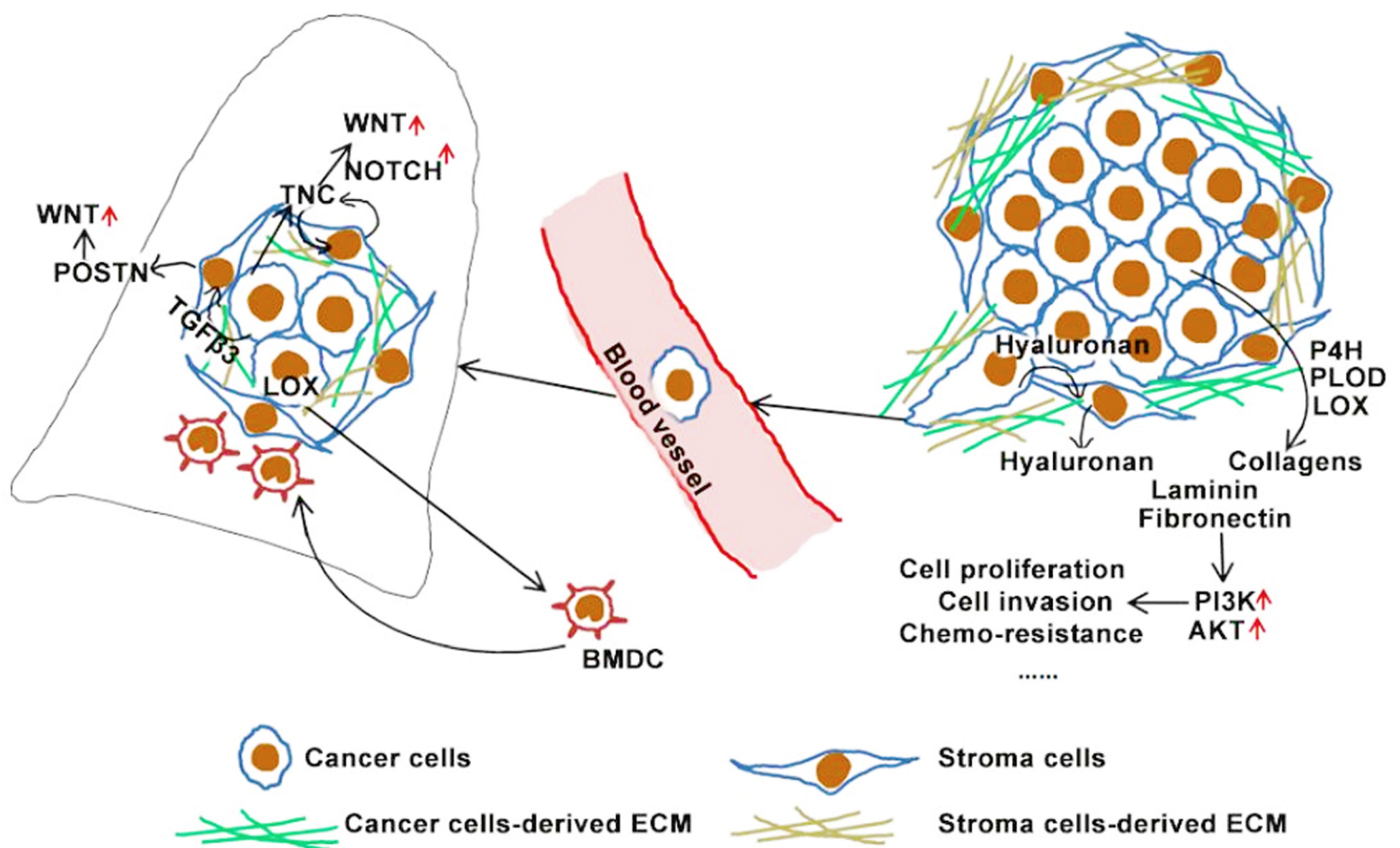


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Long non-coding RNAs as key regulators of cancer metastasis

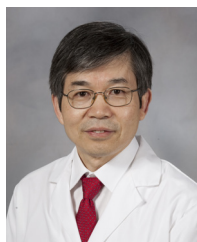
Pratirodh Koirala¹, De-Hong Zou², Yin-Yuan Mo^{1,3}

¹Department of Biochemistry and Cancer Institute, University of Mississippi Medical Center, Jackson, MS 39201, USA.

²Department of Breast Surgery, Zhejiang Cancer Hospital, Hangzhou 310022, China.

³Department of Pharmacology/Toxicology, University of Mississippi Medical Center, Jackson, MS 39201, USA.

Correspondence to: Dr. Yin-Yuan Mo, Department of Pharmacology/Toxicology, University of Mississippi Medical Center, Jackson, MS 39201, USA. E-mail: ymo@umc.edu



Yin-Yuan Mo is Professor in the Department of Pharmacology/Toxicology, Cancer Institute, at University of Mississippi Medical Center. His research interests are epigenetic regulation of genes involved in tumorigenesis and chemoresistance, cancer susceptibility due to alterations of microRNA expression, and lncRNA-mediated gene expression in cancer.

ABSTRACT

The recent advances in functional genomics have discovered that a large number of long non-coding RNAs (lncRNAs) are pervasively transcribed from the human genome. Increasing evidence further indicates that lncRNAs are important for gene expression during cell differentiation and development through various mechanisms such as nuclear organization, post-transcription regulation, alternative splicing, and epigenetic regulation. Thus, aberrant expression of lncRNAs can cause abnormality in those cellular functions and lead to various pathological conditions. One of such fatal consequences is cancer metastasis which is responsible for more than 90% of cancer-related deaths. A good understanding of how lncRNAs regulate different genetic and epigenetic changes during different stages of cancer metastasis is important not only for general cancer biology but also for identification of novel biomarkers and therapeutic targets for treatment of metastatic cancer. A significant progress has been made regarding the role of lncRNAs in cancer for past several years. In this study, we first discuss general functions of lncRNAs and then highlight recent findings of how lncRNAs impact cancer metastasis, and finally we provide our perspectives on clinical implications of lncRNAs.

Key words: Cancer metastasis; epigenetics; gene regulation; long non-coding RNA

INTRODUCTION

It is well-known now that protein-coding genes account only about 2% of the human genome,^[1] whereas the vast majority of the transcripts do not code for protein.^[2] Although these non-coding RNAs were considered “transcriptional noise”, their functions are increasingly valued for defining the cellular complexity of organisms. For instance, the number of protein-coding genes in humans is only a 2-fold more than that in worms such as *Caenorhabditis elegans* do,^[1] implying that the protein alone is not sufficient to determine the complexity of organisms. Instead, this complexity may be achieved by efficient programming, which helps in handy

expression and functioning of protein in a different context. The versatility and plasticity of non-coding RNAs help in such programming of protein function by regulating their expression and assembly in contextual cues.^[3]

Non-coding RNAs include a broad category of RNA molecules. Some of them are constitutively expressed in the cells, and they may play a housekeeping role such as ribosomal RNA, transfer RNA, small nuclear RNA, and small nucleolar RNA (snoRNA). In contrast, other non-coding RNAs may be spatiotemporally expressed, and they often play a regulatory role.

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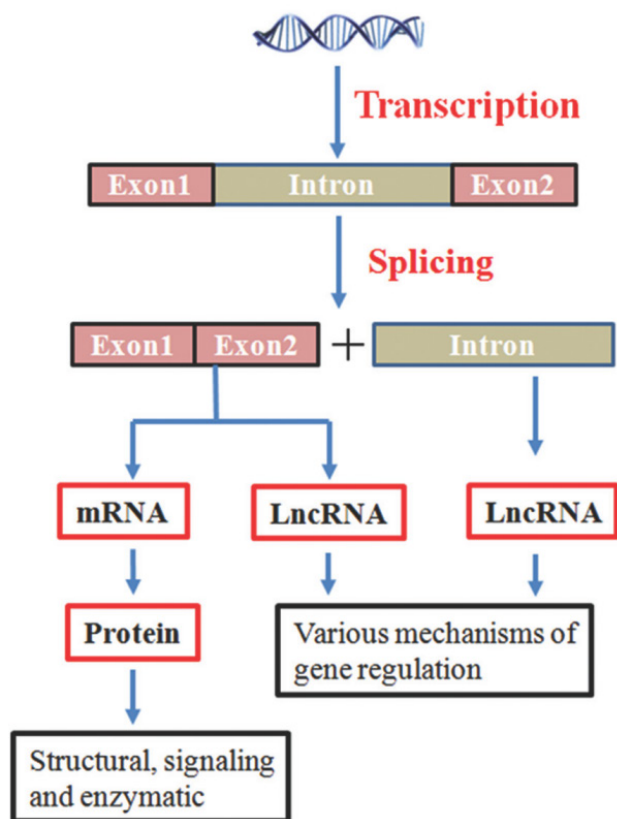


Figure 1: Flow of genetic information involving messenger RNA and long non-coding RNA

These regulatory RNAs, on the basis of their size, are arbitrarily classified into two groups. The first group is small non-coding RNAs (< 200 bp in length) such as short interfering RNA, microRNA (miRNA), and piwiRNA. The second group of non-coding regulatory RNAs is long non-coding RNAs (lncRNAs) (> 200 bp in length).^[4,5] Like protein-coding genes, most lncRNAs are polyadenylated and capped.^[6] Both protein-coding genes [messenger RNA (mRNA)] and lncRNAs carry genetic information [Figure 1]; however, their functions can be very different. Based on their locations in the genome, lncRNAs can be derived through the following means:^[7] (1) intergenic lncRNAs which are located between two genes; (2) sense or antisense lncRNAs which may overlap with an exon of another transcript in the same or opposite direction; (3) intronic lncRNAs which reside within an intron and do not overlap with any exon; (4) processed transcripts which reside in a locus where none of the transcript has an open reading frame and thus, do not fit into any other categories because of structural complexity.

To date, an overwhelming number of lncRNAs have been reported. For example, the non-code database lists over 56,000 human lncRNAs (<http://www.noncode.org>) whereas LNCipedia (<http://www.lncipedia.org/>) lists over 110,000 human lncRNAs. Although no unified source for categorizing and annotating lncRNAs is available yet, evidently, the number of lncRNAs is much larger than the number of protein-coding genes. Since lncRNA research is still at a very early stage and the majority of lncRNAs

Table 1: Function of lncRNAs

LncRNA	Function
ANRIL, XIST, HOTAIR, H-19	Transcription control by chromatin modifications
LincRNA p21	Transcriptional regulation
H-19	Precursor for miRNA
Loc285194, Gas 5,	Regulators of miRNA function
lncRNA-ATB, CCAT1	
PTENP1, KRAS1P, Gas 5	Decoy
RoR, NEAT1, TER	Scaffold

lncRNAs: long noncoding RNAs; lncRNA-ATB: lncRNA is activated by cytokine transforming growth factor- β ; CCAT1: colon cancer-associated transcript 1; TER: telomerase RNA; NEAT1: nuclear paraspeckle assembly transcript 1; ANRIL: antisense non-coding RNA in the INK4 locus; HOTAIR: Hox transcript antisense intergenic RNA; XIST: X-inactive specific transcript; miRNA: microRNA

are poorly characterized, there is a critical need for a better understanding of their functions and role in cancer, and especially, how they impact metastasis.

LNCRNAS AS MASTER GENE REGULATORS

Given that they can interact with RNA, DNA, and protein, lncRNAs have been shown to have an impact on almost every aspect of gene regulation. We list a few of examples as follows [Table 1].

Transcriptional regulation

Histone modifications such as acetylation and methylation impact chromatin structure and subsequent transcriptional activity. A large number of lncRNAs have been shown to play a role in regulation of chromatin structure. Polycomb repressive complex (PRC1 and PRC2) consists of several enzymes, including enhancer of zeste homolog 2 (EZH2), and is essential for histone methylation.^[8] Antisense non-coding RNA in the INK4 locus (ANRIL) is one of the lncRNAs that can suppress transcription by remodeling chromatin structure. In this regard, the human chromosome 9p21 harbors INK4b/ARF/INK4a locus which has 3 coding genes, p¹⁴/ARF, p¹⁵/CDKN2B, and p¹⁶/CDKN2A along with ANRIL. ANRIL is an antisense lncRNA, overlapping with p¹⁵/CDKN2B and p¹⁶/CDKN2A.^[9] Binding of ANRIL to PRC1 and PRC2 facilitates the recruitment of PRC1 and PRC2 into the INK4a/ARF locus, which causes trimethylation of the histone and reduces transcription activity of the locus.^[10] Similarly, X-inactive specific transcript (XIST) is a key regulator of X chromosome activity by chromatin structure modifications during embryonic development. XIST recruits EZH2 in X chromosome and then causes the trimethylation of histone, leading to a factual heterochromatin structure and silencing of one of the two X chromosomes.^[11] Besides, recruitment of different proteins in a gene promoter region can also change the transcription activity. For example, the enrichment of hnRNP-K in the promoters of p53 regulated genes represses the transcription

of those genes.^[12] However, lincRNA-p21 interacts with hnRNP-K and helps enrichment of hnRNP-K into these promoters, resulting in transcription suppression.^[13] On the other hand, p53 enhances transcription of lincRNA-p21, thus forming an auto-regulatory feedback loop.

LNCRNA AS A PROGENITOR OF SMALL RNAS, REGULATING THEIR FUNCTIONS

Although there is still no concrete evidence yet, lncRNAs may be post-transcriptionally processed into the small RNAs. For instance, the computational analysis indicated that exons of lncRNAs are highly enriched with small RNAs. In fact, snoRNAs are enriched 6-fold higher in their exons than any other genomic loci.^[14] Similarly, many miRNAs are derived from transcripts which are capped and polyadenylated including lncRNAs. About 20% miRNAs are overlapped with either introns or exons of lncRNAs.^[15] LncRNA H19 is one such prominent example which serves as the precursor of miRNA. The pri- and pre-miR-675 resides in H19 and expression of miR-675 coincides with H19 in murine embryo. However, miR-675 is not expressed in NIH3T3 cells that lack H19 expression. The digestion of 32P-labeled H19 clone with Drosha: DGCR8 (enzyme for miRNA biogenesis) releases 57 nucleotide long pre-miRNA 675, indicating that H19 is the parental transcript of miR-675.^[16]

LncRNAs not only serve as the precursor for the small RNAs but also regulate the expression and the function of miRNAs. The lncRNAs provide putative binding sites for miRNAs. Such interactions alter the expression and the function of mature miRNAs. For example, loc285194 is a tumor suppressor in colon cancer and it carries two binding sites for oncogenic miR-211. This interaction does not affect the pri- and pre-miR-211 level but alters the mature miR-211.^[17] Similarly, growth arrest-specific 5 (Gas5) regulates the level of miR-21 through their interaction. Apparently, Gas5 does not affect the pre- and pri-miR-21. Moreover, both miR-21 and Gas5 are found in RNA-induced silencing complex (RISC), suggesting that Gas5 regulates miR-21 through RNA interference (RNAi) mechanism.^[18]

LNCRNAS AS A DECOY

A pseudogene is a class of lncRNAs, derived from mutations in protein-coding genes. They usually have similar sequences to their parental gene with few mismatches. This resemblance in structure could entice different cellular entities as lncRNAs rather than mRNAs, impacting the cellular function. For example, PTENP1 is a mutated form of PTEN and their sequences differ by only 18 mismatches. PTEN carries a number of different sites for miRNA in its untranslated region (3'-UTR). Although PTENP1 is 1 kb shorter in the 3'-UTR than PTEN, most of the miRNA binding sites are conserved. This can trap many miRNAs to PTENP1 to compete with PTEN.^[19] A similar relationship

was also observed between Kras and its pseudogene KRAS1P. KRAS1P is amplified in most of cancers with activated Kras, indicating a positive correlation between them. Although how KRAS1P regulates KRAS level is not well-understood, it may act as a sponge for miRNAs that bind to the 3'-UTR of Kras and prevents degradation of Kras transcript.^[19] The lncRNA decoy function is not limited to miRNAs, and it can also be applied to DNA. For instance, Gas5, which is enriched in growth-arrested cells,^[20] inhibits the function of glucocorticoid receptor (GR) by competing with glucocorticoid response element (GRE) to bind GR. GR is a transcription factor and is activated by ligand and subsequently the activated GR binds to GRE to initiate transcription of downstream genes. A part of Gas5 sequence is capable of forming 6 hairpin structures; among them, hairpin structure 5 has two GRE-like structures that mimic GRE. Therefore, GR could bind Gas5 instead of GRE, and as a result, this interaction hinders the GR-mediated transcription activity.^[21]

LNCRNAS AS A SCAFFOLDING AND STRUCTURAL SUPPORT

Physical association between cellular entities is critically important for coordination of a variety of cellular functions. It is well-known that specific binding between two different cellular components can control the reprogramming of cellular signaling, leading to alternations of cell phenotype or function. Apparently, proteins can serve such function as a scaffolding and structural support.^[22] Recent studies suggest that lncRNAs can also have a similar function because they can interact with different proteins, through which lncRNAs provide a platform for the assembly of various proteins. Such interactions may affect protein localization, protein function, transcriptional activity, gene splicing, *etc.* Linc-ROR is lncRNA as a regulator of induced pluripotent cell reprogramming.^[23] Of interest, linc-ROR plays an important role in repression of p53 translation by interaction with phosphorylated hnRNP I in the cytoplasm. The physical association between linc-ROR and hnRNP I controls p53 translation and deletion of hnRNP I binding motif in linc-ROR abolishes its repression capability.^[24] The scaffolding function of lncRNAs is also essential for the formation of special architect-like paraspeckles, a nuclear body structure that appears during interphase of cell cycle. Paraspeckles are primarily composed of proteins such as paraspeckle protein (PSP1, PSP2) and p54/nrb.^[25] Although the function of paraspeckles is still not clear, components within paraspeckles are known to play an important role for transcription and alternative splicing.^[26,27] Since paraspeckles do not have any membrane structure, lncRNAs within the paraspeckle may help to establish this compartment.^[28] NEAT1 is one of the lncRNAs that interact with PSP1 and together, they may help to form paraspeckles. Importantly, the number of paraspeckles increases in vivo and in vitro with the increase in NEAT 1 expression and deletion of NEAT1 eliminates the paraspeckles, suggesting an important role of

this lncRNA in the formation of paraspeckles.^[29] Another lncRNA that provides a platform for the binding of protein is telomerase RNA (TER). TER along with telomerase reverse transcriptase (TERT) is essential for telomere synthesis. A telomere is nucleotide repeat at the end of DNA which is required for the genomic stability.^[30] TER is a template RNA for the synthesis of telomere.^[31] TER consists of various motifs; core domain is essential for template activity and CR4/CR5 domain binds with TERT.^[32] The mutation in core domain of TER disintegrates proper structure of this RNA, losing the binding capability to TERT, which leads to aplastic anemia.^[33] This indicates that a comprehensive structure of TER functions as a scaffold for telomerase to bind and work properly.

CANCER METASTASIS IS A MULTISTEP PROCESS

More than 90% of cancer death is attributed to metastasis. Cancer metastasis is a process in which cancer cells migrate from its origin to a distant site and then proliferate. The two major steps of metastasis are physical dissociation from the origin of the primary tumor and migration to distant sites and colonization of those migrating cells in the distant sites.^[34]

This multistep process is usually inefficient and only very few cells that start migrating from the origin would be able to colonize in distant organs. The disaggregation of cells from the primary tumor is the first event during the metastasis. This process is greatly enhanced by loss of E-cadherin, a protein, that helps to adhere epithelial cells together.^[35,36] The next barrier that prevents migration of the cancer cell is basement membrane. The disintegrated cancer cells can induce stroma to secrete proteolytic enzyme-like matrix metalloproteinases that dissolve the basal lamina.^[37] Detached cells also require motility to move from one place to another. Several changes in cytoskeleton, interactions between cell and matrix, and induced Rho, cdc42, and Rac signaling are important for mobility. Furthermore, epithelial-mesenchymal transition (EMT) is critical for dissemination of the cancer cell to distant site as it helps in effective motility and invasiveness and survival of the cells.^[38] To reach the distant organs, the cells also require a traveling path. The hematogenous route works as highway for this process.^[39] Although it is still unclear how the altered cells invade into the blood vessels, the high invasive capacity of metastasizing cells and chemoattractive factors in the blood may help their intravasation. Inside the blood vessels, the migrating cells endure a constant physical pressure as well as immune responses, an inclement condition for tumor cells. However, the chance of survival can be increased by adhering tumor cells to different factors such as thrombin.^[40] Tumor cells may also be able to attach to endothelial cells via protein-protein interaction (integrin $\alpha 3 \beta 1$ of tumor and laminin 5 of endothelial cells) to protect themselves from harsh condition.^[41] The adherence of cells not only prevent their possible elimination, but also help

their exit from the capillaries (extravasation). Extravasation is primarily supported by enhanced permeability of capillary. Vascular endothelial growth factor secreted by tumor cells can increase the permeability of blood vessels. In addition, other factors such as alterations in receptor expression and physical bursting may also help in the exit of tumor cells from blood vessels.^[42]

Once the cells depart the capillary, they try to colonize in distant organs. However, few of circulating tumor cells can colonize to establish micro-metastases. The colonization is dictated by the microenvironment of primary site and distant site. A number of cytokines both from tumors and the site of colonization provide mitogenic signals for the successful proliferation, survival and resist to apoptosis in alien environment, finally to develop macro-metastases.^[43]

LNCRNAS AND CANCER METASTASIS

In the previous section, we highlighted the steps during metastasis. Several genetic and epigenetic modifications during this multistep process make cells sturdy to survive the foreign ambience. Although we still do not have a clear picture as to what causes those change, increasing evidence suggests that lncRNAs play a crucial role in different stages of metastasis [Figure 1]. We list the following lncRNAs as examples.

Metastasis-associated lung adenocarcinoma transcript 1

Metastasis-associated lung adenocarcinoma transcript 1 (MALAT1) is located at chromosome 11q13 with 8.7 kb in length. It is expressed in most tissues with the highest level in pancreas and lung. The elevated expression of MALAT1 was found in metastatic cases of non-small cell lung carcinoma, and as such, it was named MALAT1. The high expression of MALAT1 in metastatic tumors predicts poor prognosis.^[44] As a nuclear lncRNA, MALAT1 plays an important role in alternative splicing.^[45] However, loss of MALAT1 does not seem to affect alternative splicing in the lung cancer tissue; rather it affects expression of different genes, including those involved in EMT (LPHN2, ABC1) and others in regulation of metastasis formation (GPC6, MCAM, PRCKE). Furthermore, tail vein injection of xenograft mice with A549 cells overexpressing MALAT1 shows diffused growth in the lung. Knockdown of MALAT1 results in either fewer tumor nodules or the cells cannot exit out of endothelial cells.^[46] Similarly, loss of MALAT1 in A549 cells inhibits expression of those genes (CTHRC1, CCT4, HMMR) that regulate folding of cytoskeleton and migration of cells.^[47] These lines of evidence indicate that MALAT1 is critically important for the cell motility and extravasation of cells from capillaries.

In addition to lung cancer, MALAT1 is also important for pancreatic and cervical cancer metastasis.^[48] In pancreatic cancer, MALAT1 enhances expression of EMT markers

(loss of E-cadherin and gain of N-cadherin, vimentin, and slug) dependent on transforming growth factor- β (TGF- β). Similarly, MALAT1 was found highly expressed in cancer stem cells (CSCs) of pancreatic cancer and knockdown of MALAT1 in those cells greatly reduces CD133+ population and sphere formation,^[49] suggesting an important role of CSC formation which may help in migration and survival of cells during metastasis.

HOX transcript antisense RNA

HOTAIR stands for HOX transcript antisense RNA with 2.2 kb in length. It is derived from *HOX C* gene (which determines anterior and posterior plane during embryonic development). High expression of HOTAIR is correlated with metastasis, and it significantly decreases the chance of survival of those patients. Overexpression of HOTAIR in different cell lines increases cell invasion and transforms non-invasive cells into invasive cells *in vivo*. Similarly, overexpression of HOTAIR in breast cancer MDA-MB-231 cells increases metastatic lung nodules by a 10-fold as compared to control.^[50]

Like many other lncRNAs, HOTAIR impacts metastasis via chromatin remodeling. HOTAIR can directly bind to PRC2 and LSD1, a demethylase that flanks HOXD. This binding coordinates enrichment of EZH2 in HOXD promoter which causes methylation of H3K27, leading to silencing of transcription through HOXD gene.^[51] A similar mechanism was also observed in the metastatic breast cancer cell lines, where HOTAIR helps in PRC2 occupancy on promoter of hundreds of genes and silences them by trimethylation of H3K27. Many of those genes are involved in breast cancer progression, cell adhesion, and metastasis.^[50]

In addition to breast cancer metastasis, HOTAIR has been attributed to enhance metastasis in oral squamous cell carcinoma by suppressing the level of E-cadherin.^[52] Moreover, HOTAIR along with miR-196a is also associated with high-risk metastasis and poor survival of a patient with gastrointestinal stromal tumors.^[53]

H19

H19 is one of the first lncRNAs identified in early 1980 and its expression is in accordance to expression of α -fetoprotein.^[54] This gene represents a maternally imprinted gene in both humans and mice. The expression of H19 gradually decreases from fetal tissue to adult, which indicates its importance in embryo development.^[55] Initial reports suggested that H19 could work as a tumor suppressor in different cancer cases;^[56] however, other studies suggested that H19 expression is high in tumor tissues.^[57,58] Despite high expression in tumor samples, overexpression of H19 in T24 bladder carcinoma cell line did not provide proliferative advantage. This implies that H19 may regulate metastasis rather than formation of primary tumor. Indeed, overexpression of H19 up-regulates genes [e.g. uPAR,

tumor necrosis factor- α , interleukin-6 (IL-6), and Ezrin] that are required for angiogenesis and metastasis in T24 cells.^[59] Furthermore, H19 is highly expressed in most cases of bladder carcinoma which subsequently metastasize compared to those that do not metastasize. Similarly, H19 level is substantially higher in invasive bladder carcinoma cell lines than non-invasive cell lines. Mechanistically, H19 recruits EZH2 in the promoter region of Nkd1 (an antagonist gene of Wnt/ β -catenin) and suppresses its transcription by hyper-methylation. This makes Wnt/ β -catenin constitutively active while E-cadherin is suppressed, leading to metastasis of bladder cancer.^[60] In addition to alterations in the gene expression pattern, H19 also enhances the interaction of the tumor cell with extracellular matrix. MDA-MB-231 cells growing in three-dimensional culture exhibit high level of H19, which helps in enhanced scattering of the cells, suggesting a role of H19 in breast cancer metastasis.^[61] In addition to bladder and breast cancer, H19 may also contribute to metastasis of colorectal cancer. For instance, H19 is highly expressed in methotrexate resistant HT-29 cells which reveal mesenchymal morphology. Overexpression of H19 increases the EMT markers vimentin, ZEB-1, and ZEB-2 and also promotes cell migration.^[62]

Nuclear factor- κ B interacting lncRNA

Nuclear factor- κ B (NF- κ B) interacting lncRNA (NKILA) is a 2.5 kb transcript mostly found in the cytoplasm and it negatively regulates the NF- κ B signaling.^[63] NF- κ B is a transcription factor which mediates inflammatory signaling pathways and is often constitutively active in various cancer cells.^[64] NF- κ B is in an active (phosphorylated) or inactive state (dephosphorylated) in the cell. In the inactive state, the dimer NF- κ B (p65 and p50) is bound with an inhibitory subunit I κ B. This complex keeps the dimer in the cytoplasm by masking the nuclear localization signal. Several external stimuli activate IKK β which phosphorylates I κ B α (a subunit of I κ B) and leads to proteasomal degradation of I κ B α . Now, the free NF- κ B dimer translocates to the nucleus where it binds to NF- κ B response element and activates transcription of different genes.^[65] Thus, NKILA adds a new layer of regulation for NF- κ B activity, by interacting with I κ B α and masking its phosphorylation site from IKK β . This prevents phosphorylation of I κ B α and translocation of NF- κ B dimer from the cytoplasm to the nucleus.

The highly metastatic breast cancer cell lines express a very low level of NKILA while less aggressive breast cancer cell lines exhibit a high level of NKILA. Furthermore, overexpression of NKILA in MDA-MB-231 cells reduces their metastasis in the lung, liver, and lymph nodes. In contrast, knockdown of NKILA in MCF-7 cells increases their metastasis to those distant sites. Clinically, loss of NKILA is associated with advanced breast cancer and distant metastases; low expression of NKILA is associated with the patient survival.^[63] Therefore, NKILA can predict the outcome of breast cancer and may serve as a prognostic marker.

LncRNA-ATB

This lncRNA is activated by cytokine TGF- β (lncRNA-ATB) that is well-known for its role in tumor metastasis. TGF- β modulates different signaling pathways involved in EMT, migration, invasion, and metastasis.^[66-68] A long time treatment of cells with TGF- β induces EMT (decreased E-cadherin and increased N-cadherin, vimentin, slug, twist1, ZEB-1 and ZEB-2). Similar treatment of hepatocellular carcinoma (HCC) cells with TGF- β activates the lncRNA-ATB in a time- and dose-dependent manner. Clinically, lncRNA-ATB level is high in HCC tumors as compared to adjacent normal tissue. Similarly, a high level of lncRNA-ATB is positively correlated with microvascular invasion and portal vein tumor thrombosis. Consistent with these observations, injection of HCC tumor cells overexpressing lncRNA-ATB into orthotopic mice promotes metastasis to different organs.^[69] One of the possible mechanisms is through enhancement of EMT by interfering the action of miR-200 which can inhibit EMT by suppressing ZEB-1 and ZEB-2.^[70] This 2.5 kb long lncRNA carries 6 binding sites for miR-200. Therefore, lncRNA-ATB traps miR-200 and prevents degradation of ZEB-1 and ZEB-2 by miR-200. The high level of ZEB-1 and ZEB-2 ultimately promotes EMT and invasiveness of different cells *in vitro* and *in vivo*. In addition, lncRNA-ATB enhances colonization of migrating cells by enhancing the function of IL-11-STAT3 signaling pathway. In this case, lncRNA-ATB binds to IL-11 mRNA and stabilizes it. The increased stability of IL-11 facilitates its secretion. As a ligand, IL-11 promotes phosphorylation of STAT3. This autocrine mitogenic signal helps in robust cell survival and effective colonization in distant organs.^[69]

LncRNA-low expression in tumor

Low expression in tumor (LET) was originally identified in HCC cells.^[71] Along with HCC, a reduced level of LET is also

found in lung squamous carcinoma and colorectal cancer as compared to adjacent normal tissue. Overexpression of lncRNA-LET suppresses metastasis of HCC and colon cancer cells *in vivo*.^[72] lncRNA-LET could limit HCC metastasis in both hypoxic and normoxic condition by different mechanisms. In hypoxic condition, lncRNA-LET interferes with the function of hypoxia-inducible factor-1 α (HIF-1 α), a transcription factor that regulates a number of genes under tumor hypoxia, and promotes angiogenesis and metastasis.^[73] The high expression of lncRNA-LET suppresses HIF-1 α through inhibiting NF90 which is required for accumulation of HIF-1 α mRNA. However, hypoxia keeps the level of lncRNA-LET low by deacetylating its promotor. As a result, HIF-1 α is increased promoting metastasis. In normoxic condition, lncRNA-LET inhibits expression of CDC42 (which is required for trans-endothelial migration) of circulating tumor cells. The low level of lncRNA-LET in HCC keeps CDC42 high and this results in profound metastasis of HCC.^[72]

Colon cancer-associated transcript 1

Colon cancer-associated transcript 1 (CCAT1) was found up-regulated in colon cancer tissue, circulating blood cells of colon cancer patient and metastasis cases, indicating its role in colon cancer progression.^[74] Besides, high expression of CCAT1 is also associated with primary tumor tissue, lymph node metastasis, and metastatic cases of gastric carcinoma.^[75] The elevated level of CCAT1 reduces the survival of HCC patients. In both gastric cancer and HCC cell lines, overexpression of CCAT1 enhances the proliferation and migration of cells driven by c-Myc, an oncogenic transcription factor required for cell survival. On one hand, c-Myc binds to promoter of the CCAT1 and up-regulates its level in cancer cells.^[75] On the other hand, CCAT1 prevents degradation of c-Myc by interaction with let-7, a known miRNA that can target c-Myc through its 3'-UTR.^[76]

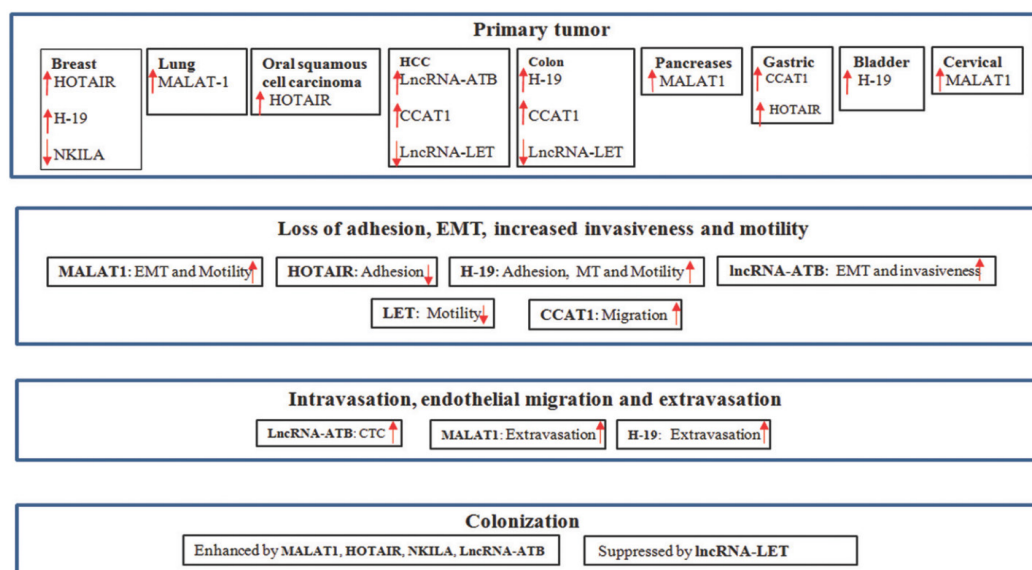


Figure 2: Long non-coding RNAs involved in different stages of cancer metastasis

DEREGULATION OF LNCRNA EXPRESSION IN CANCER

It is well-known that most of lncRNAs are transcribed through RNA polymerase II, just like protein-coding genes; they are also spliced products via canonical genomic splice site motifs, frequently ended with a poly A tail. Importantly, lncRNAs are often regulated by well-established transcription factors and are expressed in a tissue-specific manner.^[77] For example, we have shown that wild-type p53 can transcriptionally induce linc-RoR and loc285194, but mutant p53 cannot.^[17,24] On the other hand, c-Myc, as an oncogene, can regulate a group of lncRNAs.^[78] In cancer, c-Myc is often amplified or up-regulated, which may explain why some lncRNAs are often deregulated.

CONCLUSION AND PERSPECTIVE

A tremendous progress has been made in our understanding of the genes and events involved in metastasis in recent years. Moreover, emerging evidence indicates that lncRNAs have also joined this complex regulatory network and may serve as very important regulators at different stages of metastasis (e.g. EMT, invasion, migration, and colonization) often through their expression levels [Figure 2]. However, overall, lncRNA research in this field is still at the infancy stage. Given the complex interactions of lncRNAs with DNA, RNA, and protein, a systematic approach may be needed to better understand the molecular mechanism of lncRNA-mediated metastasis. With the development of advanced technology such as CRISPR/Cas9, it is now feasible to perform knockout or knockin experiments and these research tools will no doubt speed up new discovery. In this system, nuclease Cas9 assisted by a sequence-specific guide RNA (gRNA) which is functionally similar to RNAi, cuts targeted DNA sequence.^[79] Once the double strand break is made, the cell employs one of two major DNA repair mechanisms, non-homologous end joining (NHEJ), and homologous recombination (HR). Unlike HR, the NHEJ mechanism often leads to deletions or insertions, and thus it is an error-prone repair, a feature important for knockout. The HR mechanism would allow for introducing mutations or correcting a mutant sequence by knockin. Increasing evidence indicates that this technology has a potential to transform the field of cancer genetics such as the development of next-generation models of human cancer.^[80]

Given the nuclear localization nature for a number of lncRNAs, genetic manipulations at the DNA level provides a better alternative to RNAi approach which mainly works through RISC complex in the cytoplasm. Our recent study indicates that a dual gRNA/Cas9 system combined with donor vector for HR can greatly improve the efficiency of obtaining complete lncRNA knockouts in various cancer cell lines.^[81] As this field advances, we anticipate that more lncRNAs will be identified to be important players in cancer metastasis. More importantly, further

characterization of this regulatory system will reveal many of detailed mechanisms. As a result, these studies will help develop novel strategies for cancer treatment. Furthermore, lncRNAs may serve as biomarkers for diagnosis/prognosis as supported by profiling studies of clinical specimens. Finally, given their important role in metastasis, lncRNAs may also prove to be valuable targets for cancer therapy. In particular, ribonucleoprotein complexes through lncRNAs are critical to lncRNA-mediated metastasis, drugs that block or enhance such interactions may have a bright future.

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Conflicts of interest

There are no conflicts of interest.

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Roles of dysregulated Notch pathway and small DNA tumor viruses in cancer initiation and progression

Anthony G. Clementz¹, Paola Rizzo², Fernanda Martini², Mauro Tognon²

¹Department of Chemistry, College of Health and Sciences, DePaul University, Chicago, IL 60614, USA.

²Department of Morphology, Surgery and Experimental Medicine, Section of Pathology, Oncology and Experimental Biology, Laboratories of Cell Biology and Molecular Genetics, School of Medicine, University of Ferrara, 44121 Ferrara, Italy.

Correspondence to: Dr. Mauro Tognon, Department of Morphology, Surgery and Experimental Medicine, Section of Pathology, Oncology and Experimental Biology, Laboratories of Cell Biology and Molecular Genetics, School of Medicine, University of Ferrara, 44121 Ferrara, Italy.
E-mail: tgmt@unife.it

ABSTRACT

Notch pathway is a major determinant of cell fate, and research within the last 30 years has shown dysfunctions within this pathway in the majority of solid tumors and leukemias. The molecular mechanisms causing aberrant expression of Notch in cancer are still partially known. Mesotheliomas, breast, and cervical cancers are among the cancer types for which the dysregulation of Notch has been reported together with the association of simian virus 40 (SV40) or human papilloma virus (HPV) infections. In mesotheliomas and cervical cancer, there is clear evidence that these viruses cause and rely on dysregulation of the Notch pathway to promote and sustain cell transformation. The existence of a relationship in tumors between DNA viruses and Notch could have an impact on cancer therapy by implementing Notch inhibition to interfere with the growth of SV40- and HPV-positive cancers. In addition, since Notch links innate and acquired immunity and plays a key role in the regulation of the anti-viral response, targeting Notch in the presence of oncogenic viruses infections may help prevent the onset and progression of cancers associated with the exposure to these viruses.

Key words: Cancer; human papilloma virus; Notch; pathway; simian virus 40

INTRODUCTION

Notch has been identified as a critical pathway aberrantly expressed in many types of solid tumors and leukemias. Dysregulation of Notch signaling is a result of many factors including interactions with viral proteins. In this short review, we took in consideration significant articles dealing with the dysregulation of the Notch pathway and/or presence of oncogenic viruses, mainly simian virus 40 (SV40) and human papilloma viruses (HPVs), in cancer. Indeed, the proteins encoded by Notch pathway genes and the viral oncoproteins of SV40 and HPV were found in some models of study, interconnected in the cell transformation *in vitro* and tumor initiation and progression *in vivo*.

BASICS OF NOTCH SIGNALING

Beginning in the early 20th century, the discovery of a new

Type 1 transmembrane receptor came after the identification of a specific mutation in *Drosophila melanogaster*, which formed a Notch on the wing of the fly. This discovery led to the naming of “Notch” to the mutated gene.^[1] In *Drosophila*, the Notch receptor was found to encode a 300 kDa single-pass transmembrane receptor. Later, Notch-like molecules were identified from *Caenorhabditis elegans* (LIN-12) to humans, which are highly conserved and play pivotal roles in development, stem cell renewal, and differentiation in postnatal tissues.^[2] In mammals, there are four Notch Type I transmembrane receptors (Notch 1, 2, 3, and 4) and five known ligands (delta-like 1, 3, and 4 and Jagged 1, 2). Notch signaling relies on cell-cell contact to initiate its eventual signaling activation.^[3] To be primed for mature Notch signaling activation, the protein is processed first in the trans-Golgi apparatus by furin-like convertase creating a heterodimer, which is shuttled to the cellular membrane and held

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together by Ca^{2+} cations. The mature receptor is available to interact with its ligand, which subsequently stimulates through conformational changes a second proteolytic cleavage by tumor necrosis factor- α converting enzyme or a disintegrin and metalloprotease (10/17).^[4] This in turn results in shedding of the extra-cellular portion of Notch, which through receptor-mediated endocytosis, propagates signaling events in neighboring cells. The final cleavage occurs within the membrane through an associated aspartyl protease known as the γ -secretase complex composed of presenilin, nicastrin, APH 1 and PEN2.^[5] Intra-cellular Notch cleaved protein translocates to the nucleus where it binds with the transcription factor recombining binding protein-Jk or C-promoter-binding factor 1/suppressor of hairless/Lag1 (CSL)^[6] and, after displacing co-repressors and recruiting co-activators such as p300, histone acetyl transferases, and mastermind-like protein 1 (MAML1), it activates downstream pathways [Figure 1].^[7] The “canonical” Notch signaling is known to activate genes coding for transcriptional factors such as those belonging to the hairy/enhancer of split (Hes1-5), the hairy-related (Hrt), and the Hes1-5-Hrt with YRPW motif (Hey) families involved in inhibiting neuronal differentiation.^[8] The “canonical” Notch pathway is a major determinant of cell proliferation and survival through the activation of genes controlling cell cycle progression such as cyclin D1^[9] and genes belonging to the anti-apoptotic pathway nuclear factor kappa-light-chain-enhancer of activated B-cells (NF- κ B).^[10,11] Notch activation can also be attained in a “non-canonical” fashion initiated by a

non-canonical ligand or may not require cleavage of the Notch receptor.^[1] Among suggested mechanism of “non-canonical” Notch signaling are interactions of Notch with non-CSL transcription factors, such as β -catenin,^[12] hypoxia-inducible factor-1 α , NF- κ B,^[13] and estrogen receptor α (ER α).^[14] Anti-apoptotic activity independent of canonical functions has been associated with active Notch1, which signals via the kinase AKT to prevent the loss of mitochondrial function and consequent nuclear damage and requires mitochondrial remodeling proteins mitofusins-1 and 2.^[15] Notch activity is finely regulated by interactions with other key proteins and pathways, among them p53,^[16] ER α ,^[17,18] the epidermal growth factor B2 (ErbB-2)^[19] and the vascular endothelial growth factor receptors (VEGFRs),^[20] the Wingless (Wnt)^[21,22] and Hedgehog^[23] signaling pathways. Recent genome-scale studies in *D. melanogaster* have revealed an even more complex network of genes that can affect Notch activity^[24] consistent with decades of work showing that the highly conserved Notch pathway is extremely complex, and the output of its activation or its inhibition will result in differentiation, proliferation or increased survival based on the existing cellular context.

NOTCH SIGNALING PATHWAY IN TUMORS

Many reports have been published on the role of the Notch pathway in the development of the cardiovascular system,^[25,26] in regulation of stem cells functions such as survival of cardiac progenitor cells,^[27] the differentiation

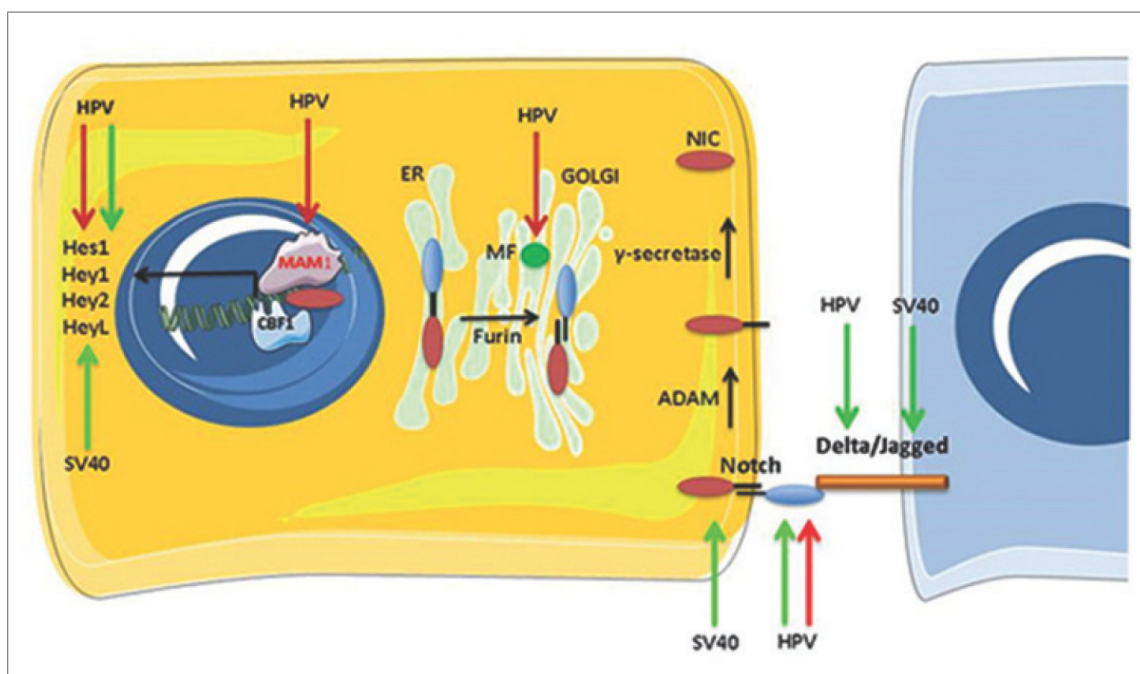


Figure 1: Schematic representation of the events leading to Notch signaling activation and the steps of this process affected by the oncogenic viruses simian virus 40 and human papillomavirus. Notch precursor is cleaved in the Golgi apparatus by a furin-like convertase and then exposed on the cell membrane. Notch ligands Delta/Jagged bind Notch extra-cellular subunit. This causes a disintegrin and metalloprotease to clip the extra-cellular portion of Notch transmembrane generating an intermediate, cleaved by γ -secretase which releases active Notch. Active Notch enters the nucleus, where it causes the dissociation of silencing mediator of retinoic acid and thyroid hormone receptor corepressor complex from C-promoter-binding factor 1/suppressor of hairless/Lag1, and recruits mastermind-like 1 coactivator complex, resulting in transcription of target genes. Simian virus 40 induces upregulation of the Notch pathway, whereas conflicting reports exist on the modulation of Notch by human papillomavirus (green arrow indicates up-regulation, red arrow indicates down-regulation or inhibitory binding)

of insulin-secreting pancreatic cells^[28] of inner ear hair cells,^[29] and intestinal crypt and goblet cells.^[30] Accordingly, the important role of the Notch pathway for normal tissues development has been proven by the identification of Notch mutations in human inherited diseases. Indeed, Notch alterations have been detected in: (1) Cerebral autosomal dominant arteriopathy, with subcortical infarcts and leukoencephalopathy (a heritable arteriopathy that leads to damaged small blood vessels and irreversible dementia); (2) spondylocostal dysostosis (characterized by abnormal development of bones in the spine and ribs); (3) Alagille syndrome affecting the liver, heart, kidney, and other systems of the body;^[31] (4) congenital heart diseases.^[32] Similarly, in the last decade, it has been demonstrated that the Notch signaling pathway contributes to the regulation of the immune system by playing a role in multiple lineage decisions of developing lymphoid and myeloid cells.^[33] Recent work has shown that Notch, through macrophage-dependent delta-like ligand 1 and 4 signaling, is critical in providing an anti-viral response by linking innate and acquired immunity during influenza^[34] and dengue^[35] viral infections.

Notch has emerged as a potent oncogene when it was first shown that a subset of T-cell acute lymphoblastic leukemias (T-ALL) contained a chromosomal translocation, t(7;9), leading to abnormal expression of the Notch1 intracellular domain,^[36] which was later shown to be able to cause T-cell neoplasm in mice.^[37] Later studies confirmed the existence of Notch1 mutation in 60% of human T-ALL.^[38] In T-cell neoplasms, Notch1 represses p53,^[38] induces c-myc,^[39] and inhibits phosphatase and tensin homolog, a downregulator of the phosphatidylinositol 3 kinase (PI3K)-AKT pathway involved in promoting cancer cell survival.^[40] Recent work has shed light on the role of Notch in T-ALL showing that in these tumors aberrant Notch activity counteracts the tumor suppression function of the transcription factor IKZF1 (IKAROS).^[41]

The major role played by Notch in breast cancer is also well established. Reports of an involvement of Notch in mammary gland development and neoplasia came from the observation of the Notch4/int3 gene as a common provirus integration site in mammary tumors of mice infected with mouse mammary tumor virus (MMTV),^[42] followed by the report that transgenic female mice carrying Notch1 and 3 activating mutations (caused by the insertion of the MMTV) developed mammary gland tumors.^[43] Notch has been found activated in ER α positive-, negative-, triple negative-breast cancer cell lines and breast cancer cell lines overexpressing the oncogene *Her2/neu*.^[18,19,44] Dysregulation of Notch has been shown in human breast cancer biopsies^[45-47] in which overexpression of Notch1 and one of its ligands, Jagged1 has been linked to poor prognosis and overall diminished survival.^[48,49] Of interest, Notch2 overexpression was instead associated with increased survival in breast cancer patients,^[49]

suggesting a role for Notch2 as a tumor suppressor gene in these cancers. In agreement with this observation, active Notch2 induces reduction in tumor take and increased apoptosis in human MDA-MB-231 (ER α , Her2 negative cell line) xenograft tumor growth.^[50] The Notch pathway is a major determinant of breast cancer stem cells survival, and Notch activation in these cells has been linked to resistance to tamoxifen.^[51,52] Consistently, Notch activation plays a role in tamoxifen resistance observed in protein kinase C- α overexpressing estrogen-responsive breast cancers^[53] and in ErbB-2-positive breast tumors.^[54]

Dysregulated expression of Notch proteins, ligands, and targets has been described in a multitude of solid tumors, including cervical, head and neck, endometrial, renal, lung, pancreatic, ovarian, prostate, esophageal, oral, hepatocellular and gastric carcinomas, osteosarcoma, mesothelioma melanoma, gliomas, medulloblastomas, and rhabdomyosarcoma.^[8] Dysregulation of Notch signaling has been reported in some hematological malignancies, other than T-ALL, including Hodgkin lymphomas, anaplastic large-cell non-Hodgkin lymphomas, acute myeloid leukemias, and B-cell chronic lymphoid leukemias multiple myeloma (for the original articles on the subject the reader is referred to).^[8]

Tumor angiogenesis is crucial for cancer growth and progression.^[55] The Notch pathway promotes cancer growth not only by enhancing the survival of cancer cells and their progenitors but also by controlling tumor vascularization. Dll4/Notch1-mediated signaling modulates VEGF-A-driven angiogenesis by affecting the number of sprouts (new branches) on endothelial cells. This interplay between Dll4/Notch1/VEGFR determines the balance between the number of tip cells (leading and guiding the blood vessel sprout) and stalk cells (proliferating cells forming the vascular lumen).^[24,56] Interference with tumor angiogenesis by inhibition of Dll4-mediated signaling has been effective in blocking cancer growth in animal models.^[57] Recently, high levels of Jagged1 have also been shown to promote tumor angiogenesis by destabilizing the tip and stalk cell fates^[58] and by regulating levels of VEGFR1, 2^[59] and activate Notch3/Hey1 in tumor cells thus promoting proliferation, survival, and epithelial to mesenchymal transition.^[59] Consistently, inhibition of experimental tumors growth has been obtained by blocking Jagged1-dependent Notch signaling.^[60]

Notch inhibitors are currently under clinical investigation, in combination with existing therapies for the treatment of several types of cancers.^[61] Considering the role of Notch in maintaining intestinal homeostasis, patients treated with Notch inhibitors require clinical monitoring of the gastrointestinal tract.^[62] Furthermore, due to the effect of Notch in promoting angiogenesis and survival of cardiac progenitor cells, cancer patients with preexisting ischemic diseases should also be monitored for possible

cardiotoxicity linked to the use of Notch inhibitors.^[63]

The mutations causing the activation of Notch signaling have been identified for T-ALLs;^[38] however, little is known about the molecular mechanism involved in dysregulating Notch in other malignancies. Few activating mutations of the Notch pathway have been found in solid tumor patients, with most being observed in non-small cells lung^[64] and head and neck cancers.^[65] In breast and lung cancers, inactivation of Numb, a protein involved in Notch1 downregulation, has also been identified.^[64,66] Rearrangements of the Notch gene families have been found in breast cancer.^[67]

THE ROLE OF SMALL DNA TUMOR VIRUSES IN THE PATHOGENESIS OF CANCER

SV40

SV40^[68] is a monkey virus, which was accidentally administered to humans, in the years 1955-1963, through contaminated poliovirus vaccines.^[69,70] However, a more recent study indicates that some oral poliovirus vaccines were contaminated with infectious SV40 in sub-sequent years.^[71] Early experiments both *in vitro* and *in vivo* classified SV40 as a transforming and oncogenic viral agent. These activities are due to SV40 large tumor antigen (Tag) and small tumor antigen (tag), which act as activated viral oncogenes.^[69,70] These studies addressed a new wave of investigations into the potential of SV40 to induce cancer in humans. To date, hundreds of molecular and epidemiologic studies aimed at investigating whether SV40 infects humans, its potential mode of transmission and its putative role in human tumors have been carried out.^[72-74]

SV40 was assigned to the family of Papovaviridae, an acronym proposed by Melnick^[75] obtained by fusing the names of the 3 representative viruses papilloma, polyoma, and vacuolating agent. However, this nomenclature at present is considered obsolete. More recently, SV40 has been enclosed among polyomaviruses, together with the human polyomaviruses (HPyV), BK Polyomavirus (BKPyV), and JC polyomavirus (JCPyV). The virion is about 45 nm, an icosahedral particle, with a density of 1.34-1.35 g/cm³. The viral genome is a circular, double-stranded DNA molecule. SV40 encodes for six main viral proteins: Two early non-structural polypeptides, Tag and tag, an agnoprotein, probably involved in the assembly of viral particles and processing of late messenger RNA (mRNA) and 3 capsid proteins, VP1, VP2 and VP3.^[76-78] The early and late genes are transcribed on different DNA strands in a way that the transcription proceeds divergently from the regulatory region. This region contains the origin of DNA replication and binding sites for the transcription factors that control viral gene expression and terminates within DNA sequences containing the polyadenylation signals. Recently, a predicted late polarity pre-microRNA to the untranslated region 3' of the polyadenylation cleavage site

in the late pre-mRNA has also been detected.^[79,80] SV40 is phylogenetically, closely related to HPyV. There is evidence of similarity with respect to size (about 5.2 Kb), genome organization, and DNA sequence. The tags of SV40, BKPyV, and JCPyV strongly cross-react with the same antisera^[81,82] while a less, strong cross-reactivity is observed in most structural antigenic determinants of the viral proteins, named VP1, 2 and 3. A genus-specific capsid antigen, located on viral peptide VP1, has been identified.^[83] The DNA sequences of SV40 share 70% homology with BKPyV,^[84] and 69% with JCPyV.^[85] The greatest homology is found in the early region coding for the Tags and tags, whereas a lower homology is detected in the regulatory region.

Transformation of rodent and human cells by SV40 is induced by the 2 oncoproteins, Tag and tag, which display multiple functions. The main activity of Tag for cell transformation^[69] and tumorigenesis is to target key cellular proteins,^[86-88] such as the tumor suppressor p53^[89-91] and retinoblastoma protein (pRB) family proteins, inactivating their functions.^[92-94] SV40 Tag may also lead to transformation by inducing mutations to the cellular genome^[95] or numerical and structural alterations of chromosomes,^[96,97] such as gaps, breaks, dicentric and ring chromosomes, chromatid exchanges, deletions, duplications, and translocations.^[98] The principal role of the tag in transformation is to bind the catalytic (36 kDa) and regulatory (63 kDa) sub-units of protein phosphatase 2A (PP2A),^[69,86] inactivating their function. Moreover, tag interacts with the centrosome and blocks mitosis in human cells,^[99] suggesting that it may disrupt cell cycle progression. Recently, it has been shown that in human mammary epithelial cells tag activates PI3K^[100] an enzyme involved in pathways crucial for cell proliferation, and transformation through phosphorylation of the hydroxyl moiety present on the phosphatidylinositol inositol ring. Aberrant regulation of EGFR upstream from PI3K through mutations in EFGR can lead to cancer promotion in glioblastoma.^[101,102] In addition, SV40 tag can enhance transcription from E2F-activated promoters of early growth response genes.^[103,104] The process of rodent cell transformation induced by SV40 typically depends on the integration of the viral DNA into the host genome where it produces a high level of expression of the major viral oncogenic proteins, Tag, and tag. However, human cells experimentally transformed by SV40 harbor viral genomes in an episomal state in addition to integrated viral DNA. SV40 immortalized^[105] and transformed human cells^[106-108] can induce tumors when implanted subcutaneously in autologous hosts.^[107] An SV40 Tag needs cooperation of the catalytic sub-unit of telomerase and the activated c-HRas oncogene, for the complete transformation of human cells, as shown in cotransfection experiment.^[109] SV40 is highly oncogenic in rodents and when inoculated subcutaneously, intra-cerebrally, or intra-venously in newborn hamsters induces soft

tissue sarcomas, osteosarcomas, ependymomas and choroid plexus papillomas, and neoplasms of the hematopoietic system, such as lymphocytic leukemia, histiocytic lymphomas and rarely, and B-cell lymphomas, respectively.^[87,110-112] Direct inoculation of SV40 into the pleural space induces malignant mesothelioma in 100% of the injected hamsters.^[111] The oncogenic potential of SV40 is confirmed by the generation of transgenic mice in which polyomavirus large Tag expression is regulated by the native viral early promoter enhancer.^[113] Furthermore, SV40-transgenic mice develop ependymomas and choroid plexus papillomas, as well as other neoplasms.^[87,114-116] Many reports were published on SV40 sequences detected, at high prevalence in human cancers of the same histotypes induced by this small DNA tumor virus in experimental animals, that is, lymphoproliferative disorders, mesothelioma, and bone and brain tumors.^[72,117,118] SV40 sequences were also detected at low prevalence in healthy subjects.^[119-121]

Most of these studies were obtained by polymerase chain reaction techniques. More recently, investigations reported the detection at high prevalence of specific antibodies in serum samples from patients affected by malignant pleural mesothelioma,^[122] glioblastoma multiforme,^[123] osteosarcoma,^[124] ocular melanoma,^[125] and non-Hodgkin lymphoma,^[126] suggesting an association of SV40 with these human cancers. Indeed, in serum samples from normal individuals^[127-129] or patients affected by tumors, and^[130,131]/other pathologies^[132,133] unrelated to SV40, the prevalence of antibodies against SV40 is lower than that detected in human cancers found to be associated with SV40. It is worth noting that taken at all, the prevalence of SV40 sequences and the prevalence of specific antibodies against SV40 in these human tumors/normal tissues and sera, respectively, are very similar. This result indicates that SV40 is also a human virus, which infection occurs at low prevalence in normal individuals. Altogether, these data suggest that this small DNA tumor virus of monkey origin seems to be associated at high prevalence with specific human cancers. It is also possible that the immunologic data are due to the cross-reactivity with a new, still undetected, human polyomavirus closely related to SV40.

HPV

HPV infection is considered to be the main oncogenic agent for the onset of female genital tumors.^[134] HPVs are non-enveloped small DNA tumor viruses, with a double-stranded genome of approximately 8.2 kb. HPVs are sub-divided into 2 classes such as low-risk, which are detected in mainly genital warts, and high-risk (HR), which are associated with invasive cervical cancer. HR HPV includes 15 types (16, 18, 31, 33, 35, 39, 45, 51, 52, 56, 58, 59, 68, 73 and 82), whereas low-risk HPV includes 12 types (6, 11, 40, 42, 43, 44, 54, 61, 70, 72, 81 and 108).^[135] However, the oncogenic potential of HPV

is mediated by the expression of the viral oncoproteins identified as E6 and E7. The role of HPV E6 and E7 oncoproteins in HPV-associated cervical carcinogenesis is mainly due to their interaction with the cellular tumor suppressor p53 and members of the pRB family, respectively.^[136-138] The mechanisms of action of HPV cause genetic instability and cell transformation resulting in cell cycle regulated escape and inhibition of apoptosis-hallmarks of cancer initiation and progression.^[139] Studies on the association between HPV and cervical neoplasia have indicated a strong link between these oncogenic virus types.^[140]

Research demonstrates that only a fraction of HPV-positive women develops genital tumors.^[141] Indeed, the majority of patients who are infected with HPV can clear these viral agents naturally within 1 year.^[142] Persistent infection with HR HPV at a high viral load in cervical mucosa is considered the main cause of the initiation and progression of genital tumors^[143] as it is a well-established cause of cervical cancer. In addition to E6 and E7 transformations, HR HPV oncogenic types 16, 18, 31, 33, 35, 45, 52, 58, and 66 are associated closely with > 95% of cases of squamous cell carcinoma of the cervix.^[144] Moreover, only genotype HPV 16 accounts for > 55% of diagnosed tumors.^[145] Although infection with HR HPV is the major risk factor associated with cervical cancer, some studies have reported a possible tumor-initiating and promoting role in cervical cancer for other DNA tumor viruses. Taken together, this interaction may synergize with HPV in a normal cell to initiate and progress a tumorigenic cell.^[146]

ONCOGENIC DNA VIRUSES AND MODULATION OF THE NOTCH PATHWAY

As previously discussed, the Notch signaling pathway influences cell fate decisions, proliferation versus differentiation, and cell survival. Similarly, viruses in infected cells promote cell survival, promote or block cell cycling and employ a variety of mechanisms to evade innate cellular anti-viral responses to ensure their own survival and multiplication. In light of these similarities, it is not surprising that several viruses hijack the Notch pathway to ensure the completion of their own life cycles.^[147]

The first report of an interaction between a virus and the Notch pathway came from studies showing that binding of Epstein-Barr virus (EBV) nuclear antigen 2 (the transcriptional activator essential for EBV-driven B-cell immortalization) to responsive promoters requires the interaction with the nuclear effector of Notch signaling CSL.^[148] More recently, also, the Kaposi's sarcoma (KS)-associated herpes virus replication and transcription activator protein (involved in controlling the switch from latency to lytic replication) has been found to activate lysis-related gene by binding to CSL.^[149] Studies using γ -secretase

inhibitors (GSI-I Z-Leu-Leu-Nle-CHO and LY-411, 575), small molecules which block Notch activation, resulted in apoptosis in KS cells and established KS cell tumors in mice, demonstrating the requirement for an active Notch signaling in KS.^[150] Notch pathway interactions have also been shown with adenoviral oncoprotein 13S E1A, which binds to CSL, displaces associated corepressor complexes, and activates CSL-dependent gene expression.^[151]

In agreement with reports of an association between SV40 infection and human mesothelioma,^[152] SV40 infection upregulates the expression of Notch1 in mesothelial cells.^[153] SV40-mediated Notch1 induction is achieved at the transcriptional level; it requires both SV40 Tag and tag and tag-induced activation of the mitogen-activated protein kinase-extracellular-signal-regulated kinase pathway. Notch activation is necessary for the growth of SV40-transformed mesothelial cells, as treatment of these cells with a Notch inhibitor leads to G2/M cell cycle arrest.^[153] Consistently, upregulation of Notch1 and ligands Jagged1 and 2 is maintained in SV40-transformed human mesothelial clones and SV40-positive mesotheliomas and derived cell lines.^[153]

Other than in mesothelial cells, Notch1 expression and signaling has been linked to SV40-mediated transformation of primary astrocytes.^[154] In both mesothelial cells and astrocytes, SV40-mediated activation of Notch signaling determines the survival of cells grown in suspension. Of interest, the archetypal (1 copy of enhancer sequence in the regulatory region) and the non-archetypal (2 copies of enhancer sequences in the regulatory region) SV40 strains are both able to transform astrocytes whether only the non-archetypal strain can transform mesothelial cells. Differences in expression levels of Notch1 and its downstream effectors (c-Myc, Hey1, Hes1 and HeyL) appear to explain these differences in SV40-mediated transformation of primary astrocytes and mesothelial cells.^[154]

SV40 tag, which forms a complex and inhibits PP2A activity, plays a critical role in the malignant transformation of human cells. Microarray analyses on human embryonic kidney cell lines overexpressing SV40 tag have identified induction of Dll1 and Jagged1 suggesting a role for SV40 tag in the activation of the Notch pathway.^[155] Of interest, in these cells, Notch signaling was found to be upregulated in association with Hedgehog and Wnt pathways but inhibition of Hedgehog and not of Notch interfered with cell survival suggesting that Notch signaling is not essential for survival in cells expressing SV40 tag.^[155] A link between SV40 tag and Notch has been observed also in human bronchial epithelial cells. Specifically, Wang *et al.* have shown that miR-27a is upregulated in SV40 tag-transformed human bronchial epithelial cells (HBERST) following the interaction between tag and PP2A. In these cells, miR-27a promotes cell cycle progression by downregulating Fbxw7, a regulator of ubiquitin-dependent proteolysis of a set of protein involved in cell cycle progression, including

Notch1. Suppression of miR-27a expression in HBERST cells leads to cell cycle arrest in the G0-G1 phase.^[156]

Both SV40 Tag and tag have been shown to induce the immortalization of mammary gland epithelial cells.^[157,158] SV40 tag expression inhibits mammary gland differentiation during mid-pregnancy and about 10% of multiparous tag transgenic animals develop breast tumors with latencies ranging from 10 to 17 months, whereas expression of N-terminal truncated Tag molecules harboring the intact p53 and pRB binding region does not have this effect.^[158] Expression of SV40 Tag in the epithelium of the mammary glands results in cancers which resemble the human disease and do not require hormone supplementation or pregnancy for insurgence.^[157] Breast cancer has been associated to SV40 infection^[159] and a specific gene signature in transgenic models of breast cancer intrinsic to the functions of the SV40 T/t-antigens has been identified which is associated with poor prognosis.^[160] It is not known whether SV40 is involved in the dysregulation of Notch signaling observed in breast cancer.^[19,152] Of interest, the Notch target gene cyclin D1 is overexpressed in the SV40 tag-positive mammary gland epithelial cells and in the breast tumor cells from SV40 tag-expressing mice.^[157]

HPV is the most significant causative agent in the development of cervical cancer. Despite its presence in almost all cervical cancers, it is widely recognized that HPV by itself is unable to transform a normal cell to a cancerous one, and additional cellular mutations are required to supplement the HPV oncoproteins E6 and E7. The activation of the Notch signaling pathway induced by HPV infection has been proposed as one of the cellular changes that cooperate with the E6 and E7 proteins to cause cervical cancers.^[161] This proposition is based on several studies showing overexpression of Notch signaling in HPV-cervical cancer or cell lines. Specifically, active Notch1 expression has been shown in high-grade cervical lesions and cancers^[162,163] and progressively increasing up-regulation of Notch3 expression with severity of disease as compared to normal cervix tissue has been reported in a set of 168 tissue biopsy samples comprising of tumor specimens, precancer, and non-neoplastic cervical tissues.^[164] Noteworthy, in the same specimens, Notch1 was found to be downregulated thus suggesting the existence of a complex interplay between Notch signaling and HPV in the context of the development of cervical carcinogenesis.^[164] Upregulation of both Jagged1 and Hes1 and downregulation of Manic Fringe, a negative regulator of Jagged1-Notch1 signaling, have been shown in squamous cell carcinoma of cervix compared to high-grade lesions and in late-passage, but not early-passage, HPV type 16-positive human cervical low-grade lesion-derived cell line W12.^[165] Overexpression of all Notch receptors, Hes1, and MAML1, the transcriptional co-activator originally identified by its role in Notch signaling, has been found in HeLa, SiHa, and CaSki, three other cell lines derived from

HPV-positive human cervical cancer.^[166] Evidence in favor of an oncogenic role for Notch in cervical cancer comes from the observation that activated Notch1 synergizes with HPV16 E6 and E7 proteins in conferring apoptosis protection through the activation of the prosurvival PI3K-protein kinase B/AKT (PI3K-PKB/AKT) pathway and in the transformation of the immortalized human keratinocytes HaCaT cell line.^[167] Furthermore, in HaCaT cells active Notch1, through the PI3K-PKB/AKT-dependent pathway, inhibits p53-induced apoptosis and sustains transformation by HPV 16 E6 and E7.^[168] Consistently with the findings of high level of Jagged1 in cervical cancer, Jagged1 but not Dll1 expression correlates with the rapid induction of PI3K-mediated epithelial-mesenchymal transition both in HaCaT cells and in a human cervical tumor-derived cell line.^[169] Microarray studies by the same authors show that Notch-PI3K oncogenic functions can be independent of CSL activation and rely instead on Deltex 1, an alternative Notch effector.^[169] The anti-apoptotic role played by Notch in cervical cancer progression has also been revealed by immunohistochemistry conducted in cervical cancer specimens in which high levels of Jagged1, Hes1, and Cdk9 were paralleled by nuclear translocation of both NF- κ B p50 and p65 and NF- κ B target genes expression (I κ B- α , B-cell lymphoma 2 and cyclin D1).^[170] An active Notch pathway is necessary for the survival and the maintenance of the neoplastic phenotype of HPV-positive cervical cancer cell lines as demonstrated by experiments in which Notch signaling was inhibited by anti-sense Notch1 oligo,^[46,171] by upregulation of Manic Fringe,^[165] by small interfering RNA against Jagged1^[165] or by inhibition of γ -secretase in combination with dominant negative MAML1, a regulator of crosstalk between the Notch and NF- κ B pathways.^[166]

Experimental evidence shows that as with SV40, HPV proteins have a direct effect on the activation of Notch signaling. Weijzen *et al.* have reported that transfection of mouse primary embryonic cells and human primary fibroblasts with HPV16 E6 and E7 upregulates Notch1 not only transcriptionally but also post-translationally by upregulating presenilin-1, a protein involved in Notch processing.^[46] Microarray analyses have revealed enhanced expression of Notch1 mRNA in HPV16 E6-expressing keratinocytes when NFX1-123 (a protein involved, together with E6, in binding and stabilization of mRNA coding for human telomerase reverse transcriptase, the catalytic subunit of telomerase) was overexpressed. A moderate increase in Notch1 mRNA was seen with overexpression of NFX1-123 alone, but with 16E6 coexpression the increase in Notch1 was enhanced.^[172] A recent study by the same group has shown that the Notch canonical pathway genes Hes1 and Hes5 were increased with overexpression of NFX1-123 in 16E6 - expressing keratinocytes, and their expression was directly linked to the activation or blockade of the Notch1 receptor. Of interest, keratin 1 and keratin 10 were also increased in this model, but in contrast to Notch target genes, their upregulation was only indirectly associated

with Notch1 receptor stimulation, and it did not lead to growth arrest, increased p21 (Waf1/CIP1), or decreased proliferative factor Ki67.^[173]

Notch signaling pathway is a key determinant of keratinocyte growth arrest and differentiation.^[174] and it has been recently shown that it promotes expression of differentiation markers acting together with the TAp63 β isoform of the p63 transcription factor.^[175] This evidence supports a role for Notch as putative tumor suppressor in HPV-associated tumorigenesis rather than an oncogene, as discussed so far. It is well established that Notch activity regulates tumor biology in a context-dependent manner and may act as an oncogene or a tumor-suppressor gene within the same tumor type. In human, esophageal keratinocytes overexpression of Notch1 induces senescence (induction of G0/G1 cell-cycle arrest, Rb dephosphorylation, flat and enlarged cell morphology, and senescence-associated beta-galactosidase activity) requiring both canonical CSL-dependent transcriptional activity and the p16INK4A-Rb pathway. Loss of p16INK4A or the presence of HPV E6/E7 oncogene products (which inactivate both the p53 and pRB) in these cells have been shown not only to prevent intracellular Notch1 (N1IC) from inducing senescence, but also to facilitate N1IC-mediated anchorage-independent colony formation and xenograft tumor growth with increased cell proliferation and reduced squamous-cell differentiation.^[176] These observations provide a possible molecular mechanism to explain and support the hypothesis of the oncogenic role on Notch in HPV-positive cervical cancer.

In agreement with a protective role of Notch against HPV-induced transformation, Talora *et al.* have reported that the expression of the endogenous Notch1 gene is markedly reduced in a panel of cervical carcinoma cells, whereas expression of Notch2 remains elevated, and Notch1 expression is reduced or absent in invasive cervical cancers.^[177] The authors show that increased Notch1 signaling, but not Notch2, causes a dramatic downmodulation of HPV-driven transcription of the E6/E7 viral genes, through suppression of AP-1 activity by upregulation of the Fra-1 family member and decreased c-Fos expression. According to the authors, the downmodulation of Notch1 expression would play an important role in late stages of HPV-induced carcinogenesis.^[177] In agreement with these observations, E6 protein from cutaneous HPVs of the β -genus, such as bovine papillomavirus Type 1 and β -HPV5 and 8, induces a repression of Notch transcriptional activation, which is dependent on an interaction with MAML1^[178-180] and it has been shown to inhibit keratinocyte differentiation.^[181]

Technical approaches (type of anti-body used) for Notch detection have been invoked to explain the differences in expression levels of Notch in HPV-positive cervical tumors linked to the different roles of Notch as an oncogene or tumor suppressor gene.^[182] As previously discussed, the opposite

roles on Notch in the context of HPV-cervical cancer have also been attributed to the cellular context. Extremely high levels of Notch1 seem to adversely affect HPV E6 and E7 expression and cellular proliferation whereas moderate levels of Notch1 and PI3K exhibit oncogenic properties that transform primary cells containing HPV16 E6 and E7 proteins.^[161] More recently, in SiHa cervical cancer cells, it was shown that moderate Notch activation contributed to increased viability and anchorage independent growth, whereas high-level Notch activation decreased anchorage independent growth. The shift in phenotypical outcome was correlated to altered AP-1 activity and complex composition.^[183]

Interactions between the Notch pathway and HPV may play a role also in the progression of head and neck squamous cell carcinoma. Exome sequencing of head and neck squamous cell carcinoma have revealed inactivating mutations in Notch1^[184] and recent work by Seiwert *et al.* has shown an enrichment in the frequency of Notch1 mutations in HPV-positive compared to HPV-negative head and neck squamous cell carcinomas.^[185]

CONCLUSION

Many reports indicate that dysregulated Notch pathway and oncogenic viruses may act together in the initiation and progression of different human tumors. More investigations are necessary to acquire new knowledge on the molecular mechanisms involved in the oncogenic process, which are regulated by oncogenic viruses-mediated Notch dysregulation [Figure 1]. These studies could lead to the identification of biomarkers or the development of targeted therapeutic approaches specific for Notch-associated malignancies characterized by the presence of the oncogenic viruses. Furthermore, considering the role of Notch in the regulation of the host immune response against viral infections, a deeper understanding of the interactions between oncogenic viruses and the Notch pathway could lead to the targeting of Notch to prevent or reduce oncogenic virus infections and, possibly, onset of cancers associated with exposure to these viruses.

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Conflicts of interest

There are no conflicts of interest.

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The farnesoid X receptor and colon cancer

Guofeng Xie, Jean-Pierre Raufman

Division of Gastroenterology and Hepatology, Veterans Affairs Maryland Health Care System, University of Maryland School of Medicine, Baltimore, MD 21201, USA.

Correspondence to: Dr. Guofeng Xie, Division of Gastroenterology and Hepatology, Veterans Affairs Maryland Health Care System, University of Maryland School of Medicine, 22 South Greene Street, Baltimore, MD 21201, USA. E-mail: gxie@medicine.umaryland.edu

ABSTRACT

Worldwide, colorectal cancer (CRC) is a leading cause of cancer death, primarily because of limited therapeutic options for those with advanced disease. The farnesoid X receptor (FXR) is a member of the nuclear receptor superfamily of ligand-activated transcription factors. Besides its prominent role in bile acid synthesis, and lipoprotein and glucose metabolism, recent data indicate that FXR also plays a key role in regulating intestinal cell proliferation and carcinogenesis. Here, we review the role of FXR as a tumor suppressor in CRC, with particular emphasis on the molecular mechanisms underlying FXR-dependent tumorigenesis and its regulation, FXR-bile acid relationships and FXR-targeted drugs as potential therapeutic agents.

Key words: Colon cancer; farnesoid X receptor; nuclear receptor; tumor suppressor

INTRODUCTION

Despite advances in screening and treatment, colorectal cancer (CRC) results in over 50,000 deaths yearly and may soon surpass lung cancer as the overall leading cause of cancer-related death in the USA alone.^[1,2] Despite increased efforts to improve access and compliance, many people neglect CRC screening. In addition, the efficacy of colon cancer screening is limited by the limited sensitivity of tests, “miss” rates on colonoscopy and other factors. Chemoprevention using non-steroidal anti-inflammatory drugs is marginally effective^[3,4] but limited by gastrointestinal (GI)^[5] and cardiovascular^[6] toxicity that led to the withdrawal of rofecoxib.^[7] Non-surgical treatments (e.g. chemotherapy and radiation) for advanced colon cancer have limited efficacy. Although the use of biologicals that target vascular endothelial growth factor and epidermal growth factor receptor (EGFR) (i.e. bevacizumab, cetuximab and panitumumab) may increase survival with advanced CRC by several months, these agents have a limited impact on 5-year survival, on the order of only 10%.^[8-10] Moreover, their use is limited by off-target toxicity that commonly reduces patient tolerance; EGFR, which is expressed widely in non-intestinal epithelial cells (e.g. dermal epithelial cells),^[11] causes skin reactions that may force

discontinuation of treatment.

FARNESOID X RECEPTOR AND ITS LIGANDS

Farnesoid X receptor (FXR) [nuclear receptor subfamily 1, group H, member 4 (NR1H4)] is a member of the nuclear receptor superfamily of ligand-activated transcription factors and acts as a bile acid sensor.^[12-14] FXR regulates the expression of genes involved in bile acid synthesis, and cholesterol and triglyceride metabolism by binding to their promoters as a homo- or hetero-dimer with a common partner of nuclear receptors, retinoid X receptor. FXR agonists include naturally-occurring bile acids (e.g. chenodeoxycholic acid [CDCA; EC50 of 10-50 μmol/L]),^[15] synthetic compounds GW4064 (EC50 of 15 nmol/L),^[16] 6E-CDCA (EC50 of 99 nmol/L),^[17] WAY-362450 (EC50 of 4 nmol/L)^[18] and fexaramine (EC50 of 25 nmol/L);^[19] FXR antagonists include plant-derived guggulsterone^[20] and synthetic AGN34.^[21] The FXR agonist fexaramine is poorly absorbed following oral administration; thus, it acts as an intestine-restricted FXR agonist without systemic side-effects.^[19] Oral administration of fexaramine results in serum levels that are an order of magnitude lower than those obtained following intraperitoneal injection of the drug, and it activates FXR target genes only in the GI tract.^[19]

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FXR EXPRESSION AND REGULATION IN NORMAL INTESTINAL MUCOSA

FXR is expressed primarily in the GI tract, liver and kidney.^[22,23] Modica *et al.*^[24] showed that murine FXR (Nr1h4) is expressed at high levels in the small intestine and colon, whereas human FXR (NR1H4) is expressed at moderate levels in the colon. FXR expression is localized primarily to fully differentiated cells lining the intestinal epithelium of the ileum and colon.^[24] In the *Apc^{min/+}* murine model of CRC, FXR messenger RNA (mRNA) levels were down-regulated in tumor tissue compared with adjacent normal mucosa. Likewise, in patients with familial adenomatous polyposis (FAP) syndrome, FXR mRNA expression was decreased in normal and neoplastic tissues. In a human CRC cell line, HT-29 cells, restoring wild-type APC protein induced FXR expression, suggesting that APC may directly or indirectly regulate FXR expression.^[24] FXR can also be regulated at the transcriptional level by the caudal-related homeobox 2.^[25] Moreover, Bailey *et al.*^[26] showed that DNA methylation and KRAS signaling silence FXR in human CRC. In approximately, 12% of human colon cancers and in several colon cancer cell lines, including SW620, FXR promoter methylation of a CpG island results in very low FXR expression.^[26] Cabrerizo *et al.*^[27] found FXR promoter methylation at two additional CpG islands (-358 and -1890 bp). Furthermore, functional analysis of the 5'-promoter region of the human FXR gene in HepG2 cells suggests that hepatic nuclear factor 1a may be a transcription factor for FXR.^[28]

FXR IS AN INTESTINAL TUMOR SUPPRESSOR

In addition to its essential role in regulating lipid metabolism, emerging evidence supports a key role for FXR as an intestinal tumor suppressor. In two mouse models of CRC, *Apc^{min/+}* and chronic colitis, Modica *et al.*^[29] showed that FXR deficiency increased adenoma size and number. In a xenograft model, they showed that FXR reactivation via adenoviral infection blocked tumor growth. Using *Apc^{min/+}* and azoxymethane-induced mouse models of CRC, Maran *et al.*^[30] confirmed that FXR was an intestinal tumor suppressor. Smith *et al.*^[31] showed that activating FXR with sodium taurocholate markedly reduced adenoma formation in *Apc^{min/+}* mice.

FXR is down-regulated drastically in colon tumors from both murine (*Apc^{min/+}*) and human FAP models of CRC.^[24] FXR mRNA expression is reduced in colon adenomas and even more profoundly in colon adenocarcinomas.^[32,33] Diminished FXR expression is associated with advanced CRC stage and an adverse prognosis.^[26,33]

Colon cancer risk increases substantially with chronic intestinal inflammation as in inflammatory bowel disease, including both Crohn's and ulcerative colitis (UC).^[34,35] FXR activation decreases the production

of pro-inflammatory cytokines, such as interleukin (IL) 1-beta, IL-2, IL-6, tumor necrosis factor-alpha and interferon-gamma, thereby reducing inflammation and intestinal permeability.^[36] Torres *et al.*^[37] showed that FXR expression was inversely correlated with neoplastic progression and the severity of colonic inflammation in UC. FXR expression is also reduced in colonic mucosa from patients with primary sclerosing cholangitis (PSC) and UC-associated neoplasia. Compared to patients with UC alone, those with PSC-UC have diminished FXR expression in the right colon suggesting they are at a higher risk of proximal colon neoplasia.^[37]

FXR AND COLON CARCINOGENESIS

Although the above observations strongly implicate FXR as a tumor suppressor, the underlying mechanism is incompletely understood. No mutations have been identified in the FXR gene in CRC.^[26] Several studies suggest the role of FXR in colon carcinogenesis is multifactorial. Modica *et al.*^[29] showed the importance of Wnt signaling and apoptosis downstream of FXR. FXR promotes Wnt signaling with the expansion of basal proliferative intestinal cells, and a concomitant reduction in the apoptosis-competent apical epithelium. When FXR is activated in CRC cells, induction of apoptosis results in the removal of genetically altered tumor cells. The same investigators showed that FXR activation increased expression of several pro-apoptotic genes, including FAS, BAK1, P21, KLF4, FADD, CAS9 and P27. Maran *et al.*^[30] showed that FXR deficiency increases intestinal cell proliferation, accompanied by up-regulation of cyclin D1 and IL-6. In addition, it was shown that sodium taurocholate inhibits intestinal tumorigenesis by activating FXR, leading to increased Shp expression and consequent down-regulation of cyclin D1.^[31]

Several other potential mechanisms may account for FXR inhibition of intestinal tumor genesis. Peng *et al.*^[38] showed that Src-mediated cross-talk between FXR and the EGFR inhibited human intestinal cell proliferation *in vitro* and growth of human colon cancer xenografts in nude mice. Yang *et al.*^[39] has showed that FXR is a transcription factor for microRNA-22, and also a tumor suppressor which silences cyclin A gene expression in colon cancer cells. In inflammation-associated intestinal neoplasia, activation of FXR is repressed by pro-inflammatory cytokines that activate intestinal nuclear factor-kB signaling;^[40] the investigators concluded that FXR not only inhibits inflammation, but also is targeted by the inflammatory response, resulting in a vicious cycle where reduced FXR activity causes less repression of inflammation. Zhou *et al.*^[41] also showed that activation of the PPAR α -UGT axis repressed intestinal FXR-FGF15/19 feed-back and exacerbates experimental colitis, thereby possibly promoting intestinal tumorigenesis. In mice, both PPAR α knockout and treatment with recombinant FGF19 strongly attenuated dextran sulfate sodium-induced colitis.^[41]

ROLE OF BILE ACIDS IN FXR-MEDIATED INHIBITION OF TUMORIGENESIS

Colon cancer is often linked to a Western diet, rich in carbohydrates and saturated fatty acids.^[42-44] Subjects who consume a Western diet and patients with CRC have elevated levels of fecal secondary bile acids, mostly lithocholic acid (LCA) and deoxycholic acid (DCA), implicating bile acids as contributing factors in colon carcinogenesis.^[45-48] Although controversial, cholecystectomy, which increases intestinal bile acid levels, may predispose persons to CRC.^[49,50] Nonetheless, recent evidence suggests that FXR inhibits intestinal tumorigenesis through a bile acid-independent mechanism. Degirolamo *et al.*^[51] showed that FXR deficiency, not elevated bile acid levels, mediated susceptibility to intestinal tumorigenesis. The tumor-promoting activity of bile acids does not occur as a function of their ability to activate FXR in the intestines.^[29,51] Raufman *et al.* showed that several bile acids, including DCA and LCA, promoted colon carcinogenesis and cell proliferation by interacting with M3 muscarinic receptors that are overexpressed in a majority of colon cancers and human colon cancer cells through transactivation of EGFR.^[52-55] Although the role of FXR as an intestinal tumor suppressor might not be directly mediated by bile acids, FXR activation can have tumor-suppressive effects by transcriptional induction of detoxifying enzymes that mediate transformation and excretion of toxic bile acids.^[51] Interestingly, FXR's role in liver cancer (hepatocellular carcinoma) as a tumor suppressor may be mediated by bile acids.^[56-59]

FUTURE DIRECTIONS

In addition to being a master regulator of bile acid synthesis, and glucose and fat metabolism, recent research data reveal a novel and important role for FXR as a tumor suppressor in intestinal carcinogenesis, cell proliferation and tumor growth. Because FXR is considerably down-regulated in colon tumor cells, restoring or reactivating FXR expression may offer a therapeutic strategy. In addition, because normal intestinal epithelial cells express high levels of FXR, pharmacological FXR agonists might be effective chemopreventive agents, particularly in high-risk populations, including those with hereditary CRC (e.g. FAP and Lynch syndrome). To avoid systemic toxicity associated with FXR activation (e.g. 6E-CDCA can cause pruritus)^[60] intestine-specific FXR agonists, like fexaramine may be especially useful.^[19]

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Conflicts of interest

There are no conflicts of interest.

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Withaferin A targeting both cancer stem cells and metastatic cancer stem cells in the UP-LN1 carcinoma cell model

Lai-Lei Ting¹, Andy Shau-Bin Chou², Chin-Hsuan Hsieh³, Shih-Chieh Hsiung³, See-Tong Pang³, Shuen-Kuei Liao^{4,5}

¹Department of Radiation Oncology, Cancer Center, Taipei Medical University Hospital, Taipei 110, Taiwan, China.

²Department of Radiology, Tzu-Chi General Hospital, Hualien 970, Taiwan, China.

³Department of Surgery, Division of Uro-Oncology, Chang Gung Memorial Hospital, College of Medicine, Chang Gung University, Taoyuan 333, Taiwan, China.

⁴The PhD Program of Cancer Biology and Drug Discovery, Taipei Medical University, Taipei 110, Taiwan, China.

⁵Department of Research and Development, Vectorite Biomedica Inc., New Taipei City 221, Taiwan, China.

Correspondence to: Dr. Shuen-Kuei Liao, The PhD Program of Cancer Biology and Drug Discovery, Taipei Medical University, Taipei 110, Taiwan, China. E-mail: liaosk@h.tmu.edu.tw



Dr. Shuen-Kuei Liao earned his PhD degree from McMaster University, Canada, and subsequently received his postdoctoral training at University of Toronto. He has been working in the areas of cancer biology and immunotherapy in Canada, USA and Taiwan over the past three decades. Currently he is Professor at Taipei Medical University with his interests in cancer stem cells and immunotherapy, and the roles of mesenchymal stem cells in transplantation.

ABSTRACT

Aim: As our understanding of cancer stem cell (CSC) biology improves, search for inhibitory agents of CSCs and metastatic CSCs (mCSCs) positive for CXCR4 is warranted. Withaferin A (WA), a withanolide extracted from the medicinal plant *Withania somnifera*, has been shown to exhibit anti-cancer effects through multiple mechanisms. Whether WA could selectively target CSCs, mCSCs, or non-CSCs of a gastrointestinal (GI) carcinoma tumor remains unclear. **Methods:** Side-population (SP) analysis, flow cytometric phenotyping and sorting, non-invasive imaging in conjunction with xenotransplantation, and immunohistochemistry were used in this investigation. **Results:** Using the lymph node metastatic GI cancer cell line UP-LN1, consisting of CD44^{high}/CD24^{low} floating (F) and CD44^{low}/CD24^{high} adherent (A) cell subsets, this study demonstrated that as compared with parental UP-LN1 cells or A cells, WA preferentially reduced F-cell proliferation, tumor sphere formation, and SP cells *in vitro* in greater efficiencies by apoptosis. This action was mechanistically mediated via the down-regulation of CXCR4/CXCL12 and STAT3/interleukin-6 axes, both of which are instrumental in the acquisition of metastatic ability. Attenuation of interferon- γ -induced CXCR4 expression in F cells by knockdown with siRNA or blocking with an anti-CXCR4 antibody, followed by Western blot analysis, showed significantly reduced metastatic potential *in vitro*. The extent of *in vitro* anti-invasive effect of WA on the IFN- γ -treated F cells was significantly greater than on the F cells without WA treatment, or F cells treated with control siRNA or with control IgG antibody. The observed *in vitro* effects of WA on the CSC and mCSC targeting were validated by data obtained with non-invasive imaging in NOD/SCID mouse xenotransplantation. **Conclusion:** WA could efficiently block the formation of both CSCs and mCSCs in the UP-LN1 cell line, suggesting that WA may be considered an effective therapeutic agent for this type of GI malignancies.

Key words: Cancer stem cells; CXCR4; gastrointestinal cancer; metastasis; metastatic cancer stem cells; STAT3; withaferin A

INTRODUCTION

Distant metastasis represents one of the few most challenging aspects in cancer management. Cancer cells progress from the primary lesion site and gain the ability to spread to

distant organs. It has been demonstrated both experimentally and clinically that the tumor microenvironment plays a pivotal role in tumor progression, particularly in the

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acquisition of metastatic potential.^[1] During the process of tumor metastasis, a cellular event termed epithelial-to-mesenchymal transition (EMT), is initiated and is believed to be a prerequisite for tumor dissemination.^[2] Accumulating evidence from neoplastic tissues indicates the presence of self-renewing, stem-like cells within tumors called cancer stem cells (CSCs) or tumor-initiating cells. CSCs, which constitute a small subpopulation of neoplastic cells within a tumor, are defined operationally by their ability to seed new tumors.^[3] Recently, a seminal study has demonstrated that disseminating cancer cells require self-renewal capability, similar to that exhibited by stem cells and has indicated that the EMT process enriched the CSC population.^[4] Collectively, these studies provided important evidence and insights into CSC biology. The existence of CSCs has been shown to contribute to many aspects of tumorigenesis, especially therapy resistance^[5,6] and metastasis.^[7] However, studying CSCs has been a great challenge due to their rarity and the accuracy of identification methods. Therefore, a reliable tumor cell model which replicates the physiological properties of CSCs and metastatic CSCs (mCSCs) becomes a valuable tool for the understanding of CSC biology.

The UP-LN1 lymph node metastatic cell line we have established previously exhibits CSC characteristics.^[8,9] UP-LN1 is a CEA-producing gastrointestinal (GI) carcinoma cell line which harbors a unique co-existence of 2 major naturally occurring cell populations, adherent (A) and floating (F) cells. Between the 2 subpopulations, the F cells were characterized to possess several CSC-like properties, including CD44^{high}/CD24^{low} phenotype, high expression of multiple drug resistance genes, and tumor-initiating ability in NOD/SCID mice with low cell numbers, depressed HLA class I expression,^[9,10] and resistance to natural killer (NK)/lymphokine activated killer (LAK)-mediated cytotoxicity, relative to CD44^{low}/CD24^{high} A cells.^[9] In addition, F and A cells were found mutually convertible with F to A cells at a faster rate. It is also conceivable that F cells may be more easily separated from the primary lesion than A cells to enter the bloodstream as circulating tumor cells and then deposit and proliferate at the new site through extravasation and intravasation, as the initial step toward metastasis. This phenomenon appears not to be restricted to GI malignancies since a similar result was recently reported with other cancer types such as breast cancer recently.^[11] Within the CSC cell population, there is an even smaller subset which could be become induced to CXCR4-positive mCSCs responsible for initiating metastatic activity in or migrate toward/invoke a new microenvironment where a greater CXCL12 gradient is present.^[9] Moreover, in response to interferon- γ (IFN- γ) or activated NK or LAK cells, the CXCR4-positive mCSCs could only be induced from CSCs, which were harbored in the highly tumorigenic CD44^{high}/CD24^{low} F subset. Thus, the UP-LN1 cell line represents an ideal *in vitro* model for studying CSCs and screening for effective anti-CSC and anti-mCSCs agents.

Withaferin A (WA), a cell-permeable steroidal lactone extracted from the Indian winter cherry, *Withania somnifera*, has been cited for its anti-cancer effects via multiple mechanisms.^[12-16] For instance, WA has been shown to elicit oxidative stress reactive oxygen species (ROS) and mitochondrial dysfunction in leukemia cells leading to apoptosis.^[14] In breast cancer, WA-induced apoptosis via the induction of Bim-s and Bim-L in estrogen-responsive MCF-7 cells and in triple-negative MDA-MB-231 cells.^[17] In another study, WA has been shown to exhibit anti-tumor and anti-angiogenesis activity by binding to the intermediate filaments vimentin and F-actin.^[18] More importantly, WA at low dosages appeared to eliminate cells expressing breast CSC markers including CD44, CD24, CD34, CD117 and Oct 4, and to down-regulate Notch1, Hes1 and Hey1 expression,^[19] suggesting the potential of WA as a CSC-targeting compound. Together, these findings provide the rationale to further explore the anti-cancer effects of WA on the UP-LN1 cell line in terms of the mechanisms involved in blocking the formation of CSCs and/or mCSCs.

In this study, we also used side-population (SP) method^[20,21] to enrich the CSC subpopulation from UP-LN1 cells, and then showed that F cells harbored the highest percentage of CSC-like cells with an elevated expression of few selected stemness-related genes. Subsequently, we demonstrated that WA treatment could inhibit the formation of tumor aggregates/spheres and induce apoptosis in F cells. More importantly, WA treatment could lead to the down-regulation of CXCR4/CXCL12 and STAT3/IL-6 axes, both being key members of a metastatic signaling pathway. Finally, using non-invasive bioluminescence imaging technique, we demonstrated that after treatment with WA, both the tumor burden and dissemination ability were significantly suppressed in NOD/SCID mice implanted with F cells.

METHODS

Chemicals and reagents

WA was purchased from Sigma-Aldrich (St. Louis, MO, USA), and its purity was > 95%. Primary monoclonal antibodies (mAbs) to Oct4, Sox2, c-Myc, Nanog, vimentin, Fas receptor, caspase-3, caspase-8, caspase-9, poly ADP-ribose polymerase (PARP), Bcl-2, survivin, Akt, ERK, GRK3/2, STAT3, and β -actin were purchased from Cell Signaling Technology (Boston, MA, USA). Additional mAbs used were as follows: Mouse anti-human CXCL12 (clone 79018, R and D Systems, Minneapolis, MN, USA), and CXCR4 (clone 15G5, R and D Systems, Minneapolis, MN, USA), Fluorescein isothiocyanate (FITC) or phycoerythrin-conjugated goat-antimouse IgG (Biollegend, San Diego, USA) were used as the secondary Ab for tracing the primary mAb. For multiple color-phenotyping, 5×10^5 cells were directly incubated with FITC-conjugated mouse anti-human CD44 (clone G44-26, BD-Pharmingen, Franklin

Lake, NJ, USA), and allophycocyanin-conjugated mouse anti-human CD24 (clone ML10, Biolegend, San Diego, USA) mAbs according to the manufactural instructions. Labeled cells were then washed 3 times by phosphate-buffered saline (PBS) plus 2% fetal bovine serum (FBS) followed by fixation with 1% paraformaldehyde. The fixed samples were then analyzed cytofluorometrically.

Cell line, subsets and culture conditions

The UP-LN1 cell line and its A and F subsets were used in this study. Unless specified, all the cell lines were maintained in the condition described previously.^[8] For the separation of A cells, we discarded all floating cells in the culture supernatant and then harvested only the adherent cells by light trypsinization to set up new cultures for A cells. To obtain F cells, we only collected the floating cells in the culture supernatant of UP-LN1 culture for the subsequent culture passage. Each of these 2 protocols was used for the enrichment of A or F cells when subculturing for 10 consecutive rounds. To maintain the parental (P) UP-LN1 cells (termed P cells), floating cells and trypsinized adherent cells were washed and pooled, then set up in a new culture. Trypan blue dye exclusion was used to determine cell viability. A and F cells were maintained in Roswell Park Memorial Institute (RPMI)-1640 medium supplemented with 10% FBS.

Characterization of UP-LN1 cells by SP analysis

To examine the existence of CSCs in the UP-LN1 carcinoma cell line, the SP cells were isolated by flow cytometry and cell sorting techniques. SP cells have been shown to express an elevated level of ATP-binding cassette transporters (ABCG2), which enhance their ability to pump out Hoechst 33342 dye. This efflux activity of Hoechst dye is similar to many drug-resistant cancer cells and can be sorted by FACS Aria flow cytometry. Therefore, we utilized SP analysis as one of the characteristics to demonstrate and analyze the population of cancer stem-like cells in our unique UP-LN1 cells. UP-LN1 cells were labeled with 2.5 µg/mL Hoechst 33342 (Sigma-Aldrich, Chemie GmbH, Munich, Germany) for 30 min at 37 °C. The control cells were incubated in the presence of 50 µmol/L verapamil (Sigma-Aldrich, Chemie GmbH, Munich, Germany). Propidium iodide (PI) 1 µg/mL was added to identify dead cells. Analysis and sorting were performed on FACS Aria flow cytometry (Becton Dickinson, San Jose, CA, USA), similar to that described by Patrawala *et al.*^[20] After sorting, SP sphere cells of UP-LN1 were placed at a density of 1,000 cells/mL under stem cell conditions by resuspension in tumor sphere medium consisting of serum-free HEScGRO medium, N2 supplement (Invitrogen, Carlsbad, CA, USA), 10 ng/mL human recombinant bFGF (Invitrogen, Carlsbad, CA, USA), and 10 ng/mL EGF (Invitrogen, Carlsbad, CA, USA), followed by culturing in ultra-low attachment plates (Corning, NY, USA) for about 1 week.

Assessment of the growth of UP-LN1 cells and subsets following WA treatment

Sulforhodamine B (SRB) dye (Sigma-Aldrich, Chemie GmbH, Munich, Germany) was used to test the effects of selective inhibitors on cell growth and viability of SP cells. The WA was dissolved in dimethyl sulfoxide (DMSO) before diluting with growth medium to a final DMSO concentration of 0.05%. The P, A, and F cells were seeded into 96 well plates in growth medium at 3,000 cells/well. After 24 h, the medium was replaced with fresh growth medium containing the WA. The cells were incubated for another 48 h. The cells were fixed with trichloroacetic acid (TCA) by gently adding 50 µL TCA (50%) to each well to a final TCA concentration of 10% with subsequent incubation for 1 h at 4 °C. The plates were then washed 5 times with tap water and air dried. The dried plates were stained with 100 µL of 0.4% (w/v) SRB prepared in 1% (v/v) acetic acid for 10 min at room temperature. The plates were rinsed quickly 4 times with 1% acetic acid to remove unbound dye and were then air dried until no moisture was visible. The bound dye was solubilized in 20 mmol/L Tris-base (100 µL/well) for 5 min on a shaker. Optical densities were read on a microplate reader (Molecular Devices, Sunnyvale, CA, USA) at 562 nm.

Apoptosis assay

Apoptosis was assessed by staining the cells with Annexin V-FITC (BD-Pharmingen, Franklin Lakes, NJ, USA), and PI and analyzing stained cells via flow cytometry. Briefly, the UP-LN1 P, A, and F cells (1×10^6 cells/mL) were grown in RPMI medium alone or in the same medium supplemented with either WA or DMSO. After 48 h, cells were washed twice with ice-cold PBS and then re-suspended in 100 µL binding buffer containing 2 µL of FITC-conjugated Annexin V and 2 µL of PI for 15 min. Following the incubation, without washing the cells with excess reagents, 400 µL binding buffer was added. Samples were then analyzed by flow cytometry. Data acquisition and analysis were done using CellQuest™ software (Becton Dickinson, San Jose, CA, USA).

Knockdown of CXCR4 by siRNA

UP-LN1 cells were transfected with Validated MISSION® siRNA (SASI_Hs01_00084886, Sigma-Aldrich Taiwan, Linkou, New Taipei City, China) according to the vendor's instructions. Transfected cells were lysed and subjected to both total RNA extraction and Western blot analysis 48 h post-transfection. CXCR4 expression was confirmed using Western blot and anti-CXCR4 antibody (SAB3500383, Sigma-Aldrich, China).

CDy1 immunofluorescence staining

UP-LN1 P, A, and F cells were cultured in a 60-mm culture dish for 24 h in the presence of 500 nmol/L

CDy1.^[22] CDy1 was a generous gift from Dr. YT Chang, Laboratory of Bioimaging Probe Development, Singapore Bioimaging Consortium Agency for Science, Technology, and Research, Singapore through Dr. Gi-Min Lai (Wan-Fan Hospital, Taipei, China). The cells were harvested by trypsin treatment, washed with PBS, and re-suspended in PBS. The cells were fixed with 4% paraformaldehyde for 10 min, permeabilized with 0.1% Triton X-100/PBS for 10 min, and blocked with 2% bovine serum albumin/PBS for 1 h. After incubation in dark at room temperature for 15 min, the cells were rinsed with PBS. The fluorescence images of the cells were acquired using fluorescence microscope (Nikon, Lewisville, TX, USA).

***In vitro* cell migration and invasion assays**

A Boyden chamber system was used to measure the invasive ability of UP-LN1 cells. Briefly, UP-LN1 P, A, and F cells were harvested, washed with PBS, and re-suspended in a serum-free RPMI medium (5×10^4 cells/200 μ L) in the presence or absence of WA. The cells were then seeded into the upper chambers of Matrigel-coated filter inserts. A serum-containing RPMI-1640 medium (500 μ L) was added to the lower chambers. After incubating for 24 h at 37 °C, filter inserts were removed from the wells, the cells that had invaded were stained with PI, and fluorescence images were taken. The number of invaded cells was determined using Analytical Imaging Station Software Package (Imaging Research, ON, Canada). The migration assay was performed accordingly but with 8- μ m pore polycarbonate filters, which were not coated with Matrigel.

Western blotting

UP-LN1 P, A, and F cells lysates were prepared using ReadyPrep Protein Extraction Kit (Bio-Rad, Hercules, CA, USA) according to the instructions provided. Total cell lysates (50 μ g) were separated electrophoretically by a 10% polyacrylamide SDS-PAGE gel and transferred to a polyvinylidene fluoride membrane using the BioRad Mini Protean transfer system. The blots were then blocked with 5% skim milk in PBST for 1 h and probed with primary antibodies overnight at 4 °C. All primary antibodies were purchased from cell signaling unless otherwise specified. The membranes were sequentially detected with an appropriate peroxidase-conjugated secondary antibody incubated at room temperature for 1 h. Blots were washed 3 times with PBS. Signals were then detected using the enhanced chemiluminescence detection system and the BioSpectrum Imaging System (UVP, Upland, CA, USA).

***In vivo* evaluation of WA-mediated anti-UP-LN1 F cell effects**

All animal studies were performed strictly under the animal experimentation protocols approved by Taipei Medical University. UP-LN1 F cells were first modified to express dual reporter system, FUW-Luc-mCherry-Puro (a

generous gift from Dr. Andrew Kung, Lurie family Imaging Center, Dana-Farber Cancer Institute, MA) according to an established protocol.^[14] Imaging-ready UP-LN1 F cells were harvested and subcutaneously injected into the left flank for NOD/SCID mice (3×10^5 cells/mouse; 5 mice/group). Tumor-bearing mice were then subdivided into control and WA-treated groups (10 mg/kg intraperitoneal injection [i.p.], 3 times a week). For intravenous (i.v.) tumor injection, 5.5×10^5 cells/mouse were injected, followed by WA i.p injection as described for subcutaneous (s.c.) tumor injected animals. For either the s.c. or i.v.-tumor injection group, WA treatment was initiated 2 weeks after tumor injection into the animals. Tumor burden was then non-invasively assessed based on bioluminescence intensity for 6 weeks using IVIS200 system (Caliper Life Sciences Inc., Hopkinton, MA, USA). Tumor autopsies were obtained at the end of the experimental period by humanely sacrificing the animals for pathological and immunohistological analyses. All experiments were conducted in accordance with the Guide for the Care and Use of Laboratory Animals of the National Health Research Institutes of Taiwan and following the Institutional Animal Care and Use Committee protocol authorized by Taipei Medical University.

Histology and immunohistochemical staining

Tumor tissues were fixed in 10% formalin and embedded in paraffin. Serial sections of the embedded specimens were deparaffinized and then rehydrated in a gradient fashion and stained with hematoxylin and eosin. For immunohistochemical staining, the deparaffinized slides were subjected to antigen retrieval and probed with anti-CXCR4 (1:100), anti-caspase-3 (1:200), anti-PARP (1:100) antibodies, or isotype IgG control. Slides were washed and incubated with biotinylated link universal antiserum, followed by horseradish peroxidase-streptavidin conjugate (LSAB 1 kit). The slides were rinsed, and the color was developed using 3,3-diaminobenzidine hydrochloride as a chromogen. Finally, sections were rinsed in distilled water, counterstained with Mayer's hematoxylin, and mounted with DPX mounting medium for evaluation. Pictures were captured with a Photometrics CoolSnap CF color camera (Nikon, Lewisville, TX, USA).

Statistical analysis

Each experiment was performed in triplicate. The results were expressed as means \pm standard deviation. The significant difference between control and experimental groups was analyzed using t-test (* $P < 0.05$; ** $P < 0.01$).

RESULTS

F subset of UP-LN1 cells are enriched with cancer stem cells

We utilized the SP method to compare and analyze the percentage of F cells in the UP-LN1 cell line. In the

absence and presence of verapamil, the percentage of SP cells in each group was calculated. Results of SP analysis from 1 of 3 independent experiments showed that parental (P) UP-LN1 cells contained an intermediate of 2.93% SP cells, A cells contained the least among the three groups at 1.07%, and F cells contained the highest of 4.20% [Figure 1a, upper frame]. The results show Hoechst 33342 dye exclusion was verapamil-sensitive; they suggest that F cells contained the highest proportion of CSCs in UP-LN1, and A cells contained the least. Quantitative results based on the 3 experiments reveal the statistical differences between F versus A and between F versus P cells in terms of the percentage of SP cells as follows: $F > A$ with $P < 0.01$, and $F > P$ with $P < 0.05$ [Figure 1a, lower frame]. To reinforce our SP data, an embryonic stem cell specific fluorescent dye CDy1^[22] was used to stain UP-LN1 cells. The red fluorescent signal was strongly associated with F cells as compared to A cells [arrowheads, Figure 1b]. Notably, red fluorescence was significantly stronger in F cell aggregates [arrowheads, Figure 1b]. To add support to F cells identity as potential CSCs, we examined the expression of stemness gene signatures such as Nanog, Oct4, Sox2, and c-Myc in the UP-LN1 cell line. F cells exhibited the highest expression level of these stemness

genes followed by P and A cells [Figure 1c], establishing F cells as the major subpopulation containing the CSCs and their CSC niche of the UP-LN1 cell line.

WA reduces SP and cell aggregates in UP-LN1 cells

We next sought to examine the potential CSC inhibitory effect of WA. Our cytofluorometric data demonstrated that WA reduced the percentage of SP cells in UP-LN1 in a dose-dependent manner [Figure 2a]. The ability of WA to affect UP-LN1 viability was then tested on F and A cells. The viability of SP in A cells was affected least among the three groups. WA preferentially targeted F cells in a dose-dependent fashion [Figure 2b]. Since F cells spontaneously formed grape-like cell aggregates, they are a close representation of the so-called tumor spheres or CSCs reported. WA treatment also prevented the formation of F-cell aggregates [Figure 2c]. At 10 $\mu\text{mol/L}$, WA reduced F-cell aggregates by approximately 80%.

WA preferentially induces apoptosis in F cells

Since WA has been shown to induce apoptosis in cervical

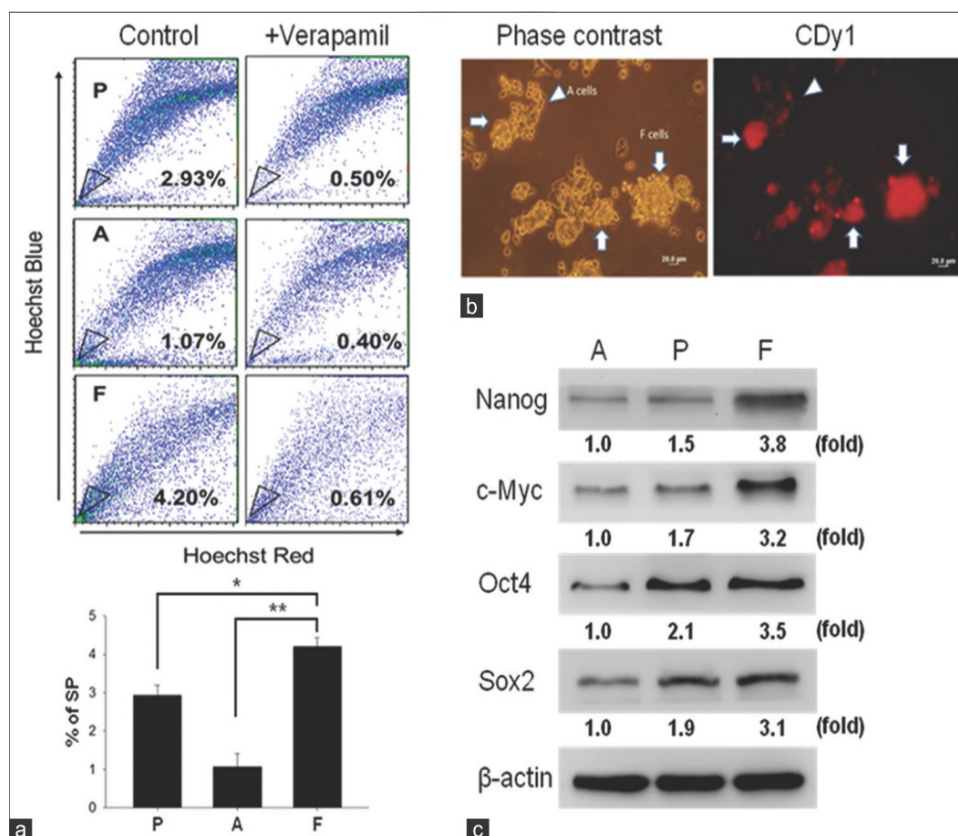


Figure 1: Characterization of cancer stem-like properties of UP-LN1 cells. (a) The side-population method was employed to compare and analyze the percentage of potential stem cell-like cells in UP-LN1 cells. With the absence and presence of verapamil, the percentage of side-population cells was calculated. In the upper frame, based on representative results of one experiment, parental (P) contained an intermediate of 2.93% side-population cells, adherent (A) cells contained the least among the three groups at 1.07%, and floating (F) cells contained the highest percentage at 4.20%. The quantitative results shown in the lower frame reveal that the percentage of side-population in F cells is significantly higher than that in A and P cells with $P < 0.01$ and $P < 0.05$, respectively; (b) to support our side-population data, an embryonic stem cell-specific fluorescent dye CDy1 was used to stain UP-LN1 cells. The red fluorescent signal was strongly associated with F cells as compared to A cells (arrowheads). Notably, red fluorescence was significantly stronger in F-cell aggregates (arrows); (c) when examined by Western blot analysis, F cells were found to express a significantly higher level of stemness genes (including Nanog, c-Myc, Oct4, and Sox2) than P and A cells. Note that the relative densities in the expression of each stemness gene among F, P, and A cells are also shown by fold difference in this figure

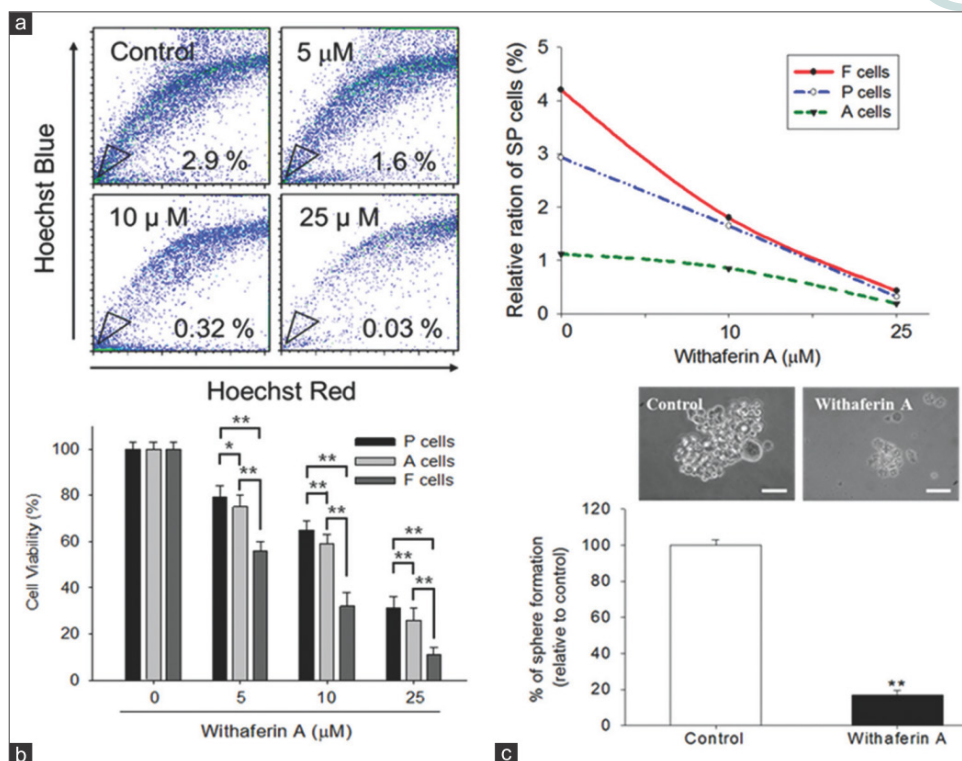


Figure 2: Withaferin A reduced side-population and cell aggregates in UP-LN1 cells. (a) Withaferin A reduced the percentage of side-population cells in UP-LN1 in a dose-dependent manner as indicated by our cytofluorometric analysis. The reduction of side-population cells in different populations of UP-LN1 cells was plotted against the concentration of withaferin A (right panel); (b) sulforhodamine B viability assay indicated that withaferin A targeted floating (F) cells most effectively and also in a dose-dependent manner as compared with adherent (A) or parental (P) cells. The difference between F and A cells is significant ($P < 0.01$), and so is the difference between F and P cells ($P < 0.01$) at each of the three withaferin A dose levels (5, 10, 25 μmol/L) tested; (c) microscopic analysis of F cells under the influence of withaferin A demonstrated a significant reduction in the formation of F-cell aggregates or spheres. At 10 μmol/L, withaferin A reduced F-cell aggregates by approximately 80%

cancer cells,^[16] we wished to determine if it exerted a similar function in UP-LN1 cells. Using Annexin V as an apoptotic indicator, we demonstrated that WA promoted apoptosis in P, F, and A cells in a dose-dependent manner [Figure 3a-c, respectively]. When analyzed quantitatively, WA appeared to preferentially target F cells and triggered apoptosis to a higher extent in F cells than in P and A cells [Figure 3b]. This observation corroborates the preference of WA in suppressing the formation of F-cell aggregates in the aforementioned section. In addition, Western blot analysis of cell lysates obtained from WA-treated F cells indicated an increased expression in some pro-apoptotic molecules including caspase-3, -8, -9 and PARP at higher concentrations [≥ 5 μmol/L, Figure 3c]. The remaining pro-apoptotic molecule, Fas receptor, and anti-apoptotic molecules such as Bcl-2 and survivin were clearly down-regulated in a dose-dependent manner.

WA treatment suppresses two major metastasis signaling pathways (STAT3 and CXCR4) in F cells

The presence of IFN-γ in tumor microenvironment or NK/LAK culture conditioned medium has been reported to promote the metastatic ability of cancer cells through the modulation of CXCR4 expression in cancer cells.^[9,23] We wished to determine if WA treatment could overcome metastatic potential induced by IFN-γ in the CSC-like

F-cell population. It was observed that the addition of IFN-γ increased the invasive ability in all 3 cell populations of the UP-LN1 cell line, with F cells at the highest efficiency.^[9] In the presence of WA, IFN-γ-induced invasion was significantly suppressed, particularly in the F-cell population [Figure 4a]. We subsequently examined the 2 major signaling axes involved in cellular trafficking, namely CXCR4 and STAT3.^[24,25] Using cytofluorometric analysis, we demonstrated, as shown previously,^[9] that the surface expression of CXCR4 was elevated in the presence of IFN-γ at concentrations as low as 10 U/mL, in terms of increased percentage of CXCR4-positive cells. Notably, each positive cell bore a relatively constant number of CXCR4 receptor sites, as revealed by a constant value of mean fluorescence intensity. The addition of a low concentration of WA (2.5 μmol/L) reduced the percentage of CXCR4-positive cells [Figure 4b], and with the addition of this agent at a higher concentration (5 μmol/L), CXCR4-positive cells could hardly be detected.

Next, we examined STAT3 and several key signaling pathways involved in cancer metastasis using Western blot analysis. WA treatment suppressed the expression of Akt/ ERK, CXCR4, STAT3, and GRK3/2 in F cells with or without the induction of mCSCs by IFN-γ treatment [Figure 4c]. A note of explanation is needed regarding the appearance of CXCR4 bands in the absence of IFN-γ added, which is

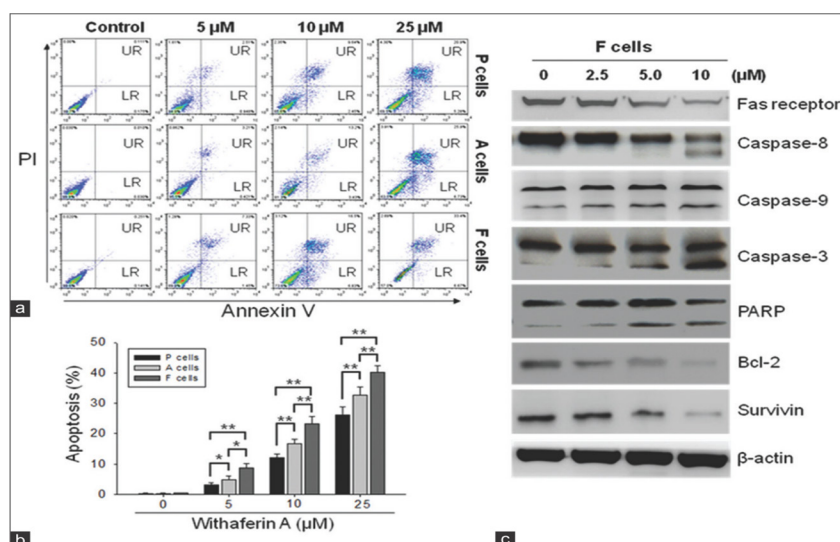


Figure 3: Withaferin A induced apoptosis in floating (F) cells. (a) Using cytofluorometric technique and Annexin V as an apoptotic indicator, we demonstrated that withaferin A promoted apoptosis in parental (P), F and adherent (A) cells in a dose-dependent manner; (b) quantitative representation of withaferin A-induced apoptosis. Withaferin A appeared to trigger apoptosis to a higher extent in F cells than in P cells ($P < 0.01$) or in A cells ($P < 0.01$); (c) immunoblots of total cell lysates obtained from withaferin A-treated F cells showed an increased expression of pro-apoptotic molecules such as caspase-3, -8, -9, and poly ADP-ribose polymerase at higher concentrations of withaferin A (5-10 $\mu\text{mol/L}$), except Fas receptor. On the other hand, anti-apoptotic molecules, survivin, and Bcl-2 were clearly down-regulated when the two higher concentrations of withaferin A were used

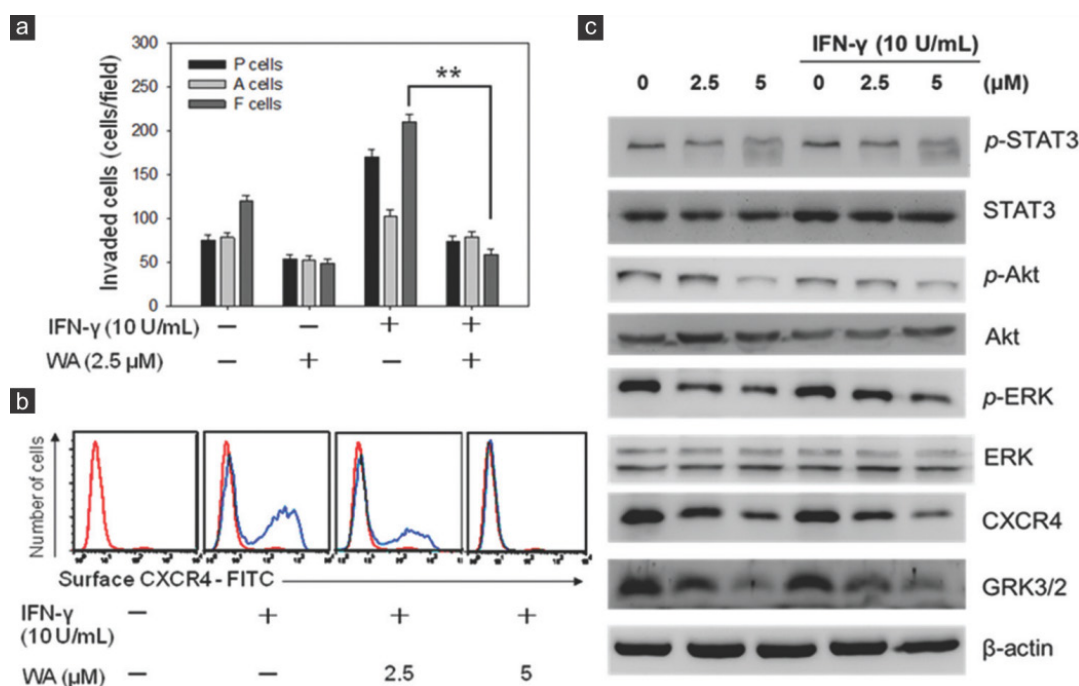


Figure 4: Withaferin A suppressed metastatic potential via modulating signaling pathways participating in invasive tumor activity. (a) Withaferin A treatment was able to suppress interferon- γ -induced invasive ability in both parental (P) and to floating (F) cells; (b) withaferin A treatment also suppressed the expression of CXCR4 expression even under the stimulation of interferon- γ . The difference between F cells treated with 2.5 $\mu\text{mol/L}$ withaferin A and F cells without withaferin A treatment is significant ($P < 0.01$) regardless of whether or not interferon- γ (10 U/mL) was used to stimulate F cells; (c) Western blot analysis of withaferin A-mediated suppression in invasive ability in F cells. Several major signaling pathways including Akt, ERK, CXCR4, GRK3/2 and STAT3, all of which are known to participate in cell mobility, appeared to be down-regulated by withaferin A treatment in a dose-dependent manner

contradictory to the results obtained cytofluorometrically. This was most likely due to the fact that in Western blotting, both surface and cytoplasmic CXCR4 molecules were detected, whereas in the cytofluorometric results, only the surface CXCR4 molecules were seen. Moreover, the inhibitory effect on the phosphorylation of STAT3 was readily noted at the highest concentration of WA (5 $\mu\text{mol/L}$) used. Collectively, we concluded that WA blocked the

formation of IFN- γ -mediated induction of mCSCs through the inhibition of both STAT3 and CXCR4 pathways.

Time course study of inhibition of IFN- γ -enhanced CXCR4 expression in F cells by WA

Vimentin is known to affect the mobility and invasiveness of cancer cells.^[26] Increasing evidence also indicates that the

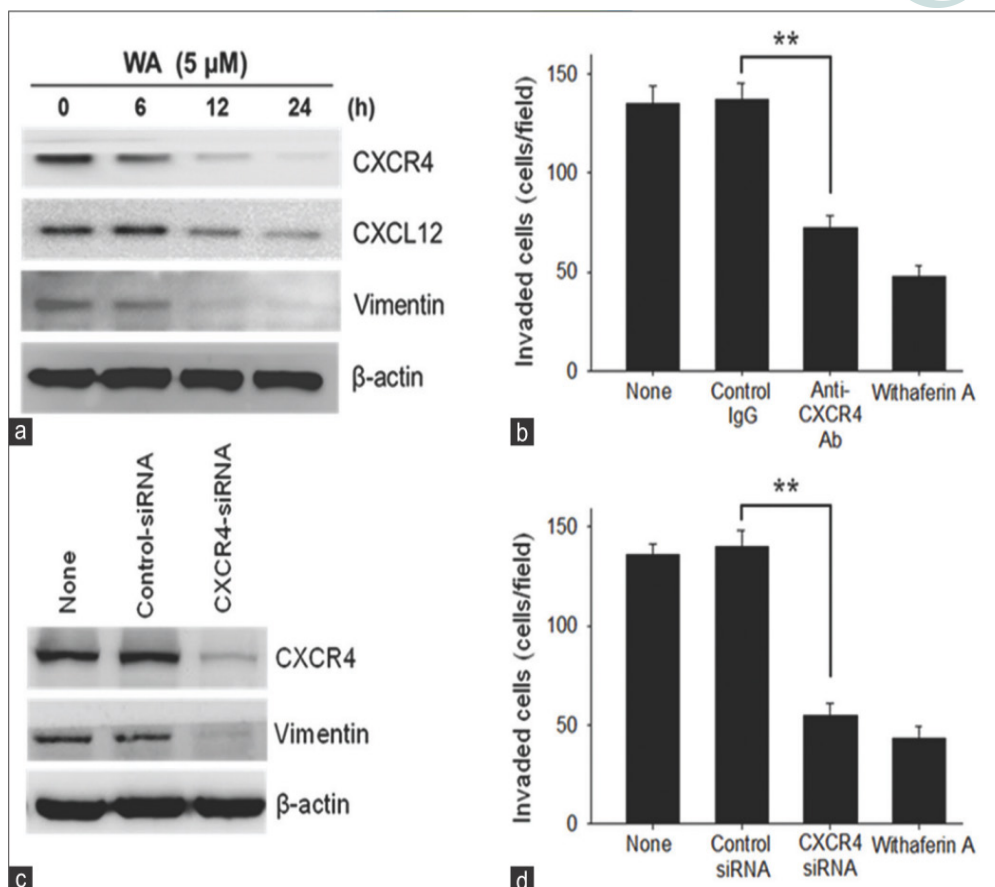


Figure 5: Inhibition of the CXCL12/CXCR4 axis expression by withaferin A in F cells which were pretreated with interferon- γ (10 U/mL for 24 h). (a) Time-dependent inhibition of CXCR4, CXCL12, and vimentin expression by withaferin A. Interferon- γ pretreated floating (F) cells were treated with 5 μ M/L withaferin A for various lengths of time as indicated, and the expression of each protein was measured by Western blot analysis; (b) suppression of cellular invasion by interferon- γ pretreated F cells by neutralizing anti-CXCR4 antibody. Interferon- γ pretreated F cells were incubated with 5 μ M/L control IgG, 5 μ M/L anti-CXCR4 antibody, or 5 μ M/L withaferin A for 24 h. Asterisks denote a statistically significant difference between anti-CXCR4 antibody treatment and control IgG treatment ($P < 0.01$); (c) knockdown of CXCR4 expression by CXCR4-siRNA. Interferon- γ pretreated F cells were transfected with control-siRNA or CXCR4-siRNA, and the expression of CXCR4 and vimentin were measured by Western blot analysis; (d) suppression of cellular invasion by interferon- γ pretreated F cells by treatment with CXCR4-siRNA. Interferon- γ pretreated F cells were transfected with control-siRNA or CXCR4-siRNA. Interferon- γ pretreated F cells were then harvested, and incubated in a chamber for 24 h. Asterisks denote a statistically significant ($P < 0.01$) difference between CXCR4-siRNA treatment and control-siRNA treatment

expression of vimentin is closely associated with CSCs and EMT positive circulating tumor cells.^[27,28] In addition, we have shown that IFN- γ induces surface CXCR4 expression on the F but not A subset of the UP-LN1 cell line, while the same treatment decreases cytoplasmic expression of CXCL12 in the F, but not the A, subset.^[9] No changes were found in the expression of CXCR4 and CXCL12 in A cells.^[9] These findings prompted us also to look into the possible correlation between vimentin, CXCL12, and CXCR4 expression by F cells pretreated with 10 U/mL IFN- γ for 48 h, followed by incubation with 5 μ M/L WA for indicated time periods *in vitro*. In Figure 4c, we showed that WA could exhibit a direct inhibitory effect on IFN- γ -mediated enhancement of surface CXCR4 expression in F cells in Western blot analysis. The expression of both CXCL12 and vimentin was inhibited in a similar manner as early as 12 h after WA treatment, although the extent of inhibition for CXCL12 was not as obvious as that for CXCR4 or vimentin [Figure 5a].

Effect of inhibition of CXCR4 expression on *in vitro* invasion of IFN- γ -treated F cells

IFN- γ -induced surface CXCR4 expression on F cells was blocked by anti-CXCR4 mAb, and cell invasion was examined by *in vitro* assay. IFN- γ -treated F cell invasion was clearly much reduced when compared to the control IgG group or the untreated group, each with $P < 0.01$ [Figure 5b]. Similarly, when the expression of CXCR4 was knocked down by CXCR4 siRNA treatment, the invasion of IFN- γ -treated F cells was again significantly reduced as compared with either the control siRNA group or the untreated group, each with $P < 0.01$ [Figure 5c and d]. Taken together, we herein clearly demonstrated that the extent of attenuation patterns of IFN- γ -induced CXCR4 expression in F cells following WA treatment was similar to that following blocking by anti-CXCR4 [Figure 5b] or that following knockdown of CXCR4 by CXCR4 siRNA [Figure 5c and d]. The observed attenuation of CXCR4 expression by F cells seemed to be accompanied by a decrease in vimentin and

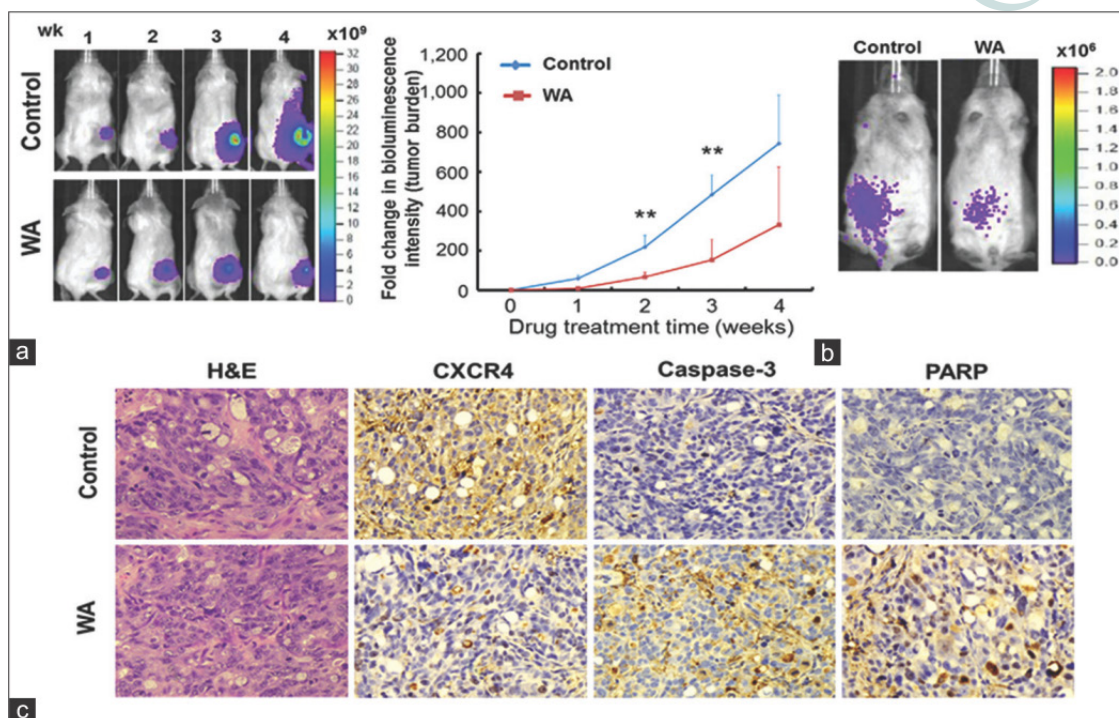


Figure 6: *In vivo* validation of withaferin A-mediated anti-cancer effects. (a) Representative bioluminescence images of control and withaferin A-treatment mice subcutaneously inoculated with floating (F) cells. Withaferin A treatment was initiated 2 weeks post-tumor implantation to allow tumor establishment. Note, inoculated F cells were found to start disseminating anteriorly during week 3-4 as compared to localized signal found in withaferin A-treated mice (left panels). The bioluminescent intensity was significantly lower in withaferin A-treated group than in the control group. The data were quantitatively represented by the fold change in bioluminescence intensity over time (right panel); (b) systematic injection of F cells mimicking metastatic model. Representative images of F cell-inoculated mice (via tail vein injection) demonstrated that withaferin A treatment given 2 weeks after intravenous tumor injection not only suppressed tumor growth but also controlled the spread of F cells; (c) immunohistochemical analysis of tumor samples harvested from both control and withaferin A-treated animals (from subcutaneous tumor samples). Representative sections of the tumor samples were stained with CXCR4, caspase-3, and poly ADP-ribose polymerase antibodies. Samples treated with withaferin A demonstrated decreased CXCR4 immunostaining, while increased caspase-3 and poly ADP-ribose polymerase immunostaining as compared with the control samples ($\times 200$)

CXCL12 expression. The similarity in the response profile between vimentin and CXCR4 expression is striking.

***In vivo* validation of WA-mediated suppression of tumor growth and metastasis**

Finally, we wished to validate the anti-cancer effects using tumor-bearing mouse model. F cells expressing dual luciferase reporter (enhanced green fluorescent protein and firefly luciferase, L2G) were subcutaneously injected into NOD/SCID mice for *in vivo* validation of WA-mediated anti-cancer effects. Tumor burden and spreading were monitored using the bioluminescent imaging technique. Tumor burden was significantly larger in the control group than in the WA-treated group [left panel, Figure 6a], reflected by the change in bioluminescent intensity. Importantly, anterior spreading of tumor cells was evident in the control animals starting from week 3, while WA-treated animals exhibited suppressed and restricted bioluminescent signal at the primary lesion site [left panel, Figure 6a]. Tumor burden was quantitatively measured as fold changes in bioluminescent intensity and plotted against time [right panel, Figure 6a]. In another model, when F cells were intravenously injected, they appeared to localize to the abdominal region of the animals, and WA treatment appeared to prevent the spreading of the F cells [Figure 6b]. Subcutaneous tumor biopsies were obtained for immunohistochemical analysis. Immunostaining

of CXCR4 was significantly less intense in the tumor sections from WA-treated mice than in control samples [Figure 6c]. In contrast, caspase-3, -8, -9 and PARP staining was markedly higher in the WA-treated xenografted tumor samples. These *in vivo* observations, while preliminary, corroborated our *in vitro* data (induction of apoptosis), suggesting that WA could indeed suppress metastatic propensity and induce apoptosis.

DISCUSSION

CSCs, which are a small subpopulation of tumor cells, are characterized by their tumor-initiating/self-renewal capacities and the ability to generate bulk populations of non-tumorigenic progenies through differentiation. CSCs have been identified in many human malignancies, and their abundance in clinical specimens has been correlated with disease progression.^[3] Importantly, clinical cancer progression driven by CSCs may contribute to the failure of both conventional and targeted therapies.^[4] CSC is targeted by a novel fluorescent dye, CDyl, which has specific affinity for pluripotent stem cells.^[22] Suspended F cells spontaneously formed tumor aggregates or spheres under normal culture conditions and gave rise to A cells with a greater differentiated phenotype, which have been shown to be more sensitive to the conventional therapeutic modalities, such as chemotherapy and/or radiotherapy. The dynamic phenotypic transition between F and A cells closely resembles CSC physiology, thereby representing an ideal *in*

vitro model. Therapeutic approaches represent translational strategies, which could improve the clinical outcome for patients with malignancies that are currently refractory to conventional treatments.^[4,5] However, developing such an agent or strategy has been hindered by the lack of an experimental CSC cell model, which could be maintained with relative ease and replicate most of the clinical CSC characteristics.

Previously, we identified the UP-LN1 cell line which was characterized by a co-existence of 2 unique cell populations.^[8,9] The CD44^{high}/CD24^{low} F cells attracted our attention particularly due to their phenotypic and cellular resemblance of CSCs in the form of tumor spheres. Using SP methods, we demonstrated that F cell population was enriched with CSCs with clearly elevated levels of stemness markers including Nanog, c-Myc, Oct4 and Sox2 noted, as compared with the CD44^{low}/CD24^{high} A-cell population [Figure 1a].

Having established this CSC cell model,^[9] we intended to examine WA as a potential anti-CSC and anti-mCSC agent. WA has been indicated for its anti-cancer effects by modulating multiple molecular pathways, predominantly through the induction of intracellular oxidative stress.^[12,13,17,29] WA dose-dependently reduced the percentage of SP cells in A, F, and parental UP-LN1 cells [Figure 2a]. Interestingly, F cells appeared to be more sensitive to WA treatment than A and parental cells [Figure 2b]. In addition, we demonstrated that WA promoted apoptosis in F cells in a dose-dependent manner as evidenced by both cytofluorometric [Figure 3a and b] and Western blot [Figure 3c] analyses via up-regulating caspases-3, -8 and -9, as well as PARP, which are collectively a family of proteins involved in a number of cellular processes, such as DNA repair and programmed cell death. It has been suggested that CSCs were more sensitive to ROS-induced apoptosis.^[26,27] F cells were found to contain a lower intracellular glutathione (GSH) level (data not shown), which could partially explain why WA eliminated F cells more efficiently. On the other hand, A cells are in general more sensitive to killing by chemotherapeutic drugs and NK/LAK cells.^[8,9]

In addition to promotion of apoptosis in F cells, WA appeared to be potent in suppressing the metastatic potential of F cells. As we observed in our previous study,^[9] we have also demonstrated here that the migratory/invasive ability of UP-LN1 cells could be stimulated by low levels of IFN- γ via an increase in only the percentage of F cells expressing CXCR4, while the number of CXCR4 density on a per-cell basis remained relatively constant [Figure 4b]. This study showed that WA suppressed IFN- γ -induced invasiveness in the UP-LN1 parental, A and F cells in a dose-dependent manner [Figure 4a], with the greatest effect on F cells, the intermediate effect on parental cells, and least effect on A cells. The WA-mediated effect was found to be through down-regulation of CXCR4 and vimentin expression [Figure 4b]. This finding suggests that the increase in the

number of CXCR4-positive F cells, also known as mCSCs, played a pivotal role in lymph node metastasis of some types of GI cancer, including UP-LN1.^[8,9,30] This observation was further supported by a recent study where the positive CXCR4 expression was shown to be significantly associated with lymph node metastases ($P = 0.028$) and higher stages III/IV ($P = 0.047$) in gastric cancer.^[27] In addition, several other major metastasis-associated pathways such as Akt/ERK, GRK3/2, and STAT3 could also be attenuated by the addition of WA [Figure 4c]. Interestingly, GRK3/2 expression, in particular, has been shown to form complexes with CXCR4 and/or FAK in human primary monocyte-macrophages and to play a part in their trafficking, upon the stimulus by inflammatory cytokines such as IL-4 and IL-13.^[30] Equally important, it has been demonstrated in small cell lung cancer where the activation of CXCR4/CXCL12 axis leads to the activation of the JAK/STAT3 pathway.^[31-34] STAT3 has been found and deposit and proliferate at the new site constitutively activated as a result of carcinogenesis in different cancer types, and aberrant STAT3 signaling has been implicated as an important process in malignant transformation^[35-37] and induction of angiogenesis.^[37] Activation of CXCR4 and STAT3 has been linked to tumor progression in different cell types, such as hematopoietic progenitor cells^[38] and small cell lung carcinoma lines^[39] with high mobility. Interestingly, STAT3 activation was shown to allow a crosstalk between tumor cells and dendritic cells which forms an immunosuppressive microenvironment favorable for tumor survival and perpetuation.^[40] Thus, the observation that WA treatment negatively regulated the CXCR4/CXCL12 chemotactic axis and molecules involved in IFN- γ -stimulated F cells, leading to making more CXCR4-positive mCSCs in cell numbers but not in increasing the number of CXCR4 sites on a per-cell basis, provides support for using WA as a potent anti-metastasis agent. The extent of the inhibitory effect of WA on invasiveness of IFN- γ -treated F cells was as great as that achieved by IFN- γ -mediated induction of CXCR4 in F cells attenuated by neutralizing anti-CXCR4 antibody [Figure 5b], or by knocking down the CXCR4 expression using siRNA [Figure 5c]. It should be pointed out that IFN- γ is not the only agent or means known to induce mCSCs,^[9] since other agents/methods such as HGF^[41] and hypoxia conditions^[42] have also been reported to be able to do the same. Interestingly, the fluctuations of vimentin and CXCR4 expression in the experiments stated above were very similar, suggesting the importance of these 2 molecules in the appearance of EMT phenotype during the process of cancer migration and invasion.^[26,27] Interestingly, a recent study by Bargagna-Mohan *et al.* indicated that WA acted as a tumor inhibitor, as well as the antiangiogenic agent through targeting the intermediate filament protein vimentin.^[18]

Based on our own current findings and those by others,^[13,15,17,19] the apoptotic process triggered by WA works through multiple mechanisms. They involved the mitochondrial pathway and associated Bcl-2 down-regulation, caspase-8,

-9 and -3 activations, DNA fragmentation, and the inhibition of both CXCR4/CXCL12 and STAT3/IL-6 pathways. To our knowledge, WA inhibition of IFN- γ -induced mCSCs via CXCR4/CXCL12 axis is reported herein for the first time. In addition, WA exerts the anti-angiogenic effect by targeting and binding vimentin, and WA cytotoxicity requires early ROS production and GSH depletion, and the inhibition of ROS increase resulting in complete suppression of a series of cellular events.^[18] Collectively, these results strongly support the therapeutic potential of WA against different types of human solid tumors including GI malignancies.

Finally, we evaluated the anti-human cancer effects of WA in a xenograft mouse model. Using bioluminescent imaging technique,^[14] we demonstrated that WA not only significantly suppressed the proliferation of F cells but also dissemination [Figure 6a]. A similar observation was made when F cells were injected intravenously [Figure 6b] that WA suppressed the dissemination of F cells around the abdominal region of the animals. Investigations on a larger panel of cancers or tumor cell lines similarly exhibiting the features of A and F subsets are warranted to confirm our current conclusion.

It is now considered that the CSC phenotype is more fluid than previously envisioned and is strongly modulated by the tumor microenvironment. This concept has been referred to as the dynamic CSC model.^[42,43] Our data imply that UP-LN1 cells represent a micro-niche where the dynamic transitions between adherent cells (A cells, with the more differentiated status) and floating cells (F cells, with the undifferentiated CSC phenotype) occur, and may closely replicate the pathophysiological characteristics of CSCs *in vivo*. Accordingly, we believe that this unique cell line is valuable for the further study of the biology of CSCs and mCSCs and drug screening. Using our unique UP-LN1 cell model, we have provided experimental evidence that WA is a potent CSC- and an mCSC-targeting agent which preferentially promotes apoptosis in F cells and suppresses tumorigenic (CSCs) and metastatic (mCSCs) activities. It achieves the latter through the metastasis-associated signaling pathways, notably of CXCR4/CXCL12 and STAT3/IL-6. Therefore, we propose that WA is considered for clinical trials in patients with a subset of GI-cancer like UP-LN1 exhibiting grape-like tumor spheres.

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Conflicts of interest

There are no conflicts of interest.

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Case Report

Hepatocellular carcinoma presenting as rapidly growing sternal mass: an unusual presentation

Rahul S. Kulkarni, Asha S. Anand, Apurva A. Patel, Sandip A. Shah

Department of Medical and Paediatric Oncology, Gujarat Cancer Research Institute, Ahmedabad 380016, Gujarat, India.

Correspondence to: Dr. Rahul S. Kulkarni, Department of Medical and Paediatric Oncology, Gujarat Cancer Research Institute, Ahmedabad 380001, Gujarat, India. E-mail: dr.rsk08@gmail.com

ABSTRACT

Hepatocellular carcinoma (HCC) is the most common malignant tumor of the liver. The most frequent sites of metastases are lungs, regional lymph nodes, adrenals and bones. However, an isolated sternal metastasis from HCC as an initial presentation has been rarely reported. A 45-year-old man presented with a progressively increasing mass over the anterior chest wall. On investigations, it was found to be arising from the sternum. Histopathology was suggestive of metastatic HCC, later confirmed by the presence of a 9 cm × 7 cm mass in the liver on abdominal computed tomography scan and a significantly elevated serum alpha fetoprotein level. Thus, metastasis from HCC should be included in the differential diagnosis of anterior chest wall mass and rapidly growing osseous metastases at unusual sites, even in the absence of signs of liver disease.

Key words: Bony metastasis, hepatocellular carcinoma, sternal mass

INTRODUCTION

Hepatocellular carcinoma (HCC) is the most common primary malignant tumor of the liver. It commonly occurs in the 6th and 7th decades of life in the western countries whereas in Asia it is more common in the 4th and 5th decades. Chronic viral hepatitis, particularly hepatitis B, has been the most common etiological factor.^[1] Hematogenous extra-hepatic metastases are commonly seen in lungs, lymph nodes, kidneys, adrenals, and bones. Though bone metastasis may occur in around 10% cases with HCC, the most frequent sites are vertebrae and pelvis, rarely sternum or ribs.^[2] However, isolated sternal metastasis as the initial presentation of HCC has been rarely reported. We hereby report a case of a 45-year-old man who presented with progressively increasing anterior chest wall swelling, which was diagnosed to be sternal metastasis from incidentally diagnosed HCC.

CASE REPORT

A 45-year-old male presented to our hospital with chief complaints of progressively increasing swelling over the anterior chest wall, associated with mild pain for

2-3 months. There was no history of fever, jaundice, abdominal pain, loss of appetite and weight. Personal and family history was not significant.

On examination, there was an 8 cm × 6 cm mass over the sternum, immobile and firm with no local rise of temperature. The overlying skin was tense, with dilated veins over the mass [Figure 1]. The remainder of the physical examination was unremarkable.

Routine blood investigations including hemogram and renal function tests were normal. However, liver function tests were altered, showing increased transaminases and alkaline phosphatase [Table 1]. Human immunodeficiency virus and hepatitis C virus were negative. However, the patient was found to be hepatitis B surface antigen-positive.

Fine needle aspiration cytology (FNAC) from the mass showed cellular smears highly suspicious of malignancy. Hence, computed tomography (CT) of thorax was done which revealed a 67 mm × 47 mm expansile, osteolytic lesion with destruction and markedly enhancing soft tissues involving the manubrium, suggestive of malignancy

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Table 1: Routine blood investigations

Investigation	Value
Hemoglobin (g/dL)	13
Total leukocyte count ($\times 10^9/L$)	9.2
Platelet count ($\times 10^9/L$)	260
Serum creatinine (mg/dL)	0.6
Blood urea level (mg/dL)	22
Serum bilirubin (mg/dL)	1.0
SGOT (mg/dL)	125
SGPT (mg/dL)	168
Alkaline phosphatase (U)	360
HIV	Negative
HBsAg	Positive
HCV	Negative
Serum AFP (ng/dL)	34,300

SGOT: serum glutamic-oxaloacetic transaminase; SGPT: serum glutamic pyruvic transaminase; HCV: hepatitis C virus; HIV: human immunodeficiency virus; AFP: alpha fetoprotein; HBsAg: hepatitis B surface antigen

infiltration. Both lung fields were clear, and there was no mediastinal adenopathy. Trucut biopsy showed moderate to large-sized polygonal cells with abundant eosinophilic cytoplasm and pleomorphic nuclei with a few cells showing characteristic inclusion bodies suggestive of metastatic carcinoma likely from an HCC [Figure 2].

Further, CT scan of the abdomen revealed a 9 cm \times 7 cm heterogeneously enhancing mass in the arterial phase in segment VIII and IV of the liver with early washout in the venous phase, suggestive of HCC [Figure 3]. Serum alpha fetoprotein (AFP) was greatly elevated at 34,300 ng/dL. In view of raised AFP, characteristic liver mass and biopsy of sternal mass, the diagnosis of HCC with sternal metastasis was confirmed. The patient was treated with local radiotherapy to sternal metastasis (20 Gray, divided into 10 fractions) and was started on entecavir, 0.5 mg daily for hepatitis B and sorafenib, 400 mg daily for HCC. One month after the start of treatment, there was a mild reduction in the size of the sternal mass. The patient is currently under follow-up.

DISCUSSION

HCC is the most common primary malignant tumor of the liver and is one of the most frequently occurring malignancies in Asia. The incidence exceeds 30 cases in 100,000 people per year in the East Asian region.^[3] The course of clinically apparent disease is generally very rapid, and, if untreated, most patients die within 3-6 months after diagnosis. HCC shows both intra-hepatic and extra-hepatic metastasis, with intra-hepatic metastases occurring more frequently. Extra-hepatic metastasis has been reported in 18% of cases.^[4] The mode of extra-hepatic spread is generally hematogenous, less commonly via lymphatics or direct spread. The most common sites of extra-hepatic involvement are lungs, lymph nodes, adrenals, and bones.^[4] Bony metastasis has been reported in 3-10% of cases.^[5] The most common bones involved are vertebrae, pelvis, ribs, long bones, skull and, very rarely, sternum.^[6] Further, bony metastases in HCC are



Figure 1: Prominent sternal mass on presentation

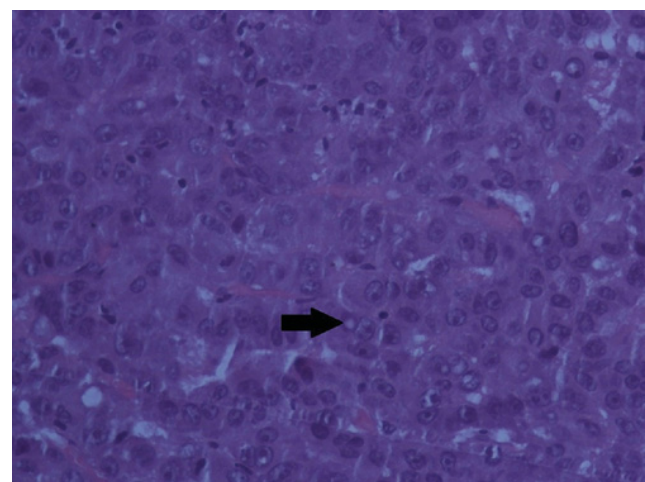


Figure 2: Hematoxylin and Eosin staining section ($\times 40$) showing moderate-to-large sized polygonal cells with abundant eosinophilic cytoplasm, pleomorphic nuclei, with few cells showing characteristic inclusion bodies (black arrow)

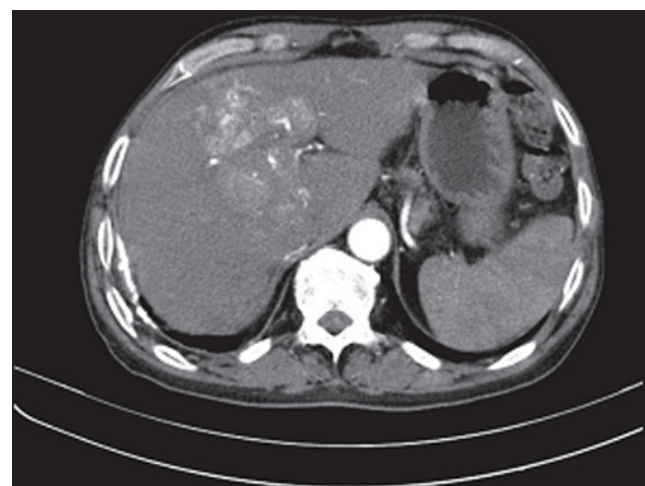


Figure 3: Computed tomography scan of abdomen showing heterogeneous enhancing mass in segment VIII and IV in liver in the arterial phase

generally multiple. An isolated bony metastasis as an initial presentation of HCC, as in our case, is rarely seen.^[7]

HCC bony metastases are characteristically osteolytic and hypervascular and thus may rupture spontaneously, causing hemorrhage. Chen *et al.*^[7] reported a case of a life-threatening hemorrhage from sternal metastasis from HCC. Similarly, Huang *et al.*^[8] have reported a case of intractable bleeding from an isolated mandibular metastasis, which was controlled by palliative radiotherapy.

Very rarely, bony metastases from an unknown primary HCC have been reported. The exact mechanism is not known, but various theories have been postulated such as metastasis from micro HCC, which is later destroyed by the immune system, spontaneous regression of HCC, or HCC developing in ectopic liver tissue.^[9] The etiology of HCC in our case was chronic Hepatitis B infection. In view of the raised AFP, a large liver mass and a characteristic osteolytic lesion in sternum with biopsy suggestive of HCC, the diagnosis was confirmed and an FNAC from the hepatic mass was not required.

Sorafenib is one of the first-line drugs used in the treatment of advanced metastatic HCC. Sorafenib is a tyrosine kinase inhibitor which inhibits cell growth in a dose- and time-dependent manner by altering the expression of genes involved in angiogenesis, apoptosis, and transcriptional regulation.^[10] Various other treatment modalities have been reported for bone metastasis such as chemoembolization as for a primary HCC, systemic chemotherapy, radiotherapy or surgical resection.^[6] Unfortunately, prognosis remains poor. Median survival for HCC with bone metastasis is reported to be 6.2 months.^[6]

To conclude, we here report an unusual presentation of HCC as an isolated sternal mass. A high index of suspicion is required to accurately diagnose the disease at this point. Thus, authors have recommended that metastatic HCC

should be included in the list of differential diagnosis of progressively growing bony lesions at unusual sites, even in the absence of signs of liver disease.

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Conflicts of interest

There are no conflicts of interest.

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Molecular and cellular aspects of extramedullary manifestations of acute myeloid leukemia

Javad Mohammadiasl¹, Abbas Khosravi², Mohammad Shahjahani², Shirin Azizidoost², Najmaldin Saki²

¹Department of Medical Genetics, Faculty of Medicine, Ahvaz Jundishapur University of Medical Sciences, Ahvaz 6135715794, Iran.

²Health Research Institute, Research Center of Thalassemia and Hemoglobinopathy, Ahvaz Jundishapur University of Medical Sciences, Ahvaz 6135715794, Iran.

Correspondence to: Dr. Najmaldin Saki, Health Research Institute, Research Center of Thalassemia and Hemoglobinopathy, Ahvaz Jundishapur University of Medical Sciences, Ahvaz 6135715794, Iran. E-mail: najmaldinsaki@gmail.com

ABSTRACT

The myeloid extramedullary tumor is a solid tumor formed by infiltration of immature myeloid cells in various tissues of the body. This tumor is also identified as chloroma or myeloid sarcoma (MS). MS is a manifestation of acute myeloid leukemia (AML) occurring at presentation or during treatment or relapse. MS is associated with multiple chromosomal abnormalities and molecular mutations since patients with these disorders bear a high potential for MS manifestation. There is a high incidence of extramedullary infiltration (EMI) in AML. AML patients with EMI have a worse prognosis than patients without it. Hematopoietic stem cells and leukemic stem cells reside in a special bone marrow microenvironment called niche, which is essential for their normal functions. Cancers are exploited dysfunctional cell-cell and matrix-cell interactions, which convert a normal niche into a neoplastic niche. This study summarizes the current knowledge on the molecular and cellular characteristics of AML with EMI and extramedullary niches in AML patients.

Key words: Acute myeloid leukemia; extramedullary infiltration; niche

INTRODUCTION

Acute myeloid leukemia (AML) is an aggressive myeloid neoplasm characterized by maturation arrest of myelopoiesis leading to an accumulation of myeloblasts in the blood and bone marrow (BM).^[1] AML is a complex and heterogeneous disease strongly associated with genetic and epigenetic changes in the hematopoietic progenitors.^[2] These changes lead to disruption of several signaling pathways that result in increased proliferation, survival and accumulation of leukemic cells.^[3]

Normal hematopoietic stem cells (HSCs) reside in a specialized area of the BM microenvironment known as niche, which regulates their survival and function. Two distinct niches exist in the BM: Vascular and endosteal/osteoblastic niche. The vascular niche is localized in close proximity to the osteoblastic niche, at the inner surface of bone cavity with abundant bone-forming osteoblasts. The vascular niche is composed of sinusoidal endothelial cells

lining blood vessels, and it promotes the proliferation and differentiation of short-term HSCs. The endosteal niche includes osteoblasts, osteoclasts, glial non-myelinating Schwann cells and regulatory T-cells, and it is located in the endosteum. The vascular niche contains CXCL12-abundant reticular cells, nestin-positive mesenchymal stem cells and leptin receptor-positive cells.^[4] HSC niches are present in different tissues during development, first in the aorta-gonad-mesonephros (AGM) region and yolk sac, then in the placenta, fetal liver, spleen and BM. After birth, the BM is the primary site of HSC maintenance and hematopoiesis, but the niche can shift to extramedullary sites in response to hematopoietic stress.^[5]

AML may present with extramedullary-AML at initial diagnosis or in relapse. Myeloid sarcoma (MS) is defined as an extramedullary mass composed of myeloid blasts occurring in anatomic sites other than BM.^[6] Extramedullary

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infiltration (EMI) is fairly common in AML patients. In addition, MS has been observed in all age groups, and may occur anywhere in the body. The most common tissues include soft tissues, bone, peritoneum, lymph nodes and gastrointestinal tract. Other occasional sites include male and female urogenital system and central nervous system (CNS).^[7] Moreover, several studies have found a worse prognosis in cases of acute leukemia with EMI, which may be explained by a poor response to chemotherapy and disease relapse.^[8] Evaluation of the cellular and molecular structures of extramedullary niches, as well as the migration and homing of leukemic cells, may help in designing diagnostic and therapeutic techniques and preventing relapse. However, there is still little information in this regard. The aim of this study was to investigate the characteristics of leukemic cells and the changes in their microenvironment that promote to EMI.

GENETIC AND MOLECULAR FEATURES OF EXTRAMEDULLARY INFILTRATION IN AML

Extramedullary leukemia (EML) is also called MS, granulocyte sarcoma and chloroma. In the WHO classification, MS is an important subgroup of myeloid neoplasia and acute leukemia. MS may occur simultaneously with, before or after the diagnosis of AML.^[9] Genetic mutations and molecular aberrations are an important tool for the evaluation of acute leukemia and assessment of prognosis. However, there is very limited information on the role of genetic mutations in MS.^[10] Although the overall incidence of MS in AML has been reported at 1.4-9%, it is particularly high in some subtypes of AML, reaching 18-24% in AML patients with t(8:21) and 25% in pediatric AML.^[11] Other genetic abnormalities diagnosed in EML patients include t(15:17), t(9:11), t(1:11), t(8:17), del(16q), del(5q), del(20q), monosomy 7, trisomy 4 and trisomy 8.^[12] Moreover, according to the French-American-British classification, some AML types are associated with EML, including M4 and M5 monocytic leukemias and the M2 subtype.^[13]

The t(8:21) has been reported as the most common cytogenetic abnormality associated with EML, occurring both at presentation and upon relapse, and is associated with orbital involvement in infants.^[10] Inv(16) is another abnormality associated with EML; it is rarer than t(8:21). According to studies, the bowel may be a target organ in men with inv(16) while breast and ovary tend to develop EML in female patients with inv(16).^[14] AML with trisomy 8 is found in nearly 5% of AML cases with a genetic abnormality. According to a study, trisomy or tetrasomy of chromosome 8 is observed in 35-65% of AML cases with leukemia cutis as a type of MS. Although confirmation of this relationship requires further evaluation, based on numerous reports, we can suggest that trisomy 8 is a risk factor for skin infiltration in AML.^[15]

Acute promyelocytic leukemia (APL) is another subtype of AML defined as having a translocation between chromosomes 15 and 17 and generation of promyelocytic leukemia/retinoic acid receptor alpha fusion protein.^[16] This fusion protein causes a block at the promyelocytic differentiation stage.^[17] APL can occur in extramedullary form, and EMI is responsible for 3-8% of cases in relapse. The most common target tissues are CNS and skin.^[18] Some studies indicate a relationship between 11q23 mixed-lineage leukemia rearrangement (MLLr) and EML. According to some studies, the involvement in this type of cytogenetic abnormalities has been limited to chest and uterus.^[19,20] Furthermore, another study suggests a link between MLLr and lymph node involvement.^[21] More studies are needed to confirm these observations.

Molecular abnormalities associated with EML have not been systematically defined; however, a well-documented molecular abnormality is a mutation in the nucleophosmin (NPM-1) gene.^[12] Nucleolar phosphoprotein or NPM-1 is localized in nuclear foci and is a multifunctional protein expressed in various cells.^[22] *NPM-1* gene mutation is the most common molecular genetic abnormality in AML, particularly AML with normal karyotype.^[23] *NPM-1* is mutated in almost 15% of cases of MS.^[24] In a survey conducted on 89 AML patients, 15 patients (18%) had extramedullary manifestation at diagnosis, and 13 of them (87%) had mutated *NPM-1*.^[25]

FMS-related tyrosine kinase 3-internal tandem duplication (FLT3-ITD) mutation is observed in 28-34% of cases of AML with normal cytogenetics. It plays an important role in cell proliferation, survival and differentiation of hematopoietic progenitor cells.^[26] Some studies have found an association between FLT3-ITD mutation and EML, so that in one study, 15% of MS patients have this mutation.^[27,28]

CD56, a neural cell-adhesion molecule, is expressed in normal, natural killer cells. Aberrant expression of CD56 in AML blasts, particularly AML with translocation t(8:21) correlates with a worse prognosis than CD56-negative cases.^[29] An association has been described between the expression of CD56 and EMI, especially in lymph nodes (lymphadenopathy).^[21,30,31] CD56 gene is in the 11q23.1 locus.^[32] Due to this fact and to the connection between 11q23 mutation and EMI, MLLr is likely associated with aberrant expression of CD56 in EMI. Some case reports and studies support this hypothesis.^[20,21,33]

Minimal residual disease (MRD) assessment is an important feature of therapy management, especially in cases whose recurrence risk is high. There is not much information on MRD in MS patients, and only one study has evaluated the correlation between continuous detection of AML1-MTG8 chimeric transcripts in BM and peripheral blood, and extramedullary relapse in t(8:21) AML.^[34]

Available information indicates that the prognosis of EML is poor with short overall survival.^[11] In an evaluation, the 5-year survival rate for patients with MS was 21%. Patients treated with chemotherapy showed longer survival than untreated patients.^[35] Although the mortality rate of acute leukemia patients has been reduced with the emergence of new therapies, many patients still suffer from refractory disease or relapse, and EMI is one of the main causes of poor prognosis in these patients.^[8]

EXTRAMEDULLARY NICHE IN AML

During development, HSCs are initially present in AGM and then migrate into the fetal liver and embryonic bone, which remains the only active site of hematopoiesis in adult life. Movement and homing of HSCs in the BM is associated with CXCL12 chemokine and its receptor CXCR4.^[36] Cancer subverts cell-cell and matrix-cell interactions and converts the normal niche to a neoplastic one.^[37]

ITD-FLT3 mutation, which is common in AML and MS patients, leads to deregulation of CXCR4 in AML leukemic cells since CXCR4 signaling is markedly decreased in patients with ITD-FLT3 compared with patients without it. It is thought that this mutation facilitates the infiltration of leukemic cells into visceral organs by reducing the homing of leukemic cells.^[38] Infiltration of leukemic cells in other organs is likely associated with chemokine receptor expression and different adhesion molecules. For example, NCAM1 or CD56, which is associated with a high incidence of MS, is highly expressed in the breast, testicular tissue, ovary and gut. This molecule is responsible for homing of leukemic cells in these tissues.^[11] Moreover, AML blasts isolated from skin show a group of specific chemokine receptors including CCR5, CXCR4, CXCR7, and CX3CR1 compared with AML blasts isolated from blood and BM. These cytokine-cytokine receptor interactions enable homing and survival of AML blasts in skin [Figure 1].^[39]

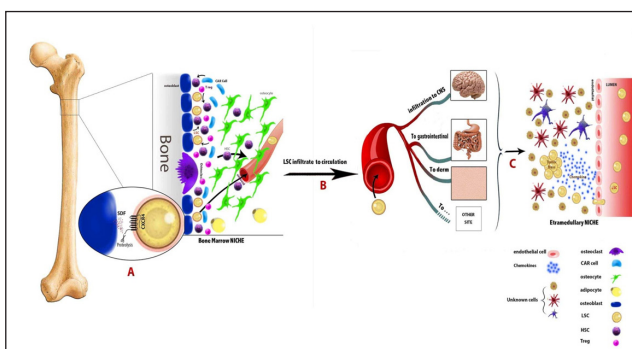


Figure 1: Extramedullary infiltration formation process in acute myeloid leukemia. (A) Within deregulation of LSCs located in BM, cell links with niche will be cut, and then they will enter into the circulation via BM sinusoids; (B) disseminated LSCs circulate in blood vessels and based on their special characteristics, enter specific tissue, like skin; (C) leukemic cells, along with new, distinct niche, initiate new tumor in metastatic tissue. The exact molecular and cellular characteristics have not been defined completely. SDF-1: stromal cell-derived factor 1; BM: bone marrow; LSC: leukemic stem cells; HSC: hematopoietic stem cell; CAR cell: CXCL12-abundant reticular cell

Expression of matrix metalloproteases may contribute to the increased incidence of EMI in some subtypes of AML. For example, in SHI-1 cells, a highly invasive human acute monocytic leukemia cell line, there is a high expression level of matrix metalloproteinase 2 (MMP-2), membrane-type 1 MMP and tissue inhibitor of metalloproteinase, which facilitate cell invasion.^[40] Moreover, it has been suggested that the specific binding of MMP-9 via its procatalytic domain to leukocyte surface I domains of beta-2 integrins is essential for precellular proteolysis and migration of AML-derived cells^[41] [Table 1].

MICRORNAS' SIGNIFICANCE IN EXTRAMEDULLARY AML

MicroRNAs (miRNAs) are small, 18-25 nucleotide non-coding RNA molecules, which regulate gene expression by hybridizing to their complementary messenger RNA. Each miRNA has the potential to regulate several different transcripts through partially complementary target sequences. miRNAs participate in cell differentiation, proliferation and carcinogenesis.^[56] Several studies have shown that miRNAs play key roles in normal hematopoiesis and various hematological malignancies. Different miRNAs are also known in myelopoiesis and myeloid neoplasias like AML.^[57] Functional studies have shown that miRNAs play an important role in the pathogenesis of AML as either oncogenes or tumor suppressors. It has also been shown that distinct miRNA expression signatures are associated with response to chemotherapy and clinical outcomes.^[58] Based on our literature and database searches, there has been no study describing miRNA signatures in MS. However, some studies show a link between miRNAs and mutation-induced MS. MiR-100 is aberrantly expressed in a number of cancer cells, including AML cells. Increased expression of miR-100 in AML is associated with maturation block. *In vitro* studies indicate that increased expression of miR-100 in AML cells inhibits retinoblastoma 1 serine phosphates from human chromosome 3 and causes the release of E2F in addition to increased levels of phosphorylated retinoblastoma. These events induced proliferation and inhibited the differentiation of granulocyte/monocyte cells.^[59] In a study performed on 106 pediatric AML patients, it showed that this miRNA was associated with AML with extramedullary manifestation.^[60]

High expression level of miR-10 family is associated with AML with mutated NPM-1.^[25,61] Furthermore, miR-424 in AML patients with NPM-1 mutation was down-modulated.^[62] In AML patients with the FLT3-ITD mutation, miR-451 and miR-144 were down-regulated while miR-155 was overexpressed.^[63] As previously mentioned, NPM-1 and FLT3-ITD mutations, as well as some other cytogenetic abnormalities, are associated with increased risk of EMI.^[25,27,28] In summary, deregulated miRNAs in these disorders can be considered as candidate markers for future studies in MS patients [Table 2].

Table 1: Evaluation of AML patients with extramedullary infiltration

Age (years), sex	Cytogenetics	Molecular test	CD markers	Extramedullary site	Subtype	Prognosis	References
51, female	46XX (50%)/45XX del (5)(q13q33), -7, add (15)(q22), -18	WT1+	MPO+, CD3-, CD20-, TdT-	Genital area	AML with multi-lineage dysplasia	Poor	[42]
16, female	PML-RAR at (15;17)		CD13+(85%), CD15+, CD33+, CD117+, CD34±, HLA-DR-	Right humerus, right proximal femur and distal tibia	APL		[43]
19, male		11q23 (MLL-AF10) rearrangement, low-level	HLA-DR+, CD4+, CD11c+, CD13+, CD15+, CD33+, CD117+, CD56+, CD45±	Pulmonary	M5		[44]
42, female	t(8;21)-RUNX1-RUNX1T1	FLT3 inhibition FLT3-ITD	MPO+, CD34+, Ki67 (60-70%)	Auditory canal	M2	Second morphologic CR	[45]
29, female	47, XX, +8, t(9;11)(p22; q23)	MLL-AF9 fusion gene	CD117+, CD33+, CD38+, CD15+, CD64+, CD4+, CD56+	Left and right breast	M4	CR	[20]
28, female	No overt cytogenetic aberration was shown	MLL-AF9 fusion gene	CD117+, CD13+, CD33+, CD34+, CD38+, MPO+, HLA-DR+	Left breast	M4	CR	[20]
12, female	t(9;11)(p22;q23)	MLL gene rearrangement	CD45+, CD33+, CD4+, alpha-1-antitrypsin+, muramidase+	Intra-abdominal and presacral	M5	CR	[46]
15, female			HLA-DR+, CD33+, CD15+, CD4+, CD11c+, CD11b+, CD9+, CD7+, CD56+, CD14+	Abdomen	M5a	Poor	[47]
3-month, female	normal female karyotype - t(9;11)(p22; q23)	MLL gene rearrangement	CD45+, CD33+, CD117+, CD4+, CD1a+	Skin	M5	CR	[47]
10, male	45, X,-Y, del(2)(p21), t(8;21)(q22;q22)		CD13+, CD34+, CD33+, MPO+, HLA-DR+	Appendix	M2	CR	[48]
38, female	t(6;21)		CD13+, CD33+, CD34+, CD15+, CD117+, CD64+, CD65+, MPO+, CD56+	Gastric	M4	Poor	[33]
57, male	47, XY, +8, t(9;11)(p22; q23)	11q23, tetrasomy 8	CD4+, CD13+, CD16+, CD33+, CD56+ and	Forearm and thigh	M5a	No relapse during chemotherapy	[33]
69, female			HLA-DR+	Eye	M1	Poor	[49]
47, male	t(8;17), t(17;17)		MPO+, CD43+, CD33+, CD34+, CD117+	Pancreas	M2		[50]
1, male	t(15;17)	PML-RARA	CD33+, CD65+, MPO+	Mandible	APL		[51]
13, female	47, XX, +21 and 46, del(x)(q22)		CD45+, CD117+, CD34+, CD43+, CD68+	Cardiac	M5	Poor	[52]

Contd...

Table 1: Contd...

Age (years), Cytogenetics sex	Molecular test	CD markers	Extramedullary site	Subtype	Prognosis	References
59, male	46, XY, dup (1) (q21; q32) in 2/20 cells and 46, XY 18/20 cell	FLT3-ITD mutation	CD34+, MPO+, CD25+	Epidural	M2	[53]
64, male	Trisomy 8		CD45+, CD68+ (KP-1), CD4+, CD56+	Skin	M5	[15]
24, male	46, XY and t (8;21) (q22; q22)		MPO+, CD56+	Stomach	M2	[54]
47, female	Deletion 17q21		CD43+, CD68+, CD56-	Eye	M4 Poor	[55]

MPO: myeloperoxidase; AML: acute myeloid leukemia; APL: acute promyelocytic leukemia; CR: complete remission; FLT3-ITD: FMS-related tyrosine kinase 3-internal tandem duplication; MLL: mixed-lineage leukemia; PML-RARA: promyelocytic leukemia/retinoic acid receptor alpha; HLA-DR: human leukocyte antigens-DR; TdT: terminal deoxynucleotidyl transferase

Table 2: MiR changes in AML with abnormalities associated with prevalence of myeloid sarcoma

Abnormality	Down-regulate	Up-regulate	References
11q23, MLL rearrangement	miR-34b, miR-15a, miR-29a, miR-29c, miR-372, miR-30a, miR-29b, miR-30e, miR-196a, miR-196b, let-7f, miR-102, miR-331, miR-299, miR-193	miR-326, miR-219, miR-194, miR-301, miR-324, miR-339, miR-99b, miR-328, miR-150, miR-17-92 cluster	[64-66]
FLT3-ITD	miR-451, miR-144	miR-155 (3.1-fold), miR-10a (2.5-fold) and miR-10b (2.27-fold)	[63,65]
NPM-1	miR-424	miR-10	[61,62]
APL	miR-181b	miR-15a, miR-15b, miR-16-1, let-7a-3, let-7c, let-7d, miR-223, miR-342 and miR-107, miR-125b	[67,68]
+8 AML	miR-496, miR-493	miR-34b, miR-370, miR-107, miR-342-3p, miR-96	[69]

AML: acute myeloid leukemia; APL: acute promyelocytic leukemia; NPM-1: nucleophosmin-1; MLL: mixed-lineage leukemia

CONCLUSION

EMI is a relatively common manifestation of AML, with increased incidence in specific subtypes.^[7] Despite advances in the diagnosis and treatment of myeloid leukemias, there is insufficient information on the diagnosis, treatment and pathogenesis of EML.^[38] Molecular and cellular studies of EML cases, as well as evaluation of the differences between AML patients with and without EMI, have revealed some features of EML. Elucidating the relationship between genetic abnormalities and sites prone to infiltration may contribute to the prevention and early detection of EML in target tissues. In many cases, MS is misdiagnosed at first, with the most common alternative diagnoses being lymphoma, melanoma, extramedullary hematopoiesis and inflammation. Given the aggressive nature of MS, early diagnosis with sensitive and specific tests is vital to these patients.^[9] Available information suggests that ITD-FLT3 mutations, which are prevalent in patients with EML, may play an important role in the pathogenesis of disease. Therefore, ITD-FLT3 mutation scan should be evaluated as a diagnostic and prognostic factor in patients. Moreover, *NPM-1* mutation, which also has a high prevalence in EML, should be evaluated as a prognostic test.

According to case report studies, common CD markers in EML include CD13, CD33, CD34, CD117, myeloperoxidase (MPO), CD56 and CD68; these should be considered in immunophenotype assessment of the disease [Table 1]. In a study conducted on MS patients, similar results were indicated, and CD68/KP1 was the most common positive

marker in 100% of patients. Other markers, in order of positive frequency, were: MPO (83.6%), CD117 (80.4%), CD99 (54.3%), CD68/ PG-M1 (51%), CD34 (43.4%), terminal deoxynucleotidyl transferase (31.5%), CD56 (13%), CD61 (2.2%), CD30 (2.2%) and CD4 (1.1%).^[70] These data can be useful to develop a diagnostic immunophenotyping panel for MS patients. Allogeneic hematopoietic stem cell transplantation (HSCT) is increasingly used as treatment procedure for AML patients, but there are no standard procedures for EML therapy. Furthermore, HSCT not only is not an effective procedure for EML, but it can also increase the risk of EML relapse in AML patients.^[71] Studies reviewed in this article suggest that cases of AML that have blasts with relatively specific characteristics have a high-risk for non-hematopoietic tissue infiltration. These features may be very helpful in distinguishing patients susceptible to EMI. Further studies are needed to develop diagnostic and therapeutic standards for patients with EMI as well as sensitive and specific prognostic biomarkers.

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Conflicts of interest

There are no conflicts of interest.

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What we have learned from urinary bladder cancer models

Carina Bernardo^{1,2}, Céu Costa^{1,4}, Carlos Palmeira^{1,3,4}, Rosário Pinto-Leite^{1,5}, Paula Oliveira⁶, Rui Freitas⁷, Francisco Amado², Lúcio L. Santos^{1,4,8}

¹Experimental Pathology and Therapeutics Group-Research Center, Portuguese Oncology Institute-Porto (IPO-Porto), Rua Dr. António Bernardino de Almeida, 4200-072 Porto, Portugal.

²Mass Spectrometry Group, QOPNA, Department of Chemistry, University of Aveiro, Campus Universitário de Santiago, 3810-193 Aveiro, Portugal.

³Department of Immunology, Portuguese Oncology Institute-Porto (IPO-Porto), Rua Dr. António Bernardino de Almeida, 4200-072 Porto, Portugal.

⁴School of Health Sciences, Fernando Pessoa University, 4249-004 Porto, Portugal.

⁵Genetic Service, Cytogenetic Laboratory, Hospital Center of Trás-os-Montes e Alto Douro, 5000-508 Vila Real, Portugal.

⁶Department of Veterinary Sciences, CITAB, University of Trás-os-Montes e Alto Douro, 5001-801 Vila Real, Portugal.

⁷Department of Urology, Portuguese Oncology Institute-Porto (IPO-Porto), Rua Dr. António Bernardino de Almeida, 4200-072 Porto, Portugal.

⁸Department of Surgical Oncology, Portuguese Oncology Institute-Porto (IPO-Porto), Rua Dr. António Bernardino de Almeida, 4200-072 Porto, Portugal.

Correspondence to: Dr. Lúcio L. Santos, Portuguese Oncology Institute-Porto (IPO-Porto), Rua Dr. António Bernardino de Almeida, 4200-072 Porto, Portugal. E-mail: llarasantos@gmail.com

ABSTRACT

Urinary bladder cancer (UBC) is a heterogeneous disease with highly variable clinical outcomes and responses to chemotherapy. Despite some advances in the molecular understanding of UBC, this knowledge still has not been translated to the clinic in terms of improvements in the prognosis and treatment of patients. Suitable urinary bladder tumor models representative of the human disease in terms of histology and behavior are needed to study factors involved in tumor initiation, progression and metastasis. Further, accurate model systems would facilitate identification of new therapeutic targets and predictive markers that could lead to optimization of existing therapies and development of new ones. Many established cancer cell lines derived from human urinary bladder tumors representing different grades and stages have been used as experimental models for UBC study. These cell lines reflect some of the genetic and morphologic alterations observed in human urothelial carcinoma and serve as simplified models to study the behavior of cancer cells *in vitro*. However, their translational potential is limited due to the artificial conditions, in which the cells are maintained, grown and tested. Animal models offer a more complex and realistic model for the establishment, development, and progression of tumors as well as to evaluate new therapeutic approaches. Over the years, the authors' group has worked with several UBC cell lines, established and characterized chemically induced UBC models, and patient-derived xenografts models. In this study, the authors will provide a summary of the UBC models developed by their group, analyze their translational potential and weaknesses, and define areas that remain to be explored.

Key words: Animal models; cell lines; tumor behavior; urothelial bladder cancer; xenografts

INTRODUCTION

Urinary bladder cancer (UBC) is a heterogeneous disease in terms of histopathology and clinical behavior. Around 70% of the patients are diagnosed with non-muscle-invasive bladder cancer (NMIBC) that often recur and, in about 10-30% of the cases progress to invasive disease despite local therapy. The remaining 30% are muscle-invasive bladder cancer (MIBC) at presentation and are associated with high risk of metastasis and progression even after radical surgery and systemic treatment.^[1] The necessity of lifelong

surveillance, repeated relapses, and chemoresistance makes UBC the malignancy with the highest lifetime treatment cost per patient.^[2] Patient treatment and surveillance are typically based on tumor histopathological features, such as histologic type, differentiation degree and anatomical extent of the disease. However, it is still challenging to predict the risk of recurrence and progression for individual patients and to identify which patients will significantly benefit from adjuvant and/or neoadjuvant chemotherapy.

