19 mRNA as a marker. Just like other solid tumors, in case of pancreatic cancer, several studies have similarly suggested the use of CTCs for not only diagnosing but also for identifying metastasis in patients and helping to select patient-specific therapies.^[117-119] A study by Kuhlmann *et al.*^[120] brings to light an importance of the molecular characterization of CTCs in ovarian cancer. They have shown that ERCC1+ CTCs can predict platinum resistance therapy in ovarian cancer which is still remains a big challenge in the treatment of ovarian malignancy.^[120] Obermayer *et al.*^[121] have shown that there are more number of cyclophilin C gene (PPIC) positive CTCs are detected usually in ovarian platinum-resistant cancer group as compared to the sensitive group than EpCAM positive CTCs in these patients. It is also related to poor outcomes of this disease.^[121] Advancements in CTCs detection techniques have given rise to newer methods, such as the one-step detection of using fluorescent silica nanoparticles for ovarian cancer.^[122] With the growing technologies and persistent work on CTCs, we are slowly channelizing the efforts to derive a clearer picture of the use of CTCs in diagnosis and treatment of various cancers.

Future Directions

Taken together, CTCs have potential to aid in the entire course of a patient's cancer journey starting from diagnosis, treatment selection, post-treatment/surgery monitoring, and follow-up. Although vast amount of research have been accelerated in the field of these disseminated tumor cells, their availability in scant

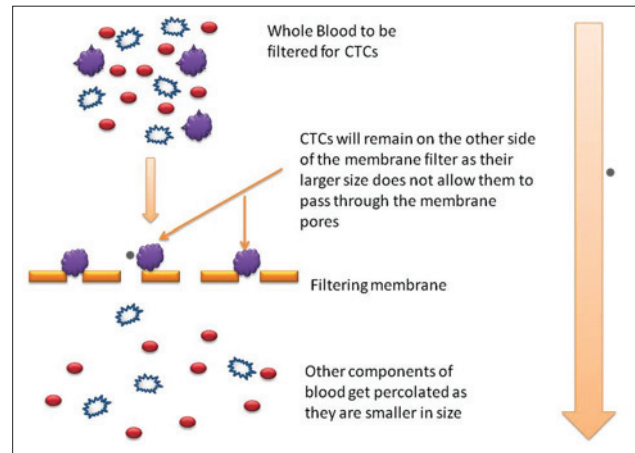


Figure 5: Size-based separation of circulating tumor cells

numbers has limited research. We anticipate the development of isolation and enrichment combination techniques which help in avoiding cell loss. A range of specific markers is also bound to enhance the enrichment results as those cells which can escape EpCAM selection could also be captured. Given their tremendous potential to help change the enigmatic situation of solid tumors, we can conclude that CTCs are sure to become an inevitable part in the near future of solid tumors malignancies.

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Urothelial bladder cancer progression: lessons learned from the bench

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ABSTRACT

Urothelial bladder carcinoma (UBC) is an intricate malignancy with a variable natural history and clinical behavior. Despite developments in diagnosis/prognosis refinement and treatment modalities, the recurrence rate is high, and progression from non-muscle to muscle invasive UBC commonly leads to metastasis. Moreover, patients with muscle-invasive or extra-vesical disease often fail the standard chemotherapy treatment, and overall survival rates are poor. Thus, UBC remains a challenge in the oncology field, representing an ideal candidate for research on biomarkers that could identify patients at increased risk of recurrence, progression, and chemo-refractoriness. However, progress toward personalized medicine has been hampered by the unique genetic complexity of UBC. Recent genome-wide expression and sequencing studies have brought new insights into its molecular features, pathogenesis and clinical diversity, revealing a landscape where classical pathology is intersected by the novel and heterogeneous molecular groups. Hence, it seems plausible to postulate that only an integrated signature of prognostic/predictive biomarkers inherent in different cancer hallmarks will reach clinical validation. In this review, we have summarized ours and others' research into novel putative biomarkers of progression and chemoresistance that encompass several hallmarks of cancer: tumor neovascularization, invasion and metastasis, and energy metabolism reprogramming of the tumor microenvironment.

Key words: CD147, lymphovascular invasion, mammalian target of rapamycin, monocarboxylate transporters, progression, Raf kinase inhibitor protein, scoring system, urothelial bladder cancer

Introduction

The urothelium, one of the slowest cycling epithelia in the human body,^[1] is constantly exposed to a variety of potential carcinogens that can stagnate in urine for a few hours before urination. For that reason, the bladder is a particularly high-risk organ for cancer development, and incidence and mortality from bladder cancer represent an important public health problem. An estimated 429,000 new cases of bladder cancer and 165,000 deaths occurred in 2012, worldwide. It was the 9th most common cancer for both sexes combined (4th in men, 15th in women). Sixty percent of cases occurred in more developed regions of the world (Europe, North America, North Africa).^[2] In these regions, more than 90% of all bladder tumors

originate from transitional cells of the urothelium, while approximately 5% and 2% are squamous and glandular variants, respectively, while the remainder comprises other rare subtypes.^[3,4] The most well-established risk factors for bladder carcinogenesis are cigarette smoking and industrial exposures in the context of a number of occupational settings.^[5]

Of all newly diagnosed cases of urothelial bladder carcinoma (UBC), 70-80% arise as non-muscle invasive (NMI). These tumors, although without aggressive histopathological features, often experience recurrence, and a subgroup of high-risk lesions frequently progress to invasive forms. Conversely, 20-30% originally present as muscle invasive (MI) disease. Invasion of the muscular wall portends common progression to metastasis. Despite radical cystectomy, radiation, and/or platinum-based chemotherapy, patients often fail treatment, so the 5-year overall survival (OS) rate is < 50%, mostly due to chemotherapy resistance and patient fragility.^[4,6-9] Repeated relapses, occurrence of progression, and chemoresistance make UBC the costliest cancer to treat from diagnosis to death.^[10] Thus, personalization of treatment could improve patients'

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quality of life, and reduce the burden on health care systems.

Clinical staging and histopathological parameters remain the “gold standards” for UBC diagnosis and prognostic prediction.^[11,12] However, they are not sufficient to characterize individual biological features and clinical tumor behavior. Understanding disease pathobiology could potentially add essential information to these classical criteria and contribute to more accurately predicting prognosis and refine treatment. Ideally, the clinical use of standardized prognostic and predictive biomarkers could allow the prediction of tumor recurrence through a non-invasive method, avoiding use of invasive techniques, such as cystoscopy and biopsy, which cause significant patient discomfort and add substantial costs.^[13] Furthermore, it could allow timely prediction of UBC progression, from NMI to MI disease, particularly for high grade or carcinoma *in situ* lesions, guiding more vigilant surveillance and refining treatment strategies.^[14] Finally, it could allow the prediction of response to conventional cytotoxic therapies typically associated with chemorefractory relapse and patient fragility.^[15]

A cancer-related biomarker may be defined as a molecule produced by the tumor or by the organism in response to the tumor, measurable in sample matrices such as tissue, blood, or urine, representative of the cancerous process, and reproducible, specific, and sensitive.^[16] A reasonable number of UBC biomarkers, namely those involved in the key molecular pathways of urothelial malignization (fibroblast growth factor receptor 3 and tumor protein p53 mutations), seem to be prognostically relevant.^[17-19] Despite this, there is a substantial delay in translation into the clinic, and clinical trials with molecularly targeted agents have been few in number and largely unsuccessful.^[20] There is the need to expand biomarker research beyond the current focus on therapies directed at deregulated oncogenic or tumor suppressor pathways, and into new molecular portraits encompassing all the hallmarks of cancer.^[21] In fact, recent medium- to high-throughput gene expression profiling technologies and sequencing studies have revealed a multifactorial scenario where additional molecular alterations seem to be involved in urothelial carcinogenesis and tumor progression.^[8]

Biomarkers of UBC Progression: Lessons Learned from the Bench

In the next sections, we will summarize the contributions of our group and of other authors to the research of three poorly explored biological events that overlap several cancer hallmarks and seem to influence UBC progression: occurrence of tumor neovascularization, loss of metastasis suppressor proteins, and metabolic reprogramming of the tumor microenvironment.

Tumor neovascularization

The leading cause of mortality from cancer is not the primary tumor itself, but the occurrence of metastasis from the primary tumor.^[22,23] Disease dissemination can occur by direct invasion of tissues and cavities surrounding the primary site. However, the preferential course for metastases is spread through blood or lymphatic vasculature. Moreover, several preclinical and clinical studies have highlighted the preponderance of the lymphatic vascular system over the blood vascular system with the involvement of the sentinel lymph node being a standard diagnostic and prognostic parameter.^[24,25]

The occurrence of “*de novo*” vascularization is a crucial step in the metastatic route. In fact, the tumor neovasculature not only supports the metabolic needs of the malignant cells, but also establishes the routes for dissemination. The malignant cells overexpress various angiogenic and lymphangiogenic growth factors that alter the normal neovascularization pattern, significantly increasing blood and lymphatic vessel density (BVD and LVD, respectively).^[26] The link between neovascularization and lymphovascular invasion significantly worsens prognosis, with numerous reports on its association with risk of tumor recurrence, progression, lymph node metastasis, and distant metastasis.^[27,28] Accordingly, several preclinical models have demonstrated a significant reduction in tumor growth and tumor associated-neovasculature when the expression of angiogenic and lymphangiogenic factors was blocked.^[29-31] Anti-angiogenic/lymphangiogenic agents and targeted inhibitors, in monotherapy or in combination with standard chemotherapeutic drugs, have already reached the phase of clinical trials, and several compounds have obtained approval from the Food and Drug Administration agency.^[32,33] While these agents have shown promising therapeutic effects, substantial evidence of primary and acquired resistance has been reported.^[34] Vessel normalization, by restoring physiological perfusion and oxygenation of tumor vasculature, has recently emerged as a promising strategy to overcome resistance to certain antiangiogenic therapies.^[35]

In the setting of UBC, angiogenesis has been extensively reported, with several studies, including large-scale approaches, indicating the independent prognostic value of high vascular endothelial growth factor (VEGF) levels and BVD counts.^[36-40] A number of clinical trials with anti-angiogenic agents are ongoing for UBC patients with NMI and MI disease.^[41] Reports on lymphangiogenesis, although fewer in number, also point to a significant role of lymphatic vessel formation in UBC spread. Overexpression of VEGF-C, VEGF-D, and VEGFR-3, the key players of lymphangiogenesis, has been demonstrated in several studies, associating with high LVD counts and lymph node metastasis, also predicting poor prognosis.^[40,42-45] *In vitro* and *in vivo*

studies have shown that VEGF-C/D blockade suppresses lymphangiogenesis and lymphatic metastasis, enhancing UBC chemosensitivity.^[46,47] Therefore, there is no doubt that both blood and lymphatic vessels participate in the metastatic process. Lymphovascular invasion (LI) has been identified as an independent prognostic factor for recurrence and OS.^[48,49] A recent meta-analysis demonstrated that LI is an important selection criterion for early cystectomy in high-grade stage T1 UBC.^[50] Also demonstrated is that the LI status helps to stratify N0 UBC patients at increased risk of UBC recurrence and death.^[51] Regardless of these important associations, LI occurrence is not included as a standard parameter in many pathology reports, mostly due to the lack of strict diagnostic criteria.^[52,53]

In our research, in 83 UBC tissue sections, we used immunohistochemistry (IHC) (CD31 and D2-40 antibodies) to assess BVD and blood vessel invasion (BVI), and lymphatic vessel invasion (LVI), respectively [Table 1].^[54] Regarding angiogenesis occurrence, although we observed an association between BVD and parameters of UBC aggressiveness and progression, we did not find a significant influence on prognosis. In fact, conflicting results exist,^[38,55,56] and it has been advocated that additional factors are necessary to determine the real impact of angiogenesis in UBC progression and dissemination.^[57] In accordance, BVI occurred more frequently in cases with high BVD and was identified as an independent prognostic factor for OS. The same correlation was observed between LVD and LVI, although LVI was identified as a prognostic factor only

by univariate analysis. Nevertheless, high LVD was significantly associated with tumor aggressiveness. These results have been corroborated by others.^[58,59] Moreover, we observed that intratumoral lymphatic vessels seemed to cooperate actively in malignant dissemination by the presence of single-malignant cells in well-preserved vessels [Figure 1b]. Although these vessels have been described as collapsed and non-functional by others,^[60,61] in our series, there was a significant proportion of cases where vessels with visible lumina were seen; edema was not observed, which would support a more efficient lymphatic flow. Accordingly, the presence of intratumoral lymphatic vessels was correlated with parameters of UBC aggressiveness in one study,^[62] and was identified as a predictive factor of pelvic lymph node metastasis in another.^[59]

Another result of our study was the validation of the use of IHC markers to separate blood and lymphatic vessels. Its usefulness was particularly important in the detection of isolated malignant cells invading lymphatic capillaries [Figure 1b]. These cells, intravasated in a milieu that flows slowly and has a composition similar to interstitial fluid, have a higher survival probability when compared to the typical rigors of the blood.^[63] LVI by isolated malignant cells was significantly correlated with a poor prognosis. The same association was observed when considering BVI, but only when malignant emboli were intravasated [Figure 1a]. Thus, these parameters represent potential biomarkers of progression that can guide therapeutic regimes, and their routine evaluation is recommended by us and others.^[53,54] We additionally

Table 1: Major findings of selected immunohistochemical studies on urothelial bladder cancer biomarkers

Reference	Cohort	n	Markers	Cut-off	Impact on clinicopathological parameters and survival
[54]	RC	83	BVD	≥ 17.6 vessels	Quantification of vessel density and identification of lymphovascular invasion significantly improved when using blood (CD31) and lymphatic (D2-40) vessel markers. High LVD associated with tumor aggressiveness. BVI and LVI significantly lowered DFS and OS. BVI remained an independent prognostic factor for OS.
			LVD	≥ 8.8 vessels	
			BVI	Malignant emboli	
			LVI	Isolated tumor cells	
[75]	RC	76	p-mTOR	≥ 10% positive cells	p-mTOR expression decreased with increasing stage and was lost from non-tumor to tumor urothelium. T3/T4 positive cases (n = 49) had significant worse DFS rate.
[85]	RC	81	RKIP	≥ 10% positive cells	RKIP expression associated with favorable clinicopathological profile. Loss of RKIP expression associated with LVI occurrence, significantly lowered DFS and OS, remaining independent prognostic factor for DFS.
[100]	RC and TUR	114	MCT1 MCT4 CD147	Percentage of immunoreactive cells*+intensity of staining [†] (positive score ≥ 4)	MCT1, MCT4, CD147 expressions significantly associated with unfavorable clinicopathological parameters and poor prognosis. In selected platinum treated-patients, OS was significantly lower for those with MCT1+CD147-positive tumors.
[101]	RC	77	Scoring model [‡]	≥ 3 positive parameters	Model stronger in predicting prognosis than individual parameters, remaining independent prognostic factor for DFS and OS. CD147 expression added significant prognostic information to the model.

*0: 0% of positive cells; 1: < 5% of positive cells; 2: 5-50% positive cells; 3: > 50% of positive cells; [†]0: negative; 1: weak; 2: intermediate; 3: strong; [‡]scoring model: includes stage, grade, BVI, LVI, CD147 overexpression. BVD: Blood vessel density; BVI: Blood vessel invasion; DFS: Disease-free survival; LVD: Lymphatic vessel density; LVI: Lymphatic vessel invasion; MCT: Monocarboxylate transporter; OS: Overall survival; p-mTOR: Phospho-mammalian target of rapamycin; RC: Radical cystectomy; RKIP: Raf kinase inhibitor protein; TUR: Transurethral resection

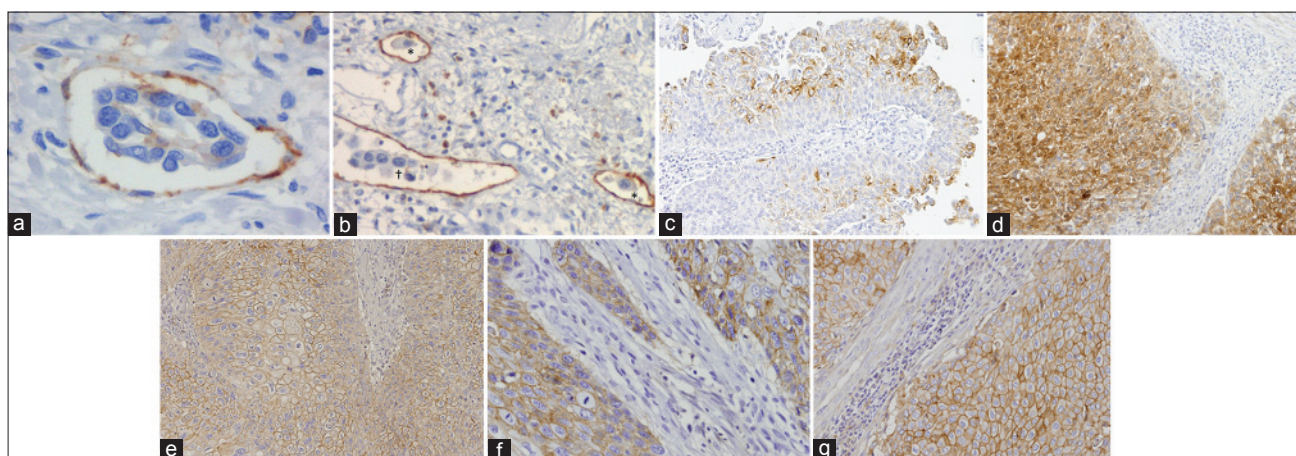


Figure 1: Representative images of immunohistochemical positive reactions for CD31, D2-40, p-mTOR, RKIP, CD147, MCT1, MCT4 in non-muscle invasive (c) and muscle invasive (a, b, d-g) urothelial bladder carcinoma (original magnifications indicated). (a) An embolus of malignant cells intravasated in an intratumoral blood vessel highlighted by CD31 (×400); (b) isolated malignant cells (*) and malignant embolus (†) invading intratumoral lymphatic vessels highlighted by D2-40 (×200); (c) heterogeneous pattern of p-mTOR immunoexpression, with the intensity of staining being lost from the luminal to the basal cell layers of the urothelium (×100); (d) heterogeneous pattern of RKIP immunoexpression, with the tumor core being more intensely stained than the invasive front (×100); (e) strong CD147 membrane immunoexpression in the inner layers of the tumor (×100); (f) immunoexpression of MCT1 in the malignant urothelium (×200); (g) immunoexpression of MCT4 in the malignant urothelium (×200). MCT: Monocarboxylate transporter; p-mTOR: Phospho-mammalian target of rapamycin; RKIP: Raf kinase inhibitor protein

suggest specific immunostaining of blood and lymphatic vessels in histologically equivocal cases that require confirmation in order to better identify lymphovascular invasion that could have been missed during routine evaluation on HE-stained tumor sections, and to allow a more accurate discrimination between the 2 forms of lymphovascular invasion.

As above, the occurrence of angiogenesis and lymphangiogenesis as potential targets for therapeutic intervention in UBC is already under clinical testing, with several compounds targeting the most relevant neovascularization signaling pathways.^[41,64] However, as with other types of cancer, the risk of refractoriness to VEGFs/VEGFRs signaling abrogation exists.^[65] Compensatory mechanisms to VEGF blockade in UBC cell lines have been described.^[66] While these anti-neovascularization compounds have clear value, additional efforts are being undertaken in the search of alternative pathways to abrogate angiogenesis/lymphangiogenesis. The mammalian target of the rapamycin (mTOR) intracellular pathway is an important signaling mediator in hypoxia-induced angiogenesis,^[67] besides transducing activator signals for promoting cell growth.^[68] UBC pre-clinical^[69] and clinical trials,^[70,71] although few in number,^[72] have shown the anti-angiogenic effects of rapamycin analogues. Nevertheless, levels of mTOR activation in UBC tissue sections have been little explored, with controversial results being found.^[73,74] We assessed phospho-mTOR (p-mTOR) levels in a series of 76 UBC tissue sections, where blood and lymphatic vessels were also specifically stained, in order to correlate angiogenesis and lymphangiogenesis with p-mTOR expression [Table 1]. Representative tumor and non-tumor (normal-like or hyperplastic) areas were

present.^[75] We did not find significant associations between clinicopathological parameters, vascular density, and p-mTOR expression. Nonetheless, p-mTOR expression [Figure 1c] decreased with increasing stage and was lost from non-tumor to tumor urothelium, particularly in muscle-invasive tumors, where immunoexpression was only observed in cell clusters. Angiogenesis was compromised in T3/T4-negative cases; conversely, the group with T3/T4-positive tumors had a quite poor outcome, as observed by others.^[73] These two patterns of expression, complete absence or presence in clusters of cells, are a possible consequence of opposing biological settings mediated by mTOR signalling. There is the need to expand the research on larger and comprehensive series of UBC patients, with molecular effectors of upstream and downstream mTOR signaling, together with reproducible IHC and molecular methodologies, and with *in vivo* and *in vitro* UBC models. This is in order to elucidate the role of the mTOR pathway in human UBC and to find more appropriate target therapeutic strategies. Accordingly, recent studies characterizing UBC genetic background revealed chromosomal alterations not seen at the same level in other types of cancers, namely, mutations of genes involved in the PI3K/AKT/mTOR signaling pathway.^[76]

Tumor metastasis

The ability of malignant cells to leave a primary tumor and to disseminate widely is commonly agreed to be the basis for metastasis formation, the mainly cause of death from cancer. As above, angiogenesis and lymphangiogenesis are integrate parts of the metastatic process, but additional steps need to occur in order for a malignant cell or a cluster of cells colonize secondary sites. These interrelated steps involve the expression of molecular promoters

and suppressors of metastasis. Moreover, the success of metastatic spread depends, not only on the intrinsic properties of the tumor cells, but also on host feedback.^[77]

Genes inhibiting metastasis without blocking the ability of the transformed cells to develop a primary tumor are included in the group of metastasis suppressors. Obviously, loss of expression of metastasis suppressor genes is part of the metastatic genetic program, and a mandatory requisite for the success of the process. After initial scepticism following the discovery of the *Nm23* gene, more than thirty protein coding/non-coding genes have been described that significantly reduce the onset of metastasis without affecting the formation of the primary tumor. Therefore, their loss occurs during cancer progression, not during transformation.^[78,79]

In the UBC setting, progression of high-risk NMI tumors (high grade Ta/T1 tumors or carcinoma *in situ*) to muscle-invasive disease and ultimately, to extra-vesical dissemination, carries a significant risk of invasion and metastasis, despite radical surgical treatment.^[4] Inhibiting biomarkers of progression and metastasis represents an attractive therapeutic approach, but restoring the function of metastasis suppressor proteins, although poorly explored, is also appealing. In this picture, the role of the metastasis suppressor Raf kinase inhibitor protein (RKIP) in cancer has been highlighted due to its ability to modulate several intracellular signaling pathways involved in cell differentiation, cell cycle kinetics, apoptosis, epithelial to mesenchymal transition, and cell migration.^[80,81] Given its pleiotropic abilities in maintaining cellular equilibrium, RKIP downregulation is associated with metastatic events in an increasing number of solid tumors.^[82,83] Its preponderance in UBC is largely unknown. In one study,^[84] low mRNA levels were reported in NMI tumors when compared with normal urothelium. In our research, and for the first time (to the best of our knowledge), we studied 81 tumor sections from UBC patients for RKIP immunostaining [Table 1].^[85] We observed tumors with a favorable clinicopathological profile, namely, NMI tumors where LVI was absent, with a homogeneous expression of RKIP. Conversely, LVI occurrence was associated with a heterogeneous pattern of RKIP expression, where expression intensity was lost from tumor center to invasion front [Figure 1d]. Low RKIP expression significantly impacted prognosis, remaining an independent prognostic factor for disease-free survival. As mentioned, similar associations concerning other aggressive cancer types have been previously reported. Clinically, a gradual decrease of RKIP expression was noted from benign to malignant tumors, and from those to metastatic sites.^[82,83] In the UBC setting, additional studies are needed in order to confirm our results and to expand research into therapeutic strategies that can potentially restore RKIP functionality. Besides acting as a biomarker of progression to metastatic disease, the

potential role of RKIP as a predictive biomarker has also been proposed, since its expression may mediate apoptosis induced by chemotherapeutic regimes.^[86,87]

Tumor metabolic reprogramming

Cancer is not only a complex genetic disease, but also a disease of deregulated bioenergetic metabolism. Elevated glycolytic rates are a common trait of malignancy.^[88] Warburg was the first to describe the metabolic switch, known as “The Warburg Effect,” whereby a tumor cell avidly consumes glucose and reprograms its metabolism, producing large amounts of lactate, even under aerobic conditions.^[89] Lactate is the main source of microenvironmental acidosis in tumors, contributing to an acid-resistant phenotype that supports increased migration and invasion abilities of cancer cells.^[90-92] Its dependence on monocarboxylate transporters (MCTs) for transport across the plasma membrane directly implicates MCTs in tumor behavior.

Monocarboxylate transporters belong to the *SLC16* gene family, comprising 14 members, of which MCTs 1-4, the proton-linked MCTs, mediate influx/efflux of monocarboxylates across the plasma membrane. MCT1 and MCT4 are the best characterized MCTs in human tissue, with MCT1 having ubiquitous distribution and MCT4 being present in highly glycolytic tissues.^[93] The proper expression of MCTs at the plasma membrane depends on their interaction with CD147,^[94] a cell surface glycoprotein implicated in extracellular matrix remodeling, angiogenesis, migration, and invasion and related to chemoresistance-promoting events.^[95] CD147 and MCTs overexpressions have been described in several cancers, associated with poor clinicopathological and survival parameters.^[95,96] Some *in vitro* models have demonstrated that CD147 downregulation sensitizes malignant cells to platinum-based therapy.^[97-99] Therefore, metabolism-related cellular pathways involved in malignancy represent potential areas of therapeutic intervention.

Biological mechanisms that reprogram cellular energetics in the setting of UBC are poorly characterized. Thus, we investigated, in a series of tumor tissue sections from 114 UBC patients, a panel of three metabolism-involved molecules [Table 1].^[100] The central protein seemed to be CD147. We had previously demonstrated the prognostic impact of its overexpression in UBC when we developed a model of UBC aggressiveness ($n = 77$) that included classical clinicopathological (stage and grade) and biological parameters (lymphovascular invasion^[54] and CD147 expression).^[101] In fact, this scoring system separated a low aggressive from the high aggressive group, remaining as an independent prognostic factor for disease-free and OS. In the group of highly aggressive tumors, CD147 positivity was 87%, clearly adding prognostic information to the model [Table 1]. Thus, we decided to re-evaluate this glycoprotein in a larger series, exploring its crosstalk with MCTs and possible role in

chemoresistance.^[100] Significant associations were found among the biomarkers, which support the chaperone function of CD147, as corroborated by others.^[94] We also observed that CD147, MCT1, and MCT4 were upregulated [Figure 1e-g], being significantly associated with a poor clinicopathological profile, namely, advancing stage, grade, type of lesion, and occurrence of LVI. Moreover, MCT1 and CD147 overexpressions were associated with poor prognosis, particularly in cases that were positive for both biomarkers. Interestingly, when we selected patients who received platinum-based chemotherapy, the prognosis was significantly worse for those with MCT1- and CD147-positive tumors. Probably, MCT1 cooperates with CD147 in the promotion of a chemoresistance phenotype and possibly, of other functions primarily attributed to CD147. In fact, it appears that CD147 maturation is affected by MCT expression.^[102] Other authors have identified CD147 expression in UBC as an independent prognostic biomarker,^[103,104] and have additionally proposed it as a predictive biomarker in the setting of cisplatin-containing regimens.^[104,105] Recently, one group^[106] demonstrated the independent prognostic significance of MCT1 and MCT4 in UBC. Those results led us to knock down CD147 expression in a UBC cell line with a cisplatin-resistant phenotype. We found that CD147 depletion was accompanied by a decrease in MCT1 and MCT4 expressions, additionally supporting its chaperone function. Notably, we also found an increase in chemosensitivity to cisplatin. To the best of our knowledge, this is the first study to assess MCT expression and correlation with CD147 in UBC tissue from platinum-treated patients, and to characterize UBC chemosensitivity to cisplatin *in vitro* upon CD147 inhibition. Our findings reveal a major role of CD147 and companions in promoting progression of a UBC-aggressive phenotype, with high glycolytic activity, contributing to microenvironmental acidification. This enables the malignant cell to demonstrate growth, migration, invasion, and chemoresistance abilities that can potentially be bypassed if new approaches of targeted therapeutic intervention are investigated. Though investigation of CD147 and its association with metabolic remodeling and chemoresistance is still in a preliminary phase in UBCs, progress has been made in other areas, and CD147-directed monoclonal antibodies have reached the phase of pre-clinical/clinical trials, namely for hepatocellular carcinoma.^[107]

Concluding Remarks

Urothelial bladder carcinoma represents about 90% of all cases of bladder cancer.^[5] Its relapsing and progressive nature, and the disparities in treatment responses, are the major concerns in patient care and have a significant socio-economic impact.^[10] In an attempt to clarify its heterogeneous natural history and clinical behavior, recent progress has been made in genomic studies.^[8,108] This should help in refining our understanding of the

pathogenesis of the disease and of the biological basis for outcome disparities. Furthermore, the consequent emergence of UBC biomarkers will allow us to identify patients at increased risk of recurrence, progression, metastasis, and/or chemorefractoryness, informing us about more efficient treatment and surveillance strategies. In addition, biomarkers may improve prediction of response to treatment and guide us to an era of personalized medicine and targeted therapy. In fact, classical diagnostic and prognostic instruments, such as risk stratification tables,^[109,110] nomograms,^[111,112] and artificial neural networks,^[113,114] would undoubtedly refine diagnosis, prognosis, and therapeutic decisions with the inclusion of prognostic and predictive biomarkers. Several studies have demonstrated the potential impact of developing risk stratification tools that combine clinicopathological and biological parameters.^[101,115,116] Moreover, it seems that integrating a molecular signature of biomarkers inherent in different cancer hallmarks improves predictive accuracy over one biomarker abnormality, as several biomarkers may help to elucidate individual biological features of tumors.^[14,101,117-120] Our previous study on a tumor aggressiveness scoring model, where we combined analysis of 2 clinicopathological parameters, stage and grade, with 3 biological parameters, BVI, LVI, and CD147 overexpression, also corroborates those premises [Table 1].^[101] In fact, the recent genomic profile of UBC revealed a more complex picture than it was previously supposed, with multiple molecular subclasses that traverse conventional grade and stage groupings.^[76,121,122] This leads us to believe that only an integrated clinicopathological and molecular signature will refine prognostication and therapeutic index for UBC patients. Therefore, it is important to transpose tests on small groups of patients to large-scale independent validation assays, involving multiple institutions so that prospective validations and randomized trials based on the retrospective outcomes may then proceed. As stated,^[123] any newly proposed anticancer strategy must integrate a personalized treatment outcome approach, ideally resulting in a predictive cancer staging system orientated toward the patient and the tumor, and a response evaluation system with multiple standardized variables.

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Detection and quantification of extracellular microRNAs in medulloblastoma

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ABSTRACT

Aim: Medulloblastoma (MB) is the most common malignant brain tumor in children. The crucial role of extracellular-microRNAs (ex-miRNAs) in cancer has been widely recognized; however, their role in MB remains unknown. This study aimed to investigate MB-driven ex-miRNAs. **Methods:** Microarray analysis was used to disclose the identity and quantity of key miRNAs excreted in culture-medium (CM) of 3 human MB cell lines and cerebrospinal fluid (CSF) of brain tumors (including MB) and leukemia patients. MiRNA expression was validated by quantitative reverse transcription polymerase chain reaction. **Results:** We have demonstrated that the 3 MB cell lines tested commonly expressed 1,083 miRNAs in their spent CM. Among them, 57 miRNAs were specific to the CM of metastasis-related cell lines which represents the aggressive group 3 and group 4 MB subtypes. A significant number (1,254) of ex-miRNAs were identified in the CSF of a MB patient. Eighty-six of these miRNAs were found to be differentially expressed in this patient's CSF compared with controls. Interestingly, 3 metastasis-associated miRNAs over-represented in CM of metastasis-related MB cell lines were found to be significantly enriched in the CSF of the MB patient. **Conclusion:** Although more samples are required to fully verify these results, our work provides the first evidence for the presence of a significant amount of miRNAs excreted extracellularly by MB cells and raises the possibility that, in the near future, miRNAs could be probed in CSF of MB patients and serve as novel biological markers.

Key words: Medulloblastoma, extracellular-microRNA, pediatric cancer

Introduction

Medulloblastoma (MB) is the most common malignant brain tumor in children.^[1] Metastatic MB carries a poor prognosis.^[2] Mechanisms that predict dissemination are poorly understood. Recently, several studies have revealed a critical role for microRNAs (miRNAs) during tumorigenesis and metastasis of several cancers, including MB.^[3-6]

Besides intracellular miRNAs with the traditional function of translation regulation, there is accumulating evidence that miRNAs exist extracellularly in body fluids, including cerebrospinal fluids (CSF).^[7,8] Several reports have described that deregulated extracellular-miRNAs (ex-miRNAs) are closely associated with the clinical course of malignant tumors.^[9,10] Interestingly, such deregulation returns to a normal level after tumor resection.^[7,8] Hence, expression analysis of ex-miRNAs is of increasing interest for diagnostic and prognostic purposes.

Every cancer investigated has a distinct miRNA signature and deregulated levels of miRNAs have been detected in body fluids of patients, including those with lymphoma,^[11] leukemia,^[12] colon,^[13] breast,^[14] prostate,^[15] ovarian,^[16] pancreatic,^[17] gastric,^[18] and lung cancer.^[19] In the context of brain tumors, recent studies have demonstrated a significant presence of certain miRNAs in CSF samples from patients with central nervous system lymphoma, glioma, and metastatic brain cancers.^[20-22] Recent miRNA profiling of CSF has enabled early detection of glioblastoma and reflected disease activity.^[22] Therefore, ex-miRNAs may represent important minimally invasive candidate biomarkers in brain tumors. The presence and biological role of ex-miRNAs in MBs, however, remain unknown. This study was conducted to gain insight into the identity and quantity of MB-related ex-miRNAs and to speculate on their possible biological function in the context of MB metastasis.

Methods

Patient characteristics and CSF

CSF samples from patients with MB ($n = 2$), control patients with leukemia with no intracranial mass lesions and/or neurologic disorders ($n = 3$), CSF samples from patients with ependymoma ($n = 3$) and glioblastoma ($n = 1$) that were collected from patients treated at the University Children's Hospital of Zürich,

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Switzerland. Written informed consent was obtained from each patient. CSF samples from patients with MB were collected 3 weeks after surgery and before start of radiotherapy or chemotherapy. CSF samples were centrifuged (500 g, 10 min, room temperature) within 60 min after collection to remove cells and debris and were stored at -80 °C until further processing.

Human MB cell lines

Human MB cell lines (DAOY and D283) were purchased from American Type Culture Collection (Manassas, VA, USA). D341 human MB cells were the kind gift of Dr. Henry Friedman (Duke University, Durham, UK). MB cell lines were cultured as previously published^[23] and maintained at 37 °C in a humidified atmosphere with 5% CO₂. To isolate RNA from cultured medium, 10.000-20.000/mL DAOY cells or 20.000-40.000/mL D341, D283, and T293 cells were plated and left to grow in their conditioned media for 72 h in 24 wells plates. Conditioned medium (2 mL) of each cell lines were centrifuged at 1,200 rpm to remove cells. The supernatant was then centrifuged at 10,000 rpm to remove debris.

RNA extraction for microarray

Total RNA from cell cultures or CSF were extracted using a mix of Qiazol, Qiagen (Qiagen, Basel, Switzerland) and chloroform directly on cells. For small RNA in conditioned medium or CSF, the addition of miRNAs extraction reagent (Toray) was performed. In both situations, a centrifugation step was required to collect aqueous phase containing RNA that was finally transferred to miRNeasy Mini spin column from miRNeasy purification kit Qiagen (Qiagen, Basel, Switzerland). After subsequent washing steps, RNAs were eluted using 30 µL of nuclease-free water and concentrated up to 3 µL with vacuum concentrator. Quality was checked on Bioanalyzer using RNA 6000 Pico Chip (Agilent Chemical Analysis, Life Sciences,

and Diagnostics, Basel, Switzerland) gel and quantified using Nanodrop Photometer [Figure 1a and b].

Labeling and hybridization

Total RNA (250 ng) extracted from cells and 3 µL of concentrated small RNA extracted from medium were used with Toray 3D-Gene miRNA labeling kit (Toray, Japan) in presence of spikes used as positive controls. Briefly, 5'-phosphates were removed from miRNA end using alkaline phosphatase and a fluorescent label was enzymatically attached to the 3'-end of the miRNA. After an enzyme inactivation step and addition of a hybridization buffer, labeled miRNA was injected on 3D-Gene Human miRNA Oligo Chips (Toray, Japan) targeting 2019 miRNA based on miRBase release 19. Finally, arrays were placed in a hybridization chamber and set into a 32 °C oven for 16 h with a shaker adjusted to 250 rpm.

Washing and scanning

Arrays were washed using 3 solutions with different stringencies to remove non-specifically bound miRNAs. Then, arrays were scanned with the 3D-Gene Scanner 3000 instrument (Toray, Japan) to measure fluorescence. Scanning was carried out using 3 different photomultiplier sensitivities (PMT gain) to allow optimizing of signal detection and checking for consistency.

Microarray analysis

Images were analyzed with the 3D-Gene Extraction software (Toray, Japan). After completion of the auto-analysis work followed on image files, raw signals, and detection calls was produced in tabular files. GeneSpring GX12 (Agilent) was then used to apply quantile normalization and differential expression analysis using modified *t*-test implemented in the software. Experimental variability was assessed with principal component analysis (PCA) [Figures 2 and 3b] and Pearson correlation matrix [Figure 3a] generated using the same software.

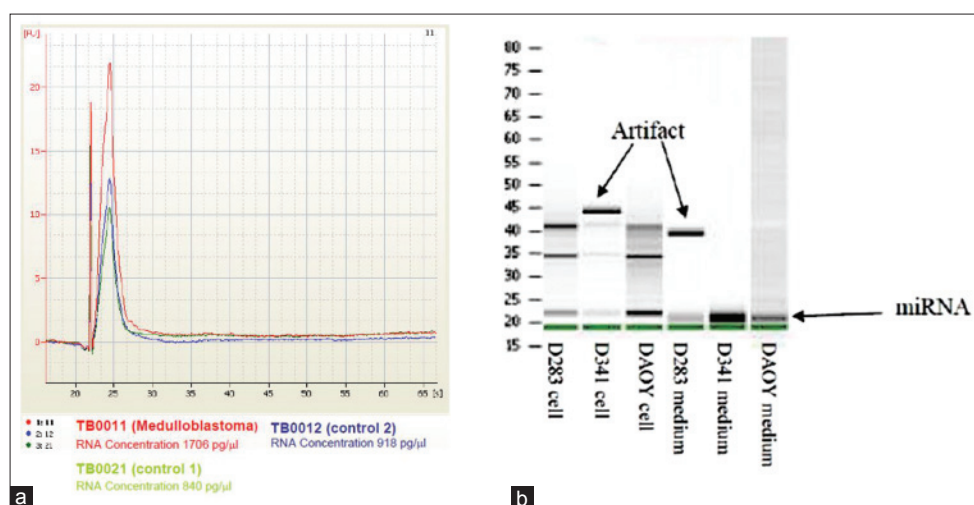


Figure 1: Quality control for RNA isolated from CSF, cell lines, and their corresponding CM measured/analyzed by (a) BioAnalyzer PicoChip (Agilent); (b) RNA gel. CM: Culture-medium; CSF: Cerebral spinal fluid

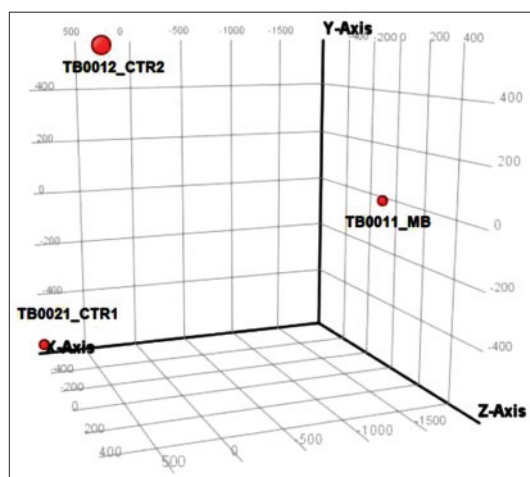


Figure 2: PCA graph showing microRNA spectra in CSF of MB patient vs. control CSFs. TB0021_CTRL1: CSF from patient with no brain tumor control 1; TB0012_CTRL2: CSF from patient with no brain tumor control 2; TB0011_MB: CSF of MB patient. MB: Medulloblastoma; CSF: cerebral spinal fluid; PCA: Principal component analysis

MicroRNA isolation for reverse transcription polymerase chain reaction analysis

For precipitation of nucleic acids, the monovalent cation concentration of the solution was adjusted to 0.5 mol/L sodium acetate. Glycogen (AM9510, Ambion, Life Technology, NY, USA) was added to a final concentration of 100 µg/mL. The solution was then mixed with 1 volume of isopropanol. The mixture was chilled for 20 min at -20 °C, then centrifuged for 20 min at 13,000 rpm. The supernatant fluid was removed, and the nucleic acid resuspended in lysis buffer. Final purification of RNA enriched for small RNAs from 600 µL of conditioned media and CSF samples was obtained using the mirVana™ miRNA Isolation Kit (Ambion, Life Technology) according to manufacturer's instructions for "Enrichment Procedure for Small RNAs." Using this approach consisting of two sequential filtrations with different ethanol concentrations, an RNA fraction highly enriched in RNA species ≤ 200 nt was obtained. First strand synthesis of mature miRNAs was followed by quantitative reverse transcription polymerase chain reaction (qRT-PCR) using miRNA-specific TaqMan MGB probes (Applied Biosystems, Life Technology). For the qRT-PCR reaction, the Gene Expression Master Mix was used and the protocol was optimized for the ABI7900HT reader (Applied Biosystems). Probe-primer solutions specific for the following miRNAs were used: miR-1290 (002863), miR-125a-3p (002199), miR-1298 (002861), miR-125b-1* (002378), miR-486-3p (002093), miR-572 (001614), miR-4476 (464702_mat), miR-615-5p (002353), and miR-3918 (464506_mat) (Applied Biosystems, Life Technology). The relative gene expression was calculated for each gene of interest using the $\Delta\Delta CT$ method, where cycle threshold values were normalized to the level of cel-miR-39-3p (4464066,

Ambion, Life Technology), which was used as spike-in by adding it during the lysis step of miRNAs extraction.

Results

Detection of ex-miRNAs in cultured medium of MB cell lines by microarray analysis

Given that some human cancer cells secrete miRNAs into their extracellular environment and body fluids,^[24-26] it was hypothesized that MB cell lines may secrete miRNAs into their spent culture medium. To test this hypothesis, 3 cell lines representing MB subtypes D341 and D283 (metastasis-related group 3 and group 4 MB subtypes)^[27] and DAOY (sonic hedgehog-related) were cultured individually for 72 h *in vitro* and miRNAs expression was analyzed in the lysates of each MB cell line and in their corresponding culture media. We identified 1,662, 1,615, and 1,199 secreted miRNAs in the culture-medium (CM) of MB cell lines D283, D341 and DAOY, respectively, among them 1,083 miRNAs that were common in the CM of the 3 cell lines. In cell lysates of D283, D341 and DAOY, on the other hand, we detected 1,787, 1,394 and 1,761 miRNA respectively, with 1,347 miRNAs found common to all 3 cell lines [Figure 4a]. Interestingly, 950 miRNAs were commonly identified in CM of both groups and in lysates of the 3 cell lines tested, indicating that the level of ex-miRNAs may well reflect the expression level of tumor miRNAs. Using a fold-change > 2, we identified a group of 156 miRNAs that are commonly enriched in CM derived from the 3 cell lines compared to their respective cell lysates [Figure 4b] and [Supplementary Table 1] and 57 miRNAs that were specific to the CM of D341 and D283, which represented the 2 metastasis-related group 3 and group 4 MB subtypes, respectively^[27] compared to DAOY-derived CM [Figure 4b] and [Supplementary Table 2]. We found 2 additional groups of miRNAs to be differentially enriched in CM of D341 and D283, represented by 60 miRNAs overrepresented and 52 underrepresented compared to DAOY-derived CM [Supplementary Tables 3 and 4]. Overall, the results of this experiment demonstrate that MB cell lines secrete miRNAs into the CM and that certain ex-miRNAs retain different enrichment levels in the CM-derived from the 2 cell lines representing the metastasis-related group 3 and group 4 MB subtypes

Detection of ex-miRNAs in CSF of MB patients by microarray analysis

We next asked whether ex-miRNAs could be detected in CSF of MB patients, to test whether it would be technically possible to use the CSF as a source for diagnostic miRNA testing. Using microarray analysis, we screened cell-free CSF from a patient with MB and compared the results to controls (CSF from two different leukemia patients with no cerebral manifestation or

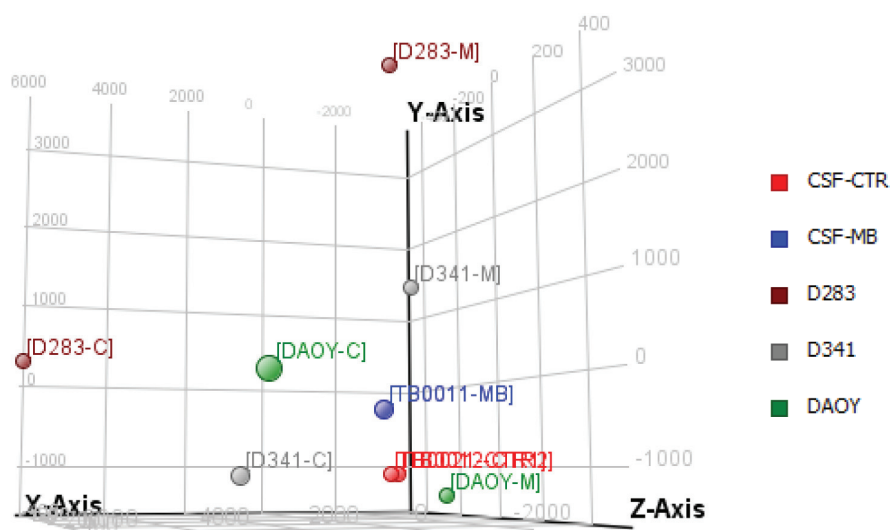
Inter-sample correlations

	D283-C	D283-M	D341-C	D341-M	DAOY-C	DAOY-M	TB0011-MB	TB0012-CTR2	TB0012-CTR1
D283-C	100.00%	21.69%	85.12%	20.54%	83.91%	17.33%	19.55%	20.36%	19.58%
D283-M	21.69%	100.00%	23.70%	91.94%	29.50%	83.96%	88.54%	88.77%	86.66%
D341-C	85.12%	23.70%	100.00%	22.64%	93.61%	18.88%	20.34%	22.13%	20.65%
D341-M	20.54%	91.94%	22.64%	100.00%	28.22%	86.76%	85.53%	85.12%	83.75%
DAOY-C	83.91%	29.50%	93.61%	28.22%	100.00%	23.71%	26.54%	26.54%	27.26%
DAOY-M	17.33%	83.96%	18.88%	86.76%	23.71%	100.00%	93.56%	93.56%	91.32%
TB0011-MB	19.55%	88.54%	20.34%	85.53%	26.54%	93.56%	100.00	98.02%	97.37%
TB0012-CTR2	20.36%	88.77%	22.13%	85.12%	28.05%	92.88%	98.02%	100.00%	98.43%
TB0012-CTR1	19.58%	86.66%	20.65%	83.75%	27.26%	91.32%	97.37%	97.37%	100.00%

Intra-and inter-type correlations :

	Cell lines	CM	CSF-MB	CSF-CTR
Cell lines	87.59%	22.91%	22.14%	23.01%
CM	22.91%	87.55%	93.56%	88.08%
CSF-MB	22.14%	93.56%	100.00%	97.69%
CSF-CTR	23.01%	88.08%	97.69%	98.43%

a



b

Figure 3: (a) Pearson correlation analysis for indicated samples; (b) PCA graph representing microRNA spectra inside MB cell lines (cell line name-C), in culture medium (cell line name-M) and in CSF of MB patient (TB0011-MB) compared to control TB0021_CTR1 and TB0012_CTR2. Graph demonstrating similarity between miRNA profile in CM and those secreted in CSF of MB patient. CM: Culture medium; PCA: Principal component analysis; MB: Medulloblastoma; CSF: Cerebral spinal fluid

neurological disease). PCA [Figure 2] showed clear separation between the miRNA spectrum in CSF of MB patient and controls. Microarray analysis identified 1,254 miRNAs in the MB-CSF sample [Table 1], of which 86 miRNAs were differentially expressed in CSF of the MB patient compared to the 2 CSF

controls [Figure 4c] and [Supplementary Table 5]. Further analysis identified 268 miRNAs over-represented (with fold-change > 2) and 6 miRNAs under-represented in MB-CSF compared with the 2 different controls tested [Supplementary Tables 6 and 7], indicating a trend toward miRNA enrichment in the MB-CSF sample.

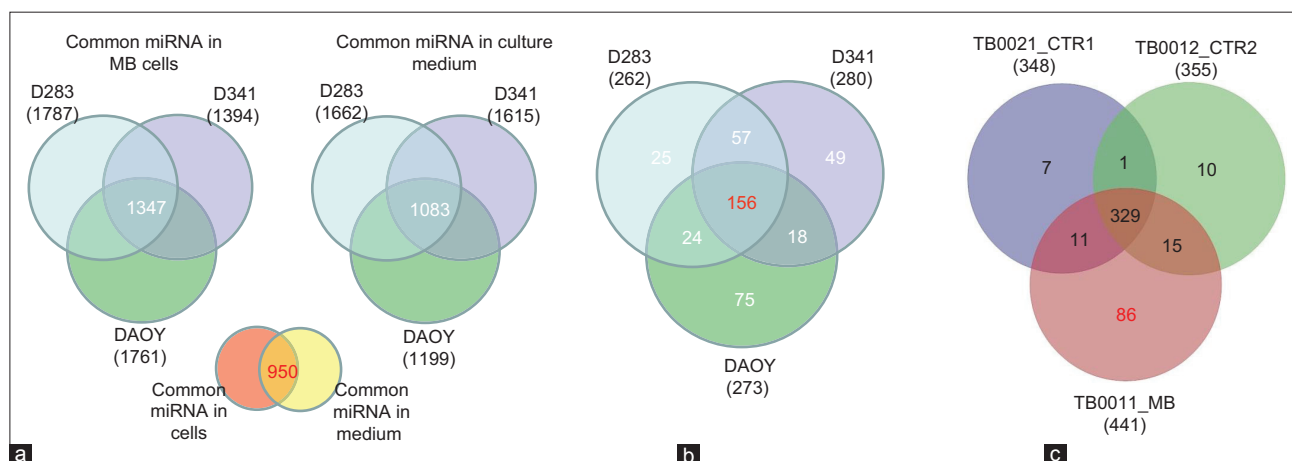


Figure 4: MiRNAs expression spectrum in MB cell lines, their corresponding CM and in CSF (a) Quantitative Venn diagram showing miRNAs commonly expressed in indicated cell lines and their corresponding CM. Differences linked to the expression level rather than detection threshold; (b) Venn diagram showing that 156 miRNAs enriched in CM of MB cell lines compared to their cell lysates and 57 miRNAs enriched in CM of the MR D283 and D341 but not in DAOY. Fold change > 2; (c) Venn diagram demonstrating miRNAs with high expression in CSF of MB patient compared to controls. TB0021_CTRL1: CSF of leukemia with no brain tumor (patient control 1); TB0012_CTRL2: leukemia with no brain tumor (patient control 2); TB0011_MB: CSF of MB patient. MB: Medulloblastoma; CSF: Cerebral spinal fluid; CM: Culture-medium; MiRNAs: MicroRNAs

Table 1: Number of miRNAs detected in CSF

CSF	Number of miRNA detected
CSF of MB patient	1,254
CSF control 1	1,004
CSF control 2	1,049

CSF: Cerebral spinal fluid; miRNAs: MicroRNAs; MB: Medulloblastoma

Comparison between miRNA expressions in CM vs. CSF samples

An overlap of the spectra of ex-miRNA candidates detected in the CSF of the MB patient and those excreted by MB cell lines into the CM would support our hypothesis of miRNA secretion by MB cells. Indeed, Pearson correlations analysis showed that ex-miRNAs profiles in MB-CSF displayed a good homogeneity with the profile of miRNAs secreted in CM of MB cell lines [Figure 3a]. This conclusion was confirmed by PCA showing clear separation of miRNAs derived from lysates of MB cell lines from those derived of MB-CSF samples or of CM derived from MB cells [Figure 3b], confirming the conclusions from Pearson correlations. Compiling expression tables allowed identification of 5 miRNAs (miR-486-3p, miR-572, miR-3918, miR-4476, and miR-615) that were significantly up-regulated in the CM of the 3 cell lines (D283, D341 and DAOY) and enriched in MB-CSF compared to control CSF [Figure 5a]. Moreover, 3 other miRNAs (miR-1290, miR-125a, miR-125b), known to be associated with metastasis, and miR-1298, were over-represented in the CM of metastasis-related cell lines (D283 and D341), but not in DAOY and were significantly over-represented in MB-CSF compared to control CSF [Figure 5b].

Validation of microarray data by qRT-PCR

In order to further verify the results of miRNA microarray analysis, we selected miR-486-3p, miR-572, miR-3918, miR-4476, and miR-615 for quantitative real-time PCR assays because of their over-representation in the CM of the 3 MB cell lines and in MB-CSF. TaqMan analysis confirmed the outcomes of miRNA microarray profiling for the 5 miRNAs tested and showed that the five were enriched in the CM of the 3 cell lines [Figure 6a]. However, only miR-615 and miR-572 were also accumulated in MB-CSF [Figure 6b]. We also chose miR-1290, miR-125a, miR-125b, and miR-1298 as other candidates for qRT-PCR due to their over-representation in CM of metastasis-related cell lines D283 and D341, as well as in CSF of the MB patients. TaqMan analysis showed an evidently increased expression level of the 4 cell line-derived miRNAs in both D283 and D341 compared to DAOY [Figure 7a]. The levels of 3 miRNAs (miR-1290, miR-125a, miR-1298) were also markedly increased in MB-CSF, thus confirming the results of the miRNA microarray analysis for 3 out of 4 of these selected miRNAs [Figure 7b].

To further validate the finding of selective enrichment of miR-1298 in MB-CSF, we tested it against an additional 5 different CSF controls from one leukemia patient, 3 ependymoma patients, and one glioblastoma patient (specifically chosen to control for brain surgery as a possible factor influencing miRNA secretion). Consistently, TaqMan analysis confirmed significant enrichment of miR-1298 in MB-CSF compared to the 5 controls [Figure 8a]. Together, using TaqMan analysis, we confirmed the microarray data result and demonstrated the feasibility of quantitative detection of miRNAs in culture medium and CSF using qRT-PCR (popular gene expression assay and efficient method for high-throughput

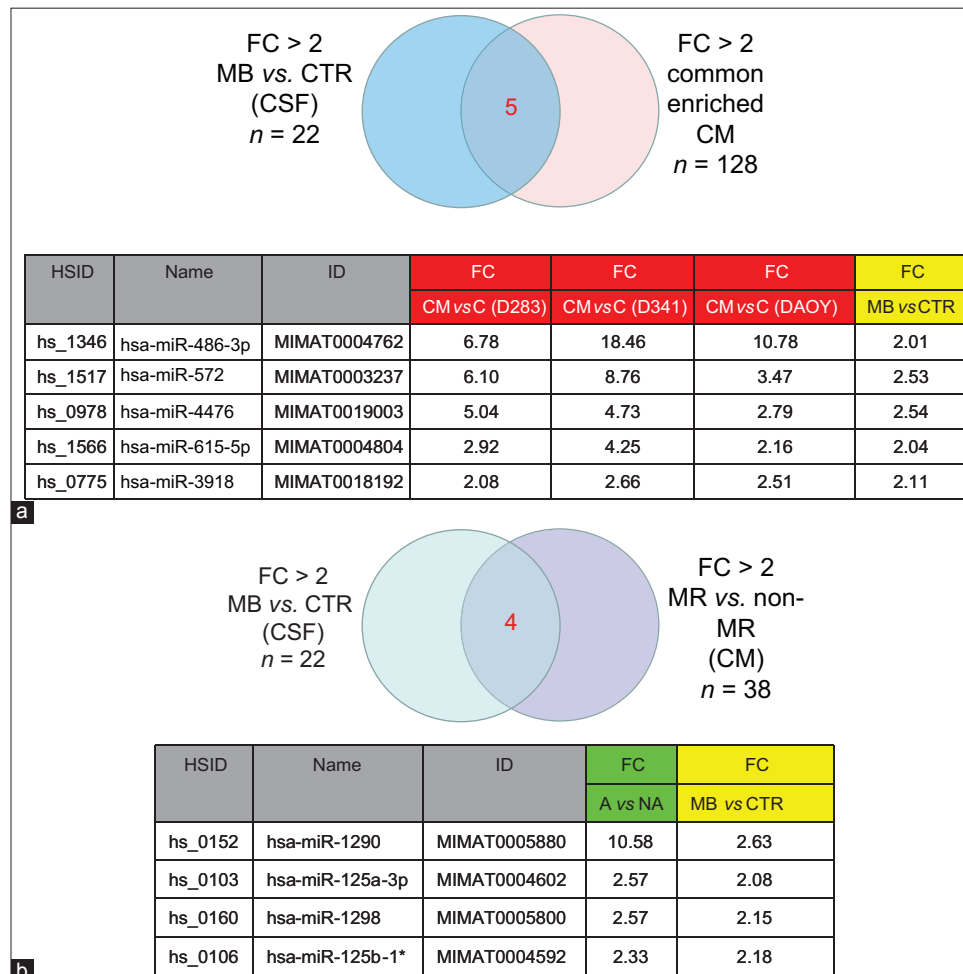


Figure 5: MiRNAs commonly enriched in CM of MB cell lines, and in CSF sample. (a) Venn diagram and table presenting 5 miRNAs commonly upregulated in CM of 3 MB cell lines and in CSF from MB patients compared to CTR; (b) Venn diagram and table representing 4 miRNAs commonly upregulated in CSF samples from MB patients and over-represented in CM of the MR cell lines D341, D283. CM: Culture-medium; MB: Medulloblastoma; CSF: Cerebral spinal fluid; MR: Metastasis related; FC: Fold change; CTR: Control; MiRNAs: MicroRNA

used in most diagnostic labs). Importantly, we could detect ex-miRNAs by qRT-PCR in CM of as few as 100-500 MB cells [Figure 8b], recommending qRT-PCR for the development of non-invasive detection of metastasis-predicting markers for MB.