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Recently, several candidate molecular biomarkers have been discovered and could be used to subdivide urinary bladder tumors in clinically relevant subsets, aiding the prognosis, and treatment selection for patients.^[3] Further analytical and clinical validation is required to integrate these markers into the clinical practice.

Several risk factors and genetic pathways have been implicated in the development of UBC. Low grade, non-invasive tumors are associated with fibroblast growth factor receptor 3 (FGFR3) and Harvey rat sarcoma viral oncogene homolog (HRAS) overexpression and/or mutations whereas carcinoma *in situ* (CIS) and high-grade/invasive UBC are associated with tumor protein p53 (TP53), retinoblastoma 1 (RB1), and phosphatase and tensin homolog (PTEN) mutations and/or loss.^[4] Chemical and environmental exposure including aromatic amides, aniline dyes, nitrites and nitrates, and tobacco smoking as well as frequent cystitis and *Schistosoma haematobium* infections are also associated with UBC etiology.^[5]

Malignant UBC consists of heterogeneous mass of interconnected cells containing tumor cells in different phases of the cell cycle, cancer stem cells subpopulations, supporting stromal cells and vasculature. Several tumor models have been used in basic science studies and clinical trials to increase our understanding of molecular mechanisms underlying tumor initiation, progression, metastasis and chemoresistance; yet none of these models can adequately mimic the clonal origin, histopathology, progression, and clinical behavior of human tumors. Therefore, a combinatorial approach based on multiple model systems studying specific aspects of this highly complex disease is required. Several models have been used in the study of UBC, ranging from cell lines (including cancer cell lines from human or non-human origin, immortalized and transformed cells, and grown in monolayer or three-dimensional systems) to animal models (including carcinogen-induced tumor models, xenografts, and genetically engineered mice). Here, we discuss the translational potential and applications of these models with particular emphasis on chemically induced UBC models, patient-derived xenografts (PDXs) models, and human bladder cancer cell lines.

CHEMICALLY INDUCED UBC

Chemically induced bladder cancer models induced by organo-specific bladder carcinogens were initially explored in the 1960s and were of the first models used to evaluate chemotherapy for UBC.^[6,7] In these models, carcinogenesis of the urothelium occurs after repeated exposure to carcinogens such as N-butyl-N-(4-hydroxybutyl) nitrosamine (BBN), N-[4-(5-nitro-2-furyl)-2-thiazolyl]-formamide (FANFT), and N-methyl-N-nitrosourea mimicking the environmental exposure known to be the leading cause of bladder cancer in

humans. The carcinogens can be delivered systemically, by gavage or in the drinking water, or locally, by injection or via instillation. The tumor subtype and the time needed for tumorigenesis depend on the carcinogen used, animal species and strains and the treatment regimen. Because these models use immunocompetent animals and are highly reproducible, they can be used to study the mechanisms involved in pathogenesis of bladder cancer and are suitable for studies on interactions between host immune system and the tumor. In addition, several rodent bladder cancer cell lines derived from carcinogen-induced bladder tumors were established and made available for *in vitro* and *in vivo* studies. These include rat bladder tumor cell lines such as Nara bladder tumor II and AY-27 derived from BBN-induced bladder tumors in Wistar rats, and Fischer 344 rats exposed to FANFT, respectively. The murine bladder tumor-2 murine bladder cancer cell line, derived from an FANFT-induced bladder tumor in C3H mice, has also been widely used.^[8-10] Rodents are exceptionally suited for these types of studies because rats and mice do not develop spontaneous urinary bladder tumors under normal conditions.^[11] The occurrence of non-neoplastic urothelial lesions, such as inflammation and hyperplasia, is also uncommon in these species.^[12]

In our studies, C3H/He mice were exposed to BBN, a complete genotoxic carcinogen metabolically derived from a compound found in tobacco smoke. Exposure to BBN in the drinking water induced the development of hyperplasia, dysplasia, low and high-grade papillary UBC, CIS, and MIBC in the urothelium of exposed rodents.^[13] The grade of cell atypia and extent of tumor invasion increased with increasing doses of carcinogen and extended period of exposure.^[12] Similar exposure to BBN resulted in papillary tumors in Fischer 344 rats.^[13] The broad spectrum of lesions mimics the major pathogenic mechanisms found in human urinary bladder carcinogenesis: pre-neoplastic lesions occur before *in situ* and muscle-invasive tumors, following different genetic pathways. These lesions are characterized by 3 different variables with recognized relationship to the natural history of UBC and patient outcome as DNA content, p53 alterations, and proliferative index measured by Ki-67 protein expression. DNA ploidy was evaluated by calculating the 5c exceeding rate, defined as the percentage of cells with values above 5 n. Alterations in these markers were detected even in non-malignant histological lesions but were more frequent in urothelial tumors with higher malignant and aggressive potential namely CIS, high-grade papillary and muscle-invasive tumors as shown in Figure 1.^[13] This abnormal biological profile represents a high level of genetic instability underlying the urothelium carcinogenesis process. This phenomenon is well known in human UBC, especially when CIS is present. The CIS surrounding normal-appearing urothelium shows a high frequency of abnormal DNA content, p53 mutated protein expression, and a high proliferative status.^[11,14]

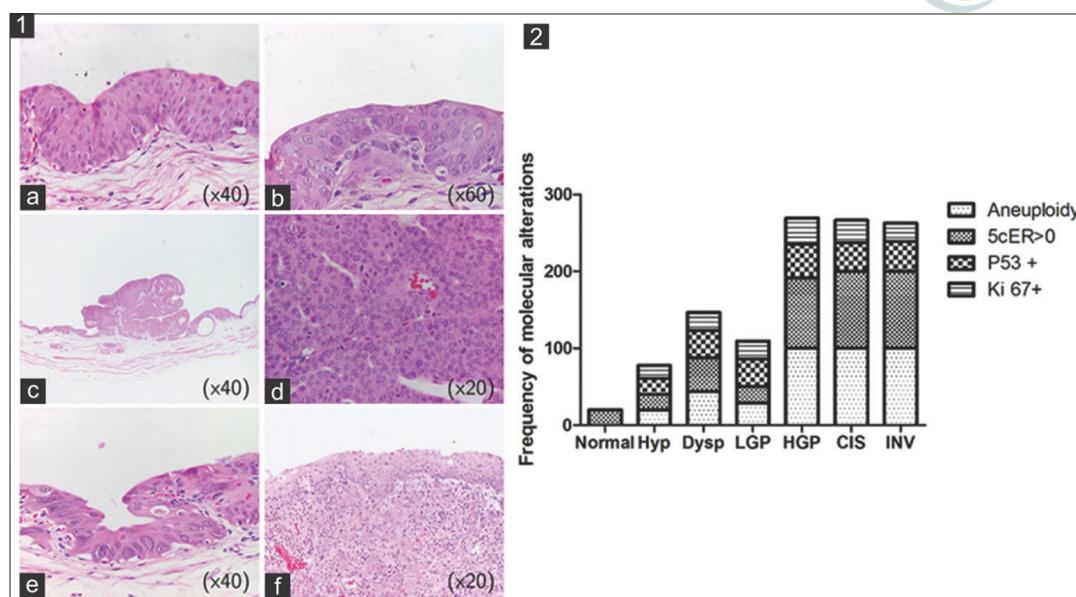


Figure 1: Urological lesions identified in rats exposed to BBN. (1) Hematoxylin and eosin stain; (2) respective molecular alterations. a: Hyp; b: Dysp; c: LGP urothelial carcinoma; d: HGP urothelial carcinoma; e: CIS; f: INV. BBN: N-butyl-N-(4-hydroxybutyl) nitrosamine; Hyp: hyperplasia; Dysp: dysplasia; LGP: low grade papillary; HGP: high grade papillary; CIS: carcinoma *in situ*; INV: invasive urothelial carcinoma; 5cER: 5c exceeding rate

Our studies revealed histopathological and biological similarities between the rodent urothelium carcinogenesis process and the corresponding process in humans.^[11] The more aggressive lesions identified in rats showed a higher rate of DNA aneuploidy, p53 immunoreexpression, and Ki-67 labeling index. This biological profile was also observed in early stage human tumors, suggesting that the rat model is more suitable to study the papillary pathway or NMIBC. These results are in agreement with William *et al.*, who purport that rodent (rat) tumors provide an accurate mechanistic model for the study of genes putatively involved in invasive and metastatic UBC.^[15] The main limitations of these models are the cost, long experimental protocol and the difficulty to monitor UBC development during the experimental protocol.

PATIENT-DERIVED UBC XENOGRAFT MODEL

PDX tumor models are primarily obtained by implanting human-derived tumor cells into immunocompromised mice. The tumors growing in these animals derive directly from patient tumor samples with minimal manipulation and recapitulate the biological characteristics of the human tumor of origin. Figure 2 presents a schematic representation of a study design to establish a direct tumor xenograft model from human samples. The expansion cohort enables the amplification of tumor tissue to establish a treatment cohort.

Tumors grown in these mice can also be stored by slow freezing in appropriate medium to replicate the model later. These models retain the cellular structure and molecular markers of the original tumors and have high predictive

power.^[16-18] PDX are suitable to evaluate the effectiveness of anticancer drugs, providing not only an investigational platform but a potential therapeutic decision-making tool based on the expression profile of individual tumors and their responsiveness to individual therapies.^[19]

As a proof of concept, our group established and characterized a patient-derived UBC xenograft model to evaluate tumors expressing translational modifications of cell surface proteins *in vivo*. A freshly collected sample of human MIBC was fragmented and subcutaneously engrafted into male nude mice and expanded until the third passage. Histology and immunohistochemistry of tumor markers [p53, p63, Ki-67, CK20 and sialyl-Tn (sTn)] were used to evaluate tumor phenotype maintenance.^[20] According to our results, the model recapitulated the histological and molecular nature of the primary tumor, including the expression of the cell-surface antigen sTn, a protein post-translational modification associated with motility and invasive capacity of UBC cells.^[21]

The main limitations of this model are the long latency period before tumor growth begins and low take rate, especially in the first passage. The stroma and blood supply are provided by the host, and the tumor is not growing in the organ of origin. The artificial tumor microenvironment may explain the rare occurrence of tumor metastasis observed in these subcutaneous models.^[22] The absence of host immune system is also an important factor to consider, as it influences tumor behavior.^[23] We were unsuccessful in establishing PDX in nude rats with none of the 7 implanted tumors grafting during a 12 months period. The explanation for this results remains to be elucidated.

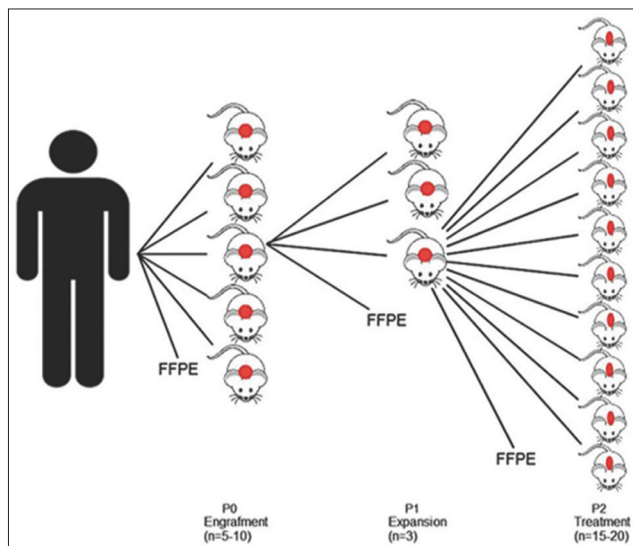


Figure 2: Schematic representation of the study design to establish a patient-derived tumor xenograft model. The original tumor and tumors grown in mice can be analyzed and compared using fresh or Formalin-fixed, paraffin-embedded tumor samples for histology and immunoeexpression of specific markers. FFPE: Formalin-fixed, paraffin-embedded

Subrenal capsule xenografting of primary bladder tumor in mice has been recently described with higher tumor take rates and retention of the genetic and morphological characteristics of the primary tumor.^[24] Both subcutaneous and subcapsular renal sites are not the orthotopic location for bladder cancer growth; however, the latter seems to be a more favorable environment for PDX survival and growth as is also observed for other cancer.^[25,26] The use of PDXs mice is feasible and allows a higher predictive power than other animal models.^[27,28] Because of this, PDX will be an increasingly valuable tool in the evaluation of human tumor response to traditional and new chemotherapeutic drugs.

UBC CELL LINES

Cell lines established from tumors and adapted to proliferate in culture have been extensively used in cancer research and their use has significantly improved our knowledge of cancer biology. Cancer cell lines have an important role in the study of the biological effects of genetic alterations in different tumor subtypes, in the identification and characterization of genes involved in cancer initiation and progression, and drug testing. *In vitro* assays using panels of cell lines are used as first line models in the preclinical development of new drugs to discover, validate, and evaluate the potential of new therapeutic agents.^[29] Gene expression can be manipulated in cell culture to introduce gain- or loss-of-function mutations to evaluate the biological effect on cell survival and proliferation, both *in vitro* and *in vivo* through the use of xenografts or syngeneic models. For more than a half-century, tumor cell lines served as a foundation for cancer research since they are easy to use and cost-effective. Within the bladder cancer field,

available cell lines represent different bladder cancer subtypes and varying degrees of genetic complexity depending on the sample of origin. However, continuous passages and culture of cells *in vitro* selects the cells better adapted to *in vitro* culture, reducing the heterogeneity, and promoting the acquisition of new mutations. Several human bladder cancer cell lines have been established and used over the years for multiple purposes. As a result of these studies, genomic and pharmacological profiles of 28 human bladder cancer cell lines are now available in the Cancer Cell Line Encyclopedia (<https://www.broadinstitute.org/software/cprg/?q=node/11>).^[30]

Our *in vitro* studies on UBC were based on three different human cancer cell lines: 5637, T24 and HT1376. These cell lines have been widely used to evaluate efficacy of anticancer drugs. Although significant heterogeneity and complexity were detected between them, their genomic profiles exhibited a similar pattern to human UBC.^[31] The NMIBC cell line 5637 and the two MIBC cell lines T24 and HT1376 cover the most frequent subtypes of UBC; T24 represents an FGFR3/Cyclin D1 subtype, while 5637 and HT1376 represent the E2F3/RB1 pathway mutational profile, with the former representing a less aggressive phenotype and the later bearing more invasive and metastatic properties.^[31] Figure 3 shows the karyogram of HT1376 cell line that presented a near-tetraploid karyotype with complex arrangements in several chromosomes.

We have used the cell lines 5637, T24, and HT1376 to evaluate the effect of everolimus and temsirolimus [sirolimus analogs and mammalian target of rapamycin (mTOR) inhibitors] in UBC cells. mTOR signaling was found to play an important role in cell growth, survival, proliferation, and angiogenesis in eukaryotic cells and its dysregulation was detected in many cancer, including in UBC, where it is believed to have potential for prognostic information and targeted therapy.^[32-34] Therapies targeting mTOR are already used in the clinics to treat renal cell carcinoma and mantle cell lymphoma.^[35] Their potential application to the treatment of other cancer is studied, particularly in combinational strategies, to overcome resistance and enhance efficacy of standard therapies. According to our studies, sirolimus analogs exert a slight interference on proliferation, apoptosis, and autophagy in these cancer cell lines. The NMIBC cell line 5637 was the most sensitive to mTOR inhibitor treatment alone.^[36,37] Considering other preclinical studies where sensitivity to mTOR inhibitors has been associated with PTEN loss,^[38] we expected that the UBC cell lines 5637 and HT1376 (both of which harbor deletion of PTEN), would respond well to sirolimus analogs. However, only 5637 was sensitive to monotherapy. More recently, in patients with MIBC treated with everolimus, PTEN loss was associated with resistance to treatment with the unhampered feedback loop driving PI3K/Akt activation suggesting

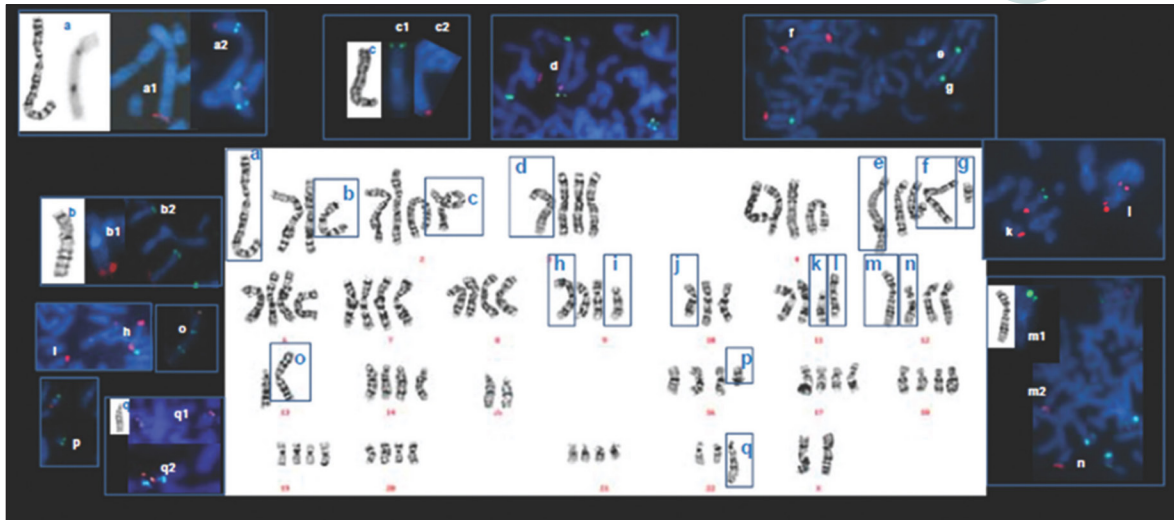


Figure 3: Karyogram of HT1376 with GTL banding. The chromosomes with changes are marked within square with letters that represent partial metaphases with GTL and FISH subtelomeric probes. GTL: giemsa trypsin leishman banding; FISH: fluorescent *in situ* hybridization

that the use of both PI3K and mTOR inhibitors would be beneficial in these cases.^[39] We further evaluated the combined effect of mTOR inhibitors with gemcitabine and cisplatin, which are currently used in the treatment of MIBC. The combined therapy resulted in enhanced inhibition of cell proliferation, increased apoptosis and autophagy, especially in 5637 and HT1376 cell lines, when compared with gemcitabine or cisplatin alone. In contrast, in the T24 cell line, the addition of everolimus or temsirolimus to cisplatin did not increase the efficacy of the latter one but, when combined to gemcitabine resulted in enhanced cell proliferation inhibition.^[40-43] This evidence supports the role of complex tumor signaling pathways in tumor behavior and response to chemotherapy and highlights the diverse and sometimes controversial results observed in preclinical studies.

To some extent, the cell lines used in our investigation reflect the tumor heterogeneity and its response to anticancer drugs. Knowing the limitations of this study material, the use of cell lines can be a very important starting point, indicating new research opportunities. However, evidence from *in vitro* studies must be further confirmed using more realistic and complex models such as xenografts.

In addition to *in vitro* assays, human cell lines have been widely used to establish xenograft models in mice. In this model, tumor cells are implanted either under the skin (heterotopic) or in the bladder (orthotopic). In orthotopic models, the tumor arises within the bladder of the recipient host allowing the study of tumor cells behavior in the normal host tissue microenvironment. Single cell suspensions of bladder cancer cell lines can be inoculated by intravesical instillation or direct injection into the bladder wall to establish xenografts or syngeneic models, if using human cell lines or mouse/rat cell lines in the corresponding background strain,

respectively.^[10,44] To achieve reliable tumor take after transurethral implantation of tumor cells, the host bladder is usually submitted to catheterization with chemical pre-treatment or mechanical traumatization. Although widely used, these methods are often associated with adverse reactions in some study animals and can lead to uncontrolled tumor growth in adjacent organs. On the other hand, the injection of tumor cells into the bladder wall frequently relies on laparotomy and mobilization of the bladder, also a morbidity-associated procedure. More recently, ultrasound guided percutaneous implantation of cells between the urothelium and lamina propria have been reported with the benefit of accurate cell delivery and a minimally invasive procedure.^[45] Bladder palpation and urine inspection are the initial approaches to identify growing tumors, followed by imaging techniques such as ultrasound, magnetic resonance imaging and bioluminescence.^[45,46] Inclusion of fluorescent or luciferase reporter genes in tumor cells prior to implantation enables *in vivo* imaging of tumors and metastases, and this method has been validated in mouse orthotopic models with promising results.^[47]

CONCLUSION

UBC is a complex disease with both genetic and environmental factors playing a role in tumor initiation, progression, and metastasis. In addition to the models used by our group, many more have been developed and are available to study the molecular biology, behavior, and chemosensitivity of UBC.

Most murine orthotopic UBC models can be obtained by three ways such as induced by a chemical carcinogen, implantation of human UBC cells in immunocompromised mice, or implantation of murine UBC cells in immunocompetent mice (allograft or syngeneic models). The model characteristics will depend on the site of

tumor cell implantation and the origin of implanted cells: traditional cell lines, primary cell culture, patient-derived tumor fragments, or tumor cells suspension. These factors influence tumor heterogeneity and the ability to model human tumor identity and behavior.^[48] Moreover, a greater understanding of UBC molecular biology has enabled the development of genetically engineered mouse models that recapitulate genetic abnormalities of human tumors and allow the study of individually altered genes in tumor behavior and response to therapy *in vivo*. These models are created by knock out or knock in of genes involved in transformation or malignancy such as HRAS, EGFR, TP53, PTEN, or RB as reviewed elsewhere.^[49] These models provide a useful platform to study genetic events associated with tumor development and progression without losing tumor microenvironment and the immune system of the host. However, they can fail to reproduce tumor heterogeneity and the genetic complexity of human tumors, which influence tumor progression and metastasis. More recently, animal models with functional immune systems are gaining attention as a platform to test emerging immunotherapies such as anti-programmed death ligand-1.^[50]

Despite the numerous existing UBC models, some mechanisms underlying the pathophysiology of these tumors remains unknown, such as in the case of *Schistosoma*-related UBC. This is mainly due to the lack of a tractable animal model. Hsieh *et al.* have developed a mouse model of *S. haematobium* urinary tract infection after microinjection of purified *S. haematobium* eggs into the urothelial bladder wall.^[51] This model recapitulates several aspects of human urothelial schistosomiasis however, the development of infection-associated UBC was not reported and remains to be explored.

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Conflicts of interest

There are no conflicts of interest.

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Prognostic factors and efficacy of GDP-R therapy in refractory/relapsed diffuse large B-cell lymphomas not eligible for high-dose therapy

Francesco Ghio¹, Giulia Cervetti¹, Nadia Cecconi¹, Matteo Pelosini¹, Sara Galimberti¹, Riccardo Morganti², Paola Ferrari¹, Andrea Nicolini¹, Mario Petrini¹

¹Department of Clinical and Experimental Medicine, University of Pisa, 67-56127 Pisa, Italy.

²Department of Oncology, University Hospital of Pisa, 67-56127 Pisa, Italy.

Correspondence to: Dr. Francesco Ghio, Department of Clinical and Experimental Medicine, University of Pisa, 67-56127 Pisa, Italy.

E-mail: francescoghio83@gmail.com

ABSTRACT

Aim: The main aim of the present study was to evaluate the overall survival (OS) and time to treatment failure (TTF) in a cohort of relapsed/refractory diffuse large B-cell lymphomas (DLBCLs) not eligible for high-dose therapy (HDT) treated with gemcitabine in association with dexamethasone, cisplatin and rituximab (GDP-R) protocol. The secondary aim was to identify the prognostic factors impacting OS and TTF. **Methods:** The authors retrospectively analyzed 45 patients with refractory/relapsed DLBCLs treated with GDP-R. **Results:** Overall response rate (ORR) was 48.8%; complete response 15/45 (33.3%), partial response 7/45 (15.5%). Response was influenced by the number of previous therapies administered and International Prognostic Index (IPI) value. Although no significant impact occurred with regard to OS, patients pre-treated with 2 or < 2 chemotherapeutic regimens had better ORR ($P = 0.014$) and a longer TTF ($P = 0.029$ in multivariate Cox model). IPI value also influenced TTF. Patients with < 2 IPI value had significantly more prolonged TTF than the other ones ($P = 0.048$ in multivariate Cox model). Treatment was well-tolerated, with the majority of patients treated on out-patient modality. GDP-R regimen represents a valid treatment for aggressive relapsed/refractory B-cell lymphoma not eligible for HDT thanks to its efficacy and good toxic profile. **Conclusion:** The number of previous chemotherapeutic regimens and IPI value select those who benefit more from this treatment.

Key words: Cisplatin; dexhametazone; GDP; gemcitabine; relapsed/refractory diffuse large B-cell lymphomas

INTRODUCTION

Diffuse large B-cell lymphomas (DLBCLs) are quite often curable with intensive combination chemotherapy. Despite the improvement of outcome with chemoimmunotherapy, 30-40% of patients relapse after the first-line treatment, and the rate of the second complete remission is lower than 30%.^[1-3] Management of these cases is not well-established. High-dose therapy (HDT) with hematological stem-cell support is the standard treatment for chemosensitive patients. Induction salvage therapies are usually based on platinum and etoposide: R-DHAP (rituximab, dexamethasone, cytosine arabinoside, and cisplatin) and R-ESHAP (rituximab, etoposide, methylprednisone, Ara-C, and cisplatin) are generally used,^[4] but they are often characterized by poor responsiveness and significant toxicity. Gemcitabine, an antimetabolite drug, has shown

significant activity in heavily pre-treated patients with NHL even after autologous stem cell transplantation (ASCT). Its favorable toxicity profile allows its use in combination regimens with other cytotoxic drugs and anti-CD20-targeted therapy with an overall response rate (ORR) of 50-60% in different phase II studies.^[5-9] In the present retrospective study, we described the experience of our institution about the use of gemcitabine in association with cisplatin, dexamethasone, and rituximab (GDP-R), in relapsed/refractory DLBCLs not eligible for (HDT) with hematological stem cell support. The principal aims of this study were to evaluate the overall survival (OS) and treatment failure (TTF) and the prognostic factors impacting OS and TTF.

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METHODS

Patients

From February 2006 to July 2014, 45 relapsed/refractory DLBCLs patients treated with GDP-R at our Institution entered into the study. Eligibility criteria were men or women aged > 18 years; documentation of unresponsiveness disease according to the Cheson criteria,^[10] after one or more chemotherapeutic regimens; absence of renal, hepatic, and respiratory failure; no evidence of active infections; HIV-negativity; at least one site of measurable disease; and written informed consent. In particular, of a total of 45 studied patients, 37 (82%) relapsed after achieving an initial complete response (CR), while the remaining 8 patients (18%) were primary non-responders (primary refractory disease). Patient evaluation included a full history and clinical examination, complete serum biochemistry with dosage of lactate dehydrogenase (LDH) and β 2-microglobulin, peripheral blood and bone marrow immunophenotyping, bone marrow biopsy, bone marrow molecular analysis, chest and abdomen and pelvic computed tomographic scan, serology for HIV, hepatitis-B virus and hepatitis-C virus. The age range of the cohort was 23-84 years. The number of previous therapies (NPTs) was also evaluated in this series. The first-line chemotherapy was R-CHOP ($n = 35$), R-DHAP ($n = 2$), and hyper-CVAD ($n = 8$). Most cases (27/45) received less than two previous chemotherapies; 20/45 cases had bone marrow involvement documented by biopsy (stage IV).

Treatment

GDP-R regimen consisted of gemcitabine (1,000 mg/m²) intravenous (IV) on the days 1 and 8; cisplatin (75 mg/m²) IV on the day 1; rituximab 375 mg/m² IV on the day 2; oral dexamethasone 40 mg on the days 1-4; this regimen was given every 3 weeks for a total of four courses. The standard anti-emetic regimen including ondansetron and dexamethasone was provided prior to chemotherapy. Chemotherapy was delayed on day 8 until recovery for a maximum of 3 weeks if the neutrophil count was < $0.5 \times 10^9/L$ and/or the platelet count was < $50 \times 10^9/L$ or if the patient showed grade 3/4 non-hematological toxicity (except for nausea, vomiting, and alopecia). The dose of cisplatin was reduced by 50% in the event of grade 2 neurological toxicity or grade 1 renal toxicity. In the event of febrile neutropenia, grade 4 thrombocytopenia or more than grade 3 non-hematological toxicity (except alopecia), treatment with 75% of the dose was given. Patient's disease was evaluated for response 1 month after the end of treatment, and then every 3 months during the first 2 years and every 6 months for further 3 years. International Workshop NHL response criteria were used to assess the response to treatment.^[10] The toxicity was estimated and graded according to the National Cancer Institute Common Toxicity Criteria version 3.0 grading system. Side-effects were described in the overall population and in each of 2 subsets that

were divided according to the International Prognostic Index (IPI) value and numbers of chemotherapeutic regimens as prognostic factors referred to OS and TTF.

Statistical analysis

Before performing survival analysis, an exploration phase was carried out. Categorical data were described by frequency and percentage, whereas continuous data by mean, median, and range.

Complete and partial response to chemotherapy

CR and partial response (CR and PR, respectively, according to the Cheson criteria) in patients with more than 2 or < 2 chemotherapeutic regimens were assessed by using the Fisher exact test.

Survival analysis

The survival was expressed as mean, median, and range. The endpoints studied included TTF (defined as the time from the beginning of treatment to further disease progression, relapse, or death) and OS (defined as the time from diagnosis to the last follow-up). Six variables (risk factors) were assessed in TTF and OS univariate and multivariate survival analysis: sex (male, female); age (≤ 65 , >65); LDH (≤ 300 , >300); stage (I-II, III-IV); IPI: (≤ 2 , >2); and NPT (≤ 2 , >2).

The results of the Cox regression were expressed using both the hazard ratios with its related confidence interval and related P value.

Survival curves were calculated using the Kaplan-Meier method and the log-rank test was used to evaluate the differences between curves. Univariate survival analysis was performed including each risk factor in a Cox regression model. All variables significantly influencing survival in the univariate analysis were analyzed together in a Cox regression model as multivariate analysis, with the aim of studying the independent contribution of each risk factor in explaining survivorship. Furthermore, the proportional hazard is always verified by using of log(-log) curves. The results of the Cox regression were expressed by hazard ratios with its related confidence interval and related P value calculated by Wald test. Regression coefficients (B) were also calculated. Statistics was applied to the overall population ($n = 45$) and to each of the two subsets that were obtained after all patients were divided according to whether the IPI value was ≤ 2 or > 2 and the number of chemotherapeutic regimens was ≤ 2 or > 2 (27 vs. 18 pts, respectively). The cut-off value for the number of previous chemotherapies and IPI was determined by a preliminary investigation considering the available data from the study.

Differences were considered significant at $P < 0.05$.

Analyses were performed using the SPSS 21 technology.

RESULTS

The principal clinical characteristics of patients are shown in Table 1. All studied patients had received 2 previous chemotherapeutic programs as median (range: 1-5). All cases were evaluable for response. ORR was 48.8%: CR 15/45 (33.3%); PR 7/45 (15.5%). At the time of this analysis, after a median follow-up of 22 months (range: 5-148), 4/22 responsive patients relapsed with a median duration of response of 10.5 months (range: 4-15). With a median follow-up of 57 months, the 2-year TTF and OS rates were 43% and 70%, respectively. No significant difference occurred with regard the OS in the 2 subsets divided according to the IPI value and numbers of chemotherapeutic regimens ($P = 0.823$ and $P = 0.389$, respectively) [Table 2]. Response was influenced by the NPTs. Of 45 patients, 27 were pre-treated with 2 or less than 2 chemotherapeutic regimens and 12 achieved CR, 5 PR, and 10 a stable/progressive disease (SD/PD), with an ORR of 17/27 (63%). The remaining 18 patients were pre-treated with more than 2 chemotherapeutic regimens. Three cases of them obtained a CR, one a PR, and the 14 remaining an SD/PD with an ORR of 4/18 (22%). Thus, patients pre-treated with 2 or < 2 chemotherapeutic regimens had better ORR ($P = 0.014$, Fisher exact test). TTF

median time was 22.2 months for patients pre-treated with 2 or < 2 chemotherapeutic regimens and 2.7 months for the other ones [Figure 1] [$P = 0.029$ in multivariate analysis; Table 3]. Even IPI value was able to influence TTF: patients with $IPI \leq 2$ had significantly more prolonged TTF than the other ones [$P = 0.048$ in multivariate analysis; Table 3].

Toxicity

No serious adverse event was observed. The treatment was generally well-tolerated, with the majority of patients treated on out-patient modality. Neutropenia grades 2, 3, and 4 were, respectively, reported in 8.9%, 4.4%, and 2.2% of cases; whereas thrombocytopenia grades 2 and 3 were reported in 4.4% and 8.8% of patients, respectively. No febrile neutropenia was observed. Grade 2 neurotoxicity occurred in 2.2%, but no grade 3/4 neurotoxicity was reported. In 6 patients, creatinine levels (which not overcame $176 \mu\text{mol/L}$) increased during treatment. Hospitalization was necessary in 1 case. As to toxicity not significant difference occurred in each subset of patients and it was not affected by the number of previous treatments. In fact, among 27 patients pre-treated with 2 or < 2 chemotherapeutic regimens, we recorded 8 cases of hematological toxicity (29%) and in the remaining 18 patients treated with more than 2 chemotherapeutic regimens, we recorded 5 hematological toxicity (28%) ($P = \text{ns}$).

Table 1: Principal clinical characteristics of patients

	Number	%
Sex		
Female	20	44
Male	25	56
Age		
≤ 65 years	36	80
> 65 years	9	20
LDH		
≤ 300 UI/L	18	40
> 300 UI/L	27	60
Stage		
I	2	4
II	12	26
III	11	25
IV	20	45
IPI		
0	2	4
1	9	20
2	16	36
3	15	34
4	1	2
5	0	0
Not available	2	4
NPT		
1	9	20
2	18	40
3	13	29
4	4	9
5	1	2

NPT: number of previous treatments; LDH: lactate dehydrogenase; IPI: international prognostic index

DISCUSSION

About 40-60% of elderly patients with DLBCL will be refractory or will experience relapse during their clinical course.^[11] These and other patients are not eligible for ASCT due to old age, or important comorbidities and management of this population is not yet standardized. Many current regimens, such as DHAP, ICE, ESHAP, show an ORR between 39% and 69%, but remarkable side-effects are frequent.^[3,4] Therefore, these regimens are not feasible for this subset of refractory/relapsed DLBCLs. Gemcitabine is a drug classified as a

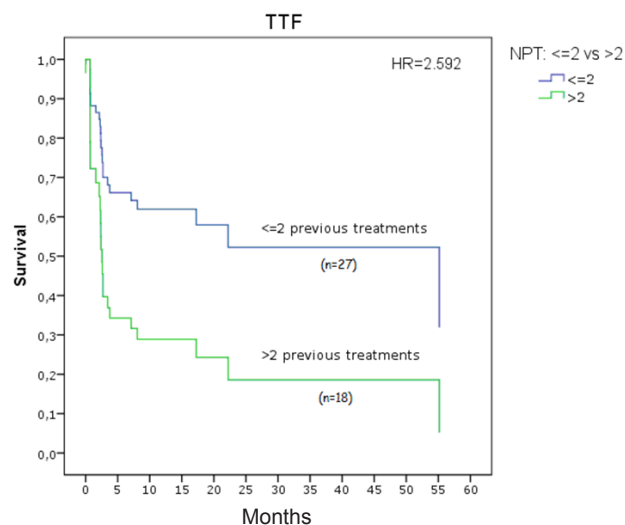


Figure 1: Time to treatment failure curves according to the number of previous chemotherapeutic regimens

Table 2: OS risk factors

	Univariate analysis		
	P	HR	IC 95%
Sex	0.555	1.31	0.53-3.24
Age	0.289	1.74	0.62-4.85
LDH	0.271	1.80	0.63-5.12
Stage	0.863	0.91	0.32-2.62
IPI	0.823	1.15	0.34-3.92
NPT	0.389	1.40	0.60-3.76

NPT: number of previous treatments; LDH: lactate dehydrogenase; IPI: international prognostic index; OS: overall survival

Table 3: TTF risk factors

	Univariate analysis			Multivariate analysis			
	P	HR	IC95%	B	P	HR	IC95%
Sex	0.551	1.28	0.57-2.87				
Age	0.536	1.37	0.51-3.66				
LDH	0.290	1.59	0.67-3.76				
Stage	0.243	1.80	0.67-4.85				
IPI	0.041	2.82	1.01-7.87	1.29	0.048	3.65	1.24-10.7
NPT	0.019	2.59	1.17-5.74	1.15	0.029	3.17	1.12-8.95

NPT: number of previous treatments; LDH: lactate dehydrogenase; IPI: international prognostic index; TTF: time to treatment failure

nucleoside analog. It is a competitive substrate with deoxycytidine for incorporation into DNA, and in this way, it inhibits DNA replication and repair. It is a derivative of cytidine and even if it is similar to cytosine arabinoside, it can be absorbed by cells faster, more effectively phosphorylated, and it remains in cells for a longer of time. Gemcitabine inhibits the DNA synthesis by preventing the activity of ribonucleotide reductase, and this conduces to a reduction of the concentration of intracellular nucleotide pool. In this way, gemcitabine has more antitumor activities and a lighter bone marrow inhibition than higher dosage of cytosine arabinoside.^[5,6] As far as we know, one previous study^[12] only has been conducted using the same GDP-R regimen of chemoimmunotherapy in patients with refractory/relapsed DLBCLs as in our report. In this study, in 50 successive patients, the 2-year OS and progression-free survival were 70% and 48%, respectively. Hence, both these end-points was the same or similar to those we have reported in our study. ORR was 72% and grade III-IV neutropenia and thrombocytopenia occurred in 34% and 40% of patients. However, the schedule adopted by the investigators in this study was different than in ours. In fact, cisplatin was given at 25 mg/m² IV on the days 1-3 instead of 75 mg/m² on the day 1 and rituximab was delivered on the day 1 instead of on the day 2. These slight differences could have affected both ORR and toxicity that were higher than in our study. Moreover, another previous study evaluated the efficacy of GDP regimen given with the same schedule as in our study but not including rituximab.^[13] In this study, the ORR was 58.3% for assessable patients, and the 1-year OS rate was 41.7%.

This last value is much lower than that we have reported at 2-year in our study (70%) and suggests that the addition of rituximab to GDP regimen significantly increases its efficacy. However, an occurrence rate for grade III/IV leukopenia of 37.5% and 25% for thrombocytopenia was found. These rates are higher than those we have observed in our study and the reason is not clear. In our study, 45 patients with aggressive refractory/relapsed DLBCLs not eligible for ASCT were treated with GDP-R achieving an ORR of 48.8% with a median duration of response of 13.59 months (range: 2.13-58.6 months). Moreover, GDP-R resulted safe: no febrile neutropenia was recorded; grade-4 neutropenia was registered in one patient, and two patients developed grade-2 neurotoxicity. These data confirm GDP-R therapy is a reasonable option for refractory/relapsed DLBCLs in patients who are not eligible for ASCT. In particular, patients pre-treated with 2 or < 2 lines of therapy had a better ORR than that of ones (63% vs. 22%) receiving more than 2 lines before GDP-R, with a median TTF of 22.2 months vs. 2.7 months ($P = 0.029$ in multivariate Cox model). Even IPI influenced TTF with a median of 17.3 months for patients with IPI value less or equal to 2 and 3.4 months for patients with IPI > 2 ($P = 0.048$ in multivariate Cox model). These data suggest that exposition to numerous different chemotherapeutic regimens selects chemoresistant neoplastic cells that are difficult to be eradicated. Moreover, they suggest that within the entire population of patients with refractory/relapsed DLBCLs not eligible for ASCT the number of previous chemotherapeutic regimens and IPI value select those who benefit more from GDP-R treatment. It is likely that the disease was intrinsically more aggressive in patients with higher IPI index and in those who required multiple chemo-treatments. In fact, tumor phenotype and its biological aggressiveness are different in any cancer. In the multivariate analyses, among the evaluated prognostic factors, the number of previous chemo-treatments and IPI index were significant variables for TTF. This finding suggests that the number of previous chemo-treatments and IPI are independent prognostic factors. Moreover, tumor phenotype can change during the progression of the disease due to genetic instability of cancer cells. This could account for the lack of a significant correlation between the number of previous chemo-treatments or IPI and OS. In fact, prognostic factors other than the number of previous chemo-treatments and IPI and inherent to tumor phenotype can prevail with the progression of the disease.

In conclusion, the shown results, even if based on a retrospective monocentric study and a small sample size, evidence that for patients with relapsed/refractory DLBCL, who cannot benefit from HDT and GDP-R is a reliable and well-tolerated therapeutic choice.

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Conflicts of interest

There are no conflicts of interest.

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Case Report

The therapeutic potential of duloxetine in prostate cancer-related fatigue

Rita De Sanctis¹, Alessandro Viganò^{2,3}

¹Department of Medical Oncology and Haematology, Humanitas Clinical and Research Center, IRCCS, 20089 Rozzano, Italy.

²Department of Neurology and Psychiatry, Sapienza University, 00185 Rome, Italy.

³Department of Anatomy, Histology, Forensic Medicine and Orthopaedics, Sapienza University, 00185 Rome, Italy.

Correspondence to: Dr. Rita De Sanctis, Department of Medical Oncology and Haematology, Humanitas Clinical and Research Center, IRCCS, 20089 Rozzano, Italy. E-mail: rita.de_sanctis@cancercenter.humanitas.it

ABSTRACT

Cancer-related fatigue (CRF) is a common polysymptomatic syndrome with no standard therapy. The authors present the case of a prostate cancer patient in whom, during hormone therapy, disabling CRF and urinary incontinence occurred. CRF was assessed according to the brief fatigue inventory (BFI). The patient received duloxetine, 60 mg daily, due to its impact on both CRF and incontinence. After 2 months, the BFI score decreased (from 9 to 2) and urinary incontinence resolved. After duloxetine discontinuation, the patient maintained a low BFI score. The authors conclude that, as a serotonin-noradrenaline reuptake inhibitor, duloxetine could be active on prostate CRF, especially with associated urinary symptoms. Therefore, a pilot placebo-controlled trial with duloxetine to treat prostate CRF may be worthwhile.

Key words: Duloxetine; fatigue; prostate cancer

INTRODUCTION

According to National Comprehensive Cancer Network guidelines, cancer-related fatigue (CRF) is a distressing, persistent, subjective sense of physical, emotional and cognitive tiredness due to cancer and/or its treatments, which is not proportional to real daily living activity.^[1] Diagnosis of CRF depends on the administration of multi-dimensional scales, albeit to date the superiority of one scale compared to the others is not yet well defined.^[2]

Generally, 60-90% of all cancer patients under specific treatment and 30-75% of cancer survivors present CRF.^[3] It has been reported that about 74% of prostate cancer patients experience fatigue.^[4] This association is due, in part, to the impact that androgen deprivation, the mainstay of pharmacological prostate cancer treatment, has on the pathophysiological mechanism of CRF.^[5]

Most prostate cancer patients receive hormone therapy (HT) during their lifetime since it is used for localized disease, as neoadjuvant/adjuvant to radiotherapy or surgery, or for biochemical relapse following radical local treatment. Furthermore, HT often constitutes the sole

treatment for localized disease in patients unsuitable for curative therapy or for metastatic disease. At early stages, 17% of patients undergoing HT complain about severe fatigue.^[6] In patients receiving both radiotherapy and HT, the prevalence of chronic fatigue is about 39%.^[7]

CRF is a complex, polysymptomatic syndrome caused by direct and/or indirect effects of neoplastic lesions, supportive care management, comorbidities and related medications, and environmental and psycho-emotional aspects. Although CRF patho-physiology is still not completely understood, each of these above mentioned factors can cooperate to lead to an abnormal production and use of adenosine triphosphate, an increase in pro-inflammatory cytokines, adhesion molecule and acute phase proteins. These metabolic changes are responsible for sleep-wake rhythm disorders and alterations of central nervous system mediators (corticotropin-releasing hormone increase, serotonin release and dopamine decrease).^[8]

In one study, only 9% of patients with CRF were treated, and the rate of success was quite low.^[9] At present, no satisfactory standard therapy for CRF is available.^[10]

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Corticosteroids can be useful but are not devoid of major side effects. Some evidence supports the use of methylphenidate over placebo, but its use is limited by side effects, and it should be administered only under expert supervision. One placebo-controlled trial on methylphenidate in prostate cancer showed a small benefit. However, 37.5% of treated subjects dropped out for severe drug-related adverse events. Secondly, the sample size was quite small, and statistical analysis was not corrected for multiple comparisons.^[11]

Also, erythropoietin may be effective but a specific dose for routine practice in CRF cannot be recommended. The aim of treatment should be to use the minimum required dose for the shortest duration.^[12] This is due to the theoretical increase in the risk of thromboembolic side effects with higher doses and protracted treatment with erythropoietin and possible cancer stimulation.^[10]

In some trials, antidepressants and psychostimulant drugs showed a small little clinical benefit.^[13] Here, we report a case of CRF treated with duloxetine. Duloxetine is a serotonin-noradrenergic reuptake inhibitor, commonly used as an antidepressant. It has been recently approved for neuropathic pain, chronic fatigue syndrome (at the dose range of 30-60 mg daily), fibromyalgia and it is also recommended for urinary incontinence in Europe (40-80 mg daily).^[14-16]

CASE REPORT

A 74-year-old man presented to our clinic complaining of more than 6 months of fatigue, which was exacerbated by activity and not relieved by rest. The patient had a history of advanced prostate cancer 3 years before, he underwent a radical prostatectomy (Gleason score 4 + 4, pT3bN0; prostate-specific antigen (PSA) 21.1 ng/mL) followed by adjuvant radiotherapy. Following treatment, only mild urinary incontinence was noted. Two years later, he had a biochemical relapse, with a PSA doubling time < 6 months for which he started on daily bicalutamide, 50 mg and monthly luteinizing hormone-releasing hormone agonist. After starting medical therapy, a progressive and significant PSA normalization was observed. However, 6 months later, he complained for recurrent moments of sadness, loss of interest in daily activities, insomnia, concentration problems and worsening urinary incontinence (from mild to moderate).

On physical examination, he was not pale or jaundiced, afebrile with normal heart rate and blood pressure. Heart, lung, abdominal and musculoskeletal examinations were normal. No hepatomegaly, gynecomastia or neurological signs were present. Eastern Cooperative Oncology Group performance status was 0-1. Laboratory tests, including a complete blood count and electrolytes, showed mild anemia (hemoglobin 11.7 g/dL) and elevated transaminase levels (alanine transaminase 83 U/L, aspartate transaminase

51 U/L) while thyroid, hepatic and renal function were normal. Serological tests for hepatitis B virus, hepatitis C virus, human immunodeficiency virus, Epstein-Barr virus and cytomegalovirus were negative. Chest radiography, abdominal ultrasound, electrocardiography and echocardiography were all normal.

Diagnosis of CRF was established as a diagnosis of exclusion. CRF was assessed according to the Brief Fatigue Inventory (BFI),^[17] and a score of 9 (BFI range, 0-10) was found.

Considering both the urinary incontinence and depression symptoms, we prescribed duloxetine at a starting daily dose of 30 mg and then, as per drug schedule, after 2 weeks, the dose was increased to 60 mg. Duloxetine was chosen due to its efficacy against urinary incontinence at a similar dose (dose range, 40-80 mg daily).

After 2 months of treatment at the dose of 60 mg, the BFI score was decreased to 2, urinary incontinence completely resolved, and the patient returned to regular activities. There were no side effects. Duloxetine was continued at the same dose for a further 2 months and then was withdrawn because of alcoholism relapse, a disorder that the patient and his relatives had omitted in medical history. After duloxetine discontinuation, the patient reported a moderate CRF worsening over 6 months. However, 4 months later, he maintained a 5 points-lower BFI score than the initial one. At the same time, he was referred to our alcohol abuse center with a progressive reduction of heavy-drinking days in a 6-month timeline follow-back.

DISCUSSION

CRF is a major problem in prostate cancer management, according to several studies, the prevalence of CRF regardless of intensity is about 74%.^[4] There is no “gold standard” treatment currently recommended, and the commonly given therapies are poorly successful. Only 2 drugs have shown some activity against CRF: methylphenidate and erythropoietin, although their regular use do have caveats.^[10]

Our patient, who was not receiving chemotherapy, showed mild anemia, thus not presenting an indication for erythropoietin. Some data show that CRF can disappear spontaneously 6-8 weeks after the end of HT, but our patient was receiving HT, and was to be continued until progression or unacceptable toxicity, so that a medical withdrawal was not indicated. Although the timing and modality of treatment of PSA-only recurrence after radical prostatectomy and radiotherapy remains controversial, our patient had high-risk disease deserving first-line treatment.^[18]

In CRF pathophysiology, a relevant impairment of neurotransmitter (serotonergic, noradrenergic and dopaminergic) systems is present. The use of different

psychoactive drugs increasing neurotransmitter concentrations shows an effect in CRF treatment. The poor outcome of serotonergic drugs, as paroxetine and sertraline, on fatigue in randomized controlled trials could be due to their selective action on the serotonin reuptake.^[13,16,19] Nevertheless, it is worth mentioning that a recent study stressed the role of tryptophan (a serotonin precursor) depletion in developing CRF and found a correlation between the higher degree of fatigue and lower tryptophan concentrations. Thus, a drug that acts on more than one mechanism could be appropriate.^[13] Modafinil, a non-amphetamine psychostimulant, reduces CRF intensity.^[12] Although the precise mechanism of modafinil action has not been elucidated, it seems to rely on the interaction of adrenergic and dopaminergic transmission in prefrontal cortices.^[20] Also, methylphenidate could facilitate excitatory synaptic signaling, mainly through strengthening catecholaminergic synaptic transmission. As a blocker of dopamine and norepinephrine transporters, it increases both extracellular dopamine and norepinephrine.^[21,22]

Considering these data, the use of a drug like duloxetine, that has a pleiotropic action on these pathways, has some rationale in the treatment of CRF. Moreover, in our case, further benefit was derived from its activity on urinary incontinence, thus promoting the patient's sense of well-being and, therefore, ameliorating his quality of life and increasing his compliance with physical therapy (mild exercise, daily walking), which has a therapeutic role in CRF and in social life.

In conclusion, a pilot placebo-controlled trial using duloxetine seems worthwhile since it acts on fatigue, urinary symptoms and depression, all often occurring in a complex and multidimensional disease as prostate cancer CRF.

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Conflicts of interest

There are no conflicts of interest.

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Case Report

Unusual skeletal muscle metastasis from carcinoma cervix

Mehlam Kausar¹, Desh Deepak Ladia¹, Abhinav Mutneja¹, Virendra Bhandari²

¹Department of Radiation Oncology, Sri Aurobindo Institute of Medical Science, Indore 452008, Madhya Pradesh, India.

²Roentgen-SAIMS Radiation Oncology Centre, Sri Aurobindo Institute of Medical Sciences, Indore 452008, Madhya Pradesh, India.

Correspondence to: Dr. Virendra Bhandari, Roentgen-SAIMS Radiation Oncology Centre, Sri Aurobindo Institute of Medical Sciences, Indore 452008, Madhya Pradesh, India. E-mail: virencancer@yahoo.co.in



Dr. Virendra Bhandari, MD in Radiation Oncology, has worked as Senior Registrar at TMH, Mumbai and then as Consultant Oncologist at Allahabad and Aurangabad, and treated solid and haematological malignancies. He has been trained in Brachytherapy, Hyperthermia, IGRT, SRT, and SBRT at Christie Hospital Manchester(UK), Utrecht Hospital and DD Hoed Hospital, The Netherlands, Henry Ford Hospital, Detroit & FROG's Clinics, Jacksonville, USA and Humanitas Hospital, Milan, Italy. Presently he is working Professor at Sri Aurobindo Medical College and P G Institute, Indore. He has 35 Publications in Indexed journals to his credit.

ABSTRACT

Cervical cancer is the most common malignancy in Indian women. It usually spreads locally or via regional lymphatics to retroperitoneal lymph nodes and hematogenous spread is rare. The occurrence of skeletal muscle metastases is a very rare event and only a few cases have been reported in literature. The authors present an unusual case of cervical carcinoma in a patient that presented with skeletal muscle metastasis 1 year after the treatment.

Key words: Cervix; skeletal muscle; unusual metastasis

INTRODUCTION

Metastasis of carcinoma cervix to skeletal muscles is a rare occurrence. Muscles are highly resistant to primary and metastatic cancer due to their high contractile activity, local changes in pH, oxygenation, accumulation of metabolites, blood flow, and local temperature.^[1] Psoas, iliopsoas, paraspinal muscles, and proximal musculature of the upper and lower limbs, represent the most frequently involved sites. Malignancies known to metastasize frequently to the muscle are melanoma, kidney, lung, thyroid cancer, lymphoma, leukemia and colon cancer.^[2] We report a case of carcinoma cervix with metastasis to paraspinal and intercostal skeletal muscle as the initial sign of dissemination.

CASE REPORT

A post-menopausal female, married for 45 years with two children, presented with a complaint of white discharge

per vagina for 3 months. On local examination, cervix was completely destroyed by a proliferative growth involving both the right and left fornix and the lower third of the vagina. Both the right and left parametrium were indurated to the lateral pelvic wall. Ultrasonography of the abdomen was suggestive of 8.6 cm × 5.3 cm large solid heterogeneous mass with scattered calcification in the cervical region. Histopathology from the growth revealed moderately differentiated squamous cell carcinoma. Metastatic workup did not show any metastatic lesion in liver or lungs. Thus, a diagnosis of carcinoma cervix IIIb (Federation of Gynecology and Obstetrics stage) was made. Then, the patient was planned for concurrent chemotherapy and radiotherapy. She was given radical radiotherapy to the whole pelvis with external beam radiotherapy to a dose of 60 Gy/30 over a period of 6 weeks. She also received 6 cycles of weekly cisplatin concurrently (30 mg/m² intravenous). Intracavitary boost was not given as both the

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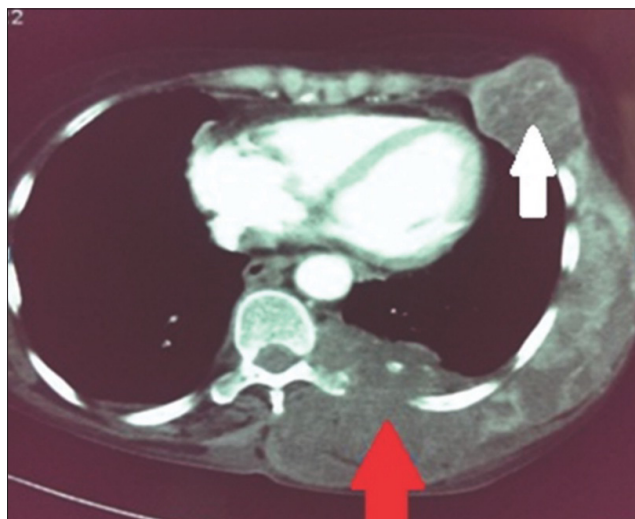


Figure 1: Contrast enhanced computed tomography-thorax showing skeletal muscle metastasis in the paraspinal and intercostal muscles invading the vertebra (red arrow) and lung metastasis (white arrow)

fornices were flushed, and vagina was small and conical. The patient remained locoregionally controlled for 1 year, after which she presented with complaints of swelling in the left side of the back along with pain. On examination, a large tender swelling was seen on left paraspinal region, size around 8 cm × 4 cm, hard and fixed. Vaginal examination performed at the time showed growth over cervix extending to involve upper 2/3 of the vagina, and both the parametrium were involved. Contrast-enhanced computed tomography (CT)-thorax revealed a huge mass in the left paraspinal muscles, involving the vertebrae along with multiple lung secondaries [Figure 1]. Fine-needle aspiration cytology (FNAC) from back mass revealed metastatic squamous cell carcinoma [Figure 2]. The patient was advised for palliative local radiotherapy to vertebra followed by chemotherapy, but patient declined treatment and went home and succumbed to the disease.

DISCUSSION

The incidence of metastasis to skeletal muscles is < 1% of all hematogenous metastasis despite the fact that the muscles represent 50% of total body mass in a person.^[2] Cancer cell survival is found more in denervated muscle, which is unable to contract rather than those stimulated one.^[3]

Skeletal muscle involvement from cervical cancer is very rare.^[4] Since 2008, only 10 cases with muscle metastasis from cervical cancer have been reported in literature. Deleted the third mention of the fact that the most common site of muscle metastasis is psoas.^[5,6] Various imaging modalities have been used to identify metastasis to muscle, but none of them are specific in differentiating carcinomas, sarcomas and other muscle disorders. CT scans show muscle metastasis as muscle enlargement but cannot specify this as malignant. Magnetic resonance imaging (MRI) in metastatic lesions show low to intermediate intensity on T1-weighted images, high intensity on T2-weighted images,

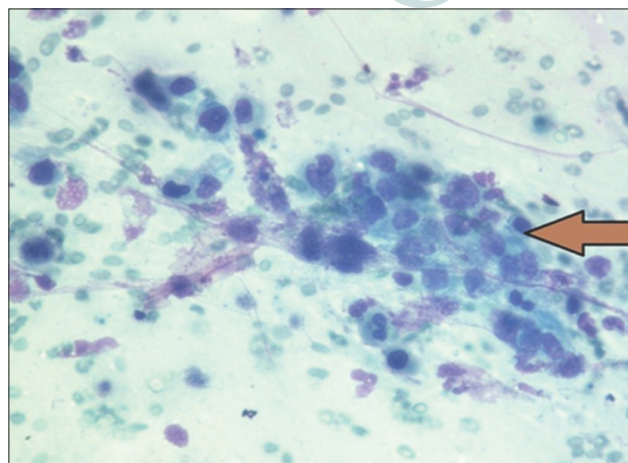


Figure 2: Fine-needle aspiration cytology from skeletal muscle mass showing nests of pleomorphic squamous cells (bold arrowhead)

and enhancement on gadolinium.^[7] Our patient declined an MRI scan. Differentiation between a primary sarcoma and metastatic carcinoma is difficult without a biopsy/FNAC.^[8] The FNAC was done, which showed metastasis from squamous cell carcinoma in the muscles. Primary squamous cell carcinoma in muscle is not recognized, and thus we have concluded that this is a metastasis from the past diagnosis of cervical cancer.

The outcome of the patients with skeletal metastasis is usually poor mostly due to diffuse metastasis and a lack of consensus on treatment options. When exercised, treatment options include radiotherapy, chemotherapy, and surgery according to the site number and extension of the lesion. In the case of a solitary skeletal muscle metastasis, metastasectomy has been performed, followed by radiotherapy.^[4] The general consideration of skeletal muscle metastasis usually requires chemotherapy, in particular, the platinum-taxane combination is often chosen because of high response rate documented with this regimen as compared to cisplatin alone.^[10] Palliative radiotherapy or combined radiotherapy and chemotherapy are effective in controlling pain and size of the metastatic lesion.^[9]

A reason for the prevalence (I have changed incidence to prevalence) of skeletal muscle metastasis in cervix cancer being low may be due to difficulty in differentiating malignant from benign lesions. Thus, in a patient with the previous history of cancer presenting with soft tissue mass, skeletal muscle metastasis should be considered and should be confirmed with imaging modalities and FNAC/biopsy. On confirmation palliative chemotherapy with or without local radiotherapy for pain should be given.

Muscular pain or weakness or just a palpable mass in a patient with a history of cervical cancer should always raise the suspicion of the metastatic muscular disease even after many years of locally controlled disease.

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Conflicts of interest

There are no conflicts of interest.

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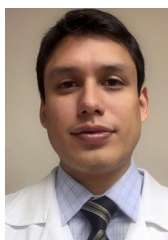
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Brain tumor surgery: supplemental intra-operative imaging techniques and future challenges

Telmo Augusto Barba Belsuzarri, Raphael Martinelli Anson Sangenis, João Flavio Mattos Araujo

Department of Neurosurgery, Pontifical Catholic University of Campinas, Campinas, São Paulo 13060-803, Brazil.

Correspondence to: Dr. Telmo Augusto Barba Belsuzarri, Department of Neurosurgery, Pontifical Catholic University of Campinas, Campinas, São Paulo 13060-803, Brazil. E-mail: telmobelsuzarri@hotmail.com



Telmo Augusto Barba Belsuzarri, MD, is a Neurosurgery Resident at the Pontifical Catholic University of Campinas (PUC-CAMPINAS). Graduated in Neurocritical Care, with thesis in Magnesium Sulphate in Neurointensive care, Dr. Belsuzarri has published papers in several areas and has special interest in Neurooncology/Cranial Base, Vascular Neurosurgery and Functional Neurosurgery.

ABSTRACT

Modern brain tumor surgery stands in the pillar of maximum safe resection. Tumor borders are always challenging, especially infiltration zones in malignant brain tumors. Novel technologies are designed for a better delineation and to increase the extent of resection (EOR) in brain tumor surgery, such as: cortical and sub-cortical mapping strategies with somatosensory-evoked potentials, awake stimulation mapping and cortical/sub-cortical stimulation for motor pathways, important for resection in eloquent areas; intra-operative imaging as functional and intra-operative magnetic resonance imaging, diffusion tensor imaging and intra-operative ultrasound are important for the tumor borders and to achieve the gross total resection; neurochemical navigation methods as 5-aminolevulinic and sodium fluorescein are important for the non-contrast-enhanced tumor border; future methods can be achieved with augmented reality surgery, new intra-operative chemical markers, and visualization methods. Nevertheless all these techniques seem to be promising, the real challenge in the future will be held in how to apply them and how they really affect the prognosis of the patients. Also, new concepts in tumor genetics will provide knowledge for the tumor behavior and will guide resection. Despite all limitations, the increasing importance of safe EOR shows the possible benefits of the novel technologies and surgical advances in brain tumor surgery, taking it to a new step of the neuronavigation era.

Key words: Brain tumor; fluorescein; intra-operative; neuronavigation; novel; technology

INTRODUCTION

Neurosurgery went through several changes over the past 50 years; technology has been applied to all fields, since the introduction of microscope and the microsurgical technique by Yasargil, until endoscopes, minimally invasive spine surgery and functional neurosurgery with deep brain stimulation implants. As we see, the neurosurgery has two important arms in this modern era: the equipment and the surgical expertise.

New imaging technologies are applied to other two different manners, pre-surgical moment and intra-operative imaging.^[1]

Modern neurosurgery lives a paradigm of concepts. Although there are insufficient proves of the real benefits and impacts of the aggressive image-guided neurosurgery,^[2] evidences show the importance of gross total resection (GTR) in the quality of treatment and the effectiveness

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increase of adjuvant therapy.^[3-5]

Neuroimaging has been playing an important role in neurosurgery in the last century and technology has come to provide details in neuroanatomy, neurological function, metabolic and metabolism, which augments the ability to increase the extent of resection (EOR) while simultaneously, minimizes the risk of damage in eloquent brain.^[1]

Increasing evidences show the importance of GTR for glioblastoma multiforme (GBM's) and adjuvant radiochemotherapy and demonstrate a 2-8 months survival benefit in patients with GTR compared to sub-total resection.^[3-5] Nevertheless, after a first impact, the focus has changed from just increasing the EOR, to increase the quality of life and safe resection; the tumor relationship critical anatomic structures and eloquent areas have become the center of the discussion.

Nowadays, molecular genetics came to open further discussions about tumor behavior, such as isocitrate dehydrogenase mutations (IDH) 1/2, 1p19q codeletion, PTEN deletions, MGMT mutation, telomerase reverse transcriptase (TERTp) mutation, EGFR and TP53.^[6-8] The IDH 1 and 2, were first described in GBM's, nevertheless further research showed that it was more expressive in grade II-III diffuse gliomas (about 70%). IDH 1/2 mutations are important biomarkers for diffuse gliomas, because they behave less aggressively and a better prognosis, than other IDH mutations (IDH wild-types), with a positive predictive value (PPV) for better progression-free survival and overall survival.^[6] The 1p/19q codeletion is found in almost 70% of histologically oligodendrogliomas, thus have an indolent progression and might be the molecular definition of oligodendroglial lineage. Also, these low-grade gliomas (LGG) tend to respond well for chemotherapy, thus have better prognosis. The MGMT is an enzyme, which repairs the DNA and interferes in temozolamide effect. Mutations in the MGMT have been correlated with improved prognosis and improved survival rate.^[6,7] On the other hand the ATRX/TP53 mutations might be the objective genetical markers of the astrocytic lineage.^[6,7]

In GBM's the most common aberrations are found in the chromosomes 7 and 10, where the PTEN and EGFR are located. Aberrations on the PTEN and EGFR amplifications are found in 80% and 30-40%, respectively, both of them strongly related to poor prognosis and aggressive progression, which reinforces the idea that these tumors with PTEN and/or EGFR amplifications are IDH-wild type tumors.^[6,7] Finally, studies points for the association of promoter region of the TERTp mutation and poor prognosis.^[8] A recent research was published comparing the TERTp mutation, 1p19q codeletion and IDH mutation in Grade II-III and GBM's with interesting findings.^[8] TERTp mutation only, was found in 347 patients with GBM's, compared to the TERT and IDH group with 11 patients, IDH

mutation only group with 32 patients and triple negative group with 80 patients. This data shows that almost 75% of the patients with GBM have only TERT mutation and have a correlation with aggressive behavior type of gliomas. Also, patients with Grade II-III with TERT mutation only (59 patients), had an aggressive course and were associated with poor survival, which suggest the need of early adjuvant therapies and special follow-up. Also only IDH mutation, was associated with lowest age of diagnosis (37 years) and the highest rates were found in the only TERT mutation group (59 years), between all the gliomas. This study opens for further research between the association of TERTp and other previously discussed mutations.^[8]

The genetic studies and imaging findings have become allies in the understanding of tumor behavior; nevertheless have also pointed questions on the efficiency of the surgical techniques to improve patient prognosis and the natural history of these tumors.

METHODS

A literature search of the Ovid Medline and PubMed databases for the period January 1980 to September 2015 was conducted using the following key words: brain tumor, borders, technology, neuronavigation, intra-operative, fluorescein, novel. Main novel technologies were selected by their relevance and were analyzed by categories.

RESULTS

Neurosurgery has rapidly changed in the past years due to new technologies and new different possible surgical approaches. These changes have modified neurosurgical concepts, from an aggressive vision to a safe EOR with good function. Since the beginning of the microsurgical era, the surgical planning has improved from an anatomy-planned surgery to an optimal non-visible tumor borders resection.^[1,2]

Several technologies were introduced in the intra-operative field such as functional monitoring with cortical and sub-cortical mapping, imaging technologies as neuronavigation, intra-operative magnetic resonance imaging (iMRI), intraoperative ultrasound (iUS), chemical biomarkers as 5-aminolevulinic acid (5-ALA), and sodium fluorescence.^[2]

Some of these advances were possible not only for the technology, but also due to anesthetic advances and better neurofunctional knowledge.^[9,10]

Nevertheless, even with the standard care in neurosurgery, the 2-year survival rate in GBM's still is about 38.4% and the 5-year rate is below 5%.^[11-13] Also, past reports have shown that even with hemispherectomies, patients could not be cured.^[14,15] Even though, the surgical technology has improved the past years, there are no consistent evidences

of improving survival rate.^[2]

In this point we have two arms, the technologies to improve resection and to increase the knowledge of tumor nature. By now it is clear that just improving resection won't provide the best result, but better understanding of the different diseases and tumor natures, will provide direction for optimal resections and better outcomes.

Awake craniotomy

Anesthetic advances permitted safer awake craniotomies to obtain brain mapping and better neurosurgical borders. However, it has a series of challenges to be analyzed before such as integration of different types of knowledge, imaging, multidisciplinary team, cooperation from several clinics sectors, application of protocols, application of specific technical adjustments, and a multidisciplinary approach. The integration of the pre-operative functional MRI (fMRI) and neuropsychological tests are the key for a good planning and patient selection. Not all tumor patients should undergo awake craniotomy, but patients with lesions close relationship with eloquent areas, in special for motor and speech.^[16,17] Talacchi *et al.* stated that intra-operative complication can vary from anesthetic (inadequate or excessive sedation, pain, nausea, vomiting); respiratory (oxygen saturation < 90%, increased CO₂, hypoventilation < 8 breaths/min, airway obstruction); hemodynamic (hyper- or hypotension, tachy- or bradycardia); and neurological (convulsions, brain swelling, new neurological deficit). From these complications, hyper- and hypotension are the most frequent in awake surgery (11% and 56%, respectively).^[16,17]

The main purpose of awake surgery is the monitoring of speech and motor pathways. This way, the physical pre-operative imaging/clinical examinations and intra-operative positive tests are important. Patients with aphasia and language disturbance seen at the physical examination, have higher risk of post-operative neurological deterioration. Intra-operative positive tests for stimulation in motor areas have also higher risk of motor deterioration, probably due to the proximity of the tumor lesion to the cortical tracts.^[16]

Shinoura *et al.* studied motor worsening after 102 motor areas glioma surgery; they have encountered motor worsening immediately after surgery and after 1 month were related to awake surgery failure and intra-operative complications. The main causes of failure of awake surgery are severe somnolence, epilepsy, air embolism, no wake up and motor neglect.^[18]

In order to analyze the hemisphere dominance, the Edinburgh, Wada or fMRI (with verb generation tasks) can be done. Also, multiple tests are applied to the language task with visual object naming tests such as the Boston naming test, Snodgrass and Vanderwart Test, DO80, and Aachner Aphasia Test. They are done to map the dominant

hemisphere and localization of speech areas. The pre-motor areas of the face are always tested to identify possible motor causes of the aphasia. Even with all protocols, intra-operative positive sites errors can range from 4.6% to 22%.^[16] The counting test is used to document a speech arrest during electrocortical stimulation and also auditory naming, verb generation and reading are commonly used tests. Additional tests can be applied such as calculation, visuospatial functions, working memory, visual pathways, eye movements, and writing.^[16,17]

One important point is that function can be found at the edge of high-grade gliomas and also within the tumor in low-grades, so it has to be analyzed for a safe EOR.^[19,20]

Awake surgery has been used for some time, but new tests and anesthetic evolution have permitted a better understanding of functional areas and also the mapping of complex brain areas.

Cortical and sub-cortical mapping

During the past years, the increase importance of the EOR and the relationship with increased overall survival has made the neurosurgeons push to the limits of the glioma surgeries, even in eloquent areas. Nevertheless, without intra-operative monitoring, morbidity increasing became fact. The objective of increasing overall survival with good functional status made the neuronavigation era a reality.^[1,21]

As imaging has increased its accuracy over the past years, neuroanatomy studies have shown a better knowledge of the sub-cortical tracts and the new mapping technologies have shown the real cortical and functional mapping, which most of the times can be changed by the lesion.^[9,22]

Intra-operative monitoring has been studied by several different methods, using somatosensory-evoked potentials (SSEP), awake stimulation, and cortical/sub-cortical direct motor stimulation. SSEP uses sub-dural electrodes to evoke potentials of gyri and to localize the central core (pre-central and post-central gyri) [Figure 1]. Awake stimulation is a

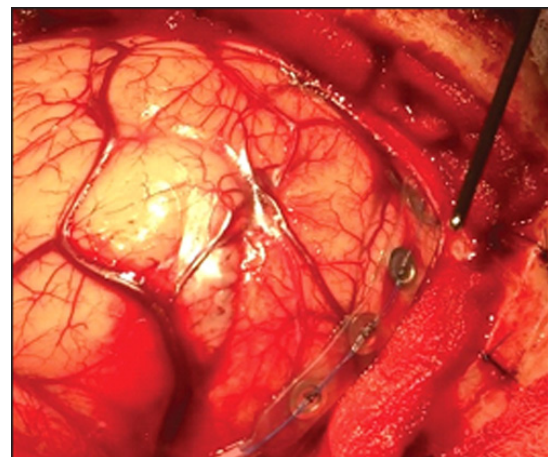


Figure 1: Direct electrical stimulation with and somatosensory-evoked potentials in motor/sensory areas

good approach for the language function and its multiple association areas, which could be more complex and even specific for different languages/cultures. Language function is the most complex and superior level function, with multiple localizations and spread connective areas. The exact language-related cortical area is not mathematical and is individual related, especially in patients with intra-cranial lesions. Patients with intra-cranial tumors can have very atypical language areas related to their brain mapping conformation and compliance to the tumor.^[22] The key for brain surgery of the dominant hemisphere is the central core and language function, which has become possible.^[22,23]

Brain mapping usually needs large craniotomies and longer time of surgical/anesthetic exposure, but provides multiple functional areas.^[23,24] Li *et al.* analyzed 91 cases of brain functional area glioma surgery under direct electrical stimulation (DES) and noticed that the most commonly observed areas of counting interruption were distributed on the posterior part of the left anterior central gyrus (47.7%), the operculum of the left inferior frontal gyrus (24.4%), the triangular part of the left inferior frontal gyrus (12.8%), and even the posterior part of the superior frontal gyri (4.7%). After surgery, 46% had no post-operative dysfunction, 42.9% a brief language dysfunction, 29.7% limb movement disorder, and 1 case had a permanent disability; this shows that DES is a non-invasive accurate method.^[22] Another positive point of DES is the mapping of the sub-cortical areas because it does not have influences on brain shift or other positioning errors.^[22]

Even though fMRI is satisfying for motor/sensitivity areas, its sensitivity is only of 59-100% and specificity of 0-97% for language areas.^[22]

De Witt Hamer *et al.* reviewed and made a meta-analysis of surgical situations of 8,091 glioma cases and found that the rate of long-term severe neurological dysfunction sub-sequent to DES was 3.4%, while the long-term severe disability rate of patients that underwent surgery without DES was 8.2%. In addition, for the patients undergoing

DES, the overall resection rate and the rate of involvement of the language functional area in the resection were significantly increased.^[23]

Event though DES is a relative novel technology, is also an important research method for higher cognitive functions, such as music, calculation, memory, complex speech processes, hemispheric ignorance, perception, visual pathways and more.^[25-29]

Neuronavigation and intra-operative magnetic resonance imaging

Magnetic resonance has changed the course of anatomical marks in neurosurgery; since its beginning in the early 80's, the pursuit of high field technologies for better images has become a challenge.^[9]

In the neuronavigation era, planning surgery has become not only a decision on craniotomies and different approaches, but also a way to prevent and predict the final surgery with minimal injuries. This way, fMRI, positron emission tomography (PET), and diffusion tensor imaging (DTI) are important technologies.^[1]

PET utilizes $H_2^{15}O$ as a blood tracer to measure flow or (^{18}F) -fluorodeoxyglucose uptake to measure cerebral metabolism.^[30]

fMRI measures blood oxygen level dependency changes due to alterations in the ratio of the oxyhaemoglobin and deoxyhaemoglobin in the most metabolically active regions^[30] [Figure 2].

Another image technology is the DTI, which visualizes the fiber tracts with the thermally driven motion, or diffusion of water and molecules through fibers.^[31,32]

DTI and fMRI allow neurosurgeons to have functional/eloquent areas and sub-cortical fibers related to the lesions in their pre-operative planning; therefore, functional

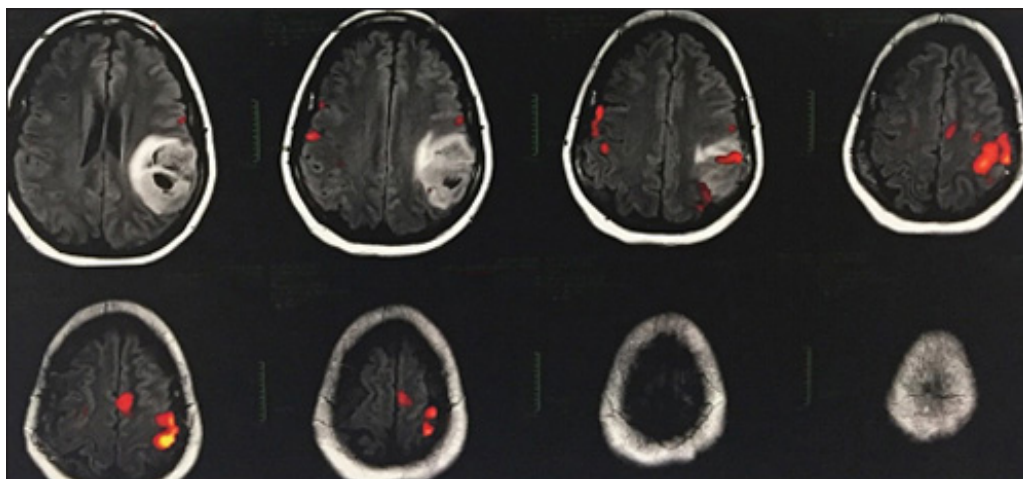


Figure 2: Functional magnetic resonance imaging and glioma: Red spots of the functional areas for the speech test near/between the tumor

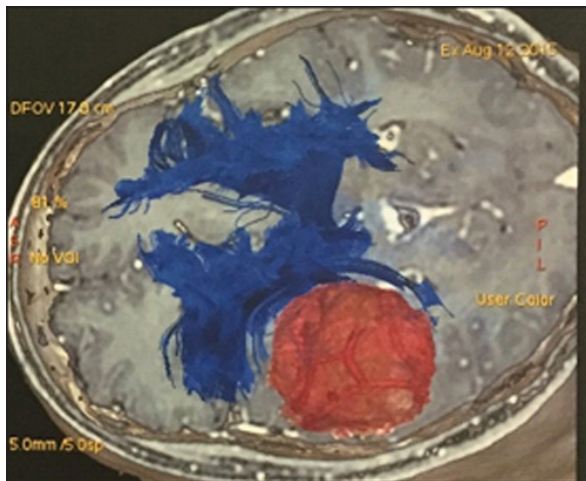


Figure 3: Tractography magnetic resonance imaging. In blue: the white fibers tracts. In red: tumor

neuronavigation has become part of the clinical decision-making, surgical approach, and EOR^[33] [Figure 3].

Nevertheless, the functional neuronavigation has not shown its clinical utility due to a lack of high evidence studies. Wu *et al.* carried out the only randomized controlled trial with an established protocol in functional neuronavigation and demonstrated a reduced post-operative motor deterioration, a higher Karnofsky Performance Scale, and an increased overall survival in study patients.^[33]

One of the worst problems in neuronavigation is the brain shift; it is the change of tissue/lesion during surgery due to cerebrospinal fluid drainage, tumor resection, and brain swelling; with estimated to be around 1 cm after opening the dura,^[34] and more than 1 cm after initial resection of tumor. Therefore, the iMRI technology has come to solve this problem and also increase the EOR. The first iMRI was performed in 1994; it presented several benefits and showed that a considerable part of patients had resectable residual tumor.

In special for LGG treatment, iMRI has led to favorable results in several studies. Reports show 30-60% of return to surgery after initial resection with iMRI.^[35-38]

Even though iMRI is an interesting method, nowadays there is only one randomized controlled trial comparing iMRI to conventional surgery; the trial found that iMRI was associated with higher rate of complete resection (96% vs. 68%) and increased progression-free survival without additional morbidity.^[5,39] Kubben *et al.* held a systematic review and showed just an evidence level II of iMRI being more effective than conventional neuronavigation in increasing EOR, quality of life or prolonging survival after GBM resection.^[38,39]

In practical analysis, iMRI has some issues for global implementation regarding costs and time. This method requires special implementation; most of the times not

only the equipment, but also revision of the local of implementation, making it a high cost technology.^[40,41] In addition, the time for image acquiring and the need of stop the surgery for it, prolong time of surgery and anesthesia.^[42-44]

Roder *et al.* studied retrospectively 117 patients after conventional surgery, after 5-ALA, and after iMRI they found that mean residual tumor volume after iMRI-assisted surgery (0.5 [0.0e4.7] cm³) was significantly smaller compared to the residual tumor volume after 5-ALA-guided surgery (1.9 [0.0-13.2] cm³; $P = 0.022$), which was significantly smaller than in conventional surgery (4.7 [0.0-30.6] cm³; $P = 0.007$). Total resections were significantly more common in iMRI (74%) than in 5-ALA-assisted (46%, $P = 0.05$) or conventional surgery (13%, $P = 0.03$). Also, the iMRI time of surgery was significantly higher compared to pre-iMRI period (213 vs. 354 min). Improvement of the EOR using iMRI was safely achievable and post-operative morbidities were comparable between cohorts. Total resections increased 6 months progression free survival from 32% to 45%. In follow-up analysis, the neuronavigation had new or worsened neurological deficits at 3 months in 18.2% of patients, compared to 45.5% of the control group. Non-neurological complications were present in both groups, 31.8% in the control group and 30.4% in the neuronavigation group. Also, the progression-free survival and survival rate was better in the neuronavigation/iMRI groups vs. control groups.^[2]

Despite it is a retrospective study with a short period of time and limited patients in different chronologic times, the great outcomes and promising results should open for new prospective studies.^[42] Further, the quality of iMRI images remains an issue; pre-operative MRI images are usually acquired by high-fields MRI with DTI and fMRI as a surgery plan, though intra-operative images are usually low-field MRI with worse definitions without DTI and fMRI; thereby the surgery plan for critical and eloquent areas is difficult and questionable after tumor resection and brain shift. Also, studies related to contrast dosage/timing and the local of resection have been done. The main challenge is to differentiate tumor border from blood brain barrier brakes and surgical tissue changes, which also have contrast-enchanted borders.^[43,44] The Cochrane review point for different patients' baselines with heterogeneous lesions and the current studies do not provide quality evidences of benefits. Also, there is no standard protocol for its use and most of the time it is used in single centers.^[2]

Intra-operative ultrasound

Intra-operative ultrasound is a dynamic method that can provide dynamic images with brain shift corrections and also the correlation between the tumor and normal brain, just as the tumor vascular nutrition and borders. In the past decades, the iUS increased the quality of images, from poor-quality images to three-dimensional (3D) imaging

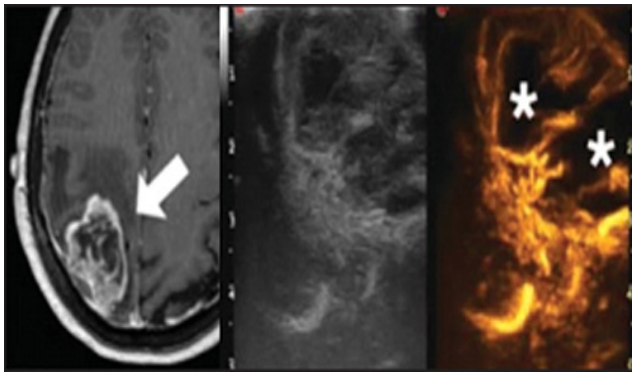


Figure 4: Contrast-enhanced magnetic resonance imaging and intra-operative ultrasound/contrast-enhanced US: High grade lesion can be compared, between the technologies. iUS can be used for the localization for most of the lesion, but with better results with cystic/heterogeneous tumors. Contrast-enhanced US has good visualization in vascularized tumors and give intra-operative vascular aspects. Images from Prada *et al.*^[51]

technology.^[45-47]

Intra-operative ultrasound is more effective with heterogeneous lesions, with cystic compartments, and lesions with different echogenicity from the cortex, important in deep lesions, more than 1 cm from the cortex. Several reports support the use of iUS with good results such as good visualization of tumor borders with 3D iUS in 88% of patients and had led to an EOR in 55%,^[48] numbers compared to the use of iMRI and 5-ALA.^[49,50]

Therefore, the use of US contrast in brain tumor surgery, called intra-operative contrast-enhanced US (ICEUS), is to determine better tumor visualization and also vascularization, is in study.^[51] The contrast agents containing microbubbles hit by low-acoustic power US waves resonate with a specific value that can be read by a US algorithm for contrast.^[52,53] There is a good correlation between the pre-operative MRI and iUS and can reach a small difference of 2 mm with the advantage of being intra-operative and dynamic [Figure 4]. Nevertheless, neither iUS nor ICEUS can provide good borders for all LGG because of the similar echogenicity between the tumor and normal tissue.^[51] Differently from the contrast-MRI, the ICEUS depends on intra-vascular micro bubbles resonance, which will not provide the interstitial aspects. Fluorescence-guided surgery such as the use of 5-ALA can highlight tumor borders, but only in high-grade gliomas. Compared with 5-ALA and iMRI, the iUS has the advantage of providing borders images not only for high grade gliomas, but also for other types of tumor such as metastasis, meningiomas and some LGG, and the relationship to normal/vascular tissue with the non-stop surgery advantage.^[51]

Fewer studies have shown the capability of the iUS and MRI,^[48] further studies are needed to evaluate the real aspect of the ICEUS and the use of combined methods with hybrid probe with MRI neuronavigation and iUS.

Fluorescence guidance

Even though intra-operative image guidance has evolved



Figure 5: Aminolevulinic acid: the use of 5-aminolevulinic acid in high grade glioma. The tumor has the pink aspect and the normal brain in dark blue

the past decades, the interface between tumor borders, remaining tumor cells, and normal tissue is challenging.

Despite several substances have been studied, there are two major promising fluorescences: 5-ALA and sodium fluorescein.

The administration of 5-ALA leads to differential accumulation of protoporphyrin in the malignant tissues, via heme-biosyntheses pathway.^[54,55]

The 5-ALA administration has proved to increase the GTR of glioblastomas (65% vs. 36%; $P < 0.0001$), smaller volume of the residual tumor (medians 0 cm³ vs. 0.7 cm³; $P < 0.0001$), and better progression-free survival in 6 months after intervention (41% vs. 21%; $P = 0.0003$) [Figure 5]. Recently, such beneficial results were corroborated by the assessment of 251 eligible cases from 18 clinics; they demonstrated greater proportions of complete resections of malignant gliomas with the use of 5-ALA (67% vs. 45%; $P = 0.000$) and progression-free survivors in 6 months after removal of glioblastoma (69% vs. 48%; $P = 0.002$), which corroborated with previous studies.^[54,56,57] Studies of fluorescence guidance combined with neuronavigation and brain mapping shows rates up to 98% of GTR in selected cases.^[58,59] In addition, the fluorescence guidance may reach beyond contrast-enhanced tumor borders and infiltrative zones that might be shown in the fluid attenuation inversion recovery (FLAIR) sequences of MRI.^[42] Although 5-ALA might be promising, it has some issues to be considered. First, we have to consider its high sensitivity and a low-specificity, in special the non-high intensity pigmentations areas of fluorescein such as in tumor border and the hyperpigmentation in non-tumoral areas (necrosis, fibrosis, astrocytes infiltration) and also other non-glial lesions as lymphoma and metastasis. Furthermore, the absence of tissue fluorescence is common in LGG due to its relatively unruptured blood-brain-barrier and other intrinsic mechanisms of fast elimination of the drug; this makes it useless for LGG surgery.^[1,60-63] Moreover, the studies with

fluorescence guidance have not studied the tumor genes and the good results could be genetically related. Further studies are needed to direct correlate the genetically aggressive tumors and the use of 5-ALA.

Another substance used for guidance is the sodium fluorescence, which accumulates in high neovascularization areas, also seen in high-grade lesions. Recent studies point to an increase of EOR and GTR, but without increasing of the overall survival rate.^[64] After review, 5-ALA had 91% sensitivity, 59% specificity, 85% PPV, and 71% negative predictive value for histopathological identification of malignant glioma.^[65] Future objectives in fluorescence guidance may lead to better microscopic visualization methods for the fluorescein such as filters, special masks or lens.^[66]

Current evidences

The Cochrane group has reviewed all the reports of image-guided surgery for brain tumor resection and found some issues. Most of the studies are not controlled and randomized; also patients' baselines and tumor aspects were heterogeneous in most of the groups and the resectability of them was different between intervention and control groups.^[2] Despite limitations and low quality of evidence, the analyses from the classical reports from Senft 2011, Stummer 2006 and Wu 2007 showed a trend for better results.^[2] Complete tumor resection was achieved with iMRI in 23/24 (96%) of participants in the intervention arm group compared with 17/25 (68%) of participants in the control arm (relative risk [RR] for incomplete resection 0.13, 95% confidence interval [CI]: 0.02-0.96, low quality evidence).^[2]

Using 5-ALA, complete resection was performed in 90/139 (65%) of the intervention arm vs. 47/131 (36%) of the control arm (RR for incomplete resection 0.55, 95% CI: 0.42-0.71, low quality evidence). Finally, neuronavigation with DTI was achieved among the 85 participants with high-grade glioma and complete tumour resections were achieved in 32/42 in the DTI arm vs. 14/43 in the control arm (RR for incomplete resection 0.35, 95% CI: 0.20-0.63, very low quality evidence). Among 129 participants with LGG, complete tumor resections were achieved in 40/61 in the DTI arm vs. 42/68 in the control arm (no significant difference).^[2] In survival analysis, the 5-ALA groups had a median survival of 15.2 months (95% CI: 12.9-17.5) in intervention group and control with 13.5 months (95% CI: 12.0-14.7). The neuronavigation-DTI arm was 21.2 months (95% CI: 14.1-28.3) vs. 14.0 months (95% CI: 10.2-17.8). Only in World Health Organization grade IV tumors analysis, neuronavigation-DTI arm was 19.3 months (95% CI: 15.2-23.5) vs. 11.1 months (95% CI: 7.3-15.2) in the control arm.^[2] In time to progression, the median time in iMRI group was 226 days (95% CI: 0.0-454) vs. 154 days (95% CI: 60-248) in control. With 5-ALA, it was 5.1 months (95% CI: 3.4-6.0) vs. 3.6 months (3.2-4.4 months)

in control.^[2] It is clear that the group analysis was not homogeneous and it might be due to a lack of protocols and a standardized approach to all lesions. Furthermore, there is need for standardization of reports for a systematic-review analysis and for future trends. Even though, the theoretical benefits of the novel techniques should impulse more randomized, controlled trials with better baselines.

Future technologies

Neuronavigation has become more popular and the localization of tumors has come to practice with the navigation instrument and the monitor. Even though, what if we had the images seen in the surgical field continuously, without navigators? The augmented reality has come to time with the objective of sending information to surgical field without monitors.

Augmented reality technique has four steps: virtual image creation; real environment; projection and registration. Thus, image can be seen in the surgical field and the virtual interface can be used. The augmented reality is important in planning surgery and having the lesion visible in the skin since the beginning of the surgery. The augmented reality can be applied not only to the surgical field to prepare a better surgical incision and approach, but also to the surgical view in the microscope, which is important when the surgeon cannot take his or her eyes/instruments from the microscopic field.^[67,68]

Moreover, the augmented reality could also include other parameters such as fiber tracts or important structures that should not be approached. As an innovation in neurosurgical surgery, there are few studies but promising applications.^[67]

Also other interesting concept is the regional vs. global DTI biomarkers for glioblastoma. Most of this lesions are heterogeneous with multiple histological features and can lead to different degrees of malignancy, thus biopsies can be different in multiple areas. DTI is routinely used to locate high-grade areas, but the development of a sensitive and specific biomarker, remains an issue. Also, the role of DTI-derived tensor metrics in normal brain and infiltrated brain is important for the distinction of tumor infiltration in non-contrast-enhanced areas. As the GBM been considered as a whole brain disease, DTI analysis of the whole brain might be more interesting than studying just the lesion areas. Roldán-Valadéz *et al.* showed that relative anisotropy, axial diffusivity (AD), CI (linear tensor), Cs (spherical tensor), were important for regional DTI tumor analysis.^[69] Also, Cortez-Conradis pointed for AD, CI, Cs and introduced the whole brain concept. The advantages of whole brain DTI analysis are: Decrease of bias associated with the analysis of just one region of interest; the tumor and edema regions are included; lesions not perceived by the radiologist's eye on conventional sequences would be included in a global assessment; it may avoid problems associated with partial volume effects, and inaccurate image coregistrations.^[70]

Furthermore, these biomarkers could also been applied for other tumors and even other neurological diseases, without any contrast addition and increase of costs.^[69,70]

For high-grade lesions with increased neo-vascularization, there was a report with use of indocyanine green (ICG) for detection of tumor borders. It is classically used by ophthalmologists for retinal vasculature and more recently for vascular neurosurgeries for aneurysms and arterio-venous malformations; however, for surgical borders for high-grade gliomas, it is a novel technique.^[71] Eyüpoglu *et al.* reported the ability of demonstrating the hypervascular areas with ICG that were not visible with the 5-ALA use. This technique was called dual intra-operative visualization approach (DIVA) with the initial approach using 5-ALA; after all initial tumor was resected, ICG was administered for visualization of remaining hypervascularization areas, with good initial results. Further studies are needed, but DIVA technique could be an interesting approach for further resection of non-fluorescein areas.^[72]

One of the most difficult tasks in glioma surgery is the low-grade lesion. Most of the low-grades have similar density, echogenicity, and macroscopic aspect. Despite the neuronavigation progression, there are few MRI methods for low-grade tumor visualization, and most of the times the lesion is not contrast-enhanced and there is just the FLAIR sequence for tumor borders.^[73] Ramakrishna *et al.* showed improvement of overall survival with aggressive resection of FLAIR tumor limits, not only in the first attempt, but also in reoperation, regardless of patient age, pathology, chemotherapy, and radiation.^[74]

The 5-ALA for LGGs is usually reported as non-visible, but it is not true for all of them. Valdés showed that 5/12 patients had at least 1 instance of visible fluorescence during surgery and 45% of the non-visible fluorescence had a higher and detectable concentration of PpIX in the tumor tissue after the 5-ALA administration. With this idea, other researches were made to accurate the visibility of the fluorescein, or guide the elevated concentration in tissue with special probes of light visualization or high-resolution microscopic techniques, but with few results by this date.^[75]

CONCLUSION

Evidences of the correlation between tumor removal and increase of survival rate have an impulse in novel technologies for safe resection and EOR. The uses of iMRI, DTI, PET, iUS, and fluorescence guidance have come to establish the neuronavigation era in neurosurgery.

Also, there is an increasing importance of the tumor genetics and behavior, which will provide crucial information and will guide tumor resection and adjuvant treatment. Despite all limitations of each technology and the lack of clear evidences, it is clear that this neurosurgeon/technology

interface has come tighter and promising. However, the best result will come with the integration between technology for resection and tumor nature knowledge.

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Conflicts of interest

There are no conflicts of interest.

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The role of the PI3K/AKT/mTOR pathway in brain tumor metastasis

Silvia Crespo*, Marcus Kind*, Alexandre Arcaro

Division of Pediatric Hematology/Oncology, Bern University Hospital, CH-3008 Bern, Switzerland.

Correspondence to: Dr. Alexandre Arcaro, Division of Pediatric Hematology/Oncology, Bern University Hospital, Murtenstrasse 35, CH-3008 Bern, Switzerland. E-mail: alexandre.arcaro@dkf.unibe.ch

*Contributed equally to the work.



Alexandre Arcaro is a group leader at Bern University Hospital. He graduated at the University of Lausanne and obtained a Ph.D. from the University of Fribourg. He was then a postdoc at the Ludwig Institute for Cancer Research, UCL and Lausanne Branch. He was then a lecturer at Imperial College London. Subsequently, he was a group leader at the University Children's Hospital Zurich.

ABSTRACT

The PI3K/AKT/mTOR (PAM) pathway is involved in a variety of cellular functions and often contributes to oncogenesis and cancer progression. It has been recognized that this pathway is frequently activated in the most common central nervous system cancers of adults and children, malignant gliomas and medulloblastomas (MB). In these tumors, the PAM network controls key functions necessary for cell invasion and metastasis, such as cell motility. This review summarizes the current knowledge about the role of PAM signaling in cell invasion and metastasis in gliomas and MB. Current approaches to inhibit cell invasion and metastasis by targeting the PAM pathway will also be discussed.

Key words: PI3K/AKT/mTOR pathway; glioblastoma; medulloblastoma; metastasis

INTRODUCTION

Tumors of the central nervous system include a broad range of neoplasms that arise from different cell lineages. The most common variants in adult and pediatric populations are malignant gliomas and MB, respectively.

Glioblastoma (GBM) is a highly aggressive tumor that arises from different glial cell types. Based on WHO classification, GBM is a grade IV astrocytoma that either develops de novo (primary GBM) or gradually from lower grade astrocytomas (secondary GBM).^[1] Due to limited therapy options, the median survival is a dismal 15 months with standard of care, which includes surgical resection, temozolomide chemotherapy and radiation.^[2]

Medulloblastomas are embryonal tumors that originate from fetal tissue due to aberrant developmental signaling.^[3] By using treatment protocols that combine chemotherapy, surgery and cranio-spinal radiotherapy, 70-80% of patients can be cured, albeit with debilitating long term side effects.^[4]

Advances in molecular biology have led to remarkable insights into the understanding of the underlying molecular pathogenesis of malignant gliomas and MB and have revealed specific pathways and signaling networks that promote tumorigenesis in these malignancies.^[5,6] These frequently feature aberrant receptor tyrosine kinase (RTK) signaling via the PI3K/AKT/mTOR (PAM) pathway.

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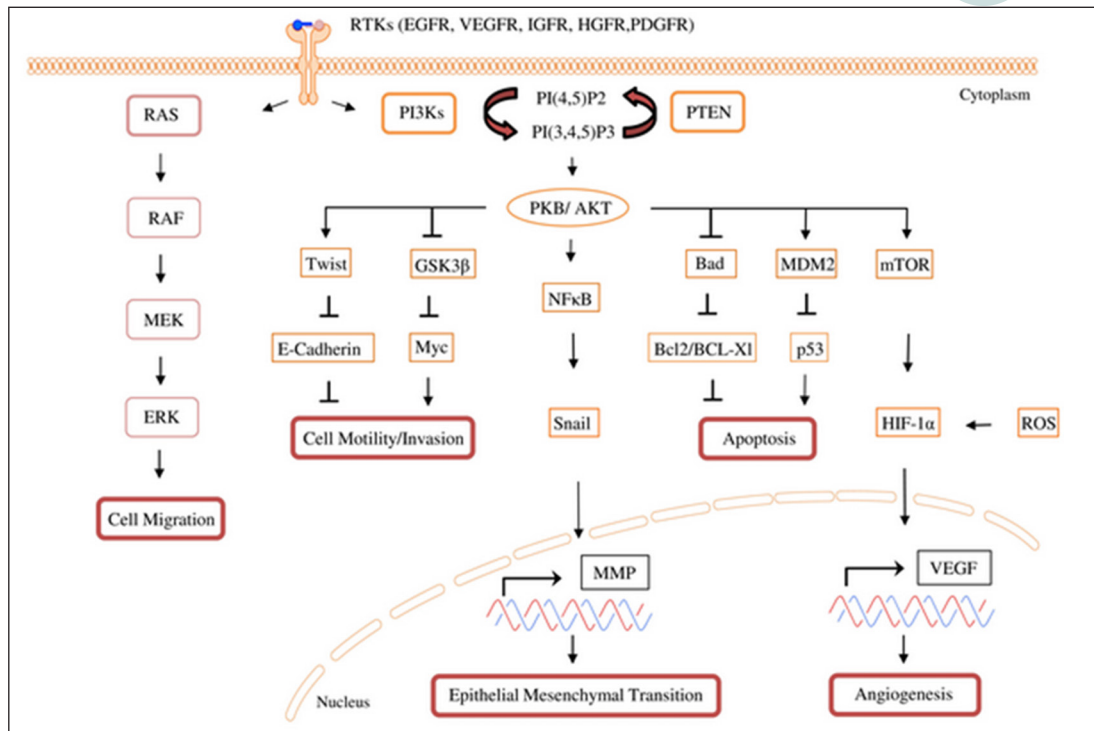


Figure 1: PAM-signaling network and effector functions associated with metastasis: In GB and MB, aberrant PAM signaling can promote tumor progression by over- inducing angiogenesis, EMT, cell migration and invasion, and also by inhibiting loss of adhesion associated apoptosis. PAM: PI3K/AKT/mTOR; VEGF: vascular endothelial growth factor; PDGFR: platelet derived growth factor receptor; IGF-1: insulin-like growth factor-1; IGFR: insulin-like growth factor receptor; NFκB = nuclear factor kappa-light-chain-enhancer of activated B cells; HIF-1α: hypoxia inducible factor 1α; PTEN: phosphatase and tensin homolog deleted on chromosome 10; PI3Ks: Phosphatidylinositol-3-kinases; MEK: mitogen-activated ERK kinase; EGFR: epidermal growth factor receptor; ERK: extracellular-signal regulated kinase

The PAM signaling axis integrates extracellular signals via RTK and G protein-coupled receptors and regulates a host of intracellular functions, such as cell cycle, metabolism, migration and apoptosis.^[7-9] Phosphatidylinositol 3-kinase (PI3K) phosphorylates the 3'-hydroxyl group of phosphatidylinositol, producing second messengers that recruit cytoplasmic proteins to the membrane. These include various modulators of small GTPase activity, TEC family tyrosine kinases and members of the AGC protein kinase family like AKT (also known as Protein Kinase B, PKB).^[10] The serine-threonine kinase mTOR, a regulator

of translation and protein synthesis, is activated by AKT signaling.

Since many hallmarks of malignancy are controlled by PAM signaling, genetic and epigenetic alterations in various components of this pathway are frequent events in central nervous system (CNS) cancers. These include gain-of-function mutations and amplifications in genes encoding RTKs such as epidermal growth factor receptor (EGFR), loss-of-function mutations of the phosphatase and tensin homolog deleted on the chromosome 10 (PTEN)

Table 1: Stage of clinical development of PAM pathway inhibitors for brain tumors^[138]

Inhibitor	Target	Stage of clinical development for brain tumors
SF-1126 (RGDS-conjugated LY294002 prodrug)	Pan-PI3K	Phase I
PX-866	Pan-PI3K	Phase II
Pictilisib (GDC-0941)	Pan-PI3K	Phase II
LY294002	Dual PI3K/mTOR	Preclinical
Wortmannin	Dual PI3K/mTOR	Preclinical
Dactolisib (NVP-BEZ235)	Dual PI3K/mTOR	Phase II
Perifosine (KRX-0401)	Akt	Phase II
KP-372-1	Akt	Preclinical
KP-372-2	Akt	Preclinical
A-443654	Akt	Preclinical
Bevacizumab (Avastin)	VEGF-A	Phase III
Aflibercept	VEGF and placental growth factor	Phase I
Cediranib (AZD2171)	VEGFR, Flt1/4, PDGFR, FGFR1, c-KIT	Phase I
Cabozantinib (XL-184)	c-MET and VEGFR2	Phase I
SGX-523	c-MET	Phase I
Osthole	IGF-1/IGF-1R and calcium channel blocker	Preclinical

PAM: PI3K/AKT/mTOR; VEGF: vascular endothelial growth factor; PDGFR: platelet derived growth factor receptor; FGFR: fibroblast growth factor receptor; IGF-1: insulin-like growth factor-1

tumor suppressor gene, and oncogenic mutations in various PI3K isoforms that lead to a constitutively activated pathway.^[11,12] Aberrant PAM signaling also favors essential steps for cell invasion and metastasis in CNS malignancies [Figure 1]. The implications of aberrant PAM signaling in angiogenesis, epithelial to mesenchymal transition (EMT) and immune response modulation is currently under intense investigation.^[13-15] Components of the PAM pathway are therefore being considered as potential drug targets [Table 1] to inhibit the often fatal events of metastasis and cell invasion.^[16-18]

ANGIOGENESIS

Angiogenesis is a process consisting of the generation of blood vessels and is essential for the growth of tumor mass beyond 1mm in diameter.^[19] This process allows tumors to become invasive by supporting them with nutrients and oxygen. Tumor and host cells synthesize and secrete pro-angiogenic factors, such as vascular endothelial growth factor (VEGF), that activate quiescent endothelial cells and induce the formation of new blood vessels from pre-existing vascular structures.^[20]

The PAM pathway plays a critical role in this neovascularization process by controlling the hypoxia-inducible factor 1- α (HIF-1 α) mediated expression and secretion of VEGF.^[21,22] In cancer cells, VEGF stimulation can be mediated by chronic stimulation by growth factors, such as insulin-like growth factor-1 (IGF-1); constitutive activation of PI3K; or constitutive activation of AKT due to inactivation of PTEN.^[23,24] The important role of the PAM pathway in angiogenesis has been confirmed in various malignancies where inhibition of pan-PI3K by LY294002 and downregulation of p110 α (or recently, PI3KC2 α) were shown to block tumor vascularization.^[15,22,25] In myeloid cells, PI3KY was reported to be involved in the activation of integrin α 4 β 1, leading to myeloid cell invasion into tumors and, in turn, to tumor angiogenesis.^[26]

In GBM, the most aggressive glioma subtype, the PAM pathway also plays a crucial role in the induction of invasion, angiogenesis and the expression of VEGF in cells.^[24,27] Therefore, new small molecule inhibitors targeting PI3K enzymes are being tested in this CNS malignancy. These include the PI3K inhibitors SF1126 (a RGDS-conjugated LY294002 prodrug) and PX-866, and the dual PI3K/mTOR inhibitor NVP-BEZ235.^[28-30] These compounds were shown to induce a substantial inhibition of the expression of VEGF, thus reducing the invasive and angiogenic capabilities of GBM cells. In fact, PX-866 has recently entered phase II studies in patients with recurrent GBM. Unfortunately, preliminary results of this trial have shown a low overall response rate.^[31]

The combined inhibition of VEGF and vascular endothelial growth factor receptor (VEGF/VEGFR) is currently thought

to be an effective way to control GBM growth.^[32-34] Examples of VEGF/VEGFR inhibitors are bevacizumab, already in phase III trial,^[35] and aflibercept, a VEGF/VEGFR inhibitor that also targets placental growth factor.^[36] Unfortunately, long-term treatment with aflibercept was reported to induce an invasive phenotype of GBM.^[37,38]

In addition, RTK inhibitors such as cediranib (an inhibitor of VEGFR, platelet-derived growth factor receptor, fibroblast growth factor receptor 1, and v-kit Hardy-Zuckerman 4 feline sarcoma viral oncogene homolog), have also been used with promising results.^[39,40] Inhibitors of c-MET such as cabozantinib are also being considered, and have been reported to induce a significant increase in overall survival of mice bearing GBM xenografts.^[41]

However, anti-angiogenic therapies targeting VEGF/VEGFR have had less of an effect than expected.^[42] This could be because, in highly vascularized tissues like the lung and brain, tumors can often proliferate around existing vessels and hijack them, a process called vessel co-option.^[43,44] These pre-existing blood vessels circumvent the need to generate new tumor vasculature, and may explain the inefficacy of anti-proliferative therapies in GBM, the most vascularized tumor in humans.^[38]

Autophagy is an evolutionarily conserved, catabolic process that maintains cellular biosynthesis through the degradation and recycling of proteins and organelles to support metabolism and survival during starvation. This process has been shown to have a complex relationship with angiogenesis induction in various malignancies. While some studies have reported that autophagy inhibits angiogenesis,^[45,46] other studies have found that induction of autophagy promoted cancer and its inhibition prevented angiogenesis.^[47,48] This illustrates the dual role that autophagy plays in cancer, acting as a pro-survival or pro-death mechanism depending on the tumor type and stage.^[49]

Autophagy is induced by different cellular stress-mediated signaling pathways, the inputs of which are integrated by the protein kinase mammalian target of rapamycin (mTOR). The mTOR complex 1 (mTORC1) is a negative regulator of autophagy and a downstream target of the PI3K/AKT pathway.^[50] Anti-cancer agents that target this pathway are able to induce autophagy, which has a cytoprotective role as well as an anti-angiogenic potential similar to the action of the dual PI3K-mTOR inhibitor NVP-BEZ235.^[51-53]

High-grade gliomas have been reported to have lower expression of autophagy-related proteins than low-grade gliomas.^[54] The amplification of EGFR, which is often found in these tumors, is known to suppress autophagy.^[55] The progression of astrocytic tumors is associated with a decrease in autophagic capacity.^[56] In most of these CNS malignancies, the modulation of autophagy sensitizes tumor cells to standard chemotherapy and radiotherapy

induced cell death.

EMT, CELL INVASION AND MOTILITY

EMT is a biological process that allows immobile epithelial cells to acquire a mobile mesenchymal phenotype, becoming detached and invasive. It was initially described in the context of embryonic differentiation.^[57] In tumor cells, this process, together with the induction of neo-angiogenesis, initiates cancer metastasis, inducing enhanced migratory properties, invasiveness and resistance to apoptosis.^[58,59]

During EMT, a variety of transcription factors are upregulated in metastatic cells, such as Snail, Slug, Twist and Zeb 1.^[60] Snail can be activated by a number of pathways, including hypoxia, HIF-1, HIF-2, Notch, nuclear factor kappa-light-chain-enhancer of activated B cells (NF- κ B), and transforming growth factor beta (TGF- β), a pro-apoptotic factor. Snail up-regulates AKT phosphorylation and Bcl-X_L countering the induction of apoptosis,^[61] and down-regulates cyclin D2, inhibiting cell cycle progression.^[62]

Twist, which promotes loss of E-cadherin mediated cell-cell adhesion and cell motility,^[63] has been linked to the PI3K/AKT pathway in various malignancies. This link is established by the AKT2 isoform, a Twist-mediated transcriptional regulator that activates Twist, constituting a positive feedback loop that promotes EMT.^[64,65] Twist also maintains hyper-activation of the PI3K/AKT pathway in breast cancer cells, through its transcriptional target TGF- β 2.^[65]

AKT hyper-activation and PIK3CA knock-in can promote EMT in various human cancers.^[61-66] The association between EMT and PI3K activation has also been reported in ER α -negative endometrial carcinomas.^[67]

Twist overexpression has also been correlated with the induction of tumor cell invasion in GBM.^[68] However, these malignancies usually do not metastasize out of the CNS, mainly due to their rapid relapse rate and poor prognosis.^[69] Even so, there are reports describing GBM metastasis^[70] involving the spread of GBM cells out the CNS through cerebrospinal fluid, blood or lymphatic vessels.^[71,72]

Medulloblastoma, on the other hand, has a high tendency to disseminate to the spinal cord and leptomeninges of the cerebellum and forebrain. These tumors are classified into 4 molecular subgroups: wingless (WNT), sonic hedgehog (SHH), group 3 and group 4.^[73] Group 3, characterized by cMYC amplification, is associated with metastatic disease.^[74]

The PI3K/AKT pathway is activated in 50% of GBMs. In the case of MB, there are a number of studies concerning alterations in this pathway.^[6,75,76] This pathway appears to facilitate an invasive phenotype of GBM and MB, especially

in terms of motility and resistance to stress.^[77]

The class I_A PI3K isoform p110 α is the most relevant PI3K isoform affecting cell growth and survival. The gene encoding this isoform, PIK3CA, is usually mutated in GBM (27%).^[78] In this malignancy, PIK3CA mutated form plays a main role in cell growth under anchorage-independent conditions. In MB, however, this PI3K isoform is typically overexpressed,^[79] promoting cell proliferation, for example, through the regulation of the leukemia inhibitory factor receptor α (LIFR α).^[80] The inhibition of p110 α impairs cancer cell growth, migration, and survival in these CNS malignancies.^[16,79]

Other class I_A PI3K isoforms are also overexpressed in brain tumors, such as p110 δ , which has been reported to be overexpressed at the mRNA level in primary GBM, controlling migration in these cells.^[81,82] The isoform p110 γ , which is overexpressed in primary MB, contributes to cisplatin resistance and has emerged as a novel target for combinatorial treatments.^[83] The class II PI3K isoform PI3KC2 β , which is overexpressed in a variety of cancers, acts as a modulator of cell migration, survival and proliferation in leukemia and brain tumors.^[84] The highly specific pan-PI3K inhibitor GDC-0941 has recently been shown to have anti-migratory, anti-proliferative and pro-apoptotic effects in MB cell lines, showing synergy with the standard chemotherapeutic drug etoposide and good clinical tolerability.^[85]

Other elements of the PI3K/AKT pathway are also being considered as potential targets to inhibit cell proliferation and migration in GBM and MB. One example is AKT, which usually shows high levels of phosphorylation in these brain tumors.^[86] Its inhibition by KP-372-1, KP-372-2, A-443654, or perifosine, was reported to inhibit cell growth and induce radio-sensitizing effects in GBM and MB.^[87-89] Clinical trials of perifosine in GBM patients are ongoing.^[90]

PTEN is a tumor suppressor usually mutated and inactivated in GBM, with an inverse correlation between its expression and glioma grade.^[91] In MB, PTEN is rarely mutated but frequently downregulated, by promoter hypermethylation and/or allelic losses, inducing AKT activation.^[86]

PTEN, together with the MAPK signaling pathway, has a primary role in the regulation of G1/S cell cycle checkpoint-defective astrocytoma invasion, and its deletion increases migration, invasion and resistance to apoptosis in GBM cell lines.^[92] PTEN controls integrin-dependent migration through the regulation of Src family kinase activation, in a PI3K/AKT-independent manner.^[93] The re-expression of PTEN in GBM cell lines increases the cellular content and activity of the p53 tumor suppressor protein inducing cell cycle arrest and increasing the sensitivity of the tumor cells to various chemotherapeutic agents such as etoposide.^[94]

Upstream regulators of EMT induction, such as insulin-like

growth Factor-1 receptor (IGF-1R), c-MET and the CXCR4 receptor, have been proposed as potential targets to inhibit GBM or MB invasion.

IGF-1R is typically overexpressed in malignant GBM,^[95] and its activation by IGF-1 contributes to Snail and Twist expression though PI3K/AKT signaling pathway activation.^[96,97] Therefore, IGF-1R tyrosine kinase inhibitors or IGF-1 inhibitors, such as osthole, have been used to inhibit GBM proliferation, migration and EMT.^[97,98] In a recent study of 218 cases of human GBM, IGF-1R overexpression was reported as an independent prognostic factor associated with shorter survival time and a less favorable response to temozolomide.^[99]

C-MET expression levels correlate with tumor grade in CNS malignancies,^[100] and its activation also mediates EMT-promoting signals in cancer cells via class I_A PI3K.^[101,102] In MB, c-MET signaling is deregulated, thus inducing tumor growth and an anaplastic histology.^[103] The use of c-MET kinase inhibitors, such as SGX523, suppressed tumor growth in GBM cell lines.^[104] This inhibition blocked the EMT induced by VEGF ablation in a GBM mouse model^[105] and induced an effective decrease in MB cell migration and invasion.^[106,107]

Stromal cell derived factor (SDF-1) or CXCL2 and its chemokine receptor CXCR4 can induce EMT in GBM via activation of PI3K/AKT and extracellular-signal-regulated kinases (ERK) pathways, and its inhibition suppressed EMT in glioma cell lines by upregulating E-cadherin.^[108]

However, single agents targeting the PAM pathway have been reported to be an inefficient approach in MB and to increase invasion in the surviving fraction of GBM.^[109] Therefore, new therapeutic approaches should be based on increasing the therapeutic window by targeting two different routes, namely the PAM and ERK pathways, or on combining PAM inhibitors with chemotherapeutic agents.^[110]

MicroRNAs have also been shown to play an important role in various CNS malignancies, and miR-142-5p and miR-25 are upregulated in all of them.^[111] In MB, miR-21 suppression inhibited tumor migration.^[112] MiR-183 has a pro-tumorigenic effect in the MYC-driven MB subgroup through the inhibition of apoptosis, deregulation of the mTOR pathway and modulation of cell motility and migration.^[113]

During the EMT process, malignant cells start to intravasate into the surrounding blood vessels in order to migrate to other parts of the body. To accomplish this, the extracellular matrix and basement membrane of blood vessels have to be degraded by matrix metalloproteases (MMP).^[114] The most relevant metalloproteases in this invasive process are MMP-2 and MMP-9.^[115]

One of the upstream pathways controlling MMP production is the PI3K/AKT pathway.^[116] As a consequence, drugs like wortmannin, a drug that inhibits the secretion of MMP-2, blocks GBM invasion through the down-regulation of the PI3K/AKT/NF- κ B signaling pathway.^[117] Since Snail induces MMP-9 expression, EMT seems to be necessary for intravasation of lymph vessels in GBM and other cancers.^[119]

PI3KS IN INFLAMMATION/MICROENVIRONMENT

The process of inflammation has been extensively linked to tumor progression, as it can stimulate immune suppression, angiogenesis and tumor metastasis.^[119,120] In response to tumor-derived growth factors and chemokines, inflammatory cells of the immune system are recruited to the tumor microenvironment. There, cells normally involved in chronic inflammation, such as mast cells, granulocytes and monocytes, provide the tumor with angiogenic factors, enzymes for extracellular matrix (EM) remodeling and growth factors to create a favorable milieu for expansion and dissemination.^[121,122]

Members of the class I PI3K family have also been implicated in tumor-associated inflammatory responses. In myeloid cells, p110 γ can be activated via tumor-derived chemoattractants, such as IL-6, IL-8, TNF- α and CSF-1. Upon activation, p110 γ promotes extravasation into the tumor microenvironment (TME) via integrin α 4 β 1 and promotes inflammation-associated tumor progression.^[26,123] This is in line with other reports indicating a crucial role of p110 γ for immune cell chemotaxis, as well as for chronic inflammation.^[124]

Microglial cells are resident macrophages of the CNS. Depending on the signaling context, these cells possess a dual role in tumor biology. By secreting cytokines like IL-6, IL-10 and immune suppressive molecules, gliomas can polarize microglia into tumor supporting M2 phenotypes that participate in matrix remodeling and cell invasion.^[125-127] In a recent study, PAM signaling was upregulated in microglial cells that were exposed to glioma derived factors, indicating that PAM signaling is needed to force microglial cells into a tumor supportive M2 state.^[128] This result was supported by a report showing that mTOR inhibition with rapamycin polarizes microglia cells to express a tumor suppressive M1 phenotype.^[129] To date, the exact molecular mechanism by which PI3K signaling contributes to M2-polarization of microglia is still unknown and should be the subject of further investigation.

The tumor microenvironment of MB is also being investigated. A recent study associated the SHH-MB subtype with high infiltration of tumor associated macrophages (TAM) and strong expression of the inflammatory genes CSF1R and CD163.^[130] It has been shown that PI3K binding

to CSF1R stimulates spreading and motility in macrophages and their enhancement of tumor cell invasion.^[131] Inhibition of p110 δ impairs CSF-1 induced macrophage spreading and their invasive capacity.^[132] Hence, it may be worth investigating whether selective inhibition of PI3Ks in the SHH-MB subtype impairs TAM-driven tumor invasiveness. The CD163 gene is a surface marker that is strongly expressed by tumor promoting M2 macrophages, but it is not clear whether or not MB cells polarize surrounding TAM via PI3K to enhance tumor invasion.

CLINICAL TRIALS OF KINASE INHIBITORS IN GLIOBLASTOMA

Oncogenic kinase signaling (e.g. via the PAM pathway) is crucial in GBM and hence attractive for targeted therapy.^[133,134] Unfortunately, the overall response rate of GBMs to kinase inhibitors in clinical trials has been poor so far.^[135] One reason for these disappointing results may be inadequate trial design. Systematic flaws such as small sample sizes, absent control groups and unverified drug activity have been reported in the past.^[135] Therefore, various changes in study design have been proposed to improve the reliability of the results. Clinical trials enriched for patients with an aberrant kinase target are likely to give a better picture of the overall performance of a particular inhibitor.^[136] In addition, the importance of monitoring target inhibition and negative feedback has been shown in a phase I trial in PTEN-deficient glioblastomas.^[137] To improve the results of clinical trials using kinase inhibitors, it appears necessary to set higher requirements for preclinical models and to verify efficacy in a broader spectrum of GBM models in order to address each model's shortcomings. Given the fact that kinase signaling pathways are often dysregulated in parallel, it may also prove worthwhile to evaluate combinations of different kinase inhibitors.

CONCLUSION

Aberrant PAM signaling can promote crucial metastatic events such as angiogenesis, EMT, and modulation of immune cells in both MB and GBM. Targeting the PAM network may be a useful way to inhibit these often fatal events. Understanding the molecular mechanisms and the context by which different components of the PAM pathway contribute to tumor progression is a prerequisite for the design of novel treatment strategies. Some of these mechanisms, such as the interaction between malignant CNS cells and TME, have only recently become the focus of investigation and are still incompletely understood. Further studies are necessary to elucidate these mechanisms and to determine which components of the PAM pathway should be targeted to inhibit the metastasis of CNS malignancies.

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Conflicts of interest

There are no conflicts of interest.

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Brain infiltration by cancer cells: different roads to the same target?

Mayra Paolillo, Sergio Schinelli

Department of Drug Sciences, University of Pavia, 27100 Pavia, Italy.

Correspondence to: Dr. Mayra Paolillo, Department of Drug Sciences, University of Pavia, 27100 Pavia, Italy. E-mail: mayra.paolillo@unipv.it



Mayra Paolillo is research associate at the Drug Sciences Department, University of Pavia, Italy. Her researches mainly deal with the study of functional effects and signal transduction pathways modulated by the pharmacological blocking of RGD-binding integrins in glioma cancer stem cells and glioma cell lines.

ABSTRACT

Brain infiltration by cancer cells is a complex process in which metastatic cells detached from the primary tumor must firstly survive in the blood flow, cross the blood brain barrier (BBB) and finally colonize a foreign microenvironment. The cells that successfully bypass the cellular barriers surrounding capillaries, proliferate to form micrometastasis and trigger the angiogenic process. Different molecular mechanisms have been proposed to explain the metastatic behaviour of solid tumors that infiltrate brain tissue; in this review the most recent findings concerning mechanisms and genes potentially involved in brain metastasis, that differ according to primary tumor types, will be discussed. The three tumors that more frequently develop brain metastasis, lung cancer, breast cancer and melanoma, will be considered and, in addition, the role of BBB and the process of endothelial to mesenchymal transition in cancer metastasis will be briefly described.

Key words: Brain metastasis; breast; epithelial-mesenchymal transition; lung; melanoma; micro-RNA

INTRODUCTION

The prognosis for cancer patients is strictly dependent on the metastatic behaviour of the tumor. Each tumor displays preferential sites where metastases more frequently develop and patients survival depends upon the possibility to perform surgery followed by oncological therapy and radiotherapy. Indeed, these approaches are usually sufficient to eradicate local oligometastases but unfortunately the picture is different in the case of brain metastases (BM), which are frequently associated with a poor prognosis.^[1] In the case of BM, stereotaxic radiosurgery is a useful tool to reduce local recurrence and achieve the same level of local control of whole-brain radiation therapy, with fewer side effects and comparable outcomes.^[2]

BM affect up to 40% of metastatic cancer patients^[1] and the

tumors that most often spread to the brain are lung cancer (30-50% of patients) and breast cancer (10-30% of patients), with melanoma ranking at the 3rd place (6-10% of patients).^[3] In many cases, the poor life expectancy associated with BM is due to other widespread metastasis but this is not true for melanoma patients, who very early display BM that make unsuccessful further therapeutic efforts.^[4]

Without treatment, median survival for a patient with BM is estimated to be about 3 months for a single lesion, although life expectancy has recently increased due to enhanced diagnostic tools that may even detect very tiny neoplastic formations.^[4]

Cancer cells traveling through the bloodstream eventually colonize a vascular place, by adhering to endothelial cells, or

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cross the brain barrier to begin the process that leads to the niche formation in the brain parenchyma. Thereafter cancer cells grow and invade the brain tissues by different mechanisms, such as expansive growth, multicellular migration and individual cell migration.^[5]

On the other side, the host tissue re-organizes its structure and vasculature recruiting stromal cells, such as fibroblasts, endothelial cells and tumor-associated macrophages, that sustain the tumor growth by producing and releasing extracellular matrix (ECM) proteins, cytokines and growth factors.^[6]

METASTASIS FORMATION: EPITHELIAL-MESENCHYMAL TRANSITION

The epithelial to mesenchymal transition (EMT), identified as one of the earliest steps of solid tumor progression, is associated with tumor growth, invasion, metastasis and contributes to the conversion of tumors from low- to high-grade malignancy.^[7] In epithelium-derived carcinoma, the EMT program induces a series of functional and structural changes aimed at the formation of tumor cells that will be able to invade surrounding tissues and lead to metastases formation. Invasion is a key step to progression toward a malignant phenotype and occurs when tumor cells translocate from the relatively constrained initial neoplastic mass into neighbouring host tissues. To accomplish this task, cancer cells must somehow detach from the primary tumor and migrate through the ECM, opening up the opportunity to penetrate the basal membrane surrounding a blood or lymphatic vessel, travel throughout the body via the circulatory system, and colonize distant sites to form metastatic foci. EMT is acknowledged to confer to cancer cells the molecular features required for these tasks. During EMT, in fact, epithelial cells undergo a developmental switch that results in decreased adhesion and loss of cell polarity, increased proliferation, motility and invasiveness; these changes are associated with the downregulation of epithelial cell surface markers and cytoskeleton components (E-cadherin, zonula occludens-1, claudins, occludins, cytokeratins) and the upregulation of mesenchymal markers (vimentin and α -smooth muscle actin) together with ECM components (collagens and fibronectin).^[8] Although the molecular changes that occur in cancer cells during EMT have been extensively documented, the molecular switches that turn on EMT still represent an open question and a crucial challenge because the possibility to inhibit this process could be of great importance in reducing metastatic spread. *In vitro* and *in vivo* model systems have identified several transduction pathways that lead to EMT and EMT-like phenotypes, many of which connect EMT to the ECM and the microenvironment surrounding tumors.^[9]

Among these pathways, integrins and transforming growth factor-beta (TGF- β) work synergistically to drive tumor cells towards EMT.^[9-11] TGF- β , in fact, is secreted as inactive precursor in a complex with 2 peptides, latency

associated peptide (LAP) and latent TGF- β -binding protein (LTBP). Its activation requires the dissociation from the complex, that may also be achieved by several integrins. The LAP peptides bound to TGF- β 1 and TGF- β 3 contain an Arginine-Glycine-Aspartate (RGD) motif that can be recognized by the RGD-binding integrins (α v β 3, α v β 5, α 5 β 1); this binding activates a driving force that leads to breaking of LAPs and LTBP binding to TGF- β and releases the active form of TGF- β .^[10,12] Integrin inhibitors with different molecular structures have been studied and are in clinical trials as anti-angiogenic agents or in support of other anti-cancer therapies, therefore integrin antagonists could represent a valuable and near-at-hand tool to inhibit the integrin dependent TGF- β activation and eventually reduce metastatic spread.^[13]

Lung, breast cancer and melanoma are tumors that display the EMT phenotype,^[14,15] and in lung cancer, the expression of markers of this transition has been associated with prognosis.^[16-18] This link between EMT and malignancy is further supported by the finding that, in other cancer types, EMT markers are overexpressed in 40% of tissue samples and are associated with vascular invasion and advanced clinical stage.^[19]

Once in the brain tissue, during reimplantation, the circulating tumor cells have been shown to undergo a mesenchymal to epithelial transition, thus reversing the EMT, to reacquire some of the original epithelial features necessary to survive in the new environment.^[20]

The knowledge of EMT mechanisms could clearly be an invaluable tool in defining molecular markers predicting tumors metastatic behaviour and prognosis and in identifying targets for new molecules that could inhibit the EMT process.

THE ROLE OF MICRO-RNAS

A number of recent studies has identified micro-RNAs (miRNAs) as key regulators of cancer cells survival and metastatic spread. Indeed, approximately 30% of human genes are likely to be regulated by miRNAs^[21] and miRNAs have been shown to regulate a variety of biological processes, including cell proliferation, cell differentiation and cell death.^[22] In this section miRNA involved in metastases, though with different molecular mechanisms are described.

miRNAs are an endogenous, highly conserved class of non-coding 20-24 nucleotides small RNAs that regulate gene expression at post-transcriptional level by binding to 3'-UTR of target mRNAs, thus leading to inhibition of mRNA translation and degradation.^[23] Several reports have elucidated the role of certain miRNAs as a class of oncogenes or tumor suppressors, depending upon their targeted genes.^[24] In addition, several studies have reported

that miRNAs genomic locations are frequently associated to genomic regions involved in cancer. It has been calculated that about 50% of known miRNAs are located inside or close to fragile sites in minimal regions of loss of heterozygosity, regions of amplifications and common breakpoints associated with cancer.^[25,26]

These studies indicate that miRNAs represent key players in cancer development and moreover, accumulating evidence demonstrates that miRNAs can also influence multiple steps of metastasis such as EMT, tumor cell migration, invasion and colonization.^[27]

The miR-200 family (miR-200a, miR-200b, miR-200c, miR-141 and miR-429) and miR-205 were the first group of miRNAs found downregulated in various tumors that underwent EMT progression. Members of the miR-200 family inhibit the EMT process by positively regulating E-cadherin expression through direct targeting of ZEB1 and ZEB2, transcriptional repressors of E-cadherin. Furthermore, expression of the miR-200 family in mammary carcinoma cells induced mesenchymal-epithelial transition by up-regulating E-cadherin expression and inhibited migration of these tumor cells,^[28] thus suggesting that downregulation of these miRNAs may be an important step in tumour progression.

miR-145 was found downregulated in several tumor types including breast, gastric, lung, ovary, prostate cancer and esophageal squamous cell carcinoma and notably, accumulating evidence indicates that the processing of miR-145 is also involved in cancer metastasis.^[22,29] In breast cancer, miR-145 suppress breast cancer cell line invasion and metastasis by targeting mucin-1, a glycoprotein that can help tumor cells to escape immunosurveillance;^[30] in addition, miR-145-dependent regulation of 3'UTR of the JAM-A and fascin decreased motility and invasiveness of MDA-MB-231, MCF-7 and other breast cancer cells.^[31]

miRNA analysis could also be instrumental for prognostic purposes: in a retrospective study on 256 melanoma patients, divided into three cohorts, four miRNAs (miR-150-5p, miR-15b-5p, miR-16-5p and miR-374b-3p) were identified as a prognostic signature that, in combination with stage, was able to distinguish primary melanomas that metastasized to the brain from non-brain metastatic primary tumors.^[32] Although at the present time the biological significance of these miRNAs dysregulation may be difficult to understand, nevertheless the notion, together with classical staging parameters, could be of great importance to clinicians to set specific therapeutic strategies.

Interestingly, miRNA can also modulate gene expression in adjacent cells within the microenvironment and even in distant cells, since miRNAs have been detected in the blood and in other body fluids;^[33] indeed, circulating miRNAs, extra-cellular vesicles and exosomes-associated miRNA

are extensively studied as potential biomarkers in different cancer types.^[34]

Exosomes are 40-100 nm vesicles secreted by a wide range of mammalian cell types, including cancer cells.^[35] miRNAs shuttled by exosomes involved in cancer metastases have been found to be implicated in angiogenesis and tumor niche formation.^[36,37]

Recently, an interesting study reported a new and unexpected mechanism by which a miRNA contributes to metastatic spread in the brain; the miR-181c contained in cancer-derived extra-cellular vesicles, carrying proteins and miRNAs, promotes the destruction of blood brain barrier (BBB) through delocalization of actin fibres via the downregulation of 3-phosphoinositide-dependent protein kinase-1 *in vitro* and *in vivo*.^[38] The breakdown of BBB triggered by miR-181c can easily open the way to brain parenchyma to circulating cancer cells.

A very recent study has demonstrated that miRNAs contained in exosomes released by human and mouse tumors that metastasize to lung, liver or brain, like breast cancer, trigger cellular changes in target organs by promoting the formation of tumoral niche and organ-specific invasion. The organ sites where tumor derived exosomes take contact and release their content is related to integrins expressed on the exosomes surface: $\alpha 6 \beta 4$ preferentially interacts with lung cells, $\alpha v \beta 5$ mediates exosomes delivery to liver.^[39]

These new findings suggest that the exosome miRNAs content and integrin expression can be useful to predict the tendency of primary tumors to metastasize and to determine the preferential organ sites of future metastases; in addition, this evidence highlights the role of integrins as potential valuable targets to inhibit exosomes interactions with metastatic sites.

CROSSING THE BBB

The key step during BM formation is the migration of cancer cells through BBB. Anatomically, the BBB is formed by brain microvascular endothelial cells (BMVECs), that form tight junctions without pores, and perivascular elements including pericytes, astrocytes, oligodendrocytes and the basement membrane. This complex structure represents a physical barrier for cells and molecules, selected on the basis of their molecular weight and charge. In addition, this barrier regulates the diffusion processes and the brain parenchyma homeostasis by highly selective transport mechanisms mediating flux of solutes and molecules and by a metabolic barrier consisting of highly specific enzymes.^[40]

Tumor cells recognize and bind to components of the vascular membrane, thereby initiating extra-vascularization and promoting the formation of the tumoral niche that will host the new neoplastic formation. The brain vascular

endothelium is therefore very important in counteracting cell extra-vasation but, nevertheless, cancer cells adopt different strategies to overcome this obstacle. Although the exact molecular mechanisms that trigger BM are still poorly understood, increasing evidence are shedding new light on the processes underlying the ability of cancer cells to cross the BBB.

In a transendothelial migration model, highly metastatic melanoma cells migration has been found to be mediated by interaction of the $\alpha 4 \beta 1$ integrin with its ligand vascular cell adhesion molecule-1 (VCAM-1) on the surface of activated endothelial cells. VCAM-1 is expressed by endothelial cells only upon activation by inflammatory stimuli like TNF- α or interferon- γ , suggesting that highly metastatic melanoma cells preferentially leave the blood vessels at sites of inflammation.^[41]

In a very similar experimental model, the matrix metalloproteinase 1 (MMP1) was found to play a critical role in BBB penetration; in parallel experiments cyclooxygenase-2 (COX2)-mediated prostaglandin synthesis promotes proliferation of tumor initiating cells by activating tumor-associated astrocytes followed by secretion of the chemokine CCL7.^[42]

The process of transendothelial migration of melanoma cells has been further investigated by other *in vitro* studies showing that the ability of these cells to cross the BBB is related to melanotransferrin expression levels on the cell surface, to the fibrinolytic system and to serine proteases released by melanoma cells.^[43-45]

This accumulating evidence indicates that inflammatory stimuli contribute to the formation of breaches in BBB and of a suitable surrounding in the brain parenchyma for cancer cells.

However, in contrast with these findings, other *in vivo* studies suggest that transendothelial cancer cells migration does not necessarily imply a damage to vascular endothelial cells: metastatic breast cancer cells, in mice, were found to cross the endothelium in correspondence of sites where the vessel wall shows discontinuity sites without causing apoptosis or hypoxia in endothelial cells.^[46]

Another interesting *in vivo* study demonstrated by multiphoton laser scanning microscopy that in the mouse brain the essential steps in melanoma and lung cancer metastasis formation were first the arrest at vascular branch points and after extra-vasation, perivascular growth in close contacts to microvessels.^[47]

In this scenario, the interactions of metastatic tumor cells with BMVECs appear to be regulated by a number of effectors and mediators and represent a key step of metastasis formation; however, the cellular mechanisms that lead to BBB extra-vasation appear to be strictly related

to cancer cells features and therefore linked to the primary tumour characteristics.^[48]

Two very recent studies have demonstrated that meningeal lymphatic vessels are present in mouse central nervous system (CNS) and display all the classical features of lymphatic vessels.^[49,50] These findings have highlighted a new path for cerebrospinal fluid flux and for immune cells leak, opening interesting avenues for future researches on BM formation.

LUNG CANCER

Lung cancer is the leading cause of cancer-related deaths worldwide and is characterized by rapid progression and metastases to brain that develop within months of diagnosis and simultaneously affect different organs besides the brain.^[51] Lung cancer is classified into two broad histological sub-types: Non-small-cell lung cancer (NSCLC), representing about 85% of diagnoses, and SCLC, accounting for the remaining 15%; NSCLC is further classified into adenocarcinoma, squamous-cell carcinoma, and large-cell carcinoma.^[52] SCLC and NSCLC are traditionally considered as different cancer types but increasing evidence supports the notion that the two histological sub-types can coexist. This mixed histology reinforces the hypothesis of common mutated precursors for the two cancer types thus complicating prognosis and therapy.^[52]

Although several mechanisms concerning lung cancer cells survival strategies have been elucidated, the early molecular processes leading to BM are still poorly understood.

In SCLC patients BM are associated with poor prognosis. Previous evidence indicated that attachment to brain microvasculature represented the first step for tumor cell extra-vasation and growth.^[53] In particular, it was demonstrated that the interaction of SCLC cells with human BMVECs triggers the disassembly of tight junctions between brain endothelial cells and contributes to SCLC cells transendothelial migration,^[54,55] thus suggesting that brain microvasculature and mechanisms that regulate cell-cell adhesion are likely to play an important role in SCLC metastasis to brain. An intriguing mechanism was highlighted in a study reporting that the interaction of SCLC cells with BMVECs induces tumor cells to secrete annexin A1 into tumor metastatic microenvironment. The secreted annexin A1, previously reported to be upregulated in human lung cancer and to be related to poor prognosis, in turn promoted SCLC cells adhesion to brain endothelium and transendothelial migration.^[56,57]

Other studies have shown that SCLC cells are abundantly surrounded by ECM components, including collagen IV, tenascin, fibronectin and laminin; high expression of these components is associated with a poor prognosis.^[58]

Adhesion of SCLC cells to the ECM components requires β 1-integrins, whose activation results in suppression of chemotherapy-induced apoptosis by stimulation of the PI3K-dependent pathway.^[59] Thus, ECM via β 1 integrin-mediated PI3K activation confers to SCLC resistance to apoptosis, allowing SCLC cells to survive even in presence of DNA damage.

Taken together, this evidence indicates that adhesion processes play important roles in SCLC cells survival strategies linked to metastasis and furthermore suggest that interference with adhesion molecules or receptors could be an interesting topic for future researches.

Approximately 40% of all NSCLC patients suffer from BM. The prognosis of patients with BM of NSCLC is remarkably poor, with a median survival time of 1-2 months for untreated patients and 6 months for those receiving surgery, radiotherapy and chemotherapy.^[60] While SCLC metastatic brain tumors do not respond to systemic chemotherapy and poorly respond to molecularly targeted therapies,^[58] NSCLC patients frequently display activating epidermal growth factor receptor (EGFR) mutations.^[61] Complete and partial response rates to tyrosine kinase inhibitors have been recorded in clinical studies with gefitinib and erlotinib^[62,63] and these treatments improved overall survival (OS) rates. However, other genes or genetic alterations have been reported to be involved in BM of lung cancer.

An interesting study performed by microarray in lung adenocarcinoma and squamous cell carcinoma samples has shown different expression profiles of EMT-related genes in primary tumors compared to tumor-derived BM.^[64] In particular, BM had significantly lower integrin α β 6 and N-cadherin expression than the primary tumors, thus supporting the hypothesis that the disseminated tumor cells, deriving from primary tumors with marked mesenchymal features, once inside the brain, undergo the reverse process of EMT called mesenchymal-to-epithelial transition.

Gene expression profiles of miRNAs in lung cancer, aimed at identifying molecular markers as predictor of patient survival, identified several miRNAs targeting genes involved in crucial pathways such as the EGFR- and KRas-dependent pathways.^[65,66]

miR-145, a miRNA involved in metastatic spread in several cancer types and discussed above, has been found to be downregulated in the BM compared to primary lung adenocarcinoma samples and its upregulation in lung adenocarcinoma cells suppresses proliferation of tumor cells.

The mechanism by which miR-145 causes these latter effects was hypothesized to be the targeting c-Myc, EGFR and NUDT1;^[67] however, *in vitro* invasion assays did not confirm that upregulation of miR-145 was implied in lung adenocarcinoma cancer cell migration and invasion.

The miR-145 expression levels were not significantly different between primary lung adenocarcinoma samples with and without lymph node involvement^[67] and recent studies have found a downregulation of miR-145 expression in lung cancer primary tumors and BM. Silencing of miR-145 was found to contribute to BM via downregulation of the fascin homolog 1 (FSCN1) protein, an actin-binding protein involved in cell migration, and upregulation of miR-145 target protein, such as EGFR, OCT-4, MUC-1, c-MY,^[68,69] that are involved in cell proliferation and survival.

miR-328 has been associated with NSCLC BM and mediates NSCLC migration. In patients with BM, the elevated expression of miR-328 in both primary and brain metastatic NSCLC samples suggests that this miRNA may be involved in driving the access of metastatic cells to the brain. In agreement with this finding, *in vitro* miR-328 overexpression in A549 and H1703 cells was shown to increase cell migration.^[70]

Another class of RNAs termed long non-coding RNAs (lncRNAs) appear to play a role in lung cancer metastasis spread. lncRNAs are a class of non-protein coding transcripts, longer than 200 nucleotides, associated with the progression of cancer. Some members of the lncRNAs family are involved in metastases formation such as the metastasis-associated lung adenocarcinoma transcript 1 (MALAT1), HOX anti-sense intergenic RNA, and anti-sense non-coding RNA in the INK4 locus.^[71] In lung cancer cells MALAT-1 was found to enhance cell motility by modulating the expression of motility-related genes^[72] and to promote lung cancer BM by inducing EMT in both *in vitro* and *in vivo* NSCLC models.^[72]

Another documented mechanism involved in BM formation is represented by integrin receptors activation. Several studies that have investigated α β 3, α β 5 and α β 6 expression in BM and corresponding primary tumors^[73,74] found that α β 3 activation strongly promotes metastatic growth in the brain by inducing endothelial cell proliferation and network formation.^[75]

Interestingly, a recent study performed on formalin fixed paraffin-embedded human primary NSCLC and BM specimen showed that expression of α β 3, α β 5 and α β 6 integrins is associated with pathological parameters such as enhanced tumor cell proliferation index and increased hypoxia-inducing factor (HIF-1 α) expression. Moreover, α β 3 and α β 5 were mainly expressed on proliferating endothelium of sprouting vessels, in agreement with previous observations that have hypothesized their involvement in neovascularization.^[76]

Among factors that stimulate vascular proliferation and vessel formation, MMP have been shown to promote endothelial cell migration and induce vascular endothelial growth factor (VEGF) release, leading to development of

angiogenic vasculature.^[77] In agreement with these findings, in lung carcinoma BM a correlation between MMP2 and angiogenesis was also found.^[78] In this study tumors expressing MMP2 display a more proliferating vasculature at the tumor-brain interface compared to MMP2-negative tumors, suggesting that MMP2 expression may be a key player in this process by enhancing both invasion and vascularization.

Fibroblast growth factor receptor 1 (FGFR1) signaling has repeatedly been described as a critical permissive factor for distant spread of cancer cells through induction of EMT, interaction with neural cell adhesion molecule neural cell adhesion molecule and N-cadherin or upregulation of osteopontin and matrix metalloproteases.^[79] FGFR1 amplifications are common in squamous cell carcinoma and rare in adenocarcinoma of the lung but a recent study found enrichment of FGFR1 amplifications, not related to patients survival, in BM of NSCLC and adenocarcinomas (5-fold more frequent than in primary tumors) suggesting a specific role of FGFR1 in metastasis formation.^[79]

In order to identify new molecular features associated to BM formation, chromosomal copy number alterations in NSCLC samples was performed; selectively amplified regions of primary lung adenocarcinomas (5q35, 10q23 and 17q23-24) were identified as significantly associated with the development of early BM within 3 months after first diagnosis of primary tumors. Interestingly, those regions were found to contain putative metastasis promoting genes, such as *NeurLIB*, *ACTA2*, *FAS* and *ICAM2*,^[80] but the biological significance of these amplifications still remains to be elucidated.

BREAST CANCER

Breast cancer types are routinely classified on the basis of clinical parameters (age, lymph node status, tumor size, histological grade) and pathological markers that usually direct clinicians for the therapy [estrogen receptor (ER), progesterone receptor (PR), human epidermal growth factor receptor 2 (HER2)]. During the last 15 years, 5 sub-types of breast cancer have been identified, on the basis of molecular markers: luminal A, luminal B, HER2-enriched, basal-like and claudin-low.^[81]

In patients with breast cancer BM are less common than bone or visceral metastases and frequently represent a late event; nevertheless, up to 16% of metastatic breast cancer patients develop clinically significant BM while autopsy studies showed that up to 30% of patients actually develop brain disease.^[3,82]

Several risk factors have been associated with the development of BM in patients with metastatic breast cancer, particularly the young age (35 or younger), HER2-enriched sub-type and triple-negative breast cancer (ER⁻,

PR⁻, HER2⁻).^[83] Patients with HER2-positive metastatic breast tumors are 2-4 times more likely to develop CNS tumors than patients with HER2-negative disease^[82] and patients with triple negative breast cancer and basal like breast cancer (BLBC) also appear to be at a high risk for developing BM.^[84]

The *HER2/neu* gene is amplified in 20-25% of primary breast cancer cases; however, gene expression profiles can vary between the primary tumor and metastatic formations and therefore it could not be correct to assume that the HER2 status of the metastatic tumor reflects that of the primary tumor.^[85] Biopsies of metastases could provide essential informations in the case of HER2 expression discordance, thus redirecting therapeutic strategies by clinicians. It was found that loss of HER2-positive status in metastatic tumors from patients with primary HER2-positive breast cancer is related to therapeutic treatments with chemotherapy with or without trastuzumab and in addition, patients with HER2 discordance between their primary and metastatic tumors have shorter OS.^[85]

Another study found that 243 genes were up or down-regulated in brain metastatic cell lines, compared to the primary tumor derived cell line^[86] and that the expression of 17 genes was correlated with brain relapse. Interestingly, the expression of these 17 genes in breast tumors was not associated with relapse to bones, liver or lymph nodes and the association with brain relapse was significant within ER-tumours and in patients who received no adjuvant therapy. A sub-set of these 17 genes that includes prostaglandin-synthesizing enzyme COX2, collagenase-1 (MMP1), angiopoietin-like 4, LTBP1 and FSCN1, the putative metastasis suppressor retinoic acid receptor responder three and heparin-binding EG plays fundamental roles in cell extra-vasation and invasion and in general, in supporting cancer cells migration and survival.^[87-91]

Among the genes upregulated in breast cancer BM, an important role is also played by the α 2,6-sialyltransferase (ST6GALNAC5) because its mRNA levels were found to be notably higher in brain metastatic cells than in parental, primary tumor derived cell lines.^[86] Sialyltransferases are a family of at least 18 different intra-cellular Golgi membrane-bound glycosyltransferases that catalyse the addition of sialic acid to gangliosides and glycoproteins. Cell-surface sialylation has been implicated in cell-cell interactions^[92] and metastatic cells overexpressing the ST6GALNAC5 messenger, compared to the parental cell lines, show a more marked adhesive behaviour to monolayers of human primary brain endothelial cells. Conversely, ST6GALNAC5-knockdown decreased the brain metastatic activity of BM derived cells.^[86]

Another gene implicated in BM is hexokinase 2 (HK2). HK2 is one of four members of the HK family that includes HK1, HK2, HK3 and Glucokinase, enzymes involved

in glycolysis by phosphorylating glucose to produce glucose-6-phosphate. A microarray study, comparing gene expression profiles of BM and primary breast tumors, found an overexpression of HK2 in BM.^[93] HK2 is overexpressed in several cancer types, compared to normal tissues, and its overexpression is generally related to a poor prognosis;^[94] its upregulation in BM suggests that it could be instrumental for cell growth under conditions of limited nutrient availability.

Also, in a recent work 86 matched BM and primary tumors were analyzed by whole-exome sequencing and the authors found that metastatic samples, though showing common features with the primary counterpart, display alterations particularly related to PI3K/AKT/mTOR, CDK, and HER2/EGFR cascade.^[95]

Another gene that appears to have a role in the metastatic behaviour of breast cancer is the Forkhead-box transcription factor C1 (FOXC1), essential for mesoderm tissue development and highly expressed in the basal-like (BLBC) and in the triple-negative breast cancer. Overexpression of FOXC1 in BLBC cells and in MCF-7 cell line increases cell proliferation, migration, invasion and anchorage-independent growth of MCF-7 cells in soft agar.^[96] The mechanism underlying FOXC1-mediated invasive behaviour is the induction of MMP7 expression in breast cancer cells and interestingly, both FOXC1 and MMP7 are overexpressed in BLBC samples, suggesting a possible new molecular target for BLBC therapy.^[97]

Finally, an analysis of circulating tumor cells (CTCs) from breast cancer patients demonstrated that CTCs, circulating as single cells or as clusters bound to platelets, express EMT markers such as TGF- β and FOXC1, thus supporting the role of EMT in metastatic cells and indicating FOXC1 as a reliable peripheral marker of breast cancer dissemination.^[98]

MELANOMA

Malignant melanoma is a frequently lethal malignant tumor that accounts for 4% of all skin cancers but it is responsible for 80% of skin-cancer deaths.^[99] BM are a frequent complication in melanoma patients, and unlike in other solid tumors, arise independently from other visceral metastasis. Many melanoma patients are cured after excision of the primary tumor but, in some cases, a disease recurrence appears in different sites as metastatic lesions^[100] suggesting that melanoma cells had already spread before excision of the primary tumor.

Melanomas are classified into four major sub-types according to their histological features: lentigo maligna melanoma, superficial spreading melanoma, acral lentiginous melanoma and nodular melanoma.^[101] A series of parameters are usually taken into account for patient prognosis: tumor thickness, tumor location, histological sub-type and ulceration. Melanoma classification based on

genetic analysis are instrumental for prognosis and targeted therapy;^[102] for example, mutations of *BRAF*, particularly the V600E and V600K mutations, have been identified both in benign melanocytic proliferations and in all stages of metastatic melanoma, with the frequency of 36-45% *BRAF* mutations in primary melanomas and 42-55% in metastatic melanomas. The presence of a *BRAF* mutation in patients with primary melanoma appears to be related to the OS and to a worse prognosis compared to patients who lack the mutation.^[103] Nearly 50% of melanoma BM display V600 *BRAF* mutation^[104] and the analysis of *BRAF* alterations in melanoma BM is of critical in the selection of patients for targeted therapy with specific inhibitors.

In a very recent and extensive study the Cancer Genome Atlas program performed a systematic characterization of 333 cutaneous melanomas at the DNA, RNA and protein levels with the specific goal to create a catalog of somatic alterations with important and potential implications for prognosis and therapy.^[105]

The first step of metastasis formation, before detachment from the primary tumor, is supposed to be represented by EMT. During this step cancer melanocytes change their adhesion properties and modify their gene expression profiles that results in changes in the amount of integrins and cadherins at protein levels,^[106] associated to an increased expression of EMT markers such as SNA1 (Snail and twist), Wnt, Notch, SPARC and Hedgehog.^[107] Early-stage melanocytes express CDH5/non-epithelial cadherin^[107,108] that leads to the loss of epithelial adhesion properties and to gain of mesenchymal progenitor cells features.

Melanoma metastatic cells are driven to lymph or blood vessels by concentration gradients of cytokines, chemokines, and growth factors.^[109] Like in other metastatic tumors, in the bloodstream most cancer cells undergo anoikis but a sub-set acquires some genetic modifications that confer survival advantages such as anoikis resistance. Deregulated activation of the PI3K/Akt pathway, in particular the increased phosphorylation of Akt3, confers resistance to anoikis to melanoma cancer cells and like in other metastatic tumors, loss of phosphatase and tensin homologue contributes to the Akt pathway deregulation related to the tumor malignancy.^[110,111]

The circulating melanoma cancer cells that have acquired the ability to survive in the circulatory system may also form microaggregates with platelets or leucocytes and travel protected in bloodstream.^[15] These microemboli, once finding a niche in very small size capillaries, promote extra-vasation in tissues displaying the appropriate feature such as brain parenchyma.

It has been found that extra-vasation of melanoma circulating cells is prompted by interleukin-8 (IL-8) secretion by melanoma cells and IL-8 summons neutrophils

to establish a connection between neutrophils and molecular cocrystals through interaction of ICAM-1 protein and $\beta 2$ integrin.^[112] Subsequently, the neutrophil-melanoma cell complex binds to endothelial cells of capillaries to promote brain tissue invasion and metastasis growth.

Neoangiogenesis, prompted by VEGF release and increase in levels of HIF-1 α , is another necessary step for the tumor growth that regulates tumor cell-microenvironment interactions.^[112]

Finally, in the brain, astrocytes forming and surrounding the tumoral niche may play a protective role towards the tumor cells growth, including melanoma BM, by priming reactive astrocytosis or protecting tumor cells from cytotoxicity induced by chemotherapeutic drugs.^[113]

CONCLUSION

The emerging picture depicted here appears quite complicated and the genes, with the related cellular mechanisms, that have been found to be involved in BM carry out a number of different but still interrelated functions. In addition, a plethora of different cell types like platelets, leukocytes, endothelial cells and astrocytes cooperate all together to sustain the survival of metastatic cells in the blood flow and in the brain parenchyma.

However, although the intrinsic complexity of BM appears to be a daunting task, recent findings may boost the efforts in the field. The development and refinement of existing *in vitro* three-dimension models of BBB, traditionally employed in the screening of drugs or molecule designed to cross the BBB, could be used as a novel approach to investigate the genotype and phenotype of cancer cells that migrate through artificial BBBs.^[114,115]

Also, the previous mentioned breakthrough discovery of the presence of lymphatic vessels in the brain could open an avenue of cutting edge experimental approaches in the study of CTCs and BM.

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Effects of Gas1 on gliomas: a review on current preclinical studies

Jose Segovia, Elizabeth Bautista, Manuel Lara-Lozano

Departamento de Fisiología, Biofísica y Neurociencias, Centro de Investigación y de Estudios Avanzados del IPN, 07360 Distrito Federal, Mexico.

Correspondence to: Dr. Jose Segovia, Departamento de Fisiología, Biofísica y Neurociencias, Centro de Investigación y de Estudios Avanzados del IPN, 07360 Distrito Federal, Mexico. E-mail: jsegovia@fisio.cinvestav.mx

ABSTRACT

Glioblastoma multiforme (GBM) is the most common and lethal brain tumor. Its prognosis remains very poor, despite the use of combined treatments such as surgical resection, radiation and chemotherapy. The major limitations for the treatment of GBM are its high invasiveness, tumor recurrence and resistance to treatments. Therefore, gene therapy appears as a relevant strategy for its treatment. Thus, we have investigated the use of growth-arrest-specific 1 (Gas1) for the treatment of GBM. Gas1 is a tumor suppressor protein that inhibits glioma growth by inducing arrest and apoptosis of tumor cells. Moreover, we have shown that a soluble form of Gas1 acting in both autocrine and paracrine manners is also effective inhibiting tumor growth in animal models, indicating its potential as an adjuvant for the treatment of GBM.

Key words: Growth arrest specific 1; glioma; serine-threonine protein kinase; glial cell-derived neurotrophic factor; extracellular signal-regulated kinases and tumor

INTRODUCTION

Gliomas are the most frequent and aggressive tumors of the central nervous system (CNS) and current treatments have not improved their prognosis. In children and adolescents, tumors of the CNS are the most common and lethal;^[1] and glioblastoma multiforme (GBM) is the most frequent malignant primary brain tumor.^[2-5] Gliomas are the main neuroepithelial tumors of the CNS that originate from mature or precursor ectodermal-derived glial cells. The World Health Organization (WHO) has classified gliomas on 4 grades from I to IV (GI-GIV) according to the histological dedifferentiation and the expression of the KI-67 protein, which indicates the rate of proliferation. WHO-GI gliomas are considered as benign tumors, since the malignant features are only present on low-grade (WHO-GII) and high-grade (WHO-GIII and -GIV) gliomas; in this respect, WHO classification correlates with the prognosis of the patient, regardless of multimodal therapeutic treatments;^[6,7] the 5-year life span rates after diagnosis are 50%, 30% and 5% for WHO-GII, -GIII and -GIV glioma patients, respectively.^[8]

Tumors of glial origin are considered gliomas and are divided into: astrocytoma, oligodendroglioma, ependymoma, mixed gliomas and not otherwise specified.^[2,3] On the other hand, The Cancer Genome Atlas (TCGA) designed a sub-classification of GBM based on their molecular signature [Table 1], which comprises: classical, mesenchymal, proneural and neural tumors.^[8,9] Classical GBM are tumors that present high expression of the epidermal growth factor receptor (EGFR) and absence of tumor suppressor proteins such as p16 and p14. In mesenchymal GBM, the phosphatase and tensin homolog (PTEN) gene is mutated and loss of its activity leads to activation of the serine-threonine protein kinase (AKT) survival pathway. Additionally, this subtype expresses chitinase 3-like-1 and MET transcripts characteristics of mesenchymal cells and shows low expression of the transcript for the tumor suppressor protein neurofibromin 1. On the other hand, proneural GBM expresses oligodendrocyte transcripts NK2 homeobox 2 and oligodendrocyte transcription factor (OLIG2), as well as high levels of platelet-derived growth factor (PDGF) receptor, alpha polypeptide, and mutations of the dehydrogenase 1 and/or P53 genes. The neural GBM is the most dedifferentiated subtype because it expresses

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Table 1: Subclassification of GBM based on their molecular signature^[9]

GBM subclassification	Genes altered	Signal pathway	Status	Physiological activity
Classical	<i>CDKN2A</i> (p14/p16)	RB	Homozygote deletion	Cell cycle: G1/S transition
	<i>EGFR</i>	EGF/TNF- α	Overexpression and mutations	Cell Cycle
	<i>NESTIN</i>		Overexpression	Neural stemness
	<i>NOTCH3, JAG1, LENG</i> <i>SMO, GAS1, GLI2</i>	NOTCH SHH		
Mesenchymal	<i>NF1</i>	AKT	Deletion and/or mutation	Survival and proliferation pathways
	<i>PTEN</i>		Mutation	
	<i>TRADD, RELB, NFRSF1A</i>	NF- κ B/TNF superfamily	Overexpression	Cell stress response
	<i>CH3IL1, MET</i>		Expression	Mesenchymal transition
Proneural	<i>PDGFRA</i>	PDGF	Overexpression and mutation	Cell cycle and angiogenesis,
	<i>IDH1</i>		Mutation	cytoplasmic NADPH production
	<i>PIK3CA/PIK3R1</i>	AKT	Mutation	Survival and proliferation pathways
	<i>P53</i>	P53	Mutation	Cell cycle: G1/S transition
Neural	<i>CDKN1A</i> (p21)		Low expression	
	<i>DCX, DLL3, ASCL1, TCF4</i>	SOX	Overexpression	CNS cell fate determination
	<i>NKX2-2</i>			
	<i>OLIG2</i>			
	<i>NEFL</i>		Expression	Neuronal markers
	<i>GABRA1</i> <i>SYTI</i> <i>SLC12A5</i>	GABA		

neuronal markers such as: negative regulatory factor (NEFK), γ -aminobutyric acid A receptor A, synaptotagmin 1 and the symporter K⁺: Cl⁻ (SLC12A5).^[8,9] The variability of the molecular signature of GBM suggests that the characterization of gliomas must be analyzed to devise specific treatments.

CLINICAL MANAGEMENT OF GLIOMAS

Advanced anaplasia in high-grade gliomas difficults the complete surgical resection of the tumor; as a consequence, tumor recurrences are unavoidable. On the other hand, postoperative radiotherapy has been the standard treatment for GBM. However, the survival after radiation is low and overall survival remains poor.^[10-12]

Concomitant and adjuvant chemotherapy for high grade gliomas include alkylating agents that damage DNA, such as: carmustine, procarbazine, lomustine, vincristine and temozolamide (TMZ). Recently, it has been reported that both bevacizumab and cediranib prevent angiogenesis by inhibiting the vascular endothelial growth factor (VEGF) signaling pathway.^[11-13] Moreover, TMZ is the drug of choice for the treatment of high-grade glioma. TMZ alkylates guanine, inducing the methylation of gene promoters and leading to apoptosis, when the mismatch repair system is intact.^[14,15] This drug is well tolerated by most patients, furthermore it has a favorable safety profile

that is associated with only mild side-effects compared with nitrosoureas.^[10,16] The addition of chemotherapy to standard postoperative radiotherapy improves in 2.5 months the median survival relative to postoperative radiotherapy alone.^[10-12] To enhance the effect of TMZ it has been proposed the use of lipid-based nanoparticles, which cross the blood brain barrier more efficiently causing an increment of brain levels of TMZ and reducing the adverse effects in other organs such as the heart and kidneys.^[16-18] Despite the above GBMs that express high levels of O⁶-methylguanine DNA methyltransferase (MGMT) protein are resistant to TMZ chemotherapy.^[19-22] Small molecule inhibitors of MGMT exist, but their use in combination with TMZ is limited due to toxicity to peripheral organs.^[23] Furthermore, the mutation in the mutS homolog (MSH) 6 mismatch repair gene facilitates resistance to TMZ and recurrence of GBM.^[14] Until now, the surgical approach is still the most effective measure to treat gliomas, followed by radiotherapy and chemotherapy; however the clinical prognosis of the patients remains very poor. Therefore, new strategies and therapeutic agents should be investigated, based on the molecular characteristics of gliomas.

MOLECULAR APPROACH AGAINST MULTI-RESISTANT GLIOMAS

Resistance to various treatments and the recurrence of tumors has been attributed to the presence of a subpopulation

of cells in gliomas with properties of stem cells, known as glioma-initiating cells (GICs). These cells express neural stem cell markers such as NESTIN, OLIG2, sex determining region Y-box 2 (SOX2) and fucosyltransferase 4.^[20,24,25] The classification of GICs is based on the expression of prominin-1 (CD133); the CD133⁺ GICs are more invasive than those that do not express the antigen, and constitute 3-29% of the glioma mass.^[26,27] Also, GICs from secondary cultures conserve the characteristics of the original tumor, even after several passages.^[28] Self-renewal, aggressiveness and stemness of GICs are associated with the expression of Cyclin E and proteins of the family of inhibitor of DNA-binding/differentiation proteins, increased activity of several signaling pathways including: transforming growth factor (TGF)- β ; protein kinase A and jagged-NOTCH;^[26,29] as well as the activation of the receptors for PDGF, epidermal growth factor (EGF) and fibroblast growth factor (FGF).^[26] Surprisingly, CD133⁺ GICs also contribute to malignant transformation of the adjacent normal glial cells through paracrine activity of PDGF- α and stimulate angiogenesis by activation of the NOTCH signaling pathway on endothelial tissue.^[26,28,30]

It is noteworthy that CD133⁺ GICs are predisposed to become resistant to chemo- and radiotherapy with respect to non-GICs cells, implicating the activation of extracellular signal-regulated kinases 1 and 2 (ERK1/2).^[20,31,32] In this context, the use of classical therapies against gliomas, facilitate the selection of multi-resistant GICs, leading to the formation of more aggressive tumors, resistant to chemo- and radiotherapy.^[33] Moreover, *in vitro* and *in vivo* studies have shown that with the adequate stimulus, GICs differentiate to either neuronal or astrocytic cells, however many of these responses are deregulated in gliomas.^[31]

Currently, the analysis of protein expression and of the transcriptome, including micro-RNAs (miRNAs), has helped to uncover molecular markers involved in the susceptibility or resistance to treatments. Indeed, differential expression patterns of miRNAs have been reported in high-grade gliomas.^[34] Even now, the association of miRNAs with the methylation of the MGMT promoter is still controversial since miRNAs are not considered as direct epigenetic regulators.^[21,35] However, overexpression of miR-222, -145 and -132 is related with TMZ resistance coupled with MGMT promoter methylation.^[35] Additionally, miR-181b and -181c are downregulated in patients that responded to radiotherapy and concomitant TMZ.^[36] However, sensibility to chemotherapy is not only dependent of the presence of MGMT.^[37,38]

Screening of molecular changes after radiotherapy showed overexpression of miR-1, -125a, -144, -150, -151-5p, -221/22, -425 and -1285.^[39-42] The ectopic expression of miR-1, -125a, -150 and -425 increases cell survival and confers radioresistance through the induction of the cell-cycle.^[39] On the other hand, TGF- α and - β , and the EGFR

also contribute to radioresistance in classical-GBM.^[43] Thus, inhibiting TGF- β has been proposed as a treatment since it induces radiosensitivity in gliomas through decreasing the expression of miR-1 and -125a.^[23,39,44] Additionally, miR-221/222 downregulate PTEN, leading to activation of proteins that promote cell proliferation or prevent cell death, such as: AKT, B-cell lymphoma 2 (Bcl-2), Cyclin-D, matrix metalloproteinase 2 and 9,^[41,45,46] Interestingly, the phosphorylation of AKT is the main mechanism for developing radioresistance, regardless of the activity of its negative regulator PTEN.^[40,41] In summary, molecular analysis could help to reconsider whether conventional treatments are suitable, or therapeutic modifications should be adapted to the requirements of the patient.

GROWTH-ARREST-SPECIFIC 1

The balance between proliferation or growth arrest is regulated by several extrinsic and intrinsic factors. Cells can exit the cell cycle and enter in the non-proliferative phase, known as the G0 phase. Particularly, in this phase six genes named growth-arrest-specific (*Gas*) genes are expressed, from 1 to 6.^[47] The *gas1* transcript is the most abundant in NIH3T3 cells arrested in the G0 phase by deprivation of serum or high cell density.^[47-49] *Gas1* induces growth arrest by inhibiting DNA synthesis in NIH3T3 cells when it is ectopically expressed.^[49]

TRANSCRIPTIONAL AND TRANSLATIONAL REGULATION

Human and mouse *gas1* genes are located in the long arms of chromosome 9 (9q21.3-q22)^[50,51] and chromosome 13^[52] respectively, with 77.04% of homology between them. *Gas1* is an intronless gene, suggesting that it probably originated from a retrotransposon.^[53]

There are few studies about the regulation of the *gas1* gene, however it has been reported that Menin and Myb-like (coded by the *men1* and *dmp1* genes respectively), induce the transcriptional repression of *gas1*.^[54,55] Also, c-Myc and Src repress *gas1* transcription, since they facilitate re-entering to the cell cycle.^[56,57] c-Myc protein requires the Myc-Box2 domain to be present on the N-terminus to repress the transcription of *gas1*. Furthermore, the basic Helix-loop-Helix leucine zipper domain located at the C-terminus of c-Myc is also necessary to induce the transcriptional repression of *gas1*, perhaps together with an accessory protein not yet identified.^[56,58] Both c-Myc and Src are key components for the proliferation, growth, and survival of glioma cells. The expression of c-Myc closely correlates both with cellular dedifferentiation and the grade of malignancy,^[59-61] since its activity induces the transcription of cyclin D1 and repression of the p21^{WAF1/CIP1} cyclin-dependent kinase inhibitor.^[62] Interestingly, the histone chaperone, Facilitate Chromatin Transcription protein complex (FACT) increases the transcription of *Myc*, a recent report showed that the downregulation or inhibition

of FACT decreased the formation of metastasis and delayed tumor growth, it also proved to be an excellent cytotoxic adjuvant.^[63] Thus, these data suggest that the inhibition both of FACT and Myc, could increase the anti-tumoral effect of Gas1. Moreover, transgenic mice that express Src under the transcriptional control of the glial fibrillary acidic protein develop hypervascularized glioblastomas with morphological and molecular characteristics of human GBM.^[64,65]

On the other hand, estrogens like estradiol, induce their biological effects through binding to intra-cellular hormone-specific estrogen receptors (ER α and ER β), and this binding produces a conformational change in the receptors, causing the activation of their transcriptional domains. Specifically, estradiol reduced the levels of *gas1* mRNA, however it is not yet known whether the *gas1* promoter has an estrogen response element.^[66]

Little is known about the transcription factors that up-regulate *gas1*. For example the transcription factor Tbox5 increases the activity of the mouse *gas1* promoter.^[67] Moreover, microarray experiments indicate that *gas1* could be a retinoic acid responsive gene.^[68,69] Since both retinoic receptors and Gas1 are expressed during embryonic development,^[70-74] we insinuate that retinoic acid may induce the expression of Gas1 to promote exit from the cell cycle and initiate the differentiation process.

Four miRNAs have been reported to interact with the human *gas1* transcript: miR-34a-5p, -148a-3p, -130b-5p and -183-5p.^[75,76] Only miR-34a, derived from the 5' arm of the pre-miRNA sequence (miR-34a-5p), has been shown to downregulate the translation of Gas1 when the miRNA interacts with nucleotides located at position 812-832 from the 3' untranslated region of *gas1*, preventing the activity of Gas1 on the phosphatidylinositol 3-kinase (PI3K)-AKT dependent cell survival pathway.^[77,78] In fact, the repression of *gas1* by miR-34a-5p promotes cell survival and proliferation, preventing apoptosis by reducing the cleavage of Caspase-3 on papillary thyroid carcinoma cell cultures.^[77]

GAS1 PROTEIN STRUCTURE AND EXPRESSION

The nucleotide sequence of the *gas1* of both the human and mouse genes reveals an open reading frame of 345 and 384 amino acids, respectively.^[49] The proteins encoded by these genes undergo post-translational modifications in the endoplasmic reticulum consisting of an N-linked glycosylation, signal peptide cleavage and addition of a glycosylphosphatidylinositol (GPI) group at the C-terminal. The mature form of the Gas1 protein has a molecular mass of about 37 kDa and is anchored to the outer cell membrane by a GPI molecule.^[49,51,79] The region of Gas1 from amino acid 182 to amino acid 234 is essential to induce growth

arrest whereas neither the GPI nor the C-terminal domain are necessary for this function.^[80]

We previously showed that Gas1 possesses significant structural homology with the glial cell-derived neurotrophic factor (GDNF) family of receptors (GFR α s). Gas1 has two domains, called D-N and D-C, which have high similarity to the D2 and D3 domains of the GFR α s. These domains have cysteines that participate in the formation of five disulfide bridges.^[81,82] It is noteworthy to mention that Gas1 binds to RET in either the presence or the absence of GDNF.^[82] Based on the above information we and other research groups showed that Gas1 inhibits the signaling pathway induced by GDNF, an aspect that we will discuss later.

Interestingly, it has been reported that a soluble form of Gas1 inhibits the proliferation of mesangial cells. Disintegrin and metalloproteinase (ADAM) 10 and 17 are responsible of cleaving the Gas1 GPI anchor.^[83,84] In glioma cells, ADAM17 increases the shedding of soluble VEGF and activates the EGFR-PI3K-AKT pathway, contributing to invasiveness, and angiogenesis.^[85] For its part, ADAM10 promotes glioma cell migration by cleaving the adhesion molecule N-cadherin from the cell surface.^[86] On the other hand, we constructed a lentiviral vector that produce a soluble and secretable form of Gas1 (tGas1), lacking the GPI consensus sequence. tGas1 induces cell arrest and apoptosis of GBM cells and inhibit glioma tumor growth *in vivo*.^[87,88] This soluble form of Gas1 acts in both autocrine and paracrine manners. However, previous data suggests that the full form of Gas1 (with GPI) can have paracrine effects, since the GPI anchor of Gas1 could be cleaved by ADAM 10 and 17 in gliomas.

Gas1 is expressed during the early stages of development in the primitive streak, somites, heart, limb, otic vesicle, kidney, lung, muscle, gonads, brain and placenta.^[51,70,72,73] Its expression is fundamental during embryonic development since Gas1 *knockout* (K.O.) mice die immediately after birth.^[89-91] The K.O. mice develop several defects including decreased cell proliferation in cerebellum, morphological alterations in the gastrointestinal tract and microform holoprosencephaly associated with multiple craniofacial defects.^[72,89-93] The defects in Gas1^{-/-} mice, are associated with the loss of the signaling induced by Sonic hedgehog (Shh). Interestingly, some patients with holoprosencephaly present mutations in the *Gas1* gene with or without additional mutations on the *Shh* gene.^[93,94] During development, Gas1 has both negative and positive effects on cell proliferation, for example: in the limbs, Gas1 promotes the death of the interdigital tissue;^[95] whereas it promotes proliferation of granular cell progenitors in the cerebellum.^[96]

Previous studies using *in situ* hybridization showed the expression of the *gas1* gene in the brain of adult mice (<http://www.brain-map.org/>; www.genepaint.org; www.stjudebgem.org). Additionally, we reported that Gas1 is

of two RET proteins on lipid rafts.^[109] Like other receptor tyrosine kinases, RET can activate various signaling pathways including ERK, PI3K/AKT, the p38 mitogen activated protein kinase and the c-Jun N-terminal kinase (JNK) pathways.^[109,110] AKT is constitutively expressed in GBM cells and its activation induces uncontrolled growth, resistance to apoptosis, and enhanced tumor invasiveness,^[111] by inactivating pro-apoptotic proteins as BAD and procaspase-9 [Figure 1], as well as the transcription factor forkhead box O (FOXO).^[111] Thus, the inhibition of AKT is an important therapeutic target for the treatment of gliomas. The activation of AKT is regulated by PI3K, a member of the intracellular lipid kinase family, which catalyzes the generation of phosphatidylinositol-3,4,5-triphosphate (PIP3) from phosphatidylinositol-4,5-triphosphate (PIP2).^[112] PIP3 recruits AKT to the plasma membrane where it is phosphorylated in Thr308 by phosphoinositide dependent kinase 1 and in Ser473 by PDK2, which results in the full activation of AKT.^[113] On the other hand, the activation of the PI3K/AKT signaling pathway is reduced when PIP3 is dephosphorylated and converted to PIP2 by the activity of PTEN.^[114] In neuroblastoma and glioma cells, Gas1 blocks cell cycle progression, inhibits proliferation and induces cell death by inhibiting the GDNF/AKT pathway.^[107,108,115,116] We showed that Gas1 prevents the phosphorylation of Ret Tyr1062 and reduces the activation of AKT [Figure 1]. This leads to the translocation of BAD to the mitochondria and the release of cytochrome-C to the cytosol which in turn induces the activation of Caspases 9 and 3.^[107,108,115-118] Recently Wang and *et al.*^[100] demonstrated that Gas1 promotes excitotoxicity in dopaminergic neurons by inhibiting the GDNF signaling pathway.

AKT phosphorylates, activates, or inhibits a number

of proteins that regulate several processes related with cell survival.^[114,119] First, AKT has anti-apoptotic effects through the phosphorylation and inhibition of pro-apoptotic proteins, such as BAD, MDM2 and members of the FOX family. Second, AKT promotes the progression of the cell cycle by blocking the degradation of cyclin D and inactivating the inhibitors of the cell cycle p21 and p27. Finally, AKT activates the mammalian target of rapamycin (mTOR) kinase by inhibiting a complex formed by the tumor suppressor proteins tuberous sclerosis 1 and 2. In turn mTOR increases protein synthesis and cell proliferation.^[114,119]

Additionally, we found that Gas1 inhibits cell growth through a RET-independent mechanism. Gas1 decreases the viability of MDA-MB-231 human breast cancer cells, interfering with the interaction between ARTN and GFR α 3, leading to a decrement of the activation of ERK1/2.^[120] In turn, the activation of the ERK pathway is triggered by a wide variety of receptor tyrosine kinases activated by growth factors and cytokines. ERK1/2 is activated by the small G protein Ras-Raf family members (Raf-1, A-Raf, B-Raf) followed by MEK1/2. ERK1/2 controls either cell survival or apoptosis by regulating the activity of anti- and pro-apoptotic transcription factors.^[121] The phosphorylations of ERK 1/2 promote cell survival by enhancing the transcription and activity of the anti-apoptotic molecules Bcl-2, myeloid cell leukemia 1 and B-cell lymphoma-extra large.^[122] Alternatively, ERK1/2 downregulate the expression and inhibit the activity of the pro-apoptotic protein Bcl2-interacting mediator. Moreover, under conditions of oxidative stress, ERK has pro-apoptotic effects;^[123] however this process it is not well understood yet.

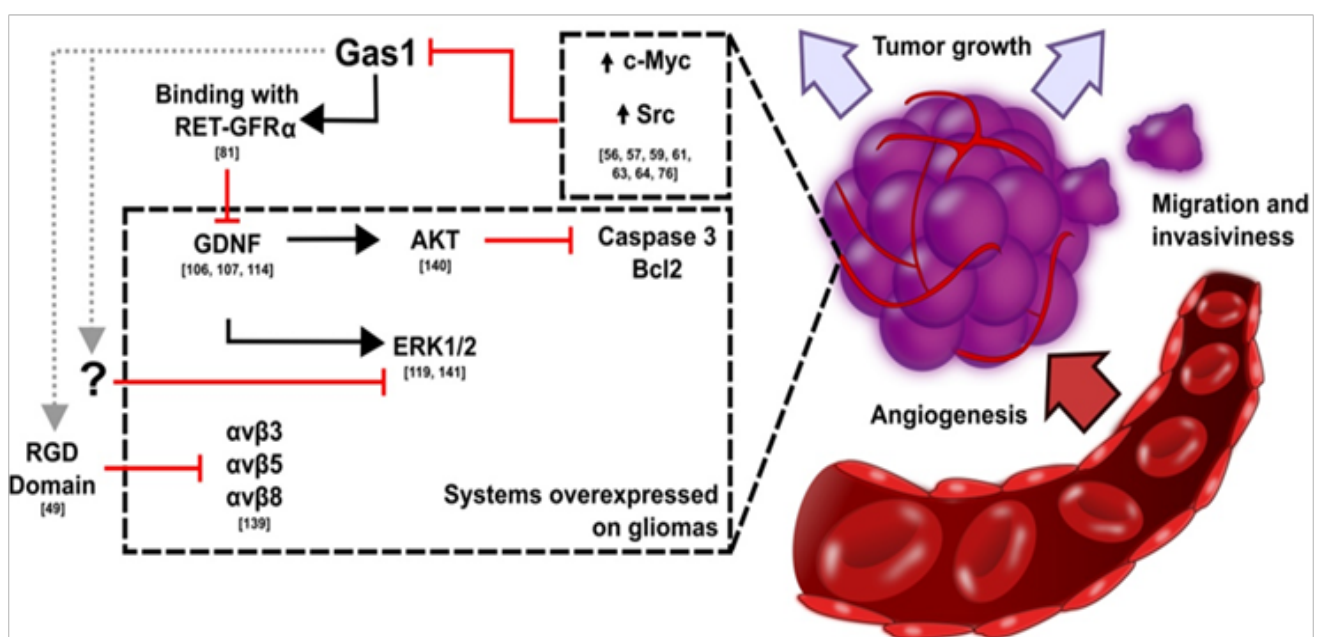


Figure 2: Pathways activated in gliomas and corresponding targets of Gas1

POTENTIAL THERAPEUTIC EFFECT OF GAS1 FOR THE TREATMENT OF GLIOMAS

Several studies suggest that GAS1 is a tumor suppressor and that its downregulation facilitates the uncontrolled growth of several types of cancer cells.^[107,108,115-118,124,125] It is worth noting that the downregulation of *GAS1* is associated with the progression of thyroid and prostate cancer and with a poor prognosis of survival.^[126,127] Additionally the loss of *GAS1* increases the metastasis of breast, prostate and gastric cancers.^[124,128] On the other hand, *GAS1* has been proposed as a molecular marker for prostate cancer.^[129] The mechanisms that regulate the expression of GAS1 in gliomas have not been identified yet; however these tumors express several transcription factors that negatively regulate GAS1 such as c-Myc and v-Src.^[56,57]

We previously showed that GAS1 induces apoptosis and inhibits cell growth in glioma cell lines and human glioma primary cultures of low and high grade.^[87,88,107,116-118] Furthermore, we demonstrated that GAS1 decreased the proliferation and induced apoptosis through the inhibition of AKT as well as the induction of apoptosis mediated by caspase 3, independently of the activity of p53 in C6 glioma cells and U373 human astrocytoma cells.^[87,88,116-118] Interestingly, GAS1 produces death of glioma cells even in the presence of the molecular machinery of Shh,^[92] suggesting that it acts through the GDNF pathway [Figure 2].

GAS1 binds to Ret in a manner independent of the presence of GDNF.^[82] On the other hand, the expression and activity of GDNF and its receptor GFR α 1 are increased by their soluble forms in gliomas.^[130-133] Based on the above, we developed a lentiviral vector in which the expression of tGAS1 is inducible.^[87,88] This soluble form of GAS1 acts both in autocrine and paracrine manners in GBM cells and inhibits glioma tumor growth *in vivo*. Subsequent to this study, we used neural stem cells as a vehicle to deliver tGAS1 into intracranial gliomas, since they have innate tropism towards tumors. We found that tGAS1 decreased tumor growth and increased the overall health and survival of nude mice implanted with GBM.^[88]

There is evidence indicating that GAS1 is a metastasis suppressor in mouse 67NR breast cancer cells and B16-F0 melanoma cells.^[128] Extracranial metastasis is a rare manifestation of GBM, this is probably due to the shortened survival of patients, that will not allow glioblastoma cells generate metastasis.^[134] On the other hand, gliomas overexpress ERK1/2 and GDNF, molecules that promote migration and invasiveness of gliomas into the brain parenchyma.^[104] There is evidence that GAS1 inhibits the migration of breast cancer cells by blocking ERK in a RET-independent manner.^[120] Moreover, GAS1 decreased tumor vascularization in a breast cancer model.^[120] All these suggest that GAS1 can be an important molecule to counter

the migration of glioma cells. Additionally, GAS1 has a RGD domain which is essential for the binding and blockade of some integrins that promote the migration and invasiveness of gliomas.^[49,135] It has been found that RGD-integrin antagonists can inhibit cell adhesion and angiogenesis.^[135] On the other hand, it has been reported that integrin α 5 β 1 (in the absence of attachment to fibronectin) decreased the proliferation of HT29 colon carcinoma cells by inducing the transcription of *GAS1*.^[136] Until now, however, there is no evidence of a relationship between the RGD domain of GAS1 and cell migration [Figure 2].