Discussion

Aberrant expression of ex-miRNA circulating in CSF of certain brain tumor patients has recently been reported to be cancer biomarkers and potential regulators of the disease.^[7,8] However, the existence and role of ex-miRNAs in MB extracellular environment are unknown. Therefore, better understanding of ex-miRNA secretion and function in MB seems crucial for the development of novel insights for its diagnosis and prognosis. This study aimed to identify key miRNAs in culture medium of 3 cell lines, representing different MB subtypes. Our results identified a significant number (1,347) of hitherto unrecognized new miRNAs commonly expressed in CM of the 3 cell lines. A significant concordance of ex-miRNA

spectra in CM and those expressed intracellularly was observed. Since deregulated miRNA expression is an early event in tumorigenesis, measuring miRNA levels in CSF may also be useful for early detection, which can contribute greatly to the success of treatment.^[28] Therefore, in order to use ex-miRNAs as biomarkers for MB, it is important to establish a signature capable of differentiating disease from healthy states. Our pilot microarray screening identified 86 miRNAs exclusively detected in CSF of MB patients but not in control CSF from patients with no brain tumor. We also identified 268 miRNAs that are over-represented and interestingly, only 6 miRNAs under-represented in MB-CSF compared with control CSF. These findings could be of great significance, providing the correlation between expression levels of these miRNAs in CSF of MB patients and their disease states can be established in future studies.

Tumor cell-derived ex-miRNAs are reported to be pro-tumorigenic.^[29] Ex-miRNAs can transfer their oncogenic activity to recipient target cells to influence

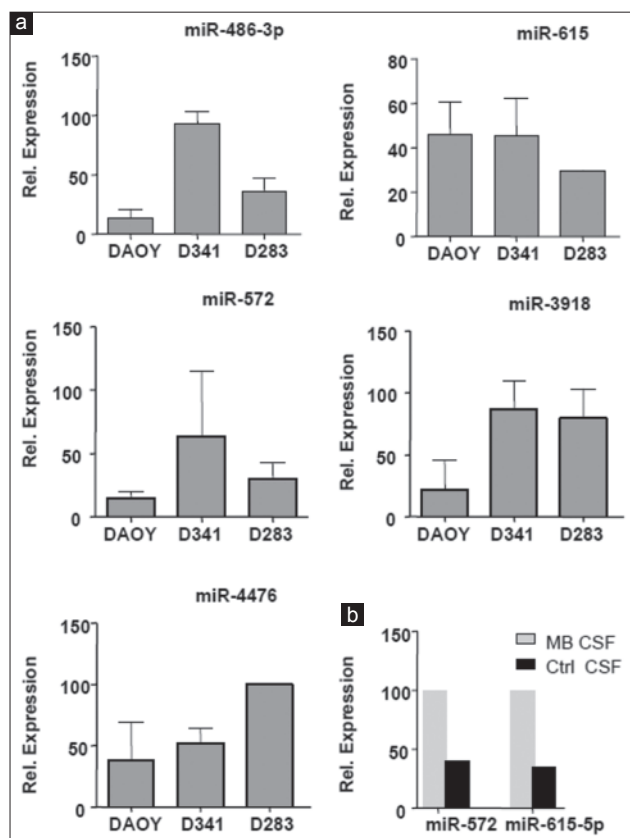


Figure 6: Expression analysis of 5 ex-miRNAs in MB cell lines CM and in CSF of MB patient. (a) TaqMan qRT-PCR analysis for miR-486-3p, miR-572, miR-3918, miR-4476, miR-615 in CM of indicated cell lines; (b) TaqMan qRT-PCR analysis for miR-572, miR-615 in MB-CSF ($n = 3$; \pm standard deviation). MB: Medulloblastoma; CSF: Cerebral spinal fluid; CM: Culture-medium; qRT-PCR: Quantitative reverse transcription polymerase chain reaction

cancer stimulatory activities, thus contributing to the formation of a pre-metastatic niche and promotion of metastasis.^[28,30] This exchange of miRNAs between primary tumors and target cells is an interesting and novel dimension to the regulation of a cell phenotype^[31-34] and may be particularly important in cancers that have a propensity for dissemination, such as MB. MB includes various subtypes with group 3 and 4 subtypes being clinically distinct with regard to metastasis and prognosis, which may also manifest in a difference in their miRNA spectra. Hence, it was not surprising to find a group of miRNAs that were uniquely over-(60 miRNAs) or under-represented (52 miRNAs) in the CM of the 2 metastasis-related cell lines D283 and D341. More importantly, we identified 4 miRNAs (miR-1290, miR-125a, miR-125b, miR-1298) that were over-represented in MB CSF and significantly enriched in the CM media of the 2 metastasis-related cell lines (D283 and D341). Remarkably, apart from miR-1298, where no functional information is publically available, the 3 other miRNAs (miR-1290, miR-125a, miR-125b) were detected in body fluids of various cancer patients, whereby their increased expression and/or secretion is associated with metastasis

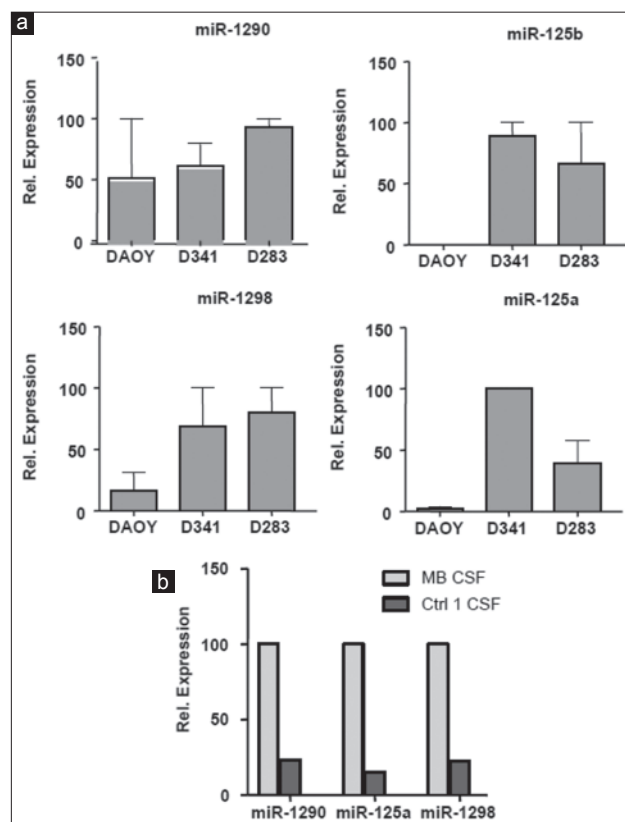


Figure 7: Expression analysis of 4 overexpressed ex-miRNAs in MB cell lines CM and in CSF of MB patient. (a) TaqMan qRT-PCR analysis for miR-1290, miR-125a, miR-1298, miR-125b in CM of indicated cell lines; (b) TaqMan qRT-PCR analysis for miR-1290, miR-125a, miR-1298 in MB-CSF ($n = 3$; \pm standard deviation). MB: Medulloblastoma; CSF: Cerebral spinal fluid; CM: Culture-medium; qRT-PCR: Quantitative reverse transcription polymerase chain reaction

of multiple malignancies.^[35-39] Consistently, detection of metastasis-related ex-miRNAs in extracellular environment of certain human malignancies, including breast and prostate cancers, were observed in other studies.^[40-44] Our observations provide indirect evidence supporting the hypothesis that ex-miRNA are possible facilitators of metastasis by modifying local or distal microenvironments.^[45] However, further studies are needed using counter-regulation of key ex-miRNA expression to determine their effect on regulation of motility, migration, and invasion of MB cells.

To the best of our knowledge, this is the first study revealing the spectra of ex-miRNAs in cell CM conditioned by MB cell lines and in CSF of an MB patient. Although the number of samples studied here is very small, our identification of key secreted miRNAs that are specifically enriched in MB-CSF provides a rationale for future investigations. Such investigations, using larger sets of MB samples could lead in the near future to the discovery of CSF-derived miRNA markers, with diagnostic and prognostic significance and ultimately, hopefully also with therapeutic potential.

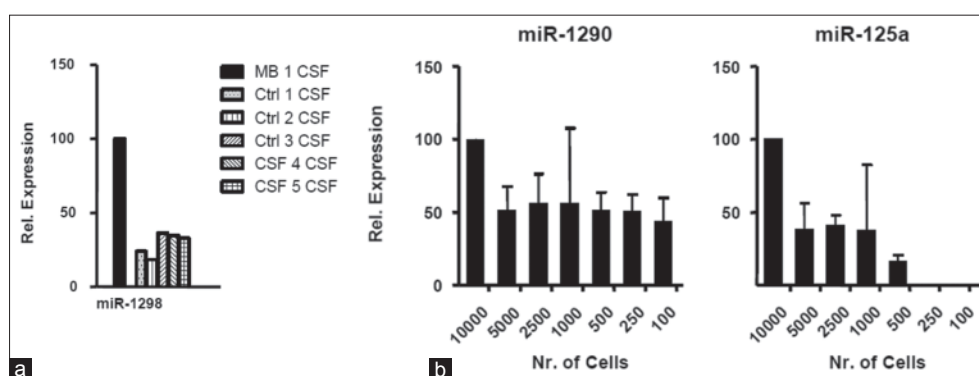


Figure 8: Relative ex-microRNAs expression analysis in CSF, MB cell lines: (a) TaqMan qRT-PCR analysis for miR-1298 compared to 5 controls: Ctrl 1 CSF from leukemia patient, Ctrl 2 CSF from glioblastoma patient, Ctrl 3-5 CSF from 3 ependymoma patients ($n = 3$; \pm SD); (b) TaqMan qRT-PCR analysis for miR-1290, miR-125a in serial dilution of D341 CM ($n = 3$; \pm SD). CM: Culture-medium; MB: Medulloblastoma; CSF: Cerebral spinal fluid; qRT-PCR: Quantitative reverse transcription polymerase chain reaction; SD: standard deviation

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Non-anthracycline chemotherapy associated with a poor outcome in elderly Egyptian patients with diffuse large B-cell non-Hodgkin lymphoma

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ABSTRACT

Aim: Rituximab plus cyclophosphamide, doxorubicin, vincristine, and prednisone (CHOP) is the standard treatment for patients with diffuse large B-cell non-Hodgkin lymphoma (DLBCL). Nevertheless, anthracyclines are contraindicated for some patients, e.g. cardiac dysfunction, severe hepatic dysfunction, jaundice. Thus, this study assessed the effectiveness of non-anthracycline chemotherapy regimen cyclophosphamide, vincristine, and prednisone (CVP) in elderly DLBCL patients vs. the standard CHOP. **Methods:** This retrospective study included 418 DLBCL patients diagnosed between 2003 and 2006 and followed until March 2014. During this period of time, rituximab was not available for all patients, particularly for patients older than 60 years. **Results:** CHOP and CVP were administered to 351 (84%) and 67 (16%) patients, respectively. Older age and comorbidities, particularly cardiovascular and diabetes mellitus, were independent determinants for not receiving CHOP. Patients received more courses of CHOP treatment than that of CVP (6 vs. 3 courses; $P < 0.001$) and developed more toxicities (48.4% vs. 23.9%; $P < 0.001$), particularly fatigue, alopecia, and gastrointestinal tract toxicities. Complete response rate was higher in CHOP than in CVP (69.9% vs. 29.9%; $P < 0.001$). Moreover, early death was significantly higher in CVP group of patients than in CHOP (43.3% vs. 8.6%; $P < 0.001$). After a median follow-up of 71 months, the median overall survival (OS) and event-free survival (EFS) were significantly better in CHOP than in CVP (49.5 vs. 3.7 months and 32.2 vs. 3.5 months; $P < 0.001$ for both, respectively). Older age, poor age-adjusted International Prognostic Index scores, not receiving CHOP or consolidative radiotherapy were independent predictors of poor OS and EFS. **Conclusion:** Use of the CVP regime to treat DLBCL patients who were unfit to the standard CHOP treatment was associated with lower remission rates and poorer EFS and OS in this group of patients.

Key words: Non-Hodgkin's lymphoma, diffuse large B-cell, anthracycline, chemotherapy, treatment

Introduction

Non-Hodgkin's lymphoma (NHL) was the 10th most commonly diagnosed cancer and the 9th cause of cancer mortality in the world in 2012.^[1] In Egypt, NHL was the 4th most common cancer in males and 5th in females and the 5th cause of cancer mortality.^[1,2] NHL is a diverse group of malignancies with different clinical and biological features.^[3] Diffuse large B-cell NHL (DLBCL) is the most common NHL type in the world, accounting for 30% of NHL and 80% of its aggressive subtypes.^[4] In Egypt, DLBCL accounts for 44.5% of lymphoid malignancies in a population-based cancer registry^[5] and 50% of NHL subtypes at the Egyptian

National Cancer Institute.^[6] DLBCL treatment mostly relies on multi-agent combination chemotherapy.^[7] The addition of the anti-CD20 monoclonal antibody rituximab to the chemotherapy combination dramatically improved overall survival (OS).^[8,9] Anthracyclines, particularly doxorubicin are an integral component of these combination chemotherapy regimens, e.g. cyclophosphamide, doxorubicin, vincristine, prednisone (CHOP); procarbazine, methotrexate, doxorubicin, cyclophosphamide, etoposide-cytarabine, bleomycin, vincristine, methotrexate; methotrexate-bleomycin, doxorubicin, cyclophosphamide, vincristine, dexamethasone; methotrexate, doxorubicin, cyclophosphamide, vincristine, dexamethasone, bleomycin, and many others.^[10] Intensive chemotherapy with more agents failed to show additional benefit, and the CHOP regimen was concluded to be the best available for patients with intermediate and high-grade NHL, including DLBCL.^[7] Reductions in dose intensity clearly determine treatment efficacy.^[11] However, patients with older age, comorbidities, particularly cardiovascular, and expected higher morbidity and mortality may hinder the use of an anthracycline.^[12,13] Compared to

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anthracycline-containing regimens, the 3-year OS is almost halved when a non-anthracycline-containing regimen is used with an absolute survival reduction of 23%.^[12]

Thus, the aim of this retrospective study was to investigate the effectiveness of non-anthracycline chemotherapy regimen on elderly DLBCNHL patients by mainly focusing on geriatric organ dysfunction, frailty and comorbidities *vs.* suboptimal treatment with the cyclophosphamide, vincristine, and prednisone (CVP) *vs.* the standard CHOP to assess the factors that impact the regimen choice.

Methods

Study population

This retrospective clinical study included 418 patients with a confirmed DLBCNHL diagnosis at Tanta Cancer Center, Gharbiah, Egypt between 2003 and 2006. Diagnosis of DLBCNHL was based on histology and immunohistochemical data on CD19, CD20, and CD 22 expression. Patients were treated with either CHOP chemotherapy regimen (cyclophosphamide 750 mg/m² intravenous (IV) on day 1, doxorubicin 50 mg/m² IV on day 1, vincristine 1.4 mg/m² (maximum 2 mg) IV on day 1 and prednisone 100 mg p.o. for 5 days) or CVP regimen (same as CHOP without doxorubicin) and followed-up until March 2014 via phone conversation. Response to therapy was assessed using the response criteria developed by the lymphoma International Working Group.^[14] OS is calculated from the date of diagnosis to the date of death from any cause or last follow-up. Event-free survival (EFS) was calculated from the date of starting treatment to the date of relapse, progression, death or last follows up.^[14] Clinicopathological data were extracted from patients' medical records. This study was approved by the Institutional Review Board of the Egyptian National Cancer Institute.

Statistical analyses

Statistical analyses were performed using IBM SPSS software version 21.0 (SPSS Inc., Chicago, IL, USA). Nominal and categorical variables were compared using the Chi-square or Fisher's exact test. Numerical variables were compared using *t*-test or Man-Whitney's test. Multivariate logistic regression was used to describe the use of CHOP or CVP, controlling for patient covariates. Unadjusted survival was estimated using the Kaplan-Meier method and groups were compared using the log-rank test. Stepwise Cox regression hazards model was used for calculating adjusted survival for each treatment, controlling for patients covariates. A probability $P \leq 0.05$ was considered statistically significant. The primary endpoint was OS. The secondary endpoint included EFS, complete response (CR) rate, and treatment-related toxicities.

Results

Patients' characteristics

CHOP and CVP were administered to 351 (84%) and 67 (16%) patients, respectively. Compared with those receiving CVP, patients receiving CHOP were significantly younger, having less comorbidity, better performance status (PS), fewer B-symptoms, and lower International Prognostic Index-risk (IPI-risk) categories [Table 1]. Logistic regression analysis assessed the impact of different baseline characteristics on the likelihood to receive CHOP or CVP. Only age and comorbidities were independent determinants of the regimen received [Table 2]. Older patients had 10.5 odds of not receiving CHOP compared to the younger patients (95% confidence interval (CI): 4.6-23.6; $P < 0.001$). Patients with comorbidities had 37.2 odds of not receiving CHOP compared to those with no comorbidities (95% CI: 12.6-109.6; $P < 0.001$).

Table 1: Characteristics of 418 DLBCNHL patients

Characteristic	Subgroup	n (%)		P
		CHOP	CVP	
n		351	67	
Age	Mean \pm SD	48.6 \pm 13.3	69.7 \pm 8.8	<0.001
	< 70	334 (95.2)	29 (43.3)	
	\geq 70	17 (4.8)	38 (56.7)	<0.001
LDH	\leq Normal	78 (22.2)	12 (17.9)	
	> Normal	273 (77.8)	55 (82.1)	0.431
Gender	Female	176 (50.1)	30 (44.8)	
	Male	175 (49.9)	37 (55.2)	0.421
Comorbidity	No	289 (82.3)	4 (6.0)	
	Yes	62 (17.7)	63 (94)	<0.001
Bulky disease	Yes	40 (11.4)	6 (9.0)	
	No	311 (88.6)	61 (91.0)	0.673
PS grouping	0-1	221 (63.0)	21 (31.3)	
	2-4	130 (37.0)	46 (69.7)	<0.001
Extra-nodal disease	No	232 (66.1)	44 (65.7)	
	Yes	119 (33.9)	23 (34.3)	0.946
Stage	1	68 (19.4)	16 (23.9)	
	2	128 (36.5)	20 (29.9)	
	3	119 (33.9)	23 (34.3)	
	4	36 (10.3)	8 (11.9)	0.701
B symptoms	A	191 (54.4)	27 (40.3)	
	B	160 (45.6)	40 (59.7)	0.034
IPI risk category	Low	85 (24.2)	3 (4.5)	
	Low intermediate	150 (42.7)	15 (22.4)	
	High intermediate	86 (24.5)	18 (26.9)	
	High	30 (8.5)	31 (46.3)	<0.001
aaIPI groups	0-1	90 (25.6)	17 (25.4)	
	2-3	261 (74.4)	50 (74.6)	0.963

DLBCNHL: Diffuse large B-cell non-Hodgkin's lymphoma; CHOP: Cyclophosphamide, doxorubicin, vincristine, and prednisone; CVP: Cyclophosphamide, vincristine, and prednisone; SD: Standard deviation; LDH: Lactate dehydrogenase; IPI: International prognostic index; aaIPI: Age-adjusted international prognostic index; PS: Performance status

Patients with diabetes mellitus, hypertension, and cardiovascular diseases (e.g. myocardial infarction, heart failure, cerebrovascular stroke) were significantly more common in the CVP group [Table 3]. Among different comorbidities, cardiovascular diseases, and diabetes mellitus were the most significant ones that guided regimen selection. The odds of not receiving CHOP were 125 times higher in patients with cardiovascular diseases compared

Table 2: Multivariate analysis of the factors that impact not receiving CHOP treatment

Variables in equation	OR (95% CI)	P
Age (≥ 60 vs. < 60 years)	10.5 (4.6-23.6)	< 0.001
Comorbidity (yes vs. no)	37.2 (12.6-109.6)	< 0.001

CHOP: Cyclophosphamide, doxorubicin, vincristine, and prednisone; CI: Confidence interval; OR: Odds ratio

Table 3: Comorbidities among DLBCNHL patients receiving CHOP or CVP

Comorbidity	Sub-group	n (%)		P
		CHOP	CVP	
Diabetes mellitus	No	330 (94.0)	43 (64.2)	< 0.001
	Yes	21 (6.0)	24 (35.8)	
Hypertension	No	345 (98.3)	60 (89.6)	0.002
	Yes	6 (1.7)	7 (10.4)	
Cardiovascular	No	340 (96.9)	15 (22.4)	< 0.001
	Yes	11 (3.1)	52 (77.6)	
Renal impairment	No	347 (98.9)	64 (95.5)	0.085
	Yes	4 (1.1)	3 (4.5)	
Liver disease	No	331 (94.3)	64 (95.5)	1.000
	Yes	20 (5.7)	3 (4.5)	
Others*	No	343 (97.7)	61 (91.0)	0.014
	Yes	8 (2.3)	6 (9.0)	

*Include bronchial asthma, chronic obstructive airway disease, thyroid dysfunction, ulcerative colitis, rheumatoid arthritis, and systemic lupus erythematosus. DLBCNHL: Diffuse large B-cell non-Hodgkin's lymphoma; CHOP: Cyclophosphamide, doxorubicin, vincristine, and prednisone; CVP: Cyclophosphamide, vincristine, and prednisone

to those without cardiovascular diseases (95% CI: 48-327; $P < 0.001$). The odds of not receiving CHOP was 9 times higher in patients with diabetes mellitus compared to those without diabetes mellitus (95% CI: 3-28; $P < 0.001$).

Treatment responses and toxicities

Patients with CHOP treatment received more chemotherapy cycles than those treated with CVP (median 6 and 3 cycles, respectively; $P < 0.001$; Table 4). CR rate was higher in CHOP-treated patients than in CVP-treated patients (69.9% vs. 29.9%; $P < 0.001$). More patients received radiotherapy after CHOP treatment achieved CR than CVP-treated patients (22.2% vs. 3%; $P = 0.001$; Table 3). Compared to CVP, CHOP was associated with significantly higher toxicities (48.4% vs. 23.9%; $P < 0.001$), particularly fatigue, alopecia, and gastrointestinal tract toxicities. However, early deaths following one or two chemotherapy courses were significantly higher in patients with CVP treatment than with CHOP treatment (43.3% vs. 8.6%; $P < 0.001$).

Overall survival and event-free survival

The median EFS was 22 months (range: 1.0-104.7 months; 95% CI: 16.7-27.4 months) in these patients [Figure 1]. The 2- and 5-year EFS rates were 47.8% and 30.4%, respectively. However, compared to CVP, CHOP was associated with significantly better EFS (median of 32.2 vs. 3.5 months; $P < 0.001$). After 5 years, no CVP-treated patients were event-free compared to 36% of CHOP-treated patients [Table 5]. The EFS was also significantly better in patients who were younger than 60 years, females had no comorbidities or B symptoms, good PS, lower stages, or lower IPI scores or those who received consolidative radiotherapy. Multivariate analysis showed that age > 60 years old, poor age-adjusted IPI (aaIPI) scores, and not receiving CHOP or radiotherapy were independent predictors for poor EFS [Table 6].

The median follow-up period of time was 71 months (range between 1.0 and 111.7 months;

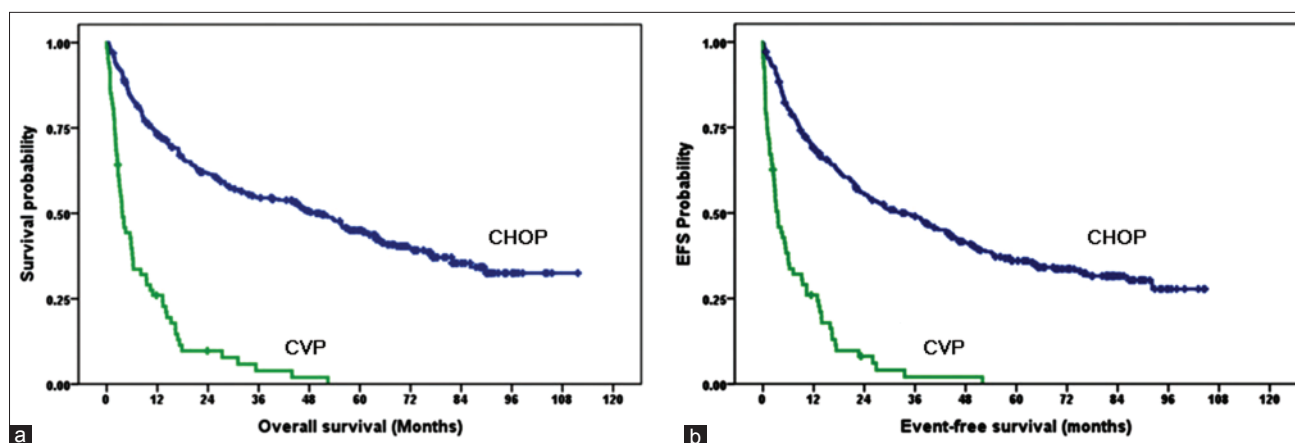


Figure 1: Kaplan-Meier curves of overall survival (OS) and event-free survival stratified by CHOP and CVP regimes. (a) OS of DLBCNHL patients after receiving CHOP or CVP treatment; (b) event-free survival of DLBCNHL patients after receiving CHOP or CVP therapy. CHOP: Cyclophosphamide, doxorubicin, vincristine, and prednisone; DLBCNHL: Diffuse large B-cell non-Hodgkin lymphoma; CVP: Cyclophosphamide, vincristine, and prednisone

Table 4: First-line treatments administered to DLBCNHL patients according to their age

Characteristic	Sub-group	n (%)		P
		CHOP	CVP	
No cycles 1st	Median (range)	6 (1-9)	3 (1-8)	<0.001
Toxicity	No	181 (51.6)	51 (76.1)	<0.001
	Yes	170 (48.4)	16 (23.9)	
Early death*	No	321 (91.4)	38 (56.7)	<0.001
	Yes	30 (8.6)	29 (43.3)	
Fatigue	No	230 (65.5)	61 (91)	<0.001
	Yes	121 (34.5)	6 (9)	
Alopecia	No	230 (65.5)	62 (92.5)	<0.001
	Yes	121 (34.5)	5 (7.5)	
Anemia	No	333 (94.9)	67 (100.0)	0.092
	Yes	18 (5.1)	0 (0)	
Neutropenia	No	317 (90.3)	63 (94.0)	0.486
	Yes	34 (9.7)	4 (6.0)	
Thrombocytopenia	No	343 (97.7)	67 (100)	0.365
	Yes	8 (2.3)	0 (0)	
GIT*	No	319 (90.9)	67 (100.0)	0.005
	Yes	32 (9.1)	0 (0)	
Skin	No	346 (98.6)	67 (100.0)	1.000
	Yes	5 (1.4)	0 (0)	
DVT	No	345 (98.3)	67 (100.0)	0.595
	Yes	6 (1.7)	0 (0)	
Liver	No	345 (98.3)	67 (100.0)	0.595
	Yes	6 (1.7)	0 (0)	
Response group	CR	245 (69.8)	20 (29.9)	<0.001
	No CR	106 (30.2)	47 (70.1)	
Radiotherapy	No	273 (77.8)	65 (97.0)	0.001
	Yes	78 (22.2)	2 (3.0)	

*Early death after 1-2 courses of chemotherapy (response was not assessed). DLBCNHL: Diffuse large B-cell non-Hodgkin's lymphoma; CHOP: Cyclophosphamide, doxorubicin, vincristine, and prednisone; CVP: Cyclophosphamide, vincristine, and prednisone; CR: Complete remission; PR: Partial remission; SD: Stable disease; GIT: Gastrointestinal toxicity in the form of either: mucositis, diarrhea or constipation; DVT: Deep venous thrombosis

95% CI: 66.3-75.0 months) [Figure 1]. At the last follow-up, 263 patients were deceased (199 in the CHOP group and 64 in the CVP group). The median OS rate was 28.6 (95% CI: 17.0-40.2) for this cohort of patients. However, the median OS rate was significantly longer in CHOP-treated patients than that of CVP-treated patients (49.5 vs. 3.7 months; $P < 0.001$; Table 5). The median OS rate was also significantly longer in young patients without comorbidities, bulky disease or B symptoms, good PS, lower stages, and IPI or aaIPI scores or patients who received consolidation radiotherapy. The multivariate analysis showed that age > 60 years, poor aaIPI scores, and not receiving CHOP or radiotherapy were independent predictors of poor OS [Table 6].

Discussion

Since its development in the late 1960's, doxorubicin

has been firmly established as the most effective single agent in the treatment of malignant lymphoma.^[15,16] The CHOP regime was invented in the late 1970's and after its efficacy in NHL was established, it became the standard of care as it produced high CR rate and durable effects.^[15,17] Its known adverse effects mainly affect the cardiovascular system.^[15,16,18] Reduction of inter-treatment intervals (CHOP-14) and the addition of rituximab (R-CHOP) were shown to improve treatment outcomes.^[16] CHOP-14 does not appear to be superior to CHOP-21 when given with rituximab, but associates with increased toxicities, including an increased risk of Pneumocystis Jiroveci Pneumonia. Use of R-CHOP-21 is recommended rather than R-CHOP-14. This is primarily due to decreased need for growth factor support, and a lack of data showing the superiority of one regimen over another in the rituximab era. More intensive chemotherapy or additional agents have failed to show additional benefit.^[7] However, elimination of anthracycline from the treatment regimen reduced the CR rate, duration of response and disease stabilization, and OS.^[12,13]

In the current study, 16% of DLBCNHL patients (67/418) did not receive anthracycline, whereas other studies showed a higher percentage (20-67%) as they only included patients aged 66 years or older.^[12,19,20] However, Link *et al.*^[18] reported a lower percentage in an older population. Different studies in the different period of time and inclusion criteria may explain this variance. The rate of anthracycline use in the treatment of DLBCNHL did not vary with time, that is, between the pre-rituximab era and the post-rituximab era.^[18] Furthermore, similar to other studies,^[18,19,21] our current study showed that older age and comorbidities were strong indicators of treatment regimen selection without doxorubicin in addition to cardiovascular diseases and diabetes mellitus but the lower relevance of kidney and liver disease.^[19] Pre-therapy heart disease, diabetes, hypertension, and older age were reported to be independent predictors of cardiotoxicity and subsequent death from the same cause.^[22-24] Our results also concur with those of van de Schans *et al.*^[25] and Peters *et al.*^[26] regarding the impact of poor PS and estimated short survival on the likelihood of treatment regimens without anthracycline. We showed that early death, that is, following 1-2 chemotherapy courses was encountered more in the non-anthracycline group (43.3% vs. 8.6%). Expected higher toxicities are another important reason. While this is difficult to assess quantitatively before therapy is given, it was confirmed by the higher rates of toxicities in the CHOP compared to the CVP group (48.4% vs. 23.9%).

The lower response rate with the CVP regimen without anthracycline than anthracycline-containing CHOP regimen confirms the established fact that anthracycline is the most active single agent in the treatment of lymphoma.^[12,13,15,16] In the current study, doxorubicin contributed almost 40% of the CRs exceeding the

Table 5: EFS and OS of 418 DLBCNHL patients

Group	n	EFS				OS			
		Median	2-year rate	5-year rate	P	Median	2-year rate	5-year rate	P
All	418	22.0	47.8	30.4		28.6	53.3	37.9	
First line chemotherapy									
CHOP	351	32.2	55.3	36.0		49.5	61.8	45.0	
CVP	67	3.5	8.1	0	<0.001	3.7	9.7	0	<0.001
Age (years)									
< 60	297	39.4	59.6	39.9		57.4	67.0	49.6	
≥ 60	121	6.3	18.2	5.7	<0.001	6.0	19.0	6.3	<0.001
Gender									
Male	212	17.8	43.6	25.0		25.0	50.0	35.6	
Female	206	26.8	52.2	35.9	0.032	43.0	56.7	40.3	0.188
Comorbidities									
No	293	35.2	56.0	36.2		53.7	63.3	46.4	
Yes	125	7.2	28.4	16.4	<0.001	8.0	28.8	16.7	<0.001
Bulky disease									
Yes	46	13.9	34.8	24.6		17.0	43.5	31.2	
No	372	23.9	49.5	31.1	0.178	31.1	54.6	38.8	0.407
B symptoms									
A	218	28.8	54.7	36.4		46.2	60.0	42.8	
B	200	16.0	40.2	32.6	0.002	18.0	45.8	32.6	0.003
PS									
0-1	242	41.2	59.7	38.6		55.9	67.0	48.8	
2-4	176	9.7	31.2	18.9	<0.001	10.6	34.2	22.7	<0.001
Extra-nodal									
No	276	22.9	48.8	29.7		31.1	55.2	38.4	
Yes	142	18.0	45.8	31.8	0.738	21.8	49.5	37.1	0.376
Stage									
1.0	84	76.7	63.0	52.2		NR	68.1	5.6	
2.0	148	20.6	45.3	29.2		28.0	52.4	36.8	
3.0	142	19.1	44.4	21.3		25.6	50.3	32.8	
4.0	44	6.9	39.5	19.1	<0.001	8.8	41.9	21.9	<0.001
Stage-group									
1-2	232	26.0	51.6	37.4		44.2	57.3	43.4	
3-4	186	16.3	43.2	20.7	0.001	21.3	48.3	30.7	0.006
IPI-group									
Low	88	NR	72.0	57.6		NR	79.1	65.4	
Low intermediate	165	28.9	54.1	31.3		45.6	62.3	42.2	
High intermediate	104	14.1	39.3	21.3		16.3	42.5	27.2	
High	61	4.6	9.2	0	<0.001	4.6	10.7	0	<0.001
aaIPI									
0-1	107	52.0	62.4	84.2		NR	68.3	54.7	
2-3	311	17.8	42.8	24.1	<0.001	20.5	46.5	32.1	<0.001
Radiotherapy									
No	338	17.2	43.5	26.6		20.0	47.9	32.9	
Yes	80	50.7	66.1	46.0	<0.001	72.5	77.5	58.8	<0.001

EFS: Event-free survival; OS: Overall survival; DLBCNHL: Diffuse large B-cell non-Hodgkin's lymphoma; CHOP: Cyclophosphamide, doxorubicin, vincristine, and prednisone; CVP: Cyclophosphamide, vincristine, and prednisone; PS: Performance status; IPI: International prognostic index; aaIPI: Age adjusted international prognostic index; NR: Not reached

combination of cyclophosphamide, vincristine, and prednisolone (from 29.9% to 69.9%) in DLBCNHL treated solely by chemotherapy. Achieving CR is crucial for long-term survival and cure.^[27] Our current study clearly shows that patients are failing to achieve CR only had a median OS of 4.4 months compared to 76.8 months in those who achieved CR with almost

11-fold higher relative risk of death. CHOP-produced CR rates is comparable to those reported by Khaled *et al.*,^[28] Burton *et al.*,^[29] Hallack Neto *et al.*^[30] [Table 7]. However, a large Egyptian study by Abdelhamid *et al.*^[6] reported a 10% higher CR rate. This latter study only included younger patients with a maximum age of 60, better PS, and lower aaIPI scores. In contrast, our current

study included older patients with a maximum age of 82, poorer PS, and higher aalPI scores. Patients that are older and have poor PS frequently received reduced doses or interrupted and delayed therapy. This reduced dose intensity is a key determinant of CR and survival.^[6,31]

In the current study, removal of the anthracycline doxorubicin from the CHOP regimen significantly reduced the median OS (unadjusted from 49.5 to 3.7 months, i.e. 45.8 months and adjusted from 44 to 9 months, i.e. 35 months) and the 3-year OS (unadjusted from 54.5% to 3.9% i.e. 50.5% and adjusted from 52% to 19% i.e. 33%) with an increase in the hazards of death by 4 times. This is similar to Tien *et al.*^[12] and Link *et al.*^[18] who showed a 22% and 16% decline in 3-year OS, respectively [Table 7]. The difference in our study (33%) may be due to the poorer outcome of patients

receiving non-anthracycline-containing regimens (19%) compared to that in the mentioned studies (29% and 33%). This may be due to the more developed health care system in the US than Egypt as the former ranks 37th and the latter ranks 63th in overall health system performance.^[33] A high performing health care system is capable of providing better supportive therapies for patients that are elderly, having comorbidities and progressing on inadequate anti-lymphoma therapy.

OS with CHOP treatment (52% at 3 years) in the current study is comparable to the 49-57% figure reported by many authors [Table 7],^[6,9,12,18] but was lower than the 60-70% OS reported by Habermann *et al.*,^[32] Burton *et al.*,^[29] and Khaled *et al.*^[28] All of these studies performed prospective trials where patients were carefully selected and generally fit to tolerate therapy. It is understandable that results from phase III studies do not always translate into corresponding outcomes in the general population.^[18]

Similar to CR and OS, our current data showed that removal of doxorubicin from the CHOP regimen significantly reduced EFS. We could not easily find information on the use of CVP in DLBCNHL to compare our EFS with the studies that comparison of anthracycline-containing regimens to non-anthracycline-containing regimens only showed OS.^[12,18] The EFS rate of CHOP treatment in our current study is similar to Sehn *et al.*^[9] and Habermann *et al.*^[32] However, it was lower than that of Khaled *et al.*^[28] and Burton *et al.*^[29] This may be explained by the difference in study settings between the well-controlled environment of a clinical trial and the community practice environment. The disease-free survival of our study (75.9% at 2 years) was similar to that of Abdelhamid *et al.*^[6] (68.8%) who used a similar setting to our study. It was higher than that reported by Hallack Neto *et al.*^[30] This retrospective Brazilian study reported on a relatively small number of

Table 6: Multivariate analysis of EFS and OS in DLBCNHL patients

Variables in equation	EFS		OS	
	HR (95% CI)	P	HR (95% CI)	P
Age (≥ 60 vs. < 60 years)	2.1 (1.6-2.9)	<0.001	2.5 (1.8-3.0)	<0.001
First line chemotherapy (non-CHOP vs. CHOP)	2.6 (1.9-3.7)	<0.001	2.6 (1.8-3.8)	<0.001
aaIPI (score 0-1 vs. 2-3)	1.8 (1.3-2.5)	<0.001	2.0 (1.4-2.7)	<0.001
Radiotherapy (no vs. yes)	1.8 (1.3-2.5)	<0.001	2.1 (1.5-3.1)	<0.001

DLBCNHL: Diffuse large B-cell non-Hodgkin's lymphoma; CHOP: Cyclophosphamide, doxorubicin, vincristine, and prednisone; CVP: Cyclophosphamide, vincristine, and prednisone; EFS: Event-free survival; OS: Overall survival; HR: Hazard's ratio; CI: Confidence interval; aaIPI: Age-adjusted international prognostic index; IPI: International prognostic index

Table 7: Comparison of treatment outcomes in DLBCNHL patients

Authors	Regimen	n	Age	CR (%)	2-year (3-year) EFS/PFS (%)	2-year (3-year) OS (%)
Our current study	CHOP	251	17-82	69.8	55.3 (46.0)	58.0 (52.0)
	CVP	67	45-87	29.9*	18.0 (12.0)*	25.0 (19.0)*
Tien <i>et al.</i> ^[12]	ACR	1090	≥ 66			(52)
	Non-ACR	267	≥ 66			(29)*
Link <i>et al.</i> ^[18]	ACR	2346	≥ 66			59 (49)
	Non-ACR	460	≥ 66			40 (33)*
Abdelhamid <i>et al.</i> ^[6]	CHOP	224	18-60	79.5	2-year DFS: 68.8	57 (57)
Hallack Neto <i>et al.</i> ^[30]	CHOP	77	< 60	68.8	2-year DFS: 61.3	5-year OS: 72.8
Habermann <i>et al.</i> ^[32]	CHOP	279	> 60		(46)	(60)
Sehn <i>et al.</i> ^[9]	ACR	140	19-86		51% (46%)	52 (50)
Khaled <i>et al.</i> ^[28]	CHOP	40	19-75	67	54 (54)	82 (71)
Burton <i>et al.</i> ^[29]	CHOP	105	22-66	70	4-year PFS: 56	4-year OS: 65
	CIOP	106	25-67	52	4-year PFS: 40*	4-year OS: 56 [#]

*P < 0.05, [#]P ≥ 0.05. EFS: Event-free survival; PFS: Progression-free survival; DFS: Disease-free survival; CHOP: Cyclophosphamide, doxorubicin, vincristine, and prednisone; CVP: Cyclophosphamide, vincristine, and prednisone; ACR: Anthracycline containing regimen; CIOP: Cyclophosphamide, idarubicin, vincristine, and prednisone; CR: Complete response; DLBCNHL: Diffuse large B-cell non-Hodgkin's lymphoma

patients ($n = 77$) with many poorer prognostic factors than ours.

DLBCNHL is potentially curable after treated with anthracycline-containing regimens; however, a significant proportion of patients do not receive anthracyclines, particularly doxorubicin for various reasons, e.g. older age, expected poor tolerance or significant comorbidities. These patients present an unmet medical need.^[12] Measures that may decrease toxicity and improve anthracycline tolerance includes adequate supports (e.g. hematopoietic growth factors), dose reductions, increase in infusion time, the addition of cardio-protectants (e.g. dexrazoxane).^[16,18,26,34,35] An alternative less-toxic and more tolerable anthracycline may be considered if feasible, e.g. liposomal doxorubicin,^[36,37] epirubicin,^[38] mitoxantrone^[39] or pixantrone.^[40] In case an anthracycline cannot be used, substitution with other agents, e.g. etoposide or gemcitabine may better than omission.^[41] Addition of the immunotherapy agent like rituximab to non-anthracycline-containing regimens significantly improves the outcomes and should be considered.^[18] Non-anthracycline-containing regimens with the addition of rituximab produced equivalent outcomes to anthracycline-containing regimens.^[12,18,19]

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Prognostic significance of transcription factors FOXA1 and GATA-3 in ductal carcinoma *in situ* in terms of recurrence and estrogen receptor status

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ABSTRACT

Aim: The aim was to analyze the expression of novel biological transcription markers, forkhead-box A1 (FOXA1), GATA binding protein 3 (GATA-3), and established markers such as Ki-67 (MIB-1) and human epidermal growth factor receptor 2 (HER2) in estrogen receptor (ER(+)) and ER(-) ductal carcinoma *in situ* (DCIS) patients with/without recurrence. **Methods:** Two hundred and ninety-one cases of DCIS were retrieved from our pathology database, with complete data available for 219 cases. The follow-up period is from 1988 to 2009. Recurrence is defined in terms of DCIS or invasive carcinoma (IC). No recurrence was seen in 88% (196/219) of cases; 12% (26/219) had a recurrence (IC: 13, DCIS: 13). We are reporting the results of biological marker expression in terms of recurrence and ER status. **Results:** Our study demonstrates strong expression of GATA-3 in the ER(+) DCIS in recurrence and nonrecurrence groups similar to previously described in IC. A reduced expression of GATA-3 was observed in ER(-) recurrence and nonrecurrence groups. A strong HER2 protein expression, as well as high proliferation index, was seen in recurrence group (DCIS and IC). FOXA1 expression is reduced across the groups though not statistically significant. **Conclusion:** This is the first study to analyze novel transcription markers FOXA1 and GATA-3 in DCIS. Further work needs to be done on a larger cohort of DCIS cases with recurrence to better understand, which variables are best able to predict recurrence and guide therapy decision strategies. Maintenance of FOXA1 and GATA-3 expression in ER(-) DCIS may offer new promising targets for therapy in future.

Key words: Ductal carcinoma *in situ*, estrogen receptor, forkhead-box A1, GATA binding protein 3

Introduction

Ductal carcinoma *in situ* (DCIS) is a heterogeneous pre-invasive carcinoma and has become a significant proportion of screen-detected breast malignancies in North American and Western Europe, since the onset of wide-spread screening mammography nearly two decades ago.^[1,2] Unlike invasive breast carcinoma (IC), DCIS is a more heterogeneous malignancy without clear prognostic indicators for recurrence, defined as either recurrent DCIS or IC. While the Van Nuys Prognostic Index, based on histopathologic indicators (high nuclear grade, necrosis, margin width, and size) has been used clinically for predicting recurrence, there has been no good biomarker(s) that predicts outcome in DCIS.^[3] Furthermore, whereas IC has been classified into distinct molecular subtypes (luminal A/B, human epidermal growth factor

receptor 2 (HER2)-like, basal-like), which confer prognostic clinical significance, to date few studies have attempted to classify DCIS into similar molecular-based subtypes.^[4-6] There is emerging evidence on limited data to suggest cDNA microarray, gene-expression profiles can segregate DCIS into similar distinct molecular subtypes as in IC;^[4] however, a significant proportion of DCIS shows tumor heterogeneity^[4,5,7] making it difficult to stratify DCIS cases into a single subtype, and thus subsequently diminishing the prognostic significance of these molecular subtypes, as compared to IC.

Estrogen receptor (ER) status has been the leading candidate biomarker in DCIS, as it plays a key role in development and influences hormonal treatment in IC patients. Absence of ER was shown to be one of the significant predictors of recurrence in IC.^[8] In addition, it is well-known that about 30% of ER(+) tumors are not hormone responsive, and about 5-15% of ER(-) tumors are responsive to anti-estrogen therapy.^[9] However, the two broad groups of IC namely ER(+) and ER(-), are yet to be well understood in DCIS.

Recently, various research groups have looked at the functional interaction between the forkhead-box A1 (FOXA1) winged helix transcription factor and GATA binding protein 3 (GATA-3), a zinc finger transcription

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factor, in their role in suppressing ER-dependent breast cancer cell growth and tumor genesis and maintenance of breast luminal-cell differentiation *in vivo*. Their use as prognostic clinical biomarkers has recently been studied in IC but not in DCIS.^[10-13] FOXA1, originally called the hepatocyte nuclear factor 3 α , is a ubiquitous transcription factor expressed in liver, breast, prostate, lung, colon, and pancreas that has both activator and repressor activity. As an activator, FOXA1 has the unique ability to bind to its target sites via altering chromatin structure facilitating ER α binding and thus promoting gene expression.^[14,15] FOXA1 may also act as a growth inhibitor via binding and activation of the p27 promoter, located within the BRCA1-responsive element,^[16,17] thus plays a critical role in suppressing ER-dependent breast cancer cell growth and tumor genesis *in vivo*.^[17]

GATA-3, a member of the zinc finger DNA binding proteins, was originally discovered in its role in T-lymphoid development into Th2 cells.^[18] In the breast, GATA-3 was initially discovered to be associated with ER expression in breast carcinomas^[19] and has subsequently been demonstrated *in vivo* to be highly expressed in the mammary luminal epithelial cells, responsible for both development and maintenance of luminal cells fate.^[20,21] Like FOXA1, GATA-3 is also promising biomarker and the complex relationship between ER α , FOXA1, and GATA-3 is being better understood in order to refine the hormone-responsive phenotype in IC, which will help both with therapy decision making and better prediction of clinical outcomes.^[21-23] GATA-3 has been identified as an upstream promoter of FOXA1 transcription.^[20] FOXA1 has been shown to be responsible for expression of at least 50% of ER α regulated genes^[24-26] and thus has been proposed as the link between GATA-3 and ER α .^[21] GATA-3 genes are involved with induction of FOXA1 expression, with increased activity in ER(+) carcinoma; therefore, the highest expression of FOXA1 in IC should be seen in association with both GATA-3 and ER α expression,^[27] which has been seen in IC.^[12] However, this relationship has not yet been categorized in DCIS. The specific aim of this study is to analyze the expression for the first time in DCIS of novel biological transcription markers FOXA1, GATA-3, along with established markers MIB-1 (Ki-67) and HER2-neu in ER(+) and ER(-) groups of DCIS. As it has been shown in IC, we will investigate if there is a similar association between FOXA1/GATA-3 with ER α in DCIS. The secondary goal is to define an expression profile of FOXA1, GATA-3 and other biomarkers that could predict recurrence in these DCIS groups and further stratify low versus high-risk patients and impact treatment decisions.

Methods

Patients

In our retrospective study, we identified 2,434 women diagnosed with DCIS from 1988 to 2009 from the

tumor registry data. Paraffin-embedded blocks and hematoxylin and eosin (HE) slides of 291 patients with initial DCIS were retrieved. Complete demographic data, menopause state, hormone therapy use, family history, prior history of pregnancy, mammography report (mass, calcifications), surgical treatment and adjuvant therapy, along with pathologic data were reviewed. All patients had undergone a core needle biopsy or needle localization excision biopsy for initial diagnosis. Two hundred and nineteen cases who had complete follow-up, glass slides and tumor blocks were chosen for the study. Recurrence was defined as DCIS or invasive breast cancer in the same breast 1-year or more after the initial diagnosis of DCIS. Nonrecurrence DCIS group included patients who had DCIS or microinvasion (invasion \leq 1 mm) or invasive cancer that was subsequently diagnosed at the time of complete excision.

Procedure

The project was approved by the University of Pittsburgh Institutional Review Board. All cases were reviewed by two pathologists with confirmation of nuclear grade, as described by conventional features observed on the HE slide. All other pathologic features were obtained from the original report. The tumor size measurement was assessed either by size from microscopic or gross description or as an estimation based on tumor volume from number of slides involved per total slides. Margins were considered clear (negative) defined as no link on the tumor and positive if less than 1 mm. Table 1 shows complete clinical, radiological and pathologic characteristics in relation to recurrence.

Immunohistochemistry (IHC) was performed on the selected paraffin-embedded tumor block of the index DCIS lesion using the following biomarkers GATA-3, FOXA1, ER, progesterone receptor (PR), Ki-67 and HER2.

Predilute rabbit monoclonal antibodies directed against ER alpha (SP1), PR (1E2) and HER2 (4B5) were purchased from Ventana Medical Systems Inc., Tucson, AZ, USA (VMSI). The manufacturer's recommended protocols were followed, utilizing CC1 for antigen unmasking, and iVIEW/DAB (Ventana Medical Systems, Inc.) for detecting the antigen-antibody complex and a biotin block to inhibit nonspecific staining of endogenous biotin. Mouse monoclonal anti-GATA-3 (L50-823), purchased from BD Biosciences was diluted 1:300 and shared the same protocol parameters as the previous mentioned. FOXA1 protein was detected using a goat polyclonal antibody from Santa Cruz. Slides were pretreated in a steamer in Target Retrieval Solution, pH 6.0 (Dako) at 95 °C for 20 min, then cooled at room temperature. Slides were then incubated with anti-FOXA1 (1:400) followed by Goat Immpress/DAB polymer for detection (Vector Labs) [Table 2].

Table 1: Clinical, radiological and pathologic characteristics in relation to each DCIS case

Characteristics	Nonrecurrence group		Recurrence group	
	DCIS	DCIS with/IC	DCIS	IC
Radiology				
Calcifications	126	41	1	10
Mass	16	7	2	2
Nipple discharge	5	1	0	1
Chemotherapy				
Positive	1	9	0	0
Negative	6	35	0	0
NA	140	2	0	0
Radiation therapy				
Positive	82	29	45	20
Negative	44	14	2	1
NA	21	6	0	0
Surgery				
Segmental (bilateral)	4	1	0	1
Segmental (unilateral)	106	25	22	12
TM (bilateral)	17	7	10	8
TM (unilateral)	28	13	15	0
Nuclear grade				
1	20	2	0	1
2	57	34	26	3
3	70	13	21	17
Size (cm)	1.8 (0.3-10)	2.0 (0.35-9.0)	1.6 (0.3-9.0)	0.8 (0.2-2.4)
Focality				
Unifocal	76	37	2	8
Multifocal	71	12	40	13
Lymph node biopsy				
Done	56	46	2	1
Not done	91	3	45	20
Margin status				
Positive	45	12	3	14
Negative	102	34	42	7
NA	0	0	2	0
Total	147	46	13	13

DCIS: Ductal carcinoma *in situ*; IC: Invasive carcinoma; TM: Total mastectomy; Segmental: Segmental mastectomy; NA: Not available

Table 2: Antibodies used for immunohistochemical characterization of ductal carcinoma *in situ*

Antibody	Clone	Dilution	Source
FOXA1	Goat polyclonal	1:400	Santa Cruz Biotechnology, Inc.; Santa Cruz, CA, USA
GATA-3	L50-823	1:300	BD Biosciences, USA
HER2-neu	485	Predilute	Ventana; Tucson, AZ, USA
Ki-67	30-9	Predilute	Ventana; Tucson, AZ, USA
ER	SP1	Predilute	Ventana; Tucson, AZ, USA
PR	1E2	Predilute	Ventana; Tucson, AZ, USA

FOXA1: Forkhead-box A1; GATA-3: GATA binding protein 3; HER2: Human epidermal growth factor receptor 2; ER: Estrogen receptor; PR: Progesterone receptor

Positive and negative control tissues were used for assessment of each marker. FOXA1, GATA-3, ER, PR,

and nuclear stains were evaluated with a cumulative “H score” based on proportionality score and intensity scores (0-10: negative; 11-150: low; 151-250: intermediate; 250-300: high). A 10% or more “nuclear” staining of the tumor cells was considered “positive.” The proliferation marker, Ki-67/MIB-1, was given a nuclear proliferation index (1-10%: low; 11-25%: intermediate; 26-50%: high; > 51%: very high); HER2 a membrane stain, was interpreted as per routine guidelines for IC (0/1 + negative, 2 + weakly positive, 3 + strongly positive) [Figure 1].

Statistical analysis

Four risk groups based on ER expression were defined separately for subsequent invasive cancer/DCIS based on the risk associated with clinical/histopathologic characteristics and biomarker expression. Groups were defined by combining biomarker expression that had similar strength associations and level of risk for subsequent tumor events. We used Fisher’s exact test to determine the dependence between clinical outcomes in the ER(+) and ER(-) groups with and without recurrence for each biomarker and calculation of *P* value. We also examined combinations of these biomarkers that were found as individual markers in univariate analyses to be statistically significantly associated with invasive cancer and/or DCIS or were previously shown to have a biological basis for association with subsequent tumors after a DCIS diagnosis or were previously reported to be associated with breast cancer survival.

Results

Of the total 219 patients selected for study, with a median follow-up of 4.5 years (range: 1-21 years); 88% (196/219) developed no recurrence. In 12% (26/219) patients who developed subsequent recurrence; 6% (13/26) recurred as IC; 6% (13/26) as DCIS. In the nonrecurrence group, 67% (146/196) were pure DCIS on both biopsy and final excision; 26% (46/196) cases had subsequent IC associated with DCIS on final excision. The IC associated with DCIS were all ductal carcinomas. Their overall nuclear grade is 1, 2 and 3, which constitution ratio is 4% (2/46), 87% (40/46), 9% (4/46), respectively. The mean tumor size in the DCIS with subsequent IC group was 0.4 cm (range 0.25-3.5 cm). Seventy percent (136/196) of nonrecurrence group and 92% (24/26) of recurrence group were treated with breast-conserving surgery alone. Fifty-eight percent (111/196) in the nonrecurrence group and 77% (20/26) in the recurrence group were treated with radiation therapy. In both the groups, negative (clear) surgical margins, defined as no ink on the tumor on final excision, were obtained in 70% (136/196) nonrecurrence group and 62% (16/26) cases of the recurrence group. The mean tumor size in DCIS with recurrence as IC group was 1.5 cm (mean 0.1-4.5 cm). Several morphologic characteristics were

reviewed, and none showed statistical significance with an increased risk of subsequent DCIS, although the index DCIS lesions with high nuclear grade, which had positive or uncertain margins showed a higher rate of recurrence.

We are reporting the results of biological markers expression in terms of recurrence and ER status. ER(-) DCIS with and without recurrence had lower expression of GATA-3 ($P < 0.05$) than ER(+) cases. A strong HER2 overexpression ($P < 0.05$) and higher proliferation index of Ki-67 ($P < 0.05$) were seen in ER(-) group. FOXA1 as an individual biomarker expressed in ER(+) and ER(-) groups was not statistically significant. Nearly all ER(-) cases retained expression of FOXA1 and GATA-3. Overall, cases with recurrence demonstrated the greater percentage of ER(-), HER2 overexpression, and high proliferation compared to nonrecurrence cases [Table 3 and Figure 2].

Discussion

This is one of the first studies to analyze novel transcription factors in DCIS patients, and we show that FOXA1 and GATA-3 expression is strongly seen in both ER(-) and ER(+) DCIS groups. We observed that strong expression of FOXA1 and GATA-3, low/intermediate Ki-67, and

low/absent HER2-neu expression were characteristically seen in our ER(+) DCIS groups, similar to previously described in IC.^[23] While there is a statistically significant lower expression of GATA-3 in the ER(-) cases, nearly all maintained expression. A significant number of ER(-) DCIS cases showed FOXA1 expression. FOXA1 and GATA-3 transcription factors have been shown to correlate highly with the luminal A molecular subtype of IC.^[10,11] Within the luminal, a subtype of IC, it has been shown that FOXA1^[10,11] and GATA-3^[22,24] can sub-classify patients into a low and high-risk groups based on their strong expression. FOXA1 via its actions on the p27 promoter,^[16,17] is thought to maintain IC in a less proliferative state, with a decreased metastatic potential,^[10-13] while GATA-3 is important in the maintenance of tumor differentiation and suppression of metastatic potential.^[21] Therefore, it is not surprising that these transcription factors are highly expressed in DCIS as well, which by definition is an *in situ* (noninvasive) carcinoma.