The recurrence of gliomas that occurs after surgical resection, is attributed to the presence of GIC's. Alternatively the activation of ERK is involved with the maintenance of the expression of MGMT and resistance to TMZ of GBM-GICs.^[32] As previously mentioned GAS1 inhibits the activation of ERK1/2, thus it may promote the elimination of the GIC's population [Figure 2]. It is relevant that the overexpression of GAS1 in human adenocarcinoma cells (A549) increases their sensibility to cisplatin, which inhibits proliferation and induces cell cycle arrest and apoptosis.^[137,138] Also, the downregulation of *GAS1* promotes resistance to epirubicin in human gastric cancer by regulating drug efflux and apoptosis.^[139] On the other hand, it was reported that GAS1 could be an important biomarker for the prognosis of gastric cancer patients, since it was found that reduced or negative GAS1 expression is associated with shorter survival time and worse patient prognosis.^[124] In conclusion, current data suggest that GAS1 is a potential adjuvant for the treatment of gliomas and other tumors. The use of GAS1 with current treatments may improve their efficacy.

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Conflicts of interest

There are no conflicts of interest.

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Tailored nanocarriers and bioconjugates for combating glioblastoma and other brain tumors

Fatema ELAmrawy¹, Amr A. Othman¹, Chris Adkins², Aliaa Helmy¹, Mohamed I. Nounou^{1,3}

¹Department of Pharmaceutics and Pharmaceutical Sciences, Faculty of Pharmacy, Alexandria University, Alexandria 21521, Egypt.

²Department of Basic Pharmaceutical Sciences, Health Sciences Center, School of Pharmacy, West Virginia University, Morgantown, WV 26506, USA.

³Department of Pharmaceutical Sciences, Appalachian College of Pharmacy, Oakwood, VA 24631, USA.

Correspondence to: Dr. Mohamed I. Nounou, Department of Pharmaceutical Sciences, Appalachian College of Pharmacy, Oakwood, VA 24631, USA.
E-mail: nounou@acp.edu



Dr. Mohamed I. Nounou serves the Appalachian College of Pharmacy (Oakwood, VA, USA) as an assistant professor of pharmaceutical sciences. His current research is focused on brain drug delivery, non-viral gene delivery, weight loss management, topical and transdermal drug delivery and quality of pharmaceuticals, nutraceuticals and cosmeceuticals.

ABSTRACT

Worldwide, the incidence of primary brain tumors is on the rise. Unfortunately, noninvasive drug therapy is hampered by poor access of most drugs to the brain due to the insurmountable blood-brain barrier (BBB). Nanotechnology holds great promise for noninvasive therapy of severe brain diseases. Furthermore, recent bioconjugation strategies have enabled the invasion of the BBB via tailored-designed bioconjugates either with targeting moieties or alterations in the physicochemical and/or the pharmacokinetic parameters of central nervous system (CNS) active pharmaceutical ingredients. Multifunctional systems and new entities are being developed to target brain cells and tumor cells to resist the progression of brain tumors. Direct conjugation of an FDA-approved drug with a targeting moiety, diagnostic moiety, or pharmacokinetic-modifying moiety represents another current approach in combating brain tumors and metastases. Finally, genetic engineering, stem cells, and vaccinations are innovative nontraditional approaches described in different patents for the management of brain tumors and metastases. This review summarizes the recent technologies and patent applications in the past five years for the noninvasive treatment of glioblastoma and other brain tumors. Till now, there has been no optimal strategy to deliver therapeutic agents to the CNS for the treatment of brain tumors and metastases. Intensive research efforts are ongoing to bring novel CNS delivery systems to potential clinical application.

Key words: Glioblastoma; brain delivery; blood-brain barrier; nanotechnology; novel treatment

INTRODUCTION

The central nervous system (CNS) was first described in the Edwin Smith papyrus about 3,600 years ago.^[1,2] Tumors and cancer were described in this papyrus, as well as in the Ebers papyrus, dating back to 1,300 BC.^[1-3] Hippocrates, the father of Western Medicine, was the first to use the terminology

“*karkinos*,” a Greek word for “crab/cancer,” because he noted that these tumors had tentacles reminiscent of the legs of a crab.^[4]

According to GLOBOCAN 2012, the number of new cases diagnosed with brain tumors were 256,000 for both sexes, out of 14.1 million total cancer cases.^[5] The incidence of brain tumors is higher in men than in women.^[5] The highest incidence rates

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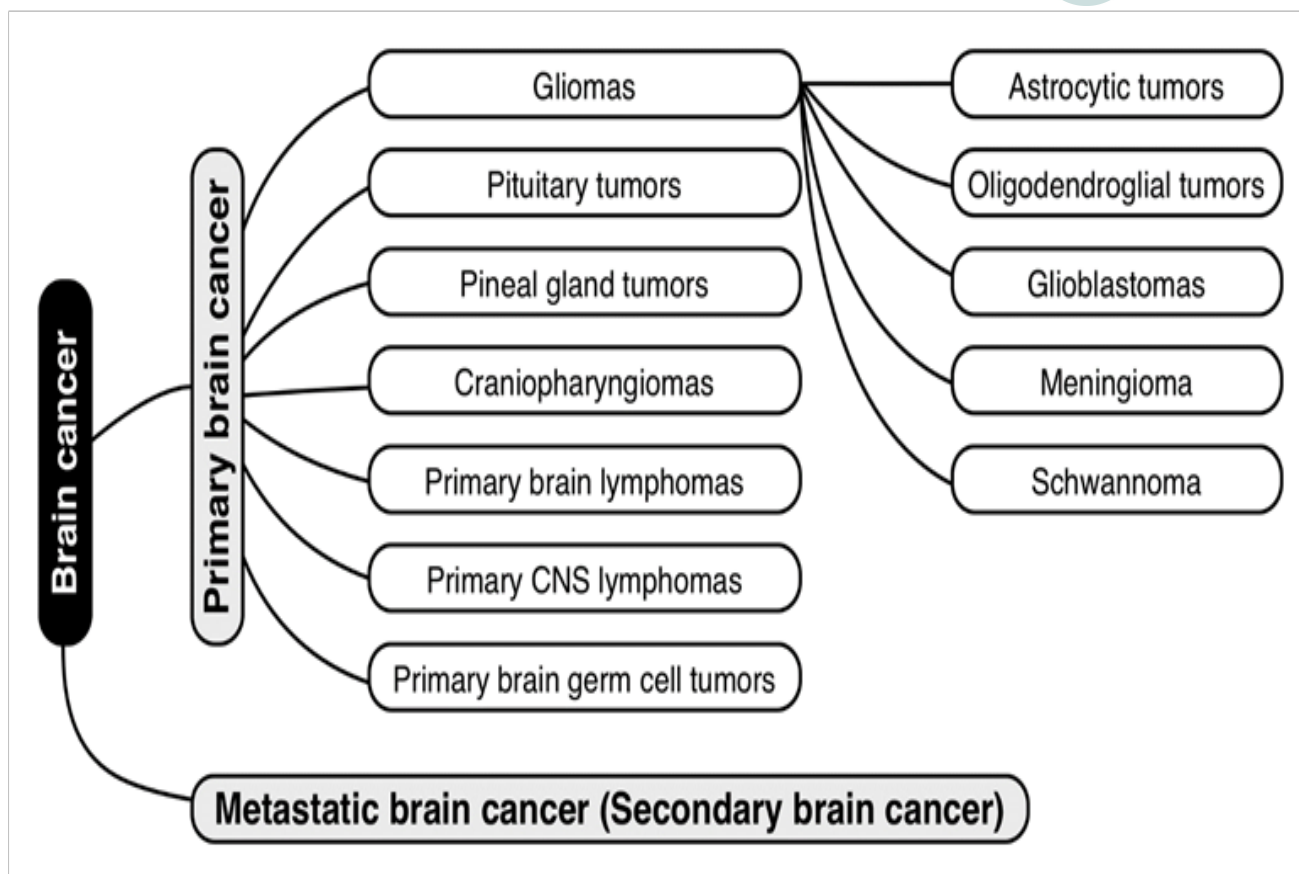


Figure 1: Most common types of brain tumors^[6-8]

occur in people between 65 and 79 years of age.^[5]

This review provides an overview of the management of primary brain tumors, especially glioblastoma multiforme. The huge surge in the development of novel strategies for management of primary brain tumors in the past 5 years will be demonstrated in this review article via recent published patents. Table 1 enumerates patents on brain drug delivery and treatment of brain tumors between 2010 and 2015 [supplement material Table 1]. This part of the review will focus on recent patents and studies using nanoparticles and bioconjugates in brain tumor treatment and diagnosis.

TYPES OF BRAIN TUMORS

Primary brain tumors originate within brain tissue. They are classified according to the type of originating tissue [Figure 1]. The most common primary brain tumors are gliomas, pituitary adenomas, and vestibular and primitive neuroectodermal tumors.^[6,7] Gliomas are tumors that begin in the glial tissue. Gliomas include glioblastomas, astrocytomas, schwannomas, oligodendrogliomas, and others.^[8]

The most common malignant brain tumor is glioblastoma multiforme (GBM, 81% of malignant CNS tumors), which is usually associated with poor prognosis.^[9-11] GBM is classified as a subtype of astrocytoma. GBM is classified as grade IV/V according to the WHO.^[11] With regard to treatment, GBM and

grade III brain tumors are managed similarly.

Any intracranial tumor, regardless of the degree of malignancy, can potentially invade or displace critical brain areas, resulting in neurologic compromises.^[12] The most common complications are seizures, peritumoral edema, venous thromboembolism, fatigue, and cognitive dysfunction.^[11-13]

GBM, is usually described in two different clinical forms, primary and secondary.^[14] Primary GBM is the most common form (about 95%); it typically arises de novo, within 3-6 months,

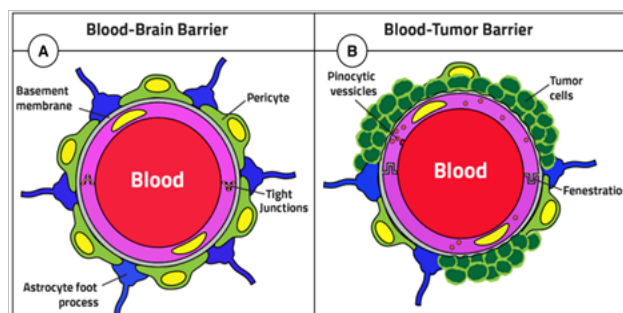


Figure 2: Diagram illustrating the difference between BBB and BBTB. BBB: blood brain barrier; BBTB: blood brain tumor barrier

in older patients. On the other hand, secondary GBM arises from prior low-grade astrocytomas over 10-15 years in younger patients.^[13] Both types respond similarly to treatment.^[13]

THE BLOOD-BRAIN BARRIER: THE BRAIN'S PROTECTION SYSTEM

The blood-brain barrier (BBB) represents a diffusion barrier system that protects the brain. BBB maintains the brain's homeostasis by controlling the influx of blood components into the brain.^[15-17] The BBB is mainly formed by brain capillary endothelial cells (BCEC), in addition to other cell types such as pericytes, astrocytes, and neuronal cells that play an important role in its function.^[17] BCEC's tight junction prevents paracellular transport of small and large water-soluble compounds from the circulation to the brain, except for some very small or gaseous molecules such as water and carbon dioxide [Figure 2].^[15,17-20]

In addition to physical barriers, several functional barriers contribute to the restrictive nature of BBB, creating major obstacles to effective drug delivery into the CNS.^[21] Besides tight junctions, a group of efflux transporters [such as P-glycoprotein (Pgp), breast cancer resistance protein, and multidrug resistance-associated proteins] are expressed on the brain tissue and collectively cause rapid efflux of large groups of lipophilic drugs from the CNS.^[22,23] Also, the presence of numerous degradative enzymes in the BBB creates another functional barrier.^[17,24,25]

The functioning and organization of the BBB can be altered under pathological conditions, such as in the case of tumors. In such a case, the barrier is called the blood-brain tumor barrier (BBTB).^[19] In low-grade gliomas, BBTB resembles BBB, while in high-grade gliomas, BBTB becomes disrupted and "leaky," characterized by major alterations of the normal vascular function manifested by contrast-enhanced MRI by Dhermain *et al.*^[19,26] However, the magnitude of this disruption is unlikely sufficient to allow drug penetration in therapeutically meaningful quantities, and thus BBTB remains a major obstacle for brain drug delivery.^[27,28]

BRAIN DRUG DELIVERY

Although BBB is difficult to bypass, inventions in the area of brain delivery in the last five years have shown promising progress and well-established techniques. There are two general strategies adopted to facilitate crossing the blood-brain barrier: invasive techniques and noninvasive techniques.^[29] Invasive techniques rely primarily on disrupting the BBB's integrity by direct intracranial drug delivery through intracerebroventricular, intracerebral, or intrathecal administration, use of osmotic pumps, or biochemical means.^[29] All these approaches are severely limited by poor distribution into brain parenchyma.^[30]

Noninvasive methods include drug modification through transformation of the drug into lipophilic analogues or prodrugs or through chemical drug delivery, carrier-mediated drug delivery, receptor/vector-mediated drug delivery, and intranasal drug delivery.^[29,31] The noninvasive techniques depend on either pharmacologic strategies (lipid-based systems), or physiologic-based strategies (nutrient or receptor-mediated systems).^[31] These techniques will be the focus of the

next sections of this review.

Receptor mediated transcytosis

Receptor-mediated transcytosis facilitates trans-BBB transport of various macromolecules after initial binding of a targeting ligand to a receptor expressed on the brain endothelial cells.^[32,33] Transferrin receptor (TfR), insulin receptor, low-density lipoprotein receptor (LDLr), acetylcholine receptor, glutathione transporter, and diphtheria toxin receptor are examples of receptors of interest.^[34] Several ligands have been studied and utilized to shuttle nanoparticles, antibodies, and drugs across the BBB and into the brain cells.^[35] For instance, the LDL receptor family can be targeted via apotinin, ApoE3 mimetic, angiopep-2, and p97 (melanotransferrin).^[36-38]

Angiopep-2, a 19-amino-acid peptide, is one of the promising vectors designed to target the LDLr-related protein to mediate transcytosis across the BBB.^[39] Angiochem Inc., in partnership with Geron Inc., developed ANG1005 (also known as GRN 1005), an Angiopep-2-PTX conjugate for treating primary (glioblastoma) and metastatic brain tumors. ANG1005 showed promise in many preclinical studies and was well tolerated in phase I clinical studies.^[32,40] However, phase II clinical trials utilizing ANG1005 are either terminated or ongoing but not actively recruiting participants, and Geron has announced that it discontinued development of GRN1005 (NCT014880583, NCT01967810, NCT02048059).^[41] Other Angiopep drug conjugates include ANG1007 (angiopep-2-doxorubicin),^[42] ANG1009 (angiopep-2-dimethylglycine etoposide), and ANG4043 (angiopep 2-trastuzumab). ANG4043 is a novel brain-penetrant peptide-mAb conjugate that is effective against HER2-positive intracranial tumors in mice, an angiopep anti-HER 2 mab conjugate. Applications of angiopep as brain targeting moiety are still under intensive research.^[43-47]

Pieter Gaillard, in a patent for "to-BBB technologies BV," suggested delivery of drugs to cells and across the blood-brain barrier by targeting them to endogenous internalizing uptake receptors for glutathione on the capillaries of the brain, without modifying or disrupting the normal function of the neuroprotective BBB.^[48] In another set of patents, Gaillard and his to-BBB technologies BV group used diphtheria toxin receptor ligand to control the blood-brain barrier vascular permeability and deliver lipopolysaccharide-sensitive nucleic acids and polypeptides across the BBB.^[49,50]

Dickerson *et al.*^[51] developed agents that modulate calcitonin-receptor related peptide (CGRP) signaling. This represents a novel target for cancer, particularly glioma and breast cancer, since CGRP stimulates cell replication and growth. In another patent, Furness *et al.*^[52] invented a method for detecting calcitonin receptor in brain cells of the subject; this method can be used for therapeutic, diagnostic, and prognostic purposes.

Due to the increased expression of the transferrin receptor in brain glioma, it is one of the most extensively studied targets for receptor-mediated transcytosis (RMT).^[53] Cedars-Sinai

Medical Center owned two patents on using anti-TfR antibodies conjugated to polycyfin-LLL to cross BBB.^[54] In the second patent, Patil *et al.*^[55] prepared polycyfin-LLL nanonjugates that could be loaded with temozolomide (TMZ) in its hydrazide form and modified with PEG.

A promising approach to enhance brain delivery is to inhibit efflux transporters by modulating their expression and/or activity.^[56,57] Clinical trial data of third-generation inhibitors (ariquidar, zosuquidar and elacridar) are awaited for possible clinical application of this treatment approach.^[58] Other naturally occurring compounds such as curcumin,^[59] quercetin,^[60,61] and kaempferol are being studied and modified for use in brain cancer therapy to overcome the problem of multidrug resistance (MDR).^[62] Barthomeuf *et al.*^[22] studied the use of curcuminoid compounds to enhance the clinical efficacy of docetaxel for the treatment of cancers including GBM. The group proposes that, in addition to reducing Pgp transport, curcumin may reduce HIF-1-dependent and HIF-1-independent angiogenesis, which in turn would inhibit tumor progression, angiogenesis, and induction of resistance.^[22] Banks *et al.*^[63] provided a method to inhibit the function of RNA- and DNA-encoding efflux transporters among other blood-brain barrier proteins using antisense compounds. The patent suggests that inhibition of Pgp expression would allow increased accumulation of chemotherapeutic drugs in the CNS and thus improve therapeutic clinical outcomes. In another patent, McChesney *et al.* used a group of taxane analogues that stabilize tubulin dimers or microtubules at G2-M during mitosis but are not substrates for MDR proteins.^[64]

The physiologic approach to target brain tumors takes advantage of endogenous receptors that are highly expressed at the BBB.^[30,31] Unfortunately, almost all the receptors are nearly nonspecific as indicated by percentage dose reaching the brain following administration compared to percentage reaching other organs such as the liver, spleen or lung.^[30] To avoid such nonselective patterns, Tosi *et al.*^[65] used double-targeting ligands to provide added targeting benefit and minimize nonselectivity. The targeting ligands used by Tosi *et al.*^[65] were sialic acid and glycopeptides. The targeting ligands were covalently conjugated to PLGA nanoparticles (SA-g7-Np).^[65]

Nanocarriers for brain drug delivery

Nano-based delivery systems have seized increased attention from formulators, as indicated by recent patents and studies [supplement material Table 1]. This can be attributed to their unique ability to deliver to therapeutic and diagnostic moieties.^[66-72] Nanocarriers are unique because of their small size (typically sub 200 nm).^[73] Nanoparticles are easily tailored in their structure and properties.^[73] They also can carry active therapeutic or diagnostic moieties of heterogeneous physicochemical properties, and their release pattern can be controlled.^[73]

A representation of possible NP structure(s) is shown in Figure 3A. NPs can be formulated from different materials including polymers, lipids, organometallic compounds, and viruses.^[74]

However, mostly amphiphilic molecule-formed liposomes and polymeric nanoparticles (chemical species having a “polar” head group and “hydrophobic” tails) have been extensively exploited for brain drug delivery.^[73,75] Long circulation time of the delivery system can be achieved by conjugating the nanoparticles with polyethylene glycol (PEG) (“PEGylation”).^[66,67] The PEG-coated nanoparticles can escape the mononuclear phagocytic system and circulate in the body for a longer time, increasing the chance of reaching the target and thereby enhancing the effect of the loaded drug.^[66,67] The effect and benefits of PEGylation are discussed later.

Unfortunately, nanoparticles can carry some serious adverse effects.^[76] Adverse effects of nanoparticles depend on individual factors such as genetics, existing disease conditions, exposure, nanoparticle chemistry, size, shape, agglomeration state, and electromagnetic properties.^[76] The key to understanding the toxicity of nanoparticles is their size.^[76] Nanoparticles are smaller than mammalian cells and cellular organelles, which allows them to penetrate these biological structures and disrupt their normal function.^[76] Examples of toxic effects include tissue inflammation and altered cellular redox balance toward oxidation, causing abnormal function or cell death.^[76]

Polymeric nanoparticles

Polymeric micelles are formed from amphiphilic block copolymers forming a core/shell nanostructure. In aqueous media, the hydrophilic heads are arranged to the outside and the hydrophobic tails to the inside to stabilize the structure, which is suitable for IV injections.^[77] Delivery of docetaxel for the treatment of brain tumors by cyclic arginine-glycine-aspartic acid (RGD)-tagged polymeric micelles was developed by Li *et al.*^[78] The authors found that RGD has affinity to bind to integrin receptor, which is overexpressed in glioblastoma tissues.^[78]

Krebs invented a novel biodegradable hydrogel polymer comprising chitin and poly(lactic-co-glycolic acid) for delivery of therapeutic agents to brain tumors.^[79] The biodegradable hydrogel detailed in Krebs’ patent would allow release of anti-VEGF to the periphery of the resected tumor site in a localized manner, with stable release rate over a sustained period. The pH-sensitive polymers which release the drug in an acidic microenvironment of solid tumors and endosomes, were the focus of a patent by Bae *et al.*^[80] Targeting ligands, such as folate, can also be attached to the mixed micelles for enhancing drug delivery into brain cells.^[56]

Zhou *et al.*^[81] in a recent patent, developed small, less aggregable brain-penetrating polymeric nanoparticles that can be loaded with drugs. In another patent, Wu *et al.*^[82] used polymethacrylic acid grafted starch (PMAA-g-St) nanoparticles containing polysorbate moieties that can target the polymer to brain tissues. Hyper-branched polymer of polyglycerol-amine (PG-NH₂) was demonstrated to accumulate in the tumor environment due to the enhanced permeability and retention effect (EPR), as described in a patent by Yerushalmi *et al.*^[83]

Tour *et al.*^[84] devised poly(ethylene glycolated) Hydrophilic Carbon Clusters Antibody Drug Enhancement System (HADES), in which nanovectors are coupled with an active agent and one of the agents that target glioma surface antigens, such as Interleukin 13 receptor (IL-13R), epidermal growth factor receptor (EGFR), and Glial fibrillary acidic protein (GFAP).

Lipid-based nanoparticles

Liposomes are the first generation of nanoparticulate drug delivery systems and consist of one or more vesicular bilayers (lamellae) composed of amphiphilic lipids, delimiting an internal aqueous compartment.^[85] The most advantageous features of liposomes are their ability to incorporate and deliver large amounts of drugs and the possibility of decorating their surface with various ligands.^[86]

Chlorotoxin-modified, doxorubicin-loaded liposomes were described by Xiang *et al.*^[87] to target chloride channel-mediated brain gliomas. Also, Li *et al.*^[88] suggested that chemotherapy using functional targeting of paclitaxel via artemether liposomes could provide a novel strategy for treating invasive brain glioma. Chen *et al.*^[89] studied lactoferrin-modified, doxorubicin-loaded procationic liposomes and showed that the system offers effective therapeutic potential for gliomas. Cationic liposomes were described in a patent by Migliore *et al.*^[90] to provide a novel, noninvasive strategy for nasal delivery of neuroactive proteins to the brain for treatment of central nervous system disorders. In another patent by Munson *et al.*^[91] PEGylated uni-lamellar vesicle liposomes were described that were appropriately sized and formulated to cross the blood-brain barrier to deliver imipramine. To overcome toxicity associated with high peak drug concentration, Redelmeier and Luz used a non-PEGylated liposomal composition comprising at least one saturated neutral phospholipid and at least one saturated anionic phospholipid encapsulating a therapeutic or diagnostic agent.^[92]

Solid lipid nanoparticles

Solid lipid nanoparticles (SLN) are stable lipid-based nanocarriers with a solid hydrophobic lipid core in which the drug can be dissolved or dispersed.^[93,94] They are made of biocompatible lipids such as triglycerides, fatty acids, or waxes.^[93,94]

Nanoparticles containing brain-derived lipids may be transported into the brain via specific receptors for these lipids. Panyam and Chavanpatil designed nanoparticles composed of a brain lipid (phospholipid), a supplemental lipid (long chain saturated or unsaturated fatty acids, stearic acid, palmitic acid, linolic acid, or linoleic acid) and a PEG-conjugated lipid (dist earoylphosphatidylethanolamine-polyethylene glycol).^[95] This nanoparticle system can deliver a drug or therapeutic compound to the brain.^[95]

Jin *et al.*^[96] used solid lipid nanoparticles made of lipids extracted from deproteinized lipoproteins and enriched with cationic cholesteryl hydrochloride and phosphatidyl-ethanolamine. The authors, after intravenous administration of such cationic NPs

for the delivery of siRNA to inhibit c-Met expression, were able to suppress the tumor growth without evident signs of systemic toxicity in an orthotopic xenograft tumor mouse model of glioblastoma.^[96]

Singh *et al.*^[97] studied lactoferrin-bioconjugated solid lipid nanoparticles as a new drug delivery system for potential brain targeting. Lactoferrin was conjugated to the surface of SLN using carbodimide coupling. SLN surface-conjugated with lactoferrin-encapsulating docetaxel maintained its complete activity and conserved its mechanism of action as characterized by cell viability and apoptosis studies.^[97]

PEGylated-liposomal formulation for enhanced pharmacokinetics (Stealth® technology)

PEGylated liposomal doxorubicin (PLD; Caelyx™, Doxil®) represents the first commercial liposomal formulation for passive cancer management with enhanced efficacy and reduced toxicity profile.^[98] PLD is superior to the conventional doxorubicin preparation, showing reduced cardiotoxicity and prolonged activity due to stealth properties imparted by its polyethylene glycol PEG layer. Despite PLD's smart passive properties in targeting cancer, its long circulation half-life and its ability to escape the reticuloendothelial system (RES) defense mechanism, it fails to manage brain tumors because of the BBB enhanced protective features.^[50]

For the PLD to cross BBB, glutathione-PEGylated liposomal doxorubicin (2B3-101) is being investigated. Based on the patent owned by BBB Therapeutics BV (formerly, to-BBB technologies), glutathione-based drug delivery system can target brain tissues by receptor-mediated transcytosis.^[50] According to the preclinical studies, 2B3-101 showed a 5-fold enhanced doxorubicin brain delivery versus PLD (Doxil®).^[99] The company held a phase I/IIa clinical study in patients with solid tumors and brain metastases or recurrent malignant glioma.^[100]

Nektar develops new drug candidates by utilizing its proprietary 3D 4-armed branched PEGylation and advanced polymer conjugate technologies to modify the chemical structure of various active pharmaceutical ingredients. It is a PEGylation technology supplier to a number of pharmaceutical companies including Affymax Inc., Amegen Inc., Merck and Co. Inc., Pfizer Inc., and UCB Pharma.^[101] Nektar Therapeutics is currently investigating the use of etirinotecan pegol (NKTR-102) for treating brain tumors.^[101,102] Furthermore, Nektar Therapeutics is conducting a phase II pilot study of NKTR-102 in patients with recurrence of high-grade glioma after bevacizumab therapy.^[102]

Bioconjugates delivery systems

The main aim of bioconjugation is to form a stable, biologically cleavable covalent link between two molecules, at least one of which is a biomolecule [Figure 3B].^[103] Bioconjugation is a form of functionalization of nanoparticles, which aims to increase stability, protect a drug from proteolysis, or enhance the targeting properties of the delivery system.^[77,103] In spite of the historic fact that bioconjugates are older than nanoparticles,

research is increasingly being diverted back to it.^[103] Factors that may encourage this resurgence of interest could include its ease of synthesis, high scale-up yield, ease of bench-to-bedside transformation, ease of formulation, and final formulation stability.^[103] Bioconjugation reactions are generally categorized by the general reactivity or the functional group involved in the conjugation process, such as amine reactions, thiol reactions, carboxylate reactions, hydroxyl reactions, aldehyde and ketone reactions, active hydrogen reactions, photochemical reactions, and cyclo-addition reactions.^[103] The design of a useful bioconjugate will depend mainly on its use, purpose, and the desired properties needed.^[104] Thus, one could choose a suitable molecule and a proper cross-linker to form the bioconjugate.^[104] The key to forming a successful bioconjugate is choosing the suitable crosslinker between the molecules.^[103]

As in any delivery system, bioconjugates are usually tailor-designed to provide the function of interest. The active drug entity can be linked to a diagnostic agent, targeting moiety, pharmacokinetics-modifying agent such as PEG, bioresponsive or stimuli-sensitive agent, an aptamer, or an antibody. Furthermore, the choice of the proper linker can impart new functions and smart characteristics to the bioconjugate system [Figure 3].

A bioconjugate was patented by Bacha *et al.*^[105] that may compromise a chimeric peptide of the structure of Formula (D-III): A-NH(CH₂)₂S-S-B (cleavable linkage), avidin-biotin-agent complex, PEG layer, and a fusion protein for targeting the brain tumor. Another bioconjugate formulation, developed by Jefferies *et al.*^[38] comprised a BBB-transport moiety linked to an antibody or therapeutic Fc-fusion polypeptide. Jefferies *et al.*^[38] modified Fc regions to facilitate the delivery of therapeutic and/or diagnostic polypeptides across the BBB and thereby treat and/or diagnose conditions associated with the CNS, including cancer.

A patent entitled “Anti-EGFR antibody drug conjugate formulations” by Tschoepe *et al.*^[106] discussed a staple formulation including: an anti-EGFR antibody or antigen-binding portion thereof conjugated to an auristatin, a sugar,

a surfactant, and histidine. In their patent Adair *et al.*^[107] described nonaggregating resorbable calcium phosphosilicate nanoparticles bioconjugated to targeting molecules that are specific for brain cells. The targeting moieties used by Adair *et al.*^[107] included antibodies, peptides, ligands, and/or receptors having sulfhydryl-group. Hutchison invented p97-antibody conjugates and related compositions that could be used in the treatment of cancers such as Her2/neu-expressing and Her1/EGFR-expressing cancers to inhibit, prevent, or delay the metastasis of an antibody-resistant cancer.^[108]

Kang *et al.*^[109] hypothesized that modification of calreticulin (CRT) peptide to poly(ethylene glycol)-poly(L-lactic-co-glycolic acid) (PEG-PLGA) nanoparticles would mediate drug transport across the BBB and enable deep penetration to the interior of the glioma by functionally mimicking iron. Their study proved that CRT-NP significantly improved the therapeutic efficacy of paclitaxel for the treatment of gliomas.^[109]

Toxins: targeting agents and a potential treatment

Disintegrins, a group of snake venom toxins, have the potential to block cancer cell migration and invasion by interaction with integrins.^[110] Contortrostatin, a snake venom disintegrin, was proven to inhibit tumor growth and angiogenesis and to prolong survival in a rodent glioma model by Pyrko *et al.*^[111] Similarly, scorpion venoms has been used in targeting brain tumors, in tumor painting, and in cell sensitization to chemotherapy.^[112-114] Chlorotoxin (CTX) is a promising tool for glioma management.^[112,115-118]

Chlorotoxin binds to metallomatrix proteins-2 and a glioma-specific chloride channel.^[119] CTX is a highly diffusible peptide that can cross the BBB or the BBTB with, to date, no evident signs of toxicity for normal human cells.^[110] Coated iron superoxide particles conjugated to CTX may be used as a MRI contrast agent as well as for delivering therapeutic agents (e.g. O6-benzylguanine and siRNA) to glioma cells.^[120-122] Other toxins such as BLZ-100 are being investigated.^[123,124]

Physically facilitated brain drug-delivery

Advanced physically manipulated systems can be used to treat diseases and allow controlled dosage of drugs. Physical manipulation can be achieved via ultrasound, electric, magnetic, or photonic-emission technologies.^[125] Davalos *et al.*^[126] applied pulsed electric fields into brain tissue of an animal to cause temporary disruption of the BBB. There are examples of using electromagnetic field pulses to induce the permeability of the BBB. Qiu *et al.*^[127] showed that electromagnetic pulses alter BBB permeability via regulating protein kinase C signaling and translocation of tight junction's protein ZO-1.

Kievit *et al.*^[122] attached chlorotoxin to an iron oxide magnetic nanoparticle (MNP) core using a short PEG linker. Similarly, *in vivo* experiments by Braun *et al.*^[128] have shown the effects of MNPs within a magnetic field on glioma cells lasting up to 100 min postexposure. A patent by Akhtari and Engel used functionalized MNP that comprise a moiety that provides

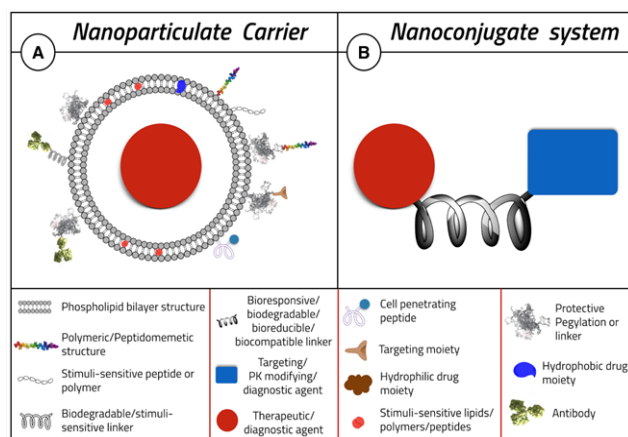


Figure 3: Diagrammatic sketch for nanoparticulate and nanoconjugate systems design strategies

selective association with cancer cells for the treatment and diagnosis of brain tumors.^[129]

Yang and David formulated magnetic iron oxide nanoparticles (MIONs) coated with a molecule that is noncovalently associated with a brain-targeting molecule. The coated MIONs comprise an anti-tumor agent linked to a cell-penetrating peptide.^[130] MIONs are oriented at the site of the brain tumor with an external magnetic field.^[130] In a patent by Dixit *et al.*^[131] gold nanoparticles conjugated with peptides against both EGFR and TfR and loaded with the photosensitizer phthalocyanine 4 have been designed and characterized. Laser was then applied to activate the photosensitizer, causing subsequent cell death.^[131]

On the other hand, nonthermal techniques to reversibly open BBB have been studied. One of these techniques is using ultrasound in the presence of microbubbles (MB).^[132,133] MB work by resonating in an ultrasound beam, rapidly contracting and expanding in response to the pressure changes of the sound wave.^[134] Inertial cavitation and destruction of microbubbles are capable of producing strong mechanical stress to enhance the permeability of the surrounding tissues and further increase the extravasation of drugs into the cytoplasm or interstitial cells.^[135] Chen *et al.*^[136] studied MB-carrying TGFβ1 inhibitor combined with ultrasound sonication to induce BBB/BBB disruption and enhance drug delivery. Pulsed-mode ultrasound exposure therapy was recently shown to enhance the antitumor effect of an EGFR-targeting chemotherapeutic drug facilitating antiangioma treatment.^[137]

NUCLEIC ACID TECHNOLOGIES

MicroRNA

MicroRNAs (miRNAs) are endogenous RNAs composed of about 22 nucleotides. The miRNAs can play important regulatory roles in animals and plants by targeting mRNAs for cleavage or translational repression.^[138,139] Currently, about 2% of known human genes encode microRNAs.^[140] A growing body of evidence shows that miRNAs are one of the key players in cell differentiation and growth, mobility, and apoptosis.^[141-143] Most microRNAs in animals are thought to function by inhibition of effective mRNA translation of target genes through imperfect base pairing with the 3-untranslated region of target mRNAs.^[138,140]

MiRNAs are appealing therapeutic targets and potential biomarkers of GBMs.^[141-143] Chan *et al.*^[144] were the first to investigate the functional properties of a single miRNA in GBM cell lines. They discovered that high expression of miR-21 is a common feature of GBM.^[144] In GBM, 15 types of miRNAs are the most studied (miR-7, miR-10b, miR-15b, miR-17, miR-21, miR-23a, miR-25, miR-124, miR-128a, miR-128b, miR-132, miR-137, miR-195, miR-221 and miR-222).^[145] In a patent by Park *et al.*^[146] hypoxia-induced angiogenesis-associated diseases including cancers was suggested to be treated by miRNA-125.

Aptamers

Aptamers are nonbiological oligonucleotides that can bind

to protein targets.^[147] Aptamers can be used for therapeutic purposes in the same way as monoclonal antibodies.^[147] However, unlike traditional methods for producing monoclonal antibodies, no organisms are required for the *in vitro* selection of oligonucleotides.^[147] For this reason, aptamers avoid the immunogenicity of antibodies while maintaining all their properties.^[147] However, there still remain largely unknown pharmacokinetic properties which make them harder to develop than any given therapeutic antibody.^[147]

Aptamers, consisting of a single-stranded nucleic acid having 100 nucleotides or less that specifically bind to tumor-initiating cancer cells, were developed and described by Rich *et al.*^[148] The aptamer specifically binds to tumor-initiating cells of GBM.^[148] Aptamers were the targeting agent of choice for a patent by Bloembergen *et al.*^[149] where they used an aptamer-biopolymer-active agent conjugate system for the treatment of cancer.

CONCLUSIONS AND FUTURE DIRECTIONS

The development cycle of new therapeutic drug entities for brain and CNS costs from \$500 million to \$1.5 billion to get to market. Such huge expense could be directly attributed to drugs failing late in clinical trials or during the post-market follow-up (Phase IV).^[150] In spite of the advances in drug discovery technologies and high-throughput screening techniques, the development cycle of new therapeutic entities is still costly and lengthy. It is challenging to ensure efficacy and safety throughout the four phases of clinical trials.^[151,152]

To overcome these problems and alleviate some of the costs associated with new drug entity letdown, pharmaceutical formulators spend effort modifying and reinventing therapeutic and diagnostic agents, giving them new characteristics with enhanced safety and efficacy profiles. The use of novel nano-sized drug delivery systems (nanoDDS) is a major approach in such reinvention process. The nanoDDS can provide methods for targeting and releasing large quantities of therapeutic agents in exact, well-defined organs or tissues. Furthermore, they can easily be tailored, decorated, and modified via various agents such as stimuli-sensitive moieties, targeting agents, pharmacokinetics-modifying mediators, diagnostic agents, cell-penetrating peptides, protective PEGylation layer, or antibodies. Such modifying moieties can provide novel functions and better efficacy or safety profiles to current therapeutic agents. Furthermore, most nanoDDSs provide both hydrophobic and hydrophilic environments, facilitating better drug solubility and enhanced physicochemical characteristics.^[153]

Despite their advantages, nanoDDS suffer from many problems such as stability issues, formulation scale-up difficulties, and short shelf life. Developing novel complexes and sophisticated systems that could never reach the market due to high cost, inability of scaling-up the system, or instability of the final formulation is a major problem. Major process and formulation development concerns exist with respect to the scale-up process of complex nanoparticulate carriers. To overcome

these problems, pharmaceutical formulators started to divert their effort from nanoDDS to simple bioconjugate techniques to directly attach old problematic active pharmaceutical agents such as stimuli-sensitive moieties, targeting agents, pharmacokinetics-modifying mediators, diagnostic agents, cell-penetrating peptides, protective PEGylation layer, or antibodies. Active pharmaceutical ingredients can be directly conjugated to antibodies against specific cell-type markers to create a hybrid smart molecule that is able to direct the active molecule to the disease tissue specifically. Consequently, many patents currently focus on simple bioconjugate structures that are easily synthesized with high yield, reduced cost, and high stability of the final formulation. This could provide a practical direction for the development of novel management tools and therapeutics for brain cancer for researchers worldwide, paving the road to affordable, scalable, stable, efficient, and safe management strategies.

All such techniques and technologies were illustrated in the recent patents analyses discussing brain drug delivery during 2010 to 2015. Despite such efforts, the development of brain drug delivery carrier system is still costly and troublesome in its transformation from bench to bedside. Such systems require huge effort in their *in vivo*, *in vitro* testing and clinical trials. Most of the research funding in academia for brain delivery research comes from investing companies. Most of the companies investing in this field are small startups such as to-BBB and BiOasis Therapeutics. If such industrial startups fail to develop a promising moiety or carrier for brain drug delivery, their existence is usually jeopardized.^[154,155] An integrated "bench-to-clinic" approach, realized through a structural collaboration between industry and academia, would strongly promote the development of brain tumor-targeted nanomedicines towards effective and safe clinical application.^[156]

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Case Report

Metastatic breast cancer: an unusual cause of diplopia

Nasser Mohammed Amer¹, Gareth Bashir², Arikoge Ogedegbe³, Ibtisam Saeed⁴

¹Department of General Surgery, University of Dammam, Al Khobar 40262, Kingdom of Saudi Arabia.

²Department of General Surgery, King George Hospital, Ilford IG3 8YB, UK.

³Department of General Surgery, King George Hospital, Ilford IG3 8YB, UK.

⁴Department of Pathology, Queens Hospital, Romford RM7 0AG, UK.

Correspondence to: Dr. Nasser Mohammed Amer, Department of General Surgery, University of Dammam, Al Khobar 40262, Kingdom of Saudi Arabia. E-mail: nmamer@uod.edu.sa

ABSTRACT

While secondary solid cancer into the eye orbit is rare, it is the most common site for primary metastasis in female breast cancer. We report a case of a sixty-six years old woman presenting to her optician with complaints of double vision. Magnetic resonance imaging revealed an invasive lesion in the superior and medial rectus muscles of the right orbit, biopsy of which confirmed this as an infiltrating breast carcinoma. Investigation of the primary lesion showed an advanced invasive ductal carcinoma of the right breast. She was then treated with radiotherapy to the orbit and a non steroidal aromatase inhibitor Anastrozol (Arimidex®). We herein review and discuss the literature, epidemiology, mechanism of tumor spread, the “seed and soil” theory, clinical presentation, pathology, and management of this uncommon presentation.

Key words: Metastatic breast cancer; diplopia; ocular metastasis

INTRODUCTION

Cancer metastasis to the eye orbit is rare^[1-4] However, female breast cancer is the commonest primary cancer metastasizing to the orbit,^[2,3,5,6] followed by, lung cancer, prostate cancer, melanoma, and genitourinary cancer in no particular order. Patients typically present with limited ocular mobility,^[6] proptosis, blepharoptosis, palpable mass, blurred or decreased vision or pain. Signs and symptoms relating to orbital metastasis are usually noted late in the disease progression, and treatment generally consists of local radiotherapy to the orbit^[6] in addition to treatment of the primary cancer, which in this case of ductal adenocarcinoma of the breast required hormonal therapy only.

CASE REPORT

A 66-year-old woman presented to her optician with symptoms of diplopia affecting the right eye only and was subsequently referred to an ophthalmologist. Physical examination showed a right sided ptosis and significant impairment in all extra-ocular muscle function with some

sparing of the lateral rectus and superior oblique muscles. The initial diagnosis was that of a partial third cranial nerve palsy. A computed tomography (CT) scan revealed increased abnormal soft tissue enhancement in the superior aspect of the orbit, with involvement of superior and medial rectus muscles. The patient was then referred to an oculoplastic surgeon who noted that the patient had a “frozen eyeball”. A magnetic resonance imaging (MRI) scan further revealed an abnormal infiltrating lesion at the orbital apex encasing the optic nerve and involving all four rectus muscles [Figures 1 and 2]. Next, a CT-guided biopsy of the lesion was performed and histology revealed a metastatic lesion, most likely from a primary breast adenocarcinoma [Figure 3]. Thus, the patient was referred to our multidisciplinary breast clinic whereupon a 10 mm palpable mass in the upper outer quadrant of the right breast was found, clinically suspicious of cancer. A mammogram and ultrasound were performed, followed by a core biopsy of the mass. The latter demonstrated features consistent with an invasive ductal carcinoma, histologically identical to the biopsy from the orbit. The tumor cells were estrogen receptor

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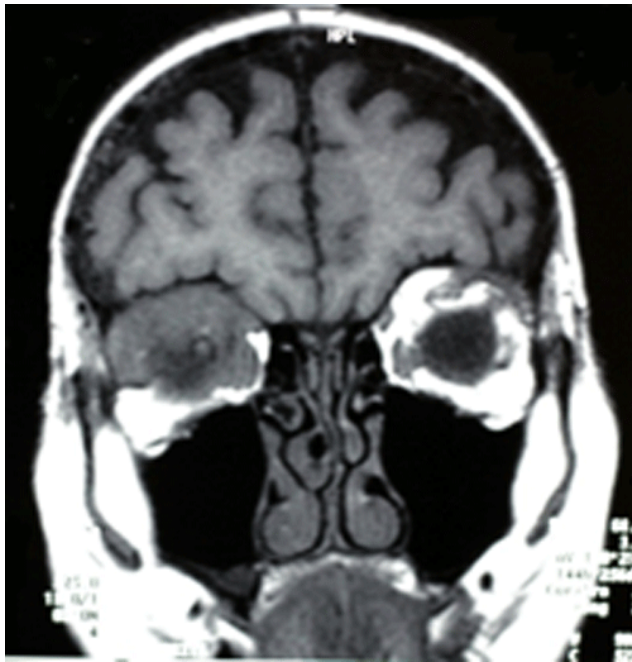


Figure 1: Axial T1 MRI of skull showing an abnormal infiltrating lesion at the right orbital apex involving all four rectus muscles

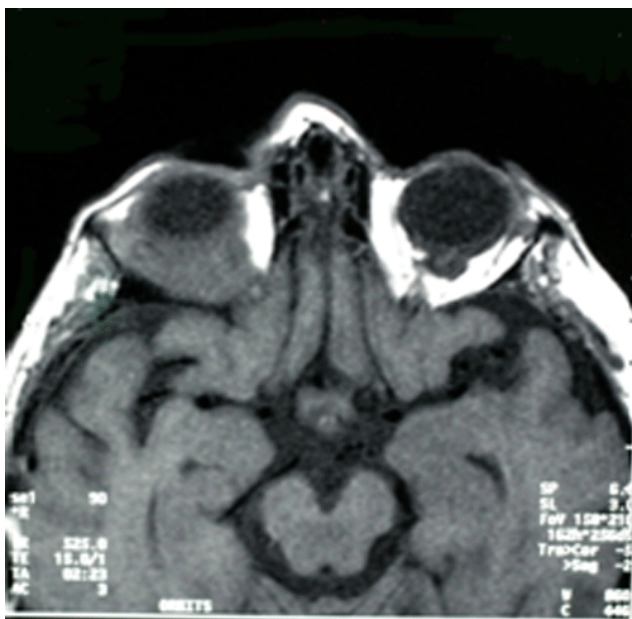


Figure 2: Coronal T1 MRI of skull shows intra- and extra-conal soft tissue signal intensity lesion encroaching upon right optic nerve, likely infiltrating and effacing retrobulbar fat

positive, progesterone receptor positive and HER2 negative (Luminal A). Staging investigation unfortunately revealed diffuse bony metastases. The consensus of our team was to treat her with primary hormonal therapy and the patient was treated with an aromatase inhibitor (Arimidex®) 1 mg once a day. Adjuvant radiotherapy to the right eye was also done under the direction of the ocular oncologist.

DISCUSSION

Orbital metastasis of solid tumors is very rare^[2-6] with most occurring in the uveal tract, especially in the posterior

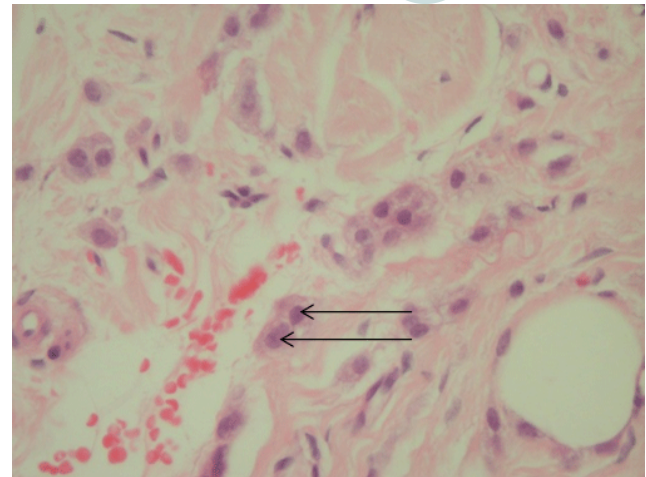


Figure 3: Histology of orbital core biopsy showing infiltration by metastatic adenocarcinoma, most likely of breast origin

part of the choroid. It has been proposed^[7] that breast cancer cells have the ability to remain viable away from their primary site. Indeed, breast cancer accounts for the highest incidence of metastasis to the orbit,^[2,3,5-9] followed by prostatic, lung, melanoma, and gastrointestinal cancers. Another study showed that lung cancer was the second most common primary source for orbital metastasis, followed by prostate cancer. Other reported sources include cancers of the thyroid, liver, pancreas, adrenal gland, salivary and choroidal melanoma.^[6]

Overall, orbital metastasis occurs in 2-3% of patients with systemic cancer.^[6] However, metastasis into the extraocular muscles is an even less frequent presentation.^[1] It did, however, occur in this reported case of breast carcinoma metastatic to the orbit, with all four recti muscles being involved. This is not in concordance with the prevailing view that skeletal muscles are considered an uncommon site for metastasis, (albeit less infrequent in malignant lymphoma and leukemia). It may be due to the fact that these muscles are in a more or less constant state of movement, thus preventing neoplastic cells from seeding them, or by producing an unfavorable chemical environment for neoplastic growth.^[5] Clinical studies have shown that different cancer types frequently display distinct metastatic patterns, with neoplasms of particular histological types tending to metastasize to specific organs.^[10] Paget^[11] first proposed the “seed and soil” hypothesis of cancer metastasis. He postulated that tumor development was a consequence of the provision of a fertile environment (the soil) in which compatible tumor cells (the seed) could proliferate. The ability or inability of specific organs to provide this favorable milieu and the success or failure of specific cells to respond to these microenvironments dictated the observed patterns of metastatic development in different cancers.^[11] In contrast to the “seed and soil” theory, there is a mechanistic explanation for secondary tumor growth patterns. Here, the organ or tissue specificity is the direct consequence of the anatomical location of primary tumors. Thus, the secondary foci of epithelial cancers,

which metastasize predominantly via the lymphatics, are subsequently found mainly in draining lymph nodes. It has been proposed that entrapment and growth of tumors might be affected by qualitative or quantitative differences in tumor cells' ability to adhere to the vascular endothelium of particular organs;^[11] organ-specific growth is thus a direct consequence of the specific localization or entrapment of circulating tumor cells. While it is tempting to speculate an immunologic basis for the propensity of breast cancer and malignant melanoma to metastasize to the extra-orbital muscles; the nature of any such site specificity remains unknown.^[12]

Although the patient did have skeletal symptoms due to widespread bony metastasis which were largely dismissed by her general practitioner as being part of her arthritis complex, the main presenting symptom in this case was diplopia. However, a review of the literature indicates that this not commonly the case. A previous study of 100 patients^[6] showed that diplopia constituted only 9% of such patients. Frequency of their presenting symptoms and signs were limited ocular mobility (54%), displacement of the globe with proptosis (50%), blepharoptosis (49%), a palpable mass (43%), blurred or decreased vision (23%), pain (17%), visible mass or swelling (17%), and enophthalmos (11%). The latter sign interestingly and paradoxically was found to be associated with scirrhous breast cancer.^[6,9,12] This may be explained by the presence of desmoplasia and fibrosis associated with the tumor, causing contracture of the orbital content, and paradoxical enophthalmous.

The diagnosis of metastatic tumor can be confirmed by CT scan^[1,2] for more than 95% of orbital metastases and CT scan can provide considerable information regarding size, location, relation to musculature and other structures, as a well as the nature of the lesion. High resolution CT imaging is also an excellent diagnostic tool for extra-ocular metastasis. Focal or nodular muscle enlargement without focal bone destruction, fossa formation, orbital enlargement, or other evidence of neoplastic extension into contiguous structures is highly suggestive of metastasis. Diffuse enlargement with feathering of the muscle edge may occur or masquerade clinically as a myositic pseudotumor.^[6] Bilateral involvement can be present despite only unilateral symptoms.^[6] MRI, on the other hand, did not add specificity to the radiographic information in our case. Any patient with an undifferentiated malignancy first discovered in the orbit should undergo a full systemic evaluation to reveal the primary tumor. Although MRI may provide the best resolution of orbital metastasis,^[6] CT is more useful in cases of suspected prostate and breast cancer as metastatic bone involvement is very common in breast cancer too.

Metastatic lesions involved the horizontal rectus muscles are more common than the vertical rectus or oblique muscles.^[6] Orbital metastasis from breast cancer tends to be diffuse and irregular, often growing along rectus

muscles and fascial planes. In contrast, orbital metastasis from carcinoid tumor, renal cell carcinoma, and melanoma tends to be more circumscribed, at least in early stages.^[6] Diagnosis is usually established by tissue biopsy, open, or fine needle aspiration to confirm a metastatic tumor lesion, and not simulating lesions, such as idiopathic orbital inflammation (also known as orbital pseudotumor). Special staining^[12] with mucicarmine and alcian blue may be helpful, not only to obtain a diagnosis but also to identify the primary neoplasm. Histological diagnosis of metastasis is typically straightforward and does not present a problem to the pathologist.^[7]

Although we did not test for Ki-67 status, (also known as MK 167) it has been used as prognostic parameter in breast cancer patients,^[14] and its presence is associated with lower disease free survival and lower overall survival. Currently neither St Gallen, nor ASCO recommendations nor the German Interdisciplinary S3 Guidelines for the diagnosis, treatment, and follow-up of breast cancer have proposed Ki-67 as a routine prognostic marker. Ki-67 has been shown to have an inverse relationship with estrogen receptor status, but a possible direct relationship with HER2 status.

Treatment of histologically proven metastatic tumors to the orbit is mainly palliative,^[12] and is comprised of orbital irradiation^[6] with approximately 35-40 Gy to the affected orbit in divided doses over 3-5 weeks. Slightly more or less radiation may be indicated depending on tumor type. Radiation^[7] may improve and preserve vision for the remaining lifespan of the patient. Regression of ocular metastasis following sterilization, adrenalectomy, or hypophysectomy has been reported in a number of cases of hormone dependent breast cancers,^[2] however, Aromatase inhibitors (Anastrozole, Letrozole, Exemestane) are now recognized as the agents of choice for the management of post menopausal women with steroid hormone positive metastatic breast cancer, in whom indications for chemotherapy are not absolute.^[13] Enucleation of the eye on the other hand should only be carried out in cases of intractable pain, most often caused by secondary glaucoma.^[2]

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Conflicts of interest

There are no conflicts of interest.

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Case Report

Metastatic high-grade neuroendocrine tumor of mandible

Jitender Batra¹, Chinmay D. Vakade², Sonal Grover², Gyanander Attresh¹

¹Department of Oral and Maxillofacial Surgery, Post Graduate Institute of Dental Sciences, Pt. B.D. Sharma UHS, Rohtak 124001, Haryana, India.

²Department of Oral and Maxillofacial Surgery, Bapuji Dental College and Hospital, Davangere 577004, Karnataka, India.

Correspondence to: Dr. Jitender Batra, Department of Oral and Maxillofacial Surgery, Post Graduate Institute of Dental Sciences, Pt. B.D. Sharma UHS, Rohtak 124001, Haryana, India. E-mail: dr.batrajatin@gmail.com

ABSTRACT

Neuroendocrine tumors of the oral cavity and jaws are exceedingly rare. They include paragangliomas, a melanotic neuroectodermal tumor of infants, small cell carcinomas, and Merkel cell carcinomas. Most have been non-functional in nature. Breast, lung, liver, colon, and prostate are the most common reported primary malignancies which can metastasize to the oral cavity. In most cases, oral metastases involve maxilla and mandible rather than soft tissues. The premolar-molar region is the most common localization. The purpose of this article is to describe a rare case of a high grade neuroendocrine tumor of the mandible which metastasized from the cervix.

Key words: Large/small cell carcinoma of mandible; metastatic tumor/carcinoma to the mandible; neuroendocrine/carcinoid tumor of mandible

INTRODUCTION

Metastatic lesions to the jaws are known to simulate periodontal and pulpal disease and other radiolucent lesions that can occur in the jaws. Breast, lung, liver, colon, and prostate cancers are the most commonly reported primary malignancies which can metastasize to the oral cavity.^[1]

Tumors of the neuroendocrine system constitute a heterogeneous group of lesions that vary in origin, location, histological appearance, the degree of differentiation, biologic behavior, functional activity and size but share certain histochemical, immunohistochemical, and ultrastructural characteristics.^[2]

Neuroendocrine tumors comprise carcinoids, islet cell tumors, medullary carcinomas of the thyroid, mastocytomas, melanomas, Merkel cell tumors of the skin, neurocytomas, oat cell carcinomas, paragangliomas, pinealomas, and pituitary adenomas.^[3]

The purpose of this article is to describe a rare case of a high-grade neuroendocrine tumor of the mandible which metastasized from the cervix.

CASE REPORT

A 35-year-old female patient reported with the chief complaint of a swelling in the right lower back region of the jaw for 20 days. Her history revealed a painful tooth in the region which was extracted 1 month earlier, followed by the appearance of swelling few days later. The swelling was initially small in size to start with but gradually progressed to the present size and was associated with loss of sensation on the right side of the lower lip. Medical history revealed that the patient had undergone a hysterectomy for small cell carcinoma of cervix 11 months earlier.

Extra-oral examination revealed a solitary swelling in the right lower third of the face measuring around 2.5 cm × 2 cm in the greatest dimensions, roughly oval in shape with diffuse borders. Right sub-mandibular lymph nodes were palpable, tender, firm-to-hard inconsistency, and fixed.

Intraoral examination revealed the presence of solitary swelling in the right mandibular molar region, measuring 1.5 cm × 1 cm. Vestibular obliteration was evident [Figure 1]. Clinically, teeth number 26, 36, 46, 47 and 48 were

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missing. Orthopantomograph revealed the presence of an ill-defined, honeycomb radiolucency in the right side of the body of mandible distal to 45, measuring 4.5 cm × 5 cm, roughly oval in shape [Figure 2]. Occlusal view showed a lingual cortical plate expansion.

Computed tomography scans were taken which demonstrated a destructive lesion in the right mandibular premolar-molar region and exhibited possible muscle infiltration. Further clinical investigations, including full bone scan, abdominal, chest and pelvic examinations, sonar ultrasonography of the abdomen and mammography, showed no space-occupying lesions. Standard hematologic investigations were within normal limits.

Incisional biopsy of the lesion was done and keeping in view the past medical history, a diagnosis of metastatic small cell carcinoma of the mandible was made. Surgery was advised to excise the tumor mass [Figure 3] and a radical right disarticulation hemimandibulectomy along with radical neck dissection on the right side was performed, and reconstruction was done with pectoralis major myocutaneous flap. She recovered well from the

procedure and was referred to the radiotherapy center for further management. Later, the patient denied a secondary procedure for reconstructive purposes.

The pathological specimen was sent for histopathological examination and revealed tumor cells arranged in a lobular pattern, rosettes-papillary pattern, solid sheets and clusters. These cells had scant eosinophilic cytoplasm with round, polygonal nuclei with stippled chromatin. There were areas of necrosis. These tumor cells were seen infiltrating into the skeletal muscle fibers. Sections from lymph nodes revealed hyperplastic lymphoid follicles and prominent germinal centers. Sinusoids were filled with histiocytes [Figures 4 and 5].

Immunohistochemical studies revealed that the tumor cells were positive for chromogranin, CD56 and synaptophysin, while they were negative for S-100, cytokeratin (CK)-5/6 and p63. Mib-1 labeling index was 50%. These findings were diagnostic markers of high-grade neuroendocrine carcinoma.

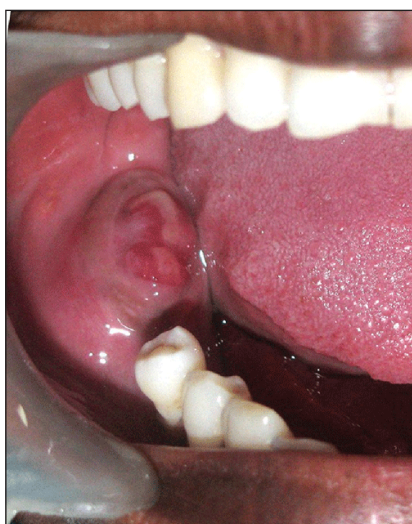


Figure 1: Intraoral photograph of the patient showing the tumor mass at the time of presentation



Figure 2: Orthopantomograph showing radiolucent changes in molar region on the right side

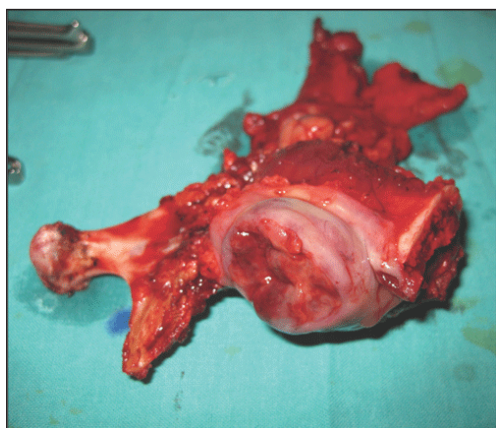


Figure 3: Excised tumor mass with safe margins

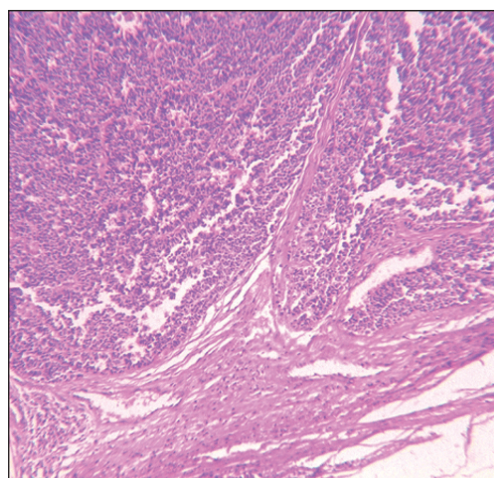


Figure 4: Photomicrograph revealing islands of carcinoma cells arranged in sheets with darkly stained nuclei (H and E, ×10)

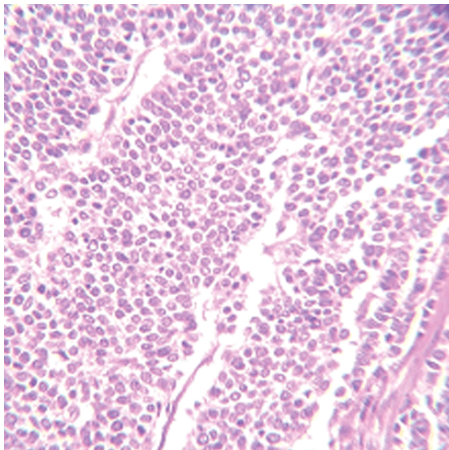


Figure 5: Photomicrograph revealing nuclei with stippled chromatin (H and E, $\times 40$)

DISCUSSION

Primary carcinomas which are reported to metastasize to the jaws include those of the breast, lung, liver, colon and prostate.^[1] The primary carcinomas having highest tendency to metastasize are different for both genders. They are as follows in decreasing order: for women, breast followed by carcinomas of the adrenal, colorectum, female genital organs and thyroid; for men, the lung followed by the prostate, kidney, bone and adrenals.^[4]

It has been reported that most metastatic oral tumors are found in patients in their fifties, sixties and seventies.^[4,5] According to Hirshberg *et al.*,^[4] metastases to the jawbones have a slight female predilection while metastases to the oral mucosa have a male:female ratio of 1.6:1.

Features that might assist in the assessment of malignancy include the site of origin, depth of invasion, degree of differentiation, functional activity and size of the tumor.^[2] The most common symptoms reported in literature are painful swelling, paresthesia, bleeding and increasing tooth mobility along with delayed healing of extraction socket, pathological fracture, masticatory difficulties, trismus, dysphagia, and dyspnea.^[6] Paresthesia of the lower lip and the chin was found in our patient. As already reported, this should be considered as ominous sign for metastatic lesions to the mandible, as this signifies deep invasion of the tumor into the bone and involvement of the inferior dental or mental nerves.^[7] Mental nerve neuropathy or the “numb chin syndrome”, in the absence of other causes, should be considered to be due to mandibular metastases until proven otherwise when seen in a patient with known malignancy.^[8]

Being very sensitive immune-markers, neuron-specific enolase, and CK are most commonly used for definitive

diagnosis. At present, in addition to ultrastructural studies, immunohistochemical techniques are the most sensitive methods available for the diagnosis of neuroendocrine tumors.^[9] Treatment modalities for neuroendocrine carcinomas of the oral cavity have included surgery, multidrug chemotherapy, and radiotherapy, alone or in combination. Currently, well-established treatment protocols do not exist. Surgery alone is inadequate because these tumors tend to progress rapidly and at the time of diagnosis, they are reported to have a metastasis rate of roughly 14-50%.^[10]

The presentation of a malignant lesion in the orofacial region may be the first indication of the existence of an unknown malignancy at a distant primary site. The presence of altered sensation in the area of the lower jaw and lip/chin region in a patient with a known non-head and neck malignancy should alert the clinician to the possibility of metastatic malignant disease. Appropriate investigations should be carried out to rule out other secondary and manage the case satisfactorily.

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Conflicts of interest

There are no conflicts of interest.

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Case Report

Metachronous bladder metastasis from papillary renal cell carcinoma

Arun Ramdas Menon¹, Nivedita Suresh², Prajwal Ravinder¹, Rajeev Thekke Puthalath¹

¹Department of Urology, K S Hegde Medical Academy, Mangalore 575018, Karnataka, India.

²Department of Pathology, K S Hegde Medical Academy, Mangalore 575018, Karnataka, India.

Correspondence to: Dr. Arun Ramdas Menon, Department of Urology, Justice K. S. Hegde Medical College Hospital, Mangalore 575018, Karnataka, India. E-mail: dr_arunmenon@yahoo.com

ABSTRACT

Renal cell carcinoma (RCC) is well known for its metastatic potential and predilection for unusual sites of metastasis. Metastasis to the bladder is rare and has been reported predominantly from clear cell RCC. We report a case of a 72-year-old male presenting with a bladder tumor which on histopathological evaluation was found to be a metastasis from papillary RCC, 7 years after radical nephrectomy. This case highlights the need to maintain a high index of suspicion to diagnose bladder metastasis in a patient with a history of RCC presenting with a bladder lesion.

Key words: Bladder metastasis; metachronous metastasis; renal cell carcinoma

INTRODUCTION

Renal cell carcinoma (RCC) metastasizing to the bladder is a rare phenomenon. It can be easily mistaken for primary bladder tumor on cystoscopy. Of the few cases reported in literature, bladder metastasis was predominantly from clear cell RCC. Here, we report a case of bladder metastasis from papillary RCC presenting 7 years after radical nephrectomy.

CASE REPORT

A 72-year-old male, a chronic smoker, presented with irritative lower urinary tract symptoms. He had undergone left radical nephrectomy for RCC (papillary sub-type, stage T3N0M0) 7 years ago. Clinical examination was unremarkable. His baseline investigations, including complete blood counts and serum biochemistry, were within normal limits. Urine analysis showed microscopic hematuria. A transabdominal sonography revealed a 3 cm hyperechoic lesion in the left postero-lateral wall of the bladder. Abdominal contrast-enhanced computed tomography (CECT) confirmed the same lesion [Figure 1]. No extra-vesical spread or pelvic lymph node metastasis was evident. The CECT also confirmed that he was post-left radical nephrectomy; there was no evidence of

local tumor recurrence or intra-abdominal metastasis. However, a chest radiograph revealed multiple cannon ball metastases. Cystoscopic examination revealed a solitary, broad-based lesion in the region of the left ureteric orifice [Figure 2]. The rest of the bladder was unremarkable. A cold cup biopsy of the lesion showed a neoplasm arranged predominantly in papillary pattern and focal areas of solid sheets, both composed of tumor cells with moderate eosinophilic cytoplasm and low-grade nuclear features. Immunohistochemistry revealed strong diffuse positivity for cytokeratin 7 (CK7), vimentin and focal positivity for cluster of differentiation 10 (CD10) [Figure 3], suggesting a metastatic bladder tumor from renal papillary adenocarcinoma.

The patient was counseled regarding his disease; he refused active therapy and was referred for palliative care. He died of progressive disease 10 months later.

DISCUSSION

RCC can be an aggressive disease with the ability to metastasize widely. Besides the common sites of metastasis, i.e. lung, liver, bone and brain, RCC can also metastasize

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to unusual sites with myriad presentations. Of the RCC sub-types, clear cell RCC is notorious for its unpredictable metastatic pattern; on the other hand, papillary RCC is

rarely associated with prodigious metastasis. This has been attributed to its hypovascular nature, owing to the lack of Von Hippel-Lindau mutations that regulate vascular endothelial growth factor, the primary proangiogenic molecule in RCC.^[1] The relative rarity of papillary RCC metastatic to the bladder was also demonstrated in a recent series of 11 cases of metastatic RCC to the urinary bladder that were detected over a span of 15 years, with only 18% (2/11) originating from papillary RCC.^[2]

The bladder is an unusual site for metastasis of RCC with an incidence of 1.6% in autopsy series.^[3] Other metastatic sites of RCC to the genitourinary tract include the ipsilateral ureter, contralateral ureter, ureteric stump and prostatic fossa. Bladder metastasis may be solitary or multiple, the latter having a worse prognosis. Both synchronous and metachronous bladder metastasis from RCC have been described. Metachronous lesions occur more commonly and have been reported to occur up to 12 years after radical nephrectomy.^[4] Synchronous lesions are more likely to be associated with the presence of metastasis in other organs.

Although a variety of possible pathways for metastasis of RCC to the bladder have been proposed, the exact mechanism is not clear.^[5] Hematogenous spread may occur through the general circulation or retrograde through the periureteric or gonadal veins. In this scenario, the metastasis is usually located within the bladder detrusor layer. Lymphatic spread may occur through the renal hilar lymphatics down the periureteral lymphatics and subsequently through the pelvic lymphatics to the pelvic organs. Transluminal spread with seeding of the distal urothelium may occur, especially in cases where the renal tumor infiltrates the pelvicalyceal system. We believe this to be the likely mechanism in our patient, considering that the site of metastasis was in the region of the left

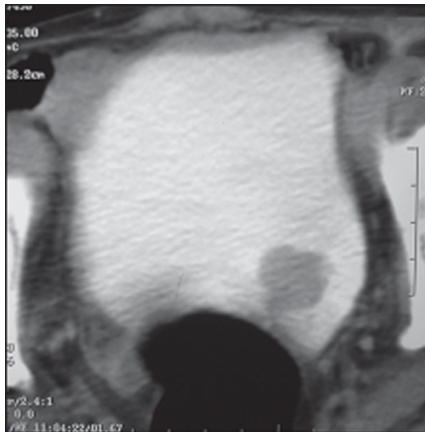


Figure 1: Contrast-enhanced computed tomography: Moderately enhancing lesion in the left postero-inferior wall of the bladder

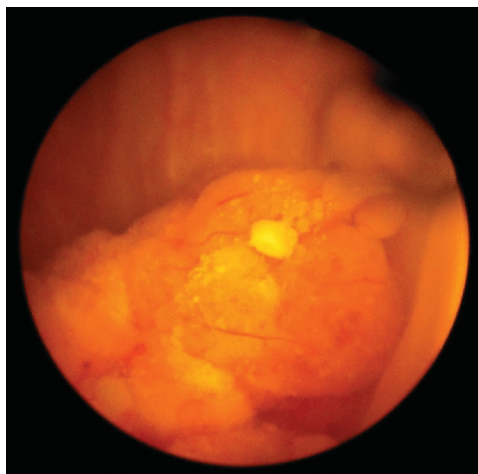


Figure 2: Cystoscopic image: Broad-based non-papillary lesion arising from the region of the left ureteric orifice

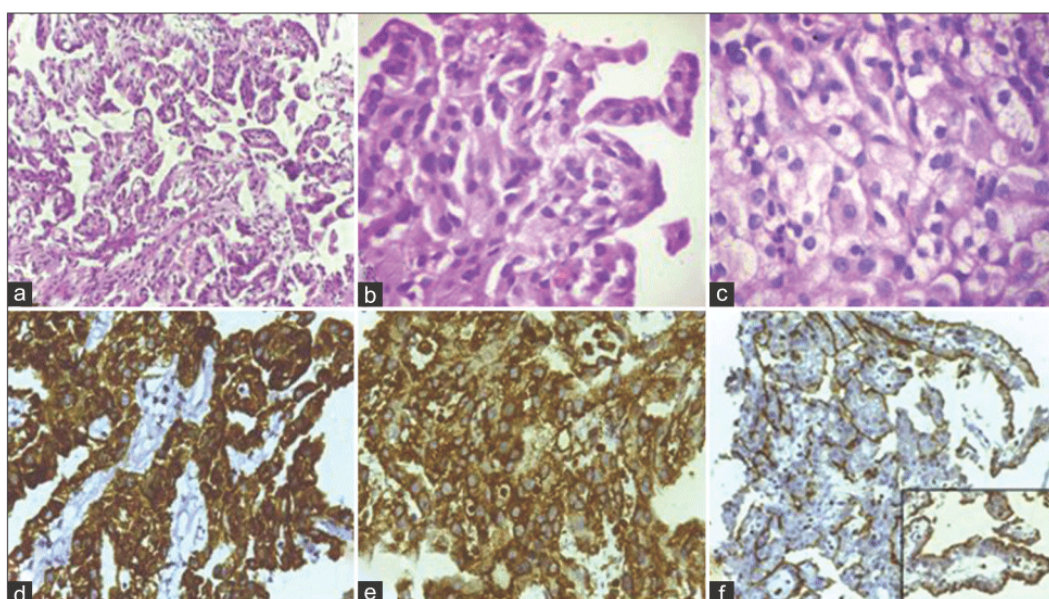


Figure 3: (a-c) Histopathology showing papillary adenocarcinoma with moderate nuclear pleomorphism and eosinophilic cytoplasm. Hematoxylin and Eosin staining section (a, $\times 4$; b, $\times 40$); (d) Immunohistochemistry shows strong positivity for cytokeratin 7; (e) vimentin; (f) focal positivity for cluster of differentiation 10

ureteric orifice and the lesion was primarily situated within the urothelium.

The most common presenting symptom of bladder metastasis is hematuria. In a patient with synchronous bladder metastasis, hematuria may be wrongly attributed to collecting system infiltration of RCC. The bladder metastasis may, thus be overlooked, only to become apparent later, when the patient continues to have hematuria post-nephrectomy. This has led some authors to recommend cystoscopic evaluation in all patients with RCC and hematuria.^[4,6]

The definitive diagnosis of bladder metastasis is made by cystoscopy and biopsy or transurethral resection. The metastasis histologically resembles their renal primary. However, a basic immunohistochemical panel is useful to differentiate metastasis from poorly differentiated bladder tumors. RCC metastasis, like their primaries, show positive staining for CK AE1/AE3, CK7, CD10 and vimentin.^[4] Well-differentiated primary papillary urothelial carcinomas are positive for CK7 and usually negative for vimentin. Urothelial carcinomas attain vimentin positivity only on sarcomatoid transformation. In diagnostically challenging cases, discriminatory immunohistochemical markers, such as PAX8 and GATA3 positivity, may be used to differentiate metastatic RCC from primary bladder urothelial carcinoma, in addition to alpha-methylacyl coenzyme A racemase positivity for papillary RCC.^[2]

Due to the rare occurrence of bladder metastasis, there are no established recommendations for management. Metastasectomy has been advocated if complete resection of all metastasis can be accomplished.^[7] Management options that have been described, include partial cystectomy or transurethral resection, either as a single modality or in combination with immunotherapy or targeted therapy.^[8]

Prognosis has been reported to be good only when a

single metastasis exists in the bladder.^[7,9] To summarise, it must be emphasized that not all papillary tumors of the bladder are primary transitional cell carcinomas. Metastasis from papillary RCC must also be considered in a patient with a history of renal malignancy presenting with hematuria or a bladder mass. As in our case, these metastases may present several years after treatment of the primary malignancy.

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Conflicts of interest

There are no conflicts of interest.

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The role of technetium Tc99m-tetrofosmin as head and neck tumor-seeking agent: a preliminary report

Chrissa Sioka¹, Thomas Exarchopoulos¹, Vlasios Skloupitotis², Vaios Papathanassiou³, Vasileios Ragos⁴, Maria Argyropoulou⁵, Anna Gousia⁶, Periklis Tsekeris², Georgios Exarchakos³, Dimitrios Assimakopoulos³, Andreas Fotopoulos¹

¹Department of Nuclear Medicine, University of Ioannina School of Medicine, University Campus, Ioannina 45110, Greece.

²Department of Radiotherapy, University of Ioannina School of Medicine, University Campus, Ioannina 45110, Greece.

³Department of Ear Nose and Throat, University of Ioannina School of Medicine, University Campus, Ioannina 45110, Greece.

⁴Department of Maxillofacial Surgery, University of Ioannina School of Medicine, University Campus, Ioannina 45110, Greece.

⁵Department of Radiology, University of Ioannina School of Medicine, University Campus, Ioannina 45110, Greece.

⁶Department of Pathology, University of Ioannina School of Medicine, University Campus, Ioannina 45110, Greece.

Correspondence to: Dr. Chrissa Sioka, Department of Nuclear Medicine, University of Ioannina School of Medicine, University Campus, Ioannina 45110, Greece. E-mail: csioka@yahoo.com

ABSTRACT

Aim: To evaluate the diagnostic value of technetium Tc99m-tetrofosmin (^{99m}Tc-TF) in primary cancers of the head and neck. **Methods:** Single photon emission computer tomography with planar imaging of the neck for primary site evaluation and whole body scanning for assessment of metastases in 12 patients with newly diagnosed head and neck cancer. Tumor-to-background index (T/Bg) was derived in patients with positive findings (tumor or lymph nodes). **Results:** The tomographic images showed increased tracer uptake in pathological sites (primary tumor or lymph node) in 9 patients (overall sensitivity 75%). Primary tumor was visualised in 7 patients (sensitivity 58%) and infiltrated lymph nodes in 4 out of 7 patients (sensitivity 57%). Mean values for T/Bg index were 5.44 ± 1.28 for primary tumor and 4.25 ± 1.67 for lymph nodes. Mean values for T/Bg index were 4.5 ± 0.71 for patients with *in situ* and grade I carcinoma and 6.68 ± 0.36 for patients with tumor grade II and III ($P = 0.034$, Mann-Whitney *U* test). **Conclusion:** The present study demonstrates that ^{99m}Tc-TF is a valuable radiotracer for head and neck cancer imaging. To determine the potential role of this imaging protocol in clinical practice will require a larger sample size.

Key words: Head and neck cancer; radionuclide imaging; single photon emission computer tomography; ^{99m}Technetium-tetrofosmin

INTRODUCTION

Malignant tumors of the head and neck are among the six most common forms of tumors in the body. Head and neck cancer include neoplasms of the upper respiratory tract (nasopharynx, oropharynx, larynx) salivary glands, and soft tissue of the neck with squamous cell carcinoma being the most frequent histological type. Diagnosis of this type of cancer is based on endoscopy and biopsy of the suspicious lesion.^[1] Staging and evaluation of the extent of disease involves the use of computed tomography (CT), magnetic resonance imaging (MRI) and positron emission tomography (PET).^[2] Follow-up evaluations after surgery and/or radiotherapy and differential diagnosis of disease relapse from radiation necrosis is more difficult to assess,

due to extensive distortion of the normal anatomy, and may require a combination of different imaging tests,^[3-7] such as PET scan.^[8-11] However, its availability is limited by the need for a cyclotron that produces ¹⁸Fluorodeoxyglucose (¹⁸FDG) and its increased cost. In addition, in many countries, PET is not widely available so other methods are needed for tumor-imaging assessment.

When a PET scanner is not available, nuclear medicine still plays a crucial role in the initial staging of the disease and for follow ups. Lymph node cancerous involvement at initial presentation may be difficult to assess by conventional

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imaging methods based only on morphology and size. Functional nuclear medicine imaging has the unique advantage of assessment of the metabolic state of lymph nodes. Currently, the agents employed are Thallium-201 (^{201}Tl) and the two technetium-99m labelled compounds Sestamibi (MIBI) and Tetrofosmin.^[2]

$^{99\text{m}}\text{Tc}$ -MIBI and $^{99\text{m}}\text{Tc}$ -tetrofosmin ($^{99\text{m}}\text{Tc}$ -TF) are two lipophilic cationic complexes, which were originally employed in myocardial perfusion imaging, but later were found to possess tumor-seeking properties in the evaluation of diverse human malignancies.^[12] The diagnostic value of $^{99\text{m}}\text{Tc}$ -TF could hold promise as a head and neck cancer tracer, although limited data exist in clinical research.^[13,15] In the present study the diagnostic utility of $^{99\text{m}}\text{Tc}$ -TF prior to surgery of head and neck neoplasms was assessed and correlated with the $^{99\text{m}}\text{Tc}$ -TF uptake of histological grade, and tumor and lymph node size.

METHODS

Prior to surgery, 12 subjects (11 males and 1 female) of median age 65.5-year-old (48-83) with suspected head and neck cancer had $^{99\text{m}}\text{Tc}$ -TF planar and Single photon emission computer tomography (SPECT) imaging of the neck and whole body scanning for metastatic evaluation. None of the patients had received treatment prior to scintigraphy, except for patient twelve, who had surgery, radiotherapy and chemotherapy, one year prior to scintigraphy. This patient had scintigraphy because of a tumor relapse at the primary tumor.

All patients were interviewed before $^{99\text{m}}\text{Tc}$ -TF imaging and the patients' age, height, weight, smoking habits, and alcohol consumption were noted. Data from the CT findings included the anatomical location and size of the tumor, as well as the existence and size of lymph nodes. Correlation of SPECT imaging with the tumor histopathology diagnosis and grade was performed after the original tumor was excised and the pathology was established. The protocol was approved by the Hospital Research and Ethics Committee. Informed consent was obtained from all patients. Clinical trial registration was not required for this small study.

Protocol

All patients were injected with a dose of 740 MBq (20 mCi) bolus $^{99\text{m}}\text{Tc}$ -TF; and immediately after injection patients drank 5 mL of lemon juice to stimulate salivary glands. Lemon juice stimulation achieves the lowest possible uptake of radiotracer in the salivary glands (normal distribution of $^{99\text{m}}\text{Tc}$ -TF) during imaging. Scintigraphy was performed with patients in a supine position. Anterior planar images were acquired 5-10 min post injection, using a zoom factor of 2.66. Tomography was acquired 15 min post-injection with the dual-head camera at 6°-angles (60 stops) and 30 s per projection (30 projection/head) over

a 360° arc, using a low-energy, general purpose collimator. Acquisition was obtained with a matrix size of $64 \times 64 \times 16$, 1.85 zoom factor, and a 15% symmetric window at 140 keV (no contour). Reconstruction method was filtered back projection (filter butterworth, cut-off frequency 0.5, power 7.0). No attenuation correction was used. Finally, whole body scan was acquired in all patients for possible distant metastases evaluation.

Two nuclear medicine specialists visually evaluated the planar, tomographic and whole body images, which were compared to the CT scans. Increased uptake in SPECT images (in a site of pathological finding on CT (primary tumor or lymph node) was considered a positive finding. A region of interest (ROI) was drawn on the relative coronal image. Background (Bg) ROI was drawn over the apex of the right lung. T/Bg index for tumor and lymph nodes was derived in all patients with positive findings. Patients with no significant uptake on pathological sites were considered negative.

Table 1: Physical characteristics of the patients and tumor anatomical location

Characteristics	Patients
Gender	11 M/1 F
Mean age (years)	65.75 \pm 11.8 (48-83)
Height (cm)	166.75 \pm 8.9 (155-184)
Weight (kg)	69.25 \pm 9.5 (55-82)
BMI (kg/m ²)	25.00 \pm 3.8 (16.2-32.4)
Smoking	11/12 (91.7%)
	* 0: 1/12 (8.3%)
	* 1: 0/12 (0.0%)
	* 2: 11/12 (91.7%)
Alcohol	8/12 (66.7%)
	** 0: 4/12 (33.3%)
	** 1: 2/12 (16.7%)
	** 2: 6/12 (50.0%)
Anatomical location of tumor	
Epiglottis	1/12 (8.3%)
Vocal cord	2/12 (16.7%)
Base of tongue	2/12 (16.7%)
Pharyngeal wall	1/12 (8.3%)
Tonsil	1/12 (8.3%)
Submandibular gland	1/12 (8.3%)
Soft palate	1/12 (8.3%)
Pyriform fossa	2/12 (16.7%)
Nasopharynx	1/12 (8.3%)
Histopathological findings	
Squamous cell	10/12 (83.3%)
Adenoid cystic cell	2/12 (16.7%)

M: male; F: female; BMI: body mass index; * Smoking habits: 0 = no smoking; 1 = less than 500 cigarettes/year; 2 = more than 500 cigarettes/year; for more than 2 decades. **Alcohol consumption: 0 = no alcohol consumption, 1 = alcohol consumption no more than 2-3 times in week; 2 = every day consumption of alcohol

Table 2: Tumor and regional lymph node characteristics in patients with head and neck cancer

Patient	Tumor diameter: cm (max)	LN diameter: cm (max)	T/Bg Index	LN/Bg index	Histological Grade*
1	3	5	5.2	5.18	I
2	2.5	No LNs	3.52	No LNs	0
3	2	No LNs	4.57	No LNs	0
4	4.5	4.5	4.72	3.17	I
5	2	2	-	-	0
6	3	3	6.44	-	III
7	2.5	2.5	-	2.55	III
8	2.5	4	-	6.1	II
9	6	No LNs	6.5	No LNs	III
10	4.5	2	-	-	III
11	2.5	No LNs	-	No LNs	0
12	5.5	No LNs	7.1	No LNs	III

D: diameter; LN: lymph node; T/Bg: tumor-to-background; LN/Bg: lymph node-to-background; *Histopathological grade 0: *in situ*

Table 3: Tumor and lymph node uptake of ^{99m}Tc -TF and uptake sensitivity according to tumor grade and size and lymph node size in patients with head and neck cancer

Patients	Patients No.	TF uptake sensitivity (%)	P	Mean T/Bg \pm SD	P
<i>In situ</i> and Grade I	4/6	66.7%		4.05 \pm 0.71	
Grade II and III	3/6	50.0%	0.343	6.68 \pm 0.66	0.034*
Total	7/12	75.0%		5.44 \pm 1.28	
Tumor size < 3cm	2/6	33.3%		4.05 \pm 0.74	
Tumor size \geq 3cm	5/6	83.4%	0.074	5.99 \pm 0.99	0.053
Total	7/12	75.0%		5.44 \pm 1.28	
LN size < 3 cm	1/3	33.3%		2.55	
LN size \geq 3 cm	3/4	75.0%	0.32	3.99 \pm 1.5	0.180
Total	4/7	57.0%		4.25 \pm 1.67	

LN: lymph node; T: tumor; Bg: background; TF: ^{99m}Tc -TF; *: $P < 0.05$

Statistical analysis

Data are presented as mean (\pm standard deviation). Uptake of ^{99m}Tc -TF (T/Bg index) in tumors, as well as sensitivity, was correlated to tumor histological grade, and the tumor and regional lymph node size. For statistical analysis, the software “SPSS for Windows” ($P \leq 0.05$ was considered as statistically significant). Non parametric statistics was applied. The Mann-Whitney U test compared means and the chi-square test to compare frequencies.

RESULTS

The characteristics of the twelve patients and the tumors are summarized in [Table 1].

Among them, 4 patients had *in situ* carcinomas, 2 patients grade I carcinoma, 1 patient grade II and 5 patients grade III carcinoma. The smallest measurable tumor was 2.0 cm, and the largest 5.5 cm. Pathological enlarged lymph nodes were noted in 7 patients with sizes between 2 cm and 5 cm [Table 2].

Tomographic images showed increased tracer uptake in pathological sites (primary tumor or lymph nodes) in 9 patients, with an overall disease detection sensitivity of 75% [Figure 1]. Primary tumor was visualised in seven patients (sensitivity 58%) and infiltrated lymph nodes in 4

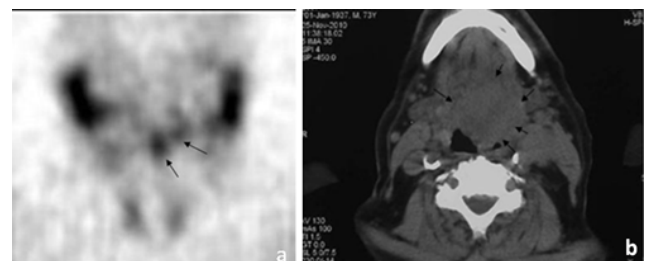


Figure 1: Patient No. 4 with base of tongue cancer: (a) SPECT coronal slice (arrows showing the ^{99m}Tc -TF uptake); (b) relevant CT slice (arrows showing the tumor)

out of 7 patients (sensitivity 57%) [Table 3]. According to histological tumor grade, 4 (66.7%) patients with *in situ* or grade I and 3 (50%) patients with grades II or III had higher ^{99m}Tc -TF tumor uptake ($P = 0.373$) than the counterparts. Scintigraphic sensitivity was lower with tumor size < 3 cm (33.3%) compared to tumor size \geq 3 cm (83.4%) sensitivity. The statistical analysis found a trend towards a positive correlation with tumor size in radiotracer uptake, although the results did not reach statistical significance ($P = 0.079$) [Table 3]. In addition, one patient with tumor size of 4.5 cm had no uptake [Table 2]. Among the 7 patients with ^{99m}Tc -TF uptake in primary tumor, 4 patients exhibited increased ^{99m}Tc -TF uptake in the regional infiltrated lymph nodes. One of them had lymph node size < 3, while the other patients had lymph node sizes larger than 3 cm. Two of the

5 patients with no primary tumor uptake had uptake in the lymph nodes [Table 2].

Mean values for T/Bg index in all patients was 5.44 ± 1.28 for primary tumor and 4.25 ± 1.67 for lymph nodes. Statistical difference was found with histological grade, after categorizing the patients according to their grade, tumor and lymph node size. Thus, concerning histological grade, patients with tumor grades 0 or I had mean values for 4.5 ± 0.71 , whereas patients with tumor grades II or III had T/Bg indexes of 6.68 ± 0.36 . Statistically significant difference between the 2 groups was found ($P = 0.034$, Mann-Whitney U test) [Table 3]. Regarding tumor size, T/Bg index was lower in tumors < 3 cm (4.05 ± 0.74) than in tumors ≥ 3 cm (5.99 ± 0.99). After statistical analysis, there was a trend towards a positive correlation of T/Bg index with increasing tumor size ($P = 0.053$) [Table 3]. Mean values for T/Bg index of lymph nodes < 3 cm were 2.55, and in lymph nodes ≥ 3 cm, was 3.99 ± 1.5 . There was no statistical difference ($P = 0.180$), possible due to the small number of cases [Table 3]. No metastatic lesions were found on whole body images.

DISCUSSION

The study showed as sensitivity of SPECT in pathological sites (either primary tumor or regional lymph nodes) of 75%. SPECT sensitivity for only primary tumor diagnosis was 58% while for infiltrated lymph nodes it was 57%. In accordance with our findings, a previous study in 10 patients with nasopharyngeal carcinoma reported the ^{99m}Tc -TF uptake in 7 out of 10 patients (70%).^[15] Fattori *et al.*^[14] studied exclusively patients with laryngeal cancer using ^{99m}Tc -TF and reported 96% sensitivity for detecting the primary mass and 50% for lymph node involvement. Variations of sensitivities using ^{99m}Tc -TF uptake in primary cancers of the head and neck between studies may be caused by a small study sample, but it may be higher in patients with exclusively laryngeal cancer according to other trials.^[14]

In another study of 21 patients with nasopharyngeal carcinoma that evaluated ^{99m}Tc -MIBI, sensitivity was 97% and specificity 100%.^[16] A study that compared ^{99m}Tc -MIBI to ^{99m}Tc -TF in nasopharyngeal cancers found that both radiotracers detected all primary tumors, ^{99m}Tc -MIBI was superior in detecting pathological lymph nodes (sensitivity 95% vs. 79%).^[13] The same authors also reported better sensitivity for ^{99m}Tc -MIBI compared to ^{201}Tl during monitoring response to radiotherapy.^[17] Similarly, another study reported a limited role of ^{201}Tl in detection of the primary tumor with a sensitivity of 54%, specificity 75% and accuracy 57%.^[18]

In contrast, Wang *et al.*^[19] reported that ^{201}Tl was more sensitive than ^{99m}Tc -MIBI, with ^{201}Tl SPECT identifying 94% of the primary lesions in head and neck cancers with

different sites, and all of the positive and two negative lymph nodes.^[20]

Another study found a higher percentages for sensitivity (88%) and specificity (94%).^[21] Shiao *et al.*^[22] reported a 64% sensitivity for ^{99m}Tc -MIBI in primary tumor detection and 73%^[23] for tumor recurrence. Shen *et al.*^[15] reported higher specificity, but lower sensitivity for ^{99m}Tc -TF, as compared to CT. Finally, other researchers suggested that both ^{201}Tl and ^{99m}Tc -MIBI have the same accuracies in locating primary, recurrent and lymph node involvement and thus could also be valuable.^[24]

In the study, no relation of the ^{99m}Tc -TF sensitivity with the pathological grade of the tumor was observed, but there was a potential correlation of ^{99m}Tc -TF sensitivity with tumor size. Thus, it appeared to be a trend toward positive uptake with increasing tumor size, which did not reach statistical significance, probably a result of a relatively small number of cases. In contrast, ^{99m}Tc -TF result from previous studies with ^{99m}Tc -MIBI did correlated tumor size, stage, or histology and it did not affect the tracer uptake.^[25] Five patients did not have any ^{99m}Tc -TF uptake and no tumor was visualized. All but one of these tumors had a size < 3 cm and were in the pharyngeal wall, with close proximity to the tonsils and the pyriform fossa. Lower sensitivity in this area could be due to the complex anatomy and physiological uptake, which make tumor distinction more difficult. Although not verified in the present study, another possibility for negative radiotracer uptake by some tumors could be a possible molecular mechanism that pumps the radiopharmaceutical out of the tumor cells.^[26] Such mechanisms attributed to membrane multidrug resistance proteins have been associated with resistance to chemotherapy,^[27-29] and or linked to limited or no radiotracer uptake in a variety of tumors.^[30-33] if this mechanism proves to be significant in head and neck cancers, then the ^{99m}Tc -TF scintigrams may be useful in therapy planning for these patients.

Although the tumor grade was not correlated to radiotracer uptake sensitivity, it was positively correlated to T/Bg index. In another study, false-positive cases were reported when T/Bg index were ≤ 1.7 .^[13] In our study, no patient with radiotracer uptake had a T/Bg index ≤ 1.7 .

In conclusion, the study showed that ^{99m}Tc -TF SPECT had an overall sensitivity for visualization of a head and neck primary cancer site of 58%. However, sensitivity was lower for certain tumor locations than others. For example, patients with tonsillar and pyriform fossa tumors didn't have any uptake (false negative exam). Since these sites are difficult to assess, even with SPECT, the results in these locations should be interpreted with caution. Generally, SPECT should be accurate for visualization of tumors of > 3 cm in any other location. The T/Bg ratio was correlated with malignancy grade. Larger studies will help to increase the statistic power (as well as comparison with

¹⁸FDG PET/CT) to establish a role for ^{99m}Tc-TF SPECT in therapy, as well as prognosis of head and neck cancers.

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Conflicts of interest

There are no conflicts of interest.

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The association of the uterine motion with bladder volume during radiotherapy in gynecological malignancies

Bhandari Virendra, Mutneja Abhinav, Gurjar Omprakash, Saadvik Raghuram, Bagdare Priyusha, Gupta Krishnlal, Singh Kanchan

Department of Radiation Oncology, Sri Aurobindo Medical College and PG Institute, Indore 452001, India.

Correspondence to: Dr. Bhandari Virendra, Department of Radiation Oncology, Sri Aurobindo Medical College and PG Institute, 401, Samyak Towers, 16/3 Old Palasia, Indore 452001, India. E-mail: virencancer@yahoo.co.in

ABSTRACT

Aim: This study was performed to assess the extent of interfraction uterine motion during radiotherapy for cervical cancer and uterine body carcinoma while maintaining a strict bladder filling protocol. **Methods:** Twenty-four patients with cervical cancer or uterine body carcinoma who were treated on a linear accelerator, were recruited. During the course of external beam radiotherapy, cone beam computed tomographic scans were taken, once at the start of treatment and then weekly until the completion of the radiotherapy course. Patients were instructed to maintain a strict bladder filling protocol. After negating the effect of patient's setup error by offline cone beam computed tomographic imaging, the position of the uterus was defined in the clinical target volume. Then the position of the uterus was compared in the following weekly scans. The position of the uterus was also correlated with the position and the filling of the bladder. This change in uterus position was measured separately in the anteroposterior (AP), superoinferior (SI), and lateral directions. **Results:** According to calculations based on weekly imaging, The mean values of shift in AP, SI, and lateral directions were respectively 0.67, 0.29, and 0.23. The mean extent of motion in the uterine position on a daily basis for individual patients ranged from -2.28 to +1.3 in AP, -0.56 to +0.71 in SI, and from -0.6 to +0.45 in lateral directions. **Conclusion:** At least once a week cone beam computed tomography might be necessary to minimize the geometrical miss and deliver the planned doses to the target tissue and normal structure provide best results with minimum toxicity by maintaining a bladder volume of about 100 mL and an empty rectum during the whole course of treatment. The daily anatomical shift and contour of the patients maintaining a bladder volume of approximately 100 mL with an empty rectum may result in asymmetrical conforming to the planning target volume and hence appropriate and adequate planning target volume margins are required.

Key words: Uterine motion; weekly computed tomographic evaluation; intensity modulated radiotherapy; inter-fraction variation; bladder volume

INTRODUCTION

External beam radiotherapy (EBRT) plays a great role in the management of female gynecologic cancers. Intensity modulated radiotherapy (IMRT) and image guided radiotherapy (IGRT) are considered the treatment of choice for cervical cancer and uterus carcinomas. These new techniques have overtaken the conventional four-field box technique as the preferred modality of treatment and have proven more efficacious in various studies.^[1-3] The IGRT further reduces the radiation dose to the organs at risk (OAR) and thus further reduces toxicities. On the other hand, IMRT has very strict clinical and planning target volumes (CTV and PTV respectively) conforming to a particular volume to

spare the adjacent organs; however, it does not account for interfraction and intrafraction motion of various organs as well as reduction in tumor volume during treatment.

This phenomenon did not need to be considered in the conventional technique as it provided a uniform dose to all the structures included in the treatment field which avoids a geometrical miss of the tumor. Therefore, motion of organs within the treatment area is a vital issue in IMRT and three dimensional conformal radiotherapy (3D-CRT). During the treatment a steep dose gradient is usually present that uses

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the newer techniques that conform to the optimum dose of the CTV or the shape of the target volume. Even a little geometrical movement could result in an underdosing to the target volume or conversely, delivering high undesirable doses to the surrounding normal tissues. These effects highlight the importance of accurate margin determination.

This pilot study was conducted to define the daily uterine shift in patients undergoing external radiotherapy on linear accelerators with IMRT technique using IGRT with the help of an on-board cone beam computed tomography (CBCT) scan taken once a week during the whole course of radiotherapy.

METHODS

We recruited 24 patients with the ages of 45 and 70 years who were diagnosed with cervical cancer and uterine body carcinoma were treated with EBRT (50 Gy in 25 fractions) from September 2010 to December 2013, and opted for the IMRT technique.

Before starting radiotherapy a six-clamp thermoplastic Orfit cast was prepared for immobilization of the pelvic region in all the patients and then contrast enhanced computed tomographic (CT) scan of pelvis was done and 3 mm slice thickness scans were acquired and transferred to the treatment planning system (TPS) (Eclipse version 8.9). The gross tumour volume (GTV), CTV, PTV, and organs at risk (OAR) such as rectum, bladder, and femoral heads were delineated on the CT images following the guidelines of the International Commission on Radiation Units and Measurements report number 83 (ICRU 83).^[4] Then IMRT plans were created with 6 Mega Volt (MV) and 15 MV photon beam and a Varian leaf motion calculator (version 8.9.08), was utilized to calculate leaf motion for dynamic dose delivery. Dose-volume optimizer was used for plan optimization. Anisotropic analytical algorithm was used to calculate doses with grid size of 0.25 cm. After approving, the plans were scheduled for 25 fractions with daily imaging by On Board Imaging system and CBCT technique.

Patients were positioned and immobilized with the orfit cast on couch and then CBCT was done with the OBI system. The anatomy matching software Portal Vision 7.5, was used to study the patient's setup deviations and to determine the spatial coordinates in the images. After patient setup and laser alignment during EBRT, a kV portal image was taken and matched with the reference image to avoid patient's setup error and a CBCT scan was performed once at the start of treatment and then weekly until the completion of treatment. This CBCT was matched with the reference CT image to see the shift of the uterus, which was noted in X, Y, and Z axes [Figures 1 and 2]. A total of 96 scans were obtained during the whole treatment period, ie, four scans for each patient. Then the patients were assessed for intracavitary brachytherapy and if they did not fit, they

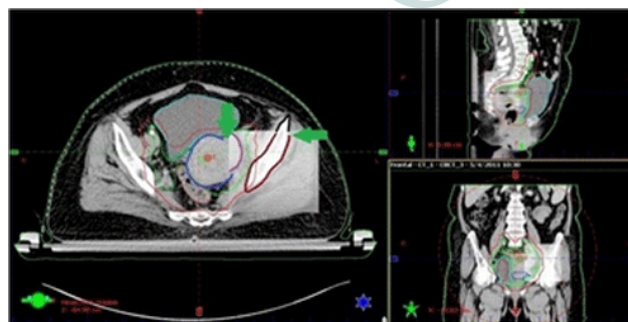


Figure 1: The perfect bone to bone matching of a patient with the reference computed tomographic image and the uterine shift between the two scans to negate the effect of the patient's setup errors

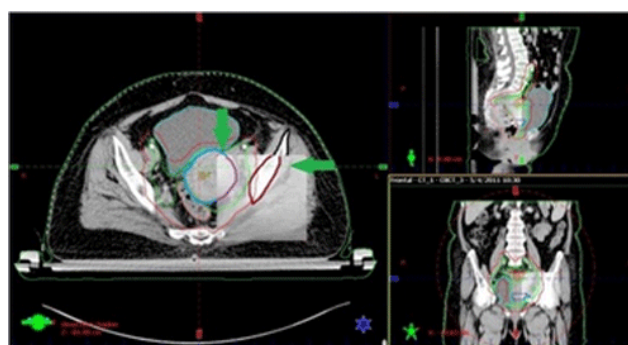


Figure 2: The soft tissue matching of the contoured uterus with the reference scan showing bone displacement after radiotherapy

were continued for boost by EBRT.

Patients were asked to maintain a strict bowel and bladder filling protocol by instructing all the patients to defecate and urinate and then to maintain strict water intake of around 200 mL of water 20 min before the procedure. The position of the uterus was defined in the CTV during delineation on axial images of the lesion for radical radiotherapy. The CTV included all the gross as well microscopic lesions. The OARs such as the bladder, rectum, intestines, and femoral heads were also delineated on axial images.

The position of the uterus was then compared in the following weekly scans on the axial images guided by sagittal, coronal and three-dimensional reconstructions. This was done by merging the weekly CT images with the reference CT image taken before the start of the treatment at the same level. For every scan, we used the lower level of the S1 vertebra. After merging the images, a preliminary bone to bone matching was done to negate the effect of patient's setup errors which was followed by soft tissue matching of the uterus in two CT images. The change in CTV position during the bone to bone matching was subtracted from the anteroposterior (AP), superoinferior (SI), and lateral changes during the soft tissue matching. The correlation between the position of uterus with the position and the filling of the bladder was also assessed. This change in uterus position was measured separately in the AP, SI and lateral directions. No additional effort on the part of the patient or the doctor was required because a part of the OBI software performed the measurements during

the treatment. The time taken for every treatment was also similar among the patients undergoing IGRT of the pelvic region. The Mean of all the obtained-values for each patient was calculated and an unpaired-one-sample student *t*-test was applied to obtain the significance. The *P* value is less than 0.001 which is highly significant.

RESULTS

The mean, standard deviation, and median of uterine motion in each plane were calculated to see its association with the bladder filling and its influence on the displacement of the uterus. As shown in the Table 1, the displacement ranges were significant depending on the patient, although the mean values of the displacement were within 1 cm. The mean values of shift in AP, SI, and lateral directions were respectively 0.67, 0.29, and 0.23 for all the 96 scans done for 24 patients over the period of EBRT [Table 2].

The mean extent of motion in the uterine position on a daily basis for individual patients ranged from -2.28 to +1.3 in AP, -0.56 to +0.71 in SI, and from -0.6 to +0.45 in lateral directions. The mean movement in all the directions was also calculated over the course of the full treatment [Figure 3], and showed more anterior and superior shift that might be due to bladder filling while the lateral deviation, although



Figure 3: The mean change of the uterine position in the lateral (X), anteroposterior (Y), and the superoinferior (Z) directions per patient over the whole course of the treatment along with its association with the bladder volume depicted in the area curve

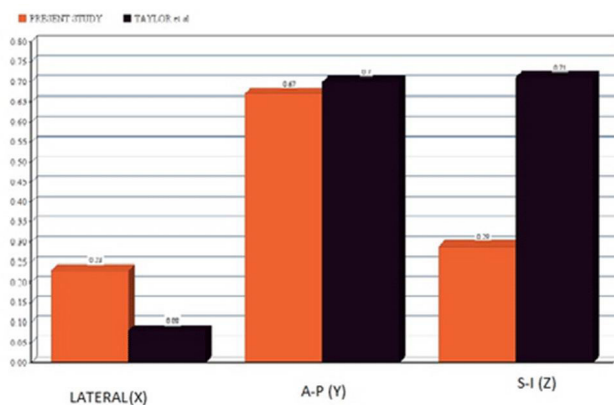


Figure 4: Comparison of different means of displacement in different directions between current study and the study of Taylor *et al.*^[11]

Table 1: Combined uterine motion in three different dimensions in patients undergoing radiotherapy

Dimensions	Mean (SD), cm	Median, cm	Range of motion, cm
Lateral (X)	0.23 (0.22)	0.2	- 0.6 to 0.45
Anteroposterior (Y)	0.67 (0.83)	0.57	- 2.28 to 1.3
Superoinferior (Z)	0.29 (0.40)	0.245	- 0.36 to 0.71

present, was minimum. The posterior shift might be due to the rectal filling or presence or absence of gas in the rectum.

The mean bladder volume was calculated to be 90.55 mL for all patients, and each patient had an average bladder volume of about 80 mL to 100 mL over the course of their treatment. This was done by maintaining a strict bladder control protocol for each patient. We found that maximum range of motion was observed when the bladder volume exceeded 100 mL as was seen in patient number 4, where a mean maximum shift in AP direction was almost up to -2.8 cm. When it was compared with their mean bladder volume, it was found to be excessive with a mean of almost up to 180 mL during the course of their treatment.

DISCUSSION

The EBRT with radiation doses of 40 to 50 Gy followed by boost with brachytherapy has been proven to be effective in the local control of cervical and uterine cancers. However, one of the major concerns with this modality of treatment has been acute or chronic small bowel toxicities with advancement in the treatment techniques of radiation therapy, it has been possible to reduce the toxicity to bowel. Nonetheless, it is essential to see that the benefits are not achieved at the cost of decreased local control due to a geometrical miss.^[5-7]

The CTV for primary cervical cancer treatment comprises the partially mobile uterus and cervix, the less mobile upper vagina, parametrium and pelvic lymph nodes located along the side walls in the pelvis. When treating with a conventional four field box technique, internal motion is less critical as the dose distribution is likely to encompass the central structures within the high dose region even if they move a little. The dose distribution in IMRT has the potential to conform more precisely to the target volume. Therefore, assessment of organ motion has become more important as there can be a geometrical miss during daily treatment.

According to the ICRU statement number 62 (ICRU 62) two margin volumes of CTV should be used to create the PTV: the internal margin to account for organ motion, and the setup margin to account for variation in patient position.^[8] Huh *et al.*^[9] and Lee *et al.*^[10] have previously reported the changes in the uterus position by comparing two magnetic resonance images taken before and during the period of radiotherapy. They showed that uterus movement and its

Table 2: The mean of movement in all directions and the mean bladder volume for each patient

Patient No.	Dimensions			Bladder volume, L
	Lateral (X), cm	Anteroposterior (Y), cm	Superoinferior (Z), cm	
1	0.16	- 0.3	- 0.06	0.031
2	0.1	- 0.133	0.1	0.111
3	0.2	1.32	- 0.24	0.151
4	- 0.6	- 2.28	0.16	0.189
5	0.25	1.15	0.55	0.157
6	- 0.08	1.04	- 0.4	0.113
7	0.45	- 0.75	- 0.225	0.062
8	0.3	1.15	- 0.05	0.125
9	- 0.06	0.6	- 0.12	0.088
10	0.36	0.55	- 0.2	0.067
11	0.4	0.3	- 0.15	0.046
12	0.1	0.04	- 0.36	0.064
13	- 0.12	0.18	0.56	0.055
14	0.1	0.3	0.71	0.049
15	0.3	0.4	0.4	0.076
16	0.31	- 1.21	- 0.35	0.140
17	- 0.1	- 0.53	0.21	0.096
18	0.2	0.67	0.6	0.090
19	- 0.45	0.45	- 0.34	0.070
20	0.15	- 0.22	- 0.56	0.040
21	0.2	0.71	0.43	0.083
22	0.3	0.51	0.25	0.066
23	- 0.21	0.81	0.16	0.100
24	0.14	0.91	- 0.22	0.105

Table 3: The comparison of the magnitude of displacements between current study and the study of Taylor *et al.*^[11]

Dimensions	Magnitude of displacement, cm		
	Mean (SD)	Median	Range
Present study			
Lateral	0.23 (0.22)	0.2	- 0.6 to 0.45
Anteroposterior	0.67 (0.83)	0.57	- 2.28 to 1.3
Superoinferior	0.29 (0.40)	0.24	- 0.36 to 0.71
Taylor <i>et al.</i> ^[11] study			
Lateral	0.08 (0.13)	0.0	0 to 0.5
Anteroposterior	0.7 (0.9)	0.5	0 to 0.48
Superoinferior	0.71 (0.68)	0.5	0 to 0.32

An assessment of interfractional uterine and cervical motion: implications for radiotherapy target volume definition in gynaecological cancer

Table 4: Adverse effects of radiotherapy in twenty-four participants

	Frequency of toxicity, %	
	Grade 1	Grade 2
Dysuria	80	20
Urinary frequency/urgency	90	10
Diarrhea	95	5

positional change were significant, which suggested the importance of accurately determining the target mobility for the conformal treatment. However, in that study, two sets of magnetic resonance images were taken in the supine position without a small bowel displacement system (SBDS) while the patients were treated in prone position with a SBDS placed under the patient's abdomen. We studied the uterine motion once a week during the full course of radiotherapy and every time, we found a significant shift in

uterine position in all directions.

In our study the mean bladder volume was 90.55 mL and it was shown that major shift occurred if the bladder volume exceeded 100 mL. We expected a mean bladder volume of 80 to 120 mL during IMRT in all our patients and this was corroborated on the weekly CBCT scans. Despite maintaining a standard bladder volume, we saw a uterine shift daily.

In a similar study by Taylor *et al.*^[11] in addition to the uterus, the movement of the cervix was assessed to determine the internal margin for radiotherapy. They concluded that an asymmetrical margin with CTV-PTV expansion of the uterus and cervix was needed during the treatment while they emphasized on the need for a strict bladder and rectum filling protocol during treatment.^[11] Very few studies have documented the changes in uterine position during the radiotherapy course. An association between bladder filling and uterine movement was reported by Buchali *et al.*^[12] that indicated no major AP change in cervical position. A maximum mean displacement in AP direction was seen in our study. In contrast, the mean displacement in SI direction was also substantial in the study by Taylor *et al.*^[11] [Table 3 and Figure 4]. The difference might be due to strict adherence to the bladder-bowel filling protocol in our study as well as once weekly CBCT instead of two imaging on two consecutive days in their study.

There is a definite association between the bladder filling and rectum emptying with uterine cervix movement. In addition, this association has been demonstrated by studies that have assessed the association of the bladder and rectum volume with the displacements of the uterus and the cervix respectively. Moreover, maximum uterine motion at the fundus emphasizes on the need to contribute a variable

margin around the fundus in the uterine shift.

The incidence of early bladder and rectal toxicities amongst all our patients were mild with all of them except two showing Grade 2 cystitis and mild diarrhea. Only one patient had Grade 2 diarrhea which was controlled with conservative measures [Table 4].

Despite maintaining a strict bladder filling and rectal emptying protocol, the interfraction movement in the uterine position during the course of radiotherapy may lead to a miss in target or overtreatment of the rectum, which lead to toxicity. We could not find any study in which uterine shift was observed during the whole course of radiotherapy hence, we were unable to compare the results with other works.

In conclusion, interfraction movement of the target organs may lead to overdosing or underdosing of the target or the normal structures during IMRT, hence at least once a week CBCT imaging might be necessary to minimize the geometrical miss of the tumor and deliver the planned doses to the target and normal structures for the best local control with minimum toxicity which is the primary aim of IMRT. This would also aid in the selection of appropriate and adequate planning target margins and provide an asymmetrical PTV conforming to the daily anatomical shift and contour of the patients. We also recommend a tapered CTV to PTV margin especially around the fundus of the uterus as maximum uterine motion is known at the fundus however further studies with larger numbers of patients and exact point localization of the uterus will be required for this purpose.

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Nil.

Conflicts of interest

There are no conflicts of interest.

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Case Report

Primary malignant melanoma of the spinal cord: a case report

Ashutosh Das Sharma, Jyoti Poddar, Ubrangala Suryanarayan Kunikullaya, Jay Prakash Neema

Department of Radiotherapy, Gujarat Cancer and Research Institute, Asarwa 380016, Ahmedabad, India.

Correspondence to: Dr. Ashutosh Das Sharma, Department of Radiotherapy, Gujarat Cancer and Research Institute, Asarwa 380016, Ahmedabad, India.
E-mail: sharmaashutoshdas@gmail.com

ABSTRACT

Primary malignant melanoma of the central nervous system is rare, and the events involving the spinal cord are even more infrequent. A 30-year-old male presented with a mass lesion of the spinal cord. After radiological workup, the mass was resected in December 2012. The histopathological examination report and immunohistochemistry suggested malignant melanoma. PET-CT scan, brain MRI, and funduscopic examination did not reveal malignant melanoma elsewhere in the body. The patient received postoperative radiotherapy until March 2013. Presently, the patient is asymptomatic with normal neurological functions.

Key words: Malignant melanoma; cervical spine; spinal cord; intramedullary; central nervous system

INTRODUCTION

Primary malignant melanoma of the central nervous system (CNS) is rare, with less than 60 cases reported in the literature;^[1] a disease that presents substantial diagnostic, prognostic and therapeutic challenges. We report a case of primary malignant melanoma of the spinal cord which was treated successfully with surgery and adjuvant radiation therapy (RT).

CASE REPORT

A 30 years old male presented with complaints of pain/stiffness in the neck and numbness/weakness in all four limbs. The symptoms had been present for 90 days and were gradually progressing.

Magnetic resonance imaging (MRI) scan of the cervical spine [Figures 1 and 2] showed an intradural extramedullary enhancing mass lesion at the second and third cervical vertebral level, compressing the spinal cord with focal cord edema and anterior-right lateral displacement of the spinal cord. The lesion measured $14 \times 17 \times 29$ mm in size and was hyperintense on T1 and hypointense on T2 weighted images. Another intradural extramedullary mass lesion was found at the craniovertebral level, indenting the cervico-medullary junction. This lesion measured $13 \times 18 \times 16$ mm

in size and was hyper to hypointense on T1 and isointense on T2 weighted images.

The patient underwent laminectomy and surgical excision and decompression on November 26th 2012. No residual lesion was found on postoperative MRI scan.

The histopathological examination showed clusters of atypical spindle cells with prominent nucleoli and eosinophilic cytoplasm [Figure 3], with evident intra- and extracellular pigment deposition. Immunohistochemistry staining showed positivity for HMB-45 [Figure 4], S-100 [Figure 5], and Melan-A [Figure 6]; all consistent with the histopathological diagnosis of malignant melanoma.

A thorough systemic survey was done including PET scan, tumor markers, ophthalmological, and dermatological examinations. These did not reveal any other foci of melanoma, leading to the diagnosis of primary spinal malignant melanoma. The patient received postoperative RT (50 Gray in 25 fractions on a linear accelerator with 6 MV photons by parallel opposed portals) from January 3rd to February 13th 2013. Since then, the patient has been on regular follow up with normal neurological functions.

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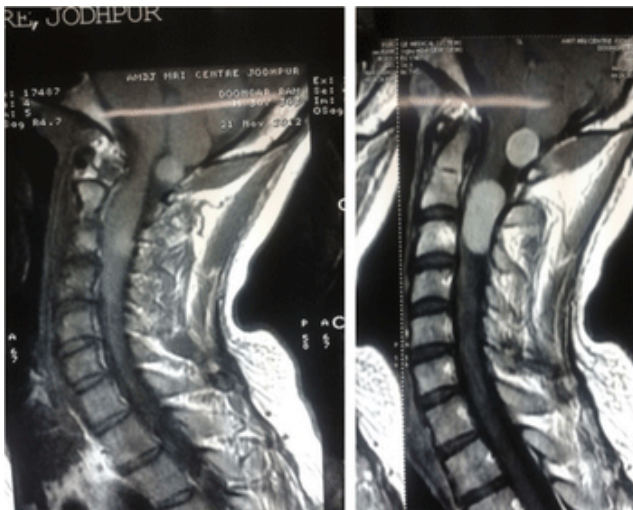


Figure 1: MRI of cervical spine, sagittal plane. MRI: Magnetic resonance imaging

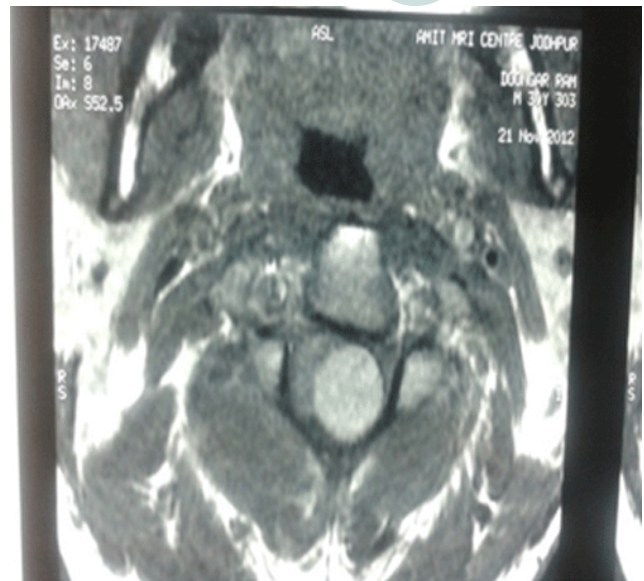


Figure 2: MRI of cervical spine, transverse plane. MRI: Magnetic resonance imaging

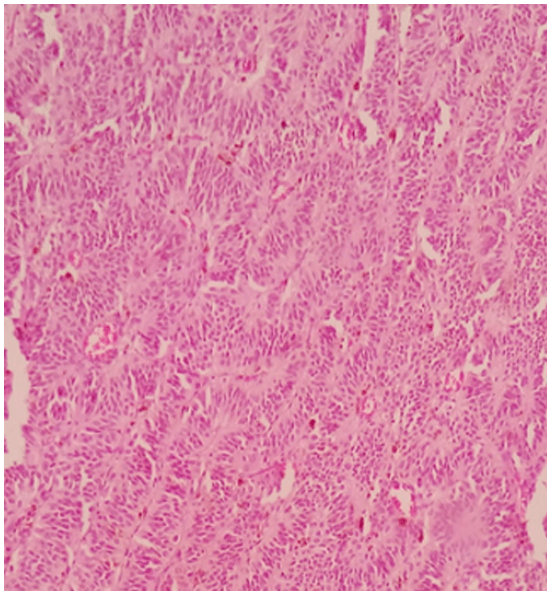


Figure 3: Low power hematoxylin-eosin staining slide (× 10)

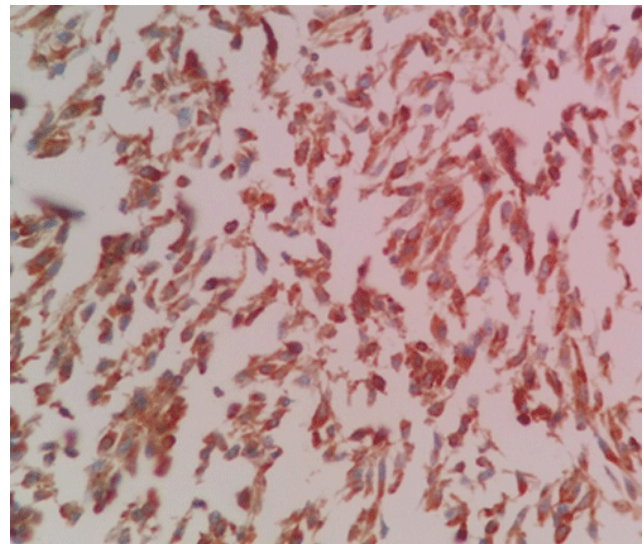


Figure 4: Histopathological slide stained with HMB-45 (× 40)

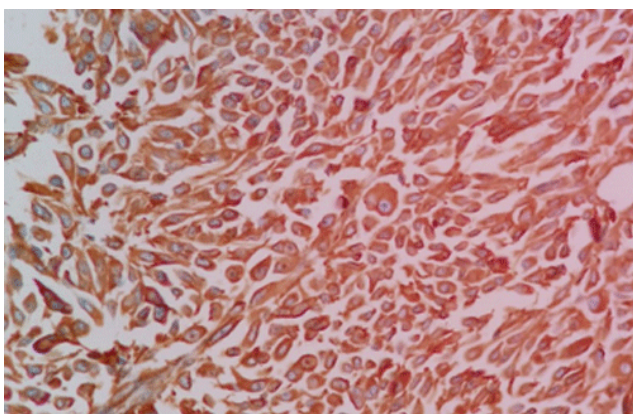


Figure 5: Histopathological slide stained with S-100 (× 40)

DISCUSSION

Primary malignant melanoma of the CNS is rare. Other

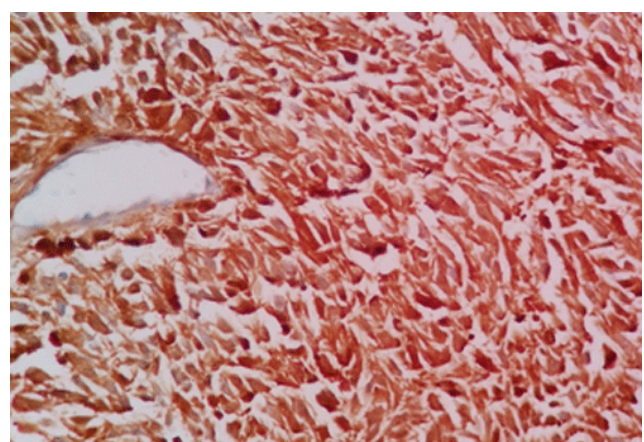


Figure 6: Histopathological slide stained with Melan-A (× 40)

noncutaneous primary malignant melanoma sites include the gastrointestinal tract and the eyes. Primary malignant

melanoma of the spinal cord most commonly involves the thoracic spine, followed by the cervical spine, and the lumbar region.^[2] The diagnosis requires exclusion of a primary cutaneous or ocular lesion, as malignant melanoma, although infrequently, can metastasize to the spinal cord.^[3]

The melanotic tumors of the CNS should be distinguished from other pigmented CNS lesions, e.g., meningioma, schwannoma, pigmented astrocytoma, and gliomas.^[4] The diagnosis can be confirmed by histology and immunohistochemistry, as per Hayward's criteria stating that "there should be no melanoma outside the CNS, and the confirmation should be done by IHC.^[5]" Melanocytic tumors are positive for S-100, HMB45, and Melan-A.

MRI of spinal cord melanoma shows characteristic features such as high signal intensity on T1-weighted images and equal or low signal intensity on T2-weighted images,^[6] due to the paramagnetic properties of melanin or the hemorrhagic elements in the tumor.^[6] Currently, there is no standard treatment for primary malignant melanoma of the spinal cord. The treatment regimen is similar to that of metastatic disease in the spinal cord, i.e., surgical resection followed by postoperative RT. Chemo- and immunotherapy have no proven clinical effects.^[7] Differentiation between primary and secondary CNS melanoma is important, because primary CNS melanoma is associated with longer overall survival (OS).^[8,9] OS in secondary CNS melanoma patients is less than one year,^[1] although complete surgical resection followed by postoperative RT does increase OS in these patients.^[10] However, lack of conclusive data renders the clinical outcome of spinal cord melanoma unpredictable.^[2]

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Nil.

Conflicts of interest

There are no conflicts of interest.

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Introduction to this special issue on brain tumor cell invasion and metastasis: anatomical, biological and clinical considerations

Michael A. Grotzer

Department of Oncology, University Children's Hospital Zürich, Steinwiesstrasse 75, CH-8032 Zürich, Switzerland.

Correspondence to: Prof. Michael A. Grotzer, Department of Oncology, University Children's Hospital Zürich, Steinwiesstrasse 75, CH-8032 Zürich, Switzerland. E-mail: michael.grotzer@kispi.uzh.ch

It is my privilege to introduce the readers to this special issue entitled "Brain tumor cell invasion and metastasis: anatomical, biological and clinical considerations". As cancer is a global epidemic which knows no borders, efforts to better understand biology and to control it should know no borders either. This issue contains a mixture of clinical and preclinical scholarly articles that have been written by scientists from America, Europe, and the Middle East. I hope that the fresh insights represented here will be appreciated by neuro-oncologists and brain cancer researchers across the translational spectrum.

The role of the PI3K/AKT/mTOR pathway in brain tumor metastasis

Alexandre Arcaro et al. (Switzerland)

This article is emphasizing the role of PI3K/AKT/MTOR pathway on glioma growth and metastasis with a specific focus on angiogenesis, glioma cell invasion and inflammation.

Dissecting brain tumor growth and metastasis *in vitro* and *ex vivo*

Martin Baumgartner et al. (Switzerland)

This article reviews the *in vitro* and *ex vivo* techniques used to study growth and dissemination of brain cancer cells including organotypic slice culture methods.

Tailored nanocarriers and bioconjugates for combating glioblastoma and other brain tumors

Mohamed I. Nounou et al. (Egypt)

This article reviews blood brain barrier hampered drug delivery and suggests new CNS therapeutics delivery

techniques by using tailored nanocarriers and bioconjugates.

Interdisciplinary management of central nervous system metastasis and neoplastic meningitis: recent developments and future perspectives

Ghazaleh Tabatabai et al. (Germany)

This article reviews advances in our understanding on the molecular mechanisms leading to invasion of tumor cells to the CNS and highlights the challenges and perspectives in the field of interdisciplinary management of CNS metastasis.

Brain tumor surgery: supplemental intraoperative imaging techniques and future challenges

Telmo Augusto Barba Belsuzarri et al. (Brazil)

This article discusses maximum safe brain tumor resection techniques including methods that are designed for a precise demarcation of brain tumors and their infiltration zones.

Brain infiltration by cancer cells: different roads to the same target?

Mayra Paolillo et al. (Italy)

This review illustrates recent findings of genes and cellular mechanisms that have been found to be involved in brain metastasis and describe the different cell types involved.

Effects of Gas1 on gliomas: a review on current preclinical studies

Jose Segovia et al. (Mexico)

This article reviews the potential therapeutic effect of the tumor suppressor Gas1 for treatment of GBM.

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Targeting cerebrospinal fluid for discovery of brain cancer biomarkers***Tarek Shalaby et al. (Switzerland)***

This review examines potential and limitations of brain tumor biomarkers in the CSF.

Gemcitabine followed by radiotherapy in treatment of newly diagnosed high-grade gliomas***Maha El-Naggar et al. (Egypt)***

This prospective single centre phase II study evaluated the

efficiency of gemcitabine as radiosensitizer in the treatment of newly diagnosed high-grade glioma patients.

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Nil.

Conflicts of interest

There are no conflicts of interest.

Dissecting brain tumor growth and metastasis *in vitro* and *ex vivo*

Michael A. Grotzer^{1,2}, Anuja Neve^{1,2}, Martin Baumgartner^{1,2}

¹Department of Oncology, University Children's Hospital Zürich, Steinwiesstrasse 75, CH-8032 Zürich, Switzerland.

²Children's Research Center, University Children's Hospital Zürich, August-Forel Strasse 1, CH-8008 Zürich, Switzerland.

Correspondence to: Dr. Martin Baumgartner, Department of Oncology, University Children's Hospital Zürich, August-Forel Strasse 1, CH-8008 Zürich, Switzerland. E-mail: Martin.Baumgartner@kispi.uzh.ch



Dr. Martin Baumgartner is a research group leader at the Children's Research Center of the University Children's Hospital Zürich and private docent at the Science Faculty of the University of Zürich. Martin Baumgartner obtained a Ph.D. from the Pasteur Institute and the Université Pierre et Marie Curie in Paris and did postdoctoral work at the University of California San Francisco and the Universities of Zürich and Bern.

ABSTRACT

Local infiltration and distal dissemination of tumor cells hamper efficacy of current treatments against central nervous system (CNS) tumors and greatly influence mortality and therapy-induced long-term morbidity in survivors. A number of *in vitro* and *ex vivo* assay systems have been established to better understand the infiltration and metastatic processes, to search for molecules that specifically block tumor cell infiltration and metastatic dissemination and to pre-clinically evaluate their efficaciousness. These systems allow analytical testing of tumor cell viability and motile and invasive capabilities in simplified and well-controlled environments. However, the urgent need for novel anti-metastatic therapies has provided an incentive for the further development of not only classical *in vitro* methods but also of novel, physiologically more relevant assay systems including organotypic brain slice culture. In this review, using publicly available peer-reviewed primary research and review articles, we provide an overview of a selection of *in vitro* and *ex vivo* techniques widely used to study growth and dissemination of primary metastatic brain tumors. Furthermore, we discuss how our steadily increasing knowledge of tumor biology and the tumor microenvironment could be integrated to improve current research methods for metastatic brain tumors. We believe that such rationally improved methods will ultimately increase our understanding of the biology of brain tumors and facilitate the development of more efficacious anti-metastatic treatments.

Key words: Primary brain tumor; metastasis; *in vitro* model system; cell migration; organotypic brain slice culture

INTRODUCTION

Impressive achievements in genomic and epigenomic analyses of tumor tissues and individual tumor cells have revolutionized our understanding of primary brain tumors. Alterations detected on the genome or transcriptome level in large patient cohorts in combination with our increasing understanding of epigenetic gene regulation have

disentangled apparently identical brain tumors as related but functionally different tumor entities.^[1-5] Within such single tumor entities, alterations detected in their respective metastases suggested potential driver mechanisms of tumor progression.^[6] This considerably more complex image we currently have is instrumental to better understand the highly heterogeneous nature of the tumor tissue itself and of the host environment interacting with it and shaping some of

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its spatial, functional and morphological manifestations. However, in order to translate this still growing knowledge into clinical applications targeting the tumor phenotype, sophisticated model systems are necessary to explore and validate potential interference strategies under physiologically relevant conditions. In addition, functional genomics and cell-based molecular analyses are indispensable in many cases to clarify whether mutated or amplified genes are necessarily contributory to an altered proteome and causative for the cancerous phenotype. Moreover, the current wealth of genomic and transcriptomic data is insufficient on its own to isolate specific signaling networks driving tumor progression from a benign lesion to a disseminated cancer. Hence, to tackle the complexity of the metastatic process it is necessary to dissect it into individual steps that can be addressed with rationally adapted model systems. In this review we focus on *in vitro* and *ex vivo* primary brain tumor model systems and discuss how they can be improved and used to develop the molecular understanding necessary for designing novel anti-metastatic therapies. While none of these model systems on its own will suffice to tackle such a complex disease as cancer, they can effectively guide our search for efficacious and less toxic therapies and instruct the design of appropriate *in vivo* studies.

THE MACHINERY: ALTERED CYTOSKELETON DYNAMICS AND CELL MOTILITY DRIVE CANCER DISSEMINATION

Dissemination of tumor cells from the primary tumor causes healthy tissue infiltration and metastatic disease, and

it hampers the efficacy of current cancer treatments. It is triggered by the transient or permanent induction of motility and invasiveness in the tumor cells. An essential prerequisite for primary brain tumor cell migration and invasion is the remodeling of the actin and tubulin cytoskeletons,^[7-9] which not only provide force, traction and rigidity but also scaffold signaling complexes in a spatially controlled manner.^[10-12] Hence, blocking motility and invasiveness by targeting pro-migratory cytoskeleton dynamics in tumor cells could prevent local tumor cell invasion, further dissemination from proximal metastases and the evolution towards a more aggressive phenotype. In a seminal review by Giese *et al.*,^[13] the dichotomy of migration and proliferation in gliomas was recognized as the consequence of antagonistic cell regulation. Consequently, the authors concluded that an approach to influence the underlying mechanisms could be the basis of novel anti-invasive therapy strategies. A computational modeling study predicts that even a small increase in the motile capability of tumor cells, and the consequent short-range dissemination, increases net tumor growth and resistance to targeted therapy^[14] [Figure 1]. Indeed, targeting tumor cell motility and invasiveness as a strategy against metastasis is an emerging theme in cancer research,^[15-17] and the pro-migratory phenotype in tumor cells has been addressed in the past by a number of approaches that impair cell autonomous migration, cell-cell communication, cell-cell or cell-matrix interaction (^[15] and references therein). This research led to the development of a number of clinical trial studies for solid tumors with approaches inhibiting various components of the aforementioned pro-migratory determinants.^[15]

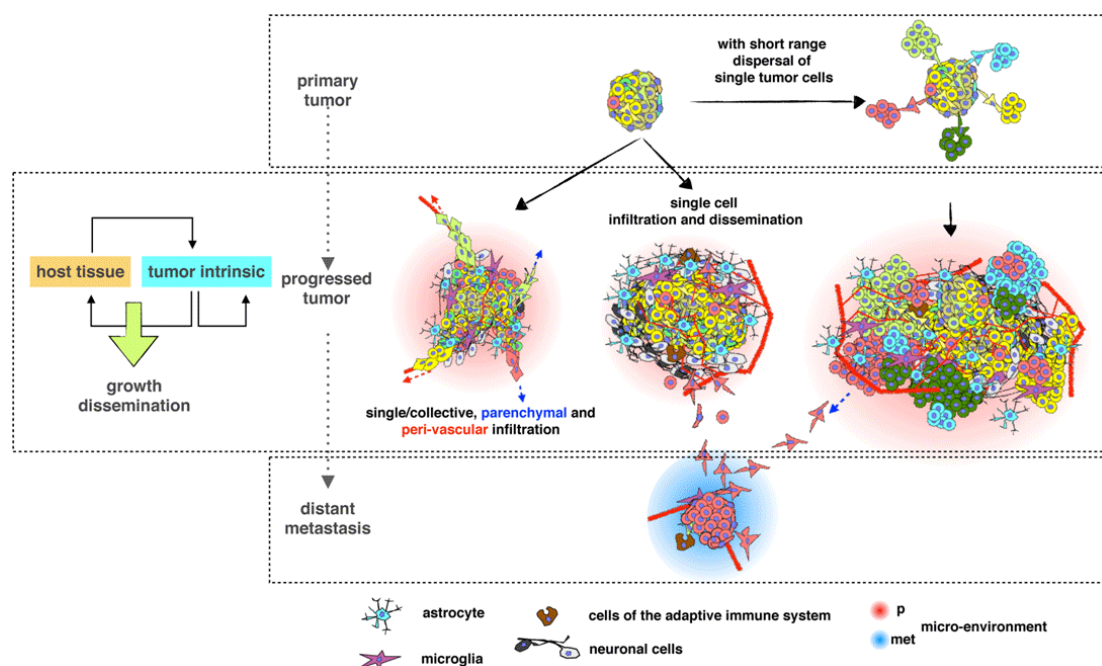


Figure 1: Model of growth, progression and dissemination of primary brain tumors. The progression of primary brain tumors from a small neoplastic lesion to a metastasizing tumor through growth and dissemination of tumor cells is schematically visualized. The mode of tumor cell growth and dissemination varies between different tumors and involves random or guided, single or collective dissemination of tumor cells. The model depicting low range dissemination at early stages and the consequent increased net tumor growth is according to Waclaw *et al.*^[14]

Despite of this, most current treatments including those against primary brain tumors still focus primarily on targeting growth and survival of the tumor cells. This lack of adequate anti-dissemination therapies is due in part to the complexity of the cell migration process itself and the redundancy of the signaling that controls its mechanics.

Additionally, tumor cells exploit mechanisms that normally direct physiological movements. However, the addition of tumor cells to druggable pathways and our increasing understanding of cell mechanics and its control offer room for therapeutic interventions targeting tumor cell dissemination specifically.

The soil: the microenvironment in the brain

To address the impact of the tumor microenvironment on tumor growth and progression *in vitro*, we need (a) to better understand the intricate interaction between a growing neoplasm and its cellular, biophysical and chemical environment and (b) to continuously implement this increasing knowledge for advancing our model systems to mimic the micro environmental parameters better. The following paragraph will briefly discuss some relevant aspects of the still poorly understood interaction between the cells of primary brain tumors and their cellular host environment.

Biophysical properties of the brain microenvironment

Mammalian cells are sensitive to biophysical and chemical signals emanating from the surrounding matrix environment, the extracellular matrix (ECM), which can influence their behavior.^[18-20] Depending on the tissue, composition and stiffness of the ECM differs markedly.^[21] The stiffness or rigidity of a material such as a meshwork of collagen I fibers, describes its resistance to deformation. It depends on the elastic modulus (or compliance) of its constituting material e.g. fibrillar polymers of the protein collagen, which describes the ability to resist a distorting influence and to return to its original size and shape when the influence is removed. Thus, the stiffness of the ECM depends on its components and their elastic modulus. As the parenchyma of the brain is mostly devoid of fibers with a high elastic modulus such as collagen or fibronectin fibrils, its stiffness is very low compared to the ECMs in other tissues of the human body.^[22] Conversely, the leptomeninges in the subarachnoid space, to where metastatic medulloblastoma tumors preferentially spread,^[23,24] are connected by a network of collagen-rich trabeculae, which likely is much stiffer than the parenchyma.

The basic constituents of the brain ECM are glycosaminoglycans with their most prominent member hyaluronan (Hyaluronic acid, HA), link proteins, lecticans and tenascins.^[25] HA acts as a backbone for the assembly of a relatively loose and flexible meshwork. The distribution and composition of these ECM components in the developing rodent brain is changing during embryonal and postnatal phases and reaches a mature stage at postnatal

day 20.^[25] However, disease-associated remodeling of the CNS ECM has been observed after injury,^[25-28] suggesting that growing primary neoplasms in the brain may also alter the surrounding ECM. Relatively little change in the expression levels of a small set of proteins in normal brain tissue and in brain tissue surrounding invasive glioblastoma was observed in a recent study,^[29] except for Tenascin-R and CD168, which were both up-regulated. Matrix stiffness regulates proliferation and motility of Glioblastoma multiforme (GBM) cells^[30] and the increase of ECM stiffness through fiber crosslinking by the product of the LOX gene causes their enhanced integrin-dependent invasion.^[31] The specific impact of matrix stiffness on cell migration was investigated in glioma and found to decrease motility in agarose-stiffened collagen gels^[19] and to increase motility in matrigel.^[32] This somewhat conflicting result may be explained by the receptors sensing the matrix environment and their underlying signaling, which markedly influence the migratory outcome. Hence, the impact of matrix stiffness on the migratory behavior should always be investigated in the context of the cognate receptors. Whether matrix stiffness could exert a selective pressure on brain tumor cells contributing to the altered genetic landscapes is still poorly understood. One potential sensor and transducer of matrix stiffness in brain tumors is the HA receptor CD44, which was identified in GBM to facilitate invasiveness in stiff matrices.^[33]

Chemical properties of the brain microenvironment

Analogous to solid tumors outside the CNS, where parallels between the inflammatory response in wounds and the host tissue response to growing neoplasms has been noted,^[34] remarkable similarities in brain tissue response after injury and in the vicinity of brain tumors exist.^[35] Tissue response is driven initially by a local repertoire of innate and adaptive immune cells that is subsequently supported by infiltrating cells of the adaptive immune system. In the brain, an immune privileged site of the human body, tissue response is driven by microglia/macrophages and astrocytes. Microglia are involved in first-line innate immunity in response to brain injury, when they convert to an active proliferating, migrating and phagocytic phenotype.^[36] Microglia and macrophages accumulate in and around glioma to which they are suspected to be attracted by glioma-secreted chemo attractants such as monocyte chemotactic protein-3 (MCP-3), colony-stimulating factor 1 (CSF-1), granulocyte-colony stimulatory factor (G-CSF), and hepatocyte growth factor/scatter factor.^[37] Besides direct stimulatory functions through secretion of growth factors or proteolytic enzymes, glioma infiltrating macrophages were also found to contribute to tumor vascularization and net tumor growth.^[38] Surprisingly, however, malignancy or primary cranial origin did not seem to determine immune cell infiltration as no significant difference in immune cell distribution was observed between different primary or secondary brain malignancies (Glioma, PNET/Medulloblastoma,

adenocarcinoma, melanoma meningioma).^[39] A more recent study correlating inflammatory gene expression with the molecular subgroup of medulloblastoma, revealed significantly increased immune cell infiltration of tumor associated macrophages and other immune cells in the SHH subgroup,^[40] suggesting a potential therapeutic relevance of immune cell targeting specifically for this subgroup.

Microglia are outnumbered by astrocytes, which account for nearly half of all cells resident in the brain. Astrocytes respond to brain injury and tumor growth in a process named reactive gliosis. On the one hand, reactive gliosis and the associated secretion of growth factors and cytokines help repairing injury in the CNS.^[41] On the other hand, the astrocytic response in the tumor microenvironment also contributes to disease progression. Of note in this context is the capability of U87MG glioblastoma cells to induce astrocyte activation through the secretion of Receptor Activator of NF- κ B ligand (RANKL), which in turn facilitates glioblastoma invasiveness *in vivo* by releasing FGF4, FGF6, TGF- β and Hepatocyte growth factor.^[42] Consistently, co-cultured astrocytes display increased expression levels of a number of growth factors and cytokines and enhance invasiveness of glioblastoma stem-like cells.^[43] Another decisive input could stem from astrocytes activated by the neoplastic lesion and the consequent up-regulation of matricellular proteins such as secreted protein acidic and rich in cysteines (SPARC) in astrocytoma^[44] and medulloblastoma^[45] or connective tissue growth factor in glioma,^[46] which jointly with additional matricellular proteins remodel neuronal tissue during development or after brain injury.^[28] Significantly, the concept of reciprocal stimulation of tumor cells and astrocytes was recently also identified in metastatic melanoma, which elicits an inflammatory cytokine response in astrocytes that facilitates brain metastasis.^[47]

Combined, these studies emphasize the importance of

incorporating environmental parameters into experimental protocols to explore their contribution to the proteomic landscape and the functional outcomes of primary brain tumors.

CURRENT *IN VITRO* MODEL SYSTEMS TO ADDRESS FUNCTIONS OF METASTATIC PRIMARY BRAIN TUMORS

Preclinical evaluation of novel anti-metastatic therapy strategies in animal models will remain an essential step towards the development of novel therapeutics. However, cell culture models are instrumental for deciphering essential morphological and functional aspects of the biology that drives neoplastic lesions into disseminated diseases. They also provide essential insights for designing appropriate animal models and help elucidating the causes that may underlie controversial outcomes of *in vivo* studies. Although a general trend towards 3D model systems can be noted, a majority of experiments in tumor-related research are still conducted in 2D settings. For a general, in depth description and comparison of 2D versus 3D culture systems, the reader is referred to Zimmermann *et al.*^[48] who emphasized the need of higher throughput approaches to understand cell dissemination capabilities on one hand and the role of the microenvironment on the other hand.

The “ideal” *in vitro* cell culture model should mimic one or several of the following characteristics of the *in vivo* tumor: proliferative capabilities and morphology of the tumor cells, cellular and phenotypic heterogeneity, a dynamic tumor microenvironment and the drug response profile. A series of excellent reviews have recently described in depth the use of 3D tissue culture model systems in pathophysiology^[49] and high-throughput drug candidate toxicity analysis,^[50] to identify tumor-specific signaling pathways and biomarkers,^[51] and to determine growth determinants for drug target discovery.^[52] These reviews delineate what parameters contribute to a disease representing, efficient

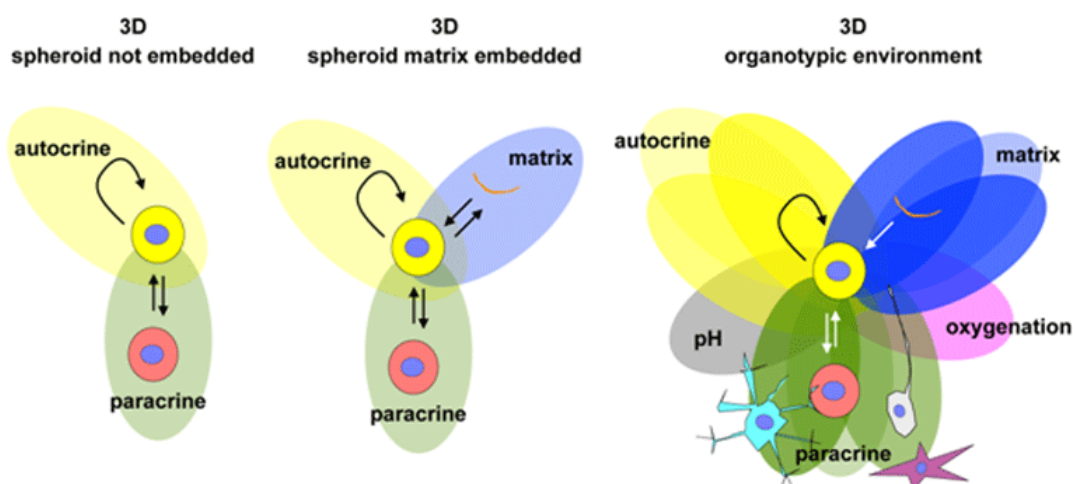


Figure 2: Tumor cell growth, survival and dissemination are governed by extrinsic and intrinsic parameters. Tumor cells are under the spheres of influence of intrinsic and extrinsic parameters. Colored ovals represent various degrees and manifestation patterns of such parameters, which dramatically increase in number and complexity in the organotypic environment

Table 1: Studies using 3D primary brain tumor model systems

Cells	Tumor type	Condition	Experiment	Matrix or scaffold	Effect	Ref.
U87MG	Glioblastoma multiforme (GBM)	2D, 3D neurospheres	Analysis of cell growth in neurospheres, wound healing after DDX6 or PHLDB1 knock-down	None	50 % reduction in neurosphere formation and migration	[83]
U-251MG, U-343MG, LN-229	Glioma	2D, 3D spheroid culture	Analysis of stable over-expression of wt and mutant proteins under different oxygenation.	None	Mutant IDH1 causes reduced cell migration and differences in growth properties in 3D spheroid cultures.	[84]
U87	GBM	2D, 3D single cell embedding	Spheroid in soft agar, 2D culture, analysis of Glioma co-culture with MSCs expressing suicide gene. Rotary cell culture to generate large cell aggregates, growth on top of matrigel.	Soft agar	Stem cell-mediated anti-tumor effect. Increased IC50 under 3D culture conditions.	[85]
KNS42, U87, Res196, T7/11, GB-1	Pediatric and adult GBM and ependymoma, pediatric mixed glial tumor	3D, long-term culture of large cellular aggregates		None	Angiogenic change and endothelial marker expression in GBM aggregates	[86]
SHSY5Y, T98G, U138MG	GBM and neuroblastoma	3D, spheroid culture	Exposure to Doxorubicin, Etoposide and Vincristine, analysis by electrochemical impedance spectroscopy.	None	Cytotoxic effect measured in 2D cannot be extrapolated to 3D. 3D cultures can also display higher sensitivity to chemotherapeutics.	[58]
U67-MG	GBM	2D	2D wound healing, tubulogenesis assay on matrigel after exposure to rapamycin or hypoxia.	None	Tube structure formation	[87]
Rat C6, NSCs adult hippocampal	Glioma	3D, spheroid culture	Comparison of different 3D tissue clearing protocols.	None	Validation of tissue clearing protocols of high resolution imaging of spheroid culture. Increased HLA-E expression in 3D culture and increased resistance to NK-mediated cytotoxicity.	[88]
U251	Glioma	2D and 3D rotary cell culture system	Proteomic comparison of 2D and 3D cell culture.	None		[89]
DBTRG, T98, U87, A172, 8MGBA, 42MGBA, DKMG, GAMG, GMS10, GSCs	GBM	2D, 3D, single cell embedding	Cytotoxicity assays using collagen I and collagen I-HA matrices in combination with receptor tyrosine kinase inhibitors.	Bovine skin collagen I, Collagen I-HA mixtures	Collagen-based 3D matrix reduces sensitivity of GSCs to receptor tyrosine kinase inhibitors.	[60]

Contind...

Cells	Tumor type	Condition	Experiment	Matrix or scaffold	Effect	Ref.
U87-MG, primary MB samples	GBM	2D, 3D culture	Comparison of 2D and 3D cultures for growth and viability after irradiation or treatment with TMZ, cisplatin or carmustin.	ExtracelTM (Polyethylene-based hydrogel with HA and gelatin).	3D cell culture is better morphological correlate to <i>in vivo</i> tumor, 3D grown GBM moderately less sensitive to irradiation.	[90]
U118-MG	GBM	3D culture, cells grown on rigid matrix	Evaluation of growth and stem cell properties	Porous chitosan-HA	Porous chitosan-HA increases growth and causes up-regulation of stem-cell markers	[63]
Patient-derived tumor material	GBM	3D, single cells	Embedding in hydrogels, morphology and cell migration analysis, single cell tracking for motility.	Hydrogels made of collagen I, III, or collagen-HA mixtures	HA causes rounded morphology and reduces motility of matrix-embedded cells.	[62]
U87-MG, U87+EGFR	Glioma	3D, single cells	Analysis of growth, metabolic activity and HIF-1 VEGF, MMP-2, MMP-9 and Fibronectin production.	GelMA or PEG4A hydrogels supplemented with methacrylated HA (HAMA) at increasing concentrations	Increasing HAMA concentrations cause up-regulation of fibronectin, VEGF and HIF-1.	[64]
A-172	Glioma	3D, single cells, microfluidic chip	Analysis of single cell viability, F-actin size and cellular orientation in embedded cells under flow and VEGF in microfluidic chip.	Acrylated HA cross-linked with MMP-sensitive or RGD peptides	F-actin reorganization and re-orientation of cells in response to flow and VEGF.	[91]
OSU-2	GBM	3D, single cells	Evaluation of matrix stiffness impact on tumor cell morphology and migratory/invasive capabilities.	Matrigel with varying stiffness	Increased matrix stiffness causes increased invasive motility.	[32]
M059K, HepG2, CYP3A	GBM, Hepatoblastoma	3D, micro-scale perfusion system	Evaluation of liver cell metabolism on cytotoxic effect of IFO and TMZ.	Polylactic acid scaffold	TMZ showed much lower cytotoxicity against GBM cells in 3D than in 2D. IFO effect dependent on metabolic activity of cytochrome P450 in hepatocytes.	[68]
U251MG, LN229 and U87MG	GBM	3D, organotypic slice culture	Evaluation of Rho-family GTPase activation during GBM invasion in brain slice and 3D matrigel culture. Use of Rho-family GTPase fluorescent protein sensors.	Matrigel, slice culture	Perivascular and intraparenchymal invasion is associated with increased Rac and Cdc42 and reduce Rho GTPase activity.	[53]
U87, U251HF, SNB19, LN2308, LN229	Glioma	2D, 3D	Comparison of protein expression in cells grown under 2D or 3D conditions and in different oxygenation.	AlgiMatrix	Differential expression of invasion, survival and hypoxia driver proteins between 2D and 3D. Effect of 3D growth dominates oxygenation.	[92]

Contind...

Cells	Tumor type	Condition	Experiment	Matrix or scaffold	Effect	Ref.
LN18, GL15, U87, A172	GBM	2D, 3D	Seeding of GBM cells on brain Hi-spots and exposure to anti-proliferative drugs Ara-C, Taxol and TMZ	Brain Hi-Spots	Increased anti-proliferative effect of TMZ on GBM cells maintained on Hi-spots.	[65]
C6 rat, U-87 MG, U-118 MG	Glioma	2D, 3D, Matrigel and chitosan-alginate scaffolds	Comparison of growth and morphology and secretion of VEGF, MMP2, fibronectin and Laminin between cells grown in 2D, in matrigel or on chitosan-alginate scaffolds.	Matrigel, Chitosan-alginate scaffolds	Growth on chitosan-alginate scaffolds reduces growth but increases secretion of VEGF, MMP2, fibronectin and Laminin.	[93]
LN18, F98, F98EGFR- vIII, C6 rat, U-87 MG,	Glioma	2D, 3D spheroid culture, transwell migration	Evaluation of SHA impact on cell growth, collagen I invasion and mRNA expression of genes relevant for cell-cell and cell-matrix interaction.	Collagen I	SAHA treatment causes reduction of invasion and the reorganization of the matrix surrounding the tumor spheroids.	[94]
U178, U251	Glioma	3D transwell	Analysis of transwell invasion and migration after compound inhibition of PKC δ .	Collagen I supplemented with Tenascin C	Tenascin-C deposition triggers glioma invasion in a PKC δ -dependent manner.	[95]
U373	Glioma	3D spheroid	Analysis of growth and dissemination in increasingly stiff collagen I gels.	Collagen I-agarose	Matrix stiffness impacts on glioma cell invasiveness. High stiffness blocks invasiveness.	[19]
U251, U178	Glioma	3D, single cells	Quantification of transwell migration of cells stimulated with TNF- α , IL-1 or a combination of both.	Collagen I	Interleukin-1 beta (IL-1b) and tumor necrosis factor-alpha (TNF- α) increase glioma cell invasiveness in 3D with parallel increased MMP-2 and MMP-9.	[96]
Primary mouse G3 MB	Medulloblastoma	3D neurospheres	Neurosphere compound toxicity assays using FDA-approved drugs and ATP-sensor dye.	None	FDA-approved Pemetrexed and Gemcitabine significantly block proliferation of G3 MB.	[55]
DAOY, UW228	Medulloblastoma	2D, 3D transwell, 3D micro beads	Quantification of collagen invasion after HGF stimulation, small compound kinase inhibitor or siRNA treatment in cells seeded on Micro-beads and embedded in collagen I matrix.	Collagen I	HGF-induced c-Met activation promotes MB cell invasion through the kinase MAP4K4.	[9]
DAOY, UW228, Med PDX1712, MedPDX411, primary MB	Medulloblastoma	2D, 3D micro beads and spheroids	Quantification of collagen invasion and cell migration after growth factor stimulation using invasion counter platform for automated quantification of motile cell behavior in different environments.	Collagen I	HGF, EGF and bFGF are strong promoters of MB cell migration and invasion	[56]

Contind...

Cells	Tumor type	Condition	Experiment	Matrix or scaffold	Effect	Ref.
DAOY, UW228	Medulloblastoma	3D, transwell migration	Quantification of VEGF-A induced, PERK-dependent transwell migration.	Matrigel	Tumor cell-derived VEGF-A promotes medulloblastoma cell migration and invasion through VEGFR2 and enhanced by PERK.	[97]
DAOY, UW228	Medulloblastoma	3D, transwell migration	Quantification of EphB1 effect on SHH medulloblastoma transwell migration using electrical impedance measurements.	None	Knockdown of Eph-B1 causes reduction in B-1 integrin expression and in growth and migration.	[98]
DAOY	Medulloblastoma	3D, μ Lane microfluidics system	Quantitative and qualitative analysis of chemotactic response of MB cells to a gradient of EGF in a microfluidic system.	Matrigel	Matrigel invasion of MB cells towards an EGF gradient is blocked by pharmacological PI3-K inhibition.	[99]
DAOY	Medulloblastoma	3D, transwell migration, xCelligence assay	Quantitative analysis of PDGFR control of CXCR4 pro-migratory signaling in SHH MB model.	Matrigel	PDGF signaling restricts expression of negative regulator GRK6 and promotes CXCR4-Src-dependent cell migration.	[100]
DAOY, UW228-3	Medulloblastoma	3D confrontation co-culture	Quantification of repulsive action of Slit-Robo signaling during MB invasion.	Collagen I	Slit represses MB invasion in collagen gels.	[101]
DAOY	Medulloblastoma	2D/3D transwell migration	Evaluation of impact of matricellular SPAR on MB cell migration and invasion	Matrigel	SPARC suppresses migration and invasion by repressing Rho-GTPase activation and by triggering Src-dependent cytoskeleton reorganization. Highly self-renewing CD271 high, CD133 low MB cell population in the core sustains tumorigenesis.	[45]
DAOY, D283	Medulloblastoma	2D spheroid outgrowth, 3D transwell migration	Comparison of invasion and self-renewal. Analysis of higher versus lower self-renewing tumor spheres and stationary versus migrating adherent MB cells with respect to CD271 and CD133 expression.	Collagen I	Commitment to migration/invasion (metastatic phenotype) is identified by reduced CD271 and increased CD133 signature.	[102]

Overview of a selection of primary brain tumor studies that used 3D cell culture technologies. Ara-C: cytosine β -D-arabinofuranoside; CXCR4: CXC-motif-chemokine receptor 4; PERK: pancreatic endoplasmic reticulum kinase; EGF: epidermal growth factor; GBM: glioblastoma multiforme; GM-CSF: granulocyte-macrophage colony stimulating factor; GSCs: glioblastoma stem cells; GRK6: g-protein coupled receptor kinase 6; HA: hyaluronic acid; HAMA: methacrylated HA; HGF: hepatocyte growth factor; IDH1: isocitrate dehydrogenase 1; IFO: ifosfamide; MB: medulloblastoma; MMP: matrix metalloproteinase; PEG: polyethylene glycol; PDGFR: platelet-derived growth factor receptor; PI3-K: phosphoinositide 3'Kinase; RGD: L-arginine, glycine, and L-aspartic acid; SAHA: suberoylanilide hydroxamic acid (or vorinostat a HDACi); SPARC: secreted protein acidic and rich in cysteine; Src: rous sarcoma kinase; TMZ: temozolomide; VEGF: vasculature endothelial growth factor; 2D/3D: two dimensional/three dimensional

model system: the system should mimic biophysical and chemical properties of the tissue environment (composition and stiffness of matrix, availability of growth factors,

cytokines, metabolites) in a well controllable manner, the cells should be observable to increase output options (morphological analysis, use of fluorescent protein^[53] and dye

sensors^[54]) and it should have a high-throughput potential.

To understand the causes and consequences during pathophysiological progression from a primary neoplastic lesion in the brain towards a metastatic cancer and to pre-clinically test potential intervention strategies, we thus require model systems that mimic not only the proteomic heterogeneity of the tumor cell itself but also the reciprocal interactions between the tumor and the receiving brain tissue [Figure 2]. The following paragraph provides an overview over some recent approaches in primary brain tumor research. It highlights the difficulty to design an optimal, tumor-adapted system and emphasizes the need to further improve currently used systems.

2D and 3D model systems in primary brain tumor research

A number of articles have been published in the last few years that used *in vitro* model systems to evaluate effects of novel potential treatment strategies on growth, viability or motile behavior of primary brain tumors [Table 1]. A general consensus has been reached in that 3D cell culture model systems reflect the specifics of the *in vivo* situation better compared to 2D model systems. On the down side of this was the lack of high-throughput capability of 3D methods that hampered until a few years ago their broader use in combination with in screening approaches. A milestone in this context was the generation of spheroid cultures in 96 or even 384 well format from primary brain tumors that allowed the parallel testing or large sample sizes.^[54-56] In these studies, diagnostic dyes and fluorescent proteins were used individually or in combination for probing cellular functions on the one hand and for discriminating specific cell populations on the other hand. A general protocol describing the reproducible establishment and microscopy-based analysis of spheroid cultures using fluorescent protein quantification in high throughput was described recently.^[57] As an alternative to fluorometric read-outs, electrochemical impedance spectroscopy was used to quantify different susceptibilities of 2D versus 3D spheroid culture of glioblastoma and neuroblastoma cell lines to cytotoxic compounds^[58] and to determine the therapeutic window of these compounds. Using different combination of dyes to separate subpopulation of cells grown in co-culture combined with diagnostic flow cytometry and two-photon microscopy allowed to further refine the selective output of 3D methods.^[54] However, high-throughput capabilities and accuracy of a selected read-out has to be carefully balanced and discriminating phenotypic differences at single cell level in 3D cultures in high throughput remains a formidable challenge.

The impact of the embedding matrix on the behavior of the tumor cell

The choice of the embedding matrix is of outmost importance for 3D cultures, in particularly for primary brain tumors that encounter *in vivo* mostly brain parenchyma and collagen-rich surfaces and structures in the subarachnoid space.^[23,24,59] Hence, the biophysical and chemical properties

of the matrix should be adjusted to those in the location of growth and metastatic dissemination of the tumor under investigation. In this context, Fernandez-Fuente *et al.*^[60] investigated the impact of different environmental conditions on glioblastoma stem cells (GSCs). They found that GSCs grown in collagen-based 3D conditions were markedly less susceptible to receptor tyrosine kinase inhibition by currently available inhibitors, suggesting that oncogene addiction of tumor cells could also be bypassed by adhesion signaling.^[61] Interestingly, matrix stiffness or the addition of hyaluronic acid (HA) did not affect the sensitivity of the GSCs in this study. Primary cells from glioma patient tumor material exposed to increasing concentrations of HA responded with rounded morphology and reduced migration, suggesting that HA concentrations may affect glioma cell behavior.^[62] Consistently, addition of HA to porous chitosan scaffolds^[63] or to artificial hydrogels^[64] increased the expression of stem cell markers and VEGF and HIF-1, respectively. However, the finding that increasing matrix stiffness - by adding agarose to a collagen I matrix - blocks glioma invasiveness,^[19] suggested that stiffness alone and independent of ligand binding acted as a critical determinant for primary brain tumor cell function. An improved *in vitro* environment for brain tumor research would consist of neuronal and brain-resident interstitial cells that secrete the brain-specific ECM components into which the brain tumor cells can then be implanted. Such an environment was established from brain tissue extracts on micro filters (Hi-spots) on which GBM cell sensitivity to anti-proliferative compounds was tested.^[65] Despite its high-throughput potential, a setback of this method is the lack of control over the cellular composition in the Hi-spots and the absence of brain-specific architectural organization. A while ago, a simple but intriguing co-culture model of medulloblastoma and leptomeningeal cells was published, and it indicated paracrine, growth-promoting effects of latter that might be instrumental for studying the notoriously difficult to grow primary tumor cells *in vitro*.^[66] The ideal “organotypic environment” for primary brain tumor research was already in development in the early seventies of the last century, when the organotypic brain slice culture (OBSC) technology was established.^[67] The advantages of OBSCs are that micro environmental parameters and a relatively correct architectural organization are maintained that mimic the *in vivo* situation (see below).

Increasing complexity: system impact and single cell behavior

Neither are tumor functions disconnected from other tissues and the organs nor can the impact of tissues or organs on drug efficacy in the targeted tumor be predicted. An interesting approach to evaluate the effect of metabolic activity on cytotoxicity of compounds and chemotherapeutics *in vitro* was tested by Ma and colleagues using a 3D micro-tissue perfusion system.^[68] TMZ and IFO were perfused through hepatocytes before exposure to GBM cells and a clear impact of hepatocyte-provided cytochrome P450 on IFO activation could be shown. Analogous experimental follow-

ups are a number of organ on a chip technologies that are currently developed for assaying different disease states^[69] and testing drug effects and metabolism.^[70]

On the opposite side of the spectrum is the need to resolve the mechanisms underlying brain infiltration of single tumor cells, which necessitates approaches allowing the quantitative analysis of molecular events in individual cells. This problem was tackled for the activation status of the important Rho family GTPase's - Rho, Rac and Cdc42 - in glioma cells.^[53] Hirata *et al.*^[53] used Rho-GTPase-FRET (Förster energy resonance transfer) probes, where spatial activation of the GTPase's was monitored by a shift in fluorescence signal. Rho-family GTPase-FRET fusion protein-expressing glioma cells were orthotopically implanted in rat brains and later analyzed inside brain slice cultures derived of these brains using two-photo microscopy. This study revealed higher Rac1 and Cdc42 and lower RhoA activities in glioblastoma cells penetrating the brain parenchyma than those advancing in the perivascular regions, and suggested that different driver mechanisms could exist for single cell dispersion in glioma.

Together, these studies highlight the need for adapting the model system to the specifics of the biological context, with the consequent inclusion of biophysical or chemical components that best reflect the *in vivo* situation. Besides high-throughput screening platforms for the identification of novel pro-metastatic key players or alternative interference strategies against metastatic dissemination, we also need improved phenotype-based single cell analysis to decipher clonal differences and micro environmental impact on tumor behavior at the single cell level.

Organotypic brain slice culture (OBSC) in primary brain tumor research

A number of causal gene(s) and associated genetic mutations, molecular changes, probable targets and treatments for a variety of primary brain tumors have been identified. Despite of this, the process of dissemination, metastasis of the tumor cells from the primary site, and tumor recurrence, which is the leading cause for brain tumor related mortality in patients, remain obscure. Total removal of the primary tumor is on many occasions impossible at the microscopic level due to the insidious infiltration of the tumor cells into the surrounding brain tissue.^[71] This majorly results in therapeutic failure and urges for model systems that allow addressing brain tumor cell invasion specifically. Standard 3D *in vitro* invasion assays use ECM macromolecules that mimic the basement membrane (e.g. matrigel) as barriers to tumor invasion. These assays (described above and in table 1) although quick, reliable, commercially available and easy to perform, have several limitations. They do not take into account the unique ECM composition in the brain and thus provide artificial environments that fail to closely mimic the normal brain tissue/tumor environment. This is further emphasized by the fact that distinct types of brain tumors

localize within specific regions of the brain, highlighting the need for different microenvironments for modeling tumor growth and invasiveness. To circumvent this, mouse models have been generated for studying tumor propagation via orthotopic or subcutaneous xenografting of tumor cells. These experiments, however, are ethically controversial if inappropriately conducted, costly, labor intensive and need lengthy time periods for animal surgery and subsequent tumor development (especially for low grade tumors). These challenges and limitations highlight the need for developing a novel system wherein living brain tissue can be used as an ideal matrix for studying tumor cell growth and invasion.^[72] One such system is the organotypic culture, where cellular constituents of organs or parts of organs are allowed to regrow into or persist as organ replacements.

An excellent overview of 3D organotypic cultures has recently been provided,^[73] which describes their potentials as experimental systems to visualize cellular mechanisms that drive tissue development, to study the genetic regulation of cell behaviors in tissues and to evaluate the role of micro environmental factors in normal development and disease. One hallmark of organotypic cultures is the tissue environment mimicking the structural and functional specifics of the organ of origin. This turns them into attractive models for cancer research to explore tumor host tissue interactions and to advance therapeutic approaches.

Organotypic brain slice culture for visualization and quantification of brain tumor cell dissemination

OBSCs allow culture, maintenance and long-term survival of sections from any tissue of the CNS. Slices are mostly cultured at an air/liquid interface by either continuous rotation using the roller tube method or on a semi porous membrane using the Stoppini method.^[74] Brain tissue slice cultures maintain their normal cytoarchitecture, complex cell relationships and biochemical and electrophysiological properties. OBSCs have been widely used in the field of neurobiology for synaptogenesis, neurogenesis, myelin formation, as models for studying neurodegeneration, for neuroprotective and neurotoxic assays, *etc.*^[67] In the field of brain tumor research, they are an ideal platform to access the tumor microenvironment under intact anatomical conditions. Indeed, Jung *et al.*^[71] established a brain tumor slice model wherein they used human white matter specimens in the upper chambers of transwell culture dishes. After 24 h, control human astrocytoma cells stably expressing enhanced GFP or GFP-RHAMM (receptor for hyaluronan-mediated motility) transfected astrocytoma cells were placed in a small centrally punched-out hole in the slice. The infiltration and migratory behavior of the GFP-expressing astrocytoma cells could be easily studied using confocal laser scanning microscopy (CF-LSM) up to 30 days post implantation. The authors were able to demonstrate that different astrocytoma cell lines display different degrees of invasion and that the migration of the human astrocytoma cells could be

stimulated or, using antisense targeting strategies, specifically blocked.^[71] In an analogous study it was demonstrated that (1) the invasive behavior of the astrocytoma cells in the brain slice co-culture is not always identical to the results obtained from 2D migration studies, (2) the tumor cells spread out multidirectionally, (3) frozen human normal brain tissue can be used for the organotypic culture, (4) there were no obvious signs of necrosis, and (5) the brain cytoarchitecture and viability was preserved for at least 14 days.^[72]

Although the human origin of the biopsies used as the host tissue in these studies excludes species-specific effects in the co-culture, slices from newborn rat or mouse brains are excellent alternatives. They offer several advantages: brain regions corresponding to the *in vivo* tumor localization can be chosen, developmental stage of the brain slice can be adjusted, multiple replicas from same brain region can be generated, and the use of transgenic animals allows modification of the cellular microenvironment. Ohnishi *et al.*^[75] established OBSCs from 2-day-old neonatal rat brains, which were transferred on double-layered membranes consisting of two different membrane types and maintained at an interface between the air and the culture medium. The slices were then co-cultured with C6 glioma cells labeled with PKH2 fluorescent dye. After 2 days of co-culture, the exogenous application of the chemotactic stimulator neural cell adhesion molecule L1 triggered tumor cell migration from the upper to the bottom membrane through the brain slice.^[75] Since this study lacked CF-LSM analysis, OBSCs were subsequently performed by the slightly modified Stoppini method, which allowed quantifying glioma cell invasion using confocal microscopy.^[76] This study revealed that the migrating cells showed a strong increase in immunoreactivity for matrix metalloproteinase 2 and 9.^[76] Analogous OBSC technology was later used for mouse brain slices to quantify the invasiveness of glioma^[77] and to correlate it with histological type.^[78] Both studies used human, DiI-stained glioma biopsy tumor fragments and GFP-expressing spheroids directly implanted in the cortex of brain slices derived from 7 day old mice. This intraslice implantation system could be maintained in culture for 2 to 4 weeks. Quantification of the distance and density of the tumor cell invasion revealed that GBMs were 2-4 times more invasive than the lower grade glioma cells (LGGs). Within the different groups and grades of GBMs and LGGs, heterogeneity in terms of invasion was seen. It was also observed that the spheroids were less invasive in comparison to the directly grafted fragments. Overall using this system, Palfi *et al.*^[77,78] and de Bouard *et al.*^[77] could successfully recapitulate, monitor and quantify the invasion of single cells and the dissemination of glioma *ex vivo*. Recently, Chadwick *et al.*^[79] developed OBSCs from postnatal day 6 mice and cultured the whole brain slices on membrane inserts coated with laminin. Tumor cells (astrocytoma and medulloblastoma) were stained with Cm-DiI for monitoring, and dispensed on the center of the slice. This co-culture system remained viable for one week and effects of drug therapies on tumor cell proliferation, cell

death or changes in protein expression were successfully analyzed. Thus, Chadwick *et al.*^[79] used the OBSC system as a qualitative and quantitative assay to calculate the fold change in the number of cells during the period of slice culture. Furthermore, they investigated either the whole brain or specific regions within the brain, to assess environmental impact on primary brain tumor cell growth.

Organotypic brain slice culture to study the microenvironmental impact

Malignant astrocytoma/GBM cause mortality by local tumor growth and brain invasion rather than systemic metastasis. GBM tumor cells diffusely infiltrate the brain parenchyma within and along the white matter tracts or around cerebral blood vessels,^[53] and rarely penetrate basal lamina structures at the glial limitans externa. Analogously, malignant medulloblastoma must also infiltrate cerebellar tissue for distal dissemination. Moreover, resection of MB tumors is inevitably followed by relapse if the patients are not treated with cranio-spinal radiotherapy and chemotherapy, suggesting the occurrence of local dissemination of tumor cells from the primary medulloblastoma. *In vitro* studies aiming at better understanding the local invasion process have been hampered by the lack of identification of the brain ECM macromolecules involved and the only poorly understood implication of the cellular microenvironment. *In vivo* approaches on the other hand, offer too little spatial and temporal resolution to monitor tumor-microenvironment interactions appropriately. Thus, OBSCs could provide an important platform to study the cross-talk between the tumor cells and normal cells in a physiologically relevant environment. OBSCs can be used for investigating the microenvironment and its impact on the growth and spread of primary brain tumors, and for testing the measures that could be taken to prevent or treat it effectively.^[79] Although, there is a lack of vascular supply to the tissue in the slices, capillaries do survive in these sections without any circulation.^[80] Despite of the fact that there is no blood flow and that the capillaries are not functional, it is likely that they are still capable of expressing and secreting various molecules,^[81] which could affect other cell types in the slice culture including the tumor cells. In addition, the intriguing exchange between tumor cells and astrocytes and the suspected tumor promoting functions of astrocytes^[41-43] urges for novel studies addressing the therapeutic potential of the astrocyte-tumor interaction, for which organotypic slice culture would be an ideal system.

Along with their use for monitoring tumor dissemination, OBSCs have also been used for high resolution imaging of cytoskeletal structures in living glioblastoma cells. For this, glioblastoma cells were transfected with GFP-actin and placed onto murine brain slices and spinal cord explants. Using live-cell imaging to visualize the cytoskeleton of the tumor cells, a major change in the gross morphology from a solid, two dimensional state to a three dimensional substrate was noted. This morphological change was characterized by long, dendritic-like processes that displayed regions

of ruffling activity and filopodial protrusions and by down regulation of stress-fibers.^[82]

Thus, OBSC is an excellent technology to address a wide range of topics in primary brain tumor research, ranging from growth- and dissemination-promoting signaling, to the intricate interrelations between the tumor and its surrounding host tissue to the evaluation of efficaciousness of novel targeting strategies.

FUTURE PERSPECTIVES

Main emphasis for improving current *in vitro* technologies should be given to the cellular composition and the biophysical and chemical environment conditions under which the experiment is performed. The microenvironment of the *in vivo* location of the tumor and the composition of the neuronal and interstitial cells resident in this location should guide the choice of the components. At the single cell and population levels, molecular sensors for specific cell functions should be used for probing tumor cell behavior and therapeutic efficacy. Finally, an increased output should be strived for to enable pharmacological and genetic screening approaches for drug target identification. Thus, an organotypic environment, specific read-outs and the high throughput capability will be the three pillars of future *in vitro* approaches. A great potential lies in organotypic slice culture, and when this technology is combined with state-of-the-art microscopy, it will allow to reveal fundamental aspects of local tumor cell infiltration, the interaction of neuronal and brain interstitial cell populations with the tumor cells and the evaluation of the efficaciousness of novel treatments.

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Conflicts of interest

There are no conflicts of interest.

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Topic: Brain tumor cell invasion and metastasis: anatomical, biological and clinical considerations

Interdisciplinary management of central nervous system metastasis and neoplastic meningitis: recent developments and future perspectives

Ghazaleh Tabatabai^{1,13,14,17}, Marilyn Koch^{1,13,14}, Cristiana Roggia^{1,13,14}, Juliane Ebert^{1,13,14}, Claus Garbe^{2,14}, Friedegund Meier^{2,14,15}, Sara Brucker^{3,14}, Eva Maria Grischke^{3,14}, Diethelm Wallwiener^{3,14}, Robert Möhle^{4,14}, Lothar Kanz^{4,14,17}, Walter Erich Aulitzky⁵, Ulrike Ernemann^{6,13,14}, Christian laFougere^{7,13,14,17}, Konstantin Nikolaou^{8,14}, Bernd Pichler^{9,14,17}, Jens Schittenhelm^{10,13,14}, Manuela Neumann^{10,13,14}, Falko Fend^{10,14}, Stefan Czemmel^{11,14,16}, Sven Nahnsen^{14,16}, Frank Paulsen^{11,13,14}, Daniel Zips^{11,13,14,17}, Maike van Lessen^{1,12,13,14}, Hans-Otto Karnath^{12,13,14}, Ulf Ziemann^{1,13,14}, Hans-Georg Rammensee^{17,18}, Constantin Roder^{1,13,14}, Marco Skardelly^{1,13,14}, Jürgen Bernd Honegger^{1,13,14}, Marcos Tatagiba^{1,13,14}

¹Interdisciplinary Division of Neuro-Oncology, Departments of Vascular Neurology and Neurosurgery, University Hospital Tübingen, Hertie Institute for Clinical Brain Research, Eberhard Karls University, 72076 Tübingen, Germany.

²Division of Dermato-Oncology, Department of Dermatology, University Hospital Tübingen, Eberhard Karls University, 72076 Tübingen, Germany.

³Department of Gynecology and Obstetrics, University Hospital Tübingen, Eberhard Karls University, 72076 Tübingen, Germany.

⁴Department of Internal Medicine (II), University Hospital Tübingen, Eberhard Karls University, 72076 Tübingen, Germany.

⁵Department of Hematology and Oncology, Robert Bosch Hospital Stuttgart, Stuttgart, Germany, Comprehensive Cancer Center, Tübingen Stuttgart, Germany.

⁶Department of Diagnostic and Interventional Neuroradiology, University Hospital Tübingen, Eberhard Karls University, 72076 Tübingen, Germany.

⁷Department of Nuclear Medicine, University Hospital Tübingen, Eberhard Karls University, 72076 Tübingen, Germany.

⁸Department of Diagnostic and Interventional Radiology, University Hospital Tübingen, Eberhard Karls University, 72076 Tübingen, Germany.

⁹Department of Preclinical Imaging and Radiopharmacy, University Hospital Tübingen, Eberhard Karls University, 72076 Tübingen, Germany.

¹⁰Department of Pathology and Neuropathology, University Hospital Tübingen, Eberhard Karls University, 72076 Tübingen, Germany.

¹¹Department of Radiation Oncology, University Hospital Tübingen, Eberhard Karls University, 72076 Tübingen, Germany.

¹²Division of Neuropsychology, University Hospital Tübingen, Hertie Institute for Clinical Brain Research, Eberhard Karls University, 72076 Tübingen, Germany.

¹³Center for CNS tumors, Comprehensive Cancer Center Tübingen Stuttgart, Germany.

¹⁴Center for Personalized Medicine, University Hospital Tübingen, Eberhard Karls University, Tübingen, Germany.

¹⁵Center for Dermato-Oncology, University Hospital Dresden, Germany.

¹⁶Quantitative Biology Center (QBiC), Eberhard Karls University, Tübingen, Germany.

¹⁷DKTK, DKFZ partner site Tübingen.

¹⁸Department of Immunology, Eberhard Karls University Tübingen, Germany.

Correspondence to: Prof. Ghazaleh Tabatabai, Interdisciplinary Division of Neuro-Oncology, Departments of Vascular Neurology and Neurosurgery, University Hospital Tübingen, Hertie Institute for Clinical Brain Research, Eberhard Karls University, 72076 Tübingen, Germany.
E-mail: ghazaleh.tabatabai@uni-tuebingen.de

ABSTRACT

The incidence of metastatic disease in the central nervous system (CNS) is rising. According to current estimates, up to a third of adult cancer patients will suffer from CNS metastasis. Clinical evidence-based data from prospective randomized trials are rare, however, because CNS metastasis patients were often excluded from clinical trial participation. The management of CNS metastasis patients is therefore rather ill-defined and an interdisciplinary challenge. Recent basic and translational science data have begun contributing to a more profound understanding of the molecular mechanisms leading to invasion of tumor cells into the CNS. This report reviews advances, challenges, and perspectives in this field.

Key words: Brain tumor; central nervous system metastases; interdisciplinary management

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INTRODUCTION

Invasion of tumor cells from primary tumors of the central nervous system (CNS) to organs outside the CNS is a highly rare event. In contrast, invasion of tumor cells arising outside the CNS to brain, spinal cord, or cerebrospinal fluid (CSF) occurs frequently, leading to CNS tumor growth and neoplastic meningitis. Moreover, in approximately every tenth patient, the diagnosis of brain metastasis is the first sign of the cancer disease.^[1]

CNS metastases are the most common intra-axial malignancies, accounting for more than 50% of all brain tumors,^[2] occurring in 20-40% of patients with cancer, and leading to symptoms during lifetime in about 60-75%.^[3] Autopsy series identified CNS metastases in 15-41% of patients with known primary cancers at the time of death.^[4-8] Most metastatic manifestations affect the brain parenchyma; 80% are found supratentorially and 20% infratentorially (15% cerebellum, 5% in the brain stem), with the spinal cord most infrequently involved. The incidence of single vs. multiple sites of CNS metastasis is approximately equal.^[9] In about 4-15% of patients with CNS disease, CSF is involved.^[10] Lung cancer, breast cancer, and melanoma are the primary malignancies that contribute to 80% of brain metastases.^[7,11,12] Moreover, there is a high incidence of asymptomatic CNS metastases, so it is hard to estimate their true prevalence. Current studies estimate that approximately a third of patients with cancer eventually develop brain metastases.^[10]

Several reasons may explain the increase in incidence of brain metastases over the past decades: Certainly, the widespread use and improvements in new imaging technologies facilitates the detection of metastatic lesions. For example, magnetic resonance imaging (MRI) of the neuraxis is currently used for the examination of approximately 60-70% of patients with cancer; 20 years ago, it was used in 2% of cancer patients.^[13] The global increase in cancer prevalence is another contributing factor, especially the increase in cancers that have a tendency to invade the CNS, such as lung cancer. Moreover, the introduction of targeted therapies that have limited bioavailability in the CNS might also have resulted in an increase of CNS metastases (e.g. the treatment of human epidermal growth factor receptor protein 2 (HER2)-positive breast cancer with trastuzumab, a compound with limited penetration from the blood to the CSF).^[14,15]

Neoplastic meningitis (also referred to as meningeosis neoplastica or, based on the underlying tumor, as meningeosis carcinomatosa, gliomatosa, or lymphomatosa) is a spread of tumor cells into the subarachnoid space. It is found in approximately 5-10% of all patients with malignant tumors and is a condition frequently diagnosed in late stage cancer.^[16] The most common associated primary tumors are lung cancer, breast cancer, melanoma

and lymphoma and leukemia.^[17]

Patients with CNS metastases present with rather unspecific clinical symptoms. Headaches (40-50%), focal neurological deficits (30-40%), and seizures (15-20%) are the most common presenting symptoms. In leptomeningeal disease many symptoms are caused by an increased intracranial pressure mainly due to hydrocephalus, which leads to nausea and vomiting, neck and back pain, and confusion.^[3]

MOLECULAR MECHANISMS OF CENTRAL NERVOUS SYSTEM METASTASIS FORMATION AND MAINTENANCE

Cancers that metastasize to the CNS need to undergo multiple steps, including detachment from the primary site, invasion, intravasation into the bloodstream, extravasation, survival, and proliferation. Even with different primary tumor origins, invasion and proliferation into the CNS appears to be associated with similar molecular programs and is highly supported and maintained by the tumor-associated brain microenvironment.^[18]

First, the growth of metastatic brain tumors is critically dependent on angiogenesis,^[19] so therapies targeting this process might be important in the prevention or management of brain metastases. In a mouse model of brain metastases [HER2-amplified breast cancer cells in an orthotopic xenografting of human BT-474 cells], extracranial disease was successfully controlled using the HER2 inhibitors trastuzumab or lapatinib, but tumor control with monotherapy in the brain failed. By adding anti-VEGFR2 antibodies, however, tumor growth in the brain was better controlled, leading to improved survival, especially with a combination of lapatinib, trastuzumab, and anti-VEGFR2 antibody treatment.^[20]

Second, astrocytes are intimately involved in maintaining normal homeostasis of the brain microenvironment, accomplished through transport of nutrients to the neurons and facilitation of neural signal transduction. In fact, activated astrocytes induced upregulation of survival genes. These mechanisms usually protect injured neurons from apoptosis, but can be abused by tumor cells (e.g. for protection from cytotoxic effects of chemotherapeutic agents).^[21,22] A very interesting study on the impact of astrocyte-derived reshaping of the brain microenvironment was recently published by Zhang and colleagues: Mouse tumor cells lost PTEN expression only after dissemination to the brain, but not to other organs, and PTEN levels in PTEN-loss brain metastatic tumor cells were again rescued after leaving the brain microenvironment. This brain microenvironment-dependent plasticity of PTEN expression is epigenetically regulated by astrocyte-derived exosomes mediating an intercellular transfer of PTEN-targeting microRNAs to metastatic tumor cells. As a

result of this adaptive PTEN loss, brain metastatic tumor cells released more chemokine CC chemokine ligand 2, leading to recruitment of IBA1-expressing myeloid cells and further enhancement of the growth and maintenance of brain metastases.^[23]

Infiltrating inflammatory host cells, including tumor-infiltrating lymphocytes or myeloid cells, are a third key component shaping the tumor microenvironment and correlating with patients' survival times in several extracranial malignancies.^[24] These cells significantly change their functional characteristics under the influence of high-grade glioma,^[25] indicating that they might also play a role for supporting CNS metastatic growth. Clinicopathological correlations of associated lymphocytic infiltrates indicate a beneficial outcome for CNS immune response.^[26] Two functional phenotypes of tumor-associated macrophages have been proposed: the M1 and the M2 phenotype. While M1 is characterized by tumor-suppressive functions, the M2 may have more tumor-promoting functions, including suppression of immune responses and promotion of adaptive immune response and migration/invasion.^[25] However, recent studies suggest that this dichotomy does not completely reflect the situation in brain tumors.^[27]

In recent years, high-throughput technologies have evolved significantly. Thus, molecular tumor profiling (e.g. by Next-generation sequencing, panel sequencing) for identifying molecular targets is in principle feasible in the short term. Data on the molecular characteristics of CNS metastases have only recently been acquired. This might be due to the fact that CNS metastatic tissues are only available from patients who are eligible for neurosurgical resection. Because craniotomies are not indicated in all patients with CNS metastases (see below), a systematic analysis of the molecular differences between CNS metastases and matched primary tumors or between CNS metastases and extracranial metastases remains challenging. Molecular profiling of matched CNS and extracranial metastases in smaller series of melanoma patients showed that CNS metastases distinguished themselves through specific molecular differences in the activation of the PI3K/mTOR/Akt or HER2 or kirsten rat sarcoma (KRAS) pathway.^[28-31] These studies highlight, for example, the potential of adding PI3K inhibitors or mTOR inhibitors as adjunct targeted therapy in the treatment of CNS metastases.

TREATMENT STRATEGIES NEED A PROFOUND INTERDISCIPLINARY DIAGNOSTIC WORKUP

In addition to the staging of extracranial disease, a thorough neurological workup including neurological examination, neurocognitive assessments, neuroimaging, and a spinal tap is in principle indicated in all patients with established malignant disease and suspected brain metastases.

Depending on clinical symptoms and neuroradiological features, one single spinal tap or up to three spinal taps can be considered. If, for example, clinical symptoms strongly suggest an underlying meningeomatosis, a single lumbar puncture might not be enough to detect atypical cells in the CSF, so serial lumbar punctures might be necessary. Diagnostic workup of the CSF includes analyses of opening pressure, protein, glucose, and lactate levels as well as cytology and immunocytology.

Standard MRI exams include T1-weighted images with or without contrast enhancement, T2-weighted imaging, and FLAIR sequences. Differential diagnosis of brain metastases includes malignant gliomas and lymphomas or nonneoplastic conditions, such as abscess, infections, demyelinating diseases, and vascular lesions. Recently, the Response Assessment in Neuro-Oncology Brain Metastases (RANO-BM) working group has proposed criteria for a harmonization of the assessment of CNS metastases.^[32] This might contribute to a standardization of techniques and assessment tools, particularly important in the era of targeted compounds. It is not yet entirely clear to what extent and how novel targeted therapies (e.g. immunotherapies and kinase inhibitors) will alter imaging characteristics. The recently published recommendations of the RANO-BM working group provide a guideline to differentiate imaging alterations during immunotherapies in brain tumors.^[33] Innovative and advanced neuroimaging techniques will certainly gain even more importance. Examples include the addition of diffusion-weighted MRI (DW-MRI), perfusion MRI, proton magnetic resonance spectroscopy (MRS), and various amino acid tracers in positron emission tomography (PET). These techniques might be especially relevant to meet the challenges of disease monitoring (e.g. the discrimination of radiation necrosis from recurrent tumor might be challenging on MRI since both conditions present with contrast enhancement on T1-weighted MR images, and the pattern of abnormal enhancement closely mimics that of a recurrent brain metastasis).^[34] In fact, small studies with perfusion MRI using CBV analysis showed the potential to differentiate between radiation necrosis and tumor recurrence with good sensitivity and specificity.^[35] Nuclear medicine techniques might contribute to answering this critical question. While an fludeoxyglucose (FDG) tracer was not sensitive enough to differentiate vital brain metastases from unspecific non-tumor changes related to therapy,^[36] the amino-acid PET tracer ¹¹C-methionine showed higher tumor-to-lesion uptake ratios in patients with recurrent metastases/glioma after radiation treatment than in patients with radiation necrosis.^[37] Furthermore, the combination of two amino acid tracers (FET and MET) identified treatment-related changes with high sensitivity and specificity.^[38]

The blood-brain-barrier is often mentioned as a challenge for diagnosis and therapy. In a very interesting preclinical study using mouse models of small metastatic breast

tumors, infusions of recombinant human tumor necrosis factor induced selective permeabilization of the blood-brain barrier to imaging tracers at sites of brain metastases. This method enabled the detection of smaller tumors that had been invisible using standard imaging techniques. Notably, this strategy even increased the delivery of radiolabeled trastuzumab to these metastatic lesions,^[39] demonstrating the translational potential of similar approaches for theranostics.

THE ESTIMATION OF PROGNOSIS IS IMPORTANT FOR CLINICAL MANAGEMENT

The most widely established risk stratification scores are the Recursive Portioning Analysis (RPA), the Graded Prognostic Assessment (GPA), and Diagnosis Specific Graded Prognostic Assessment (DS-GPA) [Table 1].^[40-43] Definitely, the presence of neoplastic meningitis in patients with solid tumors indicates a poor prognosis. Negative prognostic factors associated with leptomeningeal tumor cell dissemination are low Karnofsky performance status (KPS), increased age, uncontrolled intracranial pressure,

low glucose levels, and high protein levels.^[44-46]

RPA divides patients into three categories based on KPS, age, and primary tumor control, with patients in group I having a better prognosis than patients in group III.^[40] The GPA evaluates the prognosis of patients with brain metastases based on the primary tumor diagnosis.^[42] Histology carries prognostic significance, along with other subcategories (e.g. age and extracranial disease in lung cancer patients, or number of metastases in melanoma patients). Tumor subtype based on HER2/ER/PR status and age is prognostic for breast cancer and is expanded upon with a specific breast-GPA, currently in use in clinical trials.^[43] Other prognostic scores were defined^[47] and are summarized in Table 1. In large retrospective studies of melanoma patients with brain metastases, poor prognostic factors associated with worse survival were: > 3 parenchymal lesions, leptomeningeal disease, brain lesions developing concurrently with extracranial disease or while on systemic therapy for extracranial disease, poor performance status (KPS < 70%), elevated pretreatment LDH levels, and RPA class III.^[48,49]

Table 1: Prognostic scores

Recursive partitioning analysis				
Class	I	II	III	
	Age < 65 KPS > 70% Stable primary tumor No extracranial metastases	All patients not in Class I or class III	KPS < 70%	
Basic score for brain metastases				
Score	0	1		
KPS	50-70%	80-100%		
Control of primary tumor	No	Yes		
Extracranial metastases	Yes	No		
Score index for radiosurgery				
Score	0	1	2	
Age (years)	> 60	51-59	< 50	
KPS	< 50%	60-70%	80-100%	
Systemic disease	Progressive	Stable	Complete response or no evidence for disease	
Number of lesions	> 3	2	1	
Volume of largest target lesion	> 13 mL	5-13 mL	< 5 mL	
Graded prognostic assessment				
Score	0	0.5	1.0	
Age	> 60	50-59	< 50	
KPS	< 70%	70-80%	90-100%	
CNS metastases (no.)	> 3	2-3	1	
Extracranial metastases	Present	-	None	
Diagnosis-specific graded prognostic assessment				
i) NSCLC/SCLC				
Score	0	0.5	1.0	
Age	> 60	50-60	< 50	
KPS	< 70%	70-80%	90-100%	
Extracranial metastases	Present	-	Absent	
CNS metastases (no.)	> 3	2-3	1	
ii) Melanoma/RCC				
Score	0	1	2	
KPS	< 70%	70-80%	90-100%	
CNS metastases (no.)	> 3	2-3	1	
iii) Breast/GI cancer				
Score	0	2	3	4
KPS	< 70%	70%	80%	90%
				100%

CNS: central nervous system; KPS: karnofsky performance status; NSCLC: non small-cell lung cancer

The prognostic indices certainly play an important role in assessing the risk/benefit ratio and providing realistic advice and expectations to patients. For example, patients with poor prognosis can be offered supportive care, and those with good prognosis can be offered multimodality treatment. The prognostic scores might play a vital role in designing clinical trials as well.

Information about variables on neuroimaging, in addition to the pure number of brain metastases, might be valuable extensions to currently established prognostic scores. Spanberger and colleagues found a significant correlation between a small brain edema with an invasive tumor growth pattern, a low neo-angiogenic activity, and a low expression of HIF1 α . These findings were associated with a shorter overall survival.^[50] Further, high DW-MRI hyperintensity correlated significantly with a high amount of interstitial reticulin deposition, and this was again associated with lower survival.^[51] Similarly, pre-operative DW-MRI characteristics of cerebral metastases and their peritumoral region in 76 patients were related to patient outcome.^[52]

THERAPEUTIC APPROACHES TO CENTRAL NERVOUS SYSTEM METASTASIS

CNS metastases are, of course, a heterogeneous group with varied response to treatment and survival. Conventional treatment options usually include a combination of steroids, surgery, and radiation. Cytotoxic chemotherapy has had a limited role in the treatment of brain metastases, probably because CNS metastases often arise from heavily pretreated primary tumors and may thus have already acquired resistance to chemotherapeutics. In addition, the impaired blood-brain barrier penetration of some agents might further reduce their bioavailability in the CNS. Therapeutic decisions mainly depend on several factors related to patient clinical status (neurological deficit, neurocognitive deficit, general condition, comorbidities, *etc.*), primary disease status, extracranial metastatic disease, and CNS tumor characteristics (number, radiological aspect, size, and location).^[40] Median overall survival times after occurrence of CNS metastases might be predicted by biomarkers as shown for LDH elevation in melanoma CNS metastases.^[48] All relevant clinical factors need to be taken into account to identify the best therapeutic strategy among the available therapeutic options. We outline the currently available local and systemic therapeutic options in the following paragraphs.

LOCAL THERAPEUTIC STRATEGIES: NEUROSURGICAL INTERVENTION AND RADIATION THERAPY

Neurosurgical intervention and radiation therapy are currently the main modalities in the therapy of

symptomatic CNS metastases. New surgical modalities have expanded the indication and spectrum of tumors that can be successfully removed. Since the introduction of intraoperative monitoring and development of less invasive strategies (e.g. microsurgery, endoscopic surgery, intraoperative navigation, ultrasound, and intraoperative MRI), surgical removal of brain metastases even in deep-seated and elusive areas has become feasible without increased morbidity. To date, the strongest evidence for a survival benefit from surgery is for single CNS metastases.^[53] In 1996, Mintz *et al.*^[54] did not confirm a positive impact of surgery on overall survival in these patients. However, only 21.4% of patients in this study had a controlled extracerebral disease, and none of the patients had brain MRI assessment; therefore, comparability with other studies is rather limited. In a retrospective study of treatment modalities in 1,292 patients with CNS metastasis of lung cancer, breast cancer, and melanoma, Lagerwaard *et al.*^[55] demonstrated an increase of median OS of 1.3 months in patients who received best supportive care only, 3.6 months in patients who received RT, and 8.9 months in patients who received a combination of surgery and RT. Similar median OS benefits were also shown in a retrospective study of 1,137 melanoma patients who received best supportive care (2.1 months), RT (3.4 months), surgery (8.7 months), or combined RT and surgical resection.^[56]

Benefits of surgery include the ability to establish a tissue diagnosis and an immediate decrease of tumor mass, particularly of masses in the posterior fossa. Nevertheless, patients who might benefit from surgical resection must be carefully selected. Predictors that favor a surgical benefit include: single or few metastases, tumor location, surgical accessibility, KPS > 70, patient age < 65 years, local mass effect, control of extracranial disease, and absence of leptomeningeal involvement.^[57] Based on the therapy oncology group database, patients of RPA class I are likely to benefit from surgery, whereas patients of RPA class III are not.^[40] The primary goal of surgery is either macroscopic gross total resection or decompression dependent on the aforementioned predictors. Intraoperative neurosurgical techniques to maximize resection (e.g. image-guided surgery,^[58] ultrasonography,^[59] and introduction of fluorescence-guided surgery^[60]) and to minimize neurological deficits by electrophysiological techniques^[58] improved the likelihood of complete and safe removal of metastases. A combination of surgery plus radiation in patients with up to three CNS metastases can improve survival and preserve functional independence, as outlined in two prospective studies^[61,62] and three retrospective studies.^[63-65] Several criteria -- including tumor location, medical comorbidities, extracranial disease, and performance status -- may impact individual consideration and risk assessment for surgical resection. This is particularly relevant because evidence from studies in high-grade glioma surgery indicates that a new

Table 2: Overview of targeted compounds for central nervous system metastases that are outlined in the text

Molecular target	Compound	Compound characteristics
HER2	Trastuzumab	Humanized mAb targeting the extracellular domain of HER2
	Trastuzumab emtansine	Antibody-drug conjugate; the antibody targeting HER2 is conjugated with an antimicrotubule agent that is only released in HER2 ⁺ target cells
	Lapatinib	Small molecule tyrosine kinase inhibitor that dually targets HER1 and HER2, binding to the intracellular domain
	Neratinib	Irreversible inhibitor targeting the catalytic domain of EGFR, HER2, and HER4
EGFR	Gefitinib	Inhibitor of EGFR
	Erlotinib	Inhibitor of EGFR
ALK	Crizotinib	Inhibitor of ALK
	Ceritinib	Inhibitor of ALK
	Alectinib	Inhibitor of ALK
BRAF	Vemurafenib	Selective inhibitor of mutated BRAF ^{V600E}
	Dabrafenib	Inhibitor of mutated BRAF, wild-type BRAF, and CRAF
CTLA4	Ipilimumab	Antibody targeting CTLA-4
PD-1	Pembrolizumab	Antibody targeting PD-1 receptor
	Nivolumab	Antibody targeting PD-1 receptor

ALK: anaplastic lymphoma kinase; BRAF: serine/threonine-protein kinase B-Raf; CTLA: cytotoxic T-lymphocyte-associated antigen; HER: human epidermal growth factor receptor protein; EGFR: epidermal growth factor; PD: programmed death

postoperative neurological deficit decreases survival up to 3-4 months, and any substantial postoperative complication negatively affects functional status and the patient's ability to undergo subsequent radiation treatment, both of which are crucial factors in determining survival.^[66]

The main modalities in radiation therapy include stereotactic radiotherapy and whole brain radiation therapy. Stereotactic radiotherapy alone might be considered for patients who have a controlled systemic disease and a limited number of CNS metastases whose size is less than 3 cm. A combination of stereotactic and whole brain radiotherapy has been investigated in large clinical trials. There was no difference in overall survival, but the addition of whole brain radiation therapy significantly improved local and distant control.^[9,67] Yet, patients treated with whole brain and stereotactic radiation therapy were at higher risk of a decline in learning and memory. Of note, neurocognitive testing was only performed once at 4 months in this trial.^[68] Novel concepts of whole brain radiation therapy with an avoidance of the hippocampal region might lead to new opportunities in this treatment modality.

Radionecrosis can occur, typically within the first year after stereotactic radiotherapy. The differentiation between tumor progression and radionecrosis might be difficult, as mentioned earlier. Treatment recommendations for radiosurgery radionecrosis include bevacizumab and/or steroids.^[69]

Regarding a refinement of treatment planning for radiation therapy, the value of amino acid PET in stereotactic radiotherapy treatment planning for focal recurrence at a previously irradiated site of a brain metastasis was evaluated. In 88 patients, the authors found that the total irradiation volume was significantly smaller in the PET group and that the median survival time was significantly longer in the PET group (18.1 months) than in the MRI planning group (8.6 months).^[70]

CYTOTOXIC CHEMOTHERAPY: NO CONVENTIONAL STANDARD REGIMEN FOR CENTRAL NERVOUS SYSTEM METASTASES

To date, standard cytotoxic chemotherapy regimens have not been defined for the treatment of CNS metastases. Instead, inoperable patients are treated using the same cytotoxic chemotherapy employed for the treatment of extracranial disease. Alternatively, cytotoxic agents with good CNS penetration (such as topotecan, irinotecan, procarbazine, and carboplatin, temozolomide, or fotemustine) are also employed for empirical therapy, even in cases in which these agents are not the standard therapy for the primary tumor site. Pharmacological treatments for intrathecal therapies are ill-defined, too.

INTRATHECAL TREATMENT THERAPY FOR TARGETING THE CEREBROSPINAL FLUID

Intrathecal administration of drugs aims at targeting tumor cells in the CSF efficiently by circumventing the blood-CSF barrier while omitting systemic toxicity. Treatment can be done by repetitive lumbar punctures or through intraventricular catheter systems (i.e., Rickham or Ommaya reservoir). Among the drugs available for intrathecal treatment, methotrexate (MTX) and cytarabine are most frequently used. Alternatively, thiotriethylenephosphoramidate has been approved in some countries. Liposomal cytarabine is a sustained-release form of cytarabine and was compared with MTX in a controlled trial in patients with solid tumors and leptomeningeal carcinomatosis. Patients who were treated with liposomal cytarabine experienced a longer time until neurological progression. However, there was no difference in overall survival.^[71] Liposomal cytarabine is associated with an increased risk for radiculitis and arachnoiditis. This might be prevented by prophylactic dexamethasone application.

Supportive therapy aims at symptom relief. Steroids may help to decrease symptom burden similar to the situation in solid tumor manifestations in the brain.^[72]

TARGETED THERAPIES ARE AVAILABLE FOR A SUBSET OF CENTRAL NERVOUS SYSTEM METASTASES

With increasing insight into molecular alterations and improved CNS penetration of targeted compounds, some specific molecular-targeted compounds are available that can also be applied in CNS metastases [Table 2]. We focus here on breast cancer, lung cancer, and melanoma.

Breast cancer

HER2 is overexpressed in up to 30% of breast cancers.^[73] A retrospective analysis of 9,524 women in the pre-trastuzumab era identified HER2 expression as a risk factor for brain metastases^[74] with an incidence of CNS metastases in HER2-positive patients twice that of unselected breast cancer patients. Additionally, an increasing percentage of patients develop brain metastases, whereas their systemic disease is controlled using HER2-directed therapies.^[75] A retrospective case series reported 23 of 93 (25%) patients developed brain metastases after trastuzumab therapy, and 78% of those patients had stable or better systemic disease. A meta-analysis using data from three large phase III trials indicated the incidence of CNS disease was significantly higher in the trastuzumab-treated patients.^[15] Trastuzumab's high molecular weight, approximately 700 times that permitted by the blood-brain barrier, may create a sanctuary site in the CNS for HER2-positive tumors, and its limited CSF bioavailability hinders efficacy in treating brain metastases.^[14] Lapatinib is a dual HER1 and HER2 inhibitor that is administered orally. A single-arm phase II trial evaluated the activity of lapatinib plus capecitabine in 45 patients with HER2-positive breast cancer and brain metastases before Whole brain radiation therapy (WBRT). The CNS response rate was 67% with a median time to progression of 5.5 months.^[76] Trastuzumab emtansine (T-DM1) is an antibody-drug conjugate incorporating the human epidermal growth factor receptor 2 (HER2)-targeted antitumor properties of trastuzumab with the cytotoxic activity of the microtubule-inhibitory agent DM1. The antibody and the cytotoxic agent are conjugated by means of a stable linker.^[77] The incidence of central nervous system (CNS) metastases after treatment with trastuzumab emtansine (T-DM1) versus capecitabine-lapatinib (XL), and treatment efficacy among patients with pre-existing CNS metastases in the phase III EMILIA study was analyzed in a retrospective study. In this retrospective, exploratory analysis, the rate of CNS progression in patients with HER2-positive advanced breast cancer was similar for T-DM1 and for XL. In patients with treated, asymptomatic CNS metastases at baseline, T-DM1 was associated with significantly improved OS compared with XL.^[78]

Neratinib is an orally administered inhibitor of the ErbB receptor tyrosine kinase with antitumor activity in advanced HER2-positive breast cancer.^[79] A phase II trial is currently underway for patients with HER2-positive breast cancer and brain metastases (NCT01494662). Further aspects of CNS metastases in the breast are outlined in a recent review.^[80]

Non small-cell lung cancer

With the discovery of targetable molecular alterations in the treatment of non small-cell lung cancer (NSCLC), patients with newly diagnosed disease are currently stratified based on molecular alterations of several genes in the primary tumor, including the epidermal growth factor receptor (EGFR), Kirsten rat sarcoma viral oncogene homolog (KRAS), and translocations involving the echinoderm microtubule-associated protein like 4 (EML4) anaplastic lymphoma kinase (ALK) genes.^[81] In a retrospective study of 89 patients with NSCLC treated with stereotactic radiation therapy for CNS metastases, the addition of targeted therapies was associated with significantly better outcomes. Patients treated with targeted therapy against EGFR or ALK had a median survival of 21 months compared with 11 months for patients who did not receive targeted therapy.^[81] EGFR mutations are present in 10-25% of NSCLC. EGFR mutations in patients with brain metastases may be more common; two reports found EGFR mutations to be present in 63% and 50% of patients, raising the question whether EGFR mutations lead to an increased risk of developing brain metastases similar to HER2 overexpression in breast cancer.^[82] Patients with ALK activation, on the other hand, had no increased risk of brain metastases but did show a higher frequency of liver metastases.^[83,84]

Gefitinib and erlotinib are oral compounds and irreversible inhibitors of the intracellular domain of EGFR. Gefitinib is FDA-approved for NSCLC with EGFR mutations. Erlotinib is approved for locally advanced or metastatic NSCLC that has failed at least one prior chemotherapy regimen or for maintenance treatment for locally advanced metastatic NSCLC whose disease has not progressed after four cycles of platinum-based first-line chemotherapy. There is concern about poor BBB penetration of these agents as CNS response rates are disproportional to systemic response rates. Serum to CSF comparisons for gefitinib revealed only about 1% of the serum dose represented in the CSF.^[85] Both drugs are near the 400 kDa molecular weight range, with the BBB retaining selectivity for molecules greater than 200-400 kDa. Despite concerns for optimal bioavailability, gefitinib and erlotinib have been investigated in first-line palliative and combination settings. Two phase II trials for tyrosine-kinase inhibitors (TKI) in the first-line setting include data on patients with CNS metastases.^[86,87] Both studies do not include sequencing data for EGFR mutations but instead use the clinical indicator of never-smokers. Lee *et al.*^[86] reported 36 never-smoker patients including 10 patients with synchronous brain metastases. Seven of ten patients demonstrated an intracranial objective response to

gefitinib, one patient had stable disease, and two patients had progressive disease after a median of 48-week follow-up period. Kim *et al.*^[87] reported 23 never-smoker patients with synchronous brain metastases with a response rate to either gefitinib or erlotinib of 69% and a disease control rate of 82%. The median overall survival was 18.8 months, and time to salvage WBRT averaged 19.3 months.

Further evidence for first-line TKI comes from a retrospective analysis of 155 patients screened for EGFR mutations.^[88] The rate of CNS progression was lower in EGFR-mutant patients with advanced NSCLC treated initially with erlotinib or gefitinib compared with upfront cytotoxic chemotherapy (33% vs. 48%) at a median follow-up of 25 months, supporting a role for these drugs in prevention of CNS metastases. Median overall survival, on the order of 30 months, was not different between the two groups.

Erlotinib in combination with WBRT was evaluated in a prospective phase II trial in 40 patients with brain metastases from NSCLC regardless of EGFR status. The overall response rate was 86% and the median overall survival was 11.8 months. Of these 40 patients EGFR status was known in 17 patients. Interestingly, patients negative for EGFR mutations had a median overall survival of 9.3 months, whereas patients who were positive for EGFR mutations had a median overall survival of 19.1 months.^[89] The clinical benefit and feasibility of targeting ALK was demonstrated first with the multitargeted tyrosine kinase inhibitor crizotinib that competitively binds to the ATP-binding pocket of the ALK and MET tyrosine kinases and inhibits phosphorylation of activated ALK. This was subsequently confirmed in phase II and III trials.^[90-92] The ability of ALK-directed therapies to control and prevent the development of CNS metastases remains incompletely studied, with early reports suggesting inefficient CSF penetration of crizotinib.^[93-95]

Ceritinib is a second-generation ALK inhibitor with increased activity against common ALK point mutations. The activity of ceritinib in ALK+ NSCLC has been confirmed in phase I and II studies. Larger head-to-head trials such as the phase III, ALEX “trial comparing alectinib to crizotinib will directly investigate PFS in the CNS and may provide further information to inform treatment decisions for ALK+ patients with brain metastases.

Melanoma

Activating BRAF mutations affect up to 60% of melanoma patients; more than 95% are the p.V600E mutation, with the remainder largely being p.V600L. Constitutive BRAF signaling activates the mitogen-activated protein kinase (MAPK) pathway.^[96] Vemurafenib is an FDA-approved BRAF inhibitor. In a pilot study of 24 patients with melanoma metastatic to the CNS treated with vemurafenib, median PFS was 3.9 months, and median OS

was 5.3 months. An overall partial response rate at both intracranial and extracranial sites was achieved in 42%, and stable disease was achieved in 38%.^[97] Further data are available from individual cases^[98] and population-based studies.^[99] New trials with CNS metastases are ongoing. Dabrafenib is an oral ATP-competitive inhibitor of BRAF kinase. A multicenter clinical trial evaluated dabrafenib in 172 patients both with and without prior brain therapy for BRAF-mutated melanoma metastatic to the brain with confirmed p.V600X mutation.^[100] The primary outcome measure was overall response rate observed to be 29/74 (39.2%) in patients without prior brain therapy and 20/65 (30.8%) in patients with prior brain therapy. Thus, dabrafenib was helpful in patients with both new and pretreated brain metastases. Duration of response was 20.1 weeks for patients without prior brain treatment and 28.1 weeks for patients with prior brain treatment. Median overall survival was 33 weeks in patients without prior brain therapy and 31 weeks with prior brain therapy.

Resistance to therapy with BRAF kinase inhibitors is associated with a reactivation of the MAPK pathway. Consequently, the combination of BRAF and MEK inhibitor was assessed and showed increased efficacy compared to BRAF monotherapy alone.^[101]

Current immunotherapy approaches focus mainly on checkpoint inhibitors Ipilimumab and PD1/PDL1 inhibition. Ipilimumab is a humanized monoclonal antibody against cytotoxic T lymphocyte antigen-4 (CTLA-4); it shows activity in melanoma brain metastasis, particularly if asymptomatic, by improving overall survival.^[8,102,103] A phase 2 study of ipilimumab and fotemustine showed an overall immune disease control rate of 50% and median progression-free survival of 4.3 months, with increased incidence in hematological and nonhematological toxicity. Clinical trials for the assessment of immune checkpoint inhibition strategies in CNS metastases are ongoing.

QUALITY OF LIFE AND NEUROCOGNITION

The systematic assessment of neurocognitive function is often neglected in clinical routine but is crucial, mainly because neurocognitive function is a key feature of quality of life for patients. It is important to raise awareness and encourage more frequent use of neurocognitive monitoring tools (not only in large centers) as a regular part of the diagnostic workup. Certainly, there are multiple reasons for cognitive decline in patients with CNS metastases, including the neuroanatomical location of the lesions, symptomatic seizures, depression, distress, and potentially also the effects of neurotoxic systemic therapies and whole brain radiation therapy. It is notable that corticosteroids are a very common cause of neurocognitive decline. Steroid-induced changes in mood and sleep certainly affect cognitive function, leading to measurable effects on

declarative memory and even to decreased hippocampal volumes.^[104] The severity of memory impairment is correlated to dose and duration of use.^[105]

Primary prevention strategies might include the implementation of hippocampal-sparing whole brain radiation therapy, prophylactic use of the N-methyl-D-aspartate receptor modulator memantine, or blocking the RAAS cascade. Assessment and treatment of depression is an important strategy, including appropriate pharmaceutical or psychological treatments.

FUTURE PERSPECTIVES

With increasing incidence of CNS metastases, an improvement of existing treatment strategies is urgently needed. Important steps for meeting this important epidemiological challenge include systematic interdisciplinary multiprofessional treatment teams, thorough biosampling and biobank studies for the establishment of further biomarkers or therapeutic targets, innovative imaging tools, and innovative clinical trial designs with meaningful endpoints including survival, quality of life, and neurocognitive assessments. Any extension of progression-free or overall survival for these patients will only be meaningful if quality of life and neurocognition can be preserved. There is rising need for further definition of reliable molecular/genetic tumor markers to be implemented in routine pathology/neuropathology diagnostics, to catch up to increasing insights into molecular heterogeneity of cancer and its interaction with the local microenvironment.

An important future challenge will be to implement affordable investigations of the molecular and cellular components of the tumor microenvironment. In this regard, it will be increasingly important to visualize and monitor the expression of molecules and cell motion as well as to enhance the technical possibility of calculating cellularity, vessel permeability, vascular perfusion, metabolic and physiological changes, apoptosis, and inflammation prior to and during the course of therapy. A multimodal imaging algorithm is likely to improve sensitivity and specificity to meet these requirements. Certainly, novel multimodal algorithms will have to be prospectively investigated in multicenter trials for validation and standardization.

Since serial tissue biopsies are rarely clinically justified in CNS metastases, and in light of new upcoming targeted treatment options, noninvasive tools to measure drug penetration, pharmacodynamic effects, and efficacy are becoming increasingly important. Examples include PET-based approaches for noninvasive measuring of drug uptake with ⁸⁹Zr-trastuzumab and ⁸⁹Zr-bevacizumab.^[106,107]

Recent studies using magnetic resonance-guided focused ultrasound suggest a role for this noninvasive, radiation-free alternative for treatment of small deep-seated

brain metastases. New developments in this field could potentially further expand the treatment spectrum.^[108-111]

For meeting these challenges, interdisciplinary and integrative research strategies must combine clinical investigation, neurological workup, quality of life assessments, neurocognitive testing, imaging, and histological and molecular profiling of tumor tissue to design individualized treatment strategies tailored to patients with CNS metastases. Only then can the full potential of precision therapeutic approaches be exploited for improving outcomes for our patients.

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Targeting cerebrospinal fluid for discovery of brain cancer biomarkers

Tarek Shalaby, Federica Achini, Michael A. Grotzer

Department of Oncology, University Children's Hospital Zürich, Steinwiesstrasse 75, CH-8032 Zürich, Switzerland.

Correspondence to: Dr. Tarek Shalaby, Department of Oncology, University Children's Hospital Zürich, Steinwiesstrasse 75, CH-8032 Zürich, Switzerland.
E-mail: tarek.shalaby@kispi.uzh.ch



Dr. Shalaby obtained his MD-PhD degree in molecular and cell biology at the University of Bern, Switzerland. He was then a postdoc at neuro-oncology at University Children's Hospital, Zurich. He is now a senior scientist at the oncology department, University Children's Hospital, Zurich and the Editor-in-Chief of the *Journal of Unexplored Cancer Data*.

ABSTRACT

Central nervous system (CNS) cancer is a devastating illness with unmet therapeutic needs. Establishing biomarkers that have the potential to guide accurate CNS cancer diagnosis or are helpful in predicting disease progression or therapy response is of great interest. Cerebrospinal fluid (CSF) has been extensively targeted for the detection of molecules that might be useful markers for cancer detection. However, so far very few of such markers have found a standardized routine clinical application. This review examines the current scientific knowledge about the biochemical elements in the CSF that have been reported in the literature as brain cancer biomarkers and highlight reasons why the role of most markers is not yet established in the management of CNS tumors.

Key words: Cerebrospinal fluid; central nervous system cancers; cerebrospinal fluid cytology; biochemical markers

INTRODUCTION

Brain cancers are the leading cause of death by solid tumors in children and the cause of morbidity and mortality across a wide range of adult individuals.^[1,2] The identification of biomarkers that could allow diagnosis of brain neoplasms and could be informative for cancer spread or monitor therapy response is in great demand. Blood analysis for novel biomarkers has facilitated the timely diagnosis for patients with several malignancies such as prostate and breast cancers.^[3] However, one of the challenges that contributes to the paucity of biomarkers in the serum for central nervous system (CNS) malignancies is the blood-brain barrier, which is thought to prevent the release of tumor-specific molecules into the blood circulation. Cerebrospinal fluid (CSF) has thus been investigated in the search for brain tumor markers.

CSF is a readily accessible body fluid that is reflective of the underlying pathological state of the CNS, hence it has been widely targeted for biomarker discovery for a variety of neurological disorders. The CSF is continuously produced and recycled much like blood or lymph.^[3] The majority of CSF is produced by the choroid plexus located on the lateral, third and fourth ventricles. The rate of CSF production in humans is 0.3-0.4 mL/min and the total CSF volume is 90-150 mL in adults and 65-150 mL in children.^[4-6] CSF circulates through the ventricles, the cisterns, and the subarachnoid space at the base of the brain, then flows over the convexities of the brain and down the length of the spinal cord.^[5-7] Therefore, CSF is in contact with brain tissue and in proximity to most tumor bulks, making it an ideal reservoir of tumor-related/secreted molecules.^[8]

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It is accessible through lumbar puncture, a little invasive procedure. Any cancer cells released by brain cancer bulk or molecules that are actively secreted or passively diffused by cancer cells are likely to disperse into the CSF and therefore can be detected. Hence CSF analysis is considered to be an important tool in the evaluation of CNS malignancies. This review discusses potential and limitations of CSF analyses in brain cancer patients.

DETECTION OF CANCER CELLS IN THE CSF

Primary CNS cancers and metastases are often located in close proximity to ventricular surfaces or CSF cisterns.^[9-11] Malignant cells derived from brain cancers reach the leptomeninges by CSF spread or by direct extension from the primary tumor itself e.g. medulloblastoma, primitive neuroectodermal tumors, germ cell tumors, ependymoma, and glioma may be disseminated throughout the neuroaxis by the flow of the CSF.^[12-14] Table 1 shows the particular incidence of malignant leptomeningeal involvement in selected primary brain cancers. Currently microscopic evaluation of CSF is routinely performed in CNS malignancies with frequent leptomeningeal spread, such as medulloblastomas, PNET, pineoblastomas, germ-cell tumors and CNS lymphoma.^[15] Cancer therapy and prognosis of these groups of brain cancer are crucially determined by positive CSF cytology.^[13,14]

CSF cytoanalysis

CSF cytology, in which CSF is prepared and examined under a microscope to look for cells, is currently considered the gold standard for diagnosis of brain cancer with leptomeningeal spread and metastatic cancer to the brain.^[14] To achieve CSF cytology a sample can be obtained at the time of tumor surgery or by lumbar or intracerebroventricular (ICV) reservoir puncture.^[3] However, lumbar CSF remains the specimen of choice to detect malignant cells of primary CNS tumors.^[12,16] To avoid false positive results due to sloughing of tumor cells at the time of surgery, a recovering interval of one to two weeks is currently suggested before

performing diagnostic postoperative CSF cytologic evaluation.^[9,16,17] Accurate cytopreparatory techniques are essential criteria for successful CSF microscopic evaluations. 7.5 mL of CSF are usually withdrawn and immediately processed, as the cell counts can diminish by up to 50% within 2 h of collection.^[4,18] CSF samples are then processed by centrifugation (Cytospin[®]) at 800 g for 3-5 min, air-dried for 10-15 min and stained with May-Grunwald Giemsa (MGG) stain solution for 10-15 min.^[19] Thin-layer preparation (ThinPrep) is a relatively new liquid-based cytology method which has been suggested to better detect malignant cells in CSF from solid tumors by performing good preservation of cell morphologic features. During the ThinPrep analysis, the CSF cells are collected through high-precision filtration driven by fluid mechanics and gently absorbed onto a glass slide by using electrochemical forces. The collected samples need to be added to 10 mL preservation solution, mixed and stood for 15 min. Slides are fixed in 95% ethanol for 15 min and stained by standard Papanicolaou method.^[19]

CSF cytology, although indispensable, has many limitations Table 2. To start with it involves the pathological identification of abnormal cells in the CSF by Giemsa stain and clinicians must make judgments on the presence or absence of malignant cells. Hence, CSF cytological analysis is a pure qualitative test that bears no quantification and lacks validation.^[3,20] Another weakness is that because the shedding of malignant cells into the CSF may occur intermittently and in low numbers, inconsistent presence of cancer cells in the CSF should be expected. CSF specimens may, therefore, fail to capture malignant cells representing one of the major weaknesses of CSF cytology. It is therefore recommended that CSF analysis should be repeated if initially negative.^[21] One of the drawbacks is while CSF cytology is highly specific in detection of cancer cells, it suffers from a lack of sensitivity. A retrospective meta-analysis^[22] reported that CSF cytology sensitivity could be as low as 45% depending on how many times the lumbar puncture was repeated. False negative cytopathology is common (10-20% of patients) because of the paucity of

Table 1: Association between primary brain tumors localisation and LS incidence

Disease	Localisation	Incidence LS	Source
Medulloblastoma	Fossa posterior possible extension to fourth ventricle, brainstem, cisterna magna	30-40%	[17,85]
Supratentorial PNETs	Frontal lobes, parietal, temporal and occipital lobes	25-40%	[17,86]
CNS AT/RT	The exact incidence of CNS AT/RT is difficult to determine because the tumor has been widely recognized for only the last decade	29%	[87-89]
Retinoblastoma	Retina, possible optic nerve invasion and choroidal involvement	3-23%	[90,91]
Germ-cell tumors	Pineal-region, possible extension to third ventricle, suprasellar	22%	[92]
Primary CNS lymphoma	Cerebral hemispheres, basal ganglia, corpus callosum, cerebellum	10-20%	[93]
Brainstem glioma	Tectal plate to medullary cervical junction, possible extension to preponine cistern and fourth ventricle	3-13%	[94,95]
Pinealoblastoma	Pineal-region	10%	[96]
LGG hypothalamic	LGG can occur anywhere in the CNS	7%	[97-100]
Ependymoma	Infratentorial intraventricular (fourth ventricle's floor, lateral walls, roof) or supratentorial within the brain parenchyma	5%	[17]

LS: leptomeningeal spread; PNETs: primitive neuroectodermal tumors; CNS: central nervous system; AT/RT: atypical teratoid/ rhabdoid; LGG: low-grade glioma

Table 2: Advantages and disadvantages of different methods for brain tumors biomarkers detection in the CSF

Approach	Method	Pros	Cons
Detection of cancer cells in the CSF	CSF cytoanalysis: CSF is examined under a microscope to look for cancer cells	Highly specific ^[12-14,16]	Low sensitivity and false negative results are common ^[3,13,20,23,24]
	Flow cytometry analysis: Have the potential to provide information about cell surface protein expression	Automated method that allows rapid analysis ^[14,25] Smaller CSF volume is needed ^[9,23]	False negative and false positive results can occur (especially at low cell counts, < 25 cells/uL). Poor differential ability between mitoses and neoplastic cells is reported ^[14,23,25]
	Other tools: Measuring chromosomal content of cancer cells in the CSF using DNA single cell cytometry techniques or fluorescence <i>in-situ</i> hybridization CSF proteomic analysis: Systematic identification and quantification of the complete complement of proteins in the CSF	These techniques have the ability to detect genetic aberrations as a sign of malignancy location ^[26] Specific proteomic patterns can differentiate subtypes or grades of specific brain tumors ^[27-30]	Low sensitivity ^[26] Limited sensitivity and specificity ^[31]
Detection of biochemical molecules secreted by cancers to the CSF	CSF microRNAs analysis: Measuring microRNA Profiling of CSF	High specificity and chemical stability ^[60,101] Only small amounts of CSF samples are required for the detection of miRNAs in the CSF offers the advantage of convenient repetitive monitoring of molecular events happening in cancer in the response to treatment ^[76]	The unknown origin and factors influence their level of expression might impact their specificity as biomarkers ^[76,102-106]

CSF: cerebrospinal fluid

cells in the CSF and morphological similarities between benign and malignant cells.^[13,23,24] The lack of standardized techniques for obtaining and evaluating CSF cytology specimens and the absence of molecular analysis of tumor cells certainly contributes to the wide sensitivity range.^[3] Hence although it is currently used in the clinic, CSF cytology remains a poor surrogate marker for disease response in brain cancer/metastasis involvement.^[9,13]

Flow cytometry analysis

CSF fluid flow cytometry is a useful addition to CSF cytology. Cytology examines morphologic patterns, and flow cytometry has the potential to provide information about cell surface protein expression. It is an additional highly sensitive cytological technique capable of accurately detecting malignant CSF cells, especially in comparatively smaller CSF volume and in samples with very low cell counts when combined with multicolor fluorescent antibody labelling.^[9,23] In this method CSF must be processed similar to cytology within 1 hour of sampling however centrifugation should be minimized.^[14,23] Automated methods allow rapid flow cytometry data analysis and thereby reduce the significant time expenditures used in conventional cytology routine.^[14,25] Flow cytometry seems to provide a higher sensitivity. However the cell count and the percentage of neoplastic cells reported in the CSF by

both cytology and flow cytometry were significantly higher compared with those found to be positive by flow cytometry alone.^[23]

It has to be said that both false negative and false positive results (especially at low cell counts, < 25 cells/uL) can occur with flow cytometry too, a poor differential ability between mitoses and neoplastic cells is also reported Table 2. Therefore before flow cytometry can be recommended in a routine CSF examination in combination with the conventional cytology, standardized protocols are needed to uniform definitions of positivity and procedure.^[14,23,25] Rare cell capture technology, for example, CellSearch® is a recent technique using molecular markers to detect and enumerate circulating tumor cells in the CSF. This method is established to detect prognostic marker on different cancer cells circulating in the peripheral blood such as breast cancer and has recently attracted the interest of CSF cancer researcher.^[3,9] However, the application of CellSearch® technology for detecting primary CNS cancer cells in CSF has not been published yet.

Other tools for cancer cell detection in the CSF

Measuring the chromosomal content of cancer cells in the CSF, using DNA single cell cytometry techniques or fluorescence *in-situ* hybridization that detects genetic

aberrations as a sign of malignancy, can also give additional diagnostic information to CSF analysis, but still has a low sensitivity Table 2. PCR can also establish cancer diagnosis when cytology is inconclusive, but the genetic alteration of the neoplasia must be known for it to be amplified with this technique, and this is generally not the case.^[26]

PROTEOMIC ANALYSIS OF CSF

Proteomic profiling has become an active area of research for the biomarker discovery and the identification of new targets for therapeutic strategies. Recent studies have shown that specific proteomic patterns can differentiate subtypes or grades of human brain tumors.^[27-30] Modern technological advancements in protein quantification which provide rapid screening, low sample consumption, and accurate protein identification, have enhanced the precision of proteomic analyses and are anticipated to accelerate brain tumor biomarker discovery.^[31]

Research work on traditional sampling sources for proteomic profiling, such as blood^[31,32] and tissue lysates,^[33] have yielded a substantial amount of information on potential brain cancer biomarkers. However, the majority of these markers exhibited limited value in a clinical setting, justifying the need for the exploration of more clinically relevant sampling sources. One such a promising source for protein biomarker discovery is the CSF where protein presences might result from either secretion/leaking by tumor tissues or abnormal blood brain barrier function.^[8]

CSF proteomic analysis for detection of brain cancer markers

In the search for accurate biomarkers a number of reports have emerged over the past decade describing the analysis of different brain cancer proteome using CSF. For example the CSF level of carcinoembryonic antigen (CEA), is a protein tumor marker that is commonly increased in several human malignancies, was found recently to play an important role in differential diagnosis of primary and metastatic brain tumors^[34,35] and useful auxiliary marker in diagnosis of meningeal carcinomas.^[36-38] In a study by Khwaja *et al.*,^[39] the authors reported that proteomic analysis of CSF can discriminate malignant and non-malignant disease of the CNS and identified carbonic anhydrase protein (known to be overexpressed in many malignancies including high-grade gliomas) as a prognostic marker of brain cancer.

The most significant example of how analysis of CSF proteins has impacted the clinical management of CNS cancer is in the case of intracranial malignant germ cell tumors.^[40] Germ cell tumors are heterogeneous group of gonadal or extragonadal tumors that thought to arise from the aberrant migration and differentiation of primordial germ cells during embryogenesis. Extragonadal germ cell tumors can occur intracranial in the pineal and suprasellar regions and comprise approximately 3% of all pediatric

brain tumors. Germ cell tumors retain the molecular characteristics of their primordial lineage as they maintain the expression of embryonic proteins, such as beta human chorionic gonadotropin (bHCG) and alpha-fetoprotein (AFP).^[41] bHCG is a 36 kDa glycoprotein normally secreted by placental tissues while AFP is a 70 kDa glycoprotein normally secreted by the foetus primarily in the yolk sac, gastrointestinal tract, and liver. AFP is elevated in wide range of cancers, including colon adenocarcinoma, liver and gastric cancers while bHCG and AFP were found to be markedly elevated in the CSF of intracranial malignant germ cell tumor patients.^[42] Both markers are currently utilized clinically as diagnostic and accurate indicators of response to therapy. Assessment of AFP and total bHCG in both serum and CSF is mandatory in order to distinguish between germinoma and NGGCT non-germinoma germ cell tumors. CSF AFP > 1000 ng/mL at diagnosis, or age < 6 years, intracranial malignant germ cell tumor patients are stratified as high risk and are treated more intensively. Moreover, the verification of bHCG and AFP levels prior to surgical resection provides a reference point that can be used to assess recurrence during follow-up however their absence does not rule out a germ cell tumor. Additional CSF protein markers such as placental alkaline phosphatase (PLAP) and lactate dehydrogenase isoenzymes have been shown to be clinically useful in the diagnosis and monitoring of pediatric intracranial germinomas, however such markers are less specific.^[43] Elevated levels of s-kit, the soluble form of the c-kit receptor, a transmembrane tyrosine kinase receptor, was found to be a reliable marker for germ cell tumor diagnosis that can differentiate germ cell tumors from other CNS cancers. Miyanohara *et al.*^[44] also reported that s-kit expression is able to detect recurrence of germ cell tumors and subarachnoid dissemination.

Gliomas are the most common primary brain tumors in adults. Glioblastoma multiforme (GBM) is the deadliest glioma with a median survival of only 14 months despite the recent advances in intensive therapeutic strategies.^[45] Hence more effort was applied to study whether specific CSF proteomic profile can be generated to evaluate gliomas prognosis. Fang Shen *et al.*^[8] conducted a review of the literature on the proteomic screening for glioma-related protein biomarkers in CSF. They were able to identify 19 differentially expressed proteins, the majority exhibited increased concentrations (B2M, CA2, CA12, CALD1, DDAH1, MYCN, PPIA, SPP1, VEGFB, ALB, MAPT, SERPINA3, SPARCL1) while (GSN) was downregulated in the glioma CSF. Further functional assessments revealed several important protein networks (e.g., IL6/STAT-3) and four novel focus proteins (IL-6, galanin (GAL), HSPA5 and WNT4) and the authors reported that these proteins might be involved in glioma pathogenesis. On the same theme, Khwaja *et al.*^[46] used two proteomic techniques, two-dimensional gel electrophoresis and cleavable Isotope-Coded Affinity Tag to compare CSF proteomes in order to identify tumor- and grade-specific biomarkers in patients

bearing brain tumors of different histology and grades. By performing retrospective analyses on 60 samples derived from astrocytomas WHO grade II, III, and IV, schwannomas, metastatic brain tumors, inflammatory samples, and non-neoplastic controls, the group identified 103 potential tumor-specific markers of which 20 were high-grade astrocytoma-specific. SPARCL1, FGF14, VEGF-B, tau, b2M, bdefensin and Attractin were found as an upregulated marker in the CSF of patients with malignant astrocytoma and mediates glioma cell migration.^[47] Sampath *et al.*^[48] assessed whether vascular endothelial growth factor (VEGF) could be measured in the CSF of patients with cerebral neoplasms and used as a marker of particular brain cancer tumors. They investigated CSF samples from 27 patients with high-grade astrocytomas, 39 patients with nonastrocytic CNS neoplasms, and 14 patients with no known CNS neoplasm. In their study, VEGF was detectable in 89% of samples with malignant astrocytoma and not normal CSF samples. The levels of VEGF were significantly higher in high-grade astrocytomas than in nonastrocytic tumors indicating that detection of VEGF in CSF could be a potential marker for differentiating astrocytic from nonastrocytic tumors.

Another group applied mass spectrometry based technology to identify possible CSF peptide markers of GBM.^[49] Out of 2,000 detected CSF peptides four peptides which significantly distinguished GBM from controls were identified. They were specific C-terminal fragments of alpha-1-antichymotrypsin, osteopontin, and transthyretin as well as N-terminal residue of albumin. Interestingly the identified four molecules are constituents of normal CSF, but this group are the first to report their significant elevation in CSF of GBM patients. To detect biomarkers in high-grade astrocytomas, Ohnishi *et al.*^[50] analysed the differential expression of proteins in the CSF from two cases each of diffuse astrocytoma (grade II), and glioblastoma (grade IV) using agarose 2-D gel electrophoresis. The authors found that the expression of gelsolin protein is decreased with histological grade. To examine whether gelsolin is a useful indicator of tumor aggressiveness the group further analysed the gelsolin expression in 41FFPE astrocytomas. Gelsolin expression was found to be significantly lower in high-grade than in low-grade astrocytomas. Moreover the overall survival of patients in the low-gelsolin expression was significantly poorer than in the high expression group highlighting the usefulness of gelsolin as a potential prognostic factor in astrocytoma.

Diffuse intrinsic pontine glioma (DIPG) is not surgically resectable, resulting in a paucity of tissue available for molecular studies and, currently, there are no effective treatments. Saratsis *et al.*^[51] investigated 15 CSF specimens from patients with DIPG for proteomic analysis. Protein profiling was generated by mass spectrometry. CSF proteomic analysis revealed selective upregulation of Cyclophilin A (CypA) and dimethylarginase 1 (DDAH1) in DIPG, compared with controls. Protein expression

was further validated with Western blot analysis and immunohistochemical assays using CSF and brain tissue as well as in blood samples from DIPG. Immunohistochemical staining showed selective upregulation of secreted but not cytosolic CypA and DDAH1 in patients with DIPG. Their study indicated that detection of secreted CypA and DDAH1 in CSF and serum has potential clinical application, with implications for assessing treatment response and detecting tumor recurrence in patients with DIPG.

Primary central nervous system lymphoma (PCNSL) is another highly aggressive tumor that can lead to quick death if not diagnosed in time. The diagnosis of PCNSL can present a diagnostic challenge. It relies on histopathology of brain biopsies to the same extent as most brain tumors, while less invasive tests to detect early tumor pathogens with sufficient diagnostic accuracy are not available yet. Proteomic analysis of CSF has revealed various proteins that are differentially expressed in CNS lymphoma.^[52-54] Among these, antithrombin III (ATIII), a serine protease inhibitor that is associated with neovascularization in CNS lymphoma, has been prospectively validated.^[26] ATIII expression was reported by Roy *et al.*^[55] to be elevated in the CSF of patients with CNS lymphoma compared to those patients with control. ATIII levels higher than 1.2 g/mL made the detection of CNS lymphoma possible with >70% sensitivity and 99% specificity.^[26] Elevated antithrombin III levels significantly correlated with shorter survival rates and less response to chemotherapy. However and on the contrary a recent study from Finland, by Kuusisto *et al.*^[56] declared that ATIII is not a suitable biomarker for diagnosis of PCNSL and increased concentrations of ATIII in CSF might be due to leakage of the blood-brain barrier.^[57]

CXCL13 protein that is known to mediate chemotaxis of CNS lymphoma cells was detected within biopsy specimens from PCNSL patients^[58] raising the possibility that this chemokine may contribute to CNS tropism. Rubenstein *et al.*^[55] investigated the concentration of CXCL13 in CSF of CNS lymphoma patients and control cohorts in a multicenter study involving 220 patients. Their result demonstrated that elevated CXCL13 concentration in CSF is a highly specific marker for the detection of CNS lymphoma and can be helpful as an adjunctive diagnostic test and response to treatment assessment. Following their steps in studying chemokine in PCNSL, Sasagawa *et al.*^[59] investigated CSF from 19 patients with CNS lymphoma (15 and 26 non-lymphoma patients with various brain tumors) and reported that CSF IL-10 is a superior biomarker for initial screening for patients with CNS lymphoma.

Medulloblastoma (MB) is the most common malignant brain tumor in children. It 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 proteomic spectra. With the aim to identify putative biomarkers for MB in CSF, Rajagopal *et*

al.^[60] investigated the CSF proteome from 33 children with MB and compared it against the CSF proteome from 25 age-matched controls using two-dimensional gel electrophoresis. In their study levels of prostaglandin D2 synthase (PGD2S) were found to be six-fold significantly decreased in the CSF of tumor samples most likely representing a host response to the presence of the tumor.^[61] Usually biomarkers are often thought to be elevated in a disease state compared to normal levels however candidate negative diagnostic marker such as PGD2S could be useful for detecting MB as well as recurrence of the disease. On the other hand it has to be said that while negative biomarkers are potentially useful, their relationship to tumor biology is less direct and more highly complex in comparison to proteins that are over-expressed in tumor associated samples.^[40] Desiderio *et al.*^[62] investigated CSF from 14 children with posterior fossa tumors (6 Pilocytic astrocytoma, 5 Medulloblastoma, 3 Ependymoma and 5 nontumoral control). In their study the CSF proteomics demonstrated the potential biomarker role of the hemoglobin subunit beta fragments (peptides LVV- and VV-hemorphin-7) in posterior cranial fossa pediatric brain tumors. Both LVV- and VV-h7 were detectable in control-CSFs but absent in the patient CSFs collected before surgery (i.e. in presence of tumor). Interestingly both LVV- and VV-h7 were also absent in the CSF collected 6 days after the resection tumor in patients with tumor relapse. Their data suggest that analysis in post-surgery CSF could be used to predict patient prognosis. However, it will be interesting to evaluate the cancer specificity of LVV- and VV-h7 in relation to other forms of CNS pediatric tumors. Finally levels of polysialic-neural cell adhesion molecule (PSANCAM), considered a marker of developing neuron, were found to be significantly higher in CSF from MB patients that are refractory to treatment or those who relapsed, than patients in remission.^[63]

Atypical teratoid/rhabdoid (AT/RT) tumor is a rare, highly malignant tumor of the CNS most commonly found in children less than 5 years of age. Osteopontin (OPN) a bone matrix glycoprotein levels were found to be significantly elevated in patients with AT/RT. Clinical studies identified OPN as a potential diagnostic marker in ovarian, breast, colon, prostate, and lung cancers.^[64] Using enzyme-linked immunosorbent assay and immunohistochemical analysis, Kao *et al.*^[65] investigated plasma, CSF, and brain tissue specimens from 39 patients MB, 16; AT/RT, 8; epilepsy, 6; hydrocephalus, 9) and found that patients with AT/RT have higher plasma and CSF OPN levels in comparison with patients with MB, hydrocephalus, or epilepsy. Interestingly significant correlation between OPN levels and the risk of tumor relapse in patients with AT/RT was identified while OPN levels in the CSF were found to decrease with treatment.

Other biochemical markers

Malignant brain tumors may show an increased fraction of anaerobic LDH concentrations (LD4 and LD5) in CSF.^[52] A

number of other potential CSF protein biomarkers for CNS cancers have been reported in the literature such as Insulin-like growth factor binding protein 2 (IGFBP2), Insulin-like growth factor binding protein 3 (IGFBP3),^[66] Polysialic-neural cell adhesion molecule (PSANCAM),^[63] Total Tau (t-Tau),^[67] Tumor necrosis factor (TNF) alpha^[68] and CSF , S-100,^[69] Neuron-specific (NSE),^[70] neuron growth factor, HCG,^[71] Apolipoprotein A-II,^[72] MIC-1/GDF15,^[73] Elevated expression of such markers in the CSF was found to be relatively specific for brain cancer^[74,75] however sensitivities and specificities have widely varied.^[26]

MICRORNAS

MicroRNAs (miRNA) are short, non translated fragments of RNA that bind to 3' untranslated regions of messenger RNA and repress protein translation in several molecular pathways.^[26] The discovery of miRNAs role in controlling essential regulators of key pathways implicated in development of CNS tumors make them a powerful tool for detection of cancer, risk assessment and prognosis. During the past decades, great efforts have been made in conducting research evaluating the diagnostic value of miRNAs in CNS cancer's tissue.^[76] However, a major drawback of the tissue-based approach centers on the need for invasive surgical procedures in sample collection. MiRNAs have been found to stably coexist in several body fluids including CSF which can be collected with minimal invasiveness and permit following the disease over time.^[26] In this context several reports have described that deregulated miRNAs in CSF are closely associated with the clinical course of CNS malignant tumors.^[2,77-82]

For example Baraniskin *et al.*^[77] found that combined expression analyses of miR-21 and miR-15b were able to distinguish patients with glioma from controls with various neurologic disorders, including patients with carcinomatous brain metastases and primary CNS lymphoma with accuracy of 90% sensitivity and 100% specificity. While Teplyuk *et al.*^[2] reported that combined analysis of a group of seven CSF miRNAs enabled the discrimination between GBM and metastatic brain cancers with more than 90% accuracy. miRNA-21 and miR-10b expression levels were significantly increased only in brain tumor lesions (in patients with GBM or brain metastases) compared to nonneoplastic conditions while members of the miR-200 family were found solely in CSF of patients with brain metastases, indicating that CSF miRNAs could be used to discriminate between glioblastoma and metastatic brain tumors, an important consideration for cancer treatment.^[2] GBM is the deadliest glioma with median survival of only 14 months despite the recent advances in intensive therapeutic strategies.^[80] Due to their anatomic location and infiltrative nature, these tumors are not amenable to surgical resection or even to biopsy in some cases. The paucity of biomarkers represents a sizable gap in improving the clinical management of these patients. Analysis of CSF

Table 3: CSF biomarkers for the detection of brain cancer

Brain cancer	Marker	Method of detection	References
Medulloblastoma, primitive neuroectodermal tumors, germ cell tumors, ependymoma and glioma	Cancer cells	CSF cytology	[12-14]
Intracranial malignant germ cell tumors	bHCG and AFP	CSF proteomic analysis	[41]
Pediatric brain tumors (medulloblastoma, high-grade glioma, atypical rhabdoid tumor, astrocytoma, plexus carcinoma and anaplastic ependymoma, germ cell tumor)	Apolipoprotein A-II	CSF proteomic analysis	[72]
CNS lymphoma	CD27, AT III, chemoattractant, CXCL13, CXCL12 and IL10	CSF proteomic analysis	[55,57,107-111]
Cerebral low-grade lymphoma	Immunoglobulin G IgG	CSF proteomic analysis	[112]
Brain metastases from lung adenocarcinoma	Epidermal growth factor receptor EGFR	CSF proteomic analysis	[113]
Brain metastases from lung and breast cancers	VEGF and stromal cell derived factor (SDF)-1	CSF proteomic analysis	[73]
Medulloblastoma	PGD2	CSF proteomic analysis	[60]
Meningeal carcinomas	CYFRA 21-1, NSE and CEA	CSF proteomic analysis	[70]
Glioblastoma	MIC-1 GDF15	CSF proteomic analysis	[114]
Glioblastoma	miR-21 and miR-15b	CSF microRNA analysis	[115]
PCNSL	miR-19, miR-21, and miR-92a	CSF microRNA analysis	[115]
Glioblastoma and brain metastasis	miR-10b and miR-21	CSF microRNA analysis	[116-117]
Brain metastases from lung and breast cancers	Members of miR-200 family	CSF microRNA analysis	[116]
Glioblastoma, medulloblastoma, brain metastasis and lymphoma	miR-935, miR-451, miR-711, miR-223 and miR-125b	CSF microRNA analysis	[118]

CSF: cerebrospinal fluid; PCNSL: primary central nervous system lymphoma; bHCG: human chorionic gonadotropin; AFP: alpha-fetoprotein; AT III: antithrombin III; CXCL13: chemokine C-X-C motif ligand 13; IL10: interleukin 10; VEGF: vascular endothelial growth factor; PGD2: Prostaglandin-D2 synthase; CYFRA 21-1: cytokeratin-19 fragment; NSE: neuron-specific enolase; CEA: carcinoembryonic antigen; MIC-1: macrophage inhibitory cytokine-1; GDF15: growth differentiation factor 15

miRNA could therefore be advantageous for identifying putative disease markers for DIPGs.

An earlier work by Baraniskin *et al.*^[77] demonstrated that combined miRNA analysis of miR-19, miR-21, and miR-92a in CSF accurately discriminate patients with PCNSL from other neurologic disorders controls with diagnostic accuracy of 95.7% sensitivity and 96.7% specificity indicating significant diagnostic value. In the same theme, Scott *et al.*^[79] conducted a review of the literature on CNS lymphoma diagnosis (1966 to 2011) and extracted data regarding the usefulness of CSF cytology, proteomics and miRNAs in the diagnosis of CNS lymphoma. The authors reported low sensitivity for CSF cytology (2-32%) which is increased when combined with flow cytometry. CSF lactate dehydrogenase isozyme 5, β 2-microglobulin, and immunoglobulin heavy chain rearrangement studies have improved sensitivity over CSF cytology (58-85%) but have only moderate specificity (85%). Interestingly miRNA analysis has more than 95% specificity in the diagnosis of CNS lymphoma.

Twenty three studies with a total of 299 CNS cancer patients and 418 controls were analyzed by Wei *et al.*^[81] through systematic meta-analysis for articles in the topic diagnostic value of miRNAs for CNS cancers and comparing sensitivity of on blood-and CSF based miRNAs assays for the diagnosis of CNS malignancies. Thirteen

out of the 23 studies they analyzed focused on miRNAs as diagnostic biomarkers for glioma and 10 for PCNSL detection. The performance of miRNAs in CSF for CNS cancers detection showed more correctness in sensitivity suggesting a relatively high diagnostic accuracy. By the end of the study the authors concluded that miRNAs may be suitable as biomarkers for CNS cancers detection and that the CSF based miRNAs assays could be considered more reliable for clinical application. However, further validation based on a larger sample of patients and controls is still required.^[81]

The presence and biological role of miRNAs in the extracellular environment of medulloblastoma MB was examined recently by our lab and we found that more than one thousand miRNAs were released in the culture-medium in each of the MB cell lines tested.^[82] Among them a panel of miRNAs were specific to the culture-medium of metastasis-related cell lines (D341 and D283) which represents the aggressive group 3 and group 4 MB subtypes. Interestingly, three metastasis-associated miRNAs were over-represented in culture-medium of metastasis-related MB cell lines were found to be significantly enriched in the CSF of the MB patient. Although more samples are required to fully verify these results, our work presented the first evidence for the presence of miRNAs excreted extracellularly by MB cells and raises the possibility that 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.

HOW NEAR ARE WE TO USING CSF MOLECULAR MARKERS FOR BRAIN CANCER DIAGNOSIS IN THE CLINIC?

The promise of CSF biochemical markers Table 3, such as proteins and miRNA, to detect and monitor brain cancer has swept through the oncology research area in recent years leading to ample publications. However, most putative markers did not progress beyond their initial discovery. A striking discrepancy exists between the effort directed toward CSF biomarker, whether it is protein or miRNA, discovery and the number of markers that made it into clinical practice.^[83] Understanding the reasons why the role of these markers is not yet established in the diagnosis of CNS tumors can help accelerate the conduit between their discovery and clinical implementation. One of the confounding issues that participate in the failure of potential markers to reach the clinic is the lack of reproducibility between similar studies or low correlation of results. The most significant source for such inter-laboratory discrepancies is mainly due to differences in protein/miRNA preparation, in the analytical methods or the use of different technologies which may bias the analysis. There are various platforms/techniques which exist each with specific biases that can greatly influence the relative expression of certain molecules in the tested CSF sample and may lead to foregone conclusions. No wonder there is often a low correlation of results obtained from different platforms or even from the same labs using kits and reagents from different vendors. Yet there are no universally implemented guidelines. Hence standardization of these assays including CSF handling (collection, storage and preparation) is a challenge for the near future. Teunissen *et al.*^[84] have proposed protocols for the standardization of CSF collection to minimizing blood contamination of CSF and protocols for the standardization of CSF storage to prevent sample degradation and global proteome changes.^[40]

Another critically important consideration is that despite the fact that several advanced platforms are available the analysis of secreted proteins/miRNA in the CSF is still a very challenging task due to technical difficulties. Often the scientists working on CSF biomarker discovery have limited knowledge of the protein/miRNA isolation/detection new platforms and or the analytical requirements which may hamper the subsequent markers analysis.

Together with low sample numbers that usually result in inadequate statistical power is another general weakness and might explain why not many of these markers have been validated for clinical use.^[31,40] Taking together the successful translation of CSF biomarkers from basic research to clinical applications will likely require multi-centre standardized and coordinated efforts to facilitate

biomarkers discovery and implementation. Finally there are some other limitations to the interpretation of CSF cancer related molecules studies as biomarker. Protein/miRNA composition of CSF is dependent on patient attributes such as age, gender, the specific site of CSF access.^[40]

CONCLUSION

CSF is an invaluable diagnostic window to the pathological state of CNS. It is easily accessible by minimally-invasive standard clinical methods and can provide the necessary biological information for the diagnosis of neurological diseases. Biochemical molecules secreted by brain cancers to the CSF hold great promise as diagnostic markers for a wide range of brain malignancies owing to the significant differences that have been reported between their expression profiles in healthy individuals and those of patients. However, significant concerns remain. Despite the sizeable published number of potential diagnostic and prognostic CSF biochemical markers their reproducibility between studies is unclear, and none have been validated for clinical use. The reported sample size in the literature is small. Most data were generated by a limited number of research groups using different protocols or technologies. No universally implemented guidelines are available yet for the CSF sample collection and preparation or for protein profiling or miRNA extraction from CSF and importantly for data analysis. It is therefore premature to make specific recommendations for their clinical implementation. More research that includes multi-institutional research and longitudinal studies of large patient cohorts to validate the clinical value of putative CSF markers, as demonstrated for the field of cancer genomics, is certainly warranted. The road from CSF biomarker discovery, validation, until the translation into the clinical setting could be long and difficult however, the reward for patients, clinicians and scientists could be rather significant.

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Conflicts of interest

There are no conflicts of interest.

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Gemcitabine followed by radiotherapy in treatment of newly diagnosed high-grade gliomas

Maha El-Naggar^{1,3}, Mervat Omar¹, Ahmed Elgeriany², Godefridus J. Peters³, Amina Mostafa¹, Samir Shehata¹

¹Department of Medical oncology, Assiut University Hospital, Assiut 71515, Egypt.

²Department of Neurosurgery, Assiut University Hospital, Assiut 71515, Egypt.

³Department of Medical Oncology, VU University Medical Center, 1081 HV Amsterdam, the Netherlands.

Correspondence to: Dr. Maha El-Naggar, Department of Medical oncology, Assiut University Hospital, Assiut 71515, Egypt. E-mail: maha_elnaggar@yahoo.com

ABSTRACT

Aim: High-grade glioblastoma multiforme (GBM) has a poor median overall survival (OS). The standard treatment after surgery is temozolomide and radiotherapy (RTH). Patients with unmethylated methylguanine-methyltransferase promoter (MGMT) have no or little benefit from temozolomide and are eligible for alternative therapies. Gemcitabine is a good radiosensitizer. We aimed to evaluate the combination of gemcitabine with RTH in newly diagnosed GBM. **Methods:** The study was a prospective phase II study. Eligible patients were required to have histologically proven anaplastic astrocytoma or GBM. Patients underwent biopsies or subtotal resection. The treatment consisted of fixed-dose rate gemcitabine 175 mg/m² weekly followed after 24 h by standard cranial RTH for 6 weeks. Tumor response was evaluated by Macdonald criteria. In case of progression, patients received temozolomide (200 mg/m²/5 days every 28 days). **Results:** Thirty patients with a median age of 52 years (30-69), 73%/27% male/female, the Eastern Cooperative Oncology Group performance status 1 (range 0-2) were enrolled. Five patients had a partial-response (17%) and 13 stable-disease (43%). Median time to progression was 7.88 months (95% CI 6.1-9.69) and OS was 11.77 months (95% CI 9.97-13.56). The treatment was well tolerated with grade-3 neutropenia in 3, grade-3 anemia in 2 and impaired liver enzymes in 1 patient. **Conclusion:** Gemcitabine followed by radiotherapy is active and promising regimen in newly diagnosed GBM. Gemcitabine uptake is easy, with a long local retention of active metabolites, precluding systemic side effects of radiosensitization. In a phase III study this treatment should be evaluated in patients with unmethylated MGMT promoter who will not benefit from temozolomide.

Key words: Gemcitabine; radiation; glioblastoma multiforma; temozolomide

INTRODUCTION

Malignant gliomas grade 3 anaplastic astrocytoma (AA) or grade 4 glioblastoma multiforme (GBM) are rapidly progressing primary brain tumors, which in spite of advances in surgery, radiotherapy and chemotherapy, remain associated with high morbidity and mortality.^[1] Despite the current multimodality therapy, the overall median survival for newly diagnosed patients is 10 months for patients with GBM and 2-3 years for those with AA.^[2,3]

Standard treatment of malignant gliomas is surgery, followed by radiotherapy concomitant with temozolomide (TMZ), followed by adjuvant TMZ (Stupp *et al.*,^[2] 2005). Surgery followed by involved field radiotherapy up to total

dose of 60 Gray (Gy) significantly prolongs survival. There have been many efforts to intensify radiotherapy, including the use of radiosensitizers, brachytherapy, radioactive seeds implanted in the tumor bed, and stereotactic radiosurgery in selected cases.^[4]

Initially the routine use of chemotherapy in addition to cranial irradiation was controversial. Individual randomized, controlled studies demonstrated no significant improvement in median survival with single agent or multiple agents chemotherapy, although a significant increase in survival was noted in a meta-analysis.^[1] There was a significant

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increase in the proportion of long-term survivors with the addition of chemotherapy, from less than 5 percent to approximately 15-20 percent, regardless of the patient's performance status, the histological features of the tumor, the duration of symptoms or age (up to 65 years).^[5]

However, the combination of radiotherapy with TMZ completely changed standard therapy, since this improved the median survival and overall survival.^[2] However, not all patients benefit since an unmethylated promoter of methylguanine methyltransferase (MGMT) enables extensive repair of TMZ-adducts and these patients do not benefit from temozolomide^[6] and are eligible for development of alternative therapies. Moreover, TMZ is relatively a poor radiosensitizer compared with other cytotoxic drugs.^[7] However, the group of nucleoside analogs, including gemcitabine are excellent radiosensitizers.^[8,9] Gemcitabine has been evaluated as a radiosensitizer for several tumor types, both in model systems and patients.^[8,10] Gemcitabine, an analogue of deoxycytidine, enters the cell by the action of a nucleoside transporter^[11] and it is activated by phosphorylation in a reaction catalyzed by deoxycytidine kinase (DCK), to gemcitabine monophosphate (dFdCMP) and subsequently phosphorylated to the 5'-diphosphate (dFdCDP) and triphosphate (dFdCTP) derivatives.^[12,13] Gemcitabine exerts its cytotoxicity mainly through the irreversible incorporation of the activated triphosphate into DNA, resulting in chain termination. Due to a number of self-potentiating mechanisms, gemcitabine exhibits prolonged intracellular retention, a property likely partially responsible for gemcitabine's broad spectrum of activity.^[14,15]

The standard dose of gemcitabine (1,000 mg/m²) given over 30 min results in high gemcitabine peak levels (> 20 µmol/L), which rapidly decline below 1 µmol/L within 2 h.^[16,17] However, DCK is saturated at much lower gemcitabine levels and *in vitro* and *in vivo* sensitivity to gemcitabine is most optimal at prolonged exposure to low drug levels in the nanomolar range.^[18-20] Therefore, it was reasoned that prolonged infusion of gemcitabine, which would result in prolonged but lower plasma concentrations of gemcitabine, would be advantageous.^[16,21] The fixed dose rate of 10 mg/m²/min infusion of gemcitabine gives this pharmacodynamic advantage over the 30 min infusion,^[16] resulting in a prolonged formation and retention of gemcitabine nucleotides which favour the activity of gemcitabine. A phase II trial showed that fixed dose rate gemcitabine can improve survival in patients with pancreatic adenocarcinoma but the difference was not significant in a randomized study.^[22] Single agent studies of gemcitabine in high grade glioma did not show any benefit,^[23,24] so that development of gemcitabine for this disease was discontinued. However, gemcitabine has shown a radiosensitizing effect in a number of human glioma cell lines^[25-27] and in an animal model system.^[28]

Sigmond *et al.*^[29] demonstrated that gemcitabine could pass the blood-tumor barrier in GBM patients. In tumor samples, concentrations of gemcitabine and its active metabolite dF-dCTP were high enough to enable radio sensitization, which warrants clinical studies using gemcitabine in combination with radiation.

Weller *et al.*^[24] and Metro *et al.*^[30] indeed showed that gemcitabine combined with RTH was an active regimen, but whether it was more effective than RTH alone remained elusive. The combination gemcitabine-RTH followed by temozolomide showed promising activity.^[30] Preliminary results of another phase I study showed that gemcitabine combined with radiotherapy is efficient and tolerable in high grade glioma.^[31] The aim of our study was to evaluate the activity of gemcitabine with RTH as a treatment modality in newly diagnosed high- grade gliomas and temozolomide was administered after progression only.

METHODS

This was single center, phase II, open label, one arm non-randomized trial designed to determine the efficacy and safety of gemcitabine combined with therapy in the treatment of patients with newly diagnosed malignant glioma. The Research Ethics Board of Assiut University Hospital approved the study. All patients gave written informed consent before starting treatment.

Patients

Eligible patients had histologically proven malignant glioma (grade 3 or 4). Patients were at least 18 years of age; had the Eastern Cooperative Oncology Group (ECOG) performance status < 3, had adequate bone marrow reserves (hemoglobin > 9 g/dL, absolute granulocytes > 1.5 × 10⁹/L, platelets > 100 × 10⁹/L), and acceptable serum chemistries (serum calcium in normal range (8.8-10.2 mg/dL), serum creatinine < 1.5 × upper limit of normal, bilirubin < 1.5 × upper limits of normal and AST (aspartate transaminase) < 3 × upper limits of normal).

Patients were excluded if they were < 18 years old, had previous invasive malignancies or received previous chemotherapy or radiotherapy, had poor medical conditions, or were pregnant, nursing or not practicing effective contraception if appropriate.

Assessments and treatment plan

Pre-treatment evaluation included a history and physical and neurological examination, laboratory (complete blood picture, liver and kidney function, serum calcium level) and imaging studies (baseline CT and MRI brain) and a baseline toxicity evaluation.

After surgery of patients with malignant gliomas for either cytoreduction or a biopsy, patients received fractionated local RTH at a daily dose of 2 Gray (GY) per fraction, five days per week for six weeks (total dose of 60 GY).

RTH was applied to the gross tumor volume with 2-3 cm margin for the clinical target volume. Radiotherapy was carried out using linear accelerator with 6-15 MV photons. Gemcitabine was administered at a fixed dose of 175 mg/m² by intravenous infusion starting 24 h prior to radiotherapy in the first week and then once weekly before RTH for the whole duration of the radiotherapy. Toxicities were graded using the NCIC-CTG expanded common toxicity criteria. Evaluation during protocol treatment included history and physical examinations (including full clinical neurologic examination), biochemical profiles; and imaging studies. Contrast-enhanced (gadolinium-DTPA) MRI of the brain was uniformly adopted for tumor assessment and evaluation of response. Baseline MRI examination was performed 24-48 h after surgery and then within 1 week prior to the start of the experimental treatment, 4 weeks after the end of chemo-radiotherapy and every 8 weeks thereafter until evidence of disease progression. Toxicity assessments were done weekly during the radiotherapy and then one month from the end of the treatment then every 2 months or when clinically indicated. Toxicity was graded according to NCI-CTC version 3.0.^[32] Response was assessed using standard Macdonald criteria,^[33] but patients were not considered to have had complete or partial responses unless clinical neurologic assessment was improved or stable. Patients were monitored until death.

Statistics

The duration of response was calculated from the first day of treatment to the date of progression for patients who achieved complete or partial response. Progression-free survival, analyzed by Kaplan-Meier method including 95% CI, was defined as the period of time elapsed from the first day of treatment to the date of disease progression, relapse or death from any cause. Overall survival was defined as the interval from the first day of study treatment to the date of patient death. The survival curves were estimated by the Kaplan-Meier product-limit method. The SPSS (11.0) statistical program was used for analysis.

RESULTS

Patients

From April 2009 to April 2011, thirty patients were enrolled. Table 1 shows the characteristics of patients entered on the study. Of the 30 patients included in the analyses, 8 were female and 22 male, with a median age of 52 years (range 30-69). Patients had an ECOG performance status range 0 to 2. There were 8 patients with anaplastic astrocytoma, and 22 with glioblastoma multiform.

Outcome

All patients received concomitant dexamethasone, while anti-convulsant treatment was given on demand. All the patients completed the chemoradiotherapy treatment. Six patients responded to the treatment (20%) and 13 patients had stable disease (43%) for an overall disease control rate

Table 1: Patient characteristics

Total patients	30
Age in years, median (range)	52 (30-69)
Gender	
Male	22 (73%)
Female	8 (27%)
ECOG performance status, median (range)	1 (0-2)
Pathology	
Anaplastic Astrocytoma	8 (27%)
Glioblastoma multiforma	22 (73%)
Surgical procedure	
Subtotal resection	10 (33%)
Biopsy	20 (67%)

ECOG: Eastern Cooperative Oncology Group

Table 2: Treatment response

Response	Patient number (%)
Complete response (CR)	1 (3)
Partial response (PR)	5 (17)
Stable disease (SD)	13(43)
Progressive disease (PD)	11 (37)
Disease control rate (CR+PR+SD)	19/30 (63)
Tumor response rate (CR+PR)	6/30 (20)

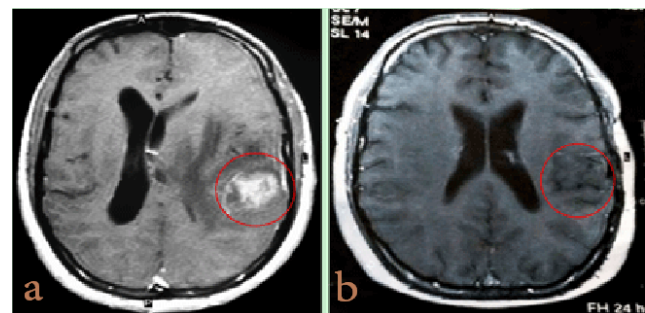


Figure 1: (a) MRI in T1 after Gd-DTPA infusion in axial plane shows an area of enhancement in GBM in the left parietal area; (b) MRI performed 1 year after the end of chemo-radiotherapy shows a dramatic response of the tumour T1 axial plane after Gd-DTPA infusion. MRI: magnetic resonance imaging; GBM: glioblastoma multiforme

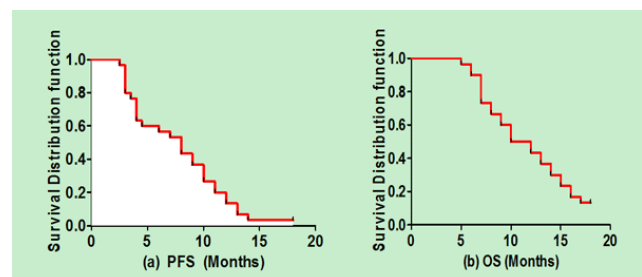


Figure 2: (a) PFS at a follow up of 18 months. The median PFS was 7.9 months (95% CI 6.1-9.7); (b) analysis of OS for 18 months, median OS was 11.8 months (95% CI 10.0-13.6). PFS: progression free survival; OS: overall survival

of 63% [Table 2] [Figures 1 and 2]. At a median follow up of 18 months median time to progression was 7.88 months (95% CI 6.1-9.69) and overall survival was 11.77 months (95% CI 9.97-13.56). According to the histology Grade 3

Table 3: Adverse events

	Grade 1 Patient number (%)	Grade 2 Patient number (%)	Grade 3 Patient number (%)	Grade 4 Patient number (%)
Heamatological toxicity				
Neutropenia	2 (7)	2 (7)	3 (10)	0
Aneamia	2 (7)	1 (3)	2 (7)	0
Thrombocytopenia	1 (3)	0	0	0
Non heamatological toxicity				
Impaired liver enzymes	2 (7)	1 (3)	1 (3)	0
Fever	2 (7)	0	0	0
Nausea/vomiting	3 (10)	1 (3)	0	0
Anorexia	2 (7)	0	0	0
Diarrhea	2 (7)	1 (3)	0	0
Fatigue	3 (10)	2 (7)	0	0
Convulsion	2 (7)	0	0	0
Headache	4 (13)	0	0	0
Insomnia	1 (3)	0	0	0
Alopecia	9 (30)	4 (13)	0	0
Otitis externa	1 (3)	0	0	0
Scalp dermatitis	2 (7)	0	0	0

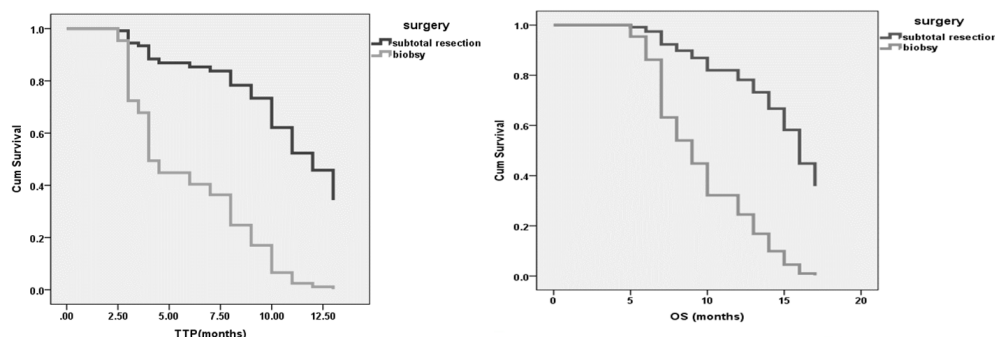


Figure 3: Subgroup analysis of PFS (depicted as Time to Progression, TTP) and OS according to surgical extension median PFS of 11 months (95% CI 8.1-13.9) for subtotal resection versus 4 months (95% CI 3.5-4.6) for a biopsied patients and median OS 15.4 months (95% CI 13.5-17.3) versus 9.5 months (95% CI 8.1-11.0) for subtotal resection versus biopsy, respectively. PFS: progression free survival; OS: overall survival

astrocytoma has more favorable PFS 11.13 months (95% CI 9.37-12.88) and OS 15.25 months (95% CI 12.54-17.96) than Glioblastoma multiform patients with PFS 6.70 months (95% CI 4.505-8.90) and OS 10.50 months (95% CI 8.41-12.59). On multivariate analysis, factors predictive of progression were performance status ($P = 0.04$) and the extent of surgery ($P = 0.02$). The latter was evaluated in a subgroup analysis, which showed that patients with a subtotal resection had a higher probability for a longer survival than those patients who were only biopsied [Figure 3].

Safety

All patients completed radiotherapy for a total dose of 60 Gy. All patients were evaluable for safety of the combination of gemcitabine and radiotherapy. Treatment-related adverse events are summarized in Table 3. Generally the treatment was well tolerated. Hematological toxicity consisted of grade 3 neutropenia in three patients (10%), while grade 3 anemia was reported in 2 patients (7%) on day 24; these patients received packed red blood cells. In the 3 cases of neutropenia, this was afebrile and occurred on day 16 in 2 cases and on day 24 in one case after the initiation of study treatment. Also, non-hematological adverse events were mostly mild (grade 1) or moderate (grade 2)

in intensity. Hypertransaminasemia was the only grade 3 non-hematological adverse event in one patient (3%), and this patient was receiving antiepileptic treatment. No treatment-related grade 4 toxicities were observed.

DISCUSSION

In this study we demonstrated that gemcitabine followed by RTH is an active regimen for treatment of high grade newly diagnosed GBM. Our study met the primary activity objective, producing a response rate of 20% and disease control rate of 63%, which was in line with earlier gemcitabine/RTH data of 17.5% and 75%, respectively.^[30] The results of these gemcitabine/RTH studies compare favorably with corresponding values for activity and disease control of 15.5% and 57.5%, respectively, obtained with nitrosurea given concurrently with radiotherapy.^[34] Furthermore, the promising values of PFS of 7.88 months and OS 11.77 months are in the same range as that observed for temozolomide plus radiotherapy 6.9 months for PFS and of 14.6 months for OS.^[2]

However, it is difficult to compare PFS and OS of the present study with those obtained in studies of radiotherapy-temozolomide with or without adjuvant temozolomide,

where 17.5-34.5% of patients had received complete tumor resection prior to study entry.^[2,35] All patients in our study had residual disease after surgery, while 67% of the patients were biopsied-only. A subgroup analysis of TMZ plus radiotherapy showed that the 93 patients who underwent only biopsy had no significant improvement in median overall survival.^[2]

Although PFS was the secondary objective in the present study, our results are not likely to be influenced by sequential temozolomide as it was only given after progression of the patients. In contrast, OS may be affected by the TMZ administered at disease progression. In another study,^[30] this might not have been the case.

The combination of gemcitabine plus radiotherapy was well tolerated. No treatment-related grade 4 toxicities were observed, while there were only 6 cases of grade 3 adverse events, including 5 patients with hematological toxicity and one with hypertransaminasemia. This was in line with toxicity reported for gemcitabine with RTH in high grade glioma patients with weekly schedule.^[30] On the whole, the treatment-related morbidity did not differ significantly from that observed with nitrosourea or temozolomide given concurrently with radiotherapy.^[2,34,35]

The standard treatment of high grade glioma patients has changed considerably since the introduction of radiotherapy combined with temozolomide.^[2,36] However, the benefit is only achieved in a subgroup of patients, who have a methylated MGMT promoter in the tumor.^[6] The patients with a hypomethylated MGMT promoter have an active MGMT enzyme, which will repair the DNA damage. Moreover, even in cells with a methylated MGMT promoter TMZ is a relatively poor radiosensitizer.^[7] Therefore these patients are eligible for an alternative treatment. Although gemcitabine as a single drug does not have an antitumor activity against GBM,^[23,24] this is not due to a poor passage of the blood-brain barrier. Normal brain depends on preformed nucleosides to enable nucleotide synthesis in the brain.^[37] Since gemcitabine resembles normal deoxynucleosides it is not unexpected that it is taken up efficiently into the brain and that the blood-brain barrier does not preclude gemcitabine's passage.^[29] Although the low dose of gemcitabine would preclude an antitumor effect by the drug itself, this dose results in sufficiently high concentrations in the tumor for radiosensitization can be reached. Since gemcitabine is an excellent radiosensitizer, low concentrations are sufficient,^[25-27,38] while it is also important to have an adequate time-period between the drug and radiation,^[8,9,39] since the active metabolite, dFdCTP, is retained for at least 24 h in tumors this will allow to maintain sufficiently high levels.^[19] This delay will also prevent cumulative toxicity. In addition to the radiosensitizing effect of gemcitabine, also its main catabolite, difluorodeoxyuridine, has been shown to be a good radiosensitizer,^[40,41] while the catabolite is

maintained at micromolar levels for days, including brain. This catabolite was recently shown to be able to inhibit thymidylate synthase.^[42] This inhibition, leading to an accumulation of deoxyuridine triphosphate (dUTP), might be basis for an additional radiosensitizing effect.^[9] Hence a dual radiosensitization might be achieved in glioma.

In conclusion, this study shows that fixed dose rate infusion of gemcitabine given before radiotherapy is clinically effective as a radiosensitizer for newly diagnosed GBM. Gemcitabine has a better cost effectiveness compared to the financial cost temozolomide. Further investigation of chemo-radiotherapy is needed and a Phase 3 trial with a higher number of patients will be initiated, to determine whether the gemcitabine radiosensitizing effect can be achieved irrespective of the methylation status of the MGMT promoter.^[30]

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Conflicts of interest

There are no conflicts of interest.

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Muscarinic receptor signaling and colon cancer progression

Guofeng Xie, Jean-Pierre Raufman

Division of Gastroenterology and Hepatology, Veterans Affairs Maryland Health Care System, University of Maryland School of Medicine, Baltimore, MD 21201, USA.

Correspondence to: Dr. Guofeng Xie, Division of Gastroenterology and Hepatology, Veterans Affairs Maryland Health Care System, University of Maryland School of Medicine, 22 South Greene St., Baltimore, MD 21201, USA. E-mail: gxie@medicine.umaryland.edu

ABSTRACT

Due to the lack of effective treatments, advanced colorectal cancer (CRC) remains a leading cause of cancer death in the United States. Emerging evidence supports the observation that muscarinic receptor (MR) signaling plays a critical role in growth and progression of CRC. MR activation by acetylcholine and bile acids results in transactivation of epidermal growth factor receptors (EGFR) and post-EGFR signal transduction that enhances cell proliferation, migration, and invasion. Here, the authors review recent progress in understanding the molecular mechanisms underlying MR-mediated CRC progression and its therapeutic implications.

Key words: Muscarinic receptor; colon cancer; epidermal growth factor receptors; bile acids; acetylcholine

INTRODUCTION

Colorectal cancer (CRC) is the second leading cause of cancer death worldwide with 1.4 million new cases and 693,900 deaths each year.^[1] In the United States, CRC is currently the third leading cause of cancer death for both men and women.^[2,3] Annually, approximately 140,000 people are diagnosed with CRC of which 50,000 will die, primarily from advanced disease.^[2,3] Although surgical and endoscopic treatment is very effective for patients with early-stage CRC, late-stage CRC is generally resistant to chemo- and radiation- therapy.

Uncontrolled cell proliferation is an integral part of CRC progression. Cancer cell proliferation is regulated by a variety of growth factors and receptors. Epidermal growth factor (EGF) and other EGF receptor (EGFR) agonists play key roles in promoting growth of many human cancers, including CRC. As a result, biologicals that target EGFR have been approved by the Food and Drug Administration for the treatment of EGFR-positive advanced CRC; this approach enhances survival by several months.^[4,5] However, the 5-year survival rate for

advanced CRC remains only 10-15%.^[4,5] New therapeutic approaches are urgently needed.

MUSCARINIC RECEPTORS IN NORMAL COLON TISSUE AND CRC

The muscarinic cholinergic family of G-protein-coupled receptors (GPCRs) consists of five muscarinic receptor (MR) subtypes designated muscarinic acetylcholine receptor subtype M1 (M1R)-M5R (for review see^[6-9]). MR is expressed in many tissue types and play important roles in progression of many cancers including breast, prostate, lung and CRC.^[10-12] M1R and M3R, expressed widely in the gastrointestinal (GI) tract, are coupled to G_{q11}, activate phospholipase C and increase cell calcium. Using reverse transcription polymerase chain reaction with primers specific to MR subtypes, radiolabeled ligand binding assays, and calcium mobilization studies, Frucht *et al.*^[13,14] reported that 60% of colon cancer cell lines they tested expressed M3R. Subsequently, Yang and Frucht^[15] reported up to 8-fold increased M3R expression in 62% of colon cancers compared to normal adjacent normal colon epithelium.

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MUSCARINIC RECEPTOR LIGANDS IN CRC (ACETYLCHOLINE AND BILE ACIDS)

Role of acetylcholine

Acetylcholine (ACh) was traditionally regarded solely as a neurotransmitter that functioned exclusively in the central and peripheral nervous systems. However, over the past decade emerging evidence indicates that ACh is also produced and released by normal and neoplastic non-neuronal cells including human keratinocytes, small cell lung cancer cells, immune cells, and intestinal epithelial cells.^[16-21] Choline acetyltransferase (ChAT) plays a critical catalytic role in the biosynthesis of both neuronal and non-neuronal ACh. Homozygous ChAT mutant embryos lack detectable ACh.^[22] Also, ACh released from nerve endings is rapidly hydrolyzed by choline esterases thereby limiting its actions to immediately-neighboring cells. As a consequence, ACh production by non-neuronal cells can play a key role in regulating the actions of cells or tissues that are not directly innervated by cholinergic neurons.

ACh can be released by enteric neurons or produced by colon cancer cells. Although ACh release from intestinal non-neuronal cells was identified more than a decade ago,^[23] its physiological relevance remains poorly understood. We showed that human colon cancer cells produce and release ACh that acts as an autocrine growth factor to stimulate cell proliferation.^[24] We demonstrated that: (1) basal colon cancer cell proliferation is inhibited by the cholinergic antagonist atropine, cholinesterases, and inhibitors of choline transport; (2) ChAT is expressed at low levels by mouse and human intestinal mucosa, and at high levels by mouse colon tumors, a majority of human colon cancer cell lines, and CRC surgical specimens; and (3) human colon cancer cell lines produce and release ACh as demonstrated by high-performance liquid chromatography with electrochemical detection. These findings strongly support a role for ACh in CRC progression.

We proposed an important role for MR activation in ultrarapid growth of CRC in a patient with pheochromocytoma.^[25] This elderly man with long-standing, unresectable pheochromocytoma experienced rapid development of rectal adenocarcinoma despite close endoscopic surveillance. We determined that the patient's CRC overexpressed M3R, whereas his pheochromocytoma expressed ChAT. These findings suggested that ACh release from the pheochromocytoma stimulated rapid growth of the rectal neoplasm. As proof-of-principle we found that culture media conditioned by pheochromocytoma cells stimulates proliferation of a human colon cancer cell line, an effect attenuated by adding the MR antagonist atropine.

Role of bile acids

Besides ACh, bile acids and its derivatives are also

important MR ligands. In 1998, we made the serendipitous observation that bile acids interact functionally with muscarinic receptors on gastric epithelial cells.^[26] Subsequently, we made several novel observations: (1) conjugated secondary bile acids interact selectively and functionally with choline containing compounds (Cho) cells expressing muscarinic receptors;^[27] (2) molecular modeling revealed a strikingly similar structural alignment of the geometry and surface electrostatic charges of bile acids and ACh;^[27] (3) bile acid binding triggers appropriate post-M3R signaling;^[27] (4) hybrid molecules created from bile acids and ACh are MR ligands;^[28] and (5) lithocholic and deoxycholic acid conjugates interact with M3R on human colon cancer cells, thereby stimulating post-receptor signaling and cell proliferation.^[29] Because muscarinic effects on colon cancer cell proliferation are mediated by transactivation of EGFR,^[30,31] results of bile acid interaction with M3R depend on the cell type examined. In Cho cells that express M3R but not EGFR, deoxycholic acid conjugates are MR antagonists.^[32] In H508 and HT-29 colon cancer cells that express both M3R and EGFR deoxycholytaurine (DCT) is a MR agonist whose effects are mediated by transactivation of EGFR.^[30,33] To our knowledge, no endogenous mammalian cholinergic agonists other than ACh and bile acids have been identified.

The observation that bile acids interact selectively and functionally with plasma membrane muscarinic receptors prompted us to examine their actions on intestinal epithelial cells. In particular, we studied H508 human colon cancer cells that co-express M3R and EGFR, and SNU-C4 cells that express EGFR but not muscarinic receptors.^[14] DCT caused dose-dependent increases in M3R signaling and H508 cell proliferation that were not observed in SNU-C4 cells.^[30] These proliferative effects of bile acids are mediated by interaction with plasma membrane M3R, not by interaction with bile acid nuclear receptors (i.e. the farnesoid X receptor) that regulate bile acid metabolism.

We demonstrated that efficacious concentrations of pro-proliferative bile acids are achieved in the intestine.^[34] Because H508 cells derive from a moderately well-differentiated cecal adenocarcinoma, cecal contents were obtained immediately post-mortem from 19 persons. Using internal controls, bile acid spectrum and concentration were determined by an enzymatic assay and gas-chromatography/mass spectrometry. Total 3 α -hydroxy bile acids were 400 ± 200 $\mu\text{mol/L}$ (mean \pm SD) and deoxycholic acid conjugates were 12 ± 28 $\mu\text{mol/L}$ (maximum, 104 $\mu\text{mol/L}$).^[33] Overall, in one-third of subjects, cecal conjugated deoxycholic acid achieved levels (10-100 $\mu\text{mol/L}$) that stimulate colon cancer cell proliferation *in vitro*.^[30,33,34] Cecal bile acid concentrations in persons with ileal disease, ileal resection, and colon cancer are not known. Additional factors suggest that bile acid interaction with GI epithelial

cell muscarinic receptors can regulate cell proliferation: (1) fecal bile acids are in contact with intestinal epithelial cells for many years (average age for developing colon cancer is > 50 years);^[35] (2) bile acids lack an ester linkage and are not hydrolyzed by tissue cholinesterases that rapidly inactivate ACh;^[28] (3) lipophilic lithocholic acid derivatives have access to muscarinic receptors in the lipid bilayer of cell membranes (i.e. the novel bile acid: ACh hybrid molecule, lithocholylcholine, interacts with muscarinic receptors on rat aortic strips);^[28] (4) neoplastic cells commonly lose polarity, thereby expanding expression of muscarinic receptors, usually restricted to the basolateral membrane, to the entire plasma membrane; and (5) increased tight junction permeability between neoplastic cells allows access of luminal bile acids to basolateral membrane receptors.^[36] We believe that this collective evidence makes a compelling argument that muscarinic receptors and MR ligands play an important role in intestinal epithelial cell proliferation and CRC progression.

Although experimental studies in rodents suggest that bile acids are intestinal tumor promoters,^[37] the role of endogenous bile acids in colon carcinogenesis remains poorly understood. Dawson *et al.*^[38] showed that in mice deficient in the ileal apical sodium-dependent bile acid transporter (ASBT, encoded by SLC10A2), fecal bile acid excretion increased more than 10-fold. To examine the development of aberrant crypt foci (ACF), the earliest histological marker of colon neoplasia, we treated WT and *Asbt*-deficient [*Slc10a2* (-/-)] male mice with azoxymethane (AOM), an intestine-selective carcinogen.^[39] We also used a combination of AOM and dextran sodium sulfate to induce colon tumorigenesis. Compared to littermate controls, we found that *Asbt*-deficient mice demonstrated significant increases in ACF, as well as colon tumor number and size. Also, *Asbt*-deficient mice had a two-fold increase in the number of colon adenocarcinomas. Finally, in murine colon neoplasia, increased fecal bile acids were associated with increased expression of M3R and EGFR, and activation of post-EGFR signaling. These observations indicate that endogenous bile acids also promote intestinal tumorigenesis.

MR SIGNALING IN CRC AND DISEASE PROGRESSION

Transactivation of EGFR

EGFR is commonly over-expressed in many epithelial malignancies and this feature often indicates a more aggressive phenotype.^[40] Likewise, as observed with M3R, EGFR is frequently over-expressed in colon cancer (in 25-77% of tumors compared to adjacent normal mucosa).^[40,41] Co-expression of M3R and EGFR in many colon cancer cell lines, and over-expression of these receptors in the majority of colon cancers suggests that the functional interaction observed between M3R

and EGFR is important for regulating colon cancer cell proliferation.^[31]

Previously, we found that in H508 cells which over-express both M3R and EGFR, but not in SNU-C4 cells that express EGFR but not M3R, ACh stimulated cell proliferation by approximately 200% compared to control.^[31] In H508 cells, both ACh and EGF stimulated calcium-dependent EGFR activation (tyrosine phosphorylation) and activation of extracellular signal-regulated kinase 1 and 2 (ERK1/2); MR antagonists and inhibitors of the mitogen-activated protein kinase (MAPK) phosphorylation blocked these effects. In addition, ACh- and EGF-induced phosphorylation of ERK1/2 MAPK and cell proliferation were abolished by EGFR inhibitors. However, in Cho cells transfected with rat M3R, which lack EGFR, ACh-induced ERK1/2 MAPK phosphorylation was not altered by EGFR inhibitors. It was concluded that, in H508 cells, cholinergic ligand interaction with M3R results in transactivation of EGFR, thereby stimulating cell proliferation.^[31] These results indicate that EGFR transactivation is a key mechanism underlying MR-mediated intestinal tumorigenesis and cancer progression.

Cell proliferation and tumorigenesis

Uncontrolled cell proliferation is a hallmark of malignancies. We showed that in human colon cancer cells ACh-induced activation of M3R stimulates robust but selective matrix metalloproteinase (MMP) gene expression.^[42] In H508 human colon cancer cells, ACh caused a striking dose- and time- dependent increase in mRNA and protein levels of MMP1, 7, and 10 by upregulating gene transcription. As a consequence, ACh stimulated MMP7-dependent cell proliferation by transactivating EGFR.

Using *in vivo* models, we showed that genetic ablation of M3R in AOM-treated mice attenuates epithelial cell proliferation, and the number of adenomas and adenocarcinomas per mouse colon (65% reduction in the number of adenocarcinomas/colon).^[43] Whereas 50% of AOM-treated wild-type (WT) animals had multiple adenocarcinomas/colon, this was not the case with any M3R-deficient animal. Moreover, in M3R-deficient mice the overall colon tumor volume was reduced by 60% compared to that in WT animals. Collectively, these observations suggest that M3R may play a role in both tumor initiation and promotion; that is, both the number and size of tumors was reduced in M3R-deficient animals.

MR antagonists are potential therapeutics in CRC. We showed that M3R gene ablation and treatment with scopolamine butylbromide, a non-subtype-selective MR inhibitor, attenuated small intestinal neoplasia in *Apc*^{Min/+} mice with aberrant beta-catenin signaling.^[44] Compared with *Apc*^{Min/+} mice, *Apc*^{Min/+}M3R^{-/-} mice showed 70% and 81% reductions in tumor number and volume,

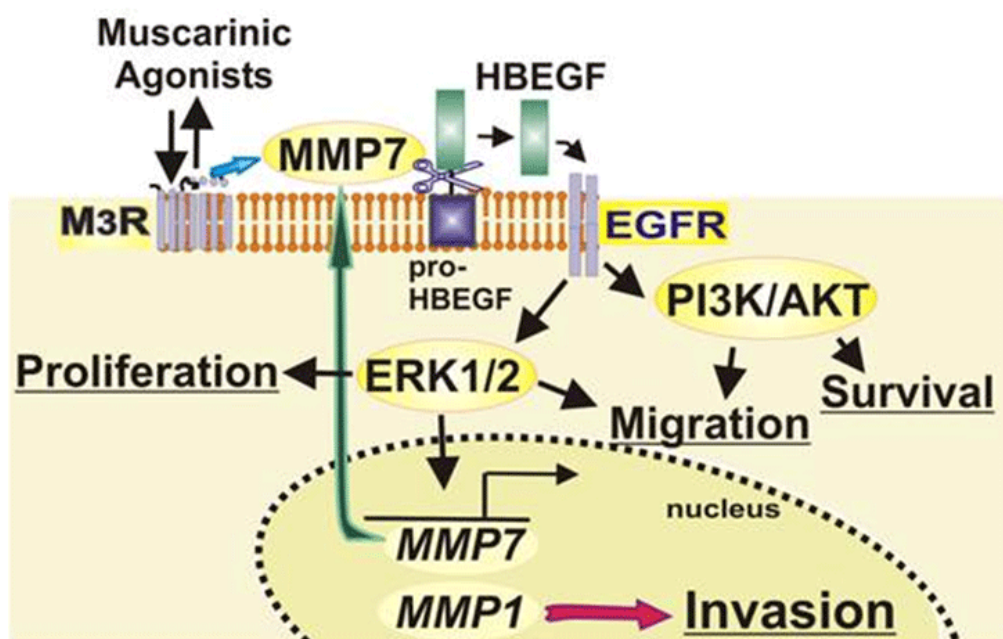


Figure 1: Muscarinic acetylcholine receptor subtype M3 (M3R) agonist-induced signaling in human colon cancer cells. Post-M3R matrix metalloproteinase 7 (MMP7) activation releases heparin binding epidermal growth factor (EGF) like growth factor (HBEGF), thereby activating EGF receptors (EGFR) signaling which promotes cell proliferation, survival and migration downstream extracellular signal-regulated kinase (ERK) activation induces *MMP7*, which replenishes *MMP7* (green arrow), and *MMP1*, which promotes colon cancer cell invasion (red arrow)

respectively. After 8 weeks of continuous treatment, scopolamine butylbromide-treated mice had a significant reduction in both tumor number and volume as compared with control mice. Overall, these findings indicate that the interplay of M3R and beta-catenin signaling is important for intestinal mucosal differentiation and neoplasia.

Because intestines express both M1R and M3R receptors, it is important to determine whether these two receptors have distinct functions. We showed divergent effects of MR subtype gene ablation on murine colon tumorigenesis.^[45] Although AOM-treated M3R-deficient mice had fewer and smaller colon tumors than control WT mice, reductions in colon tumor number and size were not observed in M1R-deficient and dual M1R/M3R-deficient mice. Microarray and real-time PCR analyses revealed a possible role for zinc finger protein (Zfp) 277 expression in mediating these different phenotypes. However, the molecular mechanism underlying the MR-dependent regulation of Zfp 277 requires further investigation.

We demonstrated cholinergic MR activation augments murine intestinal epithelial cell proliferation and tumorigenesis *in vivo*.^[46] Mice treated with the MR agonist bethanechol, provided in drinking water, had increased AOM-induced colon tumor numbers and size compared to AOM-treated mice drinking untreated water. Cell proliferation in both normal mucosa and adenocarcinomas was increased in bethanechol-treated compared to control mice. Also, in tumors, bethanechol treatment was associated with increased expression of M3R, EGFR and post-EGFR signaling molecules Myc and cyclin D1. Bethanechol treatment also increased normal colon

mucosal thickness and stimulated expression of selected MMP genes, including *MMP7*, *MMP10*, and *MMP13*. These findings confirm that MR agonists are intestinal tumor promoters.

Cell migration and invasion

Cell migration is a key mechanism of cancer invasion. Using three distinct *in vitro* models, we showed that MR activation enhances cell migration and invasion. Using a soft agar colony formation assay, we showed that ACh enhanced anchorage- and MMP-dependent growth of H508 human CRC cells. In addition, in H508 and HT29 human CRC cells, using *in vitro* wound closure and Matrigel invasion models,^[47] we showed that ACh treatment increased cell migration that was blocked by inhibiting RhoA and Rho kinase, key proteins that interact with the actin cytoskeleton. Lastly, using an electrical cell impedance sensing invasion assay, we showed that ACh stimulated MMP1-dependent invasion of H508 cells.^[48]

CONCLUSION

Muscarinic receptors are expressed in normal colon epithelial cells and overexpressed in colon tumors and colon cancer cell lines. Primary MR Ligands include ACh, deriving from both neuronal and non-neuronal tissues, and secondary bile acids. MR activation enhances colon cancer cell proliferation, cell migration, and invasion by transactivating EGFR, thereby initiating post-EGFR signaling [Figure 1]. MR antagonists and other agents that block MR activation or subsequent post-MR signal transduction have promise for CRC therapeutics.

FUTURE DIRECTIONS

It is clear that muscarinic receptors and ligands play key roles in CRC progression. Unfortunately, there are no published translational and clinical studies at this time. Future studies focused on determining overexpression of muscarinic receptors in different patient cohorts, relation to CRC staging, tumor differentiation and prognosis will shed more lights in the importance of muscarinic receptor signaling in CRC.

Another important aspect of MR signaling in CRC is therapeutic potential of MR-based therapy. Because M1R and M3R are both expressed abundantly in the intestinal epithelium, but have distinct functions,^[45] it is critical to develop M1R- and M3R-specific ligands, and test their pharmacological and therapeutic characteristics both *in vitro* and *in vivo*. These studies will lay important foundations for future human clinical trials that are focused on MR-based therapy. In addition, colon-specific drug delivery systems^[49] should be utilized to reduce unwanted systemic side-effects.

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Conflicts of interest

There are no conflicts of interest.

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The role of human papillomaviruses in cancer progression

Pinar Tulay, Nedime Serakinci

Department of Medical Genetics, Faculty of Medicine, Near East University, 999058 Nicosia, Cyprus.

Correspondence to: Dr. Nedime Serakinci, Department of Medical Genetics, Faculty of Medicine, Near East University, 999058 Nicosia, Cyprus.
E-mail: nedimeserakinci@gmail.com

ABSTRACT

The importance of human papillomavirus (HPV) infection and its role in the progress of cancer have been widely evaluated. The understanding of HPV association with certain cancers, such as cervical cancer, is very well established. A big step forward in the prevention of HPV associated cancers with the use of early detection by screening strategies has also been taken. In the last decade, development of HPV vaccination has reduced the number of cases in HPV infections and infection induced cancers. In this report, we review the HPV pathogenesis and highlight the mechanism of HPV involvement in cancer development.

Key words: Human papillomavirus; cancer; immune response; human papillomavirus vaccine

INTRODUCTION

Human papillomavirus (HPV) is considered to be one of the viral infections associated with cancers and other diseases worldwide. HPVs are non-enveloped viruses with double stranded circular DNA.^[1,2] The genome of papillomavirus constitutes three segments; early, late and genomic regions. The early region with E1, E2, E4-E8 forms half of the HPV genome. The early fragments function at different stages, in such both E1 and E2 is involved in the regulation of DNA replication, E2 in transcription (E2), E5, E6 and E7 in cell transformation [Table 1]. The late region (L) with L1 and L2 forms 40% of the genome and the genomic regulatory region forms the rest of the genome.^[3] The late region of the genome involves the structural proteins of the virion [Table 1].^[4]

HPVs are characterised according to their tissue tropism and they are subdivided into five main genera (Alpha-, beta-, gamma-, nu- and mu-papillomaviruses) depending on the DNA sequences, HPV life cycle characteristics and disease associations.^[5-8] Alpha-HPVs infect mucosal tissues, whereas beta-, gamma-, nu- and mu-papillomaviruses infects cutaneous sites causing cutaneous lesions in humans.^[9,10] However, as in recent years the number of HPV genotypes identified in healthy skin is increased, it is difficult to assign the cutaneous HPV types with a given

cutaneous pathology. The HPVs can be further subdivided according to the epidemiological classification as ones with low, intermediate and high risk oncogenic potentials depending on the viruses' ability to promote the proliferation of infected cells and lead to malignant transformations.^[1,11] The low risk HPVs including HPV6, 11, 42, 43 and 44 may cause condylomas and benign cervical lesions that do not form malignancies.^[1,4,12,13] The intermediate oncogenic risk HPVs involves HPV31, 33, 35, 51 and 52 and there is still an ongoing debate whether the intermediate oncogenic risk HPVs cause malignant transformation as much as the high risk HPV types.^[2,14] High oncogenic potential HPVs include HPV16, 18, 45 and 56 and these HPVs mostly cause neoplastic transformations.^[2,4,14] Unlike alpha-HPVs, most of the beta- and gamma-HPVs results in asymptomatic infections in immune-competent individuals and these viruses adapt to their host and complete the life-cycle without causing any apparent diseases.^[8,15-17]

Although the molecular defects caused by HPV infection leads to malignant transformation, it is not well established how they predispose to disease and whether keratinocyte^[18,19] or the immune system is being compromised.^[20,21] Therefore, although mainly the high risk HPV types cause malignant transformation and the low risks do no, it is possible that the low-risk viruses

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Table 1: List of HPV proteins and their function

HPV proteins	Function
E1	Viral DNA replication Repressive agent in transcription Inhibition of DNA replication ^[24,199] DNA replication Functions with E1, especially in HPV6, 11 and 16 ^[24] Responsible for coding proteins regulating viral DNA transcription ^[199]
E2	cell transformation, initiating and inhibiting apoptosis, transcriptional regulation, and in the modulation of the immortalizing and transformation potential of HPV ^[24] When inactive, it promotes E6 and E7 expression and influence tumor lesion development When active, it inhibits E6 and E7 transcription leading to increased p53 expression and apoptosis of infected cells ^[199]
E4	Affects the formation of the HPV-1 triggered nodules ^[24] may be involved in the cell cycle regulation ^[199]
E5	Transformation of viral DNA Viral DNA replication ^[24,199] Maintains the viral replication
E6	Synthesis of the genes via epithelium differentiation ^[200] Involved in HPV dependent malignant transformation via destructing the control of cell cycle regulation and cell maturation ^[199] Maintains the viral replication Contributes to the genetic instability of HPV-infected cells by interfering with the normal replication of centrosomes
E7	Synthesis of the genes via epithelium differentiation ^[200] Involved in HPV dependent malignant transformation via destructing the control of cell cycle regulation and cell maturation ^[199]

HPV: human papillomavirus

may also be associated with human cancers. The current understanding indicated that HPVs infect cells found in germ layers of the skin and mucous membranes, keratinocyte or cells with differentiation potential of keratinocyte. The mechanism of HPV infection is suspected to be similar among different tissues; in such the HPV infects the basal layer of the cervix causing exposure of the basement membrane, and HPV enters the basal layer of the tonsillar epithelium infecting and exposing the crypt cells.^[22,23]

TRANSMISSION OF HPV

The most common sexually transmitted infection is presumed to be the HPV infection. HPV infection can be transmitted via both sexual and nonsexual contacts. HPVs penetrate the body through the skin and epidermis injuries, mucous membranes and skin abrasions.^[24] Genital types of HPVs are mostly transmitted sexually. Generally in women, epidemiological studies have shown that the HPV infection is associated with the number of sexual partners, initial age of sexual intercourse and the likelihood of one of the sexual partners with an HPV infection.^[25,26] Therefore for HPV associated cancers, such as cervical, penile or urethra, the sexual partner plays a key role as much as the individual's own sexual behaviour.^[25,27]

More rarely, HPVs can be transmitted via perinatal transmission during birth from the mother to child that is also observed in the transmission of other microbial and viral infections.^[28,29] Horizontal transmission of HPV is also possible and it was first reported with a 5 year old boy of HPV2 infection presented as warts on the hands and

anus of the child via genital-finger transmission.^[30]

IMMUNE RESPONSES TO HPV AND VACCINE-INDUCED PROTECTION

HPVs that cause persistent visible papillomas, especially at oral and genital sites, are the main concern for the individuals. It is known that under some circumstances the virus is cleared and although the underlying mechanism of the virus clearance is not well understood, the immune response, particularly T cells, seems to play the main role.^[31-33] Lesion persistency and progression are increased in both animals and humans with genetic, iatrogenic or acquired cell mediated immune deficiencies, such as in patients with severe combined immunodeficiency,^[34] in immunosuppressed organ recipient patients,^[35] in patients with epidermodysplasia verruciformis^[35] and sun-exposed sites of patients with non-melanoma skin cancer.^[35-37] Moreover, HPVs can escape the immune system and down regulate the innate immune signalling pathways.^[38]

The clearance of high risk HPV types are believed to be harder since these types weaken the immune defences causing infection to continue and progress to neoplasias. However, it should be noted that progression from infections to cancer is a rare event and the first defence against HPV is the natural immunity. High risk HPV types are believed to destabilize the immune responses via obstructing the interferon pathway, down regulating major histocompatibility complex class I genes and changing the antigen production.^[39] High risk HPV types continue to express the E6 and E7 oncoproteins that leads to genomic

aberrations and malignancies. Furthermore, differences in cell tropism and disease progression patterns are believed to be one of the reasons of higher cancer association with certain HPV types, such as higher association of HPV18 with adenocarcinoma and in cervical intraepithelial neoplasias grade 2 (CIN2). The high risk HPV types causing adenocarcinomas may infect cells that already have a potential glandular differentiation.^[40] Therefore abortive or semipermisive infection of these cells may play an important role in the adenocarcinoma development. Recently, *in silico* models and epidemiological studies showed that the immune response may only contribute less than 20% of HPV clearance in individuals with normal immunity.^[41] Ryser and colleagues (2015) further proposed that the virus is mainly cleared by stem cell divisions in immunocompromised individuals.^[41]

Overall balance between the positive and negative immune factors may vary and these may lead to clearance of lesions. Therefore, therapeutic vaccines against HPV infections may play a strong role in prevention HPV associated lesions and cancers.^[42] In 2006, the Food and Drug Administration approved the use of recombinant quadrivalent HPV vaccine gardasil for protection against HPV6, HPV11, HPV16 and HPV18 L1 proteins in females in the age between 9 and 26 years old.^[43] It is proposed that in three doses of this vaccination at 0, 1 to 2 and 6 months, the HPV associated genital warts and the cervical cancer can be prevented.^[44] This vaccination is also proposed to protect against the vulvar and vaginal cancers as well as intraepithelial neoplasias.^[45] In 2009, the bivalent vaccine against HPV16 and HPV18 was licensed^[46] and this vaccine is intended to protect against anogenital warts, precancerous lesions and cervical cancer.^[45] Both the bivalent and quadrivalent HPV vaccines have been actively used in more than 80 countries.^[47] Both of the vaccines are shown to be safe, having enduring protection against primary infection and stable protection.^[48] These vaccines have a moderate cross-protection against high risk HPV types, HPV31, 33, 45, 52 and 58.^[49,50] However, only 70% of cervical cancer cases can be avoided by using these vaccines.^[51] Quadrivalent vaccines also protects against low risk HPV types, HPV6 and HPV11 that causes 90% of genital warts.^[43] The development of these vaccinations has brought a new era in the prevention of HPV and these vaccinations are great promise; however there is still room for much more development. In general, therapeutic vaccines have been proposed but only few of them reached clinical trials. The current vaccinations do not protect against all the HPV types and the cost of these vaccinations make them impossible to be used in some parts of the world, especially in newly developing countries. Therefore, although vaccinations enabled a tremendous step towards prevention of HPV associated diseases, more feasible and affordable vaccinations with protection against all the HPV types are required.

GLOBAL BURDEN OF HPV IN CANCERS AND DISEASES: PREVALENCE AND ROLE OF HPV

The highest HPV prevalence is observed to be 24% in Saharan Africa, 21% in Eastern Europe and 16% in Latin America.^[52] In majority of the populations, the highest prevalence of HPV is observed in women younger than 25 years. The prevalence reduces in older women with some having an increased rate in pre- or early-menopause. Although these prevalences are observed for many populations, in some others like China, the HPV prevalence is age-independent. On the other hand, HPV prevalence remains to be at a constant rate across all age groups in countries like Asia and Africa.^[53] The reason of different prevalences observed in different populations worldwide are not very well understood, but it is possible that it varies due to the age of initial sexual activity, the number of partners and the habits of the sexual activities.

Different HPV genera cause both non-cancerous and cancerous diseases. Formation of warts on the skin and uretra, mucous membranes of the oral cavity, respiratory tract, throat and genitals have been associated with HPV infections. Current data indicates that the prevalences of the genital HPV infections are considerably higher compared to the oral HPV. Globally HPV infections are associated with approximately 50% of HPV caused cancers in women and 5% in men.^[54] Different carcinogenesis is detected at different anatomical sites and at different level that is most likely because of the differences in the expression of the viral genome, in such HPV associated genital tract infections are observed at higher incidence compared to the head and neck cancer incidence. Genital HPV infections are connected with more than 99% of cervical cancers,^[55] 97% of anal cancer,^[56] 70% of vaginal cancers,^[57] 47% of penile cancers,^[58] 40% of vulval cancers,^[57] 47% of oropharynx cancers and 11% of oral cavity cancer cases.^[59]

ROLE OF HPV IN CANCER DEVELOPMENT

The mechanism of cancer progression in patients with HPV infection is not well established. However, there are a number of hypothesis on the possible routes of HPV in cancer progression. One of the hypotheses suggests that the cancer progression is associated with the increased accessibility and proliferation of the basal layers at the metaplastic epithelial site and therefore this increases the risk of metastasis. This becomes even more apparent at the puberty time and the onset of sexual activity.^[60]

The initial infection of the cell and the relation of this to the disease outcome are not well understood. Generally HPV infection causes cell destruction as well as cell transformation and tumour development. HPVs interfere with cell cycle regulation and prevent apoptosis in cells

with unscheduled DNA replication. It is possible that HPV infection mainly affects the cells located near the squamocolumnar junctions that form the stratified epithelial layers of the transformation zone as the cervix matures, such as the epithelial reserve cells.^[61,62] It is believed that the formation of the lesion starts with the infection of the basal stem cell and the formation of a persistent lesion depends on the longevity of the stem cell.^[6,63,64] This hypothesis is especially convincing for the low-risk HPV types since they do not usually lead to neoplasia and do not particularly stimulate the basal cell proliferation. The viral replication proteins E1 and E2 may play a role in the amplification of the viral genome.^[63,65,66] One of the hypotheses suggests that E2 may be possibly involved in genome partitioning where the viral transcription is regulated by E2.^[67] A viral DNA helicase, such as E1, may separate the viral DNA replication from cellular DNA replication during establishment and amplification of the genome.^[6,68] Of all the HPV proteins, E6 and E7 are the key ones associated in cancers via eliminating the tumour suppressors p53 and Rb leading to anti-apoptosis, genetic instability and formation of skin or mucosa lesions.^[22,23,69] In low-risk HPV types, the wound healing process may hold an important role in the initial proliferation of the infected cells.^[70] For the high-risk HPV types, viral proteins E6 and E7 function in the cell proliferation in the basal and parabasal cell layers. This function is particularly important at cervical sites where neoplasias may occur.^[6] The functions of viral proteins E6 and E7 vary between the high and low-risk HPV types and these are associated with different pathologies.^[71] The low risk HPV E6 and E7 proteins cause weak transformation or no transformation at all. RB1 is targeted and degraded by the high risk HPV E7 proteins, whereas E6 proteins target TP53 and stimulate telomerase (TERT). Telomerase activation is a fundamental stage for the high risk HPV type mediated cell immortalization *in vitro*.^[72] However, more studies involving animal models are required to understand the HPV integration *in vivo*. On the contrary, even though the low risk HPV E7 proteins bind to RB1, it is not involved in the degradation. Low risk E6 does not bind to TP53 and it does not stimulate TERT.^[73] The mechanism of oncogenesis associated with HPV is proposed to be through p16-INK4a expression. High risk HPV E7 triggers p16-INK4a through KDM6B histone demethylase causing p16-INK4a mediated CDK4/6 inhibition and RB1 mediated cell cycle arrest and senescence.^[74-76] More aberrations including abnormal number of centromeres, multipolar mitotic spindles, chromosome lagging and anaphase bridges are also observed in cells expressing HPV16 E6 and E7 genes.^[77] These aberrations may occur in cells with HPV infection at the early stages, but they can be easily detected in invasive cancers. Therefore, these abnormalities that originates during mitosis increases the risk of mutation accumulation that may cause malignant transformation *in vitro*. One of these aberrations is the allelic loss, such as losses in 3p and 10p that are associated with telomerase activation.

LOWER GENITAL TRACT NEOPLASIAS: CERVICAL, VAGINAL AND VULVAR CANCER

Neoplasias of the genital tract includes cervical (CIN), vaginal and vulvar intraepithelial neoplasias and a fraction of these neoplasias progresses to invasive cancers. HPV infection is detected in almost all cervical, half of the vulvar and approximately 70% of vaginal tumors.^[78]

The organisation of the life cycle of HPVs in the development of lower genital tract neoplasias is well established.^[79-82] Retrospective studies have reported that almost all the women with cervical cancers are infected with HPV and in the more severe cases, that are squamous cell carcinomas, HPV16 is the most prevalent type observed in 90% of the cases.^[40,52,83,84] Ten percent of the cervical cancers are adenocarcinomas that are mostly caused by HPV infections.^[40] Women with HPV16 (61%) and HPV18 (10%) were shown to have 200 fold higher risks for the development of cervical cancers.^[1,85] The prevalence of other HPV types are less observed in cervical cancer cases, in such HPV45 was observed in 6%, HPV31 in 4%, HPV52 in 3%, HPV35 in 2% and HPV58 in 2% of cervical cancer cases.^[86]

The risk factors for cervical cancers follow the similar parameters for the general HPV infection risks, such as high parity (more than 4 vaginal deliveries), full term pregnancy at earlier age (18 years old or earlier) and use of hormonal oral contraceptives.^[83,87] Progression of the cervical cancer can be affected by several factors including coinfection with other sexually transmitted infection, such as Chlamydia trachomatis, herpes simplex virus, HIV or tobacco smoking and immune suppression.^[55,83] Therefore, counselling adolescents at earlier age for avoiding tobacco use, initiation of sexual intercourse and limiting the number of partners may help to reduce the cervical cancer.

The HPV proteins E6 and E7 are proposed to play a role in the pathogenesis of HPV associated cervical cancers.^[88] The phenotype of the cervical neoplasia was suggested to vary depending on the expression levels of E6 and E7 were suggested to increase from cervical intraepithelial neoplasia grade 1 to 3 (CIN1 to CIN3). These interactions of HPV proteins with cellular pathways of the host cell will give a chance for potential targets for HPV based cancer treatment strategies. Additionally, E2 gene is also believed to take a part in cervical cancer since in about 35% of HPV induced cervical cancers full length viral genomes are expressed.^[89,90] The regulation of gene expression is changed when the viral DNA integrates with the cell chromosomes. This integration leads to a continuous expression of E6 and E7 proteins causing accumulation of mutations of the cellular DNA and promoting malignancies.^[77,91] These accumulations of mutations, mostly monosomies, trisomies, structural changes, chromatid gaps and breaks and double minutes,

are often detected in cervical cancers as well as other epithelial tumors.

The underlying mechanism of the progression from CIN1 through CIN2, CIN3 and eventually cancer is not well established, it may be due to the early integration events in CIN1 or due to deregulation of viral gene expression. It is also possible that the initial deregulation leads to instability of chromosomes and causes integration. It is believed that the integration arises in high grade lesions, such as CIN2 and CIN3 and the deregulation of E6 and E7 expression may increase or remain at a constitute level.^[92,93] In this scheme, flat warts can be resembled in CIN1 lesions, however the proliferation level of the cell is lower in the basal and parabasal layers.^[113] Increased expression levels of E6 and E7 in high-risk HPV type infections causes CIN2+ phenotypes. This phenotype leads genetic changes that contribute to cancer progression. These suggest that low expression levels of E6 and E7 does not affect the function of the cellular targets in CIN1 and therefore does not contribute to cancer progression. In CIN2/ CIN3+, the viral deregulation assists the viral episome into the host cell chromosome. This may further cause deregulation of E6 and E7 expression. In clinical vaccine trials it was shown that young women can have CIN2+ soon after infection^[94-97] for these cases, it is possible that deregulation of the gene expression is due to cell signaling changes^[98] or epigenetic modifications, such as viral DNA methylation.^[99]

An important step has been taken towards prevention of HPV induced cervical cancers with the use of vaccines against HPV. However, due to various reasons, including the unavailability of the vaccines in certain regions of the world or the high costs of the vaccines, the wide application of the vaccines is not available. Therefore, in case of cervical cancer development, early detection strategies and treatment play a vital role to prevent any deaths. The treatment for the early cervical cancers is usually performed by conisation or radical hysterectomy. For the more advanced tumors, cisplatin based chemo-radiotherapy is preferred that results in 65-80% survival rates. Surgical excisions are usually the standard for the HPV associated anogenital lesions.^[100] The treatment strategy for CIN is to eliminate the abnormal HPV infected precancerous cells and maintain the cervical integrity. One of the most commonly used treatments for CIN involves loop electrosurgical excision procedure, electrofulgaration and cryotherapy.^[101]

The other lower genital cancers include vulvar and vaginal cancers. Majority of the vulvar and vaginal cancers are squamous cell carcinomas.^[57] In majority of the cancers of the vagina HPV DNA is detected; approximately half of the vaginal cancers are caused by HPV16 (54%) followed by HPV18 (8%).^[57] Similarly, HPV DNA is detected in most of the vulvar intraepithelial neoplasia, however only half of these neoplasias causes cancer. HPV16 is associated with 32% and HPV18 with 4% of the cases.^[57,102-104] Therefore,

although HPV may play a role in vulvar cancer, this association is not clear.

BREAST CANCER

Several epidemiological studies reported HPV detection in breast cancer samples.^[105-109] Nevertheless the role of HPV in breast carcinogenesis is by far not certain and further randomized control trials are required to establish the definite role of HPV in breast cancer development.

HEAD AND NECK CARCINOMAS

Head and neck carcinomas involve a wide range of tumors and is one of the most common cancers worldwide.^[110] The prevalence of HPV DNA in head and neck cancers depends on the cancer site, geography and ethnicity.^[104] The most consistent prevalence of HPV infection is the oropharyngeal cancers with an association of 35-50% in developed cancers, whereas the HPV is detected in approximately 5-15% within the rest of the oral cavity.^[52,84] The overall risk factors for head and neck carcinomas include tobacco smoking and alcohol consumption.

The first cases of HPV relationships with oral cell squamous cell carcinomas were reported in 2008 for lingual cancer, tonsil cancer and oropharyngeal cancers.^[111,112] Overall the prevalence of these cancers are higher in men compared to women.^[113] Oropharyngeal carcinomas (OPCs) are the most studied and the most characterised type of head and neck carcinomas. In the last decade the incidence of HPV related OPCs have doubled in number of patients and therefore more attention has drawn to these cancer types.^[114] HPV positive oropharyngeal cancers are mainly associated with oral sex and rare p53 mutation.^[115] Interestingly HPV infection was shown to improve the prognosis of OPC with better survival is reported in HPV positive OPCs^[116] and therefore these patients may have a chance to benefit from a less intense treatment strategy.^[117] Chemotherapy using paclitaxel, cisplatin on centuximab; followed by concurrent radiation has been used in treatment of OPC patients.^[118] With the increasing number of HPV associated OPC patients, the use of antiviral and immunotherapeutic strategies show an improved outcome.^[42] Although HPV related OPC have increased through the years, the HPV negative OPCs still account for the majority of the OPC patients.

The HPVs, mostly HPV16 and HPV33, were detected in quarter of the patients with invasive laryngeal cancers and are predominantly detected in women compared to men.^[119-121] HPV is also associated with potential malignant disorders, such as erythroplakia, oral leukoplakia and oral lichen planus.^[122] Erythroplakia has the highest risk of malignant transformation. Half of the cases with erythroplakias alone is associated with HPV infection^[123] and the frequency of the HPV detection influences the severity of the lesions. In one study the HPV prevalence was 32.8% in oral lichen planus, 40.9% in oral leukoplakia

and 47.7% in oral squamous cell carcinomas.^[124] Oral leukoplakia is associated with HPV6, HPV11 and HPV16 and these may lead to malignant oral diseases.^[125-127] Similarly, HPV is detected more often with increased prevalence in oral lichen planus.^[128]

The overall prognosis of head and neck squamous cell carcinomas seems to be better with HPV infected patients. Young individuals appear to have increased risk of having HPV positive tonsillar and oropharyngeal carcinomas^[129,130] with better prognosis and lower relapse risks compared to HPV negative head and neck squamous cell carcinoma (HNSCC) patients.^[131] Approximately 6% prevalence was reported for HPV positive OSCCs.^[132] However, more than half of the patients with HNSCC (57%) were shown to have metastases to the brain where all are HPV positive.^[133]

LUNG CANCER

Lung cancer is one the foremost causes of cancer associated deaths worldwide. Although cigarette smoking plays a crucial role in lung cancer development, less than 20% of the smokers have lung cancer.^[134] Therefore, other factors including inactivation of tumour suppressor genes, such as p53, Rb and p16, and HPV infection have been proposed to be involved in the development of lung carcinogenesis.^[134,135] The possible role of HPV in lung cancer was initially proposed due to the similarities of the morphological epithelial changes detected in bronchial carcinomas with genital HPV lesions.^[136,137] HPV detection in lung cancer was confirmed in 1988^[138] and the association of HPV with lung cancer was then verified by detection of HPV DNA in lung cancer samples.^[139,140] However, the issue is debated and controversial studies have been reported.^[141,142] Some groups reported that E7 proteins of high risk HPV16 and HPV18 are detected,^[143,144] some reported that none of the HPV types are present in non-small lung cancer.^[145] An international pooled analysis of HPV association with lung cancers revealed that HPV DNA is present but in a very small number of lung tumors.^[146] Therefore, the direct relevance of lung cancer with HPV requires further analysis. A recent meta-analysis data showed that HPV infection has a strong relationship with lung cancer with significantly increased risk of lung squamous cell carcinoma upon HPV16 and HPV18 infection and in this meta-analysis, it is proposed that the HPV vaccination may lower the lung cancer risk.^[147]

Respiratory papillomatosis (RRP) is a serious condition that may spread to lungs and can progress to cancer.^[148,149] Patients with RRP have an increased risk of developing laryngeal neoplasias and carcinomas.^[150] RRP is mainly caused by the alpha-HPVs, HPV6 and/or HPV11.^[151] The transmission of upper respiratory tract infections may be passed on by sexual contact and from mother to child during child birth canal.^[4,152] Although many therapies have applied for RRP patients, such as surgical, treatment with antivirals

and chemotherapeutic drugs; there is limited success with mostly side effects.^[153] Therefore like all the other cancers, early detection and vaccines can play a crucial role in RRP. Although the present HPV vaccines protect against HPV 11, there is the need for development of vaccines for other HPV types, especially HPV6 for the prevention of RRP.

BLADDER CANCER

The first association of HPV and bladder tumors was reported in 1988.^[154] The prevalence of HPV infection in bladder carcinomas ranges from 0% to 81%.^[155-159] Overall, the involvement of bladder cancer with HPV is controversial. Although some studies reported a positive correlation between HPV infection through contribution of E6 and E7 oncogenic proteins,^[160-163] some reported no association between HPV infected bladder carcinoma.^[164,165] Furthermore, p16-INK4a was reported to be involved in the development of bladder cancer through suppressing the inactivation of Rb protein association with HPV infected bladder carcinoma.^[163,166,167] The controversy continues with the inverted papiloma of the urinary tract and urothelial carcinomas. In some reports HPV is associated with inverted papilloma of the urinary bladder^[168] and urothelial carcinomas,^[167,169] but in the others no association was reported.^[170,171]

HPVs, especially HPV16 and HPV18, were detected mostly in low grade (grade 1) tumours and never have they been reported for grade 3 carcinomas.^[163,167,172-175] Therefore potentially HPV is only associated with low grade carcinomas.

PENILE CARCINOMA AND ANAL CARCINOMA

Penile carcinomas mainly originate in the squamous mucosa of the glans, coronal sulcus or inner surface of the foreskin of the penile. Penile cancers are rare and they usually occur in uncircumcised men.^[176] About half (40-50%) of the penile squamous cell carcinomas are related to the high risk HPV infection^[52,177-180] and mostly the basaloid and warty types of penile cancers are consistently related to HPV infection, whereas HPV DNA was only detected in some of the keratinizing and verrucous penile carcinomas.^[179] Mainly HPV16 (69%) and HPV18 (13%) play a role in the development of penile squamous cell carcinomas.^[57] High risk HPV types, generally HPV16 and HPV18, are detected in Bowenoid papulosis, which resemble genital warts but with high grade squamous cell carcinoma *in situ*, can be found on the external genitalia, perineum or perinally.^[181] HPV16 and HPV18 are also associated with Erythroplasia Queyrat, which is *in situ* carcinoma of the penile mucosa. This carcinoma can also be present on the urethra, vulva, tongue and oral mucosa. Buschke-Löwenstein tumors, which cause destruction of the underlying tissues leading to transformation into squamous cell carcinoma and are

located on the penile glans, prepuce, vulva, vagina and perianal sites, are also associated with low risk HPVs, HPV6 and HPV 11.^[182,183] Additionally, in both males and females, approximately 85-95% of the anal cancers are HPV DNA positive.^[52,104] Of these, HPV16 (75%) and HPV18 (3%) are the causes for almost all the cases of anal cancers.^[56,184]

SKIN CANCER

Similar to the head and neck, bladder and breast cancers, the involvement of HPV in cutaneous squamous cell carcinoma has not been surely established. A range of nonmelanoma skin cancer forms contain DNA from beta HPV types.^[185] HPV induced skin cancers include cutaneous squamous cell carcinoma and superficial squamous cell carcinoma, such as Bowen's disease.^[186] Approximately 30% individuals with infection develop invasive squamous cell carcinomas with 90% of these tumors correlated with HPV5 and HPV8.^[36,187] Genetic susceptibility to HPV is demonstrated with epidermodysplasia verruciformis; however, HPV infection alone is not enough to develop cancerogenesis in epidermodysplasia verruciformis.^[42] Mainly, these tumors are induced by sun explosion and ultraviolet radiation. Cells with HPV5 and HPV8 E6 proteins disturb DNA double strand break repair^[188] and reduces the efficiency of base excision repair pathway^[189] causing higher sensitivity to UV-B exposure. It may be possible that because of impaired DNA repair activity, patients with acquired immunodeficiency syndrome or patients with epidermodysplasia verruciformis are more subjected for the infections and at a higher risk of developing HPV associated cutaneous malignancies.^[185,190,191] In order to reduce the prevalence of HPV induced skin cancers, diagnosis of skin manifestations caused by HPV should be routinely checked.^[186]

ROLE OF HPV IN NON-CANCEROUS DISEASES

One of the most common non-oncogenic HPV diseases involves genital warts and the clinical manifestations extend from flat and common warts and cauliflower like or filiform warts.^[186] The genital warts are mostly common in younger people with the age of less than 25 years old and the transmission is more than 60% with an incubation time between 2 to 8 months.^[192] Various clinical presentations are observed when keratinocytes respond to the HPV infection depending on the HPV type and the anatomical site. Genital warts are mainly associated with HPV6 and HPV11. Although mainly low risk HPV types, HPV6 (89%) and HPV11 (11%),^[193] both high and low risk HPV types may cause genital warts.^[194] Bowenoid papulosis is described by several flat patches in genital area. Similarly, condylomata plana are flat warts that have been associated with HPV infection.^[195] Recurrence of genital warts with progression of lesions even after 3 months are reported in one-third of individuals with presence of genital warts.^[196]

Genital warts can be found on penile shaft, base of the penis, scrotum, pubic region, glans and rectal area. In women, they are mostly present in the labia minora and vaginal opening.^[197] In the decision of the therapy strategy, many factors, such as morphology of the lesion, HPV classification and immune competent status, are taken into account. Unfortunately, none of the treatment strategies, including targeted lesion destruction or immunologic modification, are shown to clear the HPV infection or avoid the recurrence. With the use of HPV vaccines, the incidence of the warts has been decreased.^[198] If these warts remain untreated, they can either regress spontaneously or they can grow larger and become more numerous resulting in complicated cases.^[192] Therefore, prevention HPV infection and therefore formation of these warts will be the optimum goal.

CONCLUSION

In the recent years, the biology of HPV infection and its role in the progress of cancer have been widely evaluated. All the data discussed in this review point out the significance of HPV infection in several benign and malignant diseases. Although the understanding of association of HPVs with cervical cancer is very well established further studies are required to analyse the relationships between HPV and certain cancers including breast, lung, bladder, some types of head and neck cancers and penile cancers.

To improve the mortality and morbidity of HPV associated cancers and diseases, there is an enormous need for early detection and prevention strategies. Although screening programs for early detection strategies have been developed for some cancers, such as cervical, there is still a big gap to be filled for other precancerous lesions, such as for some of the head and neck carcinomas. One of the examples of these screening strategies may involve oral examination, cytology and salivary HPV DNA tests which may provide a better early diagnosis for oral and oro-pharyngeal cancers. Moreover development and spread of more cost-effective vaccines is mandatory. Availability of low cost screening may prevent the future generations to develop HPVs induced cancers. In light of this knowledge, HPV vaccines are useful in the protection against cervical, oral and oro-pharyngeal cancers. However, it should be kept in mind that the current HPV vaccines do not protect against all HPV types, particularly beta-HPV types and their associated diseases. Therefore, despite all these advances, other strategies for early detection and prevention for different HPV types are required.

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Conflicts of interest

There are no conflicts of interest.

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Changing paradigm in treatment of lung cancer

Sundaram Viswanath, Abhishek Pathak, Amul Kapoor, Anvesh Rathore, Bhupendra Nath Kapur

Medical Oncology, Army Hospital Research and Referral, New Delhi 110010, India.

Correspondence to: Dr. Abhishek Pathak, Medical Oncology, Army Hospital Research and Referral, New Delhi 110010, India.
E-mail: drabhipat@gmail.com

ABSTRACT

Lung cancer is one of the most common and deadliest forms of cancer. It accounts for 13% of all new cancer cases and 19% of cancer-related deaths. In India, lung cancer constitutes 6.9% of all new cancer cases and 9.3% of all cancer cases. There has also been a dramatic rise worldwide in both the absolute and relative frequencies of lung cancer occurrence. In 1953 it became the most common cause of cancer mortality in men. By 1985, it became the leading cause of cancer deaths in women, causing almost twice as many deaths as breast cancer. The demographic profile of lung cancer has changed greatly over the years; however, methods for diagnosing, screening, and managing lung cancer patients have improved. This is due to our growing understanding of the biology of lung cancer. It is now possible to further define lung cancer types beyond small cell lung carcinoma and non-small cell lung carcinoma. Moreover, new histology-based therapeutic modalities have been developed, and more new lung cancer biomarkers have been uncovered. Therefore, more detailed histological characterization of lung cancer samples is warranted in order to determine the best course of treatment for specific patients. This review article describes how these new molecular technologies are shaping the way lung cancer can be treated in future.

Key words: Non-small cell lung carcinoma; epidermal growth factor receptor; anaplastic lymphoma kinase

INTRODUCTION

Lung cancer is one of the most common and deadliest forms of cancer. Worldwide, it accounts for 13% of all new cancer cases and 19% of cancer-related deaths. In India alone, lung cancer constitutes 6.9% of all new cancer cases and 9.3% of all cancer-related deaths. There has been a dramatic rise worldwide in both the absolute and relative frequencies of lung cancer occurrence.^[1] By 1953, lung cancer was the most common cause of cancer mortality in men. By 1985, it was the leading cause of cancer deaths in women, causing almost twice as many deaths as breast cancer.^[2]

The demographic profile of lung cancer has changed greatly over the years; however, methods for diagnosing, screening and managing lung cancer patients have also improved. This is due to our growing understanding of the biology of lung cancer. It is now possible to further define lung cancer types beyond small cell lung carcinoma (SCLC) and non-small cell lung carcinoma (NSCLC). In 2012, the Cancer Genome Atlas (TCGA) Research Network published in Nature that characterized the lung squamous cell carcinoma

genome. The researchers found a large number and variety of DNA alterations, many of which seem to be the driving force behind the initiation and progression of lung cancer. TCGA is jointly funded and managed by the National Human Genome Research Institute (NHGRI) and the National Cancer Institute (NCI), both of which are part of the National Institutes of Health. New histology-based therapeutic modalities have been developed, and more new lung cancer biomarkers have been uncovered. As a result, more detailed histological characterization of lung cancer samples is warranted in order to determine the best course of treatment for specific patients.^[3]

For NSCLC, there are currently more than 50% of adenocarcinoma cases and around 15-20% of squamous cell carcinoma cases that need to be further characterized based on mutation analysis. Mutations in epidermal growth factor receptor (EGFR) gene strongly predict the efficacy of EGFR inhibitors, with response rates of over 70% in patients who have EGFR mutations.^[4] In

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two randomized phase III studies, the Iressa Pan-Asia Study (IPAS) and WJTOG3405, the use of gefitinib as the first-line treatment for previously untreated metastatic adenocarcinoma of the lung leads to longer progression-free survival (PFS) in patients with tumors positive for EGFR mutations, compared to platinum-based doublet chemotherapy.^[5] Similar outcomes have been observed for erlotinib in advanced NSCLC patients with *EGFR* mutations.^[6] These findings have important implications to lung cancer treatment regimes in India, where EGFR mutations have been shown to occur in 25-50% of lung cancer cases.^[7] However, the purpose of mutational studies in adenocarcinoma and squamous cell carcinoma can be very different. Researchers have made important progress in the understanding and development of treatments for adenocarcinomas, which are the most common type of lung cancer. Unfortunately, these treatments have been largely ineffective in treating lung squamous cell carcinomas. Lung squamous cell carcinomas frequently develop in the large airways in the centre of the lungs, while adenocarcinomas often arise at the edges of the lungs. Lung adenocarcinomas sometimes affect non-smokers, while lung squamous cell carcinomas arise almost exclusively in smokers.

Another example of mutation-driven therapy is the targeting of the echinoderm microtubule-associated protein like 4-anaplastic lymphoma kinase (EML4-ALK) rearrangement. The ALK gene encodes anaplastic lymphoma kinase, a member of the receptor tyrosine kinase (RTK) family. RTKs transmit signals from the cell surface into the cell through a process called signal transduction. The EML-ALK fusion leads to uncontrolled cell proliferation. This mutation occurs in about 3-7% of unselected NSCLC.^[8] In one study, NSCLC patients treated with crizotinib, a tyrosine kinase inhibitor targeting ALK, showed a response rate of 65%.^[9] In cases where there was disease progression after treatment with crizotinib, ceritinib can be used. Ceritinib is a new ALK inhibitor that has been recently approved based on its encouraging response rate of 56% in patients whose cancer has progressed after treatment with crizotinib.

MOLECULAR TESTING

The most useful biomarkers for predicting the efficacy of targeted therapy in advanced NSCLC are somatic genome alterations known as driver mutations. These mutations occur in cancer cells in genes encoding proteins that are critical to cell growth and survival. Driver mutations are typically transformative, which means that they initiate the evolution of a non-cancerous cell to a cancerous one. In addition, driver mutations often impart an oncogene addiction trait to the transformed cell. This means that the mutated protein enables the cancer cell to receive survival signals from the driver mutations. Hence, driver mutations are good biomarkers for selecting patients for targeted therapies. Whenever feasible, patients with

advanced NSCLC should have their tumours assessed for the presence of driver mutations.^[10] Guidelines by the College of American Pathologists (CAP), the International Association for the Study of Lung Cancer (IASLC), and the Association of Molecular Pathologists (AMP) recommend analysis of either the primary tumour or of a metastasis for EGFR and ALK mutations for all patients with tumours that exhibit features of an adenocarcinoma, regardless of their clinical characteristics.^[11]

The most important requirements for molecular testing modalities are that they should utilise clinically available specimens (formalin- or paraffin-embedded tissue) and that the turnaround time should be relatively short. The instrument should be semi-automated and relatively inexpensive. The most commonly used modalities are: (1) gene sequencing, the most comprehensive method for mutation testing; (2) next-generation sequencing, which uses simultaneous evaluation of multiple genes or even whole genomes; (3) allele-specific testing, which analyzes DNA for a predefined abnormality; (4) mass spectrometry, which analyzes short fragments of DNA by their mass to charge ratio and can detect fragments that have different molecular weights than expected, a mutation; (5) fluorescence *in situ* hybridisation (FISH), which is typically used to detect gene translocations, amplifications, and other rearrangements; (6) immunohistochemistry (IHC), which is considered an alternative to FISH for determining ALK translocations; and (7) multiplex genotype testing, which allows an entire panel of genotypes of interest to be queried at a single time from a single tissue sample instead of doing the tests sequentially. IHC is, however, not currently recommended for detecting EGFR driver mutations since positive or negative IHC results do not necessarily indicate the presence or absence, respectively, of an EGFR mutation. In contrast, multiplex genotype testing is the most tissue-efficient approach, particularly when dealing with small tumour samples.

Mutations associated

The identification of oncogenic activation of particular tyrosine kinases in some advanced NSCLC tumours, most notably mutations in the EGFR gene or rearrangements in of the ALK gene, has led to a paradigm shift and the development of specific molecular treatments for patients.

EGFR mutations

EGFR is a transmembrane protein with cytoplasmic kinase activity that transduces growth signals to the cell. Among Asians, the incidence of EGFR mutation is much higher, up to 62%, and occurs more frequently among non-smokers. In advanced NSCLC, the presence of an EGFR mutation confers a favourable prognosis and is strongly indicative of sensitivity to EGFR tyrosine kinase inhibitors such as erlotinib, gefitinib, and afatinib.

Nevertheless, it has been observed that most of the patients who initially respond to an EGFR tyrosine kinase inhibitor

subsequently experience a recurrence. How this acquired resistance towards tyrosine kinase inhibitors occurs is not fully understood, but secondary mutations in EGFR and amplification of the oncogene MET are common in these patients. The most common secondary EGFR mutation is the substitution of methionine for threonine at position 790 (T790M).^[12] Another characteristic of the acquired resistance is the amplification of the MET oncogene, which is detected in 5-20% of patients with progressive disease while being treated with either erlotinib or gefitinib.^[13] In some cases, only MET amplification is present, while in others, amplification and the secondary T790M mutation in EGFR are present. The absence of the MET oncogene amplification may be indicative of improved survival in patients with surgically resected NSCLC.^[14]

ALK translocation

Translocations involving ALK are present in approximately 4% of NSCLC adenocarcinomas in the United States, and occur more frequently in non-smokers and younger patients. In advanced NSCLC, the presence of an ALK translocation indicates sensitivity to ALK tyrosine kinase inhibitors such as crizotinib and ceritinib, and treatment with these agents significantly prolongs progression-free survival.

RAS mutations

Approximately 15-25% of patients with lung adenocarcinoma have oncogenic KRAS mutations. The RAS family of proteins is a central mediator for the mitogen-activated protein kinase (MAPK), signal transducer and activator of transcription (STAT), and phosphoinositide 3-kinase (PI3K) signalling pathways, which work together to control cell proliferation and apoptosis. The most common RAS mutation are missense substitutions in codons 12, 13, or 61. These mutations result in a constitutively active RAS due to malfunctioning of the RAS GTPase.

ROS1 translocation

ROS1 translocation is associated with adenocarcinoma histology, and is typically observed in younger patients and those who have never smoked. ROS1 is a RTK of the insulin receptor family. For these patients, first-line management with crizotinib is recommended, instead of platinum-based chemotherapy (Grade 1B). For patients who have received prior chemotherapy, treatment with crizotinib the preferred second-line chemotherapy (Grade 1A).^[15]

HER2 mutation

HER2 (ERBB2) encodes a RTK from the EGFR family. Mutations in HER2 have been detected in approximately 1-2% of NSCLC tumours, primarily adenocarcinomas. For patients with a HER2 exon 20 insertion mutation, a second-line targeted therapy with either afatinib monotherapy or trastuzumab in combination with single-agent chemotherapy (vinorelbine or docetaxel) is recommended, rather than single-agent chemotherapy alone (Grade 2C).^[16]

BRAF mutation

BRAF encodes a downstream signalling mediator of KRAS. The BRAF protein activates the MAPK pathway. BRAF mutations have been observed in 1-3% of NSCLC with adenocarcinoma variant and are usually associated with a history of smoking.^[17] For patients with a BRAF V600E mutation, treatment with BRAF inhibitors such as (dabrafenib and vemurafenib, or dabrafenib plus trametinib to inhibit the MAPK pathway is recommended, rather than single-agent chemotherapy.

MET abnormalities

Generally smoking-related and identified via IHC in 25-50% of NSCLC specimens, MET expression also appears to be associated with a more severe prognosis.^[18] MET encodes a RTK for hepatocyte growth factor (HGF). Abnormalities include overexpression due to gene amplification and splice site alterations at exon 14 of the gene. In such patients, treatment with a MET inhibitor (crizotinib or cabozantinib) is recommended rather than single-agent chemotherapy as second-line treatment.

RET translocation

The RET gene encodes a cell surface RTK that is frequently altered in medullary thyroid cancer. RET mutations are encountered in younger patients and non-smokers. For patients with RET rearrangements, treatment with a RET inhibitor such as cabozantinib or vandetanib is recommended rather than single-agent chemotherapy as second-line treatment (Grade 2C).^[18]

PIK3CA, AKT1, PTEN alterations

PIK3CA encodes the catalytic subunit of phosphatidylinositol 3-kinase (PI3K), which is an intracellular central mediator of cell survival signals. AKT1 acts immediately downstream of PI3K. PTEN dephosphorylates and subsequently inhibits AKT. Oncogenic alterations in this pathway include gain-of-function mutations in PIK3CA and AKT1, and loss of PTEN function. Alterations in the PI3K signalling pathway appear more frequently in patients who are smokers and with tumours of squamous histology. PIK3CA mutations also may promote resistance to EGFR inhibitors in EGFR-mutant NSCLC.^[19]

FGFR1 amplification

Fibroblast growth factor receptor-1 (FGFR1) is a cell surface RTK that mediates cell survival and proliferation. FGFR1 amplification has been detected in 13-25% of squamous tumours.^[20] FGFR1 amplification is associated with smoking and with worse overall survival.

CTNNB1 (β-catenin) mutation

The CTNNB1 gene encodes β-catenin, a protein important for the regulation of epithelial cell growth. Mutations in this gene have been observed in approximately 2% of NSCLC, particularly in tumours with secondary EGFR mutations following acquired resistance to EGFR inhibitors.^[21]

DDR2 mutation

The DDR2 gene encodes a cell surface RTK that is mutated to an active form in about 4% of squamous cell carcinomas of the lung.^[22] Dasatinib inhibits DDR2, and one patient treated with the combination of dasatinib and erlotinib had a tumour response. Clinical trials to determine dasatinib efficacy are underway.

MEK1 mutation

The MAP2K1 gene encodes the MEK1 protein, a central mediator of cell proliferation signals that is downstream of RAF in the MAPK pathway. MAP2K1 mutations may be found in approximately 1% of adenocarcinomas.^[23] The clinical response of NSCLC with MAP2K1 mutation to MEK or ERK inhibitors is currently being investigated.

The accessibility of mutation analysis is limited largely due to the high cost, as well as the lack of quality control, uniformity of techniques and standards among various laboratories. However, the cost for these tests may decrease when the reagents are purchased in bulk. The amount and quality of the tumour tissue used for molecular profiling is also an important issue to consider, especially since the tissue yield for lung cancer samples is limited by small core biopsies. Judicious use of IHC and conservation of samples for molecular testing would be helpful. Cell-free circular tumour DNA is also emerging as a useful tool for mutation testing and therapeutic monitoring.

IMMUNOTHERAPY

More than 80% of lung cancer cases are classified as NSCLC. Although there have been significant advances in the treatment of subsets of patients with molecularly defined NSCLC, for instance, NSCLC positive for EGFR mutation and ALK rearrangement, the improvement of prognosis is still modest for the majority of NSCLC patients. It is clear that a plateau has been reached with traditional chemotherapy, with minimal added benefit when chemotherapy is combined with the angiogenesis inhibitor bevacizumab.

Immunotherapeutic approaches are based on the premise that the immune system plays a key role in surveillance and the eradication of malignancy, and tumours evolve in order to elude the immune system. These approaches differ from traditional chemotherapy and targeted therapies that primarily target rapidly dividing cells and key molecular events that drive tumour growth and invasion. The goal of immunotherapy is to help the host's immune system recognize that cancer cells are foreign in order to stimulate immune response.

Historically, non-small cell lung cancer (NSCLC) was considered to be non-immunogenic. Two approaches to harness the immune system are of particular interest: immune checkpoint inhibition and vaccination.

IMMUNE ACTIVATION AND CHECKPOINT INHIBITION

Immune recognition is initiated by antigen presenting cells (APCs). When stimulated by antigens on cancer cells, APCs express B7-1 and B7-2 on their cell surface, and migrate to the lymph nodes to present the antigens to resting T cells. The B7 proteins bind to CD28 on the T cells, initiating a series of downstream signalling events that promotes the activation, survival and proliferation of the target T cells. These activated T cells then release cytolytic enzymes such as perforin and granzyme, as well as cytokines that help recruit other members of the immune system to the cancer cells. The result is tumour destruction and the creation of memory T cells. Several immune checkpoints exist to dampen the immune response to protect healthy individuals from detrimental inflammation and autoimmunity. Two well-characterized checkpoint proteins, the cytotoxic T-lymphocyte antigen 4 (CTLA-4) and the programmed death receptor 1 (PD-1), are targets in NSCLC clinical trials. The purpose of inhibiting these checkpoint proteins is to prevent their interference with the elimination of cancer cells.

Antibodies targeting CTLA-4: ipilimumab

Ipilimumab is an IgG1 CTLA-4 monoclonal antibody that prolongs overall survival in patients with metastatic melanoma. Currently there is a phase III trial that compares standard chemotherapy with carboplatin and paclitaxel with the same regimen combined with concurrent ipilimumab for patients undergoing chemotherapy for treating naive metastatic squamous cell NSCLC (NCT01285609).

Antibodies targeting PD-1 and PD-L1

Treatment of NSCLC with antibodies against PD-1 and programmed death-ligand 1 (PD-L1) has yielded encouraging results; early clinical trials showed a prolonged response to the antibody in patients with chemotherapy refractory metastatic NSCLC. Randomized phase III trials to evaluate anti-PD-1 and anti-PD-L1 antibodies for metastatic NSCLC treatment are in progress, and other studies are investigating various combination strategies.

Nivolumab is an IgG4 monoclonal antibody against PD-1 that has been approved for both advanced squamous cell carcinoma of the lung and unresectable or metastatic melanoma. Nivolumab received US Food and Drug Administration approval on March 4, 2015 for the treatment of patients with advanced squamous NSCLC with progression, either in concurrent with or after platinum-based chemotherapy. This approval was based on results from the CheckMate 017 and CheckMate 063 trials.

Pembrolizumab, an IgG4 monoclonal antibody also targeting PD-1, is a breakthrough therapeutic agent for treating advanced NSCLC that received FDA approval in late 2014. The approval was based on emerging results

from a large Phase I dose expansion trial that have since been updated.

PD-L1 expressed on cancer cells can bind to PD-1 on activated T cells to suppress the immune system.

MPDL3280A is an IgG1 monoclonal antibody against PD-L1 that targets cancer cells expressing PD-L1, thus preventing the interaction between PD-L1 and PD-1 expressed on activated T cells. It is also engineered to prevent antibody-dependent cell-mediated cytotoxicity (ADCC) and complement mediated cytotoxicity in activated T cells that may express PD-L1. Similarly, IgG1 monoclonal antibody MEDI4736 and IgG4 monoclonal BMS-936559 also target PD-L1. BMS-936559 is a fully human antibody that is an IgG1 monoclonal antibody to PDL1 with an engineered Fc domain to eliminate MEDI4736 effector function (i.e. complement mediated cytotoxicity and ADCC). BMS-936559 is a fully human IgG4 monoclonal antibody to PDL1 which has been evaluated in a dose escalation phase I trial with expansion cohorts in NSCLC, melanoma, and renal cell carcinoma.^[24]

Vaccination

Non-small cell lung cancers (NSCLCs) are characterized by several genetic alterations in neoantigens that can potentially be recognized by the immune system as foreign. Vaccination enhances the body's exposure to such antigens and immune cell priming. Randomized trials are currently focusing on approaches that couple tumour antigens or cells with immune adjuvant agents; such approaches may enhance the antigen presenting cell response to the vaccine.

One example is the melanoma associated antigen A3 (MAGE-A3) vaccine. The melanoma associated antigen A3 (MAGE-A3 gene family consists of "cancer germline" or "cancer testis" genes that are normally expressed only on testicular germ cells and placental trophoblasts.^[25] Several tumours also express MAGE-A3, including 30-50% of NSCLCs. The GSK1572932 vaccine is a recombinant MAGE-A3 protein vaccine combined with the immunological adjuvant AS15.

MUC-1 is a cell surface glycoprotein that is overexpressed and/or aberrantly glycosylated in several epithelioid malignancies, including NSCLC. Tecemotide is a vaccine consisting of the BLP25 MUC-1 lipopeptide and the adjuvant monophosphoryl lipid A, as well as cholesterol dimyristoyl phosphatidylglycerol (DMPG) and dipalmitoyl phosphatidylcholine (DPPC) as the carrier lipids that form the liposome. The primary endpoint of the trial evaluating this vaccine was overall survival. There was no significant increase in overall survival among 1239 patients receiving tecemotide compared to those receiving the placebo (median 25.8 and 22.3 months, respectively).

Belagenpumatucel-L is an allogeneic whole tumour vaccine consisting of cells from four irradiated NSCLC cell lines

modified using a TGF-beta antisense plasmid to block TGF-beta secretion. TGF-beta inhibits T and B cell activation, dendritic cell maturation and antigen presentation, as well as natural killer (NK) and lymphokine activated (LAK) activation. TGF-beta also induces immunosuppressive T regulatory cells.

In addition to the vaccines described above, there are several other types of vaccines that are currently being evaluated in phase III studies. More effort is being invested into developing new vaccines and combining vaccines with other immunologic agents, chemotherapy, or targeted agents. Advances in DNA and RNA sequencing as well as drug development may also ultimately enable the design of personalized vaccines consisting of antigens uniquely expressed by tumour cells from a specific patient.

CONCLUSION

Lung cancer is the leading cause of cancer-related mortality in the United States and worldwide. More than 80% of lung cancer cases are classified as NSCLC. In the past decade, there has been significant breakthrough in our understanding of the tumour biology of NSCLC. Signalling pathways that are vital for tumour growth have been identified and have been effectively targeted pharmacologically. This article summarizes the implications of these advances for treating lung cancer and highlights the ongoing work to improve clinical outcomes of this disease. Treatment of lung cancer has come a long way with greater use of molecular markers and targets. Nonetheless, there is still much to be done to help our fight against lung cancer.

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Department of Pathology, Army Hospital Research and Referral, New Delhi.

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Conflicts of interest

There are no conflicts of interest.

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Case Report

Osteonecrosis of the jaw in a patient with acute myeloid leukemia, who received azacitidine

Ourania Nicolatou-Galitis¹, Dimitra Galiti², Maria Moschogianni³, Sotirios Sachanas³, Beatrice J. Edwards⁴, Cesar A. Migliorati⁵, Gerassimos Pangalis⁶

¹Clinic of Hospital Dentistry, Dental Oncology Unit, Dental School, National and Kapodistrian University of Athens, 15451 Athens, Greece.

²Clinic of Oral Diagnosis and Radiology, Dental School, National and Kapodistrian University of Athens, 15451 Athens, Greece.

³Department of Hematology, Athens Medical Center-Psychikon Branch, 15451 Athens, Greece.

⁴Geriatric Medicine, Department of General Internal Medicine, Center for Research and Education, University of Texas, MD Anderson Cancer Center, Houston, TX 77042, USA.

⁵Department of Diagnostic Sciences and Oral Medicine, University of Tennessee Health Science Center College of Dentistry, Memphis, TX 37174, USA.

⁶Department of Hematology, Athens Medical Center-Psychikon Branch, 15451 Athens, Greece.

Correspondence to: Prof. Ourania Nicolatou-Galitis, Clinic of Hospital Dentistry, Dental Oncology Unit, Dental School, National and Kapodistrian University of Athens, Bouboulinas 41, N. Psyhico, 15451 Athens, Greece. E-mail: nicolatou.galitis@hotmail.com



Ourania Nicolatou-Galitis is Professor and Chair of the Dental Oncology, at the National and Kapodistrian University of Athens, Greece. She is President of the International Society of Oral Oncology and serves as Chair of the Bone Study Group, MASCC.org. Osteonecrosis of the jaw in medication is her major research interest.

ABSTRACT

The first case of osteonecrosis of the jaw (ONJ) related to azacitidine therapy was reported. A 64-year-old male with acute myeloid leukemia, who received 5-azacitidine, presented with pain and purulence of the right second premolar. An unsuccessful endodontic therapy resulted in dental extraction 6 months later. The post-extraction non-healing socket was managed with antibiotics and multiple surgical debridements without response. ONJ stage 2 was diagnosed 12 months after the initial symptoms of pain and purulence and was managed conservatively. Currently the patient is still receiving 5-azacitidine therapy, while ONJ remains asymptomatic. This case highlights the presence of alveolar bone disease prior to the appearance of ONJ. Osteonecrosis in chemotherapy, although rare, may increase as long-term survival of cancer patients, who receive those medications increases. Health care professionals need to be alert, while collaboration with an experienced oral/dental oncologist would be beneficial to the patient.

Key words: Acute myeloid leukemia; azacitidine; periodontal/dental disease and infection; dental extraction; osteonecrosis of the jaw

INTRODUCTION

Osteonecrosis of the jaw (ONJ) in cancer patients is related to antiresorptive therapy, such as bisphosphonates and denosumab, and angiogenesis inhibitors, such as bevacizumab and sunitinib.^[1-3] Medications with antiangiogenic effect, such as sorafenib, imatinib, everolimus, aflibercept, trastuzumab and pazopanib were related to ONJ in few case reports.^[4-8] Ipilimumab, a monoclonal antibody against cytotoxic T-Lymphocyte-Associated Antigen-4 (CTLA-4)

was recently associated with ONJ.^[9] In patients receiving antiresorptives, concurrent chemotherapy was reported as a risk factor, while chemotherapy alone has also been related to ONJ in three patients.^[10-12]

Dental extraction has been considered as one of the causes for ONJ.^[13-15] This myth has, however, been questioned.^[16] It is now believed that the dental extraction, indicated due

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to periodontal/dental infection and disease, will lead to exposure of the alveolar bone, which may already be necrotic and will not heal.^[17] Infection, within a unifying concept of medication-related impaired immune response was proposed to play an important role in the pathophysiology of ONJ.^[18]

In this paper we report what we believe is the first case of ONJ in a patient with acute myeloid leukemia (AML), who was treated with azacitidine. The presence of alveolar bone disease leads to the dental extraction and the subsequent diagnosis of ONJ.

CASE REPORT

A 64-year-old male, smoker was diagnosed on April 2010 with myelodysplastic syndrome (refractory anemia) of low-risk according to IPSS (normal karyotype, without cytopenia, blasts 3-4%).^[19] One year later the patient progressed to refractory anemia with excess blasts, type II (RAEB-II), normal karyotype, without cytopenia, blasts 15%.^[20] He was placed on 5-azacitidine therapy [75 mg/m² (150 mg) day 1 to day 7 on 28 days cycle] with partial remission (Hgb > 11 g/dL, Platelets > 100 × 10⁹/L, Neutrophils > 1.0 × 10⁹/L, bone marrow blasts decreased by 50% but still > 5%). Two years later, after 17 cycles of 5-azacitidine, he progressed to AML. His complete blood counts showed: Hemoglobin 9.6 gr/dL, white blood cells 21.6 × 10⁹/L, absolute neutrophil count of 4.0 × 10⁹/L, immature white blood cells (myelocytes, metamyelocytes) and blasts 5.0 × 10⁹/L, platelets 142.0 × 10⁹/L. Bone marrow biopsy revealed 25-30% infiltration of CD34 (+) cells (blasts). Cytogenetic analysis (karyotype) was normal (46XY). He received 7 + 3 induction chemotherapy [intravenous infusion of Cytarabine (200 mg/m² day 1 through day 7) and Idarubicin 10 mg/m² on 30' infusion on day 2, 4, 6]. During hospitalization the patient developed neutropenic fever, managed with empiric antibiotic treatment (piperacilin + tazobactam and amikacin) and red



Figure 1: Swelling, fistula and purulence on the post extraction non-healing socket (July 2015). Necrotic bone could be probed through the fistula

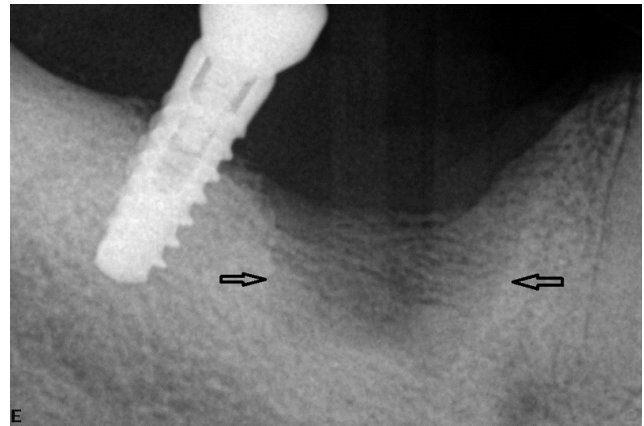


Figure 2: Radiolucency is seen in the socket (July 2015)



Figure 3: Remission of pain, swelling and purulence (August 2015)

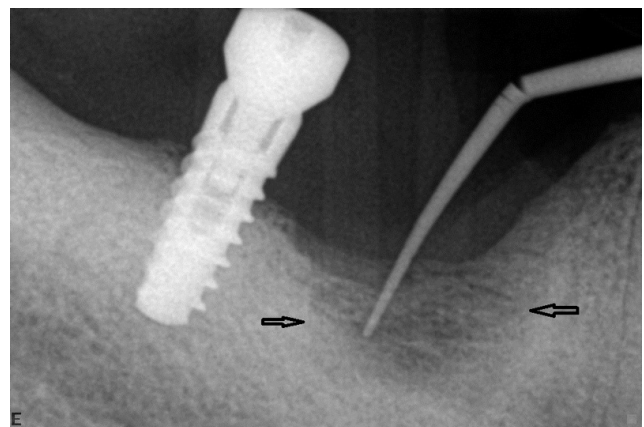


Figure 4: Radiolucency remains in the bone, socket area (September 2015). A gutta-percha cone has been inserted through the fistula

blood cell and platelets transfusions. Two months later, bone marrow aspiration and flow cytometry disclosed persistent disease.

Patient did not consent to receive induction chemotherapy and was placed on low intensity chemotherapy with hydroxyurea per os for six months. Bone marrow biopsy revealed greater than 60% blast cell infiltration, with a normal karyotype and patient was treated again with 5-azacitidine from that time to present. Bone marrow blasts dropped to 14%.

On June 2014 the patient received a restoration of the 2nd right mandibular premolar because of pain. Pain and purulence persisted in spite of endodontic therapy. Dental extraction was recommended and completed in December 2014. The post-extraction follow up revealed a non-healing socket. This area was managed with antibiotics (amoxicillin alone or combined with metronidazole) and surgical debridements (January to July 2015). A biopsy taken from the soft tissue of the socket showed granulation tissue. On July 2015 pain, swelling and purulence at the site of previous extraction [Figure 1], with necrotic bone being probed through a fistula and radiolucency, was observed on the periapical X-ray [Figure 2], leading to the diagnosis of osteonecrosis.

Management with antibiotics, ozone oil applications and low level laser therapy (LLLT) treatments [Ripamonti-11, Nicolatou-13], twice weekly, resulted in remission of symptoms [Figure 3], while the radiolucency and fistula persisted [Figure 4]. This is a retrospective case presentation from existing de-identified medical record data. Patient gave consent for the medical record review.

DISCUSSION

Azacitidine is a chemical analogue of the cytosine nucleoside and functions as a DNA demethylating agent and as an antimetabolite.^[21] Reduced cell division and growth may result from demethylation of DNA. Azacitidine, as a metabolite, can exert a direct myelotoxic and cytotoxic effect. Azacitidine, by both its demethylating and antimetabolite actions, might have negatively affected the increased need of cellular division and growth of bone remodeling and the soft tissue healing after the dental extraction in our patient. Azacitidine-related cytotoxicity and impaired immune response to infection could have also contributed to the development of alveolar bone disease and infection, which had preceded the appearance of ONJ.

Gemcitabine chemotherapy was associated with ONJ in a patient to Sezary syndrome, an aggressive leukemic form of cutaneous T-cell lymphoma.^[12] In that case, ONJ was related to the effects of gemcitabine, through suppression of vascular endothelial growth factor. Osteonecrosis was also reported in a patient with AML and in one with breast cancer, who received aggressive chemotherapy. No azacitidine was administered to the above patient with AML. Neutropenia and severe immune suppression were related to the development of ONJ in those cases.^[10] The presently reported patient developed ONJ while he was on azacitidine therapy. T-cell-related altered immune response and infection were related with ONJ in another patient, with advanced metastatic melanoma, who received ipilimumab.^[9] Pain, purulence and periodontal ligament widening indicating alveolar bone disease and infection preceded the appearance of ONJ in all those cases, including the present case. Our findings support the proposed role of

alveolar bone disease and infection in the pathogenesis of ONJ.^[17,18]

The major treatment objectives for patients with ONJ are pain and infection control and minimization of ONJ progression. Antibiotics and topical antiseptics combined with ozone oil applications and LLLT are used as best available clinical practice for early ONJ stages.^[1,5,17,22] Ozone oil has antimicrobial and healing properties, while LLLT biostimulation can improve healing.^[23,24] The patient was managed with antibiotics, amoxicillin and/or metronidazole, ozone oil applications and LLLT. The long delay (12 months) for the diagnosis of ONJ and the multiple unsuccessful dental and surgical interventions, combined with the continued azacitidine therapy, may be related to the persistent ONJ lesion.

In conclusion, this case increased the list of medications that can lead to ONJ and highlighted the importance of the presence of localized alveolar bone infection prior to the appearance of ONJ. The occurrence, though rare, of this potentially serious complication may increase with the long-term survival of cancer patients.

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Conflicts of interest

There are no conflicts of interest.

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Case Report

An unusual case of encapsulated papillary carcinoma of breast

Kriti Chauhan, Monika Garg

Department of Pathology, Maharishi Markandeshwar Institute of Medical Sciences and Research, Mullana, Ambala, Haryana 133203, India.

Correspondence to: Dr. Kriti Chauhan, Department of Pathology, Maharishi Markandeshwar Institute of Medical Sciences and Research, Mullana, Ambala, Haryana 133203, India. E-mail: kritichauhan25@gmail.com



Dr. Kriti Chauhan works as an Assistant Professor in Mmimsr, Mullana, Ambala, Haryana. She has done her post graduation in pathology from Gujarat Cancer and Research Institute, Ahmedabad. She has an interest and special inclination towards oncopathology.

ABSTRACT

Intracystic (encapsulated) papillary carcinoma of breast is a rare variant of breast cancer. It is usually a low-grade tumor showing estrogen, progesterone positivity. The authors report an unusual case of intracystic papillary carcinoma showing high nuclear grade, brisk mitosis, and necrosis with triple negativity for estrogen, progesterone, and Her-2/neu receptors, as well as negative axillary lymph nodes. Such cases need to be reported to increase awareness so that they will be managed conservatively, avoiding any overtreatment despite being high grade and triple negative.

Key words: Intracystic papillary carcinoma; high grade; triple negative

INTRODUCTION

Intracystic (encapsulated) papillary cancer (IPC) is a rare entity of breast cancer accounting for approximately 1 to 2% of all breast tumors and usually presenting in postmenopausal women.^[1] Histologically, it is characterised by an expansile papillary lesion which is surrounded by a thick fibrotic wall and an absent myoepithelial cell (MEC) lining. These lesions are known to have an excellent prognosis with only sufficient local therapy.^[2] They tend to have low-grade nuclei and low mitotic activity, and to be estrogen (ER) and progesterone (PR) receptors positive and Her-2 neu negative.^[3] To date, there are only two reported cases of IPC which are triple negative in the literature.^[2,4] Our case is unusual in being a noninvasive encapsulated grade III papillary carcinoma with negative nodes and triple-negative immunostaining.