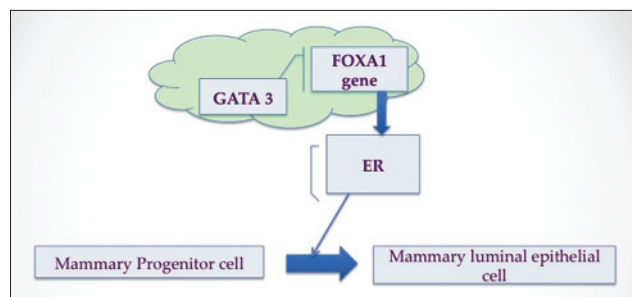


Figure 1: Pathway describing the role of GATA-3 and FOXA1 in development and maintenance of mammary luminal cells. GATA-3 promoter of FOXA1 transcription which in turn is responsible for expression of ER α regulated genes. FOXA1: Forkhead-box A1; GATA-3: GATA binding protein 3

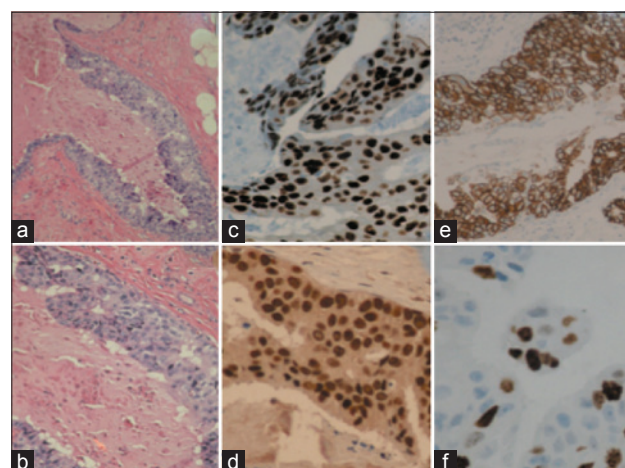


Figure 2: Representative high grade ductal carcinoma *in situ* case (x40). (a and b) Hematoxylin and eosin stain; (c) GATA-3 expression (H score 162); (d) FOXA1 expression (H score 180); (e) human epidermal growth factor receptor 2/neu expression (immunohistochemistry score 3+); (f) Ki-67 expression (15% proliferation rate). FOXA1: Forkhead-box A1; GATA-3: GATA binding protein 3

Table 3: Biomarker score in DCIS, stratified by hormone status and follow up outcome

	n (%)	ER score	PR score	P	GATA-3	P	FOXA1	P	HER2	P	Ki 67	P
Recurrence DCIS												
ER(-)	6 (46)	1	1	0.019*	150	0.12	169	0.50	3	0.032*	35	0.11
ER(+)	7 (54)	150	96		190		169		1		19	
Recurrence IC												
ER(-)	4 (31)	0	0	0.034*	165	0.021*	192	0.08	2	0.14	16	0.31
ER(+)	9 (69)	182	75		212		216		1		20	
No recurrence DCIS												
ER(-)	34 (23)	1	6	< 0.001*	158	< 0.001*	178	0.026*	3	< 0.001*	29	< 0.001*
ER(+)	113 (77)	208	96		204		195		1		16	
No recurrence IC												
ER(-)	11 (24)	0	1	< 0.001*	172	0.019*	223	0.255	3	< 0.001*	47	< 0.01*
ER(+)	35 (76)	197	92		228		211		2		26	

ER(-) represents H score < 10, ER(+) represents H score > 10. *P value with statistical significance. ER: Estrogen receptor; PR: Progesterone receptor; DCIS: Ductal carcinoma *in situ*; IC: Invasive carcinoma; FOXA1: Forkhead-box A1; GATA-3: GATA binding protein 3

There was a trend toward lower GATA-3 expression in all groups of ER(-) DCIS compared to the ER(+) cases. There was not a significant difference in the recurrence group; however, our numbers are low and may be lacking statistical power to draw meaningful conclusions. Others have shown that in IC with low/absent expression of GATA-3 expression, there is an association with absence of hormone receptor expression for ER/PR, overexpression of HER2, and most significantly, shorter disease-free survival.^[24]

For ER(+) luminal type-A invasive cancers, FOXA1 is a significant predictor of cancer survival.^[10,11] Interestingly, high FOXA1 expression in ER(-) IC has also been shown to confer a lower risk of recurrence,^[12] while loss of GATA-3 expression in ER(+) is associated with a higher rate of recurrence and/or metastasis.^[24] These data suggest that FOXA1 and GATA-3 expression in IC has a complex relationship with ER. These novel transcription factors appear to be important prognostic biomarkers associated with a well-differentiated state. These data help explain why our DCIS cases maintained such high expression of GATA-3 and FOXA1 even within the ER(-) group. It would be of importance to know the difference in the level of expression between cases at recurrence and at diagnosis, index to see if there is an incremental decrease in transcription factor expression at recurrence. In this pilot study, we were not able to perform this comparison.

Others have shown in DCIS that the loss of ER expression along with HER2-neu overexpression is a predictor of recurrence.^[8,28] Similarly, we saw this pattern in our cases, with a higher percentage of ER(-), HER2-neu positive cases in the recurrent group compared to the nonrecurrent group. It is our hypothesis that with greater statistical power and optimization of our antibody titers that we may see a small but significantly lower expression in FOXA1 and GATA-3 in recurrent cases, as was seen with greater loss of ER expression in this group.

Further work needs to be done on a larger cohort of DCIS cases with recurrence to understand better which variables are best able to predict recurrence and guide therapy decision strategies. Our study compared two novel biomarkers, along with established biomarkers and other important histopathological, clinical, and treatment factors, in a novel prediction model, to determine which factors best predict recurrence in DCIS. The maintenance of FOXA1 and GATA-3 expression in ER(-) DCIS needs to be evaluated further, as these transcription factors may offer new promising targets for therapy.

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Squamous cell carcinoma of the lung: clinical criteria for treatment strategy

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ABSTRACT

Aim: Primary lung cancer is the leading cause of human cancer deaths worldwide, and squamous cell carcinoma (SCC) is one of the most frequent histologic subtypes. The aim of our study was to analyze clinical factors potentially affecting the overall outcome of advanced lung SCC patients. **Methods:** A series of 72 consecutive patients with advanced SCC undergoing chemotherapy at our institution between January 2007 and July 2013 were eligible for our analysis. **Results:** By univariate analysis, a better overall survival (OS) was related to response to first-line chemotherapy: median OS were 19.7 vs. 7.17 months, respectively, for responders and nonresponders patients ($P < 0.0001$). Eastern Cooperative Oncology Group performance status, gender, and surgery were other prognostic factors. No significant relationship between OS and smoking status, age, body mass index, or type of treatment was found. In the third-line setting, a better OS was associated with objective response to second-line treatment ($P = 0.015$). **Conclusion:** Our results suggest that differences in OS seem strictly associated with clinical response to previous treatments. These data should be considered in the therapeutic strategy and management of patients with SCC of the lung.

Key words: Non-small cell lung cancer, prognostic factors, squamous cell carcinoma

Introduction

Squamous cell carcinoma (SCC) represents 25-30% of all non-small cell lung cancer (NSCLC).^[1] It is due to the transformation of bronchial epithelium caused primarily by cigarette smoking and shows a remarkable dose-dependence with it. Typically, SCC originates in bronchial airways, in particular, those proximal and of medium caliber while adenocarcinoma (ADC) occurs in about 50% of cases and is localized to bronchi of smaller diameter. ADC is the most frequent histological type in nonsmokers, and its pathogenesis differs from SCC.

In general, SCC tends to be locally aggressive with metastasis to distant organs occurring less frequently than in ADC. New treatment options for ADC underline the need for mandatory subtyping.^[2] In particular, mutations in the epidermal growth factor receptor (EGFR) kinase, as well as fusions involving anaplastic lymphoma kinase (ALK), have led to a remarkable improvement in personalized therapy for ADC.^[3,4] Unfortunately, activating mutations in EGFR and ALK fusions are

typically absent in SCC,^[5] and targeted agents developed for ADC are largely ineffective against SCC. The aim of our study was to analyze clinical factors potentially affecting the outcome of advanced SCC in clinical practice. This was done to identify criteria that can help physicians to select the best treatment strategy in their clinical settings.

Methods

The study includes patients with locally advanced or metastatic (tumor-node-metastasis (TNM) stage III-IV) SCC of the lung undergoing chemotherapy at our institution between January 2007 and July 2013. Age, smoking history, sex, Eastern Cooperative Oncology Group (ECOG) performance status (PS), body mass index (BMI), and pathological stage of disease (TNM) were included in recorded patient characteristics and clinical features. The following data were collected for each patient: first and second-line chemotherapy details and surgical resection or radiotherapy information if performed. Tumor response was evaluated according to the Response Evaluation Criteria in Solid Tumors (RECIST 1.1).

The statistical association between categorical variables and clinical outcome was assessed with a Chi-square test. We used Kaplan-Meier analysis to measure survival distribution. Tested variables included sex (male vs. female), age (> 65 vs. ≤ 65), ECOG PS (0 vs. ≥ 1), BMI (< 25 vs. $25-29.9$ vs. ≥ 30), smoking status (never smoker vs. smoker/former smoker), stage (III vs. IV), surgery (surgery vs. not

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surgery), radiotherapy (radiotherapy vs. not radiotherapy), type of chemotherapy (two-drug chemotherapy regimens including platinum and gemcitabine vs. gemcitabine alone vs. docetaxel vs. others), response to first and second-line chemotherapy (responders vs. nonresponders). Cox multiple regression analysis was used to assess the role of those variables resulting in significance by univariate analysis. Overall survival (OS) was defined as interval between start of chemotherapy to death or last follow-up visit. Progression-free survival (PFS) was defined as interval between start of treatment to clinical progression or death or last follow-up visit if not having disease progression. Significant differences in probability of surviving between strata were evaluated by log-rank test. A significance level of 0.05 was chosen to assess statistical significance. Statistical analyses were performed using MedCalc version v9.4.2.0 (MedCalc Software, Broekstraat 52, 9030 Mariakerke, Belgium).

Results

Between January 2007 and July 2013, 72 patients undergoing chemotherapy for advanced SCC of the lung at our institution were included in the analysis. Median age at diagnosis was 68 years (range: 45-83); male/female ratio was 60/12. The majority of patients (56%) presented with stage IV, while 32 patients had stage IIIA (29%) and IIIB (15%) stage. Twenty-three patients (32%) underwent surgery, and 12 of these had adjuvant therapy. Table 1 summarizes patient characteristics. Median OS in all patients was 12.3 months (range: 1.1-72.5). By univariate analysis, gender ($P = 0.026$), PS ($P = 0.0009$) and surgery ($P = 0.02$) were related to OS. No significant relationship was found between OS and age, type of treatment, smoking status, or BMI.

In the first-line setting, we observed partial responses (PR) in 21 patients (29%), progressive disease (PD) in 30 cases (42%), with 10 patients (14%) showing stable disease (SD). No complete remissions (CR) were obtained. In 11 cases (15%) response was not reported.

By univariate analysis, a better OS ($P < 0.0001$) and a better PFS ($P < 0.0001$) were associated with response to first-line chemotherapy: median OS was 19.7 vs. 7.17 months for responders and nonresponders patients, respectively [Figure 1]. Median PFS was 8.5 months in responders as compared to 2.9 months in nonresponders [Figure 2].

These variables, with the exception of PS, maintained statistical significance even by multivariate analysis and proved to independently affect the outcome: sex ($P = 0.019$), surgery ($P = 0.036$), response to first-line therapy ($P < 0.0001$). Thirty patients (42%) received chemotherapy as second-line therapy. Median OS in this group was 6.43 months (range: 0.6-54.4), with PFS of 3.1 months (range: 0.4-51.4). Moreover, in the second-line setting, better OS was associated

Table 1: Patients and tumor characteristics

Characteristics	n (%)
Patients (median age 68 years old, range: 45-83)	72
Stage	
III A	21 (29)
III B	11 (15)
IV	40 (56)
Sex	
Male	60 (83)
Female	12 (17)
Smoking status	
Ever smoker	66 (92)
Never smoker	6 (8)
Performance status	
0	44 (61)
1	26 (36)
≥ 2	2 (3)
Surgery	
Yes	23 (32)
No	49 (68)
Radiotherapy	
Yes	24 (33)
No	48 (67)
Chemotherapy	
Platinum + gemcitabine	48 (67)
Gemcitabine	15 (21)
Docetaxel	2 (3)
Other	7 (9)

with response to previous chemotherapy ($P = 0.015$): median OS 18.77 and 5.83 months for responders and nonresponders, respectively [Figure 3]. A significant impact in terms of different PFS was seen as a function of response to second-line therapy (5.9 vs. 2.7 months, $P = 0.007$). Finally, only 11 patients received third-line chemotherapy.

Discussion

Genomic alterations in SCC of the lung have not been comprehensively characterized, and molecular targeted therapies have mainly shown no efficacy. To date, the most important molecular and therapeutic achievements in advanced NSCLC have been mostly confined to patients with nonsquamous histology. However, recent research is focusing on identifying potential driver mutations affecting SCC patients.^[6,7] In a recent large phase III trial, necitumumab added to cisplatin and gemcitabine as first-line treatment increased survival in patients with advanced SCC.^[8,9] Nevertheless, at present, the standard frontline treatment remains exclusively chemotherapy. For patients with locally advanced or metastatic SCC, two-drug chemotherapy regimens (including cisplatin or carboplatin and a third-generation agent, such as gemcitabine, taxanes, or vinorelbine) currently remain the standard of treatment options.^[10] A single agent (mainly docetaxel) is the preferred treatment in second-line setting.^[11]

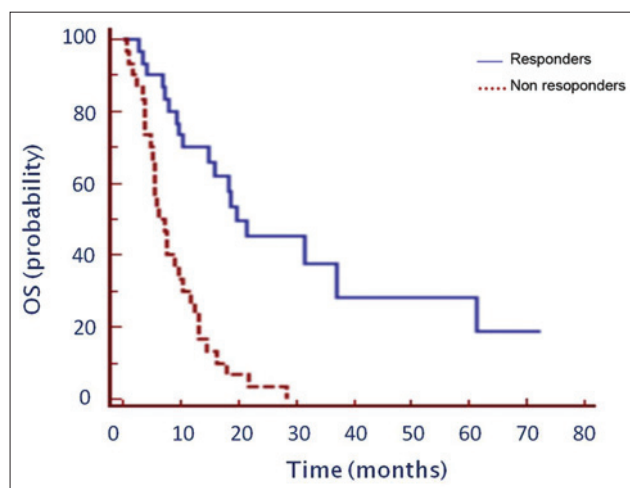


Figure 1: Median overall survival of patients as function of response to first-line chemotherapy, $P < 0.0001$

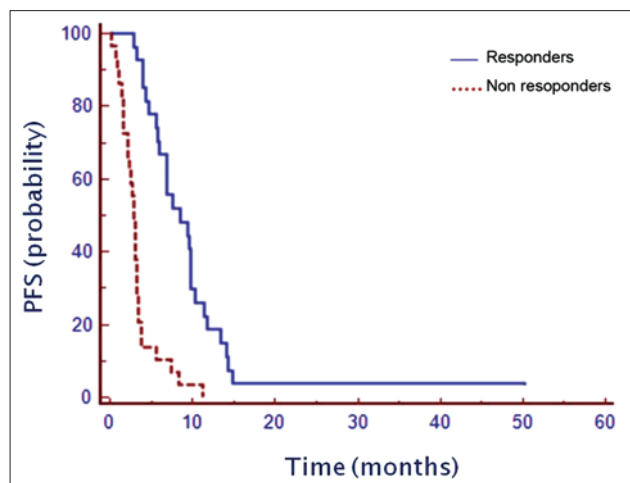


Figure 2: Median-progression free survival of patients as function of response to first-line chemotherapy, $P < 0.0001$

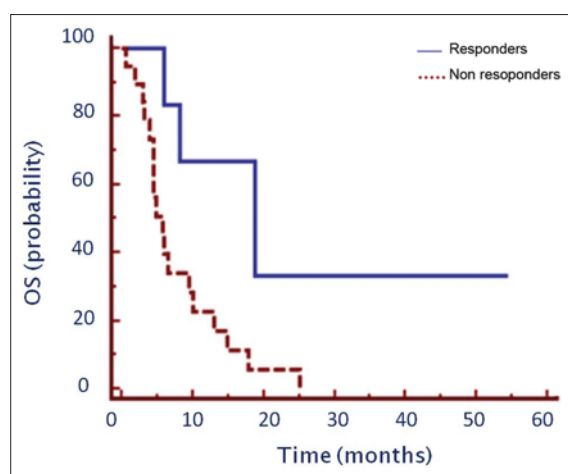


Figure 3: Median overall survival of patients as function of response to second-line chemotherapy, $P = 0.015$

In our study, we analyzed clinical factors potentially influencing the overall outcome of patients with advanced lung SCC to identify a population of patients

likely to benefit from chemotherapy with a prolonged life expectancy. Previous publications have suggested various prognostic factors involved in advanced NSCLC using heterogeneous patient populations.^[12-15] In a recent study, 245 patients were analyzed with the aim of evaluating factors associated with long-term survival (> 2 years) in patients with advanced NSCLC. Fifty-two patients (21%) had SCC. Six prognostic factors were identified: PS of 0-1 at first tumor progression, normal lactate dehydrogenase levels at diagnosis, use of maintenance therapy, surgical resection, time to progression of > 3 months, and number of chemotherapy agents received.^[13] Conversely, our study showed that a better PS at diagnosis was significantly associated with a better OS. In another large report, FLEX^[15] investigated the prognostic significance of baseline characteristics and showed that age, gender, PS, smoking status, tumor histology, and number of involved organs were independent factors of prognostic value. Interestingly, in our analysis, those factors did not show an impact on outcome while response to first-line chemotherapy was the major determinant for OS. A previous retrospective study evaluated the impact of first-line chemotherapy on results of second-line chemotherapy, using data from a large phase III study. One hundred and seventy-one (30%) of 571 patients had SCC. The study showed that gender, histology, stage at diagnosis, PS at the beginning of second-line therapy, and best response to initial therapy were associated with survival outcome. In particular, median survival was 15.8 months in cases of CR/PR, 10.5 months in cases of SD and 4.6 months for PD ($P < 0.001$).^[16]

In advanced colorectal cancer, it has been shown that patients eventually receiving all available drugs have a better OS.^[17] Similarly, in our study, patients responding to first-line chemotherapy had better OS and for those patients, receiving second-line therapy to maximize OS seemed important. Overall, despite heterogeneous treatment characteristics, our findings seem to indicate that SCC patients who responded to therapy may most benefit from additional treatments and this result could be relevant for the decision making process and the therapeutic strategy.

In conclusion, response to first-line and second-line treatments seems to have a significant prognostic impact in SCC. These observations should be considered relevant for the management of such patients, although further studies also based on biological markers are essential to better understand the prognostic factors in this population.

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***Withania somnifera* extract reduces the invasiveness of MDA-MB-231 breast cancer and inhibits cytokines associated with metastasis**

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ABSTRACT

Aim: The aim was to examine the anti-proliferative effect of a *Withania somnifera* (WS) root extract in cell cultures and nude mouse xenografts of breast cancer cell line MDA-MB-231. **Methods:** WS root extract was used to treat tumor cells at concentrations up to 100 µg and for nude mouse experiments, the mice received daily WS at 300 mg/kg by oral gavage for 8 weeks. **Results:** The WS extract reduced viability of MDA-MB-231 cells by 75% and 88% after exposure of the cells to 50 and 100 µg/mL, respectively, compared to vehicle-treated controls. WS extract caused a dose-dependent increase in the percentage of cells in the sub-G1 phase compared to untreated controls by 6% and 10% after exposure to 25 and 50 µg/mL WS extract, respectively. WS extract also inhibited proliferation of xenografted MDA-MB-231 cells. The WS extract caused reductions in xenograft size by 60% compared to the untreated control after 8 weeks of treatment. Six of ten mice in the control group showed tumor metastasis to the lung, whereas there was none in the mice treated with the WS extract. At the gene level, WS caused a 75% reduction in chemokine CCL2 expression ($P < 0.05$) in the xenografted tumors of the treated mice. **Conclusion:** WS root extract inhibited proliferation of breast cancer cells *in vitro* and *in vivo* and significantly reduced expression of the cytokine, CCL2. These results warrant further studies to assess the underlying molecular mechanism of the anti-tumor activity of the WS extract in breast cancer.

Key words: *Withania somnifera* extract, MDA-MB-231, breast cancer, metastasis, animal model

Introduction

Invasive breast cancer is considered one of the great challenges for clinicians to control and improve survival of patients. In 2013, an estimated 232,340 new cases of invasive breast cancer were diagnosed in women in the USA, along with other 64,640 cases of non-invasive breast cancer.^[1] For women under 45, deadly forms of this type of breast cancer are more common in African-American women than white women, and African-American women are more likely to die of breast cancer.^[2] Despite three decades of advances in treatment of breast cancer using hormone receptor modulators, aromatase inhibitors, and surgery,^[3-5] mortality remains high due to tumor metastasis to the lymph nodes, liver, and lung.^[6] Triple-negative breast cancer (TNBC) accounts for 10-20% of diagnosed breast cancers and is more likely to affect younger African Americans, Hispanics, and/or those with *BRCA1* mutations. TNBCs are more aggressive, difficult to treat, and more likely

to spread and recur.^[2] TNBCs are different from other kinds of breast cancer in that they are highly metastatic and resistant to conventional therapies, such as anticancer drugs and radiation.^[2]

In a search for an agent that inhibits proliferation and invasion of TNBCs, we evaluated an extract derived from an Indian herb, *Withania somnifera* (WS), which is a nightshade medicinal plant that contains active components for the treatment of a variety of ailments, including cancer.^[7-10] The use of WS root extract is practical since it contains the active compounds present in the plant. In TNBC cells, sub-cytotoxic concentrations of withaferin A, derived from WS, reduce various effectors of metastasis.^[11] In the present study, we assessed the effect of the WS extract on proliferation and metastasis of MDA-MB-231 cells, derived from a TNBC, in cell cultures, and in mice.

Methods

Preparation of WS extract

Roots of WS were ground to a paste, and then extracted with 5 volumes of 70% ethanol by stirring for 2 days. The alcoholic extract was filtered, and the solvent was evaporated under a vacuum. The extract was then dried to a powder and kept in a closed container until use.^[12] To avoid variations in the activity of different preparations, the sufficient extract was obtained in one batch for use throughout the experiments.

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Reagents and antibodies

WS roots were purchased from a local market in the USA and dimethyl sulfoxide (DMSO) from Sigma (St. Louis, MO, USA). Antibodies (anti-chemokine CCL2, CXCL1, CXCL2, CXCL3, PARP, and GAPDH) were from Cell Signaling (Beverly, MA, USA). Human breast cancer MDA-MB-231 cell line and a normal breast cell line, MCF10A, were obtained from ATCC (Manassas, VA, USA). The HCA-II human cytokine primer kit was obtained from Real Time Primers (Elkins Park, PA, USA).

Cell culture and treatment

Breast cancer MDA-MB-231 cells were maintained in Dulbecco's Modified Eagle's Medium (ATCC) supplemented with 10% fetal bovine serum and penicillin/streptomycin. MCF10A cells were maintained in complete MEGM (Lonza, Houston, TX, USA). All cell cultures were incubated at 37 °C with 5% CO₂ in a humidified incubator.

Assessment of cell viability

To assess the effect of the WS extract on regulation of cell viability, cells were seeded into 96-well, 6-well or 6-cm plates at densities of 10³, 10⁴ or 10⁵ cells per well, respectively. For experiments requiring longer than 48 h, cell numbers were reduced by one half. Viability was assessed by using the 3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium assay in 96-well plates in triplicate with CellTiter 96® AQueous One Solution cell proliferation kits from Promega (Madison, WI) according to the manufacturer's instructions. Absorbance was recorded at 490 nm using a Synergy HT multimode plate reader or PowerWave XS2 (BioTek®, Winooski, VT, USA) reader. DMSO was used as a control. To calculate the viability index, absorbance readings from DMSO-treated control wells were set at 100%, and the relative A490 was calculated as a percentage of the control.

Flow cytometry

Cells treated with the WS extract were harvested and prepared for flow cytometry as described by Samuel *et al.*,^[13] with some modifications. WS treated and untreated cells were harvested by trypsinization in 0.25% trypsin/ethylenediaminetetraacetic acid. Prior to trypsinization, floating or loose cells were harvested by gentle rocking of the culture dishes and transferring the culture medium containing the cells into centrifuge tubes. Trypsinized and detached cells were then combined and centrifuged. Cell pellets were suspended in 300 µL of phosphate-buffered saline (PBS), fixed with 700 µL of 100% ethanol with vortexing, and stored at -20 °C overnight. The fixed cells were centrifuged and stained in fluorescence-activated cell sorting staining solution (3 mg/mL RNase A, 0.4 mg/mL propidium iodide) in PBS without calcium or magnesium for 30 min at 37 °C

and then filtered through a 70-µm filter and analyzed by flow cytometry (FACScalibur® Becton Dickinson or C6 Accuri® flow cytometer). Data were analyzed with CellQuest and CFlow software (BD).

Immunocytochemistry

Breast cancer MDA-MB-231 cells were seeded in 4-well plates and grown for 16 h. The cells were then treated with DMSO (vehicle) or with 25 or 50 µg/mL of WS root extract for 18 h. After treatment, the culture medium was removed, and the cells were fixed with 10% neutral buffered formalin. Xenograft tissues were placed in an automatic tissue processor, embedded in paraffin, sectioned at 5-µm thickness, and stained with hematoxylin and eosin (HE). For immunohistochemistry, the fixed cells and tissues from xenografted tumors were stained with CCL2 antibody because this cytokine is considered to be most responsible for metastasis of breast cancer.^[14] The sections were de-paraffinized in xylene and rehydrated through a series of graded ethanol (100%, 95%, and 70%) and in water for 5 min each. The sections were then washed three times for 5 min each in PBS containing 0.05% Tween 80 (pH 7.4). Antigen retrieval was achieved by heating the sections in a microwave with 0.01 mol/L sodium citrate (pH 6.0) solution and subsequently cooling down to room temperature. Endogenous peroxidase activity was blocked by incubating the sections for 30 min in 1% hydrogen peroxide in methanol. Non-specific binding was blocked by incubating the sections for 1 h with a normal horse serum (Vector Laboratories, Inc., Burlingame, CA, USA). The sections were then incubated with mouse anti-CCL2 (MCP-1, eBioscience, San Diego, CA, USA) overnight at 4 °C. On the next day, the sections were rinsed 3 times with PBS at room temperature and then further incubated with goat anti-mouse IgG-FITC (Santa Cruz Biotechnology, Santa Cruz, CA, USA) for 1 h at room temperature. The fluorescence was then read using a wide-field fluorescent microscope (Olympus, Center Valley, PA, USA). Stained sections were reviewed and scored according to the intensity of staining (0, +1, +2 or +3) and for the percentage of tumor cells staining positive for CCL2 (0%, 0.1-30%, +1; 31-70%, +2; or > 70%, +3). The score of the intensity of immunostaining was multiplied by the score of percentage of cell staining to obtain the final staining index.

RNA isolation and quantitative reverse transcription-polymerase chain reaction

Total RNA was isolated from treated and control samples with RNeasy Mini Kits (Qiagen, Valencia, CA, USA) and reversely transcribed into cDNA using Quantitect Reverse Transcriptase Kits (Qiagen) according to the manufacturer's instructions. All primers were from SABiosciences (Valencia, CA, USA); and quantitative polymerase chain reaction (qPCR) amplification was performed using 50 ng of cDNA, 10 µL of Brilliant III Ultra-Fast SYBR Green qPCR Master Mix

(Agilent Technologies, Santa Clara, CA, USA), and 500 nM of each primer. β -Actin was used as the internal control, and the final reactions were adjusted to a total volume of 20 μ L with DNase RNase-free water (Qiagen). All qPCR amplification was performed in duplicates with a Stratagene Mx 3005P system (Agilent Technologies), and the conditions were set to initial cycle of denaturation at 95 °C for 10 min, 40 cycles of denaturation at 95 °C for 30 s, annealing at 55 °C for 1 min, and extension at 72 °C for 1 min. The final segment involved generation of a dissociation curve. This comprised one cycle at 95 °C for 1 min, followed by 55 °C for 30 s and 95 °C for 30 s. Inclusion of a dissociation curve in each qPCR run ensured specificity of the amplicon.

Microarray analysis

To determine the effect of WS extract on expression of cytokines in MDA-MB-231 cells, cells were incubated overnight with either 50 μ g/mL WS or DMSO (vehicle) as a control. The analysis was accomplished by use of HCA-II cytokine primer library II according to the manufacturer's instructions.

Experimental mice and treatments

Athymic Nude-Foxn1^{nu} mice at 6 weeks of age were obtained from Harlan Sprague-Dawley and housed in animal quarters at 22 °C with a 12 h light/dark cycle. Animals were given free access to water and food. These studies were approved by the Tuskegee University Institutional Animal Care and Use Committee. At 8 weeks of age, mice were injected subcutaneously with 0.2 mL of PBS containing 1.5×10^6 human breast cancer MDA-MB-231 cells into the right flanks. Twenty mice that developed tumor sizes of 50-200 mm³ were divided into two equal groups. The control group received 0.2 mL of 5% DMSO orally by gavage, and the treated group received 300 mg/kg/day WS root extract dissolved in 5% DMSO orally by gavage daily for 5 days a week for 8 weeks. Tumor sizes were checked weekly in each group. Tumor dimensions in mm (length and width) were measured with vernier calipers and calculated for each tumor by using the following equation: tumor volume = $1/2$ (length \times width²). At the end of the 8th week, mice were euthanized with CO₂. Tumors and lung tissues were collected and fixed with 10% formalin for histopathological and immunochemistry analysis.

Evaluation of lung metastasis

Two pathologists histopathologically evaluated lung metastases in untreated and treated groups after staining of sections with HE, and the results were reported independently. The number of metastatic foci was counted in each stained tissue section.

Statistical analyses

Student's *t*-test was used to assess differences between values for the treated and control groups. One-way analysis of variance was used with Dunnett's test.

Results

WS extract caused a dose-dependent reduction of viability of breast cancer MDA-MB-231 cells by 75% and 88% after treatment with 50 or 100 μ g/mL WS extract, respectively, compared to vehicle-treated controls [Figure 1], but WS treatment did not affect the viability of non-cancerous epithelial mammary cells, MCF10A [Figure 2]. Moreover, compared to untreated controls, WS extract caused a concentration-dependent increase in the sub-G1 phase of the cell population, by 6% and 10% after exposure to 25 μ g/mL and 50 μ g/mL, respectively [Figure 3].

Furthermore, WS extract inhibited proliferation of xenografted MDA-MB-231 cells, reducing the size of xenografted tumors by 60% compared to the untreated control after 8 weeks of treatment ($P < 0.05$) [Figure 4]. In addition, after euthanasia, six of ten mice in the control group showed tumor metastasis to the lung, whereas none of the mice in WS-treated group developed metastasized tumor lesions in the lung [Figure 5]. This finding motivated us to explore the underlying molecular mechanism by which the WS extract inhibited tumor metastases to the lung.

Microarray analysis of gene expression of cytokines was then performed. WS suppressed expression of CCL2, CXCL1, CXCL2, CXCL3, IL1B, TGFB3, and BMP4 mRNA [Figure 6]. These inhibitory effects were confirmed by quantitative reverse transcription-polymerase chain reaction analysis [Figure 7]. WS caused a 75% reduction in CCL2 expression ($P < 0.05$) in the xenografted tumors of treated mice [Figure 8].

Discussion

The current study assessed the effect of an alcoholic extract of WS roots on proliferation and metastasis of

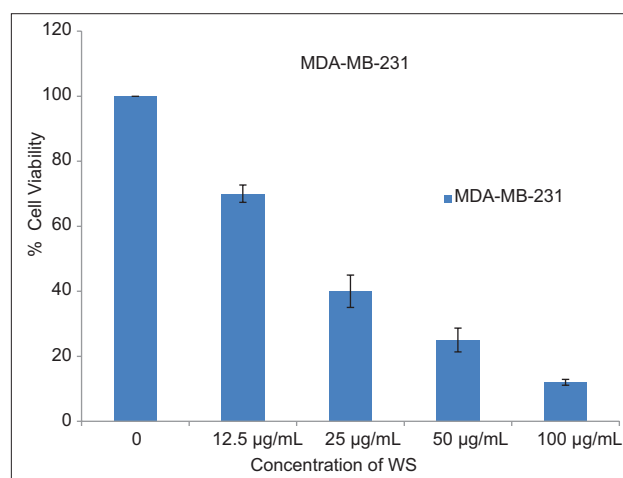


Figure 1: Effect of WS on viability of breast cancer MDA-MB-231 cells. The bars represent the mean \pm standard deviation of six 24-h treatments for the vehicle and different concentrations of WS. The results are statistically significant ($P < 0.05$) compared to the DMSO-treated (control) cells as determined by one-way ANOVA with Dunnett's test. WS: *Withania somnifera*; ANOVA: Analysis of variance; DMSO: Dimethyl sulfoxide

breast cancer MDA-MB-231 cells *in vitro* and in nude mice, respectively. WS roots have been used in ayurvedic medicine for their anti-inflammatory, analgesic, anticancer, and anti-stress properties.^[7,8] These diverse effects are attributed to the presence of active steroidal compounds that are called withanolides.^[15] Our current data showed that the WS extract inhibited proliferation and metastasis of MDA-MB-231 cells *in vitro* and in nude mice. This inhibition was greater than that caused by withaferin A.^[16] The difference in inhibition may be attributed to the fact that the whole extract contains active ingredients that have a synergistic effect against breast cancer cells.^[7,17] Since MDA-MB-231 cells are “triple-negative” form estrogen-independent tumors *in vivo*, the anti-proliferative effect of WS is apparently

estrogen-independent. The WS extract caused increases in the percentage of MDA-MB-231 cells in the sub-G1 phase, indicating that WS causes apoptosis. Withaferin A, one of the active compounds of WS, causes G (2)/M cell cycle arrest, associated with modulation of cyclin B1, p34(cdc2), and PCNA levels, decreases the levels of STAT3 and its phosphorylation at Tyr(705) and Ser(727), and alters expression levels of p53-mediated apoptotic markers-Bcl2, Bax, caspase-3, and cleaved PARP.^[18]

Results of our current mouse experiments are consistent with *in vitro* data. The WS extract, administered orally, inhibited formation and growth of MDA-MB-231 cell xenografts in nude mice, indicating that the active ingredients of the WS extract are bioavailable after oral administration.^[19] Six mice of the untreated group developed tumor metastasis to the lung, whereas none of the treated mice showed such tumor metastases. This effect may be attributed to inhibition of CCL2 in xenografted tumors after treatment with WS root extract. These results are consistent with a previous study^[20] concerning the inhibition of CCL2 in animals. Inhibition of CCL2/CCR2 signaling by anti-CCL2 antibodies blocks recruitment of inflammatory monocytes, inhibits metastasis, and prolongs the survival of tumor-bearing mice. Depletion of tumor cell-derived CCL2 also inhibits metastatic seeding. Moreover, CCL2 mediates development of cancer stem cell (CSC) phenotypes. Promotion of CSC is relevant since these cells, through self-renewal, maintain heterogeneity and give rise to metastasis of breast cancer.^[21]

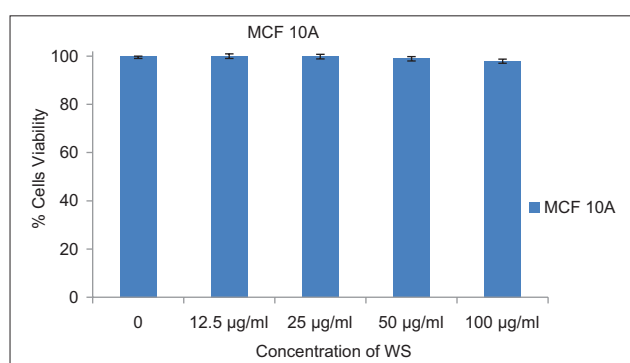


Figure 2: Effect of WS on the viability of non-cancerous epithelial mammary cells, MCF10A. The bars represent the mean \pm standard deviation of six 72-h treatments for the vehicle and different concentrations of WS. As determined by one-way ANOVA, results of treated cells are not statistically significant compared to the DMSO-treated (control) cells. WS: *Withania somnifera*; ANOVA: Analysis of variance; DMSO: Dimethyl sulfoxide

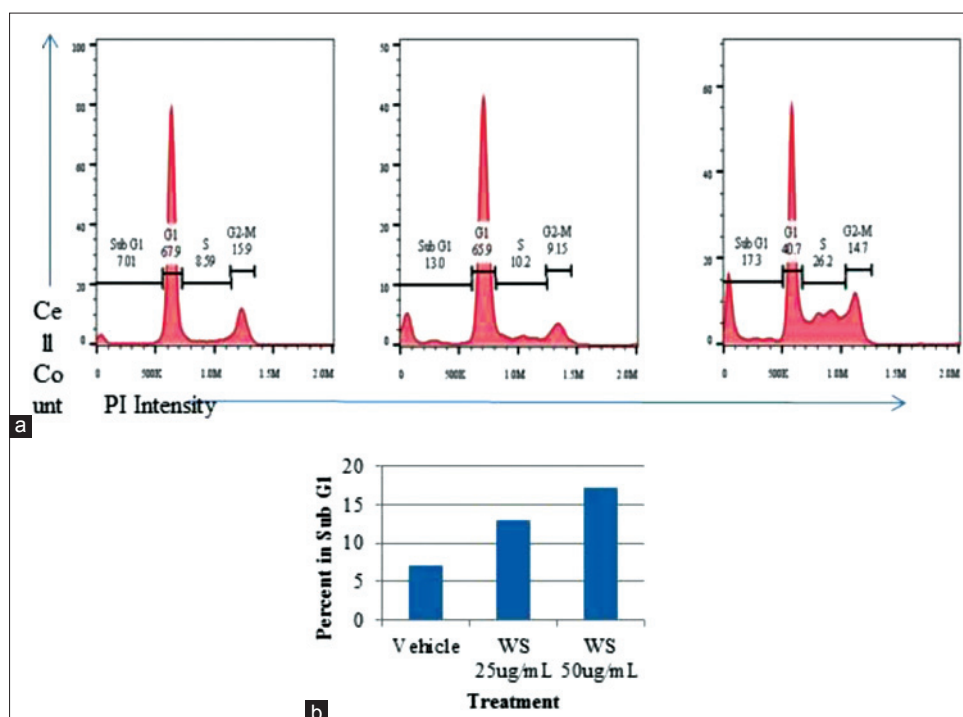


Figure 3: Effect of different concentrations of WS on the cell cycle of MDA-MB231 breast cancer cells. (a) Cell cycle histograms by treatment (vehicle, WS 25 µg/mL and WS 50 µg/mL). Range gates show cell percentage in each cell cycle stage; (b) percentage of cells in cell cycle arrest by treatment. WS: *Withania somnifera*

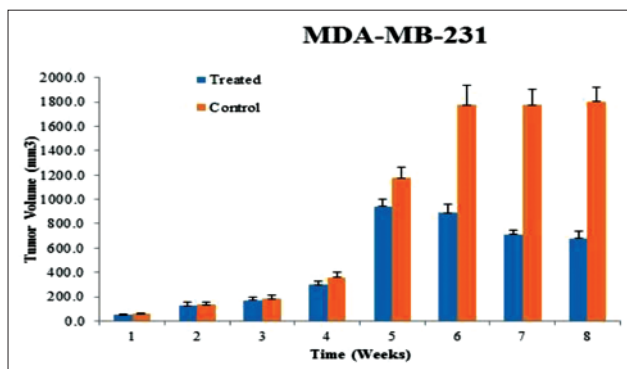


Figure 4: Effect of WS on suppression of growth of xenografted MDA-MB-231 cells in nude mice. The bars represent the means \pm standard deviations of tumor size (mm^3) ($n = 10$). The highest reduction (60%) relative to the untreated control was shown after 8 weeks of WS treatment ($P < 0.05$). Student's t -test was used to assess significant differences between treated groups and the untreated control group. WS: *Withania somnifera*

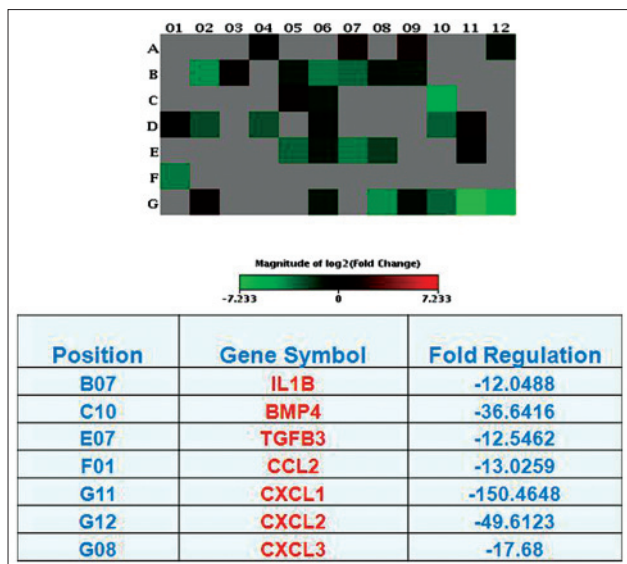


Figure 6: Effect of WS on inhibition of cytokine/chemokine expression. WS: *Withania somnifera*

Our current data are consistent with those reported by others.^[17] A root extract of WS showed dose-dependent inhibition of tumor growth and metastatic lung nodule formation with the minimal toxicity to mice.^[17] The extract apparently inhibited cancer metastasis through inhibition of the epithelial-mesenchymal transition (EMT). Furthermore, withaferin A treatment of MCF-10A cells inhibited EMT and in mice, reduced mammary cancer growth, effects of which were associated with reduced vimentin expression.^[22] In the present study, the oral dose of WS extract used to inhibit tumor metastasis to the lungs was 300 mg/kg/day body weight. This dose was extrapolated from the cell culture experiments regarding the effect of WS extract on MDA-MB-230 cells. This dose was selected based on a pilot study involving a range of doses to estimate

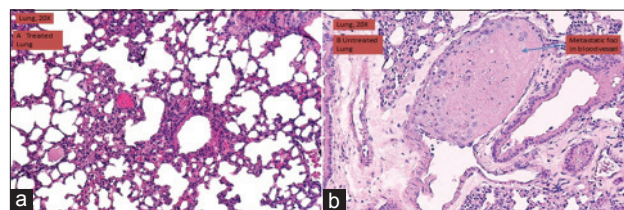


Figure 5: Effect of WS treatment on inhibition of lung metastasis in nude mice. HE staining of lung tissue sections after treatment with or without WS for 8 weeks ($\times 20$). (a) WS treated mouse lungs showed no tumor metastasis ($n = 10$); (b) six of ten mice showed tumor metastasis to the lungs, with a total of 12 metastatic foci in the blood vessels and the parenchyma of the lungs in control mice. WS: *Withania somnifera*

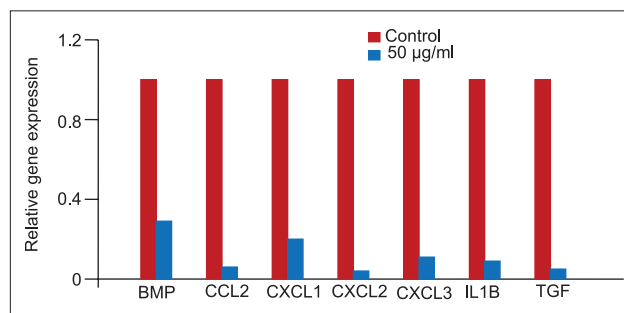


Figure 7: Effect of WS treatment on the regulation of cytokine expression. Quantitative reverse transcription-polymerase chain reaction was used to measure cytokine expression in cells treated or not treated with 50 $\mu\text{g}/\text{mL}$ WS. WS: *Withania somnifera*

the optimal dose. In addition, the *in vitro* cytotoxic concentration, ranging between 50 and 100 $\mu\text{g}/\text{mL}$, gave us an idea about the dose. In a previous study, WS root extract inhibited lung metastasis of xenografted MDA-MB-231 cells at a dose of 8 mg/kg body weight, administered 3 times a week for 4 weeks.^[19] This dose is 37.5 times less than the dose used in our current study. There is no obvious explanation for the difference in the two doses. Differences in the source of roots, age of roots, and extraction yield may contribute to different dose-responses when using crude plant extracts. However, the WS extract, at a dose of 150 mg/kg/day for 155 days, caused a 23% reduction in development of mammary tumors in rats administered the carcinogen, methylnitrosourea.^[23]

In transgenic (MMTV/Neu) mice that received a diet containing the extract (750 mg/kg of diet) for 10 months, mice in the treated group ($n = 35$) had an average of 1.66 mammary tumors, and mice in the control group ($n = 33$) had 2.48, a reduction of 33%. Moreover, in treated mice, WS caused a 50% reduction in the expression of CCL2.^[24]

WS caused *in vitro* and *in vivo* inhibition of breast cancer MDA-MB-231 cells and caused a significant reduction in expression of the cytokine, CCL2, a marker of the metastasis of breast cancer to other organs. These results warrant further studies to assess the underlying

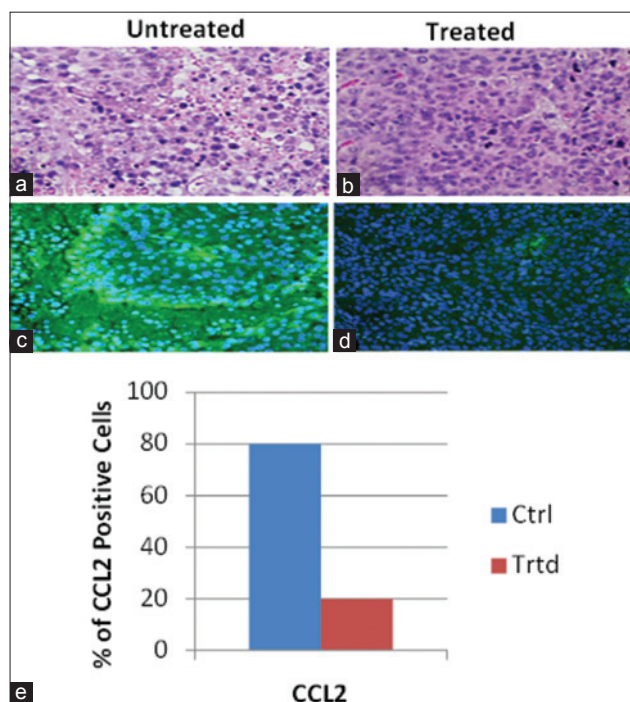


Figure 8: Effect of WS on expression of CCL2 in MDA-MB-231 xenografted tumors. (a and b, ×20) Hematoxylin and eosin sections of untreated and WS treated tumors, respectively; (c and d, ×20) immunohistochemical staining of CCL2 in untreated and WS treated tumors; (e) summarized data. There was a significant reduction ($P < 0.05$) in CCL2 expression in WS-treated tumors compared to untreated tumors as determined by Student's *t*-test. WS: *Withania somnifera*; CCL2: Chemokine (C-C motif) ligand 2

molecular mechanism of WS extract antitumor activity in the breast cancer metastasis.

Acknowledgments

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Metastatic inguinal lymph nodes with two different histological types in a case of carcinoma of unknown primary site

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ABSTRACT

Cancer of unknown primary site is a group of uncommon cancers where patients present with metastatic disease and the primary site is not identified, even after a complete workup to establish the diagnosis. Inguinal metastasis with unknown primary is even more uncommon, and histological type is the most important guiding factor to look for the primary. This report describes the rare situation of inguinal metastasis with an unknown primary site where a combination of squamous and transitional cell carcinoma was found on final histopathology. It highlights the importance of multimodality approach including an aggressive surgical resection combined with adjuvant radiation therapy to achieve an optimal outcome.

Key words: Carcinoma of unknown primary site, inguinal metastasis, squamous cell carcinoma, transitional cell carcinoma

Introduction

Cancer of unknown primary site (CUPS) is a clinical syndrome that is considered in patients where, even after extensive standard clinical, pathological and radiological evaluation, the primary site cannot be identified. Patients with CUPS account for 0.5-4% of all cancers diagnosed.^[1] Within this heterogeneous group, there is a wide variation of clinical presentations and histological types. Most present as a metastatic disease, which is often difficult to categorize using histology alone. Immunohistochemistry (IHC) is helpful in separating carcinomas from a neoplasm of other lineages. CUPS is more common in the head and neck and axillary regions, and inguinal involvement accounts for < 5% of cases.^[2,3] Metastatic inguinal lymphadenopathy mainly originates from the genitalia and anorectal areas. In this case report, we describe an uncommon case of two different histological types of metastases in inguinal nodes with unknown primary sites.

Case Report

A 49-year-old male patient, a farmer, presented in October 2012 to the surgical oncology clinic with swelling in the right groin crease for 2 years, which had been increasing progressively in size and subsequently became ulcerated. On examination the mass was hard, irregular in shape

due to the conglomeration of inguinal lymph nodes, about 5 cm in diameter, fixed to the skin and deeper structures, and superficially ulcerated [Figure 1]. Bilateral hydroceles were also present. No other enlarged lymph nodes in other regions were palpable. Per rectal examination and clinical evaluation, the genitals were normal. Fine-needle aspiration cytology of the node was suggestive of squamous cell carcinoma. For better categorization, a biopsy was performed, which was suggestive of poorly differentiated squamous cell carcinoma. On contrast-enhanced computed tomography (CT) scan, an ill-defined mass lesion of 5 cm × 4 cm was noted over right inguinal region encasing the femoral vein and having 180° contact with the femoral artery [Figure 2]. Right external iliac and obturator nodes were also enlarged. The remainder of the abdomen and chest were normal. Positron emission tomography (PET) scan showed increased tracer uptake in right inguinal, external iliac, and obturator nodes, but a primary site could not be visualized. Ultrasound evaluation of the testes was normal. Upper and lower gastrointestinal endoscopy were normal. Serum carcinoembryonic antigen, CA19-9, alpha-fetoprotein, prostate specific antigen, and beta human chorionic gonadotropin were within normal range.

With no primary site of cancer identified, the patient was taken for a right ilioinguinal lymph node dissection. The nodal mass, along with the encased segment of the femoral vein, was resected, and an autologous internal jugular vein graft was placed [Figures 3 and 4]. Lymph nodal clearance up to the aortic bifurcation was done. The postoperative period was uneventful. Histopathology was suggestive of squamous cell carcinoma with islands of transitional cells in interposed [Figure 5]. IHC stainings for CK20, CK5, CK6, and CK7 were negative. In view of the transitional cell elements, cystoscopy,

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Figure 1: Clinical appearance of right inguinal nodes

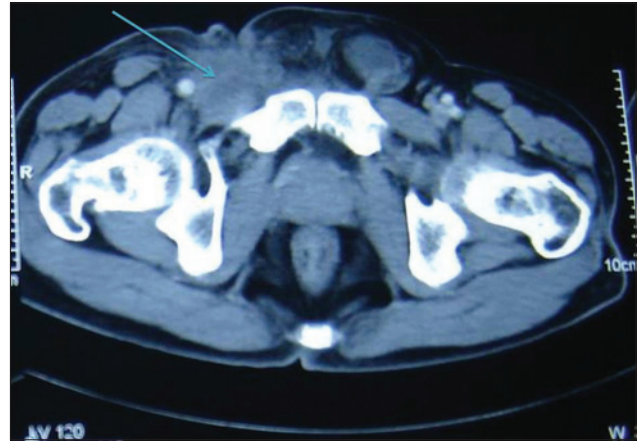


Figure 2: Contrast-enhanced computed tomography of pelvis showing right inguinal nodal mass (marked with arrow) infiltrating femoral vein

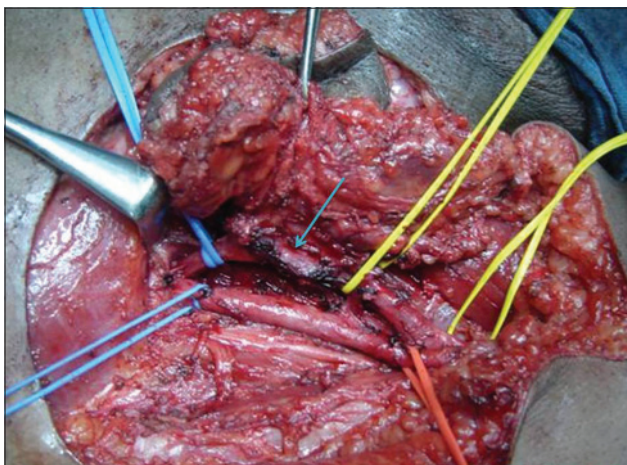


Figure 3: Intra-operative picture of nodal mass infiltrating femoral vein (site of infiltration marked with arrow)

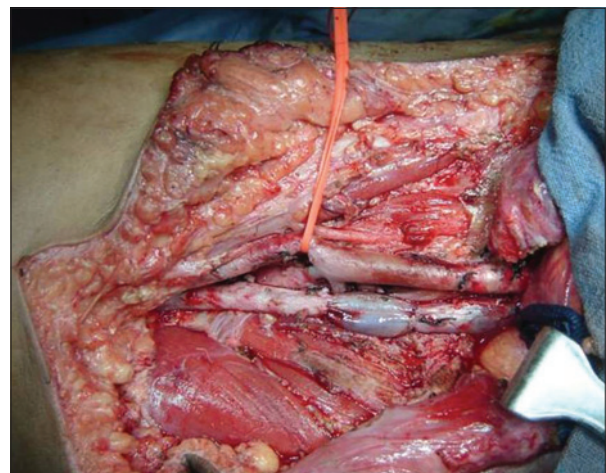


Figure 4: Reconstructed femoral vein with interposition graft from internal jugular vein

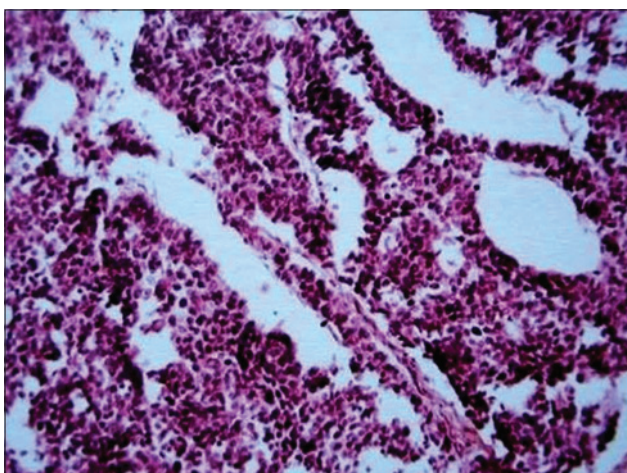


Figure 5: Histopathology picture from lymph node showing squamous cell carcinoma with areas of transitional cell carcinoma. (×40)

and urine analysis were also done, both of which were normal. Adjuvant radiotherapy to the bilateral inguinal, pelvic, and para-aortic regions with a dose of 55 Gy/25 fractions over 5 weeks was given. The patient tolerated the radiotherapy with minimal complications and was

doing well in the last follow-up one year post-surgery. All investigations and tumor markers repeated at the last follow-up in December 2014 were normal.

Discussion

The inguinal area is a relatively uncommon metastatic site of CUPS.^[4] There has been a wide variety of primary sites from where inguinal nodal metastasis has been reported. These include some sites, which are quite distant from the pelvis (nasopharynx, breast, tracheobronchial tree, salivary glands, orbit) but most originate in the pelvis, genitalia or lower limb.^[2,5,6] In one of the largest series, involving more than 2,000 patients with inguinal nodal metastasis, the primary site could not be identified in 22 (1%), even after a significant period of follow-up.^[2] In the present case, even after extensive attempts to find the primary site, the site could not be determined. The final histopathology of this patient showed a mixed picture of squamous and transitional cell carcinoma, with the former predominating. A literature search revealed no such report of two different histological types of tumors, squamous and transitional, in the same patient at the

same site, from an unknown primary site, although there are reports of mixed squamous and adenocarcinomas.^[7]

The clinical investigative approach toward CUPS patients is mainly directed according to the histopathology, and every attempt should be made to obtain a good tissue sample for detailed IHC analysis. Investigations should involve a multi-modality approach. The role of PET scan is yet to be established but has the potential to modify the treatment in some patients whose tumor was localized with CT.^[8] As early as 1979, it was emphasized that the analysis of tissue samples should help to eliminate the need for undirected investigations screening for the primary site.^[9] Since then, there have been significant advances in the molecular analysis of tumors, and so the incidence of CUPS has decreased.^[10]

Since CUPS in the inguinal region is rare, there is a paucity of literature on the management of such patients, and no clear guidelines are described. The mainstay of treatment is surgery, with complete surgical excision through systematic lymph nodal dissection being mandatory. Aggressive surgical treatment including vascular resection and reconstruction with grafting may be required to achieve tumor-free margins, as was the situation in this case. Although role of postoperative radiotherapy is not clearly defined, it is thought that, in the presence of extensive nodal involvement and/or extranodal spread of tumor, postoperative radiotherapy should be used as it would be with any known primary site with squamous cell carcinoma. A review article indicates that surgery with adjuvant irradiation was the preferred treatment for inguinal metastasis with the unknown primary site.^[11]

A diligent follow-up is required for these patients. In one case report described an occult carcinoma of the penis manifested 3 years after treatment of inguinal nodal metastasis.^[12] According to the authors, circumcision and random biopsy of glans should be a routine of such patients. The patient in the present case was also followed up clinically, radiologically, and with cystoscopy in view of the presence of transitional cell carcinoma.

Carcinoma of unknown primary with inguinal metastasis is a rare entity. Investigations should be directed to identify the primary site according to

histopathology. Although there are no clear guidelines for the management of such patients, treatment should be multimodal, including aggressive surgical resection, and postoperative radiotherapy. The possible role of chemotherapy is unknown. A diligent follow-up is a must. In the future, molecular studies may increase our ability to distinguish subtypes of CUPS and treat them differentially.

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Orbital metastasis from anorectal carcinoma

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ABSTRACT

Pulmonary and liver metastases are common sites of distant metastasis from the rectal carcinoma. Metastases to the head and neck region are uncommon from carcinoma of the rectum, and orbital metastases are extremely rare. Here, we describe a 27-year-old female, who was diagnosed as a case of anorectal carcinoma in April 2010. She underwent abdominoperineal resection followed by concurrent chemoradiotherapy and adjuvant chemotherapy with 5 fluorouracil and leucovorin on follow-up. In January 2012, she presented with gradually increasing swelling over the left temporal region and left sided proptosis. Fine-needle aspiration and a cell block were performed. Metastasis was confirmed histologically. Palliative radiotherapy to the left orbit at the dose of 3 Gy per fraction 10 fractions to a total dose of 30 Gy was given by cobalt-60. In patients with a history of rectal carcinoma, recent onset proptosis with temporal swelling, although rare, should raise suspicion of metastatic deposit.

Key words: Anorectal carcinoma, distant metastasis, orbital metastasis

Introduction

Colorectal cancer is the third most common cancer with more than one million new cases each year worldwide. However, metastases from colorectal cancer to the orbit are exceedingly rare.^[1,2] We report here, in the first patient from the India with such a presentation. The reason for the rarity of colorectal metastases to the eye and orbit is not clear but may be related to anatomical barriers and routes of metastasis.