CASE REPORT

A 60-year-old female presented with a complaint of progressively increasing swelling in the right breast since one year. The swelling was not associated with any pain or discharge. On physical examination, a 2 cm × 2 cm relatively firm lump was palpable in the upper outer quadrant. Mammography revealed a lobulated, well-defined nodular mass. Ultrasonography showed a well-delineated, heterogeneous, hypoechoic solid lesion with no axillary lymphadenopathy. Fine-needle aspiration cytology was performed, which showed sheets and occasional papillary clusters of ductal cells revealing extensive pleomorphism, vesicular nuclei, and prominent nucleoli [Figure 1]. Based on these findings, a diagnosis of high-grade ductal carcinoma was offered. Despite being a T2N0 tumor, a modified radical mastectomy (MRM)

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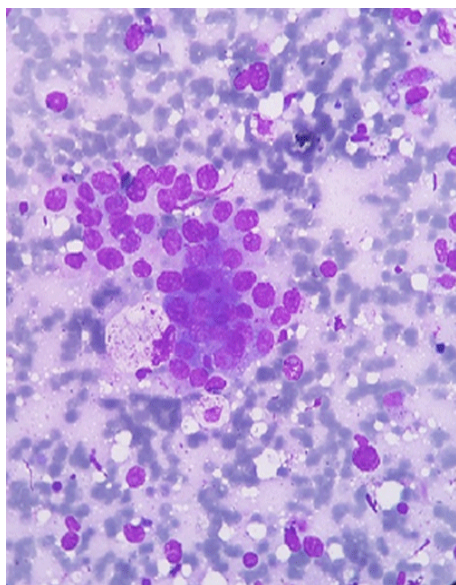


Figure 1: High power view of cytology smear showing tumor cells exhibiting pleomorphism, high N:C ratio, hyperchromatic nuclei, and prominent nucleoli. A cystic macrophage is also seen, suggesting a cystic change in the neoplasm (Giemsa ×400)

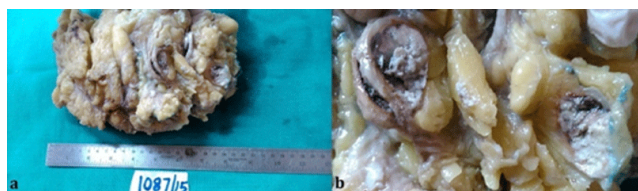


Figure 2: Gross specimen. (a) Modified radical mastectomy specimen showing tumor; (b) a closer view showing a well-defined, thick capsule surrounding a necrotic, friable tumor

was performed to rule out any invasion and to look for any micro metastasis in lymph nodes. Gross examination showed a well-circumscribed and encapsulated mass measuring 2.8 cm × 2 cm × 2 cm having a friable grey-white cut surface with areas of necrosis [Figure 2]. The base of resection and overlying skin were 2 cm and 3 cm away, respectively, and free of tumor. Sections were taken from the tumor along with the capsule. Separate sections from the adjoining breast were also taken to rule out invasion. Eleven lymph nodes were dissected from the axillary fat. Microscopic examination showed a thick fibrous capsule surrounding a neoplasm composed of blunt or delicate papillary structures with central cores. Intervening necrotic areas were also seen [Figure 3]. The cells lining the papillae showed high-grade nuclear atypia with variable N:C ratios, vesicular chromatin, and prominent nucleoli [Figure 4]. Mitotic figures were seen frequently (> 10/10 hpf). The MEC lining was absent within the papillary processes and at the periphery of tumor; this finding was confirmed by performing immunohistochemistry for smooth muscle actin (SMA) [Figure 5]. Tumor cells were negative for ER, PR, and Her-2/neu immunostains, performed with positive controls [Figure 6]. Adjacent breast tissue showed only fibrosis. No

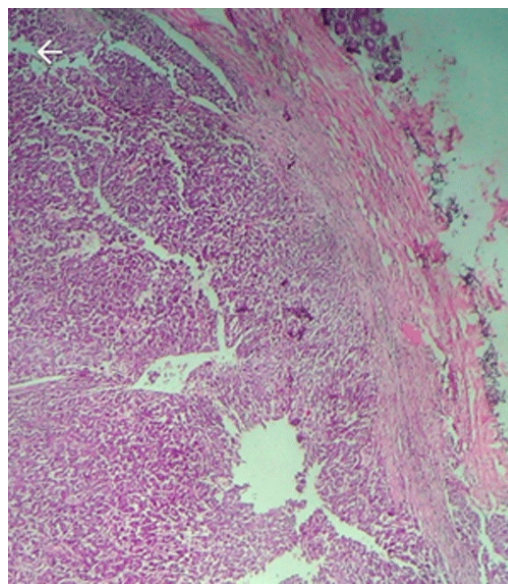


Figure 3: Scanner view showing a papillary tumor surrounded by a thick, fibrous capsule (HE, ×40)

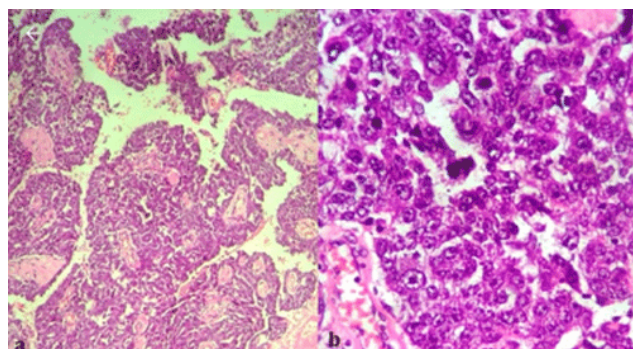


Figure 4: Microscopic view showing the tumor architecture and cytological features. (a) Low power view showing papillary structures with fibrovascular cores. (HE, ×100); (b) high power showing tumor cells exhibiting pleomorphism, vesicular nuclei with prominent nucleoli (HE, ×400)

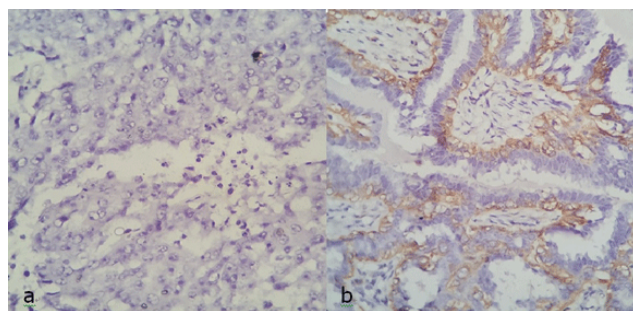


Figure 5: High power view showing tumor cells negative for: (a) smooth muscle actin immunostains; (b) performed with a positive control in a benign papillary tumor (SMA ×400)

invasive malignancy was seen. All the dissected axillary lymph nodes were free of metastasis (0/11). A diagnosis of encapsulated (intracystic) papillary carcinoma, high grade and triple negative, was rendered. Post-surgery on follow-up, the patient is disease free to date (4 months).

DISCUSSION

Papillary lesions of the breast are usually difficult to

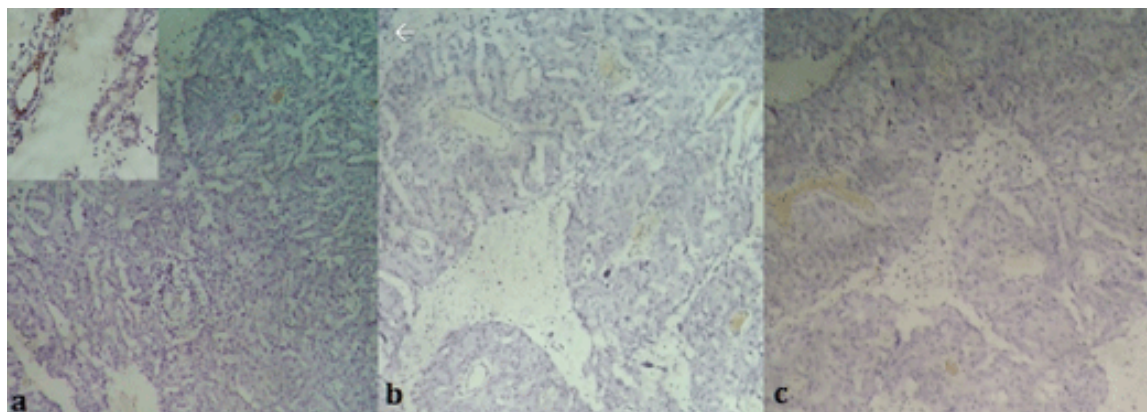


Figure 6: Low power showing negative immunostaining for: (a) inset showing positive internal control; (b) progesterone; (c) Her-2/neu (HE, ×100)

differentiate. Papillary carcinomas of the breast are divided into invasive and noninvasive types. The noninvasive type is further divided into the diffuse form (papillary variant of ductal carcinoma *in situ*) and the localized form (intracystic or encysted papillary carcinoma).^[5] Encapsulated papillary carcinoma is characterised by the presence of papillary carcinoma within an apparent cystically dilated duct. Myoepithelial cells are present neither in the papillae of IPC nor at the periphery, in contrast to papillary ductal carcinoma *in situ* (DCIS), in which there are MECs at the periphery of involved spaces.^[6] Several IHC stains like SMA, CD10, or S-100 can be used to confirm the presence of myoepithelial cells. IPCs have been considered to be a form of low-grade invasive carcinoma with an expansile growth pattern, or part of a spectrum of progression from in-situ to invasive disease.^[6] IPCs may occur alone, but more often the surrounding breast tissue contains foci of low- or intermediate-grade DCIS, usually with a cribriform or micropapillary pattern.^[7] Areas of invasive carcinoma may also be seen in association with them. These tumors are usually of low or intermediate nuclear grade with no evidence of necrosis and are strongly ER positive and Her-2/neu negative,^[1] unlike our case, which shows high-grade nuclear features and is triple negative. Also, these tumors are well delineated, remain quiescent, and are best regarded as intraductal papillary carcinomas.^[8] The patients with IPC are much less likely to die than those diagnosed with other types of breast cancer. At 10 years, the survival rate has been found to be greater than 95%.^[5] Lefkowitz *et al.*^[9] have reported a 100% survival rate and 91% disease-free survival rate at 10 years. The treatment options can involve breast-conserving surgery in the form of wide local excision with or without adjuvant radiotherapy or mastectomy.^[10] Low-grade tumors are less likely to recur or metastasize and are best treated by local excision in the absence of invasion. On the other hand, patients with higher-grade tumors have an increased risk of recurrence and metastasis.^[1] It is for this reason that a MRM was performed in our case because cytology

showed high-grade nuclear features and invasion could not be excluded. Axillary interventions include sentinel lymph node biopsy and/or axillary dissection.^[11] The low yield for metastasis and vascular invasion makes chemotherapeutic intervention not mandatory.^[10] This treatment modality is considered only in cases associated with lymphovascular invasion. Adjuvant radiotherapy and endocrine therapy (tamoxifen) has been recommended in younger patients (< 50 years) and in patients having IPC associated with invasion and/or DCIS.^[12]

In our case, no DCIS or foci of invasive carcinoma were seen in the surrounding breast. In addition to that, our case showed high-grade morphology (Nottingham's histologic score = 8/9, grade III) with triple-negative immunostaining, which is a very rare finding.^[2]

To conclude, the unusual high-grade adverse histomorphological features of IPC, with triple-negative immunostaining and no invasive foci, as seen in our case, is a rare finding. The management and prognosis in such a case remains questionable.

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Case Report

Carcinoma cervix with fat attenuating skull metastases

Anuradha Kapali¹, Atmakuri Sateesh Kumar¹, Mukunda Malathi², S. D. Shamsundar³

¹Department of Radiology, Kidwai Memorial Institute of Oncology, Bangalore 560029, India.

²Department of Pathology, Kidwai Memorial Institute of Oncology, Bangalore 560029, India.

³Department of Radiotherapy, Kidwai Memorial Institute of Oncology, Bangalore 560029, India.

Correspondence to: Dr. Anuradha Kapali, Department of Radiology, Kidwai Memorial Institute of Oncology, Bangalore 560029, India.

E-mail: kapali.anuradha@gmail.com



Dr. Anuradha Kapali, Assistant Professor in Department of Radiology, Kidwai Memorial Institute of Oncology, Bangalore, Karnataka, India. Special interest in oncology and women imaging.

ABSTRACT

Skeletal metastasis in carcinoma cervix occurs in about 0.8-23% of cases. These lesions are usually radiographically lytic. Very few cases of metastases to the skull have been identified, about 5 cases to the best of our knowledge. We present a case of adenosquamous cell carcinoma of cervix with fat attenuating skull metastases in a 38-year-old lady that is not reported till date. The lesion was lytic, expansile and with negative attenuation of -15 to -30 Hounsfield units corresponding to fat. Metastases must be included in the differentials of scalp lesions. A history of recent onset of swelling and associated lytic areas in calvarium on contrast enhanced computed tomography with multiplicity can give a clue to metastatic nature of disease.

Key words: Carcinoma cervix; metastases; skull; fat attenuating

INTRODUCTION

Bone metastases in carcinoma cervix can be due to local extension, however, distant metastases are due to hematogenous dissemination. The metastatic sites are commonly the spine, followed by pelvic bones rarely it can involve the skull where the appearance is of an expansile lytic lesion. High index of clinical suspicion is required for the diagnosis of skull metastases and should be included in the differentials of scalp lesions in a known primary. Till date fat attenuating metastasis to skull has not been reported.

CASE REPORT

A 38-year-old lady presented with severe neck pain for which she underwent contrast enhanced computed tomography (CECT) spine examination elsewhere that revealed multiple lytic lesions in the vertebra suggestive of metastases. She underwent biopsy of the same, which revealed metastatic adenosquamous carcinoma. The lady was diagnosed with metastases of unknown origin and referred to our institution for work up. At the time of admission the patient was bed ridden with altered sensorium. She later had one episode of vaginal bleeding for the first time according to history with

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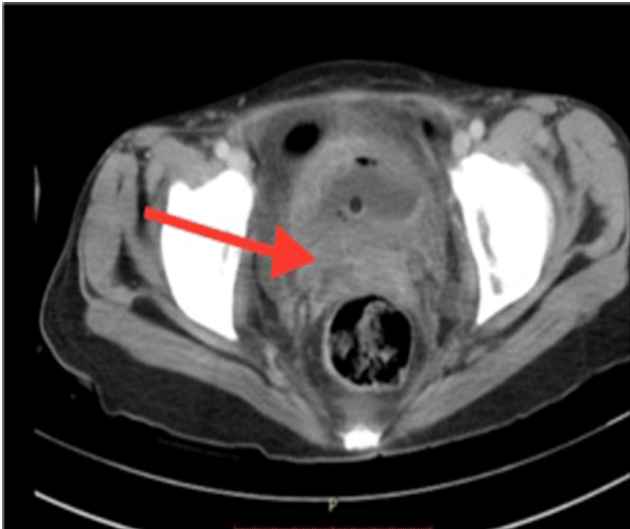


Figure 1: Heterogeneously enhancing cervical mass infiltrating the urinary bladder with bladder base thickening. Laterally parametrial stranding extends to the medial 2/3rd. Fat plane with rectum is lost posteriorly. Left obturator node is seen

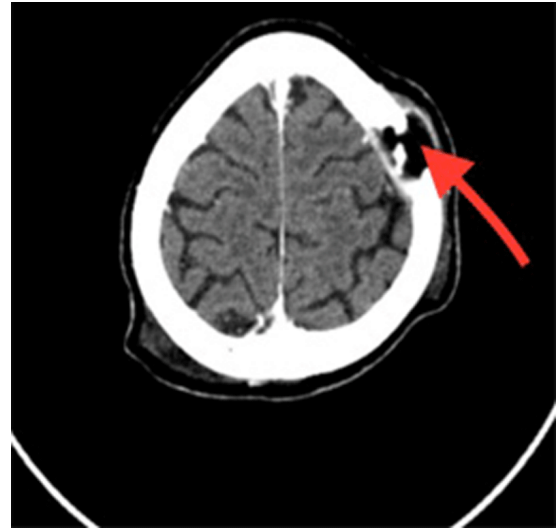


Figure 2: Expansile lytic lesion in the left frontal bone with average fat attenuation of -27 Hounsfield units (arrow tip). Underlying brain parenchyma is normal. The lesion causes corresponding scalp bulge in frontal region along the external surface

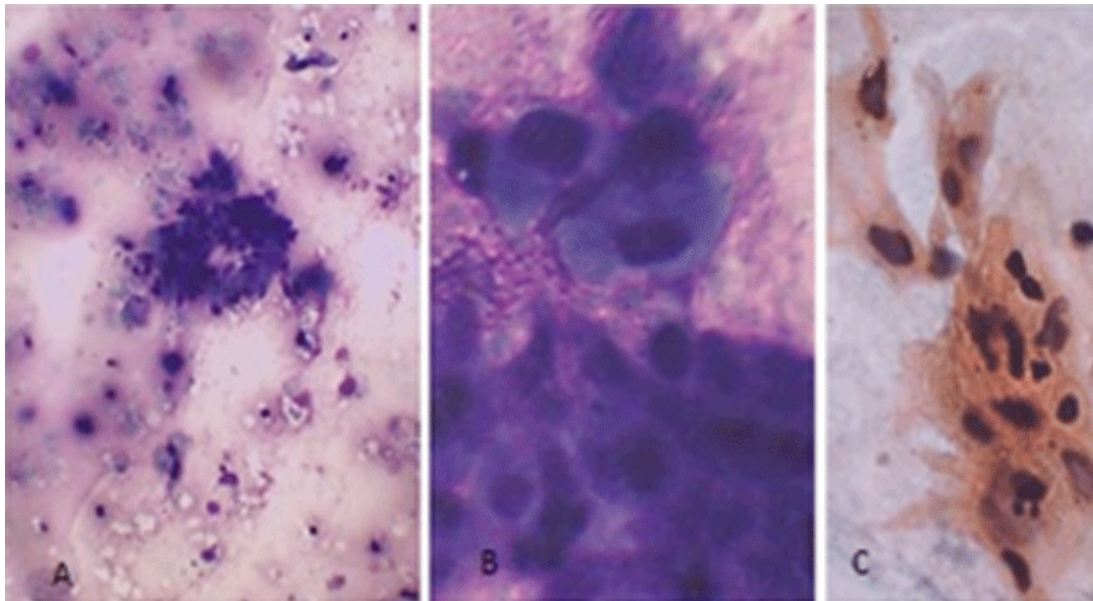


Figure 3: Photomicrograph of smear of FNAC scalp showing clusters of neoplastic squamous cells admixed with fat globules. (A) power view MGG (×100); (B) power view MGG (×400); (C) power view PAP (×100). FNAC: fine needle aspiration cytology; MGG: May-Grunwald Geimsa stain; PAP: papanicolaou stain

no other relevant clinical history. On general examination she had multiple soft scalp swellings, which were of recent onset, no other significant findings were present. She had a proliferative growth in the cervix measuring 3.5 cm × 3 cm, rest of the systemic examination was unremarkable. CECT exam of the whole body confirmed the cervical mass [Figure 1] that was infiltrating the urinary bladder. Multiple vertebral, pelvic and skull metastases were detected. The skull metastases were lytic with negative attenuation of -15 to -30 Hounsfield units [Figure 2] corresponding to fat. The metastases in spine and pelvis were of soft tissue attenuation. One of the calvarial lesions infiltrated the right transverse sinus causing thrombosis. She underwent a biopsy of cervix, which showed primary adenosquamous carcinoma. Fine needle aspiration cytology of the scalp

swelling was performed which demonstrated metastatic squamous cells with presence of fat globules [Figure 3]. She had an Eastern Cooperative Oncology Group score of 4. Due to advanced nature of the disease she was treated palliatively with supportive care including intravenous fluid administration, pain management and antibiotics. There was no role of active treatment. The clinical status of the patient deteriorated rapidly and she succumbed due to the disease.

DISCUSSION

Carcinoma cervix is the most common malignancy in Indian women, with an incidence of 19-44% per 100,000 women.^[1]

Apart from local spread, the disease spreads to the pelvic and para-aortic lymph nodes and then by hematogenous route to the supra and infradiaphragmatic viscera.^[2] About 5-35% eventually develops pulmonary metastases,^[3,4] 3% develops liver metastases,^[5] 16% develops bone metastases.^[4]

Skeletal metastasis in carcinoma cervix occurs in about 0.8-23%^[1] of cases. Bone metastases can be due to local extension, however, distant metastases are due to hematogenous dissemination. These lesions are usually radiographically lytic, and patients have recurrent or advanced disease with other sites of metastases.^[6] The metastatic sites are commonly the spine, followed by pelvic bones. Very few cases of metastases to the skull have been identified, about five cases to the best of our knowledge.

Yanuck *et al.*,^[7] reported a 21-year-old woman with stage IV cervical cancer that presented with a mass on the frontal bone, other cases of skull metastases reported are Niloofar Ahmadloo *et al.*,^[8] Mohanthy *et al.*,^[9] Abhishek *et al.*,^[10] and Zilberlicht *et al.*^[11]

The case reported by Abhishek *et al.*^[10] was similar to our case where the histological subtype was adenosquamous carcinoma and patient also had superior sagittal sinus thrombosis. Our patient had transverse sinus thrombosis. The other cases were of squamous cell carcinoma. Rath *et al.*^[12] and Agrawal *et al.*^[1] reported cases with multiple metastases to the scalp (skin).

In a recent analysis of 813 patients with stage IB disease, Look *et al.*^[13] noted a poorer survival for patients with adenosquamous lesions. Also, more patients developed extrapelvic recurrences than those with squamous or adenocarcinoma cell types. Neuroendocrine cervical tumors and glassy cell tumors have also been associated with hematogenous spread with early-stage disease. The aggressive disease in our patient could be explained by the adenosquamous cell type of carcinoma she had.

No reports have been published till date demonstrating fat containing metastases to skull as seen in our case. The spine and pelvic metastases were not fat attenuating.

In a patient with carcinoma of cervix, metastases must be included in the differentials of scalp lesions. The lesions may mimic sebaceous cysts and lipomas (in our case, they were soft on clinical exam and fat containing on computed tomography mimicking lipoma). A history of recent onset of swelling (present in our case) should prompt

imaging, associated lytic areas in calvarium on CECT with multiplicity can give a clue to metastatic nature of disease. In our patient, the disease was detected at an advanced stage as the presenting symptom itself was metastases to vertebrae presenting as neck pain she had no symptoms relating to carcinoma cervix until later.

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Introduction to the Special Issue “Cancer Stem Cells: Impact on Treatment”

Ira-Ida Skvortsova

Laboratory for Experimental and Translational Research on Radiation Oncology (EXTRO-Lab), Department of Therapeutic Radiology and Oncology, Innsbruck Medical University, 6020 Innsbruck, Austria.

Correspondence to: Dr. Ira-Ida Skvortsova, Laboratory for Experimental and Translational Research on Radiation Oncology (EXTRO-Lab), Department of Therapeutic Radiology and Oncology, Innsbruck Medical University, 6020 Innsbruck, Austria. E-mail: Ira.Skvortsova@i-med.ac.at

Despite the fact that currently existing therapeutic approaches are highly effective and can markedly improve clinical outcome in cancer patients with even advanced diseases, the problems of treatment resistance, therapy recurrences and unfavorable disease progression are still not solved. It is generally believed that the small population of the intratumoral carcinoma stem cells (CSCs) is responsible for poor clinical outcome, because CSCs are considered as a reason for the tumor heterogeneity, diminished sensitivity to chemo- and radiotherapy and enhanced abilities for metastatic spread.^[1-5] Investigation of the biological properties of CSCs is a hot topic in cancer research. In order to know more about CSC behavior, it is necessary to possess the CSC-specific molecular patterns distinguishing CSCs from non-CSCs. Using currently existing surrogate CSC biomarkers [CD133 (prominin-1), CD44, CD24, Bmi-1, Notch family members, Hedgehog, aldehyde dehydrogenase 1 (ALDH1), nestin, *etc.*], subpopulations carcinoma cells with stem cell properties can be isolated for further investigations.^[2] Recent studies have demonstrated that a variety of intracellular pathways are affected in CSCs: CSC metabolism is characterized by activation of glycolytic pathways^[6] and intracellular redox potential is dysregulated;^[1,7,8] molecular mechanisms governing cell cycle, cell proliferation and cell death development are also disrupted. Thus, there is a hypothesis that one of the reasons of CSC insensitivity to chemotherapeutics and ionizing radiation is the slower CSC proliferation and CSC quiescence.^[1] It is known that chemotherapeutic agents and radiotherapy eradicate fast dividing and proliferating carcinoma cells more effectively than the slower dividing cells.^[1] Therefore, it is logical to suggest that quiescent CSCs should be changed in their intracellular signalings underlying cell cycle regulation and cell division. Indeed, Gardane *et al.*^[9] and Vaidya^[10] in their article have clearly demonstrated that low doses

of curcumin can accelerate proliferation of the leukemic cells and application of 5-fluorouracil becomes more effective compared to the treatment with 5-fluorouracil without curcumin. These findings help to assume that administration of the compounds affecting quiescence of carcinoma cells can improve therapy results in cancer patients with malignant tumors containing a high number of quiescent CSCs.

Review article by Kim *et al.*^[11] highlights therapeutic opportunities to target CSCs and to reach better treatment results in cancer patients. Recent years have seen an increased number of research reports on the CSC-related intracellular and intratumoral molecular pathways that can be effectively blocked in order to reach better survival rate in cancer patients. This review article provides an analysis of different strategies that can be introduced into the clinical practice in order to improve therapy outcome in patients with unfavourable prognosis.

The Guest Editor and contributors to this special issue of the journal *Journal of Cancer Metastasis and Treatment* hope that basic researchers and clinicians will read these articles with great interest.

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Therapeutic strategies for targeting cancer stem cells

Yu Jeong Kim^{1#}, Elizabeth L. Siegler^{2#}, Natnaree Siriwon³, Pin Wang^{1,2,3}

¹Department of Pharmacology and Pharmaceutical Sciences, University of Southern California, Los Angeles, CA 90089, USA.

²Department of Biomedical Engineering, University of Southern California, Los Angeles, CA 90089, USA.

³Mork Family Department of Chemical Engineering and Materials Science, University of Southern California, Los Angeles, CA 90089, USA.

[#]Authors contributed equally to this work

Correspondence to: Dr. Pin Wang, Mork Family Department of Chemical Engineering and Materials Science, University of Southern California, 3710 McClintock Ave, RTH509, Los Angeles, CA 90089, USA. E-mail: pinwang@usc.edu



Dr. Yu Jeong Kim is a PhD candidate in the Department of Pharmacology and Pharmaceutical Science at University of Southern California (USC) mentored by Dr. Pin Wang. Her research focuses on liposomal nanoparticle-based drug delivery for combination therapy such as co-delivering two inhibitors to target two distinct populations within tumor bulk. Current studies involve the combination of immunotherapy and existing chemotherapeutic drug by utilizing Chimeric Antigen Receptor (CAR)-engineered T cells and Natural Killer (NK) cells and pharmaceutical drugs loaded crosslinked multilayer liposome vesicles for targeted cancer therapy.

ABSTRACT

The therapeutic limitations of conventional chemotherapeutic drugs present a challenge for cancer therapy; these shortcomings are largely attributed to the ability of cancer cells to repopulate and metastasize after initial therapies. Compelling evidence suggests that cancer stem cells (CSCs) have a crucial impact in current shortcomings of cancer therapy because they are largely responsible for tumor initiation, relapse, metastasis, and chemo-resistance. Thus, a better understanding of the properties and mechanisms underlying CSC resistance to treatments is necessary to improve patient outcomes and survival rates. In this review, the authors characterize and compare different CSC-specific biomarkers that are present in various types of tumors. We further discuss multiple targeting approaches currently in preclinical or clinical testing that show great potential for targeting CSCs. This review discusses numerous strategies to eliminate CSCs by targeting surface biomarkers, regulating CSC-associated oncogenes and signaling pathways, inhibiting drug-efflux pumps involved in drug resistance, modulating the tumor microenvironment and immune system, and applying drug combination therapy using nanomedicine.

Key words: Cancer stem cells; targeted cancer therapy; drug resistance

INTRODUCTION

Cancer stem cells (CSCs) are a small subset of cancer cells with the ability to self-renew and initiate tumor growth. They were first discovered in acute myeloid leukemia (AML) in the late 1990s.^[1] Since then, CSCs have been discovered in many solid tumors.^[2-6] Within the last two decades, CSCs have become a subject of intense research as a potential target for cancer therapeutics.

The discovery of CSCs led to a major shift in cancer modeling. Previously, cancers were thought to be made up of equipotent malignant cells which either renewed or differentiated stochastically, giving rise to a heterogeneous tumor. In contrast, the CSC model suggests that a hierarchy

exists among tumor cells, with CSCs at the top, producing the bulk of the tumor cells while maintaining their own renewal.^[5] A third model, clonal evolution, states that heterogeneity comes from genetic or epigenetic changes that arise during cancer progression. The CSC and clonal evolution models are not mutually exclusive, as CSCs can also evolve over time, generating different clonal subpopulations within the tumor.^[6]

CSCs share a number of properties with normal stem cells (SCs). Both typically make up a small percentage of the total number of cells in a tissue, they are largely quiescent, and, most notably, they are multipotent and

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can self-renew indefinitely. Many pathways vital to SC function, such as Wnt, Hedgehog, Notch,^[7] and PI3K/Akt,^[8] are dysregulated in CSCs, potentially contributing to neoplastic transformation. For example, cases of multiple myeloma have displayed abnormal signaling in response to elevated levels of Hedgehog ligand secreted by tumor stromal cells,^[9] and upregulated Notch4 signaling has been implicated in drug-resistant breast CSC activity.^[10] Like SCs, CSCs are able to repair damaged DNA more quickly and overexpress drug-efflux pumps such as ATP-binding cassette (ABC) transporters. In a glioblastoma model, aberrant Akt signaling contributed to overactivation of the ABC transporter ABCG2 in CSCs, leading to increased drug expulsion and rendering them resistant to mitoxantrone.^[11]

CSCs may also contribute to metastasis. During normal wound healing, cells are able to migrate to the wound site through the epithelial to mesenchymal transition (EMT) process. CSCs may also undergo EMT when migrating from the primary tumor site. Another theory hypothesizes that the CSC microenvironment -- including the surrounding vasculature -- facilitates metastasis.^[12] While the exact mechanisms have not been discovered, there are many reports of CSC-driven metastasis. In fact, numerous studies have used breast CSC-rich cell lines such as MDA-MB-231 to first produce primary tumors and then seed lung metastases.^[13,14]

Studies of CSC-targeted therapy depend on the isolation and enrichment of CSCs. They can be identified, isolated, and characterized by several methodologies, including flow cytometric analysis of CSC-specific cell surface markers, detection of side-population (SP) phenotypes by Hoechst 33342 dye exclusion, changes in aldehyde dehydrogenase (ALDH) enzymatic activities using an aldefluor assay, ability to grow as suspension spheres in serum-free medium, SC-related gene expression, and auto-fluorescence.^[6,15-17] There are no widely accepted techniques solely developed to isolate CSCs, necessitating the use of combination markers and methods rather than single strategies.

Surface marker-based assays have become the mostly commonly used method.^[18] Table 1 summarizes the list of cell surface phenotypes of CSCs in different tumors. The detection can be performed with specific antibodies in flow cytometry, competitive ELISA, or magnetic beads.^[19] Dick and coworkers showed the first evidence of the presence of CSCs in human AML by the flow cytometric display of the CD34⁺CD38⁻ surface marker phenotype.^[20] A breast CSC subpopulation was identified and isolated by

the combination of CD44 and CD24 markers.^[2]

Functional CSC properties like intracellular ALDH enzymatic activities and ABC transporter efflux activities of vital DNA dyes such as Hoechst 33342 have been used for CSC isolation.^[21,22] Increased aldehyde dehydrogenase isoform 1 (ALDH1) activity has been used to identify and analyze different types of CSCs. Furthermore, CSCs have a distinct efflux mechanism, stemming from their high expression of ABC transporter proteins.^[15] These cells, referred to as the “side population” (SP), are able to actively transport fluorescent dyes such as Hoechst 33342 out of the cells. Flow cytometric SP analysis has been performed with numerous cancer cell lines and the SP has shown enriched CSC activities.^[21]

A subpopulation of CSCs exhibit intrinsic autofluorescence and were shown to be exclusively linked to a functional CSC phenotype in different epithelial tumors. These autofluorescent cells had CSC characteristics such as high self-renewal, long-term tumorigenic capacity, invasiveness, and chemoresistance. These cells have intrinsic autofluorescence with excitation wavelengths at 488 nm and emission at about 520 nm. This new marker has been proven to be a more reliable and accurate way to identify and characterize CSCs.^[16,23]

Another important functional property of CSCs, as well as normal stem SCs, is the ability to produce sphere-forming colonies from a single cell in serum-free medium or in soft agar medium, as differentiated cells cannot survive and proliferate in this environment.^[24] Thus, several studies have used the sphere formation assay as an efficient method for isolating, enriching and maintaining CSCs from various primary tumors. Generally, these CSC-driven spheres are greater in both number and size as compared to ones generated from non-CSCs.^[18,25] These spheres clearly demonstrated stem-like properties and expressed characteristics of CSCs.^[16]

Here, we will focus on cancer therapeutics which can target CSCs. The development of various strategies that can act effectively against CSCs has been categorized into six groups, as shown in Figure 1.

REGULATING CSC SIGNALING PATHWAYS

Many signaling pathways are deregulated in CSCs and are potential targets in anti-CSC therapies. Overactivation

Table 1: Cancer stem cell surface markers in human cancers

Tumor types	Surface marker on the CSCs	References
Breast	CD44+/CD24-, CD133+, EpCAM+	[2,17,18]
Colon	CD133+, EpCAM+, CD44+	[17,18,36]
Glioma (brain)	CD133+, CD15+, CD49f+, CD90+	[3,17,18]
Leukemia (AML)	CD34+/CD38-, CD123+	[1,17,18]
Lung	ABCG2, CD133+	[16-18]
Melanoma	ABCB5, CD133+, CD20+, CD271+	[18]
Ovarian	CD44+, CD117+, CD133+	[39]

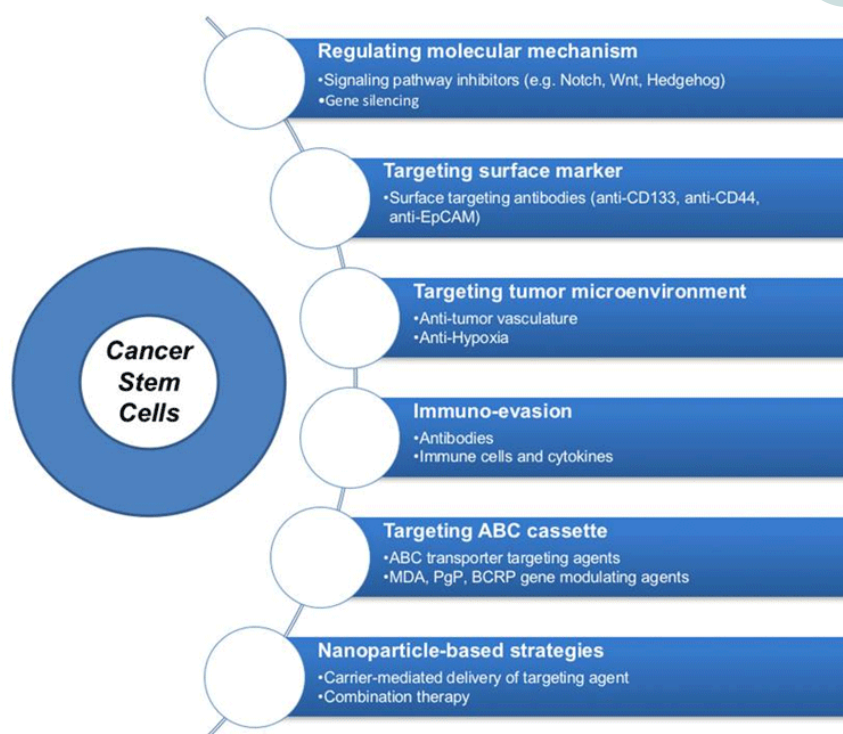


Figure 1: Novel therapeutic strategies for targeting cancer stem cells

of the Notch pathway has been implicated particularly in breast CSCs, possibly by influencing the EMT and contributing to the invasiveness of the CSCs.^[26] One group investigated the effects of the bioactive compound psoralidin on Notch signaling in a breast cancer model. The plant-derived drug inhibited Notch signaling in both bulk tumor and CSCs, resulting in decreased mammosphere formation, upregulation of pro-apoptotic proteins, and inhibition of CSC proliferation.^[27] Other studies have demonstrated that inhibiting Notch signaling resensitizes breast cancer cells to doxorubicin and docetaxel.^[28]

The Hedgehog signaling pathway may also contribute to CSC formation. Hedgehog signaling controls cell fate and proliferation in embryonic SCs, but dysregulation of the pathway has been associated with CSC generation. Cyclopamine was the first Hedgehog antagonist to be identified and its effects have been studied in many cancers.^[29] Cyclopamine depleted CSCs and induced tumor regression in a chronic myeloid leukemia model,^[30] decreased tumor growth rate in a medulloblastoma model,^[31] and inhibited proliferation of pancreatic CSCs.^[32]

Hedgehog abnormalities are linked to aberrant Wnt pathway activity. It is believed that Wnt plays a role in maintaining the self-renewal capabilities of CSCs. CD44, a CSC marker, is an important target for Wnt signaling, and CD44 knockdown resulted in decreased tumor formation in an intestinal cancer model.^[33] Another group tested several small molecule antagonists of Wnt and reported reduced mammosphere formation *in vitro* and halted tumor growth *in vivo* in a breast cancer model.^[34]

SILENCING ONCOGENES

RNAi is biological processes in which small interfering RNAs (siRNA) cause complementary target mRNA to be degraded, thereby silencing the gene. While there are many RNAi-based strategies which target bulk tumor cells, fewer studies have shown CSC-specific RNAi. One group used lentiviral short hairpin RNA (shRNA) to silence the human papillomavirus gene E6 in CSC-enriched cervical cancers. They discovered that after shRNA exposure, CSC growth and sphere formation were dramatically inhibited. E6 is upregulated in cervical CSCs, and E6 silencing also led to decreased CSC self-renewal through TGF- β modulation.^[35] Another study also used shRNA to silence HMGA1, an oncogene overexpressed in CD133⁺ colon CSCs. HMGA1 knockdown restored normal SC properties to CSCs, including quiescence, increased asymmetric division, and decreased self-renewing division. HMGA1 silencing was also linked to increased p53 expression.^[36] Both E6 and HMGA1 may be viable targets for anti-CSC therapy.

The application of RNAi in the clinic has been hampered by the inability to deliver high enough doses to the tumor site. One group has used targeted siRNAs to downregulate CSC oncogenes *in vivo*. They used PEGylated EpCAM aptamers to guide siRNA to EpCAM-overexpressing breast CSCs. The siRNA accumulated at the tumor site and resulted in an 80% knockdown of the survivin gene, which inhibits apoptosis and promotes chemoresistance in CSCs. When combined with doxorubicin, the aptamer-siRNA chimera improved survival rates of tumor-bearing mice,^[37] demonstrating the effectiveness of anti-CSC RNAi *in vivo*.

as well as *in vitro*.

TARGETING CSC SURFACE MARKERS

One potential CSC therapeutics approach is targeting CSC surface markers. One of the most established and commonly used CSC biomarkers is CD44, which is a cell-surface extracellular matrix receptor.^[6] Many studies represented CD44 antibody therapy as the major anti-CSC approach. The first of these studies showed that H90 anti-CD44 therapy successfully eradicated AML.^[38]

CD133 is a transmembrane glycoprotein and is another well-known CSC marker in several tumors such as glioblastoma, hepatocellular and colon cancers. CD133⁺ CSCs have shown resistance to chemotherapy and radiotherapy due to their slower cell cycle, lower proliferation, higher expression of DNA repair and anti-apoptotic genes.^[39,40] In a study by Carter *et al.*,^[38] the AC133 antibody was conjugated to a potent cytotoxic drug, monomethyl auristatin, using a protease cleavable linker. This antibody drug conjugate was efficiently internalized, co-localized with the lysosome and showed high effectiveness against hepatocellular cancer cells.

EpCAM has been discovered as a CSC marker in solid tumors and is correlated with all the characteristics of CSCs. EpCAM⁺/CD44⁺/CD24⁻ population in breast cancer had a significantly higher frequency of tumor-initiating cells. Moreover, ovarian cancer cells with high EpCAM expression were involved in EMT, leading to metastasis.^[2,41] Humanized EpCAM antibodies have been successful in both preclinical and early clinical studies, showing potent anti-tumor activity.^[38,41]

INHIBITING ABC TRANSPORTERS

CSC chemoresistance is due in large part to the overexpression of drug efflux pumps such as ABC transporters. Several pharmacological agents have demonstrated inhibitory or neutralizing effects on these transporters. There are three generations of inhibitors of one of the main ABC transporters, P-glycoprotein (P-gp). However, none have been approved for clinical use due to a lack of specificity and adverse side effects. Recently, a more specific P-gp inhibitor, vardenafil, has shown promise in mitigating the effects of P-gp overexpression. Vardenafil appeared to directly block P-gp-mediated drug efflux and resulted in increased intracellular concentration and cytotoxicity of paclitaxel and vincristine.^[42]

RNAi has also been used to silence ABC transporter genes. siRNA targeting P-gp reversed drug resistance in a doxorubicin-resistant breast cancer model. Doxorubicin-resistant cell lines are enriched with CSCs upon prolonged doxorubicin exposure.^[43] Exposing the resistant, CSC-enriched cells to P-gp siRNA resulted in downregulation of P-gp gene expression and led to increased intracellular

accumulation of doxorubicin and a 4-fold resensitization.^[44]

Nanotechnology can be used alone or in combination with drugs or RNAi of ABC transporters. Triblock copolymers by themselves have been shown to resensitize P-gp-overexpressing tumors to chemotherapeutic drugs; one group incorporated such a copolymer into polylactic acid micelles and reported overcoming multidrug resistance (MDR) in a paclitaxel-resistant breast cancer cell line.^[45] One ABC transporter inhibitor, ritonavir, was conjugated with copolymer nanoparticles to increase uptake into tumor cells and enhance the cytotoxic effect of doxorubicin in drug resistant murine leukemic cells.^[46] Another study suppressed P-gp using siRNA-loaded dextran polymeric nanoparticles in conjunction with doxorubicin treatment.^[47]

ENHANCING IMMUNE RESPONSES

It is hypothesized that CSCs are able to evade cancer immunosurveillance due to phenotypic and functional properties that allow them to survive in immunocompetent hosts. Antitumor immune cells are detectable and relevant to disease prognosis. Tumor associated antigens (TAA) are encoded by lineage specific genes and are often present or overexpressed on tumor cells.^[48] In patients with metastatic melanoma, circulating CD8⁺ T cells targeting the TAA MART-1 were detected, although they were functionally unresponsive.^[49,50] These TAA-specific T cells may be rendered anergic *in vivo*, and it is also plausible that CSCs downregulate their expression of human leukocyte antigen class 1 molecules or TAAs as another means of immunoevasion.^[49,50] Consequently, immunotherapy has become one of the most promising treatments for patients with metastatic cancer. Examples of strategies developed to enhance the host immune system are nonspecific immunomodulation to activate the host's immune response, and adoptive cell transfer of *ex vivo* expanded lymphocytes, such as T cells and natural killer (NK) cells.^[48,51]

Nonspecific immunomodulation includes treatment of patients with metastatic cancer using FDA-approved cytokines such as IFN α and IL-2.^[48,52] Administration of high doses of IL-2 into experimental animals was reported to reduce lung and liver metastases, and further investigation was conducted in human patients with metastatic melanoma, which demonstrated 15-20% objective clinical response.^[53,54] Several researches have attempted to explain the role of IL-2 in immunomodulation, and proposed that IL-2 induces expansion of T cells with major histocompatibility complex (MHC) -specific recognition of TAA to eliminate target cells.^[54,55] One disadvantage of this nonspecific antitumor immune activation is it also upregulates the CD4⁺CD25^{hi} Foxp3⁺ regulatory T cell (T_{reg}) population, which impedes general antitumor T cell function and contributes to tumor immunoevasion.^[52,56]

Another promising strategy in targeting cancers *in vivo* is adoptive transfer of chimeric antigen receptor (CAR) engineered T cells, which can specifically target any TAAs or cancer stromal antigens with high binding affinity. Preclinical models have been developed as a proof of concept that CARs could also be used to target CSCs.^[57] Deng *et al.*,^[58] for instance, demonstrated that CAR T cell therapy could inhibit tumor growth of highly metastatic prostate cancer that expresses low levels of EpCAM. Other CSC-targeting adoptive T cell therapies include CAR T cells which bind to a CSC-specific N-glycosylation-dependent epitope of CD133,^[59] high-molecular weight melanoma associated antigen or chondroitin sulfate proteoglycan 4-specific CAR T cells that were reported to specifically eliminate melanoma with a CSC phenotype,^[60,61] and epidermal growth factor receptor variant III (EGFRvIII) specific CAR T cells which target glioma SCs.^[62]

Cells in the tumor microenvironment was also found to express several negative immune regulators such as programmed cell death 1 (PD-1) and its ligand (PD-L1), cytotoxic T lymphocyte associated 4 (CTLA-4), and transforming growth factor β (TGF- β).^[63] Engagement of CTLA-4 attenuates activation of downstream inflammatory cytokines, which contribute to T cell antitumor immunity, such as IL-2 and IFN- γ .^[64,65] In T_{reg}s, the engagement of CTLA-4 is required for immune suppression.^[66] Antibodies blocking CTLA-4 engagement were developed and tested for their cancer therapeutic potential.^[67] Wu *et al.*^[68] showed that CTLA-4 monoclonal antibody (mAb) was able to inhibit early stages of tumor growth in a murine mesothelioma model and improved tumor infiltration of CD8⁺ and CD4⁺ T cells. In human patients with advanced melanoma, the CTLA-4 mAbs ipilimumab and tremelimumab prolonged T cell activation. However, only ipilimumab demonstrated improved survival in phase III study of patients with previously treated melanoma and gained FDA approval for treatment of metastatic melanoma in 2011.^[69] Future applications of mAb CTLA-4 will most likely come in the form of combination therapy to modulate the host immune system in a more effective synergistic fashion.^[67,68,70,71]

The second T cell regulatory pathway is the PD1/PD-L1 axis, which inhibits lymphocyte activation. PD-L1 or B7 homolog 1 (B7-H1) is expressed in many tumors including melanoma and cancers of the lung, colon, ovarian, liver and breast.^[63,72] PD1/PD-L1 binding triggers apoptosis of B and T cells in the tumor microenvironment. In tumors with upregulated PD-L1 expression, there is decreased T cell infiltration, activation, and expansion, effectively shielding CSCs against the host's immune response.^[63,72,73] Most recently, the new immunotherapy drug which has been approved by FDA in May 2016. Tecentriq is a monoclonal antibody that targets the PD-1/PD-L1 pathway by directly binding with a PD-L1 protein expressed on tumor cells and tumor-infiltrating immune

cells. This immune checkpoint inhibitor will help the body's immune system fight against cancer cells.^[74]

In addition to previously reported suppressor molecules, CD200 (OX-2) is another immunosuppressive factor that may have an important role in CSC's immunoevasion.^[75] CD200 is co-expressed with CSC markers such as CD133⁺ glioblastoma, colon and melanoma CSCs, CD44⁺/CD24⁻ in breast CSCs and CD44⁺ prostate CSCs.^[76,77] Upregulation of CD200 negatively correlates with the levels of Th1 cytokines required for effective T cell activation, such as IL-2 and IFN γ .^[78-80] Shifting of Th1 to Th2 cytokine production is observed in the progression of many cancer types and is a characteristic of the tumor microenvironment, especially in carcinomas with poor prognosis.^[78]

TARGETING THE TUMOR MICROENVIRONMENT

The tumor microenvironment of CSCs has three major characteristics: (1) chronic inflammation and secretion of inflammatory cytokines,^[81] (2) hypoxia,^[82] and (3) perivascular niches that regulate the capacity of proliferation and differentiation.^[83] Inflammatory cytokines such as IL-1 β , IL-6 and IL-8 activate the Stat3/NF- κ B pathways in tumor and stromal cells to further secrete cytokines in a positive feedback loop that prompts CSC self-renewal, angiogenesis, and metastasis.^[81,84] Moreover, the CSC population along with other cells which coevolved in the tumor microenvironment are near blood vessels that form a niche characterized by severe hypoxia and increased angiogenesis.^[82,83] These aspects of the tumor microenvironment have been explored as possible pharmaceutical targets of CSCs.

Recent studies have demonstrated decreased tumor growth after blocking IL-6 and/or IL-8 cytokine signaling.^[85,86] One of the pharmaceutical molecules tested was repertaxin, a non-competitive inhibitor of IL-8 and CXCR1 signaling, which decreased tumor size and increased efficacy of chemotherapy.^[87] However, the effects of blocking single cytokines is limited as both IL-6 and IL-8 are critical for xenograft tumor growth and the combined expression of these genes correlates with poor prognosis in patients with breast cancer. Therefore, co-inhibition of both IL-6 and IL-8 was suggested to be a more advantageous method to induce substantial effects on tumor growth.^[88]

Tumor hypoxia is another intriguing method for attacking CSC niches. Hypoxia activates the hypoxia inducible factor (HIF) pathway and upregulates HIF-1 α , which mediates multiple biological effects of hypoxia in tissues and increases resistance against chemotherapy and radiation.^[89] Several small molecule inhibitors of the HIF pathway have been pursued in clinical trials, although only a few of them were successful. Bortezomib

(Velcade®, PS-341) was approved by the FDA in 2003 for use against multiple myeloma, followed by Temsirolimus (Torisel®, CCI-779), approved in 2007 for use against renal cell carcinoma. The majority of the other drugs -- including Perifosine, 2-methoxyestradiol, Echinomycin, Geldanamycin -- were terminated in either phase I or II trials when they failed to show significant advantages.^[89]

Lastly, targeting tumor vasculature is another way to disrupt the CSC niche. Several agents blocking the activity of vascular endothelial growth factor, which drives the migration of endothelial cells and stimulates angiogenesis, are being tested in initial phases of clinical therapy with moderate success. These include Bevacizumab (Avastin®), Cediranib (AZD2171), Sunitinib and Vandetanib.^[82,90-93]

NANOMEDICINE IN COMBINATION THERAPY

Frequently after treatment, surviving CSCs induce new tumor formation and metastases in which cancer reappears in an even more aggressive form. With this phenomenon in mind, an increasing number of CSC-targeted therapeutic agents have been developed over the past several years such as salinomycin, curcumin, thioridazine hydrochloride, sulforaphane, miR-34a, and miR-130b.^[94-97] Despite their therapeutic potential in targeting CSCs, their clinical application has been hindered by their hydrophobicity, poor specificity and poor pharmacokinetics (PK) profiles.^[98-100]

Recent developments in nanoparticle delivery systems have provided new strategies to efficiently deliver therapeutics that can overcome the challenges posed by CSCs and improve therapeutic efficacy of CSC-targeting agents by controlling release kinetics, prolonging circulation time and improving bio-distribution. In a study by Zhou *et al.*,^[98] they used HPMa polymeric nanoparticles to deliver a Hedgehog pathway inhibitor that efficiently eliminated CD133⁺ cells within prostate tumors. Mamada and coworkers designed mesoporous silica nanoparticles to deliver a potent inhibitor of the Notch signaling pathway. Their nanoparticle drug treatments efficiently targeted CSC populations in the tumor. Furthermore, in the study done by Wei and colleagues, salinomycin was conjugated to a hyaluronic acid-based nanogel to target CD44⁺ drug resistant cells which enhanced the therapeutic efficacy of salinomycin.^[97,98]

Another advantage of using nanoparticles is the additional capability to modify their surfaces with targeting agents such as mAbs and peptides. High target selectivity and internalization can be achieved by surface modification of nanoparticles with targeting moieties. As previously discussed, CSCs are characterized by certain surface markers; this allows specific targeting of CSCs as a therapeutic strategy for drug delivery. Swaminathan

et al.^[101] demonstrated that their targeted nanoparticles induced a significant tumor volume reduction compared to untreated control and non-targeted groups in an *in vivo* MDA-MB 231 xenograft tumor model by developing paclitaxel-loaded polymeric PLGA nanoparticles conjugated with CD133 mAb. In another study done by Dou *et al.*,^[94] myeloma CSCs were treated with silver nanoparticles decorated with anti-ABCG2 antibodies on the surface along with vincristine. Despite these advances in the laboratory, targeted nanoparticle approaches in the CSC field are still in the early preclinical development stage due to limitations such as potential systemic toxicity, unwanted side effects, and poor extravasation and exposure to their targets.^[97,98,102]

It has been shown that using a CSC-targeted inhibitor alone is not very effective in reducing the tumor bulk due to the fact that these inhibitors are not highly cytotoxic as compared with conventional chemotherapeutics. Thereby, dual targeting nanoparticles loaded with CSC inhibitors and conventional cytotoxic agents can improve clinical outcomes by effectively eradicating both CSCs and bulk tumor cells at the same time. When compared with the free drugs, the nanoparticle formulated drugs were significantly more effective and less toxic both *in vitro* and *in vivo*.^[103-105]

CONCLUSION AND FUTURE PERSPECTIVES

It is clear now that conventional chemotherapy is not enough to overcome the abilities of CSCs to self-renew and metastasize. A combination of surface markers and their functional properties have been used to identify and isolate CSCs. Despite this progress, there is still a lack of reliable and accurate CSC markers. This must be overcome in order to develop therapeutic strategies with higher specificity and fewer side effects.

Using either small molecule inhibitors or RNAi to target CSC-associated oncogenes and signaling pathways have resulted in decreased functionality and numbers of CSCs and tumor regression in several pre-clinical models. CSCs develop resistance to conventional chemotherapeutics, but targeting ABC transporters resensitizes CSCs to those same drugs. Several studies have shown greater CSC targeting effects by employing antibodies against CSC-specific biomarkers. Anti-CSC approaches such as CD44 and EpCAM antibodies could selectively induce differentiation and inhibit proliferation.

Modulating the immune system and tumor microenvironment as a means of targeting CSCs has shown encouraging results. However, the efficacy of immunotherapy alone may be inadequate to produce clinical results. Therefore, combination therapy with conventional modalities as well as with immunomodulatory agents may be of future interest to enhance therapeutic effects.

As discussed above, nanocarriers enhanced the delivery and cytotoxic activity of CSC-inhibitors. Several studies introduced active targeting strategies of nanoparticle surfaces to increase their specificity and cellular uptake by CSCs. Lastly, researchers have been focusing on nanoparticle-mediated drug combinatorial therapy. One important advantage of nanocarriers is their capability to incorporate multiple therapeutic agents in one carrier system, allowing co-delivery of cytotoxic drugs and CSC inhibitors to simultaneously target both bulk tumor and CSCs. Patient cures will rely on the ablation of the entire tumor. Ultimately, nanoparticle mediated combination therapy may prove to be the most successful in eradicating whole tumors.

The CSC field is relatively new, and CSC-targeting therapeutics is in their early stages. While many advances have been made in CSC research, many of these studies have been performed *in vitro* only, and none are past the early clinical stages. Important factors such as effective dosages and side effects must be elucidated before employing cancer treatment plans that target both differentiated tumor cells and CSCs. There is need to improve the existing methods to precisely isolate, identify and target CSCs. As mentioned, increasing amount of nanomedicine have been evaluated about their application potentials in CSC therapy, but only a small amount of them can be approved to translate to clinical treatment. With the fact that every cancer acts differently in different patients, the development of personalized combinational therapies may serve as a key to successful treatments. Furthermore, it is important to realize that the combination of nanomedicine and immunotherapy may present a novel direction which shows great potential in personalized cancer therapy.

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Conflicts of interests

There are no conflicts of interest.

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The quintessential quiescence of cancer stem cells: a struggle towards better treatment

Anuradha Vaidya

Symbiosis School of Biomedical Sciences, Symbiosis International University, Symbiosis Knowledge Village, Pune 412115, India.

Correspondence to: Dr. Anuradha Vaidya, Symbiosis School of Biomedical Sciences, Symbiosis International University, Symbiosis Knowledge Village, Pune 412115, India. E-mail: dydirector@ssbs.edu.in

It has been almost two decades, since the existence of leukemic stem cells (LSCs) were first demonstrated in acute myeloid leukemia (AML) using xenogenic transplant models.^[1,2] Although LSCs were the first type of cancer stem cell (CSC) to be described experimentally, CSCs have been identified in a variety of malignancies and extensive efforts have been made to understand and characterize specific biomarkers associated with the various types of cancers.^[3] However, it still remains unclear whether these transformed cells arise as a result of the normal cells undergoing a malignant change or whether they are the differentiated malignant cells that have re-acquired stem-like characteristics.^[4] Irrespective of the conundrum regarding the origin of the LSCs, studies have highlighted that there exists remarkable heterogeneity to the LSC compartment at both the cellular and molecular level.^[5] Such intratumoral heterogeneity has been associated with the failure of many chemotherapeutic agents and progress to a refractory state, also known as the state of secondary resistance.^[6] Furthermore acquired quiescence has offered the CSCs to evade being killed by conventional chemotherapy and radiotherapy, leading to cancer relapse and metastasis.^[4]

Cancer immune surveillance is considered to be an important host protection process to inhibit carcinogenesis and to maintain cellular homeostasis. It has been shown that deregulation of the tightly controlled immune response may result in immune escape of CSCs, and there has been a growing interest in understanding the mechanisms that regulate the immune modulatory properties of the CSCs in order to develop more effective therapy that can eradicate these quiescent cells. Some of the signs of immune tolerance projected by CSCs include downregulation of positive co-stimulatory molecules, higher expression of negative co-stimulatory molecules, and secretion of soluble factors that induce regulatory T

cells, such that they inhibit the productive activation of effector T cells.^[7]

Other than the intrinsic factors such as the signaling pathways and the very recently stated micro RNA (miR-126) that drives quiescence and self-renewal within the LSCs,^[8] extrinsic factors such as the tumour microenvironment also plays a central role in the progression of cancer. The microenvironment has been implicated as a source of chemoresistance and disease relapse. Recent advances strongly indicate that the leukemic cells target the microenvironment to create an environment that is more suitable for the progression of cancer.^[9] In fact quiescence has been described as a survival strategy adopted by CSCs to resist harsh environmental conditions and cytotoxic insults.^[10]

Thus, cancer stem cells are the “unscathed successors” that progressively deteriorate the condition of the patient. Their inherent quiescent “status quo” along with the complex interplay of several factors (as those discussed above) contribute towards sustaining and propagating the malignant disease. Eradicating CSCs, the root of cancer origin and recurrence, has therefore been thought as a promising approach to improve cancer survival or even to cure cancer patients.^[11] Nevertheless, a major challenge thwarting the eradication of CSCs is that their identification and isolation has been hampered due to the non-specificity of their cell surface biomarkers^[3] and also by the fact that the commonly used fluorescent markers are not stable, and hence do not allow tracking over an extended period of time.^[12]

A study conducted by Gardane *et al.*,^[13] published in this issue demonstrates that low concentrations of

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curcumin sensitize the quiescent leukemic cells (QLCs) towards more effective killing by the anti mitotic drug, 5-fluorouracil. Curcumin pushes the QLCs into the cell cycle, thereby sensitizing them through the induction of proliferative responses. Similar observations have been reported by other studies wherein it has been implicated that the induction of cell cycle entry of the QLCs enhances apoptosis and elimination of human primary AML cells *in vivo*.^[14,15] Such studies underscore the essential role of cell cycle regulation in LSC function.

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Conflicts of interest

There are no conflicts of interest.

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Curcumin sensitizes quiescent leukemic cells to antimetabolic drug 5-fluorouracil by inducing proliferative responses in them

Anagha Gardane, Mariyah Poonawala, Anuradha Vaidya

Symbiosis School of Biomedical Sciences, Symbiosis International University, Lavale, Pune 412115, India.

Correspondence to: Dr. Anuradha Vaidya, Deputy Director, Symbiosis School of Biomedical Sciences, Symbiosis International University, Symbiosis Knowledge Village, Gram: Lavale, Taluka: Mulshi, District: Pune 412115, India. E-mail: dydirector@ssbs.edu.in



Dr. Anuradha Vaidya is the Deputy Director of Symbiosis School of Biomedical Sciences (SSBS), Symbiosis International University (SIU), Pune. Broadly, her area of research is microenvironment-mediated regulation of stem cell fate. Areas of expertise include Stem Cell Biology, Niche Biology, Experimental Hematology, Cancer Biology and Signal Transduction.

ABSTRACT

Long-term quiescence or dormancy is a fundamental feature of cancer stem cells (CSCs) that are genetically identical to the malignant clone but constitute the only cells with tumor propagation potential within the overall tumor population. These quiescent cells show significant resistance to radiation and antiproliferative chemotherapy due to distinctive properties that seem to be related to their stem cell-like character. Hence, successful anticancer therapy must consist of approaches that can target not only the differentiated cancer cells, but also the CSCs. Using serum-starved KG1a cell line as an experimental model system of quiescent leukemic cells (QLCs), the present study demonstrates that QLCs exposed to low concentrations of curcumin show high proliferative potential. Furthermore, when subjected to a combination therapy consisting of low concentrations of curcumin and 5-fluorouracil (5-FU), the QLCs displayed a high kill with an increase in the levels of nitric oxide (NO) and reactive oxygen species. These results were further consolidated with the observation of high caspase-3 activity in cells subjected to the combination therapy. This may suggest that low concentrations of curcumin stimulate the QLCs to become mitotically active, thereby sensitizing them to killing by the antimetabolic drug, 5-FU.

Key words: Acute myeloid leukemia; KG1a cell line; curcumin; 5-fluorouracil; quiescent leukemic cells

INTRODUCTION

Acute myeloid leukemia (AML) is a heterogeneous clonal disorder of hematopoietic progenitor cells that is characterized by a blockage of differentiation and an accumulation of immature non-functional myeloid cells in the blood.^[1] It is the most common malignant myeloid disorder among children and adults.^[2] The mainstream approach for AML treatment is chemotherapy, radiation, or surgery.^[1,3] However, the association between conventional therapy and severe toxicity followed by a tendency to relapse or metastasize cannot be ignored.^[3,4] In many cases resistance to therapy develops, leaving AML patients with no alternative but to undergo bone marrow transplantation (BMT) for a disease-free survival.^[2,4]

According to cancer stem cell (CSC) theory, CSCs are responsible not only for tumor initiation, development, and metastasis but also for therapeutic resistance.^[3,5-7] These cells were first identified by Bonnet and Dick^[8] in AML. Following their findings many other groups have identified these cells in various solid tumors, such as brain, breast, pancreas, and prostate.^[9-12] Standard chemotherapy and radiotherapy target only the active tumor cells. Quiescent CSCs evade therapy and remain unharmed, a major concern for the development of insensitivity towards therapy leading to relapse associated with leukemia.^[3,5,7] Furthermore, the release of inflammatory

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cytokines -- particularly interleukin (IL)-6, IL-8 and IL-1 -- as a consequence of induced cancer cell death has been shown to stimulate replication of CSCs,^[13,14] and also affect at multiple sites along CSC pathways such as Wnt, Notch, Hedgehog, and focal adhesion kinase (FAK).^[7,12,13] CSCs that are generated as a result of chemotherapy-induced tumor cell death that stimulates the release of inflammatory cytokines have been reported to be more refractory to therapy.^[13,15,16] This suggests that, for therapy to be consistently effective, it must eliminate both CSCs and non-stem cell cancer cells.

Currently research is being done to harness the medicinal properties of natural compounds for treating leukemia.^[17,18] Natural compounds are cheap, are easily available, and do not cause any adverse effects.^[17,18] Curcumin is a well-known dietary polyphenol^[19-21] and is an active ingredient of turmeric that possesses antioxidant and anti-inflammatory activities.^[19,21] Its safety and tolerability has been well-established by numerous clinical studies.^[19,21] It has been shown that curcumin has significant cytotoxic and apoptotic effects on the promyelocytic cell line, HL-60, suggesting that it may have a potential therapeutic role for human leukemia.^[22-25] A study conducted by Fong *et al.*^[26] showed that curcumin inhibited the side population (SP) phenotype of the rat C6 glioma cell line, demonstrating for the first time *in vivo* that curcumin has anticarcinogenic and antimetastatic activity in the brain. Another study demonstrated that curcumin is able to target breast stem/progenitor cells, as evidenced by suppressed mammosphere formation along serial passage and by a decrease in the percent of aldehyde dehydrogenase (ALDH)-positive cells.^[27-29]

To summarize, several cell and animal studies have demonstrated and corroborated the apoptotic activity and anticancer effect of curcumin in different types of cancers,^[30-34] and recent research has shown that curcumin can also target CSCs.^[35] In the present work we demonstrate that curcumin, at low concentrations, induces proliferative responses in QLCs, thereby sensitizing them to the antimitotic drug, 5-fluorouracil (5-FU).

METHODS

Reagents

Curcumin, Fetal Bovine Serum (FBS), Griess Reagent, Dichloro-dihydro-fluorescein diacetate (DCFH-DA), Propidium Iodide (PI), 3-(4, 5-Dimethylthiazol-2-yl)-2, 5-Diphenyltetrazolium Bromide (MTT), 5- Fluorouracil (5-FU), Dimethyl Sulfoxide (DMSO), RNase-A were purchased from Sigma-Aldrich, USA; Iscove's Modified Dulbecco's Media (IMDM), L-Glutamine, Antibiotic Solution (Penicillin + Streptomycin), Trypan Blue Dye, Phosphate Buffer Saline (PBS) were purchased from Himedia, India; Caspase-3 Colorimetric Assay Kit was purchased from RayBiotech.

Cell culture

KG1a cell line was procured from National Centre for Cell Science (NCCS), Pune, India and was maintained under standard conditions as per the ATCC guidelines. KG1a is a variant sub-line of KG1 that is morphologically and functionally less mature than KG1. It does not respond to colony-stimulating factors in soft agar assays. Cells were starved in low serum (0.5% FBS) medium overnight to prepare Quiescent Leukemic Cells (QLCs) from them.^[36]

QLCs were subjected to various treatments as follows:

1. Treatment with only curcumin (CU): QLCs were treated with various concentrations of curcumin ranging from 10 µg to 100 µg/mL in growth medium (IMDM supplemented with 20% FBS) for 48 h.
2. Treatment with only 5-fluorouracil (5-FU): QLCs were exposed to varying concentrations of 5-FU (6-100 µg/mL) in growth medium for 24 h.
3. Combinatorial treatment: QLCs subjected to curcumin treatment (10-100 µg/mL) for a period 48 h (step 1) were harvested and resuspended in fresh growth medium containing 6 µg/mL of 5-FU. The cells were further incubated for a period of 24 h after which they were subjected to various biochemical assays as described below.

3-(4,5-Dimethylthiazol-2-yl)-2, 5-Diphenyltetrazolium Bromide (MTT) assay

QLCs exposed to various treatments were subjected to a standard MTT assay as discussed before, and the percent proliferation was determined.^[36]

Growth curve experiment

Growth curve experiment was performed to determine the doubling time. QLCs were treated with 10 µg/mL curcumin, and viable cell counts were taken at specified time intervals using trypan blue dye exclusion method.

Flow cytometric analysis of cell cycle

Cell cycle analysis using propidium iodide (PI) staining was performed to distinguish the cells in various stages of the cell cycle. Briefly, the QLCs exposed to various treatments were stained with PI, after which analysis of the cell cycle was performed using BD FACSCalibur™ (BD Biosciences, USA).

Nitric oxide (NO) assay

Nitric oxide assay was performed using modified Griess reagent for the colorimetric detection of NO production by QLCs subjected to curcumin and/or antimitotic drug treatment(s) at 540 nm using BioTek™ Eon™ Microplate Spectrophotometer (USA).

2', 7'-dichlorofluorescein diacetate (DCFDA) assay

QLCs exposed to curcumin and/or antimitotic drug treatment(s) were checked for the generation of Reactive

Oxygen Species (ROS) by DCFDA assay. QLCs were incubated with 10 μ M DCFDA for 30 min at 37°C. After incubation, 2', 7'-dichlorofluorescein (DCF) was measured at 495-529 nm by using a fluorometer (Fluoroskan Ascent, Thermo Fisher Scientific, USA).

Apoptotic assay

This assay was performed as per manufacturer's instructions (RayBiotech) to estimate the caspase-3 activity of the QLCs. The intensity of the color was measured at 400/405 nm by using a spectrophotometer reader (BioTek™ Eon™ Microplate, USA).

Statistical analyses

The data were analyzed by One-way Repeated Measure Analysis of Variance (One-Way RM ANOVA). The Standard Error of Mean (S.E.M.) values were used for plotting the error bar graphs, using the SigmaPlot software (version 13.0). Level of significance was denoted as follows: * $P \leq 0.05$, ** $P \leq 0.01$ and *** $P \leq 0.001$.

RESULTS

Low concentrations of curcumin induce proliferation of quiescent leukemic cells

It has been shown that curcumin inhibits cell proliferation, causes cell cycle arrest, and initiates apoptosis in several human cancer cell lines.^[37,38] We first wanted to determine the concentration(s) of curcumin that would be most effective against the QLCs. Hence we cultured the serum-starved KG1a cells for 48 h with various concentrations (10-100 μ g/mL) of curcumin. We were expecting to see a dose-dependent inhibitory effect of curcumin on the QLCs. Interestingly, however, we observed that low concentrations of curcumin (10 μ g/mL and 20 μ g/mL) stimulated the cells to undergo proliferation, whereas at all other concentrations of curcumin (30 μ g/mL to 100 μ g/mL) imparted inhibitory effects [Figure 1]. Our proposition is that since KG1a cells are known to contain leukemia-like stem cells,^[16] low concentrations of curcumin could have induced the leukemia-like stem cells to proliferate.

Curcumin results in an increased cell yield by reducing the doubling time of QLCs

Since low concentrations (10 μ g/mL and 20 μ g/mL) of curcumin led to proliferation of the QLCs, we next wanted to know whether treatment with curcumin would alter the cell cycle kinetics. Serum-starved KG1a cells were treated with 10 μ g/mL of curcumin, and viable cell counts using trypan blue dye exclusion method were taken at every 24 h interval for a period of 6 days. As seen in Table 1, it was observed that till day 4, the doubling time of QLCs treated with curcumin was reduced to almost half that of the untreated cells. The minimum doubling time of 6.14 h was observed on the second day. It is also important to note that the doubling time of curcumin-treated cells was lower than that of untreated cells for all 6 days [Figure 2].

Table 1: Doubling time of untreated versus treated QLCs

Day	Time interval in hours	Doubling time in hours	
		Untreated QLCs	QLCs + curcumin (10 μ g/mL)
1	0-24	23.99	11.99
2	24-48	11.99	6.14
3	48-72	29.74	13.97
4	72-96	36	18.15
5	96-120	44.8	28.2
6	120-144	35.3	20.5

QLCs: quiescent leukemic cells

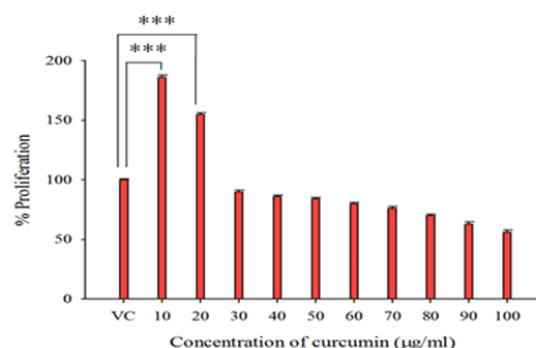


Figure 1: QLCs undergo proliferation in response to low concentrations of curcumin: Serum-starved KG1a cells were treated with varying concentrations of curcumin (10-100 μ g/mL) for 48 h and were subjected to MTT assay. Treatment with low concentrations (10 μ g/mL and 20 μ g/mL) of curcumin led to proliferation of QLC cells as compared to the vehicle control (VC) cells. The data represent mean \pm S.E.M. of three independent experiments (** $P \leq 0.001$)

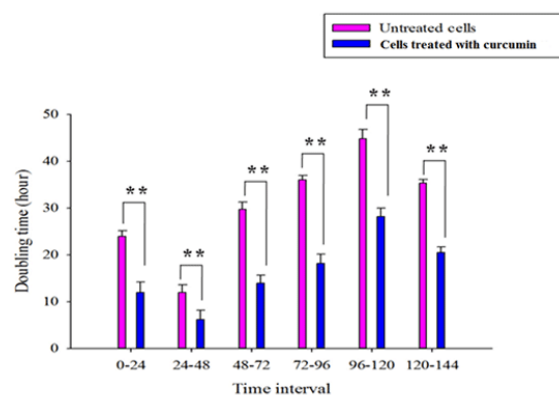


Figure 2: Treatment with curcumin reduces the doubling time of QLCs: QLCs were treated with 10 μ g/mL of curcumin. Viable cell count was taken at an interval of 24 h for 6 days. Although minimum doubling time (6.14 h) was observed at Day 2 (24-48 h), the overall doubling time of curcumin-treated cells was always lower than the doubling time of vehicle control (VC). The data represent mean \pm S.E.M. of three independent experiments (** $P \leq 0.01$)

5-Fluorouracil inhibits the proliferation of QLCs in a dose-dependent manner

The presence of leukemic stem cells (LSCs), also known as cancer stem cells (CSCs), is a major problem in the treatment of leukemia. The LSCs are refractory

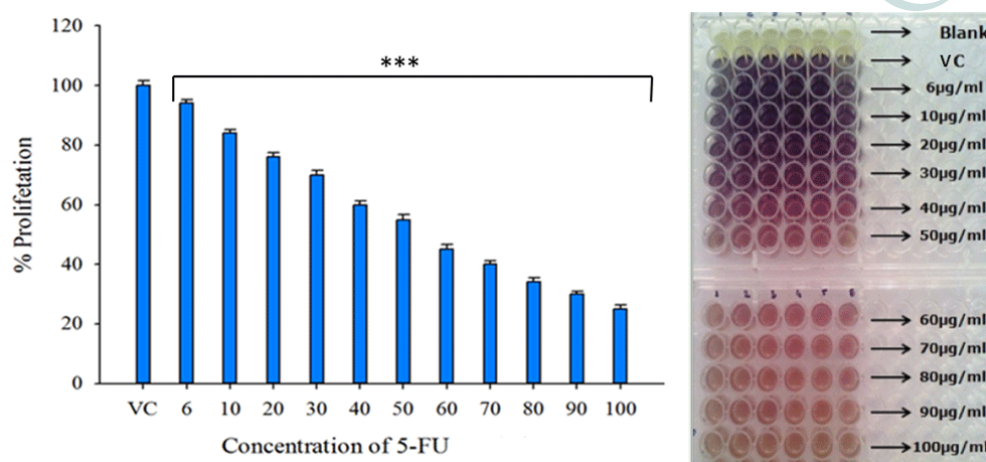


Figure 3: 5-Fluorouracil imparts its cytotoxic effect on quiescent KG1a cells in a dose-dependent manner: Serum-starved KG1a cells were incubated with different concentrations of 5-FU (6 µg, 10 µg to 100 µg/mL) for 24 h and were subsequently subjected to MTT assay. As seen in the graph, QLCs displayed a dose-dependent response to increasing concentrations of 5-FU. The data represent mean \pm S.E.M. of three independent experiments (** $P \leq 0.001$)

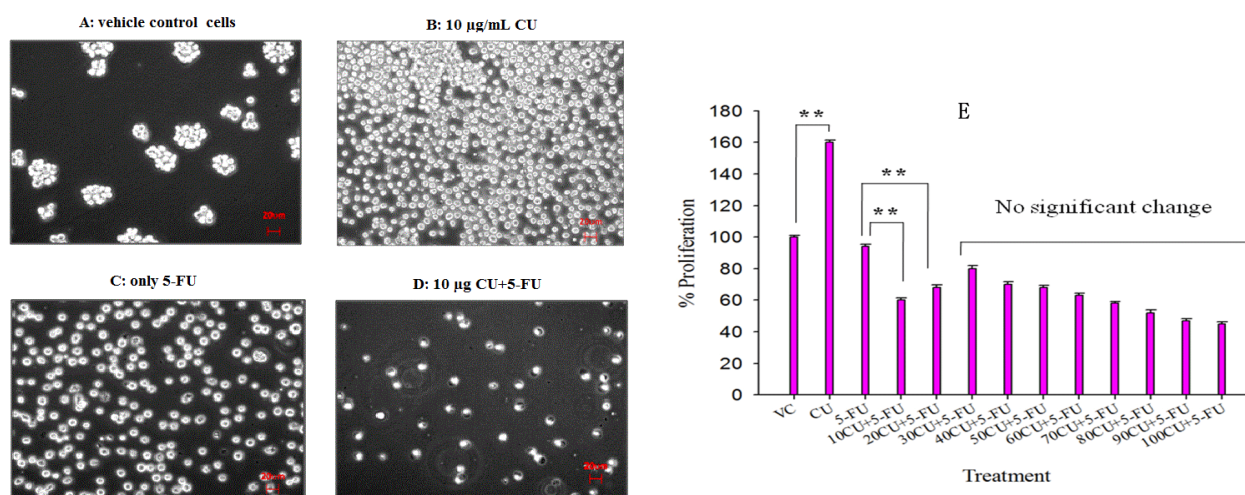


Figure 4: Treatment with low concentrations of curcumin sensitizes the QLCs to the antimitotic agent, 5-FU: Serum-starved KG1a cells were incubated with different concentrations of curcumin (10-100 µg/mL) for 48 h. After 48 h, the QLCs were further incubated with 5-FU (6 µg/mL) for another 24 h. When the cells were subjected to MTT assay (E), it was observed that the percent proliferation of QLCs exposed to the combination treatment (10CU + 5-FU, 20CU + 5-FU) was lower than for the cells treated with only 5-FU. The data represent mean \pm S.E.M. of three independent experiments (** $P \leq 0.01$). (A-D) They represent phase contrast images using a 20 \times objective of an inverted microscope (Carl Zeiss, 200 \times magnifications) of QLCs exposed to curcumin and/or 5-FU. (A) Vehicle control QLCs growing in clumps; (B) QLCs exposed to 10 µg/mL curcumin, showing maximum proliferation; (C) QLCs exposed to only 5-FU; (D) QLCs exposed to 10 µg/mL curcumin and 5-FU, showing fewer cells, indicating that the QLCs have been more efficiently killed by 5-FU; (E) graphical representation of QLCs subjected to MTT assay

to treatment, and their presence is associated with relapses.^[13,15,16] In 2008, Vaidya *et al.*^[36] showed that inhibition of p38 mitogen-associated protein kinase (MAPK) sensitizes the QLCs to antimitotic agents 5-FU and cytosine arabinoside (Ara-C). Since the treatment of QLCs with low concentrations of curcumin was pushing the cells into proliferation, we conjectured that this proliferative response could be translated to increase the sensitivity of the leukemic cells to antimitotic agents. Hence, we first reconfirmed whether serum-starved KG1a cells were a good model system to study the effects of antimitotic drugs. We exposed the quiescent KG1a cells to different concentrations of 5-FU and subjected them to MTT assay. As shown in Figure 3, 5-FU induced dose-dependent killing of the quiescent KG1a cells, thereby validating it as a good model system for testing the efficacy of antimitotic drugs.

Quiescent leukemic cells prepared from KG1a get sensitized to low levels of 5-FU when treated with low concentrations of curcumin

Based on our previous studies,^[36] we wanted to examine whether the proliferative response induced by low concentrations of curcumin treatment would make the quiescent leukemic cells more susceptible to the mitotic inhibitor 5-FU. KG1a cells that were made quiescent by serum deprivation were first treated with low concentrations of curcumin (10 µg/mL and 20 µg/mL) and then exposed to 6 µg/mL (lowest concentration) of 5-FU.^[36] MTT assay was then carried out to assess the percent proliferation of the cells. It was observed [Figure 4E] that QLCs that were treated with a combination of 5-FU and low concentrations of curcumin were more effectively killed (low percent proliferation: 60% and 65% for 10 µg/mL and 20 µg/mL, respectively) as compared to

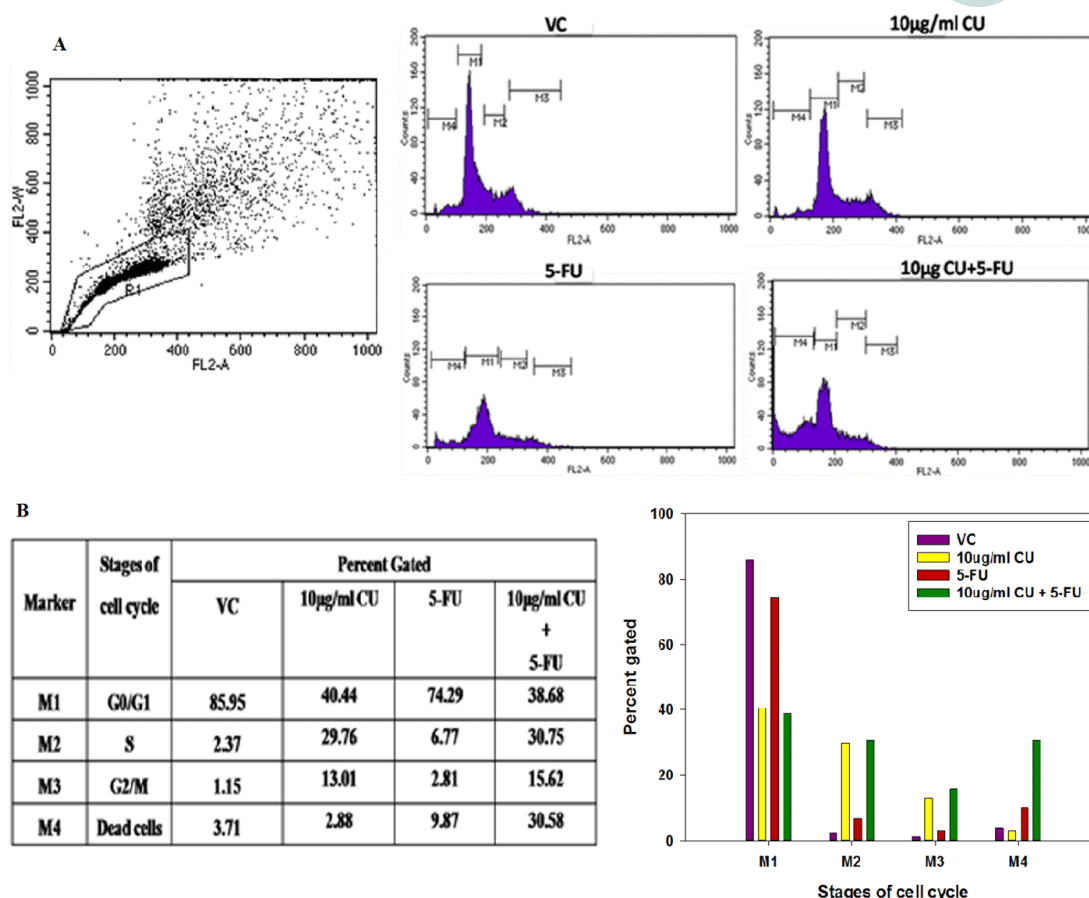


Figure 5: Cell cycle analysis showing that curcumin causes greater migration of QLCs from the G₀/G₁ phase to other stages of the cell cycle: QLCs were treated with curcumin (10 µg/mL) for 48 h. After 48 h the quiescent cells were incubated with 5-FU (6 µg/mL) for another 24 h and then subjected to PI staining. The stained cells were acquired and analyzed using BD FACSCalibur™. As compared to the vehicle control (VC), a much higher percentage of cells treated with curcumin were pushed into the S and G₂/M phase. More importantly, the QLCs that were exposed to curcumin were more effectively killed by 5-FU than cells that were not. (A) Side scatter plot of untreated vehicle control cells (QLCs), where R1 is the gated population of QLCs that is positive for PI. The histograms demonstrate a distinct pattern of the different phases of the cell cycle marked as M1 (G₀/G₁ phase), M2 (S-phase), M3 (G₂/M phase) and M4 (Dead cells) of untreated QLCs (VC) and of QLCs subjected to curcumin and/or 5-FU treatments; (B) a tabular and graphical representation of Figure 5A, depicting the percentage of gated QLCs in each stage of the cell cycle in response to treatment with curcumin and/or 5-FU

the cells that were treated with 5-FU alone (higher percent proliferation: 90%). Figure 4 (A-D) represents phase contrast images of QLCs that were untreated [Figure 4A] or exposed to only curcumin [Figure 4B], only 5-FU [Figure 4C] and both curcumin and 5-FU [Figure 4D]. It is clearly seen that QLCs that were exposed to only curcumin underwent high proliferation and were more susceptible to the antimitotic effects of 5-FU.

Curcumin pushes the QLCs into the S phase of the cell cycle which sensitizes them to killing by the antimitotic drug 5-FU

The antimitotic drug, 5-fluorouracil (5-FU) selectively kills the cells in the S-phase of the cell-cycle, leaving the quiescent leukemic cells (QLCs) unharmed.^[36,39] Hence, our next step was to check whether the induction of proliferative responses in presence of low concentration(s) of curcumin was pushing the QLCs into the S phase of the cell cycle.

In this set of experiments, serum-starved KG1a cells were treated with curcumin and/or 5-FU, after which they were taken for propidium iodide (PI) staining. Figure 5B shows

that, as compared to the vehicle control (VC) cells (M2 = 2.37%), a greater number of curcumin-treated cells (M2 = 29.76%) migrated from G₀/G₁ phase towards S phase of cell cycle. It was also observed that as a consequence, fewer number of curcumin-treated cells (M1 = 40.44%) were present in G₀/G₁ phase than in the untreated cells (M1 = 85.95%). In QLCs that were exposed to combination treatment (curcumin + 5-FU), a profile similar to curcumin-only cells was observed in M1, M2, and M3 stages. However, a striking difference was seen at the M4 stage between the cells that were subjected to combination treatment (M4 = 30.58%) (green bar at M4 of Figure 5B) and those that were treated with 5-FU only (M4 = 9.87%) (red bar of at M4 Figure 5B). The difference indicates that the proliferative responses induced by curcumin sensitized the QLCs to killing by the antimitotic drug 5-FU.

QLCs exposed to combination treatment show higher caspase-3 activity

The flow cytometric analysis of the cell cycle effectively demonstrated that the exposure to curcumin was helpful in improving the outcome of antimitotic drug therapy [Figure 5B]. However, we wanted to confirm whether the

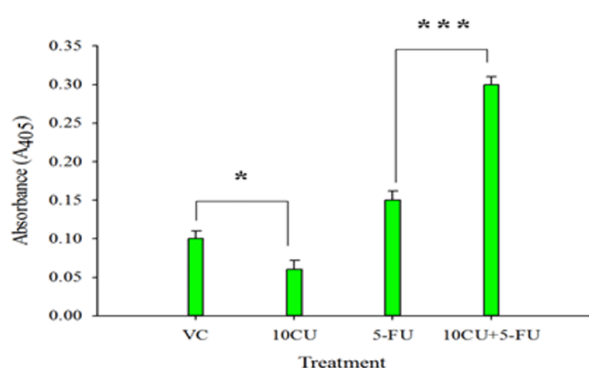


Figure 6: Combination therapy promotes higher caspase-3 activity in quiescent leukemic cells. QLCs were treated as represented in the graph. The treated cells were harvested and subjected to caspase-3 assay. Intensity of the color was measured at 400/405 nm by using microplate spectrophotometer. In the graph, VC represents vehicle control; 10CU represents the cells treated with curcumin only (10 µg/mL); 5-FU represents cells treated with 5-FU only (6 µg/mL), and 10CU + 5-FU represents combination treatment of 10 µg/mL of curcumin and 6 µg/mL of 5-FU. Serum-starved KG1a cells treated with 10 µg/mL of curcumin induce minimum caspase-3 activity as compared to VC. Cells treated with a combination of 10 µg/mL of curcumin and 6 µg/mL of 5-FU induces maximum caspase-3 activity as compared to the cells exposed to only 5-FU. The data represent mean \pm S.E.M of three independent experiments (* $P \leq 0.05$, *** $P \leq 0.001$)

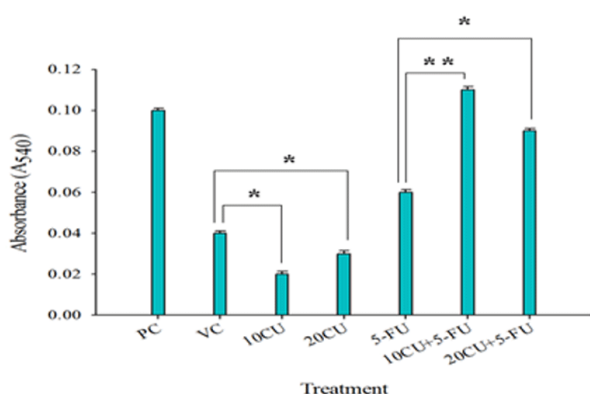


Figure 7: Combination treatment with low curcumin concentrations and 5-FU leads to higher generation of NO in quiescent KG1a cells: QLCs were subjected to various treatments as shown in the graph. After treatment, the cells were spun, supernatant was collected into fresh plates and to the supernatant was added an equal volume of Griess reagent. The plate was incubated for 15 min in dark at room temperature (RT) and the intensity of color was measured at 540 nm using microplate spectrophotometer. PC represents positive control, that is sodium nitrite solution (50 µM). VC represents vehicle control; 10CU and 20CU represent the concentrations of curcumin used, that is cells treated with only 10 µg/mL and 20 µg/mL respectively; 5-FU represents cells treated with only 6 µg/mL of 5-FU; 10CU + 5-FU, and 20CU + 5-FU represents cells treated with combinatorial treatment of curcumin (10 µg/mL or 20 µg/mL) and 5-FU (6 µg/mL). Serum-starved KG1a cells treated with 10 µg/mL and 20 µg/mL of curcumin generated minimum amount of nitrites as compared to VC, whereas cells treated with a combination of 10 µg/mL or 20 µg/mL of curcumin and 6 µg/mL of 5-FU, respectively, showed maximum levels of nitrite as compared to the cells treated with only 5-FU. The data represents mean \pm S.E.M of three independent experiments (* $P \leq 0.05$, ** $P \leq 0.01$)

QLCs that were being targeted by 5-FU were undergoing apoptosis. To check the apoptotic profile of QLCs treated with both curcumin and 5-FU, we performed caspase-3 assay as per manufacturer's instructions. As seen in Figure 6, there was indeed a high caspase-3 activity in cells treated with combination therapy when compared to those treated with only 5-FU. This confirms that curcumin sensitized the QLCs to undergo apoptosis in presence of the antimetabolic

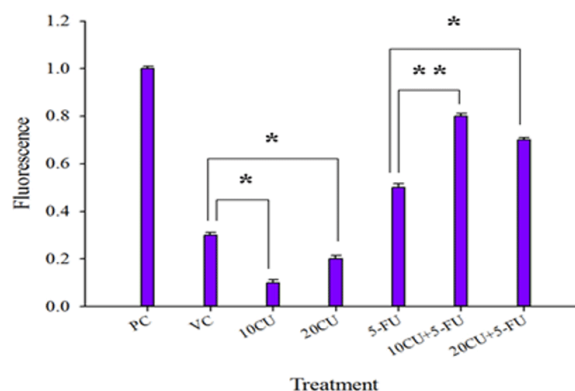


Figure 8: QLCs subjected to combination treatment generate higher levels of ROS: Quiescent cells generated from KG1a cells were incubated with low concentrations of curcumin only (10 µg/mL and 20 µg/mL), 5-FU only (6 µg/mL) and a combination of curcumin and 5-FU, as shown in the graph. The treated cells were collected and spun, and the cell pellet was incubated with 100 µL of DCFDA (10 µM) for 30 min at 37°C. After incubation the cells were centrifuged, and the pellet was resuspended in 1 \times PBS. The fluorescence was measured at 495-529 nm. In the graph, PC represents positive control that is hydrogen peroxide (50 µM), VC represents vehicle control; 10CU and 20CU are cell samples treated with curcumin only (10 µg/mL and 20 µg/mL, respectively), 5-FU represents 5-FU only (6 µg/mL) treated cells, and 10CU + 5-FU, 20CU + 5-FU represents combination treatment of curcumin (10 µg/mL or 20 µg/mL) and 5-FU (6 µg/mL), respectively. Quiescent KG1a cells treated with only 10 µg/mL or 20 µg/mL of curcumin generated minimum amount of ROS, as compared to VC. QLCs treated with a combination of 10 µg/mL or 20 µg/mL curcumin, respectively, and 6 µg/mL of 5-FU showed maximum ROS generation as compared to the cells treated with only 5-FU. The data represent mean \pm S.E.M of three independent experiments (* $P \leq 0.05$, ** $P \leq 0.01$)

drug 5-FU.

Low concentrations of curcumin and 5-FU together increase the levels of nitric oxide in QLCs

Since low concentrations of curcumin caused the proliferation of QLCs that were sensitive to 5-FU treatment, we wanted to check whether the kill seen in QLCs was being mediated by the expression of nitric oxide (NO). NO is known to react with superoxide at a high rate ($k \geq 6.7 \times 10^9 \text{ M}^{-1} \text{ s}^{-1}$) to form peroxynitrite, which is far more reactive and damaging than its precursors.^[40] The downstream products of superoxide, including hydrogen peroxide and peroxynitrite, are potent oxidants that induce oxidative injury of cells, resulting in apoptosis.^[41,42] Nitric oxide assay determines nitric oxide based on the enzymatic conversion of nitrate to nitrite by nitrate reductase.^[43,44] The reaction is followed by a colorimetric detection of nitrite as an azo dye product of the Griess reaction, which absorbs light at 540 nm. As seen in Figure 7, cells that were treated with a combination of both curcumin and 5-FU expressed higher levels of NO than cells that were treated with only curcumin or only 5-FU.

Combination treatment of quiescent KG1a cells with low concentrations of curcumin and 5-FU stimulates higher generation of reactive oxygen species

Generally, the production of ROS by mitochondria is a consequence of the blockade of the electron transfer chain. It has been well documented that NO can inhibit the activity

of several enzymes of the mitochondrial respiratory chain including complex I, complex II-III, and complex IV in the cells.^[41,45,46] The inhibition of mitochondrial respiration by NO may increase the electron leakage and cause the formation of endogenous ROS (mainly superoxide anion), which can be observed in submitochondrial particles.^[46-48] High levels of ROS may cause the oxidative damage of various cellular components and finally result in cell death.^[45,46] ROS is capable of causing oxidative damage to biomacromolecules, leading to lipid peroxidation, oxidation of amino acid residues (especially cysteine residues), formation of protein-protein cross-links, and DNA oxidative damage.^[41,49] Since we had seen a high expression of NO in cells treated with the combination of curcumin and 5-FU, we were interested in finding out whether these cells would also generate high levels of ROS. The QLCs treated with a combination of curcumin and 5-FU were subjected to DCFDA assay. It was observed [Figure 8] that combination treatment [curcumin (10 µg/mL and 20 µg/mL) + 5-FU (6 µg/mL)] led to an increase in the level of ROS generation (10 µg/mL = 80%, 20 µg/mL = 60%), as compared to that generated by only 5-FU treated cells (40%).

DISCUSSION

AML is a hematological malignancy that results from transformation of multipotent hematopoietic progenitors and leads to accumulation of immature myeloid cells in the bone marrow. Many studies have shown that curcumin demonstrates antiproliferative, antioxidative, cytotoxic, pro-oxidant, and antitumor activity in many human cell lines,^[1,19,21] including T and B leukemia, in a dose-dependent manner.^[50,51] Although it has been reported that curcumin induces apoptosis in human leukemia HL-60 cells,^[24] the exact pathway that leads to apoptosis of the HL-60 cells remains unclear. Another study has demonstrated that ROS is involved in the apoptosis induced by curcumin in HL-60 cells.^[25] Additionally, it has been shown that curcumin may inhibit proliferation and induce apoptosis of leukemic cells by arresting them in various phases of the cell cycle.^[13,14] It has also been suggested that curcumin may induce apoptosis in tumor cells by a mitochondria-dependent mechanism, suggesting that curcumin can activate cytochrome c caspase-3.^[37,38,45,46,48]

In the last few years, progressive studies have underscored the importance of a combination approach in the development of effective therapies against leukemia. It is becoming obvious that the molecular basis for most leukemias is far more complex than can be addressed by use of a single-target or single-drug approach. On one end, there are cycling cancer cells that are receptive to antimetabolic drugs; on the other end, there are mitotically inactive leukemic stem cells that evade traditional anticancer therapies. As a result, it is imperative to adopt a multitarget-based drug development paradigm for the treatment of complex human diseases that

work by different mechanisms of action. In the present study, we have demonstrated that low concentrations of curcumin push the quiescent leukemic cells into the cell cycle, thereby sensitizing them to the antimetabolic drug 5-fluorouracil. Although the molecular mechanism(s) behind the inhibitory effect of the combination therapy on leukemic cells have yet to be explained, this approach could be exploited to selectively target leukemic stem cells that are responsible for relapse associated with leukemia, and to develop novel anticancer therapies for the treatment of leukemia. Simultaneously, this approach could also be combined with other strategies employed in the *ex vivo* expansion of normal hematopoietic stem/progenitor cells.

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Nil.

Conflicts of interest

There are no conflicts of interest.

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EGFR mutation -- a commonly neglected mutation in squamous cell lung carcinoma

Rajeev Saini, Ullas Batra, Akhil Jain, Chaturbhuj Agrawal

Department of Medical Oncology, Rajiv Gandhi Cancer Institute and Research Centre, Sector 5, Rohini, New Delhi 110085, India.

Correspondence to: Dr. Rajeev Saini, Department of Medical Oncology, Rajiv Gandhi Cancer Institute and Research Centre, Sector 5, Rohini, New Delhi 110085, India. E-mail: rajeev.rajeev7@gmail.com

ABSTRACT

Lung cancer is the leading cause of cancer-related death worldwide. Advances in molecular biology have unveiled various targetable mutations with epidermal growth factor receptor (EGFR) being most common. EGFR testing is recommended for all locally advanced or metastatic adenocarcinoma lungs but recommendation in squamous histology is uncertain. However, just on the basis of histology, EGFR testing should not be withheld in patients diagnosed as squamous cell cancer on small biopsy, in females, never smokers and Asians. We report two cases with squamous cell lung cancer diagnosed on small biopsy, in non smoker females with EGFR mutations emphasizing the importance of testing in such population.

Key words: Epidermal growth factor receptor mutation; squamous cell lung carcinoma; never smoker; small biopsy

INTRODUCTION

Lung cancer is the most common cancer in the world accounting for 12.9% of total cases and 19.4% of total cancer related mortality.^[1] Advances in molecular biology have led to the identification of mutations within the epidermal growth factor receptor (EGFR), and the finding that these mutations make tumors exquisitely sensitive to EGFR tyrosine kinase inhibitors (TKIs), has revolutionized treatment of non-small cell lung cancer (NSCLC). EGFR mutations are more common in never-smokers, in patients with Asian ethnicity, and in patients with adenocarcinoma histology.^[2] However, solely on the basis of histology, EGFR testing should not be excluded in patients with squamous cell cancer, especially females, never smokers, Asian ethnicity and squamous histology diagnosed on small biopsy as adenocarcinomatous component cannot be ruled out on small biopsy specimens.^[3] We present two cases of squamous cell lung cancer diagnosed on small biopsy in non smoker females with EGFR mutations who benefitted with oral TKIs.

CASE REPORT

Case 1

A 56-year-old female, diabetic, hypertensive, non smoker presented with history of cough, weight loss and right sided weakness. Magnetic resonance imaging brain showed two hypodense lesions in left frontal lobe. Positron emission

tomography-computed tomography revealed right lung mass with mediastinal lymph nodes, brain, adrenal, pancreatic and bone lesions. A core needle biopsy from lung mass revealed squamous cell carcinoma, p40 positive and thyroid transcription factor (TTF) negative [Figure 1]. In view of symptomatic brain metastasis, she was treated with whole brain radiotherapy followed by two cycles of gemcitabine and carboplatin based chemotherapy. However, in view of poor tolerability due to grade 4 neutropenia and poor performance status, chemotherapy could not be given. Her biopsy was reassessed for EGFR mutational analysis and showed L858R mutation positive. She was started on erlotinib and imaging studies after 2 months of therapy demonstrated significant tumor response in the pulmonary lesions and in the metastatic sites.

Case 2

A 44-year-old female, diabetic, hypertensive, non smoker presented with history of breathlessness. Bronchoscopy showed malignant intermediate bronchus obstruction. Bronchial biopsy revealed squamous cell carcinoma expresses p40 [Figure 2] but negative for TTF1. In view of her being non smoker EGFR mutation was tested and was positive for exon 21L858R mutation. After 3 cycles of gemcitabine and cisplatin based chemotherapy there was partial response and now she was on maintenance erlotinib with good disease control.

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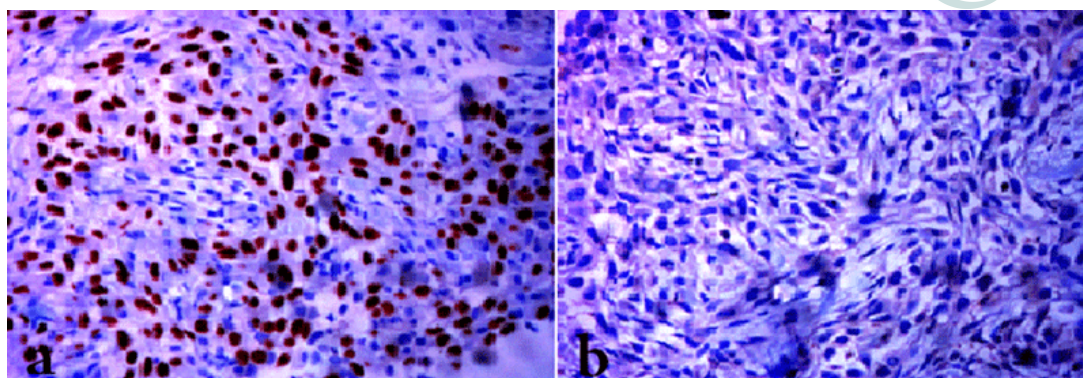


Figure 1: Squamous cell carcinoma positive for p40. (a) Lacking expression of thyroid transcription factor; (b) immunohistochemistry (magnifications, $\times 40$)

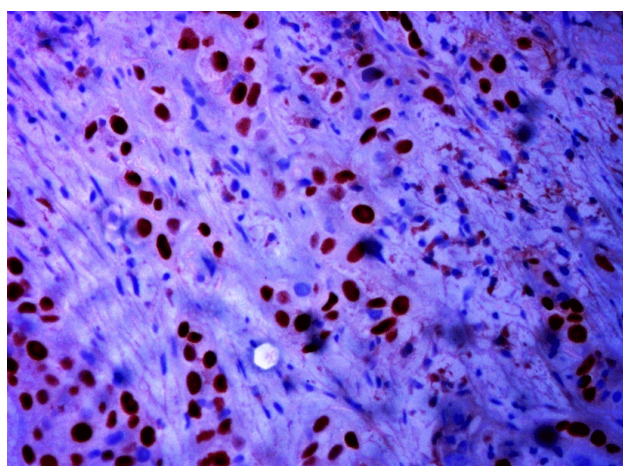


Figure 2: Immunohistochemistry (magnification, $\times 40$) on biopsy for case 2 positive for p40

DISCUSSION

Deeper understanding of the pathobiology of NSCLC has led to the development of small molecules that target genetic mutations known to play critical roles in the progression to metastatic disease. EGFR mutation is one of the most common targetable mutations in NSCLC particularly in non squamous histology. The incidence of EGFR mutations in NSCLC varies by ethnicity, with studies estimating a range from 10-15% of Caucasians to 40-50% of Asians.^[4] In India the frequency of EGFR mutations has been found to be between 25-40% in various studies.^[5] However, EGFR mutation occurs only in less than 5% patients of squamous cell histology. We present two cases of squamous cell carcinoma of lung with activating EGFR mutation. Thus, just on the basis of histology, patient should not be deprived of potentially beneficial non toxic therapies and can derive same benefits with oral TKIs as in patients of adenocarcinoma histology,^[6] especially in light of some important caveats regarding exclusion of testing in all cases diagnosed as squamous cell carcinoma. First, a small biopsy sample showing squamous morphology does not exclude the possibility of an adenocarcinomatous component elsewhere in the lesion. Second, the distinction between adenocarcinoma and squamous cell carcinoma can be extremely challenging in some cases.^[7] EGFR

mutations have been found to be more common in female patients, never-smokers, and patients of Asian ethnicity irrespective of histology. In conclusion, EGFR testing should be tested in patients with squamous cell lung cancer, especially in females, never smokers, Asian ethnicity and squamous histology diagnosed on small biopsy.

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Conflicts of interest

There are no conflicts of interest.

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Malignant eccrine acrospiroma with nodal and bone metastasis

Burhan Wani, Shiekh Aejaaz Aziz, Mohamad Hussain Mir, Gull Mohammad Bhat, Abdul Rashid Lone

Department of Medical Oncology, Sher-i-Kashmir Institute of Medical Sciences, Srinagar 190011, Kashmir, India.

Correspondence to: Dr. Burhan Wani, Department of Medical Oncology, Sher-i-Kashmir Institute of Medical Sciences, Srinagar 190011, Kashmir, India. E-mail: burhan4187@gmail.com

ABSTRACT

Acrospiromas are cutaneous tumors of sweat duct differentiation. Although various eccrine sweat gland tumours including benign acrospiroma are widely reviewed, malignant acrospiroma is rarely reported. Clinically, they resemble other cutaneous lesions and the primary treatment is wide local excision with or without lymph node dissection. The efficacy of adjuvant chemotherapy and radiation therapy requires further investigation.

Key words: Acrospiroma; metastasis; chemotherapy; radiotherapy

INTRODUCTION

Acrospiroma represents a group of benign ductal tumors of the eccrine sweat glands that sometimes are connected to the skin, ranging from solitary plaques to exophytic papules or dermal nodules.^[1] Malignant acrospiroma (Syn: malignant nodular/clear cell hidradenoma, malignant clear cell acrospiroma, clear cell eccrine carcinoma, primary mucoepidermoid cutaneous carcinoma) comprises a group of rare epidermal, juxta-epidermal, and dermal ductal carcinomas that may coexist with their benign counterparts and have the potential for regional lymph node and, very rarely, distant metastases.^[2] The primary treatment is wide local excision with or without lymph node dissection.^[3] We describe a case of a malignant acrospiroma involving inguinal region with metastases to inguinal lymph nodes and bones in a 37-year-old man despite initial wide local excision. Although various eccrine sweat gland tumors including benign acrospiroma have been widely reviewed, malignant acrospiroma is rarely reported and thus the literature on their response to chemotherapy is limited.

CASE REPORT

A 37-year-old man presented at our medical oncology outpatient department with complaints of a mass in the right inguinal region for over 1 year with no history of antecedent trauma. The mass gradually increased in size and was associated with mild discomfort. There was no skin ulceration or discharge. Examination revealed a rounded mass adherent to skin with diameter of 4 cm in

right inguinal region. The swelling was firm in consistency and mildly tender. There was another mass 2 cm below this measuring 3 cm × 2 cm, firm in consistency, mobile, non-tender with normal overlying skin, felt to be a lymph node clinically.

The patient was operated on and excision of the mass along with inguinal nodal dissection. Pathology revealed dermal appendage neoplasm (acrospiroma -- of hydra adenoma type), well-circumscribed, with mitotic figures (< 2/hpf). No necrosis was seen. Nodal tissue showed metastasis from the same tumor [Figures 1 and 2]. The patient was put on regular follow up and no adjuvant chemo/radiotherapy was given in view of lack of clear benefits from either of these modalities.

However, patient was lost to follow up and presented 1 year later with swelling in same area. The swelling had appeared 4 months earlier and gradually increased in size, associated with mild discomfort. Examination revealed a firm, smooth swelling, not adherent to skin, round in shape with dimensions of 6 cm × 5 cm in the right inguinal region.

Computed tomography (CT) with contrast of chest, abdomen and pelvis revealed a well-defined soft tissue density lesion in the right inguinal region, with minimal fat stranding. The lesion showed mild heterogenous enhancement [Figure 3].

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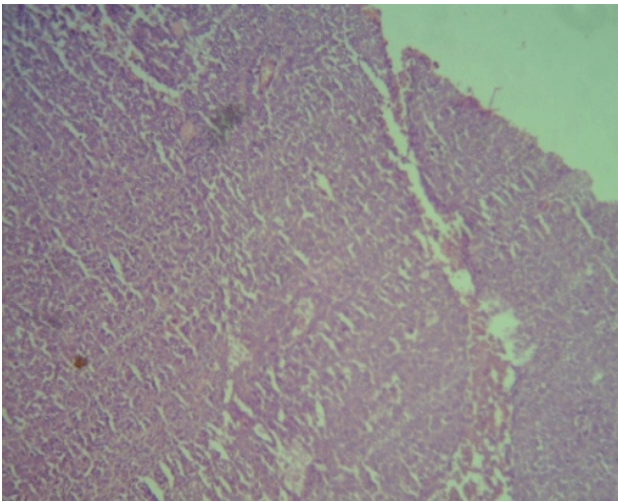


Figure 1: Light microscopy showing dermal appendage (acrospiroma) (magnification, × 10)

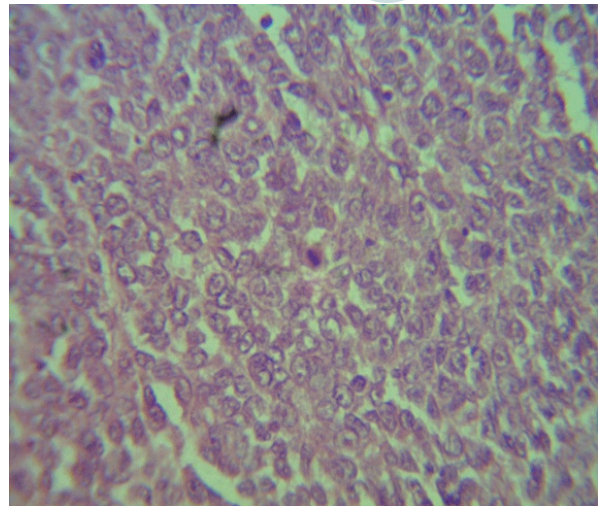


Figure 2: High power view of malignant acrospiroma (magnification, × 100)

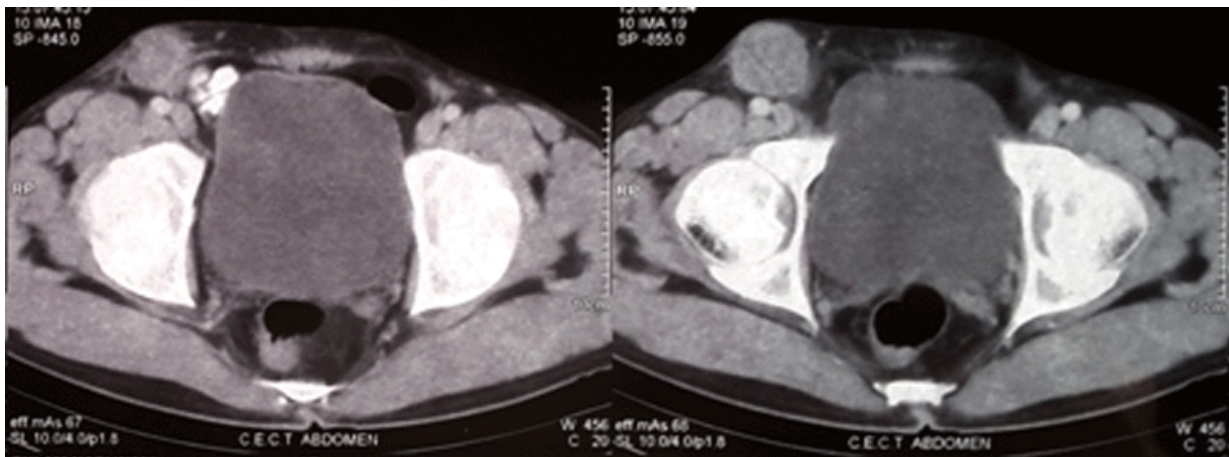


Figure 3: Computed tomography pelvis showing nodal mass involving right inguinal region

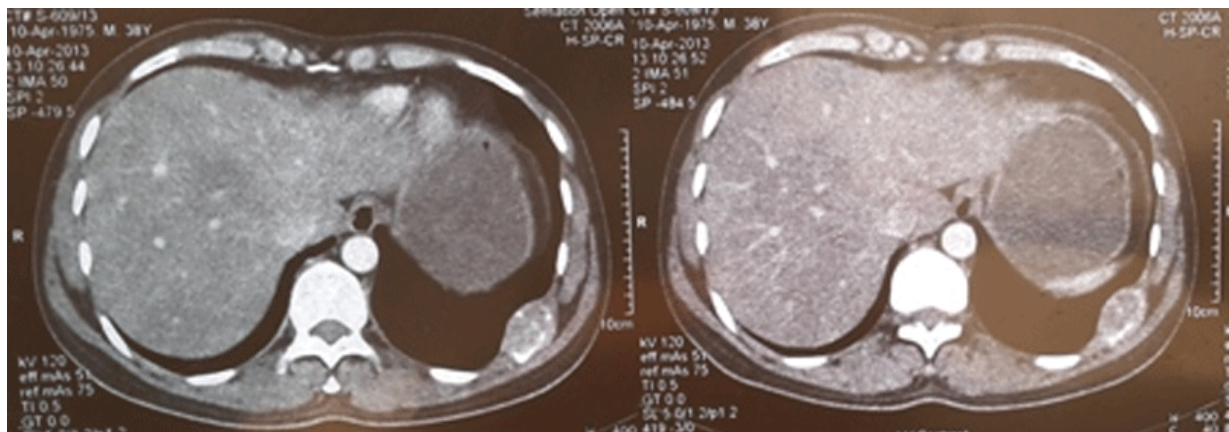


Figure 4: Computed tomography chest showing destructive lesion involving posterior end of 9th rib

FNAC smears of right inguinal mass revealed features of metastatic deposits from a round cell tumor. Immunohistochemistry on the cell block showed strong positivity for vimentin, focal positivity for NSE, and negative staining for desmin, MIC-2, and synaptophysin such features favouring a diagnosis of malignant acrospiroma.

The patient went to surgery and a right inguinal dissection with excision of the mass was done. Grossly, the specimen revealed a fibro-fatty, globular, soft tissue lesion measuring

15 cm × 8 cm × 6 cm. On serial sectioning a globular, encapsulated grey-white area measuring 5 cm × 5 cm was identified, with 6 nodes being removed. Microscopic examination again revealed a metastatic cutaneous adnexal tumor (malignant acrospiroma) with 2 of 6 nodes involved.

In view of only a locoregional recurrence, the patient was planned for external beam radiotherapy to right inguinal region with a total of 45 Gy in 20 fractions. However, after 13 treatment days, the patient developed ulceration of local site and further radiotherapy was withheld. The

patient was lost to follow-up but and presented 1 year later, again with a firm nodule at the right inguinal region measuring 1 cm × 1 cm. Repeat CT of chest/abdomen/pelvis revealed a soft tissue thickening with a solitary, round lesion in the right inguinal region along with an expansile soft tissue density lesion with bone erosion involving the left 9th rib, suggestive of metastases [Figure 4].

Patient was again subjected to wide local excision of right inguinal lesion and also of 9th rib mass. Three nodes also were dissected, with the largest measuring 3 cm × 3 cm, along with a 4 cm × 5 cm mass present over and adherent to 9th rib postero-laterally. Microscopy revealed sections from both the rib lesions as well as groin nodes showing infiltration by malignant sweat gland tumor. Marrow of rib bone revealed infiltration of same tumor. Bilateral iliac bone marrow aspiration and biopsy were negative for tumor.

Patient was subsequently given adjuvant chemotherapy consisting of paclitaxel 175 mg/m² and cisplatin 80 mg/m² every 3 weeks for 6 cycles. The patient is on regular follow up and in clinical remission for the past 18 months.

DISCUSSION

Acrospiromas are cutaneous tumors of sweat duct origin and differentiation. They usually present as slowly enlarging 1 cm to 2 cm nodules in middle-aged or older adults without site predilection. The term eccrine acrospiroma was first coined by Johnson and Hewig, in 1969, because, by histologic and histochemical studies, the cells were believed to mimic those of the eccrine sweat gland.^[4] Histologically, these lesions are subclassified according to the location of the tumor in relation to the epidermis, with those confined primarily to epidermis as epidermal acrospiroma and those involving both epidermis and dermis as juxtaepidermal acrospiroma or just eccrine poroma. Those which are confined exclusively to dermis or have minimal connection to epidermis are termed dermal acrospiroma or hidradenoma.^[1]

Malignant acrospiroma comprises a group of rare epidermal, juxtaepidermal, and dermal ductal carcinomas occurring over the head and neck, anterior trunk, or extremities.^[5,6] One series described an incidence of only five cases in a group of 750,000 evaluated individuals over an eight-year period.^[7] They follow a predictable pattern from the initial tumor site to regional lymph node and ultimately to systemic spread.^[3,8]

In the present case, the lesion recurred multiple times despite initial wide local excision and adjuvant radiotherapy, carried out following the first recurrence. Secondly, the lesions were slowly growing with delayed recurrent nodal and bone metastases and hence the need for prolonged follow up.

Malignant acrospiromas are treated by wide local excision, but with a local recurrence rate of around 50%.^[9]

In one case there was described the use of wide local excision with adjuvant radiotherapy for malignant eccrine acrospiroma of the scalp and left parotid, which eventually had local recurrence in the parotid region after 2 years.^[3] In another case there was described a more radical surgical approach of amputation of the leg with regional lymph node dissection. This was required for clinical control of extremity acrospiroma.^[10] In another reported case, a 66-year-old female with a recurrent malignant acrospiroma of the chest treated by wide radical resection, including chest wall excision, followed by reconstructive surgery and radiotherapy. After 16 months, there was no evidence of local recurrence or distant metastasis.^[11]

One group described the role of radiotherapy in malignant eccrine acrospiroma, wherein 3 cases of malignant acrospiroma were treated with postoperative radiotherapy with doses of 71-76 Gy to the primary surgical bed and 50 Gy to the draining lymph node basin, with modest disease-free survival (27 and 35 months) in 2 of the 3 cases. They suggested that certain histological features such as dermal lymphatic invasion, nerve sheath involvement, deep structural infiltration, positive resected margins, and extracapsular lymph node extension may identify a high risk of recurrence and merit postoperative radiotherapy.^[12] The role of chemotherapy in eccrine sweat gland carcinomas, and especially malignant acrospiromas, is not clear. Various case reports and case series have reported on the use of a multitude of drugs in various sweat gland carcinomas including cyclophosphamide and doxorubicin, bleomycin, cisplatin, mitomycin C, with partial response and a median duration of response of 4 to 16 months.^[13-16] There are also isolated reports of response to taxanes (docetaxel and paclitaxel).^[17]

Analyzing all the available literature, we conclude that wide local excision is the treatment of choice for these rare skin appendage tumors when localized, while adjuvant radiotherapy may provide some additional benefit in local control. Poly-chemotherapy is thought to be an option for more extensive lesions and paclitaxel-containing regimens could provide a viable option for palliation. However, more evidence in the form of case series and case reports is needed to establish its usefulness.

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Conflicts of interest

There are no conflicts of interest.

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Evaluation of anti-metastatic effect of chitosan nanoparticles on esophageal cancer-associated fibroblasts

Pravin D. Potdar, Aashutosh U. Shetti

Department of Molecular Medicine and Biology, Jaslok Hospital and Research Centre, Mumbai 400026, India.

Correspondence to: Dr. Pravin D. Potdar, Department of Molecular Medicine and Biology, Jaslok Hospital and Research Centre, Mumbai 400026, India. E-mail: ppravin012@gmail.com



Dr. Pravin D. Potdar's present interest is to study molecular profiling of Circulating Tumor Cells (CTC), Circulating Tumor DNA, Cancer Associated Fibroblasts and Cancer Stem Cells involved in metastatic process of cancers, and to see how this process can be reverted back to normal by using innovated technologies which include nanotechnology and nanomedicine.

ABSTRACT

Aim: Esophageal cancer is one of the major types of cancers, causing death of approximately 5% of all cancer deaths. This is due, in large part, to both relatively ineffectual and unavailable treatment. In order to develop an effective treatment strategy against esophageal cancer, it is important to target metastatic genes. In the present study, we have used a cancer-associated fibroblast (CAF) cell line derived from culturing peripheral blood mononuclear cells from a metastatic esophageal cancer patient to see whether chitosan nanoparticles (Ch-Np) treatment can modulate the metastatic phenotype of CAF cells by using various cellular and molecular markers. **Methods:** A CAF cell line was developed from peripheral blood mononuclear cells (PBMC) from a metastatic esophageal cancer patient. The cells were treated with 100 µg/mL of chitosan nanoparticle *in vitro* for the morphological and oncogenic characteristic studies, along with the expression of various genes involved in process of tumor development and metastasis. Techniques such as Light and Phase Contrast Microscopy, cell growth rate, Scratch metastatic assay, and molecular profiling were carried out to see changes in CAF cells before and after Ch-Np treatment. **Results:** It was observed that CAF cells grew in monolayer and had a doubling time of 25 ± 0.38 h. Morphologically, the cells had a fibroblastic appearance. After treatment with 100 µg/mL of Ch-Np *in vitro*, there was an increased doubling time to 30 ± 0.83 h. Similarly, Scratch Assay showed an inhibition in the metastatic property of these cells. These findings were confirmed with gene expression studies. It was also observed that there was complete down-regulation of metastatic genes MMP1 and MMP9 and chemokines such as CXCR-4, CXCR-7, CCR-5, and SDF-1, indicating that Ch-Np inhibited the metastatic characteristic of CAF cells. **Conclusion:** This study has shown that there was an inhibition of metastatic properties of CAF cells after treatment with Ch-Np, suggesting that Ch-Np can be a delivery system used for targeting cancer cells for treatment of esophageal cancer.

Key words: Cancer-associated fibroblast; molecular markers; metastasis; chitosan nanoparticle; anti-metastatic; metastatic genes

INTRODUCTION

The tumor microenvironment plays a crucial role in development and progression of cancers. The microenvironment is mainly comprised of specialized stroma cells known as fibroblasts, also called cancer-associated fibroblast (CAF) or myofibroblast. CAFs secrete various tumor promoting factors as well as angiogenic factors which accelerates tumor growth.

Tumor Growth Factor β (TGF- β) and Hepatocyte Growth Factor (HGF) are the mediators released by CAFs. These cause increased cell proliferation, more angiogenesis, and reduced apoptosis.^[1] CAFs have been found to play an important role in a variety of cancers, including breast, pancreatic, prostatic, and esophageal cancers.^[2,3]

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Esophageal cancer is an aggressive cancer, affecting 450,000 patients. In esophageal squamous cell carcinoma (ESCC) expression of HGF and fibroblast growth factor (FGF) in CAFs has been found to be related to tumor cell proliferation.^[4] CAF-derived wnt2, an important signaling molecule, was able to enhance a process called epithelial mesenchymal transition (EMT). This EMT involves loss of intracellular adhesion and polarity by tumor cells of epithelial origin. These cells can be transformed into mesenchymal cells with the capability of migration and invasion.^[5] Protein levels of CAF are also related to a poor prognosis of patients with esophageal cancer. Also, proteins such as α smooth muscle actin, CD-10, and periostin have been found to be related to the poor patient survival.^[3] Thus, it is evident that CAFs are important to tumor cells in esophageal cancer since they are associated with invasion, migration, and a poor prognosis. Many drug trials are carried out in order to develop a successful treatment strategy against esophageal cancer, but the role of CAF has been neglected. Hence, it is essential to attempt to target their CAF cells in order to prevent tumor progression.

In current cancer research nanoparticles are replacing traditional chemotherapeutic drugs because of their specificity, small size, and permeability into cells. Nanoparticles made up of biodegradable material such as chitosan have appeal since they are cheaper, do not involve toxic chemicals in their preparation, and have low cell cytotoxicity.^[6] The chitosan nanoparticle has shown therapeutic significance in various cancers, including breast, gastric, and oral cancers. Chitosan nanoparticles have not been explored in esophageal cancer and their effect on CAFs has not yet studied. The current project was designed to understand the effects of chitosan nanoparticle on human peripheral blood-derived CAF by performing gene expression studies. We have attempted to demonstrate that chitosan nanoparticles alter expressions of genes involved in esophageal tumors and have found that these nanoparticles effectively reduced the metastasis of CAF cells. These results suggest that using chitosan nanoparticles targeting esophageal CAFs could be a potential therapeutic strategy against esophageal cancers.

METHODS

Materials

Low Molecular weight Chitosan ($\geq 75\%$ deacetylation), sodium Tri-polyphosphate (sTPP), Acetic Acid, 1N NaOH, D/W, Low-glucose Dulbecco's Modified Eagle Medium (DMEM), Fetal Bovine Serum (FBS), Penicillin Streptomycin (PenStrep), L-Glutamine, Vitamin C, Phosphate Buffer Saline (PBS), Trypsin EDTA, TRIZOL reagent, cDNA Preparation kit (Applied Biosystem, USA), Agarose, Primers for Actin, Keratin18, Vimentin, VEGF, MMP1, MMP9, E-cadherin, CXCR-4, CXCR-7, CCR5, Sdf1 α , Oct4, Nanog, SOX-2 were purchased from Sigma Chemicals, USA.

Development and maintenance of esophageal CAF

Peripheral blood from an esophageal squamous cell carcinoma (ESCC) patient was taken for extraction of CAF. Ficol-gradient was performed and separated cells cultured in RPMI supplemented with 10% FBS, Penstrep, and glutamate. After 24 h of culture the media were replaced with complete DMEM supplemented with 10% FBS, 1% Penstrep, 0.2% Glutamate, and Vit C. CAFs were seen after about 34 days of culturing. The cells were confluent within a week, then stored in -80°C while some were maintained in culture. These cells were labeled as esophageal CAF. Frozen cells were revived and cultured in growth medium at passage number 31. Culture dishes were incubated at 37°C with 5% CO_2 and the media removed on alternate days, followed by washing of cells with PBS and supplementing with new media. After reaching confluency cells were trypsinized with 1% trypsin and transferred into fresh flask for expansion.

Staining of CAF cell line (Giemsa, Alizarin Red, and Oil Red staining)

Culture plates were washed with PBS, fixed with methanol (50%), and incubated for 30 min at 4°C . The plates were then washed with D/W to remove the methanol. Fixed and washed plates of CAF were stained with Giemsa stain. Alizarin Red staining was required for the methanol-fixed cells, which were stained with 2% Alizarin stain (pH 4.2) for 30 min. After oil red staining, the fixed and washed cells were incubated with 60% isopropanol for 5 min. This was followed by removing the isopropanol and then staining with the 0.3% oil red for 5 min. After staining the plates were washed with D/W to remove excess stain.

Phase contrast microscopy

Inverted Phase contrast Microscope (Carl Zeiss Co.) was used for studying morphology of the cultured cells. The microscope was attached to the computer having TS View software for observing and capturing the images. The cells were monitored regularly with the use of phase contrast microscope and images captured.

Chitosan nanoparticle (Ch-Np) preparation

Ch-Np was prepared using the ionic gelation method. Low molecular weight chitosan was dissolved in 1% acetic acid under constant stirring conditions. Zero point one percent sTPP prepared in D/W was added in the chitosan solution, drop by drop, under constant stirring. A solution change from clear to turbid was taken as confirmation for nanoparticle formation. pH of the chitosan solution was adjusted to 7 using 1N NaOH. Formed nanoparticles suspended in the solution were separated by centrifuging at 2000 g for 3 min. The supernatant was discarded and the nanoparticles were washed with DMEM and again centrifuged in order to remove any chemical residue. The nanoparticles were then suspended in the media for later use.

Growth curve for control vs. treated CAF

Cells were plated at a density of 5×10^4 per well in a 6 well plate and fed with the DMEM medium. The cells were collected from each well at different time intervals, i.e., 24 h, 48 h, 72 h, and 96 h. For each time point the cells were washed with $1 \times$ PBS and trypsinized. The trypsinized cells were mixed with equal amount of Erythrocin B. The cell count was taken by using Neubauer hemocytometer. The cell growth rate was carried out for control and 100 μ g treated Ch-Np. The experiment was repeated three times and average growth and Standard Deviation were calculated for each time point.

Cellular morphology of CAF cells

CAF cell morphology was observed before and after treatment with Ch-Np. Sixty-five millimeter petri dishes were seeded with 5×10^4 cells per plate. Two plates were taken, one as untreated control whereas another dish was treated with 100 μ g/mL Ch-Np. Cell morphology was observed under phase contrast microscopy for after 24 h, 48 h, and 72 h of treatment and compared to control cells.

Molecular marker analysis

Two 65 mm petri dishes were seeded with 20×10^4 cells per plate. The cells were then washed with PBS and fed with new DMEM daily. After 2 days one plate was treated with 100 μ g/mL Ch-Np. After 24 h of treatment the cells were washed with PBS and RNA extraction from cells was carried out using TRIZOL method (Invitrogen). cDNA was

prepared from extracted RNA by using cDNA Reverse transcriptase kit. Gene expression studies were performed using PCR. The PCR mix consisted of ammonium sulphate buffer including 1.5 mm $MgCl_2$, 200 μ m of each of the dNTPs, 200 ng/ μ L each primer, 1U *Taq* Polymerase, and 5 μ L cDNA. Pluripotency markers (Oct-4, Nanog, SOX2), differentiating markers (Keratin 18, Vimentin, E-Cadherin, VEGF), chemokine and cytokine (CXCR-4, CXCR-7, CCR5 and Sdf-1 α), and metastatic markers (MMP1, MMP9) were used. Primers and annealing temperatures used for these genes are mentioned in Table 1. Initial denaturation was carried out at 95°C followed by denaturation at 94°C; annealing (specified in Table 1), extension at 72°C and final extension at 72°C for 7 min. Forty cycles were run for each PCR followed by gel loading and observation under UV-illuminator and photographed.

Scratch assay for evaluation of CAF migration

Two 65 mm plates were initially seeded with 5×10^4 cells per plate. The cells were then allowed to reach confluency. After reaching confluency, both dishes were scratched with the help of a sterile scalpel. Care was taken to scratch equal areas in both culture plates. This caused a loss of cells on the scratched area. The scratched control plate was kept as it is whereas other the scratched plate was treated with 100 μ g/mL Ch-Np and incubated at 37°C at 5% CO_2 . The scratched area was observed under the phase contrast microscope after 24 h, 48 h, and 72 h of treatment and photographed for cell migration. This experiment was

Table 1: Primer sequence, annealing temperature and size of band for molecular markers

Name	Primer	Annealing (°C)	Size (bp)
Actin	Upstream	55	417
	Downstream		
Oct4	Upstream	55	310
	Downstream		
Nanog	Upstream	55	256
	Downstream		
SOX2	Upstream	57	264
	Downstream		
Keratin	Upstream	55	357
	Downstream		
Vimentin	Upstream	62	426
	Downstream		
VEGF	Upstream	62	422
	Downstream		
E-Cadherin	Upstream	60	422
	Downstream		
MMP1	Upstream	55	427
	Downstream		
MMP9	Upstream	64	405
	Downstream		
CXCR-4	Upstream	60	273
	Downstream		
CXCR-7	Upstream	60	293
	Downstream		
CCR-5	Upstream	60	261
	Downstream		
Sdf-1 α	Upstream	60	188
	Downstream		

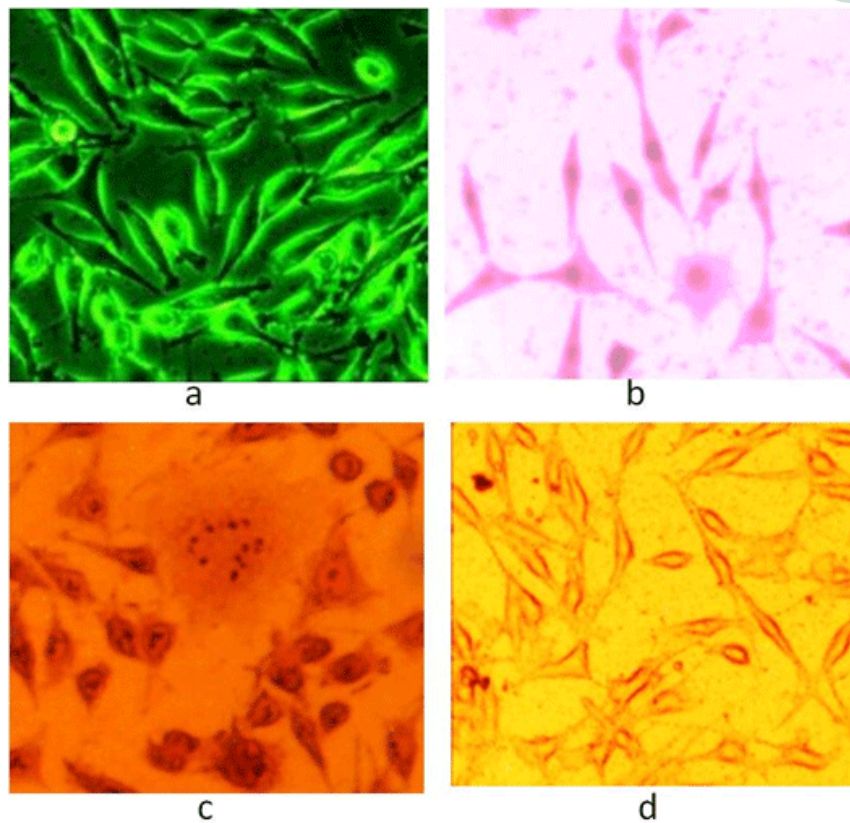


Figure 1: (a) Phase contrast microscope image of CAF; (b) giemsa staining; (c) alizarin staining; (d) oil red staining of CAF. CAF: cancer associated fibroblast

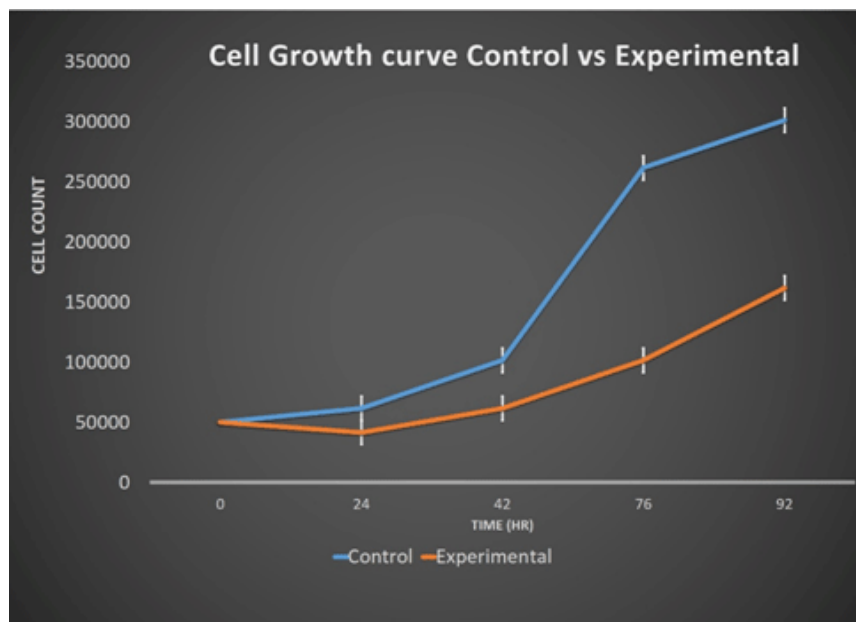


Figure 2: Cell growth curve for control untreated cells vs. Ch-Np-treated cells effect of Ch-Np on alignment of CAF. CAF: cancer associated fibroblast

repeated twice with the same number of cells and using same scalpel for making the scratch.

RESULTS

Morphological characterization of CAF cells by phase contrast microscopy

Esophageal CAF showed fibroblast-like appearance,

having extended cellular filaments as shown in Figure 1a. Giemsa Stain stained the nucleus of CAF, as shown in Figure 1b, making the nucleus completely visible and showing clear cytoplasm. Calcium granules within CAF cytoplasm of were stained by Alizarin Stain as shown in Figure 1c. Oil Red staining did not impart any color on the cells as shown in Figure 1d, indicating that there were no adipocytes.

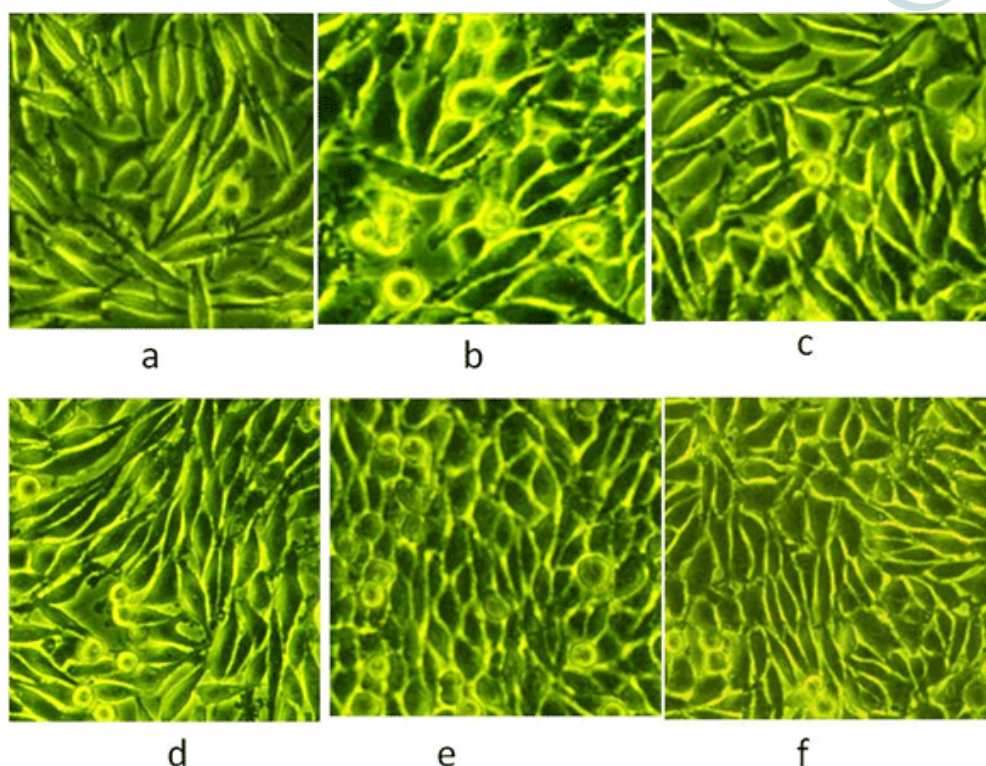


Figure 3: (a) Randomized alignment of control cells after 24 h; (b) randomized alignment of control cells after 48 h; (c) randomized alignment of control cells after 72 h; parallel alignment of Ch-Np-treated cells [(d), (e), (f)]

Growth curve for control vs. treated CAF

Growth curve for untreated CAF showed a gradual increase in the number of cells during 0-24 and 24-48 h. After 48hr the number of cells almost doubled. However, the 72-96 h time duration did not show a doubling of cells. This experiment was repeated three times and overall doubling time for untreated cells was 25 ± 0.38 h [Figure 2]. In the case of treated cells, during first 24 h, the cell count was less than the initially seeded cells. Then, cells showed a gradual increase in number. Importantly, the doubling rate of treated cells was increased because the number of cells after 92 h of culturing in the treated plate was less than that of control cells, as shown in Figure 2. This experiment was repeated three times and the overall doubling time for treated cells was 30 ± 0.83 h [Figure 2].

Cellular morphology of CAF cells

Phase-contrast morphology of untreated and Ch-Np-treated esophageal CAF was observed at 24, 48 and 72 h. Untreated CAF cells showed random growth and cells were overlapping with each other, as shown in Figure 3a, 3b, and 3c, whereas in the case of Ch-Np-treated plates, the cells exhibited monolayers with equal gaps and looked parallel to each other, as shown in Figure 3d, 3e, and 3f. These cells did not overlap with each other as was observed in the control CAF cells. This seems to indicate that they changed their malignant phenotype towards a normal phenotype by Ch-Np treatment.

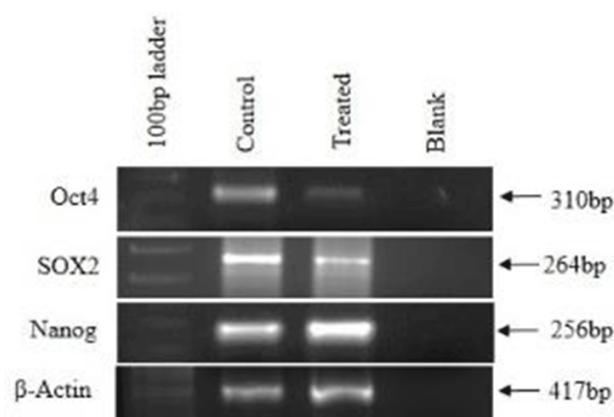


Figure 4: Expression of pluripotency markers in control and treated cells

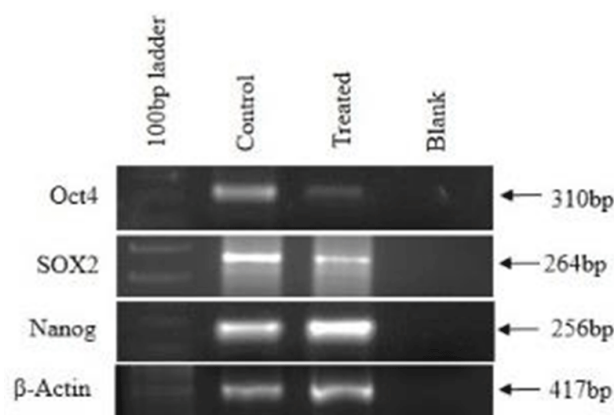


Figure 5: Expression of differentiating markers in control and treated cells

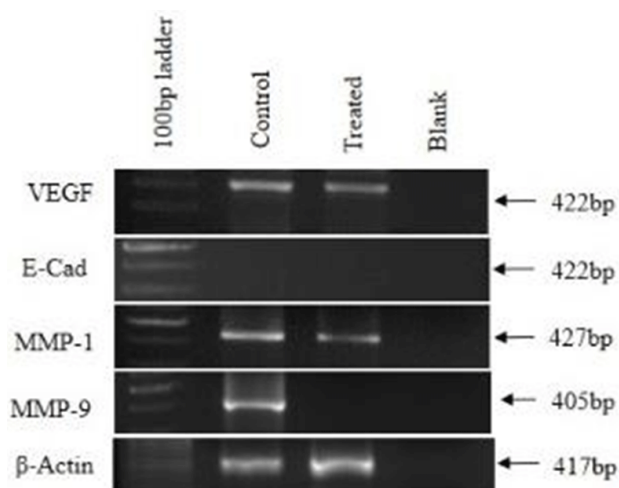


Figure 6: Expression of metastatic markers in control and treated cells

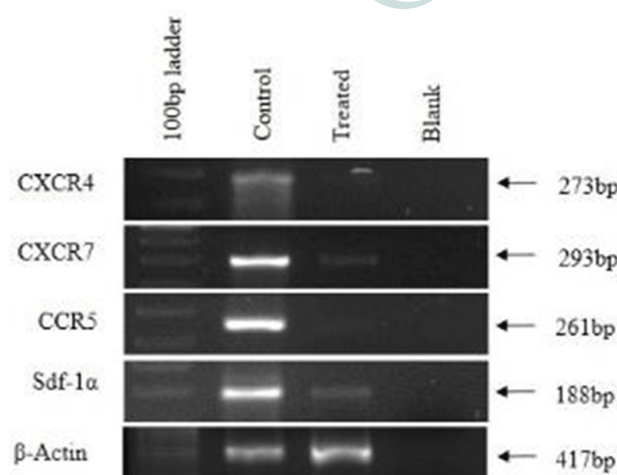


Figure 7: Expression of chemokine and chemokine receptors

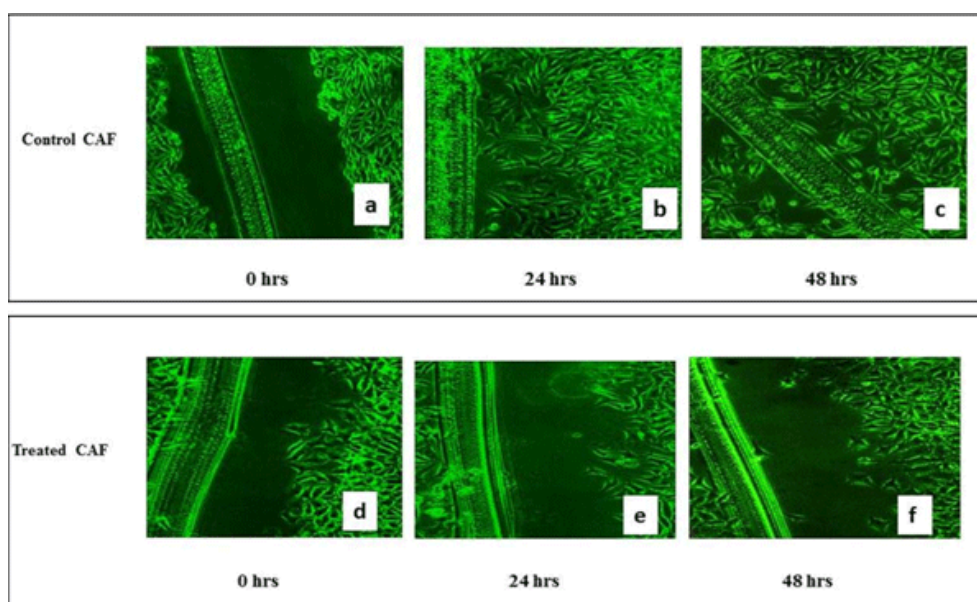


Figure 8: (a) Scratched Assay performed on control CAF after 24 h; (b) Scratched Assay performed on control CAF after 48 h; (c) Scratched Assay performed on control CAF after 72 h; (d) Ch-Np-treated cells after 24 h; (e) Ch-Np-treated cells after 48 h; (f) Ch-Np-treated cells after 72 h

Molecular marker studies

This study was undertaken to evaluate mRNA expression of pluripotency, differentiation, metastatic spread, and chemokine markers in CAF cells before and after treatment with Ch-Np by using specific primers as described in Table 1.

Pluripotency markers in CAF and Ch-Np-treated CAF

Pluripotency of cells is defined as properties of stem cells which allow cells to proliferate indefinitely. Oct4, Nanog, and Sox2 are known as such markers. These markers were studied in untreated and treated CAF cells. It was shown that these CAF cells normally expressed Oct4, Nanog, and Sox2, indicating their proliferative activity as transformed cells. However, in the Ch-Np treated cells, Oct4 and Sox2 genes were down regulated and Nanog remained unchanged, as shown in Figure 4.

Differentiating markers in CAF and Ch-Np-treated CAF

Keratin18 and Vimentin were the two differentiating markers studied in CAF- and Ch-Np- treated cells. Figure 5 shows prominent expression of Keratin18 and Vimentin. However, both these genes were down-regulated in Ch-Np-treated cells.

Metastatic markers in CAF and Ch-Np-treated CAF

VEGF, MMP1, and MMP9 were studied as metastatic genes in CAF and Ch-Np-treated CAF. There was slight down-regulation of VEGF and MMP1 genes in Ch-Np-treated CAF. However, complete down-regulation of MMP9 was observed in Ch-Np-treated CAF [Figure 6]. Also studied was E-Cadherin, an adhesive molecule and actively involved in the EMT (define EMT) process. It was observed that there was complete-down regulation of E-Cadherin in CAF as well as in Ch-Np-treated CAF,

as shown in Figure 6.

Chemokines in CAF- and Ch-Np-treated CAF

CXCR4, CXCR7, and CCR5 are chemokine receptors mainly involved in the process of metastasis where Sdf-1 α is a ligand to CXCR4. Their expressions were studied in CAF and Ch-Np-treated CAF. Figure 7 shows complete down-regulation of CXCR4 and CCR5 in Ch-Np treated CAF cells. Also, there was slight expression observed in CXCR7 and Sdf-1 α in Ch-Np treated CAF cells as compared to control CAF cells, as shown in Figure 7. Control CAF cells expressed all these genes normally [Figure 7].

Evaluation of metastatic potency of CAF cells by scratch assay

Scratch assay is mainly useful to evaluate migration potencies of metastatic cells. As CAFs were isolated from a metastatic patient, the scratch assay was used to study the metastatic potency in control CAF cells and Ch-Np-treated CAF.

This effect was studied at two time points, i.e, 24 and 48 h after Ch-Np treatment, as shown in Figure 8. It was observed that there was good migration of these cells in a scratch area in control CAF dishes, even at 24 h. In 48 h many cells were seen in scratch area in control CAF [Figure 8a, 8b, and 8c], whereas CAF cells treated with Ch-Np showed few cells migration after 24 h and 48 h, as shown in Figure 8d, 8e, and 8f. These observations indicated that Ch-Np treatment affects cell motility after 48 h of treatment.

DISCUSSION

CAFs are some of the most important stromal cells involved in tumor initiation, progression, and its metastasis in ESCC.^[7] It is important to attempt to target these cells along with their cancer cells when developing a drug against the cancer. Recent trends in drug development involve the use of nanoparticles which are efficient in drug delivery. Chitosan nanoparticle is one such nanoparticle which is being explored in various cancers and other diseases.^[8] Our study focused on evaluating the anti-metastatic effect of Ch-Np on CAF isolated from the peripheral blood of a patient with metastatic esophageal cancer.

CAFs have shown extensive growth proliferation and multiply at a doubling time of 25 ± 0.38 h. However Ch-Np-treated cells have inhibited the growth of these cells, indicating an inhibitory effect of Ch-Np on CAFs. Studies have shown that Ch-Np inhibited the growth of breast cancer cells *in vitro*.^[9] Similarly, another group found^[8] that Ch-Np effectively inhibited proliferation of a human gastric carcinoma cell line, indicating its potential beneficial activity against human gastric cancer. The present study also indicated the potential use of Ch-Np

for inhibition of growth of metastatic esophageal cancer.

Cancer cells are resistant to contact inhibition, a common phenomenon in normal cells.^[10] In current study CAF cells showed random overlapping cell growth in the control plates, whereas in Ch-Np-treated cells they were aligned in parallel fashion and showed clear cut monolayer cells, as if they were having normal phenotypic growth. The results further showed that these cells seem to have better contact inhibition than control CAF cells, which had metastatic potential.^[11] This might be the reason for the random growth of CAF since they were isolated from metastatic PBMC cells of the esophageal cancer patient. These phenotypic changes were confirmed by molecular markers studied in this project.

Several studies have shown that Oct4, Nanog, and Sox2 are excellent pluripotency markers in cancer cells.^[12,13] In the present study mRNA expression of Oct4, Nanog, and Sox2 in control CAF and Ch-Np-treated CAF were studied. Prominent expression of this pluripotency marker in control CAF was found. According to one study,^[14] Oct4 played an important role in promoting carcinogenesis and also in preventing cancer cells from undergoing apoptosis. Another group^[15] found that expression of Oct4 and Sox2 was altered in ESCC and together they impart a poor prognosis in the disease. In the present study down-regulation of Oct-4 and Sox2 in CAF after Ch-Np treatment was observed. Thus, clinical inhibition in expression of these genes may give hope a better outcome in ESCC.

In order to understand the characteristics of CAFs, the expression of keratin18, an epithelial marker in cancer cells, was studied. Keratin18 is currently mainly studied to understand prognosis in cancer patients.^[16] Recently it was^[17] noticed that there was up-regulation of keratin18 in breast cancer patients. This coincides with the observation in the current study where there was a higher expression of Keratin18 in CAF cells. Furthermore, in the current study there was a complete down-regulation of keratin18 after treatment of Ch-Np, implying the acquisition of a normal cell phenotype and loss of tumor progression capability. Vimentin was also studied in the present study and its expression was noticed on CAFs. This is accordance with a previous study.^[18] Over-expression of Vimentin in cancer cells has been associated with increased invasion and metastasis in tumor. According to one study^[19] inhibition of vimentin expression reduced cancer cell migration. Down-regulation in expression of vimentin was observed in the Ch-Np-treated CAF, suggesting that Ch-Np can reduce cell migration and, ultimately, metastasis. Metastasis is an important characteristic of cancers which is governed by genes such as VEGF, MMP1, MMP9, and E-Cadherin. MMP1 and MMP9 have been found to be associated with cancer cell metastasis in ESCC.^[20] Down-regulation in these two important genes, MMP1 and MMP9, observed

in Ch-Np-treated cells, may thus reduce the metastatic ability of cancer cells.

As mentioned earlier, CAFs are derived from the tumor by the process of EMT, which involves loss of intracellular adhesion. The absence of E-cadherin in control CAFs supports the process of EMT and loss of intracellular adhesion.^[5,21] The other important molecular marker, VEGF, which plays a role of angiogenesis, was also not affected by Ch-Np treatment.^[22] Hence, future studies have to be done with Ch-Np on CAFs so as to target E-cadherin and VEGF. Tumor development involves a variety of chemokines which are secreted by cancer cells. CXCR4, CXCR7, CCR5, and Sdf-1 α are some such chemokines. Prominent expression of CXCR4 in esophageal cancer has been shown to have a poor long term prognosis and involvement in tumor spread.^[23] CXCR4 and its ligand, Sdf-1 α , were found to be involved in the metastasis of esophageal cancer in an *in vivo* model.^[24] The role of CXCR7 and CCR5 in esophageal cancer is poorly understood but in breast cancer they are involved in proliferation and metastasis.^[25,26] Hence, these chemokines serve as important metastatic genes in the case of esophageal cancer. Down-regulation was noticed in expression of CXCR4, CXCR7, CCR5, and Sdf-1 α in Ch-Np-treated CAF, implying anti-metastatic activity of Ch-Np. Further support to our hypothesis of anti-metastatic activity of Ch-Np was provided by the Scratch Assay in which treated CAFs did not spread in the scratched area, indicating loss of metastatic activity.

In conclusion, chitosan is a biopolymer which has been extensively studied for its ability to encapsulate the drug molecule within it. In our study we have shown anti-tumor and mainly anti-metastatic ability of Ch-Np on esophageal CAF. Decrease in the various genes by chitosan shows that it is a promising drug molecule in the treatment of metastatic cancer. Hence, chitosan should not be considered only as a carrier of drug molecules but should be considered as a drug itself. Also, in order to better treat ESCC, it is important to study the stromal cell fraction and its molecular mechanism so as to develop molecular targeted therapy. Encapsulation of an anti-cancer drug within Ch-Np could work as a dual stratagem against cancer, targeting both cancer and CAFs. Hence, future clinical and pharmacological studies with Ch-Np need to be done.

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Nil.

Conflicts of interest

There are no conflicts of interest.

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Oxidative stress and breast cancer biomarkers: the case of the cytochrome P450 2E1

Subir Singh¹, Ramkumar Rajendran², Kengo Kuroda³, Emiko Isogai³, Marija Krstic-Demonacos⁴, Constantinos Demonacos¹

¹Manchester Pharmacy School, University of Manchester, Manchester M13 9PT, UK.

²School of Pharmacy, International Medical University, Bukit Jalil, 57000 Kuala Lumpur, Malaysia.

³Laboratory of Animal Microbiology, Tohoku University Graduate School of Agricultural Science, Sendai, Miyagi, 981-8555, Japan.

⁴School of Environment and Life Sciences, University of Salford, Peel Building, Salford, M5 4WT, UK.

Correspondence to: Dr. Constantinos Demonacos, Manchester Pharmacy School, University of Manchester, Stopford building, Oxford Road, Manchester M13 9PT, UK. E-mail: cdemonacos@manchester.ac.uk



Dr. Demonacos joined the University of Manchester, Manchester Pharmacy School in 2003 where he is involved in the investigation of the role of ROS in cellular energy metabolism and breast carcinogenesis. In addition, Dr. Demonacos' laboratory explores the signaling events that facilitate cancer cells to evade immunosurveillance.

ABSTRACT

Aim: The aim of the study is to investigate the impact of the cytochrome P450 2E1, which is the most efficient CYP450 family member in generating reactive oxygen species (ROS), on cellular energy metabolism of breast cancer cells and therefore the effects of CYP2E1 on breast carcinogenesis. **Methods:** The estrogen receptor positive MCF-7 and the triple negative MDA-MB-231 breast cancer cells were used as experimental system to estimate ROS generation in these cells overexpressing CYP2E1 and treated with the glycolytic inhibitors 3-bromopyruvate or 2-deoxyglucose in the presence or absence of the CYP2E1 inhibitor chlormethiazole. Adenosine triphosphate (ATP) assay was used to measure ATP production and lactate assay to quantify the efflux of lactic acid in breast cancer cells treated with the CYP2E1 inhibitor chlormethiazole, the mitochondrial membrane potential and cell viability assays were employed to assess the pathway of cellular energy production and cellular death respectively after treatment of MCF-7 and MDA-MB-231 with the CYP2E1 activator acetaminophen or the CYP2E1 inhibitor chlormethiazole. **Results:** The results indicated increased ROS generation in breast cancer cells overexpressing CYP2E1. ROS generation was differentially regulated in breast cancer cells upon treatment with the CYP2E1 inhibitor chlormethiazole. Chlormethiazole treated MCF-7 cells exhibited reduced lactate efflux implying that CYP2E1 directly or indirectly regulates the glycolytic rate in these cells. Furthermore the mitochondrial membrane potential of both MCF-7 and MDA-MB-231 cells was differentially affected by the CYP2E1 activator acetaminophen versus the CYP2E1 inhibitor chlormethiazole providing additional support for the involvement of CYP2E1 in energy metabolic pathways in breast cancer. **Conclusion:** Results presented in this study provide evidence to suggest that CYP2E1 regulates cellular energy metabolism of breast cancer cells in a manner dependent on cell type and potentially on the clinical staging of the disease therefore CYP2E1 is a possible breast cancer biomarker.

Key words: Reactive oxygen species; cytochrome P450 2E1; glycolysis; breast cancer

INTRODUCTION

Reactive oxygen species (ROS) such as superoxide, hydroxyl radical, and hydrogen peroxide are metabolic by-products leaking from the complexes I and III of the mitochondrial respiratory chain.^[1] Generation of high ROS levels is

detrimental for the cells as it can lead to DNA damage and oxidation of proteins and lipids changing their functions.^[2] Accumulating evidence indicates that apart from their

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harmful effects ROS act as second messenger signalling molecules regulating numerous pathways including cell cycle,^[3] autophagy,^[4,5] apoptosis,^[6] endoplasmic reticulum (ER) stress^[7] and cellular energy metabolism.^[8,9]

Sources of intracellular ROS generation include both organelles such as mitochondria, ER and peroxisomes as well as enzymes such as the NADPH oxidases, xanthine oxidase, lipoxygenases and cytochrome P450 enzymes, which produce ROS through their enzymatic activities.^[10] CYP450 enzymes are mainly involved in the phase I metabolism of a wide range of exogenous and endogenous compounds oxidizing them to form more hydrophilic molecules thereby facilitating easier clearance.^[11] In the case the monooxygenation reaction catalysed by CYP enzymes is uncoupled from the NADPH reaction instead of a monooxygenated substrate production of ROS occurs.^[12] The CYP450 family member CYP2E1 is the most active enzyme of the family in terms of generating ROS sometimes inducing production of oxygen radicals even in the absence of substrates.^[13]

Apart from the liver CYP2E1 gene expression has been detected in other tissues such as breast, lung, kidney and hematopoietic tissues^[13] and has been reported to be over expressed in malignant compared to normal tissues.^[14-17] CYP2E1 overexpression in cancer is attributed to the inflammatory conditions present in the tumor microenvironment characterised by increased inflammatory cytokine production which affects CYP2E1 gene expression.^[18-20] Several CYP2E1 dependent mechanisms contributing to tumorigenesis have been suggested including formation of toxic intermediate derivatives and activated carcinogens.^[21-23] CYP2E1 mediated ROS generation could also contribute to tumor development through pathways in which ROS play vital role such as DNA damage, enhanced angiogenic responses^[24] autophagy^[4,25,26] ER stress^[27] and unfolded protein response (UPR).^[28] Furthermore, research in our laboratory has indicated that CYP2E1 is differentially expressed in a manner dependent on the genetic background and the stage of breast cancer, regulating oxidative stress response and metastasis.^[29]

Cancer cells produce energy predominantly through aerobic glycolysis -- a phenomenon also called Warburg effect -- rather than oxidative phosphorylation even in the presence of oxygen and functional mitochondria.^[30] The Warburg effect is induced in cancer cells by increased cellular glucose uptake stimulated by ROS mediated upregulation of gene expression of glucose transporters such as GLUT-1.^[31] On the other hand, experimental evidence supports the view that increased glycolytic conversion to pyruvate leads to ROS generation^[32] suggesting the existence of an interrelation between ROS generation with glycolysis and vice versa.^[9,33]

Taken together, the above mentioned observations allow

the hypothesis that overexpression of CYP2E1 and the resultant elevated ROS production might regulate cellular energy metabolism in cancer cells pointing out CYP2E1 as a potential cancer biomarker. The understanding of the interplay between CYP2E1 -- ROS generation -- cellular energy metabolism can provide important conclusions towards establishing novel breast cancer biomarkers and overcoming drug resistance. The estrogen receptor-positive MCF-7 and the triple negative MDA-MB-231 [estrogen receptor-negative, progesterone receptor-negative and human epidermal growth factor receptor 2 (HER2)-negative] breast cancer cells were used in this study to evaluate the impact of the CYP2E1 mediated ROS generation on the energy metabolism of these cells.

METHODS

Cell culture

The human breast carcinoma cell lines MCF-7 and MDA-MB-231 [obtained from the European Collection of Cell Cultures (ECACC)] were maintained in Dulbecco's modified Eagle's medium (Sigma-Aldrich, Gillingham, UK), supplemented with 10% foetal bovine serum (Gibco, Paisley, UK) and 1% penicillin/streptomycin (Lonza, Allendale, NJ, USA) at 37°C in a humidified atmosphere containing 5% CO₂. Cells were treated with 100 µM 3-bromopyruvate (3BP) (Sigma-Aldrich) for 3 h, 20 mmol/L 2-deoxyglucose (2DG) (Sigma-Aldrich) for 24 h, 2.5 mmol/L acetaminophen (APAP) (Sigma-Aldrich) for 3 h and 20 µM chlormethiazole (CMZ) (Sigma-Aldrich) for 16 h.

Transient transfection

Transient transfections were carried out using the polyfect transfection reagent (Qiagen, Crawley, UK), according to the manufacturer's instructions. Constructs used for ectopic expression included the pcDNATM3.1 (Invitrogen) and the pCI-neo-CYP2E1 (kindly provided by Dr. Cederbaum, Mount Sinai School of Medicine, New York).^[29]

Measurement of ROS

Cells were grown until they reached 80% confluence prior to transient transfection and different treatments. ROS levels were measured using flow cytometry as described previously.^[29] Cells were transiently transfected with the indicated constructs and 16 h after transfection they were harvested and incubated with 1 mL of APC-H7-conjugated CD20 antibody (BD Biosciences, Franklin Lakes, NJ, USA) to detect only the cells ectopically expressing CYP2E1. Cells were then incubated with H2DCFDA (Invitrogen, Carlsbad CA, USA) in the dark at 37°C for 30 min and subjected to flow cytometry using CYAN-ADP flow cytometer (Dako, Glostrup, Denmark) following the fluorescence profile of the H2DCFDA and APC-H7 probes.

Adenosine triphosphate (ATP) assay

ATP levels were measured using the ViaLight plus kit

(Lonza, Slough, UK), based on the bioluminescent measurement of ATP present in cells. ATP monitoring reagent (AMR plus) was prepared by adding assay buffer into the vial containing the lyophilized AMR and incubated at room temperature for 15 min for complete rehydration. Cells were lysed in 50 μ L of cell lysis reagent for 10 min. A total volume of 100 μ L of cell lysate was added to a luminometer plate and 100 μ L of AMR plus was added to the appropriate well. The plate was then incubated at room temperature for 2 min and values were obtained from the luminometer.

Lactate assay

To measure the lactate efflux MCF-7 and MDA-MB-231 breast cancer cells were grown in 6 well plates and left untreated or treated with CYP2E1 specific inhibitor CMZ.

Media was collected in a 96 well plates after treatment. Two microlitre of this media was mixed with 60 μ L of lactate reagent and incubated at room temperature for 15 min and the absorbance was recorded at 540 nm. Lactic acid standard solutions (Trinity Biotech, Ireland) were used to plot the standard curve and the concentration of lactic acid present in the media was calculated accordingly. Lactate production rates were expressed as mmol/L.

Mitochondrial membrane potential

Mitochondrial transmembrane potential ($\Delta\psi_m$) was measured using the cationic dye JC-1 (5, 5, 6, 6-tetrachloro-1,1,3,3-tetraethylbenzimidazolcarbocyanine iodide) (ChemoMetec, Allerod, Denmark) using the NucleoCounter[®] NC-3000[™] system. Cells were grown in 6-well plates and treated with the CYP2E1 activator APAP

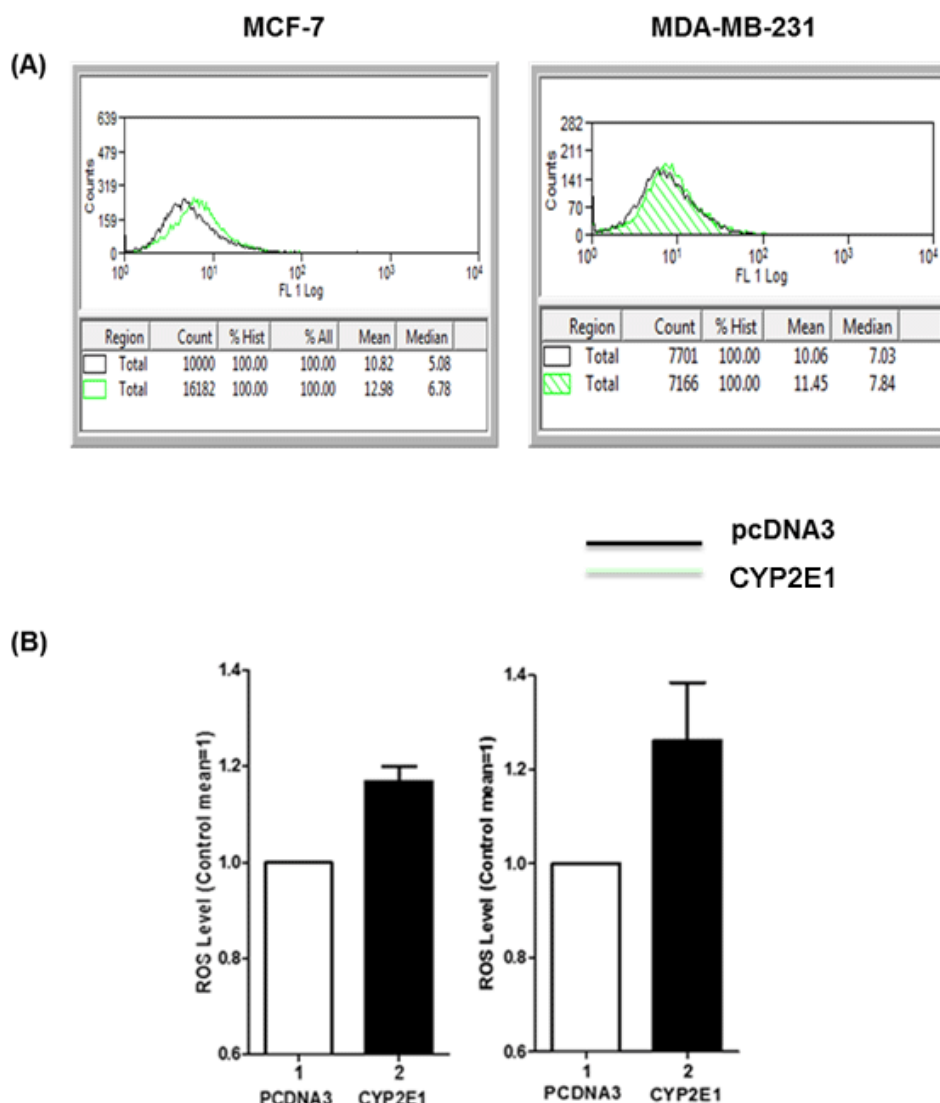


Figure 1: ROS generation in MCF-7 and MDA-MB-231 cells ectopically expressing CYP2E1. MCF-7 and MDA-MB-231 cells were transiently transfected with a CYP2E1 expressing or the empty vector PCDNA3. ROS levels were determined using H2DCFDA fluorescent dye and flow cytometry only in the cells ectopically expressing CYP2E1 (co-transfected with CD20). FACS data were analyzed using Beckman Coulter Summit 4.1 software. (A) Histograms displaying ROS levels after transient transfection of CYP2E1 or pcDNA3 as indicated. Green coloured histograms represent ROS levels in cells transfected with CYP2E1 and black histograms represent ROS levels in cells transfected with PCDNA3; (B) bar graphs representing ROS levels generated in cells transfected with PCDNA3 and CYP2E1 as indicated. Data are average of three independent experiments. ROS: reactive oxygen species; CYP: cytochrome P450

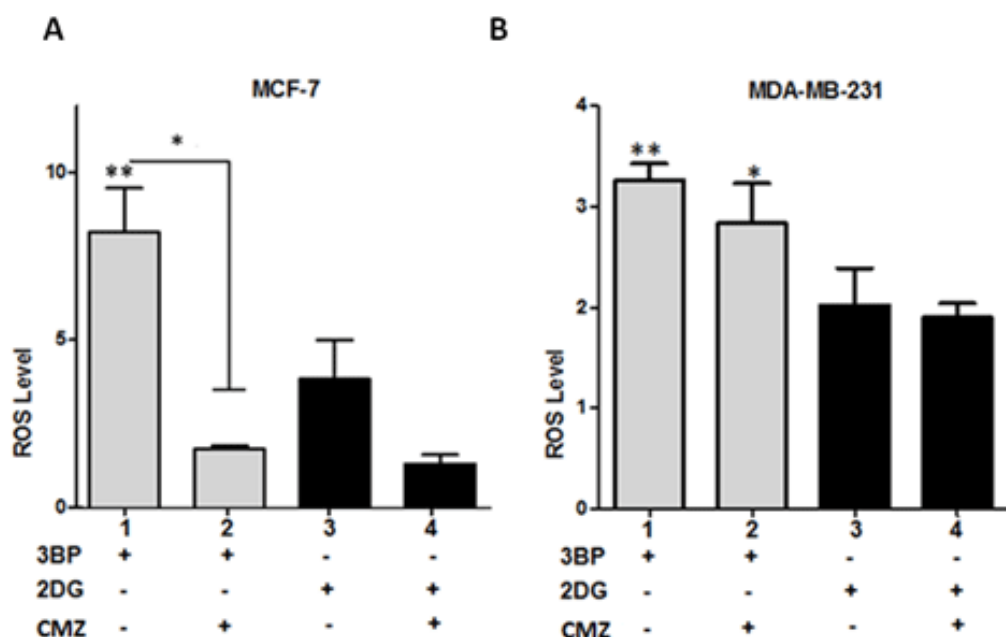


Figure 2: CYP2E1 mediated ROS generation in breast cancer cells under diverse stress conditions. Graph indicating ROS levels generated in 3BP, 2DG and CMZ treated MCF-7 (A) and MDA-MB-231 (B) cells. Error bars represent mean \pm SEM from three independent experiments. Statistical analysis was performed by one-way ANOVA followed by Tukey post hoc for multiple pair-wise comparisons. One asterisk indicates $P < 0.05$ and two asterisks $P < 0.005$. ROS: reactive oxygen species; CMZ: chlormethiazole

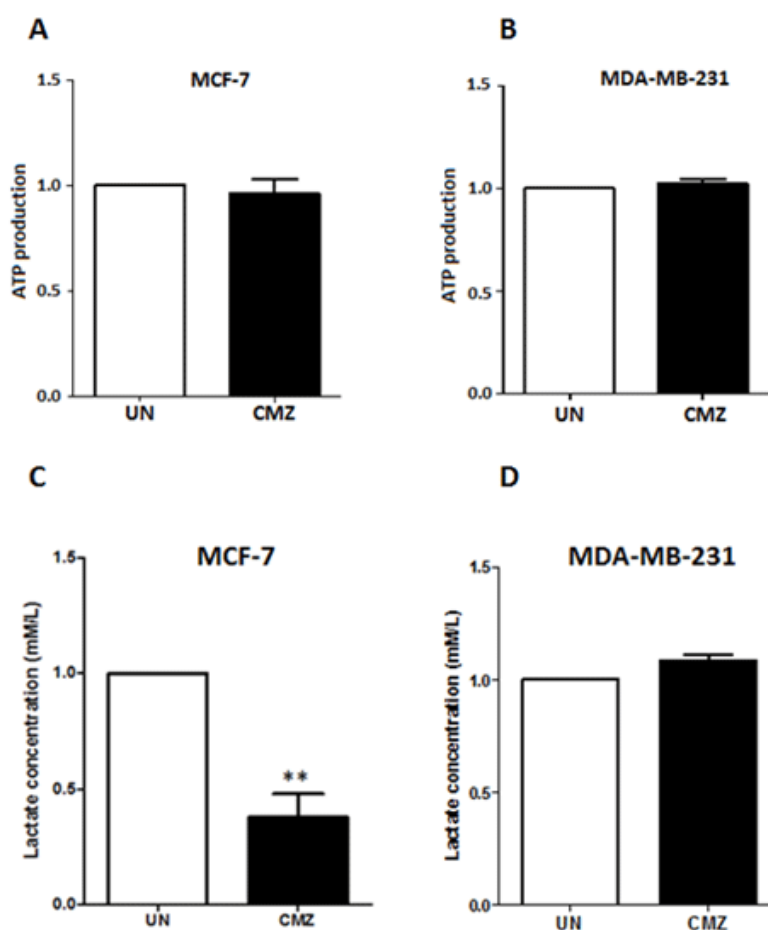


Figure 3: ATP production and lactate efflux in MCF-7 and MDA-MB-231 cells treated with the CYP2E1 inhibitor CMZ. MCF-7 and MDA-MB-231 cells were either left untreated or treated with the CYP2E1 specific inhibitor CMZ. ATP production (A and B) was determined using the ViaLight™ plus kit (Lonza, Slough, UK) and lactate efflux (C and D) using the lactate reagent (Trinity Biotech, Dublin, Ireland). Data are average of three independent experiments \pm SEM; ** $P < 0.005$. ATP: adenosine triphosphate; CMZ: chlormethiazole

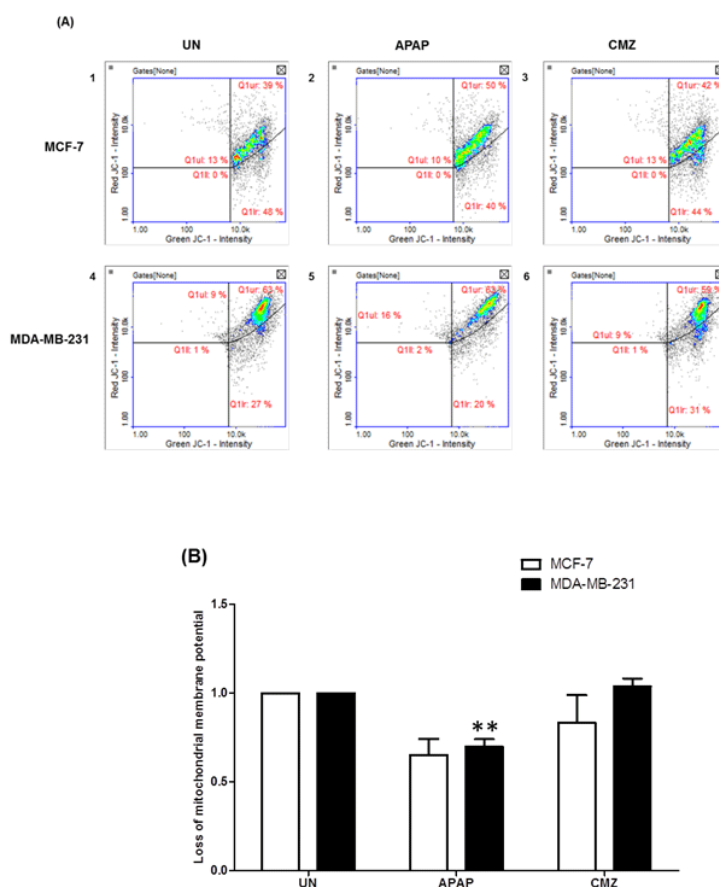


Figure 4: Mitochondrial membrane potential ($\Delta\psi$) in breast cancer cells treated with the CYP2E1 activator APAP or the CYP2E1 inhibitor CMZ. Breast cancer cells were left untreated or treated with either the CYP2E1 inducer (APAP) or the CYP2E1 inhibitor (CMZ). Mitochondrial membrane potential ($\Delta\psi$) was determined using JC-1 and DAPI fluorescent dye (ChemoMetec, Allerod, Denmark) and the NucleoCounter NC3000. Data were analyzed using NucleoView software. (A) Histograms representing the mitochondrial membrane potential ($\Delta\psi$) in breast cancer cells under different stress conditions; (B) bar graphs representing the effect of APAP and CMZ treatments on mitochondrial membrane potential ($\Delta\psi$) in breast cancer cells. Error bars represent mean \pm SEM from three independent experiments. Two asterisks indicate $P < 0.005$. APAP: acetaminophen; CMZ: chlormethiazole

or the CYP2E1 inhibitor CMZ. After treatment, cells were stained with JC-1 and DAPI (ChemoMetec, Allerod, Denmark). Cellular JC-1 monomers and aggregates are detected as green and red fluorescence, respectively. Mitochondrial depolarization and apoptosis are revealed as a decrease in the red/green fluorescence intensity ratio. Necrotic and late apoptotic cells are detected as blue fluorescent (DAPI) cells. After staining cells were loaded on an 8-chamber NC-Slide A8™ and samples were analysed using the NC-3000™ system and the amount of blue, green and red fluorescence of the individual cells was quantified.

Cell viability assay

Cell viability was measured using the NucleoCounter® NC-3000™ system. Cell viability assay was used to detect changes in the intracellular level of (reduced) thiols. Cells were seeded in 6 well plates and cultured until they reached 80% confluence prior to different treatments. After the treatments, cell culture medium was aspirated and 500 μ L of cell dissociation buffer was added to cells for dissociation from culture plates. Five hundred microlitre of complete culture medium was added to quench the toxicity of dissociation buffer after cell dissociation.

Then cells were stained with solution 5 as described by the manufacturer. Solution 5 (ChemoMetec, Allerod, Denmark) contains three different stains, each one of them staining either all nucleated cells (DAPI), dead cells (Propidium iodide) or viable cells (VB-48) (ChemoMetec, Allerod, Denmark) and the intensity of the stain depends on the GSH level. After staining, cells were loaded into an 8-chamber NC-slide. Samples were analysed using the NC-3000™ system.

RESULTS

The role of CYP2E1 in ROS generation in breast cancer cells has been investigated by our and other groups indicating that overexpression of this cytochrome P450 family member in breast cancer cells coincides with elevated ROS levels implying that CYP2E1 is one of the intracellular sources of ROS.^[29,34] To confirm that this is the case in the triple positive MCF-7 and the triple negative MDA-MB-231 cells CYP2E1 expressing vectors were transiently transfected and the ROS levels in mock and ectopically expressing CYP2E1 cells were followed as described in Materials and Methods. Increased ROS levels were recorded in both cell lines ectopically expressing

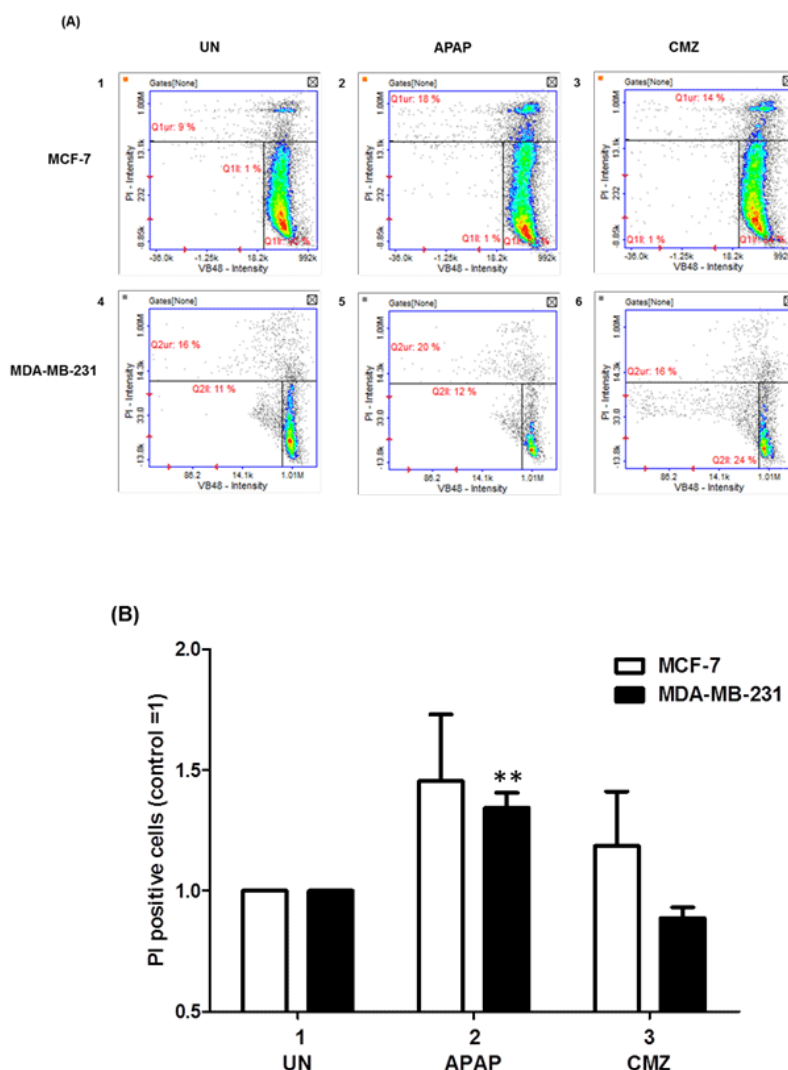


Figure 5: Cell viability of breast cancer cells treated with the CYP2E1 activator APAP or the CYP2E1 inhibitor CMZ. Breast cancer cells were left untreated or treated with either the CYP2E1 activator APAP or the CYP2E1 inhibitor CMZ as indicated. Cell viability was calculated using the Vitality kit Assay (ChemoMetec, Allerod, Denmark). (A) Histogram representing cell viability under different stress conditions. Dead cells stained with PI are shown in the Q1ur gates; (B) bar graph representing the PI positive breast cancer cells treated with either APAP or CMZ. Error bars represent mean \pm SEM from three independent experiments. Two asterisks indicate $P < 0.005$. APAP: acetaminophen; CMZ: chlormethiazole

CYP2E1 compared to mock transfected cells [Figure 1B, compare bars 2 to bars 1 respectively].

To explore further the effects of CYP2E1 on the glycolytic pathway of energy production the glycolytic inhibitors 3BP and 2DG were used to inhibit glycolysis in MCF-7 and MDA-MB-231 cells either individually or in combination with the CYP2E1 inhibitor CMZ and the ROS generated under these conditions were monitored as described in Materials and Methods. Treatment of MCF-7 cells with 3BP generated higher ROS levels compared to MCF-7 cells treated with 2DG [Figure 2A, compare bar 1 to bar 3]. Combination of 3BP or 2DG treatment with CMZ resulted in dramatic decrease of ROS levels in MCF-7 cells [Figure 2A, compare bar 2 to bar 1 and bar 4 to bar 3]. In contrast, in MDA-MB-231 cells CMZ had marginal effect on that observed when cells were treated with the glycolytic inhibitors 3BP and 2DG alone [Figure 2B, compare bars 2 and 4 to bars 1 and 3 respectively] providing additional

evidence that CYP2E1 exerts cell type dependent effects in ROS generation in a manner dependent on the genetic background and potentially their invasive potential.

Accumulating evidence supports the notion that ROS generation is associated with cellular energy production.^[31,32,35] Results shown in Figure 1 indicate that CYP2E1 overexpression led to elevation of ROS in MCF-7 and MDA-MB-231 breast cancer cells implying a potential role of CYP2E1 in cellular energy metabolism. To test this hypothesis MCF-7 and MDA-MB-231 cells were treated with the CYP2E1 inhibitor CMZ and the levels of ATP produced under these conditions were determined as described in Methods. CMZ treatment of both MCF-7 and MDA-MB-231 cells did not have any significant effect on the ATP produced under these conditions [Figure 3A and 3B]. To test whether the ROS levels' profile observed in breast cancer cells was related to lactate production, MCF-7 and MDA-MB-231 cells were treated with the CYP2E1

inhibitor CMZ^[36,37] and lactate production was monitored as described in Methods. Results shown in Figure 3C indicate that inhibition of CYP2E1 in MCF-7 cells resulted in reduction of lactate production in these cells whereas inhibition of CYP2E1 in MDA-MB-231 cells did not have any effect on lactate production [Figure 3D] reiterating the concept that CYP2E1 effects are cell type dependent.

It is known that oxidative stress can trigger the mitochondrial permeability transition and $\Delta\psi$ collapse leading to defects in ATP production.^[38] Taking into account these observations, we next assessed potential changes in the mitochondrial membrane potential in breast cancer cells treated with the CYP2E1 activator APAP or the CYP2E1 inhibitor CMZ. APAP treatment of both MCF-7 and MDA-MB-231 cells led to decrease of $\Delta\psi$ compared to untreated cells [Figure 4B, compare APAP white (0.652) and black (0.698) bars to UN white (1) and black (1) bars]. In contrast, CMZ treatment did not exert any effects on $\Delta\psi$ which remained the same as that observed in the untreated cells [Figure 4B, compare CMZ white and black bars to UN white and black bars]. These results indicate a potential role of CYP2E1 mediated ROS generation in the process of alterations of mitochondrial membrane potential.

Alterations in $\Delta\psi$ might in some cases lead to cell death^[39] and in order to explore whether that was the case in breast cancer cells treated with APAP or CMZ MCF-7 and MDA-MB-231 cell death was determined by PI staining upon treatment with APAP or CMZ. APAP treatment of MCF-7 and MDA-MB-231 cells led to increased cell death in both cell lines (MCF-7 cells from 9% to 18% and MDA-MB-231 cells from 16% to 20%) [Figure 5B, compare APAP white and black bars to UN white and black bars]. CMZ treatment of both MCF-7 and MDA-MB-231 cells did not exert any effects on cell death as it did not affect the percentage of PI positive compared to untreated MCF-7 and MDA-MB-231 cells [Figure 5B, compare CMZ white and black bars to UN white and black bars]. Taken together these results indicate that at least in part CYP2E1 mediated generation of ROS alters collapse of mitochondrial membrane potential determining cell survival or death in a cell type dependent manner.

DISCUSSION

Accumulating evidence supports the view that ROS generated by CYP2E1 activity mediate cell signalling events that promote alterations in the cellular physiology and disease development.^[40-43] Studies in our and other laboratories have indicated diverse levels of CYP2E1 gene expression in a manner dependent on the genetic background and the migratory potential of these cells.^[16,17,29,34] CYP2E1 overexpression in breast cancer cells is involved in the alteration of numerous pathways linked to the disease such as cell cycle control, apoptosis, autophagy, ER stress and UPR.^[29,44,45] In addition to these,

another pathway that is regulated by the cellular redox state is cellular energy metabolism. The interrelation between ROS and aerobic glycolysis which is the main pathway through which cancer cells produce energy has been extensively investigated.^[31,32] Since cytochrome P450 enzymes are one of the endogenous sources of ROS^[46,47] it was hypothesized that CYP450s might be involved in the regulation of cellular energy metabolism.

The role of the CYP2E1 mediated ROS generation in the energy metabolism of breast cancer cells was investigated in the estrogen receptor positive MCF-7 and estrogen receptor negative MDA-MB-231 cells.^[48] Given that CYP2E1 gene expression is under the transcriptional control of factors responsive to inflammation^[19,29,49,50] it was theorized that inhibition of glycolysis in breast cancer cells bearing diverse genetic background would lead to alternative CYP2E1 cellular levels and hence dissimilar ROS.^[42] On the other side inhibition of glycolysis would lead to increased ROS generation^[31] that could be altered by CYP2E1 enzymatic activity.^[42,51-53] In accord with published results treatment of both MCF-7 and MDA-MB-231 cells with the glycolytic inhibitors 3BP and 2DG increased ROS levels in the two cell lines.^[33,54,55] Combination of either 3BP or 2DG with the CYP2E1 inhibitor CMZ reduced dramatically the oxygen radicals' levels in the MCF-7 but not in the MDA-MB-231 cells [Figure 2] implying that the dissimilar genetic background in the two cell lines (wild type ER and p53 in MCF-7 and defective ER and mutated p53 in MDA-MB-231 cells) determines the differential response of these cells to the glycolytic and CYP2E1 inhibitors.^[56]

The observation that CMZ decreased ROS generation stimulated by 3BP and 2DG treatment in MCF-7 cells prompted our interest to explore the possibility that CYP2E1 is involved in the process of energy metabolism in breast cancer cells. The potential link between CYP2E1 and energy metabolism was investigated in the MCF-7 and MDA-MB-231 cells by estimating the ATP production after treating these cells with the CYP2E1 inhibitor CMZ. Results shown in Figure 3 indicate that CMZ treatment did not significantly affect ATP generation in the two cell lines implying that if CYP2E1 had inhibitory effect on one of the pathways of ATP generation another pathway compensates for the loss facilitating cells to meet their energy requirements,^[57] or CYP2E1 is not involved in ATP production in these cells. To answer these questions the lactate concentration was determined in CMZ treated MCF-7 and MDA-MB-231 cells.^[58] CMZ treatment reduced lactate efflux in MCF-7 but not in MDA-MB-231 cells [Figure 3C and 3D] implying that CYP2E1 exerts cell type dependent effects on energy metabolism. These results are in line with those shown in Figure 2 indicating reversion of the effect of the glycolytic inhibitors 3BP and 2DG on ROS levels by CMZ in MCF-7 cells, and published studies reporting that high ROS levels induce hypoxia inducible factor 1 alpha (HIF-1 α) thereby

inducing lactate dehydrogenase (LDH-A) gene expression and hence lactate efflux.^[58]

Depolarization of the mitochondrial membrane is determined by the gradient of protons across the mitochondrial membrane. Opening of the mitochondrial permeability transition pore (PTP) permitting influx or efflux of protons can lead to mitochondrial membrane depolarization. Proteins involved in the regulation of the PTP opening are susceptible to redox modifications therefore high levels of ROS may lead to PTP opening and induce mitochondrial membrane depolarization.^[59] Given that overexpression [Figure 1] or inhibition of CYP2E1 altered the redox state of MCF-7 and MDA-MB-231 cells [Figure 2] we were interested to study potential changes of the mitochondrial membrane potential in breast cancer cells attributed to the activation or inhibition of the CYP2E1 enzymatic activity. Reduced mitochondrial membrane potential was observed in both MCF-7 and MDA-MB-231 cells treated with the CYP2E1 activator APAP whereas no changes in mitochondrial membrane depolarization were recorded in these cells treated with the CYP2E1 inhibitor CMZ [Figure 4].

Cell death through the intrinsic pathway of apoptosis is triggered by sustained mitochondrial membrane depolarization.^[59] To investigate the potential role of CYP2E1 in inducing cell death by mediating alterations in the mitochondrial membrane depolarization MCF-7 and MDA-MB-231 cells were treated with either the CYP2E1 activator APAP or the CYP2E1 inhibitor CMZ and cellular viability was assessed. APAP induced cell death in both MCF-7 and MDA-MB-231 cells while CMZ induced cell death only in MCF-7 cells [Figure 5] reiterating the view that CYP2E1 effects are cell type specific.

Taken together results presented in this study provide evidence to support the concept that CYP2E1 regulates cellular energy metabolism in a cell type dependent manner affecting predominately this pathway in less invasive and early stages of breast cancer represented by the MCF-7 cells. Although these results require validation in an *in vivo* system they endorse the conclusion that CYP2E1 cellular levels can be a prognostic indicator and a potential breast cancer biomarker.

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Conflicts of interest

There are no conflicts of interest.

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Neuroendocrine tumors: a multidisciplinary approach for a complex disease

Rossana Berardi

Medical Oncology Unit, Università Politecnica delle Marche, Azienda Ospedaliero-Universitaria Ospedali Riuniti Umberto I, GM Lancisi, G Salesi di Ancona, Via Conca 71, 60126 Ancona, Italy.

Correspondence to: Dr. Rossana Berardi, Medical Oncology Unit, Università Politecnica delle Marche, Azienda Ospedaliero-Universitaria Ospedali Riuniti Umberto I, GM Lancisi, G Salesi di Ancona, Via Conca 71, 60126 Ancona, Italy. E-mail: r.berardi@univpm.it

Neuroendocrine neoplasms include a heterogeneous group of neoplasms, representing a spectrum of rare neoplasms arising in different organism sites with different malignant potential and behavior. They typically occur in gastrointestinal and bronchopulmonary tracts.

The incidence and prevalence of these neoplasms showed a significant increase in the last four decades leading to a rising interest in these tumours with remarkable progresses in their both treatment and management. Nevertheless, they are still considered rare diseases with a global clinical incidence of 3.65 cases/100,000 per year according to the National Cancer Institute SEER (Surveillance Epidemiology and End Results) registry.^[1]

Surgery still remains the primary treatment approach mainly depending on tumour size, stage and patients performance status. However in loco-regional unresectable and/or metastatic disease, curative surgery is generally not possible, therefore medical therapy is usually primarily considered. Several treatment options are available and to date the management of neuroendocrine tumors within clinical practice is based on a multimodal therapeutic strategy including surgery and other loco-regional therapies, somatostatin analogs (SSAs), peptide receptor radionuclide therapy (PRRT), cytotoxic agents, biological agents (including angiogenesis inhibitors such as sunitinib and inhibitors of mammalian target of rapamycin as everolimus) with a multidisciplinary approach.^[2]

SSAs, including octreotide and lanreotide, represent effective options in the presence of carcinoid syndrome, but they also have an antiproliferative effect in secreting and nonsecreting neuroendocrine tumors.^[3,4]

PRRT is an emerging treatment modality for advanced neuroendocrine tumors. It is performed in the treatment

of neuroendocrine tumors, where somatostatin analogues (DOTATOC, DOTATATE) are radiolabeled with ¹⁷⁷Lu, ⁹⁰Y, or ¹¹¹In for pre-therapeutic and therapeutic purposes.^[5]

There are many cumulative evidences about the effectiveness and tolerability of this therapeutic approach, especially in gastro-entero-pancreatic neuroendocrine tumors.

Neuroendocrine neoplasms therapy also includes cytotoxic agents, especially in symptomatic patients, in progressive disease, in case of moderate or poor differentiation and more aggressive features. Chemotherapy schedules used in this setting include alkylating agents (streptozotocin, dacarbazine, and temozolomide), antimetabolites (5-fluorouracil, capecitabine), etoposide and platinum derivatives (including cisplatin and oxaliplatin).^[6]

The availability of new targeted agents, such as everolimus and sunitinib, which are effective in advanced and metastatic pancreatic neuroendocrine tumors, has provided new treatment opportunities.

Despite comprehensive and interesting medical progress, the current available therapeutic options are still inadequate for gastrointestinal and lung neuroendocrine tumors, mainly due to the lack of in-depth knowledge of molecular mechanisms and predictive factors.

Prognostic evaluation is mainly based on their morphologic features and proliferation index, according to WHO classification.^[7]

Due to the usually long life-expectancy of these patients, many different lines of therapy are performed according to

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difference status of the disease as well as on timing. Thus, despite the sequencing of different therapies represents a true challenge in real life, a standard therapeutic sequence is still lacking and it is a matter of debate.

Therefore novel strategies are needed, especially for refractory and/or recurrent neuroendocrine neoplasms that present a poor prognosis. Personalized approaches are currently being developed and molecular targets are emerging.

Several driver pathways have been investigated and they may represent important factors in the carcinogenesis process and, therefore, potential targets for new anticancer therapies.

In particular, activating mutations have been identified several genes, including those of the epidermal growth factor receptor, platelet-derived growth factor receptor, vascular endothelial growth factor, basic-fibroblastic growth factor, transforming growth factor, insulin-like growth factor-1, and their receptors, stem cell factor receptor. New drugs (including immunotherapy) and several combination regimens with new biological agents are being developed and studied in recently conducted and ongoing trials.

Further investigations could increase our knowledge about molecular mechanisms responsible for the neuroendocrine neoplasms heterogeneity, about tumor interactions with adjacent healthy tissue and as regard its variegated response to treatments, to guarantee the development of new promising therapies.

This special issue on neuroendocrine neoplasms aims to summarize the present knowledge about the treatment of these tumors highlighting available evidences as well as new biological perspectives on biological and targeted therapies, also including case reports.

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Neuroendocrine tumors: current therapies, notch signaling, and cancer stem cells

Judy S. Crabtree, Lucio Miele

Department of Genetics and Stanley S. Scott Cancer Center, Louisiana State University Health Sciences Center, New Orleans, LA 70112, USA.

Correspondence to: Dr. Judy S. Crabtree, Department of Genetics, Louisiana State University Health Sciences Center, 533 Bolivar Street, New Orleans, LA 70112, USA. E-mail: jcrabt@lsuhsc.edu

ABSTRACT

Neuroendocrine tumors (NETs) encompass a broad spectrum of malignancies all derived from neuroendocrine cell lineage, affecting many different organs including the gastrointestinal (GI) tract, the endocrine pancreas, the thyroid, the skin and the respiratory tract. These tumors as a group are very heterogeneous, with varying characteristics attributed to each tissue of origin and tumor subtype. The pathogenesis of the different subtypes of NETs is not fully understood, but recent studies suggest the Notch signaling pathway may be dysregulated in these tumors either by under or overexpression of Notch receptors and/or ligands, or by disruption of pathway functionality through other means. Notch receptors can function as tumor suppressors in some cellular contexts and oncogenes in others which may, in part, account for the wide range of phenotypes present in NETs. Cancer stem cells are present in these tumors and may be responsible for the high rate of chemotherapy resistance, recurrence and metastasis. The heterogeneity of NETs suggests that to fully understand the role of Notch signaling and the therapeutic implications thereof, a comprehensive and systematic analysis of Notch expression and function across all NET subtypes is required. Here we outline the current knowledge base with respect to current therapies and Notch signaling in neuroendocrine tumors of the lung, skin, thyroid, GI tract and endocrine pancreas.

Key words: Neuroendocrine tumor; Notch; small cell lung carcinoma; medullary thyroid carcinoma; merkel cell carcinoma; pancreatic NET; carcinoid

INTRODUCTION

Neuroendocrine tumors (NETs) are a heterogeneous group of neoplasms that arise from the neuroendocrine cells of the gastrointestinal (GI) tract, endocrine pancreas, thyroid, skin, lung, adrenal gland and other tissues. These tumors are typically slow-growing, yet pose a significant threat due to high metastatic potential. In many cases, patients initially present with advanced metastatic disease resulting in poor outcomes and low 5-year survival rates. An understanding of the mechanism(s) of tumorigenesis and metastasis is required for target identification and new therapeutic development, since many NET subtypes have no curative options beyond surgical resection.

In recent years, studies have suggested that the Notch signaling pathway may be involved in the pathogenesis of NETs. Notch signaling has been studied for many years in the context of cancer and as these pathways are dissected, the complexity of Notch signaling becomes

more and more evident. Notch signaling is classified into two broad categories: 1) canonical signaling, wherein Notch receptors regulate transcription through CSL (CBF-1/Suppressor of Hairless/LAG-1), also known as RBP-Jk, and can play an oncogenic or tumor suppressive role depending on context, or 2) non-canonical, which functions through interplay with other signaling networks including phosphatidylinositol 3' kinase (PI3K)/Akt, mTOR, NF- κ B and beta-catenin.^[1-6] In NETs, interactions with these pathways as well as complexes between canonical Notch target hairy enhancer of split 1 (Hes1) and achaete-scute complex-like 1 (ASCL-1) have been reported.^[7-14] Many of these pathways can be pharmacologically modulated for translational research and eventually for experimental therapy of NETs, once the role of Notch signaling in these tumors is more clearly elucidated. Here we review the current state of NET therapies, the role of canonical and non-canonical Notch signaling in these tumor types,

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and the role of cancer stem cells in NET pathogenesis, chemoresistance and recurrence.

NOTCH SIGNALING

The Notch signaling pathway is an evolutionarily conserved, critical component of basic cellular processes such as proliferation, stem cell maintenance, and differentiation during both embryonic and adult development. The canonical Notch signaling pathway has been well-studied and typically depends on the binding of a Notch receptor to its ligand residing on a neighboring cell. This ligand binding promotes the separation of the extracellular subunit from the transmembrane subunit, which is followed by cleavage of the receptor's transmembrane subunit by ADAM metalloproteases (primarily ADAM-10) and gamma secretase. The latter cleavage releases the active form of Notch, the Notch intracellular domain (NICD). The NICD then translocates into the nucleus and binds to the transcription factor CSL (CBF-1/Suppressor of Hairless/LAG1), also known as RBP-Jk, to control expression of Notch-regulated genes.^[15-18] Ligand-independent activation of Notch cleavage has been reported in some contexts, notably breast cancer stem cells, where it is mediated by activation of ADAM-17 via the Sphingosine 1-phosphate pathway.^[19]

Different species contain different numbers of Notch isoforms. *Drosophila* contains one Notch receptor, *C. elegans* has two redundant receptors, and mammals contain four Notch receptors, Notch1-4. Notch receptors contain an extracellular domain that includes multiple epidermal growth factor (EGF)-like repeats in varying numbers that are involved in ligand binding. The intracellular portion of Notch transmits cellular signals and contains an RBP-Jk Association Module (RAM) domain, a nuclear localization signal (NLS), a seven ankyrin repeat (ANK) domain and a transactivation domain that contains conserved proline/glutamic acid/serine/threonine-rich (PEST) motifs. For a comprehensive review of known Notch ligands, see.^[17] In mammals, Notch ligands include Delta-like1 (DLL1), Delta-like3 (DLL3) and Delta-like4 (DLL4), which are homologous to *Drosophila* Delta, along with Jagged1 (JAG1) and Jagged2 (JAG2), which have homology to *Drosophila* Serrate. Notch ligands have multiple EGF-like repeats in their extracellular domains and all contain an N-terminal DSL (Delta/Serrate/LAG2) motif that, along with the first two EGF-like repeats is required for ligand-receptor interaction. Jagged ligands contain almost twice the number of EGF repeats as well as an additional cysteine rich region compared to DLL ligands. The intracellular portion of all Notch ligands lacks major homology with the exception that some, but not all, ligands contain multiple lysine residues and a C-terminal PDZ (PSD-95/Dlg/ZO-1) domain.

In addition to the well-studied canonical signaling, Notch signaling can also occur in a non-canonical fashion that

is independent of CSL and can be ligand-dependent or independent.^[1,20] Compared to canonical Notch signaling, knowledge of non-canonical Notch mechanisms is limited, with the majority of studies performed in cancer and immune system cells.^[1] Non-canonical Notch pathways present an interesting new avenue of study and may reveal new targets for therapeutic intervention in the translational setting.

One mechanism of non-canonical Notch signaling occurs through the Wnt/ β -catenin pathway in cancer and the immune system. The Wnt/ β -catenin pathway regulates cell pluripotency and cell fate decisions, and aberrant functions or mutations in β -catenin have been associated with a number of cancers and other human diseases. Non-canonical Notch signaling can result in an antagonistic interaction between Notch signaling and Wnt/ β -catenin^[2,20,21] that disrupts the regulation of developmental and disease processes.^[20] This results in an inverse relationship between elevated levels of membrane-bound Notch and lower levels of active β -catenin^[20] leading to negative regulation of Wnt signaling.^[4] One example of this crosstalk is the loss of Notch1 in the epidermis of mice, which results in activated Wnt/ β -catenin signaling and the formation of hyperplasia and cancer -- both of which can be reversed by the introduction of exogenous NICD.^[22]

Non-canonical Notch signaling is also involved in the activation and proliferation of CD4⁺ T cells in the immune system as well as in the tumor-promoting effects of interleukin-6 (IL-6).^[1,23] These events rely on NF- κ B and demonstrate crosstalk with other cellular pathways in the absence of canonical Notch signaling. Studies have demonstrated that even in the absence of CSL, CD4⁺ T-cell activation and proliferation through NF- κ B requires NICD playing a major role in the signature CBM complex (CARMA1, MALT1 and BCL10).^[24] The NICD can also activate a non-canonical signaling cascade via mTORC2 and Akt as a means of transmitting extracellular nutrient sensing cues to promote cell survival.^[5,25] Notch signaling, both canonical and non-canonical, is regulated by a myriad of known and unknown binding partners as well as posttranslational modifications. Comprehensive reviews of Notch signaling are available.^[18,26,27]

NETs - ENTEROPANCREATIC

The annual incidence of enteropancreatic NETs is 2-5/100,000 patients in the United States and recent studies suggest that this incidence will continue to rise in the coming years.^[21,28-30] Overall survival (OS) for metastatic pancreatic and small bowel NETs is 24 and 56 months, respectively.^[29] Enteropancreatic NETs, or NETs that form in the pancreas or the gut (also called carcinoids), can be categorized as functional or non-functional depending on their level of hormone release. Pancreatic NETs can hypersecrete insulin (insulinoma), glucagon (glucagonoma), somatostatin (somatostatinoma), pancreatic polypeptide

(PPoma) or vasoactive peptide (VIPoma) and those in the GI tract can secrete high levels of gastrin (gastrinoma). The classification of NETs clinically is based on immunohistochemical staining for low molecular weight keratins, chromogranin and somatostatin, as well as an assessment of Ki-67 index from within the region of highest mitotic density.^[31] Other observable factors such as anatomical site, histology, grade, level of differentiation and hormone secretion are also used but this phenotypic classification system has led to confusion in both the clinical and research settings due to the molecularly heterogeneous nature of these diseases. For clinical trial purposes, enteropancreatic NETs have historically been grouped together in clinical trials, with enrollment open to all patients with gut NETs regardless of subtype. It is now recognized that NETs must be subdivided into pancreatic and non-pancreatic subgroups to reduce heterogeneity in clinical trials^[32] and that progression free survival (PFS) may be a more relevant primary endpoint in clinical trial design than OS because most patients have indolent disease.^[33] Additionally, a key predictor of outcome in enteropancreatic NETs is the degree of tumor differentiation. Well-differentiated tumors have a better prognosis than poorly differentiated tumors, which can have a 5 year overall survival of less than 4%.^[30]

Enteropancreatic NETs are relatively slow-growing and traditional chemotherapy regimens have limited efficacy.^[34] The selection of therapy is driven by the staging, location of the tumor and symptom profile. Surgery is often used in the management of NETs for both curative (localized disease) and palliative care (widespread metastases). First line therapy for enteropancreatic NETs is somatostatin analogs (SSAs),^[34] with VEGF pathway inhibitors, mTOR inhibitors or peptide receptor radionuclide therapy (PRRT) as additional options. Many of these compounds are currently in clinical practice and/or clinical trials and have exhibited moderate success. SSAs such as octreotide, lanreotide and pasireotide help control symptoms of hormone hypersecretion (carcinoid syndrome), and more recently have been noted to have anti-proliferative effects on well or moderately differentiated NETs.^[35,36] For example, the PROMID trial (NCT00171873) examined metastatic midgut NETs^[37] and the CLARINET trial (NCT00353496) focused on pancreatic, midgut or hindgut NETs,^[38] both noting prolonged PFS in the SSA treatment arms compared to placebo. The NETTER-1 trial (NCT01578239) uses radiolabeled SSA ([¹⁷⁷Lu-DOTA⁰, Tyr³] octreotate) in PRRT for a localized anticancer therapy in patients with inoperable, somatostatin receptor positive metastatic midgut NETs with the primary endpoint of PFS. The RADIANT-3 trial (NCT00510068) demonstrated an increased median PFS in patients treated with the mTOR inhibitor everolimus/RAD001 (11 months compared to 4.6 months for placebo) in patients with advanced pancreatic NETs.^[39] Finally, the oral tyrosine kinase inhibitor sunitinib was studied in a prospective trial in patients with advanced, well differentiated pancreatic NETs. PFS was

11.6 months in the sunitinib group compared to 5.5 months in the placebo arm.^[40] The RADIANT-3 and the sunitinib study both resulted in FDA approval of these drugs for patients with pancreatic NETs. The RADIANT-4 trial (NCT01524783) further confirmed the role of everolimus in adult patients with advanced, progressive, well-differentiated, non-functional endocrine tumors of the lung or gastrointestinal tract.^[41] Patients receiving everolimus had a 7.1 month increase in PFS compared to placebo.^[41] A comprehensive review of carcinoid and NET clinical trials is available.^[33] The heterogeneity of NETs requires a deeper understanding of tumorigenic mechanisms and drug function that will guide future therapeutic development, patient management strategies and eventually, genomics-driven clinical trial design.

Genetic syndromes account for 15-20% of NETs. The most common syndromes include multiple endocrine neoplasia type 1 and type 2A/B (MEN1 and MEN2A/B), von Hippel-Lindau syndrome (VHL), neurofibromatosis type 1 (NF1) and tuberous sclerosis complex (TSC), and in each of these syndromes, specific loss- or gain-of-function mutations have been identified in causative genes. The remaining 80-85% of NETs is considered sporadic and genome-wide studies have been performed in an attempt to understand driver genetic mutations. Jiao *et al.*^[42] performed whole exome sequencing of 10 pancreatic NETs that resulted in the identification of somatic mutations in a number of known cancer-associated genes including *MEN1*, *DAXX*, *ATRX*, a number of genes involved in the mTOR pathway, and to a lesser extent *TP53*. Banck *et al.*^[43] studied forty-eight well-differentiated, small intestinal NETs (carcinoids) by whole exome sequencing and also identified somatic mutations in many cancer-associated genes including *FGFR2*, *MEN1*, *HOOK3*, *EZH2*, *MLF1*, *CARD11*, *VHL*, *NONO*, *SMAD1*, *FANCD2* and *BRAF*, yet only 21 genes were in common with a subsequent study that analyzed an additional 55 well-differentiated small intestinal NETs.^[44] Upon further comparison with the Jiao study,^[42] only 17 genes with somatic mutations found in small intestinal NETs were in common with pancreatic NETs.^[44] These data highlight that this group of tumors needs to be carefully studied, subgrouped and analyzed to account for heterogeneity in terms of site of origin, level of differentiation and underlying driver mutations. Interestingly and despite the somewhat disparate results, all of these studies highlight the putative role of chromatin remodeling, perhaps in concert with Notch signaling, in the etiology of enteropancreatic NETs.

A popular model of cancer formation is that tumors are dependent on a subset of highly tumorigenic cells, so-called cancer stem cells, for initiation, maintenance and propagation.^[45] Cancer stem cells have been identified in a number of solid tumors^[46-48] and leukemias,^[49] and are noted for their pluripotency, unique complement of cell-surface antigens, ability to self-renew, and ability to form xenografts in immunocompromised mice from

very small numbers of cells. Cancer stem cells are often chemoresistant, mediate tumor recurrence, and recruit the host immune system through a variety of mechanisms to support tumor cell growth and metastasis.^[45]

Cancer stem cells have been identified in gastrointestinal^[50] and pancreatic NETs.^[51] In gastrointestinal NETs, a population of stem cells was identified based on ALDH positivity which is required for chemoresistance and enhances self-renewal.^[50] ALDH+ cells exhibit anchorage-independent growth and have elevated expression of Src, Erk, Akt and mTOR. Because therapies directed towards the Akt/mTOR pathway are already clinically validated in NETs, the investigators focused on Src and treated mouse xenografts with anti-Src siRNA. This treatment resulted in a 91% decrease in tumor mass and suggested an additional treatment avenue for gastrointestinal NETs.^[50] In pancreatic NETs, stem cells have been isolated that co-express the cell-surface protein CD90 and aldehyde dehydrogenase A1 (ALDH1), as well as CD47 which serves as a flag to evade the immune system.^[51] These stem-like cells form tumors in mice and the treatment of tumor-bearing mice with anti-CD47 antibody therapy inhibits tumor growth, prevents metastasis and prolongs survival. Combination therapy with anti-CD47 and anti-EGFR (expressed by the majority of pancreatic NETs) in the preclinical setting demonstrated improved efficacy over anti-CD47 antibody therapy alone^[51] and supports the notion that treatment of human pancreatic NETs with stem cell specific antigens will yield clinically significant results.

NETs in general remain significantly understudied with respect to molecular mechanisms of pathogenesis, and particularly Notch signaling. Mechanistically, Notch may contribute to carcinogenesis by inhibiting differentiation, promoting cellular proliferation and/or inhibiting apoptosis, yet few studies have comprehensively examined these endpoints with respect to the four Notch receptors and their ligands in NETs. The available studies suggest a tumor suppressive function for Notch1 in cells derived from the neuroendocrine lineage. This is consistent with role of Notch in *Drosophila* neurogenesis, where Notch restricts differentiation towards the neuronal lineage. The loss of Notch in *Drosophila* embryos results in uncontrolled ectodermal differentiation down the neuronal lineage.^[52,53] It is plausible that loss of Notch signaling would allow NET cells to acquire or maintain a partially differentiated neuroendocrine phenotype while retaining the ability to proliferate. For example, recent studies^[11,12,54-57] report that Notch1 signaling is minimal or absent in gut carcinoids, medullary thyroid carcinoma (MTC) and pulmonary typical and atypical carcinoids. Yet these same cancers express high levels of human achaete-scute homolog 1 (hASH1), a basic helix loop helix transcription factor that is regulated by Notch signaling. The aberrant expression of hASH1 and the arrest of NET cells at an early stage of differentiation may be due to decreased Notch1-activated expression of Hes1 and Hes5 which both facilitate

degradation of hASH1.^[57] Transient overexpression of NICD in BON1 cells resulted in increased proliferation and dose-dependent increases in Hes1. In contrast, immunohistochemistry for Notch1, Hes1, Hey1, pIGF1R and FGF2 antibodies on a tissue microarray of 120 well differentiated NETs arising from the pancreas ($n = 74$), ileum ($n = 31$) and rectum ($n = 15$), demonstrated elevated Notch1 expression in 100% rectal, 34% of pancreatic, and 0% of ileal NETs, and Hes1 expression in 64% of rectal, 10% of pancreatic and 0% of ileal NETs,^[58] exhibiting significant variability in Notch1 signaling across different tissue types. There is limited information on other Notch receptors or the ligands involved in Notch signaling in NETs and a comprehensive analysis of Notch expression patterns across all enteropancreatic NET subtypes is required to fully understand the variability and potentially redundant functions of Notch receptors and ligands.

The ability of Notch to behave as an oncogene or tumor suppressor depending on cellular context is driven in part by the availability of coactivators and corepressors. CSL coactivators such as MAML, SKIP and p300 are well known to activate transcription of Notch target genes by binding to NICD. Conversely, in the absence of NICD, corepressors also regulate transcription in specific ways and canonical Notch corepressors include SMRT,^[59] SIRT^[60] and LSD1 (histone lysine demethylase),^[61] among others (reviewed in^[62]). Epigenetic regulation by Notch activator and repressor complexes containing histone acetyltransferases, histone demethyltransferases, histone methyltransferases, *etc.* actively remodel the chromatin at Notch-responsive target genes and provide an additional layer of reversible regulation.^[63] Chromatin sites accessible to Notch NICDs are also influenced by transcriptional regulators that can act as cofactors or inhibitors.^[64-66] A recent report by Liefke *et al.*^[63] demonstrates that the histone demethylase KDM5A/RBP2 is a key component of the CSL repressor complex. Data from our laboratory demonstrates that RBP2 is upregulated in gastrointestinal NETs and in liver metastases from primary NET tumors, suggesting that RBP2 may be actively repressing canonical Notch activity (Crabtree, *et al.* 2016 Oncogenesis in press).

NETs - PULMONARY

Pulmonary NETs are an equally diverse set of NETs that fall on a continuum from well-differentiated typical carcinoid (TC), to less differentiated atypical carcinoid (AC), to highly malignant, poorly differentiated small cell lung carcinoma (SCLC) and large cell neuroendocrine carcinoma (LCNECs).^[67] Features distinguishing these groups include size, with TC and AC defined as ≥ 0.5 cm, and histologic characteristics such as organoid growth patterns with uniform cytologic features. These tumors contain a moderate amount of eosinophilic cytoplasm and nuclei containing finely granulated chromatin, which is coarser in AC than in TC. Prominent nucleoli are also present in AC, but not in TC. New 2015 WHO

clinicopathological criteria also define the mitotic index of these tumors (number of mitoses per 2 mm² in the area of highest mitotic activity with the most viable tumor cells).^[68,69] The mitotic index of typical carcinoid is < 2, atypical carcinoid is 2-10, whereas SCLC and LCNECs have mitotic indices > 10.^[67,68] Lung tumors can also be distinguished by grade, with TC classified as low grade, AC as intermediate grade and SCLC/LCNECs as high grade.^[68,69] Identity of these tumors is typically confirmed by immunohistochemistry using the cellular proliferation Ki-67, as well as neuroendocrine markers such as synaptophysin, chromogranin A and neural cell adhesion molecule (NCAM) to distinguish SCLC from non-small cell lung cancer (NSCLC). TC have no necrosis and Ki-67 ≤ 5%, AC can have focal necrosis and Ki-67 ≤ 20% and SCLC have Ki-67 > 50%. Pulmonary NETs may also exist, albeit at much lower incidence than other pulmonary NETs, as heterogeneous, combination tumors consisting of mixtures of SCLC and LCNEC, or SCLC and NSCLC with neuroendocrine differentiation.^[67] These mixed phenotypes may indicate clonal selection and/or phenotypic plasticity of a pluripotent cancer stem cell.

Pulmonary NETs have a low incidence in the US, with a rate of 1.6/100,000 individuals. TCs comprise 1-2% and ACs make up only 0.1-0.2% of all pulmonary tumors, whereas SCLC and LCNET make up 20% and 1.6-3%, respectively. Overall survival is good for the well-differentiated TC tumors (92-100% OS) and moderate for AC (61-88% OS), whereas the higher grade, poorly differentiated SCLC and LCNET have a grim prognosis with OS as low as 5%.^[70] There are limited treatment options for pulmonary NETs and the only curative therapies for TC and AC is surgery. These tumors are historically refractive to chemotherapy and exhibit response rates as low as 22%.^[71] In the case of advanced disease, such as that seen with patients initially presenting with SCLC and LCNEC, surgery is rarely performed and systemic chemotherapy is the first line treatment. Combination etoposide plus carboplatin chemotherapy has high response rates (about 90%) but within 1 year the majority of tumors recur and are refractory to further treatment.^[71] mTORC1 inhibitors (everolimus, temsirolimus) have been used in combination with standard of care chemotherapy, but these compounds exhibited only moderate efficacy with the liability of dose-limiting toxicities.^[72] mTOR inhibitors have also been combined clinically with SSAs in the RADIANT-2 trial (NCT00412061) that included enteropancreatic NETs as well as pulmonary TC and ACs. Subgroup analyses from this study found a median PFS of 5.6 months for the few TC and AC patients who received only the octreotide LAR and no advantage for the patients receiving the combination therapy.^[73] A follow-up trial called the LUNA trial (NCT01563354) is a prospective, randomized, open-label, three-arm design to study advanced lung (TC and AC) and thymic NET response to pasireotide LAR, everolimus or both in combination. The RADIANT-4 trial (NCT01524783) enrolled adult patients with advanced,

progressive, well-differentiated, non-functional endocrine tumors of the lung or gastrointestinal tract to receive everolimus or placebo with the primary endpoint of PFS.^[41] Patients receiving everolimus had significantly improved median PFS of 7.1 months compared to placebo.^[41] Sunitinib was studied in a phase II trial in patients with relapsed or refractory SCLC and the treatment was poorly tolerated and resulted in limited gain in PFS.^[74] Tyrosine kinase inhibitors such as imatinib have also been studied in pulmonary NETs with disappointing results.^[75]

The genetic basis of pulmonary NET formation has been explored in recent years. There are many cases of targeted analysis identifying inactivating mutations in *TP53*, *RBI* and *PIK3CA* genes.^[76-79] Genome-wide studies have been performed^[80-83] to identify copy number alterations, somatic single nucleotide variants and alterations in gene expression associated with SCLC. From these studies, potential driver mutations were identified in cancer-associated genes such as *TP53*, *RBI*, *CREBBP*, *EP300*, *MLL* and the *SOX* family. A separate study conducted whole genome sequencing of 110 SCLC and identified biallelic inactivation of *TP53*, *RBI*, *CREBBP*, *EP300*, *TP73*, *RBL1/2*, as well as inactivating mutations in Notch family genes in 25% of cases.^[83,84]

As with pancreatic NETs, cancer stem cells provide a plausible mechanism for drug resistance, recurrence and metastasis of SCLC. However, due to limited availability of human clinical samples, the majority of the work to identify markers of SCLC has been performed in cell lines by isolating side populations of cells with stem-like features. Using the SCLC cell lines NCI-H82, H146 and H526, Salcido *et al.*^[85] isolated a population of cells with high rates of proliferation, efficient self-renewal and decreased cell surface expression of CD56 and CD90. These isolated cells also overexpress many genes associated with cancer stem cells and drug resistance, including genes involved in the Notch signaling pathway.^[85] In a separate study, a side population of cells was isolated from lung cancer cell lines established from primary tumors.^[86] This side population was strongly positive for CD44 and co-expressed CD90, while having mesenchymal morphology, resistance to irradiation, and increased expression of stem cell related genes Nanog and Oct4.^[86] CD133 is a common cell surface antigen in SCLC stem cell populations and was upregulated in cell populations as one of several stem cell markers in six separate studies from various SCLC cell lines.^[87-92] In one of these studies, it was found that CD133+ cells express increased neuropeptide receptors which revealed an avenue for therapeutic intervention.^[90] Subsequent testing of neuropeptide receptor antagonists revealed that one of the analogs, Peptide 1, decreased cell growth and increased apoptosis in SCLC cell lines. Further, Peptide 1 produced a significant reduction in tumor volume in mouse xenograft models, exhibiting very few CD133 positive cells after treatment, compared with tumors treated with etoposide.^[90] In other studies, inhibitors

were selected due to known pathway involvement in SCLC. For example, a dual mTORC1/2 and class I PI3K inhibitor VS-5584 was tested in SCLC xenograft models and a PDX model established from a SCLC lymph node metastasis, resulting in significant decreases in tumor burden, decreased tumor-initiating frequency and marked depletion of cancer stem cells.^[93]

The Notch signaling pathway is of increasing interest in SCLC and as with enteropancreatic NETs, Notch signaling in the lung is tissue type and cell context dependent. Notch signaling can promote the growth of NSCLC, yet inhibit the growth of SCLC.^[94,95] The tumor phenotype in SCLC may be driven via Notch3 expression, which is decreased in SCLC compared to non-tumor lung tissue as measured by immunohistochemistry.^[96] SCLC may be the result of deregulated Notch in cell fate decisions that determine differentiation towards the epithelial Clara, ciliated and pulmonary neuroendocrine cell lineages.^[97] In mouse models with allelic series deletion of Notch1, 2 and 3, all three Notch receptors were required in an additive manner to regulate the abundance of neuroendocrine cells in the lung, whereas only contribution from Notch2 was required for Clara/ciliated cell development.^[98]

Over the years, many targeted therapies have been developed to modulate the Notch signaling pathway, including neutralizing antibodies, decoy ligands, blocking peptides, natural compounds and -secretase inhibitors (reviewed in^[18]). The Notch 2/3 neutralizing antibody tarextumab, inhibits tumor growth in mice in a variety of epithelial tumors, but also in SCLC xenograft tumors,^[99] suggesting that Notch2 and/or Notch3 inhibition can be therapeutic in the clinical setting. A novel way of exploiting decreased Notch signaling therapeutically is by targeting Notch ligands that are frequently overexpressed even in tumors with low or absent canonical Notch signaling. This approach was pioneered in SCLC, which frequently expresses high levels of DLL3. Because DLL3 can function as a Notch inhibitor by retaining Notch receptors in the cytoplasm or by cis-inhibition, a DLL3 mAb conjugated with a DNA damaging toxin was used as a highly effective chemotherapeutic in preclinical PDX models of SCLC. These experiments resulted in complete, durable responses 5 months post treatment. The naked mAb had no therapeutic activity, suggesting that DLL3 inhibition alone is not sufficient for tumor regression in SCLC.^[14] In other studies, it has been proposed that, in addition to the primary SCLC progression as a result of TP53 and RB1 alterations, secondary transitions from non-small cell lung carcinoma to SCLC can occur following chemotherapy. This implies phenotypic plasticity from an epithelial to a neuroendocrine lineage can occur under treatment-imposed selection. A recent publication by Meder *et al.*^[13] demonstrates that this process is mediated by the Notch-ASCL1-RB-P53 signaling axis.

Paralog-specific effects add yet another layer of complexity

to Notch signaling, since not all Notch receptors are created equal. Notch receptors are not always redundant and in some cases their functions are not only independent but opposite. Notch1 and Notch2 have opposite effects on Akt in NSCLC.^[100] In Luminal B breast cancer, Notch1 and Notch4 have similar effects on endocrine resistance but act through completely different sets of downstream genes and produce different cellular phenotypes^[101] (Espinoza and Miele, unpublished). Notch1, 3, and 4 are oncogenic in the breast, while Notch2 has been described as a tumor suppressor in breast cancer cell lines.^[102] The mechanism of these paralog-specific effects is unknown but may involve non-canonical signals, such as the inhibitory role of Notch4 on SMAD^[103] or the stimulatory role of Notch1 on NF- κ B.^[104] The oncogenic activity of Notch4 in the mouse mammary gland is independent of CSL and is therefore completely or at least partially non-canonical.^[105] Another explanation for paralog-specific effects may be in quantitative signal intensity of the different Notch ligands. For example, constitutively activating mutations in Notch1 and Notch2 are equally oncogenic in a subset of triple negative breast cancer (TNBC),^[106] despite the fact that Notch2 has been described as a tumor suppressor in TNBC cell lines.^[102] Therefore, the absolute number of NICD molecules available as a result of overproduction or decreased turnover may dictate different phenotypic consequences. Additionally, paralog-specific effects may also be achieved by selective activation of chromatin sites with different affinity for Notch NICDs, epigenetic modifications by NICD binding partners that alters binding site availability, or by a combination of canonical and non-canonical effects that depends on NICD abundance. In short, the role of paralog-specific effects has been poorly characterized in NETs and is an area in need of further study.

NETs - SKIN

Merkel cell carcinoma (MCC) is a rare, aggressive cutaneous NET that occurs most frequently in the elderly and/or the immunosuppressed, although more than 90% of MCC patients have no known immune dysfunction.^[107] It is seen primarily in light-skinned individuals and has a male predominance of 2:1.^[108] MCC occurs most frequently in sun-exposed areas of skin, particularly the head and neck, followed by extremities and then the trunk. In 80% of cases, MCC is associated with the Merkel cell polyomavirus (MCPyV).^[109,110] Infection with MCPyV is not sufficient to induce tumorigenesis^[111] and additional events including loss of cellular immune surveillance are required for oncogenic transformation. The MCPyV large T-antigen is oncogenic in MCC by binding the retinoblastoma protein and promoting cell cycle progression.^[112] The small T-antigen of MCPyV acts downstream of the mTOR signaling pathway by maintaining hyperphosphorylation of 4E-binding protein (4EBP1), resulting in dysregulated cap-dependent translation in MCC.^[113] Patients with MCPyV negative MCC tumors have increased DNA

damage signatures at the genetic level, presumably as a result of UV exposure.^[114,115]

MCC is highly metastatic and the 5 year survival rate is dependent on the stage at which original diagnosis is made. Patients with local disease at diagnosis have a 5 year OS of 63-87%, those with regional nodal involvement 39-42% and 0-18% for patients with widespread, distant metastases.^[116] The annual incidence of MCC in the US is increasing, with an estimated 1,600 patients diagnosed per year.^[117] The increase in incidence is attributed to population aging, more known risk factors associated with this cancer (such as increased aggregate sun exposure), and increased diagnostic power with cytokeratin 20 immunohistochemical staining, which is positive in 88-100% of MCC cases.^[118]

There are no FDA-approved agents for the treatment of MCC, nor are there established, standard of care chemotherapy regimens.^[109] Current first line therapies for localized disease include surgical resection followed by postoperative radiation therapy. Radiotherapy plays a significant role in both the curative setting, and palliative care setting when used as a monotherapy in advanced metastatic MCC.^[119] Systemic chemotherapy regimens used for SCLC are employed and typically include a combination of a platinum agent (cisplatin or carboplatin) and topoisomerase inhibitor (etoposide)^[120-122] or combination cyclophosphamide, doxorubicin and vincristine therapy (CAV therapy).^[122] Cytotoxic chemotherapies do not produce durable responses and are associated with significant toxicity, highlighting the need for targeted, mechanism-based therapies. Immunohistochemical analysis of MCC tumors has led to development and use of several new mechanism-based therapies including SSAs (octreotide, lanreotide),^[123,124] pan-receptor tyrosine kinase inhibitors (pazopanib),^[125] PI3K inhibitors,^[126,127] vitamin D receptor agonists,^[128] small molecules to downregulate Survivin,^[129,130] anti-PD-L1 antibody therapy,^[131] and an antibody conjugate linking a maytansinoid microtubule assembly inhibitor to CD56 (lorvotuzumab mertansine).^[132] Many of these are now in clinical trials for MCC and an excellent review of future potential therapeutic options and current clinical trials for MCC can be found in ref.^[118]

In addition to immunohistochemistry, genomic studies have also been applied to MCC to identify new therapeutic targets and understand the mechanism of tumorigenesis in both MCPyV positive and negative cases. Gene panel studies on 15 MCPyV negative and 12 MCPyV positive MCC samples identified mutations in *TP53*, *KIT*, *PIK3CA* and *EGFR* genes, with *RBI* mutations only identified in the virus negative samples, suggesting that the dysregulation of the RB pathway may be a critical step in tumorigenesis.^[133] Targeted sequencing of 17 MCC patient samples with unknown virus status, identified mutations in *TP53*, *RB* and *NOTCH1*, among others.^[134]

Exome sequencing studies performed on small numbers of formalin-fixed, paraffin-embedded MCC samples and also identified *RBI* in MCPyV negative tumors.^[135] Another small study conducted on 4 MCPyV positive tumors identified somatic mutations in *PDE4DIP*, as well as genes within the DNA damage response (*PRKDC*, *AURKB*, *ERCC5*, *ATR* and *ATRX*) and epigenetic modifying enzymes (*MLL3*).^[136] Harms *et al.*^[115] performed a slightly larger study of whole exome sequencing of 9 MCPyV negative and 7 MCPyV positive MCC samples. Known mutations were identified in *TP53*, *RBI* and *PIK3CA* along with novel activating mutations in oncogenes like *HRAS*, loss-of-function mutations in *PRUNE2* and *NOTCH* family genes, and mutations disrupting the PI3K signaling pathway in the MCPyV negative tumors.^[115,137] Further, the MCPyV negative tumors also had a higher overall mutational burden and were characterized by a prominent UV-signature pattern with C > T transitions making up 85% of the mutations. MCPyV positive tumors had a much lower mutational burden and were lacking the UV signature, suggesting that MCPyV negative tumors have increased susceptibility to UV damage.^[115] The most comprehensive study to date included exome sequencing of 49 MCC samples (21 positive, 27 negative).^[114] This study confirmed the previous report that the signature of MCPyV negative tumors is very different than the MCPyV positive tumors. MCPyV negative tumors have a higher mutation burden, frequent mutations in *TP53* and *RBI* and additional mutations in genes involved in chromatin modification (*ASXL1*, *MLL2* and *MLL3*) and DNA damage pathways (*ATM*, *MSH2*, *BRCA1*). Interestingly, both MCPyV positive and negative tumors have mutations predicted to activate the PI3K pathway (*HRAS*, *KRAS*, *PIK3CA*, *PTEN* and *TSC1*) and to inactivate the Notch signaling pathway (*Notch1*, *Notch2*),^[114] suggesting these pathways as putative points for intervention in MCC regardless of viral status.

As discussed for SCLC and enteropancreatic NETs, another possible point of intervention is by targeting cancer stem cells. However, in the case of MCC, the cell of origin is still under debate. Based on early observation of MCC and the similarity of expression patterns for neuroendocrine and epithelial markers, it was presumed that MCCs arise from the Merkel cell, part of the somatosensory system located within the basal epidermis. However, with the observations that Merkel cells and MCC are found in different regions of the skin and exhibit differential expression of marker proteins, new data are challenging the concept that MCCs arise from Merkel cells.^[138] One theory, based on pathologic diagnosis of MCC suggests a role for pluripotent stem cells in the dermis as the cells of origin, facilitated by UV irradiation and MCPyV infection.^[139] Another study proposes that MCCs arise from pro/pre-B or pre-B cells based on terminal deoxynucleotidyl transferase and PAX5 expression, as well as the preference for polyomaviruses to preferentially infect undifferentiated stem cells or progenitor cells.^[140] However, in the absence

of experimental evidence supporting a stem cell origin, more lineage tracking studies are needed to identify the cellular origin of MCC.

Notch signaling has been an area of active investigation in MCC as a result of the genome-wide studies that have highlighted the Notch pathway as one of key interest, with somatic single nucleotide variants identified in Notch1, and Notch2 that were independent MCPyV status.^[114] The inactivating mutations detected in Notch genes were located in the EGF-like and ankyrin repeat regions, consistent with loss-of-function events characterizing a tumor suppressive role for Notch in MCC.^[115] Further, the data on Notch and other genes dysregulated in MCC are common with SCLC, suggesting that these pathways are also cornerstones of neuroendocrine differentiation in epithelial cells.^[114] Another study examined the Notch signaling pathway as a target of microRNA-375, which is highly overexpressed in well-differentiated MCC cell lines yet strikingly downregulated in highly aggressive, undifferentiated MCC cell lines.^[141] miR-375 overexpression caused post-transcriptional repression of Notch2 and RBPJ resulting in decreased cell proliferation, migration and invasion *in vitro*. This led to the conclusion that miR-375 is a putative regulator of cancer cell aggressiveness through inhibition of Notch signaling.^[141] In contrast, Panelos *et al.*^[142] performed immunohistochemical studies of Notch1 expression in MCC and found 30/31 cases had Notch1 cytoplasmic and membrane expression in greater than 50% of cells. These data contradict the data in other NETs, including other data on MCC, which suggest Notch1 is a tumor suppressor in MCCs.

NETs - THYROID

Medullary thyroid carcinoma (MTC) is a NET that originates from the thyroid C-cells and express high levels of calcitonin, chromogranin A, synaptophysin and achaete-scute complex-like 1 (ASCL1). MTCs are relatively slow growing tumors that comprise 1-2% of all thyroid cancers and have a 10 year median survival of 65%.^[143,144] The majority of these tumors are sporadic, but they can be hereditary and arise with other NETs as a part of MEN2A/2B or as familial MTC. Gain-of-function mutations in the RET tyrosine kinase gene (most commonly M918T) are the known driver mutation in the majority of these tumors.^[145,146] Those tumors that are RET mutation negative frequently have RAS mutations – and the presence of these mutations appears mutually exclusive.^[147,148] As with other NETs discussed above, there are no curative therapies for MTC. Surgery is the first line of treatment for localized disease, but there are no therapeutic options for patients who present with regional or widespread metastases, highlighting the critical need for additional therapeutics.

Several promising new directed therapies for MTC are in development or clinical trials. As with other NETs, SSAs

and mTOR inhibitors have been studied in MTC, and have shown preliminary efficacy in small trials.^[149,150] One ongoing trial (NCT01625520) is examining the efficacy of SOM230/pasireotide alone and in combination with everolimus in progressive metastatic or postoperative persistent MTC. More recently, new drugs that targets both PI3K and mTOR have been developed, with BEZ235 showing efficacy in preclinical studies of thyroid cancer.^[151] Antibody therapy is also in development for MTC. Carcinoembryonic antigen or CEA is an antigen expressed by MTC cells and an anti-CEA monoclonal antibody combined with autologous hematopoietic stem cell rescue has shown promise in a phase 1 study in rapidly progressing metastatic MTC.^[152]

Tyrosine kinase inhibitors are also in development and AMG706/motesanib was studied in locally advanced or metastatic, progressive or symptomatic MTC in a single-arm phase 2 study.^[153] Despite the 81% of patients in this trial that achieved stable disease, there was no placebo or standard of care arm, making the interpretation of drug efficacy and toxicity a challenge. Axitinib was also studied in a small trial of locally advanced MTC ($n=6$), and resulted in 5/6 or 83% of patients with stable disease > 16 weeks.^[154] However, as with the motesanib trial, the single-arm study design, as well as the small number of MTC patients included makes the trial results difficult to interpret. The ZETA and EXAM trials studied two additional compounds, vandetanib and cabozantinib, in advanced, unresectable, locally advanced or metastatic MTC. The first randomized, double-blind, placebo controlled study (ZETA trial; NCT 00410761) tested vandetanib and detected an increase in PFS (30.5 vs. 19.3 months for placebo) in the 331 patients recruited to the study. Stratification of the patients by RET mutation suggested that there was an improved response in patients with RET M918T mutation and also in MTC cases with no RET mutation identified.^[155] These data led to FDA and EMA approval for vandetanib for the treatment of symptomatic or progressive, unresectable, locally advanced or metastatic MTC. The EXAM trial (NCT00704730) was a randomized, double-blind, placebo controlled study of cabozantinib in advanced and progressive MTC. This study recruited 330 patients and reported a median PFS of 11.2 months for treatment versus 4.0 months in controls.^[156] The responses in this trial were similar regardless of RET mutational status, and the results from this trial led to FDA and EMA approval of cabozantinib for progressive, metastatic MTC. Another tyrosine kinase inhibitor, regorafenib which has been approved for treatment of metastatic colorectal cancer, is now being studied as a second or third line therapy in MTC (NCT02657551). For recent, more comprehensive reviews of new molecular therapies and thyroid cancer clinical trials including those for MTC, see.^[143,157]

Although the genetic gain-of-function RET mutations are well established as the basis for MTC, additional genetic studies have been performed to understand the etiology of

RET mutation negative MTCs, and endocrine syndrome-related MTCs. Exome sequencing of 17 sporadic MTCs identified the expected mutually exclusive RAS and RET mutations, but no other commonly occurring driver mutations.^[148] Exome sequencing of MTCs associated with MEN2A also identified the expected RET mutations, but also suggested that low frequency mutations such as those found in EIF4G1 may also play a role in MEN2A-associated tumorigenesis by indirectly altering the RET pathway.^[158] A similar study was undertaken by Smith *et al.*^[159] in MTCs lacking an identifiable RET mutation. Interestingly, this group found a recurrent mutation in the ESR2 gene which encodes the estrogen receptor beta (ER). Estrogen receptor alpha (ER) and ER can form heterodimers and bind to estrogen response elements to regulate gene expression.^[160] Alternatively, ER can antagonize the transcriptional activity of ER.^[161-163] The RET gene contains three ERE sites that were shown to be actively regulating RET gene expression *in vitro*. The authors propose that this may be a novel mechanism by which the RET gene is regulated in RET mutation-negative familial MTC.^[159] Heilmann *et al.*^[164] performed genomic profiling of MTC cases during the course of clinical care and in addition to the expected RET mutations, also identified amplifications of CCND1, FGF3, FGF19 and CDKN2A. The authors propose that these may be cooperating driver mutations impacting chemoresistance and disease outcomes.

Cancer stem cells have been identified in MTC cell lines that are strongly positive for the cell surface antigen CD133 by immunohistochemistry.^[165] Interestingly, cell lines with the M918T RET mutation produce the highest number of CD133⁺ stem-like cells.^[165] This population of stem-like cells may also be involved in chemoresistance. In a study by Kucerova, CD133⁺ cells from MTC cell lines were no more chemoresistant than the parent population of cells. However, once the CD133⁺ cells were implanted in mice as xenografts and treated with 5-fluorouracil (5-FU), there emerged a new CD133⁺ stem-like cell population that was resistant to subsequent 5-FU therapy and retained these chemoresistant properties in culture.^[166] MTCs are relatively resistant to the radioactive iodine therapies used for follicular and poorly differentiated thyroid cancers, and one group treated MTC stem cells with all-trans-retinoic acid (ATRA) to sensitize these cells to radioiodine therapy. The stem cells identified and treated with ATRA increased their uptake of iodine by 8 fold, suggesting that ATRA pre-treatment followed by radioactive iodine therapy may be a new treatment modality for MTC.^[167] Finally, co-expression of CD133 and CD44 in MTC by immunohistochemistry was correlated with decreased overall survival in a cohort of 51 MTC patients, compared to those with no co-expression of these two markers implying that CD133 and CD44 can be used as prognostic markers for overall survival.^[168]

At the molecular level, MTC cells express a variety of

proteins including calcitonin and chromogranin A, as well as ASCL1 (also important in pulmonary NETs). Notch is one of the pathways regulating the production of ASCL1, especially during development. Notch1 expression is absent in MTC and overexpression of the Notch intracellular domain decreases proliferation of MTC cell lines,^[55] consistent with its role as a tumor suppressor. Activation of Notch in MTC by pharmaceutical means became possible when valproic acid was reported to activate Notch in neuroblastoma cells^[169] and subsequent work demonstrated that valproic acid increased Notch1 signaling and induced apoptosis in MTC cells.^[170] Using a mouse model system, Jaskula-Sztul *et al.*^[171] demonstrated that activation of the Notch signaling pathway may be a therapeutic strategy for MTC. This same group expanded our knowledge of Notch signaling in MTC by upregulating Notch3 *in vitro* and *in vivo* via NICD3 and the pharmacological HDAC inhibitor ABA3. They demonstrated that Notch3, like Notch1, can alter the neuroendocrine phenotype in MTC, resulting in decreased proliferation and loss of NET markers.^[172] Resveratrol treatment of MTC cells suppresses growth, induces apoptosis and reduces expression of chromogranin A and ASCL1 as a result of upregulation of Notch2.^[173] In similar studies, thiocoraline treatment *in vitro* increases the expression of Notch1 and Notch2 isoforms, as well as the downstream Notch target genes HES1, HES2 and HEY1, while expression of HES6 decreased.^[174] Taken together, these studies indicate a clear role for Notch signaling in MTC therapy.

CONCLUSION

The role of Notch signaling in NETs remains incompletely understood. Further study is required to understand how this pathway impacts tumorigenesis and chemoresistance in this diverse tumor group. There is evidence that different Notch isoforms act as tumor suppressors in some NETs but not others and paralog specific effects are understudied and remain unclear. The significant genetic heterogeneity of NETs suggests that individual molecular subtypes must be studied separately to dissect the roles of Notch signaling components and their potential therapeutic implications.

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Conflicts of interest

There are no conflicts of interest.

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Management of hepatic metastases of well/moderately differentiated neuroendocrine tumors of the digestive tract

Anna La Salvia¹, Stefano Partelli², Marco Tampellini¹, Domenico Tamburrino³, Massimo Falconi², Giorgio V. Scagliotti¹, Maria Pia Brizzi¹

¹Division of Medical Oncology, Department of Oncology, University of Turin, San Luigi Gonzaga Hospital, Regione Gonzole 10, 10043 Orbassano, Italy.

²Pancreatic Surgery Unit, San Raffaele Hospital, Vita-Salute San Raffaele University, 20132 Milan, Italy.

³HPB Unit, Royal Free Hospital, London NW32QG, UK.

Correspondence to: Dr. Maria Pia Brizzi, Division of Medical Oncology, Department of Oncology, University of Turin, San Luigi Gonzaga Hospital, Regione Gonzole 10, 10043 Orbassano, Italy. E-mail: mariapia.brizzi@email.it

ABSTRACT

In neuroendocrine tumors (NETs), liver metastases (LM) represent the most crucial prognostic factor, irrespective of the primary tumor site. At diagnosis, about 65-95% of gastroenteropancreatic neuroendocrine tumors (GEP-NETs) show hepatic metastasis. Management strategies of LM are heterogeneous and range from systemic therapy to liver-directed procedures. The type of systemic therapy used is dependent on the grade and proliferation of the tumor and includes somatostatin analogues, interferon, m-Tor and tyrosine kinase inhibitors, and chemotherapy. Angiographic liver-directed techniques, such as transarterial embolization/chemoembolization and selective internal radiation therapy, offer excellent palliation for patients with liver-predominant disease. In highly selected cases, liver transplantation and peptide receptor radionuclide therapy are considered. The relatively low disease incidence and the diversity of presentation have led to a lack of well-conducted randomized controlled trials comparing the efficacy of different treatment options. Experience indicates that surgery is the only treatment that offers potential for cure. For unresectable lesions, the absence of data from rigorous trials limits the validity of many publications that detail management. In this review we will discuss the existing approaches for hepatic metastases from GEP-NETs.

Key words: Gastroenteropancreatic carcinoids; metastases; systemic treatment

INTRODUCTION

Neuroendocrine tumors (NETs) are rare neoplasms originating from diffuse neuroendocrine cells. Even though site of origin could sometimes be unknown, NETs frequently involve any part of the gastrointestinal tract (including endocrine pancreas), bronchopulmonary tree, thyroid, and thymus and have a wide range of malignant potential. The rapid evolution of clinical and pathological findings has hampered a systematic classification of this inhomogeneous family of tumors. The last World Health Organization (WHO) classification was published in 2010.^[1] Basically, NETs are classified according to tumor differentiation and site of occurrence. Highly aggressive, poorly differentiated neoplasms were defined as Grade-3 neuroendocrine carcinomas (NECs) when originating from the gastrointestinal tract, or as small- or large-cell NECs when appearing in the lung.^[2] Well- to moderately differentiated neuroendocrine neoplasms (WMD-NEN)

are a highly heterogeneous group of tumors comprising low-grade (G1) and intermediate-grade (G2) NETs of the gastrointestinal tract, typical and atypical carcinoids of the lung and thymus, and other cancers such as medullary thyroid carcinoma and pheochromocytoma/paraganglioma.^[1,2] Finally, NETs could be associated with paraneoplastic syndromes or with a supranormal production of hormones responsible for specific syndromes.

The gastroenteropancreatic NETs (GEP-NETs) are the most common NETs. Due to their relatively indolent course, they are frequently diagnosed in an advanced stage,^[3,4] with the development of liver metastases (LM) being the most frequent clinical occurrence.^[3-5] Metastatic spread to the liver may be accompanied by a wide spectrum of clinical presentations, from asymptomatic disease incidentally

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discovered during radiologic workup for other reasons, to debilitating symptoms caused by acid hypersecretion, serotonin syndrome, or carcinoid syndrome. In any case, the vast majority of patients with hepatic involvement will die of liver failure.

The management of patients with LM from GEP-NETs remains a matter of debate. It involves several specialties: surgery, medical oncology, radiotherapy, interventional radiology, and nuclear oncology. Despite the great number of options, there is no general consensus on the optimal treatment sequence in metastatic patients.

In this review, we focus on the most recent findings about management of LM from GEP-NETs.

SURGERY

Patients with pancreatic NETs frequently present with LM.^[6] Treatment for LM includes a wide panel of treatments with the aim of achieving the best long-term result in overall survival (OS). NETs LM have been classified morphologically as type I (single metastasis), type II (isolated bulk metastasis accompanied by smaller deposits), or type III (disseminated metastatic spread).^[7] Surgery can play a role for type I LM, whereas medical treatment is always the treatment of choice for type III LM.^[7,8] The management of type II LM should be carefully evaluated, tailoring treatment to each individual patient. In metastatic pancreatic NETs (pNETs), 5-year survival rate is around 40-60%.^[9-11]

Radical surgery, including resection of primary tumor and LM, improves survival rate up to 46-86% at 5 years and 35-79% at 10 years.^[6,12,13] Nevertheless, only 15-20% of patients with LM are suitable for radical resection due to the multifocality of the lesions or the inability to preserve an adequate amount of parenchyma following resection.^[14] Nowadays in referral centers, resections of up to 70% of total liver volume may be carried out with relatively low mortality rate (0-5%) and acceptable morbidity (30%).^[15] For surgery with curative intent, the European Neuroendocrine Tumor Society (ENETS) have proposed the following criteria: (1) resectable G1-G2 liver disease with acceptable morbidity and less than 5% mortality; (2) absence of right heart insufficiency; (3) absence of unresectable lymph node and extra-abdominal metastases; (4) absence of diffuse or unresectable peritoneal carcinomatosis.^[16] Neuroendocrine carcinomas (NECs) that are G3 are usually not amenable to resection owing to their aggressive biology, high recurrence rates, and the consequent need to establish disease control.^[17] In the presence of unresectable metastatic disease, the role of debulking surgery (R2) is still controversial. In selected cases surgery may improve the quality of life or relief from symptoms when medical treatment has failed.^[18] Several nonrandomized series have documented the benefits of either complete or cytoreductive surgical

resection, compared with nonresectional treatment. They show a 74% 5-year survival for resection, compared with 30% for angiographic techniques. The Cochrane systematic reviews^[19,20] did not identify benefit of liver resection, either in terms of complete resection (R0 or R1) or cytoreduction (R2). Despite poor data, surgery is the main treatment of choice because it is the only approach with intent to cure. Whether cytoreductive surgery (90% resection) should be done when alternative nonsurgical treatment options are available is unknown.^[8]

In case of bilateral liver disease, different surgical approaches can be performed, including a 2-staged liver resection. Another technical option is occlusion of the portal vein in the tumor-bearing liver lobe, either by radiological portal vein embolization than with surgical portal vein ligation before surgery.^[21,22]

LIVER TRANSPLANTATION (LT)

In patients affected by NETs with unresectable LM, LT can be proposed due to the relatively low biological aggressiveness and slow growth of the majority of low-grade NETs. In the last 15 years, short-term outcomes have improved because of better selection of transplantation candidates, refinement of surgical techniques, and the introduction of novel immunosuppressive regimens. Moving from their former experience with hepatocellular cancer, the Milan group observed improved outcomes of LT for NETs LM patients, prospectively applying strict inclusion criteria: (1) well-differentiated NETs (Ki67 < 5%); (2) portosystemic tumor drainage; (3) patient age < 55 years; (4) stable disease for at least 6 months; (5) pretransplant R0 primary tumor resection; (6) hepatic tumor involvement < 50% of total liver volume; and (7) absence of extrahepatic disease.^[23] The two largest retrospective multicenter studies have shown that in the absence of poor prognostic factors, LT is associated with satisfactory outcomes. In particular, a European multicenter study included a large retrospective cohort of 213 patients who underwent LT for NET LM from 1982 to 2009. At a median follow-up of 56 months, 17% of patients died from early or late complications of LT, and the 5-year OS rate was 52% with a disease-specific survival rate of 30%.^[24] A study from the United States included 85 patients who underwent LT from 1988 to 2012. One, three, and five-year survival rates were 83%, 60%, and 52%, respectively, and half of deaths were due to recurrent disease. Synchronous major primary tumor resections (i.e. pancreatoduodenectomy, small bowel resection with distal pancreatectomy, multivisceral transplant) appeared to contribute to worse outcomes.^[25] In other single-center series, the 5-year OS rates ranged from 33% to 90%, and disease-free survival rates ranged from 11% to 77% at 5 years.^[26-29] Despite these experiences, firm evidence on this issue is still scarce because only 0.3% and 0.2% of transplants are performed for such indications (data from the European Liver Transplant Registry and the United

Network for Organ Sharing database.^[30] Moreover, the optimal timing of transplantation (e.g., whether stable disease needs to be observed for a certain amount of time) and selection criteria (including development of patient-specific biomarkers to identify those who gain a long-term benefit from the procedure) are still debated.

THERMAL ABLATION (TA)

The most widely applied TA modalities in the liver include radiofrequency (RF), microwave (MW), laser, cryoablation, and high-intensity focused ultrasonography. TA is often used alone or in conjunction with operative resection in the treatment of both primary and secondary hepatic malignancies. RF and MW ablation involves direct insertion of ablation probes into the region of a tumor, followed by application of several cycles of hyperthermic energy to induce cell death. MW ablation is thought to be more effective than RF ablation because a shorter time is needed for each ablation, and higher intratumor temperatures can be reached. Use of TA requires real-time ultrasonography guidance. The United States Food and Drug Administration has approved TA for the treatment of primary and metastatic tumors of the lung and liver.^[31]

RF ablation has been used for relief of symptoms of hepatic metastases of insulin- or serotonin-secreting NETs^[32] and favorable 5-year survival rates after liver resection.^[33] More than a dozen lesions can be treated in a single patient, and many patients tolerated repeated ablations for recurrent disease.^[33] To date, no randomized trials have been undertaken to study whether surgical techniques such as liver resection and/or RF ablation are more effective than hepatic artery embolization or radio embolization, peptide receptor radionuclide therapy (PRRT), or medical systemic treatments in patients with NET and LM.

PERCUTANEOUS LIVER-DIRECTED TECHNIQUES WITH A CYTOREDUCTIVE AIM

In NET patients with liver disease only or with liver-dominant metastases, loco-regional approaches such as ablative techniques or intra-arterial therapies can be proposed in place of upfront surgery with a cytoreductive aim, leading to lesion resectability and a 5-year survival rate of 50%.^[34-36]

In particular, it is well known that NET hepatic metastases are characterized by a high rate of vascularization, as opposed to many other liver primary or secondary malignancies. Vascularization of NETs LM depends mostly on the hepatic artery, whereas normal liver parenchyma has a unique dual blood supply from both the proper hepatic artery (20-40%) and the portal vein (60-80%).^[37]

Arterially directed interventional strategies, such as transarterial embolization (TAE) and transarterial

chemoembolization (TACE) with a radiologically controlled percutaneous technique have been widely investigated and adopted during the past decade for the treatment of NETs LM. These strategies have generated encouraging outcomes in term of survival, response, and quality of life.^[38] Indications included well-differentiated or moderately well-differentiated (Grade 1 or 2) unresectable symptomatic liver lesions (due to tumor bulk), excessive hormone production, and rapid progression of liver disease.^[39] Hepatic TAE, usually performed using lipiodol, obtains ischemia and necrosis of neoplastic cells by selective catheterization and obstruction of the hepatic artery supplying tumor lesions.^[40] On radiologic evaluation, TAE has been shown to improve biophysical markers, palliate symptoms, and shrink tumor lesions.^[41] In contrast to TAE, TACE combines blockage of the tumor blood supply with intra-arterial administration of cytotoxic drugs. In clinical practice, TACE is preferred over TAE in patients with NET with the worst prognostic factors, such as foregut origin (lung or pancreas) and poorly differentiated NETs.^[42] Several different chemotherapeutic agents have been used in this setting (doxorubicin, streptozotocin, gemcitabine, mitomycin C, 5-FU, or cisplatin) along with either a transient or permanent embolic agent like ethiodized oil or lipiodol.^[43] This treatment has shown effective results in patients with metastatic liver disease, with reported OS values of 3-4 years and objective response of about 75%. Notably, response to TACE is higher when treatment is used as a first-line therapy and liver involvement is lower. Combining results obtained with TAE and TACE, the rates of symptomatic responses ranged from 39 to 95%.^[44-47]

An accurate multicentric retrospective review on 100 patients with NETs LM who submitted to TACE ($n = 49$) or TAE ($n = 51$) showed comparable rates of symptom control (88% vs. 83%, respectively), similar toxicities, and comparable survival outcomes (median OS: 25.7 vs. 25.5 months, respectively). These data suggest that the two techniques should be considered comparable.^[48] Future trials focusing on the evaluation of either the efficacy of different intra-arterial techniques or the role of a combination of loco-regional approaches with systemic therapies are needed.

SELECTIVE INTERNAL RADIOTHERAPY (SIRT)

Percutaneous angiographic techniques should be used in patients with Grade 1 or 2 tumors who have liver-predominant disease. The best treatment effect is achieved in patients with < 50% hepatic involvement and no extrahepatic disease. SIRT is a targeted approach that delivers glass or resin microspheres labeled with ⁹⁰Yttrium (Y-90) that is primarily a beta particle emitter. Y-90 hepatic arterial administration is emerging as a promising treatment modality in the management of NETs patients with LM.^[49,50] Down-sizing/down-staging of hepatic tumors as a bridge to subsequent surgical treatment

appears promising. Even though Y-90 radio-embolization may achieve a survival benefit, especially in patients presenting with significant tumor shrinkage, however, this technique is not easily available, especially in outlying hospitals.^[51]

Long-term outcome analysis after SIRT indicated treatment response in 62.7% of the patients, disease stabilization in 32.5%, and a survival rate of 45.0% at 3 years. Findings from an international multicenter prospective treatment registry showed that safety and response rates of SIRT and TACE were similar when evaluated at 6 months.^[52] At 12 months, the group receiving SIRT had a significantly lower response rate than the group receiving TACE (46% vs. 66%).^[46] It should be noted that portal vein thrombosis and impaired liver function are not considered contraindications to SIRT, as they are for TACE and TA. Adverse events associated with SIRT included lung shunting of beads, radiation gastritis, duodenal ulceration, and hepatic fibrosis. Finally, the SIRT procedure is not considered pharmaco-economically advantageous.^[53]

PRRT

PRRT is a form of molecular targeted therapy which uses a small peptide (a somatostatin analog similar to octreotide) coupled with a radionuclide emitting beta radiation. This therapy can be proposed only to patients with somatostatin receptor expressing NETs. In phase II studies PRRT was demonstrated to obtain objective response rates in 20-35% of treated patients.^[54-57] Thus, it could have a potential role as a cytoreductive preoperative therapy, as demonstrated by several case reports in patients with GEP-NETs.^[55-57] The most important positive predictive factor for response to PRRT was the ratio of radiolabel uptake on diagnostic scans (normal to tumor). In a retrospective analysis, complete and partial tumor remission was reported in 2% and 28% of 310 patients, respectively, who received 177Lu-DOTATATE treatment for various histologic types of metastasized NETs.^[58] Of those patients, 89% had hepatic metastases, with extensive and moderate liver involvement in 27% and 62%, respectively. The median time to progression was 40 months, and the median OS from the first treatment cycle was 46 months. The OS from initial diagnosis was 128 months, yielding a survival benefit of 40 to 72 months compared with historical cohorts.^[58] Extensive hepatic metastatic involvement is a significant negative predictive factor for progression-free survival (PFS) or OS with PRRT.^[58] A phase-3 trial comparing PRRT and octreotide was presented at the 2015 18th-ECCO-40th-ESMO Congress.^[59] In this first prospective randomized study in patients with progressive metastatic midgut NETs, 177Lu-DOTATATE was superior to octreotide 60 mg in terms of PFS (not reached vs. 8.4 months, $P < 0.0001$) and overall response rate (19% vs. 3%, $P < 0.0004$). Interim analysis suggests increased OS (13 vs. 22 deaths), to be confirmed by final analysis. The combination of PRRT with radiosensitizing chemotherapy has been considered

a promising strategy to enhance resectability of metastatic lesions.^[60] 5-FU or capecitabine has been used in many of the numerous trials investigating the effects of external beam radiotherapy with chemotherapy. Also, Y-90 labeled antibody radioimmunotherapy in combination with 5-FU as radiosensitizer was found to be feasible and safe.^[18] The combination therapy of PRRT and oral everolimus was less effective than 177Lu-DOTATATE only in the rat pancreatic CA20948 tumor model.^[61] Despite the low toxicity, a caveat is the limited access to this therapy in Europe, the USA, and Japan. Rare side effects of treatment can adversely affect the kidney and bone marrow.

SYSTEMIC TREATMENT

Immunotherapy

A potential role of interferon alpha (IFN α) has been explored in several studies: an older comprehensive review reported an overall response rate of 20%,^[62] whereas some small-sized retrospective and randomized trials have reported an improvement of PFS and OS.^[63-65] However, these benefits in outcome were not confirmed in other studies.^[66] The combination of IFN α with continuous infusion of 5-fluorouracil was explored in a phase-II study of patients with rapidly progressive NETs, and an overall response rate of 41.6 was achieved.^[67] Other studies enrolling limited patient series have demonstrated the role of immunotherapy/immunochemotherapy in obtaining a significant shrinkage of LM from NETs.^[68-70] However, further investigations are needed to better define whether immunotherapy or immunochemotherapy could have a role as a neoadjuvant strategy in NETs.

Biotherapy

In well- and moderately differentiated somatostatin receptor expressing NETs, the mainstay of treatment consists of somatostatin analogue (SSA) administration, made manageable with long-acting repeatable (LAR) formulations.^[71] Therapy with SSAs represents the standard of care in patients with metastasized, nonresectable midgut NETs, pancreatic NETs, or NETs of unknown origin, whether associated or not with hormone hypersecretion and regardless of the hepatic tumor burden. Randomized phase-III, multicenter trials demonstrated that LAR octreotide and lanreotide depot can significantly prolong PFS in a heterogeneous population of patients with GEP-NETs.^[72,73] Therapy with SSAs, however, did not demonstrate reduction of tumor load. The best clinical response obtained in all these studies was disease stabilization.

PROMID trial enrolled 85 treatment-naïve patients with well-differentiated G1 advanced midgut or unknown origin NETs, randomizing them to receive either placebo or intramuscular octreotide LAR every 4 weeks (Sandostatin LARTM). Patients treated with octreotide LAR presented a longer time to tumor progression (14.3 vs. 6 months) and a higher disease stabilization rate (66.7%

vs. 37.2%).^[72] CLARINET trial, a double-blind, phase-III study, randomized 204 patients with well- or moderately differentiated, Octreoscan-positive, nonfunctioning GEP-NETs to receive lanreotide depot 120 mg monthly versus placebo. SSAs therapy obtained a significant improvement in PFS, with a median time not reached in the experimental arm versus 18 months in the placebo group. The estimated rates of PFS at 24 months were 65.1% in the lanreotide group and 33% in the placebo group. No information on disease control rate was reported.^[73]

Recently, the clinical activity of the new SSA pasireotide has been evaluated in an open-label, phase-II study enrolling advanced pancreatic and extrapancreatic Grade 1 and 2 NETs.^[74] Median PFS of the 29 treated patients was the primary endpoint of the study and was 11 months. According to the RECIST criteria, one patient obtained a partial response and 17 experienced disease stabilization, for a disease control rate of 64%. In all the above-reported trials, treatment with SSAs resulted in low cytoreductive activity as demonstrated by the low objective response rates reported (around 5%). This finding was recently confirmed in an extensive review.^[75] Thus, while SSAs can be considered the mainstay of treatment in well- or moderately well-differentiated NETs, both functioning or not, when a disease control is needed, there is no evidence to support the use of SSAs in the “neoadjuvant” setting.

Targeted therapies

Recently, novel targeted therapies such as everolimus and sunitinib have been introduced in the clinical management of G1 and G2 NETs.

Following exciting preclinical data demonstrating mTOR signaling pathway activation in NET cells, everolimus was extensively studied in cancer patients.^[76-78]

A randomized, phase-III, double-blind study (RADIANT-3) enrolled 410 patients with locally advanced or metastatic well- to moderately differentiated pancreatic NETs, comparing the PFS of patients treated with everolimus 10 mg/day to that of patients receiving placebo. The study met its primary endpoint as patients treated with everolimus presented a longer median PFS (11.0 vs. 4.6 months). Response rate was low, with only 5% of the patients randomized to receive everolimus achieving a partial response.^[79] Similar encouraging results have been obtained in the phase-III placebo-controlled RADIANT-2 study enrolling patients with well- and moderately differentiated locally advanced or metastatic NETs and carcinoid syndrome. Patients receiving everolimus plus SSA (octreotide LAR) presented a longer PFS than those treated with octreotide LAR plus placebo (16.4 vs. 11.3 months, $P = 0.026$). Overall response rate was similar in both groups, with 2% of patients achieving a partial response and 82% disease stabilization.^[80] The advantages of treating patients with everolimus have recently been confirmed in a randomized, double-blind, placebo-controlled, phase-

III RADIANT-4 trial. The study evaluated everolimus efficacy in patients with advanced, well-differentiated NETs of different origin and with nonfunctional disease. Patients in the everolimus arm of the study presented a significant improvement in PFS (11.0 vs. 3.9 months).^[81] Interestingly, according to subgroup analysis, the positive treatment effect was confirmed irrespective of the extent of liver metastasis. Objective responses were recorded in four (2%) patients receiving everolimus and in one patient (1%) receiving placebo. Disease stabilization was the best overall response in 165 patients (81%) in the everolimus group, compared with 62 patients (64%) in the placebo group. The findings of these three studies were consistent with the role of everolimus in prolonging PFS and not in achieving tumor shrinkage. Thus, everolimus cannot be proposed as a preferred therapy in the neoadjuvant setting.

The activity of sunitinib, a multityrosine kinase inhibitor of vascular endothelial and platelet-derived growth factor receptors, was explored in a double-blind, placebo-controlled, phase-III trial enrolling 171 patients with advanced, well-differentiated progressing pancreatic NETs.^[82] The study met its primary endpoint, as median PFS of patients receiving sunitinib was significantly longer than that of patients treated with placebo (11.4 vs. 5.5 months). In contrast to what was observed in patients with renal cell carcinoma,^[83] tumor shrinkage rate in patients with pancreatic NET was low; only 9% of those treated with sunitinib achieved an objective response according to the RECIST criteria.

The high rate of vascularization of NETs led to initial interest in angiogenesis inhibition as a promising field of research. Furthermore, an overexpression of vascular endothelial growth factor (VEGF) has been observed in both carcinoid and p-NET (either in serum or in tissue), thus making VEGF and VEGFR excellent targets to be inhibited.^[84] The anti-angiogenic agent bevacizumab has been investigated combined with IFN α in a randomized phase-II trial of 44 patients with advanced (unresectable or metastatic) carcinoid tumors. Patients were randomized to receive 18 weeks of single agent bevacizumab or IFN. At disease progression or after 18 weeks of treatment, patients were allowed to receive the combination of these two treatments. The results obtained in the bevacizumab arm were encouraging; a partial response was achieved in 18% of the patients, with a better 18-week PFS than in the IFN group (95% vs. 67%, respectively).^[85] However, even though bevacizumab monotherapy has been associated with improvement in response rate and survival, the results obtained in terms of tumor shrinkage were not encouraging, probably because of the cytostatic rather than cytotoxic effect of antiangiogenic therapies. Therefore, the role of bevacizumab-based combination therapy has been evaluated, mostly with chemotherapy agents or with mTOR inhibitors in the management of advanced GEP-NETs. In the randomized phase-II study CALGB80701 (Alliance), patients with metastatic pNETs were randomly

treated with everolimus or everolimus plus bevacizumab. The overall response rate was 31% and 12% for the combination treatment and everolimus alone, respectively. The current evidence from this available clinical trial suggests that combination strategy was more active but not more effective in terms of PFS.^[86]

Chemotherapy

While chemotherapy is the standard of care for aggressive, poorly differentiated (G3), advanced, or metastatic NECs,^[87] it could represent a therapeutic option in symptomatic and progressive well- or moderately differentiated NETs. Notwithstanding a relatively high number of agents which have been demonstrated to be active in this latter tumor setting (platinum salts, 5-fluorouracil, doxorubicin, streptozotocin, temozolomide, and capecitabine), the best chemotherapeutic strategy remains controversial.^[88]

As far as unresectable or metastatic pancreatic NETs are concerned, polychemotherapy was more active than monotherapy, with a response rate in this latter group lower than 20%. A retrospective study evaluating the combination of streptozotocin (STZ) with doxorubicin and 5-fluorouracil (5-FU) reported a response rate of 39%, with a median response duration of 9.3 months. The 2-year PFS rate was 41%, and the 2-year OS rate was 74%. Tumor burden clearly affected survival outcomes in both univariate and multivariate analyses. In fact, the PFS rate at 2 years for patients with LM involving $\leq 75\%$ of the parenchyma was 41%, whereas all 12 patients with LM involving more than 75% of the organ had experienced disease progression by 14.2 months ($P = 0.01$). At 2 years, the OS rate for patients with LM $\leq 75\%$ was 83%, whereas all 12 patients with LM more than 75% had died at 15.5 months ($P = 0.0001$).^[89]

The combination of temozolomide with capecitabine was demonstrated to be more active and better tolerated than STZ-based regimens. In a retrospective study enrolling metastatic pancreatic NETs, objective response rate of temozolomide combination was reported to be 70%. It has to be noted, however, that in this study only 30% of the patients had moderately differentiated (G2) tumors.^[90] The combination of octreotide LAR 20 mg, metronomic capecitabine, and intravenous bevacizumab was explored in the XELBEVOCT phase-II study enrolling 45 patients with well- to moderately differentiated NETs from various primary origins (pancreas, intestinal tract, lungs, and unknown site). Objective response rate was 17.8% with a median PFS of 14.9 months. This study demonstrated that the combination of SSA plus capecitabine and bevacizumab was active and well tolerated in this group of patients.^[91]

Finally, a retrospective study evaluated the combination of 5-fluorouracil, dacarbazine, and epirubicin in patients with well-differentiated NETs originating from pancreas, intestine, stomach, gallbladder, kidney, or an unknown

site. Chemotherapy was well tolerated and outcome results were encouraging. Tumor shrinkage was obtained in 44% of the patients, with a median duration of response of 12 months. Objective response rates recorded in pancreatic, gastrointestinal, and extradigestive NETs were 58%, 25%, and 36%, respectively. Interestingly, disease control was achieved in 83% of the patients progressing at the time of study inclusion. Median PFS was 11 months and OS was 21 months.^[92]

Notwithstanding this body of evidence, the number of patients enrolled in each study was relatively low, thus preventing any definitive conclusion on which could be the best chemotherapeutic strategy for each subset of patients. New multicenter, well designed, randomized clinical trials are needed.

CONCLUSION

About one in seven patients diagnosed with digestive NETs presents with metastatic disease at the time of diagnosis, with the liver being the most frequently involved organ. Moreover, 25% to 90% of patients who are nonmetastatic at diagnosis are expected to develop metastases during the course of the disease. In clinical practice, hepatic failure represents the primary cause of death in these patients. Surgery is the only technique that may permit curability of liver involvement. Thus, all treatments should primarily be focused on tumor shrinkage, especially when unresectable liver lesions could become resectable if reduced in size. When complete resection is not possible, treatment goals should be tumor control and symptom relief.

Complete resection of primary and metastatic disease (when possible) and surgical debulking of symptomatic diseases are standard procedures for G1 and G2 NETs. To patients with Grade 1 or 2 NETs (either pNETs or gastrointestinal NETs) with LM and without extra-abdominal metastasis and peritoneal carcinomatosis, surgery permits the best results in terms of recurrence-free survival and outcome. Unfortunately only 10-25% of patients can be directly submitted to surgical resection. These considerations suggest that “neoadjuvant strategies” should be explored in patients with liver-confined metastatic disease. Despite the proven efficacy of different systemic treatment strategies for metastatic NETs (SSAs, PRRT, chemotherapy, or target therapies such as everolimus, sunitinib, and bevacizumab), none of these approaches resulted in significant tumor shrinkage. Few studies have explored systemic therapies in the neoadjuvant setting. Unfortunately, trial designs, inhomogeneous inclusion and exclusion criteria, and the relatively low number of patients have hampered definitive conclusions in this patient setting.

Further research is needed to determine the value of these medical treatments as a cytoreductive strategy against LM from NETs. Moreover, loco-regional approaches to LM, such as radiofrequency ablation, laser ablation, or intra-

arterial therapies (embolization/chemoembolization), may be useful in reducing tumor burden only in selected cases. Application of the concept of tumor response as defined by RECIST or WHO criteria in patients with metastatic NETs is worthy of mention. Often it is difficult to select the target lesions to be monitored over time. Furthermore, necrosis or hemorrhage within other clinical occurrences may be misinterpreted as a stable disease instead of a response.

In conclusion, while surgical management of resectable LM from NETs is a standardized procedure, there is no consensus on the best therapeutic strategy for all other patients. For example, it is a matter of debate whether incomplete surgical resection of bulky but asymptomatic metastasis from NETs is preferable to systemic biotherapy. Extremely promising recent data have been reported in the Radiant 4 trial, suggesting that novel therapies (in particular the mTOR inhibitor everolimus) will play an increasingly important role in the management of advanced LM irrespective of the extent of liver metastasis.

Large prospective studies are needed to evaluate the optimal management of hepatic metastases from NETs, defining common guidelines and allowing the choice of the best treatment strategy for each individual patient.

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Controversies in the treatment of digestive neuroendocrine tumors

Maria Rinzivillo, Francesco Panzuto, Gianfranco Delle Fave

Department of Digestive and Liver Disease, Digestive Neuroendocrine Unit, Sant'Andrea Hospital, Sapienza University of Rome, 00189 Rome, Italy.

Correspondence to: Prof. Gianfranco Delle Fave, Department of Digestive and Liver Disease, Digestive Neuroendocrine Unit, Sant'Andrea Hospital, Sapienza University of Rome, Via di Grottarossa 1035-39, 00189 Rome, Italy. E-mail: gianfranco.dellefave@uniroma1.it

ABSTRACT

Gastroenteropancreatic neuroendocrine tumors (NETs) have an incidence of 2.39 per 100,000 inhabitants per year, and a prevalence of 35 cases per 100,000 inhabitants; the gap between these rates is due to the relatively long survival time of these tumors, which can be thus considered as chronic oncological diseases. Recently, more therapeutic options have become available, but criteria for defining timing, priority and sequence of different therapeutic options are still debated. This review offers an overview of pancreatic and small bowel NETs, critically underlining the issues that still need to be clarified and some controversial issues on the therapeutic approach for NET patients.

Key words: Neuroendocrine tumors; therapeutic strategy; surgical treatment; medical therapies

INTRODUCTION

Gastroenteropancreatic neuroendocrine tumors (NETs) are a heterogeneous group of neoplasms derived from the diffuse endocrine system in the gastrointestinal tract and pancreas. The WHO classification classifies these tumors into three principal categories with different malignant behavior: NETs with Ki67 \leq 2% (G1 NETs), NETs with Ki67 3-20% (G2 NETs) and neuroendocrine carcinomas (NECs) with Ki67 $>$ 20% (NECs G3).^[1]

In the last few decades, the increasing incidence of these diseases has aroused much interest resulting in improvements in available therapeutic options and new clinical trials. In fact, treatment options for NETs have increased in number and this is definitely an advantage for patients. However, criteria for defining timing, priority and sequence of different therapeutic options are still debated.

The optimal therapeutic sequence should be based on the evaluation of at least three major issues:

(a) Tumor characterization:

Primary site: pancreatic and small bowel NETs should be considered different diseases in terms of both risk of tumor progression and overall survival;

Histological diagnosis: conventional immunohistochemistry evaluation and Ki67 assessment are needed to classify the disease according with WHO classification, as well as define tumor grading;

Disease staging: conventional contrast enhanced computed tomography (or magnetic resonance imaging) should be performed together with functional imaging (68 Ga-PET or Octreoscan) to stage the disease according with the ENETS staging system.

(b) Patient's clinical status:

Performance status;

Presence of symptoms resulting from tumor-related secretion of active substances, in the case of a "functioning tumor";

Prior treatments and comorbidity, which may reduce therapeutic options.

(c) Defining the objectives of care:

The only curative option is represented by radical surgery;

In most patients, since curative surgery is not feasible, medical treatment is needed to treat advanced

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unresectable disease;

In “functioning tumors” the symptomatic control is a major therapeutic goal;

In advanced end-stage disease, palliative symptomatic therapies are required to maintain patient’s quality of life.

In the present paper, some controversial issues on the therapeutic approach for NET patients will be discussed.

SURGICAL THERAPY

Small incidental non-functioning pancreatic NETs (pNETs): should they always be removed?

Surgical treatment of pNETs must always be planned and adapted to each individual patient considering several variables, including patient characteristics and disease stage. Some studies have recently suggested tumor diameter as the main criterion for surgery with radical intent.^[2,3] For pNETs ≤ 2 cm, and in the absence of symptoms and/or suspected metastatic lesions, a conservative wait-and-see approach may be adopted in selected cases, scheduling a clinical and radiological follow-up.^[4-7] Pancreatic NETs ≤ 2 cm of diameter have a risk of “malignancy” of about 6%, while 5-year disease mortality is 0%.^[4] In the small and sporadic non-functioning pNETs, the mean overall tumor growth (difference between size at last follow-up and initial size) was 0.37 (+/-1.67) mm.^[7] Mean growth per month was 0.010 (+/-0.051) or 0.12 mm per year corresponding to a growth percentage of 1.5% (+/-5.5) from the initial tumor size per year. The incidental diagnosis and the absence of symptoms seem to correlate with a better prognosis in this subgroup of patients.^[2] Histological confirmation of tumor neuroendocrine origin by endoscopic ultrasonography with tissue sampling is required before planning a patient’s management. The primary tumor localization is an additional major factor to determining the surgical approach. Finally, the patient’s comorbidities and willing should always be considered in the surgical management of pNETs.

Despite recent progress, morbidity remains significant, indeed, it is necessary to carefully evaluate the type of surgery, the risks of surgery and the risks related to tumor growth in advance. Based on these considerations, conservative non-surgical management may be proposed in selected patients with small, incidental non-functioning pNETs.

Pancreatic NETs with liver metastases: should the primary tumor be resected?

The presence of metastases is the main factor associated with mortality in pNET patients. Surgical options for patients, including those with metastatic disease, include different procedures such as curative liver and pancreatic resection, primary resection, local ablative techniques, and liver transplantation. In these cases, patient selection

must be meticulous and consider several prerequisites including: (a) the presence of well - differentiated lesions; (b) the absence of extra - abdominal disease; and (c) the absence of diffuse peritoneal carcinomatosis.^[8]

In the literature, clinical studies suggest that there is a possible benefit in terms of survival when performing surgical removal of primitive pNETs if metastases are present.^[9] However, in the retrospective studies that evaluated the role of surgery in pNETs with unresectable liver metastases, there is a selection bias for patients related to the localization of primary tumors and the type of surgical approach, the patient status in terms of comorbidity, age and performance status.^[9] In the Partelli *et al.*^[10] paper, the 5-year overall survival (OS) after surgical resection was 76% with an increase to 88% after curative resection. Although palliative surgery was associated with an improved outcome, surgical management should be reserved in highly selected patients due to the high risk of peri/postoperative complications.

Small intestinal NETs (SI-NETs) with liver metastases: should the primary tumor be resected?

Surgical treatment of SI-NETs is affected by disease clinical presentation. For SI-NETs diagnosed as stage I-III, the choice of therapy is always surgical bowel resection with lymphadenectomy.^[11-13] Curative resection of the primary tumor and regional lymph node metastasis site improves long-term outcome, with a 100% 5-10 year survival for patients with stage I and II tumors and more than 80% for patients with stage III jejuno-ileal NETs.^[14] In the presence of synchronous liver lesions, surgical treatment is still highly debated. A recent systematic review^[15] analyzed the studies in the literature on the surgical resection of the primary tumor in patients with SI-NETs and distant metastases. Although it was not possible to conduct a meta-analysis of these works, the conclusions suggest improved survival after surgical removal of the primary tumor in patients with metastatic unresectable disease and a reduction in local complications (bleeding, perforation, and occlusion). In association with the intestinal resection, cholecystectomy should be performed in order to prevent gallstones due to long-term treatment with somatostatin analogue.^[16]

MEDICAL THERAPY

Being characterized by a relatively long OS, multiple sequential therapies are adopted in digestive NETs although the best sequence for these patients is not well defined.

Somatostatin analogs (SSAs): are they indicated for all NET patients?

SSAs clearly represent the first-line treatment for patients with functioning NETs. As far as non-functioning tumors are concerned, SSAs can control tumor proliferation, as shown by two randomized clinical trials. The PROMID

study^[17] described, in 42 metastatic patients treated by octreotide long-acting repeatable (LAR) 30 mg, a median progression-free survival (PFS) of 14.3 months vs. 6 months of the 43 cases enrolled in the placebo group. The more recent CLARINET trial^[18] showed, in 101 patients with digestive NET using lanreotide 120 mg, a median PFS not reached vs. 18 months of the 103 included in the placebo group. Both studies highlight the increased anti-proliferative activity of these drugs in patients with low Ki67 (G1 NETs or G2 NETs with Ki67 < 10%), stable slow-growing disease, and high somatostatin receptor expression as assessed by functional imaging. Alternative medical treatments should be considered if these criteria are not satisfied.

Peptide receptors radionuclide therapy (PRRT): is there a place as a first-line approach?

PRRT acts with the same molecular mechanism as SSAs, but the somatostatin analog is radiolabeled with Y90 or Lu177, performing an “*in loco*” radiotherapy. This well-tolerated treatment is able to inhibit tumor growth in up to 50-70% of digestive NETs.^[19-21]

Results from the first Phase III, multicenter randomized clinical trial (RCT) comparing Lutathera® vs. Octreotide in patients with inoperable, progressive, somatostatin receptor-positive G1-G2 small intestinal NETs (NETTER-1 trial) have been recently presented at the last ECC (Vienna, September 2015) (www.clinicaltrials.gov NCT01578239).^[21] They showed that, in 230 patients enrolled, the median PFS was not reached in the PRRT-treated group vs. 8.4 months obtained by SSA [hazard ratio (HR): 0.21, $P < 0.0001$]. This data supports the benefit of this therapy in metastatic small intestinal NETs, and hopefully will help achieve official registration of this drug.^[21]

All international guidelines (ENETS, NANETS, ESMO, and NCCN) consider PRRT as a valid option in patients with advanced NETs; however, there are no solid data supporting where PRRT should be placed in the therapeutic sequence. A recent multicenter Italian study on the compassionate use of everolimus in advanced NETs highlighted the increasing risk of severe toxicity in patients who had been previously treated with PRRT or chemotherapy, thus suggesting the early use of everolimus in patients with advanced NETs.^[22] Furthermore, Bajetta *et al.*^[23] treated patients with everolimus in combination with octreotide LAR as first line approach in advanced NETs and showed that in this setting, this combination treatment is very effective with disease control being reached in 92% of patients. This therapy also has an excellent safety profile, with only one single grade 4 adverse event in the population of 50 patients enrolled.^[23] Conversely in a relatively small series of NET patients treated with everolimus after previous failure of PRRT, Kamp *et al.*^[24] reported an overall safety profile similar to that presented in the randomized clinical trials. However in this trial,

severe kidney toxicity was observed in 4.2% of patients, a toxicity not reported in the regulatory trials, where no patients pre-treated with PRRT had been enrolled. To date, no conclusive data on the optimal therapeutic sequence involving PRRT is available and caution should be used when considering everolimus therapy in patients who have previously received PRRT.

Targeted therapies: everolimus or sunitinib first?

Another relevant option for digestive NETs is targeted therapy. Recent trials have demonstrated the activity of the mTOR inhibitor everolimus (RAD001, Afinitor®, Novartis Oncology) against tumor growth. In the RADIANT-3 trial,^[25] a phase III placebo-controlled study enrolling advanced pNETs, everolimus provided a significant prolongation in median PFS vs. placebo (11 and 4.6 months; 207 and 203 patients, respectively). The results of this trial led to approval by the U.S. Food and Drug Administration (FDA) and the European Medicines Agency (EMA) for the treatment of locally advanced, metastatic or unresectable pNETs.^[24] Its activity has also been reported in progressive, well-differentiated, non-functioning lung and non-pancreatic digestive NETs, based on the findings of the RADIANT-4 RCT.^[26] This study showed a significant benefit with everolimus in these patients, with median PFS being 11 months in the treatment arm ($n = 205$) vs. 3.9 months in the placebo group ($n = 97$) (HR: 0.64, $P = 0.037$).^[26] The most common adverse events reported in the phase III RCTs (Radiant 3-4) (> 30%) were stomatitis (62%, 64%), rash (37%, 49%), fatigue (31%, 31%) and diarrhea (27%, 34%), while grade 3/4 treatment-related adverse events were stomatitis (7%, 7%), anemia (1%, 6%), and hyperglycemia (5%, 5%). Overall, grade 3-4 toxicity was reported in approximately 5-8% of patients. This data suggests caution when using everolimus in patients with diabetes, in whom an optimal glucose control is mandatory before beginning the treatment.

Sunitinib (Sutent®, Pfizer) is another targeted therapy, effective for the treatment of advanced pNETs. It is an antiangiogenic, pan-receptor tyrosine kinase inhibitor, acting against multiple targets including VEGFR, PDGFR, c-KIT, Flt-3 and RET. In the phase III RCT published in 2011, it prolonged PFS to 10.2 months vs. 5.4 with placebo.^[27] The most common adverse events reported in the sunitinib trial were diarrhea (59%), nausea, fatigue, vomiting (35%) and fatigue (32%), while the most frequent grade 3/4 treatment-related included neutropenia (12%), hypertension (10%), and palmar-plantar erythrodysesthesia (6%). Notably, because patients with severe cardiac comorbidities had not been enrolled in this study, caution should be exercised when using sunitinib in patients with a significant cardiac history (e.g., arrhythmia, coronary artery disease, cardiomyopathy, uncontrolled hypertension). Grade 3-4 toxicity was present in up to 12% of patients.

The choice of which targeted agent should be used first still remains a challenge for physicians dealing with advanced pNETs. No comparative study of everolimus versus sunitinib in this setting is available yet. Thus, since phase III trials have demonstrated a similar efficacy in terms of PFS, the choice is mainly based on the evaluation of other elements, including the toxicity profile, patients' comorbidity, and physician's expertise with these drugs. An additional point of interest that should be considered, besides the physician's personal clinical experience when managing these drugs, is the larger population of NET patients treated with everolimus in comparison with sunitinib reported in the literature. In fact, more than 600 advanced NET patients have been treated in the RADIANT trials,^[25,26,28] in comparison with the 86 patients included in the sunitinib trial.^[27]

G3 NECs: is platinum-based chemotherapy always required?

According with the WHO 2010 classification,^[1] the group of G3 NECs were identified with a proliferation index (Ki67) > 20% (or > 20 mitotic count per 10 HPF). International guidelines^[29] suggest the use of platinum-based systemic chemotherapy in G3 NEC patients due to the rapidly metastatic behavior of these tumors, and the extremely poor prognosis in comparison with other NETs with lower proliferative activity (G1 and G2). However, this category constitutes a heterogeneous group of diseases, including both well-differentiated and poorly differentiated tumors based on morphological features, with different implications in terms of patients' prognosis and therapeutic approach.^[30,31] Overall, median PFS reported with platinum-based first-line approach ranges from 4 to 9 months.^[31] However, this data mostly derives from non-randomized trials, with small series of patients evaluated by a retrospective design approach, and usually enrolling a heterogeneous series of patients in terms of therapeutic schedules and biological features of the tumor (primary site, staging, Ki67 index).

Data reported by the Nordic group study^[31] proposes to consider G3 NECs with Ki67 < 55%, as a different entity that exhibits less aggressive behavior and responds well to platinum-based chemotherapy, in comparison with other G3 NECs. This specific subgroup of patients might be considered as a separate disease in which therapeutic approaches other than platinum-based should be tested. Indeed, the role of everolimus in G3 NECs is under investigation in phase II trials in several different clinical settings (MAVERIC- EudraCT: 2014-003951-72, www.clinicaltrials.gov, NCT0211380, www.clinicaltrials.gov NCT02248012).

Further prospective studies are required before considering therapeutic options based on targeted agents as the standard treatments in G3 NECs.

Locoregional therapies: is there an impact on patients' survival?

In some cases (especially with a functional syndrome) when a complete resection is not possible, debulking surgery can be performed to improve prognosis and quality of life. This approach can be based on the combination of surgery on primary and secondary tumors and loco-regional treatments (i.e., trans-arterial liver embolization, TAE; trans-arterial chemoembolization, TACE; radiofrequency ablation). Embolization is contraindicated in patients with portal vein thrombosis, liver insufficiency, biliary obstruction or prior Whipple procedure. The presence of portal vein occlusion or ascites hepatic tumor burden > 75% of the total liver are considered relative contraindications.^[32] In a retrospective study in patients with pNETs, chemoembolization showed better results when compared with bland embolization (response: 50% vs. 25%, respectively).^[33] However, no clear difference between TAE and TACE in terms of clinical outcome has been reported so far.

Another experimental approach to metastatic disease is selective internal radiation therapy (SIRT), based on the intra-arterial deliver of Yttrium-90 microspheres to the lesions. Although results seem appealing, they are from retrospective series, and a recent study comparing this technique to TAE and TACE over a 10-year period did not show any advantages of SIRT in terms of time to disease progression.^[34]

The wide range in response rates and survival duration in various studies in terms of patient population and tumor profile, the extent of liver involvement, and the presence of extra-hepatic metastases is reflection of the heterogeneous tumor biology of this disease. Gupta *et al.*^[33] found that patients treated with liver embolization with carcinoid tumors had a higher response rate (66.7% vs. 35%; $P < 0.0001$), longer time to progression (TTP) (22.7 months vs. 16.1 months, $P < 0.046$), and better OS (33.8 months vs. 23.2 months; $P < 0.012$) compared to patients with pNETs. Roche *et al.*^[35] found non-pancreatic NETs ($P < 0.006$), absence of extra-hepatic lesions ($P < 0.03$), unresected primary ($P < 0.012$) and TACE as first-line ($P < 0.028$) were significant for complete response to liver embolization, and less hepatic involvement (< 30%) significantly improved morphological response ($P < 0.016$). There is no conclusive evidence in the literature that the loco-regional therapies improve survival rate.

CONCLUSION

Despite recent advances in the knowledge of digestive NETs, there are still many controversial aspects about the management of these patients. There is a dire need for further multicenter studies designed to clarify gray areas such as the sequence of medical therapies in patients with advanced disease, the opportunity for a conservative

follow-up in small incidental tumors of the pancreas, the optimal approach to NEC G3 tumors with well differentiated morphology, liver ablative therapies, and surgery in the context of metastatic disease.

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Merkel cell carcinoma responsive to Etoposide: a case report and brief literature review

Chiara Ancona¹, Andrea Caff², Giovanni Manfredi Assanto¹, Stefano Cordio³

¹Department of Medical Oncology, Policlinico P. Giaccone, University of Palermo, Via del Vespro 129, 90127 Palermo, Italy.

²Endocrinology, Department of Clinical and Experimental Medicine, Garibaldi-Nesima Medical Center, University of Catania, Via Palermo 636, 95122 Catania, Italy.

³Department of Oncology, Garibaldi-Nesima Medical Center, University of Catania, Via Palermo 636, 95122 Catania, Italy.

Correspondence to: Dr. Andrea Caff, Endocrinology, Department of Clinical and Experimental Medicine, Garibaldi-Nesima Medical Center, University of Catania, Via Palermo 636, 95122 Catania, Italy. E-mail: caff.andrea@gmail.com

ABSTRACT

Merkel cell carcinoma (MCC), first described in 1972, is an aggressive primary cutaneous carcinoma able to incorporate both epithelial and neuroendocrine features. MCC mainly appears in individuals in their eighth decade and it is related to a high mortality rate. The etiology of this rare disease is not well-understood but ultraviolet radiation exposure, immune suppression, and aging have a consistent role in its pathogenesis. Usually, clinical lesions appear as asymptomatic coloured dermal nodules. The tumour can involve lymph nodes but further evaluation with imaging is recommended. The common approach for localized disease is surgical. This work reports a case of an 86-year-old man with locally advanced MCC where, based on clinical experience, oral mono-chemotherapy with single-agent etoposide was chosen as first-line therapy. A complete objective response was achieved in 2 months.

Key words: Merkel cell carcinoma; neuroendocrine; chemotherapy; etoposide

INTRODUCTION

Merkel cell carcinoma (MCC) is a rare, aggressive, neuroendocrine carcinoma of the skin that originates from Merkel cells of the dermoepidermal junctions, although some recent work proposes pluripotent dermal stem cells to be origin of this neoplasm.^[1]

The annual incidence is 0.6 per 100,000 persons^[2] but is apparently increasing in the last years thanks to more accurate diagnostic pathology techniques, an aging population, increased sun exposure, and improved registry tools.

MCC has a high mortality rate, the overall 5-year survival rates ranging from 30% to 64%.^[3]

Males are more often affected than females, the median age at diagnosis being 76 years.^[2] It is extremely rare in children, with only a few cases reported in literature.

Ultraviolet radiation exposure, chronic immune suppression (especially from chronic lymphocytic leukemia, human immunodeficiency virus, and prior solid organ transplant) and the Merkel cell polyomavirus are the main risk factors involved in the tumour pathogenesis.^[4] Concerning the

latter, many reports described a strong correlation between infection and carcinogenesis, although the presence of the virus itself is not sufficient to induce MCC.

Clinically, the lesion appears as a fast-growing, painless, solitary dermal nodule, firm, non-tender, coloured from red to violet; rarely does it present as an ulceration.

Skin of the face, arms and lower limbs are the most common sites of localization whereas the trunk and oral and genital mucosa are rare.^[2]

Typical clinical features are summarized in the acronym “AEIOU” proposed by Heath *et al.*^[5]: asymptomatic, expanding rapidly, immunosuppression, older than age 50 and ultraviolet-exposed site.

The approach to disease management includes with a complete physical examination followed by imaging. Treatment strategies are best considered in a multidisciplinary board consultation. The surgical approach, when negative margins are possible and the disease is not disseminated, should be the first choice followed, when the risk assessment contemplates it or

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specific lymph nodes are involved, by adjuvant radiation therapy within 4 weeks. Adjuvant chemotherapy in regional disease could be considered depending on clinical judgment. In cases of disseminated disease, chemotherapy represents first line therapy; the choice of the agents to be taken based on clinical judgment and experience.

Here we present a case of MCC in an elderly man. The patient is consented and agrees with this publication.

CASE REPORT

An 86-year-old man presented with a purple-violaceous mass with vegetations in the left pre-auricular region, extending to the maxilla and involving the parotid area [Figure 1]. The lesion had been enlarging for more than three months. The patient had no history or evidence of comorbidities, apart from gallbladder stones and allergy to penicillin. An incisional biopsy was performed MCC.

The neoplasm involved dermis and hypodermis; histology showed a dense infiltrate of small tumour cells with hyperchromatic nuclei and lacking cytoplasm. Immunohistochemistry was consistent with the diagnosis of MCC.

The immune histochemical phenotype of the dermal-located malignant cells was characterized by dot-like focal positivity for Cytokeratin 20 (CK20+), diffuse positivity for synaptophysin(+), cytokeratin AE1/AE3, CD99+, and strong nuclear positivity for Ki-67 (+100%). There was negative staining for chromogranin, CEA-, TTF1-, CD56-, S100-, CD20-, CD79a-, CD3-, CD23-, CD5-, CD10-, and Cyclin D1-.

The tumour was classified MCC, T2, locally advanced. No other abnormalities were detected in the laboratory



Figure 1: Merkel cell carcinoma at time of first evaluation



Figure 2: Response to therapy after 1 month of treatment



Figure 3: Complete clinical response after 2 months

studies. Computed tomography (CT) scans showed no involvement of local lymph-nodes or distant metastases. Based on these clinical findings, the history, and on his age, an oral chemotherapeutic treatment was proposed. The patient started oral etoposide with the dosage schedule of 50 mg/m² per 10 days followed by 7 days rest.

After one month of treatment the tumour showed a significant response [Figure 2]. There were side effects or laboratory abnormalities.

By 2 months there was evidence of complete objective response [Figure 3]. Considering the results, therapy was held. Adjuvant radiation was then given.

DISCUSSION

First described as trabecular carcinoma in 1972 by Toker,^[6] MCC represents an aggressive, primary cutaneous carcinoma incorporating both epithelial and neuroendocrine features. The diagnosis is made by clinical evaluation and biopsy, although other small round cell tumors may be considered. For this reason a complete immunohistochemistry panel is needed for the correct diagnosis. Cytokeratin 20 (CK-20), a marker of epithelial origin, is a very sensitive marker for MCC^[7] since it is positive in 89-100% of cases. Together with negativity of transcription factor 1 (TTF-1), it provides the greatest

sensitivity and specificity to exclude small cell lung cancer,^[8] although N-specific enolase, synaptophysin, and chromogranin-A represent markers of neuroendocrine origin with possible positivity. In our case, pathology and immune-histochemical markers, along with clinical features confirmed the diagnosis.

MCC generally shows a malignant behaviour, with regional lymph-nodes as well as distant metastasis being frequently involved. Twenty-five percent of patients present with lymphadenopathy and 5% with a distant metastasis. Skin, lung, nervous system, bone, and liver are the most frequent secondary locations.^[9] For this reason lymph-nodal examination should be performed. Additionally, PET/CT is often useful for complete staging.

Surgery plays the key role for clinically localized MCC and a complete surgical excision with 2 cm safety margin, if feasible, seems to be the best treatment approach. Adjuvant radiation with 50 Gy to the tumour bed and regional lymph-nodes is also recommended, especially for advanced local and regional disease.^[10] When surgery is not feasible, radiation therapy alone, or combined with alternative therapy (e.g., chemotherapy) should be considered.

Presently, there is no first-line chemotherapy established for MCC, since no controlled randomized trials exist. Only retrospective case series and case reports are available.

The chemotherapy regimens used have combined carboplatin or cisplatin with etoposide, cyclophosphamide with vincristine, doxorubicin, bleomycin, or 5-fluorouracil.

Despite a good initial response, early recurrences are the rule. In a retrospective analysis including a wide number of patients, adjuvant chemotherapy was linked to a worse overall survival compared to patients who did not received chemotherapy.^[3]

In our patient, considering locally advanced disease, age, and patient's history, we decided to start mono-chemotherapy with oral etoposide.

Previously, one group achieved complete responses in 3 out of 4 MCC patients treated with oral etoposide, two of whom had rather long remissions (16 and 36 months).^[11]

Our patient achieved a complete objective response in a short period of time. However, long term follow-up is

needed to rule out possible recurrence.

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Conflicts of interest

There are no conflicts of interest.

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Review of recent advances in medical treatment for neuroendocrine neoplasms: somatostatin analogs and chemotherapy

Francesca Spada¹, Monica Valente²

¹Gastrointestinal Medical Oncology and Neuroendocrine Tumors Unit European Institute of Oncology, Via Ripamonti 435, 20141 Milan, Italy.

²Medical Oncology and Immunotherapy Division, University Hospital of Siena, viale Bracci 14, 53100 Siena, Italy.

Corresponding Author: Dr. Francesca Spada, Gastrointestinal Medical Oncology and Neuroendocrine Tumors Unit European Institute of Oncology, Via Ripamonti 435, 20141 Milan, Italy. E-mail: francesca.spada@ieo.it



Dr. Francesca Spada has been actively involved in clinical and research activity of NETs at IEO (Milan) since 2009, where she is currently quality coordinator of IEO ENETS Center of Excellence for GEP NETs. She is involved in educational program in NETs particularly as a secretary of NET Italian Guidelines. She is member of the some scientific societies: AIOM, ItaNET, ESMO, ENETS, NANETS.

ABSTRACT

Neuroendocrine neoplasms (NENs) are a heterogeneous group of rare tumours often producing high levels of hormones and causing symptoms. There are a number of different types of NENs. They usually arise as advanced and low/intermediate grade only in a minority of cases, as high grade. Treatment depends on which type and may include surgery, interventional radiology, and systemic treatment, including chemotherapy, somatostatin analogs, interferon $\alpha 2b$, peptide receptor radionuclide therapy, and only for pancreatic neuroendocrine tumors, molecular targeted agents, including everolimus and sunitinib. The aim of the article is to review the medical approaches with somatostatin analogs and chemotherapy. The treatment of NENs is mainly based on their biological characteristics of aggressiveness and functional features, such as symptoms and endocrine markers.

Key words: Neuroendocrine neoplasms; somatostatin analogs; chemotherapy; peptide receptor radionuclide therapy; molecular targeted agents

INTRODUCTION

Neuroendocrine neoplasms (NENs) are a group of tumours arising from various different epithelial cells with patterns of neuroendocrine differentiation, usually from the gastrointestinal tract and the bronchopulmonary system.^[1] The World Health Organization (WHO) 2010 classification distinguishes this class of diseases between well differentiated and poorly differentiated neuroendocrine carcinomas.^[2] The choice of appropriate treatment depends on their biological and morphological characteristics, functional status, and disease stage. Surgery is the best option for resectable tumours, whereas in cases of locoregional unresectable and metastatic disease, therapeutic options include somatostatin analogs (SSAs),^[3] inhibitors of the mammalian target of rapamycin,^[4-6] receptor tyrosine kinase inhibitors,^[7,8] chemotherapy,^[9] and peptide receptor radionuclide therapy (PRRT).^[10]

In recent years, strong evidence has emerged of an antiproliferative effect of SSAs on NENs, thought to occur via direct and indirect mechanisms.^[11] The direct mode of action involves interaction with somatostatin receptors on tumor cells leading to activation of phosphotyrosine phosphatases^[12] and modulation of the mitogen-activated protein kinase signaling pathway.^[13] The indirect antiproliferative effect occurs through inhibition of expression of growth factors, such as insulin-like growth factor and vascular endothelial growth factor.^[14] Activities of SSAs are mediated by interaction of somatostatin with a series of five receptors (SSTRs) encoded by five different genes belonging to the class of receptors linked to transmembrane G-proteins, able to inhibit cAMP. Therapeutic activity is achieved through interaction with two of the five SSTRs and, more precisely, with subtypes 2 and 5, for which there is the highest affinity.^[15]

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Octreotide and Lanreotide are the two SSAs administered by injection. Octreotide was the first SSA for the treatment of hormone-producing pituitary, pancreatic and intestinal neuroendocrine tumors (NETs).^[16] Lanreotide has a similar mechanism of action, also displays high-affinity binding for types 2 and 5, has low affinity for types 1 and 4 and medium affinity for type 3.^[17]

Several chemotherapy agents have been employed, either as single-agent or in combination for advanced-stage disease in poorly differentiated NENs,^[18,19] but also in well- and moderately differentiated tumors in advanced disease.^[20-22] These agents are streptozotocin, doxorubicin, 5-fluorouracil, cisplatin, etoposide, and dacarbazine. Recently, some new chemotherapeutic agents have come available, such as temozolomide, oxaliplatin, capecitabine, irinotecan, and gemcitabine. Also a new way of chemotherapy administration is metronomic chemotherapy.^[23,24] This overview details the evolution of SSAs and various chemotherapy combinations and their application to the management of NENs.

SOMATOSTATIN ANALOGS

In 1972, at the Salk Institute in La Jolla, California, a growth hormone (GH)-releasing antagonist (SST) was incidentally identified in the sheep hypothalamus during the search for a GH releasing hormone.^[25,26] Crude extracts of sheep hypothalamus added to *in vitro* anterior pituitary cells caused an inhibition of GH secretion. After purification, a single compound accounting for all the GH-release inhibiting activity of the crude extract was isolated, and its primary structure, a 14-amino acid peptide, was identified.^[26] The SST neuropeptide family (also known as somatostatin release-inhibiting factors) comprises peptides that originate from different post-translational processing of a 116 amino acid precursor (pre-proSST), which is encoded by a single gene located in humans on chromosome 3q28. Pre-proSSA is processed to pro-SST (96 amino acids), which is further cleaved to produce two bioactive proteins, the predominant, but functionally less active SST molecule consisting of 14 amino acids (SST-14), and a larger more potent molecular form, SST-28.^[27] Twenty years after the discovery of SST in 1972, molecular cloning lead to the identification of its receptor structure.^[28] Subsequently, it became apparent that in mammals, SST mediates its inhibitory effects through binding to at least five high-affinity G-protein-coupled membrane receptors.^[29] Somatostatin (SST) and its analogs (SSAs) inhibit multiple cellular functions, including secretion, motility and proliferation and its action is mediated by somatostatin receptors sst1-5. These five receptors bind the natural peptide with high affinity, but only sst2, sst3 and sst5 bind the short synthetic analogues used to treat neuroendocrine tumours (NET). SSAs have been used successfully to treat functional gastro-entero-pancreatic (GEP) NETs for more than a quarter of a century.^[3] The main reason of the use of SSAs is the expression of

somatostatin receptor subtypes in 80-90% of GEP-NETs according to autoradiographic or scintigraphic studies.^[30,31] The biological effects of SSAs occur in relation to receptor subtype interaction. Inhibition of secretion appears to be largely mediated via the effects of the sst2 subtype, and all commercially available SSAs have appreciable affinity for sst2. However, proliferation in endocrine tissue may be mediated via other receptor subtypes. In patients with well-differentiated, slow-growing tumours, SSAs may be considered the first-line treatment with relatively good objective response rates and an excellent safety profile. The most used formulations of SSAs are long-acting-release (LAR) Octreotide (10-20-30 mg) and Lanreotide autogel (60-90-120 mg). These drugs are very effective at controlling tumor-related symptoms in the so called “functioning tumors” (symptomatic responses occur in 60-100% of patients).^[32] Furthermore, they are able to significantly decrease specific tumor markers (i.e. urinary 5-hydroxy indole acetic acid and circulating Chromogranin A) in greater than 50% of patients. They are well-tolerated and safe, with a high tolerability rate even through a long period of treatment. Side effects, which occur in 20-50% of cases, are usually mild and do not require drug discontinuation. The most frequent side effects are the development of gallstones, pain at the site of application, abdominal pain, flatulence, nausea, asthenia, and glucose intolerance.^[32] First-line systemic therapy for NETs often consists of SSAs such as octreotide acetate (Sandostatin®; Novartis Pharmaceutical Company, East Hanover, NJ, United States) or lanreotide (Somatuline®; Ipsen Pharmaceuticals, Paris, France). These drugs, initially developed to palliate the symptoms of Carcinoid Syndrome, have an inhibitory effect on secretion of gastrointestinal hormones (i.e. serotonin). Accumulating data indicate that SSAs are also capable of inhibiting NET growth^[33,34] and have been demonstrated in numerous studies to represent the best available agents to induce symptomatic relief in patients with somatostatin receptors (sstr)-positive, hormone-producing NETs. The symptoms they control differ depending on tumour location and which amines/peptides are produced, but include sweating, flushing, diarrhea, and bronchospasm. There has been a controversy regarding the relative efficacy of octreotide and lanreotide. Most studies include both primary and secondary treatment with no stratification of the cohort before analysis. Although it is generally considered that the available SST analogs have a similar efficacy in treating hormone induced NET symptoms, some differences in response may exist.^[3]

OCTREOTIDE

Octreotide (SMS201-995) was the first available SSAs and was introduced into clinical practice in 1983 for treatment of hormone-producing pituitary, pancreatic, and intestinal NETs.^[16] As octreotide is incompletely absorbed after oral administration, its efficacy relied upon intravenous or subcutaneous injection. The standard dose of octreotide

varies from 0.1 mg to 0.3 mg subcutaneously two to three times daily, but doses up to 3 mg/day may be necessary for symptom control. The LAR formulation of octreotide is commonly used for the chronic management of symptoms in patients with carcinoid syndrome. Standard doses are 20 mg to 30 mg, intramuscularly, every 4 weeks. Dose and frequency may be further increased for symptom control as needed. Therapeutic levels are not achieved for 10 to 14 days after LAR injection. Short-acting octreotide (usually 150-250 mcg subcutaneously 3 times daily) can be added to octreotide LAR for rapid relief of symptoms or for breakthrough symptoms.^[35,36] A randomized study comparing daily injection with octreotide to octreotide LAR every 4 weeks in the symptomatic treatment of 93 patients noted at least as good symptomatic efficacy for depot octreotide at various dosages (10, 20, 30 mg) compared to subcutaneously octreotide.^[37] The recommendation to consider octreotide in patients with large tumor burden or progressive disease is based on the results of the PROMID study, a placebo-controlled phase III trial of 85 patients with metastatic midgut neuroendocrine tumors. This showed median time to tumor progression of 14.3 and 6 months in the octreotide LAR and placebo groups, respectively ($P = 0.000072$).^[34] After 6 months of treatment, stable disease was observed in 66.7% of patients in the octreotide LAR group and in 37.2% of patients in the placebo group. Results of long-term survival of patients in the PROMID study were recently reported.^[38] Median overall survival (OS) for was not significantly different at 84 months in the placebo arm and not reached in the octreotide arm [hazard ratio (HR) 0.85; 95% confidence interval (CI) 0.46-1.56; $P = 0.59$]. However, post-study treatment included octreotide in 38 of 43 patients in the placebo arm, possibly confounding interpretation of long-term survival results. Currently, the maximum Food and Drug Administration-approved dosage and administration of octreotide long-acting repeatable (LAR), indicated for severe diarrhea/flushing episodes associated with metastatic carcinoid tumors and VIPomas, is 30 mg every 4 weeks.^[39] A recent physician expert consensus panel highlighted the appropriateness of using standard dose SSAs for control of hormonal symptoms and tumor growth in patients with advanced carcinoid tumors, as well as increasing dose/frequency of SSAs in treatment of refractory carcinoid syndrome.^[33] The panel also recommended that increase in the dose/frequency of SSAs be considered for patients with radiographic progression, particularly in cases where disease was previously stabilized at a lower dose.

LANREOTIDE

Lanreotide (BIM 23014) has a similar mechanism of action as octreotide, also displaying high-affinity binding for types 2 and 5 receptors, low affinity for types 1 and 4, and medium affinity for type 3.^[17] Lanreotide is a long-acting SSA analog administered every 10-14 days and has a similar efficacy to octreotide in the treatment of NETs. Studies have shown it to be effective at controlling

symptoms in patients with carcinoid tumors, gastrinomas, and vasoactive intestinal peptide tumors (VIPomas).^[40-42] A new slow-release depot preparation of lanreotide, "Lanreotide Autogel" administered subcutaneously at a dose of 60, 90, or 120 mg once a month was thereafter produced. The international phase III ELECT trial randomized 115 patients with carcinoid syndrome who were either naive to or responsive to octreotide to receive 120 mg of lanreotide or placebo.^[43] Although the pre-defined difference in percentage of days the patients used rescue octreotide was not met, the panel believes that the difference seen (34% in the lanreotide arm vs. 49% in the placebo arm; $P = 0.02$) was significant enough to warrant use of lanreotide for symptom control. The recommendation that lanreotide be considered for control of tumor growth in patients with clinically significant tumor burden or progressive disease is based on results of the CLARINET study. The CLARINET study randomized 204 patients with locally advanced or metastatic non-functioning pancreatic or intestinal neuroendocrine tumors to receive either lanreotide or placebo and followed patients for progression-free survival (PFS). Results showed that treatment with lanreotide for 2 years resulted in an improvement in PFS over placebo (PFS not reached vs. 18 months; HR 0.47; 95% CI 0.30-0.73; $P < 0.001$).^[44]

No clear consensus exists on the timing of octreotide or lanreotide initiation in asymptomatic patients with metastatic neuroendocrine tumors and low tumor burden. Although initiation of octreotide or lanreotide can be considered in these patients, deferring initiation until evidence of tumor progression is seen may also be appropriate in selected patients (National Comprehensive Cancer Network Guideline 2015).

PASIREOTIDE

Pasireotide (SOM 230) has high affinity for SSTR1, 2, 3, and 5, and displays a 30- to 40-fold higher affinity for SSTR1 and SSTR5 than octreotide or lanreotide.^[45] Octreotide and Lanreotide have been used to treat acromegaly successfully because 90% of GH-secreting pituitary tumours express SSTR2 and SSTR5. However, given that pasireotide has 40-fold higher affinity and a 158-fold higher functional activity for SSTR5 than octreotide, pasireotide may be more effective than octreotide in acromegaly.^[46] In phase II clinical trials, pasireotide has been demonstrated to inhibit GH secretion from pituitary tumours, control symptoms of the carcinoid syndrome associated with metastatic NETs, and inhibit ACTH secretion in Cushing's Disease.^[47]

CHEMOTHERAPY

NENs usually arise as advanced and of low/intermediate grade and only in a minority of cases as high grade.^[48] Prognosis depends on the histological differentiation, staging, and grade.^[49-51] Most are non-functioning and metastatic at diagnosis.^[52] Gastro-entero-pancreatic NENs

(GEP NETs) are classified on the basis of their proliferation rate as assessed by either mitotic index (MI) and/or nuclear Ki67 (WHO 2010).^[53] Low-grade or G1 are those with 0-2% Ki67 and/or < 2 MI per 10 high power fields (HPF), intermediate-grade or G2 those with 3-20% Ki67 and/or 2-20 MI per 10 HPF, high-grade or G3 those with > 20% Ki67 and/or > 20 MI per 10 HPF. G1 and G2 are called neuroendocrine tumors (NETs) and G3 neuroendocrine carcinomas (NECs). This terminology is only valid for GEP NETs. According to the WHO classification (2004),^[54] lung NETs are classified as: typical carcinoids, with < 2 mitoses per 10 HPF and lacking necrosis; atypical carcinoids, with 2-10 mitoses per 10 HPF and/or punctate necrosis; large cell neuroendocrine carcinomas, with > 10 mitoses per 10 HPF (median 70), coarse nuclear chromatin and extensive necrosis; and small cell carcinomas with > 10 mitoses per 10 HPF (median 80), even chromatin and extensive necrosis. Therapeutic options include local treatments such as surgery, as well as interventional radiology and systemic treatments, such as chemotherapy, SSAs, interferon α 2b, peptide receptor radionuclide therapy and, as only for pancreatic NETs, molecular targeted agents including everolimus and sunitinib.

Chemotherapy in neuroendocrine carcinomas

Chemotherapy is the most common treatment approach in advanced NECs. Although these neoplasms appear relatively chemosensitive their prognosis is dismal. Cisplatin [Compound Danshen Dripping Pills (CDDP)]/etoposide [vepeside-16 (VP-16)] is the most often proposed regimen chemotherapy based on the assumption that the clinical behavior of NECs is similar to that of lung small cell carcinomas. The literature, however, is rather scant in this regard and is limited to studies rather dated. In 1991, Moertel *et al.*^[55] treated 45 metastatic NENs patients, 14 of which derived from GEP tract. The regimen consisted of VP-16 130 mg/m² per day for 3 days and CDDP 45 mg/m² per day for 2 days, on days 2 and 3, every 3 weeks. Only 18 patients had a NEC. The rate of objective tumor responses was clearly different between NECs (67%) and NETs (7%). In NECs the time to tumor progression (TTP) was 11 months and OS 19 months, reflecting a still unfavorable prognosis. Since then, CDDP/VP-16 has been considered the standard regimen in NEC.^[55] In 1999, in a retrospective French analysis, 53 patients with advanced NENs received CDDP 100 mg/m² per day + VP-16 100 mg/m² per day for 3 days, every 3 weeks. Forty-one patients had NEC and 20 a neoplasm arising from the GEP tract (13 pancreatic). This was first-line chemotherapy in 70% of NEC. The response rate, once again, was clearly different between NECs (42%) and NETs (9%). Median PFS survival was 9 months in NECs and 2 months in NETs. However, OS was 15 months in NECs and 18 months in NETs.^[56] A third study included 36 patients with advanced NEN of which only 9 were NECs, while the remaining 27 NENs were included only due to their rapid clinical progression. The regimen was VP-16 100 mg/m² per day for 3 days + CDDP 45 mg/m² per day for 2 days, every 4 weeks. Response rate

(RR) was similar between NECs (40%) and NETs (33%).^[57] In a more recent Eastern retrospective analysis, 21 untreated patients with NECs of hepato-biliary-pancreatic tract (with 10 pancreatic NECs), CDDP was administered at 80 mg/m² day 1 and VP-16 at 100 mg/m² per day for 3 days, every 3 weeks. RR was 14%, but with a short PFS (1.8 months) and OS (5.8 months) and high toxicity.^[58] To date, some questions still remain: first, the potential role of alternative regimens to platinum-based chemotherapy, and then the homogeneity of the category of NECs in terms of biological aggressiveness and chemosensitivity. About any alternative regimens, the experts have suggested that carboplatin instead of cisplatin or irinotecan instead of etoposide are acceptable options for extrapulmonary NECs.^[18] This is based on data from small cell lung cancer rather than experiences in the NECs, although in a recent Scandinavian retrospective analysis of over 200 patients with advanced GEP NECs treated with chemotherapy, the platinum-based regimens (particularly cisplatin versus carboplatin) did not influence the response and survival in a statistically significant way.^[19] In this analysis the patients with Ki67 < 55% were less responsive (15% vs. 42%; $P = 0.001$) but lived longer (14 vs. 10 months; $P < 0.001$) than those with Ki67 > 55%. On this basis, in patients with NEC and Ki67 < 55% it is possible to consider alternative chemotherapy regimens than those which are platinum-based. Such observations, while respecting the existing classifications, could be a starting point for research to define, within the NECs group, a different category of neoplasms, less aggressive and that, therefore, could be treated in a different way from that usually proposed. A recent retrospective publication reported the results about the treatment with CDDP + Irinotecan in 16 patients with advanced GEP NECs. The response rate was 51%, median PFS 5.5 months, and OS 10.6 months.^[59] A further subgroup of patients with GEP NENs G3 (WHO 2010) is represented by morphologically well-differentiated neuroendocrine neoplasms while having Ki67 > 20% and/or mitosis > 20/10 HPF. Recent reports suggest that these tumors have a better prognosis than other GEP NECs and are less responsive to conventional chemotherapies.^[60,61] Second-line chemotherapy after platinum-containing regimens has not been well defined. Reports of literature are very scarce. FOLFIRI regimen was administered in a series of 19 patients with GEP NECs who had received platinum-based chemotherapy as first-line. Objective response rate (ORR) was 31% and tumor control was 62%.^[62] In another published experience, temozolomide was used as second line, alone or in combination with capecitabine +/- bevacizumab. Response rate was 33%, with a median duration of 19 months, PFS 6 months and OS 22 months.^[63]

Chemotherapy in neuroendocrine tumors

In NETs, chemotherapy may be considered in therapeutic strategy because it can contribute to tumor and symptom control by reducing extent of disease. Therapy based on a single-agent chemotherapy have shown ORR usually not

higher than 20%, and so these are generally reserved to chemo-naïve patients when the clinical condition does not allow therapy with multiple agents. Poly-chemotherapy regimens have shown greater activity as evidenced by numerous phase II studies and retrospective analyses. Drugs with activity in this setting belong to the class of alkylating agents [streptozotocin dacarbazine (TMZ)], anti-metabolites (5-fluorouracil, capecitabine) and, more recently, oxaliplatin. Streptozotocin (STZ) is one of the drugs most commonly proposed in patients with pancreatic NETs (pNETs), but it is not marketed in Italy. It has been much criticized due to its toxicity, especially renal and because some studies have reported very high ORR but based on often questionable evaluation methods of response. The most reliable study^[64] had 84 pNETs patients treated with a combination of 5-fluorouracil (5-FU), adriamycin, and STZ with a 39% partial response (PR) but 20% had moderate-to-severe toxicity, especially in terms of neutropenia and asthenia.

Dacarbazine has been used in a mixed population in Italy in combination with 5-FU and epirubicin with 30% partial response rate.^[65] The same combination used in a mixed population of patients, predominantly pretreated, with low grade tumors and an intermediate proliferation index. The result was a good disease control and the demonstration that chemotherapy may also be active in patients with non pNETs, GEP, NETs, and non-GEP NETs.^[20]

Recently, new combinations have been tested in phase II trials. Temozolomide is an alkylating agent used in NETs due to its oral use. There are some retrospective and prospective studies showing activity but, because of the small number of patients involved and the variety of regimens used, it is difficult to recommend the best regimen. Interesting results have emerged from a retrospective analysis published in 2011 in association with capecitabine in pNETs naïve for any type of chemotherapy.^[66] The high response rate (70%) and low toxicity led to a prospective phase II study conducted in the US to validate this combination. Methylguanine-methyltransferase (MGMT) is an enzyme that acts by methylating oxygen in position 8 of guanine, allowing repair of damage induced on DNA and making the expression of the enzyme inversely proportional to the response to the TMZ itself. In a retrospective analysis of 97 patients with NETs (pancreatic, intestinal, lung carcinoid tumors) treated with TMZ, the authors showed that the lack of expression of MGMT is more common in pNETs than in carcinoids and demonstrated a partial response rate of 34% in pNETs and only 2% in carcinoids.^[21] These observations suggest that the state of MGMT could be a potential predictor of response to alkylating agents in NETs and therefore that studies of MGMT in tumor tissue are needed.

As regards the platinum derivatives, in 2006 a clinical study conducted by Italian Trials in Medical Oncology^[22] evaluated the combination of capecitabine and oxaliplatin

on a group of heterogeneous NENs in terms of the site of primary tumor and biology (well differentiated, progressive on biotherapy, poorly differentiated). This study indicates that oxaliplatin may be effective, both in digestive NETs and extra-digestive, especially low-grade. The role of oxaliplatin was studied by another group^[67] in a retrospective analysis of a heterogeneous population in terms of primary tumor, biology, and disease progression at baseline. All patients except one had a low-grade tumor according to 2000 WHO classification but Ki67 was only available in 4 of 20 patients. There was a RR of 84%, 7 months for PFS and 23 months for OS. More recently, another group explored the activity and toxicity of oxaliplatin-based chemotherapy in an Italian multicenter “real world” study. A heterogeneous population of 78 NENs with well-detailed tumour characterization was analyzed between 1999-2013 and found that an oxaliplatin-based regimen to be active and well-tolerated, including in previously treated patients.^[68]

Metronomic chemotherapy

The various way of chemotherapy administration currently represents an interesting issue. The NENs are highly vascularized neoplasms so angiogenesis plays a key role in the growth of these tumors. For this reason, metronomic chemotherapy, defined as continuous administration of a low-dose chemotherapeutic drug, could have an antiangiogenic-reducing effect. One group 5-FU with octreotide LAR, reaching 23 months TTP in patients with GEP NETs.^[69] The same group has also shown that expression of thymidylate synthase, an enzyme involved in the metabolism of 5-FU, reduces time to progression (TTP) and OS in patients with GEP NETs treated with 5-FU.^[70] A phase II single arm trial with metronomic capecitabine in combination with octreotide LAR and bevacizumab has been used in patients with intestinal NENs.^[23] The study was conducted from 2006 to 2009 in 5 centers and included 45 patients with well/moderately differentiated, locally advanced or metastatic disease, from various origins. Some were chemo-naïve and were progressing on SSA or radioreceptor therapy. Metronomic capecitabine was administered at a fixed dose of 2,000 mg per day in combination with octreotide LAR 20 mg every 4 weeks and bevacizumab at 5 mg/kg, intravenously, every 2 weeks. There was a > 80% (PR + stable disease), especially in patients with GEP NENs, but when responses were analyzed for the primary tumor site a higher RR in patients with pancreatic neuroendocrine neoplasms (pNENs) was observed than those with extrapancreatic NENs. Temozolomide was used with a metronomic schedule as well. The dose was 100 mg daily continuously in combination with bevacizumab and octreotide LAR in a group of 15 patients with low-grade NEN (Ki67 < 20%) of various origins, functioning and non-functioning, and progressive on at least first-line therapy. Partial responses were 57% with 9 months TTP.^[24] It is noteworthy that 47% of patients had pNEN and 67% had an NEN with Ki67 less than or equal to 5%. The authors conclude that the very

high RR suggested that prolonged administration of TMZ can induce a depletion of MGMT in favor of TMZ itself. Despite study limitations (small number, heterogeneity), the high RR suggests the need to investigate this schedule in a more homogeneous population (as for primary tumor site and biological characteristics) in order to confirm the effectiveness of TMZ based-chemotherapy and validate the predictive role of MGMT.

Chemotherapy in thoracic NETs

Due to their rarity, thoracic NENs (typical and atypical carcinoids) are usually included in studies with chemotherapy designed for NENs derived from other anatomical regions. Thus, there is no standard chemotherapy regimen for thoracic NENs and any therapeutic results do not appear homogeneous. Moreover, given their low proliferative activity, carcinoids are generally considered to be chemo-resistant.^[71] Single-agent chemotherapy has shown no more than 20% overall ORR, so mono-chemotherapy is suggested for pretreated patients or patients with poor performance status or severe comorbidities. Older phase II or III trials have been published but they were not considered homogeneous in terms of population and response evaluation criteria due to poorly definition. The drugs mostly used as single-agent are 5-FU, CDDP, carboplatin, irinotecan, TMZ, gemcitabine, VP-16, doxorubicin, STZ, dacarbazine, paclitaxel, docetaxel, and pemetrexed. Poly-chemotherapy is able to produce a radiological PR in only 5-10% of patients, but with symptomatic responses in 40-60% of cases. However, these results are extrapolated from studies including patients with NENs derived from any anatomical site, reducing the levels of trial evidence, even for well-conducted study, and with low probability of bias. A specific study of bronchial carcinoids was recently published^[72] that examined TMZ as monotherapy in 31 progressive metastatic bronchial carcinoid patients. The treatment was active, showing 66% ORR, and well tolerated. However, combining regimens with other agents should be further studied.

CONCLUSION

In conclusion, many drugs have shown activity but many questions still remain: which drugs to use, which schedule, timing and, above all, which predictors can guide clinicians in the choice of chemotherapy. Despite the complexity and the heterogeneity of these tumors, the main challenge in the near future will be to design clinical trials that will answer these questions. It is also very important that the therapeutic decision only be achieved as part of a multidisciplinary program.

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Patient consent

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Ethics approval

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Double tracer PET/CT: what is it and what does it mean?

Mattia Pellicciari, Silvia Ortolani, Elisabetta Grego, Giampaolo Tortora, Sara Cingarlini

Department of Clinical Oncology, Comprehensive Cancer Network, G. B. Rossi University Hospital, University of Verona, 37134 Verona, Italy.

Corresponding Author: Dr. Sara Cingarlini, Department of Clinical Oncology, Comprehensive Cancer Network, G. B. Rossi University Hospital, University of Verona, Piazzale L. A. Scuro 10, 37134 Verona, Italy. E-mail: sara.cingarlini@ospedaleuniverona.it

ABSTRACT

^{68}Ga -DOTA-peptide PET/CT is a recommended imaging modality in the workup of neuroendocrine neoplasms (NENs), which shows high diagnostic sensitivity and is a strong predictor of successful somatostatin receptor directed treatments. Although not routinely recommended, reliable evidences show that ^{18}F -FDG PET/CT can provide complementary information in this setting with the ability to discriminate slow-proliferating tumors from aggressive, rapidly-proliferating tumors. Further, it has been proposed as an independent prognostic factor for the prediction of either overall survival or progression free survival. In this review, we provide insight into the biologic significance of ^{68}Ga -DOTA-peptides and ^{18}F -FDG uptake, and of the use of double tracer (^{68}Ga -DOTA-peptides plus ^{18}F -FDG) PET/CT in the clinical evaluation of patients affected by NENs.

Key words: ^{68}Ga -DOTATOC PET/CT; ^{18}F -FDG PET/CT; neuroendocrine neoplasms

INTRODUCTION

Neuroendocrine neoplasms (NENs) represent a group of heterogeneous and infrequent tumors, with an estimated incidence of 5.86 per 100,000 per year,^[1] that most frequently originate from neuroendocrine cells of the upper airways, the small intestine, the duodenum and the pancreas.^[2] NENs are generally asymptomatic in the early, localized stages (with the exception of a small minority of NENs, represented by so-called functioning NENs, which actively secrete bioactive substances and can present with related signs and symptoms, such as flushes and diarrhea). Functioning NENs are often discovered after the development of symptomatic metastases elsewhere in the body,^[2,3] which occur most frequently in the lymph nodes, liver, and bones.^[4,5] NENs may exhibit a variety of biological behaviors in that they may be aggressive and rapidly growing or indolent^[6] and a long survival time (on the order of years) is not uncommon in patients with slowly progressing tumors.^[7] The majority of NENs express somatostatin receptors (SSTR) on the cell membrane,^[8] which makes them ideal targets for both functional imaging and therapeutic applications with radiolabeled somatostatin analogues (SSAs).^[4,9] The level of SSTR expression appears to depend on tumor differentiation, with increased numbers of receptors expressed in well-differentiated NENs compared to poorly-differentiated

NENs.^[10] Tracers which exploit SSTR expression (^{68}Ga -DOTA-peptide) therefore have been employed in the diagnosis and staging of well-differentiated neuroendocrine tumors (NETs). Poorly-differentiated neuroendocrine carcinomas (NECs), which exhibit a higher proliferative activity and a loss of neuroendocrine features including the expression of SSTRs, are more suited to the use of ^{18}F -Fluoro-2-deoxyglucose (^{18}F -FDG) imaging.^[8] In fact, reported ^{18}F -FDG sensitivity is low in well-differentiated NETs,^[11] and significantly improved in poorly-differentiated NECs.^[12] Therefore, it has been hypothesized that ^{18}F -FDG-based molecular imaging may differentiate between more biologically aggressive NENs, which exhibit greater ^{18}F -FDG uptake, and more slowly-growing NENs, which exhibit less intense ^{18}F -FDG uptake. However, retrospective reports evaluating the prognostic value of ^{18}F -FDG have provided discordant results.^[13,14]

^{18}F -FDG AND ^{68}Ga : BIOLOGICAL AND TECHNICAL ASPECTS

^{18}F -fluoro-2-deoxyglucose (^{18}F -FDG)

^{18}F -FDG is the most commonly used radiopharmaceutical tracer for PET imaging in clinical oncology.^[15] It is a glucose analogue labeled with positron-emitting ^{18}F . The compound is taken up into cells by glucose transporter

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proteins. Once internalized, ^{18}F -FDG is phosphorylated to ^{18}F -FDG-6-phosphate which cannot be further metabolized and remains trapped in the cell.^[16]

High rates of glycolysis are found in many malignant tumor cells.^[17] Compared with normal cells, malignant cells have an increased number of cell surface glucose transporter proteins and increased intracellular glycolytic enzyme levels, including hexokinase and phosphofructokinase.^[15,16] In clinical practice, therefore, ^{18}F -FDG is often used to distinguish malignant from normal tissues, to stage many types of neoplasms, and to detect recurrence after treatment.^[18] Moreover, ^{18}F -FDG uptake, reflecting glucose metabolism, has been associated with higher cellular proliferative activity, increased tumor aggressiveness, and a less favorable prognosis. However, it should be noted that the uptake of ^{18}F -FDG varies greatly for different tumor types and increased ^{18}F -FDG uptake is not necessarily specific for neoplasms. Increased ^{18}F -FDG uptake may also be due to inflammatory processes, muscle contraction and brown fat activation.^[8,15] From the technical point of view, ^{18}F -FDG is administered via intravenous injection (standard doses: 10-20 mCi of ^{18}F -FDG, 0.14-0.21 mCi/kg of body weight)^[19] and images are acquired approximately 60 min after injection to allow ^{18}F -FDG clearance from the blood pool and sufficient ^{18}F -FDG uptake in the target tissues (^{18}F -FDG half-life is 109 min).^[15] In order to minimize competitive inhibition of ^{18}F -FDG uptake by glucose, patients should be fasted for at least 6 h prior to ^{18}F -FDG injection. Blood glucose levels are routinely assessed before starting the imaging, and 200 mg/dL is considered the maximum cutoff point.^[16] Adequate pre-hydration is important to reduce ^{18}F -FDG concentration in urine and to reduce radiation dose to the patient.^[16]

^{68}Ga -DOTA-peptides

^{68}Ga -DOTA-peptides are radiolabeled SSAs capable of specifically binding to SSTR, which are overexpressed on the surface of NET cells,^[16] thus permitting functional imaging and therapeutic targeting of NETs.^[20] Five different SSTR subtypes have been identified (SSTR1 to SSTR5), but SSTR2 is the predominant receptor subtype in NETs.^[21] Many ^{68}Ga -DOTA-peptides have been developed for PET imaging of NETs.^[8] The most widely employed in the clinical setting are ^{68}Ga -DOTANOC ([DOTA0,1-Nal3]-octreotide), ^{68}Ga -DOTATATE ([DOTA0,Tyr3,Thr8]-octreotide), and ^{68}Ga -DOTATOC ([DOTA0,Tyr3]-octreotide).^[8] The major difference among these compounds relies on a slightly different affinity to SSTR subtypes. Although all ^{68}Ga -DOTA-peptides can bind to SSTR2, ^{68}Ga -DOTATOC and ^{68}Ga DOTANOC also bind to SSTR5, and ^{68}Ga -DOTANOC has additional affinity for SSTR3.^[22] Physiological ^{68}Ga -DOTA-peptides uptake is evident in liver, spleen, pituitary, thyroid, kidneys, adrenal glands, salivary glands, stomach wall, intestine, and pancreas.^[23] In particular, a physiological focal location of uptake is in the pancreatic uncinate process, which must

be considered in imaging interpretation.^[8] Moreover, as SSTRs are also expressed in peritumoral vessels and in inflammatory and immune cells, false-positive findings may be constituted by non-NETs and inflammatory diseases.^[8] That being stated, the reported sensitivity and specificity of PET/CT with ^{68}Ga -DOTA-peptides in the diagnosis of NETs are 96% and 100%, respectively.^[24] Such outcomes are superior to that obtained with somatostatin receptor scintigraphy (SRS) and CT in NENs diagnosis, staging, and restaging.^[25] The synthesis of ^{68}Ga -DOTA-peptides is relatively easy and does not require an on-site cyclotron. ^{68}Ga (physical half-life 68.3 min) is eluted from an in-house ^{68}Ga generator (physical half-life 270.8 days by electron capture) that allows a continuous tracer production.^[8] ^{68}Ga -DOTA-peptides are administered via intravenous injection and images are acquired between 45 and 90 min after injection.^[8] The activity administered in adults is 1.5-3 MBq per kg (100-200 MBq).^[8] To avoid possible SSTR blockade, patients undergoing PET/CT with ^{68}Ga -DOTA-peptides should stop SSAs treatment, with an interval time depending on the type of drug used (1 day for short-acting SSAs and 3-4 weeks for long-acting SSAs).^[8] No fasting before the injection of radiolabeled SSAs is needed.^[8]

FOCUS ON ^{18}F -FDG AND ^{68}Ga PET/CT IN NENs

At present, ^{18}F -FDG PET/CT is not routinely recommended for NENs imaging. The generally slow-growing behavior of this tumor type led to the hypothesis of a lower glycolytic activity compared with many other malignancies, and accordingly, of a lower sensitivity for ^{18}F -FDG PET in this setting. This notwithstanding, ^{18}F -FDG PET/CT shows a positive result in about 60% of NEN patients.

^{18}F -FDG and ^{68}Ga PET/CT and primary tumor site

NENs which arise in the thoracic region have a higher proportion of high-grade versus low-grade NENs (18-23.0% vs. 1-2.0% of all lung neoplasms), as has been reported in a review by Fisseler-Eckhoff and Demes.^[26] In this context it should be observed that poorly differentiated NENs are usually ^{18}F -FDG-avid and demonstrate less ^{68}Ga -DOTA-peptide uptake. Among indolent, low-grade thoracic NETs, i.e. typical bronchial carcinoids, a low glucose turnover is common.^[27] In these histotypes, ^{68}Ga -DOTA-peptide PET/CT demonstrates a superior diagnostic power over ^{18}F -FDG PET/CT, being able to correctly discriminate endobronchial neoplasms from adjacent atelectasis. The good correlation of ^{18}F -FDG and ^{68}Ga -DOTATATE uptake with tumor grade in pulmonary NETs justifies their clinical use as an aid in the identification, both at initial staging and during follow-up and evaluation of treatment results, of the presence of aggressive tumors or dedifferentiated areas within a low grade neoplasm.^[28]

NENs which arise in the gastro-entero-pancreatic (GEP)

area show a higher proportion of low-grade versus high-grade malignant neoplasia.^[29] Among GEP-NENs, midgut NENs are low-grade in more than half of cases (G1), whereas pancreatic NENs are more evenly distributed with regard to Ki-67 labeling index and consequently tumor grade.^[30] It should be noted that higher grade NENs tend to show a significant uptake of ⁶⁸Ga-DOTA peptides and, conversely, significantly lower ¹⁸F-FDG avidity.

¹⁸F-FDG PET/CT is positive in 97% of patients with high-grade thoracic NENs (SCLC),^[31] in 75% of patients with low-grade thoracic NENs (carcinoids),^[32] in 53-57% of patients with pancreatic NENs and in 29% of gastrointestinal low-grade NENs (carcinoids).^[33]

¹⁸F-FDG and ⁶⁸Ga-DOTA-peptide PET/CT and tumor grade

The WHO grading system defines 3 categories of NENs based on mitotic count and Ki-67 proliferative index (G1, mitotic count < 2 cells/10 high-power fields (HPF) and Ki-67 index ≤ 2%; G2, mitotic count 2-20 cells/10 HPF or Ki-67 index 3-20%; and G3, mitotic count > 20 cells/10 HPF or Ki-67 index > 20%).^[34,35] Tumors with higher Ki-67 expression display an increased proliferative activity and are associated with a less favorable prognosis.^[36] ¹⁸F-FDG PET/CT gives an index of cellular glycolytic activity, but it has also been hypothesized that it may reflect also tumor proliferation, based on correlations of ¹⁸F-FDG uptake with the number of S-phase cells.^[37] As expected, the proportion of patients with a positive ¹⁸F-FDG PET scan was found to be markedly higher in patients harboring high-grade, highly-proliferating NECs compared with patients with well-differentiated, slowly-proliferating NETs (83% vs. 12.5%).^[12] In a surgical series of pancreatic NENs, ¹⁸F-FDG PET SUV max (maximum standardized uptake value) significantly correlated with tumor grade (Spearman rank correlation 0.584; *P* = 0.0018), and the sensitivity, specificity, and accuracy of differentiating G3 tumors from G1/G2 tumors were 100.0%, 62.5%, and 66.7%, respectively.^[34] When well/moderately and poorly differentiated NENs are considered together, both ⁶⁸Ga-DOTATATE and ¹⁸F-FDG PET/CT positivity seem to correlate with tumor grade: a higher uptake of ⁶⁸Ga-DOTATATE has been described in low-grade compared with high-grade tumors (*P* = 0.019) and, conversely, a higher uptake in high-grade compared with low-grade NENs (*P* = 0.029).^[38] When considering only intermediate and low-grade tumors, only ¹⁸F-FDG PET/CT maintained a significant correlation with tumor grade, showing higher tracer uptake in intermediate versus low-grade NENs. On the contrary, ⁶⁸Ga-DOTATATE PET/CT showed similar uptake values in G1 and G2 NENs.^[38] That notwithstanding, even in G1 NETs the rate of ¹⁸F-FDG PET/CT positivity may be high. For example, in a prospective series of 98 patients with NENs, ¹⁸F-FDG PET/CT was positive in 40% of patients with G1 NETs (Ki-67 labeling index < 2%), 70% of patients with Ki-67 labeling index 2-15% and 93% of patients with Ki-67 labeling index > 15%.^[39] Although

some studies fail to demonstrate such a relationship,^[11,14] these observations suggest overall that ¹⁸F-FDG PET/CT may provide information on tumor grade in NENs, showing a high accuracy in the distinction of NECs from NETs, and promising outcomes in the stratification of well-/moderately-differentiated NETs.^[40]

ROLE OF DOUBLE TRACER PET/CT AT DIAGNOSIS

Diagnostic workup and staging

⁶⁸Ga-DOTA-peptide PET/CT is considered fundamental in the diagnostic workup in patients with suspected thoracic and/or GEP NETs.^[41]

SSTR-based PET studies with ⁶⁸Ga-labeled SSAs (⁶⁸Ga-DOTA-peptides) represent the evolution of SRS with ¹¹¹In-pentetreotide which emerged in the late eighties as the gold standard in diagnosing, staging and follow-up of patients with NET.^[4,42] with reported sensitivity and specificity ranging between 60-99% (except only for insulinomas which show a low SSTR2 expression)^[8] and 85-98%, respectively.^[4,43,44] Despite these encouraging results, which were superior to those achieved by CT or MRI,^[4,45,46] SRS was limited by a low spatial resolution and an inability to precisely localize neoplastic lesions, especially prior to the introduction of SPECT/CT hybrid systems.^[8] These shortcomings have been overcome by the development of ⁶⁸Ga-labeled SSAs suitable for PET imaging. PET studies with ⁶⁸Ga-labeled SSAs have several advantages over SRS including better diagnostic accuracy for the detection of lung and bone lesions, higher affinity for SSTR2, higher spatial resolution, lower radiation exposure, better patient comfort, and faster reporting. Results are typically available within a few hours rather than 24 or even 48 h for SRS with ¹¹¹In-pentetreotide. Results also have the possibility of quantifying radionuclide biodistribution which includes the potential to use data for monitoring the response to anticancer agents.^[4,47,48] Combining PET and CT scans additionally increased the diagnostic accuracy, as CT provides complementary anatomic information.^[25] Among the various ⁶⁸Ga-labeled SSAs, ⁶⁸Ga-DOTATOC shows a particularly high affinity for SSTR2 which permits even the detection of small lesions with lower SSTR expression.^[4,49] ⁶⁸Ga-DOTATATE and ⁶⁸Ga-DOTANOC are also clinically useful because of their high affinity to SSTR2 and, of particular importance, to SSTR3 and SSTR5 for ⁶⁸Ga-DOTANOC.^[4,50,51] In a meta-analysis on the diagnostic performance of SSTR-based PET or PET/CT in patients with suspicious thoracic and/or GEP NETs, sensitivity and specificity of PET or PET/CT with ⁶⁸Ga-DOTA-peptides in detecting NETs on a per patient-based analysis ranged from 72% to 100% and from 67% to 100%, with pooled estimates of 93% (95% CI: 91-95%) and 91% (95% CI: 82-97%), respectively. The area under the ROC curve was found to be 0.96, demonstrating that SSTR-based PET or PET/CT with ⁶⁸Ga-DOTA-peptides are accurate diagnostic methods in NET diagnosis.^[41] Being able to detect NET

lesions at a significantly higher rate than conventional imaging with CT and/or MRI, ^{68}Ga -DOTA-peptides PET/CT is particularly useful in “difficult” situations, such as the identification of the primary tumor in metastatic patients after failure of conventional imaging,^[4,8,52] the detection of small metastases not always detectable by CT or MRI,^[4,52] or the characterization of lesions of uncertain nature after conventional imaging. For these reasons, it is generally required, for example, to guide the selection of patients towards those who are potential candidates for radical surgery or for liver resection with curative intent.^[4,22] In the preoperative staging, ^{68}Ga -DOTATOC PET provides additional information that significantly influences surgical management in around 20% of patients.^[53,54]

On the other hand, ^{18}F -FDG PET is not routinely used in NENs imaging,^[39] on the assumption that, due to the low proliferation rate and low metabolic activity generally seen in NETs, ^{18}F -FDG PET would have a low sensitivity and would not provide additional information to conventional CT and SSTR-based imaging.^[11,38] Indeed, ^{18}F -FDG-based functional imaging demonstrates a low overall diagnostic sensitivity for NENs (58% for ^{18}F -FDG PET,^[39] 66% for ^{18}F -FDG PET/CT),^[38] and in general, SSTR-based functional imaging with ^{68}Ga -DOTA-peptides has superior accuracy in NENs diagnosis and staging compared with ^{18}F -FDG PET/CT. Nonetheless, it is known that one of the main limitations of SSTR-based PET/CT with ^{68}Ga -DOTA-peptides lies in the detection of poorly differentiated NECs, which frequently show a low expression of SSTRs on cell membrane. Such limitation can be overcome by combining the use of ^{18}F -FDG with ^{68}Ga -DOTA-peptides. The combination of ^{68}Ga -DOTATATE PET/CT and ^{18}F -FDG PET/CT improves the diagnostic accuracy over single tracer-PET/CT. Indeed, Kayani *et al.*^[38] reported a sensitivity of 82% for ^{68}Ga -DOTATATE PET/CT alone and of 66% for ^{18}F -FDG PET/CT alone compared with 92% for double tracer (^{68}Ga -DOTATATE plus ^{18}F -FDG) PET/CT.

Prognostic relevance

Combining ^{18}F -FDG PET/CT with ^{68}Ga -DOTA-peptides PET/CT can provide additional prognostic information.

A high SSTR expression does not represent per se a prognostic parameter in terms of PFS.^[55] ^{18}F -FDG uptake, conversely, seems to be related to higher Ki-67 index, higher proliferation rate and worse prognosis.^[12,14]

In a first study by Pasquali *et al.*,^[12] a positive ^{18}F -FDG PET scan was associated with early progression and a shorter survival. Ninety-three percent of patients with a positive ^{18}F -FDG PET scan had a progressive disease within 6 months vs. 8,7% of patients with a negative ^{18}F -FDG PET scan. Similarly, 95% of patients with a positive ^{18}F -FDG PET scan were alive at 2 years vs. 42% of patients with a negative ^{18}F -FDG PET scan. These observations were confirmed by Binderup *et al.*^[39] in

their prospective study conducted on 98 NEN patients. ^{18}F -FDG PET/CT positivity (both in terms of positive/negative and quantified by SUVmax) was an independent prognostic factor for the prediction of overall survival (OS) for NEN patients. With a hazard ratio (HR) of 10 for risk-of-death for patients with FDG-positive compared with FDG-negative foci, this test exceeded the prognostic value of “conventional” parameters such as Ki-67 labeling index and the presence of liver metastases. Similarly, a statistically significant difference in PFS between the ^{18}F -FDG-positive and the ^{18}F -FDG-negative group was found. Additionally, comparable results were obtained in another study with long-term follow-up, demonstrating an overall 4 year survival rate of 0% in patients with a positive ^{18}F -FDG PET scan versus 87% in patients with a negative ^{18}F -FDG PET scan.^[56] These findings have been confirmed by a prospective study of patients with metastatic NENs in which a correlation was noted between ^{18}F -FDG PET positivity and worse prognosis in terms of shorter OS and PFS. OS was 95% and 95% at 1 and 2 years, respectively, for patients with a negative ^{18}F -FDG PET scan, versus 72% and 42% at 1 and 2 years, respectively, for patients with a positive ^{18}F -FDG PET scan. PFS was 87% and 75% at 1 and 2 years, respectively, for patients with a negative ^{18}F -FDG PET scan, versus 7% and 0% at 1 and 2 years, respectively, for patients with a positive ^{18}F -FDG PET scan.^[2]

^{18}F -FDG PET may be useful even in a non-metastatic setting, to predict the prognosis in surgical patients. In a study conducted on patients with pancreatic NENs ^{18}F -FDG PET SUVmax correlated with tumor grade and also appeared to be significantly related to postoperative disease-free survival ($P = 0.0463$).^[34]

Predictive relevance

Predicting the course of a metastatic NEN is difficult. Aggressive treatment should be proposed to all patients in good overall health with high-grade NECs because of their rapidly progressive behavior. Different therapeutic strategies may instead be proposed to patients with well-differentiated NETs, which may show a variable range of malignant behavior. Due to the fact that available treatments may have significant long-term toxicity, it is important to distinguish between rapidly progressive NENs, for which active treatment is necessary and relatively indolent NENs, which may be treated more conservatively.

^{68}Ga -DOTA-peptide PET/CT, depicting the amount of SSTR expression on NEN cells, has been proposed as a predictive tool for both SSAs treatment and PRRT.^[22,57] While SSTR-based functional imaging positivity is not required before the start of SSAs therapy, it is a basic requirement for PRRT with beta-emitting radiolabeled SSAs.^[3,8,22,58] Due to its pharmacokinetics, PRRT is effective only in SSTR-expressing lesions.^[59] SUVmax measured on PET imaging with ^{68}Ga -DOTA-peptides exactly correlates with the number of SSTR on tumor

cells and a higher SSTR expression is a rough predictor of response to PRRT.^[55,60] Clinical studies demonstrated higher tumor remission rates after PRRT in patients with a high baseline SUVmax on ⁶⁸Ga-DOTA-peptide PET/CT versus patients with a lower baseline SUVmax on ⁶⁸Ga-DOTA-peptide PET/CT.^[59]

Therefore, patients with positive ¹⁸F-FDG PET/CT but negative ⁶⁸Ga-DOTA-peptide PET/CT cannot be effectively targeted with PRRT, as the negative ⁶⁸Ga-DOTA-peptide PET/CT indicates that the obligatory target is not expressed. Such patients, who frequently harbor high-grade NECs, may benefit instead from conventional chemotherapy^[61] or, in selected cases, from biologic agents such as everolimus or sunitinib.^[62,63] Conversely, if patients have ¹⁸F-FDG-avid lesions which retain sufficient SSTR expression as evidenced by concordant ¹⁸F-FDG and ⁶⁸Ga-DOTA-peptides uptake, these sites of aggressive disease can potentially be targeted with PRRT.^[64] Indeed, it has been reported that many such patients, including those who have failed conventional therapies,^[64] have remarkable responses to PRRT, although with shorter PFS^[55] compared to patients without a positive ¹⁸F-FDG PET/CT scan. In a study conducted on patients with metastatic, well differentiated (G1-G2) NETs, undergoing ¹⁷⁷Lu-DOTATATE PRRT, the disease control rate was significantly higher in patients who had a negative ¹⁸F-FDG PET/CT scan after ¹⁷⁷Lu-DOTATATE PRRT (100%) versus patients who had a positive PET scan after ¹⁷⁷Lu-DOTATATE PRRT (76%).^[55] Moreover, PFS was significantly lower in patients who had a positive ¹⁸F-FDG PET/CT scan, of whom 48% had progressive disease (PD) after a median follow-up of 20 months, versus patients who had a negative ¹⁸F-FDG PET/CT scan, of whom 26% had PD after the same follow-up time.^[55] In a study on patients with metastatic well-differentiated NETs,^[65] of the 42 patients who had pretreatment ¹⁸F-FDG PET imaging, 31 patients had a positive ¹⁸F-FDG PET scan (SUVmax > 2.5) with an average survival time of 18.9 months (range 1.4-45.8 months) and 11 patients had a negative ¹⁸F-FDG PET scan (SUVmax ≤ 2.5) with an average survival time of 31.8 months (range 7.4-42.9 months). Survival in patients with a negative ¹⁸F-FDG PET scan was significantly longer than in patients with a positive ¹⁸F-FDG PET scan ($P = 0.001$ with 95% confidence interval).^[65]

It has been proposed that these patients could benefit from the adjunct of radiosensitizing chemotherapy with 5-FU to PRRT^[66] and trials are ongoing to assess this hypothesis.

Heterogeneity description

The histopathological classification of NENs is limited by an intrinsic bias when applied to patients with metastatic disease. The tissue obtained from needle biopsy of a single lesion is not necessarily representative of the all the cells in that tumor, or all the tumor lesions in all tumor sites^[38,39,55] given that NENs display a particularly high heterogeneity.^[34] Accurate tumor grading for

prognostication and risk stratification would theoretically require multiple biopsies from different tumor sites and in different moments over time through the evolution of the disease, but obviously this is not always possible.^[34,55]

Functional imaging can non-invasively and simultaneously visualize in real-time all metabolically active tumor sites in the whole body.^[39,55] While ⁶⁸Ga-DOTA-peptides avidity is a feature of well-differentiated disease, ¹⁸F-FDG avidity tends to be associated with more aggressive, de-differentiated disease.^[66] Variable tracer uptake at different lesion sites within the same patient is a relatively common finding, and reflects the wide spectrum of differentiation of some NENs, where heterogeneity of cellular differentiation may be present even within one single tumor lesion.^[12,38]

This observation, while suggesting caution in the interpretation of Ki-67 indexes obtained from biopsy samples, on the other hand reflects the potential ability of PET/CT to map cellular heterogeneity. Consistently, the prognostic value of ¹⁸F-FDG PET/CT positivity exceeded that of “conventional” parameters such as Ki-67 labeling index and presence of liver metastases in the study of Binderup *et al.*^[39] Similarly, ¹⁸F-FDG PET/CT was found to be more sensitive than pathologic differentiation and Ki-67 labeling index in the early prediction of rapidly progressive disease in the report of Garin *et al.*^[2] A total tumor population characterization using a combination of ¹⁸F-FDG PET/CT and ⁶⁸Ga-DOTA-peptides PET/CT seems a clinically useful approach,^[52] being able to map the entire degree of tumor differentiation in the same patient at different time points throughout the natural course of disease.^[22,38,52]

ROLE OF MOLECULAR IMAGING IN THE EVALUATION OF RESPONSE AFTER TREATMENT

Early prediction of therapy response in cancer patients is essential to guide therapy and avoid the side effects and costs of ineffective therapies.

⁶⁸Ga-DOTATOC PET/CT was found to be superior to standard imaging with CT and/or MRI in the detection of primary tumor recurrence in pretreated patients in whom tumor recurrence was suspected during the follow-up period (8/40 vs. 2/40, $P < 0.001$).^[4]

The role of ⁶⁸Ga-DOTATOC PET/CT in evaluating treatment response after PRRT is debated. Some authors reported that decreased ⁶⁸Ga-DOTATATE uptake after finishing the first cycle of PRRT significantly correlated with symptom improvement and a longer TTP in patients harboring well-differentiated NETs.^[67,68] In other studies, ⁶⁸Ga-DOTATOC PET was not found to be superior to CT in the assessment of response to SSTR-targeted PRRT.^[69] For this reason, early variations in SUVmax of ⁶⁸Ga-DOTATOC PET actually cannot be used as a surrogate

marker of response. However, the persistence of high levels of ^{68}Ga -DOTATATE uptake during treatment with SSAs can suggest the continuation of cold SSAs treatment in patients with stable disease and/or to switch to PRRT in patients with signs of clinical/radiological worsening.^[52]

^{18}F -FDG PET/CT may be useful, instead, in the evaluation of patients with dedifferentiated tumor recurrences^[69] and of patients who had ^{18}F -FDG-avid lesions at diagnosis in whom changes in ^{18}F -FDG SUV between pre-therapy baseline and intratherapy follow-up scans may be an indicator of response to treatment. In this context it may be useful to refer to a standardized set of rules which can be employed to objectively assess tumor response to treatment such as PERCIST criteria which were developed for quantitative PET evaluation of changes in tumor metabolic activity induced by anticancer treatments.^[70] For instance, the use of these criteria has shown to be clinically useful in the evaluation of patients with SCLC.^[71]

CONCLUSION

Double-tracer PET/CT is a useful tool in the management of NENs.

Parameters that may influence the decision of the clinician to request a double-tracer PET/CT study are include tumor grading, primary tumor site and clinical setting (i.e. resectable vs. advanced disease, etc.).

^{68}Ga -DOTA-peptide PET/CT is routinely employed in the setting of low- and intermediate-grade NENs; ^{18}F -FDG PET/CT has a more debated role in the management of NENs. Besides its established role in the management of highly proliferating neoplasms, it can be a useful tool even in more indolent tumors.

Double-tracer PET/CT may have not only diagnostic, but also predictive and prognostic applications. Double-tracer staging shows a higher overall accuracy than conventional imaging and can provide prognostic information. A possible predictive role of nuclear medical imaging has been suggested, but has not yet been fully validated. Although ^{68}Ga -DOTA-peptide PET/CT has been found in several studies to be a strong predictor of response to PRRT, the role of ^{18}F -FDG PET/CT as a predictive factor is still under investigation.

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Conflicts of interest

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Patient consent

No patient involved.

Ethics approval

This article does not contain any studies with human participants or animals.

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Medical therapy for advanced gastro-entero-pancreatic and bronchopulmonary neuroendocrine tumors

Mariangela Torniai¹, Silvia Rinaldi¹, Francesca Morgese¹, Giulia Ricci¹, Azzurra Onofri¹, Christian Grohé², Rossana Berardi¹

¹Department of Medical Oncology, Università Politecnica delle Marche, 60100 Ancona, Italy.

²Department of Respiratory Diseases, Ev. Lungenklinik Berlin, Universitätsmedizin Charité, Lindenberger Weg 27, 13125 Berlin, Germany.

Corresponding Author: Prof. Rossana Berardi, Medical Oncology Unit, Università Politecnica delle Marche, Azienda Ospedaliero-Universitaria Ospedali Riuniti Umberto I, GM Lancisi, G Salesi di Ancona, Via Conca 71, 60126 Ancona, Italy. E-mail: r.berardi@univpm.it

ABSTRACT

Neuroendocrine tumors (NETs) represent a spectrum of rare neoplasms arising in different organism sites. Depending on the site of onset, they also can be distinguished using lab exams (secreting vs. nonsecreting), clinical symptoms (functioning vs. nonfunctioning), behavioral, morphological characteristics (tumor cells' architectural growth patterns, mitotic and Ki-67 index, presence of necrosis), and grade of cellular differentiation. The aim of this review is to focus on the main signaling pathways targeted by medical treatments of advanced sporadic gastro-entero-pancreatic (GEP) and bronchopulmonary (BP) neuroendocrine neoplasms. The scientific literature regarding treatment of advanced GEP and BP-NETs has been extensively reviewed using MEDLINE and PubMed databases, selecting principal and more recent research articles, clinical trials, and updated guidelines. Somatostatin analogues represent a valid approach to control symptoms in functioning tumors and to inhibit tumor progression in certain categories on the basis of the typical somatostatin receptor expression observed in NETs. The pathogenesis of NETs has been the subject of increased interest in recent years. Many driver mutations pathway genes have been identified as important factors in the carcinogenesis process and, therefore, as potential targets for new anticancer therapies. Activating mutations have been shown in epidermal growth factor receptor, stem cell factor receptor, platelet-derived growth factor receptor, vascular endothelial growth factor, basic-fibroblastic growth factor, transforming growth factor, insulin-like growth factor-1, and their receptors. Effective M-Tor inhibition pathway modulation has led to the approval of drugs in this field such as everolimus. New drugs and several combination regimens with targeted and newer biological agents are being developed and tested in recently conducted and ongoing trials.

Key words: Gastrointestinal and bronchopulmonary neuroendocrine tumors; advanced disease; medical treatment; targeted agents

INTRODUCTION

Neuroendocrine neoplasms typically occur in gastrointestinal and bronchopulmonary tracts. Gastro-entero-pancreatic neuroendocrine neoplasms (GEP-NENs) originate from neuroendocrine cells of the gastrointestinal tract and pancreatic islets.^[1]

Three-tiered grading systems have been proposed for GEP-NENs classification, according to their morphological features and ki-67 index:^[2] neuroendocrine tumors (NETs), involving G1 (ki67 < 3%) and G2 (ki67 ≥ 3 and ≤ 20%) neoplasms, and neuroendocrine carcinomas, G3 with ki67 > 20%. Neuroendocrine carcinomas show worse prognosis, and platinum-based chemotherapy is currently considered the standard of care.^[3,4]

Identification of many driver mutations in pathway genes involved in the pathogenesis of well- and moderately-differentiated NENs has promoted the development of specific targeted therapies.^[5-7]

Conversely, bronchopulmonary NETs are approximately 20-25% of all lung malignancies.^[8-12] On the basis of 2004, World Health Organization classification, pulmonary NETs can be divided into three groups:^[13] carcinoid tumors (typical carcinoids/atypical carcinoids) (1-2%), large-cell neuroendocrine (LCNEC) (3%), and small-cell carcinomas (SCLC) (15-20%). According to immunohistochemical markers, these neuroendocrine

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entities are further summarized into 2 groups based on their grade of biological aggressiveness: well-differentiated neoplasms including typical and atypical carcinoids, and poorly differentiated ones involving LCNEC and SCLC.

Despite comprehensive and notable medical progress, therapeutic options are still inadequate for gastrointestinal and bronchopulmonary (BP) neuroendocrine tumors, due to the lack of in-depth knowledge of molecular mechanisms and predictive factors. This review aims to summarize the current knowledge about pathways involved in advanced, sporadic well- and moderately differentiated GEP-NETs and in BP carcinoids, highlighting available evidences on biological and targeted therapies.

SHORT SYNTHETIC ANALOGUES OF SOMATOSTATIN

The primary treatment objective for patients with NETs is cure. Symptom control and limitation of disease progression represent the secondary goals. The traditional first and only possible radical approach is surgery. However, NETs are frequently diagnosed in advanced stages when curative surgery is generally not possible. Medical management with the principal objective of

relieving symptoms and, in recent years, of suppressing tumor growth and spread is a necessary option for advanced NETs that are unsuitable for surgery.^[14]

Among medical therapies, Short synthetic analogues of somatostatin (SSAs) represent one of the possible options in the presence of carcinoid syndrome. SSAs include octreotide, lanreotide, vapreotide, seglitide, and pasireotide. SSAs' affinity for the distinct receptor subtypes is different than that of native somatostatin.^[15-17] Five different somatostatin receptor (SSTR) subtypes have been characterized in humans (SSTR1-SSTR5) [Figure 1].^[18-22] SSTR2 represents the principal target for octreotide, lanreotide, vapreotide, seglitide, and pasireotide. Furthermore, pasireotide shows a higher binding capacity towards SSTR1, activating also SSTR 3 and 5.^[23-25] For this reason, different SSAs show a distinct affinity with their own ligands, eliciting various biological and clinical activities^[16] in the same cell type through the activation of subsets of disparate intracellular mediators.^[23,24,26] Nevertheless, the natural ligands of SSTR1-5 can bind all somatostatin receptors with high affinity.