Case Report

A 27-year-old female initially presented in April 2010 with complaints of bleeding per rectum for 8 months, altered bowel habit and spurious diarrhea for 4 months. Rectal examination revealed a polypoidal mobile growth 3 cm from anal verge on the lateral and posterior wall of the rectum. Colonoscopy showed a friable circumferential growth in the rectum. Anorectal margin appeared to be involved by the tumor. Biopsy showed features consistent with adenocarcinoma, with surface ulceration. Contrast-enhanced computed tomography (CT) of the abdomen revealed an irregular wall thickening and enhancement involving the anorectal region with perifocal fat stranding and small volume (6 mm × 5 mm) lymph node in pelvis on left with involvement of

anorectal sphincter. Permanent sigmoid colostomy and abdominoperineal resection were done. Intraoperative findings were an ulceroproliferative, circumferential growth of 6 cm × 5 cm in the lower rectum, 4 cm from anal verge; there was no evidence of lymph node involvement and no ascites. Post-operative histopathology showed well-differentiated adenocarcinoma, extending into serosa, pT3, pN2 (7/11), 5 cm × 5 cm × 1 cm, 7.5 cm from proximal margin, 4 cm from distal margin, with foci of perineural invasion, and lymphovascular invasion. Carcinoembryonic antigen (CEA) was 32.5 ng/mL (normal 4-7 ng/mL). Post-operative adjuvant chemo-radiotherapy was given to the whole pelvis in anteroposterior and posteroanterior fields 2 Gy per fraction, 25 fractions to a total dose of 50 Gy by cobalt-60. During radiotherapy, 2 cycles of concurrent chemotherapy with 5 fluorouracil plus leucovorin were given on D1-D5 and D21-D25, followed by 4 cycles of adjuvant chemotherapy, with the last cycle given in November 2010. CEA (January 8, 2011) was 3.4 ng/mL. Twenty-one months later, she presented with swelling over the left temporal region and left eye proptosis [Figure 1]. On examination, there was a 5 cm × 3 cm × 4 cm swelling over the left temporal region, with ill-defined borders on palpation, firm-to-hard in consistency and with no signs of local inflammation. Asymmetrical proptosis of the left eye was noted. The vision was normal in both eyes. No focal neurological deficit was noted. CT scan of the skull, soft tissues, and brain showed a mixed density mass along the lateral wall of the left retro-orbital area, adherent to the optic nerve [Figure 2]. Fine-needle aspiration cytology and biopsy were suggestive of metastatic adenocarcinoma [Figure 3]. Bone scan showed increased uptake in the left orbital region, right sacroiliac joint

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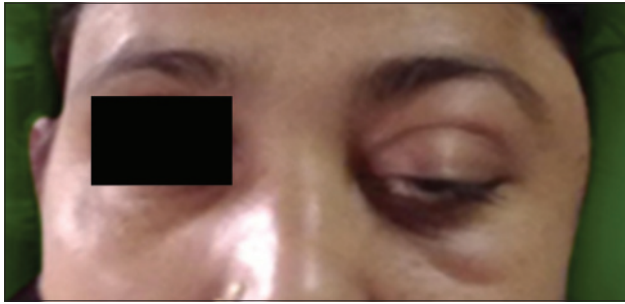


Figure 1: 5 cm × 3 cm × 4 cm swelling over left temporal region



Figure 2: Computed tomography scan showing a mixed density mass along lateral wall of left retro-orbital area, adherent to optic nerve

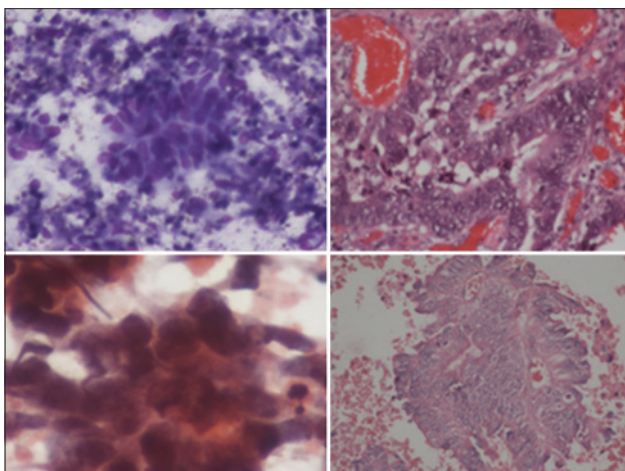


Figure 3: Morphology showing papillae and acinar pattern of columnar cells with moderate cytoplasm, oval nucleus, increased nuclear-cytoplasmic ratio

and second lumbar vertebral body, and suggestive of metastatic disease. Therefore, the patient was diagnosed with rectal carcinoma with multiple distant metastases. Palliative radiotherapy to the left orbit, lumbar spine, and right hemipelvis was given. The patient agreed to publish her pictures and signed the consent form.

Discussion

Rectal carcinoma in the young is increasing in incidence. This may be associated with familial adenomatous polyposis and hereditary non-polyposis colorectal cancer (Lynch) syndrome. Metastatic tumors to the orbit are rare and most commonly are from lung, breast, prostate, and kidney primaries. Only 5% are from the gastrointestinal tract.^[3,4] A review of the literature revealed only 6 cases reported of primary colorectal malignancy metastasizing to the orbit, with only three showing histopathology.^[5] When gastrointestinal cancers metastasize to the orbit, this is usually combined with multiple disseminated metastases.^[6] Management of metastatic orbital tumors requires a multidisciplinary team approach including radiotherapy, chemotherapy, and surgery.

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Spinal intradural mature teratoma in an elderly patient

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ABSTRACT

Spinal intradural mature teratomas are rarely encountered in adults. In this report, one of the oldest patients ever reported to harbor an intradural mature teratoma of the conus medullaris is presented, and the relevant literature concerning the teratoma's origin, clinical presentation, radiological features, and treatment modalities is reviewed. A previously healthy 70-year-old woman presented with a 2-month history of left sciatica. Her neurological examination was normal and the magnetic resonance imaging of the thoracolumbar spine showed an intradural, partially cystic mass extending from T12 to L3 level. The patient underwent a T11-L4 laminectomy. After opening the dura, a yellowish vascular tumor attached to the conus medullaris came into view. Meticulous dissection allowed for subtotal tumor removal. Only a thin part of the tumor wall, tightly attached to the conus medullaris, was left. The tumor was diagnosed as mature teratoma by histological study, and no adjunctive therapy was administered. The pain experienced by the patient disappeared postoperatively. Her condition remained unchanged with no radiological recurrence through the most recent follow-up examination, 3 years after surgery. The present study outlines that mature teratoma can arise from the conus medullaris, even in older adult patients. Functional preservation is of utmost importance, and long-term follow-up is mandatory to spot recurrences early.

Key words: Conus medullaris tumor, mature teratoma, surgery

Introduction

In 1863, an unprecedented finding, Rudolf Virchow described the “Krankenhaften Geschwülst,” an intraspinal “monstrous tumor” better known today as teratoma. One hundred and fifty years later, the origin, natural history, and occurrence of teratoma in elderly individuals are still subjects of debate. Spinal teratoma is, in fact, an extremely rare entity representing only 0.1-0.6% of all spinal tumors^[1] and mainly diagnosed during the first two decades of life. With only 31 described cases, adult forms are most uncommon. This report contributes to the understanding of these rare tumors by presenting an exceptional case of a conus medullaris mature teratoma in a 70-year-old woman and reviewing the relevant literature concerning its origin, clinical characteristics, radiological features, and treatment modalities.

Case Report

A previously healthy 70-year-old woman presented to our department in July 2011 with a 2-month history of left sciatica, with progressive aggravation. On admission,

her neurological examination was unremarkable and the physical examination including detailed clinical examination of the trunk and the extremities, did not find any patchy area of abnormal hair or dimple on her back. Anteroposterior and lateral radiographs were normal, and spinal magnetic resonance imaging confirmed the presence of a partially cystic and enhancing intradural lesion extending from T12 to L3 level [Figure 1].

A T12-L4 laminectomy was performed. The dura was opened in the midline, and a yellowish, vascular tumor came into view. The tumor originated from the conus medullaris and developed freely between the nerve roots. Fatty substance was present outside the tumor, and mucous substance, bony fragments, and hair were found inside the tumor.

Curettage of the cystic content was performed and progressive dissection allowed for a subtotal resection. A thin rim of the capsule that was tightly adherent to the conus medullaris was left. This was considered safer than performing a radical resection that would probably lead to a severe neurological deficit. The patient improved remarkably postoperatively. Her pain disappeared immediately. Histological examination showed a large number of glandular formations covered by mature, prismatic, and flattened cubic epithelial cells. These structures were embedded between fibrovascular tissue and mature fat tissue, which included some cystic formations. Three germ cell layers (including ectodermal, mesodermal, and endodermal elements)

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were observed, and the final histological diagnosis was mature teratoma [Figure 2]. No adjunctive therapy was administered. The patient has shown no clinical or radiological sign of progression through the most recent follow-up examination, 3 years after surgery [Figure 3].

Discussion

Teratoma is one of the rarest intraspinal neoplasms, representing 0.1-0.6% of all spinal tumors.^[1] It typically affects young individuals in their first or second decade of life and is frequently associated with spinal dysraphic defects. Adult cases are exceptional. Their rarity and the use of various terms to describe them led to limitations in understanding of the disorder and speculation about their true origin and natural history. We searched the PubMed and Medline databases for adult intradural mature teratomas and present here a comprehensive review of the literature concerning these rare tumors.

The total number of adult spinal intradural mature teratomas reported to date is 31. They are summarized in Table 1. The mean age at presentation was 36 years in men and 44 years in women, with a slight female predominance (sex ratio: 0.8). These lesions were predominantly located in the lower thoracic and thoracolumbar spine. Only five cases of conus medullaris mature teratoma were reported.^[14-16,26,27]

The review indicates that, in sharp contrast to pediatric cases, adult cases typically presented with subtle, nonspecific symptoms like back pain or radiculopathy. Furthermore, although these patients commonly experienced a certain degree of neurological disorder, motor weakness was not always obvious.^[30,31] Associated dysraphism, commonly seen in young patients, was found in only 6 adult patients (19%).^[3,5,13,14,21,22]

Radiologically, displaced pedicles, erosions, thinned laminae or calcifications on conventional X-rays are of significance and should be followed by a more extensive investigation.^[21] However, as demonstrated in this case, these findings are not always present. Computed tomography provides an optimal assessment of bony structures, but it is of limited interest for the exploration of the spinal cord and the extent of the tumor.

Magnetic resonance imaging is regarded as the gold standard imaging technique because it best delineates the tumor characteristics and depicts the degree of spinal cord involvement. The morphological presentation varies according to the location of the tumor. Intradural teratomas are commonly oval or lobulated, heterogeneous masses. Cystic components are usually found in adult cases, which can lead to confusion with more common epidermoid and dermoid cysts.^[17]

In the present case, a well-demarcated and strongly enhancing tumor was found, compounding the diagnostic challenges and highlighting the unspecific radiological aspect of spinal mature teratomas in

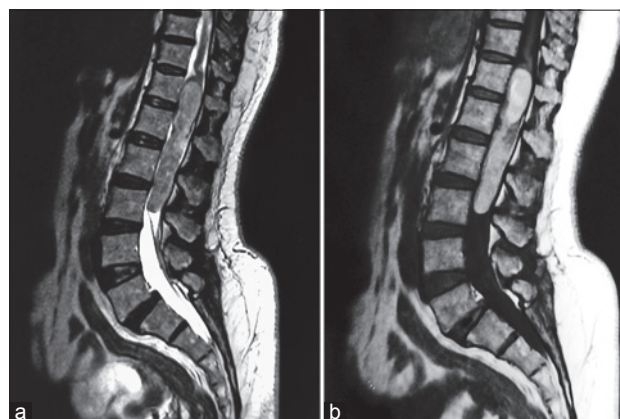


Figure 1: Sagittal T2-weighted (a) and T1-weighted post-gadolinium (b) magnetic resonance images showing a partially cystic and enhancing intradural mass located between T12 and L3 spinal levels

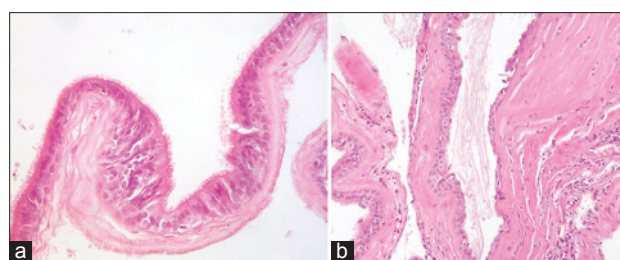


Figure 2: Photomicrographs of the tissue obtained intraoperatively. (a) A slightly disorganized cartilage surrounded by respiratory mucosa complete with bronchial glands and ciliated columnar epithelium (H and E, ×20); (b) cystic formations covered by multiple levels of keratinous squamous epithelial cells containing keratin lamellae (H and E, ×20). H and E: Hematoxylin and eosin stain



Figure 3: Sagittal T1-weighted post-gadolinium follow-up magnetic resonance imaging showing the stabilized tumor remnant at the conus medullaris level

elderly individuals. From the histological point of view, Russel and Rubinstein^[32] describe teratomas as tumors that contain a mixture of the three germinal layers of ectoderm, endoderm, and mesoderm. The review of the literature revealed that in a number of cases, only two of the three germinal layers were observable, perhaps because the derivatives of one or two of the layers may overgrow others.^[10,19] Spinal mature teratomas do not differ from extragonadal ones and are composed exclusively of fully differentiated “adult type” tissue

Table 1: Adult intradural spinal mature teratoma cases previously reported in the literature

Author, year	Age	Male/female	Spinal level	Associated dysraphism	Extent of surgery
Kubie and Fulton, ^[2] 1928	27	Female	C3-C4	No	Incomplete
Hosoi, ^[3] 1931	24	Male	L2-L3	L5-S1 spina bifida	Incomplete
Sullivan, ^[4] 1948	32	Female	L1-L3	No	Complete
Bakay, ^[5] 1956	65	Female	L1-L2	L1 and L2 vertebral body	Incomplete
Sloof <i>et al.</i> , ^[6] 1964	20	Male	L1	No	Complete
Rewcastle and Francoeur, ^[7] 1964	34	Female	T10	No	Incomplete
Hansebout and Bertrand, ^[8] 1965	47	Male	L1-L3	No	Complete
Eneström and Von Essen, ^[9] 1977	36	Male	T11-L1	No	Incomplete
Rosenbaum <i>et al.</i> , ^[10] 1978	49	Male	T9	No	Complete
Garrison and Kasdon, ^[11] 1980	23	Male	L2	No	Complete
Padovani <i>et al.</i> , ^[12] 1983	33	Female	T12-L1	No	Complete
Pelissou-Guyotat <i>et al.</i> , ^[13] 1988	33	Male	L4	L4 spina bifida occulta	Complete
Nicoletti <i>et al.</i> , ^[14] 1994	47	Male	Conus medullaris	Conus medullaris caudal exophy	Incomplete
Caruso <i>et al.</i> , ^[15] 1996	41	Male	Conus medullaris	No	Complete
al-Sarraj <i>et al.</i> , ^[16] 1998	35	Male	Conus medullaris	No	Incomplete
Poeze <i>et al.</i> , ^[17] 1999	23	Male	T12-L1	No	Incomplete
Fan <i>et al.</i> , ^[18] 2001	43	Female	L2	No	Complete
Nonomura <i>et al.</i> , ^[19] 2002	37	Female	T12-L1	No	Incomplete
	56	Male	T12-L2	No	Incomplete
Hejazi and Witzmann, ^[20] 2003	45	Female	T11-L3	No	Complete
	20	Male	L2-L4	No	Complete
Fernández-Cornejo <i>et al.</i> , ^[1] 2004	43	Male	L1-L2	No	Complete
Ak <i>et al.</i> , ^[21] 2006	43	Female	C2-C3	C3 spinal bifida, C5 level nodule	Complete
Makary <i>et al.</i> , ^[22] 2007	46	Female	C1-C2	C1-C2 dysraphic congenital spinal malformations	Complete
Biswas <i>et al.</i> , ^[23] 2009	28	Male	L2-L4	No	Complete
Ghostine <i>et al.</i> , ^[24] 2009	65	Female	C1-C2	No	Incomplete
Ijiri <i>et al.</i> , ^[25] 2009	68	Female	L1-L2	No	Complete
Jian <i>et al.</i> , ^[26] 2010	57	Male	Conus medullaris	No	Complete
Musil <i>et al.</i> , ^[27] 2011	60	Female	Conus medullaris	No	Incomplete
Li <i>et al.</i> , ^[28] 2013	23	Female	T12-L2	No	Complete
Vanguardia <i>et al.</i> , ^[29] 2014	41	Male	Cauda equina	No	Incomplete

elements. Because their capsule typically adheres to the spinal cord, radical removal carries a high risk of neurological deficits.

The pathogenesis of spinal intradural teratomas is still a subject of debate. The two widely held theories regarding the origin of intraspinal teratomas are the misplacement germ cell theory and the dysembryogenic theory.^[7,16] The traditional theory is the misplacement germ cell theory. It suggests that certain pluripotent primordial germ cells of the neural tube are misplaced during their migration to the gonadal ridges from the primitive yolk sac, resulting in spinal teratoma formation.^[7] In our situation, there is enough evidence to support the rationale of this theory, since we found that only 16% of adult cases were associated with spinal dysraphism^[16,21,26] and that the lower thoracic vertebrae and the conus medullaris region, which are adjacent to the caudal cell mass, represent the most common locations in adult population.

The alternative explanation comes from the dysembryogenic theory. This theory indicates that spinal teratomas arise from pluripotent cells which, in a locally

disturbed developmental environment like a primitive streak or a caudal cell mass, differentiate chaotically, and create spinal teratoma.^[33] The dysraphic malformations and the occurrence of a neurenteric cyst without dysraphism^[34] support this theory, and the tridermal anomaly, under such considerations, represents the primary event that will further affect the spinal closure.^[33]

The indications for surgery in an adult with intraspinal mature teratoma are controversial. Radical resection should be the goal in symptomatic cases with radicular pain and/or progressive signs due to mass effect or cord tethering.^[17] On the other hand, asymptomatic patients and those having longstanding minor and stable neurological deficits may be treated conservatively, because prophylactic surgery can be associated with a high surgical risk in adult patients with no growing or very slow-growing lesions.

Some authors advise the removal of the capsule as a potential source of regrowth.^[35] However, an epidemiological study guided by Allsopp *et al.*^[36] showed that recurrence rates for complete and gross resection

were extremely similar (9% and 11%, respectively). Many authors no longer recommend radical resection as a preferred management policy for all cases,^[19,20] since the rate of adherence to the cord reaches 50% in this region, and any attempt at total resection may result in inadvertent damage to the conus.^[10] In the present case, complete resection was not feasible without potential injury of the conus medullaris, so it was judged safer to realize a gross total resection in order to preserve nerve integrity.

Due to the extremely low incidence of adult mature spinal teratoma and limited knowledge of this disease, there is little evidence to support the use of adjunctive therapies.^[36] Radiation therapy is not justified since mature teratomas are benign tumors, and the efficacy of chemotherapy has not been demonstrated.^[36] Given the slow-growing nature of these tumors, patients will require serial clinical and radiological follow-up examinations for several years. Tumor markers such as B human chorionic hormone and alpha fetoprotein are of little interest in the follow-up of patients operated for mature intraspinal teratomas since a recurrence can take place from nonsecreting parts of the tumor.^[36]

In conclusion, mature intradural teratomas in adults are rare, with few accompanying spinal anomalies. Their location in the conus medullaris is exceptional and can present with sciatica. Although complete resection is the primary goal, subtotal removal can be considered as a safe and effective option to manage these lesions, with the understanding that a small number of these patients may need reoperation. Long-term follow-up is mandatory.

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Introduction to Volume 1 Issue 3 of *Journal of Cancer Metastasis and Treatment*

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It is a great honor for me to introduce the third issue of the *Journal of Cancer Metastasis and Treatment*. This is a special issue focusing on recent advances in research and treatment for gastrointestinal (GI) malignancies. Cancers arising from the GI tract, including esophagus, stomach, colorectum, liver, gallbladder and pancreas, are frequently observed all over the world. According to the global cancer statistics 2012, there were approximately 407,000 newly diagnosed cases with GI cancers and 304,000 deaths among them.^[1] They accounted for 29% and 37% of all cancers excluding non-melanoma skin cancer respectively. Recent progress in molecular biological techniques facilitated the understanding of the mechanism of cancer development and progression. This issue contains nine review articles concerning the topics which attract a lot of attention in the field of GI malignancies.

Epigenetic alterations regulate gene expression through mechanisms other than changes in the DNA sequence. DNA methylation abnormality, a major epigenetic process observed in many types of cancers, is characterized by global hypomethylation and site-specific CpG island hypermethylation. Shigaki *et al.* summarize the accumulated evidence for clinical application to use aberrant DNA methylation levels in GI cancers. MicroRNAs (miRNAs), which are small non-coding RNA molecules, also regulate gene expression at the post-transcriptional level and play important roles in modulating various biological processes. Some miRNAs act as onco-miR through attenuating the expression of tumor suppressor genes, while others act as tumor suppressor miR through suppressing the expression of oncogenes. Owing to the stability in plasma as well as formalin-fixed paraffin embedded samples, various miRNAs have been reported to be biomarkers in human cancers. Hiyoshi *et al.* document the utility of miRNAs as novel diagnostic/prognostic tools as well as therapeutic targets in GI cancers.

Adenocarcinoma of the esophagus or esophagogastric junction has dramatically increased in Western countries for several decades and recently an increasing trend is also observed in Asian countries. Although Barrett's esophagus is well known as a precursor of esophageal adenocarcinoma, the molecular mechanism has remained unclear until recently. Imamura *et al.* demonstrate

recent progress in the understanding of the molecular mechanisms of Barrett's esophagus and esophageal adenocarcinoma.

Chronic inflammation is known to induce carcinogenesis. Among GI cancers, adenocarcinoma arising from Barrett's esophagus, gastric cancer from chronic gastritis due to *Helicobacter pylori* infection, and colitic cancer from inflammatory bowel disease, are well known as tumors related to chronic inflammation. Ida *et al.* summarize molecular mechanisms that link chronic inflammation and GI cancers.

Cancer metastasis develops through multiple steps, including invasion, vascular permeation, circulation, arrest and extravasation, proliferation and angiogenesis. Epithelial-mesenchymal transition (EMT) is considered to be essential for tumor invasion and metastasis. Okabe *et al.* review the mechanisms of EMT as well as molecules, which play important roles during EMT in GI cancers. Recent advances in technology enabled not only to detect circulating tumor cells (CTCs) but also to elucidate the characteristics of CTCs. Iwatsuki *et al.* summarize the recent advances in methodology for detecting CTCs and discuss the implication of CTC analysis in clinical and translational research.

Cancer stem cells have the abilities for self-renewal and differentiation, and are responsible for cancer metastasis and chemoresistance. Recently, it has been revealed that cancer cells can change their characteristics reversibly from stem cells to non-stem cells, under the genetic and epigenetic regulations as well as the influence of microenvironmental factors. On the other hand, environmental factors such as chronic inflammation, obesity, metabolism and nutrition have been reported to influence carcinogenesis and the progression of colorectal cancer. Izumi *et al.* document how microenvironmental factors affect maintaining stem cell properties in colorectal cancer. In tumor cells, genetic mutations and tumor microenvironment, such as hypoxia, cause alterations in multiple signaling pathways and then the altered signals affect cellular metabolism. The most famous metabolic phenotype characteristics of cancer cells are the Warburg effect: ATP are generated through glycolysis instead of

oxidative phosphorylation, even under normoxic conditions. Aberrant metabolism in tumor cells can generate the abnormally high levels of reactive oxygen species (ROS), which induce senescence or apoptosis. To counter such oxidative stress, cancer cells exert tight regulation of ROS and antioxidants in such a way that the cell survives and the levels of ROS are reduced to moderate levels.^[2] Sawayama *et al.* review the molecular mechanism of cancer metabolism, and demonstrate possible therapeutic strategies targeting metabolism.

Molecular targeted drugs block the pathways specifically involved in tumorigenesis and the progression of cancers. Several targeted drugs have already been introduced into cancer treatment, and vascular endothelial growth factor and epithelial growth factor receptor are two major targets in the treatment for colorectal cancer. An antibody targets HER-2 has become a standard choice for gastric cancer with HER-2 overexpression/amplification. Currently, many candidate-targeted agents are under clinical or preclinical study. Eto *et al.* summarize trends in the clinical use of targeted drugs for GI cancers.

All of nine, first authors of review articles in this issue are young researchers who had started their research career at the Department of Gastroenterological Surgery, Graduate School of Medical Sciences, Kumamoto University. I would like to express my sincere appreciation to Prof. Hideo Baba and all members in his department for their guidance and support for this project. I am very happy if you enjoyed this special issue.

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Conflicts of interest

There are no conflicts of interest.

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Epigenetic changes in gastrointestinal cancers

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ABSTRACT

Epigenetic alterations, including DNA methylation, histone modification, loss of genome imprinting, chromatin remodeling and non-coding RNAs, are associated with human carcinogenesis. Among them, DNA methylation is a fundamental epigenetic process to modulate gene expression. In cancer cells, altered DNA methylation includes hypermethylation of site-specific CpG island promoter and global DNA hypo-methylation. Detection of aberrant gene promoter methylation has been applied to the clinic to stratify risk in cancer development, detect early cancer and predict clinical outcomes. Environmental factors associated with carcinogenesis are also significantly related to aberrant DNA methylation. Importantly, epigenetic changes, including altered DNA methylation, are reversible and thus, used as targets for cancer therapy or chemoprevention. An increasing number of recent studies reported DNA methylation level to be a useful biomarker for diagnosis, risk assessment and prognosis prediction for gastrointestinal (GI) cancers. This review summarized the accumulated evidence for clinical application to use aberrant DNA methylation levels in GI cancers, including colorectal, gastric and esophageal cancer.

Key words: Colorectal cancer, DNA methylation, epigenetic alterations, esophageal cancer, gastric cancer

Introduction

Epigenetics refers to heritable changes in gene expression that, unlike mutations, are not attributable to alterations in genomic DNA sequences. Epigenetic changes, such as DNA methylation, histone modifications, and altered expression of microRNAs, can regulate gene expression through mechanisms other than changes in genomic DNA sequence. Among them, genomic DNA methylation is a major epigenetic mechanism to mediate the X-chromosome inactivation, imprinting and repression of endogenous retroviruses.^[1-4] DNA methylation is the covalent post-replicative addition of a methyl group (-CH₃) to the 5-carbon of the cytosine ring in CpG dinucleotides. CpG dinucleotides are non-uniformly distributed throughout the human genome.^[2-4] Regions of the genome that are rich in sequences of a cytosine preceding a guanine (CpG dinucleotide) are known as CpG islands, which in particular, exist in the promoter regions of approximately half of all coding genes.

Altered DNA methylation in human cancers includes hypermethylation of site-specific CpG island promoter and global DNA hypo-methylation.^[1-4] DNA methylation in gene promoter CpG islands results

in its transcriptional inactivity and silence of protein expression. Thus, hypermethylation of a gene promoter is now recognized as a means of silencing tumor suppressor genes with effects similar to those of mutation or allelic loss in the development of cancer or other diseases.^[3] Another DNA methylation alteration in human cancer is genome-wide DNA hypo-methylation.^[5] Genome-wide DNA hypo-methylation appears to play an important role in genomic instability, leading to cancer development.^[6-8] Previous experimental studies demonstrated that DNA hypo-methylation of repetitive sequences, that is, short interspersed transposable elements (SINE or Alu elements) or long interspersed transposable elements (LINEs) may predispose cells to chromosomal defects and rearrangements, resulting in genetic instability.^[6] As *LINE-1* constitutes a substantial portion (approximately 17%) in the human genome, levels of *LINE-1* methylation are regarded to be surrogate markers for global DNA methylation.^[9] Thus, epigenetic regulation of gene expression has emerged as a fundamental way in pathogenesis of numerous malignancies, including cancers of the digestive system.

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In fact, many exciting discoveries in epigenetics have emerged from the study of gastrointestinal (GI) cancers. In this review, we summarized the accumulated evidence supporting the clinical application of DNA methylation level in diagnosis of esophageal, gastric and colorectal cancers.

Altered DNA Methylation in Esophageal Cancer

Esophageal cancer can be classified into two histological types, esophageal squamous cell carcinoma (ESCC) and esophageal adenocarcinoma (EAC). Their incidences vary notably by geographic distribution. ESCC accounts for approximately 90% of the esophageal cancers in East Asian countries,^[10,11] whereas the highest number of EAC is found in Northern and Western Europe, North America and Oceania.^[12] These two subtypes also have different epigenetic alterations. Growing evidence suggests that there is a field of epigenetic changes in esophageal cancer^[13-15] by particularly emphasized significance of promoter hypermethylation of 14 specific genes (*SFRP1*, *SFRP2*, *DCC*, *APC*, *p16*, *p14*, *APBA1*, *APBA2*, *APBA3*, *CACNA1G*, *PTGS2*, *DAPK1*, *MLH1* and *MGMT*) in non-cancerous mucosae from ESCC patients vs. mucosae from healthy volunteers,^[13] indicating that aberrant methylation or these 14 gene promoters in esophageal mucosae is associated with ESCC development. An overview of different previous studies of clinical implications of DNA methylation in esophageal cancer is provided in Table 1. Aberrant promoter methylation of tumor suppressor genes has also been used to predict clinical outcomes following curative ESCC resections. For example, promoter methylation of *APC* has been associated with reduced survival of ESCC patients after

esophagectomy.^[16] Ling *et al.*^[17] showed that *MSH2* promoter hypermethylation in circulating tumor DNA was a valuable predictor of disease-free survival of ESCC patients after esophagectomy. Aberrant methylation of *FHIT* was also reported to be associated with exposure to tobacco smoking and individuals with early-stage ESCC whose tumors exhibited *FHIT* hypermethylation had poor prognoses.^[18] *CDH1* hypermethylation was detected in 14-61% of ESCC, which was associated with recurrence of early-stage ESCC.^[19] Moreover, aberrantly methylated gene promoters were also detected in plasma or sera of ESCC patients. Hibi *et al.*^[20] showed that *p16* promoter methylation in ESCC specimens had this same methylation change in their serum DNA in 23% the of patients, which implied that detection of serum DNA *p16* promoter methylation could serve as a tumor marker. However, few studies have addressed or detected DNA hypo-methylation in ESCC. *LINE-1* methylation is regarded as a surrogate marker for global DNA methylation. To better understand DNA methylation in ESCC tissues, our group measured their *LINE-1* methylation using the pyrosequencing technology. Chronic tobacco smoking and heavy alcohol drinking are established as risk factors for ESCC development.^[21-25] *LINE-1* hypo-methylation is significantly associated with tobacco smoking, which supports its plausibility as a surrogate marker for an epigenetic field defect.^[26] *LINE-1* methylation is highly variable among ESCC specimens (25-92%) and its hypo-methylation is strongly associated with poor ESCC prognosis.^[27] Moreover, loss of insulin-like growth factor 2 (*IGF2*) imprinting has been found in ESCC and loss of *IGF2* methylation is associated with shorter survival of patients.^[28]

Table 1: Association of gene promoter methylation with clinical outcomes of esophageal cancer patients

Gene	Histological type	Correlation with clinical outcomes	Reference
DNA hypermethylation			
<i>APC</i>	ESCC	Associated with poor prognosis	[16]
<i>CDH1</i>	ESCC	Associated with poor prognosis	[19]
<i>p16</i>	ESCC	Associated with poor prognosis, serum promoter methylation	[20,94]
<i>Claudin-4</i>	ESCC	Associated with poor prognosis	[95]
<i>FHIT</i>	ESCC	Associated with poor prognosis and tobacco/alcohol consumption	[18,96]
<i>Integrin α4</i>	ESCC	Associated with poor prognosis	[19]
<i>MGMT</i>	ESCC	Association with lymph node metastasis	[97]
<i>MSH2</i>	ESCC	Associated with poor prognosis	[17,98]
<i>AKAP12</i>	Barrett/BAC	Progression prediction in Barrett's esophagus	[31]
<i>CDH13</i>	Barrett/BAC	Progression prediction in Barrett's esophagus	[31]
<i>p16</i>	Barrett/BAC	Progression prediction in Barrett's esophagus	[31,99]
<i>HPP1</i>	Barrett/BAC	Progression prediction in Barrett's esophagus	[31,99]
<i>NELL1</i>	Barrett/BAC	Progression prediction in Barrett's esophagus	[31]
<i>RUNX3</i>	Barrett/BAC	Progression prediction in Barrett's esophagus	[31,99]
<i>SST</i>	Barrett/BAC	Progression prediction in Barrett's esophagus	[31]
<i>TAC1</i>	Barrett/BAC	Progression prediction in Barrett's esophagus	[31]
DNA hypomethylation			
<i>IGF2</i>	ESCC	Associated with poor prognosis	[28]
<i>LINE-1</i>	ESCC	Associated with poor prognosis and tobacco consumption	[26,27]

ESCC: Esophageal squamous cell carcinoma; Barrett/BAC: Barrett's esophageal adenocarcinoma

In EAC, methylation patterns of promoter CpG islands in several genes, such as tumor suppressor genes (*APC*, *TIMP3*, *SFRP1*, *SFRP2*, *WIF1*, *AKAP12*, *RUNX3*, *SOC1* and *SOC3*) and DNA repair genes (*MGMT*), have been reported previously.^[29] In Barrett's esophagus, a pre-malignant condition that can lead to EAC development, aberrant DNA methylation has also been shown to occur in promoters of tumor suppressor genes, adhesion molecules and DNA repair genes (*AKAP12*, *APC*, *CDH13*, *DAPK1*, *GPX*, *GST*, *MGMT*, *NELL1*, *REPRIMO/RPRM*, *p16*, *SFRP*, *SOC3*, *SST*, *TAC1*, *TIMP3* and *WIF1*).^[30] Jin *et al.* reported that promoter hypermethylation of eight genes (*p16*, *RUNX3*, *HPPI*, *NELL1*, *TAC1*, *SST*, *AKAP12* and *CDH13*) could predict neoplastic progression risk in Barrett's esophagus.^[31] However, in the study of DNA hypo-methylation in Barrett's EAC (BAC), Alvarez *et al.* reported a predominance of DNA hypo-methylation rather than DNA hyper-methylation in early-stage of BAC carcinogenesis. They also detected DNA hypo-methylation in a series of genes associated with the immune system such as chemokines (*CXCL1* and *CXCL3*).^[32]

Altered DNA Methylation in Gastric Cancer

Gastric cancer is the fourth most frequently diagnosed cancer and the second leading cause of cancer-related deaths in the world.^[33] Gastric adenocarcinoma accounts for 90-95% of gastric cancer and has two histological subtypes (intestinal and diffuse) based on microscopic observation and tumor growth patterns, which differ

widely in molecular pathogenesis.^[34] Nonetheless, epigenetic alterations play important roles in the development of both gastric carcinoma types. Gene promoter methylation has been reported to associate with gastric cancer development, such as *CDKN2A*, *CDK2AP2*, *CDH1*, *MGMT*, *RASSF1*, *RUNX3*, *DLC1*, *ITGA4*, *ZIC1*, *PRDM5*, *PCDH10*, *TFPI2*, *RUNX3*, *SPINT2*, *BTG4*, *SFRP2*, *hMLH1*, *DKK-3*, *TCF4*, *GRIK2*, *RAR*, *CHFR*, *BNIP3*, *RASSF1A*, *LRP1B* and *SFRP5*, promoter of which was more frequently methylated in gastric cancer tissues than those of the corresponding normal gastric tissue.^[35,36] Furthermore, promoter methylation of many genes with different biological functions has been associated with the clinicopathological characteristics and prognosis of gastric cancer [Table 2].^[37] Of these genes, promoter hypermethylation of *CDH1*^[38] and *MGMT*^[39,40] was associated with worse outcomes of gastric cancer patients after surgery. However, patients with hypermethylated *IGF2* in blood leukocyte DNA reportedly had a significantly better survival rate than those with hypo-methylated *IGF2*.^[41] Additionally, DNA methylation of detected in body fluids that can be obtained non-invasively, such as serum and gastric washes, may have a clinical application for gastric cancer; for example, detection of aberrant DNA methylation of *CDH1*, *DAPK*, *GSTP1*, *p15*, *p16*, *RARβ*, *RASSF1A*, *RUNX3* and *TFPI2* in serum may be a useful biomarker for gastric cancer.^[42]

Environmental factors also significantly affect DNA methylation. Etiological studies have closely associated two distinct infectious agents, *Helicobacter*

Table 2: Association of gene promoter methylation with clinical outcomes of gastric cancer

Gene	Correlation with clinical outcomes	References
DNA hypermethylation		
<i>BNIP3</i>	Association with poor prognosis	[100,101]
<i>CACNA2D3</i>	Correlation with lymph node metastasis	[102]
<i>CDH1</i>	Association with poor prognosis, <i>H. pylori</i> infection, and EBV infection	[38,46,49-51]
<i>DAPK</i>	Correlation with cell differentiation, lymph node metastasis	[100,103]
<i>FLNc</i>	Association with poor prognosis	[104]
<i>GPX3</i>	Correlation with lymph node metastasis	[105,106]
<i>HAI-2/SPINT2</i>	Correlation with cell differentiation, lymph node metastasis	[107]
<i>HoxD10</i>	Association with poor prognosis	[108]
<i>LOX</i>	Association with poor prognosis and <i>H. pylori</i> infection	[45]
<i>MGMT</i>	Association with poor prognosis	[103,104,109]
<i>MLH1</i>	Association with poor prognosis	[104]
<i>p15</i>	Association with EBV infection	[49-51]
<i>p16</i>	Association with poor prognosis, <i>H. pylori</i> infection and EBV infection	[38,46,49-51,102,104]
<i>p73</i>	Association with EBV infection	[52]
<i>PAX6</i>	Association with poor prognosis	[100]
<i>RASSF1A</i>	Association with poor prognosis	[100,103]
<i>RASSF2</i>	Association with poor prognosis	[104]
<i>RUNX3</i>	Correlation with TNM stage and <i>H. pylori</i> infection	[110,111]
DNA hypomethylation		
<i>LINE-1</i>	Association with poor prognosis and <i>H. pylori</i> infection	[55,56]
<i>SURF</i>	Association with poor prognosis	[57]

H. pylori: *Helicobacter pylori*; EBV: Epstein-Barr virus

pylori and Epstein-Barr virus (EBV) with gastric carcinogenesis.^[43,44] Previous prospective studies showed that *H. pylori* infection had an essential role in gastric carcinogenesis^[43] and the mechanisms, underlying gastric carcinogenesis due to *H. pylori*-induced DNA methylation, had been indicated. *H. pylori* infection induced aberrant promoter methylation in tumor-suppressor genes, such as *RUNX3*, *p16*, *LOX* and *CDH1*.^[45,46] Furthermore, *IL-1β* is thought to be especially significant as a specific single-nucleotide polymorphism of *IL-1β* in association with increases in both gastric cancer risk and incidence.^[47,48] EBV infection occurs at a very early-stage in cancer development and plays an important role in gastric carcinogenesis. Aberrant methylation of tumor suppressor genes, such as *CDH1*, *p15*, *p16* and *p73*, is frequently observed in EBV-associated gastric cancer but is less frequently detected in adjacent non-neoplastic mucosa,^[49-52] which suggests that aberrant methylation is a critical mechanism of EBV-related gastric tumorigenesis. Regarding the molecular mechanisms underlying host DNA methylation during early-stage EBV infection in gastric epithelium, *LMP2A* expression was upregulated through STAT3 phosphorylation, which further induced DNA methyltransferases during EBV infection.^[53]

However, few studies addressed or detected DNA hypo-methylation in gastric cancer. In gastric cancer, global genomic hypo-methylation has been found in premalignant stages of the disease.^[54] In our previous study that assessed 203 resected gastric cancer specimens, we found gastric cancer tissues had significantly lower *LINE-1* methylation levels than that of their matched normal gastric mucosa. *LINE-1* hypo-methylation in gastric cancer was also associated with shorter survival of patients.^[55] Moreover, *LINE-1* hypo-methylation of non-cancerous gastric mucosae in gastric cancer patients significantly correlated with *H. pylori* infection.^[56] Hur *et al.* reported that gastric cancer tissues had conspicuously higher expression of *SULF1* regulated by promoter hypo-methylation than that of the normal mucosa. *SULF1* is also an independent prognostic factor, and LN is a metastasis predictive factor in gastric cancer patients.^[57]

Altered DNA Methylation in Colorectal Cancer

Aberrant DNA methylation was reported as an important hallmark of colorectal cancer. Colorectal cancer is a heterogeneous disease and molecularly, it can be classified into three major molecular subtypes, that is, microsatellite instability (MSI), chromosomal instability and CpG island methylator phenotype (CIMP).^[58] In 1999, Baylin and Issa *et al.* coined the term “CpG island methylator phenotype” or CIMP, in which promoter of tumor suppressor genes was methylated to contribute to tumorigenesis at least in theory through progressive genetic silence, possibly even in the absence of any genetic mutations.^[59] According to epigenetic and clinical

profiles, primary colorectal cancer is divided into three distinct subclasses, that is, CIMP1, CIMP2 and CIMP-negative. CIMP1 tumor often shows mutations of MSI (80%) and *BRAF* (53%) while CIMP2 tumor often shows *K-RAS* mutation (92%) but rarely shows MSI or *BRAF* or *TP53* mutations. Non-CIMP tumor has a high frequency of *TP53* mutations (71%).^[60] CIMP1 has a favorable prognosis, whereas CIMP2 is associated with poor prognosis.^[60] Cancer CIMP status has been assessed as a predictive marker for 5-FU responsiveness.^[61]

Colorectal cancer with CIMP is distinct from those with chromosomal instability, and there are two forms of nuclear derangement represented alternative pathways for colorectal cancer development,^[62,63] which overlap somewhat as hypermethylation can occur in *APC* and is part of the chromosomal instability pathway,^[64] or in the *MLH1* gene, triggering MSI.^[65] *MLH1* accounts for approximately 40% of the cases of the hereditary colorectal cancer and Lynch syndrome.^[66] Detection of *MLH1* methylation is currently used to discriminate between sporadic colorectal cancer with MSI and familial forms (Lynch syndrome).^[67] Methylation of *MGMT* promoter also occurs during colorectal cancer progression in either pathway and may facilitate the accumulation of point mutations as tumors evolve.^[65]

The CpG island methylation affects a number of genes in colon cancer, and significance of these epigenetic alterations in colon cancer pathogenesis has been widely reported.^[68,69] Hundreds of gene promoters have been found to be aberrantly methylated in the average colorectal cancer genome and their number is ever-growing, including genes of the Wnt signaling pathway such as *APC*, *AXIN2*, *DKK1*, *SFRP1*, *SFRP2* and *WNT5A*, the DNA repair genes *MGMT*, *hMLH1* and *hMLH2*, cell cycle-related genes such as *p14*, *p15* and *p16*, RAS signaling genes *RASSF1A* and *RASSF1B* and many more.^[70,71]

Several DNA methylation markers have been proposed as useful early biomarkers for colorectal cancer early detection and prediction of prognosis. For instance, methylation of *MLH1* can be detected in colorectal cancer tissue samples^[72] or blood^[73] to help interpret MSI because its presence helps to exclude diagnosis of Lynch syndrome. The presence of aberrantly methylated *SEPT9* (which encodes a GTPase that is involved in dysfunctional cytoskeletal organization) in plasma is a valuable and minimally invasive blood-based polymerase chain reaction test with a sensitivity of almost 70% and a specificity of 90% in colorectal cancer detection.^[74-78] In fact, an assay that detects hypermethylated *SEPT9* is now being commercialized and offered in some parts of Europe to screen colorectal cancer. Moreover, detection of aberrant methylation of vimentin in fecal DNA was reported in colorectal cancer when compared with normal control;^[79] the sensitivity and specificity of methylated vimentin for colorectal cancer were 88%

and 87%, respectively.^[80] Kamimae *et al.* have recently shown that detection of DNA methylation in mucosal wash fluid from patients undergoing colonoscopy may be a good molecular marker for predicting invasiveness of colorectal tumors.^[81]

Promoter hypermethylation of *MLH1*, *MGMT* and *HIC1* can be detrimental and lead to cancer progression.^[82-85] Seven additional genes (*TIMP3*, *CXCL12*, *ID4*, *IRF8*, *CHFR*, *IGFBP3* and *CD109*) were frequently methylated in late-stage colorectal cancer and could have a role in colorectal cancer progression and metastasis.^[71,86,87] Yi *et al.* observed that colorectal cancers that have silenced (methylated) genes in the extracellular matrix-remodeling pathway, such as *IGFBP3*, *EVL*, *CD109* and *FLNC*, showed worse survival, suggesting that methylation of this pathway-related genes might represent a prognostic signature for colorectal cancer patients.^[87] Moreover, hypo-methylation of the IGF2 differentially methylated region in colorectal tumors was

associated with poor prognosis.^[88] However, all of these possible markers need to be further validated before they are used clinically.

Global hypo-methylation may influence tumor progression by making chromosomes more susceptible to breakage and causing disruption of normal gene structure and function, leading to reactivating previously silenced retrotransposons.^[89-91] Most recent research on *LINE-1* methylation levels in GI cancers has focused on colorectal cancer; Ogino *et al.* reported *LINE-1* methylation levels widely occurred and approximately normally distributed (range: 23.1-90.3%) in a cohort of 869 colorectal cancer patients.^[92] *LINE-1* hypo-methylation was inversely associated to the MSI and CIMP;^[92,93] these findings suggest that CIMP/MSI and genomic hypo-methylation represent different pathways in colorectal cancer development. A summary of reported gene methylation in stool, blood and tissue samples of patients with colorectal cancer is shown in Tables 3 and 4.

Table 3: Association of gene promoter methylation with diagnosis of colorectal cancer

Gene	Specimen type	Correlation with clinical outcomes	References
DNA hypermethylation		Diagnosis	
<i>AGTR1</i>	Stool	Diagnosis of CRC	[112]
<i>ALX4</i>	Blood	Diagnosis of colorectal adenomas and cancers	[113]
<i>APC</i>	Blood	Diagnosis of CRC	[114]
<i>BMP3</i>	Stool	Diagnosis of colorectal adenomas and cancers	[115]
<i>BMP3</i>	Tissue	Diagnosis of colorectal adenomas and cancers	[112]
<i>CNIP1</i>	Stool	Diagnosis of CRC	[116]
<i>DAPK</i>	Blood	Diagnosis of CRC	[117]
<i>FBN1</i>	Stool	Diagnosis of CRC	[116]
<i>GATA-5</i>	Stool	Diagnosis of CRC	[118]
<i>IGFBP7</i>	Cells	Diagnosis of CRC	[119]
<i>INA</i>	Stool	Diagnosis of CRC	[116]
<i>MAL</i>	Stool	Diagnosis of CRC	[116]
<i>MGMT</i>	Blood	Diagnosis of CRC	[114]
<i>MLH1</i>	Blood, cells	Diagnosis of sporadic MSI CRC	[73]
<i>NDRG4</i>	Stool	Diagnosis of CRC	[120]
<i>NDRG4</i>	Stool	Diagnosis of colorectal adenomas and cancers	[115]
<i>NEUROG1</i>	Blood	Diagnosis of CRC	[121]
<i>NGFR</i>	Blood	Diagnosis of CRC	[74]
<i>p16</i>	Blood	Diagnosis of CRC	[122]
<i>RASSF2</i>	Stool	Diagnosis of CRC, distinction from gastric cancer	[123]
<i>RASSF2A</i>	Blood	Diagnosis of CRC	[114]
<i>RUNX3</i>	Blood	Diagnosis of CRC	[124]
<i>SDC2</i>	Blood	Diagnosis of CRC	[125]
<i>SEPT9</i>	Blood	Diagnosis of CRC	[74,75]
<i>SFRP2</i>	Stool, blood, tissue	Diagnosis of CRC, distinction from gastric cancer	[123]
<i>SLIT2</i>	Stool	Diagnosis of CRC	[112]
<i>SNCA</i>	Stool	Diagnosis of CRC	[116]
<i>SPG20</i>	Stool	Diagnosis of CRC	[116]
<i>TFPI2</i>	Stool	Diagnosis of colorectal adenomas and cancers	[115]
<i>TMEFF2</i>	Blood	Diagnosis of CRC	[74]
<i>Vimentin</i>	Stool, blood	Diagnosis of colorectal adenomas and cancers	[126]
<i>WIF1</i>	Blood	Diagnosis of CRC	[114]
<i>WNT1</i>	Stool	Diagnosis of CRC	[112]

CRC: Colorectal cancer; MSI: Microsatellite instability

Table 4: Association of gene promoter methylation with prognosis of colorectal cancer

Gene	Specimen type	Correlation with clinical outcomes	References
DNA hypermethylation		Prognosis	
<i>APC</i>	Tissue	Associated with poor prognosis	[127]
<i>CD109</i>	Tissue	Associated with poor prognosis	[87]
<i>EVL</i>	Tissue	Associated with poor prognosis	[87]
<i>FLNC</i>	Tissue	Associated with poor prognosis	[87]
<i>HLTF</i>	Blood	Associated with poor prognosis	[128]
<i>HOPX-β</i>	Tissue	Worse prognosis of stage III CRC	[129]
<i>HPP1</i>	Blood	Associated with poor prognosis	[128]
<i>IGFBP3</i>	Tissue	Associated with poor prognosis	[87]
<i>MLH1</i>	Blood	Associated with favorable prognosis	[130]
<i>p16</i>	Tissue	Associated with poor prognosis	[127]
<i>RASSF2A</i>	Tissue	Associated with poor prognosis	[131]
<i>TFPI2</i>	Blood	Associated with poor prognosis	[132]
DNA hypomethylation		Prognosis	
<i>IGF2</i>	Tissue	Associated with prognosis	[88]
<i>LINE-1</i>	Tissue	Associated with worse OS	[133]

CRC: Colorectal cancer; OS: Overall survival

Conclusion

In this review, we have summarized the main epigenetic alterations in GI cancer-global DNA hypo-methylation and site-specific CpG island promoter hypermethylation with clinical characteristics in patients with GI cancers. Epigenetic signatures have a potential usefulness in early diagnosis, screening, monitoring and prediction of prognoses or therapy responses for GI cancer patients. Further investigation in this field would increase our knowledge of epigenetic alterations of GI cancer and help to develop novel therapeutic strategies for GI cancers.

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Conflicts of interest

There are no conflicts of interest.

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Overview of genetic and epigenetic alterations in the pathogenesis of esophagogastric junctional adenocarcinoma and esophageal adenocarcinoma: recent findings by next generation sequencing

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ABSTRACT

Esophagogastric junctional adenocarcinoma is commonly treated as esophageal adenocarcinoma (EAC) and has dramatically increased in Western countries for several decades. The similar trend has been observed in Asian countries (not in China). Barrett's esophagus (BE) is a widely accepted precursor of EAC. Recent advances of next-generation sequencing could provide researchers with a better understanding of genetic and epigenetic alterations in the carcinogenesis of EAC. In this review, we have summarized the recently reported major genetic and epigenetic alterations in both BE and EAC. Sonic hedgehog/bone morphogenetic protein axis, which is a key signaling for esophageal development, plays an important role in BE intestinal metaplasia. Single nucleotide polymorphisms related to esophageal organogenesis, such as *FOXF1* and *FOXP3*, are frequently detected in BE patients. During the progression of BE to adenocarcinoma, lacking of normal function of TP53 and CDKN2A by loss of heterozygosity (LOH), mutation, or promoter methylation has been frequently observed. LOH at 9p (coding *CDKN2A*) is an earlier event to EAC carcinogenesis compared to that at 17q (coding *TP53*) LOH. In order to further elucidate the pathogenesis of BE and EAC, it will be necessary to analyze these genetic/epigenetic alterations in combination with clinical data in a large-scale cohort.

Key words: Barrett's esophagus, carcinogenesis, epigenetic, esophageal adenocarcinoma, esophagogastric junctional adenocarcinoma, genetic, intestinal metaplasia

Introduction

Esophagogastric junctional (EGJ) adenocarcinoma is classified as I to III, based on the location of the tumor center or tumor mass, by Rudiger Siewert *et al.*^[1] EGJ cancer is considered to be an esophageal cancer, according to the 7th edition of Union for International Cancer Control tumour, node, metastasis classification.^[2] EGJ adenocarcinoma/esophageal adenocarcinoma (EAC) has dramatically increased by 600%, mainly in Western countries, over the past few decades, although the current incidence rate shows only a moderate increase.^[3] Currently, a similar trend was reported in Asian country.^[4] EGJ adenocarcinoma often presents at a late stages despite recent improvements in diagnostic technology and multidisciplinary treatment. The 5-year survival rate is reported to be about 20% and median survival less than one year.^[3,5]

Barrett's esophagus (BE) is a widely accepted precursor of EGJ adenocarcinoma/EAC, although the reported risk

is around 0.5% per year.^[6] Epidemiological studies have revealed that adenocarcinomas occur from BE through multistep morphological changes, such as low-grade to high-grade dysplasia.^[6,7] BE and EGJ adenocarcinoma/EAC share poly-genetic/epigenetic alterations.^[8] BE can be described as mucosal replacement of normal squamous epithelium with metaplastic columnar mucosa, known as specialized columnar metaplasia, in response to chronic gastroesophageal reflux disease (GERD).^[9] Understanding the pathogenesis of BE and EGJ adenocarcinoma/EAC is important in prevention and thus the development of molecular targeting therapy. Here, we review the pathogenesis of EGJ adenocarcinoma/EAC, including BE, focusing on molecular alterations. We use the term EAC and include EGJ adenocarcinoma.

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Barrett's Esophagus

BE is defined by American Gastroenterological Association as “BE is the condition in which any extent of metaplastic columnar epithelium that predisposes to cancer development replaces the stratified squamous epithelium that normally lines the distal esophagus.”^[10] This means a specialized columnar epithelium characterized by columnar cells, goblet cells, and a villous-like structure.^[11,12] However, another classification includes two types of BE. One is “junctional or cardiac type,” consisting of the predominantly foveolar surface containing mucous glands and resembling cardiac mucous glands. Another one is “gastric-fundic type,” containing both parietal and chief cells with atrophic fundic glands.^[11-13] Thus, the histological definition of BE remains controversial.

The cell of origin of BE has not yet been elucidated. Six cell types are currently considered as potential origins, including transdifferentiation of esophageal squamous cells,^[14] gastric cardia cells,^[15] esophageal submucosal gland cells,^[16] esophageal progenitor cells,^[17] circulating bone marrow cells,^[18] and residual embryonic cells at squamo-columnar junction (SCJ).^[19]

There are some reports suggesting an association between p63 and intestinal metaplasia. *p63* null embryos have idiopathic metaplasia in SCJ.^[20] It has been shown that genetic alterations in metaplastic cells in mice lacking p63 were similar to those in human BE.^[21] It has also been suggested that epithelium with such genetic changes may originally exist at SCJ. Also, lack of SRY (sex determining region Y) box 2 (SOX2) induces columnar changes in esophageal epithelium in mice models.^[22] Both p63 and SOX2 are essential for squamous epithelial formation during organogenesis. Although these findings were based on studies using rodent esophagus, there are structural differences in the esophageal between rodents and human. For example, in rodents, the esophagus lacks submucosal glands and SCJ is located in mid-stomach. Therefore, findings in rodent models may not be applicable to human BE.

Molecular and Genetic Alterations Related to Intestinal Metaplasia and Intestinal Differentiation

Sonic hedgehog (SHH)/bone morphogenetic protein (BMP) signaling plays an important role in the development of columnar metaplasia, being associated with organogenesis, especially of the esophageal. These are critical molecules for separating trachea from the esophagus^[23] and are involved in the development of cell-renewable epithelium.^[24] Expressions of SHH and BMP4 are usually low in human squamous epithelia. In BE tissue, however, SHH/BMP4 signaling induces SRY (sex determining region Y) box 9 (SOX9).^[25,26] SOX9 subsequently induces CDX2 and MUC2 expression,

which are related to an intestinal phenotype.^[27] Furthermore, BMP4 shifts the gene expression profile of normal squamous cells into columnar cells. Because cytokeratin (CK) is a major cytoskeleton molecule, it can be regarded as a representative phenotype of certain cells. CK 13/14 expressions are highly expressed in squamous cells, whereas CK 7, 8, 18, and 20 expressions elevated in BE epithelium.^[28] It has been shown that expression of SOX9, but not CDX2 or BMP4, induces squamous epithelial cells formation toward columnar-like epithelium with expression of CK 8.^[29] SHH/BMP signaling were also activated in a mouse model with interleukin-1 β overexpression. After one year of continuous inflammation, intestinal metaplasia occurred at the SCJ, and the gene expression pattern of those metaplastic cells was similar to those in human BE.^[30]

Recent advances of next-generation sequencing have provided the opportunity to elucidate genetic alterations such as single nucleotide polymorphisms (SNPs). The association between SNPs and BE has been clarified. It has been reported that chromosomes 2p24 (rs3072), 12q24 (rs2701108), 6p21 (rs9257809), and 16q24 (rs9936833) are related to risk of BE development.^[31,32] Among these SNPs, rs9936833 at 16q24 is located close to *FOXF1*, which is a transcription factor in the SHH signaling pathway. Interestingly, *FOXF1* is associated with embryonic development of gastrointestinal tract formation, especially the esophagus.^[33] Also, the importance of *FOXP3*, at 3p14 (rs2687201), which is also known to possess a role in esophageal organogenesis, is based on analyzing datasets of BE or EAC cases.^[34] 19p13 (rs10419226) and 9p22 (rs11789015), with significant relation to BE and EAC, has also been identified. rs10419226 SNPs at 19p13 are known as an intronic variant of cAMP-regulated transcriptional co-activators (CRTC1). CRTC signaling exerts oncogenic activities when activated by loss of LKB1 through transcriptional activation of LYPD3, which contributes to esophageal tumor progression.^[35] rs11789015 SNP at 9p22 is located at the intron region of *BARX1*. *BARX1* is a transcription factor involved in tracheal and foregut organogenesis in developing mouse embryos.^[36,37] These findings suggest that key molecules in BE development may overlap with those in esophageal development.

Wnt/ β -catenin, and Notch are critical signaling for intestinal differentiation. Wnt family is one of the fundamental mechanisms of cell proliferation, polarity, and differentiation.^[38] Wnt signaling pathways include Wnt/ β -catenin canonical pathway and Wnt/calcium or Wnt/planar cell polarity non-canonical pathway. Among these, Wnt/ β -catenin pathway is associated with intestinal type gene expressions.^[39,40] Wnt signaling also regulates *CDX* gene expression, which controls intestinal differentiation, and homeostasis.^[41] Notch signaling

also plays an important role in intestinal differentiation in cell proliferation, apoptosis, and normal cell differentiation.^[42,43]

SHH, BMP4, SOX9, and CDX2 are key molecules for the development of intestinal metaplasia. SHH/BMP4 axis, which is a key signaling for esophageal development, plays an important role in the intestinal metaplasia of BE. In addition, SNPs that are related to esophageal organogenesis, such as *FOXF1* and *FOXP3*, are frequently observed in BE patients [Table 1].

Genetic Alterations in Progression of BE to EAC

Few cases of BE will develop high-grade dysplasia or adenocarcinoma. The widely accepted molecular events during progression of BE to adenocarcinoma are loss of normal TP53 and CDKN2A function. Mechanisms underlying this have been explained by loss of heterozygosity (LOH), mutation, or promoter methylation. Tumor suppressor genes, *TP53* and *CDKN2A*, are located at 17p and 9p, respectively.^[44] 17p LOH occurs frequently in EAC,^[45-47] while *TP53* mutation possesses malignant transformation potential during EAC carcinogenesis.^[48] 9p LOH has been reported to be the important factor driving to EAC.^[44] Somatic mutation of *CDKN2A* has also been detected in EAC cases.^[49] In addition, tumors harboring promoter methylation in *CDKN2A* showed a higher risk of EAC progression.^[50,51] Although 9p LOH is an earlier event during EAC carcinogenesis compared to 17q LOH, patients with BE harboring 9p LOH experienced much higher incidence of EAC compared to those with 17q LOH.^[44]

Comprehensive genetic analysis has provided new insights in the genetic landscape of BE-to-EAC. One group has shown that most mutations in EAC had already occurred in matched BE, using comprehensive genetic analysis on 11 cases with EAC and 2 of BE. Another group analyzed the mutations in selected 26 genes and reported that around half of the cases with BE without dysplasia already possessed mutations. Also, there was no significant difference in frequencies of those mutations between BE without dysplasia, BE with high-grade dysplasia, and EAC.^[52] Of note, they also examined associations between frequencies of mutations in the 26 genes and disease stage. They also found that only *TP53* and *SMAD4* mutations significantly increased

with progression of BE to high-grade dysplasia or EAC.