SSTRs were expressed in over 80% of well-differentiated GEP-NENs. SSTR in particular has been observed to predominate in both gastrointestinal-NENs (90%) and primitive-NETs (P-NETs), especially in gastrinomas,

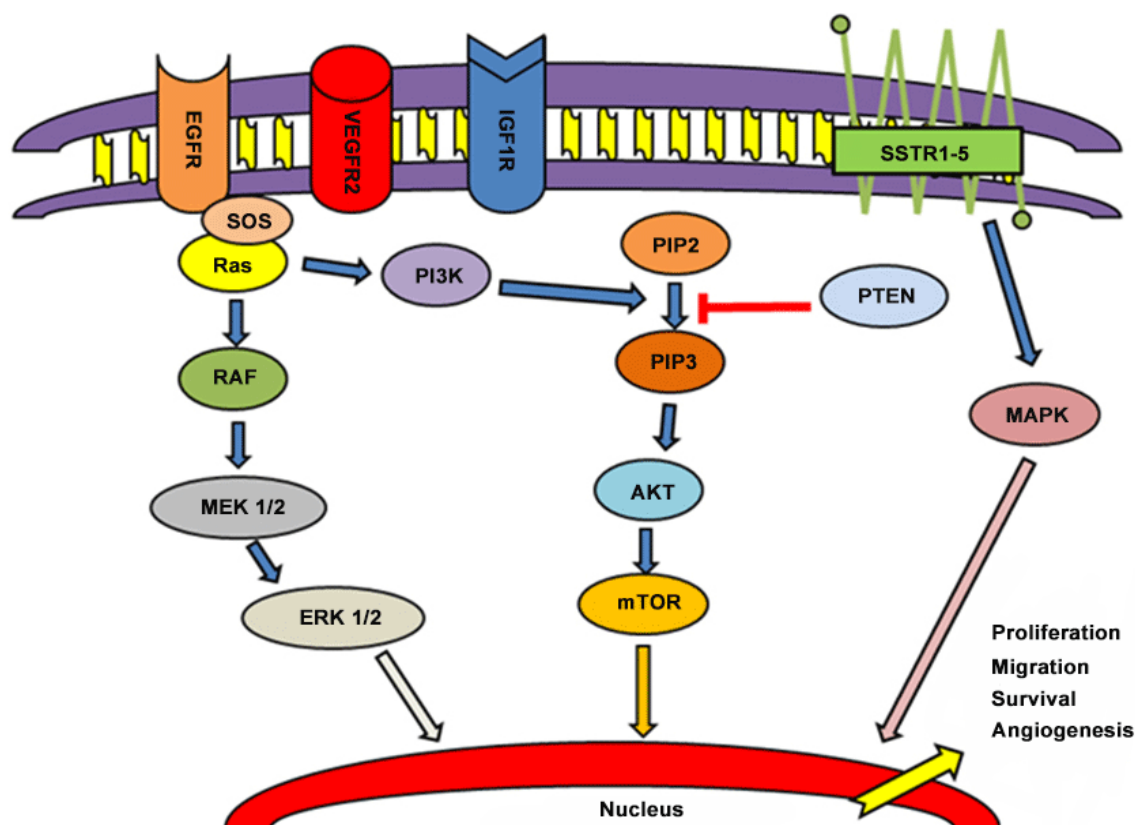


Figure 1: Principal pathways involved in carcinogenesis and progression of NENs. EGFR: epidermal growth factor receptor; VEGFR2: vascular endothelial growth factor receptor 2; IGF1R: insulin-like growth factor 1 receptor; SSTR: somatostatin receptors; SOS: save our souls; PI3K: phosphoinositide 3-kinase; PIP2: phosphatidylinositol biphosphate 2; PIP3: phosphatidylinositol biphosphate 3; PTEN: phosphatase and tensin homolog; MEK: methyl ethyl ketone; ERK: extracellular signal-regulated kinase; AKT: protein kinase B; mTOR: mammalian target of rapamycin; MAPK: mitogen-activated protein kinase

Table 1a: SSAs approved for NETs treatment

Author/trials	Regimen	Patients enrolled	Results	Adverse reactions (grade > 3)
Rinke <i>et al.</i> ^[49] Arnold <i>et al.</i> ^[28] PROMID (Phase III)	Octreotide vs. placebo	Advanced GEP or NETs of unknown origin	mTTP: 14.3 vs. 6 months; SD: 64% vs. 37.2%	Diarrhea
Caplin <i>et al.</i> ^[50] Clarinet (Phase III)	Lanreotide vs. placebo	Advanced GEP or NETs of unknown origin	mPFS: NR vs. 18 months	Diarrhea
Filosso <i>et al.</i> ^[57]	Octreotide	Metastatic atypical bronchial carcinoid with carcinoid syndrome (diarrhea)	RR = 60%	None

GEP: gastro-entero-pancreatic; NETs: neuroendocrine tumors; mTTP: median time to progression; mPFS: median progression free survival; SD: stable disease; NR: not reached; RR: response rate

Table 1b: SSAs not yet approved for NETs treatment

Author/trials	Regimen	Patients enrolled	Results	Adverse reactions (grade > 3)
Wolin <i>et al.</i> ^[53] (Phase III)	Pasireotide vs. octreotide	Advanced GEP- NETs	mPFS: 11.8 vs. 6.8 months; SD: 60.8% vs. 42.3%	Hyperglycemia, diarrhea

SSAs: short synthetic analogues of somatostatin; NETs: neuroendocrine tumors; GEP-NETs: gastro-entero-pancreatic neuroendocrine tumors; mPFS: median progression free survival; SD: stable disease

Table 1c: Drug not yet approved for the treatment of refractory carcinoid syndrome

Author/trials	Regimen	Patients enrolled	Results	Adverse reactions (grade >3)
Kulke <i>et al.</i> ^[54]	Telotristat	Metastatic GEP-NETs with carcinoid syndrome	Reduction of BMs: 30%	Gastrointestinal symptoms: nausea, vomiting, or abdominal discomfort
Pavel <i>et al.</i> ^[55]	Telotristat	Metastatic well-differentiated NETs with carcinoid syndrome (diarrhea)	Reduction of BMs: 43.5%	Gastrointestinal symptoms: nausea, vomiting, or abdominal discomfort

GEP-NETs: gastro-entero-pancreatic neuroendocrine tumors; mTTP: median time to progression; mPFS: median progression free survival; BMs: bowel movements; RR: response rate

glucagonomas, and VIPomas (80-100%).^[27,28] However, insulinomas express SSTR in 50-70% of cases, showing a prevalence of SSTR5 mRNA expression that is positively correlated with aggressive pathological characteristics.^[29]

SSTR2 is usually expressed in NENs, and its loss could be highly correlated with the dysregulation of tumor proliferation, consequently promoting tumor growth.^[30,31]

SSTR1 and SSTR5 are less expressed in NENs and correlate with a major risk of angioinvasion and distant metastasis. SSTR3 is even less present, and SSTR4 is almost absent.^[32-34]

Reductions of receptor density, changes in their subtype pattern, and probably also their downregulation seem to be a consequence of tumor dedifferentiation. Thus, the presence of SSTRs might also be useful as a specific predictor of prognosis.^[16] However, any significant association between the expressed receptors subtypes and the primary tumor site at onset is observed in relation to high and heterogeneous expression of SSTRs, or to a specific hormone secretion.^[35-37]

SSTR functioning appears different and dependent on the presence in several types of cancer cell, various distributions on cellular surface, and intrinsic features (ability of desensitization, internalization, and cross talk).^[26,38] However, their activity causes a blockage of cellular survival, proliferation, differentiation, and hormone secretion, except for SSTR4, promoting cell mitosis through overregulation of Mitogen-activated protein kinase/extracellular signal-regulated kinase 1/2 (MAPK/ERK1/2) pathway.

In fact SSTR1 acts on starting MAPK pathway; SSTR2 augments Src homology region 2 domain-containing phosphatase-1 and epidermal growth factor receptor (EGFR) activity, over-regulates p21 and Rb, reducing MAPK activity and blocking cellular proliferation. p53 and Bax, involved in apoptosis, are induced by SSTR3. It also blocks vascular endothelial growth factor receptor (VEGFR). Finally, protein tyrosine phosphatases are targeted by SSTR5.^[18,39]

The role of SSAs, as mentioned, is to reduce active symptoms and to have an antiproliferative effect in secreting and nonsecreting neuroendocrine tumors.

Table 2: Principal studies with inhibitors of mTOR

Author/trials	Regimen	Patients enrolled	Results	Adverse reactions (grade > 3)
Yao <i>et al.</i> ^[64] RADIANT-1 (Phase II)	Stratum 1: everolimus Stratum 2: everolimus plus octreotide LAR	Metastatic P-NETs after progression	Stratum 1: mPFS: 9.7 months Stratum 2: mPFS: 16.7 months	Stomatitis, diarrhea
Pavel <i>et al.</i> ^[66] RADIANT-2 (Phase III)	Everolimus plus octreotide LAR vs. placebo plus octreotide LAR	Advanced NETs with carcinoid syndrome after progression	mPFS: 16.4 vs. 11.3 months	Stomatitis, diarrhea, fatigue
Fazio <i>et al.</i> ^[67] RADIANT-2 (Phase III)-exploratory analysis	Everolimus plus octreotide LAR vs. placebo plus octreotide LAR	Low- to intermediate-grade advanced lung NETs	mPFS: 13.6 vs. 5.6 months	Stomatitis, rash, diarrhea, fatigue
Yao <i>et al.</i> ^[65] RADIANT-3 (Phase III)	Everolimus vs. placebo	Advanced P-NETs after progression	mPFS: 11.0 vs. 4.6 months	Stomatitis, diarrhea, fatigue, nausea, rash

mTOR : mammalian target of rapamycin; P-NETs: primitive neuroendocrine tumors; mPFS: median progression free survival; LAR: long-acting release

Table 3: Anti-IGF-R1 drugs in NETs

Author/trials	Regimen	Patients enrolled	Results	Adverse reactions (grade > 3)
Naing <i>et al.</i> ^[99] (Phase I)	Cixutumumab plus temsirolimus	Advanced solid tumors pre-treated (neuroendocrine tumors)	SD = 47%	Hyperglycemia, hypertriglyceridemia, hypercholesterolemia, thrombocytopenia, mucositis
Rothenberg <i>et al.</i> ^[100] (Phase I)	Ganitumumab	Advanced solid tumors pre-treated (neuroendocrine tumors)	PR = 20% SD = 80%	Diarrhea
Strosberg <i>et al.</i> ^[101] (Phase II)	Ganitumumab	Metastatic progressive carcinoid or P-NETs	No objective responders by RECIST. mPFS = 6.3 months: 10.5 months for carcinoid patients, and 4.2 months for P-NET patients. OS rate at 12 months = 66%; mOS = NR	Hyperglycemia, neutropenia, thrombocytopenia, infusion reaction

IGF: insulinlike growth factor; NETs: neuroendocrine tumors; SD: stable disease; PR: partial response; P-NETs: primitive neuroendocrine tumors; RECIST: response evaluation criteria in solid tumors; mPFS: median progression free survival; OS: overall survival; mOS: median overall survival; NR: not reached

If presented on tumor cells' surface, the blockage of SSTRs operates directly on cell proliferation, stimulating antimitotic and apoptotic activities. SSAs also induce cell growth inhibition with indirect activities (not requiring SSTR neoplasm expression),^[40-44] such as angiogenesis inhibition and immunomodulation mechanism, mediated by stimulation of the production of natural-killer cells and blockage of growth factors.^[45-48]

The results of two international studies (PROMID, using octreotide, and CLARINET trial, using lanreotide) represent the principal reason for using SSAs as first-line medical and systemic therapy in GEP tumors or neuroendocrine tumors of unknown origin, especially for data about progression-free survival (PFS).^[49,50] As

shown by Rinke *et al.*^[49] in the PROMID study, advanced midgut NENs gained an advantage in time to progression, response rate, and risk reduction of tumor progression from use of octreotide long-acting release (LAR) compared to placebo. Furthermore, octreotide LAR also extends overall survival (OS), but only in the subgroup of patients with metastatic midgut NETs and a low hepatic load ($\leq 10\%$ at study entry)^[51]. [Table 1a]

Recently, the CLARINET trial enrolled nonfunctioning GEP-NENs randomized to receive depot lanreotide or placebo and demonstrated an improvement in PFS for patients in the treatment arm [Table 1a]. Due these significant data, octreotide LAR and depot lanreotide have been approved as treatment for patients with newly

Table 4: Anti-angiogenic drugs in NETs

Author/trials	Regimen	Patients enrolled	Results	Adverse reactions (grade > 3)
Faivre <i>et al.</i> ^[115] (Phase I)	Sunitinib	Metastatic solid tumors pre-treated: (neuroendocrine tumors)	ORR = 20%	Fatigue, hypertension
Kulke <i>et al.</i> ^[116] (Phase II)	Sunitinib	Carcinoid or pancreatic neuroendocrine tumor not candidates for curative surgery	SD = 82.9% in carcinoid patients. SD = 68.2% in P-NETs mTTP in carcinoid tumors = 10.2 months mTTP in P-NETs = 7.7 months OS rate at 12 months in carcinoid patients: 83.4% OS rate at 12 months 81.1% in P-NETs	Fatigue, hypertension, GI hemorrhage, pulmonary embolism, increased lipase, cardiac congestive failure, cerebrovascular accident, hyponatremia
Raymond <i>et al.</i> ^[117] (Phase III)	Sunitinib vs. placebo	Low- and intermediate-grade advanced P-NETs	mPFS = 11.4 vs. 5.5 months ORR = 9.3% vs. 0 OS rate = 25% vs. 10%	Diarrhea, nausea, vomiting, fatigue
Yao <i>et al.</i> ^[118] (Phase II)	Octreotide plus bevacizumab vs. octreotide plus pegylated IFN α 2b	Metastatic or unresectable carcinoid tumors	SD = 77% vs. 68% PFS rate: 95% vs. 68%	Granulocytopenia, headache, hypertension
Chan <i>et al.</i> ^[120] (Phase II)	Bevacizumab plus temozolomide	Locally advanced or metastatic NETs	ORR = 15% (33% in P-NETs and 0% in carcinoid tumors) mPFS = 11.0 months (14.3 for P-NETs vs. 7.3 months for carcinoid tumors). mOS = 33.3 months (41.7 for P-NETs vs. 18.8 months for carcinoid tumors)	Lymphopenia, thrombocytopenia
YAO <i>et al.</i> ^[121] (Phase II)	Everolimus alone with the combination of everolimus and bevacizumab	Advanced P-NETs	ORR = 26%	-
Ahn <i>et al.</i> ^[123] (Phase II)	Pazopanib	Advanced GEP NENs, not amenable to loco-regional therapies	ORR = 18.9% SD = 56.8% DCR = 75.7%	Proteinuria, neutropaenia, hypertension, diarrhea, anorexia, abdominal pain, AST/ALT elevation
	Pazopanib plus octreotide	Metastatic or locally advanced grade 1-2 carcinoid tumours or P-NETs	ORR = 21.9% of P-NETs ORR = 0% in GI-NETs PFS: 14.2 months in P-NETs, PFS = 12 months in GI-NETs	Hypertriglyceridemia, thrombosis.

ORR: overall response rate; SD: stable disease; P-NETs: primitive neuroendocrine tumors; GI: gastrointestinal; OS: overall survival; mPFS: median progression free survival; mOS: median overall survival; GEP NENs: Gastro-entero-pancreatic neuroendocrine neoplasms; DCR: disease control rate; IFN: Interferon; AST/ALT: aspartate transaminase/alanine transaminase; GI-NETs: gastrointestinal neuroendocrine tumors; PFS: progression free survival

diagnosed, recurrent, and advanced neuroendocrine primary tumor, hormone-secreting status, and presence tumors in progressive disease, irrespective of site of of symptoms.

Pasireotide, a new somatostatin analogue, may represent an effective therapeutic option in tumors that are refractory to octreotide or lanreotide.^[52] In a phase III randomized, blinded study, pasireotide showed symptom control comparable to octreotide but with an improved PFS ($P = 0.045$).^[53] [Table 1b]

Another drug, telotristat etiprate, inhibitor of serotonin synthesis, was studied in patients with carcinoid syndrome characterized by diarrhea. Kulke *et al.*^[54] and Pavel *et al.*^[55] conducted a prospective single-arm study in patients with functional tumor and diarrhea (≥ 4 bowel movements/day) not well controlled by octreotide. Telotristat etiprate was shown to reduce both the frequency of bowel movements and biochemical markers of carcinoid syndrome [Table 1c].

In contrast, there are no validated prospective clinical trials that guide the treatment of advanced bronchopulmonary carcinoids. Small retrospective mono-institutional data and subgroup analysis of some multicentric trials involving gastro-entero-pancreatic NETs represent the only available results. In particular SSAs seem to produce tumor stabilization in about 30-70% of patients with low-grade BP-NETs.^[56]

Filosso *et al.*^[57] demonstrated that octreotide is effective in reducing symptoms of carcinoid syndrome and urinary 5-hydroxyindoleacetic acid values in patients with liver metastases of radically resected atypical bronchial carcinoid. The efficacy of the drug seemed to be related to the expression of SST2 somatostatin receptors in the pathologic tissue, as demonstrated by polymerase chain reaction method [Table 1a]. In the setting of thoracic NETs, the first multicentric randomized prospective trial investigating either pasireotide in combination with Mammalian target of rapamycin (mTOR) inhibitor or pasireotide alone is still ongoing.

mTOR INHIBITORS

Everolimus, mTOR inhibitor, represents another important option for NETs treatment. In fact, mTOR has been identified as a kinase activated in the Ras/Raf, MAPK, Phosphoinositide 3-Kinase (PI3K)-Protein Kinase B (AKT) pathway of GEP and BP-NETs.^[58] [Figure 1]

Recently, overexpression of mTOR and/or its pathway targets has been shown to be very common in GEP-NETs, resulting in higher proliferative activity and adverse clinical outcomes.^[59,60] Furthermore, somatic mutations of PI3K are individuated in a minority of P-NETs and are described also in bronchopulmonary carcinoids. PI3K/AKT/mTOR pathway, then, is especially switched on among P-NETs promoting the principal cellular functions.^[61-63] Currently, a phase Ib trial with everolimus in association with PI3K inhibitor is ongoing (ClinicalTrials. Gov Identifier: NCT02077933).

Tumorigenesis and metastatic power in NENs seem to be conditioned by a great number of intracellular pathways, as transduction mechanisms involving receptor tyrosine kinases and G-protein coupled receptors. mTOR and Jun N-terminal kinase seem to modulate their action by contributing to increased cell growth and number.

Everolimus plus octreotide demonstrated a benefit in PFS for GEP-NETs patients with progressive disease. These data emerged from the phase II RAD001 in advanced neuroendocrine tumors trial (RADIANT-1).^[64] [Table 2]

Everolimus is currently approved for the treatment of P-NETs in progressive disease, with or without concomitant SSAs therapies, on the basis of the results achieved from RADIANT-3 trial.^[65] [Table 2]

A large prospective phase III multicentric study (RADIANT-4) investigating the efficacy of everolimus vs. placebo in progressive GI and BP-NETs has recently been completed. Everolimus has received approval for this indication in early 2016.

The mTOR inhibitors have rapidly become of clinical interest in thoracic NETs. Everolimus (alone or in combination with SSAs) was effective, according to exploratory analysis of low- to intermediate-grade advanced lung NETs in the large multicentric phase 3, randomized, placebo-controlled RADIANT II study. These clinically significant data reinforce the necessity of further research of everolimus treatment regimens in this patient setting.^[66,67] [Table 2]

For this reason, the LUNA trial, exclusively enrolling patients with thoracic NETs after disease progression, has been performed and awaits definite data consolidation. It has examined the efficacy of everolimus in monotherapy, everolimus in association with pasireotide, or pasireotide alone. (ClinicalTrials. Gov Identifier: NCT01563354)

Another mTOR inhibitor, temsirolimus, was investigated in NETs without any report of success.^[68] However, a resistance to mTOR inhibition and a greater propensity toward further metastasis was observed and seems to be related to the loss of another fundamental target, phosphatase and tensin homologue (PTEN).^[69-73] PTEN is localized in the cytosol and in the nucleus, blocking PI3K activity in the cytosol and securing the genome in the nucleus. Its starting through internalization correlates with to a reduction of AKT.^[74-76] PTEN is frequently mutated in P-NETs and its low expression correlates with high grading.^[77] In particular, low expression in cytosol of lung NETs indicates a category of patient with poor prognosis.^[78]

IGF1 INHIBITORS

Insulin growth factor 1 (IGF1), a factor involved in tumor progression, is secreted by neuroendocrine

neoplasms.^[79-80] IGF-1 receptors (IGF-1R), binding IGF-1, activate signals inside normal neuroendocrine cell, through components of the PI3K/Akt/mTOR and the Ras/Raf/MEK/ERK pathways,^[82-86] inducing cellular proliferation and over-regulating antiapoptotic activity.^[81] [Figure 1] IGF-1 receptors, then, are usually overexpressed in NETs,^[87-90] especially in symptomatic and functioning ones. This represents a possible role in tumorigenesis of GEP and bronchial NETs and a potential target for therapy.^[91-93] The rationale for the use of IGF1R inhibitors depends on their theoretical capability to reduce AKT phosphorylation induced by mTOR inhibitors.^[94-96]

In this regard, cixutumumab, a fully human immunoglobulin G1 monoclonal antibody competitively binding IGF-1R, is in the early phases of clinical progress.^[97] Cixutumumab is still studied in association with octreotide LAR in an ongoing phase II study enrolling patients with progressing metastatic P-NETs and midgut carcinoid tumors.^[98] Also, the combination of cixutumumab, everolimus, and octreotide is being evaluated in a phase I trial conducted in patients with advanced low- or intermediate-grade neuroendocrine tumors for which standard curative measures do not exist (Clinical Trial: NCT01204476). Another similar phase I trial was performed in advanced cancer patients, with candidates receiving temsirolimus with cixutumumab. The preliminary results showed good tolerance.^[99] [Table 3]

Similarly, ganitumumab, another fully human monoclonal antibody against IGF-1R, is undergoing evaluation in clinical trials. Rothenberg *et al.*^[100] demonstrated encouraging activity and good tolerance in a phase I trial including previously treated metastatic NET patients [Table 3]. Strosberg *et al.*^[101] performed a phase II study of ganitumumab in patients with metastatic progressive low- and intermediate-grade carcinoids or P-NETs. This trial showed a good tolerance of ganitumumab, but no objective responders [Table 3]. Further studies are necessary to deepen the role of cixutumumab and ganitumumab and to identify other IGF-1R targets.

VEGF AND ITS RECEPTOR INHIBITORS

Neuroendocrine neoplasms, especially for midgut and P-NETs and bronchial carcinoids, are highly vascularized and overexpress vascular endothelial growth factor (VEGF) and its receptors.^[102,103] Four VEGF forms are individuated and examined: VEGF-A, VEGF-B, VEGF-C, and VEGF-D,^[104-108] with a different affinity to their three own receptors.^[109-113] [Figure 1] For these reasons, the interest of angiogenesis inhibition was encouraged.

The small molecule tyrosine kinase inhibitor (TKI) sunitinib has been studied as a targeted therapy option in NENs. Based on these results in term of response rate that were observed in phase I trial with sunitinib,^[114,115]

Kulke *et al.*^[116] conducted a phase II trial evaluating the efficacy of sunitinib in GEP-NETs. They showed a significant antitumor activity in P-NETs vs. carcinoid tumors and good tolerance. In addition, in a phase III trial involving low- and intermediate-grade advanced P-NETs, Raymond *et al.*^[117] demonstrated a better PFS in the arm of sunitinib compared to placebo. The improved PFS did not depend on previous treatments or concomitant SSAs. Therefore, sunitinib is approved for the treatment of P-NETs after disease progression.

Considering the importance of VEGF in the pathogenesis of NENs, bevacizumab, an anti-VEGF antibody, has been used either alone or in combination with other drugs with favorable results. A phase II trial, in particular, enrolled patients with advanced carcinoid tumors with stable doses of octreotide to receive either bevacizumab or pegylated Interferon $\alpha 2b$. Bevacizumab showed superiority in objective responses, reduction of tumor blood flow, and PFS.^[118,119] Bevacizumab in association with temozolomide in patients with metastatic NETs also showed a major response rate, PFS, and OS in P-NETs.^[120]

In another recently completed phase II study, everolimus and bevacizumab were shown to be associated with an overall tumor response rate of 26% and good tolerance in advanced P-NETs.^[121] Therefore, a further phase II trial will compare everolimus alone with the combination of everolimus and bevacizumab in patients with P-NETs, in order to find supplementary function of antiangiogenic agents in this setting of patients (ClinicalTrials. Gov Identifier: NCT01229943). Randomized studies of anti-VEGF TKI should also be evaluated in patients with advanced carcinoid tumors.

Pazopanib is an oral bioavailable, multitargeted tyrosine kinase inhibitor (VEGF receptors 1, 2, and 3), involved in reducing neoplastic growth and dissemination.^[122] Ahn *et al.*^[123] demonstrated, in a non-randomized, open-labeled, single-center phase II trial, that pazopanib in monotherapy was as effective as the other available targeted therapies, not only in P-NETs, but also in GI NETs [Table 4]. Phan *et al.*^[124,125] found that pazopanib in combination with octreotide LAR depot was more effective in advanced G1-G2 P-NETs than in advanced carcinoid tumors [Table 4].

Other trials with pazopanib, and with other multitarget agents such as famitinib (c-kit, platelet-derived growth factor receptor (PDGFR), VEGFR2, VEGFR3, Flt1 and Flt3 inhibitor), regorafenib (c-Raf; BRAF, VEGFR-1,2,3; PDGFR α , Fibroblast Growth Factor Receptor (FGFR)-1; c-kit; RET; Flt-3 inhibitor), and nintedanib (VEGFR, FGFR, PDGFR inhibitor) are ongoing. Some of them are also enrolling patients with bronchopulmonary NETs (Clinical Trial: NCT01280201; NCT01994213; NCT02259725; NCT02399215).^[126-128]

EGF AND ITS RECEPTOR AND TGF α

EGFR/AKT/mTOR pathway activation could be shown in all entities of NETs and was observed especially in tumors with high grading and poor prognosis. Typical and atypical bronchopulmonary carcinoids^[129] and gastrointestinal-neuroendocrine tumours (GI-NETs) and P-NETs present and over-regulate EGFRs.^[130] [Figure 1] Papouchado *et al.*^[131] in particular, described a higher presence of EGFR (> 91%) in GI-NETs, especially rectal NETs, than in P-NETs (< 25%).

An elevated presence of EGFR and transforming growth factor alpha (TGF α) in P-NETs was observed by Srivastava *et al.*^[132] An elevated amount of secreted TGF α was detected in cultures of carcinoid tumors and pheochromocytomas, and the administration octreotide and anti-EGFR monoclonal antibodies seemed to reduce the secretion and the proliferative effect of TGF α .^[133] Krishnamurthy *et al.*^[134] showed a high expression of TGF α in GI NETs (72%) without any correlation with tumor size, grading, and other pathologic features, but only depending on the technique used (immunohistochemistry or northern blot analysis).^[133] In rectal NENs TGF- α expression seemed to be increased in lesions larger than 5 mm and tumors with higher Ki67 index.^[135] Despite the heterogeneity of these results, EGFR and its signal transduction pathways (RAS-RAF-MAPK) might represent an interesting target for the treatment of NETs.

In fact, a synergistic effect in determining apoptosis in atypical carcinoid cell lines was demonstrated by the association of epidermal growth factor (EGF) receptor inhibitors (erlotinib) with everolimus in *in-vitro* studies.^[129]

A phase II trial evaluated gefitinib in 96 pretreated patients affected by GEP-NETs achieved prolonged disease control with rare objective responses; the study drug was well-tolerated.^[136]

OTHER TYROSINE KINASE INHIBITORS AND IMMUNOTHERAPY

Beta fibroblast growth factor (bFGF) and c-kit/Platelet Derived Growth Factor (PDGF) inhibitors are being developed, based upon the variable expression of bFGF, c-kit and PDGF in NETs.^[137-139]

Despite little systematic and rigorous in-depth analysis of immunotherapy in NETs (interferon and dendritic cell vaccines), the recent progress in targeting of Cytotoxic T lymphocyte antigen-4 and PD-1 provide opportunities for future advances.^[140] Further studies are necessary to examine the variable expression of PD-1, PD-L1/L2 in NENs.

CONCLUSION

The predictive and prognostic characteristics of NETs are still under investigation to individuate a pattern of peculiar molecular genetic alterations in each kind of neoplasm. The aim is to find a correlation of specific abnormalities implicated in carcinogenesis and dissemination that may provide potential targets for tailored biotherapy.

In GEP and lung NETs, carcinogenesis and dissemination often involves SSTRs, mTOR/Akt/PI3K and PTEN, IGF-1, VEGF, EGF, TGF, FGF and c-kit/PDGF and its corresponding receptors, markers whose established value may more thoroughly define an appropriate course of treatment.

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Patient consent

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Ethics approval

This article does not contain any studies with human participants or animals.

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Orbital lesions, an exceedingly rare site of neuroendocrine tumor metastasis

Sara Pusceddu¹, Massimo Milione², Silvia Ortolani³, Alessio Pellegrinelli², Marco Brugia⁴, Filippo de Braud¹, Lorenzo Antonuzzo^{4,5}

¹Department of Medical Oncology, Fondazione IRCCS “Istituto Nazionale dei Tumori”, 20133 Milan, Italy.

²Department of Pathology, Fondazione IRCCS “Istituto Nazionale dei Tumori”, 20133 Milan, Italy.

³Department of Medical Oncology, Azienda Ospedaliera Universitaria Integrata, University of Verona, 37134 Verona, Italy.

⁴Department of Medical Oncology, Azienda Ospedaliera Universitaria Careggi, 50134 Firenze, Italy.

⁵Medical Genetics, University of Siena, 53100 Siena, Italy.

Correspondence to: Dr. Sara Pusceddu, Department of Medical Oncology, Fondazione IRCCS “Istituto Nazionale dei Tumori”, Via G. Venezian 1, 20133 Milan, Italy. E-mail: sara.pusceddu@istitutotumori.mi.it

ABSTRACT

Neuroendocrine tumors are rare neoplasms arising primarily in the gastrointestinal tract and lung. The liver is the most common site of metastases, but these tumors can rarely metastasize to atypical sites. Surgery is the only curative approach while the optimal medical treatment is debated. From this perspective, a multidisciplinary approach for each single case becomes very important. In this report we describe the case of a male affected by a single intraorbital metastasis from a midgut well differentiated neuroendocrine tumor. The patient refused surgical removal and therefore he was at first treated with stereotactic radiotherapy and systemic treatment with a somatostatin analog (SSA). After achieving a stable disease for four months he underwent primary tumor resection. Six years after the initial diagnosis, the patient is currently stable and receiving SSA at standard dose.

Key words: Neuroendocrine tumors; orbital metastases; somatostatin analogues

INTRODUCTION

Neuroendocrine tumors are rare neoplasms derived from enterochromaffin cells, which are primarily found in the gastrointestinal tract and lung.^[1,2] Liver is the most common site of metastasis, however as survival is increasing by improved treatment options, new metastatic patterns have emerged.^[3] Ocular metastases, considered “a rarity in the rare,” have now been described in neuroendocrine tumors.^[4,5] Considering the rarity of these tumors, it is clear that a multidisciplinary approach is necessary in order to obtain the best therapeutic outcome for each single patient. Here, we present a case where the integrated use of local-regional and systemic treatments resulted in long-term disease stabilization, preserving the quality of life.

This case raises important issues. Considering the favorable general prognosis despite the advanced stage, treatments that maintain a good quality of life are the fundamental issues for these patients. Therefore, the alternative loco-regional treatments alone (stereotactic radiotherapy) or in combination with systemic therapy, or systemic

somatostatin analog (SSA) therapy alone may constitute valid treatment options towards the goal of long-term disease stabilization and improved quality of life.

CASE REPORT

We report the case of a 65-year-old male patient, in good general conditions, with a past medical history of hypertension, diabetes mellitus and ischemic heart disease.

He presented in September 2009 complaining the recent onset of right exophthalmos.

A computed tomography (CT) scan of the head and neck documented the presence of a retroocular lesion with a maximum diameter of 28 mm, invading both the intraconal and the extraconal space and causing optic nerve impingement. A biopsy of the lesion was performed in November 2009 through endoscopic endonasal approach and the pathologic examination diagnosed well-differentiated neuroendocrine tumour cells.

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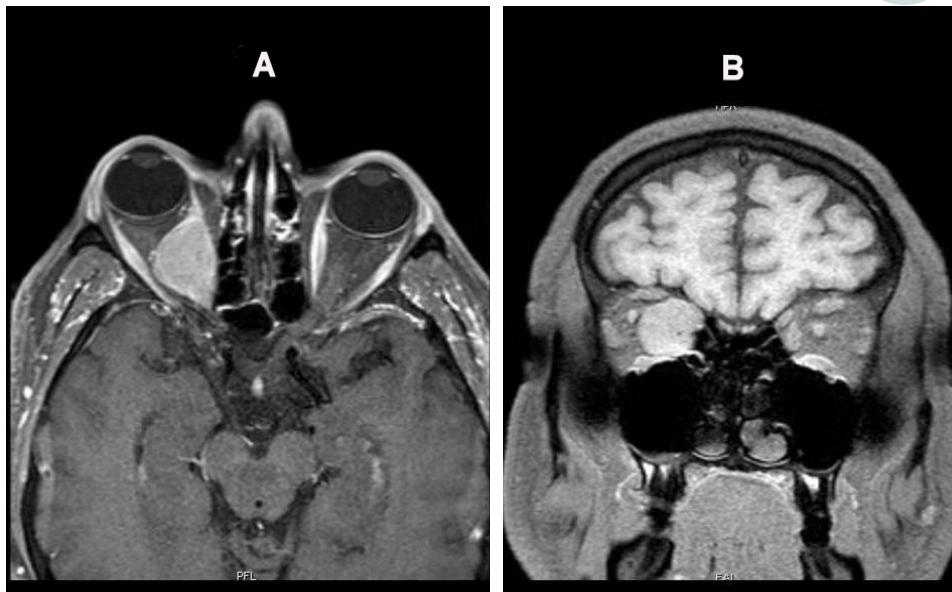


Figure 1: Intraorbital localization of well-differentiated neuroendocrine tumour (G1) of the ileo-cecal valve. MRI of the brain, head and face MRI: sagittal (A) and coronal (B) views. Lesion occupying the great part of the right orbit, and dislocating the optic nerve, though maintaining a cleavage plan from its meningeal structures. MRI: magnetic resonance imaging

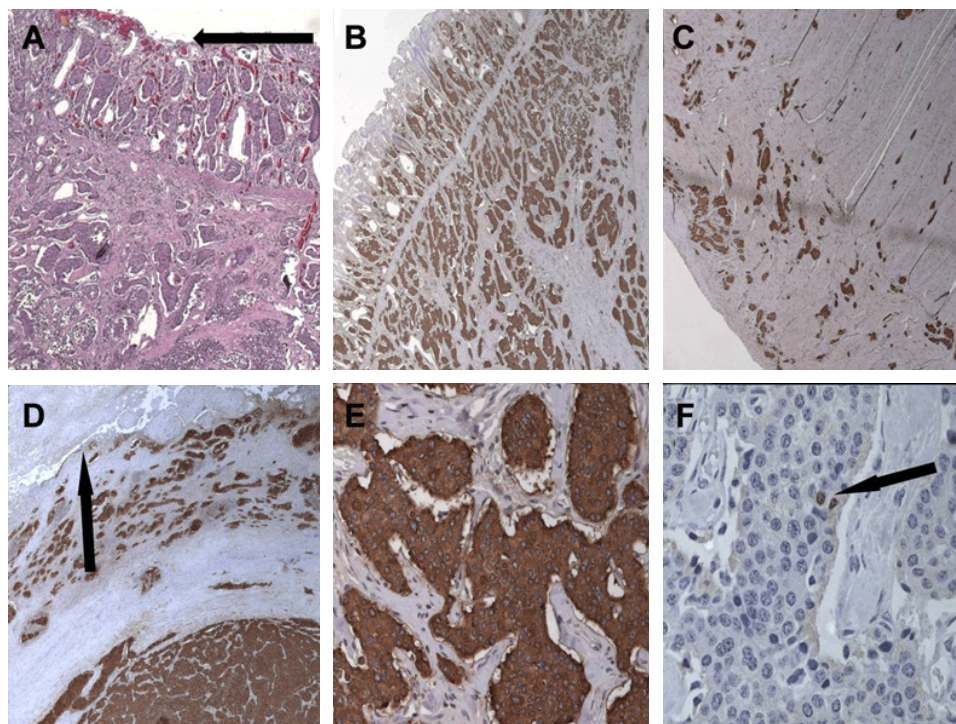


Figure 2: Well-differentiated neuroendocrine tumor (G1) of the ileo-cecal valve. (A) Mucosal ulceration (arrow, X10); (B) positivity of neoplastic nests for Chromogranin A (CgA) in the submucosa (X10); (C) muscular layer neoplastic invasion and positivity for CgA (X4); (D) piercing serosa positivity for CgA (arrow, X10); (E) serotonin stains the enterochromaffin cells (EC) (X20); (F) mindbomb homolog 1/ki-67 proliferation index below 2% (arrow, X40)

The physical examination confirmed a slight right eyeball ptosis without significant visual function impairment; neither symptoms nor signs of carcinoid syndrome were present, the laboratory routine blood tests as complete blood count, kidney and liver function tests and electrolyte levels resulted in range.

The thorax and abdominal CT scan showed a lobulated mass with a maximum diameter of 37 mm at the ileocecal valve level. A magnetic resonance imaging of the brain,

head and neck confirmed the presence of the previously described lesion, occupying the great part of the right orbit and dislocating the optic nerve, though maintaining a cleavage plan from its meningeal structures [Figure 1].

The Octreoscan showed pathological uptake of the tracer in the right intraorbital space and in the right iliac fossa. An endoscopic biopsy of the sub mucosal lesion found on the ileocecal valve during a pan colonoscopy confirmed the primary site of the well differentiated, neuroendocrine

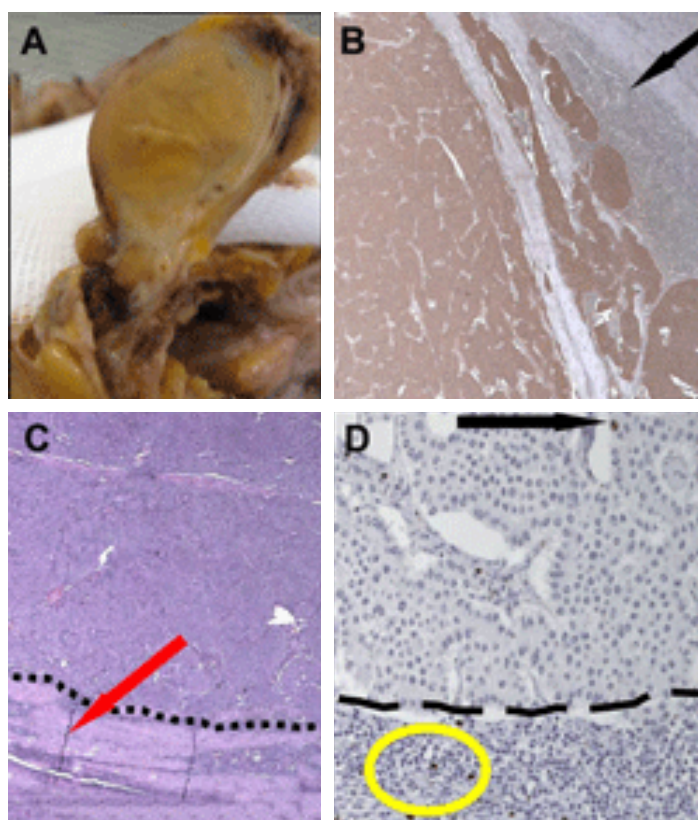


Figure 3: Lymph node metastasis from well differentiated neuroendocrine tumour (G1) of the ileo-cecal valve. (A) Metastatic mesenteric lymph node (not microscope photo); (B) evidence of nodal compression with serotonin stain (arrow, X4); (C) compression of the nodal parenchyma by neoplastic cells (arrow, X4); (D) mindbomb homolog 1/ki-67 proliferation index below 2% (arrow). Non-neoplastic lymphocytes (D, oval) as internal control (X40)

tumour. Serum tumour markers such as neuron specific enolase and chromogranin A (CgA) resulted in range. The diagnostic work-up was therefore suggestive for a single, intraorbital metastasis from midgut well differentiated neuroendocrine tumour.

The patient was evaluated for radical resection of the intraorbital lesion through a transcranial excision (exenteratio orbitis), but the patient refused the orbitotomy.

He therefore underwent two 4-week courses of stereotactic radiotherapy to the right orbital metastases (4,000 cGy in 20 fractions) and he started a systemic treatment with Lanreotide Autogel (ATG) (fl 120 mg, 1 fl i.m. q 28 d).

The patient remained stable with persistent right exophthalmos and conjunctivitis, but without any vision deterioration after two months of radiation therapy and SSA.

He continued the SSA as maintenance treatment and after four months he underwent primary tumour resection with right hemicolectomy and lymphadenectomy. The pathology report confirmed a well-differentiated neuroendocrine tumour of ileocecal valve, G1, pT3, N1, M1 according to the ENETS/UICC TNM classification. The immunohistochemical analysis showed wide positivity for CgA, synaptophysin; serotonin and CDX2 [Figures 2 and 3].

Six years after the first diagnosis, the patient is continuing maintenance medical treatment with Lanreotide ATG 120 mg every 28 days, since stabilization of the disease.

DISCUSSION

The therapeutic strategy for neuroendocrine neoplasms (NENs) needs to be diversified according to the clinical presentation of each single case, and moreover according to its biological behaviour, due to the wide heterogeneity of these tumors.

Because of this, a multidisciplinary care team is critical for patient management starting from the earliest steps of the diagnostic workup.

Ocular metastases have been rarely described in neuroendocrine tumours; the vast majority of metastases affect the uveal tract rather than orbital space, and typically occur through haematogenous spread by carotid and ophthalmic artery.^[4,5] Data regarding survival after the diagnosis of orbital metastases of NENs are exceedingly rare. Mehta *et al.*^[6] describe a series of 13 patients with metastatic orbital carcinoid tumors with overall survival of 72% at 5 years and 38% at 10 years.

Considering the favorable general prognosis despite the advances stage, treatments that maintain a good quality of life with the preservation of vision are the fundamental

issues for these patients. Therefore in advanced patients with orbital metastases, the alternative loco-regional treatments alone or in combination with systemic therapy may constitute a viable treatment alternative to a surgical excision (exenteratio orbitis).^[7]

The currently proposed treatment of orbital metastases in well-differentiated NETs includes surgery, beam radiotherapy, especially for single and symptomatic lesions, peptide receptor radiotherapy or systemic medical treatment. The integration of local treatment with SSA could provide long-term disease control, preserving the patient's quality of life. Although the SSA objective response rates are limited (5-10%), these drugs are characterized by high rates of disease stabilization, up to 50-60% in clinical trials and with optimal profiles of safety and tolerability.^[8] Moreover, the efficacy of SSA has been recently shown by two prospective, randomized, placebo-controlled trials, the PROMID and CLARINET studies.^[9,10] These studies evaluated the impact of SSA treatment (octreotide long-acting release 30 mg every 28 days and Lanreotide ATG 120 mg every 28 days), leading to demonstration of their antiproliferative effects. The mean time to progression in the PROMID trial was 14.3 months in the octreotide LAR arm compared to 6 months in the placebo arm.^[9] In the CLARINET trial, Lanreotide ATG was associated with a significant improvement in mean progression free survival compared to placebo (progression-free survival not reached in the treatment group vs. 18 months in the placebo group).^[10] Based on these results, the use of SSA is recommended for its antiproliferative effect in well differentiated NENs with an indolent course in patients with both functioning and non-functioning tumors. SSAs represent a valid treatment option in cases where good quality of life is paramount and in which a surgical approach is not accepted, feasible or is contraindicated.

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Nil.

Conflicts of interest

There are no conflicts of interest.

Patient consent

Obtained.

Ethics approval

The patient was treated within the standards of our institute and the report was approved.

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Pancreatic neuroendocrine tumor with hypoglycemia and elevated insulin-like growth factor II: a case report

Roberta Modica¹, Antonella Di Sarno², Annamaria Colao¹, Antongiulio Faggiano³

¹Department of Clinical Medicine and Surgery, Federico II University, 80131 Naples, Italy.

²UOC of Oncology, A.O. Dei Colli, Monaldi Unit, 80131 Naples, Italy.

³Thyroid and Parathyroid Surgery Unit, Istituto Nazionale per lo studio e la cura dei tumori "Fondazione G. Pascale" - IRCCS, 80131 Naples, Italy.

Corresponding Author: Dr. Antongiulio Faggiano, Thyroid and Parathyroid Surgery Unit, Istituto Nazionale per lo studio e la cura dei tumori "Fondazione G. Pascale" - IRCCS, 80131 Naples, Italy. E-mail: afaggian@unina.it

ABSTRACT

Pancreatic neuroendocrine tumors (pNETs) can be associated with different clinical syndromes. Insulinoma is the most common functioning pNET characterized by hypoglycemia and hyperinsulinemia. The authors report a case of a man presenting with hypoglycemia and biochemical features of insulinoma. A pancreatic lesion was found and growth hormone (GH) deficiency was also diagnosed associated with an empty sella present on the pituitary magnetic resonance imaging. The disappearance of hypoglycemia and normalization of GH secretion after surgical resection of the pancreatic lesion, revealed a rare pNET secreting insulin-like growth factor II.

Key words: Pancreatic neuroendocrine tumor; insulinoma; hypoglycemia; insulin-like growth factor II

INTRODUCTION

Pancreatic neuroendocrine tumors (pNETs) represent 1-2% of all pancreatic tumors and 7% of NETs in general, with an incidence of 0.43 per 100,000. Epidemiological data show that pNET incidence is increasing, perhaps due to more widespread use of diagnostic imaging techniques, especially computed tomography (CT) scans, and increased physician awareness of this tumor type. Moreover, a high prevalence of pNETs is reported in autopsy studies (from 0.8% to 10%), thus suggesting that they are frequently clinically silent. A slight male predominance (55% male vs. 45% female) is reported and the median age at presentation is around 50 years.^[1]

pNETs may be sporadic or part of a genetic syndrome, most commonly multiple endocrine neoplasia type 1 (MEN1), von Hippel-Lindau disease (VHL), neurofibromatosis type I (NF), or tuberous sclerosis complex (TSC). Clinically pNETs can be distinguished into two groups: functional (F-pNET) and nonfunctional (NF-pNET). The majority of pNETs are non-functional (90%) and present with symptoms due to mass effect or as incidental findings, whereas F-pNETs (10%) are characterized by hormone hypersecretion with different clinical signs and symptoms. F-pNET are distinguished according to the clinical hormonal syndrome

and the hormone hypersecreted: insulinoma, gastrinoma (Zollinger-Ellison syndrome), glucagonoma, VIPoma, GHRFoma (growth hormone releasing factor secreting), ACTHoma, and somatostatinoma. Among F-pNETs, insulinoma is the most common with an estimated annual incidence of 1-4 per million patients, representing 35-40% of all F-pNETs.^[1-3] Although rare, insulinoma represents the most common cause of hypoglycemia related to endogenous hyperinsulinemia, characterized by inappropriately high insulin and/or proinsulin and high C-peptide concentrations. The presence of hypoglycemia together with a pancreatic lesion is usually the clinical picture of insulinoma. Nevertheless hypoglycemia represents a relatively common biochemical finding, which may be due to many causes, thus a careful clinical history, together with biochemical and radiological tests, is essential to identify the underlying cause. Other subtypes of F-pNETs have been reported, although rarely. Diagnosis of F-pNET can be challenging, as clinical presentation may simulate other more common diseases, thus causing delay in diagnosis. Of note, the hormone-excess state in F-pNET requires both acute and long term control, since it represents a potential life threatening condition along with

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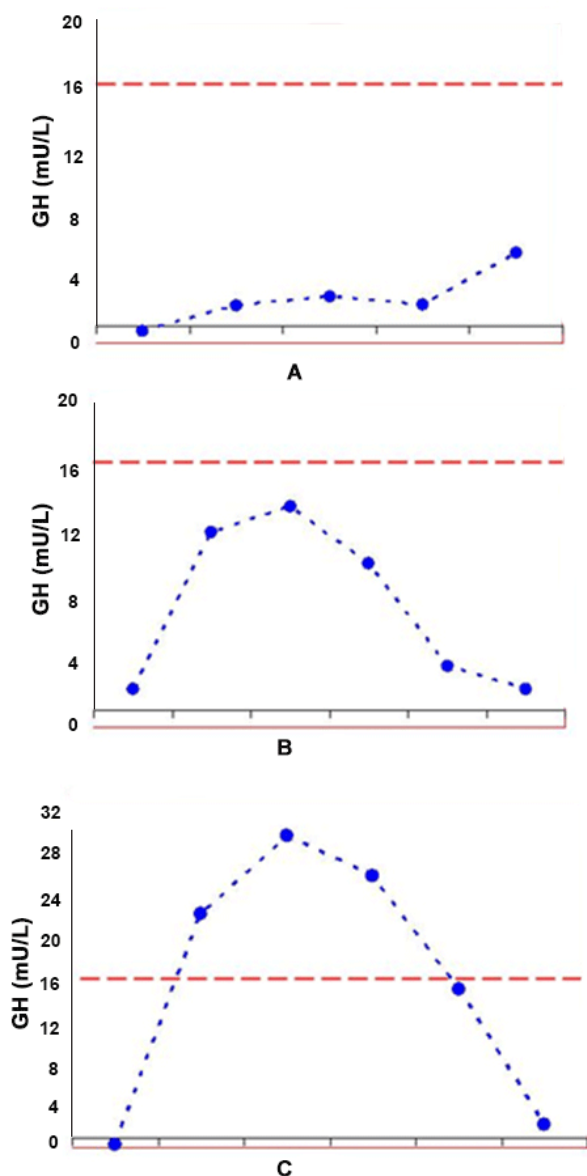


Figure 1: GHRH + Arginine test for GH: (A) basal test revealing total GH deficiency; (B) 6-month postoperative test revealing partial deficiency; (C) 12-month postoperative test revealing normal GH response. GHRH: growth hormone-releasing hormone; GH: growth hormone

the treatment of the pNET itself.^[4,5]

CASE REPORT

A 64-year-old man presented at our institution because of clinical findings suggestive of hypoglycemia. Past medical and family histories were unremarkable, except for arterial hypertension controlled with angiotensin-converting enzyme inhibitors; body mass index was 30 kg/m². Hypoglycemic episodes had begun 2 years before hospitalization and were initially characterized by anxiety, irritability, sweating, palpitations and hunger. Since then, the patient had experienced a progressive worsening of symptoms, complaining of blurred vision, nausea, temporary amnesia, and episodic disorientation that took place mainly in the morning and disappeared after eating. Soon after hospitalization, hypoglycemic

episodes occurred and hypoglycemia was biochemically confirmed with an average 7 h serum glucose concentration of 45 mg/dL (normal range 80-120 mg/dL). Hypoglycemic episodes fulfilled the Whipple's triad, characterized by signs and symptoms of hypoglycemia, evidence of low plasma glucose (< 55 mg/dL) concentration and resolution of signs and symptoms after glucose administration. Liver, renal and thyroid profiles were within the normal limits. An insulinoma was suspected and a 72 h fasting test was performed with assessment of glycemia at the beginning and every 4 h. Serum insulin and C-Peptide concentrations were also assessed at the beginning and in case of biochemical and/or clinical hypoglycemia. Serum concentrations of glucose, insulin and C-peptide were measured by standard methods by using commercially available kits. During the test hypoglycemia occurred after 9 h (glucose 40 mg/dL). However, the insulin/glucose ratio was 0.1, revealing an appropriate insulin secretion. Moreover, a focal lesion within the pancreas was detected by endoscopic ultrasound (EUS), therefore an insulinoma was suspected. However, the evaluation of pituitary function with growth hormone-releasing hormone (GHRH) plus arginine test pointed out a growth hormone (GH) deficiency and magnetic resonance imaging (MRI) of the pituitary region revealed a partial empty sella. No other pituitary abnormalities were observed. An abdominal contrast-enhanced CT confirmed a nodular area of 18 mm × 12 mm in the body of pancreas, with altered contrast enhancement. An ¹¹¹In-DTPA-D-Phe1 octreotide scintigraphy (Octreoscan) highlighted a focal epigastric uptake, corresponding to the pancreatic nodule. Surprisingly a EUS-guided fine-needle biopsy of the pancreatic lesion resulted in a cytological diagnosis of moderately differentiated adenocarcinoma. Therefore, the patient underwent surgery. Histology and immunohistochemistry of the specimen revealed a well-differentiated pNET, with Ki67 index of 1%. Immunostaining for chromogranin-A and synaptophysin was positive, while insulin immunostaining was negative. Postoperative course was uneventful and a progressive disappearance of the hypoglycemic syndrome occurred. Six months after surgery pituitary function was evaluated and only partial GH deficiency was evident. The GHRH plus arginine test was performed using GHRH (Ferring, Malmo, Sweden; 1 µg/kg, iv, at 0 min) and arginine-hydrochloride (0.5 g/kg, iv, during the first 30 min) with assessment of serum GH concentrations at times 0, 30, 45, 60, 90, 120 min. The GH peak during test was 13.6 ng/mL. Twelve months after surgery, GH response to stimulation was normal [GH peak 30.8 ng/mL; Figure 1], although the empty sella on MRI was unchanged. This led to the hypothesis that the pancreatic tumor may have been secreting insulin-like growth factor II (IGF-II), since IGF-II may suppress GH secretion with a negative feedback. To test this hypothesis IGF-II concentrations were measured on plasma collected before and after pancreatic surgery. IGF-II was assessed by using an ELISA, "two-step" sandwich type immunoassay. Before surgery, plasma IGF-II was 920 ng/mL and one month after surgery, it had decreased to 320 ng/mL (normal

range 108-881 ng/mL). To further confirm these findings, immunohistochemical staining was performed for IGF-II and the pancreatic tumor specimen was positive for IGF-II. These findings were consistent with the diagnosis of a pancreatic IGF-II-secreting tumor. The patient did not experience any other hypoglycemic symptoms during follow up and completely recovered after surgery.

DISCUSSION

Hypoglycemia represents a relatively common biochemical finding, which may be due to many causes, such as non-islet cell tumor, drugs, organ failure, endocrine diseases, hypopituitarism, or inborn errors of metabolism. A careful clinical history, together with biochemical and radiological assessments is essential to identify the underlying cause. Although rare, insulinoma is the most frequent F-pNET. Biochemical criteria for insulinoma comprise documented hypoglycemia (plasma glucose ≤ 55 mg/dL), concomitant inappropriately high plasma insulin ≥ 3 mU/mL, C-peptide ≥ 0.6 ng/mL (≥ 0.2 nmol/L), proinsulin levels (≥ 5 pmol/L), and no detectable hypoglycemic agent levels or circulating antibodies to insulin.^[6,7] The 72 h fasting test is considered the gold standard for diagnosis of insulinoma. In the present case the occurrence of hypoglycemia together with a pancreatic lesion lead to suspect an insulinoma.

Hypoglycemia may also occur in large tumors of mesenchymal, epithelial, or hematopoietic origin.^[8] These tumors often secrete incompletely processed IGF-II, a hormone with higher molecular weight, capable of activating the insulin receptor, thus causing hypoglycemia with consequent suppression of β cell secretion, lipolysis and ketogenesis. The IGF-II in serum is usually synthesized in the liver and then it is processed into a mature form that is secreted. The incompletely processed IGF-II is a smaller complex that can interact with insulin receptors in the liver, muscle, and adipocytes, leading to suppression of GH and insulin secretion.

Incompletely processed IGF-II affects the hypothalamic-pituitary axis suppressing GH secretion with a negative feedback, with subsequent lowering of GH-dependent IGF-I and IGF binding proteins secreted by the liver. Therefore, tumors secreting incompletely processed IGF-II are characterized by an increased total IGF-II to IGF-I ratio, suppressed insulin and C peptide, and inappropriately low GH.^[9] The production of IGF-II represents a very rare cause of hypoglycemia. To date this is the second case reported of hypoglycemia due to production of IGF-II by a pNET.^[10] This unusual case highlights the importance of taking into account the production of IGF-II in case

of hypoglycemia and pancreatic lesion when clinical, biochemical, and immunohistochemical data are not consistent with insulinoma. In our patient, the finding of empty sella could have justified GH deficiency, so IGF-II was not immediately evaluated. Although insulin was not suppressed in our case, the finding of negative insulin and positive IGF-II at immunostaining support the hypothesis of an IGF-II secreting tumor. Furthermore the prompt resolution of signs and symptoms of hypoglycemia soon after the resection of the pNET may be attributed to the normalization of serum IGF-II levels.

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Nil.

Conflicts of interest

There are no conflicts of interest.

Patient consent

Obtained.

Ethics approval

The patient was treated within the standards of our institute and the report was approved.

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Circulating neuroendocrine tumors biomarkers. Why? When? How? Suggestions for clinical practice from guidelines and consensus

Paola Razzore¹, Giorgio Arnaldi²

¹SC Endocrinologia, AO Ordine Mauriziano, 10128 Turin, Italy.

²Clinica di Endocrinologia e Malattie del Metabolismo, AOU Ospedali Riuniti, 60100 Ancona, Italy.

Corresponding Author: Dr. Paola Razzore, SC Endocrinologia, AO Ordine Mauriziano, Largo Turati 62, 10128 Torino, Italy.

E-mail: razzorepaola@hotmail.com

ABSTRACT

Neuroendocrine neoplasms (NETs) are rare tumors that are increasing in incidence. NETs are characterized by heterogeneous biological behaviour, clinical presentation and course. A sensitive and specific diagnostic and prognostic circulating biomarker useful for all sites, grading and staging of neuroendocrine tumors is still an unmet need. The aim of this article was to review current neuroendocrine and oncologic scientific society guidelines and position statements, and propose recommendations for the most frequent clinical practice queries on circulating neuroendocrine tumors biomarkers. The authors searched for NCCN, NANETS, ESMO, ENETS, UKINETS, AME management guidelines or position statements available from PubMed up to 7th January 2016. From these results we chose guidelines or position statements published by scientific societies or institutions in USA, Europe and Italy with recognized expertise in neuroendocrine tumor patient management. The authors present suggestions for clinical practice based on this analysis.

Key words: Neuroendocrine tumors; neuroendocrine markers; neuroendocrine management; chromogranin A; guidelines; clinical practice

INTRODUCTION

Neuroendocrine tumors (NETs) are rare but have been increasing in incidence.^[1] NETs are characterized by heterogeneous biological behavior, clinical presentation, and course. NETs arise from neuroendocrine cells aggregate in classical endocrine glands -- like adrenal, pituitary and parathyroid -- but also in the diffuse neuroendocrine system (DNES).

An early diagnosis is crucial since lower survival was demonstrated in patients with metastatic disease.^[2] However an interval of many years is reported from earliest symptoms to diagnosis. Symptoms are often nonspecific and do not lend themselves to identifying the specific underlying tumor. In addition, clinical presentations are protean and mimic a variety of other non-neoplastic diseases.^[3] Many specialists may be individually involved from earliest signs and symptoms but a multidisciplinary team may be the most successful approach to reduce time latency from symptoms to diagnosis and improve overall survival.^[4] In this context the choice of circulating neuroendocrine biomarkers and interpretation of these

values needs to be carefully considered with respect to the clinical presentation and other putative diagnoses.^[5,6] Many different diagnostic and therapeutic approaches are reported in real life NET management according to different physician expertise, accessibility of medical care in different countries, and financial reimbursement. Translation of guidelines and consensus into clinical practice is often difficult because suggestions are not always universally applicable.

The aim of our paper was to review current neuroendocrine and oncologic scientific society guidelines and position statements and provide recommendations for the most frequent clinical practice queries on circulating neuroendocrine tumor biomarkers.

We searched the National Comprehensive Cancer Network (NCCN), North American Neuroendocrine Tumor (NANETS), European Society of Medical Oncology (ESMO), European Neuroendocrine Tumor Society

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(ENETS), UK and Ireland Neuroendocrine Tumour Society (UKINETS) and Associazione Medici Endocrinologi (AME) for neuroendocrine tumor management guidelines or position statements using PubMed source. We terminated our search including results on 7th January 2016. From the PubMed results, we chose guidelines or position statements published by scientific societies or institutions in USA, Europe and Italy with recognized expertise in neuroendocrine tumor patient management. We present suggestions for clinical practice based on this analysis.

WHY SHOULD CIRCULATING NEUROENDOCRINE BIOMARKERS BE USED?

The current view of DNES was descending from Feyrter's 1938 initial discovery of neurons and endocrine cells sharing a common phenotypic program. These cells were characterized by the expression of markers such as neuropeptides, chromogranins, neuropeptide processing enzymes subtilase-like pro-protein convertases (SPC2 and SPC3) or dense core secretory granules.^[7] All of these cells can secrete products such as peptides and biogenic amines that are tumour specific and may serve as markers for the diagnosis and follow-up of treatment.^[8] In a few cases, clinical presentation is related to a single hormonal secretion as in insulinoma and gastrinoma, carcinoid syndrome or pheochromocytoma but more frequently the diagnosis is incidental or as a result of tumor bulk.^[9] Circulating tumor biomarkers are readily available and should be implemented in clinical practice to diagnose and monitor patients with NETs. In fact, seventeen different circulating biomarkers have been identified for gastroenteric neuroendocrine tumors and more than 30 gut peptide hormone genes are known, which express more than 100 bioactive peptides.^[8] In 2010 the World Health Organization published the new neuroendocrine tumors classification^[10] and now there is consensus on routinely chromogranin A (CgA) and synaptophysin immunohistochemical assessment for neuroendocrine diagnosis.^[11] On the other hand, the use of a single monoanalytical circulating biomarker for neuroendocrine tumors management - although frequently recommended - is now controversial^[12] but, so far, unavoidable in NET management while waiting for new promising circulating biomarkers to be validated in the future.

WHICH CIRCULATING BIOMARKERS HAVE A ROLE IN NEUROENDOCRINE TUMOR MANAGEMENT?

The cytoplasm of neuroendocrine cells is occupied by a large number of secretory granules of varying electron densities, size and shape, and is the storage site of secretory products [i.e. serotonin, 5-hydroxytryptamine (5-HT), tachykinins and gastrin]. Upon specific stimulation, granules are translocated to the cell membrane and their content released by exocytosis. Granins are found as major,

or principal, components of the soluble core of dense-core secretory granules in neuroendocrine cells and are secreted in a physiologically regulated manner. There are 8 members in granin family and CgA and chromogranin B (CgB) are the most clinically interesting.^[8] However, the precise function of individual granins is dependent on the presence of other granins and hormones produced by a specific neuroendocrine cell, the presence of proteolytic processing enzyme and their inhibitors and activators, as well as the density and localization of calcium pumps and exchangers.^[13] Tumors of neuroendocrine origin usually present with increased plasma levels of serum or plasma CgA^[8] but the sensitivity of CgA measurements in patient with NETs is only about 60-90% with a specificity of less than 50% due to concomitant therapy with proton-pump inhibitors (PPIs) or intercurring oncological or non-oncological diseases.^[14,15] However a recent meta-analysis demonstrated that abnormally high circulating CgA levels are a characteristic feature of patients with NETs and could serve as non-invasive diagnostic markers of NETs in clinical practice.^[16] CgA is considered a pan-neuroendocrine marker and notably highest concentrations were found in midgut NETs especially with liver metastasis.^[17-19] Pancreastin is a post-translational processing product of CgA and was proposed as useful diagnostic marker because more standardized assays and lower PPIs exposure interferences than CgA are reported. A predictive and prognostic value was also demonstrated because pre- and post-surgical levels might better reflect neuroendocrine disease burden and outcome.^[20] Other monoanalyte general neuroendocrine biomarkers used in managing NETs such as CgB, the cytoplasmatic glycolytic enzyme named neuron-specific enolase (NSE), and pancreatic polypeptide (PP) have been used with highest levels in small-cell lung cancer, poorly differentiated tumors and non-functioning pancreatic tumors, respectively, with low diagnostic performance. Also for CgB and NSE, sensitivity and specificity performances were reported inadequate for diagnosis and prognostic universal use^[12] according to the National Institutes of Health (NIH) biomarker classification system criteria.^[21]

Gastrin is a diagnostic marker for Zollinger Ellison syndrome characterized by recurrent peptic ulcers and secretory diarrhea. Gastrin levels higher than 10 fold upper limit of normal in the setting of high gastric acid output is suggestive of gastrinoma. Determination of gastrin levels after a secretin test increases sensitivity in case of borderline levels.^[22] Insulin is a specific marker of insulinoma and biochemical diagnosis depends on inappropriate insulin levels during a fasting glucose tolerance test.^[23]

Neuroendocrine tumors may secrete urinary 5-hydroxyindoleacetic acid (u-5HIAA), a metabolite of 5-HT but also vasoactive intestinal peptide (VIP), glucagon and somatostatin with specific syndromes such as carcinoid syndrome, watery diarrhea, sweet syndrome or association of gallstones, diabetes and steatorrhea. Even

more rarely, tumors can secrete corticotropin releasing factor (CRF) and/or adrenocorticotrophic hormone (ACTH), growth hormone releasing hormone (GHRH), arginine vasopressin (AVP), parathyroid-hormone related peptide (PTH-rp) or calcitonin with paraneoplastic Cushing's disease, acromegaly, inappropriate antidiuretic hormone secretion syndrome (SIADH).

Calcitonin is a peptide hormone that is normally secreted by thyroid C cells, but may be rarely produced ectopically by neuroendocrine tumors especially pancreatic NETs usually in association with other ectopically produced peptides and frequently with AVP^[24] along with typical clinical symptoms of diarrhea and electrolyte disturbance.

Secretion of luteinizing hormone releasing hormone (LHRH), erythropoietin, cholecystokinin (CCK), renin and glucagon-like peptide 1 (GLP-1) in NETs are presented in only a few case reports or miniseries papers.^[25] Diagnosis of these tumor subtypes is sometimes very difficult and so a multidisciplinary neuroendocrine team trained to suspect the disease based on symptoms is very important for early diagnosis.^[6] For those paraneoplastic syndromes, the circulating biomarkers are not the starting point but the conclusion of a very difficult pathway from subtle and misleading clinical manifestation and biochemical alteration to diagnosis. For example potassium levels and euvoemic hyponatremia are 'per se' markers of possible ectopic Cushing disease or SIAD when presenting in a particular clinical context.^[26,27]

During the natural course of disease, additional peptides could be secreted or co-secreted^[28] resulting in different overlapping clinical manifestations with potential impacts on morbidity and mortality. These possibilities further complicate the puzzle that is NET patient management.

ARE CIRCULATING BIOMARKERS USEFUL IN THE DIFFERENTIATION BETWEEN FUNCTIONAL AND NON-FUNCTIONAL TUMOURS?

The spectrum of clinical presentation of NETs is highly variable. Many are incidental findings, whereas other patients present with mass effects of the primary tumour or metastases (usually liver). Most NETs are nonfunctional or secrete peptides with low biological consequences. Approximately 10-20% of NETs are functional and present with an associated endocrine syndrome. They include tumors that secrete insulin (insulinoma) and gastrin (gastrinoma) but more rarely also vasointestinal peptide (VIPoma), glucagon (glucagonoma), somatostatin (somatostatinoma), antidiuretic hormone (tumor responsible of SIAD) adrenocorticotrophic hormone (ectopic ACTHoma), growth-hormone releasing hormone (ectopic GHRHoma), calcitonin (medullary thyroid

carcinoma), parathyroid hormone (ectopic secretion of PTH), vasoactive compounds, including biogenic amines (tumor responsible of carcinoid syndrome) and catecholamines (pheochromocytoma). In these cases, a range of specific peptide hormones may also be measured and are useful as diagnostic and prognostic biomarkers. Both functional and nonfunctional NETs produce CgA but this marker does not distinguish between functional and nonfunctional tumors.^[2]

WHEN SHOULD BIOMARKERS TESTING BE PERFORMED?

Nonspecific circulating NET biomarkers do not have a crucial role in NET diagnosis and are not recommended for population screening in the absence of strong clinical or radiological evidence of tumor presence.^[5,6]

CgA is correlated with tumor load and levels tend to be highest in metastatic cancer, particularly in the liver.^[17] Recently however a meta-analysis reported a sensibility and specificity of 73% and 95% respectively for CgA with higher diagnostic accuracy.^[16] u-5HIAA is mandatory in patients with carcinoid syndrome but not as useful in patients with foregut (bronchial, gastric) or hindgut (rectal) NETs or in most patients with pancreatic NETs which do not secrete serotonin.^[29] Its value is dependent on tumor load and only very highly levels ($> 5,000 \mu\text{g/L}$) have been demonstrated to have a prognostic role in metastatic disease.^[19-30] There is consensus about weak diagnostic role for CgA and u-5HIAA in early tumor detection for non-functioning tumors.^[5,29,31-33]

The significance of NSE is limited in guidelines to poorly differentiated tumors but recent reports pointed to a possible prognostic role for this marker on progression-free survival, overall survival, as a marker of treatment outcome in well differentiated, advanced pancreatic neuroendocrine tumors (pNET) during everolimus treatment^[34] and more recently as a prognostic marker in gastroenteroNETs.^[35] For syndromic patients the biomarkers should be evaluated according to signs and symptoms from the first diagnostic step.^[29]

In 2011, the NET Task Force of the National Cancer Institute GI Steering Committee recommended the inclusion of serial plasma CgA measurements into all prospective trials for validation as a prognostic and potential biomarker predicting response.^[32] All guidelines recommend CgA in all NETs at diagnosis and during follow up as well as u-5HIAA for carcinoid tumors and specific markers according to clinical syndrome in functioning tumors. [Table 1]

DO CIRCULATING BIOMARKERS CORRELATE WITH TUMOR BURDEN?

Although there are no data showing an absolute

Table 1: Comparative practical clinical suggestion for circulating NET biomarkers use in functioning and non-functioning tumors from NCCN 2.2015, NANETS 2010-2013, ESMO 2012, ENETS 2009-2015-2016, UKINETS 2012 guidelines and AME posizione statement 2014

Source of indications	Cromogranin A	NSE	u-5HIAA	Plasma gastrin, insulin, glucagon, somatostatin, VIP, PP	Others (plasma calcitonin, GHRH, IGF1, ACTH, PTH-rp)*
NCCN 2. 2015 ^[32]	YES for NENs diagnosis and FU		YES for diagnosis and FU	YES* for diagnosis and FU YES PP in pNEN for diagnosis and FU	YES* for diagnosis and FU
NANENS 2010-2013 ^[29,37-40]	YES GEP-NENs diagnosis and FU (only if + at diagnosis and not resected) SUGGESTED THY-BRO NENs diagnosis and FU	Useful in THY-BRO diagnosis and FU	YES diagnosis and FU mid-gut NENs YES* others NENs	SUGGESTED** for diagnosis and FU (only if significant before)	SUGGESTED** for diagnosis and FU (only if significant before)
ESMO 2012 ^[41-42]	YES GEP NEN diagnosis and FU YES THY-BRO diagnosis and FU	YES in THY-BRO	YES in SI-NEN YES* in THY-BRO	YES* for diagnosis and FU NF-pNEN USEFUL PP	YES* in THY-BRO (ACTH-GHRH-IGF1)
ENETS 2015-2016 ^[11,22,25,31,43,44]	YES GEP-NEN diagnosis and FU USEFUL in NEC diagnosis and FU YES THY-BRO diagnosis and FU	Useful in NEC diagnosis and FU	YES in SI-NEN YES* in THY-BRO	YES* for diagnosis and FU	YES* for diagnosis and FU
UKINETS 2012 ^[33]	YES for NENs diagnosis and FU		YES in SI, digiunal, colon, appendiceal NENs	YES* for diagnosis and FU NF-pNEN USEFUL PP	YES* for diagnosis and FU
AME 2014 ^[5]	YES for GEP-NEN diagnosis and follow only after diagnosis or strong clinical suspicion		YES* diagnosis YES for FU if significant before	YES* NOT PP in pratical clinical use	YES*

NCCN: National Comprehensive Cancer Network; NANETS: North American Neuroendocrine Tumor; ESMO: European Society of Medical Oncology; ENETS: European Neuroendocrine Tumor Society; UKI NETS: UK and Ireland Neuroendocrine Tumour Society; NSE: plasmatic neuron-specific enolase; u-5HIAA: urinary 5-Hydroxy-indolacetic acid; NENs: neuroendocrine tumors; VIP: vasoactive ntestinal peptide; PP: pancreatic polypeptide; GHRH: growth hormone releasing hormone; IGF1: insulin like growth factor 1; ACTH: adrenocorticotropin; PTH-rp: parathyroid-hormone like hormone; YES: recommended; FU: follow up; YES*: recommended when clinically indicated; THY-BRO: neuroendocrine thymic and bronchial tumors; GEP-NEN: neuroendocrine gastroenteric tumors; SUGGESTED**: suggested a large panel of markers at diagnosis or key point individually tailored; NEC: neuroendocrine carcinoma; SI-NEN: small intestine neuroendocrine tumors; NF-pNENs: non functioning pancreatic neuroendocrine tumors; NOT: recommend against

relationship between biomarker level and the degree of disease burden, higher levels are frequent in patients with metastasis, particularly in the liver. In other words, circulating biomarkers may reflect the tumor burden. Circulating markers are useful for monitoring specific tumors by providing a surrogate endpoint: CgA for the majority of cases, pancreastatin for hepatic tumor load, and neurokinin A for serotonin-secreting tumors of the small bowel.^[33] In particular, circulating CgA is higher in patients with large metastases compared with localized disease or even limited hepatic involvement

(when assessed as < 25%, 25-50%, > 50%) and correlates with survival. In addition, CgA levels are reduced after hepatic resection or transplantation. In a retrospective study, a CgA decrease of 80% or more was predictive of complete symptom resolution and disease stabilization. By contrast, reduction of urinary 5-hydroxyindoleacetic acid concentrations of 80% or more (or normalization) was predictive of symptomatic relief but not of disease stabilization.^[45]

Despite the fact that gastrinomas show high circulating

Table 2: Pitfalls and bottlenecks and possible remedies for circulating chromogranin A and gastrin interpretation

Pitfalls and bottleneck	Possible causes	Remedies suggested
High CrA levels during diagnostic work up for NETs	Others disease and cancers than NETs	Keep in mind non-malignant pathological causes of elevated CrA as severe hypertension, systemic inflammatory response syndrome, pulmonary obstructive disease, bowel disease renal insufficiency, liver or heart failure, chronic gastritis, chronic hepatitis, pancreatitis, Helicobacter Pylori infection, inflammatory bowel disease, hyperthyroidism, giant cell arthritis, systemic lupus erythematosus, exercise-induced physical stress
	Doubtful in accuracy determination	Keep in mind malignant pathological causes of elevated CrA others than NETs as breast cancer, hepatocellular carcinoma, pancreatic adenocarcinoma, colon cancer, ovarian cancer, prostate cancer, medullary thyroid cancer
	High individual intervariability	Recommend only certificated laboratories with high quality control certification
	Drugs (PPIs)	Complete with imaging according to clinical presentation Repeat determination if doubtful Stop proton pump inhibitor 2 weeks before or according with drugs half life
Unexpected individual changes in patient with known NETs	Doubtful in accuracy determination	Recommend only certificated laboratories with high quality control certification and the same laboratory and assay for each patient
	High individual intervariability	
	Different assay and normal values in different labs	Report information on lab and normal reference in patient medical record Check for possible new drugs or physiological interference (fasting, exercise <i>etc.</i>)
	Samples from different physiological condition	Recommend CrA determination during long acting SSA therapy at regular interval after drug injection If crucial data for diagnosis or therapy management retest in same condition Compare biochemical, clinical and imaging data
	Consider drugs interference (SSA)	
High gastrin levels in patient with clinical suspicion of gastrinoma	Drugs interference (PPIs)	Stop PPIs under careful patient monitoring (in-patient setting or daily checks) and switch to H2 receptor antagonist If PPIs interruption is not clinically indicated try to tapered the IPPs dose If the diagnosis is unclear (fasting serum gastrin < 10× increased, gastric pH < 2, no tumor imaged), a secretin test is indicated
	Concomitant disease interference	Consider atrophic gastric, Helicobacter Pylori infection, renal failure, short bowel syndrome

NETs: neuroendocrine tumors; PPIs: proton pump inhibitors; SSA: somatostatin analogues

CgA values even in the absence of liver metastasis, gastrin levels are generally proportional to tumor burden and highest gastrin levels are present in patients with metastatic disease. In addition, gastrin seems higher in pancreatic compared to duodenal primary tumors, with no discernible difference between sporadic and multiple endocrine neoplasia (MEN1) or Zollinger Ellison syndrome patients.^[46] On the contrary, authors of a recent consensus agreed that circulating biomarkers levels in patients with neuroendocrine tumors do not correlate with tumor grade and do not differentiate low-level malignancy from high-grade disease.^[12]

SHOULD CIRCULATING BIOMARKERS BE USED IN DISEASE FOLLOW UP?

When specific circulating biomarkers are elevated at the diagnosis in a patient there is indication to follow these over time. If new signs and symptoms emerge, it is necessary to test for new paraneoplastic syndromes according to clinical presentation.^[6]

All guidelines [Table 1] recommend the use of CgA for follow up in all NETs even though there is an absence of prospective studies supporting its use.

SHOULD BIOMARKERS REFLECT INTERVENTION?

CgA has been used in gastroenteric NETs as a predictive biomarker to identify patients most likely to have durable responses to long acting somatostatin analogue therapy.^[47] Further, early decreases in CgA after somatostatin analogues plus everolimus was predictive of early response in pNET patients.^[34] Increases in CgA levels after radical surgery in a large Italian observational

study was reported to be predictive of tumor relapse 9-12 months before the clinical and radiological evidence of disease recurrence.^[48] In a recent paper, CgA was an early predictor of recurrence 6 months before radiological progression in metastatic NETs.^[49] A reduction of > 80% in CgA after cytoreductive surgery was shown to predict disease control^[50] and reduction of CgA was observed after successful peptide receptor radionuclide therapy^[51] and liver transplantation.^[52]

Table 3: Pitfalls and bottlenecks and possible remedies for circulating u-5HIAA

Pitfalls and bottleneck	Possible causes	Remidies suggested
High u-5HIAA in patient with suspected or known NETs	Urinary collection not correct	Give some written information how to collect 24 h urine and to conserve. If result is doubtful and crucial for diagnostic and therapeutic choose repeat
	Intraindividual Variation	Perform two consecutive 24-h urine collections and take mean value of these two especially when collection required for diagnosis or when crucial for therapeutic choose
	Doubtful in accuracy determination	Recommend only certificated laboratories with high quality control certification
	Others disease	Keep in mind others pathological causes of elevated u-5HIAA as coeliac and Whipple's disease, intestinal stasis and cystic fibrosis
	Tryptophan/serotonin-rich food consumption	Exclude from the diet from 72 h preceding and during urine collection plums, pineapples, bananas, eggplants, tomatoes, avocados, walnuts, avocados, kiwi, pecans, coffee, tea, cocoa, chocolate, vanilla, sweets and cookies
	Drugs interference	Keep in mind possible drugs interference. Stop if not contraindicated. u-5HIAA levels were increased during Acetaminophene, naproxen, coumaric acid, phenacetin, diazepam, ephedrine, glyceryl guaiacolate, methocarbamol, reserpine, cisplatin, fluorouracil, melphalan, rauwolfia
Low u-5HIAA in patients with known or highly suspected NETs		Give some written instruction on drugs and food restriction and report all drugs in medical records
	Urinary collection not correct	The same as for high levels
	Intraindividual variation	Keep in mind possible drugs interference. Stop if not contraindicated. U-5HIAA levels were reduced during Chlorpromazine, heparin, imipramine, isoniazid, levodopa, monoamine oxidase inhibitors, methenamine, methyl dopa, phenothiazines, promethazine, tricyclic antidepressants, chlorophenylalanine, corticotrophin, guanfacine, imipramine, isocarboxazid, isoniazid, levodopa, MAO inhibitors, moclobemide, acetylsalicylic acid, streptozotocin uses
	Doubtful in accuracy determination	
	Drugs interference	Ethanol reduce u-5HIAA
	Alcohol addiction	SSA is known to decrease u-5HIAA. Assays for diagnostic purposes should be made in patients not on somatostatin analogues therapy
	Possible inhibitory roles of SSA	In the follow up setting urinary samples need to be collected on stable or comparable SSA doses
		Report in patient medical record type of somatostatin analogue and frequency of administration and eventually subcutaneous octreotide performed in the last 24 h before determination

NETs: neuroendocrine tumors; PPIs: proton pump inhibitors; SSA: somatostatin analogues; u-5HIAA: urinary 5-Hydroxy-indolacetic acid

HOW TO AVOID MISINTERPRETATION OF CGA, GASTRIN AND U-5HIAA IN CLINICAL PRACTICE?

There are many conditions that interfere with CgA and u-5HIAA measurements. For CgA there is no universally accepted CgA assay and the different methodologies can lead to confusing results. Many physiological conditions as stress, pregnancy or exercise can increase circulating CgA levels and the same is true for many drugs and non-neuroendocrine diseases. U-5HIAA measurements also have inherent pitfalls since they require a 24 h urine collection and are subject to interference by dietary habits.^[2,5,8,9,13-15,29,31,33] Tables 2 and 3 show the most important pitfalls and bottlenecks and possible remedies in CgA, gastrin and u-5HIAA interpretation and provide suggestions to reduce interference in circulating biomarker measurements for more accurate tumor management.

MONOANALYTE OR MULTIANALYTES?

The identification of effective biomarkers in patients with NETs is a high priority. In a recent Delphi consensus, the panel of neuroendocrine experts agreed that an acceptable standard for a diagnostic biomarker should have a sensitivity of at least 80%, specificity of at least 90%, and positive and negative predictive values of each at 80% or more.^[12] In addition, the biomarker should be able to provide information regarding the proliferative and metastatic capacity of a tumor, the identification of surgical and medical treatment effectiveness and correlate with patient survival. Unfortunately current universal circulating biomarkers are not able to provide this standard and, in particular, the role of CgA in the diagnosis of neuroendocrine tumors is decreasing.

The principal limitation in the measurement of circulating CgA is the absence of a gold standard assay and wide variability of results from different kits and laboratories. In addition, false positive results are reported as a result of other neoplasia (prostate and breast cancer and hepatocellular carcinoma) and common conditions (kidney, liver or heart failure, chronic gastritis, inflammatory bowel disease, PPI use, essential hypertension and physical stress). In addition, the current biomarkers used for gastroenteropancreatic NETs are inadequate for bronchopulmonary NETs and vice versa. For these reasons, a multianalyte approach would likely be more effective compared to a monoanalyte circulating biomarker. To this end, a specific multianalyte assay with algorithmic analyses (MAAA) named NETest has recently been developed. NETest is a PCR-based, 51-transcript signature that is based on correlating and normalizing multiple sets of variables that represent gene clusters specific to NETs and their biological behavior. The use of this blood-based test is proposed to facilitate early detection of disease recurrence and to predict therapeutic efficacy. The diagnostic performance of MAAAs was

better when compared to CgA (93-98% vs. 50-80%)^[53,54] exceeding the performance criteria proposed by an expert panel convened to evaluate NET biomarkers. MAAAs and NETest in particular may improve diagnostic accuracy and offer better interdisciplinary perspective than single analyte testing.

IS THERE A CLINICAL ROLE FOR NOVEL BIOMARKERS?

Recently, several novel biomarkers for NETs have been developed using an integration of genomics and technology platforms. In addition to gene transcript by MAAAs, circulating tumor cell (CTC) and microRNA (miRNA) analyses have been proposed.^[12]

Khan *et al.*^[55] showed that the number of CTC detected in patients with neuroendocrine tumors was comparable to other tumors in which CTC have been shown to have prognostic relevance. In this study, 47% of patients with midgut ($n = 101$) and 24% of patients with pancreatic ($n = 42$) tumors had \geq two CTC detected. Presence of CTC was clearly associated with increasing tumor burden and weakly with tumor grade. In a more recent, large prospective study, the same group demonstrated that changes in CTC were associated with response to treatment and overall survival in metastatic neuroendocrine tumors, suggesting CTC may be useful as a surrogate marker to direct clinical decision making.^[56] Although there is an increasing interest in CTC as a biomarker, recent consensus concluded that CTC analyses have several technical limitations and need further validation before being adopted into routine clinical practice.^[12]

There is also increasing interest in miRNAs as clinical biomarkers of tumorigenesis, treatment response and outcomes, but to date clinical data are scarce and clinical application challenging. Similarly, there are several novel monoanalyte assays (i.e. connective tissue growth factor for carcinoid heart disease (CCN2) or paraneoplastic Ma antigen 2 (PNMA2) for small intestinal neuroendocrine tumors, but these analyses are not available in clinical practice.^[12] Further, panelists of the recent Delphi consensus gave the strongest support to the use of emerging biomarkers in multianalyte technology based on genomics.^[12]

CONCLUSION

To date, the identification of sensitive, specific and reproducible NET circulating biomarkers for the prediction, diagnosis, prognosis and classification of NETs and to evaluate changes during therapy has been limited^[12] and remains an unfulfilled unmet medical need as defined by the 2007 National Cancer Institute NET meeting.^[57] There are no specific circulating monoanalyte biomarkers for neuroendocrine tumors that fulfill the NIH recommended criteria and the search continues for

markers with diagnostic and prognostic capabilities. Since Feyrter have discovered the neuroendocrine equivalent of Pandora's Box, a unique relationship between these various neuroendocrine peptides and different tumors has not been found yet.^[7] We are hopeful that in the era of Precision Medicine, specific circulating markers or a multianalyte panel for specific tumor types can be developed for NETs giving more reliable diagnostic and prognostic information. The road is long and new, robust prospective studies in different neuroendocrine tumors settings are required before new accurate biomarkers are validated and implemented into routine clinical practice.

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Patient consent

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Ethics approval

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Function of cancer cell-derived extracellular matrix in tumor progression

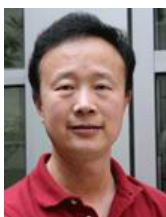
Gao-Feng Xiong¹, Ren Xu^{1,2}

¹Markey Cancer Center, Lexington, KY 40536, USA.

²Department of Pharmacology and Nutritional Sciences, University of Kentucky, Lexington, KY 40536, USA.

Correspondence to: Dr. Ren Xu, Department of Pharmacology and Nutritional Sciences, University of Kentucky, BBSRB, 741 S. Limestone, Lexington, KY 40536, USA. E-mail: ren.xu2010@uky.edu

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Dr. Ren Xu is an Associated Professor at the Markey Cancer Center, University of Kentucky. Research in his group focuses on the biological function and regulation of ECM microenvironment in normal tissue and cancer development. His recent findings reveal the crucial function of cancer-cell derived-ECM in breast cancer progression.

ABSTRACT

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Extracellular matrix (ECM) is an essential component of the tumor microenvironment. Cancer development and progression are associated with increased ECM deposition and crosslink. The chemical and physical signals elicited from ECM are necessary for cancer cell proliferation and invasion. It is well recognized that stromal cells are a major source of ECM proteins. However, recent studies showed that cancer cells are also an active and important component in ECM remodeling. Cancer cells deposit a significant amount of collagen, fibronectin, and tenascin C (TNC). Recent studies demonstrate that these cancer cell-derived ECM proteins enhance cancer cell survival and promote cancer cell colonization at distant sites. ECM-related enzymes and chaperone proteins, such as prolyl-4-hydroxylase, lysyl-hydroxylase, lysyl oxidase, and heat shock protein 47, are also highly expressed in cancer cells. Inhibition of these enzymes significantly reduces cancer growth, invasion, and metastasis. These factors suggest that the cancer cell-derived ECM is crucial for cancer progression and metastasis. Therefore, targeting these ECM proteins and ECM-related enzymes is a potential strategy for cancer treatment.



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INTRODUCTION

Cancer development and progression require extensive reorganization of extracellular matrix.^[1,2] Extracellular matrix (ECM) is a complex mixture of structural proteins, glycoproteins, and proteoglycans, which provide not only essential physical scaffolds to maintain tissue structure but also various biochemical signals to modulate cellular function.^[3-5] Altering the fine balance of ECM signal is sufficient in the long run to induce breast cancer development and progression. Increased deposition of collagen and other ECM molecules enhances the cancer tissue stiffness.^[6-9]

Collagens are the most abundant protein in the ECM.^[10,11] Collagen fibril has critical function for tumor cell growth, migration and metastasis.^[12-14] Other ECM components, such as hyaluronan, TNC, and periostin (POSTN), are also highly expressed in metastatic tumor and play important roles in tumor metastasis niche.^[8,15-18]

Fibroblasts are considered the major source for ECM in both normal and malignant tissue.^[19] Surprisingly, recent studies showed that cancer cells also produce a significant quantity of ECM protein during cancer progression.^[20,21] Dr. Hynes's laboratory, utilizing an elegant proteomic experiment, demonstrated that ECM molecules in cancer tissue are deposited by both cancer cells and stromal cells.^[20,21] ECM proteins, such as laminin 5, hyaluronan, and TNC, are highly expressed in invasive cancer cells.^[22-27] Gene expression analysis has identified that ECM protein genes are upregulated in drug-resistant cancer cells.^[28] Collagen modification enzymes, including prolyl-4-hydroxylase (P4H), lysyl-hydroxylase (PLOD), and lysyl oxidase (LOX), as well as molecular chaperone heat shock protein 47 (HSP47), are highly expressed in cancer cells and are associated with tumor metastasis.^[29-33]

This review summarizes recent findings about ECM microenvironment in solid tumor. The primary focus is on the role of cancer cells in ECM synthesis and the function of cancer cell-derived ECM in tumor progression.

THE EXTRACELLULAR MATRIX

ECM can be classified into two groups: the interstitial matrix and the basement membrane.^[34] Basement membranes are thin layers of ECM that form the supporting structure under epithelial and endothelial cells.^[35] Basement membrane has a distinctive composition containing type IV collagen, laminins, entactins, and proteoglycans.^[7,36] The interstitial matrix,

which is primarily produced by stromal cells, fills in the interstitial space between cells. The interstitial matrix is rich in types I, III, V, VI, VII, and XII collagens, as well as proteoglycans and various glycoproteins such as TNC and fibronectin.^[37]

Collagen is the most abundant protein *in vivo*. Forty-four collagen genes have been identified in the human genome; they generate at least 28 different types of collagen. From precursor procollagen to final collagen fibril, collagen synthesis process involves several important modification enzymes.^[10,38] Proline and lysine hydroxylation are well characterized modifications on procollagen, which are catalyzed by two different enzymes: P4H and PLOD. Collagen P4H catalyzes the formation of 4-hydroxyproline, which is essential to the proper folding of newly synthesized procollagen chains.^[39,40] PLOD catalyzes the hydroxylation of lysyl residues in collagen-like peptides, which is critical for the formation of intermolecular crosslinks.^[41,42] LOX is enzyme-catalyzing formation of aldehydes from lysine residues in collagen after collagen secretion, which is required for collagen fibril formation.^[43,44] HSP47 is a molecular chaperone that promotes maturation of collagen molecules by inhibiting the aggregation of collagen in endoplasmic reticulum (ER).^[45-47] The expression of collagen-modification enzymes and molecular chaperone is often associated with increased collagen deposition in cancer tissue.^[30-33,48-51] Enhanced enzyme activities are often associated with increased collagen deposition in cancer tissue.

ECM PLAYS IMPORTANT ROLES IN TUMOR PROGRESSION

ECM is a major component of tumor microenvironment and plays critical roles in cancer development and progression. Increased ECM proteins deposition and crosslink provide necessary biochemical and biophysical cues to promote cancer cell proliferation, migration, and invasion.^[12,52-54] Laminin-322 is specifically localized in the dense fibrotic zone around invasive ductal carcinoma, providing a specialized microenvironment for guiding tumor invasion.^[52] Gamma 2 chain of laminin 5 (laminin 5 $\gamma 2$) is highly expressed in invasive mammary, colon, melanoma, and sarcoma cancer cells. Laminin 5 plays a role in establishing focal adhesions of cancer cells and contributes to cancer dissemination.^[24-26]

ECM molecules, such as POSTN, fibronectin, and hyaluronan, are important components of the metastatic niche.^[7] POSTN is a secreted extracellular matrix protein originally identified from mesenchymal cells.^[8,16,17] Deletion of POSTN has little effect on normal

Table 1: Stroma cells and cancer cells-derived ECM proteins and ECM regulators

	Stroma cells	References	Cancer cells	References
Collagens	Collagen I	[20,21,66]	Collagen I	[20,21,53,65]
	Collagen II	[20,21]	Collagen II	[20,21]
	Collagen III	[20,21,66,67]	Collagen III	[20,21,53]
	Collagen IV	[20,21]	Collagen IV	[20,21,28,65,68]
	Collagen V	[20,21,53,66,67]	Collagen V	[20,21,53,63]
	Collagen VI	[20,21,66]	Collagen VI	[20,21,28,53,68]
	Collagen VII	[21]	Collagen VII	[20,21,68]
	Collagen X	[20,21,66]	Collagen VIII	[20,53,63]
	Collagen XI	[20,21,66]	Collagen IX	[20,68]
	Collagen XII	[20]	Collagen X	[20,21,53,63]
	Collagen XIV	[20,21,66]	Collagen XI	[20,21,53,63,68]
	Collagen XV	[20,21]	Collagen XII	[20,21,31,63,65]
	Collagen XVI	[20]	Collagen XV	[20,21,65,68]
	Collagen XVIII	[20,21]	Collagen XVI	[20,21,28,65]
	Collagen XVIII	[21]	Collagen XVIII	[20,21,65]
	Collagen XXIV	[20,21]	Collagen XIX	[20,21]
	Collagen XXVIII	[20]	Collagen XXII	[20,21,63]
			Collagen XXIV	[20,21,68]
Other ECM glycoproteins	Fibrinogen	[20,21]	Laminin α 4	[20,21,28,65]
	Dermatopontin	[20,21]	Laminin β 1	[20,21,28,65,68]
	Elastin	[20,21]	Laminin β 2	[20,68]
	Fibronectin1	[20,21,66]	Laminin γ 2	[20,21,66,68]
	Laminin α 2	[20,67]	Fibronectin1	[20,21,28,65,68]
	Laminin β 2	[20,21]	Elastin	[20,21]
	Nidogen-1	[20,67]	LTBP1	[20,21,68]
	Nidogen-2	[21,66]	LTBP4	[20,21]
	ECM 1	[21]	Nidogen-1	[20,21]
	Fibulin 2	[20,21]	Nidogen-2	[20,21]
	LTBP2	[20,21]	ECM 1	[20,21,28,68]
	Tenascin N	[20]	Peroxidasin	[20,21]
	EMILIN2	[20,21,66]	TINAGL1	[20,21]
	TNC	[20,66,67]	TNC	[20,21,66]
	POSTN	[21,66]	Hyaluronan	[20]
	Hyaluronan	[21]	Thrombospondin-1	[20,21]
	Thrombospondin-1	[20]	SPARC	[20,53,65,68]
	SPARC	[21,66,68]		
	Vitronectin	[20,21]		
Proteoglycan	Asporin	[20,21]	Biglycan	[20,21,28]
	Biglycan	[20,66]	HAPLN1	[20,65]
	Decorin	[20,21,67]	Decorin	[20,21,53,65,68]

Continued...

	Stroma cells	References	Cancer cells	References
ECM regulators	Cathepsin B	[20,21]	Cathepsin B	[20]
	ITIH1	[20,21]	Osteonectin	[20,68]
	ITIH2	[20,21]	P4HA1	[20,21,31,32]
	Plasminogen	[20,21]	PLOD1	[20,21]
	P4HA1	[50]	PLOD2	[20,21,30]
	P4HA2	[50]	PLOD3	[20,21]
	PLOD2	[50]	LOX	[20,21,65]
	PLOD3	[20,21]	LOXL2	[20,21]
	HSP50	[20,21]	LOXL4	[20]
	LOXL1	[21]	HSP50	[20,21,33]
Secret factors	TGF β 1	[20,21,66]	S100-A13	[20]
	S100-A9	[21]	S100-A4	[20,21]
			S100-A6	[20,21]
			TGF β 1	[20,21,65]

ECM1: extracellular matrix protein 1; EMILIN2: elastin microfibril interfacier 2; LTBP1: latent transforming growth factor beta binding protein 1; LTBP2: latent transforming growth factor beta binding protein 2; LTBP4: latent transforming growth factor beta binding protein 4; ITIH1: inter-alpha-trypsin inhibitor heavy chain H1; TINAGL1: tubulointerstitial nephritis antigen-like 1; HAPLN1: hyaluronan and proteoglycan link protein 1

tissue development and primary tumor growth, but it significantly suppresses breast cancer metastasis.^[8,17] POSTN promotes cancer stem cell maintenance and lung metastasis by enhancing the WNT signaling pathway.^[8,17] Fibronectin, a marker of epithelial-mesenchymal transition, enhances cancer metastasis through Src kinase and extracellular signal-regulated kinase/mitogen-activated protein kinase pathway.^[55] Hyaluronan expression is upregulated in breast cancer, lung cancer, pancreatic cancer, melanoma cancer, and myeloma cancer.^[22,23,27] Upregulation of hyaluronan is also associated with tumor progression and poor prognosis.^[15,56,57] Hyaluronan receptor CD44 promotes survival of disseminated cancer cells during metastasis.^[58] TNC is an oligomeric glycoprotein composed of individual polypeptides with molecular weights ranging from 180 kDa to 300 kDa. Expression of TNC in breast tumor is associated with lung metastasis.^[8,16,18] Recent studies reveal that TNC is a critical component of metastatic niche and supports survival of disseminated cancer cells at secondary organs.^[8,16,18]

Collagen is the major structural ECM protein in tumor tissue. It has been shown that women with dense breasts have a four- to six-fold increased risk of developing breast cancer, and the dense breast correlates with increased collagen deposition and crosslink. In addition, the crosslinked and orientated collagen in cancer tissue is a reliable marker associated with poor survival, regardless of tumor grade and size, tumor subtype, ER or PR status, and node status.^[12,59]

The abnormal deposition of collagen in tumor stroma promotes cancer progression. Increased collagen VI deposition stimulates cancer cell proliferation.^[59-61] Col5A2 and Col11A1 are highly expressed in invasive ductal carcinoma compared to ductal carcinoma in situ. Both of them are involved in triggering cancer cells to disseminate.^[62,63]

Collagen production and deposition is regulated by a variety of enzymes, including P4Hs, PLODs, and LOXs. Collagen deposition is regulated by hypoxia in tumor tissue.^[47,48,61] Collagen modification enzymes, P4Hs, PLOD, and LOX, are activated by HIF-1 α in cancer cells.^[27,28,40,48] Expression of collagen P4H is significantly upregulated in breast cancer. Knockdown of P4HA inhibits mammary tumor growth and metastasis to lungs, and decreased P4HA activity depresses cancer cell alignment along collagen fibers.^[31,32,50] PLOD2 expression is also associated with increased risk of mortality in breast cancer patient. PLOD2 is critical for breast cancer cell metastasis to lymph nodes and lungs because it increases fibril collagen formation and increases tumor stiffness.^[30] In sarcoma cancer, inhibition of PLOD enzymatic activity suppresses metastases.^[64] Secretion of LOX by metastatic breast cancer cells is upregulated in metastasis niche. Increased activity of LOX recruits bone marrow-derived cells (BMDs) to metastasis niche. BMDs are important in creating a microenvironment for metastatic cancer-cell invasion and growth.^[43] Increased LOX expression results in increased ECM stiffening, which is essential for cancer cell expansion.^[7] Inhibition

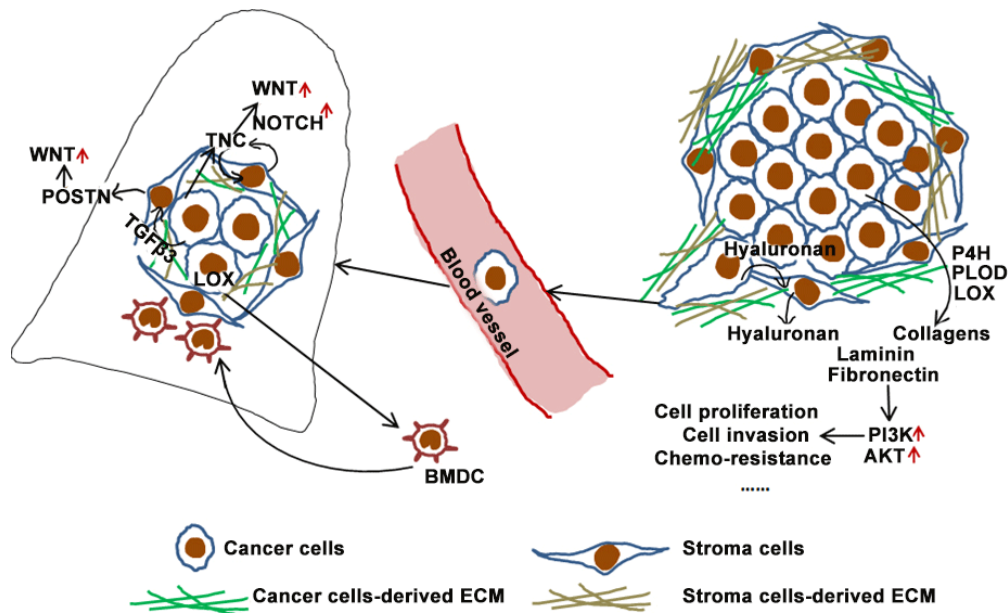


Figure 1: Stroma cell-derived extracellular matrix (ECM) and cancer cell-derived ECM collectively support cancer cell proliferation, invasion, and metastasis. ECM: extracellular matrix; BMDC: bone marrow-derived cells; PLOD: lysyl-hydroxylase; LOX: lysyl oxidase

LOX activation reduces collagen fibril formation and ECM stiffness, which depresses focal adhesions and PI3K activity, and consequently suppresses cancer cell invasion.^[54] These results indicate that collagen modification enzymes P4Hs, PLODs, and LOXs play critical roles in cancer cell metastasis.

CANCER CELLS ARE CRITICAL SOURCES OF TUMOR ECM

The cellular components of tumor stroma include fibroblasts, endothelial cells, fat cells, and immune cells. It has been shown that cancer-associated fibroblasts produce and regulate the ECM remodeling in cancer tissue, and the roles of cancer cells in ECM deposition have not been appreciated until recently. Dr. Hynes's laboratory investigated matrisome (ECM and ECM-associated proteins) in colon tumor tissues, lung tumor tissues, and human breast cancer tissue.^[20,21] They found that ECM components in tumor matrix are derived from cancer cells and stromal cells, and many of them are only expressed by cancer cells, including Col19A1, Col22A1, Col7A1, LAMA4, LAMB1, LTBP1, LTBP3, LTBP4, TINAGL1, and ECM regulators galectin 1 (LGALS1) and PLOD1.^[20,21] Gene expression analysis of drug-resistant breast cancer cells has found that 25 ECM components' genes (including collagen, fibronectin, syndecan, and laminin) and integrin ligands are upregulated in drug-resistant breast cancer cells.^[28] Gene expression analysis of drug-resistant ovarian cancer cells also discovered that molecules in ECM networks, including COL3A1, COL5A2, COL15A1, and LOX, among others, are very

significantly upregulated.^[65] Gene expression profile studies from other labs also reveal that expression of genes involved in synthesis and organization of ECM are upregulated in the epithelium of invasive cancer cells.^[53,63,66-68]

LAMC2 (gamma 2 chain gene of laminin 5) is highly expressed in invasive cancer cells in mammary, colon, melanoma and sarcoma tumors.^[24-26,69] Hyaluronan synthesis is increased in a variety types of cancer cells, including breast tumor, melanoma tumor, and myeloma tumor.^[22,23,27] Thrombospondin-1 is expressed in the stroma and cancer cells.^[70] TNC, a key metastatic niche molecule required for the metastasis initiation, is also expressed in breast tumor cells and stroma cells.^[8,16,18] Collagens are mainly synthesized by cancer-associated fibroblasts in breast cancer, but cancer cells are also an important source of the collagen.^[63] In addition, the expression of collagen synthesis regulating enzymes P4H and PLOD is induced by the HIF-1 pathway in cancer cells.^[30,31,51,64] We have summarized ECM proteins and ECM-related enzymes derived from the stroma cells and cancer cells in Table 1. This evidence clearly shows that cancer cells are a major source of tumor ECM.

CANCER CELL-DERIVED ECM IN CANCER PROGRESSION AND METASTASIS

ECM deposited by cancer cells is crucial for cancer progression and metastasis. It has been shown that inhibition of LOX expression in cancer cell represses cell adhesion, migration, and invasion.^[29,71] Hyaluronan

deposited by cancer cells promotes cell proliferation, migration, invasion, metastasis, multidrug resistance, and tumor-associated angiogenesis.^[15,56,57] TNC that is derived from disseminated tumor cells promotes lung metastasis by enhancing NOTCH and WNT signaling pathways [Figure 1].^[8,16,18] In addition, cancer cell-derived ECM proteins (fibronectin, collagen, and laminin) protect cancer cells from chemotherapy-induced apoptosis via activation of the PI3k/AKT pathway [Figure 1].^[72,73]

Cancer cell-derived ECM proteins mediate the cancer cell-stromal cell crosstalk. Hyaluronan production by stroma fibroblasts is stimulated by factors secreted by cancer cells.^[74,75] Metastatic niche molecule POSTN is secreted by stroma fibroblasts of breast tumor under stimulation from the tumor cells that are produced TGF- β 3 [Figure 1].^[8,16-18] Cancer cells also remotely recruit stromal cells to create a premetastatic niche before metastasis. Cancer cells-derived TNC initiates cancer cell metastasis, and then it stimulates stroma cell-derived TNC synthesis. Ablation of TNC expression in cancer cells at an early time in the metastatic process inhibits the outgrowth of lung metastases. Interestingly, inhibition TNC expression in cancer cells at a late stage of metastasis does not affect micrometastases expanding to macrometastases, because metastatic cancer cells have already induced TNC expression in stromal cells to promote tumor growth.^[8,16,18] These results indicate that cancer cell-derived ECM molecules are critical regulators of the initiation of metastasis outgrowth through activating the stromal cells in the secondary organs [Figure 1].

CONCLUSION

In summary, tumor cells play critical roles in ECM deposition and remodeling during cancer development and progression. Accumulated evidence demonstrates that ECM molecules deposited by cancer cells promote cancer progression by enhancing cell survival and proliferation. However, it largely remains to be determined how cancer cell-derived ECM is regulated and how those ECM proteins function in tumor microenvironment remodeling. Answering those questions is critical for developing potential cancer treatment strategies by targeting the cancer cell-derived ECM and ECM-related enzymes.

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Conflicts of interest

There are no conflicts of interest.

Patient consent

No patient involved.

Ethics approval

This article does not contain any studies with human participants or animals.

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Review

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The features of peritoneal metastases from gastric cancer

Gianni Mura¹, Beatrice Verdelli²

¹Department of Surgery, Valdarno Hospital, 52100 Arezzo, Italy.

²Department of Radiology, Valdarno Hospital, 52100 Arezzo, Italy.

Correspondence to: Dr. Gianni Mura, Department of Surgery, Valdarno Hospital, Via Cimabue n.19, 52100 Arezzo, Italy.
E-mail: gianmura@gmail.com

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Dr. Gianni Mura is a Surgical Oncologist, General and Emergency Surgeon, and Gastro-intestinal Endoscopist. Since 1996 he focused his interest on surgical oncology, with experience in: loco-regional therapies for advanced-stage abdominal cancers as the Early Post-operative Intraperitoneal Chemotherapy (EPIC) and the Hypoxic Chemo-perfusion (Stop-Flow); Cytoreductive Surgery and Hyperthermic Intraperitoneal Chemotherapy (HIPEC) for peritoneal carcinomatosis; Sentinel Node Biopsy for early gastric cancer; extended nodal dissection and neo-adjuvant chemotherapy for advanced gastric cancer; surgery and adjuvant therapies for metastatic melanoma; clinical research.

ABSTRACT

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Peritoneal Carcinomatosis (PC) from metastasization of Gastric Cancer (GC), either present at first diagnosis of GC or as recurrence, is considered a fatal disease with no hope of definitive cure. Although newer agents like S1 and docetaxel have shown some promise, the median overall survival with the current first line chemotherapy is only 8 to 14 months, and is not greatly improved by adding targeted therapy. A multi-modal approach with cytoreductive surgery (CRS) associated with hyperthermic intraperitoneal chemotherapy (HIPEC) has been developed along the last two decades in order to tackle this problem. It's an aggressive, combined treatment still under investigation. Studies coming from Europe and Far East reported long-term survival with 5-year survival rates up to nearly 25% in case of complete cytoreduction. Prophylactic/adjuvant setting is the most evidence-based indication for HIPEC in advanced-stage GC patients without PC, in order to prevent peritoneal recurrence and to improve overall survival. The rationale for immuno treatment in patients with gastric PC is strong. A randomized phase II study, combining complete CRS with intraperitoneal catumaxomab is on-going. The detection of free peritoneal cancer cells is the more realistic and practical way for the identification of patients at risk of carcinomatosis after surgery. The routine use of techniques of molecular detection in peritoneal washing appears to be the more sensitive method. Such patients are potential candidate for multimodal and locoregional treatments in order to prevent the peritoneal recurrence.



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INTRODUCTION

The regional metastatic spread of gastric cancer (GC) usually results in peritoneal carcinomatosis (PC). When GC patients are explored for potentially curative resection, 10-20% of them are found to have peritoneal metastases.^[1] Furthermore, in case of cancer infiltration of the serosal layer of the stomach, PC is present at first diagnosis of the cancer in 15-50% of cases and peritoneal recurrence develops in 35-60% of such patients after radical resection. PC is the only site of metastasis in 40-60% of patients.^[2,3] Therefore, peritoneal metastases alone usually result in death for 20-40% of patients with GC.^[4]

Conventional surgery is not adequate for PC; current treatments are systemic chemotherapy and palliative therapy, with no hope of cure. In selected cases and in experienced centers, the association of more aggressive surgery with multimodal loco-regional treatments has shown to achieve prolonged survival and reduced peritoneal recurrences.^[5-7]

PHYSIO-PATHOLOGICAL FEATURES OF PC

The molecular mechanisms by which GC undergoes PC are not completely clear. Chemokines (CXC) are surely involved. They are small secretory proteins controlling migration and activation of leukocytes and other types of cell through interactions with a group of seven trans-membrane G protein-coupled receptors. CXC may also promote growth/survival and metastasis of several malignancies.^[8-11] There is evidence that the axis between CXCL12 (highly expressed in peritoneum than in the liver or lymphnodes) and the receptor CXCR4 plays a role in the development of PC from GC.^[12,13] The CXCR4 antagonist AMD3100 prevents experimental PC by NUGC4 cells in nude mice. In human, the CXCR4 expression in primary tumors of patients with advanced GC significantly correlates with the occurrence of PC. Furthermore, CXCR4-expressing GC cells are preferentially attracted to the peritoneum cavity where its ligand CXCL12 is abundantly produced. The CXCL12/CXCR4 axis is influenced by interaction with the vascular endothelial growth factor (VEGF).^[14] VEGF is markedly elevated in malignant ascites and is one of the essential elements in the development of PC.^[12] Such results suggest that the expression of CXCR4 in biopsy specimen from primary gastric tumors may be useful for preoperative evaluation of risks for the occurrence of PC. Evaluation of CXCL12 levels in intraoperative washing of abdominal cavity in patients with advanced GC has been proposed as a predictive molecular marker for the risk of PC.

Peritoneal dissemination of free cancer cells happens through exfoliation from the tumor and leads to direct invasion of the mesothelium. Surgery itself may produce intra-operative dissemination of cancer cells by severed lymphatics, intraperitoneal blood loss, trauma at narrow margins of resection etc.. According to the “tumor cell entrapment hypothesis” proposed by Sugarbaker PH, immediately after a surgical procedure the endoperitoneal free cancer cells which are spontaneously exfoliated or iatrogenically disseminated adhere to the damaged surface created by surgery; they are trapped by fibrin and stimulated by growth factors of the wound healing and inflammation processes, with tumor cell implant on the visceral and parietal peritoneum. The nodule of carcinomatosis in this way becomes a hypoxic, and relatively immune to systemic chemotherapy, environment.^[15]

Tumor cells can also diffuse through the “milky-spots”, little cribriform “stomata” present on the peritoneal surface, communicating between peritoneal cavity and lymphatic vessels, with the function of re-absorption of abdominal fluids. Milky spots are mainly composed of macrophages and B1 cells; there are compelling data to consider the milky spots as unique secondary lymphoid organs.^[16] The peritoneal free cancer cells are trapped during their passage through the spots and attacked by inflammatory and immuno-response cells, forming a hypoxic nodule.^[17] The milky spots are mainly localized in the omentum and in the sub-diaphragmatic areas, which are in fact the preferential sites of distribution of peritoneal metastases.^[18]

THE TREATMENT OF PC

The PC arising from GC has ever been considered as a final stage of the disease, with no chances of cure but palliation. The prognosis of PC for GC is very poor, worse than that of other metastatic sites,^[19,20] with a median survival after diagnosis of only 3-7 months and 5-year survival of 0%.^[1,3] The traditional approach by surgeons is just palliation, whenever possible.

Systemic chemotherapy

In last 15 years systemic chemotherapy (adjuvant or neoadjuvant)^[21-26] and adjuvant chemo-radiation^[27,28] do not have significantly lowered the rate of distant metastases, including peritoneal recurrence. In metastatic GC, systemic chemotherapy improves median survival to only 8-14 months,^[29-31] without great improving by adding targeted therapy.^[32,33] GC patients with PC have a significantly reduced rate of tumor response to chemotherapy with reported rates of response of 14-25%.^[34,35] The poor response of PC to systemic chemotherapy is due to the presence

of the “plasma-peritoneal barrier” which isolates the peritoneal cavity from the effects of intravenous chemotherapy.^[36] Although newer agents like S1 – not available for Western Countries patients – and docetaxel have been reported to have better results against peritoneal metastases, yet the median survival even with these drugs is only 18 months.^[37,38]

Cytoreductive surgery and hyperthermic intraperitoneal chemotherapy

A poor response to systemic therapy provides the rationale for a local-regional strategy for treatment. The concept is that carcinomatosis is not to be considered as systemic but compartment disease, which can be attacked by cytoreductive surgery (CRS) associated with loco-regional treatments such as the hyperthermic intraperitoneal chemotherapy (HIPEC).^[5-7] During CRS are used well-codified peritonectomy procedures with the removal of all visible cancer with the affected peritoneum through “peritoneal stripping”, always attempting to achieve a complete cytoreduction [Figure 1].^[39] The aim of CRS is the complete macroscopic cytoreduction as precondition for HIPEC. The residual disease is classified intra-operatively using the completeness of cytoreduction (CC) Score. The efficacy of intra-peritoneal chemotherapy reaches its highest degree in absence of visible residual disease (CC-0) or in the presence of neoplastic residuals that are less or equal to 2.5 mm (CC-1).^[40,39] The main theoretical advantage of intraperitoneal chemotherapy is that it allows the direct application of high local concentration of potentially effective drugs with minimal systemic exposure and toxicity.^[2,5,7]

The neoplastic cells are more sensitive to the heat than the normal cells. Hyperthermia has a direct cytotoxic effect and an indirect effect by enhancing the action of several anti-neoplastic drugs. Experimental studies demonstrated that 42-43°C hyperthermia may have an important therapeutic effect on tumor tissue when applied alone; moreover hyperthermia synergically enhances the chemosensitivity of neoplastic cells to various antimitotic agents and allows deeper penetration of drugs into tumor tissue.^[41,42] During procedure of HIPEC, the chemotherapeutic agents are added into the extra-corporeal circuit as soon as the abdominal temperature reaches 41.5-42.5°C [Figure 2].^[40]

Postoperative mortality after CRS and HIPEC is 2-4%, comparable to that following major gastrointestinal surgery. Morbidity is relatively high (25-41%) and seems to be related to the extension of CRS rather than to the HIPEC itself.^[43,6,7] The anastomoses of total or subtotal gastrectomy in combination with CRS and

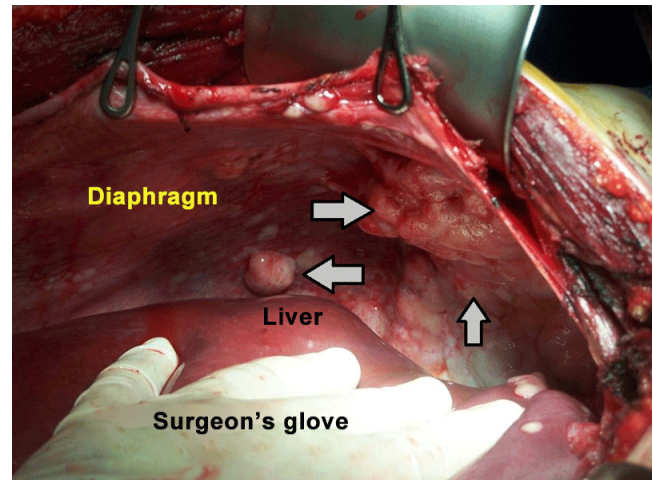


Figure 1: A phase of peritonectomy of diaphragmatic peritoneum; the arrows point to some nodules of carcinomatosis



Figure 2: HIPEC procedures for gastric carcinomatosis. Two different models of surgical auto-retractors and two different HIPEC-dedicated devices are shown. HIPEC: hyperthermic intraoperative intraperitoneal chemotherapy

HIPEC are relatively safe.^[44,45]

Currently, CRS with HIPEC is increasingly being used as a curative treatment of pseudomyxoma peritonei, peritoneal mesothelioma and selected patients with PC from colo-rectal or ovarian cancer.^[46,5,7] The CRS + HIPEC in PC arising from GC is a treatment still investigational. Several studies coming from Europe and Far East show the possibility of long-term survival with up to nearly 25% 5-year survival rates in case of complete cytoreduction [Table 1]. Glehen *et al.*^[54] published in 2010 the results of a retrospective French study of 1,290 patients with PC treated with HIPEC; 159 of them had PC of gastric origin. In patients with a complete cytoreduction the 1-, 3-, and 5-year survival rates were 61%, 30%, and 23%, respectively. Completeness of cytoreduction was the principal independent prognostic factor at multivariate analysis.^[54,58] In a systematic review of

Table 1: Survival analysis in GC patients with PC treated with CRS and HIPEC

Authors	Patients No.	Agent used in HIPEC	Mortality/morbidity (%)	Survival
Fujimoto <i>et al.</i> ^[47]	15	MMC	–	7.2 ± 4.6 mo
Yonemura <i>et al.</i> ^[48]	41	MMC + CDDP	0-29.3	3-year 28.5%
Fujimoto <i>et al.</i> ^[49]	48	MMC	–	5-year 31%, 8-year 25.4%
Hirose ^[50]	17	Etoposide	5.8-35.2	1-year survival: HIPEC vs. control: 44.4% vs. 15.8%, <i>P</i> = 0.04
Glehen <i>et al.</i> ^[44]	49	MMC	4-27	5-year survival (overall: 16%, CC0/1: 29.4%)
Hall <i>et al.</i> ^[51]	34	MMC	0-35	2-year 45%, (CC0/1) 8% (CC2/3)
Yonemura <i>et al.</i> ^[52]	107	MMC + CDDP	2.8-21.5	5-year 6.7%
Scaringi <i>et al.</i> ^[53]	37 (26 with PC)	CDDP	3.8-27	median survival: CCR0 vs. CCR2- 15 mo vs. 3.9 mo, <i>P</i> = 0.007
Glehen <i>et al.</i> ^[54]	139	MMC ± CDDP or LOHP ± irinotecan	6.5-27.8	5-year 13%, CC0/1 23%
Yang <i>et al.</i> ^[55]	RCT: 34 vs. 34 no HIPEC	MMC + CDDP	0-14.7	3-year 5.9%, CC0/1 23%
Magge <i>et al.</i> ^[56]	23	MMC + CDDP	4.3-52.2	1-year 50%, 3-year 18%
Rudloff ^[57] GYMSSA trial	RCT: 9 CRS+HIPEC+cht vs. 7 cht	Oxaliplatin	-	Median OS 11.3 months in HIPEC arm and 4.3 months in the cht arm. No patient in the cht arm lived beyond 11 months

GC: Gastric cancer; CRS: cytoreductive surgery; HIPEC: hyperthermic intraoperative intraperitoneal chemotherapy; PC: peritoneal carcinomatosis; RCT: randomized controlled trial; MMC: mitomycin C; CDDP: cisplatin

10 published studies including 441 patients who underwent CRS and HIPEC in GC carcinomatosis, Gill *et al.*^[43] reported median overall survival of 7.9 months after HIPEC, increasing to 15 months in case of complete cytoreduction. The 5-year survival of all patients was 13%. Yang *et al.*^[55] showed in a phase III randomized clinical trial the importance of connecting CRS with HIPEC, in the treatment of PC of gastric cancer origin. The CRS-HIPEC association vs. CRS alone significantly increased median survival: 11 vs. 6.5 months. The prospective randomized clinical trial GYMSSA compared patients treated with CRS-HIPEC and systemic chemotherapy vs. systemic chemotherapy treatment alone, demonstrating a benefit in terms of survival. With the limitation of a small number of patients, it showed a longer median overall survival (11.3 vs. 4.3 months) for CRS-HIPEC treatment trial arm. No patient in the systemic-chemotherapy-alone arm lived beyond 12 months.^[57]

Those are unexpected outcomes until some years ago indeed. Anyway, the results are worse than in case of other types of carcinomatosis treated with CRS and HIPEC.^[5,7] The gastric is a more aggressive carcinomatosis, and complete cytoreduction is more difficult to achieve. The correct indication is probably

the limited and resectable PC, where CC-0 is achievable.^[54]

HIPEC in adjuvant setting

Perhaps the most promising indication for HIPEC is its use in case of advanced GC without carcinomatosis in patients at risk of peritoneal recurrence. It's the adjuvant (or prophylactic) setting.

PC develops in 60% of patients with serosa-invading tumors after curative resection.^[59,4] In late '90s some prospective RCTs evaluated adjuvant HIPEC after potentially curative GC resection. In Fujimoto's 141 patients, HIPEC significantly reduced the incidence of peritoneal recurrence (*P* < 0.001) and improved the survival rate (*P* = 0.03).^[60] Yonemura randomized 139 patients in three arms, surgery alone, surgery plus HIPEC, and intraperitoneal chemotherapy without hyperthermia. The 5-year survival was 61% in the HIPEC group compared to 43% and 42% in the other two groups.^[61] Two meta-analysis of RCTs (including 1648 and 1062 patients, respectively) on HIPEC as adjuvant therapy in GC have been published.^[62,63] The patients, presenting GC with macroscopic serosal invasion but without distant metastases or PC, were randomly assigned to receive surgery combined

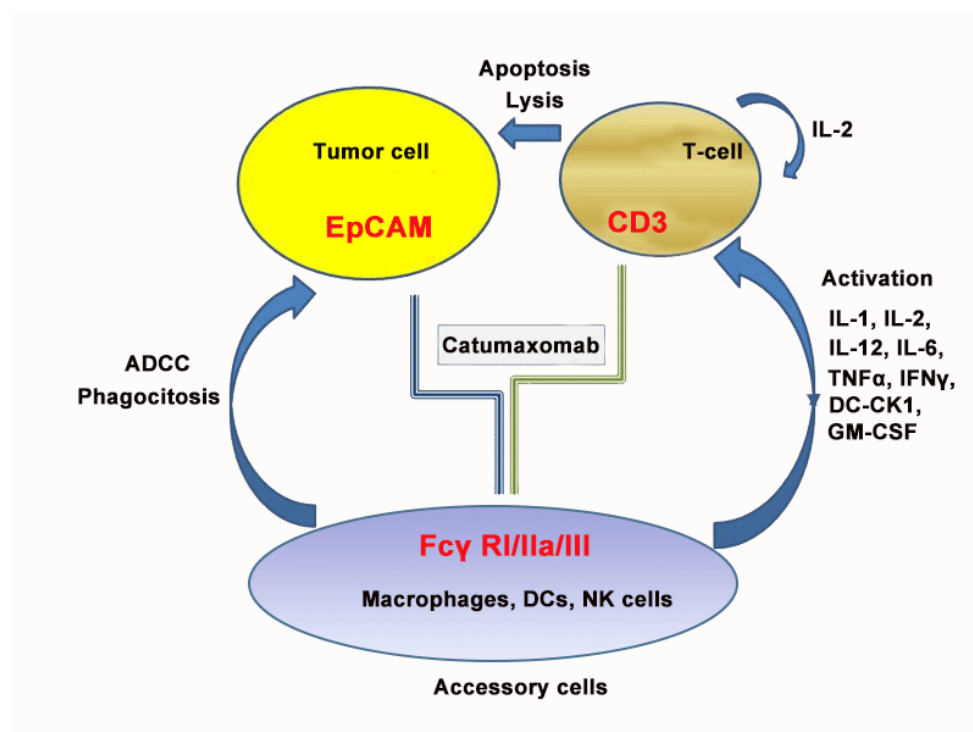


Figure 3: Catumaxomab, is a chimeric antibody, consisting in a mouse-derived anti-EpCAM Fab (fragment antigen-binding) region and a rat-derived anti CD3 Fab

with intraperitoneal chemotherapy or surgery without intraperitoneal chemotherapy. In both analyses a highly significant improvement in survival and in peritoneal recurrence rate was demonstrated for the HIPEC group compared to the control group. Recently, a meta-analysis on effects of intraperitoneal chemotherapy in advanced GC was reported by Coccolini *et al.*^[64] They imported the data from 20 prospective studies involving 2,145 patients. Overall survival was increased when intraperitoneal chemotherapy was added to surgery; intraperitoneal chemotherapy was found to reduce the incidence of peritoneal recurrence and distant metastases. In the German S3-guidelines “Diagnosis and treatment of esophagogastric cancer” HIPEC as adjuvant treatment is reported with Level of Evidence I, grade A.^[65]

Most of data anyway come from studies that have been conducted in Far-Eastern countries, with scarce contribute from the western world. Two RCTs about adjuvant HIPEC in GC patients are currently on-going in Europe. The “GASTRICHIP” is a phase III randomized multicentre study evaluating the role of HIPEC with oxaliplatin in patients with GC who have either serosal infiltration and/or lymph nodal involvement and/or positive peritoneal cytology treated by a curative gastrectomy.^[66] Another trial is being conducted by the European network of excellence for gastric cancer. In this trial, patients with high risk GC will receive 3 cycles of neoadjuvant systemic chemotherapy followed by

a D2 gastrectomy and then randomized to receive HIPEC or no HIPEC.^[67] Prophylactic/adjuvant setting is the more evidence-based indication for HIPEC in advanced-stage GC patients. No peritonectomy procedures are needed; post-operative morbidity and mortality are the same than surgery alone. Anyway a better and “standardized” identification of subset of patients at high risk of peritoneal recurrence is necessary.^[68]

Intraperitoneal immunotherapy

Survival results for the treatment of PC from GC remain disappointing even with HIPEC, with 5-year survival rates of less than 25% in selected cases only. Innovative therapies such as intraperitoneal immunotherapy have been recently proposed.

The Chimera it's a legendary fire-breathing monster comprised of a lion, a goat, and a serpent. And chimera in genetics is a single animal organism with genetically distinct cells from two different zygotes. Chimera, or fusion protein, is called in biochemistry a hybrid protein made by the splicing of two genes. Catumaxomab is a chimeric antibody, consisting in a mouse-derived anti-EpCAM Fab (fragment antigen-binding) region and a rat-derived anti CD3 Fab [Figure 3]. It is characterized by its ability to bind to three different types of cells: tumour cells expressing the epithelial cell adhesion molecule (EpCAM), T lymphocytes (CD3) and also accessory cells (Fcγ receptor). In nearly 90% of GC

the EpCAM antigen is expressed; on the contrary the peritoneal mesenchymal cells do not express it.^[69] The rationale is strong, more evidence on results is needed.

In a randomised study, a clinical effect was obtained after intraperitoneal infusion of catumaxomab in patients with symptomatic malignant ascites secondary to EpCAM+ carcinomas, 66 out of 258 notably from GC.^[69] Heiss and coll randomly assigned the patients to paracentesis alone, or to paracentesis plus intraperitoneal catumaxomab. Puncture-free survival was significantly longer in the group treated with catumaxomab compared to that in the control group (46 vs. 11 days, $P < 0.0001$) but median overall survival was similar between the two groups: 72 days in the catumaxomab group vs. 68 days in the control group (n.s.).^[69]

Elias and his team from Gustave Roussy Institute (Villejuif, France), with long-date experience in HIPEC for PC, recently proposed a randomized phase II study, combining complete cytoreductive surgery with intraperitoneal immunotherapy.^[70] The main inclusion criteria of the protocol are PC of minimum or moderate extension and macroscopic resection of all the lesions: they just match the experience-based indications for HIPEC in PC from GC.^[54] As requested for HIPEC, the complete resection of all macroscopic disease before starting the intra-peritoneal administration of catumaxomab is necessary. The immunotherapy could therefore efficiently treat microscopic residual disease.

DIAGNOSIS OF INTRA-PERITONEAL FREE CANCER CELLS AND IDENTIFICATION OF PATIENTS AT RISK OF PERITONEAL RECURRENCE

The methods of detecting peritoneal free cancer cells represent an area in evolution. It's well known that the positive peritoneal cytology is according to the depth in invasion of the gastric wall, and that it has a prognostic value.^[71,72] In the same way, it's well known that cumulative risk of peritoneal recurrence is based on the infiltration of the gastric serosa.^[73] Cytological examination of peritoneal washing at the time of primary tumor resection is frequently positive. Free peritoneal cells are associated with an average survival of 4 months vs. 21 months for patients with negative cytology.^[71,74]

According to the 7th edition of the American Joint Committee on Cancer positive cytology in the absence of visible peritoneal implants is considered as M1 disease.^[75] Peritoneal washing for cytology (better during a staging laparoscopy) is mandatory in staging/

treatment algorithm of advanced GC.^[76]

The identification of patients at risk of peritoneal recurrence and the diagnosis of intra-peritoneal free cancer cells are probably two aspects of the same problem. The majority of patients with positive cytology on peritoneal washing develop PC, although it also may occur in patients with negative cytological results. These observations indicate that conventional cytology lacks sensitivity for the detection of residual cancer cells and the prediction of peritoneal spread. Many reports have emphasized the clinical significance of molecular diagnosis using reverse transcriptase-polymerase chain reaction analysis for more sensitive detection of GC cells in peritoneal washing. Fujiwara^[77] analyzed the survival of 123 patients with serosa-invading GC. The prognosis of the 29 patients with positive cytology in the peritoneal washing was very poor, and most of them died within 1 year after surgery. Among the 93 patients with negative cytology (CY0), 49 had a positive genetic diagnosis and a significantly poorer prognosis than those with negative genetic results. More than half of the patients with positive PCR and CY0 developed peritoneal recurrence after surgery, while almost all patients with negative PCR and CY0 had no peritoneal recurrence after surgery.^[77] These results have been confirmed by many studies. All the authors concluded that molecular diagnosis based on peritoneal washing is useful to predict peritoneal recurrence for patients with serosal invasion of GC; PCR positivity has significant correlation with overall survival and with peritoneal recurrence rate.^[78-81] Up 2 patients of 3 with negative cytology can be positive to PCR detection; in other terms, when surgeons perform R0 surgery (i.e. no macroscopic, microscopic and cytologic residual disease) for advanced GC, there is high probability that it's not true.

Molecular biological techniques are anyway time- and labour-intensive, and without yet diffuse application in clinical practice. A new rapid gene detection system, One-step nucleic acid amplification has been recently proposed.^[82] It shows potential for routine use in the clinical laboratory because of its simplicity and rapidity. On the other hand, the molecular detection of intraperitoneal GC cells is not only an independent prognostic factor, but also provides valuable clinical information for choosing the appropriate treatment for cytology-negative patients: such patients are potential candidate to intraperitoneal therapy, such as HIPEC, immunotherapy or both.

CONCLUSION

The peritoneal metastatic spread of GC leads to PC, a

very aggressive disease with very poor prognosis. In selected GC patients with low peritoneal tumor burden, more aggressive multi-modal strategy with CRS plus intraperitoneal treatment as HIPEC may achieve long-term survival results with up to 25% 5-year survival rates in case of complete cytoreduction. Moreover, there are strong evidences for HIPEC in adjuvant setting after radical surgery for preventing PC in high risk GC patients. Intraperitoneal immunotherapy, when associated with radical surgery, may open very interesting perspectives for the future. The detection of free peritoneal cancer cells is the more realistic and practical way for the identification of patients at risk of carcinomatosis after surgery. The routine use of techniques of molecular detection in peritoneal washing appears to be the more sensitive method. Such patients are potential candidate for multimodal and locoregional treatments in order to prevent the peritoneal recurrence.

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Conflicts of interest

There are no conflicts of interest.

Patient consent

Obtained.

Ethics approval

The patient was treated within the standards of authors' institute and the report was approved.

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Case Report

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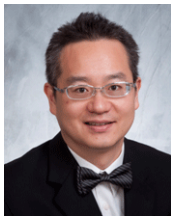
Pediatric gliomatosis cerebri presenting with intratumoral hemorrhage leading to poor outcome

Hiromasa Adachi, Masashi Kitagawa, Toshinari Kawasaki, Takafumi Wataya

Department of Neurosurgery, Shizuoka Children's Hospital, Shizuoka 420-8660, Japan.

Correspondence to: Dr. Takafumi Wataya, Department of Neurosurgery, Shizuoka Children's Hospital, 860 Urushiyama, Aoi-ku, Shizuoka 420-8660, Japan. E-mail: watayatakafumi@gmail.com

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Dr. Takafumi Wataya is a chief neurosurgeon in Shizuoka Children's Hospital, Japan, and a council member of Japanese Society for Pediatric Neurosurgery. He had fellowships of skull base brain tumor surgery and pediatric neurosurgery in North America. He got his PhD under professor Yoshiki Sasai, RIKEN institute, with inventing methods to create hypothalamic neurons from pluripotent stem cells (SFEBq method).

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ABSTRACT

Gliomatosis cerebri (GC) is an uncommon disease, defined as diffuse infiltration of neoplastic glial cells involving at least three cerebral lobes. GCs in young population are rare. We described a case of 14-year-old woman with GC who did not receive any recommended treatment, because the patient's family refused. The patient had a rapid deterioration in 5 months after first symptoms due to intratumoral bleeding. This is the first case report of intratumoral bleeding after diagnosis of GC is made, resulting in poor outcome. GC may acquire possibility of intratumoral hemorrhage through its development.

INTRODUCTION

Gliomatosis cerebri (GC) is an uncommon primary brain tumor that has quite malignant behavior. It is

characterized by diffuse infiltration of glioma cells, and defined with tumor invasion into more than three cerebral lobes.^[1,2] It often infiltrates into bilateral hemispheres, in some cases, even into the brainstem,



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cerebellum, and spinal cord, affecting both gray and white matter. It is classified as grade IV in World Health Organization 2007 criteria, regardless of its histopathological features.

GCs in most cases are seen in the adult population, rarely suffering young age group. There are two peaks of patients' age distribution in the second decades and forties.^[3]

We report here a pediatric patient with GC who refused any treatment, subsequently followed by rapid deterioration with intratumoral bleeding.

CASE REPORT

A 14-year-old woman presented with generalized tonic-clonic seizures following to history of morning headache, and mild cognitive deteriorations. She had noticed slowly progressing weakness on her right face and upper extremity, and numbness on her right side.

Neurological examination on admission revealed right facial droop and pronator drift on the right side. The patient originally was right-handed active softball player, but grasping power was weak with 21 kg on the right and 29 kg on the left at the time. Decreased proprioception and touch sensation was observed both in upper and lower extremities on the right. She was previously healthy and achieved normal developmental milestones and scholastic achievement up to an onset, but had experienced a decline in cognition. Wechsler Intelligence Scale for Children (WISC)-IV score shows intelligence quotient (IQ) 50.

Fluid-attenuated inversion recovery (FLAIR) and T2-weighted sequences of magnetic resonance imaging (MRI) showed hyperintense signal lesion in the white matter of the left frontal, parietal, and temporal lobes [Figure 1A and 1B]. Follow up MRI in 2 months later showed development of the lesion into the frontal lobe, as well as the brainstem and corpus callosum [Figure 1C and 1D]. MR spectroscopy at the time shows high peaks in both choline (Cho)/N-acetylaspartate (NAA) (2.9) and Cho/Creatine (Cr) (2.46), suggesting high grade glioma [Figure 1E].

Open biopsy was performed targeting occipito-temporal mass near posterior horn of the left lateral ventricle, which shows solid swelling without contrast enhancement on MRI. Hematoxylin and eosine staining of specimen shows marked cellularity, with marked hyperchromatism and pleomorphism [Figure 2A]. Neither necrosis nor vascular proliferation was

detected. Tumor cell infiltration in the peripheral zone of a tumor was found [Figure 2B]. Immunohistochemistry revealed positive staining for glial fibrillary acidic protein, and nuclear staining of p53. MIB-1 proliferation index was about 50% [Figure 2C and 2D]. With these results, histopathological diagnosis was made as anaplastic astrocytoma (grade III). The final clinical diagnosis was determined as gliomatosis cerebri due to invasion into 3 cerebral lobes and brainstem.

Considering potential poor prognosis of the disease, the patient's parents refused either radiation or chemotherapy, and only oral corticosteroid and rehabilitation was given to the patient. Five months after

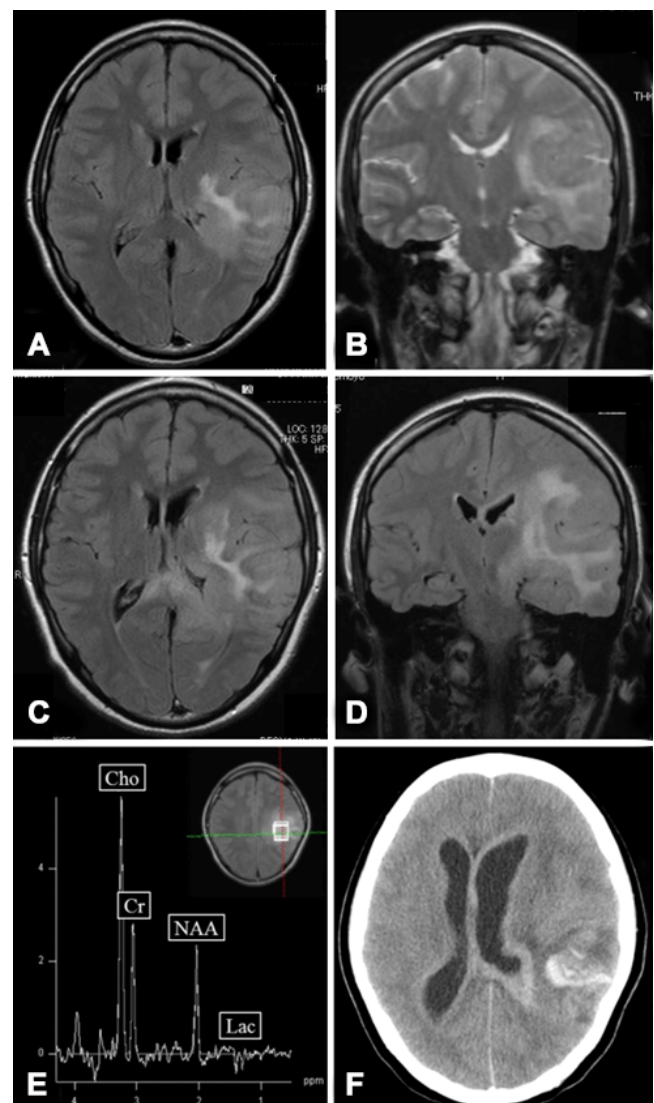


Figure 1: FLAIR and T2 weighted (T2WI) MR images demonstrating hyperintense area into 3 cerebral lobes, corpus callosum, and brainstem (A: axial FLAIR; B: coronal T2WI); follow-up FLAIR MRI took 2 months after initial symptoms (C: axial; D: coronal); MR spectroscopy suggesting high-grade glioma (E); axial CT scan demonstrating intratumoral hemorrhage 5 months after initial symptoms (F). FLAIR: fluid-attenuated inversion recovery; MRI: magnetic resonance imaging; CT: computed tomography

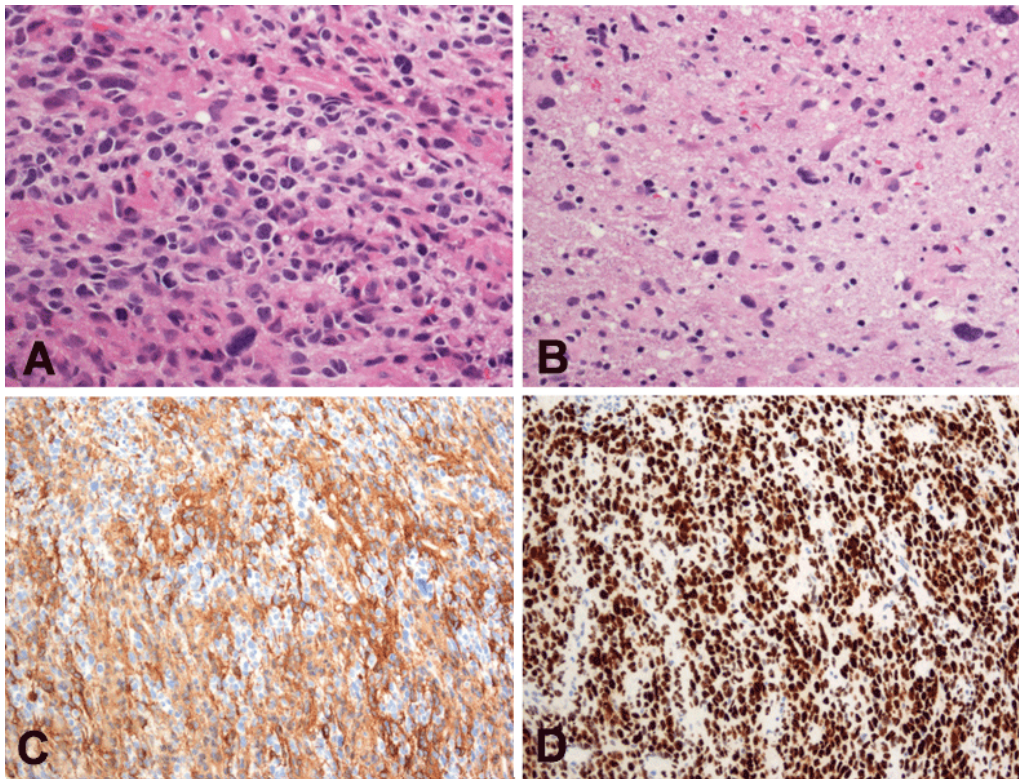


Figure 2: Hematoxylin and eosine staining of biopsy specimen. Diffuse cellular proliferation with hyperchromatism and pleomorphism (A, x400); neither necrosis nor vascular proliferation was detected. Infiltration into brain parenchyma was found (B, x400); positive staining for glial fibrillary acidic protein (C, x200), and p53 (D, x200)

the initial diagnosis, the patient had sudden respiratory arrest with uncal herniation due to intratumoral bleeding, and deceased [Figure 1F].

DISCUSSION

A case of pediatric GC which had poor clinical course with fatal intratumoral bleeding is presented. Although GCs in pediatric ages are rare, and there are no reports with large patients groups, a report with 13 GC patients under 18 years old showed that 2 years survival rate is 67%, and median overall survival is 27 months.^[4] The report also shows 2 years survival rate of patients under 10 years old is only 19%. As such, pediatric GC has extremely poor prognosis.

As for treatment of GC in general, whole brain radiation therapy (WBRT) with 45-50 Gy is considered as standard therapy.^[2] Retrospective study of WBRT with 54.9 Gy in average shows improvement of both overall survival (OS: 27.5 vs. 6.5 months) and progression free survival (PFS: 16.5 vs. 4.5 months).^[2]

Chemotherapy for GC had not been considered as effective treatments even combined with radiation therapy (RT),^[5] but recent growing publications support its efficacy to certain extent.^[6] Chemotherapy with temozolomide is widely used recently, because of

its safety. It has, however, insufficient effect to GC, and combination with RT is considered essential. Therefore, RT is recommended even for children who potentially have higher susceptibility for radiation.^[6]

In the present case, the patient deceased in 5 months after initial diagnosis, which is just as short as reported in the study in the group without WBRT.^[2] This case supports the necessity of RT in order to accomplish better OS or PFS.

Another reason why this patients had rapid deterioration was intratumoral bleeding. GC is classified into 2 types; type 1 (classical GC) shows infiltration of gliomatous cells with no mass lesions, and type 2 is categorized ones which develop tumor mass after type 1 infiltration.^[7] In the MRI of GC type 1, it has no or very small tumor enhancement with gadolinium and low relative cerebral blood volume (rCBV) value in perfusion study.^[8,9] Both indicate that the tumor has low vascularity; therefore it is expected to have small chance of intratumoral hemorrhage. To our knowledge, there is no report of intratumoral hemorrhage of type 1 GC. On the other hand, contrast enhancement can be seen in some cases of type 2 GC, with increased rCBV.^[9] From these aspects, type 2 GC may have higher possibility of bleeding than type 1.

This is the first case report of intratumoral hemorrhage of GC after its diagnosis is made. Although not frequent, the possibility of intratumoral hemorrhage of GC must be kept in mind, especially when no treatment was given, or once it become uncontrollable even with RT or chemotherapy.

A pediatric case of GC with intratumoral bleeding is reported. GC may acquire possibility of intratumoral hemorrhage through its development, and may lead to catastrophic outcome.

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Conflicts of interest

There are no conflicts of interest.

Patient consent

Obtained.

Ethics approval

The patient was treated within the standards of authors' institute and the report was approved.

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Original Article

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Do elderly NSCLC stage IV patients benefit from chemotherapy as well as younger? An analysis from clinical practice data

Regina Gironés, Pedro López, Rebeca Chulvi, Mamen Cañabate

Medical Oncology Unit, Lluís Alcanyis Hospital, 46800 Xàtiva, Spain.

Correspondence to: Dr. Regina Gironés, Medical Oncology Unit, Lluís Alcanyis Hospital, Crta Xàtiva a Silla km 2, 46800 Xàtiva, Valencia, Spain.
E-mail: girones_reg@gva.es

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Dr. Regina Gironés works at regional academic hospital named Lluís Alcanyis in Xàtiva, Valencia, Spain since 2004. Her Medicine Graduate has been undertaken in the Faculty of Medicine of the University of Valencia (1992-1998); Doctor's degree the Autonomous University of Barcelona. She works in oncogeriatrics since 2004, and had become a SIOG member in 2007. She publishes her articles at Critical Review Oncology Hematology, Journal of Geriatric Oncology, Lung cancer and Clinical Translational Oncology, among others.

ABSTRACT

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platinum-combinations

Aim: The aim of this study was to evaluate the efficacy of treatment related to age in metastatic non-small cell lung cancer (NSCLC). We compared young and elders (> 70) in the setting of a regional Spanish hospital. We hypothesized that elder benefit as much as younger patients from chemotherapy in stage IV NSCLC. The study was limited to performance status 0-2. **Methods:** Clinical and demographic characteristics were reviewed from medical records. Type of treatment was collected and compared, as well as benefit from treatment, in terms of overall survival. **Results:** 322 patients (162 young, 160 aged) Elderly patients received less active treatment (63% vs. 86%, $P = 0.001$). Elderly received less chemotherapy, less cisplatin-doublets, more carboplatin-combinations and monotherapy ($P = 0.035$). The benefits of treatment were similar, regardless of age. Smoking status demonstrated a prognosis impact for elder patients treated with chemotherapy. Those who remained active smokers had a lower overall survival in the aged group. In a multivariate analysis, the Eastern Cooperative Oncology Group, active treatment and non-smoking history were favorable prognostic factors for elder patients. Smoking had not impact on young patients. **Conclusion:** Elderly patients were undertreated in clinical practice. Treatment showed similar overall survival despite of age. The impact of smoking seems to be more significant in the elderly population.



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INTRODUCTION

Lung cancer remains the most frequent cause of cancer-related death worldwide.^[1] Elderly patients make up a substantial proportion of non-small cell lung cancer (NSCLC) patients and their numbers are expected to increase.^[2] They've been significantly underrepresented in clinical trials, making it difficult to extrapolate clinical trial data.^[3] Despite the clear benefits to survival, most elderly patients with advanced NSCLC are under-treated or do not receive chemotherapy.^[4,5] In general there is an expectation that elderly patients have poor tolerance to treatment.^[6] Physicians may be reluctant to offer treatment known to provoke troublesome side effects due to the unwarranted assumption that elderly patients do not benefit from cytotoxic therapy.^[7,8] Consequently, elderly patients are frequently under-treated, and only one quarter of elderly patients (> 65 years) with advanced NSCLC are reported to receive palliative chemotherapy.^[4,9] Advanced age has been a prevalent reason for not administering treatment, contrary to established guidelines.^[10-12]

Platinum-based doublet chemotherapy is considered to be standard of care for elderly patients with an Eastern Cooperative Oncology Group Performance Status (ECOG PS) score of 0-1.^[13-14] The association of a platinum compound with a third-generation agent improves survival,^[15-16] and seems to be the most effective therapeutic choice in such cases. Recently, several elderly-specific trials showed that chemotherapy is effective and feasible for elderly patients with NSCLC.^[17-21] National Comprehensive Cancer Network guidelines recommend platinum-doublet chemotherapy in patients with good performance status regardless of age.^[22] The European Organization for Research and Treatment of Cancer/International Society for Geriatric Oncology also recommend the use of carboplatin-based doublets in fit elderly patients and single-agent treatment for less fit patients.^[12] Despite recent developments in treatment recommendations for elderly patients, little is known about use of these in clinical practice, and very limited data are available for elderly patients outside of clinical trials. Limited data exist regarding real-world treatment patterns and outcomes with respect to patients with metastatic NSCLC treated at Spanish regional hospitals.

We hypothesized that elder benefit as younger patients from chemotherapy in stage IV NSCLC. Therefore, in this study, we aimed to evaluate the proportion of elderly advanced NSCLC patients attended at clinical practice who are candidates for standard systemic

chemotherapy, the actual proportion of patients who receive chemotherapy, the actual treatment they received and clinical outcome in these patients.

METHODS

We collected on a prospective manner of all patients with advanced NSCLC (stage IV) seen at the Regional Medical Oncology Unit from the Hospital Lluís Alcanyis, Xàtiva since January 2004, creating a data base register. Patients collected for this analysis accomplished the following conditions: histological or cytological confirmation of NSCLC (although we accepted radiological diagnosis without histological confirmation) in stage IIIB (pleural effusion, prior TNM stage), or stage IV of the disease. Outpatient and those are suitable for treatment (PS 0-2). We collected data on baseline demographics, clinical characteristics and a detailed treatment history. Our study period covers January 2004 until December 2014. Tumor histology was classified on the basis of the 2004 WHO classification.^[23] Patients were classified respect to smoking habits into 3 groups: never smoker, active smoker and ex-smoker (if they had quit smoking a year or more prior to diagnosis). Data on drug-sensitive epidermal growth factor receptor (EGFR) mutations was collected, since June 1 2010 using peptide nucleic acid-locked nucleic acid polymerase chain reaction clamp-based testing. When testing was not performed the data was recorded as "unknown". Anaplastic lymphoma kinase (ALK) translocations have been determined via fluorescence *in situ* hybridization since June 2012. Studies of K-RAS mutation are not performed as part of standard care. For surviving patients, final follow up was recorded on 15th December 2014. Survival time was calculated from the time of diagnosis until death or final follow up.

Statistical evaluation was performed using SPSS version 20.0 software; unpaired Student's *t*-test, Chi-squared, and Fischer exact test were used according to data type. Statistical significance was defined as $P < 0.05$; variables were considered to be independent for the statistical analysis; continuous data was expressed as mean \pm standard error. Statistical analyses of categorical variables were performed using Pearson's Chi-square test or Fisher's exact test as appropriate. Survival analysis was performed using the Kaplan-Meier method, and groups were compared using the log-rank test. Univariate and multivariate analyses were performed using the Cox proportional hazard regression analysis.

The institution's ethical review board approved the data base on 2004. Also it approved the review of

Table 1: Clinical characteristics of the patients; comparison between age groups

	Group 1: < 70 years old 162 (50.3%)	Group 2: > 70 years old 160 (49.7%)	P
Age, years	59 (34-69)	76 (70-91)	
Mean, range			
Gender			
Men	139 (87%)	142 (88%)	P = 0.266
Women	23 (13%)	18 (12%)	
PS 0-1	136 (84%)	98 (61%)	P = 0.00001
PS 2	26 (16%)	62 (39%)	
Histology, n (%)			
Unconfirmed	4 (2%)	10 (6%)	P = 0.025
Squamous	55 (34%)	70 (44%)	P = 0.023
Adenocarcinoma	88 (55%)	59 (37%)	P = 0.0322
Large cell carcinoma	10 (6%)	15 (10%)	P = 0.53
Untyped carcinoma	5 (3%)	6 (3%)	P = 0.6
Smoking habits:			
Never smoker	13 (8%)	28 (18%)	P = 0.0001
Active smoker	112 (69%)	33 (20%)	
Ex-smoker	37 (23%)	99 (62%)	
EGFR status			
Unknown	51 (31%)	65 (40%)	P = 0.0001
Mutated	12 (7%)	17 (11%)	
Wild-type	99 (62%)	78 (49%)	
EGFR status in adenocarcinoma (147)	(88)	(59)	P = 0.0005
Unknown	8 (9%)	8 (13%)	
Mutated	12 (14%)	17 (29%)	
Wild-type	68 (77%)	34 (58%)	

EGFR: epidermal growth factor receptor; PS: performance status

the records. Informed consent was obtained from all individual before inclusion in the data base.

RESULTS

From January 2004 until December 2014, 322 patients (162 patients in Group 1 and 160 patients in Group 2) were included in the analysis. Clinical characteristics and comparison between age groups are shown in Table 1. In the elderly group, 30% were octogenarians. More elderly patients had a PS of 2 (39% vs. 16%, $P = 0.00001$); and were derived without histological confirmation (6% vs. 2%, $P = 0.025$). Squamous cell carcinomas predominate on the elderly (44% vs. 34%, $P = 0.023$). The majority of patients had had an smoking history (92% of younger patients vs. 82% of the elderly, $P = 0.001$). Most of the elderly patients were ex-smokers (62%) while the younger patients tended to be active smokers (69%). Smoking habit was related to squamous histology in the elderly group. Younger smokers developed both squamous cell and adenocarcinoma meanwhile, in the elderly group, we found a link between the following characteristics: female gender, adenocarcinoma, no history of smoking and EGFR-mutation ($P = 0.00001$); 99% of aged women were never smokers and there were no elderly women with squamous histology. Smoking status was unrelated to PS.

No patient in our series had ALK rearrangement (analysis started on June 2012). In terms of EGFR mutations, 116 patients had unknown status (patients diagnosed prior to 2010). In both groups all EGFR-mutations were found in adenocarcinoma ($P = 0.00001$). No mutations were found in women smokers; never smoking predicted EGFR status in women in both age groups, while in men this only occurred in the elderly ($P = 0.0001$). In younger men, smoking habit did not predict mutation status [Figure 1].

There was a higher percentage of EGFR-mutations in the elderly group. In the global series, 25% of adenocarcinoma were found to be mutated; 13% in younger group, 28% in elderly group ($P = 0.01$).

Treatment data: Table 2 shows differences in patterns of treatment. Patients without histological diagnosis didn't receive treatment in either age group. Of the 102 elderly patients who received first-line treatment, 71 (70%) were treated with chemotherapy, 17 (16%) with EGFR TKI and 14 (14%) with radiotherapy. Elderly patients had received less active treatment ($P = 0.0001$). PS influenced whether treatment was administered or not in both groups ($P = 0.0001$). Performance status was an independent predictor, as patients with PS of 2 did not receive chemotherapy in either group. The same proportion of patients with a PS of 2 received

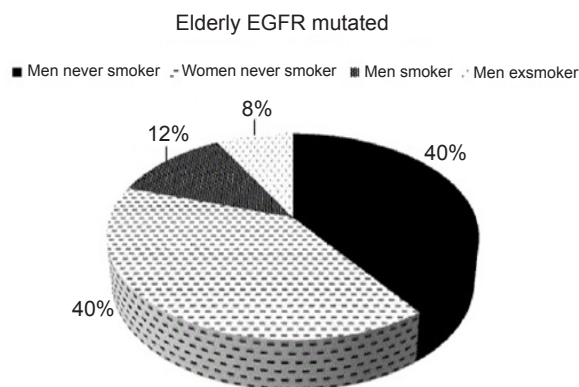


Figure 1: EGFR mutation and smoking habit in elderly patients. EGFR: epidermal growth factor receptor

palliative brain radiation in the two groups (3 and 6; 10%). Elderly patients received less chemotherapy ($P = 0.0001$) and were more likely to receive palliative radiation as sole treatment (81% vs. 5%). In the younger group, of the 124 patients with PS 0-1 suitable for chemotherapy (excluding 12 patients with EGFR mutation); 118 (95%) were treated with chemotherapy. In the elderly group, 71 of 98 patients (72%) suitable for chemotherapy received this treatment. More elderly patients with good PS received palliative radiotherapy as sole treatment (4% vs. 8%, $P = 0.0001$). Overall, of the 189 patients that received chemotherapy (58.6% of the global series), 62.5% were in the younger group vs. 37.5% who were elderly ($P = 0.0001$). In terms of chemotherapy, the elderly received more carboplatin combinations (34% vs. 60%), monotherapy (6% vs. 30%) and were less likely to receive bevacizumab combinations (2% vs. 18%) ($P = 0.035$). All patients with EGFR mutation received first line EGFR TKI. Only one patient with an EGFR mutation in the elderly group had PS 2.

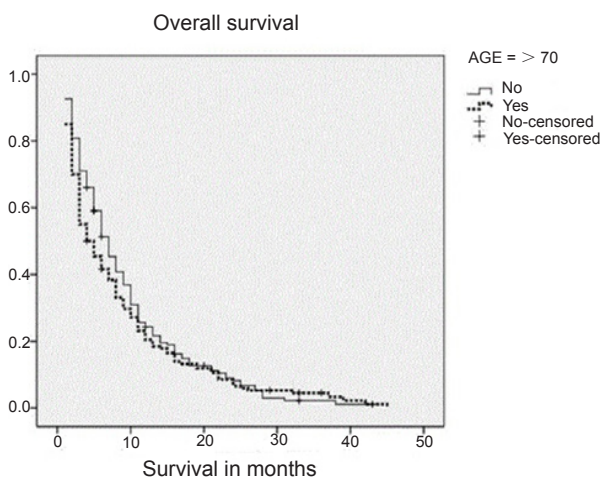


Figure 2: Comparison on overall survival between age groups

Table 2: Treatment date and comparison between age groups

	Young (162)	Elders (160)	P
Treatment			
Yes	139 (86%)	102 (63%)	$P = 0.0001$
No	23 (14%)	57 (37%)	
Treatment and PS			$P = 0.52$
PS 0-1	136	98	
Yes	136 (100%)	95 (97%)	
No	0	3 (3%)	
PS 2	26	61	
Yes	3 (12%)	7 (11%)	
No	23 (88%)	54 (89%)	
Kind of treatment and PS			
PS 0-1	136	95	
Chemotherapy	118 (86%)	71 (74%)	$P = 0.0001$
Radiotherapy	6 (4%)	8 (8%)	
EGFR-TKI	12 (10%)	16 (18%)	
PS 2	3	7	
EGFR-TKI	0	1 (15%)	
Radiotherapy	3 (100%)	6 (85%)	
Kind of chemotherapy	118	71	$P = 0.035$
Cisplatin-combination	48 (41%)	4 (5%)	
Carboplatin-combination	41 (34%)	42 (60%)	
Monotherapy	8 (7%)	21 (30%)	
Bevacizumab combination	21 (18%)	2 (2%)	

EGFR: epidermal growth factor receptor; TKI: tyrosine kinase inhibitor; PS: performance status

For the global series, overall survival was 8.979 months [95% confidence interval (CI) 7.949-10.08] and there was no difference between age groups (9.42 vs. 8.48 months; $P = 0.0238$) [Figure 2].

According to the univariate analysis using Cox proportional hazard regression analysis, the following factors were related to better survival: female gender, ECOG PS 0-1, adenocarcinoma histology, no history of smoking, presence of EGFR mutation, administration of treatment, chemotherapy and EGFR-TKI therapy.

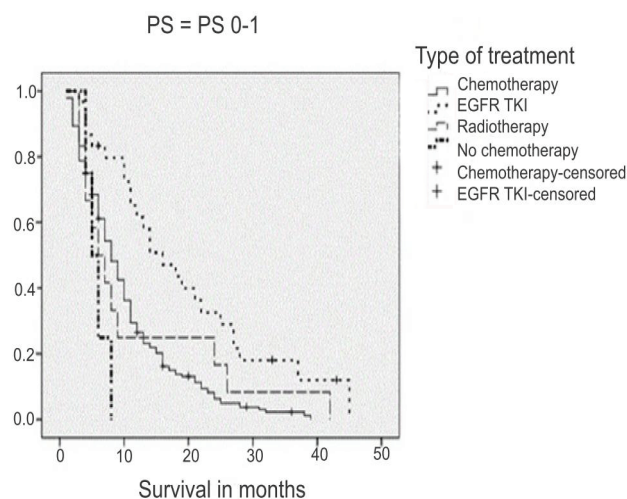


Figure 3: Comparison: treated versus untreated young patients. EGFR: epidermal growth factor receptor; TKI: tyrosine kinase inhibitor; PS: performance status

Table 3: Multivariate analyses

	Young (162)	Elders (160)	P
Age	9.4 (8.0-10.7)	8.4 (6.9-10.00)	0.238
Male	N: 139; SG: 8-8 (7.4-10.2)	N: 142; SG: 7.9 (6.4-9.4)	0.263
Female	N: 23; SG: 12.8 (8.8-16.9) $P = 0.069$	N: 18; SG: 12 (6.7-17.3) $P = 0.014$	
PS 0-1	N: 136; SG: 10.7 (9.2-12.2)	N: 98; SG: 12.3 (10.2-14.5)	0.259
PS 2	N: 26; SG: 2.5 (1.3-3.7) $P = 0.0001$	N: 62; SG: 2.3 (1.9-2.7) $P = 0.0001$	
Never smoker	N: 13; SG: 15.7 (10.4-21.1)	N: 28; SG: 13.3 (8-18.7)	0.098
Smoker	N: 112; SG: 8.7 (7.2-10.2)	N: 33; SG: 5.4 (3.5-7.3)	
Ex-smoker	N: 37; SG: 8.8 (6.1-11.5) $P = 0.041$	N: 99; SG: 8.2 (6.4-10) $P = 0.001$	
EGFR unknown	N: 25; SG: 9.9 (6.8-12.9)	N: 33; SG: 8.1 (5.6-10.6)	0.112
EGFR mutated	N: 12; SG: 21.0 (13.7-28.3)	N: 17; SG: 16.8 (10.2-23.3)	
EGFR wild type	N: 125; SG: 8.1 (6.8-9.4) $P = 0.002$	N: 110; SG: 7.3 (10.2-23.3) $P = 0.0001$	
Squamous	N: 55; SG: 7.6 (5.8-9.3)	N: 70; SG: 7.5 (5.6-9.4)	0.612
Adenocarcinoma	N: 88; SG: 10.9 (8.8-13) $P = 0.018$	N: 59; SG: 10.1 (7.1-13.1) $P = 0.002$	
Treated	N: 139; SG: 10.5 (9-12)	N: 102; SG: 11.8 (9.7-13.9)	0.19
No treated	N: 23; SG: 2.5 (1.1-3.8) $P = 0.00001$	N: 57; SG: 2.5 (2-3) $P = 0.00001$	
Chemotherapy	N: 117; SG: 18.0 (11.9-24)	N: 71; SG: 10.6 (8.5-12.8)	0.365
Radiotherapy	N: 8; SG: 3.0 (1.89-4.1))	N: 13; SG: 3.6 (2.4-4.7)	
EGFR TKI	N: 13; SG: 19.7 (12.5-26.9) $P = 0.0001$	N: 18; SG: 17.8 (11.4-24.2) $P = 0.0001$	
Combined chemotherapy	N: 89; SG: 9.2 (8.2-13.1)	N: 48; SG: 10.7 (8.2-13.1)	
Monotherapy	N: 8; SG: 5.4 (3.3-7.4)	N: 21; SG: 10.1 (6.3-13.9)	
Bevacizumab	N: 21; SG: 14.5 (10.3-18.6) $P = 0.024$	N: 2; SG: 19.0 (12-42) $P = 0.248$	

EGFR: epidermal growth factor receptor; TKI: tyrosine kinase inhibitor; PS: performance status; SG: study group

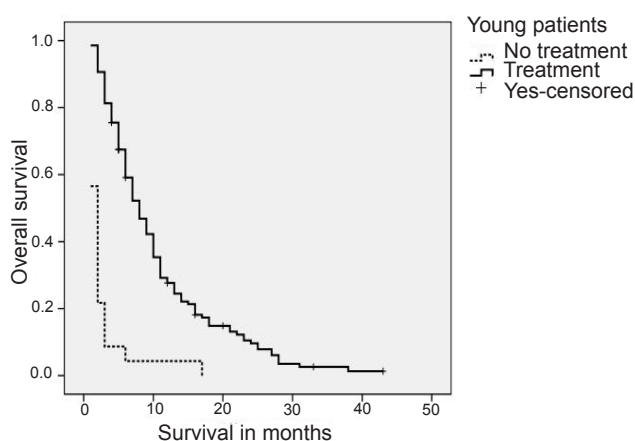
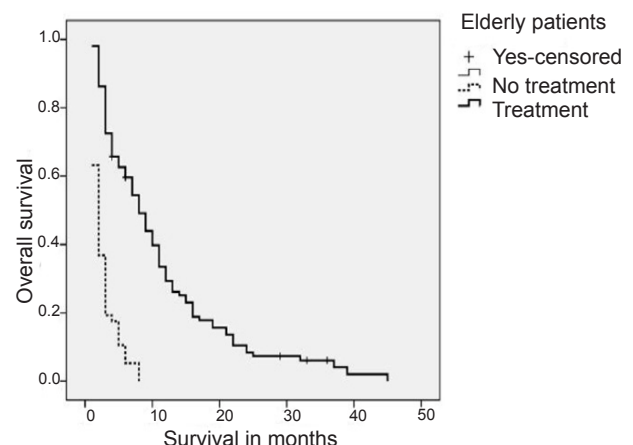
For female patients, never smoking and adenocarcinoma histology were related to EGFR-mutation and EGFR-TKI; only EGFR-mutation remained significant in multivariate analysis for overall survival [Table 3].

When we excluded these confounding factors, PS 0-1 and systemic chemotherapy were independently associated with better survival in both groups.

There was a clear benefit associated with administration of some treatment in both groups (10.5 vs. 2.5 months in the younger group, $P = 0.0000$ and 11.8 vs. 2.5

months in the elderly group, $P = 0.000$) [Figures 3 and 4]. For those patients suitable for treatment (PS 0-1), radiotherapy, chemotherapy and EGFR treatment when appropriate were also found to provide benefits [Figure 5]. No impact on overall survival was found with respect to treatment for patients with a PS of 2 (3 vs. 2.6 vs. 2.3 months; radiotherapy vs. chemotherapy vs. EGFR treatment respectively).

Elderly patients were found to benefit slightly from chemotherapy (9.9 vs. 10.6 months; no chemotherapy vs. chemotherapy respectively, $P = 0.42$). In terms of

**Figure 4:** Comparison: treated versus untreated elder patients**Figure 5:** Benefit of treatment in terms on overall survival for elderly patients

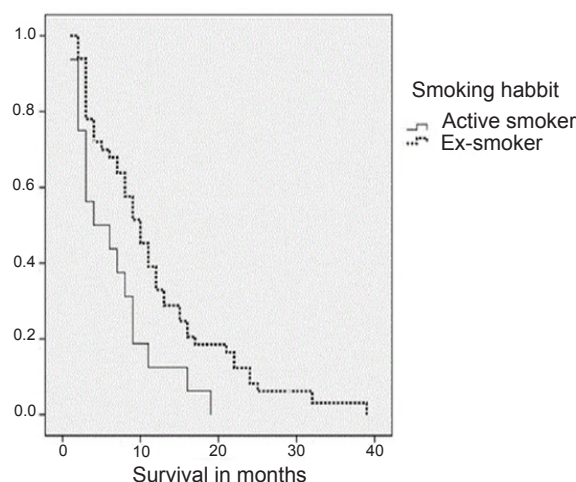


Figure 6: Differences in overall survival by smoking status in elderly patients treated with chemotherapy

platinum-combinations vs. monotherapy, the same benefit was found for younger versus elderly patients ($P = 0.14$). Platinum-combinations were found to be more effective in younger patients (9.5 vs. 5.7 months; platinum vs. non-platinum combinations respectively), but no differences were found in the elderly group (10.5 vs. 10.2 months; combination vs. monotherapy). Few patients were treated with bevacizumab and we are therefore unable to draw conclusions. No differences in survival were found with respect to distinct platinum combinations.

Smoking habit had impact on overall survival in elderly patients that received chemotherapy ($P = 0.006$) [Figure 6]. Median overall survival for active smokers during chemotherapy treatment was 6.5 (95% CI 3.9-9.1) vs. 12.1 months (95% CI 9.5-14.7), $P = 0.011$ for those who had quit smoking. In addition, smoking had an impact on the outcomes of patients who received combination therapy in the elderly group; smokers treated with platinum-combination had median overall survival of 6.9 months (95% CI 4.1-9.6) vs. 12.7 months in ex-smokers (95% CI 9.4-15.9). Median overall survival for patients receiving monotherapy in the elderly group was 6.8 months for active smokers (95% CI 3.2-13.1) vs. 10.8 months in ex-smokers (95% CI 6.4-15.1) ($P = 0.014$). The relationship between smoking status and chemotherapy was not significant in the younger group.

DISCUSSION

In this study, we analyzed all elderly patients with advanced NSCLC who visited our outpatient hospital over a 10-year period, and found that 64% ($n = 102$) received active treatment. The elderly patients treated benefit in a similar way that younger counterparts.

Smoking had an important impact on elderly patients treated.

Elderly patients tended to have poorer PS. All of the elderly women except one were never smokers. With the increasing number of female smokers, it is uncertain whether in the future we will see greater numbers of elderly women smokers.^[24-27] Most probably, due to the high sensitivity of women to tobacco carcinogens, the tendency will be towards an increase of younger female lung cancer patients.^[28] In any case, never smoker status was significantly related to EGFR-mutation in elderly and younger women. A higher prevalence of EGFR mutations in the elderly has already been described,^[29] and, as in our series, older age at diagnosis has been reported to be an independent predictor of EGFR mutations in female never-smokers with adenocarcinoma.^[30] In this study, in males never smokers, smoking habit was related to EGFR mutation in the elderly group only; it was not a predictor of EGFR status in the younger group. Smoking was related to histology in the elderly; squamous cell lung carcinoma was the main histology in this group. Adenocarcinoma related to smoking was more predominant in the younger group. It should be taken into account that the elderly patients were mostly ex-smokers. The high incidence of smoking history in the elderly has already been described.^[31]

Most elderly patients with metastatic NSCLC do not receive chemotherapy, as database analyses have shown.^[4] In our series, elderly patients were less likely to receive chemotherapy than younger patients; however on analysis of those elderly patients suitable for chemotherapy, almost 85% received chemotherapy. This is a probably a higher rate of treatment than reported in published data.^[34] For example, one analysis performed by SEER-Medicare,^[4] which considered elderly patients as those > 66 years, showed that only 25.8% received first-line chemotherapy. In that study, multivariate analyses indicated that with increasing age, comorbidity and poor PS, treatment with any chemotherapy and platinum-based doublet regimens is less likely to be used. In our series, the elderly patients were older than those in the SEER analysis (> 70). From a total of 160 elderly patients, 71 (44.3%) received chemotherapy. This is a higher figure than reported in other studies. Platinum-doublet chemotherapy regimens have been shown to extend survival in fit patients with advanced non-small-cell lung cancer.^[4] At our study, both, cytotoxic chemotherapy and EGFR TKI treatments are feasible and prolong survival when comparisons are made with patients who do not receive chemotherapy in both groups. It seems that the benefit of treatment of elderly patients

is similar (indeed a little better) to those obtained by their younger counterparts.

Doublet platinum-based chemotherapy regimens are the standard of care for both adult and elderly fit advanced NSCLC patients, with good tolerance and only minor effects on quality of life (QoL).^[32,33] In our study, a high percentage of elderly patients with PS 0-1 suitable for chemotherapy did in fact receive chemotherapy. Since 2006 we have used geriatric assessments to determine suitability for treatment.^[34,35] All young patients with good PS were treated; but there were no differences in overall survival for those elderly. Are elderly patients undertreated? Or are younger patients overtreated?

The elderly were less likely to receive cisplatin-combinations and more likely to receive monotherapy. Surprisingly we did not find any differences when comparing platinum-combinations to monotherapy. Monotherapy has been for several years the recommended palliative treatment for elderly patients with advanced NSCLC.^[36] Factors that influence whether a patient receives a platinum-doublet or single-agent are unclear in the elderly. Over the period of study we have found a tendency to prescribe monotherapy, probably due to doubts about the benefit of platinum-combination until recently. Probably, these elderly patients were more carefully selected, and we do not know whether they would have benefited from a platinum-combination. Other authors found that platinum-doublet chemotherapy provides greater benefits than single agents in the elderly.^[4]

It is difficult to make conclusions in the sense that this is not a randomized study. Bevacizumab has not been specifically studied in older patients.^[37] As few elderly patients were treated with bevacizumab we are unable to draw conclusions. Probably the two patients suitable for first line bevacizumab were carefully selected. At present we are exploring bevacizumab in elderly patients selected using geriatric assessment (ClinicalTrials.gov identifier: NCT01980472). For chemotherapy combinations (vinorelbine, gemcitabine, paclitaxel, pemetrexed, docetaxel) we did not find any differences in elderly patients, which leads us to draw the conclusion that, as in younger patients, the benefits of chemotherapy have reached a plateau.^[6,38]

Our results indicate that chemotherapy treatment is strongly associated with greater survival. Furthermore, the magnitude of this benefit is comparable with that seen in clinical trials, or even more so. The closeness of these estimates suggests that with adequate adjustments for patients' characteristics, observational studies can provide very useful information on the

effectiveness of treatment.

The same prognostic factors were found for in the elderly and younger patients; PS 0-1, active treatment, never smoker and EGFR mutation, regardless of age. For elderly patients, smoking has impact on benefit from chemotherapy, as ex-smokers benefit more from both combination and monotherapy.

Our analysis raises several questions that deserve future study. In particular, we have noted that despite gains in treatment rates during the study period, overall survival remains poor and smoking continues to be a major factor in determinant treatment outcomes, although only for the elderly. Our survival results indicate that appropriate patients, regardless of age, can benefit from aggressive treatment. Additional work on smoking is needed to further elucidate the role of smoking on age and treatment outcomes.

Our study has several limitations. First, this analysis was conducted in a single center, so we cannot extrapolate our results to the overall population with lung cancer. Secondly, we have an important selection bias, as we only collected data on ambulatory patients. However, these are the patients that benefit most from chemotherapy. Thirdly, some variables have not been collected (median number of chemotherapy cycles, chemotherapy lines, and progression-free survival). Also, EGFR mutation test and ALK rearrangement tests were not fully performed in most patients.

However, this study also has strengths. All data was collected from the same oncology unit, and patients were all attended by the same oncologist (Dr. Gironés). Possible confounding factors for treatment (physician bias) have been prevented.^[39] The number of cases was relatively high. To date, most studies of elderly lung cancer patients have been from subgroup analysis of phase III studies or were specific studies for elderly patients with fewer patients. Studies with high numbers of patients were retrospective.^[6,38]

In conclusion, patients do benefit from aggressive chemotherapy regardless of their age. Our observational data provide an opportunity to understand the effects of treatment when applied in routine practice and assess whether outcomes are comparable to those obtained in clinical trials. Approximately 45% of the elderly patients with advanced NSCLC seen at our routine clinical practice received active treatment with chemotherapy, and this prolonged survival in a similar way to in their younger counterparts. The most significant advances in median overall survival have been in cases of lung cancer unrelated to smoking (EGFR-mutations). Unfortunately, smoking remains the main cause of

lung cancer in elderly patients. Efforts to prevent the initiation of the smoking habit and also to quit smoking should be made, regardless of age.

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Nil.

Conflicts of interest

There are no conflicts of interest.

Patient consent

Obtained.

Ethics approval

Ethics approval was obtained prior to the commencement of the study.

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Case Report

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A small solitary fibrous tumor of the bladder

Guido Petracco, Carlo Patriarca

Division of Surgical Pathology, Azienda Ospedaliera Sant'Anna, Via Ravona, San Fermo d/B, 22020 Como, Italy.

Correspondence to: Dr. Carlo Patriarca, Division of Surgical Pathology, Azienda Ospedaliera Sant'Anna, Via Ravona, San Fermo d/B, 22020 Como, Italy. E-mail: carlo.patriarca@hsacomio.org

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Dr. Carlo Patriarca served as a Staff Pathologist in Genova and Milan and now he serves as Director of the Division of Anatomic and Surgical Pathology of S. Anna Hospital, Como, Italy. He focused his past interests on the role of adhesion molecules in oncology and his present interests on uropathology.

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ABSTRACT

The authors present a case of histologically benign and incidentally discovered millimetric solitary fibrous tumor of the bladder, invisible to radiologic imaging and clinically benign. The case came to our attention because of repeated episodes of renal colic. As opposed to the present case, solitary fibrous tumor are generally discovered when they reach certain dimensions, being slow-growing, painless masses. Such a tumor of the bladder is a very rare finding, with less than 20 cases reported, and it has yet to be described with such a small size. The main differential diagnoses are discussed. Such tumors with histological features of malignancy are also described in the literature. However, the present case had a bland appearance so a conservative approach with an excision was adopted. No signs of recurrence are present at follow-up.

INTRODUCTION

Solitary fibrous tumor (SFT) is a rare type of mesenchymal tumor described in the years originally as hemangiopericytoma, later as SFT.^[1-3] A malignant histology has been also described in different organs.^[4] Nevertheless, SFT involving the urinary bladder is a very

rare finding. We present one case of bladder primary SFT of a few millimeters, incidentally discovered.

CASE REPORT

A 72-year-old male patient with repeated episodes of renal colic was admitted to the emergency room of our



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hospital. He underwent an echographic investigation that showed only grade 1-2 hydronephrosis. Moreover, leucocytosis and elevated C-reactive protein was observed. An expulsion therapy was performed.

After 1 week, a computed tomography scan showed hydronephrosis with a 10 mm × 8 mm ureteral calculus located 4 cm from the bladder neck. The patient underwent an endoscopic lithotripsy. During the procedure, a 4 mm bladder nodule was seen on the mucosa surface, thus removed by the urologist and submitted for histologic examination.

This showed a mesenchymal proliferation with low cellularity [Figure 1], without atypia [Figure 2] and a mitotic index below 1/10 high power field. Immunohistochemistry demonstrated strong CD34 positivity [Figure 3], weak B cell lymphoma (BCL2)

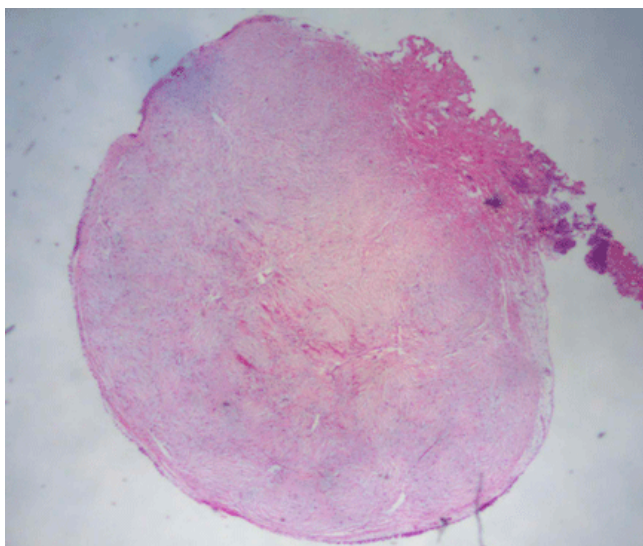


Figure 1: Nodular, small solitary fibrous tumor of bladder mucosa. Complete excision was performed (HE, ×10)

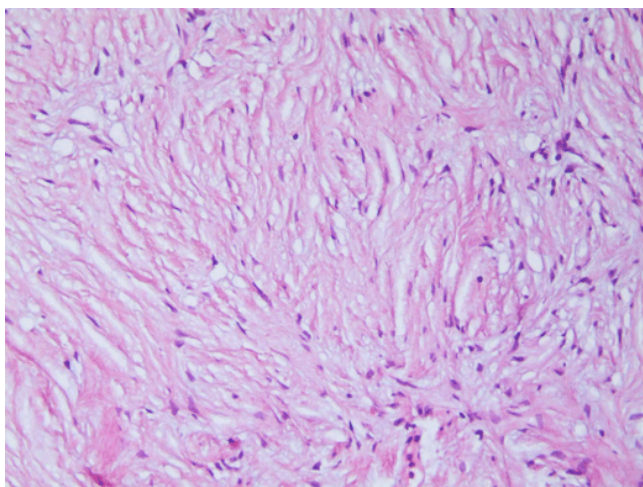


Figure 2: Mesenchymal proliferation with low cellularity, without atypia, and without mitotic activity (HE, ×40)

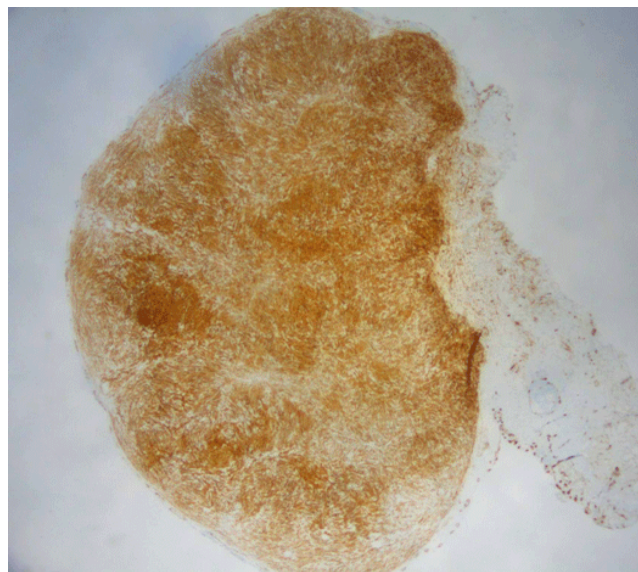


Figure 3: CD34: strong, diffuse immunostaining of entire lesion (ABC perox, ×10)

positivity, and negativity for both S100 and smooth muscle actin. Hence, a diagnosis of solitary fibrous tumor was formulated.

Among the differential diagnoses, inflammatory fibroblastic tumor was ruled out because of poor cellularity, activin receptor-like kinase 1 (ALK1) negativity, and absence of an inflammatory component. Likewise, spindle cell nodule and benign neoplasms such as leiomyoma or neurofibroma were excluded for morphophenotypic features. Ten months after excision the patient had no ecographic sign of recurrence.

DISCUSSION

Extrapleural SFTs are anatomically ubiquitous, as documented also by the present case report, and occur equally in males and females, primarily in adult life, with a wide range of ages, 20 to 70 years.^[4,5] The ubiquity of SFT supports its mesenchymal origin (with fibroblastic/myofibroblastic features).^[3] Most present as a slow-growing, painless masses. In cases of bladder SFT, the most frequent symptoms, such as pain, palpable mass, abdominal distention, urinary retention, haematuria, constipation, and bowel obstruction, are related to compression and local invasion of nearby structures.

In the English literature 15 cases have been reported,^[6,8-11] all with symptoms related to tumor volume (up to 12 cm in diameter), and presence of radiologic findings.^[12-16] Sometimes, a diagnosis of malignant soft tissue tumor was considered. Actual malignant bladder SFT has been described,^[7] while to the best of our knowledge, this is the first case of a

SFT of just 4 mm.