ARID1A is another key molecule driving BE to EAC.^[53] *ARID1A* is a member of SWI/SNF family of chromatin remodeling. This molecule has been examined mainly in gastric cancer and reported to be associated with microsatellite instability.^[54,55] *ARID1A* mutation was detected around 15% of BE with high-grade dysplasia and EAC. The frequency of loss of *ARID1A* by immunohistochemistry correlated with disease progression from BE to EAC. The EAC cell line, OE33, showed phenotypes of increased proliferation and aggressive invasion, as the gastric cancer cell line also did.^[53,54] In addition to *ARID1A*, the other members of chromatin remodeling factors encoding genes, *ARID2*, and *SMARC4A* mutations, were also reported.^[56]

Rho family GTPase activation is an important molecule in gastric cancer and EAC. Rho family consists of Cdc2, Rac1, and RhoA. These molecules are master regulators of actin cytoskeleton rearrangements, promote cancer cell invasion, and cell survival. In gastric cancer, a mutation of *RhoA* is frequently associated with diffuse-type gastric cancer. It has been reported that mutations in *ELMO1* and *DOCK2* are frequently noted in cases with EAC. These are intracellular mediators of RAC1. *ELMO1* and *DOCK2* promote tumor cell invasion and seem to be associated with EAC carcinogenesis.^[57] It was observed that 6% of EAC cases analyzed had mutations in *ELMO1* and 13% in *DOCK2*. Other genes encoding Rac1 activating enzymes were *ECT2* (1%), *TIAM1* (3%), *TRIO* (3%) and *VAV2* (1%) although these frequencies were lower than those in *ELMO1* and *DOCK2*. Taken together, around 30% of *Rac1*-activating mutations occurred in EAC patients. Also reported in EAC were frequent transversions of A to C at AA sites (T to G at TT sites).^[56,58] One possible explanation was that low pH due to GERD induces 8-OH-dG, resulting in A to C transversion at AA sites.^[59,60] Further studies also needed to clarify this interesting finding.

Epigenetic Changes and microRNA Status in BE and EAC

Recent global methylation profiling revealed that broad epigenetic alterations occur in both BE and EAC and are associated with carcinogenesis in EAC.^[61-64] CpG island promoter hypermethylations are a common feature of cancer, and regulate (traditionally down-regulate) downstream gene expression. On the other hand, DNA hypomethylation increases gene expression.^[62] As for specific CpG island promoter methylations, *CDKN2A*, *APC*, *CDHI*, *MGMT*, *TIMP-3* and *ESR1* have been evaluated in several reports.^[51,65-68] *CDKN2A* hypermethylation has been considered to occur in early steps in EAC carcinogenesis. One study suggested that 4 genes, *SLC22A18*, *PIGR*, *GJA12* and *RIN2*, were highly methylated in EAC compared to BE.^[63]

Table 1: Major molecular alterations reported across malignant progression of BE

Morphological status	Key molecular alterations
BE	SHH, BMP4, SOX9 and CDX3
Esophageal adenocarcinoma	Loss of function of CDKN2A or TP53 (by loss of heterozygosity, or mutation); ARID1A, SMAD4

SHH: Sonic hedgehog; BE: Barrett's esophagus; BMP: Bone morphogenetic protein; SOX9: SRY (sex determining region Y) box 9

Micro RNA (miRNA) is a small non-coding RNA related to post-transcriptional gene expression and silencing. Generally, up-regulation of oncogenic-miRNA or down-regulation of tumor-suppressor miRNA is identified as tumor-related miRNAs. Mir-21 up-regulation has been observed in BE and EAC compared with normal squamous cell epithelium and was associated with carcinogenesis.^[69] miRNA-194 was also induced in BE and EAC and found to be related to intestinal metaplasia and metastasis.^[70,71] miRNA-143, which suppresses transcription of *KRAS*, was down-regulated in EAC and associated with *TP53*.^[72,73] miRNA-31 and miRNA-375 were found to be down-regulated in EAC and are early and late-stage markers of EAC carcinogenesis.^[74]

Conclusion

Recent advances of next-generation sequencing have provided researchers with better understanding of genetic and epigenetic alterations in EAC carcinogenesis. However, little study has examined those genetic and epigenetic alterations in combination with clinicopathological factors. In order to elucidate the pathogenesis of BE and EAC and to find molecules for biomarkers and targeting therapy, it will be necessary to analyze those genetic alterations in combination with clinical data in a large-scale cohort.

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Conflicts of interest

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The clinical significance of circulating tumor cells in gastrointestinal cancer

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ABSTRACT

Circulating tumor cells (CTCs) are originated from the primary tumor lesion into the blood stream. CTCs could lead to recurrence of gastrointestinal (GI) cancers, even after a curative resection and colonizing in the distant organs to facilitate tumor distant metastasis; however, it has been challenging in clinic to detect CTCs for a long time, such as detection methodology or molecular markers for identification of CTCs. This review discussed the recent technical advances and biomarkers in the detection of CTCs and the molecular mechanism of CTC in cancer progression and metastasis. Moreover, novel concepts, such as cancer stem cells and epithelial-mesenchymal transition, could lead to CTCs and tumor progression and metastasis. Nevertheless, the involvement of CTCs varies greatly among cancer types in the GI and much remains to be learned. Thus, further study will provide more insightful information from a clinical and translational viewpoint to use CTCs for cancer early diagnosis or prediction of tumor recurrence and investigation of tumor progression and metastasis as well.

Key words: Cancer stem cells, circulating tumor cells, epithelial-mesenchymal transition, gastrointestinal cancer, tumor progression and metastasis

Introduction

Tumor recurrence often occurs in patients with gastrointestinal (GI) cancers, even after a curative resection, which may be because undetectable tumor cells depositor enter into the blood stream at the time of operation. In some cases, tumor recurs despite adjuvant chemotherapy after curative surgery suggesting that chemotherapy failed to eradicate all cancer cells that persist after curative surgery. Thus, tumor cells could be disseminated before surgery. The concept of the circulating tumor cells (CTCs) has been, therefore, established and indicates that tumor cells are in blood stream, which will facilitate tumor progression and metastasis although detection of CTCs in peripheral blood was described more than a century ago.^[1] Recent advance on research of CTCs largely contributed to diagnosis and treatment of GI cancers. However, the clinical relevance of CTC detection in GI cancers is still the subject of controversies, and their biology is poorly understood.

Indeed, detection of CTCs becomes a promising means to early diagnosis and prediction of prognosis and tumor recurrence for several types of human cancer.^[2-5] Furthermore, the study of CTCs could also elucidate the molecular biological profile of CTC and lead to better understanding of cancer metastasis. To date, standard procedures of CTC detection have to be established, and the clinical relevance should be confirmed by a large-scale clinical study. In this review, we updated and discussed recent progress regarding CTCs in GI cancer. These new data could improve our understanding of the mechanisms of cancer progression and metastasis as well as therapy resistance. This information may also lead to the development of novel clinical targets and improve the clinical management of GI cancer.

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Methodology in Detection of Circulating Tumor Cells

In general, methodology in the detection of CTCs consists of two steps, that is, enrichment and detection process. The enrichment process is required because of the rarity of CTCs in peripheral circulation (one CTC per 1×10^6 to 1×10^7 mononuclear cells). To enrich CTCs from blood mononuclear cells, density gradient centrifugation (Ficoll-Hypaque or OncoQuick separation), immunomagnetic or size filtration procedures are used.^[6,7] After enrichment, the identification of CTCs is then performed. For identification techniques, nucleic acid methods and cytometric methods are usually used. Recently, the development of molecular techniques can make molecular and genetic analysis of CTCs after enrichment and identification of CTCs, leading to developing CTC characterization.

Enrichment Techniques

Cell morphologic-based enrichment

Isolation of CTCs using the size of epithelial tumors is based on the size of tumor cells without functional modification and complex enrichment procedures. It is usual to utilize 5-8 μm probe filters to enable and to separate small leukocytes from the large epithelial cell and the isolation sensitivity threshold is approximately one tumor cell per milliliter.^[8,9] These techniques have a valuable advantage in isolation of CTCs without damaging cells and enable further immunocytochemical or immunofluorescence evaluation of CTCs. Although it is easily handled and cheap, it is considered to be not highly sensitive and poorly specific.

Furthermore, density gradient separation using Ficoll-Hypaque is an alternative technique to separate CTCs and mononuclear cells from other blood cells and granulocytes. However, Ficoll-Hypaque can be toxic to CTCs. CTCs can also be easily to lose due to the migration of cells to the plasma layer. OncoQuick was developed to avoid the cross-contamination of different layers, resulting in higher recovery rate of CTCs.^[10,11] Recently, RosetteSepTM (Stem Cell Technologies, Vancouver, British Columbia, Canada) developed a novel method based negative selection to improve the specificity of standard gradient separation.^[12,13]

Immunomagnetic circulating tumor cell enrichment

The immunomagnetic CTC enrichment technique is a magnetic bead-based separation method. To date, there have been two methods to identify CTCs expressing targeting-specific biomarkers. One is using the epithelial cell-specific marker, e.g. epithelial cell adhesion molecule (EpCAM) or cytokeratin (CK) expressed on the surface of tumor cells from epithelial origin. Another is using the tumor-specific markers, such as α -fetoprotein,

Her2-neu, or carcinoembryonic antigen (CEA) expressed on a particular type of cancer cells. Immunomagnetic isolation technique utilizes monoclonal antibodies that are labeled magnetic microbeads and separates CTCs from the leukocytes background by magnetic force. To separate leukocytes, the negative selection is performed using an anti-CD45 antibody recognizing surface marker of leukocytes. Based on this technique, the Magnetic Activated Cell Sorting System (MACSTM Miltenyi Biotec, Bergisch Gladbach, Nordrhein-Westfalen, Germany) is a useful technology for detecting and analyzing CTCs because it avoids cell lysis and enables cell count by immunocytochemistry and immunofluorescence assay.^[14] CellSearch SystemTM (Veridex, Warren, NJ) approved by the US Food and Drug Administration (FDA) is a semi-automated analyzer enriching the CTCs with ferrofluid nanoparticles coated with anti-EpCAM antibodies. This system is proved to be useful for detecting and analyzing CTCs with patients with breast, colorectal and prostate cancer in the clinic.^[15] However, Alunni-Fabbroni and Sandri argued that this technology has two possible limitations, that is, there is no “universal marker” available for each type of tumor, while epithelial marker (EpCAM) could be down-regulated in epithelial tumor cells after tumor cells undergo epithelial-mesenchymal transition (EMT).^[16] Thus, this method could only detect selected CTCs.^[17,18]

Enrichment Techniques

Nucleic acid-based analysis

Reverse transcriptase polymerase chain reaction (RT-PCR) based techniques can increase the specificity of the molecular methods to discriminate between the higher levels of molecular changes in cancer patients and the low background level in normal cells. Expressions of epithelial or tumor-specific markers are detected using RT-PCR to evaluate and identify CTCs. Nowadays, multiplex RT-PCR approach has been established to screen at the same time more than one single biomarker. Furthermore, quantitative RT-PCR improves the specificity of detection for CTCs by defining a cut-off value for biomarker expression. However, there are some limitations of this method: (1) contamination of non-malignant epithelial cells such as skin cells; (2) false positive due to unspecific markers; and (3) amplification of cell-free nucleic acids. Therefore, it is essential to select the appropriate marker that is expressed specifically by tumor cells to boost the specificity and reliability of CTC detection.

Cytometric-based analysis

Cytometric-based technique can isolate and count CTCs using monoclonal antibodies against various antigens. To detect CTCs, CK and EpCAM are most commonly used. It enables to keep CTCs intact during analysis because cell lysis is not necessary. Furthermore, this technique provides information of high statistical

precision and subpopulation quantification with high specificity due to simultaneous analysis using multiple parameters. However, in contrast to RT-PCR technique, the disadvantage of this technique has a lower sensitivity.

Fiber-optic Array Scanning Technology, a rapid and accurate CTC location cytometric system, is a scanning technology characterized by a large field of view.^[19] It allows analyzing large volumes of samples without any purification step and minimizing the risk of cell loss. Additional scanning systems such as ACIS (Automated Cellular Imaging System, DAKO, Spatial Technology, USA) and ARIOL (Applied Imaging Corp, Wetzlar, Germany) are available on the market.

Recent Advances in Detection of Circulating Tumor Cells

As discussed above, the detection of CTCs is involved in two steps of enrichment and identification; thus, the development of automated techniques could offer at the same time enrichment, staining and scanning of the samples. The Cell Search System® enriches the CTCs with ferrofluid nanoparticles coated with anti-EpCAM antibody. The enriched EpCAM⁺ population is stained with phycoerythrin-conjugated antibodies against CK-8, -18 and -19 with allophycocyanin-conjugated antibodies specific for leukocytes (anti-CD45 antibody) and with the nuclear dye 4', 6-diamino-2-phenylidole (DAPI) for the nucleic acids staining. The CK⁺/DAPI⁺/CD45⁻ cells are then counted as CTCs using CellSpotter analyzer (Veridex, Warren, NJ), a four-color semi-automated fluorescent microscope.^[20] More recently, CTC-chip based on a microfluidic platform has also developed to isolate a high rate of CTCs.^[21] CTC-chip consists of an array of 78,000 microspots coated with anti-EpCAM antibodies. Whole blood is pumped through this chip, and EpCAM⁺ cells are captured and detected by cameras identifying their morphology, viability and the expressions of tumor markers.^[14] However, the relevance of this technology in clinical setting remains unclear and clinical validation study is required. Finally, based on enzyme-linked immunospot assay technology, epithelial immunospot (EPISPOT) assay can identify CTCs by detecting specific CTC-secreted proteins (CK, mucin or prostate specific antigen).^[22,23] EPISPOT makes it possible to detect the only viable CTCs because dying CTCs are unable to secrete an adequate amount of proteins to be detected. Sensitivity of EPISPOT is superior to ELISA assay in a two-order magnitude while detecting the release of CK-19 from tumor cells.^[24]

Recent Development of Molecular and Genetic Characterization of Circulating Tumor Cells

The next desirable step is to elucidate the molecular and genetic characterization of CTCs after enrichment and isolation of CTCs. This step may help us to comprehend the mechanism of cancer metastasis, leading to the development of treatments of tumor metastasis. However, the molecular and genetic characteristics of CTCs are not

fully clarified when compared with corresponding tumors in GI cancer. The molecular and genetic characteristics of CTCs are usually analyzed by PCR-based methods, fluorescent *in situ* hybridization (FISH) or comparative genomic hybridization (CGH). There have been no reports about CTCs characterization analyzed by FISH and CGH in gastric cancer, whereas abnormal copy number alteration was detected in CTCs from patients with metastatic prostate cancer.^[25-27] Using PCR-based methods, conventional detection system with epithelial markers such as CEA and CK has been previously performed to show the clinical significance of CTCs in gastric cancer.^[28-32] However, Mimori *et al.*^[33] showed in a large-scale study that CTCs circulate even in early stages of the disease indicating that the simultaneous presence of CTC and VEGFR-1 expression is clinically significant for disease progression. It is also well known that there is discordance of expression profile between CTCs and primary tumor, and several markers for regulating metastasis and prognosis have been determined by PCR-based methods.^[34-37] Furthermore, a comprehensive molecular profiling using the cDNA microarray was performed to identify novel genes to predict gastric cancer metastasis, recurrence and prognosis, suggesting that expression of MT1-MMP in peripheral blood identified by the cDNA microarray technique in gastric cancer was a powerful indicator of distant metastasis, especially for peritoneal dissemination.^[38] van de Stolpe *et al.*^[39] reported that CTCs were heterogeneous and differed among different cancer types. In addition to differences across cancer types, CTCs have heterogeneity within the same patient. Although the heterogeneity of primary tumors has been known, Klein *et al.* showed that early disseminated cancer cells are genomically very unstable, as well as the primary tumor.^[40] In this case, gastric cancer is well known to have histological heterogeneity in primary lesion. Various histological types and differentiation of gastric cancer cells are frequently observed in the same specimens.^[41] Therefore, histological heterogeneity may make it difficult to the molecular and genetic characterization of CTCs in GI cancer.

Clinical Relevance of Circulating Tumor Cells in Gastrointestinal Cancer

To date, there are a number of methodologies in the detection of CTCs and the clinical relevance of GI cancer have been reported. In clinical setting, the detection of CTC is expected to be useful in early diagnosis of cancer, monitoring of treatment responses and disease progression. In the following, we summarized a comprehensive update of the studies with more than 50 patients or with an outcome analysis and discussed their clinical implications in selected GI cancers.

Esophageal cancer

There are only a few studies of esophageal cancer available as compared to gastric and colorectal cancers

[Table 1]. In esophageal cancer, RT-PCR was the main technique to detect CTCs in previous studies.^[42-45] As for available molecular markers, CEA and SCC are useful predictive markers for tumor recurrence and survival. Most recently, a large-scale of study using CellSearch System®, morphological technique are also reported.^[46,47] Matsushita *et al.*^[46] revealed that CTC detection by CellSearch System® was useful to evaluate the clinical efficacy of chemotherapy and chemoradiation therapy on esophageal cancer patients. Reeh *et al.*^[47] reported that patients with positive CTCs had significantly poorer overall survival and progression-free survival rate; therefore, preoperative CTC detection by CellSearch System® was an independent prognostic indicator for patients with esophageal cancer. However, most of previously reported patients had esophageal squamous cell carcinoma. There are some differences of biological behaviors between esophageal squamous cell carcinoma and adenocarcinoma; therefore, further study of esophageal adenocarcinoma is needed.

Gastric cancer

A number of studies of CTC detection in patients with gastric cancer have been reported previously as summarized in Table 2. Although the several methodology of CTC detection including RT-PCR and CellSearch System® [Table 2], it remains unclear which is the best method and molecular marker for detection of CTCs in gastric cancer patients. Recently, various meta-analyses demonstrated that presence of CTCs was associated with poor prognosis and advanced clinicopathological factors.^[48-50] It has been reported that detection of CTCs in gastric cancer may be useful in early diagnosis and monitoring of treatment responses and prognosis. However, as for diagnosis, a recent meta-analysis showed that CTC detection alone cannot be recommended as a screening test for gastric cancer because of lower and inconsistent sensitivity estimates for CTC.^[51] Furthermore, Mimori *et al.*^[33] showed that CTCs occurred in early stages of the disease, and CTC

Table 1: Clinical relevance of CTC in esophageal cancer

Author	Year	Case	Method	Molecular marker	Clinical relevance
Kaganoi	2004	70	RT-PCR	SCC	Prediction of recurrence
Setoyama	2006	106	RT-PCR	CEA	Prediction of recurrence
Liu	2007	53	RT-PCR	CEA	Prediction of recurrence
Hashimoto	2008	147	RT-PCR	CEA	Prediction of recurrence and prognosis
Cao	2009	108	RT-PCR	Survivin	Prediction of haematogenous recurrence and prognosis
Tanaka	2010	244	RT-PCR	CEA, SCC	Predictor for hematogenous and local recurrences
Yin	2012	72	RT-PCR	CEA, CK-19, Survivin	Clinical efficacy of RT
Matsushita	2014	90	CellSearch	EpCAM, CK-8, 18, 19	Clinical efficacy of CT or CRT
Reeh	2015	100*	CellSearch	EpCAM, CK-8, 18, 19	Prediction of recurrence and prognosis

*Esophageal adenocarcinoma is included. RT: Radiotherapy; CRT: Chemoradiation therapy

Table 2: Clinical relevance of CTC in gastric cancer

Author	Year	Case	Method	Molecular marker	Clinical relevance
Wu	2006	64	MAH	hTERT, CK-19, CEA, MUC1	Associated with recurrence
Pituch-Noworolska	2007	57	ICC	CK-8, 18, 19	No prognostic impact
Ito	2010	65	ICC	GFP, EpCAM,	Shorter OS
Majima	2000	52	RT-PCR	CK-19, 20	Shorter OS
Miyazono	2001	57	RT-PCR	CEA	Associated with liver metastasis, recurrence
Sumikura	2003	106	RT-PCR	CEA	Associated with recurrence
Illert	2005	70	RT-PCR	CK-20	Shorter OS
Ikeguchi	2005	59	RT-PCR	CEA	No association with recurrence
Uen	2006	52	RT-PCR	MUC1, c-Met	Shorter OS
Koga	2008	101	RT-PCR	CK-18, 19, 20	Shorter OS (CK-19 is better)
Yie	2008	55	RT-PCR, ELISA	Survivin	Predictive marker for DFS
Mimori	2008	810	RT-PCR	CK-7,19, 20, VEGFR1	Associated with hematogenous metastasis
Bertazza	2009	70	RT-PCR	Survivin	Predictive marker for OS
Qiu	2010	123	RT-PCR	CEA	Predictive marker for DFS
Arigami	2010	94	RT-PCR	B7-H3	Shorter OS
Arigami	2011	95	RT-PCR	B7-H4	Shorter OS
Cao	2011	98	RT-PCR, ELISA	Survivin	Predictive marker for DFS
Arigami	2013	93	RT-PCR	STC2	Shorter OS
Matsusaka	2010	52	CellSearch	EpCAM, CK-8, 18, 19	Predictive marker for PFS (CTC level after Cx)
Uenosono	2013	148	CellSearch	EpCAM, CK-8, 18, 19	Predictive marker for PFS and OS

MAH: Membrane-array hybridization; ICC: Immunocytochemistry; hTERT: Human Telomerase reverse transcriptase; ELISA: Enzyme-Linked Immunosorbent Assay; DFS: Disease free survival; OS: Overall survival; PFS: Progression free survival

alone can not be a predictor of cancer metastasis in a large-scale study. This study also revealed that elevated expression of VEGFR-1 facilitated the establishment of hematogenous metastases of gastric cancer and that the simultaneous presence of CTC and VEGFR-1 expression at premetastatic sites was clinically significant in disease progression.

Colorectal cancer

To date, there are a large number of studies of CTC detection in colorectal cancer as compared to other GI cancers as summarized in Table 3. RT-PCR and the CellSearch System® have been mainly reported methods to detect CTC in colorectal cancer and data showed that CTCs were associated recurrence and overall survival of patients. For example, Cohen *et al.*^[12] revealed that the number of CTCs detected by the CellSearch System® before and during treatment was an independent predictor of PFS and OS in 430 patients with metastatic colorectal cancer in a prospective multicenter clinical trial. The CellSearch System® using in colorectal cancer

was the first CTC detection system that was approved by US FDA.^[2] Furthermore, a previous meta-analysis reported the prognostic significance of CTC detected by the CellSearch System® has been reported.^[48] Eleven studies including 1,847 colorectal cancer patients were analyzed in this study and the presence of CTCs was significantly associated with overall and progression-free survival as reported by the previous meta-analysis.^[52] In a previous prospective study of non-metastatic colorectal cancer, preoperative CTC detection was a strong and independent prognostic marker.^[53] The treatment response rate was significantly lower in CTC-positive patients than that of CTC negative patients at base line and during treatment.^[23,54-57] Another previous study demonstrated potentially clinical application in detection of KRAS mutational in CTCs for selecting metastatic colorectal cancer patients for cetuximab therapy.^[58] In addition, recent development of molecular and genetic characterization of single-CTC demonstrated that there was intra- and inter- heterogeneity of EGFR expression and genetic alterations of EGFR, KRAS and PIK3CA,

Table 3: Clinical relevance of CTC in colorectal cancer

Author	Year	Case	Method	Molecular marker	Clinical relevance
Wong	2009	132	ICC	CK-20	Predictive marker for OS
Taniguchi	2000	53	RT-PCR	CEA	Predictive marker for DFS
Yamaguchi	2000	52	RT-PCR	CK-20, CEA	Shorter OS
Hardingham	2000	94	RT-PCR	CK-19, 20, MUC2	Shorter DFS
Bessa	2001	68	RT-PCR	CEA	No prognostic impact
Ito	2002	99	RT-PCR	CEA	Shorter DFS
Bessa	2003	66*	RT-PCR	CEA	No prognostic impact
Sadahiro	2005	93	RT-PCR	CEA	No prognostic impact
Koch	2006	90	RT-PCR	CK-20	Shorter DFS
Douard	2006	121	RT-PCR	CK-20, CGM2	No prognostic impact
Iinuma	2006	167	RT-PCR	CK-20, CEA	Shorter DFS and OS
Katsumata	2006	57	RT-PCR	CK-20	Strong relation to LN metastasis and OS
Allen-Mersh	2007	113*	RT-PCR	CK-20, CEA	Shorter DFS
Wang	2007	157*	RT-PCR**	CK-19, 20, CEA, hTERT	Shorter DFS
Uen	2007	194	RT-PCR**	CK-19, 20, CEA, hTERT	Shorter DFS
Sadahiro	2007	200*	RT-PCR	CEA	Shorter DFS
Uen	2007	438*	RT-PCR	CK-19, 20, CEA, hTERT	Shorter DFS
Yie	2008	86	RT-PCR, ELISA	Survivin	Predictive marker for DFS
Lu	2011	141*	RT-PCR**	CK-19, 20, CEA, hTERT	Shorter DFS and OS
Iinuma	2011	735	RT-PCR	CK-19, 20, CEA, hTERT	Shorter DFS and OS
Pilati	2012	50	RT-PCR	CK-19, 20, CEA, CD133, VEGF, EGFR, Survivin	Predictive marker for OS (CD133 CTC)
Cohen	2008	430	CellSearch	EpCAM, CK-8, 18, 19	Predictive marker for OS (CTC ≥ 3)
Matsusaka	2011	64	CellSearch	EpCAM, CK-8, 18, 19	Predictive marker for PFS and OS (CTC level after Cx)
Tol	2010	467	CellSearch	EpCAM, CK-8, 18, 19	Predictive marker for PFS and OS (CTC level before Cx)
Sastre	2012	180	CellSearch	EpCAM, CK-8, 18, 19	Predictive marker for PFS and OS (CTC level before Cx)
Aggarwal	2013	209	CellSearch	EpCAM, CK-8, 18, 19	Predictive marker for OS (CTC level before Cx)
Gazzaniga	2013	119	CellSearch	EpCAM, CK-8, 18, 19	Predictive marker for OS (CTC ≥ 1)
Kuboki	2013	63	CellSearch	EpCAM, CK-8, 18, 19	Shorter DFS and OS
Sotelo	2015	472	CellSearch	EpCAM, CK-8, 18, 19	No prognostic impact (stage III)
Seeberg	2015	194	CellSearch	EpCAM, CK-8, 18, 19	Predict nonresectability and impaired survival

*Post-operative mesurement. **Membrane array. ICC: Immunocytochemistry; ELISA: Enzyme-Linked Immunosorbent Assay; hTERT: Human Telomerase reverse transcriptase; LN: Lymphnode metastasis; DFS: Disease free survival; OS: Overall survival; PFS: Progression free survival; Cx: Chemotherapy

which possibly explained the variable response rates to EGFR inhibition in patients with colorectal cancer.^[59] Therefore, the information on the molecular status of CTCs might be useful for stratification of molecular-directed therapy.

Future Perspectives

Epithelial-mesenchymal transition

There are two main approaches in the detection of CTCs, that is, immunological assays using monoclonal antibodies and PCR-based molecular assays, exploiting tissue-specific transcripts.^[60] Immunocytochemical detection of epithelial or tumor-associated antigens is widely accepted.^[61] Recent studies have shown that EMT plays a critical role in cancer progression and metastasis in epithelial malignancies including gastric cancer.^[62] Our previous study implied that vimentin-positive tumor cells were able to survive in the peripheral circulation and in the bone marrow and that vimentin-positive cancer cells that invade intratumoral vessels must have undergone mesenchymal transition. We assume that not all detected CTCs but rather only a few, which have undergone EMT could give rise to tumor metastasis or recurrence.^[17] Most recently, Wu *et al.*^[63] reported that mesenchymal CTCs classified using EMT markers were more commonly found in patients in metastatic stages of the disease in different types of human cancers. Therefore, it is possible that conventional detection system using epithelial markers fail to detect that population of CTCs.

Cancer stem cell

Furthermore, the concept of rare subpopulations of cancer stem cells (CSCs) has created a novel focus in cancer research but arises a question whether CTCs have CSC property. It is expected that CTC with CSC property may be disseminated from the primary tumor lesion to a distant metastatic site. This hypothesis is supported by the similarities between the properties of CTCs and CSC and suggests that the founder cells of metastases arise from the CTC population. It has been reported that stem cell markers are frequently overexpressed in the CTCs of patients with metastatic breast cancer, and the most CTCs have stem cell phenotypes that are non-proliferating and resistant to chemotherapy. For example, Iinuma *et al.*^[64] revealed that multi genetic markers of CSC, CEA/CK/CD133 in peripheral blood samples could be a useful predictor for recurrence and prognosis. Pilati *et al.*^[65] reported that CD133-positive CTCs might represent a suitable prognostic marker to stratify the risk of patients who undergo liver resection for CRC metastasis.

Conclusion

An increasing number of studies have shown that CTC is associated with GI cancer progression, metastasis and resistance to pharmacotherapy. However, the clinical evidence supporting the role of CTC in cancer

progression still remains inconclusive. Therefore, further analysis and clinical trials are required to achieve clinical utility of CTC detection in GI cancers.

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Conflicts of interest

There are no conflicts of interest.

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Chronic inflammation and gastrointestinal cancer

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ABSTRACT

Chronic inflammation has been identified as an important risk factor in the development of the gastrointestinal (GI) tract cancers, and the underlying molecular mechanisms have been studied extensively. Chronic inflammation is able to trigger cellular events to promote malignant transformation of normal epithelial cells in the GI tract to cancer. Host inflammation responses in carcinogenesis are through multiple mechanisms such as reactive oxygen and nitration species from mononuclear phagocytes and leukocytes, immune response and pro-inflammatory cytokines. Nuclear factor- κ B (NF- κ B) has been considered as the central mediator of the immune response. Activation of NF- κ B by phosphorylation leads to translocation of NF- κ B protein to the nucleus, and in turn regulates the transcription of several pro-inflammatory cytokines and chemokines. Furthermore, chronic inflammation creates an environment for genomic and epigenetic changes. In this review, we summarize the important molecular mechanisms that link chronic inflammation and GI tract cancer, including esophageal, gastric and colonic cancers, focusing on infective and noninfective agents such as gastroesophageal reflux disease, *Helicobacter pylori* gastritis and inflammatory bowel disease.

Key words: Cancer, gastrointestinal tract, immune response, inflammation

Introduction

It is now widely accepted that inadequately resolved chronic inflammation could increase cancer risk. The etiology of inflammation varies and could result from infection with viruses, bacteria or parasites. Alternatively, it may be noninfective but caused by a physical or chemical irritant. For example, hepatitis B and C viruses account for more than 80% of hepatocellular carcinoma cases in the world, while human papillomavirus infection is the leading cause of anogenital cancer, and *Helicobacter pylori* has been considered as the major cause of gastric adenocarcinoma and is known to significantly increase the risk of gastric mucosa-associated lymphoid tissue lymphoma. Moreover, there are numerous examples of noninfective agents being associated with inflammation and development of cancers. Several pathological conditions in the gastrointestinal (GI) tract such as gastroesophageal reflux disease (GERD), inflammatory bowel diseases (IBDs), chronic pancreatitis, and cholangitis-related cholangiocarcinoma illustrate this link.^[1] As a barrier to the environment and as the main organ system for digestion and absorption of food, the GI tract is exposed to many substances and stimulants.

Some of these, such as alcohol and acid, can cause GI cancers by linking to chronic inflammation [Table 1].^[2,3] Thus, in this review, we discussed emerging concepts and provided specific examples for the role of chronic inflammation in the development of GI cancers, including esophageal, gastric and colonic cancers, since they have been investigated most thoroughly.

Role of Chronic Inflammation in Cancer Development

Immune response and cytokines in cancers

Chronic inflammation is characterized by the infiltration of mononuclear cells, such as macrophages, lymphocytes and plasma cells in damaged tissue, together with tissue destruction and attempts to repair. In this inflammatory state, local activation of the immune system occurs. Natural killer cells, monocytes, macrophages, dendritic cells, mast cells and granulocytes usually elicit the first immune response and initiate inflammation. Of the many cell types active during chronic inflammatory response,

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Table 1: Gastrointestinal malignancies linked to chronic inflammation

Organ	Tumor type	Chronic inflammation
Esophagus	Squamous cell carcinoma Adenocarcinoma	Cigarette smoking, alcohol and hot beverages GERD
Stomach	Adenocarcinoma MALT lymphoma	<i>H. pylori</i> , autoimmune <i>H. pylori</i> , HCV
Colorectal	Colorectal cancer	Ulcerative colitis, Crohn's disease
Liver	Hepatocellular carcinoma	HBV, HCV and cirrhosis (alcohol, NAFLD)
Pancreas	Pancreatic ductal adenocarcinoma	Chronic pancreatitis
Biliary system	Gallbladder carcinoma Cholangiocarcinoma	Chronic cholecystitis PSC, chronic cholangitis and liver cirrhosis

GERD: Gastroesophageal reflux disease; *H. pylori*: *Helicobacter pylori*; HBV: Hepatitis B virus; HCV: Hepatitis C virus; NAFLD: Non-alcoholic fatty liver disease; PSC: Primary sclerosing cholangitis; MALT: Mucosa-associated lymphoid tissue

macrophages are one of the key players.^[2] Recent studies showed that tumor-associated macrophages (TAMs) were dispersed throughout tumor lesions and contributed to tumor growth, invasion and metastasis by producing various mediators.^[4,5] In general, TAMs are found within and surrounding most tumor cells and can, when activated, release numerous factors to influence the behavior of tumor cells and the local tissue microenvironment. Interferon (IFN)- γ induces “classical” activation of macrophages, while anti-inflammatory mediators such as interleukin (IL)-10, IL-4 and IL-13 provoke “alternative” activation of macrophages, which are referred as M1 and M2 macrophages respectively.^[6,7] M2 macrophages are oriented toward promoting tumor progression, tissue repair and angiogenesis as well as suppressing adaptive immunity in tumors, whereas M1 macrophages, as classically or alternatively activated macrophages, are activated by lipopolysaccharides and IFN- γ , and can secrete high levels of IL-12 and low levels of IL-10.^[4,8-10]

Reactive oxygen species, nitric oxide and cyclooxygenase-2

Chronic inflammation creates a microenvironment locally to induce genomic instability in cells. At the site of chronic inflammation, cells are exposed to oxygen and nitrogen radicals from mononuclear phagocytes and leukocytes. These radicals can cause DNA damage. For example, nitric oxide and its products may exert oncogenic effects via several mechanisms, including inhibition of DNA mismatch repair, protein damage, induction of hypermethylation, inhibition of apoptosis, mutation of DNA and disruption of cellular repair functions such as those involving the p53 pathway.^[11-13] Release of reactive oxygen and nitrogen species is enhanced by pro-inflammatory cytokines such as tumor necrosis factor (TNF), IL-1 β and IFN- α .

Another inducible enzyme with carcinogenic properties that is active in inflamed and malignant tissues is cyclooxygenase-2 (COX-2). Strong epidemiological evidence implicates that COX-2 plays a role in the pathogenesis of a number of epithelial malignancies, including esophageal, gastric and colorectal

cancers (CRCs). Several mechanisms of COX-2-mediated intestinal carcinogenesis have been elucidated. These include inhibition of apoptosis, modulation of cellular adhesion and motility, promotion of angiogenesis and immunosuppression.^[14-16] Among the most potent inducers of COX-2, there are key pro-inflammatory cytokines, IL-1 α , IL-1 β and TNF- α . COX-2 is significantly overexpressed in malignancies, and non-steroidal anti-inflammatory drugs are associated with a reduction in the incidence of a variety of GI cancers.^[17,18]

Nuclear factor- κ B

Inflammatory responses contribute to carcinogenesis through multiple mechanisms. As mentioned above, reactive oxygen species, COX-2 and some cytokines interact with each other in a complex manner during development and progression of an inflammatory environment. One such mediator is the transcription factor nuclear factor- κ B (NF- κ B), which is a key mediator of inflammation and involved in the regulation of apoptotic and oncogenic gene expression and activation.^[19] NF- κ B has often been described as the central mediator of the immune response and as being critically involved in cancer-associated inflammation and the tissue repair response.^[2,20] Aberrant activation of NF- κ B protein was associated with inflammation and cancer in mouse models and in human GI cancers.^[21-23] Activation of NF- κ B plays an important role in integrating multiple stress stimuli and regulating immune responses.^[23,24] Bile acids, particularly deoxycholic acid, have been shown to activate the NF- κ B pathway.^[25] NF- κ B activation through phosphorylation leads to translocation into the nucleus, and in turn regulates the transcription of several pro-inflammatory cytokines such as TNF- α , IL-1 β , IL-6, IL-8, and chemokines such as CXCL-1 and CXCL-2.^[24,26]

Thus, chronic inflammation could lead to carcinogenesis by sustaining pro-inflammatory oncogenic signaling, angiogenesis and immune suppression.

Esophageal Cancer

There are two major histological subtypes of esophageal cancer, that is, esophageal squamous cell carcinoma

(ESCC) and esophageal adenocarcinoma (EAC). Tobacco smoking and alcohol consumption are the two major risk factors in ESCC,^[27] with a risk of heavy smokers/drinkers for 50 times greater in the induction of ESCC.^[28] Tobacco smoking and alcohol consumption have been associated with the field of cancerization in the upper aerodigestive tract. For example, Oka *et al.*^[29] demonstrated that tobacco smoking was likely to induce global DNA hypomethylation and site-specific CpG island promoter hypermethylation in the normal-appearing esophageal mucosa. Both these mutations are representative of DNA methylation alterations occurring in cancer cells. In addition, we also previously reported that global DNA hypomethylation in normal esophageal mucosa was observed in ESCC patients who habitually smoked,^[30] suggesting epigenetic field defect after exposure to risk factors. Recently, deficiency in the enzyme aldehyde dehydrogenase 2 (ALDH2), which causes the so-called alcohol flushing response, has been revealed to increase the risk of alcohol-related ESCC.^[31] In East Asian populations, there is a variant of ALDH2 in which the glutamate at position 487 is replaced with lysine, resulting in an inactive protein.^[32] Consumption of hot beverages is also suspected to cause chronic inflammation in esophageal squamous cell mucosa.^[33] In addition, the influence of human papillomavirus in increasing ESCC risk is still under debate.^[34]

Gastroesophageal reflux disease (GERD), cigarette smoking and obesity are all risk factors in EAC.^[35] EAC develops through chronic exposure to gastroesophageal reflux, Barrett's esophagus, dysplasia and adenocarcinoma as a sequence.^[36,37] Increased exposure of the esophagus epithelium to refluxed gastric and bile acid, particularly deoxycholic acid, has a critical role in promoting the development of Barrett's esophagus and EAC. NF- κ B is a key regulator of the inflammatory process that has been shown to be activated in EAC. Several studies report that NF- κ B was activated by bile acid components and subsequently involved in the development of metaplasia of Barrett's esophagus and cancer.^[25]

Gastric Cancer

Gastric adenocarcinoma is the second leading cause of cancer-related death in the world.^[38] *H. pylori* causes chronic gastritis, and the relationship between *H. pylori*-induced chronic inflammation and cancer is one of the best-elucidated factors. Indeed, *H. pylori* induces active chronic gastric inflammation, which progresses to gastric adenocarcinoma, resulting in approximately 660,000 worldwide new cases of gastric cancer per year.^[39] However, only a few percentage of infected persons do develop neoplasia.

Several recent studies described that cytotoxin associated gene A (CagA)-positive *H. pylori* strains were identified to be particularly carcinogenic. Compared to

CagA-negative strains, *H. pylori* strains that harbor the CagA pathogenicity islands (PAI) are associated with a significantly increased risk of distal gastric cancer.^[40] After attached to gastric epithelial cells, *H. pylori* CagA-positive strains eject the CagA protein directly into the gastric epithelial cells. After translocation, CagA undergoes tyrosine phosphorylation by Src and Abl kinases and the tyrosine phosphorylated-CagA binds to the Src homology 2 (SHP-2) domain, leading to morphologic alterations such as cell scattering and elongation.^[41] Furthermore, CagA-activated SHP-2 deregulates the MAP kinase signaling cascade.^[42] The CagA protein of certain *H. pylori* strains can stimulate expression of IL-8 by activating NF- κ B,^[43] thereby contributing to neutrophil infiltration in the gastric mucosa. In addition, chronic inflammation caused by *H. pylori* infection contributes to neoplastic transformation by establishing a positive feedback loop via the signal transducer and activator of transcription (STAT) 3-dependent COX-2 induction, which in turn influences STAT3 regulation via IL-6.^[44]

Another mechanism of *H. pylori*-induced gastric carcinogenesis is genomic alteration and gene mutation. For example, prevalence of the *TP53* mutation in gastric cancer is, on average, approximately 40%.^[45] Previous studies have shown that various genetic alterations occur in the gastric mucosa during chronic gastritis,^[46,47] suggesting an importance of the accumulated genomic mutations induced by *H. pylori* infection in the development of gastric cancer. Activation-induced cytidine deaminase (AID), a member of the cytidine deaminase family that functions to edit genomic DNA, is an enzyme essential for somatic hypermutation and class-switch recombination in immunoglobulin genes.^[48] However, inappropriate AID expression acts as a genomic mutagen to contribute to tumorigenesis.^[49,50] Infection with CagA PAI-positive *H. pylori* ectopically induced high expression of AID via NF- κ B activation in human gastric epithelial cells, leading to multiple mutations in the host genome, such as those found in *TP53*. The accumulation of nucleotide alterations will lead to the development of gastric cancer.^[51]

Recently, exciting data showed an association of *H. pylori* infection with cancer stem cell population. The leucine-rich repeat-containing G-protein coupled receptor (Lgr5) is known as the stem cell marker of GI cancers, including gastric cancer. Lgr5-positive epithelial cells have higher levels of oxidative DNA damage than in Lgr5-negative cells from patients with *H. pylori*-positive gastric cancer, indicating that *H. pylori* specifically targets Lgr5-positive epithelial cells.^[52]

Other inflammatory risk factors that either act independently of *H. pylori* infection or further enhance its effects have been also identified. For example, chronic gastritis caused by bile reflux can cause intestinal metaplasia as a neoplastic precursor lesion in gastric cancer. Moreover, T-cell-mediated autoimmune

gastritis fosters the development of intestinal type gastric cancer.^[53,54] Thus, these risk factors lead to a state of chronic inflammation and then development of gastric cancer.

Colorectal Cancer

CRC is one of the leading causes of cancer-related deaths in the world. CRC is one of the most serious complications of IBD, including ulcerative colitis and Crohn's disease. The relative risk of CRC in patients with colitis is two to eight times higher than the general population.^[55] Although it is clear that chronic inflammation is a CRC risk factor, pathogenesis of colitis-associated cancer (CAC) is still uncertain.

CAC develops in chronically inflamed mucosa and is believed to develop in a colitis-dysplasia-carcinoma sequence. The chronic inflammation in IBD often results in increased re-epithelialization of cells and cell turnover in the colonic mucosa and thus, leads to increased risk of errors in DNA repair and cell cycle regulation. Oxidative stress and impaired DNA mismatch repair are combined with proliferation, invasion and angiogenesis, thereby promoting cell growth signaling. In contrast with sporadic CRC, *p53* mutations occur in the early stages and APC mutations occur in the late stages of the genesis of CAC.^[56,57]

Moreover, obesity-related inflammation has been considered to be a plausible link between obesity and cancer.^[58] In general, survival of cancer cells is critically dependent on their interaction with neighboring non-malignant cells.^[59] The contribution of the tumor stroma to cancer cell survival has been widely studied. The adipocytes surrounding tumor lesions are one of the major components of the tumor stroma. Furthermore, adipose tissue can secrete signaling molecules such as adipocyte-derived cytokines (termed adipokines), pro-inflammatory cytokines, proangiogenic factors and extracellular matrix constituents.^[60] From a clinical viewpoint, obese individuals are at an increased risk of developing colon cancer, in addition to the fact that increased adiposity is associated with morbidity and mortality.^[58,61] In IBD, many inflammatory cytokines are involved in carcinogenesis, as evidenced by the elevated circulating levels of IL-6 and TNF. TNF is highly elevated in the colon of C57/BL6 mice fed with a high fat diet.^[62] Moreover, treatment with TNF-neutralizing monoclonal antibodies decreased growth of colon cancer xenografts and tumor incidence in azoxymethane (carcinogen)-treated leptin-deficient mice.^[63] These studies demonstrated that local inflammation mediated by TNF had a key role in tumor initiation in obese rodents.

Most recently, the gut microbiota has been also implicated in the initiation and promotion of CAC.^[64,65] It is thought that microbe-driven intestinal inflammation as an etiological factor contributes to CAC development;

however, better understanding of the underlying molecular mechanism needs further investigation.

Conclusion

In this review, we have discussed the links between chronic inflammation and cancer development, with special reference to GI cancers. Future studies will determine the role for this novel anti-inflammation treatment modality in the prevention of GI cancers.

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Conflicts of interest

There are no conflicts of interest.

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MicroRNAs in gastrointestinal cancer: a novel biomarker and its clinical application

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ABSTRACT

Gastrointestinal (GI) cancers remain one of the most common malignancies and are the major cause of cancer deaths worldwide. Significant advancements have improved our understanding of the pathogenesis and pathology of GI cancers, but high mortality rates, an unfavorable prognosis, and lack of clinical predictive biomarkers provide an impetus to investigate novel diagnostic/prognostic markers and therapeutic targets for GI cancers. MicroRNAs (miRNAs) are short (19-24 nucleotides), non-coding RNA molecules that regulate gene expression at the post-transcriptional level, thus playing an important role in modulating various biological processes. This includes developmental processes, proliferation, apoptosis, metabolism and differentiation, all involved in initiation and progression of various human cancers. Aberrant miRNA expression profiles have been observed in various cancer types at different stages, suggesting their potential as diagnostic and prognostic biomarkers. Due to their tumor- and tissue-specific expression profiles, stability, and the availability of robust clinical assays for their detection in serum as well as in formalin-fixed tissue samples, miRNAs have emerged as attractive candidates for diagnostic and prognostic applications. This review summarizes recent research supporting the utility of miRNAs as novel diagnostic/prognostic tools and therapeutic targets, thus potentially illuminating future treatment strategies for GI cancers.

Key words: Biomarker, gastrointestinal cancer, microRNA, therapeutic target

Introduction

Gastrointestinal (GI) cancers represent malignant tumors of the GI tract and accessory organs of digestion including esophagus, stomach, liver, biliary tract, pancreas, small intestine, large intestine and rectum. GI cancers are collectively the major cause of cancer-related morbidity and mortality worldwide.^[1] Current multimodal treatment strategies including surgery, radiotherapy, and/or chemotherapy have marginally improved curative expectations and quality of life of patients; however, the effectiveness of these new tools depends largely on the stage in which tumors are detected. Previous investigators have tried to identify more specific and sensitive novel biomarkers and therapeutic targets for better diagnosis and management of lethal GI cancers.

MicroRNAs (miRNAs) are short, non-coding RNA molecules of approximately 19-24 nucleotides involved in post-transcriptional regulation of gene expression. miRNAs bind to the 3'-untranslated region of mRNA, leading to either translational repression or

mRNA degradation initiated by miRNA-guided rapid deadenylation.^[2] It has been estimated that 60% of human protein coding genes are subject to regulation by miRNAs.^[3] They act as master regulators for many important biological processes including ontogeny, cell proliferation, apoptosis, migration, differentiation, metabolism, stress, viral infection, cancer initiation and progression and drug resistance.^[4-7] In addition, several miRNAs may also be useful for diagnostic, prognostic and therapeutic applications in GI cancers.^[8-12]

Numerous investigations on screening for altered expression of miRNAs in various types of cancer have been conducted during the past decade, with more and more functional validations in recent years. Although the majority of such studies have so far focused on miRNA profiling to identify specific miRNA species and determining their role in the biology of GI cancers, another great potential for miRNA profiling lies in their

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use as biomarkers, either in diagnosis or in prediction/monitoring of therapeutic responses. This review focuses on the most recent advances in studies on some extensively investigated miRNAs in GI cancers, particularly with regard to their potential as novel biomarkers or therapeutic targets.

Esophageal Cancer

The incidence and mortality of esophageal cancer (EC) are high, ranking eighth and sixth respectively, all types of cancer, affecting more males than females.^[13] Esophageal squamous cell carcinoma (ESCC) and esophageal adenocarcinoma (EAC) are the two main EC subtypes. Due to the potential characteristics of invasion and metastasis in esophageal carcinoma cells, the overall 5-year survival rate is poor despite advanced treatment.^[13,14] Recent discoveries have shed new light on the involvement of miRNA in EC.^[15]

miRNAs as novel diagnostic and prognostic biomarkers in EC

Guo *et al.*^[16] found aberrant expression of 46 miRNAs in EC tissues, of which 7 miRNAs may be used as biomarkers to distinguish malignant EC lesions from adjacent normal tissue. Moreover, miR-335, miR-181d, miR-25, miR-7 and miR-495 are associated with the pathological type of EC (fungating vs. medullary). miR-25 and miR-130b are associated with the degree of differentiation of EC and miR-103/107 expression level negatively correlated with survival rates miR-25 and miR-130b may also be used for early diagnosis as well as gene therapy targets for EC. Feber *et al.*^[17] found that miR-203 and miR-205 were down-regulated compared with normal epithelium in EC, while miR-21 was overexpressed in the two types of EC. miR-200c, miR-194 and miR-192 were up-regulated in EAC. Mathe *et al.*^[18] have demonstrated that the overexpression of miR-21 in non-cancerous tissue of ESCC and down-regulation of miR-375 in cancerous tissue of EAC with Barrett's esophagus (BE) were markedly associated with a worse prognosis. miR-196a was highly expressed in EAC, BE, benign and malignant junctions and highly malignant tissue and thus be used as a biomarker for screening EC.^[18-20] Among others, the overexpression of miR-129 was identified as a significant and independent prognostic factor in surgically treated ESCC patients.^[21] The expression level of miR-1322 was higher in ESCC tissue, making it possible to distinguish ESCC from healthy samples.^[22] Expressions of miR-31 and miR-142-3p correlated with histological differentiation, with high miR-142-3p expression being associated with poor prognosis. Therefore, this may be a potential independent prognostic ESCC factor.^[23] Furthermore, expression profiles of miRNAs have been found to be altered in progressive stages of EC neoplastic development, with expression levels of miR-31 and miR-31* being frequently down-regulated in EAC.^[24]

Other significant miRNAs with dysregulated expression are miR-16-2 and miR-30e, which were associated with a shorter overall and disease-free survival in all EC patients.^[25]

Several studies have demonstrated that miRNAs are consistently detectable in the circulation. The expression level of miR-21 was up-regulated and miR-375 was down-regulated in plasma of ESCC patients compared with healthy controls; patients with high plasma levels of miR-21 had greater vascular invasion and showed high correlation with recurrence.^[26] A panel of 7 serum miRNAs (miR-10a, miR-22, miR-100, miR-148b, miR-223, miR-133a and miR-127-3p) was up-regulated in ESCC and could clearly distinguish stage I/II ESCC patients from controls.^[27] Supporting the role of miRNAs in the circulation, Zhang *et al.*^[28] have found that miR-31 levels were significantly higher than controls in 523 serum ESCC samples. In addition, patients with higher levels of serum miR-31 had a poorer prognosis for relapse-free survival. miR-1322 was significantly highly expressed in ESCC serum and could be used to distinguish ESCC from healthy patients.^[22] Thus, circulating miRNAs may be used as potential biomarkers, not only for diagnostic, but also for prognostic and predictive markers in EC.

Clinical application of microRNAs in EC

Hummel *et al.*^[29] examined the impact of chemotherapy on miRNA expression in EC cells and found that 13 miRNAs were deregulated following treatment with cisplatin or 5-fluorouracil (5FU). miR-141 was most highly expressed in the cisplatin-resistant ESCC cell lines, the target of miR-141 is YAP1, which is an apoptosis-inducing gene in DNA-damaging agents.^[30] miR-296 and miR-27a are overexpressed in EC, and knockdown of miR-296 and miR-27a was found to be capable of increasing sensitivity to both P-glycoprotein-related and P-glycoprotein-non-related drugs, in turn promoting ADR-induced apoptosis in EC cells.^[31,32] Overexpression of miR-200c significantly correlated with response to chemotherapy, with this effect being associated with the Akt pathway.^[33] miR-148a up-regulation significantly increased the sensitivity to chemotherapy in the majority of cells.^[34] miR-200b/200c/429 were up-regulated in endometrial cancer and EC, and their overexpression correlated with resistance to cisplatin treatment.^[35]

Gastric Cancer

Gastric cancer (GC) is the second leading cause of cancer-related death worldwide. Approximately one million new GC cases per year were estimated to have occurred in 2010.^[36] *Helicobacter pylori* infection, Epstein-Barr virus infection, gastrin levels, germline mutations, dietary factors, and other chronic gastric conditions are all factors felt to be involved in GC development. GC is often diagnosed at an advanced

stage, accompanied by extensive invasion and lymphatic metastasis.^[37] Thus, it is important to increase the sensitivity and specificity of diagnostic markers and/or to establish methods for GC treatment and prevention of GC.^[38-40]

miRNAs as novel diagnostic and prognostic biomarkers in GC

Novel methods, such as circulating miRNA profiling, have been suggested to be useful tools for the non-invasive diagnosis of GC. Three serum miRNAs (miRs-221/744/376c) were found to distinguish GC patients from healthy controls with 82.4% sensitivity and 58.8% specificity.^[41] Moreover, miR-221 and miR-376c demonstrated significantly positive correlations with poor GC differentiation.^[41] In a validation experiment, plasma levels of miR-451 and miR-486 were higher in patients with GC compared with healthy controls, with high area under the curve (AUC) values (0.96 and 0.92).^[42] A genome-wide miRNA profile identified high serum levels of miR-378 in patients with GC, and validation yielded a high AUC (0.86).^[43] Quantitative real-time polymerase chain reaction analysis identified 5 serum miRNAs (miR-1, miR-20a, miR-27a, miR-34, miR-423-5p) to be biomarkers for GC, and their levels correlated with tumor stage.^[44] Plasma concentrations of miRNAs miR-17-5p, miR-21, miR-106a and miR-106b were higher, whereas let-7a was lower in GC patients. AUC as high as 0.879 for the miR-106a/let-7a ratio assay was achieved.^[45] High levels of miR-17 and miR-106a in peripheral blood of GC patients confirmed in another study, in which the AUC value for the combined miR-17/miR-106a assay was 0.741.^[46] These findings suggest that miRNAs are useful biomarkers for early GC diagnosis.

miRNAs have recently been used to predict the outcome of patients with GC. For example, a seven-miRNA signature (miR-10b, miR-21, miR-223, miR-338, let-7a, miR-30a-5p and miR-126) was shown to be closely associated with relapse-free and overall survival (OS) among patients with GC.^[47] High expression of miR-20b, miR-150^[48] and miR-93^[49] or down-regulation of miR-451^[50] or miR-218^[51] was also associated with poor survival, whereas there was a correlation between miR-27a and lymph node metastasis.^[48] In addition, Ueda *et al.*^[52] recently reported that miR-125b, miR-199a and miR-100 represent a progression-related signature, whereas the low expression of let-7g and high expression of miR-214 were associated with shorter OS independent of the depth of invasion, lymph node metastasis and stage.

Circulating miRNAs have been suggested to be useful prognostic markers of GC. High expression of circulating miR-17-5p/20a was an independent poor prognostic factor.^[53] Low-level expression of let-7a/let-7g/let-7f was associated with a poor prognosis.^[54,55]

miR-181b and miR-182 were also found to be novel poor prognosticators.^[56] Low expression of miR-125a-3p was associated with enhanced malignant potential, such as tumor size, lymph node, and liver metastasis, and poor prognosis, and this study suggested that miR-125a-3p is a potent prognostic marker in GC.^[57] Furthermore, miR-409-3p was found to be frequently down-regulated in patients with GC, and its expression was associated with distant metastasis.^[58]

Clinical application of miRNAs in GC

Some miRNAs have been shown to impact chemotherapy sensitivity if their levels were artificially up-regulated, others if they were down-regulated. For instance, up-regulation of miR-21 or miR-106a increased cisplatin resistance of GC cells,^[59] and Deng *et al.*^[60] showed that the up-regulation of miR-195 or miR-378 led to enhanced 5-azacytidine resistance in normal gastric cells. Up-regulation of miR-449 or miR-508-5p was demonstrated to positively impact sensitivity toward cisplatin (miR-449) or vincristine or doxorubicin (miR-508-5p).^[61,62] Interestingly, in accordance with these findings concerning the modulation of the sensitivity toward chemotherapeutic drugs via miRNAs, Bandres *et al.*^[50] reported that the up-regulation of miR-451 led to increased sensitivity of cancer cells toward radiotherapy by down-regulating macrophage migration inhibitory factor MIF. Only one research group reported the effect of miRNA down-regulation on chemotherapy resistance; Zhao *et al.*^[63] found that increased doxorubicin sensitivity in GC cells was connected with down-regulation of miR-27a.

Colorectal Cancer

Colorectal cancer (CRC) is the 3rd most common cancer and the 3rd leading cause of cancer-related death in the world, with an estimated incidence of one million new cases and a mortality of > 500,000 deaths annually.^[13] The pathogenesis of CRC typically follows a protracted stepwise progression from benign adenoma to malignant adenocarcinoma and distant metastasis, rendering screening and early diagnosis as preferred options to ease the disease burden.^[64] This also highlights the need for the development of novel screening tools and diagnostic biomarkers.

miRNAs as novel diagnostic and prognostic biomarkers in CRC

Ng *et al.*^[65] were the first to report that circulating levels of miRNAs differed in the blood plasma in CRC cases and the controls. It was found that miR-92 was expressed at higher levels in the plasma from CRC cases and could distinguish cases from healthy control patients with 70% specificity and 89% sensitivity. Another study of 120 cases and 29 controls validated these findings, showing that levels of miR-92 can discriminate between CRC cases and controls with 65% sensitivity and 82% specificity.^[66]

A similar study found that levels of miR-141 were elevated in metastatic CRC and its expression was associated with a poor prognosis, suggesting that this miRNA may be used in conjunction with carcinoembryonic antigen to detect CRC with distant metastases.^[67]

Measuring miRNAs in stool offers another non-invasive approach to detect CRC. One small study of 29 CRC cases and 8 healthy controls found that stool from CRC cases expressed higher levels of miR-21 and miR-106a.^[68] A larger study of 197 cases and 134 healthy controls investigated miRNA expression patterns of colonocytes isolated from feces and was able to demonstrate that miRNA expression patterns could distinguish cases from controls with 74% sensitivity and 79% specificity.^[69] A similar strategy found that miRNA methylation patterns from DNA isolated from stool may be promising as a screening tool for CRC.^[70] The hypermethylation pattern of miR-34b/c in stool samples could distinguish CRC cases from controls with 75% sensitivity and 84% specificity. Further tests are warranted to determine whether miRNA expression or methylation patterns in stool can be utilized, either alone or in combination with a fecal occult blood test, as an effective screening strategy for CRC.

The elevated expression of miR-21 has a robust and reproducible association with the CRC prognosis. Schetter *et al.*^[71] first reported that elevated miR-21 expression in tumors was associated with a worse survival prognosis and therapeutic outcome. The association of elevated miR-21 expression and worse survival outcomes in CRC has been validated in at least three additional studies. These include the studies of 156 Japanese CRC patients,^[72] 46 CRC patients from the Czech Republic,^[73] and 130 tumor node metastasis stage II colon cancer patients from Denmark.^[74] Additional studies have identified miRNA expression patterns that are associated with either prognosis or therapeutic outcome. Expression levels of miR-106b,^[75] miR-320,^[76] miR-498,^[76] miR-125b,^[77] miR-145,^[78] miR-185,^[79] miR-133b,^[79] miR-215^[80] and miR-17^[81] have each been reported to be associated with prognosis or therapeutic outcome. An elevated expression of Dicer, an important gene encoding an RNA nuclease involved in miRNA processing, is associated with poor prognosis in CRC.^[82] Further validation of these associations is warranted and may reveal additional prognostic classifiers.

Clinical application of miRNAs in CRC

Schetter *et al.*^[71] have shown that miR-21 expression is associated with therapeutic outcome with 5FU-based therapies. This association, in combination with the known oncogenic role for miR-21, suggests that increased miR-21 expression is, in part, responsible for resistance to 5FU. Elevated miR-21 induces resistance to 5FU in colon cancer cell lines by down-regulating DNA repair protein MutS homolog 2.^[83] Exposure of colon cancer

cells to 5FU leads to increased miR-21 expression, and this may be a response to genotoxic stress to help cells overcome the effects of 5FU.^[84] Additional *in vitro* data support the roles for altered expression of miR-140,^[85] miR-215,^[86] miR-224^[87] and miR-20a^[88] in developing chemoresistance. Further studies are warranted to determine whether expression of these miRNAs can predict response to chemotherapy and if those miRNAs can be used as therapeutic targets themselves. MiRNA replacement involves reintroducing synthetic miRNA mimics or expression vectors that will produce the miRNA of interest. This has shown promise in preclinical murine models where the reintroduction of miR-145 and miR-33a had an antitumor effect in a model of colon cancer.^[89]

Hepatocellular Carcinoma

Hepatocellular carcinoma (HCC), the most common primary liver cancer, is the 5th most frequent cancer and the third cause of cancer-related mortality worldwide.^[90] The incidence of this disease is > 600,000 cases annually.^[91,92] HCC usually develops as a consequence of underlying liver disease and is often associated with cirrhosis.^[93] Hepatitis B virus (HBV) and hepatitis C virus (HCV) viral infections, the major risk factors for HCC development, lead to liver cirrhosis and account for 75% of HCC cases.^[94,95] miRNAs have been widely reported to be involved in HCC development and may be new targets for HCC therapy.^[96-98]

miRNAs as novel diagnostic and prognostic biomarkers in HCC

Many miRNAs are dysregulated in HCC; thus, it is to be expected that circulating miRNA levels are also affected by HCC progression. The high stability of miRNAs in circulation makes them excellent biomarkers, especially for early detection.^[99] It is interesting that circulating miR-21,^[100,101] miR-222^[101] and miR-223,^[102] were up-regulated in serum/plasma of HCC patients associated with HBV or HCV. Circulating miR-21 levels were significantly higher in HCC patients than in those with chronic hepatitis and healthy controls. A receiver-operating characteristic analysis of miR-21 yielded an AUC of 0.773 when differentiating HCC from chronic hepatitis, and an AUC of 0.953 when differentiating HCC from healthy controls. Both sets of values were superior to alpha-fetoprotein (AFP) as an HCC biomarker.^[102] At the same time, the serum levels of miR-1, miR-25, miR-92a, miR-206, miR-375 and let-7f were also significantly elevated.^[103]

Serum miR-15b and miR-130b levels were also up-regulated in HCC.^[104] MiR-130b had the largest AUC (0.913), with a sensitivity of 87.7% and specificity of 81.4%, and miR-15b had the highest sensitivity of miRNAs examined (98.3%), although its specificity was very low (15.3%). The high sensitivity of circulating

miR-15b and miR-130b as biomarkers holds promise for patients with early-stage HCC, who may have low AFP levels despite the presence of disease. Similarly, serum miR-16 was found to be a more sensitive biomarker than serum AFP and Des- γ -carboxyprothrombin (DCP).^[105] The combination of miR-16, AFP, AFP-L3%, and DCP yielded the optimal combination of sensitivity (92.4%) and specificity (78.5%) for HCC overall and when the analysis was restricted to patients with tumors smaller than 3 cm.^[106] In addition, a recent meta-analysis of 8 studies showed the diagnostic value of miRNAs as follows: Pooled sensitivity 0.87 (0.72-0.98), pooled specificity 0.90 (0.76-1.00), pooled positive likelihood ratio 8.7 (3.52-97.45), pooled negative likelihood ratio 0.13 (0.02-0.31), and pooled diagnostic odds ratio 86.69 (19.06-2646.00).^[107]

Although sensitivity and stability of miRNAs as biomarkers are suitable for a clinical setting, appropriate controls must be used in a research setting because HCC is often accompanied by viral hepatitis, cirrhosis, or other underlying liver conditions.^[108] When assessing the specificity of an miRNA for detecting HCC, it is critical to ensure that patients and controls are matched, not only by age and sex, but also by etiology and severity of the underlying liver disease.

Clinical application of miRNAs in HCC

Recently, miravirsin, a locked nucleic acid-modified DNA phosphorothioate antisense oligonucleotide against miR-122, became the first miRNA-targeting drug to receive permission for clinical use.^[109] It was developed to target HCV, as the stability and propagation of this virus are dependent on a functional interaction between the HCV genome and miR-122.^[110,111] Miravirsin resulted in a dose-dependent reduction in HCV levels, without major adverse events and with no escape mutations in miR-122 binding sites of the HCV genome.^[109] The success of miravirsin is promising, not only as a novel anti-HCV drug, but also as the first trial of miRNA-targeting therapy. In addition to miravirsin, a clinical trial of MRX34 (miRNA Therapeutics, Austin, TX, USA) as a mimic of miR-34 is underway. MRX34 is a liposome-formulated mimic of the tumor suppressor, miR-34. Further study of MRX34 is being conducted by miRNA Therapeutics, which initiated a Phase I study in May 2013 to examine effects of MRX34 on unresectable primary liver cancer or advanced or metastatic cancer with liver involvement. If these oligonucleotide therapies are successful, then therapeutic options based on the numerous miRNAs deregulated during hepatocarcinogenesis appear to be promising.^[112]

Cholangiocarcinoma

Cholangiocarcinoma (CCA) is one of the most common malignancies derived from bile duct epithelial cells.^[113] Due to slow growth, late metastasis, and lack of effective

screening methods, CCA is rarely diagnosed during early stages of the disease when surgical procedures are most effective.^[114] Histopathological analyses suggest that the presence of primary sclerosing cholangitis, chronic biliary irritation, or choledochal cysts may predispose individuals to CCA.^[115] More recently, studies have identified a role for miRNAs in the development of CCA by altering different cholangiocyte features such as cell cycle, proliferation, migration and apoptosis.^[116-118]

miRNAs as novel diagnostic and prognostic biomarkers in CCA

The study by Meng *et al.*^[119] was the first to hint at the potential of miRNAs as biomarkers. It was found that miR-21 and miR-200b expression levels were predictors of gemcitabine resistance.

By sequencing and comparing the small RNA libraries of two CCA cell lines to one of a normal biliary epithelial line, Kawahigashi *et al.*^[120] identified and confirmed miR-22, miR-125a, miR-127, miR-199a/a0, miR-214, miR-376a and miR-424 as specifically expressed in normal biliary epithelial cells, but down-regulated in CCA cell lines, suggesting their use as biomarkers for diagnosis. Chen *et al.*^[121] took the approach of using miRNA sensor constructs to compare spatiotemporal activity of six miRNAs (miR-21, miR-200a, miR-200b, miR-146a, miR-155 and miR-221) in primary tissue blocks of CCA and normal control tissue grown from three patients using adeno-associated viral infections. They were unable to identify a definitive pattern between activity of each miRNA and presence of CCA over the entire time frame but when focusing on 24 h post-infection the miRNA profiles, displayed significant differences between CCA and control as well as between patients, suggesting these miRNAs play an active role in CCA. Karakatsanis *et al.*^[122] evaluated the expression levels of several miRNA species in intrahepatic cholangiocellular carcinoma and their prognostic significance. Although miR-21, miR-31, and miR-223 were found to be up-regulated and miR-122, miR-145, miR-200c, miR-221, and miR-222 to be down-regulated, the group was unable to find any correlation with clinical or pathological features. McNally *et al.*^[123] tried to investigate the predictive role of miRNAs on survival in resected CCAs and found 2 of 43 miRNA species evaluated to have the best correlation with survival. Up-regulation of miR-151-3p (41.5 months vs. 12.3 months) correlated better than down-regulation of miR-126 (21.9 months vs. 15.1 months). However, concomitant dysregulation of both showed the best overall correlation with survival (58.7 months vs. 15.1 months).

In a novel approach using bile, Li *et al.*^[117] were able to demonstrate the presence of miRNAs in extracellular vesicles in bile and analyzed the miRNA expression of 74 different species that could be reliably amplified. By using a multivariate organization of the combinatorial

alterations, they were able to establish a miRNA-based panel of five different miRNA species that were able to distinguish CCA from primary sclerosing cholangitis or other biliary obstructions. This may offer an original way to make an early diagnosis of CCA.

Clinical application of miRNAs in CCA

Meng *et al.*^[119] provided the first evidence for the involvement of miRNAs, as well as its first therapeutic use in CCA. When they compared the miRNA expression patterns between malignant and non-malignant human cholangiocytes, miR-21, miR-141 and miR-200b were highly overexpressed in malignant cells and inhibition of miR-21 and miR-200b sensitized cells to gemcitabine. Treatment of xenografts with gemcitabine changed several miRNA expression levels and modulated phosphatase and tensin homolog-dependent PI3 kinase signaling. Okamoto *et al.*^[124] sought to investigate the role of miRNAs in chemoresistance and compared miRNA profiles of two CCA cell lines distinguished by gemcitabine resistance. They identified miR-29b, miR-205 and miR-221 whose ectopic overexpression could restore gemcitabine sensitivity in the resistant cell line. They showed that knockdown of two predicted targets, PIK3R1 (miR-29b and miR-221 target) and MMP-2 (miR-29b target only), via small interfering RNA conferred the same level of gemcitabine to the resistant cell line. They showed that miR-125a-5p was up-regulated in the resistant cell line and that inhibition of miR-125a-5p inhibited cell proliferation in that cell line independent of chemoresistance.

Pancreatic Cancer

Pancreatic ductal adenocarcinoma (PDAC) is a lethal malignancy with a poor prognosis due to advanced stage disease at initial diagnosis, frequent recurrence, and the absence of treatment strategies that specifically and effectively target these tumors.^[125] Only 15% of PDAC patients are candidates for surgical resection at the time of diagnosis.^[126] Chemotherapy is considered to be the main treatment option for unresectable cases, while chemoradiotherapy may improve the survival and quality of life.^[127,128] However, PDAC is still extremely resistant to the currently available regimens. Exploring miRNAs as therapeutic targets and biomarkers for the diagnosis and prognosis of PDAC is of interest.^[129-131]

miRNAs as novel diagnostic and prognostic biomarkers in pancreatic cancer

Kong *et al.*^[132] found that three serum miRNAs, including miR-196a, were differentially expressed in PDAC compared with control groups. Another investigation by Wang *et al.* showed that the expression levels of four miRNAs in plasma (miR-21, miR-210, miR-155 and miR-196a) were significantly higher in patients with PDAC.^[133] Li *et al.*^[134]

measured 735 circulating miRNAs in PDAC cases, and controls sera and miR-1290 were found to show the best diagnostic performance. Kawaguchi *et al.*^[135] found that plasma miR-221 concentrations were significantly higher in PDAC patients than those with benign pancreatic tumors and controls. In recent studies, miRNAs were also found to be useful as diagnostic markers for precursor lesions of PDAC. Caponi *et al.*^[136] found that miR-21 and miR-155 were up-regulated in invasive intraductal papillary mucinous neoplasms (IPMNs) compared with non-invasive IPMNs. Further multivariate analyses showed that high miR-21 expression emerged as an independent prognostic biomarker in invasive IPMNs with a poor survival. Lubezky *et al.*^[137] also found miRNAs were useful in identifying IPMN with high risk for malignant transformation. The expression levels of 15 miRNAs, including miR-217, miR-216a, miR-21 and miR-155, were significantly different between two IPMN subgroups (low/moderate-grade dysplastic IPMNs vs. high-grade dysplastic IPMN) and invasive cancer with IPMN. Pancreatic cysts are a group of lesions with heterogeneous malignant potential. Farrell *et al.*^[138] showed miR-21 and miR-221 in pancreatic cyst fluid was associated with invasive cancer.

With regard to survival, strong miR-21 expression was predictive of poorer outcomes compared with absent or faint/focal miR-21 expression in patients with node-negative PDAC.^[139] Jamieson *et al.*^[140] found that high expression of miR-21 and reduced expression of miR-34a were significantly associated with a poor OS in global miRNA microarray expression profiling. Frampton *et al.*^[141] found that a high level of a combination of miR-21, miR-23a and miR-27a was associated with shorter survival times after surgical resection. While strong expression of miR-21 predicted limited survival in PDAC patients, high expression of miR-200c, a member of the miR-200 family, is a good prognostic sign.^[142,143] Elevated levels of miR-155, miR-203, miR-210 and miR-222 expression in PDCA were significantly associated with an increased risk of death compared to patients with reduced expression of these miRNAs.^[144] A subgroup of six miRNAs (miR-452, miR-105, miR-127, miR-518a-2, miR-187 and miR-30a-3p) was found to identify long-term survivors with node-positive disease from those dying within 24 months.^[145] In addition, increased expression levels of miR-155, miR-203, miR-210 and miR-222 were found to be significantly associated with poorer survival.^[144,146] In some recent studies, reduced expressions of miR-218^[147] and miR-130b^[148] in PDAC tissues were found to correlate with a poor prognosis.

Clinical application of miRNAs in pancreatic cancer

Some prognostic miRNAs also play a role in the efficacy of anticancer therapy, thus presenting themselves as new therapeutic possibilities. For instance, PDAC cells

expressing elevated levels of miR-21 are chemoresistant to gemcitabine and reduce the efficiency of apoptosis induction.^[148,149] miRNAs can be targeted. For example, inhibition of mir-21 by its antagonist led to the cessation of tumor growth and induction of apoptosis *in vitro* and *in vivo* (animal experimental model).^[150] Another therapeutic option comes from a possibility-of-function recovery of miR-34a, a potent pro-apoptotic component involved in p53-mediated apoptosis. As shown by Ji *et al.*,^[151] restoration of miR-34a may substitute the function of inactivated TP53 gene. It has been shown that miR-10a promotes the metastatic behavior of the pancreatic cancer (PC) and that its expression is regulated by retinoids.^[152] The use of retinoic acid receptor antagonists inhibit miR-10a expression and stop metastasis of PDAC cells.^[152] In contrast, miR-146a suppresses invasion of PC cells. However, its expression is lowered in PC compared to normal pancreatic tissue. Finally, use of non-toxic natural compounds which increase expression of miR-146a is also considered to be a promising approach to block both invasion and metastases.^[153]

Perspective

Many miRNAs can be used in the diagnosis of cancer, in determining the patient prognosis or as therapeutic targets. We must develop more stringent protocols for collecting and analyzing samples (to avoid variations in sample processing or histologic characterization) and to validate each finding in large independent cohorts. This approach will lead to the selection of the best candidate miRNAs for further study, the development of highly reproducible results, and reduction in inter-study discrepancies. New miRNAs are continuously being discovered, and profiling technologies are rapidly changing. Thus, creating a standardization process for integrating data will be challenging. Development of effective *in vivo* delivery systems is also required if miRNAs are to be used as therapeutics.

Although many additional studies must be performed, miRNAs appear to have many useful clinical applications for patients with GI cancers and other GI disorders. The GI community should look forward to these studies with great anticipation.

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Conflicts of interest

There are no conflicts of interest.

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Molecular insights into colorectal cancer stem cell regulation by environmental factors

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ABSTRACT

Colorectal cancer remains a significant cause of cancer-related mortality worldwide, mainly because of tumor relapse and metastases. Cancer stem cells (CSCs) are considered to be the main cause of resistance to chemotherapeutic agents, as well as being responsible for distant metastases. Although CSCs themselves possess innate abilities for self-renewal and differentiation, the environment surrounding CSCs provides oxygen, nutrients and secreted factors, and also supports angiogenesis, thus it's responsible for maintaining their CSC properties. Furthermore, extensive investigations have revealed that obesity, accompanied by excess visceral adipose tissue, induces chronic inflammation, and is linked to the risk and progression of several gastrointestinal cancers, through modulating the capacities of the CSCs. This review presents the evidence linking colorectal CSCs and their environment and summarizes our current understanding of the molecular mechanisms underlying this relationship.

Key words: Cancer stem cells markers, colorectal cancer stem cell, nutrient, obesity, tumor microenvironment

Introduction

Colorectal cancer (CRC) is the fourth-leading cause of cancer-related deaths worldwide.^[1] Although the incidence of CRC has started to decline in developed countries, it continues to increase in developing countries.^[2] Environmental factors, including chronic inflammation, obesity, metabolism and nutrition, have become recognized as major contributors to the development of CRC.^[3-6] Dietary fat intake and obesity have been shown to be significantly involved in CRC progression through an increased risk of gene mutation, epigenomic alterations, and effects on the equilibrium of various adipokines.^[7-11] Chronic inflammation is also considered to be a risk factor for CRC,^[6] and inflammatory mediators and substances such as interleukin (IL)-6, tumor necrosis factor- α (TNF- α), and reactive oxygen species have been shown to affect CRC development.^[12-15] The clearest link between chronic inflammation and CRC is seen in patients with inflammatory bowel disease, which has been reported to promote tumorigenesis by altering the microbial composition in the gut and supporting the expansion of microorganisms with genotoxic capabilities.^[16]

Cancer stem cells (CSCs) are tumor cells that possess capabilities for self-renewal, clonal tumor

initiation and clonal long-term repopulation.^[17,18] The discovery of colorectal CSCs highlighted the existence of intratumoral heterogeneity, revealing the presence of tumor cells expressing markers characteristic of immature cells and with increased abilities to resist chemotherapy and to seed secondary tumors.^[19-21] CSCs were initially considered to be a cell population with well-defined phenotypic and molecular features. However, emerging evidence has revealed that certain cancer cells exhibit plasticity, and can change reversibly from stem to non-stem cells under the regulation of genetic, epigenetic and microenvironmental factors.^[22-25] In this review, we focused on accumulating new evidence indicating that microenvironmental factors maintained colorectal CSC properties responsible for promoting tumor development and metastasis.

Markers for Colorectal CSCs

CSCs have been isolated from cancer tissues using flow cytometry with specific surface markers. Several molecules have been proposed as colorectal

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CSC markers, including CD133, CD44, CD24, CD166, Lgr-5, and aldehyde dehydrogenase 1 (ALDH1) [Table 1].^[26] CD133, a pentaspan transmembrane glycoprotein,^[27] was one of the first colorectal CSC markers to be identified.^[19,20] However, although selecting CRC cells based on AC133 positivity, an epitope of the CD133 protein identifies the tumorigenic and clonogenic population.^[28] CD133 expression has been detected throughout the normal gastrointestinal tract and is not restricted to the stem cell compartment.^[29,30] In addition, both CD133+ and CD133- metastatic CRC cells were able to form new tumors, suggesting that CD133 may not be a reliable marker of CSCs.^[29]

The cell adhesion molecule CD44 has been identified as a cell surface marker associated with CSCs in several types of tumor.^[31] CD44+ cells exhibited CSC properties, and a single cell could form a sphere *in vitro*, and a xenograft tumor resembling the original lesion *in vitro*.^[32] Overexpression of CD44 in CRC has been associated with depth of invasion and lymph node involvement and is shown to be an independent predictor of overall survival.^[33] Although CD44, like CD133, is not a specific marker for colorectal CSCs, it is possible that a combination of these two markers may be more reliable for detecting colorectal CSCs than either marker alone.^[34]

In addition to cell surface markers, activities of certain pathways or enzymes may also act as markers of stemness. For instance, normal colorectal stem cells can be identified by the activity of ALDH1, a detoxifying enzyme that oxidizes intracellular aldehydes.^[35,36] ALDH1+ cells were sparse and restricted to the bottom of normal crypts, where stem cells reside but were increased in number and distributed further up the crypts during progression from normal epithelium to adenoma.^[37] In addition, implantation of ALDH1+ colon cancer cells into NOD/SCID mice generated xenograft tumors, whereas ALDH1- cells did not.^[37] These findings indicate that ALDH1 activity may be a useful colorectal CSC marker.

Other markers include CD166, epithelial cell adhesion molecule, CD29, CD24, CD26, Msi-1, Lgr-5, and Wnt activity/ β -catenin.^[38-42] The presence of these molecules has been associated with stemness characteristics both *in vitro* and *in vivo*. These markers were also used to enrich isolated CSCs further to enhance their tumorigenic ability. The transcription factors Oct-4 and Sox2 are also promising CSC markers, given their roles in cell renewal. Oct-4 and Sox2 levels have been shown to be elevated in CRC and to correlate with increased CSC proliferation and poor prognosis.^[43,44] Other pluripotency genes, Nanog, Lin-28, Klf-4, and c-myc, are regarded as promising surrogate markers, given that they appear to facilitate a shift towards an undifferentiated state.^[45]

Table 1: CRC stem cell markers

Marker	General function	Significance	References
CD133 (Prominin-1)	Pentaspan transmembrane glycoprotein	Tumor initiation in xenografts, colony formation, correlation with: poor prognosis, survival, metastasis, resistance to therapy	[28-31,41,43,45]
CD44	Cell adhesion molecule, hyaluronic acid receptor	Tumor initiation in xenografts, colony formation, association with tumor stage, lymph node infiltration, survival	[32-36,41,43,45]
ALDH1-	Detoxifying enzyme	Tumor initiation in xenografts, further enrichment, transition from colitis to cancer, mitochondrial isoform is increased in CRC	[37-39,41]
CD166 (ALCAM)	Cell adhesion molecule	Tumor initiation in xenografts, colony formation, further enrichment, correlation with prognosis and survival	[41,45]
EpCAM	Cell adhesion molecule	Expression in CD133 ⁺ or CD44 ⁺ cells	[41]
CD29 (β 1-integrin)	Receptor for ECM	Colony formation elevated in CRC, association with tumor stage	[41,45]
CD24	Cell adhesion molecule	Clonogenic ability, multilineage potential, further enrichment, correlation with invasiveness, differentiation, and survival	[41,45]
CD26	Cell surface glycoprotein	Tumor initiation and metastasis formation in a mouse model	[43]
Msi-1	Maintenance of the undifferentiated state	Expression in CD133 ⁺ cells and spheroid cultures, association with tumor stage	[22]
Lgr-5	Wnt target gene, crypt base restriction	Tumorigenicity, poor prognostic factor, metastasis formation, adenoma development in APC knockout mice	[40-42,44,45]
Wnt activity/ β -catenin	Maintenance and proliferation of the SC reservoir	Associated with clonogenicity and tumorigenicity, detection of low stage CRC cases with high risk of relapse	[40-42,44,45]
Oct-4, Sox2, Nanog, Lin-28, Klf-4, c-Myc	Transcription factors	Correlation with poor prognosis, relapse, distant recurrence, resistance to therapy	[46-48]

ALDH-1: Aldehyde dehydrogenase-1; CRC: Colorectal cancer; ALCAM: Activated leukocyte cell adhesion molecule; EpCAM: Epithelial cell adhesion molecule; ECM: Extracellular matrix; Lgr-5: Leucine-rich repeat containing G protein-coupled receptor 5; Msi-1: Musashi-1; SC: Stem cell; APS: Adenomatous polyposis coli

Colorectal CSCs Niche in the Tumor Microenvironment

Tissue stem cells reside in their surrounding microenvironment, known as the stem cell niche, and play an essential role in maintaining tissue homeostasis through their abilities of self-renewal and differentiation.^[46,47] 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.^[48] The tumor microenvironment surrounding cancer cells contains multiple cell types including immune cells, endothelial cells, and fibroblasts, in addition to the extracellular matrix. Recent evidence suggests that cancer cells interact with their microenvironment and each other by secreting growth factors, cytokines, and proteases. Furthermore, the properties of the CSCs depend on the CSC niche, which regulates their proliferation and differentiation, as well as those of the tissue stem cells.

Mesenchymal stem cells (MSCs) have been shown to be recruited into the tumor stroma, and to enhance tumor growth and metastasis in CRC.^[49] MSCs are considered as potential precursors of carcinoma-associated fibroblasts (CAFs, also known as tumor-associated fibroblasts), which play a key role in tumor progression in various types of cancer, including CRC.^[50-52] Carcinoma-cell-derived IL-1 was shown to induce prostaglandin E2 (PGE2) secretion by MSCs, and the resulting PGE2 then acted in an autocrine manner with ongoing paracrine IL-1 signaling to induce expression of cytokines by the MSC, thus creating a CSC niche.^[53] A recent study demonstrated that CRC cells can induce adjoining bone-marrow-derived MSCs to exhibit the typical characteristics of CAFs *in vitro*, and activated Notch signaling mediates transformation of bone-marrow-derived MSCs to CAFs through the downstream TGF- β /Smad signaling pathway.^[54] Cytokines secreted by CAFs, including hepatocyte growth factor, osteopontin, and stromal-derived factor 1 α , increase CD44v6 expression in colorectal CRCs, which in turn promote migration and metastasis.^[55] Another study demonstrated that CSCs were resistant to conventional chemotherapy and that chemoresistance was also increased by CAFs. In this study, chemotherapy-treated human CAFs promoted CSC self-renewal and *in vivo* tumor growth associated with secretion of cytokines and chemokines, including IL-17A.^[56]

The Wnt/ β -catenin signaling pathway has been shown to play critical roles during the transition from normal colorectal mucosa to adenocarcinoma.^[57-59] The tumor microenvironment may play a central role in malignant transformation by locally modifying β -catenin activity in tumor cells, thus contributing to tumor growth and cancer stemness.^[60] Likewise, myofibroblast-secreted factors, especially hepatocyte growth factor, activated Wnt signaling and restored the CSC phenotype in more differentiated tumor cells both *in vitro* and *in vivo*.^[61]

Several studies have reported that CSCs reside in perivascular niches in certain types of cancer.^[62-64] Endothelial-cell-derived, soluble Jagged-1 led to Notch activation in colorectal CSC cells in a paracrine manner, thus promoting the CSC phenotype.^[65]

Hypoxia is known to play pivotal roles in cell survival, angiogenesis, tumor invasion and metastasis, and is involved in the maintenance of self-renewal and the undifferentiated state of CSCs in various types of tumors.^[66-68] According to a study of colorectal cell line-derived CSCs, hypoxia maintained their stem-like phenotype and prevented differentiation of enterocytes and goblet cells by regulating CDX1 and Notch1.^[69]

Obesity, Nutrients, and Colorectal CSCs Properties

Obesity and visceral adiposity are closely related to disorders such as diabetes, cardiovascular disease, and increased risk of various cancers, including CRC.^[4,70,71] Although a meta-analysis showed that an increase in the body mass index in men was associated with a relative CRC risk of 1.24,^[72] the relationship between increased body mass index and CRC risk in women is inconsistent. It is possible that the insulin and the insulin-like growth factor-1 axis may play different roles in colorectal carcinogenesis in men and women.^[4,73]

Visceral obesity is associated with increased infiltration of inflammatory cells such as macrophages and T-cells into the adipose tissue, together with low-grade inflammation.^[74-77] Adipose tissues produce various growth factors, hormones, and cytokines known as adipocytokines, including leptin, resistin, visfatin, adiponectin, and numerous inflammatory mediators such as TNF- α , IL-6, IL-8, IL-10, and IL-1 receptor agonists. These adipose-derived factors have demonstrated an intimate involvement in increased risk of CRC.^[4] In addition to adipocytokine-mediated inflammation, dyslipidemia, insulin resistance, and activation of the renin-angiotensin system may also contribute to CRC development.^[78]

Colorectal CSC clones have been reported to express leptin receptors and to respond to leptin by cell proliferation, activation of the ERK1/2 and PI3K/AKT signaling pathways, enhanced growth in soft agar, and improved sphere formation associated with E-cadherin overexpression. Moreover, leptin counteracted the cytotoxic effects of 5-fluorouracil.^[79] Other authors reported that leptin acted as a growth factor for carcinogen-induced colorectal tumors in a mouse model of obesity. They also showed that leptin receptor expression levels were markedly increased in colorectal tumors compared with normal epithelium, in association with activation of Wnt signaling.^[80]

Chronic inflammation is considered to be a risk factor for CRC, and an obvious association has been demonstrated between the incidence of CRC and inflammatory

bowel diseases, such as ulcerative colitis and Crohn's disease.^[81,82] A recent study showed that the inflammatory lipid mediators leukotriene D4 and PGE2 increased the ALDH+ cell population, colony formation capacity, and tumor growth in a xenograft model of colon cancer.^[83]

A high-fat diet can cause changes in the composition of the intestinal microbiota, and affect gut immune and inflammatory effectors implicated in intestinal tumorigenesis.^[84-86] In contrast, omega-3 polyunsaturated fatty acids (PUFAs) have shown substantial benefits in patients with the chronic inflammatory disease. In a placebo-controlled, randomized controlled trial, administration of omega-3 PUFAs decreased polyp number, size, and overall burden in patients with familial adenomatous polyposis.^[87] Omega-3 PUFAs were shown to inhibit proliferation and angiogenesis, and exert a pro-apoptotic effect in several *in vitro* models of CRC.^[88-91] One possible molecular mechanism involves the G-protein-coupled receptor 120, which functions as an omega-3 fatty acid receptor/sensor in pro-inflammatory macrophages and mature adipocytes and represses the production of TNF and IL-6, as well as macrophage-induced tissue inflammation.^[92,93] Furthermore, omega-3 PUFAs down-regulated the expression of CRC stem-like cell marker CD133, and up-regulated the colorectal epithelium differentiation markers cytokeratin 20 and mucin 2.^[94] A recent study revealed that the low-cytotoxic combination of eicosapentaenoic acid-free fatty acid, epigallocatechin-3-gallate, and grape-seed extract (GSE) inhibited mammalian target of rapamycin signaling and thus reduced cell proliferation and induced apoptosis in CRC cells.^[95] GSE pre-treatment of adipocytes decreased their growth-promoting effects on CRC cells. In addition, adipocyte-conditioned media collected after chronic and acute pre-treatment with GSE significantly reduced the chemotactic properties of adipocytes toward CRC cell invasion. Finally, GSE decreased the expression of CD44 and inhibited adipocyte-mediated pro-tumorigenic signals in CSC-enriched colonospheres.^[96] Overall, these findings indicate a close link between obesity and chronic inflammation, leading to CRC progression through enhanced colorectal CSC properties, whereas some nutrients decrease the expression of CSC markers and attenuate the properties of CSCs.

Conclusion

The microenvironment surrounding cancer cells forms the CSC niche, allowing them to give rise to a hierarchy of proliferative and differentiating cells. Targeting the innate pathways and molecules between colorectal CSCs and their environment may thus represent a promising therapeutic strategy, and may provide a complementary approach to conventional therapies that target the malignant cells themselves. Anti-tumorigenic agents related to nutrients in the microenvironment may have particular potential to eliminate the population of

colorectal CSCs. Further understanding of the molecular mechanisms underlying the regulation of CSC properties by environmental factors may lead to the development of potential therapeutic targets for patients with CRC.

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Conflicts of interest

There are no conflicts of interest.

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Trends in clinical use of targeted therapy for gastrointestinal cancers

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ABSTRACT

Targeted drugs therapies that block the molecular pathways involved in the development and progression of gastro-intestinal (GI) cancers have recently gained considerable attention. In addition to agents targeting vascular endothelial growth factor (VEGF), epidermal growth factor receptor, the multi-kinase inhibitor, and regorafenib have also become available for the treatment of metastatic colorectal cancer patients. Currently, trastuzumab, an antibody targeting human epidermal growth factor receptor-2 (HER-2), in combination with cytotoxic drugs is considered as the standard treatment for patients with HER-2 positive gastric cancer (GC). The efficacy of ramucirumab, a human monoclonal antibody that inhibits VEGF from binding to its receptor in GC, has also been recently demonstrated. At present, a great number of novel targeted drugs are in pre-clinical or clinical studies. In this review, we summarize trends in the use of molecularly targeted drugs that have proven to be effective for treating GI cancers, with a focus on emerging strategies for personalized treatment.

Key words: Gastro-intestinal tumors, molecular pathways, molecular targeted drug

Introduction

Many targeted drugs have been studied to target the molecular pathways involved in the development of gastro-intestinal (GI) cancers. Targeted drugs therapies that block the molecular pathways involved in the development and progression of GI cancers have recently gained considerable attention. Several molecular pathways were reported. Vascular endothelial growth factor (VEGF) and epidermal growth factor receptor (EGFR), the multi-kinase inhibitor, regorafenib, have also become available for the treatment of metastatic colorectal cancer (mCRC) patients. Currently, trastuzumab, an antibody targeting human epidermal growth factor receptor-2 (HER-2), in combination with cytotoxic drugs is considered to be the standard treatment for patients with HER-2 positive gastric cancer (GC). The efficacy of ramucirumab, a human monoclonal antibody (mAb) that inhibits VEGF from binding to its receptor in GC, has also been recently demonstrated.

Although the above improvements have reduced GI cancers mortality in the past few decades, there is sufficient evidence suggesting that the majority of patients undergoing drug therapy will not benefit and will instead experience severe and even lethal adverse drug events. Therefore, new and better molecular targeted

therapies are needed. At present, a great number of novel targeted drugs are in pre-clinical or clinical studies.

The aim of this review is to provide a comprehensive overview of the state of art, focusing on the new emerging strategies in the personalized treatment of GI cancers and discussing about the possible implications for GI cancers therapy.

The Main Pathways Targeted in Gastro-intestinal Tumors

Many targeted drugs that block the molecular pathways involved in the development and progression of GI tumors have been studied. Some of these agents are most efficacious in combination with conventional chemotherapy regimens. The molecular targeted drugs that have been approved for the treatment of GI cancers are summarized in Table 1. We have reviewed representative pathways that serve as targets in GI cancers.

Vascular endothelial growth factor pathway

Angiogenesis is the process of new capillary formation from pre-existing blood vessels, and it

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Table 1: Approved molecular targeted drugs in advanced gastro-intestinal tumors

Primary cancer site	Targets	Drugs	OS (month)	Reference
GC	HER-2	Trastuzumab	13.8 (first-line)	[51]
	VEGFR-2	Ramucirumab	9.6 (second-line)	[53]
CRC	VEGF	Bevacizumab	20.3 (first-line)	[63]
		Aflibercept	13.5 (second-line)	[84]
	VEGFR, BRAF, KIT, RET, PDGFR EGFR	Regorafenib	6.4 (third-line)	[81]
		Cetuximab	24.9 (first-line)	[75]
		Panitumumab	26.0 (first-line)	[78]

HER2: Human epidermal growth factor receptor 2; VEGFR-2: Vascular endothelial growth factor receptor-2; VEGF: Vascular endothelial growth factor; VEGFR: Vascular endothelial growth factor receptor; BRAF: V-Raf murine sarcoma viral oncogene homolog B1; KIT: Mast/stem cell growth factor receptor; RET: Rearranged during transfection; PDGFR: Platelet-derived growth factor receptor; OS: Overall survival; EGFR: Epidermal growth factor receptor; CRC: Colorectal cancer; GC: Gastric cancer

plays an important role in the growth and spread of cancers.^[1] Neovascularization promotes tumor growth by supplying nutrients, oxygen and growth factors that promote tumor cell proliferation.^[2,3] VEGF was first isolated in 1983 as a factor that increases vascular permeability in tumors.^[4] The VEGF family of proteins comprises VEGF-A, -B, -C, -D and -E, and structurally resembles the platelet-derived growth factor (PDGF) and placenta growth factor (PLGF) families of proteins. These growth factors bind selectively, but with different affinity, to at least five distinct receptors.^[5-7] Many cytokines and growth factors, including PDGF, tumor necrosis factor, transforming growth factor (TGF)- α , TGF- β , fibroblast growth factor (FGF)-4, keratinocyte growth factor/FGF-7, EGF, interleukin (IL)-1 α , IL-1 β , IL-6 and insulin-like growth factor (IGF)-1, are involved in upregulating *VEGF* gene expression.^[8] Overexpression of VEGF has been associated with increased microvessel density, tumor invasion, metastasis and thus with poor prognosis in many types of cancers.^[9]

Epidermal growth factor receptor pathway

The EGFR family consists of four homologous receptors: The EGFR (ErbB1/EGFR/HER-1), ErbB2 (HER-2/neu), ErbB3 (HER-3) and ErbB4 (HER-4).^[10] EGFR is a 170 kDa cell surface tyrosine kinase (TK) transmembrane receptor that initiates signaling cascades leading to cell proliferation, motility, adhesion, invasion, cell survival and angiogenesis.^[11] Mutation in the TK domain of the *EGFR* gene has been found in several types of cancers and has become a therapeutic target in non-small cell lung cancer.^[12] Overexpression and/or amplification of HER-2 has been observed in various cancers,^[13-15] including breast, esophageal and GCs at 7-34% frequency,^[16,17] and several studies have shown that HER-2 is an important biomarker and a key driver of tumorigenesis.^[18] Therefore, blockade of the EGFR family should lead to the inhibition of cell growth, thereby constituting an effective anti-cancer therapy.^[19] However, cross-talk between the various ErbB receptors that may induce drug resistance has been demonstrated.^[20] Because the intra-cellular space is vastly complex, targeting more

than one signaling pathway or blocking multiple targets within a single pathway may be necessary to effectively suppress cancer growth.

Phosphatase and tensin homolog-phosphoinositide 3-kinase-AKT-mammalian target of rapamycin pathway

Phosphoinositide 3-kinases (PI3Ks) are a family of lipid kinases that phosphorylate the 3'-hydroxyl group of phosphoinositides with the conversion of phosphatidylinositol-4, 5-bisphosphate to phosphatidylinositol-3, -4, 5-trisphosphate (PIP3). PIP3 is a critical second messenger that activates protein kinase B (AKT) through phosphorylation. Once activated, phospho-AKT phosphorylates up to 100 other proteins, including the mammalian target of rapamycin (mTOR), which is part of the mTOR complex (mTORC) 1 and mTORC 2.^[21,22] The activation of mTOR increases cellular proliferation and survival and decreases apoptosis. In normal tissue, this pathway is negatively regulated by the tumor suppressor phosphatase on chromosome 10 (phosphatase and tensin homolog), which targets the lipid products of PI3K for dephosphorylation.^[23]

Ras-Raf-MEK-extra-cellular-signal-regulated kinase pathway (MAPK pathway)

The Raf/mitogen-activated protein kinase (MAPK)/extra-cellular-signal-regulated kinase (ERK) pathway is an important pro-survival signaling pathway, that is, primarily involved in cell growth and survival and regulation of cellular differentiation. This pathway transduces extra-cellular signals from membrane-bound TK receptors, such as EGFR, VEGF receptor (VEGFR), IGF receptor (IGFR), hepatocyte growth factor receptor (c-MET) and PDGF receptor (PDGFR), to the nucleus. Binding of growth factors results in receptor phosphorylation, which activates an adapter molecule complex. This sequence in turn activates the Raf/mitogen-extra-cellular protein kinase (MEK)/ERK pathway, which triggering a cascade of specific phosphorylation events.^[24] Within this pathway, the small GTPase Ras and the serine/threonine kinase Raf are the key signal regulators.^[25] Intermediate

signaling is regulated by MEK1 and MEK2, which are responsible for phosphorylating and activating the final downstream signaling molecules ERK1 and 2.^[23] ERK1/2 regulates cellular activity by acting on more than 100 substrates, both in the cytoplasm and nucleus. Ras also regulates the PI3K/AKT/mTOR, the phospholipase C/protein kinase C, and the Ral guanine nucleotide dissociation stimulator pathways.^[26,27]

Wnt pathway

Extensive descriptions of the roles of Wnt signaling in development and disease can be found in recent reviews.^[28,29] The canonical Wnt/ β -catenin signaling pathway involves the sequestration of β -catenin from a destruction complex, which consists of adenomatous polyposis coli glycogen synthase kinase 3- α , casein kinase 1 and axin. The activation of Wnt/ β -catenin signaling is important for both the initiation and progression of cancers in various tissues.^[30] Therefore, the disruption of Wnt/ β -catenin signaling represents an opportunity for rational cancer chemoprevention and therapy.^[30] In CRC, 90% of all tumors have a mutation in a key regulatory factor of the Wnt/ β -catenin signaling pathway that results in pathway activation, and up to 80% of tumors exhibit nuclear accumulation of β -catenin.^[31-33]

Nuclear factor- κ B pathway

In recent years, several studies have revealed the connection between inflammation and carcinogenesis.^[34,35] In chronic inflammation, cytokines and chemokines produced by inflammatory cells propagate a localized inflammatory response and enhance the survival of pre-malignant cells by activating the nuclear factor- κ B (NF- κ B) pathway. NF- κ B is aberrantly activated in 50% of CRC patients and those with colitis-associated tumors, and mouse studies have established that NF- κ B plays a role in the development of colitis-associated cancer.^[36,37] As the NF- κ B pathway plays a pivotal role in apoptosis, tumor promotion and maintenance, inhibitors of this signaling pathway would be useful in CRC therapy. Non-steroidal anti-inflammatory drugs (NSAIDs) exhibit anti-neoplastic activities in the colon.^[38] Stimulation of NF- κ B expression is inhibited by various NSAIDs, indicating that NSAIDs may act as chemopreventive agents. Several studies, including randomized trials, have shown that regular use of NSAIDs is associated with decreased CRC incidence and mortality.^[39,40]

Clinical Application of Targeted Drugs

Esophageal cancer

Esophageal cancer is the eighth most frequent cause of cancer death and is increasing worldwide.^[41] This malignancy comprises two major histologic types, esophageal squamous cell carcinoma (ESCC) and esophageal adenocarcinoma (EAC). ESCC and EAC

differ substantially in their underlying etiology and tumorigenesis. A tri-modal treatment strategy consisting of radiotherapy, chemotherapy and surgery is standard for patients with local and/or advanced cancer of the esophagus.^[42,43] Unfortunately, as the 5-year survival rate remains < 15% the majority of patients at advanced stages of the disease fails to benefit from these treatments,^[44] and more effective therapies are eagerly awaited. Therefore, clinical trials of targeted drugs as monotherapy or in combination with conventional chemotherapy have been recently conducted for patients with esophageal cancer. However, a recent randomized Phase III trial demonstrated that the addition of cetuximab, a humanized mouse EGFR mAb, to capecitabine-cisplatin provided no additional benefit to chemotherapy alone in the first-line treatment of advanced esophagogastric AC.^[45] Similarly, the addition of panitumumab; another EGFR mAb to epirubicin; oxaliplatin and capecitabine did not increase overall survival (OS) of patients with advanced esophagogastric AC.^[46] However, nimotuzumab, a humanized EGFR mAb, in combination with standard chemotherapy (cisplatin plus 5-fluorouracil [5-FU]), has shown a good therapeutic response in a pilot study of patients with ESCC.^[47]

VEGF is up-regulated in EAC, and overexpression of VEGF protein has been reported as a negative prognostic marker in ESCC.^[9] Therefore, VEGF may be a potential therapeutic target in esophageal cancers. Although Phase II trials demonstrated that the addition of bevacizumab to conventional chemotherapy improved response rates (RRs) in patients with esophagogastric AC,^[48] no Phase III trial has demonstrated a survival benefit of bevacizumab.^[49]

The efficacy of molecular targeted drugs for esophageal cancer is still controversial. Further investigations to elucidate molecular mechanisms of esophageal cancer are needed to establish effective targeted treatment strategies.

Gastric cancer

GC is the fourth most commonly diagnosed cancer and the second leading cause of cancer mortality worldwide.^[50] Despite the recent progress in cancer treatment, the prognosis of patients with advanced GC remains poor. The understanding of molecular pathways involved in gastric carcinogenesis offers novel treatment options. When compared with chemotherapy alone, the HER-2-targeting antibody trastuzumab in combination with capecitabine/cisplatin was shown to improve the survival of advanced GC patients harboring HER-2 overexpression caused by gene amplification.^[51] Another agent with promising results in clinical trials is ramucirumab, an antibody targeting VEGFR-2.^[52,53] However, clinical trials have failed to demonstrate the benefit of agents targeting EGFR (cetuximab, panitumumab),^[45,46] VEGF-A (bevacizumab)^[54] or mTOR (everolimus).^[55] The results of Phase III trials to

evaluate the efficacy of molecular targeted drugs in GC are summarized in Table 2.

Trastuzumab

Trastuzumab is a recombinant humanized mAb directed against the extra-cellular domain of HER-2. Amplification or overexpression of HER-2 has been observed in 7-34% of GC.^[16,17,56] A recent large-scale Phase III study (the ToGA trial) demonstrated that trastuzumab combined with cisplatin and capecitabine provided a significant survival advantage over chemotherapy alone in patients with HER-2-positive GC and confirming that HER-2 is a crucial therapeutic GC target.^[51] The median OS was 13.8 months in the trastuzumab plus chemotherapy group ($n = 294$) and 11.1 months in the chemotherapy alone group ($n = 290$; hazard ratio [HR]: 0.74; 95% confidence interval [CI]: 0.60-0.91; $P = 0.0046$). In the subgroup with high HER-2 expression (defined as immunohistochemistry 2+ and fluorescence *in situ* hybridization positive, immunohistochemistry 3+), the median OS was 16.0 months in the trastuzumab plus chemotherapy group and 11.8 months in the chemotherapy alone group (HR: 0.65; 95% CI: 0.51-0.83). Trastuzumab is the first molecularly targeted drug that has been proven efficacious against GC.

Ramucirumab

Ramucirumab is a human mAb that binds to VEGFR-2 and works as a receptor antagonist blocking the binding of VEGF to the receptor. A Phase I trial demonstrated its anti-tumor activity and anti-angiogenic effect over a wide range of doses, suggesting clinical efficacy.^[57] In the REGARD Phase III randomized trial, 355 patients were treated with best supportive care plus ramucirumab or placebo in a second-line setting. Both the median OS (5.2 *vs.* 3.8 months; HR: 0.776; 95% CI: 0.603-0.998) and the median progression-free survival (PFS) (2.1 *vs.* 1.3 months; HR: 0.483; 95% CI: 0.376-0.620) were significantly longer in the ramucirumab than the placebo group, and the safety profile of the drug was acceptable.^[52] In the RAINBOW Phase III trial, ramucirumab was used as a second-line treatment in addition to paclitaxel (665 patients).^[53] The

addition of ramucirumab resulted in a significant survival benefit; the median OS increased from 7.4 to 9.6 months (HR: 0.807; 95% CI: 0.678-0.962), and the median PFS increased from 2.9 to 4.4 months (HR: 0.635; 95% CI: 0.536-0.752).^[53] Currently, a randomized Phase II trial investigating the efficacy of ramucirumab as a first-line treatment in GC is ongoing.^[58]

Colorectal cancer

Estimated new cases of CRC exceed 1.2 million/year worldwide, with more than 600,000 deaths/year.^[59] Liver metastases are observed in 25% of CRC patients at the time of diagnosis and recurrence after surgery is often encountered. The 5-year survival rate of patients with distant metastases diseases is only 10-20%, although that of patients without lymph node metastasis is more than 80%.^[60] The majority of CRC occurrences are sporadic, without the existence of family history or genetic pre-disposition, and the etiological factors for CRC tumorigenesis appear to be complex and heterogeneous. There has been significant progress in identifying distinct molecular pathways leading to CRC that include either increased function of oncogenes or loss of tumor suppressor genes.^[61] Currently, the recent introduction of molecular targeted drugs has improved the treatment of advanced CRC. Cetuximab and panitumumab (EGFR mAbs) and bevacizumab (VEGF₁ mAb) have ushered in a new era of targeted therapy for CRC.^[62-65] Table 3 summarizes molecular targeted drugs used to treat CRC.

Bevacizumab

Bevacizumab, developed in the early 1990s, is a recombinant, humanized immunoglobulin G1 (IgG1) mAb that effectively disrupts the interactions of all isoforms of VEGF-A with VEGFRs.^[66] Pre-clinical studies have demonstrated that bevacizumab exhibits a broad range of anti-tumor activity.^[67] The AVF2107 study, a trial to investigate the efficacy of bevacizumab combined with irinotecan, bolus 5-FU and leucovorin (LV) (IFL) for patients with previously untreated mCRC,^[63] demonstrated that the addition of bevacizumab to IFL improved the RR and prolonged OS. In another

Table 2: Results of completed Phase III trials with molecular targeted therapy in advanced GC

Target	Trial	Regimen	Patients (n)	OS (month)	P
HER2	ToGA	Cisplatin, capecitabine or FU \pm trastuzumab	584	13.8 versus 11.1 (first-line)	0.0046
HER2	LOGIC	Capecitabine, oxaliplatin \pm trastuzumab	545	12.2 versus 10.5 (first-line)	0.35
HER2	TyTAN	Paclitaxel \pm lapatinib	261	11.0 versus 8.9 (first-line)	0.21
EGFR	EXPAND	Cisplatin, capecitabine \pm cetuximab	679	9.4 versus 10.7 (first-line)	0.95
EGFR	REAL3	Oxaliplatin, capecitabine, epirubicin \pm panitumumab	553	8.8 versus 11.3 (first-line)	0.013
VEGFR-2	REGARD	BSC \pm ramucirumab	355	5.2 versus 3.8 (second-line)	0.047
VEGFR-2	RAINBOW	Paclitaxel \pm ramucirumab	665	9.6 versus 7.4 (second-line)	0.017
VEGFR-A	AVAGAST	Cisplatin, capecitabine or FU \pm bevacizumab	774	12.1 versus 10.1 (first-line)	0.10
mTOR	GRANITE-1	BSC \pm everolimus	633	5.4 versus 4.3 (second- or third-line)	0.12

HER2: Human epidermal growth factor receptor 2; EGFR: Epidermal growth factor receptor; VEGFR-2: Vascular endothelial growth factor receptor-2; VEGFR-A: Vascular endothelial growth factor receptor-A; mTOR: Mammalian target of rapamycin; OS: Overall survival; FU: Fluorouracil; BSC: Best supportive care; GC: Gastric cancer

Table 3: Results of completed Phase III trials with molecular targeted therapy in advanced CRC

Target	Trial	Regimen	Patients (n)	OS (month)	P
VEGF	AVF2107	IFL ± bevacizumab	402	20.3 vs. 15.5 (first-line)	< 0.001
VEGF	NO16966	FOLFOX4 or XELOX ± bevacizumab	701	21.3 vs. 19.9 (first-line)	0.077
VEGF	TREE1/2	mFOLFOX6 or XELOX ± bevacizumab	260	26.1 vs. 19.2 (mFOLFOX6 first-line) 24.6 vs. 17.2 (XELOX first-line)	
VEGF	VELOUR	FOLFIRI ± aflibercept	1,226	13.5 vs. 12.1 (second-line)	0.0032
VEGFR, BRAF, KIT, RET, PDGFR	CORRECT	Regorafenib or placebo	760	6.4 vs. 5.0	0.0052
EGFR	CRYSTAL	K-Ras WT FOLFIRI ± cetuximab	348	23.5 vs. 20.0 (first-line)	0.0093
EGFR	FIRE-3	FOLFIRI ± cetuximab	592	28.7 vs. 25.0 (first-line)	0.017
VEGF		FOLFIRI ± bevacizumab			
EGFR	PRIME	K-Ras WT FOLFOX4 ± panitumumab	656	23.9 vs. 19.7 (first-line)	0.17
EGFR	Update PRIME	K-Ras WT/MT other Ras FOLFOX4 ± panitumumab	108	17.1 vs. 18.3 (first-line)	0.31

VEGF: Vascular endothelial growth factor; VEGFR: Vascular endothelial growth factor receptor; BRAF: V-Raf murine sarcoma viral oncogene homolog B1; KIT: Mast/stem cell growth factor receptor; RET: Rearranged during transfection; PDGFR: Platelet-derived growth factor receptor; EGFR: Epidermal growth factor receptor; OS: Overall survival; CRC: Colorectal cancer; IFL: 5-fluorouracil and leucovorin

Phase III clinical trial performed,^[68] patients with mCRC were randomly assigned to receive one of three different irinotecan-containing regimens: irinotecan plus infusional 5-FU and LV (FOLFIRI), modified IFL and irinotecan plus oral capecitabine and FOLFIRI plus bevacizumab. This latter group showed a higher RR and a longer PFS and median OS than patients receiving FOLFIRI without bevacizumab. Subsequent trials with oxaliplatin-based regimens produced less robust differences.^[69-71] In the Phase III trial NO16966,^[71] the effect of capecitabine and oxaliplatin was compared with that of infused 5-FU, LV and oxaliplatin (FOLFOX), with or without bevacizumab. As compared to chemotherapy alone, treatment with bevacizumab in addition to oxaliplatin-based therapy significantly improved OS and PFS. Another Phase III trial, the TREE study^[70] investigated the tolerability of oxaliplatin in combination with three different 5-FU regimens (continuous infusion, bolus and oral) with or without bevacizumab as a first-line therapy. The study showed that as compared to patients who received chemotherapy alone, patients treated with FOLFOX6 plus bevacizumab experienced improvements in overall response, OS and PFS.

However, there is a controversy regarding the use of adjuvant treatments in CRC. The NSABP PROTOCOL C-08 trial showed that the addition of bevacizumab for 1-year to a modified FOLFOX6 adjuvant regimen did not significantly prolong disease-free survival (DFS) in Stage II and III CRC.^[72] Similarly, the AVANT trial showed that bevacizumab did not prolong DFS when added to adjuvant chemotherapy in resected Stage III CRC, and OS data suggested a potential adverse effect with bevacizumab plus oxaliplatin-based adjuvant therapy.^[73]

Cetuximab

Cetuximab is a recombinant, chimeric, human/murine IgG1 mAb that binds specifically to the extra-cellular domain of EGFR in normal and tumor cells, promoting receptor internalization and degradation without receptor phosphorylation and activation.^[74] In the pivotal Phase II study, the BOND trial, patients with mCRC were randomized to various treatment groups.^[62] As compared to cetuximab alone, the combination of irinotecan and cetuximab significantly improved overall patient response, median OS and PFS. Retrospective analysis of *KRAS* status in the CRYSTAL trial has recently shown statistically significant differences in PFS and overall response between patients with wild-type *KRAS* and those with mutant *KRAS* treated with FOLFIRI plus cetuximab.^[75] In the Phase III study, the FIRE-3, by Heinemann *et al.*^[76] patients with mCRC were randomly assigned to FOLFIRI plus either cetuximab or bevacizumab. Patients in the cetuximab and bevacizumab arms had similar times to disease progression, but those treated with cetuximab had a significantly improved OS. One of the problems of cetuximab treatment is an increased risk of severe adverse events. A meta-analysis to investigate severe adverse events in CRC patients, reported the most common severe adverse events to be neutropenia, diarrhea and rash. However, cetuximab was not associated with an increased risk of fatal adverse events.^[77]

Panitumumab

Panitumumab is a fully human, recombinant IgG2 mAb that binds specifically and with high affinity to the extra-cellular domain of EGFR in normal and tumor

cells. Through competitive binding to EGFR ligands, panitumumab prevents EGFR dimerization, auto-phosphorylation and signaling, thereby inhibiting proliferation and promoting apoptosis.^[78] A Phase III study, the PRIME trial, evaluated the combination of FOLFOX4 with panitumumab or FOLFOX4 alone as first-line treatment.^[79] As compared to chemotherapy alone, the combination therapy significantly improved PFS and increased RR in patients with wild-type *KRAS*. A non-significant increase in OS was also observed. In order to assess the efficacy and safety of FOLFOX4 with panitumumab as compared to FOLFOX4 alone according to *KRAS* (exon 2-4) and *NRAS* (exon 2-4) mutation status, data from the PRIME trial were analyzed.^[80] Patients without any Ras mutation who were treated with panitumumab had a significantly longer OS and PFS than those treated with chemotherapy alone.

Regorafenib

Regorafenib is an inhibitor of PDGFRs, c-KIT, FGF receptor and VEGF1-3.^[81] In the pivotal Phase III study, the CORRECT trial, patients with mCRC who had progressed after undergoing treatment with approved drugs were randomly assigned to regorafenib or placebo.^[82] As compared to placebo, treatment with regorafenib significantly prolonged OS and PFS, suggesting a potential new line of therapy with survival benefits for patients who have progressed after all standard therapies.

Aflibercept

Aflibercept is a recently developed, multiple angiogenic factors trap that inhibits not only VEGF-A, VEGF-B and PLGF, from activating their native receptor (VEGFR-1).^[83,84] Aflibercept has a higher VEGF-A binding affinity than bevacizumab. The velour trial evaluated FOLFIRI plus aflibercept receptor FOLFIRI alone after progression on an oxaliplatin-based chemotherapy.^[85] As compared to chemotherapy alone, the addition of bevacizumab significantly improved OS.

Conclusion

The clinical application of molecular targeted drugs has improved the survival of patients with GI cancers. We believe that both the identification of novel targets and the development of new drugs targeting several important pathways such as c-MET, rearranged during transfection, MEK and IGF/IGFR will contribute to further improvements in treatment results and the realization of personalized treatments for GI cancer.

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Conflicts of interest

There are no conflicts of interest.

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Cancer metabolism in gastrointestinal cancer

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ABSTRACT

Cancer cells exhibit altered glucose metabolism, mitochondrial dysfunction, anaerobic glycolysis and upregulation of the pentose phosphate pathway (PPP). Recent genetic and metabolic analyses have provided insights into the molecular mechanisms of genes that are involved in the alteration of cancer metabolism and tumorigenesis. Hypoxic induced factor 1 regulates the reciprocal relationship between glycolysis and oxidative phosphorylation, and p53 also modulates the balance between the glycolytic pathway and oxidative phosphorylation. Mitochondria function in cancer differs from that in normal cells owing to mutations of mitochondrial DNA and alterations of metabolism. Overexpression of transcription factors, metabolite transporters and glycolytic enzymes is observed and associated with poor prognosis, and it may be associated with chemoradiotherapy resistance in multiple cancer cell types. The PPP plays a critical role in regulating cancer cell growth by supplying cells with ribose-5-phosphate and nicotinamide adenine dinucleotide phosphate for detoxification of intra-cellular reactive oxygen species (ROS), reductive biosynthesis and ribose biogenesis. ROS levels increase during carcinogenesis owing to metabolic aberrations. This review discusses alterations of mitochondrial metabolism, anaerobic glycolysis, the PPP and control of ROS levels by the endogenous anti-oxidant system in cancer, as well as the novel small molecules targeting these enzymes or transporters that exert anti-proliferative effects.

Key words: Anti-oxidants, cancer metabolism, mitochondria, pentose phosphate pathway, reactive oxygen species, Warburg effect

Introduction

In 1926, Otto Warburg found the conversion of glucose to lactic acid in the presence of adequate oxygen as a specific metabolic abnormality of cancer cells.^[1,2] Warburg further hypothesized that cancer results from a defect of mitochondrial metabolism that leads to aerobic glycolysis. The role of dysfunctional glucose metabolism in cancer is now firmly established. Recent genomic and proteomic research has provided insights into the molecular mechanisms of cancer metabolism.

Two main pathways generate adenosine triphosphate (ATP) required for cell proliferation and survival. The first is glycolysis, which metabolizes glucose to pyruvate in the cytoplasm to produce a net two ATP molecules from each glucose molecule. The other is the tricarboxylic acid (TCA) cycle, which uses pyruvate formed from glycolysis to donate electrons via nicotinamide adenine dinucleotide (NADH) (reduced form of NADH) and

flavin adenine dinucleotide (FADH₂) (reduced form of FADH₂) to the respiratory chain complexes in mitochondria. The electron transfer system generates 36 ATP molecules per glucose across the mitochondrial inner membrane. Under limited oxygen conditions, such as muscles under prolonged exercise, pyruvate is not used in the TCA cycle and is converted into lactic acid by lactate dehydrogenase (LDH) in a process termed anaerobic glycolysis.

Recent genetic and metabolic analyses have provided insights into the molecular mechanisms of the genes that contribute to anaerobic glycolysis and tumorigenesis. The direct mechanistic links between activated oncogenes and altered glucose metabolism are regulated by phosphoinositide 3-kinase (PI3K),^[3] Akt,^[4] p53,^[5,6] AMP-activated protein kinase (AMPK),^[3,7] c-Myc and hypoxia-inducible factor (HIF). The c-Myc and HIF-1A transcription factors target many of the same glycolytic enzyme genes, including hexokinase 2 (HK2),

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pyruvate kinase type M2 (PKM2), LDH-A and pyruvate dehydrogenase kinase, isozyme 1 (PDK-1).

The pentose phosphate pathway (PPP) is a major pathway for glucose catabolism. The PPP directly or indirectly provides reducing power to fuel the biosynthesis of lipids and nucleotides and sustains anti-oxidant responses to support cell survival and proliferation. Abnormal respiratory metabolic pathways influence energy balance and the reactive oxygen species (ROS) balance in cancer cells. The increase in ROS generation from metabolic abnormalities and oncogenic signaling in cancer cells triggers a redox adaptation response to maintain ROS levels below the toxic threshold. Cancer cells would be increasingly dependent on the anti-oxidant system.

In this review, significant molecular insights into mitochondrial metabolism, anaerobic glycolysis and the PPP in cancer are discussed. We also review the control of ROS levels by the endogenous anti-oxidant system and the therapeutic strategies targeting cancer metabolism.

Mitochondria in Cancer Cells

As the main energy producers, mitochondria produce ATP using the TCA cycle and oxidative phosphorylation. However, they also generate ROS during this process, which are harmful to the cell if produced in excess. In addition, mitochondria play a crucial role in the regulation of cell death pathways and intra-cellular Ca^{2+} homeostasis. Mitochondria activate apoptosis by regulating the release of pro-apoptotic proteins from the mitochondrial intermembrane to the cytosol, and they also play a crucial role in non-apoptotic cell death.^[8] Key regulators related to cell death in the mitochondria are frequently altered in cancer cells,^[9] and the function of mitochondria in cancer cells is different from that in normal cells.^[10]

The mitochondrial mechanism in cancer cells is different from that in normal cells using oxidative phosphorylation. In oxidative phosphorylation, ATP synthesis requires significant amounts of oxygen, which leads to the continuous production of ROS such as superoxide anion, organic peroxide and hydrogen peroxide.^[11] If the redox regulating system does not eliminate the generated ROS, the excessive ROS may cause cellular damage. Mitochondria have redox defense systems for the elimination of hydrogen peroxide. Glutathione (GSH) and glutathione peroxidases require nicotinamide adenine dinucleotide phosphate (NADPH) for the elimination of H_2O_2 and other peroxides generated in the mitochondria. The mitochondrial complex V (ATP synthase) produces ATP from ADP and inorganic phosphate. As an anti-oxidant defense system, peroxiredoxin (Prx) 3, Prx5, superoxide dismutase 2 (SOD2) and thioredoxin 2 eliminate ROS produced in mitochondria.^[12,13] Prx3 knockout (KO) mice exhibit metabolic dysregulation and induction of oxidative damage,^[14] thioredoxin 2 KO mice

show an embryonic lethal phenotype^[15] and SOD2 KO mice die within 3 weeks of birth because of mitochondrial oxidative damage and severe neurodegeneration.^[16,17]

Mutations in mitochondrial DNA (mtDNA) occur at a high frequency in human tumors. Tumor mtDNA somatic mutations range from severe insertions/deletions and chain termination mutations to mild missense mutations. A total of 190 tumor-specific somatic mtDNA mutations have been reported and 72% of them are also mtDNA sequence variants found in the general population. They include 52% tumor somatic mRNA missense mutations, 83% tRNA mutations, 38% rRNA mutations and 85% control region mutations. Germline mtDNA mutations at nucleotides 10,398 and 16,189 have been associated with breast cancer,^[18] esophageal cancer^[19] and endometrial cancer.^[20] The mtDNA conferring high metastatic potential contained G13997A and 13885insC mutations in the gene encoding NADH dehydrogenase sub-unit 6. These mutations produced a deficiency in respiratory complex I activity and were associated with overproduction of ROS.^[21] Severe mutations can inhibit oxidative phosphorylation, increase ROS production and promote tumor cell proliferation; milder mutations may permit tumors to adapt to new environments.^[22]

Recent investigations have revealed that p53 can modulate the balance between the glycolytic pathway and mitochondrial oxidative phosphorylation.^[23] The key component in this regulation is the gene encoding synthesis of cytochrome c oxidase 2 (SCO2), in conjunction with the SCO1 protein. Analysis of potential p53 target genes that can influence mitochondrial function showed that SCO2, but not SCO1, was induced in a p53-dependent manner. SCO2 is critical for regulating the cytochrome c oxidase (COX) complex, the major site of oxygen use and is required for the assembly of COX.^[24] Mutation of p53 in tumor cells leads to inhibition of mitochondrial respiration as a result of COX deficiency and a shift of cellular energy metabolism toward glycolysis. Inhibition of glycolysis by glucose withdrawal leads to the activation of p53. Under conditions of cellular stress, activation of p53 could increase SCO2 expression and stimulate mitochondrial respiration and ATP production. Another newly discovered target of p53 is TP53-induced glycolysis and apoptosis regulator (TIGAR). Expression of TIGAR lowered fructose-2,6-bisphosphate levels in cells, resulting in the inhibition of glycolysis while stimulating NADPH generation through the pentose phosphate shunt.^[25] The expression of TIGAR in primary tumors is significantly correlated with standardized uptake values max, and low expression of TIGAR may predict a worse clinical outcome in patients with non-small cell lung cancer.^[26]

HIF-1 plays an important role in the upregulation of enzymes stimulating glucose use. Recent investigations demonstrated that HIF-1 suppresses mitochondrial function in tumor cells and modulates the reciprocal relationship between glycolysis and oxidative

phosphorylation. The balance between glycolysis and oxidative phosphorylation is controlled by the relative activities of two enzymes: pyruvate dehydrogenase (PDH) and LDH. The activity of PDH is negatively controlled by PDK-1, and HIF-1 can inactivate PDH by inducing PDK-1. Inactivation of PDH leads to suppression of mitochondrial respiration.^[27,28] HIF-1 also stimulates expression of LDH-A, which facilitates the conversion of pyruvate into lactate,^[10] which decreases use of pyruvate by mitochondria and suppresses mitochondrial respiration. In addition, HIF-1 can also modulate COX expression. Under hypoxic conditions, the sub-unit composition of COX is changed to optimize its activity. The expression of the COX4-2 sub-unit is increased and optimizes the activity of COX under aerobic conditions.^[24]

Another important consequence of the glycolytic shift in tumor cells is their acquired resistance to apoptotic cell death. The two major apoptotic pathways include the extrinsic (receptor-mediated) pathway and the intrinsic pathway. The extrinsic pathway engages initiator pro-caspase-8, which activates pro-caspase-3 and other effector caspases. The intrinsic pathway involves permeabilization of the outer mitochondrial membrane (OMM) followed by the release of cytochrome c and other proteins from the intermembrane space of mitochondria. Permeabilization of the OMM is considered to be a crucial event during the early phase of the apoptotic process. Multiple proteins, including B-cell lymphoma 2 (Bcl-2) family,^[29,30] hexokinase,^[31,32] Akt^[33,34] and loss of p53,^[35,36] support the glycolytic shift. These proteins render tumor mitochondria less susceptible to the permeabilization of the OMM and the mitochondrial pathway of apoptosis.

Alteration of Protein Expression in the Warburg Effect

Cancer cells exhibit altered glucose metabolism, which is described by the increased uptake of glucose and the conversion of glucose to lactate in cancer cells under adequate oxygen tension. HIF-1A and c-Myc transcription factors cooperatively induce a transcriptional program for glycolysis by targeting many glycolytic enzyme genes, including HK2, PKM2, LDH-A and PDK-1. Key regulatory sub-units of HIF include HIF-1A and endothelial PAS domain protein 1 (EPAS1; HIF-2), and these proteins are differentially overexpressed in cancer cells.^[37,38] Many studies demonstrated that HIF-1A positive expression was significantly associated with poor outcome of diverse human cancers.^[38-43] Low expression of HIF-1A may be associated with a favorable outcome of 5-fluorouracil (5-FU)-based adjuvant chemotherapy in gastric cancer patients.^[44,45] High expression of HIF-2A was associated with poor survival in gastric cancer patients,^[46] but not colorectal cancer (CRC) patients.^[42,47] The MYC protein affects the expression of approximately 15% of the genes in the human genome,^[48] and thus MYC deregulation may result in alterations in

various biological pathways involved in cancer initiation and progression.^[49] The expression of MYC genes is often elevated or deregulated in human neoplasms, and c-Myc seems to be at the crossroads of several important pathways and processes involved in carcinogenesis. MYC overexpression and promoter hypomethylation may have a role in the gastric carcinogenesis process. MYC deregulation was mainly associated with poor prognostic features.^[50]

The GLUT family proteins are glucose transporter-like proteins that have been well characterized. The 14 GLUTs are categorized into three classes based on sequence similarity: Class 1 (GLUTs 1-4 and 14); Class 2 (GLUTs 5, 7, 9 and 11) and Class 3 (GLUTs 6, 8, 10, 12 and HMIT).^[51] Several studies have been published on GLUT family members, especially GLUT 3,^[52-54] but GLUT 1 has been the main focus of the investigation.^[55-57] GLUT 1 comprises 492 amino acid residues and possesses a single N-linked glycosylation site at N45,^[58] and its crystal structure has been reported recently.^[59] GLUT 1 is transcriptionally regulated by HIF-1A^[60] and c-Myc.^[61] A recent investigation showed that GLUT 1 was upregulated in cells with KRAS or BRAF mutations,^[62] and GLUT 1 expression in CRC cells was positively correlated with FDG accumulation and KRAS/BRAF mutation.^[63] MAPK signaling induces phosphorylation of Ser 37 in PKM2, and nuclear-phosphorylated PKM2 then induces c-Myc expression, resulting in the upregulation of GLUT 1.^[64] Overexpression of GLUT 1 in a mammary tumor cell line with low levels of endogenous GLUT 1 results in both a decrease in apoptosis and an increase in proliferation.^[65]

Hexokinases catalyze the phosphorylation of glucose to glucose-6-phosphate (G6P). This is the first and rate-limiting step in glucose metabolism. HK2 is one of four members of the hexokinase family. The hexokinase isoenzymes (HK1, HK2, HK3 and glucokinase) are structurally similar; however, only HK1 and HK2 are functionally similar. HK2, but not HK1, is overexpressed in several cancer types compared with normal tissue, and overexpression of HK2 was reported in hepatocellular carcinoma (HCC).^[66-68] HK2 localizes to the outer membrane of the mitochondria and is the major hexokinase isoform expressed in cancer cells.^[69]

PK is a glycolytic enzyme that catalyzes a reaction generating pyruvate and ATP from phosphoenolpyruvate and ADP. Four isoforms of PK (L, R, M1 and M2) are present in mammals. Splicing of PKM is regulated by splicing repressors, and the expressions of those repressors are induced by MYC oncoprotein.^[70,71] M2 is expressed in embryonic cells, adult stem cells and cancer cells and is necessary for aerobic glycolysis and that this metabolic phenotype provides a selective growth advantage for cancer cells *in vivo*.^[72] Mutation of the S37 ERK phosphorylation site in PKM2 blocked translocation of PKM2 to the nucleus,^[64] which suggested

that PKM2 moves into the nucleus as a monomer. Tumor cells have multiple ways to regulate PKM2 for cell growth and survival, including controlling PKM2 expression, localization, post-translational modification and allosteric regulation. PKM2 also has non-metabolic functions as a transcriptional coactivator and protein kinase. PKM2 is considered an attractive target for cancer treatment.^[73] Further studies are needed before inhibitors and activators of PKM2 can be used as therapeutic interventions.^[74]

PDK regulates PDH, which links glycolysis to the TCA cycle by reversible phosphorylation. Phosphorylation of PDH by PDK inactivates PDH and halts pyruvate use in the TCA cycle.^[75] Four PDK isoforms have been verified in human tissue, and the expression of these isoforms was organ specific. PDK-1 positivity was associated with poor prognosis in gastric cancer;^[76] however, expression of PDK-1 was decreased in colon cancer compared to normal tissue. PDK-3 expression was detected in colon cancer, and PDK-3 positivity was associated with poor prognosis.^[77] Only a few studies have reported the relation between PDK positivity and prognosis, and the clinical significance of PDK expression has remained unclear. LDH is a tetrameric enzyme comprising two major sub-units, A and/or B, resulting in five isozymes (A4, A3B1, A2B2, A1B3 and B4) that can catalyze the forward and backward conversion of pyruvate to lactate. LDH-A (LDH-5, MLDH or A4), which is the predominant form in skeletal muscle, kinetically favors the conversion of pyruvate to lactate, controlling the conversion of pyruvate to lactate of the cellular glycolytic process.^[78] Many studies have shown that human cancers have higher LDH-A levels compared with normal tissues.^[79] Previous studies showed that 661 intestinal-type gastric cancer (ITGC)^[80] and 128 CRC^[81] specimens with high LDH-A expression are associated with poor prognosis. LDH-A is specifically phosphorylated at Y10 in various cancer cell lines, head and neck squamous cell carcinoma (SCC), lung cancer, breast cancer and prostate cancer cells and by diverse oncogenic tyrosine kinases, including FGFR1, ABL, JAK2 and FLT.^[82] LDH-A reduction using si-RNA for LDH-A can suppress the tumorigenicity of ITGC cells^[80] and HCC.^[83]

The Pentose Phosphate Pathway

The PPP is a major pathway for glucose catabolism. Glucose is a common fuel for multicellular organisms, entering cells through GLUTs and then being phosphorylated by HK to form G6P. G6P can be further metabolized by both the glycolytic pathway and the PPP.^[84] The PPP generates ribose 5-phosphate (R5P), a critical sub-strate for nucleotide synthesis. The PPP plays a critical role in regulating cancer cell growth by supplying cells with not only R5P but also NADPH for detoxification of intra-cellular ROS, reductive biosynthesis and ribose biogenesis.

Fructose-6-phosphate is isomerized to G6P in cells, and this accumulated G6P is diverted into the PPP, an alternative metabolic pathway that can provide substrates for the later steps in glycolysis. Glucose-6-phosphate dehydrogenase (G6PD) is mediated by various signals, and it acts as a sensor of cellular NADP⁺ levels. Increased NADP⁺ activates G6PD by competing with NADPH for binding to this enzyme (G6PD), and determines the amount of NADPH by controlling the metabolism of glucose via the PPP.^[85] The increased flow through the PPP lowers apoptosis because of an increased generation of reduced GSH and removal of ROS in cells.^[25] Elevated levels of G6PD in association with higher levels of PPP-derived metabolites suggest a prominent role of this pathway in metabolic alterations of human cancer.^[86,87] G6PD inhibition decreases cancer cell survival, NADPH levels and increases production of ROS, suggesting that the PPP plays an important role in the regulation of redox homeostasis.^[88,89] G6PD is associated with adriamycin resistance in breast cancer cells using proteomics analysis.^[90]

The PPP is positively regulated by K-ras^{G12D}, PI3K,^[91] mTORC1,^[92] Tap73,^[93,94] HSP27,^[95] SREBP,^[92] the ataxia-telangiectasia mutated kinase, protein kinase A, NADP and glycolytic inhibition (TIGAR,^[25] PKM2 and PGAM). The PPP is negatively regulated by p53, PTEN,^[96] AMPK,^[3] cyclic adenosine monophosphate, cyclic AMP-response element modulator and aldosterone.^[97] Tap73, the transcriptionally competent isoform of the p53 family protein p73, was identified as a transcriptional regulator of G6PD.^[94]

The PPP is a well-established metabolic pathway, but the mechanism that activates the PPP has yet to be identified. TIGAR, a target of p53, inhibits glycolysis and diverts the carbon flux into the PPP, resulting in the passive promotion of PPP activity. NADPH production pathway is targeted by nuclear factor E2 p45-related factor 2 (Nrf2).^[98] Nrf2, a bZIP transcription factor, plays a central role in the regulation (basal and/or inducible expression) of phase 2 genes by binding to the anti-oxidant response element in their promoters. A previous study focused on the cytoprotective aspect of the PPP by analyzing NADPH production as reducing equivalents for ROS elimination.^[99] The PPP genes are strongly activated by Nrf2 in proliferating cells in which the PI3K-Akt pathway is active, and increased expression of the PPP genes contributes to cell proliferation.^[98]

Under basal conditions, Keap1 binds to the ETGE and DLG motifs in Nrf2 and recruits Nrf2 to the Keap1-Cul3-E3 ubiquitin ligase complex, leading to ubiquitination and subsequent degradation of Nrf2. Oxidative stress or electrophiles can cause a conformational change in the Keap1-Cul3-E3 ubiquitin ligase by acting on specific cysteine residues in Keap1.^[100] These changes disrupt Nrf2-Keap1 binding at

the DLG domain, resulting in stabilization of Nrf2 and translocation of free Nrf2 to the nucleus.^[101] Nrf2 is aberrantly accumulated in many types of cancer, and its expression is associated with a poor prognosis in patients.^[102-106] In addition, Nrf2 expression is induced during the course of drug resistance in gastric cancer,^[107] CRC^[108] and esophageal SCC.^[109]

ROS and Energy Metabolism in Cancer Cells

Oxygen free radicals are highly reactive with biological molecules, including DNA, proteins and lipids. The free radical reaction could cause oxidative modification of these biomolecules and alter their functions. Mitochondria generate ROS that are thought to augment intra-cellular oxidative stress. In all cells, the majority of ROS are by-products of mitochondrial respiration. Approximately, 2% of the molecular oxygen consumed during respiration is converted into the superoxide anion radical, the precursor of most ROS. Mitochondria possess at least nine known sites that are capable of generating superoxide anion, a progenitor ROS.^[110] A mild increase in the level of ROS may result in transient cellular alterations, whereas a severe increase of ROS in cells could cause irreversible oxidative damage, leading to cell death.^[111] In normal cells, the ROS level is tightly controlled by the endogenous anti-oxidant system. However, energy metabolism and ROS homeostasis in cancer cells are different from those in normal cells. During the transition phases from normal tissue to invasive carcinoma, ROS levels increase because of metabolic aberrations.^[112]

Severe accumulation of cellular ROS under various endogenous and exogenous stress stimuli may induce fatal damage in cells that have inadequate stress responses or adaptation. In cancer cells, ROS stress may induce adaptive stress responses, including activation of redox-sensitive transcription factors, such as nuclear factor κ B and Nrf2. These responses lead to an increase in the expression of ROS-scavenging enzymes, such as SOD and glutathione (GSH), elevation of survival factors such as Bcl-2 and MCL1, and inhibition of cell death factors, such as caspases.^[111,113,114] ROS-mediated DNA mutations or deletions promote genomic instability and thus induce an additional mechanism for stress adaptation. All these events contribute to the survival of cells with high levels of ROS and maintain cellular viability.^[115] As these transcription factors also have roles in regulating the expression of genes that are responsible for proliferation, senescence evasion, angiogenesis and metastasis, and thus the redox adaptation processes may promote cancer development.^[116,117] The increase in GSH during the redox adaptation can enhance the export of certain anti-cancer drugs and their inactivation. This altered drug metabolism, together with enhanced cell survival, may render cancer cells more resistant to chemotherapeutic agents.^[113,118,119] Activation of oncogenes, aberrant metabolism, mitochondrial dysfunction

and loss of functional p53 are intrinsic factors known to cause increased ROS production in cancer cells.^[111] In chemotherapy, 5-Fluorouracil (FU) generates mitochondrial ROS via a p53-dependent pathway.^[120] Tumor cells which adapt to oxidative stress by increasing the production of SOD2, Prx1 and Bcl-2 are resistant to 5-FU.^[121] Products of oxidative stress can slow cell-cycle progression of cancer cells, cause cell-cycle checkpoint arrest and interfere with the ability of anti-cancer drugs to kill cancer cells.^[122] The capacity of some chemotherapeutic agents to cause an imbalance in ROS levels offers a therapeutic opportunity for treating cancer.

Considering that cancer cells have increased ROS levels, they may be selectively sensitive to the damaging effects of further increasing ROS. Cancer cells frequently have increased expression of anti-oxidants to maintain homeostasis. Inhibiting anti-oxidants to expose cancer cells to endogenously produced ROS may be a therapeutic approach.^[123] In support of this model, several small molecule screens have identified compounds that specifically inhibit the growth of transformed cells. Piperlongumine increases ROS and apoptotic cell death in both cancer cells and normal cells engineered to have a cancer genotype, irrespective of p53 status, with little effect on dividing primary normal cells.^[124] Beta-phenylethyl isothiocyanate (PEITC) is a natural compound found in consumable cruciferous vegetables with chemopreventive activity. PEITC increases ROS and selectively kills cancer cells.^[125] Malignant cells are often resistant to conventional anti-cancer drugs. These cells are under intrinsic ROS stress, so using small molecules that induce ROS to kill such malignant cells may exert a therapeutic effect.

Cancer Treatment

Novel small molecules targeting metabolic regulators and glycolytic enzymes have been reported to exert anti-proliferative effects.^[126] Phloretin, a natural product with GLUT inhibitory activity found in apples and pears, exerts anti-tumor effects in HCC and color cancer cell lines.^[127,128] The WZB117 small molecule inhibitor of GLUT 1 was effective in inhibiting cancer cell growth both *in vitro* and *in vivo*.^[129] The widely used 3-bromopyruvate (3-BrPA)^[130] depletes cellular ATP. A previous study showed that 3-BrPA inhibits HK2 expression and exhibits anti-proliferative effects when combined with daunorubicin in CRC cell lines^[131] and when combined with protein disulfide isomerase in HCC cell lines.^[132] DCA, a PDK-1 inhibitor, has reduced lactate production and increased responsiveness to 5-FU in MKN45 cells^[76] and CRC cell lines.^[133] DCA treatment exerts anti-proliferative effects and sorafenib resistance in HCC cell lines *in vivo*.^[134] Oxamate, a LDH inhibitor, combined with phenformin, has exhibited cytotoxic effects in diverse cancer cell lines, including colon cancer.^[135] Future studies should examine whether

inhibitors of glycolytic enzymes and metabolite transporters are effective in preclinical or clinical settings and evaluate adverse effects and feasibility for clinical practice.

The Nrf2 transcription factor is an important modifier of cellular responses to oxidative stress. Stable RNAi-mediated knockdown of Nrf2 in human colon cancer cells suppressed tumor growth in a xenograft model with a reduction in blood vessel formation and VEGF expression. The Nrf2-inhibited cancer cells failed to accumulate HIF-1A protein under hypoxic conditions.^[136] HIF plays a crucial role in cellular adaptation to hypoxia and regulates the expression of genes responsible for glucose metabolism, angiogenesis and cell survival. Conventional anti-cancer therapies typically target actively dividing cells near the vasculature, though they function poorly in hypoxic regions.^[137] Cells in hypoxic regions are relatively quiescent, and these cells also tend to be refractory to agents targeting rapidly proliferating cells.^[138-140]

Novel therapeutic agents targeting the resistant hypoxic zones may provide additional anti-tumor activity and clinical benefit when combined with conventional treatments. Tirapazamine is a bioreductively activated, hypoxia-selective anti-tumor agent of the benzotriazine series; it is 35-450 times more cytotoxic to hypoxic cells than to well-oxygenated cells.^[141] Standard cisplatin chemoradiotherapy plus tirapazamine has not been superior to cisplatin chemoradiotherapy in either progression-free survival or overall survival in locally advanced cervix cancer.^[142] TH-302 is a novel therapeutic agent and a hypoxia-activated, cytotoxic prodrug with a 2-nitroimidazole component designed to release the DNA cross-linker bromo-isophosphoramidate mustard when reduced by intra-cellular reductases in the setting of severe hypoxia.^[143] The phase II study by Borad *et al.*^[144] evaluated treatment of TH-302 in patients with either locally advanced or metastatic pancreatic cancer and found that the addition of TH-302 to gemcitabine resulted in a near doubling of progression-free survival and objective response with acceptable toxicity.

Many anti-cancer treatments regulating ROS levels have been demonstrated. NOV-002 is a GSH disulfide mimetic that alters the intra-cellular GSH/GSSG ratio by increasing GSSG levels via the induction of S-glutathionylation.^[145] NOV-002 modulates signaling pathways involved in tumor cell proliferation and metastasis and enhances anti-tumor immune responsiveness. NOV-002, in combination with neoadjuvant AC in patients with HER-2 negative breast cancer, was well tolerated and resulted in a favorable pCR rate in a phase II study.^[146] Sulfasalazine inhibits xCT (a cystine/glutamate transporter) and reduces the intra-cellular transport of cysteine required for GSH synthesis.^[147] Sulfasalazine in combination with conventional anti-cancer agents may be an effective therapy for refractory pancreatic cancer^[148] and small

cell lung cancer.^[149] The small molecule 968 is identified to block glutaminase activation and inhibit the growth of cancer cells, and this enzyme shows potential as a therapeutic strategy against cancer.^[150]

Conclusions and Perspective

This review describes recent investigations in mitochondrial metabolism, anaerobic glycolysis and the PPP in cancer. We also discussed the control of ROS levels by the endogenous anti-oxidant system. Key regulators related to cell death in the mitochondria are frequently altered in cancer cells, and mitochondria in cancer differ functionally and structurally from those of normal cells. Mitochondria dysfunction in cancer is associated with the activation of oncogenes and inactivation of tumor suppressors. Recent genetic and metabolic analyses have revealed the molecular mechanisms of genes that are involved in cancer metabolism and tumorigenesis. The alterations of gene expression in glycolysis are associated with poor prognosis and may be associated with chemoradiotherapy resistance. The PPP is positively regulated by oncogenes and regulates cancer cell growth by supplying cells with R5P and NADPH. Direct regulators that activate the PPP have been identified. ROS levels are increased during carcinogenesis from metabolic aberrations. Cancer cells frequently have increased expression of anti-oxidants to maintain homeostasis. Anti-cancer agents targeting ROS status may exert therapeutic effects. Novel small molecules targeting metabolite transporters, glycolytic enzymes and ROS status have been reported. However, further studies should examine whether these inhibitors are useful in cancer therapy and evaluate adverse effects and feasibility for use in clinical practice.

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Conflicts of interest

There are no conflicts of interest.

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Epithelial-mesenchymal transition in gastroenterological cancer

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ABSTRACT

Epithelial-mesenchymal transition (EMT) was first reported as an essential process in embryonic cells and later showed that cancer cells, regardless of the context, exhibited a similar phenomenon that was crucial for tumor progression. Epithelial cells lose their adhesive characteristic capacity which is necessary for their functions but gain a mesenchymal phenotype. This change from epithelial to the mesenchymal phenotype of cancer cells makes it difficult to understand the mechanism underlying cancer biology and tumor progression. A number of transcription factors involved in tumor cell EMT and microRNA-regulated EMT have been reported. This review discussed recent findings and new players in EMT in gastrointestinal cancers. Since the molecular mechanisms of tumor progression are sometimes context-dependent, the recent findings of EMT have been reviewed in a context-dependent manner.

Key words: Epithelial-mesenchymal transition, gastrointestinal cancer, microRNA, transcription factor

Introduction

Epithelial-mesenchymal transition (EMT) is a well-known phenotype and essential for tumor invasion and metastasis.^[1-3] The phenotype change in EMT is drastic, so the theory has fascinated many investigators, and several mechanisms have been reported to date. However, the number of factors essential for EMT is increasing; thus, it is challenging to integrate those factors to understand their networking. In this review, we briefly updated the recent EMT findings in a context-dependent manner, because the mechanisms underlying a disease substantially depend on the original function of the affected organ. Theoretically, the concept of EMT explains various cancer characteristics including tumor cell invasion, metastasis, chemo resistance and stem cell phenotype; therefore, it has considerable clinical significance. Thus, this review explores both the molecular mechanism of EMT and its clinical significance.

Although many EMT players, such as transcription factors and microRNAs (miRNAs) have been introduced so far such as transcription factors and miRNAs, their roles are to some extent-dependent on the context. Therefore, we discussed the role of each molecule in a context-dependent manner to clarify the specific role of each player.

Esophageal Cancer

Esophageal cancer (EC) has two distinct histological subtypes, that is, esophageal squamous cell carcinoma (ESCC) and esophageal adenocarcinoma (EAC).^[4] The former commonly occurs in Asia, whereas the latter is common in the United States and Western countries. Transforming growth factor- β 1 (TGF- β 1) was reported to induce EMT in EAC via the mothers against decapentaplegic homolog (SMAD) 4 pathway and this signaling was inhibited by bone morphogenetic protein 7, another member of the TGF- β 1 superfamily.^[5] Using immortalized esophageal keratinocyte, TGF- β 1 was shown to regulate mitochondrial superoxide dismutase 2 (SOD2) which possesses antioxidant activity, to convert CD44_{low} to CD44_{high} cells. Expression of SOD2 was transcriptionally regulated by NF- κ B and zinc finger E-box binding homeobox 2 (ZEB2), but not ZEB1.^[6] In the same cells, it was also reported that TGF- β 1-mediated EMT required *p53* mutation accompanied by up-regulation of ZEB1 and the loss of epithelial growth factor receptor (EGFR)-dependent

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senescence program.^[7] Epithelial cell adhesion molecule (EpCAM), a well-known marker for circulating tumor cells in many solid tumors, is down-regulated in TGF- β 1-mediated EMT. However, EpCAM expression in disseminated tumor cells (DTCs) was associated with lymph node metastasis and decreased overall survival of patients with EC. The conflicting evidence that DTCs need the process of EMT but express epithelial cell marker EpCAM is supported by the result that high expression of EpCAM promoted tumor outgrowth after xenotransplantation of esophageal carcinoma cells, suggesting that EpCAM expression changes dynamically over the course during cancer progression.^[8]

A notable EMT inducer that has recently been reported is interleukin-23 (IL-23). IL-23 is mainly produced by Th17 cells that infiltrate in the tumor microenvironment and contributes to EMT via activation of the Wnt/ β -catenin pathway in esophageal squamous carcinoma.^[9] Eukaryotic initiation factor 5A2 (eIF5A2) was first isolated as an oncoprotein and was later found to be involved in EMT. Increased expression of eIF5A2 induced ESCC metastasis and angiogenesis via the hypoxia inducible factor-1 signaling pathway in esophageal squamous cell lines.^[10] The clinical investigation revealed Snail overexpression in 40% of patients with SCC tissue samples, which was associated with vascular invasion, advanced clinical stage and the EMT phenotype.^[11]

Gastric Cancer

Distinct carcinogenetic pathways have been reported for intestinal and diffuse type gastric carcinoma, but EMT has been mainly discussed for the latter phenotype.^[12] The link between EMT and gastric adenocarcinoma could be partly because of the *H. pylori* cytotoxin-associated gene A (CagA) oncoprotein, which is responsible for the “hummingbird” phenotype *in vitro*, which mimics EMT.^[13] CagA overexpression in gastric cancer (GC) cells up-regulated the expression of mesenchymal markers and CD44, which is a cancer stem cell marker in GC.^[14] CagA overexpressing cancer cells also showed high tumorigenic ability *in vivo*. Immunohistochemical analysis of samples from individuals with *H. pylori* infection confirmed high CD44 expression and expression of different mesenchymal markers.^[15] Tissue microarray analysis of samples from 385 GC patients revealed three miRNAs (miR-200c, miR-200b and miR-125b) to be significantly associated with survival. Functional experiments in a mouse model demonstrated that miR-200b suppressed ZEB1 and E-cadherin and inhibited cell migration and tumor growth.^[16] *In vitro* analysis revealed that overexpression of miR-200b also down-regulated ZEB2 expression, which in turn significantly reduced cellular proliferation,

migration and invasion in GC cells.^[17] miR-7, which is down-regulated in highly metastatic GC cell lines, was found to be involved in metastasis by regulating its direct target, insulin-like growth factor-1 receptor. Overexpression of miR-7 was able to suppress Snail expression, increase E-cadherin expression and partially reverse EMT.^[18] Several other EMT inducers have been reported recently. For example, erythropoietin-producing hepatocellular (Eph) A2 overexpression resulted in up-regulation of the EMT markers N-cadherin and Snail, and the Wnt/ β -catenin targets TCF4, Cyclin-D1 and c-Myc. In contrast, Eph A2 silence by short hairpin RNA had the opposite effect.^[19] SALL4, a zinc-finger transcriptional factor for embryonic stem cell's self-renewal and pluripotency, has been suggested to be involved in tumorigenesis. SALL4 overexpression induced EMT with increased expression of Twist1 and N-cadherin, and decreased expression of E-cadherin.^[20] Telomerase activation through induction of human telomerase reverse transcriptase (hTERT) induced malignant transformation by stabilizing telomeres. hTERT overexpression could promote EMT and stemness of GC cells. TGF- β 1 and β -catenin-mediated EMT was abolished by depletion of hTERT.^[21] In the gastric epithelium, the runt domain transcription factor RUNX3 functions as a key mediator of the TGF- β pathway. Loss of RUNX3 in gastric epithelial cells results in EMT and production of tumorigenic stem cell-like subpopulation expressing gastric stem cell marker Lgr5. Loss of both RUNX3 and *p53* caused gastric epithelial cells to be sensitized to TGF- β -induced EMT, during which the resultant induction of Lgr5 is enhanced by aberrantly activated Wnt pathway.^[22]

Colorectal Cancer

EMT is critical in transdifferentiation of polarized epithelial cells to an invasive mesenchymal phenotype. The function of EMT transcription factors in colorectal cancer (CRC) has been reported. Snail, an activator of EMT, was expressed at high levels in CRC colonospheres. Overexpression of Snail in CRC cells induced colonosphere-forming property and cell dedifferentiation. Blocking IL-8 expression or activity disrupted the Snail-induced stem cell-like features of colonospheres.^[23] Snail directly induced zinc finger protein 281 (ZNF281) transcription and repressed miR-34a/b/c, thereby protection of ZNF281 mRNA from direct down-regulation by miR-34. Furthermore, *p53* activation resulted in miR-34a-dependent repression of ZNF81.^[24] Syngeneic Twist1-positive colon carcinoma cells (CT26) that invaded tissues surrounding tumors demonstrated the mesenchymal phenotype.^[25] Genotype also affected the mechanism of EMT. TGF- β 1 induced changes in cell morphology, gene expression, motility and invasion consistent with EMT in microsatellite stable colon cancer cells, whereas cells exhibited

IL-6-dependent activation of signal transducer and activator of transcription 3 (STAT3), a conserved and direct target of miR-34a.^[26] Stimulation of EMT results in the nuclear translocation of pyruvate kinase M2 (PKM2) in colon cancer cells. EMT stimulation causes direct interaction of PKM2 in the nucleus with TGF- β -induced factor homeobox 2, a transcriptional cofactor repressor of TGF- β signaling.^[27] The roles of miRNA in EMT in CRC have been reported. For example, liver metastatic tissues showed higher expression of miR-200c than that of the primary tumor, and miR-200c overexpression was significantly associated with hypomethylation of the miR-200c promoter.^[28] Overexpression of miR-212 inhibited CRC cell migration and invasion *in vitro* and intrahepatic and pulmonary metastasis *in vivo*. Manganese SOD (MnSOD) was identified as a direct target of miR-212, and an inverse correlation has been observed between the level of miR-212 and MnSOD protein in colorectal tumor samples. MnSOD was required for down-regulation of epithelial markers and up-regulation of mesenchymal markers in CRC cells.^[29]

Hepatocellular Carcinoma

TGF- β is a major microenvironmental factor to affect hepatocellular carcinoma (HCC) dedifferentiation, inducing EMT and acquisition of metastatic phenotypes. Transcriptomic analysis on human HCC tissue samples revealed that TGF- β signaling was activated in a subpopulation of HCC, called Wnt-TGF- β subclass.^[30,31] Sequential transcriptome analysis suggested that TGF- β signaling was a late event accompanied with extensive gene alterations.^[32] TGF- β has been shown to induce hepatocyte nuclear factor-4 α (HNF-4 α) post-translational modifications that correlate with the early loss of the ability of HNF-4 α to bind to target gene promoters via glycogen synthase kinase-3 β (GSK-3 β) kinase during EMT.^[33] The receptor tyrosine kinase Axl binds to 14-3-3 ζ as a result of phosphorylation of the linker region of SMAD3 at Ser213, which causes the up-regulation of TGF- β target genes such as PAI1, MMP9 and Snail.^[34] The function of EMT transcription factors have been updated recently. Accumulative data on non-coding RNA have revealed a novel mechanism of EMT in HCC. For example, miR-200c was down-regulated in HCC with bile duct tumor thrombus, which occurred in 30 out of 1,240 patients, and regulated ZEB1 expression as well as an invasive phenotype.^[35] The miR216a/217 cluster induced EMT and its direct targets, phosphatase and tensin homolog and SMAD7 were identified.^[36] miR-331-3p-mediated inhibition of PH domain and leucine-rich repeat protein phosphatase resulted in stimulation of protein kinase B (AKT) and subsequent EMT.^[37] miR-424-5p reversed resistance to anoikis, blocked EMT progression and inhibited its direct target ICAT/CTNBP1, a novel β -catenin-interacting protein.^[38] A non-coding antisense transcript, ZEB1-antisense1 (ZEB1-AS1), promoted

EMT and metastasis in HCC. The zeb1-as1 promoter was hypomethylated in human HCC samples and resulted in tumor specific up-regulation of ZEB1-AS1.^[39] lncRNA-AL589182.3 (ENST00000493038), which can be activated by TGF- β , up-regulated ZEB1 and ZEB2 through competitively binding to the miR-200 family and induced tumor cell EMT and invasion.^[40] Interestingly, hepatitis C virus (HCV) has also been found to contribute to EMT. HCV core protein down-regulated secreted frizzled-related protein 1 (SFRP1) expression by inducing hypermethylation of the SFRP1 promoter.^[41] A previous transgenic mouse study demonstrated that overexpression of HCV core protein in HCC cells increased active TGF- β levels in culture supernatants and induced SMAD2/3 phosphorylation. HCC cells expressing HCV core protein could activate stellate cells in co-culture and this activation was TGF- β -dependent.^[42] CD44s, a known cancer stem cell marker in many malignancies, mediated TGF- β -induced EMT, and regulated mesenchymal phenotype in HCC.^[43,44]

Cholangiocarcinoma

Since this disease is not common, clinical and basic research on human cholangiocarcinoma (CCA) samples is limited. CCA is one of the solid cancers that have no effective molecular targeted therapy to date. Gemcitabine plus platinum is the only chemotherapeutic drug that to some extent inhibits CCA progression.^[45] Several EMT-related molecules are also known to play pivotal roles in CCA. Inactivation of miR-200c is reported to induce the expression of mesenchymal markers and NCAM1, a known hepatic stem/progenitor cell marker.^[46] STAT3-driven expression of small proline-rich protein 2a suppressed the interaction of miR-200c/141 with ZEB1.^[47] Although the efficacy of the EGFR tyrosine kinase inhibitors, erlotinib and cetuximab, has not been confirmed in CCA treatment,^[48] activation of the EGF-EGFR axis is known to abolish gefitinib-mediated EMT progression.^[49] ANXA8 was found to be involved in EGF-forkhead box protein O signaling-mediated EMT progression.^[50] The sonic hedgehog ligand is highly expressed in human CCA, and treatment with the hedgehog inhibitors, cyclopamine and 5E1, suppressed cell proliferation, migration and invasion by down-regulating the target genes hepatoblastoma 1 and 2. Furthermore, these inhibitors have been shown to attenuate EMT.^[51] In addition to the above-mentioned molecules, some unique molecules have also been linked to EMT recently in CCA, which include 4 histamines (H1-H4) and their receptor (HR). Loss of H3HR expression or overexpression of H4HR has been shown to significantly decrease CCA proliferation and disrupt EMT progression.^[52]

Pancreatic Cancer

Pancreatic cancer is one of the worst solid cancers in terms of prognosis and treatment outcome, because there is no promising molecular target identified to

date. EMT was first reported in this malignancy two decades ago, and the major functional interactions of the EMT-transcription factors have also been reported. The genomic landscape of pancreatic cancer has been partially unveiled.^[53] However, the role of each key molecule involved in EMT remains to be elucidated, an effective therapeutic molecular target is yet to be identified for pancreatic cancer. The epigenetic analysis revealed that the Class I histone deacetylase inhibitor mocetinostat suppresses ZEB1 and induces miR-203 re-expression, thus, leading to the repression of stemness properties and drug resistance.^[54] TGF- β 1 was highly up-regulated in pancreatic cancer.^[55] TGF- β 1 has been shown to induce EMT, SMAD2/3 phosphorylation, restoration of retinoblastoma 1 expression and SMAD-dependent up-regulation of Wnt7b in KRC cell line. In *in vivo* orthotopic models, inhibition of TGF- β 1 signaling suppressed those effects, resulting in tumor regression and decrease in metastasis.^[56] The calcium-/calcineurin-responsive nuclear factor of activated T cells, a transcription factor expressed during inflammation, drives EMT in a sex determining region-box 2-dependent manner and loss of *p53* induced EMT, and acquisition of cancer stem cell-like properties by down-regulating miR-200c.^[57] Ataxia telangiectasia Group D complementing gene, which is highly expressed in pancreatic cancer,^[58] up-regulated CD44 in mouse and human PanIN lesions via activation of β -catenin signaling. This in turn results in the induction of EMT phenotype and expression of ZEB1 and Snail1.^[59]

Perspectives

Increasing evidence supports the role of EMT in cancer progression, metastasis and drug resistance. Recent studies of EMT transcription factors and microRNAs are shown in Tables 1 and 2 respectively. In a tumorigenic mouse model, it was shown that EMT precedes pancreatic tumor formation.^[60] However, whether EMT occurs in the early stage or late stage of tumor formation remains to be confirmed. The mesenchymal phenotype is essential for tumor cell migration and invasion. The epithelial phenotype might be required for cancer cells to spread to other organs. Cancer cells tend to acquire both phenotypes under specific conditions, and the functional aspect of each phenotype regarding chemoresistance remains elusive.^[61] EMT has been categorized into three types: developmental (Type I), fibrosis and wound healing (Type II), and cancer (Type III). Of these, Type III EMT is the least well understood.^[62] If Type III EMT can be classified further into subgroups based on the molecular mechanisms, it would be possible to develop personalized cancer therapeutic approaches based on the specific EMT stage.

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Table 1: EMT TFs in gastroenterological malignancies

TFs	EC	GC	CRC	HCC	PDCA	CCA
ZEB1/2		[16,17]	[63]	[64]	[54]	
Twist1			[25]	[65]		
Snail	[11]		[23,24,66]	[65]		
SHP-1				[67]		
SMAD3/4	[5]				[56]	
FoxC1				[68]		
FoxC2		[69]				
FoxM1					[70,71]	
FoxQ1				[72]		
NFATc1					[57]	

EC: Esophageal carcinoma; GC: Gastric cancer; CRC: Colorectal cancer; HCC: Hepatocellular carcinoma; PDCA: Pancreatic ductal adenocarcinoma; CCA: Cholangiocarcinoma; SMAD: Mothers against decapentaplegic homolog; ZEB1/2: Zinc finger E-box binding 1/2; TFs: Transcription factors; EMT: Epithelial-mesenchymal transition; NFATc1: Nuclear factor of activated T cells; SHP-1: Small heterodimer partner-1

Table 2: MicroRNA in gastroenterological malignancies

MicroRNA	Targets	EC	GC	CRC	HCC	PDCA	CCA
miR7	IGF1R		[18]				
miR9	CDH1	[73]					
miR34a/b/c	ZNF281			[24]			
miR125b	SMAD2/4						
miR130b	IRF1				[74]		
miR146a	Numb			[66]			
miR148a	Snail				[75]		
miR200b/c	ZEB1, NCAM1		[16,17]	[28]	[35]	[76]	[46]
miR212	MnSOD			[29]			
miR216a/217	PTEN/ SMAD7				[36]		
miR223	Fbw7					[77]	
miR331-3p	PHLPP				[37]		
miR338-3p	ZEB2		[78]				
miR424-5p	ICAT/ CTNNBIP1				[38]		
miR655	ZEB1	[79]					
lncRNA	ZEB1/2				[40]		

EC: Esophageal carcinoma; GC: Gastric cancer; CRC: Colorectal cancer; HCC: Hepatocellular carcinoma; PDCA: Pancreatic ductal adenocarcinoma; CCA: Cholangiocarcinoma; PHLPP: PH domain and leucine-rich repeat protein phosphatase; SMAD: Mothers against decapentaplegic homolog; PTEN: Phosphatase and tensin homolog; IGF1R: Insulin-like growth factor-1 receptor; ZNF281: Zinc finger protein 281; IRF1: Interferon regulatory factor 1; ZEB: Zinc finger E-box binding; NCAM1: Neural cell adhesion molecule; MnSOD: Manganese superoxide dismutase

Conflicts of interest

There are no conflicts of interest.

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Growth factor receptors: promising drug targets in cancer

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ABSTRACT

Genetic, epigenetic and somatic changes deregulate the expression of growth factor receptors (GFRs), leading to cancer initiation and progression. Tumor cell growth and survival are orchestrated by clonal expansion and evasion of apoptotic signals in cancer cells. The growth of cells is further supported by angiogenesis and metastasis to distant organs. High expression of GFRs also contributes to the development of resistance. Therefore, therapeutics to target GFRs is a potentially attractive molecular approach to treat cancer more effectively. In this review, we have discussed the contribution of GFRs to cancer development and addressed molecular approaches undertaken to inhibit GFR-mediated pathways. A wide number of monoclonal antibodies (mAbs) and protein kinase inhibitors targeting these GFR-mediated functions are in clinical trials to treat human malignancies. However, most drugs that target GFRs lead to the development of drug resistance and generate adverse effects. Nucleic acid-based therapeutics, e.g. short interfering RNA (siRNA) could be harnessed to selectively silence GFR genes in cancer cells. Different polymer, liposome-based nanocarriers, and the most recently developed pH-sensitive inorganic carbonate apatite nanoparticles have been used in cell culture and preclinical trials for cytoplasmic delivery of the siRNAs targeting different GFR genes. siRNA-based therapeutics have been shown to have significant potential to suppress GFR expression and functions and thus could be developed as molecular therapeutics. Multi-targeting of tumors at different levels by combining various approaches along with chemotherapy would be a promising therapeutic approach to fight the disease. Suitable nanocarriers capable of entrapping siRNA, mAb, GFR inhibitors and classical drugs targeting GFR have potential therapeutic applications.

Key words: Carbonate apatite nanoparticles, growth factor receptor, monoclonal antibodies, protein kinase inhibitor, short interfering RNA, tyrosine kinase inhibitor

Introduction

The heterogeneous nature of cancer is characterized by continuous clonal expansion and uncontrolled growth of mutated cells, intravasation and extravasation of blood and lymphatic vessels, dissemination, and finally metastasis into distant organs. In the tumor microenvironment, cells are supplied with nutrients by the formation of disorganized blood vessels with leaky vasculature by the process of angiogenesis. Growth factor receptors (GFRs), expressed on cell membranes or in the cytoplasm, have profound roles in cell growth, survival, angiogenesis and metastasis. Amplification of GFRs generates inherent and acquired resistance to classical chemotherapies and targeted molecules. Escalated growth signals cross-talk differently with death signals to inhibit apoptosis that is programmed cell death. Accordingly, signals mediated by GFRs function in collaboration to enhance the complexity of the tumor microenvironment.

Here, we have discussed the involvement of GFRs at different stages of cancer progression and the molecular therapeutic approaches to target GFRs.

GFR Involvement in Cancer Progression

Epidermal growth factor receptor

The epidermal growth factor receptor (EGFR) family encompasses four receptor proteins, namely ErbB-1/EGFR-1 to -4 (also called HER 1-4) that are expressed on cell surface and exhibit tyrosine kinase activities. These proteins have similar structures and are comprised of three domains: an extracellular domain with ligand binding site, a transmembrane domain, and an intracellular domain with kinase activity [Figure 1a]. There are 11 different growth factors, each possessing a conserved EGF domain

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that can bind with those four receptors. Upon ligand binding, the receptors form homo-or hetero-dimers, promoting activation, relaying signals for proliferation, survival, migration and differentiation and thus playing major roles in cancer progression [Table 1]. Overexpression and/or gene amplification of *EGFR* confer malignancy to diverse tissues. Moreover, constitutively active mutants of EGFR are found in different cancers, where they are often associated with poor prognosis [Table 2].

Insulin-like growth factor receptor

The insulin-like growth factor receptor (IGFR) family consists of two cell membrane receptors, IGF1R and IGF2R. IGF1R (that also forms a heterodimer with the insulin receptor [IR]) binds to insulin-like growth factor 1 (IGF1) with higher affinity and IGF2 with comparatively lower affinity to elicit the growth signals required for foetal and postnatal development. The post-translationally modified IGF1R

is a polypeptide containing one α - and one β -chain that are connected by a disulfide bond and expressed on the cell surface [Figure 1b]. The α -chain and portion of the β -chain comprise the extracellular domain followed by transmembrane and cytoplasmic domain in β -chain.^[6-8] The mature IGF1R is a homodimer comprising the α_2 and β_2 chains linked by disulfide bonds. The intracellular domain has tyrosine kinase activity that auto-phosphorylates the receptor and a number of downstream proteins upon binding to the ligands. The notion of involvement of this receptor in tumorigenesis came from the studies of *IGF1R*-transfected cells and the effects of *IGF1R* gene mutation.^[9-11] Overexpression of *IGF1R* gene is implicated in cellular proliferation, transformation, and metastasis in several carcinomas [Table 1]. Amplification of *IGF1R* gene in breast cancer and melanoma and overexpression of *IGF1R* gene in pediatric cancer has been reported [Table 2].

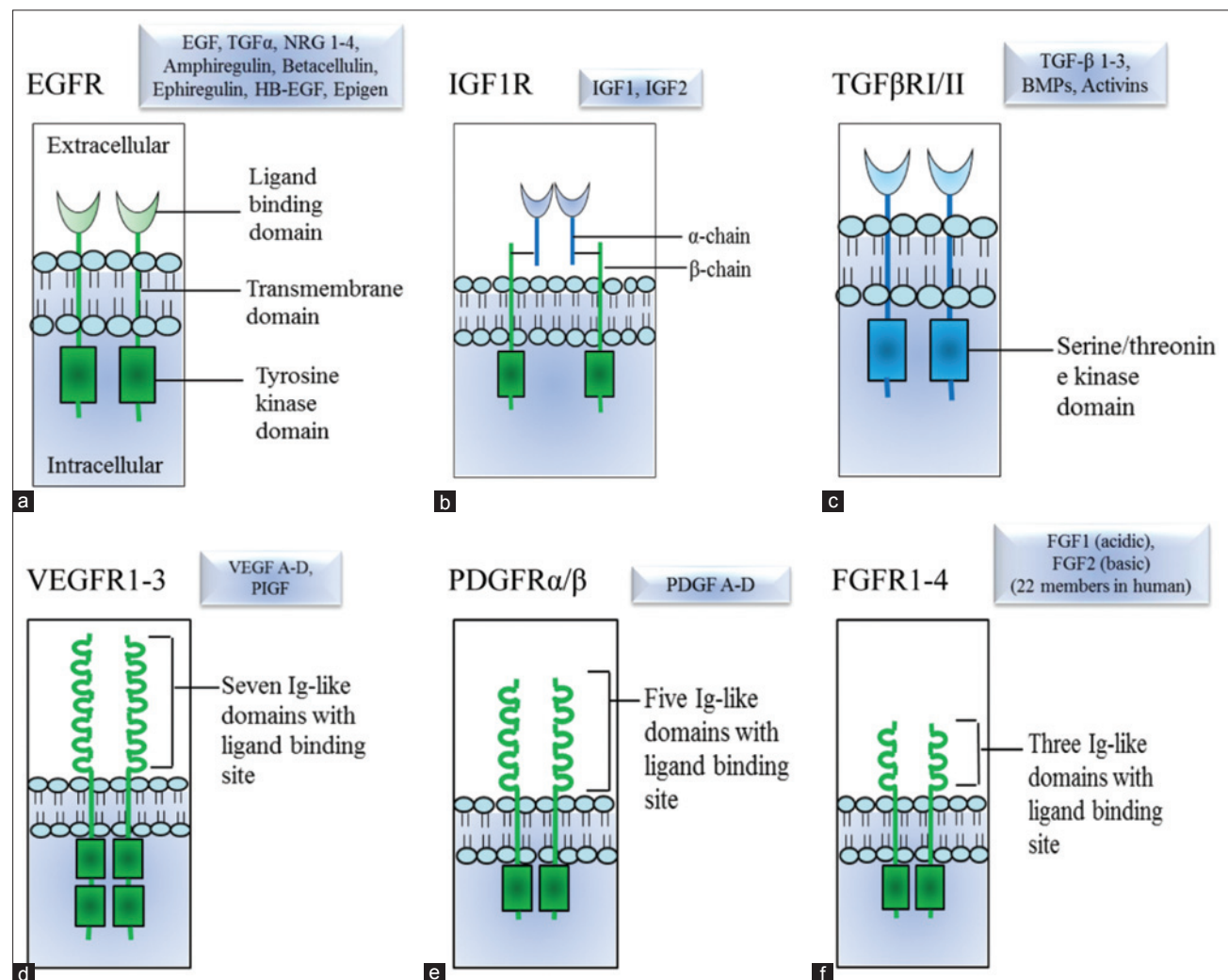


Figure 1: Schematic diagrams of membrane bound growth factor receptors and their ligands involve in cancer progression. Ligands are shown in boxes. (a) Epidermal growth factor receptor (ErbB/Her); (b) insulin-like growth factor receptor; (c) transforming growth factor-beta receptor; (d) vascular endothelial growth factor receptor; (e) platelet derived growth factor receptor, and (f) fibroblast growth factor receptor. ErbB2 (HER2) binds no known epidermal growth factor-like ligands, and ErbB3 shows no tyrosine kinase activity. They relay signals by forming heterodimer with other ErbB proteins from EGFR family. TGFβRIII does not pose any intracellular tyrosine kinase domain

Table 1: Involvement of GFRs in cancer progression

Family	Receptors	Tumor growth	Metastasis	Angiogenesis induction	Cell survival/death	Chemoresistance
EGFR/ErbB/HER	ErbB1	+	+	Pro-angiogenic	Pro-survival signals	+
	ErbB2	+	+			+
	ErbB3	+	+			?
	ErbB4	+	+			—
IGFR	IGF1R	+	+	Pro-angiogenic	+	+
	IGF2R	Suppress growth	—		—	—
TGF-βR (TβR)	TβR I-II	Dual role (contextual)	+	+	Dual role	+
VEGFR	VEGFR1	—	?	+	+	—
	VEGFR2	+	+	+	+	+
	VEGFR3	+	+	+	+	+
PDGFR	PDGFR (α/β)	+	+	+	+	+
FGFR	FGFR1-4	+	+	+	+	+

+/-: Yes/no involvement of growth factor receptor; ?: Not known yet. EGFR: Epidermal growth factor receptor; HER: Human epidermal growth factor receptor; IGFR: Insulin-like growth factor receptor; VEGFR: Vascular endothelial growth factor receptor; PDGFR: Platelet-derived growth factor receptor; FGFR: Fibroblast growth factor receptor; TGF-βR: Transforming growth factor-beta receptor; GFR: Growth factor receptor

Table 2: GFR expression in cancer

Family	Cancer	References
EGFR/ ErbB/ HER	Amplification and overexpression of <i>Her1</i> gene were found in breast (14-91%), bladder, lung, glial (50%) cancer patients; <i>HER2</i> gene related to poor prognosis was observed in different malignancies; aggressive metastatic breast (15-30%), gastric (10-30%), ovarian (20-30%), endometrial (1-47%), esophageal (0-83%), lung (20%), and invasive urothelial bladder (0-80%) carcinomas Constitutive expression of active truncated EGFR vIII that lacks extracellular domain was found in breast cancer (20-78%) associated with aggressiveness of tumor Mutations in <i>HER2</i> gene in lung, <i>Her3</i> gene (somatic) in breast, colon, gastric, <i>Her4</i> gene in melanoma, colorectal, gastric, lung, and breast cancer were observed in patients	[1]
IGFR	Amplification of <i>IGF1R</i> gene was reported in small number of breast and melanoma cases Mutations in <i>IGF2R</i> gene was found in squamous cell carcinomas of the lung	[1]
TGF-βR	<i>TGF-βRII</i> gene is mutated in colon (58-82%) and pancreatic (4%) cancer, absent in prostate (24%), and down-regulated in breast and lung cancer	[2]
VEGFR	High expression of <i>VEGFR1-3</i> genes was reported in a wide number of malignancies like bladder, brain, breast, colon, gastric, lung, ovarian, prostate, and head and neck carcinomas	[3]
PDGFR	Overexpression of <i>PDGFRα</i> gene was found in 20% of glioblastoma Germline point mutation (gain of function) in <i>PDGFRβ</i> gene was observed in 8 families with infantile myofibromatosis	[4]
FGFR	Amplification of <i>FGFR1-3</i> genes was observed in different cancer. For example, <i>FGFR1</i> gene in lung (20%), breast (10%), ovarian (~5%), bladder (3%); <i>FGFR2</i> gene in gastric (10%), breast (4% in triple negative); <i>FGFR3</i> gene in bladder and salivary adenoid cystic cancer Mutations in <i>FGFR1-4</i> genes were observed. For example, <i>FGFR1</i> gene in melanoma (rare), glioblastoma; <i>FGFR2</i> gene in endometrial (12%), lung (5%), gastric (rare); <i>FGFR3</i> gene in bladder (50-60% in nonmuscle invasive, 10-15% muscle invasive), cervical (5%), prostate (3%), colorectal; <i>FGFR4</i> gene in rhabdomyosarcoma (7-8%) cancer	[5]

EGFR: Epidermal growth factor receptor; HER: Human epidermal growth factor receptor; IGFR: Insulin-like growth factor receptor; TGF-βR: Transforming growth factor-beta receptor; VEGFR: Vascular endothelial growth factor receptor; PDGFR: Platelet-derived growth factor receptor; FGFR: Fibroblast growth factor receptor

The relatively simpler IGF2R (also called mannose-6 phosphate receptor, M6P) comprises a single polypeptide chain, and functions as a “scavenger receptor” for IGF2. It suppresses tumor growth, modulates invasiveness, and blocks angiogenesis [Table 1].^[12] Mutations in IGF2R locus have been observed in lung cells^[12] and identified as an early event in hepatocellular carcinoma in different populations.^[13]

Transforming growth factor-beta receptor

The transforming growth factor-beta receptor (TGF-βR) family comprises three membrane receptors (TβRI, TβRII and TβRIII) which are expressed in diverse types of cells and regulate distinct cellular functions by the signals transduced upon TGF-β ligand binding. TβR and TβRII are single pass serine/threonine kinases with

N-terminal ectodomains and C-terminal kinase domains. T β RIII (also known as betaglycan) is a cell surface proteoglycan > 300 kDa in molecular mass and does not possess an intracellular kinase domain [Figure 1c]. T β RIII binds with TGF- β ligands and presents them to T β RII or the ligands bind directly with T β RII depending on cell types. After binding, T β RII recruits and trans-phosphorylates T β RI, which in turn activates SMAD proteins. SMAD complexes translocate into the nucleus and function as transcription factors for TGF- β responsive genes and thus regulate cell proliferation, survival, migration and differentiation [Table 1]. TGF- β R-mediated signals play context-dependent dual roles in cell growth.^[1] Under physiological conditions, TGF- β prevents cell growth, stimulates apoptosis or differentiation. During tumorigenesis, TGF- β R-mediated signals promote cell growth due to genetic and epigenetic changes. Mutations and dis-regulation of *TGF- β R* genes were observed in different cancers [Table 2], for example, down-regulation of *TGF- β RII* gene in breast and lung cancer^[14,15] and different mutations in colon and pancreatic cancer.^[16-18]

Vascular endothelial growth factor receptor

This family consists of three membrane receptors (VEGFR1-3), predominantly expressed on endothelial cells and few additional cell types. VEGFRs are single pass protein with seven immunoglobulin (Ig)-like domains on the extracellular site and two split tyrosine kinase domains in the intracellular site [Figure 1d]. They bind with the disulfide-linked homodimer of VEGF isoform (VEGFA-D) ligands and placenta growth factors (PlGF1 and 2) to form homodimers or heterodimers of VEGFR-1 and-2 and relay the signal inside cells. The signals transduced by VEGFR are different between these receptors. For example, VEGFR2 (also known as KDR/flk-1) induces mitogen-activated protein kinases (MAPK)-dependent cell proliferation whereas VEGFR1 (flt-1) does not induce cell growth. However, activation of VEGFR1 by VEGF stimulates cell migration, a response that is also triggered by VEGFR2 activation. These VEGF-VEGFR interactions are well-known for their key roles in vasculogenesis and angiogenesis. VEGFR3 (flt-4) that is expressed on lymphatic vessels interacts with VEGF-C and VEGF-D and is thought to promote lymphangiogenesis. VEGFRs are thought to be responsible for blood and lymph vessel formation in tumor microenvironment and thus promote tumor growth and progression [Table 1]. High expression of *VEGFR* gene is observed in many different types of malignancies [Table 2]. Moreover, somatic mutations in *VEGFR2* and *VEGFR3* genes were identified in the most common infants' malignancy, juvenile hemangioma.^[19]

Platelet derived growth factor receptor

The platelet-derived growth factor receptor (PDGFR) family contains two receptors (PDGFR- α and- β) that are encoded by two different genes and are expressed on

the membrane of different cell types. These single chain receptor proteins have five Ig-like extracellular domains and a tyrosine kinase domain [Figure 1e]. Dimerization of receptors occurs upon binding to homo/heterodimers of PDGF (A-D) ligands, leading to conformational changes in receptors, activating them to trans-phosphorylate and stimulate downstream proteins. This relays the signals into receiving cells via mainly MAPK and PI3K pathways and thus regulates cell proliferation, differentiation, growth, migration, and survival. They have roles in angiogenesis and thus support tumor growth [Table 1]. Overexpression and mutations in the *PDGFR* genes are associated with diverse cancers [Table 2]. Aberrant expression of PDGFR due to amplification and/or overexpression of *PDGFR α* and *PDGFR β* genes were reported in human glioblastoma multiforme.^[4,20] Moreover, mutations and genetic translocation in *PDGFR α* gene were observed in gastrointestinal stromal tumors and chronic leukemia respectively.^[21,22] A germline point mutation (gain of function) in *PDGFR β* gene was found in the most common fibrous tumor of infancy, myofibromatosis.^[23]

Fibroblast growth factor receptor

The fibroblast growth factor receptor (FGFR) family consists of four closely related transmembrane proteins (FGFR1-4) and their different isoforms with altered ligand specificity due to differential splicing of FGFR mRNA. These single chain receptors contain one extracellular domain with three immunoglobulin repeats (Ig I-III) with ligand binding capacity, one transmembrane domain and one intracellular domain with kinase activity at the carboxy-terminus [Figure 1f]. There are 18 different FGF ligands that can bind to different FGF receptors. Upon binding, dimerization of FGFR leads to auto-phosphorylation and kinase activation. Phosphorylated FGFRs in turn phosphorylate a number of proteins and/or serve as molecular docking sites for many effectors, thus orchestrating context-dependent cellular functions including cell proliferation, growth, differentiation, migration, vascular repair, wound healing, and cell survival. FGF-FGFR interactions have pivotal roles in tumorigenesis [Table 1] as the downstream mitogenic growth signals (MAPK) and anti-apoptotic PI3K/AKT signals lead to uncontrolled growth and inhibition of cell death, respectively. The PLC/PKC pathway downstream of FGFRs also converges to the MAPK pathway to support cell growth.^[24-26] These receptors have been shown to exert profound roles in angiogenesis both in paracrine and autocrine fashions. FGFR expression causes tumor cells to acquire resistance to several drugs, especially inhibitors targeting other growth factor receptors (EGFR, PDGFR and VEGFR) because of their extensive cross-talks. Amplification and mutations in *FGFR* genes that lead to constitutive activation/up-regulation of receptors are found in different types of malignancies, including breast, ovarian, gastric and lung cancers [Table 2].

Targeting GFR-mediated Signals with Cancer Therapeutics

Accumulated understanding over the last 30 years of signaling pathways mediated by different GFRs and their relationship with cancer progression has led to the development of targeted agents for cancer treatment. There are at least 6 approaches to target these pathways: (1) monoclonal antibodies (mAbs) against GFRs; (2) protein kinase inhibitors; (3) nucleic acid-based therapeutics for gene silencing (use of antisense RNA or short interfering RNA [siRNA] to block receptor expression); (4) soluble receptors for growth factor ligands (“Traps”); (5) inhibitors of heat shock proteins and (6) antagonists of signaling pathway proteins.

Among these, the first three strategies including mAbs, protein kinase inhibitors and nucleic acid-based therapeutics are designed to target the GFRs, and thus are highlighted in this review. Radiological responses to targeted molecular agents used as monotherapy are typically more limited compared to the conventional chemotherapy and radiotherapy. However, effects on progression-free survival have been observed. Nowadays, targeted molecular agents are often combined with chemo- or radiotherapy, mainly for two reasons: combination with chemo- or radio-therapy improves efficacy and the molecular specificity of mAbs aids to target tumor selectively.

Monoclonal antibodies against GFRs

The development of hybridoma technology to produce mAbs is the first step in the process of turning the dream of “magic bullets” for targeted treatment of cancer a reality. A wide number of mAbs-based therapies have been approved by the Food and Drug Administration (FDA) for the treatment of different malignancies and many more are in clinical trials.^[27] These therapeutics act by directly blocking the function of GFRs and/or by antibody-dependent cytotoxicity, mediated by Fc fragment recognizing immune cells. Antibody-drug conjugates are designed for targeted drug delivery to cells expressing their cognate GFR. A variety of mAbs are used to modulate GFR functions in different indications [Table 3]. For example, the humanized mAb, trastuzumab designed to target ErbB2 and approved by FDA in 1998, is successfully used to treat HER2+ metastatic breast cancer patients.^[28] The approval of the drug was further expanded in 2006 for women with cancer in breast and lymph node region as early stage therapy after primary therapy (lumpectomy, mastectomy). In October 2010, it was approved to use for HER2-overexpressing, gastroesophageal junction adenocarcinoma in combination with either cisplatin or fluoropyrimidines in patients who have not received any prior treatment.

As VEGFR plays a key role in new blood vessel formation, different antibodies against VEGFR are designed to prevent angiogenesis and growth in cancer.^[29-32] The combination of mAbs with different antineoplastic drugs often increases effectiveness. Diverse combinations of mAbs conjugated with different types of drugs are currently in clinical trials.

Development of resistance against GFR-targeted mAbs is the foremost limitation to their clinical use. Resistance to these antibodies can be either primary or acquired which develops within few months to years of treatment.^[33] Patients treated with trastuzumab monotherapy showed intrinsic (66-88%) and acquired resistance within one year (15%) which resulted in loss of effectiveness.^[34] Although in most cases the underlying mechanisms of resistance remain poorly understood, in few cases resistance have been linked to the compensatory pathways mediated by other GFRs or mutations in downstream signaling pathways. For example, enhancement of IGF1R-mediated signal in anti-erbB2/HER2 (trastuzumab) antibody-treated breast cancer patients confers resistance to treatment.^[35] Overexpression of membrane-associated glycoprotein MUC4, PTEN-PI3K signaling pathways, and elevated HER2 extracellular domain in serum are also involved in trastuzumab resistance.^[34] Mutations in K-Ras are responsible for primary resistance against anti-EGFR antibodies in colorectal cancer.^[36] Treatment regimen containing multiple mAbs against different GFRs may conceivably increase resistance but may be unacceptably toxic. For example, cross-talk between EGF- and IGF-mediated signaling pathways plays major roles in acquired resistance. Preclinical data suggest that simultaneous blockade of the two pathways could be advantageous in treatment.^[37-40] However, a phase II trial in colorectal cancer patients treated with cetuximab and cixitumumab failed to show any additional antitumor activity, and so this treatment regimen was eliminated from consideration in colorectal cancer patients refractory to EGFR inhibitors.^[41,42] Mechanism-based combinations of GFR mAbs will require case-by-case validation in preclinical and pilot clinical studies.

Protein kinase inhibitors

Structural and functional analyses have paved the way to discovery and development of numerous protein kinase inhibitors, especially tyrosine kinase inhibitors (TKIs) that inhibit the cytoplasmic kinase activity of growth receptors and subsequently their downstream signaling cascades into the cells. Most of these compounds are hydrophobic in nature and, therefore, are orally bioavailable. The majority of these agents rapidly cross cell plasma membranes and compete with phosphate donor adenosine tyrosine phosphate (ATP), phosphorylation substrates, or both.^[43] Many such compounds have shown cytostatic effects in cancer cells and animal models.^[44] Because of the “druggability” of

Table 3: Some mAbs against GFRs for treating cancer

Name of mAB	Target	Targeted stages	Indication	Mechanism of action	Resistance (known mechanism)	Status (highest level)
Cetuximab (human IgG1) (use as single or conjunction with radiotherapy)	ErbB1	Suppresses cell growth and metastasis	Metastatic colorectal; head and neck carcinoma	Inhibition EGFR signaling (down regulates active EGFRVII) and ADCC	Yes (mutations in a number of diverse genes are blamable for intrinsic resistance)	Approved
Panitumumab (human IgG2) (use as single agent)	ErbB1	Suppresses cell growth	Metastatic colorectal cancer	Prevents EGFR activation		Approved
Trastuzumab (humanized IgG1) (use as single or as adjuvant for chemotherapy)	ErbB2	Suppresses cell growth and angiogenesis, induces cell death	HER2+ metastatic breast cancer	Inhibition ErbB2 signaling and ADCC	Yes (overexpression of membrane associated glycoprotein MUC4; increase IGF1R signaling are some of the reasons that confer resistance)	Approved
Ganitumab (human IgG1) (use as single or combined with different neoplastic drugs)	IGF1R	Inhibits cell growth, delays tumor progression	Non-Hodgkin lymphoma, metastatic pancreatic cancer, metastatic Ewing family of tumors	Blocks IGF-1 and-2 binding to IGF1R without crosslinking with IR, inhibits activation of IGF1R homodimer and IGF1R/IR heterodimer	Yes (calcium dependent proliferation effects acquire resistance in prostate cancer cells)	Clinical trials (passed phase II)
Cixutumumab (human IgG1) (use as single or combined with different neoplastic drugs)	IGF1R	Induces cancer cell apoptosis, decreases cell proliferation	Solid tumors, Ewing sarcoma family tumors	Prevents IGF1 binding to receptor and subsequent activation of PI3K/AKT survival pathway, mediates receptor internalization and degradation	NA	Clinical trials (phase I-II)
PF03446962/ Anti-Alk1 (human)	TGF-βR	Prevents angiogenesis (dose dependent)	Transitional cell carcinoma of bladder	Disrupts co-localization of endothelial cells with perivascular cells and reduces blood flow	NA	Clinical trials (phase II)
Ramucirumab (human IgG1): use as single or with neoplastic drugs	VEGFR2	Inhibits tumor angiogenesis and growth	Hepatocellular, renal cell, and ovarian carcinomas	Blocks VEGF binding to the receptor and thus VEGF-signaling and subsequently angiogenesis	Yes (VEGF-axis dependent pathway is involved for resistance)	Clinical trials (phase II)
1B3 (used in combination with mAB against antitumor/ anti-angiogenic agent)	Mouse PDGFRβ	Inhibits angiogenesis	Pancreatic and a nonsmall cell lung tumor xenograft models	Blocks PDGFR binding with receptor, ligand-stimulated activation of PDGFRβ and downstream signaling molecules in tumor cells	NA	Preclinical trial
IMC-2C5 (human)	Both mouse and human PDGFRβ	Delays growth (cell specific), inhibits angiogenesis			NA	Preclinical trial

Clinical phase studies are checked in www.clinicaltrial.gov site. The targeted stages of mAbs in cancer progression are given based cell line studies and preclinical trials. ADCC: Antibody-dependent cell-mediated cytotoxicity; NA: Not available data; EGFR: Epidermal growth factor receptor; VEGF: Vascular endothelial growth factor; IGF: Insulin-like growth factor; PDGFR: Platelet-derived growth factor receptor; mAbs: Monoclonal antibodies

kinases, 30% of new efforts by pharmaceutical companies are dedicated to develop new kinase inhibitors with many already approved or in clinical trials. Few examples of TKIs for GFRs are enlisted in Table 4. Chemo- or radio-therapeutic agents are often combined with TKIs to increase therapeutic efficacy. The IGF1R inhibitor, BMS-754807 showed higher efficacy when combined

with cytotoxic, hormonal or targeted agents. BMS-754807 in combination with docetaxel showed more than additive efficacy in triple negative breast cancer.^[43] There are now several clinical trials (phase I and II) ongoing with BMS-754807-including combinations. Co-targeting of IGF1R and IR with OSI-906 inhibitor showed superior antitumor activity compared to targeting IGF1R

Table 4: Some TKI for GFRs for treating cancer

Name of TKI	Target	Targeted stages	Indication	Mechanism of action	Resistance	Status (highest level)
Gefitinib	ErbB-1, -2 and -3	Inhibits anti-apoptotic signals, induces anti-angiogenic activity	Breast and lung cancers (effective in cancers with mutant and overactive EGFR)	Binds reversible to the ATP binding site of receptor and inhibits formation of phosphotyrosine residues in receptor	Yes (overexpression FGF2/FGFR1 signal was found to be accountable for resistance in NSCLC cell lines)	Approved
Erlotinib	ErbB1	Induces cell cycle arrest and apoptosis, inhibits angiogenesis	NSCLC, pancreatic cancer (more effective in cancers with mutant and overactive EGFR)	Reversibly inhibits IGF1R phosphorylation	Yes (overexpression of PDGFR α is found to be responsible for acquiring resistance)	Approved
BMS-754807 (single or coupled with cytotoxic/hormonal/targeted agent)	IGF1R (and insulin receptor)	Inhibits growth of tumors, induces apoptosis, plays significant role in mitogenesis, angiogenesis and tumor cell survival, enhances therapeutic efficacies of attached drugs	Neoplasms, breast cancer, advanced metastatic solid tumors			Clinical trials (phase I-II)
Axitinib (single or combined with drugs)	VEGFR 1-3 (thought to act on PDGFR also)	Inhibits cell growth in xenograft models, enables to inhibit angiogenesis, vascular permeability and blood flow, decreases metastasis	Breast cancer, renal cell carcinoma, hepatocellular carcinoma, advanced pancreatic cancer, glioblastomamultiforme	Binds with VEGFR and inhibits receptor activation through phosphorylation	Yes (multi-drug transporter proteins, ABCB1, and ABCG2 may play role)	Approved for metastatic renal cell carcinoma and advanced pancreatic cancer, clinical trials (phase I-II) are ongoing for different malignancies
Sunitinib/SU11248 (single or attached with drugs)	VEGFR; PDGFR β (also target some other RTK)	Inhibits cell growth and angiogenesis, delays tumor progression	Renal cell carcinoma, gastrointestinal tumor, colorectal neoplasm, metastatic breast cancer	Selectively inhibits VEGFR2 and PDGFR β phosphorylation (in a time- and dose-dependent manner)	Yes (activation of sphingosine kinase 1 is account for acquired resistance in renal cell carcinoma)	Approved for renal cell carcinoma, clinical trials (phase I-II) for other cancer
TKI258	VEGFR; PDGFR; FGFR	Inhibits cell motility and growth, delays established tumor growth, inhibits metastasis, suppresses angiogenesis	Mammary tumors, multiple myeloma, colon cancer, pancreatic cancer	Competes with ATP for the binding site, inhibits GFR-mediated signals	NA	Preclinical

Clinical phase studies are checked in www.clinicaltrial.gov site. The action of TKIs on cancer progression stages (targeted stages) and mechanism of action are given based on studies of cell line studies and animal models. TKI: Tyrosine kinase inhibitor; NA: Not available data; NSCLC: Nonsmall cell lung cancer; EGFR: Epidermal growth factor receptor; VEGF: Vascular endothelial growth factor; PDGFR: Platelet-derived growth factor receptor; RTK: Receptor tyrosine kinase; ATP: Adenosine tyrosine phosphate; FGFR: Fibroblast growth factor receptor; VEGFR: Vascular endothelial growth factor receptor; GFR: Growth factor receptor

alone by mAbs^[46] and enhanced the antitumor effect of doxorubicin in a colorectal cancer model.^[47]

However, the development of resistance to TKIs makes their therapeutic use quite challenging.^[48] Overexpression of PDGF α in cells has been found to be responsible for acquiring resistance against BMS-754807. Different mechanisms have been identified which account for TKI resistance (both acquired and inherent) in cancer cells. These are: (1) somatic, genetic or epigenetic mutations within kinase domains; (2) overexpression and amplification of GFRs genes to overrule the inhibitors' function; (3) modifications in signaling pathways to bypass the signal mediated by specific receptor; and (4) overexpression of ATP-binding cassette transporters proteins (ABC-transporters) which transport TKIs outside of cells, limiting achievable intracellular concentrations.

TKIs with broad spectrum activity that inhibit a number of GFRs are less specific but often more effective compared to highly specific inhibitors. For example, a multi-targeted TKI against VEGFR, PDGFR and FGFR (TKI258) is more potent in inhibiting angiogenesis in pancreatic cancer cells as the signals mediated by these three receptors are crucial for the blood vessels formation.^[49] This inhibitor is efficacious in delaying cancer growth and inhibiting metastasis in a pancreatic cancer model^[49] and clinically used for advanced renal cell carcinoma and breast cancer.^[50,51] Broad spectrum TKIs would be less susceptible to acquired resistance.

Nucleic acid-based therapeutics to block GFR expression

The clinical applications of current chemotherapeutic drugs are often limited by their toxic effects on healthy dividing cells. Dose reductions due to toxicity can limit efficacy and select drug-resistant cancer cell clones. Advances in cancer molecular and cell biology have led to the identification of numerous potentially actionable genes, not all of which encode druggable targets. These genes and their transcripts are potential targets for nucleic acid therapeutics. Gene silencing both at transcriptional and translational levels is a promising tool to treat cancer more effectively.^[52] Among available technologies, RNA interference (RNAi) using double stranded siRNA or short hairpin RNA (shRNA) is a promising candidate technology, provided that pharmacokinetic obstacles to quantitative delivery are overcome.

RNAi is a biological posttranscriptional regulatory process in which small endogenous RNA (microRNA) inhibit gene expression by hybridizing with mRNAs and either causing their degradation or preventing translation. Mimicking physiological RNAi, siRNAs are designed exogenously to deliver to cancer cells for selective mRNA targeting. There are two fundamental

techniques of executing RNAi: nuclear delivery of gene expression constructs to express shRNA and cytoplasmic delivery of siRNA. Silencing by synthetic siRNA, RNA oligonucleotides 21-23 nucleotides long, is more expedient than shRNA due to the difficulty of constructing shRNA expression systems^[53] and the requirement for nuclear delivery.^[54] The potential gene silencing ability of siRNAs in animal models has made them promising investigational drug candidates and some siRNAs are in clinical trials. However, no siRNA against GFRs have been approved yet for cancer treatment. There are few siRNAs against GFRs, which have been used in cell culture and animal models [Table 5]. The primary challenge to the clinical use of RNAi is the need to deliver a relatively small molecule in sufficient quantities to tumor cells after systemic administration. Nucleic acid therapeutics delivery is an area of very active investigation.

Concerns and Future Perspectives

The anionic nature of siRNA prevents its diffusion through cellular membrane posing a difficulty in delivering siRNA into cells. Moreover, systemically administered naked siRNA is subjected to degradation by endogenous nucleases, renal clearance, and non-specific bio-distribution. Accordingly, a smart carrier is essential for functional delivery of siRNAs into the system. A wide number of genetically engineered viral vectors or synthetic polymer/liposome-based nanovectors are in use to deliver siRNAs in different cells and animal models. However, there remain concerns surrounding the safety and efficacy of these nanovectors. The successful clinical application of siRNAs will require nanosized cargos with higher binding affinity for siRNAs and possibly other drugs, fast release of bound siRNA in the cytoplasm, versatility to be engineered for targeting tumors, *in vivo* stability, lack of immunogenicity and minimal toxicity. A pH-sensitive inorganic carbonate apatite nanocarrier system has recently been developed that could provide an attractive solution to the challenges presented by other carriers. This carrier has been used to transport siRNAs against *ErbB2*, *IGF1R*, and *Bcl-2* genes as well as wild-type *p53* gene that inhibited the growth of established tumors in syngeneic mouse models.^[56,58] This platform could be used to target one or more GFRs in tumors.

Conclusion

Targeting multiple GFRs offers significant therapeutic promise in cancer therapy. As overexpression of GFRs is also responsible for resistance to different drugs, combination regimens may prevent or alleviate resistance. Nanoparticles-mediated siRNA delivery may have significant clinical applications once clinically suitable delivery platforms are identified and validated.

Table 5: Some siRNAs designed against GFR genes for treating cancer. The vehicles to carry these siRNAs, their functionality in tumor progression and the experimented cell lines are listed

Targeted genes	Delivery vehicle	Functionality in tumor progression	Cell/animal models	References
<i>EGFRvII</i> (encapsulated with Erlotinib or SAHA)	Cyclodextrin-modified dendritic polyamines (DexAMs) (cationic biodegradable polymer)	Decreases cell proliferation, induces apoptosis	Brain cancer cells (U78 glioblastoma)	[55]
<i>ErbB2</i> (encapsulated with wild p53 gene)	Carbonate apatite nanoparticles (inorganic pH sensitive)	Decreases cell viability, delays tumor growth	4T1 cells induced breast cancer mouse	[56]
<i>ErbB2</i>	Folate linked lipid-based nanoparticles (cationic liposome)	Inhibits tumor growth	KB (cervical adenocarcinoma-overexpresses folate receptors) cells induced nasopharyngeal cancer mouse	[57]
<i>IGFR1R</i> (encapsulated with wild p53 gene and anti Bcl-2 siRNA)	Carbonate apatite nanoparticles (inorganic pH sensitive)	Inhibits cellular growth/proliferation, regresses tumor growth, enhances drugs' sensitivity	4T1 cells induced breast cancer mouse	[58]
<i>IGF1R</i>	Lipofectamine 2000 (cationic liposome)	Lowers cell proliferation. increases sensitivity of drug (adriamycin), induces apoptosis	Liver cell lines (HepG2 and Huh7 cells)	[59]
<i>PDGFRα</i>	siLentFect (cationic liposome)	Sensitizes cells to AKI treatment	Pancreatic cancer cells	[60]
<i>FGFR1</i>	Lipofectamine/oligofectamine (cationic liposome)	Reduces cell viability, increases sensitivity of cells to drug (4-hydroxytamoxifen)	Breast cancer cells (MDA-MB-134)	[61,62]
<i>EGFR1, IGF1R</i>	Lipofectamine	Induces cell death, increases sensitivity of adriamycin	Liver cell lines (Huh7 cells with mutated p53 gene)	[63]

GFR: Growth factor receptor; SAHA: Suberoylanilide hydroxamic acid; AKI: Aurora kinase inhibitor; siRNA: Short interfering RNA

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Conflicts of interest

There are no conflicts of interest.

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Ten years of lung cancer in a single center: gender, histology, stage and survival

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ABSTRACT

Aim: The aim was to describe, in a prospective manner, the clinical, histopathological and epidemiological characteristics of lung cancer patients who attended as outpatients at the Lluís Alcanyis, Xàtiva Medical Oncology Hospital, València, Spain from January 2004 to July 2014. We also analyzed survival and compared our data with that reported in the literature. **Methods:** Clinical and demographic characteristics were analyzed for the entire series and trends were compared by year of diagnosis. Changes in epidemiology were examined and compared. **Results:** There were 701 patients (91.4% were men, mean age 67.6). Main histology was squamous cell carcinoma (41.5%). Squamous cell carcinoma prevailed in men (45.5%) and adenocarcinoma (ADC) in women (60.3%). The percentage of men with lung cancer and of patients with squamous cell carcinoma was higher than in the reported worldwide data and remained throughout the 10 years period. Mean survival was low, with < 10% survivors at 5 years. Stage of disease remained the main prognostic factor for survival. **Conclusion:** Squamous cell carcinoma continues to be the most frequent histological type in our area. Male and smoking is associated with lung carcinoma while ADC more often occurs in females. Over the time, our epidemiological and histological patterns have not changed, possibly in relation to maintenance of smoking habits.

Key words: Epidemiology, histology, lung cancer, smoking, survival

Introduction

Epidemiological changes in smoking habits are affecting the pattern of lung cancer patients, with perhaps an increasing number of non-smokers, women involved, and variation in the occurrence of adenocarcinoma (ADC).^[1-3] Despite treatment advances in lung cancer, it continues to be one of the most lethal cancers worldwide.

Lung cancer still ranks as the leading cause of tumor-related death in the world.^[1] Some important epidemiological factors are age, gender and histology, and these have markedly changed in the past few years.^[4] Reasons could be non-smoking policies, population aging, women now smoking, improvement in histological and imaging diagnosis, etc.^[2-6] The patterns of change vary, mainly given the heterogeneity of smoking habits in different countries. There is scattered information available concerning the various epidemiological and clinical aspects of lung cancer today, especially in Spain and in daily clinical practice.^[7] In order to describe how lung cancer patients

are managed at our regional hospital, since 2004, all such patients who were seen at our outpatient oncology unit were prospectively registered into a hospital-based cancer registry. The aim of the present review is to describe their epidemiologic characteristics, focusing on gender, histology and stage. Trends through years were also analyzed.

The primary objective was to describe the lung cancer characteristics of patients followed for up to 10 years, from 2004 to 2014, and to study the evolution of the disease over these years. In addition, we herein describe their epidemiologic characteristics, correlations, and prognostic factors through these years.

Methods

Patients and methods

This was a single-center study, prospectively performed at the Medical Oncology Unit of our hospital. All

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patients seen by our medical consultation (not all with a diagnosis of lung cancer) were prospectively included in our database registry. During the study period, our hospital served a predominantly rural community, with a population of about 210,000 in which geographic mobility was low. Our lung cancer medical unit treated and monitored patients by the same oncologist. Candidates for surgery and radiotherapy were referred to other hospitals for treatment, as our hospital does not offer this specialty.

From the records of patients who attended during the study period (2004-2014), the following information was gathered: date of diagnosis, age at appointment, gender and tumor histology (2004 WHO classification).^[8] Immunohistochemical markers (CK7, CK20, TTF-1 and p63) have been used at our hospital since 2007.^[9-11] Tumor-node-metastasis (TNM) stage by American Joint Committee on Cancer, Seventh Edition, was also utilized.^[12] Patients with non-small cell lung cancer (NSCLC) were classified by clinical parameters (clinical TNM), with small cell lung cancer (SCLC) also being classified by TNM system. Dates of death were included, although when the date was undefined based on records, family was contacted. We reported the cause of death (death without disease; death with the disease). For patients still alive, the last follow-up was recorded as July 15, 2014. Survival time was calculated from the time of histological/radiological diagnosis. Patients had to have at least 1-month of follow-up.

Genetic testing, when performed, was for the epidermal growth factor receptor (EGFR) and other NSCLC-driving mutations. Screening for drug-sensitive *EGFR* mutations was conducted as part of a clinical assistance program, since June 2010, by peptide nucleic acid-locked nucleic acid polymerase chain reaction clamp-based testing. Those who were not analyzed have been recorded as "not determined." Anaplastic lymphoma kinase (ALK) translocations were determined via fluorescence *in situ* hybridization since June 2012. We did not study K-RAS mutations as part of the standard of care. Other aspects relevant to prognosis, such as the Eastern Cooperative Oncology Group score, treatment type, weight loss and smoking habits, were not recorded.

Statistical analysis

Results were expressed as means (standard deviation) and percentages. The relationships between different variables were evaluated. Statistics of contrasts, such as Chi-square, Mann-Whitney *U*-test or Kruskal-Wallis *H*-test, were used for comparisons of two variables. Estimations are accompanied by 95% confidence intervals. Statistical significance was set at a value of $P < 0.05$. Survival time was defined as the period from the date of first visit to the date of mortality or last follow-up. Survival date was updated on July 15, 2014. In addition to the estimation of the survival rates by Kaplan-Meier method,

patients were classified into groups for comparison of their demographic and clinical characteristics as follows: gender, stage and histology. The same classification was made for comparison of survival by year of diagnosis. Statistical analysis was performed using SPSS 12.

Study approval

The institution's ethical review board approved the study, and all patients provided written informed consent and gave permission before study entry to collect their clinical data for scientific purposes and publication.

Results

From January 1, 2004 to June 15, 2014, 701 patients were included at our series. Patients' characteristics are shown in Table 1. We found an aged and male-predominant population (mean age: 67.6; 91.4% were male).

Table 1: Patient characteristics (n = 701)

Characteristics	n (%)
Gender	
Men	642 (91.6)
Women	59 (8.4)
Age, years	
Mean, range	67.6 (34-94)
Median	69
Mode	70
Histology	
Unconfirmed	24 (3.4)
Small cell	120 (17.1)
Non-small cell	556 (79.4)
Squamous	291 (41.5)
Adenocarcinoma	187 (26.7)
Bronchoalveolar	1 (0.1)
Large cell carcinoma	43 (6.1)
Carcinoma not typed	15 (2.1)
Sarcoma-squamous (carcinosarcoma)	3 (0.4)
Neuroendocrine tumors	11 (1.6)
Mesothelioma	6 (0.9)
Stage at diagnosis	
0	1 (0.1)
I	71 (10.1)
II	53 (7.6)
III	171 (24.4)
IV	405 (57.8)
Survival (months)	
Mean, range	25.58 (22.1-29)
Median	11
Situation at last follow-up (July 15, 2014)	
Alive without disease	42 (6)
Alive with disease	115 (16.4)
Death without disease	23 (3.3)
Death with disease	521 (74.3)
Situation at last follow-up (July 15, 2014)	
Alive	157 (22.4)
Death	544 (77.6)

Trends in gender by years

Only 59 (8.4%) patients were women [Figure 1]. This low incidence was maintained across the years. We found a slight increase in women patients from 2010 to 2014 ($P = 0.045$). A ratio of almost 9:1 was maintained across the years. Female was related to younger age ($P = 0.001$), histology (ADC and small cell: $P = 0.001$), Stage IV ($P = 0.02$) [Table 2].

Distribution by histological type

Histology related to smoking habit (SCLC and squamous cell lung cancer [SQCLC]) was predominated (121, 17.2% SCLC and 291, 41.5% SQCLC) [Table 1].

Histologic trends by years

Trends through years showed a decline in SQCLC. Although it was the main histology (incidence 37-45%), in later years, we found a significant increase in ADC (32-40%) and a significant and relevant increase in SCLC (last date near 20-25%) ($P = 0.0001$) [Figure 2].

Stage trends by years

A tendency of an increase of earlier stages in the last years is shown in Figure 3 ($P = 0.063$). There was also a decrease in Stage IV and an increase of Stage III patients. Stage was related to gender (female and Stage IV, $P = 0.024$). For histology and stage, we found a relationship ($P = 0.03$) between squamous cell and Stage III and between ADC and Stage IV. When we studied correlations between stages

and histology, we found more early stage patients with NSCLC ($P = 0.015$). SCLC was related to the advanced stage. Genetic testing for *EGFR* and other NSCLC-driving mutations was performed only for Stage IV patients, with only *EGFR* and *ALK* being analyzed. Only ADC had *EGFR*-activating mutations (4% of all ADC, 3.2% all NSCLC, Stage IV). Female ADC was related to *EGFR* mutation (11 patients, 36% of women). Only 1.7% of ADC in men had *EGFR* mutations. No patients in our series had *ALK* rearrangement.

Overall survival

Survival time was ascertained for all patients. Median overall survival (OS) for the entire series was

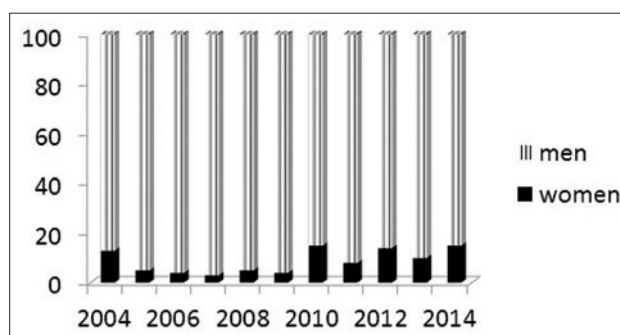


Figure 1: Trends in gender by year

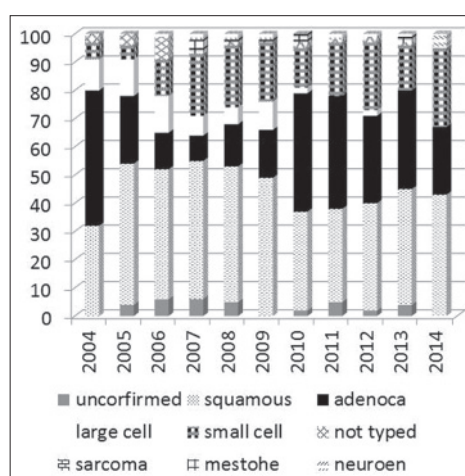


Figure 2: Trends in histology by year

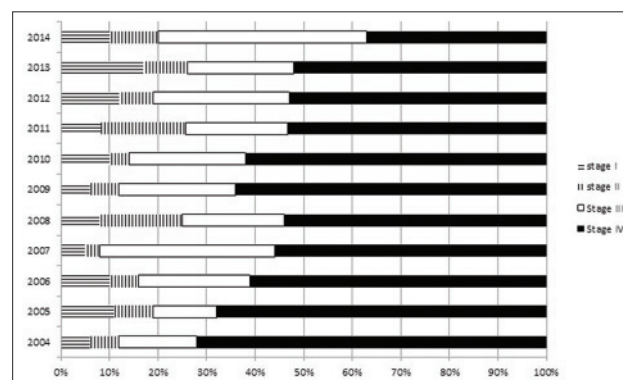


Figure 3: Stage trends by years

Table 2: Comparison between men and women

	Male <i>n</i> (%): 633 (90.3%)	Female: <i>n</i> (%): 68 (9.7%)	Chi-square Pearson
Age, years			
Mean (range)	68 (34-94)	63.3 (34-88)	$P = 0.000$
Median	70	61	
Mode	70	48	
Histology, <i>n</i> (%)			
Unconfirmed	23 (3.6)	1 (1.5)	$P = 0.000$
Small cell	109 (17.2)	10 (14.7)	
Non-small cell			
Squamous	288 (45.5)	4 (5.9)	
Adenocarcinoma	146 (23.1)	41 (60.3)	
Bronchoalveolar	0	1 (1.5)	
Large cell carcinoma	39 (6.2)	4 (5.9)	
Carcinoma not typed	9 (1.4)	6 (8.8)	
Sarcoma-squamous (carcinosarcoma)	3 (0.5)	0	
Neuroendocrine tumors	10 (1.6)	1 (1.5)	
Mesothelioma	6 (0.9)	0	
Stage at diagnosis			
0	0	1 (0.6)	$P = 0.02$
I	66 (10.4)	7 (10.3)	
II	48 (7.6)	4 (5.9)	
III	162 (25.6)	8 (11.8)	
IV	357 (56.4)	49 (71.4)	

25.5 months (22-29) [Figure 4]. At last follow-up, 22.4% of patients were alive and 77.6% were deceased. There were 6.1% (43) alive without disease, 16.5% (115) alive with disease, 3.3% (23) were dead without disease and 74.1% (520) were dead from lung cancer. The only significant prognostic factor for OS was stage ($P = 0.000$). Stage was a predictive factor for better survival and remained significant through all years. It shows OS by stage [Figure 5]. Histology was unrelated to survival by stages, except for Stage IV ($P = 0.003$). Through the years, survival for Stages I and II decreased, it maintained for Stage III, and had an increase of 2 months for Stage IV [Figure 6]. As death by other cause is important for OS, we analyzed causes of death. Only for Stages I (26.8%) and II (5.8%) there were deaths without disease. For Stages III and IV, lung cancer was the main cause of death for all patients. Gender and histology were only related to survival for Stage IV. Women with ADC and neuroendocrine differentiation had better survival ($P = 0.021$), while men with squamous cell carcinoma had better survival ($P = 0.044$), both groups in Stage IV. Also, molecular prognostic factors, in particular, mutated *EGFR* was related to better survival for Stage IV (17.3 [10.3-24.3] months vs. 10.4 [9-11] months; $P = 0.02$) but when we analyzed females, there was no difference in survival with women with *EGFR*-mutated vs. wild type or unknown ADC (*EGFR*-mutated [16.7 months] vs. wild type or unknown [14.8 months], $P = 0.54$)).

Longer survival for Stage IV

Median OS for Stage IV patients was nearly 12 months and there were 100 patients with median OS of 12 months or more in this stage (24.6%). Median OS for those with < 12 months was only 5 months (4.6-5.3). For those surviving more than 1-year, OS was 26.5 months (23-30 range) ($P = 0.0000$). Prognostic factors related to longer survival with Stage IV were: female ($P = 0.000$), histology (ADC and neuroendocrine), and *EGFR* mutation for men only. Longer survival was statistically significant, related to the year of diagnosis (2011 and 2012, $P = 0.006$).

Discussion

After the analysis of our 10 years database, we have found that lung cancer in our region remains a disease of smoker men. The predominant cause of lung cancer in men is active cigarette smoking. From our date, we cannot check the hypothesis that women are more susceptible than men to smoking-induced lung cancer. What we have found is that young women are smokers and elderly are non-smoker lung cancer patients. However, aspects of lung cancer in men and women continue to indicate potential male and female differences in the etiology of lung cancer, which based on several observations. Among non-smokers, women have higher lung cancer incidence

rates than men. There are different clinical characteristics of lung cancer in women compared with men, such as the higher percentage of ADC in non-smokers, the greater prevalence of *EGFR* gene mutations in ADCs among non-smokers, and better prognosis. Our study reports on the variation in lung cancer patterns and trends across 10 years in a single center registry.^[13-16] Special attention has been given to gender, histology, stage and survival. We found a high incidence of lung cancer in men that maintained across the years. The majority of patients were diagnosed at an advanced stage and OS remained

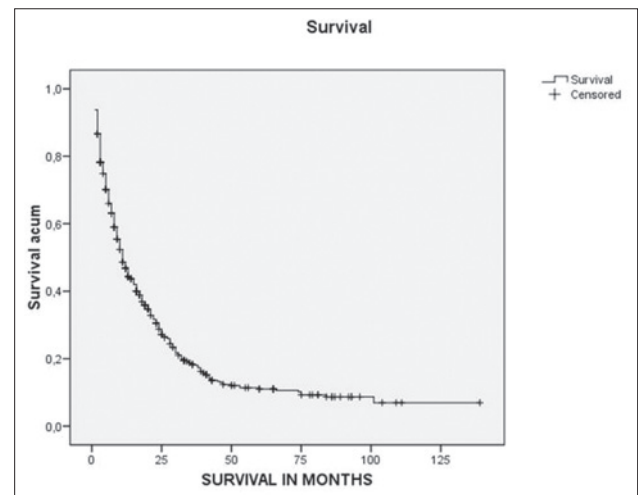


Figure 4: Overall survival for all the series

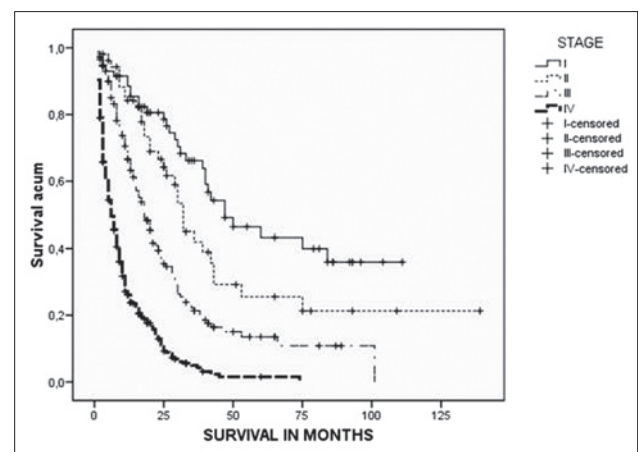


Figure 5: Overall survival by stages

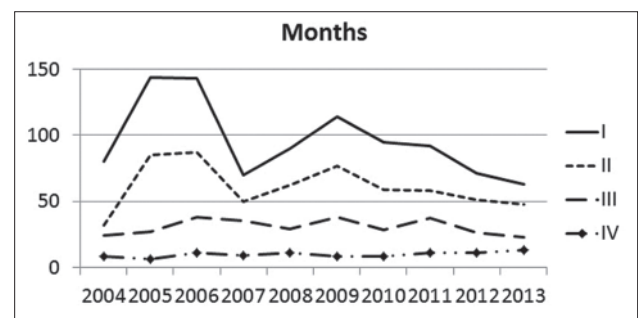


Figure 6: Evolution of median survival for stages across the years

poor for all the series. Stage was the main prognostic factor for survival.

The predominance of lung cancer men in our series has been reported yet in Spain.^[17-20] The current male-to-female ratio in the USA is close to one.^[21,22] Our ratio of males to females with lung cancer is still high,^[23-25] similar to other Spanish rural areas. In any case, the male-to-female ratio is still substantially higher in Spain (8.5 in 2003)^[26,27] than in other Western countries, where it varies between 1.3 and 4.5 likely due to the much more rare habit of smoking in women than in men.^[2,4,28] It is known that over 95% of Spanish male lung cancer patients smoke.^[3] Anti-tobacco policies had been introduced in Spain only in recent years.^[20,28] So, the reason for the predominance of male patients in our area could be explained by tobacco use. We have not specified tobacco habits of our series, but almost 90% were likely current or former smokers.^[29] There is a popular type of manufactured cigar, named “caliqueño,” without filter, and not low-tar so that smokers maintained the profile of tobacco users (not inhaling deeper, so generating central tumors, as squamous and small cell cancers).

In our rural area, in the non-smoking population, the incidence of lung cancer is higher among women.^[30] For lung cancer women, those elderly, always non-smokers, developed ADC. The younger female patients, usually smokers, developed lung cancer related to smoke, such as SCLC. Recent findings also suggest that women may be somewhat more susceptible to the carcinogenic effect of tobacco,^[6,31] although this remains a matter of debate.

Respect to histology, in our area we found a predominance of squamous and small cell cancer through the years. This distribution is different from those worldwide. Worldwide, the trend is toward an increase in the proportion of adeno- and a decrease in squamous cell carcinomas, although the rate of change varies across different geographical areas.^[18] This change has mainly been attributed to the decline in the number of smokers and the more widespread consumption of filtered cigarettes in USA. In spite of the proportional decline over the last 20-30 years, squamous cell carcinoma is still the most common histological subtype among males in several European countries (37% in France, 44% in Poland and 45% in Holland).^[5] In Spain, squamous cell carcinoma is the most common subtype with percentages varying between 24% and 50% in local and regional registries and SCLC still accounts for some 20% of cases in most Spanish registries. In United States, ADC (40%) is the most common subtype, followed by squamous cell carcinoma (25%) and large cell carcinoma (10%).^[26] Incorporation of women into tobacco use worldwide and smoking filtered cigarettes that are low in nicotine could partially explain the rise in the rate of ADC worldwide.^[28,32] We

found a relationship between histology and gender as squamous and small cell cancers prevailed in males and ADC in females. In our series as in most series, Stage IV remained the most common stage.^[26] However, our series distribution by stage has changed through the years, with a decrease in Stage IV and increase in Stage III. We have not implemented yet the lung cancer screening. We can not explain this tendency change on stage across years.

OS for our entire series remained low. Overall 5-year survival rate for all stages of lung cancer, is 17% worldwide.^[27] Our 5-year OS for all series was 15% and the only factor related to survival was stage. For Stage IV, a median increase of 2 months was seen through years with a better prognosis in the latest years. When we analyzed Stage IV patients, we found a double distribution. Those who did not survive more than 12 months had a median OS of only 5 months. Although long survival in lung cancer has been described and is a matter of interest, shorter survival patients are still more common and should be a matter of study to know why there are so many patients with this discouraging OS. For long survival at our database, the main factor was a molecular prognostic factor (*EGFR* mutation). There was an interaction between female gender, ADC histology and *EGFR* mutation, as women with ADC were nearly all mutated. Our series had a low percentage of patients with *EGFR* activating mutations. However, most patients had not been tested for *EGFR* status. Probably, the low rate of men with ADC *EGFR* mutations (only 1.7%) could be explained by the high rate of smoking habit in this population.

Our work has some weak points. Despite the importance of a long follow-up time, our work is not as accurate as it could have been. In fact, first we presented a hospital-based cancer registry of outpatient service; therefore a selection bias could not be excluded. Population-based cancer registries should be preferred. However, in Spain, in spite of many efforts^[17-20] not more than 26% of the Spanish population (28% in the case of childhood cancer) is covered by cancer registries and the distribution of them is not random.^[22] Second, we have not considered other prognostic factors, such as performance status or treatment. Thus, we must be cautious with conclusions. Treatment could be one of the explanations of why women with ADC had no differences on survival depending on *EGFR* status. In our country, lung cancer patients have access to *EGFR* TKI on the second and third line, regardless on *EGFR* status.^[33] and most of these patients may have received *EGFR* TKI. Despite this bias and weaknesses, we believe on the value of having own clinical real data if all admit that cancer is an individual disease, and probably, lung cancer is different according to epidemiology characteristics.

Conclusion

This single center analysis suggests that at least at our region, lung cancer remains a men disease and tobacco-related cancer. Advances and improvements on OS seemed to have been achieved only in those tumors unrelated to smoking (non-smoker women *EGFR* mutated patients). Efforts to reduce tobacco use and carry on with improvement in treatment could modify this disappointing survival for our patients.

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Nil.

Conflicts of interest

There are no conflicts of interest.

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Is pregnancy wise after 12-year chemotherapy of chronic myeloid leukemia woman?

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ABSTRACT

Diagnosis of leukemia during pregnancy is a dramatic event that poses challenges to the pregnant woman, the family, and physicians. Chronic myeloid leukemia (CML) comprises up to 10% of pregnancy-associated leukemia. There is no specific guideline for CML management in pregnant women. This study reported a case of successful pregnancy after 12 years of chemotherapy including tyrosine kinase inhibitor for CML. Pregnancy after 12 years of continuous chemotherapy is rare, which also led a challenge for medical oncologists and patient as well. This study described the assessment of the risk balance and benefit and management of such a patient.

Key words: Chronic myeloid leukemia, imatinib, pregnancy, tyrosine kinase inhibitor

Introduction

Chronic myeloid leukemia (CML) occurs as a result of a reciprocal translocation between chromosome 22 and chromosome 9.^[1] The discovery of tyrosine kinase inhibitor (TKI) imatinib has revolutionized the management of a once fatal disease to transform it into a treatable condition. CML patients in reproductive age and being treated with imatinib showed to have contemplating reproductive opportunities that would not have otherwise been possible in the pre-imatinib era. The management of CML during pregnancy is a unique challenge for medical oncologists and requires a balance between maternal survival and fetal health during the entire pregnancy. Because imatinib was teratogenic in rats, it was strongly advised that effective contraception should be used during therapy to prevent pregnancy.^[2] There are still sparse safety data on newer generation TKIs such as nilotinib, dasatinib, or bosutinib to be used to treat a patient during pregnancy. In this study, we are reporting the outcome of a CML patient who became pregnant after receiving chemotherapy for 12 years.

Case Report

As an 11-year-old girl, she was presented with loss of weight and dragging sensation in the left hypochondrium

for 2 months in 2001. Out of patient clinic exam showed that she had a splenomegaly and moderate hepatomegaly and peripheral blood smear, bone marrow aspiration, and cytogenetic tests all showed abnormalities and, therefore, she was diagnosed as CML in chronic phase. Hydroxyurea treatment was started and maintained the treatment until 2006, during which period of time she was in complete hematological remission (CHR). In March 2006, imatinib was given to the patient through Gleevec International Patient Assistance Program at a daily dose of 300 mg and increased to 400 mg in September 2007. In 2008, BCR-ABL was 2.29%. In 2009, she lost her CHR. Due to financial limitations, imatinib resistance mutation analysis (IRMA) was not done. In March 2010, her BCR-ABL level gone up to 36.76% and the IRMA was performed, but did not show any imatinib resistance mutations. The imatinib dose was escalated to up to 600 mg daily. Due to intolerance, her imatinib dose was decreased to 400 mg daily. In February 2011, she got married, but in August 2011, she lost CHR and BCR-ABL had increased to 67.5%; thus interferon (IFN)-alpha 5 MU was added to the treatment remedy in an alternate day until July 2012. In October 2012, her BCR-ABL

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was 0.25% and IFN-alpha was discontinued and imatinib was continued at 400 mg daily until January 2013. In the January 2013 visit, she had conceived and had amenorrhea for 3 months. She was then informed about the teratogenic effects of imatinib and the possible consequences. She elected to continue the pregnancy, so imatinib was stopped in view of its teratogenic effects, and IFN-alpha 5 MU subcutaneously on alternate days was restarted. The pregnancy was uneventful. She was in CHR and BCR-ABL at the second trimester was 2.14%. On July 16, 2013, after 9 months of pregnancy, she delivered a healthy boy with a birth weight of 2.25 kg by caesarean section without any congenital abnormality. The post-partum period was uneventful. One month after delivery, the patient requested that IFN therapy be discontinued. Imatinib 400 mg was then restarted. She was advised not to breastfeed while on imatinib. At the last follow-up in February 2015, the patient was in remission, and her baby was healthy.

Discussion

One in 1,000 pregnancies is reported with the malignant disease.^[3,4] The incidence of leukemia in pregnancy is one in 75,000-100,000 pregnancies.^[5] The majority of leukemia cases diagnosed during pregnancy are acute myeloid leukemia, followed by acute lymphoblastic leukemia. Although 15% of adult leukemia is CML, only a limited number of patients are diagnosed with childbearing age and CML accounts up to 10% of pregnancy-associated leukemia, with an annual incidence of one per 100,000 pregnancies.^[5]

Overall, malignancy during pregnancy is a unique challenge for the medical oncologist. There is another significant problem when a patient becomes pregnant during or shortly after receiving chemotherapy or radiotherapy due to the side-effects of most chemotherapeutic agents and radiotherapy.^[6-8] Side effects of chemo- and radiotherapy have been reported mostly from animal studies, but there is relatively little information on humans. The diagnosis of CML during pregnancy may be made more complicated because of physiological changes in body fluid including hematological parameters. These may temporarily mask the symptoms of malignancy.

For a male CML patient, there is no formal contraindication for fathering a baby while on TKIs, and the data available suggest that in most instances, babies born to such patients had no known abnormalities.^[9-11] Although most of the prior data on the effects of imatinib on pregnancy have shown satisfactory outcomes, they do not support a determination that imatinib can be safely administered during the first trimester of gestation. Pye *et al.*^[10] demonstrated the most comprehensive data on 180 women with CML exposed to imatinib during pregnancy. Outcome data were available for 125 (69%) patients and of those with known outcomes, 50% delivered healthy babies, 28% underwent

elective terminations, and 14% had miscarriages. Three terminations occurred due to fetal anomalies. There were a total of 12 (9.6%) infants identified to have physical abnormalities, including different congenital abnormalities, either single or in combinations, e.g. craniosynostosis, hypoplastic lungs, exomphalos, duplex or absent kidney, hemivertebrae, shoulder anomalies, hypospadias, pyloric stenosis, and scoliosis. One infant was born with complex abnormalities, i.e. communicating hydrocephalus, cerebellar hypoplasia, and cardiac defects.^[10] Cole *et al.*^[12] showed 217 CML patients with pregnancy, of whom 78% carried their pregnancies to term, 11% had spontaneous abortions, and 28.5% of patients with an unknown outcome. Among the 109 pregnancies (78%) with known outcome, 33% had complications, including spontaneous abortion in 22% of patients, still birth in one patient, malformations in 9 patients and low birth weight in 2 patients.^[12] There are limited data on pregnancy using the second generation TKI. Cortes *et al.*^[11] described the outcomes of pregnancies of 8 women who conceived while receiving dasatinib. Three had therapeutic abortions, two had spontaneous abortions, and three full-term delivered. The authors concluded that women in reproductive age with dasatinib therapy should take effective contraception. Conchon *et al.*^[13] reported a case of successful pregnancy and delivery in CML patient under dasatinib treatment. Experimental studies on nilotinib in rabbits showed treatment-associated mortality, abortion, or low gestational weights. However, the data on human are limited.^[14] Conchon *et al.*^[15] published a case report of a successful pregnancy of a patient with CML on nilotinib.

Usually, conception during chemotherapy is not recommended; thus, couples in childbearing age must be consulted to use proper contraception and inform the risk of fetal malformations for fetuses conceived while on treatment. There are limited data regarding alternatives to TKI in the successful management of CML during pregnancy. Most of these data were from case reports using leukapheresis or hydroxyurea in the third trimester of gestation and low-dose IFN-alpha as maintenance therapy.^[9] Pegylated IFN-alpha is contraindicated in pregnancy due to the accumulation of polyethylene glycol. The recommended management of CML in pregnancy (as reported at American Society of Hematology 2011) is summarized in Table 1.

A large series of the study indicated that an adequate response after restarting imatinib only occurred in patients with a major molecular response (MMR) before drug discontinuation. Therefore, for women who choose to become pregnant despite the risk, a minimal MMR should be achieved to reduce a risk of treatment failure after the reintroduction of therapy.^[16] TKI therapy should be discontinued at least 3 months before conception for planned pregnancy. Quantitative reverse-transcriptase polymerase chain reaction analysis of peripheral blood at 6-weekly intervals is useful to

Table 1: Recommendation of treatment as per American Society of Hematology 2011

Prior to conception	No negative data on imatinib in male patients IFN for male and female patients
First and second trimester	Low-dose IFN 3 million IU three times /week, adjusted to cell counts and tolerability Avoid PEG-IFN, leukapheresis in case of high leukocytes
Third trimester	IFN Hydroxyurea if loss of hematologic response
Breast feeding period	IFN

IFN: Interferon; PEG: Pegylated

check for loss of molecular response.^[17] During lactation, TKI is contraindicated, if the patient is on TKI; then she should avoid breastfeeding. In our patient, she was on chemotherapy with either hydroxyurea IFN or imatinib for the last 12 years. Successful pregnancy after continuous chemotherapy for 12 years is very rare. Our patient was diagnosed as CML at age 11, which is also quite uncommon. Her young age may have contributed to a better clinical outcome.

The treatment of leukemia in pregnancy poses a significant challenge for medical oncologists as well as patients. Our patient received all possible treatment options and achieved a long-term remission. Although this experience is limited to a single patient, data on this patient indicate that CML management during pregnancy may be individualized based on a balance between relative risks and benefits to the patient and fetus.

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Conflicts of interest

There are no conflicts of interest.

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Journal articles ahead of print	Odibo AO. Falling stillbirth and neonatal mortality rates in twin gestation: not a reason for complacency. <i>BJOG</i> 2018; Epub ahead of print [PMID: 30461178 DOI: 10.1111/1471-0528.15541]
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: https://www.fda.gov/NewsEvents/Newsroom/PressAnnouncements/ucm574058.htm . [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.
Conference paper	Christensen S, Oppacher F. An analysis of Koza's computational effort statistic for genetic programming. In: Foster JA, Lutton E, Miller J, Ryan C, Tettamanzi AG, editors. <i>Genetic programming. EuroGP 2002: Proceedings of the 5th European Conference on Genetic Programming</i> ; 2002 Apr 3-5; Kinsdale, Ireland. Berlin: Springer; 2002. pp. 182-91.
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.

For other types of references, please refer to U.S. National Library of Medicine.

The journal also recommends that authors prepare references with a bibliography software package, such as EndNote to avoid typing mistakes and duplicated references.

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