The World Health Organization classification of tumors of soft tissue in 2013 identifies the SFTs among the tumors with a rarely unpredictable behavior.^[17] Indeed, although most cases are slow-growing and benign, behavior can sometimes be aggressive and distant metastasis may occur.^[5] However, features more frequently related with a poor prognosis are tumor size over 10 cm, malignant histology such as high mitotic count and necrosis, while the feature more frequently related with local recurrence is the presence of positive surgical margins if resectability is difficult.^[11]

This is the first description of a very small, subcentimeter SFT reported in the literature. Since, in our case, no malignant characteristics were present, a conservative approach after an endoscopic complete resection was adopted. Currently the patient is being followed but without recurrence.

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Nil.

Conflicts of interest

There are no conflicts of interest.

Patient consent

Obtained.

Ethics approval

Ethics approval was obtained prior to the commencement of the study.

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Case Report

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Isolated breast metastasis mimicking as second primary cancer - a case report

Manjari Shah¹, Umang Mithal², Sandeep Agarwal¹, Sweetey Gupta¹, Disha Tiwari³, Shashank Srinivasan¹,
Asheesh Jain⁴, Ritu Chandra¹

¹Department of Radiation Oncology, Max Hospital, Vaishali 201012, Delhi NCR, India.

²Department of Surgical Oncology, Max Hospital, Vaishali 201012, Delhi NCR, India.

³Department of Radiation oncology, King George Medical University, Lucknow 226003, Uttar Pradesh, India.

⁴Consulting Histopathologist, Asheesh Pathology Lab, Uttar Pradesh 250002, India.

Correspondence to: Dr. Manjari Shah, Department of Radiation Oncology, Max Hospital, Vaishali 201012, Ghaziabad, Uttar Pradesh, India.
E-mail: manjarishah29@gmail.com

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Dr. Manjari Shah works with Max Super Speciality Hospital, Vaishali, Delhi NCR, India. She is a lifetime member of Association of Radiation Oncology of India (AROI). She is the Founder Member and Health Forum Co-ordinator at Teachgirls, an organisation which works for empowerment of underprivileged young girls. She has been active participants at various Oncology Conferences and presented posters in National and International conferences. She has won the Best Poster award in ACOS, 2016 (12th International Conference of the Asian Clinical Oncology Society, New Delhi). She sees herself as a learner and is always enthusiastic towards researching and writing.

ABSTRACT

Primary carcinoma of breast is common but breast is a rare site of metastasis and metastases from extramammary sites are even rarer. Metastasis to breast from rectal carcinoma is very unusual and till now 19 cases of breast secondaries from colorectal carcinoma have been reported in literature which include 14 cases where the primary site was colon and remaining 5 were from the rectum. Here the authors report a case of adenocarcinoma anorectum who had completed treatment and after 4 months developed a lump in her left breast which was metastatic. Metastatic lesions of breast are usually part of a widely disseminated disease but this case presented as a solitary breast metastasis which mimicked as second primary cancer of the breast.

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INTRODUCTION

Breast is an unusual site of metastasis.^[1] Contralateral breast is the most common site from which breast

metastases are seen, followed by extramammary sites, viz. leukemia, melanoma, lymphoma, ovary, lung and stomach cancer.^[2,3] Breast metastasis from extra mammary tumor is rare and accounts for



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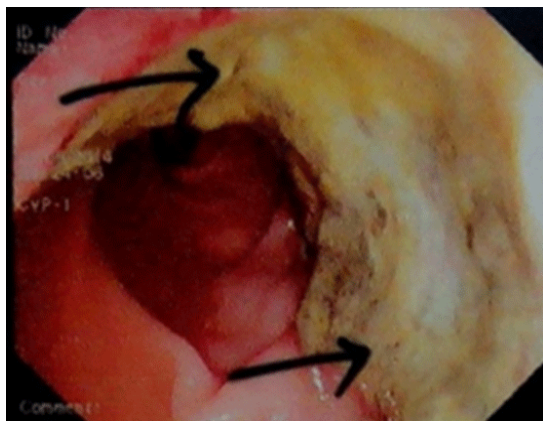


Figure 1: Colonoscopy reported circumferential ulcerative growth in distal rectum and anal canal as the arrows indicated

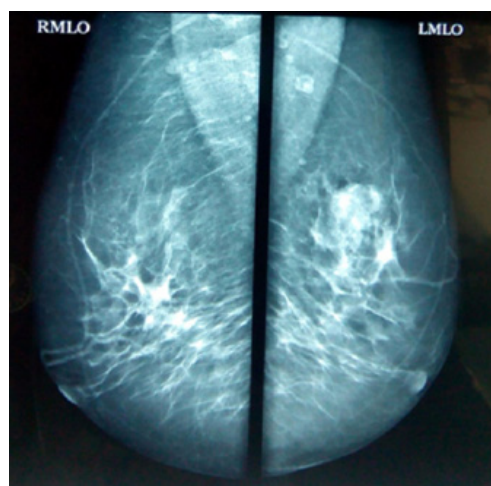


Figure 3: Digital mammography of bilateral breast showing oval hyperdense mass lesion with lobulated margins in upper outer quadrant of left breast

approximately 1.3% to 6.6% of all malignant tumors of breast.^[4] Metastasis from the colon to the breast were first reported by McIntosh *et al.*^[5] and from the rectum by Lal *et al.*^[6] in 1999. It is important to differentiate metastatic disease to the breast from primary breast carcinoma because the management differs in both the scenarios.

CASE REPORT

A 49-year-old female presented to oncology out patient department with complaints of bleeding per rectum and alteration of bowel habit since 1 month. The patient was well built and had Eastern Cooperative Oncology Group performance score of 1. General physical examination was unremarkable. Per-rectal examination revealed ulcero-proliferative growth involving posterior wall of anal canal was palpable at 4 cm from the anal verge. Colonoscopy was done which reported circumferential ulcerative growth in distal rectum and anal canal [Figure 1]. Contrast enhanced computed tomography

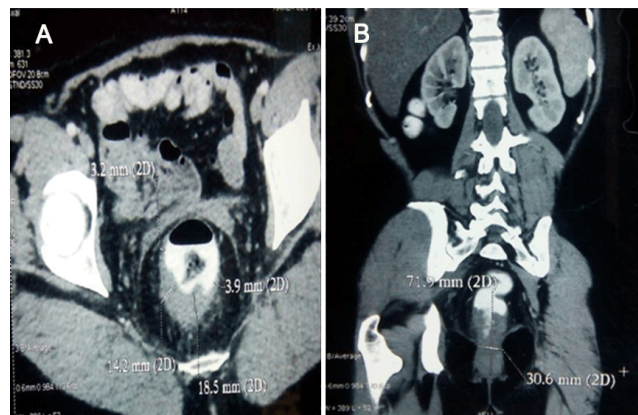


Figure 2: (A) Computed tomography scan of abdomen in axial section showing semi-circumferential mass lesion in anorectal region predominantly involving posterior wall; (B) computed tomography scan of abdomen in coronal section showing 71.9 mm mass lesion in anorectal region

Table 1: IHC markers results in our patient

IHC markers	Results
CK7	Negative
CK20	Positive in majority of tumor cells
mCEA	Positive in majority of tumor cells
ER	Negative, normal breast is positive
GCDFP-15	Negative
MUC-2	Positive in many tumor cells
CDX-2	Positive in many tumor cells
Ki-67	30%

IHC: immunohistochemistry; CK: cytokeratin; mCEA: carcinoembryonic antigen; ER: estrogen receptor; GCDFP-15: gross cystic disease fluid protein; MUC-2: mucin-2; CDX-2: Caudal type homeobox-2

scan of the whole abdomen was done which showed semi-circumferential mass lesion (length 71.9 mm; width 30.6 mm; thickness of mass 3.2 mm to 18.5 mm) in anorectal region predominantly involving posterior wall [Figure 2A and 2B]. All other baseline investigations including a complete hemogram, kidney function tests, liver function tests, and chest X-ray were within normal limits. Biopsy from anorectal mass revealed signet ring adenocarcinoma. She underwent pre-operative external beam radiotherapy 50.4 Gy in 28 fractions with concomitant 5-fluorouracil and leucovorin based chemotherapy followed by radical surgery (abdomino-perineal resection with permanent colostomy) and then adjuvant 5-fluorouracil and leucovorin based chemotherapy. Patient was disease free for 4 months after completion of treatment, and 4 months after completion of treatment, she noticed a lump in her left breast. On clinical examination a lump was palpable approximately 2 cm × 2 cm size in the upper outer quadrant of left breast with no axillary and supraclavicular lymphadenopathy. Digital mammography of bilateral breast was done which revealed oval hyperdense mass lesion with lobulated margins in upper outer quadrant of left breast [Figure 3]. She then underwent

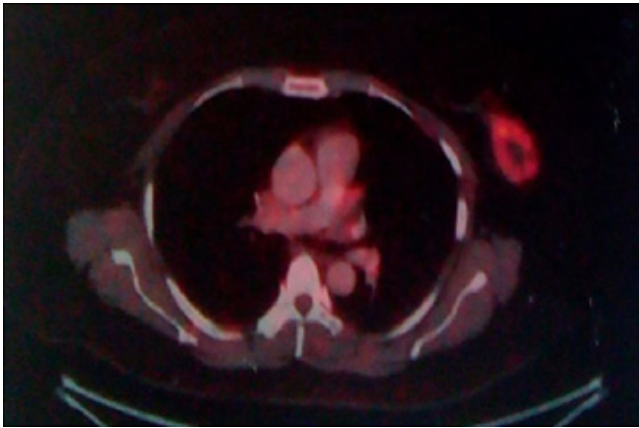


Figure 4: FDG-PET scan showed FDG avid soft tissue density lesion (size 4.2 cm × 2.8 cm SUV_{max} 13.2) in left breast. FDG-PET: fluorodeoxyglucose-positron emission tomography; SUV: standardized uptake value



Figure 5: FDG avid hypermetabolic right inguinal lymph node SUV_{max} 5.1. FDG: fluorodeoxyglucose; SUV: standardized uptake value

a whole body 18-fluorodeoxyglucose (18-FDG) positron emission tomography scan which showed FDG avid soft tissue density lesion of size 4.2 cm × 2.8 cm with standardized uptake value (SUV_{max}) 13.2 in left breast [Figure 4] and hypermetabolic right inguinal lymph node SUV_{max} 5.1 [Figure 5] with no other hypermetabolic focus elsewhere in body. Fine needle aspiration cytology (FNAC) from left breast lump showed single population of atypical epithelial cells suggestive of adenocarcinoma. FNAC from right inguinal node was also done which reported metastasis from adenocarcinoma. Her carcinoembryonic antigen (CEA) and carbohydrate antigen-15.3 was done which was 26.8 ng/mL (Normal 0-4 ng/mL) and 17.2 u/mL (Normal 0-35 u/mL) respectively. In view of isolated breast lesion it was considered as second primary of the breast and the patient was taken up for left modified radical mastectomy. Right iliac and inguinal node dissection was also performed for regional lymph node recurrence from carcinoma anorectum. Post-operative histopathology from left modified radical mastectomy specimen showed mucin secreting signet ring adenocarcinoma with lymphovascular emboli and lymphocytic infiltration. Nine out of 16 dissected left axillary lymph nodes showed metastasis of signet ring adenocarcinoma. Six out of 8 right inguinal lymph nodes and 2 out of 4 right iliac lymph nodes showed metastasis from anorectal carcinoma. Immunohistochemistry (IHC) was performed to ascertain whether the lesion was a primary carcinoma of the breast or metastasis from anorectal carcinoma. Result of IHC markers was as shown in Table 1 and Figure 6. IHC combined with morphology favored signet ring cell metastatic carcinoma to breast.

DISCUSSION

Breast metastases from colon cancer are very rare and

they are usually associated with poor prognosis, due to disseminated disease.^[7] It is of utmost importance to distinguish metastatic carcinoma to the breast from a primary breast carcinoma.^[8] Metastatic spread from anorectal cancer occurs both by lymphatic and hematogenous routes. Owing to the venous drainage into the portal system from the superior hemorrhoidal vein, the liver is the most common site of distant metastasis. Systemic drainage into the inferior vena cava from the inferior hemorrhoidal plexus may lead to metastatic involvement of the lung and bone. Metastases to the breast from anorectal carcinoma without involvement of any of these organs is a rare phenomenon. Schaekelford *et al.*^[8] reviewed 19 cases of colorectal carcinoma metastasizing to the breast and reported a majority of cases with metastases to the left breast 55%, with the right breast 30% and 3 cases with bilateral breast metastasis. In our case, patient had left breast metastasis similar to the observation by Schaekelford *et al.*^[9] The most common site is the upper outer quadrant of the breast. They can occur as synchronous lesions or may follow the primary by months to years. Metastatic breast lesions are typically mobile, well demarcated, firm, rapidly growing, discrete masses and may be confused with benign breast disease due to their often well-circumscribed nature. Rarely these lesions may be multiple or bilateral. The interpretation is difficult in some cases so a history of previous malignancy is important for the radiologist in order to evaluate these breast lesions.^[10,11] Other features suggestive of metastasis to breast are location of the lump in either fat or subcutaneous tissue, lack of micro-calcification in mammogram and lack of *in situ* disease on histopathological examination.^[12,13] The correct diagnosis is therefore crucial in these patients so as to decide the further management of these patients. Histopathology for metastatic lesion may be invasive adenocarcinoma, often with mucinous or signet-ring cell features, but unlike primary lesion of the

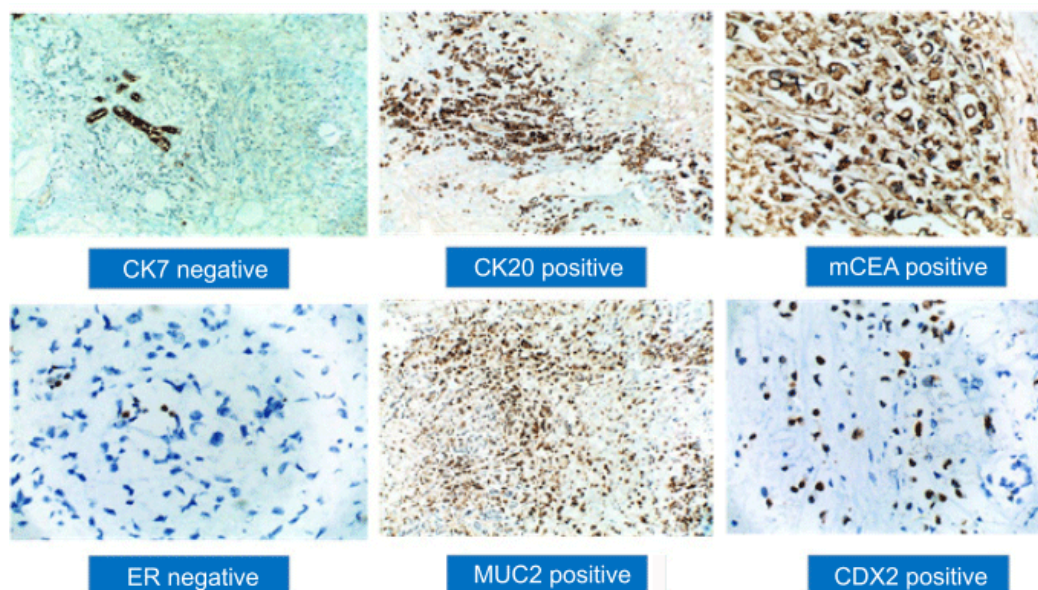


Figure 6: Immunohistochemistry markers results in our patient. CK: cytokeratin; mCEA: carcinoembryonic antigen; ER: estrogen receptor; MUC2: mucin2; CDX2: Caudal type homeobox2

breast they lack an *in situ* component. Lymphovascular space invasion may be prominent. This type of unusual histopathology in breast with previous history of malignancy are suggestive of metastasis. But the final diagnosis is established after studying the cytokeratin pattern. IHC when performed, tends to be positive for colorectal markers like caudal type homeobox-2 (CDX-2), cytokeratin (CK20), and CEA, and negative for breast markers CK7, estrogen receptor, progesterone receptor, human epidermal growth factor receptor-2, and gross cystic disease fluid protein-15.^[14,15] Expression of CK7 and CK20 is considered to be most helpful in identifying the origin of adenocarcinomas.

Most importantly, the great majority of primary breast tumors are CK7-positive and CK20-negative, while colorectal carcinomas are usually CK7-negative and CK20-positive.^[16,17] IHC markers used in our case were consistent with these findings as shown in Table 1. The strong nuclear positivity with CDX-2 is highly sensitive and specific for colonic cancers.^[18] In addition, estrogen and progesterone receptors are usually negative in metastatic breast cancers. A patchy reaction for CK5/6 and comedo like necrosis can mimic ductal carcinoma *in situ* disease. Histological features such as epithelial stratification, high nuclear atypia, significant mitotic activity, and positive reactions for CK20 and CDX-2 can help to overcome this difficulty. Metastatic carcinomas in the breast are associated with a poor prognosis with a survival rate of less than 12 months from the time of breast tumor diagnosis.^[16,19,20]

Metastatic disease in the breast is a marker for disseminated metastatic spread, and therefore

indicates a poor prognosis. Metastases to the breast are rare in themselves, and such metastasis occurring secondary to a previous anorectal carcinoma makes this case very unusual. The liver, lungs and bone are the usual sites of spread from colorectal cancers. Breast metastases with sparing of these organs is unlikely but possible. Our patient presented with an isolated breast lump and without any other complaints. She was managed considering the lesion to be second primary cancer of the breast but post operative histopathology with IHC showed it to be metastases. On the basis of histopathology showing adenocarcinoma and history of previous malignancy alone, the diagnosis of lesion being metastasis to breast should not be arrived upon and in such patients the importance of IHC to exclude the diagnosis of primary breast lesion cannot be undermined.

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Nil.

Conflicts of interest

There are no conflicts of interest.

Patient consent

Obtained.

Ethics approval

Ethics approval was obtained prior to the commencement of the study.

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Original Article

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Sunitinib effectiveness and safety as first line treatment in metastatic renal cell carcinoma, in the Costa Rican population

Esteban Gonzalez¹, Silvia Alfaro², Allan Ramos-Esquivel^{3,4}, Denis Ulises Landaverde^{1,4}

¹Department of Hemato-Oncology, Hospital Mexico, La Uruca, San Jose 10107, Costa Rica.

²Department of Hemato-Oncology, Hospital Calderon Guardia, Guadalupe, San Jose 10801, Costa Rica.

³Department of Hemato-Oncology, Hospital San Juan de Dios, Distrito Hospital, San Jose 10103, Costa Rica.

⁴Department of Medicine, Universidad de Costa Rica, San Pedro de Montes de Oca, San José 2060, Costa Rica.

Correspondence to: Dr. Denis Ulises Landaverde, Department of Hemato-Oncology, Hospital Mexico, CCSS, 76th Street, 41st Avenue, La Uruca, San Jose 10107, Costa Rica. E-mail: denislandaverde@gmail.com; denis.landaverderecinos@ucr.ac.cr

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Dr. Denis Ulises Landaverde completed his medical degree and postgraduate training in Medical Oncology at the University of Costa Rica and also completed a fellowship in Breast Cancer at the University of Toronto, Canada. Currently he is the Chief of Medical Oncology Division at Mexico Hospital, in San Jose, and Professor of Medicine at the University of Costa Rica.

ABSTRACT

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Sunitinib,
renal cell carcinoma,
effectiveness,
Latin America,
Costa Rica

Aim: Tyrosine kinase inhibitors are part of the armamentarium to treat metastatic renal cell carcinomas (mRCC). Costa Rica has approved sunitinib in the first line setting. The authors conducted a retrospective study to address the effectiveness and safety profile of sunitinib in our population in terms of overall survival (OS) and progression free survival (PFS). **Methods:** The authors analyzed all patients who were treated with sunitinib diagnosed with mRCC in the three National Hospitals (Hospital Mexico, Hospital San Juan de Dios, and Hospital Calderon Guardia) from February 2007 to June 2015. Demographics, safety profile, and efficacy (OS and PFS) were obtained from medical records. OS and PFS were calculated using the Kaplan Meier method and a Cox Proportional Model Analysis was used when OS and PFS were compared in subset of patients. **Results:** Seventy-seven patients were included; mean age was 58.9 years. Fifty-four patients were male (70.1%). The most common histologic type was clear cell carcinoma (87%), followed by papillary (9.1%) and chromophobe (2.0%) types. Median OS was 21.0 months [95% confidence interval (CI): 13.42-28.58]. Median PFS was 13.7 months (95% CI: 11.24-16.16). Patients aged 65 years or older experienced worse PFS and OS than younger patients (median PFS: 8.2 vs. 17.6 months; $P = 0.011$) (median OS: 19.0 vs. 29.0 months; $P = 0.022$). Sunitinib was well tolerated and no serious side effects were reported. **Conclusion:** This is the first study in Central America showing that sunitinib, first line, in mRCC is as effective as reported in pivotal clinical trials and expanded use studies in terms of PFS and OS.



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INTRODUCTION

Renal cell carcinoma (RCC) accounts for about 3% of all adult cancers, is the 8th most common cancer in Central America and the 10th worldwide, and the clear-cell RCC (ccRCC) is its most frequent histologic subtype.^[1-4]

Surgery remains the standard of care for localized disease, and can often be curative.^[5,6] Unfortunately, metastatic RCC (mRCC) is found in approximately one third of patients.^[7] Furthermore, RCC is extremely resistant to conventional chemotherapy.^[8] That is why different treatment strategies had been developed, taking into account improvements in understanding RCC biology and tumor behavior. RCC is highly vascularized due to overexpression of vascular endothelial growth factor (VEGF) induced by alterations of the tumor suppressor gene, Von Hippel-Lindau (VHL), leading to the increase of hypoxia-inducible factors 1 alpha and 2 alpha, ending in angiogenesis.^[9] This has allowed the development of VEGF inhibitors such as tyrosine-kinase inhibitors (TKIs), monoclonal antibodies against VEGF, and mammalian target of rapamycin (mTOR) inhibitors.^[5]

In Costa Rica the National Health Care System (Caja Costarricense de Seguro Social, CCSS) has authorized the use of sunitinib to treat mRCC in first line setting.^[10] Sunitinib is a multiple TKI, including the VEGF receptors (VEGFRs) and platelet-derived growth factor receptors, producing a strong antitumor action in mRCC^[11] and is approved worldwide as upfront line treatment of mRCC, with the reporting of significant objective response rates and also superiority over interferon-alfa in progression-free survival (PFS), with a trend to increase overall survival (OS).^[12,13]

In this retrospective study we evaluated the effectiveness of sunitinib in the Costa Rican population in terms of median overall survival (mOS), median progression free survival (mPFS) and its safety profile.

METHODS

Patients and study design

This is a retrospective study reviewing the medical records from a total of 77 patients treated with sunitinib as first-line therapy in mRCC. Data were collected between February 2007 and June 2015 in the three major hospitals (Hospital San Juan de Dios, Hospital Calderon Guardia and Hospital Mexico) in San Jose, Costa Rica. All patients were required to be at least 18 years of age and to have histologically confirmed mRCC (regardless of histologic subtype). The Ethics

Committees in each hospital approved this study. All patients received oral sunitinib maleate, 50 mg once daily for 4 weeks of a 6-week treatment cycle (4 weeks on, 2 weeks off). The dosage was reduced in some cases to 37.5 mg daily. Sunitinib was given until disease progression or unacceptable toxicity. Physical examination and clinical laboratory tests were performed approximately one or two days before each cycle. Adverse events were registered according to the National Cancer Institute (NCI) common terminology Criteria for Adverse Events (CTCAE), version 3.0. Tumor evaluation was performed according to Response Evaluation Criteria in Solid Tumors (RECIST) version 1.0, this assessment being done in accordance with local practices at each hospital. PFS was defined as from time of starting sunitinib to disease progression or death from any cause (death could occur within one month of the last treatment dose and was included in the PFS analysis). OS was defined as the time from start of sunitinib to death from any cause.

Statistical analysis

In this retrospective study we included all patients who received sunitinib during the observational period of time in Costa Rica. For that reason there were neither pre-specified sample sizes nor pre-established hypotheses to evaluate. Categorical variables are presented as percentages. Continuous variables are presented as the mean \pm standard deviation. To assess the PFS and OS the Kaplan-Meier method was used. A Cox Proportional Model Analysis was employed to determine differences in the outcome variables according to age less or higher than 65 year. In addition univariate and multivariate analyses were used to explore the association between OS and PFS with prognostic factors. A *P* value less than 0.05 was considered statistically significant. Data were analyzed using SPSS for Mac version 20.0 (SPSS, Chicago, IL).

RESULTS

A total of 77 patients were included in the study. Patient characteristics are described in [Table 1](#). All patients received sunitinib as first line treatment, while none was previously treated either with cytokines or TKIs. With a median follow-up of 18.9 months, mPFS was 13.7 months [95% confidence interval (CI): 11.24-16.16 months], and mOS was 21.0 months (95% CI: 13.42-28.58 months) [[Figure 1](#)].

A statistically significant difference was found in terms of PFS and OS according to patient age, risk of progression as well as risk of death by disease. This was higher in patients 65 years or older in comparison

Table 1: Patient characteristics

	All patients (n = 77)
Median age (years, range)	58.9 (47.4-70.4)
Patients older than 65 years (%)	25 (32.4)
Gender (%)	
Female	23 (29.9)
Male	54 (70.1)
ECOG/PS (%)	
0	60 (77.9)
1	10 (12.9)
2	7 (9.2)
Histological variant (%)	
Clear cell carcinoma	67 (87.0)
Papillary	7 (9.1)
Chromophobe	2 (2.6)
Collecting duct carcinoma	1 (1.3)
MSKCC risk classification (%)	
Low	47 (61.0)
Intermediate	25 (32.4)
High	5 (6.5)
Site of metastasis (%)	
Lung	55 (52.8)
Bone	19 (18.3)
Liver	16 (15.3)
Central nervous system	10 (9.6)
Other	4 (3.8)

ECOG: Eastern Cooperative Oncology Group; PS: performance status; MSKCC: Memorial Sloan Kettering Cancer Center

to those with less than 65 years. mPFS was 17.6 months (95% CI: 10.2-25.0 months) vs. 8.2 months (95% CI: 0.1-16.4 months); hazard ratio (HR): 1.93 (95% CI: 1.2-3.2); $P = 0.011$; mOS was 29.0 months (95% CI: 11.4-46.5) vs. 19.0 months (95% CI: 11.0-26.9, HR = 1.82; 95% CI = 1.1-3.1); $P = 0.022$ [Figure 2]. These findings were confirmed in univariate and multivariate analyses [Tables 3 and 4], showing that age was an independent prognostic factor either for PFS or OS.

There was no difference in PFS by gender or histological

variant [Table 2]. However, a significant difference was found in mOS according to histological subtype in favor of ccRCC when compared with non-clear cell carcinoma: 26.8 months (95% CI: 20.1-30.5) vs. 14.2 months (95% CI: 0-29.0); HR: 3.41 (95% CI: 1.6-7.3; $P = 0.001$) [Figure 3]. When univariate and multivariate analyses were performed, it was found that ccRCC was an independent prognostic factor in terms of OS but not PFS [Tables 3 and 4].

Sunitinib was, in general, well tolerated. There were 17 patients (22%) who received a dose reduction to a 37.5 mg daily schedule due to grade 1 or 2 toxicities; no grade 3 or 4 toxicities were registered. Diarrhea and hand-foot syndrome were the most commonly adverse reactions described [Table 5].

DISCUSSION

According to international RCC treatment guidelines, sunitinib is currently one of the preferred options to treat metastatic clear cell renal cell carcinoma (mccRCC).^[14,15] Its efficacy and safety have been evaluated in a phase III pivotal study and the global expanded-access trial (GEAT).^[16-18] There are few data in Latin America regarding the effectiveness of sunitinib. In the GEAT trial, it was reported that a subset analysis of 348 Latin American patients showed a mPFS and a mOS of 12.1 and 16.9 months, respectively.^[19,20] The final analysis of this global trial including more than 4,500 patients demonstrated a mPFS of 9.4 months and a mOS of 18.7 months.^[18] In the present study we obtained a mPFS of 13.7 and a mOS of 21.0 months, very similar to the results reported globally. This strongly suggests that sunitinib has the same effectiveness in the Latin American population as previously assessed in the pivotal trial and the GEAT, supporting the use of

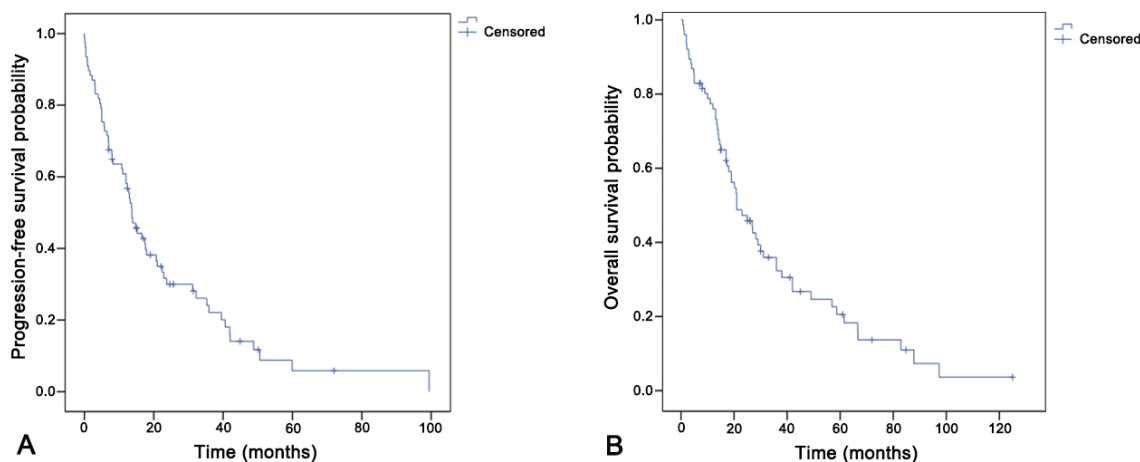


Figure 1: (A) Probability of progression-free survival in all patients: 13.7 months (95% CI: 11.24-16.16 months); (B) probability of overall survival in all patients: 21.0 months (95% CI: 13.42-28.58 months). CI: confidence interval

Table 2: Progression-free survival and overall survival by gender and histological variant

		mPFS	mOS
Gender	Female	10.8 months (95% CI: 3.1-18.5)	18.0 months (95% CI: 13.2-22.8)
	Male	15.2 months (95% CI: 10.9-19.5)	23.0 months (95% CI: 16.2-29.7)
		(HR: 1.21; 95% CI: 0.71-2.0; $P = 0.49$)	(HR: 1.23 95% CI: 0.71-2.11; $P = 0.46$)
Histology	Clear cell carcinoma	15.2 months (95% CI: 10.8-19.7)	26.8 months (95% CI: 20.1-30.5)
	Non-clear cell carcinoma	8.2 months (95% CI: 0-19.5)	14.2 months (95% CI: 0-29.0)
		HR: 1.84 (95% CI: 0.9-3.76); $P = 0.089$	HR: 3.41 (95% CI: 1.6-7.3); $P = 0.001$)

CI: confidence interval; HR: hazard ratio; mPFS: median progression-free survival; mOS: median overall survival

Table 3: Univariate and multivariate analyses of potential prognostic variables for overall survival

Variable	Univariate hazard ratio (95% CI)	P value	Multivariate hazard ratio (95% CI)	P value
Male sex	0.77 (0.44-1.36)	0.372	0.88 (0.49-1.55)	0.659
Clear cell histology	0.29 (0.13-0.63)	0.002*	0.34 (0.16-0.76)	0.008
Age ≥ 65 years	2.15 (1.26-3.69)	0.005*	1.97 (1.14-3.04)	0.015*

CI: confidence interval

Table 4: Univariate and multivariate analyses of potential prognostic variables for progression-free survival

Variable	Univariate hazard ratio (95% CI)	P value	Multivariate hazard ratio (95% CI)	P value
Male sex	0.82 (0.48-1.41)	0.497	0.81 (0.46-1.42)	0.464
Clear cell histology	0.54 (0.26-1.12)	0.096	0.62 (0.27-1.31)	0.214
Age ≥ 65 years	2.21 (1.31-3.72)	0.003*	2.21 (1.30-3.76)	0.003*

CI: confidence interval

Table 5: Sunitinib-related toxicities*

	Frequency (%)
None	24 (31.2)
Diarrhea	12 (15.6)
Fatigue	1 (1.3)
Hand-foot syndrome	11 (14.3)
Hypertension	2 (2.6)
Not reported	27 (35.1)

*Only grade 1 and 2 toxicities were reported

this drug as the standard of care in first line mRCC in Costa Rica.

Surprisingly, when analyzed by age, it was found that patients aged ≥ 65 years, experienced worse PFS and OS than younger patients (< 65), mPFS: 8.2 vs. 17.6 months; ($P = 0.011$) and mOS: 19.0 vs. 29.0 months

($P = 0.022$). This was seen, as well, when univariate and multivariate analyses were performed. These findings have not been previously reported. Another study^[21] published a retrospective pooled analysis from 1059 patients in six prospective trials. The authors found that, across the entire pooled sunitinib-treated population in the first line setting, PFS and OS were not different in younger and elderly patient aged 70 and ≥ 70 years, respectively: mPFS was 9.9 vs. 11.0 months with a HR of 0.89 (95% CI: 0.73-1.09; $P = 0.2629$), while mOS was 23.6 vs. 25.6 months, with an HR of 0.93 (95% CI: 0.74-1.18; $P = 0.5442$). Also, the GEAT study was not able to identify differences among patients by age, either regarding OS or PFS.^[18] There is no clear explanation to these findings. However,

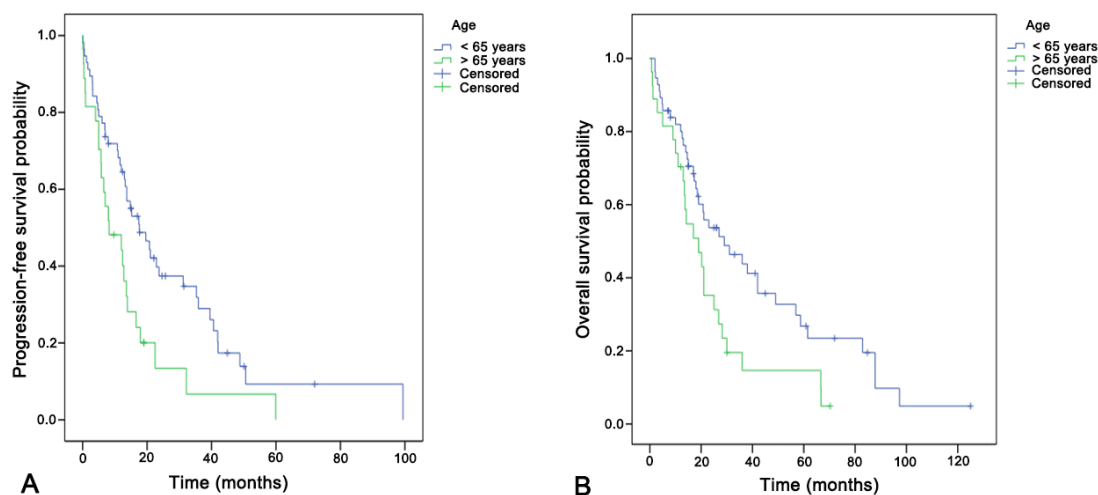


Figure 2: (A) Probability of progression-free survival according to age: less than 65 years: 17.6 months (95% CI: 10.2-25.0) and 8.2 months (95% CI: 0.1-16.4) in patients older than 65 years. HR = 1.93 (95% CI: 1.2-3.2); $P = 0.011$; (B) probability of overall survival according to age, 29.0 months (less than 65 years) (95% CI: 11.4-46.5) vs. 19.0 months (older than 65 years) (95% CI: 11.0-26.9) (HR = 1.82; 95% CI: 1.1-3.1); $P = 0.022$. CI: confidence interval; HR: hazard ratio

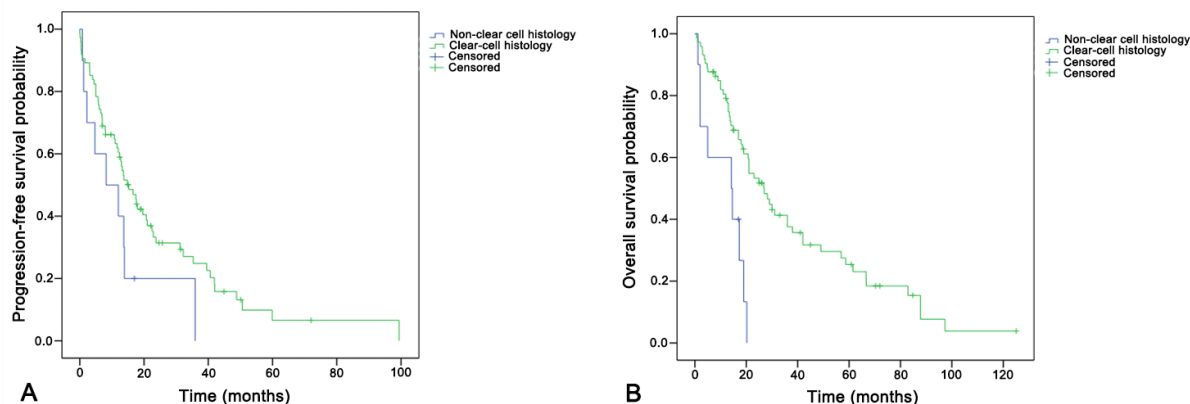


Figure 3: (A) Probability of progression-free survival according to histology. Median PFS: 15.2 months (clear cell histology) vs. 8.2 months (non-clear cell histology) HR = 1.84 (95% CI: 0.9-3.76); $P = 0.089$; (B) probability of overall survival according to histology: 26.8 months (clear cell histology) (95% CI: 20.1-30.5) vs. 14.2 months (non-clear cell histology) (95% CI: 0-29.0) HR = 3.41 (95% CI: 1.6-7.3; $P = 0.001$). PFS: progression-free survival; CI: confidence interval; HR: hazard ratio

this could be due to intrinsic characteristics of the Costa Rican population. To address this observation, a different statistical analysis in this subset of patients was performed, including performance status, dosage received, and MSKCC risk. However, it was not possible to find a strong correlation with any of these factors. Thus, it could be possible that this is specific for Latin Americans. Further study might be warranted.

In accordance the pivotal sunitinib phase III trial, the GEAT study, and other mainly retrospective studies involving small number of patients^[16,18,22-25] no differences by gender in terms of OS or PFS in the present study were found.

Sunitinib has shown only modest activity for the treatment of advanced and/or metastatic non-clear cell RCC, mPFS reported from 11 of 12 studies in a recently published systematic review ranged from 1.6 to 8.9 months and mOS in 9 studies in the same review ranged from 12 to 22 months. Both mOS and mPFS are less than reported for mCCRCC.^[26] Interestingly, the present study obtained, in non-clear cell RCC, a mPFS of 8.2 months and a mOS of 14.2 months, keeping in line with the global literature. However, when an exploratory analysis comparing PFS and OS by histological variant was performed, mPFS for mCCRCC was not statistical different from non-clear cell mRCC. Nevertheless, mOS was significantly superior in favor of mCCRCC (26.8 months vs. 14.2 months), a finding also confirmed in univariate and multivariate analyses. The explanation of this PFS, taking into account numerous confounders such small number of patients in the non-clear cell mRCC arm and possible patient selection bias, is that 7 patients had papillary histology and 1 had a chromophobe type, both histologies having demonstrated to be responders to TKIs.^[27,28] With these findings, the use of sunitinib in

either non-clear cell mRCC or mCCRCC in the Costa Rican population can be supported.

Regarding the safety profile, sunitinib was well tolerated, with diarrhea and hand-foot syndrome being the most common adverse events, with no grade 3 or 4 toxicities. In the GEAT study, diarrhea and fatigue were the most common side effects reported, and hand-foot syndrome was only in the 8th position.^[18]

Although this study has some limitations due to its retrospective design and relatively small sample size, it provides real-world effectiveness of this treatment in this particular population.

In conclusion, sunitinib exerts important activity in mRCC in the Costa Rican population, demonstrated a mPFS and a mOS similar to pivotal and expanded access trials. Sunitinib seems to be more effective in younger patients than in patients aged 65 or more years. It is also well-tolerated regardless patients age.

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Nil.

Conflicts of interest

There are no conflicts of interest.

Patient consent

Patient consent was obtained from the patients.

Ethics approval

Ethics approval was obtained prior to the commencement of the study.

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Case Report

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Metastatic clostridial myonecrosis secondary to perforated metastatic bowel cancer

Nasser Mohammed Amer¹, John Karayanis²

¹King Fahad Hospital of the University, Al Khobar 31952, Saudi Arabia.

²Locum Consultant General Surgery, Hereford County Hospital, Hereford HR1 2ER, United Kingdom.

Correspondence to: Dr. Nasser Mohammed Amer, King Fahad Hospital of the University, P O Box 40262, Al Khobar 31952, Saudi Arabia.
E-mail: nasser@nasseramer.com

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ABSTRACT

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Metastatic gangrene,
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Spontaneous metastatic clostridial myonecrosis is a rare condition caused by *Clostridium septicum*. The underlying lesion is usually either a colonic neoplasm or leukemia. The authors reported a 67-year-old female who presented with acute abdomen secondary to a perforated sigmoid cancer and who developed gas gangrene in her right leg. Unfortunately, despite all resuscitative measures, she died. The authors reviewed the literature; the diagnosis of metastatic myonecrosis was based on a high index of suspicion, development of bullae containing gram-positive rods, and subcutaneous crepitus (although this was a late sign). Treatment involves aggressive fluid replacement, high doses of intravenous penicillin, high concentration of oxygen, and surgical debridement, and/or amputation. The mortality remains very high, despite all the above measures.

INTRODUCTION

Metastatic clostridial myonecrosis is an uncommon complication of malignancy, particularly of the gastrointestinal tract, and of leukemia. Without treatment the mortality rate reaches 100% within 48 h.^[1-4] A number of reports have demonstrated the association between atraumatic clostridial infection and internal malignancy.^[5]

We reviewed the literature, which demonstrated the paramount importance of early diagnosis and institution

of early aggressive management. We reported a case of a 67-year-old woman who developed sudden myonecrosis in her right thigh secondary to perforated large bowel cancer.

CASE REPORT

A 67-year-old female was admitted to a district general hospital via a general practitioner referral, complaining mainly of pain in the epigastrium for the past four days. Pain became worse and more constant on the day of



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admission, radiating to her chest. The patient claimed milder episodes of similar attacks for the past four months. The pain was associated with nausea, but no vomiting, no change in her bowel habits, and no significant weight loss.

Her past medical history involved rheumatoid arthritis, hypertension, and rheumatic heart disease. She was receiving azathioprine and bendroflouazide for her hypertension.

On examination, the patient appeared toxic, pale but not jaundiced, and very restless. She had a temperature of 38.7°C, her blood pressure was 108/61 mmHg, and her pulse was 100 per minute, regular but weak. Examination of her heart revealed a fine diastolic murmur and a small splinter hemorrhage in the right ring finger. There was no evidence of heart failure. Abdominal examination revealed tenderness in the epigastric area and guarding in the right upper quadrant along with a palpable left lobe of the liver. Rectal examination was normal with no evidence of blood.

Results of the patient's biochemical tests showed sodium 128, potassium 3, urea 5, creatinine 83, aspartate transaminase 66, alanine transaminase 25, layered double hydroxide 781, alkaline phosphatase 150, C reactive protein 351, bilirubin 30, hemoglobin 8.8, and white blood count 5,300. The chest and abdominal radiographs were normal with no evidence of air under the diaphragm. The initial impression was of possible acute cholecystitis or peritonitis. The patient was resuscitated with intravenous fluids and oxygen and was given intravenous penicillin, gentamicin, and clindamycin.

Despite the aggressive resuscitation, the patient's condition deteriorated. A small area of dusky blue discoloration about 6 cm × 4 cm appeared in the right popliteal fossa; this area was noticed to expand gradually. The leg became increasingly painful, and bullae appeared in the same area. Fine subcutaneous crepitation was noted in the same leg and was confirmed by plain X-ray [Figure 1], demonstrating gas in the soft tissue. Diagnosis of gas gangrene was established based on an aspirate from one of the blisters, which revealed gram-positive rods. Orthopedic involvement was sought; a decision was made to take the patient for hind-quadrant amputation, along with an exploratory laparotomy and possible Hartmann's procedure. Unfortunately, the patient did not survive the anesthesia and experienced cardiac arrest during induction.

The postmortem study revealed a perforated 4-cm sigmoid colon cancer with evidence of peritonitis, and

a second primary cecal cancer 6 cm in diameter. There was evidence of metastases in liver, lungs, and para-aortic nodes. The report also confirmed the presence of gas gangrene in the right thigh as well as the presence of subacute bacterial endocarditis.

DISCUSSION

Nontraumatic clostridial myonecrosis secondary to an underlying bowel cancer is a fulminant and often fatal infection caused by *Clostridium septicum*.^[4,6] This is in contrast to most other cases of clostridial septicemia which are caused by *Clostridium perfringens*.^[1,7] The mortality is high even with aggressive management, and it can reach 100% if not treated within 48 h.^[3,4]

In a review by Kornbuth et al.^[7] of 162 cases of spontaneous *C. septicum* infection from the years 1945 to 1987, 34% of patients had colorectal cancer while 40% had a hematologic malignancy. In 37% of the patients, the malignancy had not been diagnosed. Distant myonecrosis had an even greater association with occult colon cancer (see Table 1 for causes of metastatic clostridial gangrene^[8]).

Pathogenesis

Myonecrosis is caused by Clostridia organisms, which are gram-positive rods that sporulate and are found in the soil.^[1,3,7] *C. septicum* is more aerotolerant^[8] and the inoculum required for infection is 300 times smaller than that of *C. perfringens*.^[7] It is commonly found as a normal inhabitant of the gastrointestinal and genitourinary tracts.^[1,9] The spores usually exist for years and vegetate when conditions become optimal. *C. septicum*, however, is an opportunistic pathogen in humans, gaining entry to the bloodstream via breaches in the mucosa. This may be due to tumor necrosis^[4] or necrotizing colitis in patient with leukemia

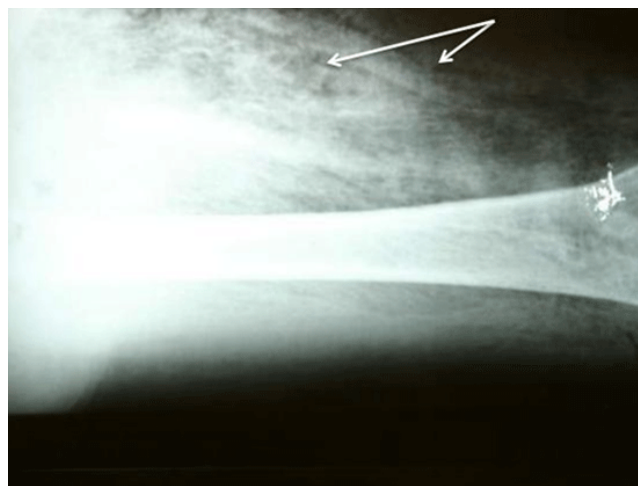


Figure 1: Plain radiograph of the right thigh. Arrows show subcutaneous gas from gas-forming organism

Table 1: Underlying conditions in nontraumatic clostridial gangrene^[8]

Gastrointestinal factors	
Colon tumor	
Ulceration of mucosa	
Chemotherapy	
Radiation	
Instrumentation	
Bowel infarction	
Intestinal surgery	
Diverticulitis	
Necrotizing enterocolitis	
Ileitis or colitis	
Fecal impaction	
Intussusception	
Volvulus	
Straining at stool	
Systemic factors	
Leukemia	
Lymphoproliferative disorder	
Diabetes mellitus	
Metastatic tumor (nongastrointestinal) with chemotherapy	
Neutropenia	
Cirrhosis	

or cyclic neutropenia.^[5] Presumably, the anaerobic glycolysis and the acidic milieu within a tumor provide a favorable environment for the germination of the clostridial spores.^[5] The affinity for necrotic tissue is not specific for malignant tissue only, as demonstrated by Thiele *et al.*^[5] where spores of *Clostridia* were injected in necrotic tissue other than tumor and were found to germinate. This study may explain the predilection of *C. septicum* for patients with necrotic colon cancer, spontaneous bowel perforation, chemotherapy treatment, surgery, or medical procedure such as endoscopy or barium enema. Once established, *C. septicum* may either cause a locally invasive infection or spread via the bloodstream to distant skeletal muscle (causing myonecrosis) or to other organs^[4] (producing abscesses that may be indistinguishable from metastasis).^[3] Clostridial organisms produce toxins that are responsible for the rapid spread and systematic toxicity of these infections.^[3] *C. septicum* is believed to produce four toxins, one of which is hemolysin, which is oxygen stable. In addition, it also produces a deoxyribonuclease, a hyaluronidase, and oxygen labile hemolysins. Secondary toxicity may result from the products of tissue breakdown such as creatinine phosphokinase (CPK).^[3] Diabetics,^[5,6,9] on the other hand, seem susceptible to developing spontaneous gas gangrene. This is most likely due to their propensity to develop focal tissue ischemia and acidosis secondary to atherosclerosis and microangiopathic vascular disease, which allow the circulating *Clostridia* organism to propagate in the hypoxic area. On the other hand, suppurative infection without signs of myonecrosis or toxemia is the most common form of clostridial disease.^[5]

The following is a histotoxic classification of gas gangrene (based on MacLennan's monograph):^[5]

I. Traumatic:

- A. Simple contamination (no clinical evidence of sepsis)
- B. Anaerobic cellulitis (local gas gangrene, with healthy muscle not invaded, e.g. pressure sores, diabetic foot ulcer)
- C. Anaerobic myonecrosis (classical, with invasion of living muscle)

II. Nontraumatic or idiopathic^[9,11] (typically arising from visceral intra-abdominal catastrophes, such as perforated cecal cancer)

- A. Anaerobic cellulitis
- B. Contiguous myonecrosis
- C. Metastatic myonecrosis

Clinical course

Gas gangrene is a rapidly spreading infection.^[7] It can advance as fast as 2 cm per hour. The incubation period varies from 6 h to 2 days.^[1] The bacilli produce several exotoxins, which can destroy the host tissue and increase permeability. The resultant necrosis, edema, and ischemia favor clostridial reproduction in which more toxins are released, and a cycle ensues.

Carbon dioxide and hydrogen are liberated during the process, which opens fascial planes and facilitates spread. The pathogenesis of subcutaneous emphysema from disruption of the gastrointestinal tract depends on localized bowel wall weakness,^[12] the anatomic site, and an increased pressure gradient between the bowel lumen and extramural tissue. The perforation occurs at a point of weakness in the bowel wall where vigorous peristaltic movement produces a large pressure gradient, precipitating rupture of the disease site. When subcutaneous emphysema occurs, it is usually confined to the anterior abdominal wall. From there it passes to the lower extremity via the femoral canal or along the iliopsoas muscle to its insertion into the lesser trochanter of the femur. The gas then spreads freely along the fascial planes towards the knee.

The two main types of gas-forming inflammatory processes^[11,13] are:

1. Emphysematous cellulitis; accounts for the vast majority of gas-forming infection in hospital practice.
2. Emphysematous myositis (gas gangrene).

Patients usually complain of severe pain,^[2] disproportionate pain,^[3,6,14] and sometimes describe the sensation as "heavy".^[3,15] Patients are usually

anxious,^[2] restless, apprehensive, and tachycardic but normotensive. Gas in the tissue is a late sign;^[2,6,15] it may be absent altogether.^[2,5,14] Gas in the tissue is neither a sensitive nor a specific sign of clostridial infection.^[5] It can be found with *E. coli*, *Streptococcus*, *Proteus*, *Enterococcus*, *Staphylococcus*, *Klebsiella*, *Pseudomonas*, and *Bacteroides* species, particularly in patients with diabetes. The skin shows bronze coloration around the area involved,^[2,15] and bullae develop that are filled with mousy-smelling fluid containing gram-positive rods. Patients usually experience profound metabolic acidosis, and hemolysis caused by the exotoxins may cause fever, hypotension, disseminated intravascular coagulation, and renal failure.

Diagnostic feature

Gram stain from the bullae renders gram-positive bacilli^[6] without spores and very low leukocyte count. The skin^[8] around the bullae is purple, reflecting the vascular compromise that results from diffusion of bacterial toxins in the surrounding tissues. X-ray of the affected limb reveals soft-tissue gas^[2,3] [Figure 1]; however, CT scan has been shown to be a more sensitive test.^[13] Clinically, the muscle looks dark and cooked, and it does not contract when incised.^[5] Other lab results may reveal evidence of hemolysis, hyperbilirubinuria, hyperkalemia, and anemia as a result of the release of toxins.^[3]

Management

Initial treatment involves high oxygen concentration and aggressive volume expansion with intravenous isotonic crystalloid fluid.^[3] Volume status should be monitored via urinary output and central venous pressure. Blood should be given sparingly, since it will be hemolyzed rapidly. Brummelkamp^[16,17] advised delay in transfusion till exotoxin production and hemolysis are brought under control with hyperbaric oxygenation. Vasopressors should be avoided, and severe acidosis should be treated with bicarbonate. Antibiotics should be started, with penicillin G being the antibiotic of choice,^[4,5,7,13,15] given in high doses of 20–40 million units. Alternatively, in the event of penicillin allergy, cephalothin, clindamycin, or metronidazole can be used. Sodium penicillin is preferable to potassium penicillin^[2,3,13] because the patient is already at risk of hyperkalemia from tissue breakdown.

The use of hyperbaric oxygen for the treatment of gas gangrene remains controversial.^[7] Brummelkamp reported that 21 (81%) of 26 patients with clostridial infection who received hyperbaric oxygen survived.^[8,16] Results from more recent study showed survival of 70% of patients treated with hyperbaric oxygen and 30% not treated with hyperbaric oxygen. The rationale

behind this treatment is that, due to the hypovascularity of the infected site, an extremely high concentration of dissolved oxygen is necessary to raise the tissue pO₂. Hyperbaric oxygen is believed to reduce the general toxicity of circulating clostridial toxins^[8] and to limit the spread of infection.^[2] In addition, hyperbaric oxygen reduces the spore granulation rate and aids eradication of the organism both *in vitro* and *in vivo*.^[15] The α toxin production is suppressed at an oxygen tension of 250 mmHg. This is achieved by the production of oxygen free radicals.^[8] Hyperbaric oxygen is also believed to protect the viability of healthy tissue surrounding an area of progressive necrosis. The accepted treatment now is five hyperbaric sessions at three atmospheric pressure within the first 48 h,^[3] up to a total of seven to ten sessions.

Surgical treatment

Surgery remains the critical life-saving intervention and should not be delayed in the interest of transferring a patient to a facility with hyperbaric oxygen.^[7] In the absence of adequately debrided wound, antibiotic will not prevent gas gangrene.^[15] Surgery ranges from simple fasciotomy to radical debridement/amputation.^[3,4] In a study on dogs, Domello^[18] showed that surgery alone or with hyperbaric oxygen left no survivors, whereas surgery with antibiotics left 70% survivors. Antibiotics alone left 50% survivors.

There is a better outcome when patients undergo one hyperbaric session prior to initial debridement, and further debridement can be planned between subsequent hyperbaric treatments. The deferment has the following advantages:

1. The patient has better improved general condition.
2. Surgery is limited to the removal of necrotic tissue alone.
3. Necrotic tissue is better demarcated.^[2]

When fasciotomy is necessary, the procedure should always be performed prior to hyperbaric O₂ treatment.^[7] Even if the diagnosis is in doubt, it is better to begin antibiotic and hyperbaric oxygen treatment promptly rather than to take a wait-and-see approach.^[15] Antitoxin has been shown to be of no value in preventing the spread of clostridial infection.

Finally, patients who do survive should be screened for colonic or hematologic malignancy.^[4]

In conclusion, clostridial myonecrosis is a rapidly spreading infection which is fatal unless recognized early. Purple discoloration in a limb of a sick patient, with or without crepitation, should be taken as a sinister sign, and early aggressive treatment with

fluids, oxygen, antibiotics, and surgical debridement/ amputation should be instituted as soon as the diagnosis is suspected.

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Patient consent was obtained from the patient.

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Ethics approval was obtained prior to the commencement of the study.

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Review

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Nanocarrier drugs in the treatment of brain tumors

Tereza Cerna^{1,4}, Marie Stiborova¹, Vojtech Adam², Rene Kizek³, Tomas Eckschlager⁴

¹Department of Biochemistry, Faculty of Science, Charles University, Albertov 2030, CZ-128 40 Prague 2, Czech Republic.

²Department of Chemistry and Biochemistry, Laboratory metallomics and nanotechnology, Mendel University in Brno and Central European Institute of Technology, Brno University of Technology, Zemědělská 1, CZ-613 00 Brno, Czech Republic.

³Department of Human Pharmacology and Toxicology, Faculty of Pharmacology, University of Veterinary and Pharmaceutical Sciences Brno, Palackého 1, CZ 612 42 Brno, Czech Republic.

⁴Department of Pediatric Hematology and Oncology, 2nd Faculty of Medicine, Charles University, and University Hospital Motol, V Uvalu 84, CZ-150 06 Prague 5, Czech Republic.

Correspondence to: Prof. Tomas Eckschlager, Department of Pediatric Hematology and Oncology, 2nd Faculty of Medicine, Charles University, and University Hospital Motol, V Uvalu 84, CZ-150 06 Prague 5, Czech Republic. E-mail: tomas.eckschlager@lfmotol.cuni.cz

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Prof. Tomas Eckschlager, Deputy head for education, head of Laboratory of biology of solid tumors, works in Department of Pediatric Hematology and Oncology, 2nd Medical Faculty, Charles University and University Hospital Motol. His main interests are: molecular biology and genetics of pediatric cancer; experimental therapy of cancer and research of cancer cell chemoresistance; clinical pediatric oncology and late effects of children cancer therapy.

ABSTRACT

Nanoparticle-mediated targeted delivery of drugs might significantly reduce the dosage and optimize their release properties, increase specificity and bioavailability, improve shelf life, and reduce toxicity. Some nanodrugs are able to overcome the blood-brain barrier that is an obstacle to treatment of brain tumors. Vessels in tumors have abnormal architecture and are highly permeable; moreover, tumors also have poor lymphatic drainage, allowing for accumulation of macromolecules greater than approximately 40 kDa within the tumor microenvironment. Nanoparticles exploit this feature, known as the enhanced permeability and retention effect, to target solid tumors. Active targeting, i.e. surface modification of nanoparticles, is a way to decrease uptake in normal tissue and increase accumulation in a tumor, and it usually involves targeting surface membrane proteins that are upregulated in cancer cells. The targeting molecules are typically antibodies or their fragments; aptamers; oligopeptides or small molecules. There are currently several FDA-approved nanomedicines, but none approved for brain tumor therapy. This review, based both on the study of literature and on the authors own experimental work describes a comprehensive overview of preclinical and clinical research of nanodrugs in therapy of brain tumors.

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INTRODUCTION

Brain tumors are divided into two groups: (i) primary, originating and residing within the brain and (ii) secondary (metastatic), originating from a primary cancer outside the central nervous system and spreading into the brain. Metastatic tumors are more frequent than primary tumors in adult patients while primary ones are the most frequent solid tumors of childhood. The histological spectrum of brain tumors in children and adolescents differs from that in adults.^[1]

Primary brain tumors represent a heterogeneous group as classified according to WHO. According to the Central Brain Tumor Registry of the United States (CBTRUS) 2005-2009 report, the incidence in the US of CNS tumors was 20.6 cases per 100,000 persons/year, the incidence of malignant tumors was 7.3/100,000 persons/year and the incidence of low-grade tumors was 13.3/100,000 persons/year.^[2]

The most frequent brain tumors in all age groups are tumors originating from glial cells - gliomas that represent a wide spectrum of tumors ranging from slow growing to highly aggressive tumors. WHO classifies gliomas within four grades: grade I (pilocytic astrocytoma), grade II (diffuse astrocytoma), grade III (anaplastic astrocytoma), and grade IV (glioblastoma multiforme). The grade III and IV are considered high-grade gliomas (malignant gliomas) and are associated with very poor prognosis. In particular, 5 year survival rate of glioblastoma multiforme, which accounts for half of primary brain tumors, is less than 10%.^[3] Brain metastases are the most common intracranial tumors in adults, with more than 150,000 cases in the USA. In adults with cancer, 8-10% develop brain metastases, although the incidence of metastases varies considerably among different primary tumor types. Lung, breast, colorectal, renal cell cancer or melanoma can metastasize to the brain and 70% of brain metastases are due to lung and breast cancer.^[4,5] High-grade brain tumors, such as glioblastoma, and brain metastases are often lethal because of their invasiveness and resistance to surgical procedures as well as chemo- and radiotherapy.^[6] The urgent need for novel therapies has led to great emphasis on the development of new anticancer drugs including nanoparticles as cytostatic drug delivery vehicles.

Nanoparticles are structures between one and several hundred nanometers in diameter. There are three major physical properties of nanoparticles: (i) they are highly mobile in the free state; (ii) they have large surface areas; and (iii) they may exhibit quantum effects due to the movement of electrons. They have

unique material characteristics, and manufactured nanoparticles may find practical applications in a variety of areas, including medicine. The nanoparticle-mediated targeted delivery of drugs might significantly reduce the dosage required, increase drug specificity and bioavailability, overcome chemoresistance and reduce side effects.

The history of therapeutic nanoparticles began in the 1950s with a polymer-drug conjugate designed by Jatzkewitz, followed by Bangham who discovered the liposomes in mid-1960s. In 1972, Scheffel and colleagues first reported albumin based nanoparticles, which formed the basis of albumin-bound paclitaxel (Abraxane).^[7]

Targeted delivery in cancer therapy is an important challenge for oncologists. Nanovectors for drug delivery typically contain a core material or matrix, a therapeutic payload, and surface modifications in some cases. Possible advantages of nanoparticle delivery systems over conventional anticancer chemotherapy include: (i) protection of drugs from degradation in the body; (ii) enhanced absorption into tumor cells; and (iii) decreased interaction of drugs with normal cells.^[8] Ideal properties of nanoparticles for drug delivery are shown in Table 1. Nano-based drug delivery carriers, or nanocarriers, can consist of a wide variety of materials, both organic (polymeric, lipid, protein, or viral) and inorganic. The largest nanocarriers are liposomes (80-200 nm diameter), polymeric nanoparticles (40-100 nm) or micelles (20-60 nm); the smallest ones are dendrimers (< 10 nm diameter).^[9] There have been several reports describing the delivery of multiple anticancer agents using nanocarriers, some having been evaluated in clinical trials. Some nanodrugs have been FDA approved.^[10] The approved nanodrugs for anticancer therapy are given in Table 2.

The blood-brain barrier (BBB) protects brain neural tissues and works as a diffusion barrier that impedes the influx of toxins and other compounds, including

Table 1: Ideal properties of nanoparticles for drug delivery. Modified from^[78,79]

Ideal properties of nanoparticles for drug delivery
Non-toxic
Biocompatible
Biodegradable
Physically stable in blood
Prolonged time in circulation
Non-immunogenic/non-activating neutrophils/non-inflammatory
Non-trombogenic/non-aggregating platelets
Avoidance of reticuloendothelial system
Amenable to small molecules, peptides, proteins and nucleic acids
Inexpensive/easy manufacturing

Table 2: FDA-approved anticancer nanodrugs. Modified from^[80]

Name	Description	Indication	Approval (year)
DaunoXome	Liposomal daunorubicin	HIV-related Kaposi sa	FDA 96
DepoCyt	Liposomal cytarabine	Lymphomatous meningitis	FDA 96
Oncaspar	PEG asparaginase	Acute lymphoblastic leukemia	FDA 94
Abraxane	Albumin-bound paclitaxel nanospheres	Various cancers	FDA 05 EMEA 08, FDA 13
		Pancreatic ca	
Myocet	Liposomal doxorubicin	Breast ca	Europe + Canada
Marqibo	Liposomal vincristin	Acute lymphoblastic leukemia	FDA 12
Genexol	Paclitaxel loaded polymeric micelle	Breast ca, small cell lung ca	Europe + Korea
Onivyde	Liposomal irinotecan	Pancreatic ca	FDA 15

sa: sarcoma; ca: carcinoma

drugs, from blood to the brain.^[11] Its main components are brain endothelial cells, basal membranes, pericytes embedded in the basal membrane, and astrocytic end-feet. The BBB is characterized by the presence of tight intercellular junctions, minimal pinocytotic activity, and a lack of fenestrations, qualities that distinguish BBB endothelial cells from peripheral cells. Endogenous and exogenous compounds including drugs may cross the BBB by passive diffusion, carrier-mediated transport, endocytosis, or active transport. The efflux and influx transporters of BBB comprise transporters like ATP-binding cassette transporters and solute carrier transporters.^[12] The different types of transport across the BBB are shown in Figure 1.

The inability of drugs to cross the BBB is one of the major impairments to developing treatments for neurological diseases.^[13-16] This highly restrictive, physiologic barrier prevents 98% of small-molecule drugs and virtually 100% of large-molecule drugs from reaching the central nervous system from blood circulation. Numerous methods to bypass the BBB have been investigated, such as transient disruption of the BBB, inhibition of efflux pumps, or transport using endogenous transcytosis systems, including receptor-mediated transcytosis. Nanodrugs are another

approach to overcoming this obstacle to brain tumor treatment.

This review presents a comprehensive overview of preclinical *in vitro* and *in vivo* research and clinical studies of nanodrugs in therapy of brain tumors.

NANOCARRIERS FOR ANTICANCER DRUGS

Drug nanodelivery has gained a great deal of attention from researchers.^[17-19] However, some difficulties related to drug delivery may occur, such as troublesome solubility and biological availability, short time in circulation, and inconvenient biodistribution to the target organ. The key features of anticancer nanoparticles are principally large size, surface properties (e.g. hydrophobicity), and in some cases also targeting ligands. The development of a broad range of nanoparticles with varying size, composition, and functionality has provided a significant resource for nanomedicine.

Although nanoparticles avoid renal clearance, they tend to accumulate in the mononuclear phagocyte system (MPS).^[20] Surface conjugation with polyethylene glycol (PEG) and other polymers improves particle

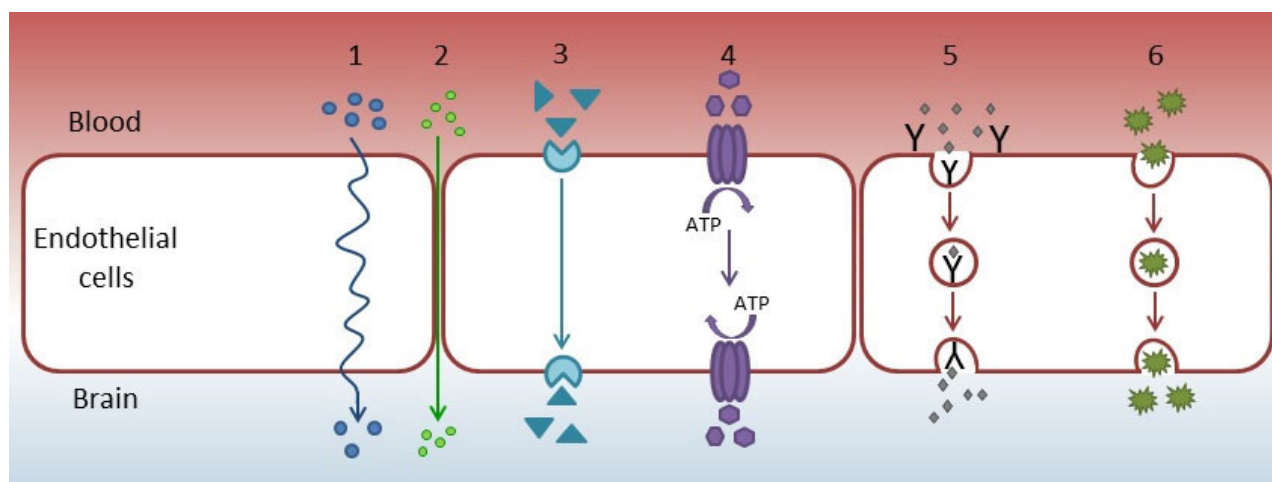


Figure 1: Mechanisms of transport across the blood-brain barrier. (1) Transcellular diffusion (small hydrophobic molecules); (2) paracellular diffusion (small water soluble molecules); (3) carrier-mediated transport (e.g. glucose, amino acids, vinca alkaloids); (4) active efflux transport; (5) receptor-mediated transport (e.g. insulin, leptin, transferrin); (6) adsorptive-mediated endocytosis (e.g. albumin, plasma proteins). ATP: adenosine triphosphate

circulation by reducing uptake into the MPS. The requirements for nanoparticle properties also depend on tumor characteristics, including cancer type, stage of disease, and location. Delivering multiple agents *in vivo* is complicated because of their independent pharmacokinetics, biodistribution, and clearance. A delivery system also has to transport a drug with high efficiency to target cells, with minimal toxicity and immune response. Drug toxicity can be reduced by encapsulating the free drug (e.g. liposomes) or by local activation of a pro-drug.^[21]

Nanoparticles designed for cancer therapy consist of various components, generally a nanocarrier and an active agent.^[22] Drug-carrier nanoparticles are considered as submicroscopic colloidal systems that may act as drug vehicles, either as nanospheres (the matrix system in which the drug is dispersed) or nanocapsules (reservoirs in which the drug is confined in hydrophobic or hydrophilic core surrounded by a single polymeric membrane).^[23]

Nanoparticles as carriers for anticancer drugs make them promising candidates to overcome chemoresistance of cancer cells, because nanoparticles loaded by cytostatic drugs promote their cellular uptake and considerably decrease their efflux, prolong drug systemic circulation lifetime, and enable targeted drug delivery.^[26] These particles can be modified with various types of materials including biomolecules. Altering the organizations of atoms can modify the properties of nanoparticles, such as elasticity, plasticity, strength, and conductivity.

Nanoparticle systems have unique properties that allow for both passive and active targeting of tumors.^[27] Tumor neovasculature has abnormal architecture and vessels are highly permeable. The tumor mass has also poor lymphatic drainage, allowing for accumulation of macromolecules greater than approximately 40 kDa within its microenvironment. Nanoparticles utilize this feature, known as the enhanced permeability and retention (EPR) effect, to target solid tumors. The ideal size range to benefit from the EPR effect is between 10 and 200 nm. Outside this range, smaller particles will be cleared by the kidney, preventing accumulation within the tumor site, while larger particles will not adequately penetrate the tumor vasculature and interstitial space. However, some clinical trials have not shown the efficacy of the EPR effect.^[28] One possible cause of EPR effect failure could be increased interstitial pressure in the tumor microenvironment. It has also been assumed that the EPR effect cannot be employed after an operation. Attempts have been made to increase the efficiency of the EPR effect by

induction of hypertension, by repairing the abnormal vasculature, or by targeting of perivascular cells.^[28]

Targeting molecules

Active targeting, i.e. surface modification of nanoparticles, is a method to decrease uptake in normal tissue and increase accumulation in a tumor. Strategies for active targeting of tumors usually involve targeting surface membrane proteins that are upregulated in cancer cells.^[25] Targeting molecules are typically antibodies or their fragments, aptamers, small molecules, or oligopeptides. Nanoparticles coupled with surface ligands or antibodies can localize to tissue, expressing the associated receptors or antigens and improving delivery efficacy.^[10] Some ligand receptor interactions will facilitate receptor-mediated endocytosis, further enhancing payload delivery. Surface ligand or antibody coupling can achieve densities high enough to interact efficiently with target sites, qualities well suited to cancer therapies.

Monoclonal antibodies, particularly IgG, are frequently used for targeting. Antigen binding sites represent only a small part of the overall size of antibodies. F(ab)2 fragments retain both antigen binding sites of the antibody, coupled by disulfide linkages. Many tumors up-regulate growth factor receptors, such as HER2/neu in certain breast cancers, which can be targeted with anti-HER2/neu surface antibodies.^[29] Liposomes modified with monoclonal antibodies against glial fibrillary acidic proteins or human insulin receptors have been studied to determine if they cross the BBB.^[30] Transferrin receptor (TfR) is another primary target investigated for receptor-mediated transcytosis across the BBB because of its high expression on BBB endothelium.^[31]

Aptamers are folded single strand oligonucleotides, 25-100 nucleotides in length, that bind to molecular targets.^[32] For example, EpCAM-fluoropyrimidine RNA aptamer-modified doxorubicin-loaded PLGA-b-PEG nanoparticles, which bond specifically to the extracellular domain of epithelial-cell adhesion molecules, have been investigated in non-small lung cancer model. Aptamer-conjugated nanoparticles *in vitro* have displayed increased cytotoxicity and decreased volume of xenografts compared with non-targeted nanoparticles.

Small molecules used for targeting include peptides, growth factors, carbohydrates and receptor ligands. Specific examples of small molecules include folic acid, transferrin and the RGD peptides. Example of small-molecule targeting protein is an HER2/neu ligands (AHNP) for targeting of poly (lactide-coglycolide) nanoparticles with docetaxel, which has

been investigated *in vitro* with HER2+ breast cancer cells.^[33]

Folic acid (FA) is essential for DNA synthesis, DNA repair, and methylation of DNA and is therefore necessary for cell survival and proliferation. The human folate receptor (FR), a glycosylphosphatidylinositol-anchored membrane protein of 38 kDa, has high affinity for FA, and is currently considered an essential component in the cellular accumulation of FA required in chemotherapy. FR expression is very low or undetectable in most normal cells and tissues, but it is upregulated in ovarian, breast, brain, lung, colorectal cancers as well as brain tumors.^[34,35] Through the process of endocytosis, ligand-bound receptor is internalized and released from the receptor through intravesicular reduction in pH.^[36] Ligand-free receptor is then recycled to the cell surface. Interestingly, covalent conjugation of small molecules, proteins and even liposomes to the gamma-carboxyl moiety of FA does not alter FA ability to bind to the FR and undergo endocytosis by receptor bearing cells. FR-mediated liposomal delivery has been shown to enhance the antitumor efficacy of doxorubicin both *in vitro* and *in vivo*, and to overcome P-glycoprotein-mediated multi-drug resistance.^[37]

Transferrin (Tf) is a single-chain iron-transporting glycoprotein that supplies iron into cells via receptor-mediated endocytosis. The TfR is expressed at low levels in most normal tissues but is overexpressed in many tumor types. The crucial aspect of Tf for molecular targeting applications, the binding of Tf to TfR on the external surface of tumor cells, is 10 times to 100 times more effective in tumor cells than in normal cells.^[38] Drug delivery systems can take advantage of this feature, most often by labeling the surface of the drug carrier with Tf, which is recognized by, and actively transported into, tumor cells. Therefore, Tf-modified liposomes, nanoparticles and dendrimers have been widely investigated in recent years. Despite the perceived potential of anti-TfR antibody-drug conjugates, a BBB-permeable drug using this approach has not yet been introduced for clinical use.^[16]

Ferritin protein also self-assembles naturally into a hollow nanocage called apoferritin, useful for encapsulation of any molecule of interest.^[39] Apoferritin can be modified with recognition ligands to achieve tumor-specific targeting. These extra surface modifications can avoid renal clearance and ensure EPR effect; however, they also eliminate the intrinsic tumor-specific binding of natural ferritin and disturb its *in vivo* performance and biocompatibility due to altered surface physicochemical properties of ferritin.

The authors have studied antibody targeted apoferritin mediated transport of doxorubicin, in which the surface of apoferritin can be modified with antibodies to enhance its targeting ability. These studies compared the cytotoxic effect of doxorubicin-loaded apoferritin, with and without surface targeting antibody anti-GCPII (PSMA), with that of free doxorubicin *in vitro* on prostatic cancer cell line (LNCaP) expressing PSMA as well as human umbilical vein endothelial cells (HUVEC) as a model of nonmalignant cells. The effect of doxorubicin-loaded apoferritin nanocarriers on cancer and healthy cells was similar to that of free doxorubicin. However, the real-time impedance-based platform demonstrated lower toxicity to HUVEC with doxorubicin loaded apoferritin than with free doxorubicin [Figure 2]. Entry of doxorubicin-loaded apoferritin nanocarriers with and without targeting antibody was higher into LNCaP than into HUVEC (Cerna *et al.*, unpublished results).

Oligopeptides are also molecules used for targeting. The RGD (Arg-Gly-Asp) oligopeptide is a component of the extracellular matrix protein fibronectin and promotes cell adhesion and regulates migration, growth, and proliferation.^[25,40] RGD is known to serve as a recognition motif in multiple ligands for several different integrins. RGD-containing peptide can be internalized into cells by integrin-mediated endocytosis. Recently, integrin-mediated carriers have been investigated as gene vehicles to enhance gene transfection and as vehicles to deliver anticancer agents. The upregulation of integrins is known to be promoted by angiogenic factors in several cancer types.

NANOPARTICLES IN THERAPY OF BRAIN TUMORS

Nanoparticles represent one of the possibilities of overcoming the BBB and delivering anticancer drugs to the brain. Therapy for brain tumors, particularly glioblastoma, using nanoparticles has been the subject of several preclinical experiments and clinical studies, but no nanodrug is as yet approved for brain tumor therapy.

Preclinical studies in brain tumors

Lipid nanoparticles loaded with doxorubicin have been investigated as a potential drug carrier to the brain, although doxorubicin cannot cross the BBB. The pharmacokinetics and tissue distribution of doxorubicin were studied in healthy rats, using i.v. administration of either free doxorubicin or doxorubicin incorporated into solid lipid nanoparticles (NANO DOX) in equivalent doses.^[42] Several blood samples and tissue samples of liver, spleen, heart, lung, kidney, and brain were collected. The mean peak plasma concentrations of

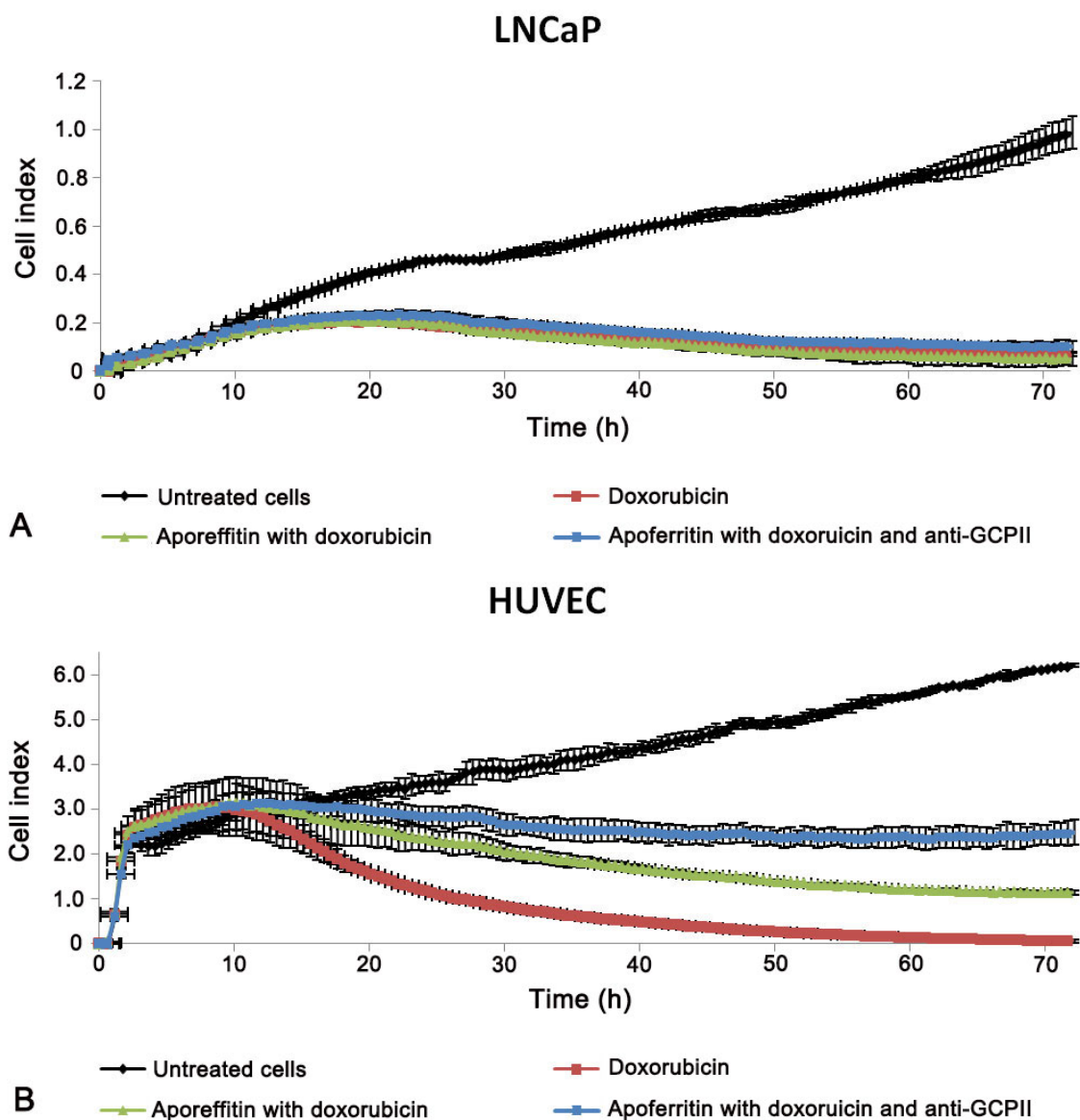


Figure 2: Cytotoxic effect of doxorubicin loaded apoferritin with and without targeting antibody anti-GCPII (PSMA) on its surface and free doxorubicin on (A) prostatic cancer cell line (LNCaP) expressing PSMA and (B) human umbilical vein endothelial cells (HUVEC)

free doxorubicin were lower than after NANO DOX treatment. In all rat tissues except the brain, the amount of doxorubicin was always lower after the injection of NANO DOX than after the injection of free doxorubicin. In the brain, however, NANO DOX increased the doxorubicin concentration significantly. The same study design, repeated in healthy rabbits, showed similar pharmacokinetic behavior and tissue distribution parameters.^[43] Docetaxel-incorporated albumin-lipid nanoparticles (DNPs) *in vitro* induce apoptosis of several cancer cell lines, and *in vivo*, accumulate at the experimental glioma site.^[44] This phenomenon is believed to be due to EPR effect. Liposomes containing temozolomide (TMZ) combined with anti-transferrin receptor single-chain antibody fragments were found to be more effective than

free TMZ in both TMZ-resistant and TMZ-sensitive glioblastoma cells in mouse models.^[45] Moreover, these liposomes showed significantly reduced toxicity. These results show that these liposomes may be an efficient vehicle for delivering BBB-impermeable drugs to the brain.

Biodegradable polymer-based nanoparticles and gold nanoparticles have both shown promise for delivering drugs across the BBB to treat glioma.^[46] Gromnicova *et al.*^[47] found that glucose-coated gold nanoparticles cross brain endothelium three times faster than non-brain endothelium. Huwyler *et al.*^[48] investigated daunorubicin-loaded liposomes with anti-transferrin receptor antibody, using an animal model, and found increased brain daunorubicin concentration compared with free drug.

Nanoparticles show promise for specific and efficient intracerebral delivery of drugs for the treatment of glioma.^[49] A two-dose regimen of topotecan non-PEGylated liposomes, locally administered with paramagnetic gadodiamide nanoparticles, increased survival rates in a U87MG glioblastoma intracranial xenograft model compared with controls; the effect was topotecan dose-dependent.^[50]

Gadolinium nanoparticles enhance MRI monitoring and are well tolerated. These nanoparticles can penetrate the BBB and be uptaken by the brain tumor parenchyma.^[51] Metal nanoparticles are also frequently integrated with other techniques such as microwave-induced hyperthermia to further increase their cellular transduction.^[52] The α -helical right handed coiled coils associated with platinum (PtIV) compound showed higher toxicity to human malignant glioma cells compared with free Pt(IV) *in vitro* and *in vivo*, without affecting healthy astrocytes *in vitro*.^[53]

Carrier-mediated transport (CMT) can transport small molecules from the blood to the brain. Receptor-mediated transport (RMT) systems are expressed on the BBB and provide transport of large endogenous biomolecules^[54] [Figure 1]. During RMT, macromolecules move across the endothelial cells into the brain, due to the expression of several peptide-specific receptors, e.g. neonatal Fc receptor,^[55] low-density lipoprotein receptor-related protein receptor, transferrin receptor,^[56] lactoferrin receptor,^[57] and insulin receptor.^[58] Some of the above-mentioned receptors have been used for drug delivery as a molecular “Trojan horse”. Shilo *et al.*^[59] demonstrated that insulin-targeted gold nanoparticles cross the BBB after systemic administration.

Gao *et al.*^[60] investigated transferrin-folate doxorubicin-loaded liposomes. The amount of doxorubicin transported across the BBB in the transferrin-folate doxorubicin-loaded liposome group of glioma bearing rats was sevenfold higher than in the non-targeted doxorubicin-loaded liposome-treated group. Boado *et al.*^[61] found that fused lysosomal enzyme with anti-human insulin receptor monoclonal antibody could deliver fusion protein across the BBB at therapeutic levels, while free lysosomal enzyme did not cross the BBB. Yang *et al.*^[62] tested dual peptide-modified (using low-density lipoprotein receptor-related protein receptor and neuropilin-1 receptor) liposomes loaded with vascular endothelial growth factor siRNA and docetaxel; the target was human glioblastoma xenografts in mice. These dual-modified liposomes showed the highest uptake compared with single modified or non-modified liposomes.

In another study, cetylalcohol/polysorbate nanoparticles loaded with paclitaxel were more cytotoxic to glioblastoma cells and had higher brain uptake in an experimental animal model than paclitaxel alone.^[63] The investigators speculated that nanoparticles may limit binding of paclitaxel to p-glycoprotein, causing higher brain and tumor cell uptake.

Coated poly (butylcyanoacrylate) (PBCA) nanoparticles have been studied as a delivery system for drugs in the brain.^[64,65] Polysorbate 80 was found to be the most efficient modifier of nanoparticles. Transport across the BBB of polysorbate 80-coated nanoparticles has been presumed to involve receptor-mediated endocytosis by endothelial cells. Polysorbate 80 absorbs plasmatic apolipoprotein E (Apo-E) and nanoparticles coated with Apo-E are internalized by the LDL uptake system.^[66] In one study in rats, PBCA nanoparticles with doxorubicin increased brain doxorubicin concentrations to levels more than 60 times that of free drug, while heart levels were very low.^[67] In another rat brain model, polysorbate 80 coated poly-lactic-co-glycolic acid nanoparticles loaded with methotrexate-transferrin conjugates were investigated and showed better penetration, lower organ toxicity and higher anti-tumor activity as compared with non-targeting nanoparticles.^[68]

Doxorubicin bound to polysorbate-coated nanoparticles was associated with significantly longer survival of glioblastoma-bearing rats compared with groups treated with free doxorubicin or noncoated nanoparticles with doxorubicin.^[69] Poly-lactic-co-glycolic acid (PLGA) camptothecin-loaded nanoparticles were investigated in orthotopic murine glioma. Nanoparticles were well tolerated and effective against glioma.^[70] Cetuximab-magnetic iron-oxide nanoparticles (IONP) that bind to both wild-type EGFR+ and mutated EGFR+ patient-derived glioblastoma cells are internalized by tumor cells and promote internalization of the EGFR, resulting in enhanced apoptosis. Treatment with cetuximab-IONPs proved efficacious in orthotopic glioblastoma xenografts in mouse and rats, and showed a favorable safety profile, as no toxicity to healthy immunocompetent mice was observed.^[71]

The *in vitro* and *in vivo* studies described above seem promising for the treatment of brain tumors, particularly glioblastoma, the tumor with the worst prognosis. The inclusion of the most efficacious and safe nanoparticles designed for cancer therapy in clinical studies is warranted. Nevertheless, despite the successful results of preclinical experiments, the progress in applying these strategies in brain tumors is still modest when compared with treatments in other types of tumors.

Clinical studies in brain tumors

A phase I clinical study of paclitaxel-Angiopep-2 peptide-drug conjugate that binds to the low-density lipoprotein receptor-related protein-1 receptor (GRN1005) has been carried out in patients with recurrent glioma grade 2-4. The clinical data show that GRN1005 facilitated the penetration of paclitaxel into tumor tissue.^[72] However, interim analysis of the phase II trial did not show therapeutic response.^[73]

Transferrin conjugated with diphtheric toxin (Tf-CRM107) demonstrated *in vitro* and *in vivo* toxicity to glioma cells and was effective when administrated locally to xenografts. Using local administration, low toxicity and tumor response were demonstrated in patients with recurrent high grade brain tumors in phase I and II clinical trials. The response rate was 35% and overall survival of responders was 74 weeks.^[74] Unfortunately, an early phase III clinical trial using this therapy had to be terminated due to disappointing preliminary results.^[75]

In a clinical study of liposomal doxorubicin in patients with high-grade gliomas, Fabel *et al.*^[75] found improved overall survival than in past trials using conventional therapies. Hau *et al.*^[77] demonstrated that pegylated liposomal doxorubicin in patients with recurrent high-grade glioma was efficacious and well tolerated.

These results presented above suggest that some nanodrugs may be efficient in therapy of high grade brain tumors, a topic of great potential interest for clinicians.

FUTURE DIRECTIONS AND CONCLUSIONS

Although the available clinical trial data are limited, evidence suggests that nanoparticles have potential in diagnosis, operative management and adjuvant therapy for brain tumors. Because the field of nanotechnology is young, the long-term health effects of nanoparticles are currently unknown. More study of nanoparticle biodistribution, pharmacokinetics, toxicity and role in therapeutic protocols is warranted if nanoparticles are to attain regular clinical use.

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Conflict of interest

There are no conflicts of interest.

Patient consent

No patient involved.

Ethics approval

This article does not contain any studies with human participants or animals.

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Correspondence to: Prof. Dr. Ira-Ida Skvortsova, Laboratory for Experimental and Translational Research on Radiation Oncology (EXTRO-Lab), Department of Therapeutic Radiology and Oncology, Innsbruck Medical University, Anichstr. 35, A-6020 Innsbruck, Austria.
E-mail: Ira.Skvortsova@i-med.ac.at; Ira.Skvortsova@tirol-kliniken.at

A1

Proteomics identification of protein biomarkers and signaling pathways for prostate cancer radioresistance therapy

Lei Chang^{1,2}, Peter Graham^{1,2}, Jingli Hao^{1,2}, Valerie Wasinger^{3,4}, Jie Ni^{1,2}, Julia Beretov^{1,2,5}, Junli Deng^{1,2}, Joseph Bucci^{1,2}, David Malouf⁶, David Gillatt^{6,7}, Yong Li^{1,2}

¹Cancer Care Centre, St. George Hospital, Kogarah, Australia;

²St. George and Sutherland Clinical School, Faculty of Medicine, UNSW, Kensington, Australia;

³Bioanalytical Mass Spectrometry Facility, Mark Wainwright Analytical Centre, UNSW, Kensington, Australia;

⁴School of Medical Science, UNSW, Kensington, Australia;

⁵SEALS, Anatomical Pathology, St. George Hospital, Kogarah, Australia;

⁶Department of Urology, St. George Hospital, Kogarah, Australia;

⁷Australian School of Advanced Medicine, Macquarie University, Sydney, Australia

Background: Radioresistance is a major problem in prostate cancer (CaP) radiotherapy (RT). The mechanisms of CaP radioresistance are still unclear. We have recently developed CaP-RR (radioresistant) cell lines which display more aggressive characteristics including increased colony formation, invasion ability, sphere formation capability, and enhanced epithelial mesenchymal transition (EMT) and cancer stem cell (CSC) phenotypes. In addition, we found the PI3K/

Akt/mTOR pathway is closely linked with EMT and CSCs expression. Therefore, these CaP-RR cells, representative of the source of recurrence after RT, provide a very good model to mimic the clinical radioresistance condition to find biomarkers and signaling pathways for CaP radiotherapy.

Aim: The objective of this study was to identify candidate proteins and the main signaling pathways involved in CaP radioresistance, validate the identified potential biomarkers in CaP-radioresistant (RR) cell lines and animal xenografts, and perform the functional study from a selected candidate. **Methods:** The differential proteins from CaP parental cell lines (PC-3, DU145 and LNCaP) and CaP-RR sublines (PC-3RR, DU145RR and LNCaP-RR) were analyzed using LC-MS/MS and identified by a label-free ion count approach. Pathways enriched as a result of radioresistance were assessed. Identified potential markers were validated in CaP-RR cell lines and subcutaneous (s.c) animal xenografts by Western blotting and immunohistochemistry. In addition, the protein fructose-bisphosphate aldolase A (ALDOA) was identified as a key protein in radioresistance and was selected for radiosensitivity study. **Results:** A total of 309 signaling pathway proteins were identified to be significantly different between CaP and CaP-RR cells



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($P \leq 0.05$, fold differences > 1.5 , $> 80\%$ power). Among these proteins, nineteen are common among three paired CaP cell lines and associated with metastasis, progression, signaling pathways and radioresistance. The PI3K/Akt, VEGF, metabolism and ERK pathways were identified to be associated with CaP radioresistance. The expression of key proteins from the identified pathways was found to be significantly increased in CaP-RR cells and s.c animal xenografts compared to controls. Furthermore, the downregulation of ALDOA combined with RT effectively reduced colony capability, induced more apoptosis and increased radiosensitivity in CaP-RR cells. **Conclusion:** CaP radioresistance is associated with EMT and enhanced CSC phenotypes via activation of the PI3K/Akt/mTOR signaling pathway. CaP radioresistance is caused by multifactorial traits and several signaling pathways. Downregulation of ALDOA increases radiosensitivity in CaP-RR cells. Our findings indicate that interfering EMT/CSCs, ALDOA and signaling pathways, in combination with RT is promising for CaP radiotherapy.

Key words:

Prostate cancer, radiation therapy, radioresistance, cancer stem cell, LC-MS/MS, signaling pathway

A2

Tumor-associated fibroblast-conditioned medium induces CDDP resistance in HNSCC cells

Teresa Bernadette Steinbichler, Jozsef Dudas, Herbert Riechelmann

Medical University of Innsbruck, Innsbruck, Austria

Aim: EMT contributes to tumor progression and metastasis. We aimed to investigate the effects of EMT on Cisplatin resistance in HNSCC (head and neck squamous cell carcinoma)-cells. **Methods:** EMT was induced in HNSCC cells using conditioned medium from a tumor cell/fibroblast co culture and confirmed with vimentin and E cadherin expression analysis at RNA and protein level. The tumor cells were alternatively treated with 1 ng/mL TGF- β 1. The response to Cisplatin was evaluated with viability and clonogenic assays. **Results:** Treatment with conditioned medium induced a mesenchymal phenotype and increased the viability of the tumor cells. Moreover, it doubled the IC50 of Cisplatin of SCC-25 cells from 6.2 μ mol/L to 13.1 μ mol/L ($P < 0.001$). The IC50 of Cisplatin of Detroit 562 cells was increased following treatment with conditioned medium from 13.1 μ mol/L to 26.8 μ mol/L ($P < 0.01$). Treatment with TGF- β 1 induced similar phenotypic changes as co-culture conditioned

medium, but decreased tumor cell viability and did not alter Cisplatin resistance. **Conclusion:** Cell free medium from an epithelial tumor cell/fibroblast co-culture was able to induce EMT in HNSCC cells. Co-culture treated HNSCC cells revealed increased viability and were less sensitive to Cisplatin treatment. TGF- β 1 also induced a mesenchymal phenotype, but decreased tumor cell viability and did not alter resistance to CDDP in HNSCC cells.

Key words:

Cisplatin resistance, tumor-associated fibroblasts, tumor microenvironment, EMT

A3

Identifying metabolic biomarkers of paediatric glioma cancer stem cells in tumour development and drug resistance

Alice Agliano, Maria Vinci, Chris Jones, Gabriela Kramer-Marek, Martin Leach, Nada Al-Saffar

The Institute of Cancer Research, London, UK

Background: Paediatric glioblastoma multiforme (pGBM) is one of the most aggressive forms of cancer of the central nervous system in children. There is increasing evidence that cancer stem cells (CSC) can contribute to the current poor outcome of pGBM since CSC play an important role in tumour initiation and drug resistance. Much effort has been directed at identifying biomarkers able to recognize and select CSC. However, this has proven challenging due to their continuous evolution during tumour progression. Metabolism has been recognized as an important regulator of several functions in stem cells and even though metabolic aspects of tumour development are widely studied, little is known about CSC metabolism. Nuclear magnetic resonance (NMR) and positron emission tomography (PET) are powerful non-invasive imaging tools that can be used to evaluate aspects of tumour cell and CSC metabolism.

Aim: To characterise metabolic differences between CSCs and non-CSCs that are detectable by NMR and PET and to determine how cell signalling pathways alter CSC metabolism in order to identify possible therapeutic targets to develop CSC-targeted therapies for pGBMs. **Methods:** Cancer cell lines with stem-like features (CSLC) have been created following culture of a panel of paediatric cell lines, such as SF188 and KNS42, and primary cells on a laminin substrate with specific CSC media supplemented with growth factors. CSLC cell lines have been compared to the correspondent parental cell line (non-CSLC) grown

under standard culture conditions. Metabolism was evaluated by NMR and radionuclide uptake. **Results:** *In vitro* ¹H NMR and ¹⁸F-FDG-PET uptake studies showed that newly established pGBM CSLC express a different metabolic signature compared with non-CSLC cell lines. Differences have been observed in the levels of several metabolites, including lactate, glutamine and several lipids involved in the membrane turnover such as phosphocholine, glycerophosphocholine and cholesterol. These findings correlated with changes in the expression of metabolism- and cell division-associated proteins and genes. The increased gene expression of the glycolytic enzymes LDH-A, HK2, and Glut-1, suggested that the CSLCs rely mainly on aerobic glycolysis. This increased reliance on glucose metabolism together with a low mitochondrial activity reduced the levels of ROS in CSLCs, a feature that has been associated with EMT and pluripotency. Moreover, contrary to non-CSLCs, treatment with the PI3K/mTOR inhibitor NVP-BE235 did not affect cell viability or changes CSLCs metabolic signature, indicating induced drug resistance of the CSLCs upon this treatment. **Conclusion:** We have shown that pGBM CSLCs have different metabolic features from non-CSLCs. Improved understanding of mechanisms related to CSC drug resistance to PI3K/mTOR inhibitors could guide the identification of potential targets, leading to development of more effective treatments for pGBM.

Key words:

Cancer stem cells, metabolic biomarker, paediatric glioblastoma multiforme, nuclear magnetic resonance, positron emission tomography, drug resistance

A4

Cancer stem cells in melanoma: a complex problem

Caterina A.M. La Porta

University of Milan, Milan, Italy

Cancer progression in humans is difficult to infer because we do not routinely sample patients at multiple stages of their disease. The identification of cancer stem cell (CSC) subpopulations inside tumors opens a new perspective on cancer development, since it implies that tumors can only be eradicated by targeting CSCs. Several markers have been proposed in the literature to identify CSCs both in breast and melanoma but no consensus has been reached, leading to the hypothesis that the CSC phenotype might be dynamically switched. Herein we provide quantitative evidence of CSCs in melanoma discussing the complex

network regulating their biological functions.

Key words:

Cancer stem cells, melanoma, complexity, miRNA

A5

Low extracellular pH inhibits glycolysis and decreases transcription factor activity responsible for stemness in induced pluripotent stem cells

Anja Wilmes¹, Caroline Rauch¹, Giada Carta¹, Georg Kern¹, Florian Meier², Wilfried Posch¹, Doris Wilflingseder¹, Lyle Armstrong³, Majlinka Lako³, Mario Beilmann², Gerhard Gstraunthaler¹, Paul Jennings¹

¹Medical University of Innsbruck, Innsbruck, Austria;

²Boehringer Ingelheim, Ingelheim, Germany;

³University of Newcastle, Newcastle, UK

Induced pluripotent stem cells (iPSC) have the potential to revolutionize biological experimentation and thus the uptake of this new technology is widespread. However, culturing iPSC is both time consuming and expensive as they require daily medium exchange. Our study investigates the reason for this high demand on frequent medium replacement.

Two human iPSC lineages were fed at different intervals up to 72 h either in a full growth area (FGA) or a restricted growth area (RGA). The FGA consisted of a well of a 6 well plate coated with Matrigel™ and the RGA consisted of a coated coverslip placed in a well. Medium was sampled every 24 h and glucose, lactate, pH were measured. In addition, flow cytometry was employed to investigate cell cycle alterations and TransAM assays utilized for cMYC, FOXO1 and p53 activity.

FGA cultured iPSC that were not fed every 24 h had significantly reduced growth rates by day 2 and showed increasing cell death by day 3. In contrast, RGA cultured cells grew to confluence over 3 days. Surprisingly, glucose was not exhausted under any condition. Instead, the extracellular pH reached 6.8 after 72 h in FGA cultures. Reducing medium pH to 6.8 also inhibited glycolysis, initiated a cell cycle block in G0/G1 and decreased in cMYC and FOXO-1 transcriptional activity.

This study demonstrates that iPSC are susceptible to cell culture medium acidification, a likely limiting factor in maintenance of proliferative and pluripotent status. Culturing iPSC in RGA prevents rapid extracellular acidification, by limiting cell numbers, while still maintain optimal oxygen diffusion rates and allows

longer feeding cycles whilst still ensuring pluripotency. These results may provide critical information for scale up procedures, e.g. the use of bioreactors, careful control of extracellular pH will be important.

Key words:

iPSC, pH, glucose, FOXO1, cMYC, growth arrest

A6

Mechanisms of radioresistance in prostate cells

Fabian Guggenberger¹, Holger Erb², Ira-Ida Skvortsova³, Zoran Culig¹, Frédéric R. Santer¹

¹Division of Experimental Urology, Medical University of Innsbruck, Innsbruck, Austria;

²YCR Cancer Research Unit, Department of Biology, University of York, York, UK;

³Department of Therapeutic Radiology and Oncology, Medical University of Innsbruck, Innsbruck, Austria

Background: Prostate cancer (PCa) is one of the most commonly diagnosed malignancies in men in Western nations. Among androgen deprivation therapy (ADT), radiation therapy is an approved treatment either for early stage local PCa, but also for metastatic M1 stage PCa. However, tumour relapse is a frequent event that affects about 80% of patients undergoing prior treatment. There is increasing evidence that the occurrence of cancer stem cells (CSC) may play an important role in therapy resistance, in particular also in radioresistance. The occurrence of CD133-positive CSCs within the basal, less differentiated layer of the malignant prostatic epithelium was demonstrated. Those cells were shown to have increased colony forming efficacy and less double-strand breaks after irradiation due to a higher DNA repair capacity when compared to more differentiated populations. Moreover, aldehyde dehydrogenase 1 expressing (ALDH1+) cells derived from PCa cell lines were shown to be more radioresistant than ALDH1- cells and ALDH1 inhibition lead to increased radiosensitivity. However, our knowledge on the mechanisms of radioresistance of the prostatic basal layer is still limited.

Aim: The aim of this study is to identify a novel molecular mechanism underlying radioresistance in the prostate basal cell layer containing stem cells.

Approach: To investigate the effect of irradiation exposure on prostate basal cells we irradiated benign PrEPs and an immortalised benign, basal prostatic cell line (EP156T) for 21 times following a therapeutic schedule with either 0, 0.5 or 1 Gray for 5 times per week using a linear particle accelerator (LINAC). Total RNA was isolated and NextGen transcriptome sequencing followed by bioinformatical analysis

(including pathway analysis) and literature research will be performed. Finally, a regulated candidate gene will be chosen for further experiments. **Methods/Results:** PrEPs obtained from 5 patients receiving radical prostatectomy were successfully isolated via collagenase digestion from prostate tissue specimens and cultured in the presence of a feeder layer. PrEPs and EP156T have been characterised by clonogenic assays and by label retention assay using FACS analysis subsequent to PKH67 membrane labelling, which might indicate the presence of stem cells within the cultures. The repeated exposition to irradiation using the protocol described above for both, PrEPs and EP156T cells, is finished and gained total RNA from the cultures will be sequenced by the company Microsynth. Bioinformatical analysis will be done in collaboration with the cancer Computational Biology Center (Erasmus MC, Rotterdam, NL). **Outlook:** Once the bioinformatical analysis is finished a careful review of literature is performed. Based thereon a candidate gene, whose expression is shown to be strongly altered by irradiation, is chosen for further experiments (e.g. clonogenic assay, knock down, over-expression, inhibition) that will test the candidate's involvement in radioprotection and which may have the potential as a possible target for radiosensibilisation. This may improve co-treatment strategies for future irradiation therapy.

Key words:

Prostate cancer, cancer stem cells, primary cells, radioresistance

A7

Cysteine cathepsins and their inhibitors as regulators of cancer stem cell dormancy and differentiation

Janko Kos^{1,2}, Milica Perišić Nanut², Mateja Prunk², Urša Pečar Fonović¹, Anja Pišlar¹, Ana Mitrović¹, Jerica Sabotič², Špela Magister², Anahid Jewett³

¹University of Ljubljana, Faculty of Pharmacy, Ljubljana, Slovenia;

²Jožef Stefan Institute, Department of Biotechnology, Ljubljana, Slovenia;

³University of California, School of Dentistry, Los Angeles, USA

Cysteine cathepsins are lysosomal peptidases involved in different processes of tumor development and progression. There is increasing evidence that these enzymes may regulate also homeostasis and differentiation of cancer stem cells. In particular, cysteine cathepsins K and X were shown to be involved in cytokine-induced niche dormancy as well as in mobilization process that release cancer stem cells from their niches. Cathepsin X has been proposed to participate in proteolytic processing of CXCL-12

chemokine (SDF-1), which is involved in adhesion and migration of cancer stem cells. Additionally, cathepsin X cleaves C-terminal ends of enolase and profilin, another two proteins that were suggested to alter cancer stem cells adhesion, migration and their metastatic potential. Cathepsin X also cleaves beta-2 chain in Mac-1 and LFA-1 integrin receptors and binds to alpha-v-beta 5 integrin receptor via RGD motif in the pro-region, affecting in this way the adhesion of endothelial and tumor cells and presumably cancer stem cells. Cathepsin X knock out accelerates a progress to senescence *in vitro* and *in vivo* via p16, p21 and p53 signaling pathway.

The activity of cysteine cathepsins is regulated by endogenous protein inhibitors cystatins. Of these, cystatin F is the only cystatin that is localized in endosomal/lysosomal vesicles. In cytotoxic T cells and NK cells its main role is the control of progranzyme convertase activity of cathepsins C and H and consequently, the granzyme dependent cytotoxic function. It is known that cytotoxic function of NK cells is suppressed after their interaction with tumor cells or cancer stem cells, the status is termed split anergy. The mechanism includes cytokine cross-talk, however, target cells may also secrete inactive dimeric cystatin F which after internalization to NK cells enters endosomal/lysosomal vesicles and after activation/monomerisation inhibits cathepsins C and H and down-regulates cell cytotoxicity. Anergized NK cells cause differentiation of cancer stem cells by secreted cytokines and as a result, differentiated tumors become resistant to NK cell-mediated cytotoxicity.

Our results show that some of cysteine cathepsins and their endogenous inhibitors have specific roles in cancer stem cell functions designating them as potential therapeutic targets for improving anticancer therapy.

Key words:

Cathepsin, cystatin, anergy, SDF-1, profilin, enolase

A8

Hypoxia and expression levels of cancer stem cell markers are prognostic for loco-regional control after radiochemotherapy in locally advanced head and neck squamous cell carcinoma

Fabian Lohaus^{1,9}, Annett Linge^{1,10}, Steffen Löck¹⁰, Volker Gudziol¹⁹, Alexander Nowak²⁰, Cläre von Neubeck^{1,10}, Inge Tinhofer^{2,11}, Volker Budach^{2,11}, Ali Sak^{3,12}, Martin Stuschke^{3,12}, Panagiotis Balermipas^{4,13}, Claus Rödel^{4,13}, Melanie Avlar^{5,14}, Anca-Ligia Grosu^{5,14}, Amir Abdollahi^{6,15},

Jürgen Debus^{6,15}, Claus Belka^{7,16}, Steffi Pigorsch^{7,17}, Stephanie E. Combs^{7,17}, David Mönlich^{8,18}, Daniel Zips^{8,18}, Gustavo B. Baretton^{1,21}, Frank Buchholz^{1,22}, Michael Baumann^{1,9}, Mechthild Krause^{1,9}

¹German Cancer Research Center (DKFZ), Heidelberg and German Cancer Consortium (DKTK) partner site: Dresden, Germany;

²German Cancer Research Center (DKFZ), Heidelberg and German Cancer Consortium (DKTK) partner site: Berlin, Germany;

³German Cancer Research Center (DKFZ), Heidelberg and German Cancer Consortium (DKTK) partner site: Essen, Germany;

⁴German Cancer Research Center (DKFZ), Heidelberg and German Cancer Consortium (DKTK) partner site: Frankfurt, Germany;

⁵German Cancer Research Center (DKFZ), Heidelberg and German Cancer Consortium (DKTK) partner site: Freiburg, Germany;

⁶German Cancer Research Center (DKFZ), Heidelberg and German Cancer Consortium (DKTK) partner site: Heidelberg, Germany;

⁷German Cancer Research Center (DKFZ), Heidelberg and German Cancer Consortium (DKTK) partner site: Munich, Germany;

⁸German Cancer Research Center (DKFZ), Heidelberg and German Cancer Consortium (DKTK) partner site: Tübingen, Germany;

⁹Universitätsklinikum Carl Gustav Carus der TU Dresden, Dresden, Germany;

¹⁰OncoRay - National Center for Radiation Research in Oncology, Faculty of Medicine and University Hospital Carl Gustav Carus, Technische Universität Dresden, Dresden, Germany;

¹¹Department of Radiooncology and Radiotherapy, Charité University Hospital, Berlin, Germany;

¹²Department of Radiotherapy, Medical Faculty, University of Duisburg-Essen, Essen, Germany;

¹³Department of Radiotherapy and Oncology, Goethe-University Frankfurt, Frankfurt, Germany;

¹⁴Department of Radiation Oncology, University of Freiburg, Freiburg, Germany;

¹⁵Heidelberg Institute of Radiation Oncology (HIRO), National Center for Radiation Research in Oncology (NCRO), University of Heidelberg Medical School and German Cancer Research Center (DKFZ), Heidelberg, Germany;

¹⁶Department of Radiotherapy and Radiation Oncology, Ludwig-Maximilians-Universität, Munich, Germany;

¹⁷Department of Radiation Oncology, Technische Universität München, Munich, Germany;

¹⁸Department of Radiation Oncology, Faculty of Medicine and University Hospital Tübingen, Eberhard Karls Universität Tübingen, Tübingen, Germany;

¹⁹Department of Otorhinolaryngology, Faculty of Medicine and University Hospital Carl Gustav Carus, Technische Universität Dresden, Dresden, Germany;

²⁰Department of Oral and Maxillofacial Surgery, Faculty of Medicine and University Hospital Carl Gustav Carus, Technische Universität Dresden, Dresden, Germany;

²¹Institute of Pathology, Faculty of Medicine and University Hospital Carl Gustav Carus, Technische Universität Dresden, Dresden, Germany;

²²University Cancer Center (UCC), Medical Systems Biology, University Hospital Carl Gustav Carus, Technische Universität Dresden, Dresden, Germany

Aim: The outcome after curative treatment of locally advanced head and neck squamous cell carcinoma (LA-HNSCC) remains unsatisfactory. Except of HPV-infection status there is no further established biomarker for treatment stratification. Currently, treatment decisions are mainly based on the tumor site and the TNM system regardless of the heterogeneous biology of HNSCC. For identification and validation of biomarkers, the German Cancer Consortium Radiation Oncology Group (DKTK-ROG) initiated a multicenter retrospective/prospective biomarker trial to explore their impact on locoregional control (LRC) after postoperative (PORT-C) and primary radiochemotherapy. **Methods:** In the multicenter retrospective part of the study, 355 patients with HNSCC of the oral cavity, oro- and hypopharynx were included. All patients received

cisplatin-based radiochemotherapy (RCTx) between 2005 and 2012. The postoperative cohort consisted of 195 patients, who were all treated with PORT-C because of clinical high risk parameter. The second cohort consisted of 160 patients treated with primary RCTx. FFPE-material, radiotherapy treatment plans and images were centrally collected. Tumor volume was segmented on CT-based radiotherapy treatment plans. HPV status (p16 overexpression) and CD44 expression were analysed by immunohistochemistry. Gene expression analyses were performed for hypoxia-associated genes and the potential cancer stem cell (CSC) markers SLC3A2, MET and CD44. Results of the biomarker analyses, clinical parameters and tumor volume were correlated with the clinical outcome. Primary endpoint was LRC. **Results:** Multivariate analysis (MVA) revealed the impact of hypoxia and expression of CSC markers in HPV(-) HNSCC on LRC after PORT-C (hypoxia gene signature: HR 4.54, $P = 0.006$; MET: HR 3.71, $P = 0.016$; SLC3A2: HR 8.54, $P = 0.037$; CD44: HR 3.36, $P = 0.054$). For primary RCTx a significant impact of tumor volume, HPV status and expression of CSC markers (tumor volume: HR 2.63, $P = 0.003$, SLC3A2: HR 2.03, $P = 0.021$; HPV: HR 0.35, $P = 0.086$) on LRC was seen in MVA. A significant impact of hypoxia associated gene expression was only seen in small tumors (< 25 ccm) (HR 9.2, $P = 0.38$). **Conclusion:** We demonstrated that the expression of CSC markers and hypoxia-associated genes are prognosticators for LRC in addition to the HPV-infection status in patients suffering from LA-HNSCC, who were treated with PORT-C or primary RCTx. After validation of these promising results in the currently ongoing part of the prospective trial of the DTK-ROG, along with established clinical parameters, they may help to further stratify patients for individualized escalation and de-escalation strategies.

Key words:

HNSCC, PORT-C, hypoxia, radiochemotherapy, biomarker

A9

Epidermal growth factor receptor activity is elevated in glioma cancer stem cells and is required to maintain chemotherapy and radiation resistance

Lisa Y. Pang, Lauren Saunders, David J. Argyle

University of Edinburgh, Edinburgh, UK

Glioblastoma remains among the most aggressive of all human and canine malignancies, displaying high mortality rates and limited treatment options.

We propose that given the similarities between canine and human gliomas, such as incidence of occurrence, histopathology, molecular characteristics, and response to therapy, that canine gliomas are a natural model of the human disease. A range of human and canine tumours have been shown to harbor specific subpopulations of cells with stem cell-like properties that initiate and maintain neoplasticity while resisting conventional therapies. Here, we show that both canine and human glioma cell lines contain a small population of cancer stem cells (CSCs), and by molecular profiling highlight the important role of the epidermal growth factor receptor (EGFR) pathway in canine CSCs. EGFR signaling is crucial in the regulation of cancer cell proliferation, migration and survival. To date EGFR-targeted interventions alone have been largely ineffective. Our findings confirm that specifically inhibiting EGFR signaling alone has no significant effect on the viability of CSCs. However inhibition of EGFR did enhance the chemo- and radio-sensitivity of both canine and human glioma CSCs, enabling this resistant, tumourigenic population of cells to be effectively targeted by conventional therapies.

Key words:

Glioma, cancer stem cells, comparative oncology, EGFR

A10

MET inhibition overcomes radiation resistance of glioblastoma stem-like cells

Francesca De Bacco¹, Antonio D'Ambrosio¹, Elena Casanova¹, Francesca Orzan¹, Roberta Neggia¹, Raffaella Albano¹, Federica Verginelli¹, Manuela Cominelli², Pietro L. Poliani², Paolo Luraghi¹, Gigliola Reato¹, Serena Pellegatta³, Gaetano Finocchiaro³, Timothy Perera⁴, Elisabetta Garibaldi¹, Pietro Gabriele¹, Paolo M. Comoglio¹, Carla Boccaccio¹

¹Candiolo Cancer Institute, FPO-IRCCS, University of Torino, Torino, Italy;

²Department of Molecular and Translational Medicine, University of Brescia, Brescia, Italy;

³Unit of Molecular Neuro-Oncology, Fondazione IRCCS Istituto Neurologico C. Besta, Milano, Italy;

⁴Octimet Oncology Ltd., Oxford, UK

Glioblastoma (GBM), the most aggressive and common primary brain tumor, usually remains refractory to the best standard of care, entailing radiotherapy as a mainstay, and, often, as the only treatment option. GBM radioresistance has been associated with distinctive properties of the GBM stem-like subpopulation (GSC): after irradiation, while bulk-cells accumulate DNA damage and die, stem-like cells efficiently activate DNA repair mechanisms and survive, driving tumor recurrence. A deeper understanding of the mechanisms

of GSC radioresistance is needed, in order to identify druggable targets for radiosensitization and long-term effective therapeutic response.

By analyzing a large panel of GSCs propagated *in vitro* as neurospheres, we provide evidence that radioresistance is significantly higher in GSCs than in their differentiated counterpart (including cells derived from GSC pseudodifferentiation). We show that the levels of radioresistance are similar in GSCs displaying different genetic alterations or transcriptional profiles, which are characteristic of distinct GBM subtypes (classical, proneural, mesenchymal). However, in a subset of neurospheres, radioresistance is associated with expression of MET, the HGF tyrosine kinase receptor. MET expressing GSCs are positively selected by ionizing radiation *in vitro* and, possibly, also *in vivo*, as assessed in a cohort of human patients including 20 cases of surgically removed primary GBMs and their matched recurrences.

We elucidate that MET promotes GSC radioresistance through a novel mechanism, relying on AKT activity and leading to (i) sustained activation of Aurora kinase A, ATM kinase, and the downstream effectors of DNA repair; (ii) phosphorylation and cytoplasmic retention of p21, which is associated with anti-apoptotic functions. We show that MET pharmacological inhibition causes DNA damage accumulation in irradiated GSCs, and their depletion *in vitro* and in GBMs generated by GSC xenotransplantation. Preclinical evidence is thus provided that MET inhibitors can radiosensitize tumors and convert GSC positive selection, induced by radiotherapy, into GSC eradication.

Key words:

Glioblastoma, glioblastoma stem-like cells, MET oncogene, MET inhibitor, radiotherapy, radiosensitization

A11

Transmembrane protein as potential CD9 is glioblastoma stem cell theranostic

Tamara Lah Turnšek

National Institute of Biology, Faculty of Chemistry and Chemical Engineering, University of Ljubljana, Ljubljana, Slovenia

Glioblastomas, the most aggressive brain tumour, is presumably maintained by a sub-population of stem-like tumor cells (GSC) that divide asymmetrically, sustaining pool of highly stable stem cells, resisting therapy. Targeting these cells thus represents more selective approach a need to define specific markers that characterize GSC. In the present study, we performed transcriptomic analysis of glioblastoma

tissues compared to normal brain tissues revealing sensible up-regulation of CD9 gene.^[1] CD9 encodes the transmembrane protein tetraspanin which is involved in tumor cell invasion, apoptosis and resistance to chemotherapy. We validated CD9 gene and protein expression showing selective up-regulation in GSC from primary biopsies and in primary organotypic glioblastoma spheroids as well as in U87-MG and U373 glioblastoma cell lines, whereas no or low CD9 gene expression was observed in their normal counterparts. CD9 silencing in three CD133+ subtypes of GSC lines^[2] (NCH644, NCH421k and NCH660h) led to decreased cell proliferation, survival, invasion, and self-renewal ability, and altered expression of the stem-cell markers CD133, nestin and SOX2. Moreover, CD9-silenced glioblastoma stem cells showed altered kinase signaling patterns. Orthotopic xenotransplantation of CD9-silenced GSC into nude rats promoted prolonged survival. Finally using the public REMBRANDT database for brain tumors, we confirmed the prognostic value of CD9, whereby a more than two fold up-regulation correlates with shorter patient survival. Therefore, we propose CD9 for further evaluation as a target for GBM treatment.

Key words:

Biomarker, CD9, glioblastoma stem cells, neural stem cells, tetraspanin

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A12

Cancer stem cells: the seeds for recurrent ovarian cancer

Nuzhat Ahmed^{1,2}, Emily Chan³, Chantel Samardjiza³, Khalid Abubaker⁴, Ardian Latifi⁴, George Kannourakis^{1,2}, Jock Findlay⁵

¹Fiona Elsey Cancer Research Institute, Victoria, Australia;

²Federation University Australia, Ballarat, Australia;

³Department of Obstetrics and Gynaecology, University of Melbourne, Victoria, Australia;

⁴Department of Surgery, University of Melbourne, Victoria, Australia;

⁵Hudson Institute of Medical Research, Clayton, Victoria, Australia

Aim: The treatment of ovarian cancer (OC) with chemotherapy leaves resistant cancer cells which in

a short time re-grow as recurrent cancer. A diverse array of resistance mechanisms for chemotherapy has been described but none have proven to be viable targets in a clinical setting. Cancer stem cells (CSCs) are increasingly accepted as the putative mediators of chemoresistance and relapse of cancer. This study aimed to understand the molecular mechanisms involved with chemoresistance and recurrence by investigating the roles of CSCs and their associated pathways in OC cell lines and tumor cells isolated from the ascites of OC patients obtained prior (chemonaive, CN) to and after chemotherapy treatment (recurrent, CR). **Methods:** Ascites collected from CN and CR OC patients diagnosed with advanced-stage serous OC were cultured using a novel *in vitro* method to obtain a distinct population of epithelial tumor cells. Flow cytometry and immunofluorescence were used to characterize the tumor population. High-resolution label-free quantitative proteomic profiling was used to define significantly differentially expressed proteins between CN and CR tumor cells. KEGG and DAVID software's were used to determine pathways associated with CR cells. The mechanisms of survival of *in vitro* cisplatin or paclitaxel treated ascites-derived tumor cells as well as cultured OC cell lines were determined by *in vitro* assays and in mouse xenografts. In another approach, the expression of embryonic stem cell factor Oct4A in primary OC tumors as well as CN and CR ascites-derived tumor cells was determined by qPCR. The functional role of Oct4A was investigated using *in vitro* assays and *in vivo* mouse models with stable knockdown (shRNA) of Oct4A in an OC cell line. **Results:** Proteomic profiling of CN and CR tumor cells showed significant differences in proteins encoding for immune surveillance, DNA repair mechanisms, cytoskeleton rearrangement, cell-cell adhesion, cell cycle pathways, cellular transport, and proteins involved with glycine/proline/arginine synthesis in tumor cells isolated from CR relative to CN patients. Pathway analyses revealed enrichment of metabolic pathways, DNA repair mechanisms and energy metabolism pathways in CR tumor cells. The treatment of ascites-derived OC cells with chemotherapy *in vitro* resulted in a CSC-like residual population with increased activation of JAK2/STAT3 pathway. Both JAK2/STAT3 activation and CSC-like characteristics were suppressed by a low dose JAK2 specific inhibitor, Momelotinib, *in vitro* and *in vivo*. This also resulted in a significantly reduced tumor burden, increased disease-free survival periods in mice *in vivo*. In another approach, stable knockdown of Oct4A resulted in the decreased expression of CSCs in OC cells and was consistent with decreased cell proliferation, migration and chemoresistance *in vitro*. *In vivo* Oct4A knockdown cells produced a significantly reduced tumor burden

in mice resulting in a significantly increased survival period compared to vector control cells. **Conclusion:** The above studies suggest that targeting the CSCs may prove a therapeutic option for advanced-stage OC patients.

Key words:

Ovarian cancer, metastasis, ascites, cancer stem cells, chemoresistance, recurrence

A13

Targeting of the epigenetic reprogramming for prostate cancer cell radiosensitization

Anna Dubrovskaya^{1,2,3}, Monica Cojoc¹, Linda Hein¹, Anna Tyutyunnykova¹, Claudia Peitzsch¹

¹OncoRay-National Center for Radiation Research in Oncology, Medical Faculty and University Hospital Carl Gustav Carus, Technische Universität Dresden and Helmholtz-Zentrum Dresden-Rossendorf, Dresden, Germany;

²German Cancer Consortium (DKTK) Dresden, Dresden, Germany;

³German Cancer Research Center (DKFZ) Heidelberg, Heidelberg, Germany

Radiation therapy is one of the mainstays of curative prostate cancer treatment. Nevertheless, the doses needed to eradicate prostate cancer are very high bearing the potential of side effects in normal tissues, and the risk of recurrence after radiotherapy still remains substantial in locally advanced disease. Tumor relapse after radiotherapy is attributed to the population of cancer stem cells (CSCs) which survived the treatment. Therefore, analysis of the CSC populations might be an important predictive tool for radiotherapy and individualized treatment selection. However, compelling evidence suggests a high plasticity of CSCs imposed by tumor treatment. Our study revealed that irradiation causes long-term upregulation in the expression of stem cell markers and induces tumor cell reprogramming. Furthermore, radioresistant and tumorigenic cell populations undergo a phenotypic switch during the course of radiotherapy. This phenotypic plasticity is associated with genetic and epigenetic changes induced by irradiation. Our results indicate that irradiation drives methylation of histone H3 on the promoter sequence of aldehyde dehydrogenase 1A1 (ALDH1A1) leading to the activation of gene transcription. We found that inhibition of H3 methylation with DZNep triggers apoptosis and inhibits tumorigenicity of the radioresistant prostate cancer cells as well as leads to their radiosensitization. Our studies suggest that radioresistant properties of prostate cancer cells are dynamic in nature and that combination of irradiation with therapeutic agents which prevent tumor cell reprogramming may enhance the effectiveness of treatment.

Key words:

Radiation therapy, cancer stem cells, epigenetic reprogramming, radiosensitization

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A14

Cytokine profile involved in the maintenance of radioresistant prostate cancer stem cells

Claudia Peitzsch¹, Monica Cojoc¹, Linda Hein¹, Ina Kurth^{1,3}, Anna Dubrovskaya^{1,2}

¹OncoRay - National Center for Radiation Research in Oncology; Dresden, Germany;

²German Cancer Consortium (DKTK) Dresden, Dresden, Germany;

³Nationales Centrum für Tumorerkrankungen (NCT) Dresden, Dresden, Germany

Aim: There is increasing evidence that human prostate cancer is driven by a malignant subpopulation with stem-like properties. These cancer stem cells (CSC) contribute to tumor-initiation, metastasis, therapy-resistance and tumor relapse. We hypothesize that the determination of CSC-related biomarker in pre-treatment biopsies of prostate cancer patients is correlating with treatment outcome and can be used for patient stratification and treatment selection.

Methods: We generated isogenic radioresistant prostate cancer cell lines by applying several fractions of 4 Gy over a certain period of time until a total dose > 56 Gy (RR). These radioresistant sublines exhibit higher expression of CSC marker (e.g. ALDH, CD133, CXCR4, ABCG2), epithelial-to-mesenchymal transition (EMT) phenotypes, higher self-renewal properties (sphere-formation), higher tumorigenicity and higher migratory activity. We applied several comparative-omic approaches, such as genomic, proteomic, metabolomic, epigenomic and secretome analysis, comparing aldehyde dehydrogenase (ALDH)-positive CSCs with the RR sublines to identify novel biomarker for prostate cancer radioresistance and to unravel the contributing molecular mechanisms. **Results:** Within

our first proof-of-principle study, we could show that ALDH-positive CSCs are radioresistant and maintained directly by the Wnt/ β -catenin signaling pathway.^[1] In addition, we found that irradiation is inducing in a dose- and time-dependent manner several CSC marker and CSC properties. This irradiation-induced CSC-plasticity was attributed to the modulation of the histone methylation code.^[2] Within the presented study we analyzed a panel of secreted cytokines in the medium of the radioresistant sublines and found for example the CXCR4-CXCL12 signaling to be involved in the CSC maintenance and the induction of radioresistance in prostate cancer. This was proven in a s.c. xenotransplantation model *in vivo* and *in ex vivo* treated primary prostate cancer biopsies. **Conclusion:** The CXCR4-CXCL12 signaling axis is involved in the maintenance of prostate CSCs and is contributing to their radioresistant properties.

Key words:

Radiotherapy, cancer stem cells, cytokines, plasticity

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A15

Oxidative stress, metastasis and melanoma stem cell - *in vitro* and *in vivo* analysis

Telma Lisboa-Nascimento, Milene Ormanji, Darcy Marinho, Michele Longoni Calió, Vera Lúcia Rigoni, Clélia Rejane Antônio-Bertoncini, Alice Teixeira Ferreira, Francisco Ribas Bosco

Federal University of Sao Paulo, Sao Paulo, Brazil

Aim: Cancer stem cells play an essential role to maintain the tumor size or fuel its growth. In an advanced stage of melanoma, the presence of a subpopulation of melanoma stem cells (MSC) is reflected in the resistance to the therapies and development of the metastasis. MSC exhibit an altered metabolism when compared to normal melanocyte. This alteration increases in the presence of reactive oxygen species (ROS), such as superoxide anion

(O₂·⁻), which stimulating all three stages of cancer: initiation, promotion and progression. In this work, we analyzed the effects of superoxide anion in MSCs *in vitro* and *in vivo*. **Methods:** The study was approved by Ethical Committee of the Federal University of Sao Paulo, process number: 1523/2008. In this work, the melanoma lineage TM5, was cultured in long term under specific medium containing: bFGF, EGF, LIF and retinoic acid, giving rise to the MSC as we describe in literature. We analyze the viability, proliferation and survivability of MSCs through techniques such as MTT (3-(4, 5-dimethylthiazolyl-2)-2, 5-diphenyltetrazolium bromide), flow cytometry, neubauer camera using biomarkers as: Brd-U, Bcl-2 and Ki-67, for after, evaluate the presence of superoxide anion, through dihydroethidium (DHE) and its function *in vitro* and *in vivo* using the same mentioned techniques and confocal microscopy and immunohistochemistry. **Results:** Long-term culture induced the development of cells with some stem cells characteristics. We named these cells as melanoma stem cells; these form an adherent and nonadherent spheres as well as they showed an increased pigmentation. MSC possess an enhanced ability to survive and adapt after changing the culture medium (epigenetic effect). *In vitro*, MSC shows an outgrowth and quiescence state and self-renewal. MSC presented less expression of Ki-67 when compared with melanoma control cells and both: MSC and control cells presents good viability. The levels of the O₂·⁻ in MSCs are increased significantly compared to the melanoma control cells, suggesting a possible protection against apoptosis in MSCs, because occurred a concomitant increase of expression of the anti-apoptotic protein Bcl-2 and these cells presented more survival capacity. *In vivo*, occurred a decrease in the levels of O₂·⁻, this reduction may be involved in increased of malignancy of melanoma. It was observed an increasing of the expression of the Ki-67 e decreased of the Bcl-2. The microenvironment, *in vivo*, could change the behavior of the MSC, increasing its proliferation and migration and invasion capacity to the neighboring tissues. **Conclusion:** These results indicate two different conclusions: *In vitro*, O₂·⁻ increases and protects MSC against apoptosis, and participates in the survival of these cells and differentiation process. *In vivo*, was observed the less expression of: O₂·⁻ and Bcl-2, and the greater expression of Ki-67, suggesting that *in vivo*, the O₂·⁻ play an important role in cellular proliferation due its interaction with the microenvironment, acting in ways that promotes progression and invasion tumor. Finally, our results imply that the different levels of O₂·⁻ acts in different signaling cascades to promote cell proliferation or differentiation.

Key words:

Superoxide anion, cancer stem cell, melanoma, oxidative stress

A16

Non-alcoholic fatty liver disease and risk for hepatocellular carcinoma - do cancer stem cells matter?

Jürgen Borlak

Hannover Medical School, Hannover, Germany

Hepatocellular carcinoma (HCC) is a frequently diagnosed cancer worldwide and a leading cause of cancer mortality. This malignancy results primarily from viral liver disease, alcoholic injury, aflatoxins and to a lesser extent from genetic disorders such as hemochromatosis. Clinical epidemiology studies suggest an association between non-alcoholic fatty liver disease (NAFLD) and risk for liver cancer. Given the epidemic in fatty liver disease the risk for HCC appears to be particularly increased in NASH cirrhosis patients.^[1]

The mechanisms leading to tumor growth in fatty liver disease are unknown, nonetheless may involve the complex interplay of adipokines and cytokines in promoting hepatocarcinogenesis.^[2,3] Importantly, research identified a decisive role of hepatic stem cells in the development of liver cancer. However, it remains enigmatic why stem cells become cancerous (CSC). Several landmark papers evidence dysregulation of signalling pathways in the control of self-renewal and differentiation of hepatic stem cells and include PI3K/Akt, JAK/STAT, Wnt/β-catenin, hedgehog, Notch, NF-κB and ABC transporters to influence stemness of CSCs.^[4,5] Knowledge on these pathways permits the development of molecularly targeted therapies.

In my presentation I will report recent findings on the regulation of cancer stem cells in steatotic human hepatoma cells to mimic the condition of NAFLD. I will particularly focus on signalling pathways either linked to WNT and Hedgehog signalling, cell cycle regulation and chromatin organisation and provide an overview on the surplus of putative targets obtained from a wide range of cell biology, life cell imaging, genomics and computational biology studies. I will also discuss the possibilities for therapeutic intervention studies in preventing the induction of CSC in NAFLD patients and this includes some preliminary findings with dual kinase inhibitors obtained from the Botta-lab of Siena, Italy.

Key words:

Non-alcoholic fatty liver disease, hepatocellular

carcinoma, steatotic hepatoma cells, tumor growth signalling pathways, dual kinase inhibitors

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A17

Metastatic prostate cancer cells are endowed with cancer stem cell properties and interact with host cells to establish a proinflammatory microenvironment conducive to metastasis

Kiera Rycaj¹, Hangwen Li², Mahipal Suraneni², Xin Chen¹, Collene Jeter², Tammy Calhoun-Davis², Hseuh-Ping Chao², Jianhua Hu², Dean Tang¹

¹Roswell Park Cancer Institute, Buffalo, USA;

²MD Anderson Cancer Center, Houston, USA

Prostate cancer (PCa) is the second leading cancer in American men with an estimated 220,800 new cases and 27,540 deaths in 2015. Androgen deprivation therapy (ADT) is currently the mainstay for advanced PCa patients; unfortunately, most treated patients eventually develop the castration-resistant disease (CRPC). Like most other solid tumors, the worst outcome for PCa patients, whether treated or not, is development of distant metastasis. Although many PCa cell-intrinsic molecules and end-organ factors have been implicated in the metastatic dissemination of PCa cells, the role of primary tumor microenvironment and the nature of the metastatic PCa cells remain poorly defined. Here we attempt to address the two questions by combining experimental PCa metastasis models in NOD/SCID mice and cDNA microarray-based expression profiling followed by extensive functional studies. We first show that PCa cells implanted orthotopically (i.e. in the prostate) metastasize much more extensively and widely than those implanted ectopically (i.e. subcutaneously or s.c). Microarray-based gene expression profiling reveals that the orthotopically implanted human PCa cells upregulate several classes of genes that have been implicated in metastasis and include those involved

in: proteolysis/invasion/angiogenesis, inflammation/cytokine signaling, and developmental pathways/stem cell signaling. Remarkably, mouse-specific microarray analysis shows that several classes of host (mouse) genes, which include those related to myoepithelial and myofibroblast phenotype/cytoskeleton/motility, extracellular matrix/matrix remodeling, inflammation/immune functions, and development/stem cells, are significantly upregulated in the orthotopic prostate tumors. These findings suggest that the implanted human PCa cells reciprocally interact with the host prostatic cells (both epithelial and non-epithelial) to establish a proinflammatory microenvironment highly conducive to PCa metastasis. Further, we provide multiple pieces of evidence that metastatic/metastasizing PCa cells have cancer stem cell (CSC) properties. This data not only advances our understanding of the biology of PCa development and progression but also lays a foundation for developing novel therapeutics to target the tumor microenvironment as well as rare tumorigenic and metastasis-initiating CSCs.

Key words:

Prostate cancer, tumor microenvironment, metastasis, cancer stem cell

A18

EGF/EGFR pathway is sufficient to induce aggressiveness and expression of pluripotency markers of patients-derived glioblastoma cells

Fabien Almairac^{1,2}, Laurent Turchi¹, Denys Fontaine², Philippe Paquis^{1,2}, Hervé Chneiweiss⁴, Marie-Pierre Junier⁴, Fanny Burel-Vandenbos^{1,3}, Thierry Vioille¹

¹University Nice-Sophia Antipolis, CNRS, INSERM, institut Biologie Valrose, Nice, France;

²Neurosurgery Department, University Hospital of Nice, Nice, France;

³Pathology Department, University Hospital of Nice, Nice, France;

⁴University Pierre et Marie Curie, UMCRI8, Neurosciences Paris Seine, Paris, France

Aim: Glioblastomas are the most devastating adult brain neoplasms. Recurrences are ineluctable, and presumed to be due to the glioma-initiating cells (GiCs). GiCs exhibit stem-cells properties such as self-renewal and pluripotency, and are highly tumorigenic. EGFR is a hallmark of infiltrative gliomas, and is overexpressed in almost 50% of glioblastomas. Our objectives were to demonstrate that glioblastoma cells are able to interconvert from a differentiated state to a stem-like state and conversely depending on their environment, and to explore the underlying biological mechanisms, particularly the EGF/EGFR/

ERK pathway. **Methods:** Tumor cells were dissociated from freshly resected human glioblastomas. They were characterized for the stem-like markers (CD133, Sox2, Oct4, Nanog) as well as for the differentiation markers (GFAP) by immunostaining and flow cytometry methods. Functionally, cells were assessed for their clonal properties *in vitro*, temozolomide sensitivity *in vitro*, and tumorigenicity *in vivo* using orthotopic xenotransplantations on NOD/SCID mice.

Results: Spontaneously, most of the cells harbored differentiated properties. Functionally, these cells did not have clonogenic properties, were sensitive to temozolomide (mean surviving cells = 20%), and were not able to form a tumor in brain mice. After 48 h of culture in an EGF enriched medium, the differentiated cells acquired stem-like properties. They expressed Sox2/Oct4/Nanog, displayed long term self-renewal in a clonogenic single-cell assay, and became more resistant to temozolomide (mean surviving cells = 60%). The xenotransplantation of few of these cells led to the development of large tumors after 3 months. Interestingly, the de-differentiation process was quickly reversible, after only 4 days of culture in a serum medium. Also, we demonstrated that the de-differentiation process was inhibited by the adjunction of an anti-EGFR antibody (cetuximab) in the EGF medium, suggesting a pivotal role of the EGF/EGFR/ERK pathway. This pathway was strongly activated during the dedifferentiation process. **Conclusion:** The cellular plasticity concept contrasts with the unidirectional commitment originally described in the cancer stem cell model. It supplies a new comprehensive level on the CiGs' origins, on their resistance mechanisms to genotoxic stresses, and on the tumor heterogeneity. These results emphasize the importance of targeting specifically the stem-like properties of the tumor cells, not only the GiCs, to prevent further tumor enrichment in GiCs.

Key words:

Glioblastoma, cancer stem-cell, glioma initiating cells, plasticity, EGF, cetuximab

A19

Cancer stem cell regulatory mechanisms change at late stages of skin squamous cell carcinoma progression

Victoria da Silva-Diz¹, Pilar Simón-Extremera¹, Adrià Bernat-Peguera¹, Jana de Sostoa¹, Maria Urpi¹, Rosa M Penín², Diana Pérez-Sidelnikova³, Oriol Bermejo³, Joan Maria Viñals³, Annie Rodolosse⁴, Eva González-Suárez¹, Antonio Gómez-Moruno¹, Miguel Angel Pujana¹, Manel Esteller¹, Alberto Villanueva¹, Francesc Viñals¹, Purificación Muñoz¹

¹IDIBELL, Barcelona, Spain;

²Pathology Service, Hospital Universitario de Bellvitge/IDIBELL, Barcelona, Spain;

³Plastic Surgery Unit, Hospital Universitario de Bellvitge/IDIBELL, Barcelona, Spain;

⁴Institute for Research in Biomedicine (IRB Barcelona), Barcelona, Spain

Cancer stem-like cells (CSC) play key roles in long-term tumor propagation and metastasis, but their dynamics during disease progression are not understood. Tumor relapse in patients with initially excised skin squamous cell carcinomas (SCC) is characterized by increased metastatic potential, and SCC progression is associated with an expansion of CSC. Here, we used genetically and chemically-induced mouse models of skin SCC to investigate the signaling pathways contributing to CSC function during disease progression. We found that CSC regulatory mechanisms change in advanced SCC, correlating with aggressive tumor growth, a strong induction of the EMT program and enhanced metastasis. β -catenin and EGFR signaling, induced in early SCC CSC, were downregulated in advanced SCC. Instead, autocrine FGFR1 and PDGFR α signaling, which have not been previously associated with skin SCC CSC, were upregulated in late CSC and promoted tumor growth and metastasis, respectively. Finally, high-grade and recurrent human skin SCC recapitulated the signaling changes observed in advanced mouse SCC. Collectively, our findings suggest a stage-specific switch in CSC regulation during disease progression that could be therapeutically exploited by targeting the PDGFR and FGFR1 pathways to block relapse and metastasis of advanced human skin SCC.

Key words:

Cancer stem cells, skin carcinoma, metastasis, EMT, PDGFR, FGFR

A20

The role of $\alpha 2\beta 1$ integrin on prostate cancer cell stemness

Marjaana Ojalil¹, Elina Taipalus¹, Johanna Jokinen¹, Pekka Taimen¹, Peter J. Boström², Jyrki Heino¹

¹University of Turku, Turku, Finland;

²Turku University Hospital, Turku, Finland

Tumor microenvironment is acknowledged to be a critical component for tumor formation, consisting of cancer and stromal cells together with extracellular matrix (ECM). During tumor development the normal ECM is reorganized and new components synthesized by cancer associated fibroblasts (CAFs), this may create favorable conditions for cancer cell proliferation, invasion and a niche for cancer stem cells. In prostate

epithelial/cancer stem cells are identified as CD44+, CD133+, $\alpha 2\beta 1$ integrin high. Integrin $\alpha 2\beta 1$ is a collagen receptor, which proposes that cell-collagen interaction is important for the stem cell biology in prostate. However the role of specific ECM proteins and cellular receptors in regulating prostate stem cells remains poorly understood.

Here, we have used primary cultures of prostate-derived fibroblastic cells, allowed them to generate ECM, these matrices and specific matrix proteins (collagen I and fibronectin) were used to examine prostate cancer cell-line DU145 proliferation and drug resistance to widely used anti-mitotic chemotherapy drug docetaxel. Sorting cells to sub-populations based on their expression of $\alpha 2\beta 1$ integrin allowed us to investigate whether higher expression of $\alpha 2\beta 1$ integrin is in consistence with theory that cells with high $\alpha 2\beta 1$ integrin are more resistant to cytotoxic drugs. To study thoroughly the role of $\alpha 2\beta 1$ integrin on prostate cancer stem cells, we created $\alpha 2$ integrin knock-out of DU145 cell-line by CRISPR/Cas9 system and compared it with wild-type DU145 cells and rescued cells, which were transfected with plasmid carrying $\alpha 2$ integrin construct.

Our results present that ECM-cancer cell interaction reduced proliferation of cancer cells however according to EC50 values had no effect on the resistance to docetaxel. The EC50 values on collagen I were 18.8 ± 0.3 ; on fibronectin: 19.5 ± 0.2 ; on fibroblast-derived ECM: 19.6 ± 0.3 . Based on collected data we concluded that there was no protective feature of fibroblast-derived ECM to DU145 cancer cells from docetaxel induced cell death. Cells that survived docetaxel treatment presented significantly higher expression of $\alpha 2$ integrin and CD44, suggesting enrichment of stem-like cell population. Indeed, the sub-population with high expression of $\alpha 2\beta 1$ integrin had slightly better survival rates. The ongoing study on whole genome sequencing of CRISPR/Cas9 modified DU145 cells will reveal the differences in gene expression and may bring to light some new properties of integrin signaling.

Key words:

Prostate cancer stem cells, $\alpha 2\beta 1$ integrin, drug resistance, CRISPR/Cas9 system, extracellular matrix, cancer associated fibroblasts

A21

YAP/TAZ, transcription factors at the roots of cancer

Luca Azzolin, Michelangelo Cordenonsi, Stefano Piccolo

Department of Molecular Medicine, University of Padova, Padova, Italy

Tumors are complex tissues and cancer is a disease characterized by aberrant differentiation as much as it is of disturbed proliferation. Tumor cells are phenotypically plastic, and an unsolved issue in cancer biology is to what extent the expansion of cancer stem cells representation that accompany tumor progression is caused by expansion of pre-existing stem cells or, rather, by a differentiation block or even de-differentiation of more differentiated tumor cells. Indeed, the molecular mechanisms that preserve differentiation or induce cell plasticity in neoplastic or normal tissues, as in the case of acquisition of stem-cell traits by more mature cells during tissue repair, remain unknown. At this meeting, I will present new evidence indicating the role of YAP e TAZ, transcriptional effectors of Hippo- mechano- and Wnt-signaling, in regulating cell plasticity. It appears that these properties of YAP/TAZ are independent of acquisition of a mesenchymal phenotype, require interaction with chromatin and are shared by multiple cell type, including non-epithelial ones. Notably, in mouse models, YAP/TAZ are essential for normal stem cells of breast, pancreas and neural tissues when these are activated by tissue damage *in vivo*, or for growth as organoids *ex vivo*. In these are other tissues, this correlate with the genetic requirement of YAP/TAZ to initiate tumorigenesis. The modalities of YAP/TAZ regulation by Wnt and other upstream cues will be also discussed.

Key words:

YAP/TAZ, cancer stem cells, Wnt signalling, cell plasticity

A22

Use of 3D spheroid cultures to screen for drugs targeting cancer stem cells

Juan Gumuzio, Olatz Leis, Angel G. Martin

StemTek Therapeutics, Derio, Spain

The cancer stem cell (CSC) concept has important implications not only for our understanding of carcinogenesis, but also for the development of cancer therapeutics. There is a growing body of preclinical evidence showing that cancer stem cells contribute to chemotherapy and radiation resistance in breast cancer. The use of drugs that interfere with stem cell self-renewal represents the strategy of choice for novel effective anti-cancer treatments, but also a great challenge because cancer stem cells and their normal counterparts share many pathways.

The biology of cancer stem cells has proven complex and difficult to translate into effective therapeutic

strategies. In order to monitor the effect of test compounds on cancer stem cells, a panel of markers, preferably easy-to-measure surface markers, must be defined. This is, however, cumbersome, since for many tumor indications that marker panel is not clearly defined, with often non-overlapping combinations of markers defining cell populations with cancer stem cell activities or tumor initiation ability. This is most likely reflecting the changing nature of the stemness capacity in tumor cells.

Thus, the question arises as: how do we test compounds for anti-cancer stem cell activity? The answer is: phenotypic screening. There are indeed several functional assays well validated in the scientific literature that have been used for years associated to the ability of cancer cells to demonstrate stem cell behavior. The most relevant is the 3D tumor spheroid assay. This assay has been used to uncover and culture stem cells from many tissues as well as from tumors. There are multiple reports now that show that spheroid derived cells are enriched in tumor initiating or cancer stem cells, derived from cell lines and from natural fresh tumors as well. There are several conceptual considerations that need to be taken into account in order to apply this assay to cancer stem cells:

1. This assay may be used to assess stem cell content only when 3D spheroids are formed through cell growth from single cells, not by aggregating cells. Aggregation does not impose the growth restriction necessary to allow stem cells to differentially survive.

2. Not every tumor and cell line is amenable to spheroid growth. This is related to cell adhesion expression profile, as some cells will not form spheroids but disperse cell clusters. Therefore only validated cell lines may be used on the spheroid assay.

3. Care must be taken with cytotoxic compounds. A non-specific cell toxic drug will kill cells regardless of culture conditions, thus only non-toxic concentrations may be used.

The tumor spheroid assay can measure stem cell number in the parental population, by looking at the number of spheres obtained or sphere forming cell frequency through limiting dilution assay. But it can also measure effect on stem cell proliferation by measuring sphere diameter upon time.

Here we describe the use of 3D spheroid models to profile compound activity against cancer stem cells. Furthermore, a case of compounds preventing hypoxia-inducible transcription factor (HIFs) activity is presented. Recently, HIF transcription factor biology

has been linked to pathways that regulate stem cell self-renewal and pluripotency, suggesting a new mechanism whereby HIF proteins may drive tumor growth, through the generation of tumour-initiating cells or cancer stem cells. Therefore, targeting the HIF pathway may provide a novel therapeutic avenue to target cancer stem cells. We demonstrate that interfering with HIF pathway activation prevents mammosphere formation, validated through independent confirmation through Sox2 promoter activation, Aldefluor® assay and *in vivo* proof-of concept experiments targeting tumor initiation.

The assays performed in this work are part of StemTek Therapeutics portfolio of research services, specialized in targeting cancer stem cells for drug discovery.

Key words:

Cancer stem cells, 3D spheroids, drug development

A23

CD34+ cells in blood and primary tumor foci in head and neck squamous cell carcinoma patients receiving radiotherapy

Elena Selivanova, Vyacheslav Andreev, Sergey Makarenko, Irina Zamulaeva

A. Tsyb Medical Radiological Research Centre - branch of the National Medical Research Radiological Centre of the Ministry of Health of the Russian Federation, Obninsk, Russia

The aim of this work was to elucidate the regularities of changes in the frequency of CD34+ hematopoietic stem cells (HSCs) circulating in peripheral blood and accumulating in primary tumor focus in patients with head and neck squamous cell carcinoma (HNSCC) after their exposure to the low-LET ionizing radiation receiving radiotherapy.

A study group consisted of 35 patients with HNSCC in age from 44 to 85 years (mean age 59.5 ± 1.3 years). The frequency of HSCs were determined in patients' biopsies and blood cell samples before treatment and at 24 h after receiving of the subsequent local dose of γ -irradiation of 10 Gy with conventionally fractionated dose of 2 Gy daily. Stem cell viability and a number of HCS were evaluated in the disrupted and dissociated tumor pieces and in blood cell samples by three-color FACS analysis using DNA binding dye Hoechst33342 and anti-CD45 and CD34 antibodies.

The high individual variability in the frequency of CD45lowCD34+HSCs was found in the tumor biopsies and blood obtained from patients before treatment. The median frequency of CD34+ cells before treatment was in the blood of $4.9 \cdot 10^{-4}$, in biopsy material is $1.6 \cdot 10^{-2}$.

This indicates a significant accumulation of the studied cells in the primary tumor. Correlation between initial frequency of the HSCs and clinical-morphological characteristics of the tumor was not observed. There was a tendency to the decreased frequency of CD34+ cells in the blood samples to $3.6 \cdot 10^{-4}$ ($P = 0.1$ in comparison with the pretreatment frequency) after irradiation. High statistically significant correlation was observed at the individual level between the frequency of these cells before and after exposure ($R = 0.82$, $P < 0.0001$). The median frequency of CD34+ cells in the tumor, in contrast, was increased to $2.7 \cdot 10^{-2}$ and did not correlate with the initial frequency of these cells before treatment in this group of patients. The results obtained indicate the decrease in migration of hematopoietic cells after the first sessions of radiation therapy, probably, due to the reduction of signals produced by the tumor to the chemotaxis and mobilization of these cells from the bone marrow. The regulation of the number of intra-tumoral hematopoietic cells is a complex process, including their differentiation in different types of cells. It is known that ionizing radiation disturbs the differentiation process of hematopoietic cells which may be to a cause of the observed increase in the frequency CD34 + cells in the majority of HNSCC patients. In the future, it would be of great interest to evaluate the predictive value of the number of CD34 + cells before treatment and changes in this index during radiotherapy.

Key words:

Head and neck squamous cell carcinoma, low-LET ionizing radiation

A24

The therapeutic targets of miRNA in hepatic cancer stem cells

Sabrina Bimonte, Maddalena Leongito, Antonio Barbieri, Francesco Izzo

IRCCS Istituto Nazionale dei Tumori Fondazione G. Pascale, Napoli, Italy

Hepatocellular carcinoma (HCC) is the fifth most common cancer worldwide malignancy, and the third leading causes of cancer death in patients. Several studies demonstrated that hepatic cancer stem cells (HCSCs), also called tumor-initiating cells, are involved in the regulation of HCC initiation, tumor progression, metastasis development and drug resistance. Despite the extensive research, the underlying mechanisms by which HCSCs are regulated remain still unclear. MicroRNAs (miRNAs) are able to regulate several biological processes such as self-renewal and pluripotency of HCSCs,

representing a new promising strategy for treatment of HCC chemotherapy-resistant tumors. In this review, we synthesize the latest findings on therapeutic regulation of HCSCs by miRNAs, in order to highlight the perspective of novel miRNA-based anticancer therapies for HCC treatment.

Key words:

Hepatic cancer stem cells, hepatocellular carcinoma, signaling pathway, microRNA

A25

Targeting metalloproteinases to sensitize breast cancer stem cell to radiation

María Auxiliadora Olivares-Urbano¹, Sandra Ríos-Arrabal^{1,2}, María Escarlata López³, María Isabel Núñez^{1,2,4}

¹Department of Radiology and Physical Medicine, University of Granada, Granada, Spain;

²Institute of Granada (ibs.GRANADA), University Hospitals of Granada-University of Granada, Granada, Spain;

³Department of Radiation Oncology, Oncosur, Granada, Spain;

⁴Biopathology and Medicine Regenerative Institute (IBIMER), University of Granada, Granada, Spain

Aim: The use of adjuvant or neoadjuvant radiotherapy improves local control and survival rate while maintaining normal tissue toxicities at acceptable levels. Nonetheless, cell radiation resistance is a limiting factor for this treatment. Radioresistance is due, in part, to cancer stem cells (CSCs) not killed by radiation. Thus CSCs isolation from breast tumours will be important for a better understanding of molecular mechanisms involved in their origin, self-renewal, differentiation into tumour cells, resistance to radio- and chemotherapy, invasiveness and metastatic ability. **Methods:** Two breast cancer cell lines (MCF-7 and MDA-MB-231) were maintained in monolayer culture and then cultured in sphere medium. At 24 h after seeding in monolayer, cells were irradiated at 2, 4 or 6 Gy, maintaining a non-irradiated control. From the general population of cells cultured in sphere media, positive and negative CSCs were separated with flow cytometry. Stem cell markers considered were ALDH1, CD24 and CD44. The genes measured in both positive and negative sub-populations were: MMP-1, MMP-2, MMP-3, MMP-9, MMP-13, HDAC-1, HDAC-2, HDAC-4, TIMP-1 and TIMP-2. **Results:** After ionizing radiation treatment, the proportion of CSCs markers varied according to the dose and cell type. ALDH positivity was increased at 4 Gy in MCF-7 cells, while ALDH1 and CD44 were increased at 6 Gy in the MDA-MB-231 line. More genes were expressed in the MDA-MB-231 versus MCF-7 line. MMP-1, MMP-2, HDAC-4, and TIMP-1 genes were expressed in both cell lines. MMP-3, MMP-9, MMP-13, HDAC-2 and TIMP-2 were

also expressed in MDA-MB-231 cells. In general, genes were more expressed in the negative population than in the positive, highlighting the positive CSCs expression of MMP-2 and MMP-9, which are related to the formation of secondary tumors. **Conclusion:** Radiation enhances the population of CSCs in different ways according to the dose and cell line. Post-radiation, a larger number and higher proportion of genes were expressed in the MDA-MB-231 cell line, which is more radioresistant in comparison to MCF-7. The increased MMP-2 and MMP-9 expression after radiation would contribute to modifying the cell survival capacity and differentiation status of MDA-MB-231 cells and may correspond to a phenotype linked to carcinogenesis. The increase in this expression with higher radiation dose in the positive CSC population may play a role in the development of secondary tumors, facilitating ECM degradation by the cells that survive radiation. Administration of inhibitors of these MMPs to patients undergoing radiotherapy may be useful to avoid the radiation-induced development of a more aggressive phenotype that promotes tumor progression. Both MMP-2 and MMP-9 may be considered novel therapeutic targets in cancer treatment.

Key words:

CSCs, ionizing radiation, MMPs, breast cancer, HDAC

A26

CD57 defines a novel maker of glioblastoma stem cells that have greater invasive potential than CD133+ tumor cells

Lin Qi, Yulun Huang, Mari Kogiso, Hua Mao, Holly Lindsay, Patricia Baxter, Jack Su, Laszlo Perlaky, Ching Lau, Murali Chintagumpala, Xiao-Nan Li

Baylor College of Medicine, Houston, USA

Glioblastoma multiforme (GBM) is the most aggressive and lethal brain tumor that occurs both in children and adults. Diffuse invasion into normal brain tissue is one of the important biologic features that make GBM refractory to conventional therapies. While existing studies on GBM invasion are primarily conducted using tumor core tissues from surgical resections, it is unclear whether unresectable, infiltrative GBM cells would be more informative for studying their invasive nature compared to those in the resected tumor cores. More importantly, little is known if and which cancer stem cell populations are driving glioma invasion. To address these fundamental issues, we utilized our panel of 7 (6 pediatric and 1 adult) patient tumor-derived orthotopic xenograft mouse models of GBM to isolate invasive GBM cells (infiltrating normal mouse

brain parenchyma) and tumor core GBM cells and directly compared their biological differences. Our result showed that the invasive cells have stronger neurosphere forming efficiency *in vitro* in a serial dilution assay and increased tumorigenic capacity after *in vivo* transplantation (particularly at 100 cells/mouse) compared to the tumor core cells. A screening of putative cancer stem cell markers (CD133, CD15, CD24/CD44, CD57 and CD117) showed that invasive GBM cells are enriched (> 2 folds) with CD57+ cells compared to the tumor core cells, and these infiltrating cells were predominantly CD57+/CD133-. Even the CD133+ cells were frequently dual-positive with CD57 (CD33+CD57+), not only in the xenograft tumors but also in a separate set of patient GBM samples. Mechanistically, we found that CD57+ cells expressed high levels of self-renewal genes and tend to stay in G0/G1 phases. In conclusion, we showed that invasive GBM cells are biologically different from the matched tumor core cells and identified CD57 as a novel stem cell marker that is associated with GBM infiltration. Our findings suggest that new anti-invasion therapies should target CD57+ cells in addition to CD133+ cells in GBM.

Key words:

CD57, GBM, xenograft

A27

Investigating radiation induced changes in stem cell population of non-small cell lung cancer (NSCLC) models following stereotactic ablative radiotherapy (SABR)

Charlene Junkin¹, Victoria Dunne¹, Gerry Hanna^{1,2}, Kevin Prise¹, Karl Butterworth¹

¹Queen's University Belfast, Belfast, UK;

²Clinical Oncology, Northern Ireland Cancer Centre, Belfast, UK

SABR is emerging as a powerful clinical technique for the treatment of localised and inoperable NSCLC. Despite improvements using radical chemoradiotherapy, overall 5 year survival rates remain low at between 7-20%, better strategies are urgently needed to improve control rates. Radiotherapy failure can be attributed to resistance as a result of heterogeneity within the tumour which may include a sub-population of cancer stem cells (CSCs), these may impact the ability of the tumours to recur following radiotherapy. Furthermore, radiation exposure may impact CSC populations causing differentiated cells to acquire stem-like properties or normal stem cells to transform into CSCs due to genetic alteration or changes in the normal stem cell microenvironment. The underlying

process of radiation induced stemness in NSCLC remains to be fully understood and may present novel opportunities for therapeutic intervention in combination with SABR that could potentially improve overall survival in NSCLC.

In this study, a panel of NSCLC cell lines (A549, H460, H157) were exposed to total doses of 6, 12 and 24 Gy delivered in 3 fractions over a 9 day period using a XRAD 225 X-ray generator (PXI, Inc.). Flow cytometry analysis of the irradiated cell populations showed significant dose dependent increases in the populations of cells bearing the putative stem cell markers CD44 and CD133. This effect was greatest in the A549 cell model which showed a population increase of 9.8, 13.9 and 24.7% at corresponding doses of 6, 12 and 24 Gy. We have identified interferon regulatory factor-7 (IRF-7) as a potential mediator of radiation induced plasticity and resistance. Quantitative PCR revealed a tumour-specific change in IRF-7 RNA expression upon irradiation, with a general relative increase in expression with increasing radiation dose. These data were compared with a normal bronchial epithelial cell model, which contrastingly showed a relative decrease in IRF7 RNA expression levels upon increasing doses of radiation. Flow cytometry analysis and IRF7 RNA quantification was repeated using the A549 model with the addition of acute hypoxia at the time of irradiation. I have not found a significant difference in the percentage of cells expressing these stem cell markers upon addition of hypoxic conditions, however, IRF-7 RNA expression levels appear to decrease by nearly 40% upon addition of hypoxic conditions without irradiation.

Our results demonstrate radiation induced cellular plasticity following exposure to hypofractionated schedules in models of NSCLC with a potential role of IRF-7. Ongoing work in our laboratory seeks to further investigate the underlying radiobiological mechanisms of CSC plasticity and the role of both acute and chronic hypoxia. We ultimately aim to identify novel therapeutic strategies, to prevent acquisition of stem-like properties, reduce treatment failure and improve outcomes.

Key words:

Cancer stem cells, stereotactic ablative radiotherapy, non-small cell lung cancer, CD133, CD44, interferon regulatory factor 7

A28

The JNK-STAT3 pathway as a therapeutic target in neuroblastoma

Mayumi Higashi

Kyoto Prefectural University of Medicine, Kyoto, Japan

Aim: Neuroblastoma is one of the most challenging tumours in children, and the 5-year survival rate of progressive neuroblastoma remains around 30-40% regardless of improvement of intensive therapies. We studied signal transduction cascades as targets for neuroblastoma therapy. It has been reported that one of major MAPKs JNK regulates STAT3, which is another pathway of STAT3 activation different from JAK-STAT3, by phosphorylating a specific amino acid residue. It has been reported that JNK or STAT3 maintains the stemness of cancer cells, although the role of this pathway is not clearly revealed. In our study, we found remarkable anti-tumour effects of inhibiting JNK-STAT3 pathway, by disrupting the stemness of neuroblastoma. **Methods:** Neuroblastoma cell lines IMR5, NLF and SK-N-AS were treated with JNK inhibitor SP600125 or JNK-IN-8, or STAT3 inhibitor Niclosamide in various concentrations. Cell viability was analysed using CellTiter® (Promega). Immunofluorescence was performed with β -III tubulin antibody (SIGMA) to stain neural processes. Gene expression was analysed by THUNDERBIRD SYBR-Green real-time PCR system (TOYOBO). **Results:** Phosphorylation of STAT3 serine 727, but not tyrosine 705, was suppressed by JNK inhibitor SP600125 or JNK-IN-8, dose-dependently. JNK inhibitors SP600125, JNK-IN-8 and a STAT3 inhibitor Niclosamide induced resembled effects on neuroblastoma cells, although the effects were cell growth dependent. All inhibitors induced remarkable decrease in cell viability in fast proliferating cell lines IMR5 and NLF, while they induced deceleration of proliferation and cell differentiation with increased β -III tubulin staining of neural processes in slower proliferative SK-N-AS. Low dose of Niclosamide also induced neural differentiation in any cells. Expressions of cancer stem cell markers SOX2 and CXCR4 were decreased by treatment with inhibitors. **Conclusion:** Inhibition of JNK-STAT3 led cells to differentiation or cell death, which indicates the disruption of cancer stemness in neuroblastoma cells. Our findings show that inhibition of JNK or STAT3 is an ideal way of tumour suppression induced by disrupting homeostasis of cancer cells. Our results indicate that JNK-STAT3 pathway is a promising target for the novel treatment of neuroblastoma.

Key words:

JNK STAT3 neuroblastoma

A29

The role of secreted frizzled-related protein 4 (sFRP4) in chemo-sensitisation of cancer stem cells

Abhijeet Deshmukh¹, Frank Arfuso¹, Philip Newsholme²,
Arun. M. Dharmarajan¹

¹Stem Cell and Cancer Biology Laboratory, School of Biomedical Sciences,
Curtin Health Innovation Research Institute, Curtin University, Perth, Australia;

²School of Biomedical Sciences, Curtin Health Innovation Research Institute,
Curtin University, Perth, Australia

Background: Cancer stem cells (CSCs) are the unipotent cell population present within the tumour mass. CSCs are known to be highly chemo-resistant and, in recent years, have gained intense interest as key tumour-initiating cells that play an integral role in cancer recurrence following chemotherapy. **Aim:** The study investigates molecular signals essential to sustain CSCs and target their activity using secreted frizzled-related protein 4 (sFRP4) alone or in combination with chemotherapeutic drugs. **Methods:** **Cancer stem cells isolation:** CSCs isolated from Breast (MDA231/MCF7), Ovary (A2780 P/ADR/Cis), and Prostate (PC3/LnCap) tumour cell lines in serum-free conditions and enriched with growth factors (EGF/FGF/B-27). **Chemo-sensitisation/drug treatment:** Sensitisation with sFRP4 was performed by adding sFRP4 to the CSCs culture alone or in combination with chemotherapeutic agents (Doxorubicin/Cisplatin) for 24 h. **Viability assay:** MTT based was used according to the manufacturer's protocol to measure cell metabolic viability. **Cell surface markers:** To assist in determining their identity, cell surface markers (CD44⁺/CD24⁻/CD133⁺) were examined in both monolayers and CSCs by flow cytometry, using CellQuest data acquisition and analysis software. **RNA isolation and cDNA synthesis:** Total RNA was isolated from cells using TRIzol reagent followed by chloroform extraction, isopropanol precipitation, and a 75% (v/v) ethanol wash and further transcribed into cDNA using a High Capacity cDNA kit. **Western blotting:** Total proteins were extracted from cells using RIPA denaturing buffer. The protein extracts were estimated using BCA Kit and 20 µg of proteins were separated by 12% SDS-PAGE and transferred onto a nitrocellulose membrane. Immunoblotting was performed by blocking the membrane in 5% Non-Fat Dry Milk (NFDM) solution and incubating the membrane in 5% NFDM/BSA containing primary antibodies overnight at 4°C. The membranes were incubated in 3% NFDM containing secondary antibodies for 1 h at RT after three washes with PBS containing 0.1% Tween 20. Signals were detected on a Chemi-Doc imaging analyser using ECL Western Blotting Substrate. **Caspase assay:** The intracellular levels and activation of caspase-8 and caspase-3 were followed by Western blotting using antibodies specific for the proenzymes and activated species. Caspase-3 activity was measured using the EnzChek Caspase-3 Assay Kit II (Molecular Probes,

Invitrogen). **Results:** The MTT assays conducted showed the chemo-sensitisation effect of sFRP4 when used in combination with tumour-specific drugs. The post-transcription data (Gene-Expression) collected from CSCs that have undergone combinatorial treatment with sFRP4 and chemotherapeutic drugs suggests there is downregulation of drug transporters and upregulation of angiogenic/apoptotic/cell death markers. The post-translational modification (protein expression) of CSCs shows the chemo-sensitisation effect of sFRP4, when used in combination with tumour-specific drugs, by downregulating the cell-survival and oncogenes signals and upregulation pro-apoptotic signals. In tumour cell lines, sFRP4 in combination with doxorubicin/Cisplatin, reduced the proliferative capacity of CSC population *in vitro*. **Conclusion:** Wnt/β-catenin signalling is important for proliferation and self-renewal of CSCs in association with human tumorigenesis. The silencing of this signalling pathway by the application of sFRP4 suggests potential for improved *in vivo* chemo-responses.

Key words:

Cancer stem cells, chemo-sensitisation, Wnt signalling pathway, Wnt antagonist, secreted frizzled related protein 4

A30

Induction of radioresistance and cisplatin resistance in HNSCC cell line after ionizing radiation

Vesna Todorovic¹, Ajda Prevc¹, Martina Niksic Zakelj¹,
Blaz Groselj¹, Primož Stojan¹, Maja Cemazar^{1,2}, Gregor Sersa¹

¹Institute of Oncology Ljubljana, Ljubljana, Slovenia;

²University of Primorska, Faculty of Health Sciences, Izola, Slovenia

Head and neck squamous cell carcinoma (HNSCC) constitutes approximately 6% of all cancers worldwide. The risk of HNSCC is strongly associated to habitual exposure to tobacco or alcohol. In addition, oropharyngeal SCC (OPSCC) can arise also from infection with human papilloma virus (HPV). Management of HNSCC is complex and is in part correlated to risk factors. Namely, HPV-positive OPSCC has a greater response to radiation or chemoradiation than tobacco/alcohol related HNSCC. In addition, a significant problem of HNSCC is its recurrence, specifically in previously irradiated areas, due to induced radioresistance and radiation tolerance limits of already irradiated normal tissues.

The aim of our study was to investigate the response of three HNSCC cell lines to ionizing radiation and

exposure to cisplatin *in vitro*. DNA damage was evaluated in all cell lines in response to ionizing radiation by quantification of H2AX foci. HNSCC cell lines used in the experiments were FaDu, a HPV-positive cell line 2A3, derived from FaDu cells, and a radioresistant cell line FaDu-RR, established from FaDu cells by repeated exposure to ionizing radiation. Radioresistant FaDu-RR cells were recovered after exposure to fractionated ionizing radiation (a total dose of 120 Gy).

The selected HNSCC cell lines responded differently to ionizing radiation and cisplatin. FaDu-RR cells were the most radioresistant with a dose modifying factor (DMF) at ED_{50} 1.66 compared to FaDu cells. Contrary, a HPV-positive 2A3 cells were more sensitive to ionizing radiation than FaDu cells (DMF at ED_{50} 0.47). In addition, FaDu-RR cells also displayed cross-resistance to cisplatin (1.8-fold potentiation in IC_{50} value) compared to FaDu cells, whereas 2A3 cells were more sensitive to cisplatin compared to FaDu cells (2.9-fold reduction in IC_{50} value).

DNA damage after ionizing radiation was quantified by detection of H2AX foci, which correlates to DNA double strand breaks. In all three cell lines, a peak of γ H2AX foci was detected as early as 30 min after ionizing radiation. Interestingly, at this time point a maximum

number of γ H2AX foci was detected in radiosensitive 2A3 cells (more than 20/nuclei), whereas in FaDu cells 11 γ H2AX foci/nuclei were detected. In radioresistant FaDu-RR cells, only 7 γ H2AX foci/nuclei were detected 30 min after ionizing radiation. The level of γ H2AX foci 24 h after ionizing radiation was reduced in all cell lines, however only in radioresistant FaDu-RR cells the number of residual γ H2AX foci was similar to the level of γ H2AX foci in unirradiated cells. In FaDu and 2A3 cells, the number of residual γ H2AX foci was reduced approximately by half.

A different response to ionizing radiation and exposure to cisplatin was observed in tested HNSCC cell lines. Namely, repeated exposure to ionizing radiation induced radioresistance and resistance to cisplatin in FaDu-RR cell line, whereas 2A3, a HPV-positive cell line, were more sensitive to ionizing radiation and cisplatin compared to parental FaDu cell line. The observed differences in radiosensitivity of HNSCC cell lines can be at least partly contributed to induction and clearance of γ H2AX foci after ionizing radiation indicating differences in DNA damage repair.

Key words:

Radioresistance, head and neck, squamous cell carcinoma, chemoresistance, cisplatin, H2AX foci

Nibrin expression in oral squamous cell carcinoma: association with clinicopathological parameters

Jigna H. Dave, Hemangini H. Vora, Trupti I. Trivedi, Nandita R. Ghosh

Division of Molecular Endocrinology, Department of Cancer Biology, the Gujarat Cancer and Research Institute, Asarwa, Ahmedabad 380016, India.

Correspondence to: Dr. Nandita R. Ghosh, Division of Molecular Endocrinology, Department of Cancer Biology, the Gujarat Cancer and Research Institute, Asarwa, Ahmedabad 380016, India. E-mail: nandita.ghosh@gcriindia.org

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ABSTRACT

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Key words:

Nibrin protein expression,
oral squamous cell carcinoma,
clinicopathological parameters

Aim: The present study sought to discover the role of Nibrin protein in 100 patients with oral squamous cell carcinoma (OSCC) and its potential relationship with clinicopathological parameters. **Methods:** Nibrin expression was evaluated immunohistochemically using the modified H-score method. **Results:** The present study included 20% of patients with stage I disease, 22% of patients with stage II disease, 18% of patients with stage III disease, and 40% of patients with stage IV disease. Nibrin showed a significant positive correlation with moderately/poorly differentiated tumor tissues ($P = 0.028$), while significant inverse correlation of Nibrin expression was observed with tumor size ($P = 0.018$) and tumor stage ($P = 0.039$). Further, using univariate survival analysis it was observed that strong Nibrin expression was significantly associated with disease relapse in early stage OSCC patients ($P = 0.049$). **Conclusion:** Thus, the present study revealed that Nibrin could be used as a prognostic marker in patients with early stage OSCC.

INTRODUCTION

Carcinomas of the oral cavity, including cancer originating from the buccal mucosa and tongue are of 10 most common cancers in the world with an increasing trend of incidence.^[1,2] Squamous cell carcinoma (SCC) is the most common type of oral cancer which accounts for more than 90% of oral malignancies which is characterized by an aggressive growth pattern, high-degree of local invasiveness, and cervical lymph node spread.^[1,3] In India, oral squamous

cell carcinoma (OSCC) is the leading cause of death which stands for 35-40% of all malignancies which is owed to the increased prevalence of lifestyle habits like chewing areca-nut/betel nut quid/tobacco and smoking with heavy alcohol consumption serving as a potent cofactor.^[4-6] The survival of patients with oral cancer has remained unchanged even with the improved therapeutic modalities, over the last 3 decades.^[4] The resultant poor prognosis is owed to a late stage diagnosis, low response rate to current therapeutic strategies, high risk of primary site recurrence and



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aggressive metastases to loco-regional lymph nodes, strongly suggestive of an urge to improve the treatment efficacy and diagnostic capabilities. Over the last decade, scientific research related to the specific pathways which are relevant to the development and progression of this disease has been performed to investigate biological, diagnostic and prognostic parameters.^[7-10]

DNA damage is one of the underlying causes for mutations which is very numerous and appears to be a fundamental problem for life leading to cancer. In human cells, the estimated average number of DNA damages occurring per hour is about 800 which reach to 19,200 per day.^[11] If, such DNA damages are not repaired in dividing cells, cause errors during DNA synthesis leading to mutations which can give rise to cancer. Thus individuals are often at increased risk of cancer with an inherited damage in DNA repair capability.^[12]

Nijmegen breakage syndrome (NBS) is a chromosomal instability syndrome associated with cancer predisposition, growth retardation, microcephaly, radiosensitivity and immunodeficiency.^[13-15] The NBS1/Nibrin/p95 is a member of the DNA double-strand break (DSB) repair complex (hMre11 complex) which is a product of the defective gene in NBS (the *NBS* gene) located on human chromosome 8q21.^[14-16] The Nibrin containing protein complex [Mre11-Rad50-Nbs1(MRN) complex] binds to the edges of the DNA double stranded break and remains attached to this site until the break gets repaired.^[17] Nibrin is also involved in various signaling cascades other than DSBs induced by irradiation such as mitotic V(D)J rearrangements in T and B lymphocytes, maintenance of telomere function and meiotic recombination.^[18,19] Once ataxia-telangiectasia mutated protein phosphorylates NBS1, it then carries out its checkpoint functions following ionizing radiation.^[20-22] However, in certain types of human cancer rare or no mutations of NBS1 have been studied.^[23-25] In addition, during the process of carcinogenesis NBS1 is expressed in highly proliferating tissues.^[26] On the basis of this information, the aim of this study was to assess whether the Nibrin expression would relate to clinicopathological variables and if it could predict survival or recurrence in OSCC.

METHODS

Study population

A total of 100 untreated patients with histopathologically confirmed OSCC of tongue and buccal mucosa evaluated between 2011 and 2013 at our institute were included in this study. Formalin fixed and paraffin

embedded primary tumor tissue blocks (buccal mucosa: $n = 39$, tongue: $n = 61$) and histologically confirmed adjacent normal tissue blocks were collected from the histopathology department of our institute. The detail clinical history of the patients [age, gender, tobacco habit, site of disease, tumor-node-metastasis (TNM) stage, histopathological findings, treatment given, etc.] was obtained from the case files maintained at our institute. In patients with OSCC the disease was staged according to the criteria of the American Joint Committee on Cancer pTNM classification. Thus, the present study included 20 patients with stage I disease, 22 patients with stage II disease, 18 patients with stage III disease and 40 patients with stage IV disease. This study was approved by our institutional review committee for dissertation/thesis/publications/conference presentations and institutional ethics committee.

Immunohistochemistry

Immunohistochemistry of Nibrin was performed using the avidine-biotin complex technique in which formalin fixed paraffin embedded tissue sections (4 μ m) were mounted on 3-aminopropyletriethoxy silane coated glass slides. The sections were first deparaffinized using xylene and then rehydrated using graded alcohol. Endogenous peroxidase activity was blocked using 3% hydrogen peroxide prepared in methanol for 15 min. Antigenicity of the processed tissue sections was retrieved by cooking the sections in 10 mmol/L tri-sodium citrate buffer (pH 6.0) solution with 0.05% tween-20 for 20 min in a pressure cooker. Sections were then allowed to cool at room temperature. For Nibrin immunostainings, a commercial mouse monoclonal antibody (clone 1D7, Santa Cruz Biotechnology, Santa Cruz, USA) at dilution of 1:100 prepared in tris buffered saline was applied to the sections and were then incubated overnight at 4 °C. For immunohistochemical detection of the antibody reaction, we used Novolink polymer detection kit from Novocastra. Sections were then dehydrated, cleared in xylene and mounted in dibutylphthalate xylene. As positive controls, formalin-fixed paraffin-embedded tissue sections with intense staining for a given marker were included with each staining procedure.

Assessment of Nibrin expression

All sections were scored independently by two independent researchers in a blinded fashion. The staining intensities and the percentage of positive cells were separately assessed in primary tumor tissues ($n = 100$) and their corresponding adjacent normal squamous epithelium ($n = 100$). As the Nibrin expression was not uniform in different parts of the epithelium or cancerous tissue, we used modified

histoscore (H-score) method to combine the staining intensity and percentage of Nibrin expressing cells. More specifically, the staining intensity was assessed with a four-point scale from negative (0); weak (1); moderate (2); and strong intensity (3). The extent of the staining was expressed as percentage of positive cells (0-100%) by 10% intervals. The Nibrin histoscore was counted by multiplying the intensity level by percentage of positive cells resulting in a value between 0 and 300. Data were divided into groups by histoscore levels. Accordingly the cancer and their corresponding adjacent normal specimens were grouped by Nibrin expression score based on the median score value of cancerous and adjacent normal tissues respectively into “weak expression” (Tumor: scores 0-209 and Normal: scores 0-144) and “strong expression” (Tumor: scores 210-300 and Normal: scores 145-300).

Follow-up and disease status of OSCC patients

Out of total 100 OSCC patients, for overall survival analysis, only 90 patients could be followed for a period of 24 months or until death within that period. On the other hand, for relapse-free survival study, 78 of 100 patients with or without recurrence within that period were considered. The remaining 12 patients could not be included for relapse-free survival study due to presence of persistent disease.

Statistical analysis

The data were analyzed statistically using SPSS software version 17.0 (Chicago, IL, USA). The two tailed chi-square test was used to assess associations between two parameters. Correlations between two parameters were calculated using spearman's correlation coefficient (r). To compare the Nibrin expression in cancerous and adjacent normal tissues, paired sample t -test was used. Univariate survival analysis was performed using Kaplan-Meier survival function and differences in survival were tested for statistical significance using the log-rank statistics. P

values ≤ 0.05 were considered significant.

RESULTS

Nibrin expression in OSCC

Of the tongue and buccal mucosa cancer tissue, Nibrin protein expression was evaluated with nuclear location of the immunoreactions, Nibrin was expressed in 99% of tumors and 92% of the adjacent normal squamous epithelium [Figure 1]. H-score varied from 0 to 300 in both OSCC and adjacent normal tissues. Median H-score for tumor tissues was 210 while that for the adjacent normal tissue was 145. The tissues expressing Nibrin below the median H-score was consider as a weak expression and tissue expressing Nibrin above the median H-score was considered as a strong expression.

Relation of Nibrin expression with clinical and histopathological parameters

Two tailed chi-square test and spearman's correlation coefficient (r) were used to assess correlation between the Nibrin protein expression and clinicopathological parameters in tumor tissues. The relations of Nibrin immunoreactivity with clinical and histopathological parameters are depicted in Table 1, respectively. In tumor tissues an inverse correlation of Nibrin expression was found with tumor size ($\chi^2 = 5.622$, $r = -0.237$, $P = 0.018$) and tumor stage ($\chi^2 = 6.600$, $r = -0.194$, $P = 0.039$) while, significant positive correlation was found with strong Nibrin expression and moderately/poorly differentiated tumor tissues ($\chi^2 = 4.857$, $r = +0.220$, $P = 0.028$). No significant association was detected with Nibrin expression and other clinicopathological parameters.

Univariate survival analysis

According to Kaplan-Meier survival analysis similar incidence of death observed in total patients with strong (42%, 20/48, log-rank = 0.112, df = 1, $P = 0.737$)

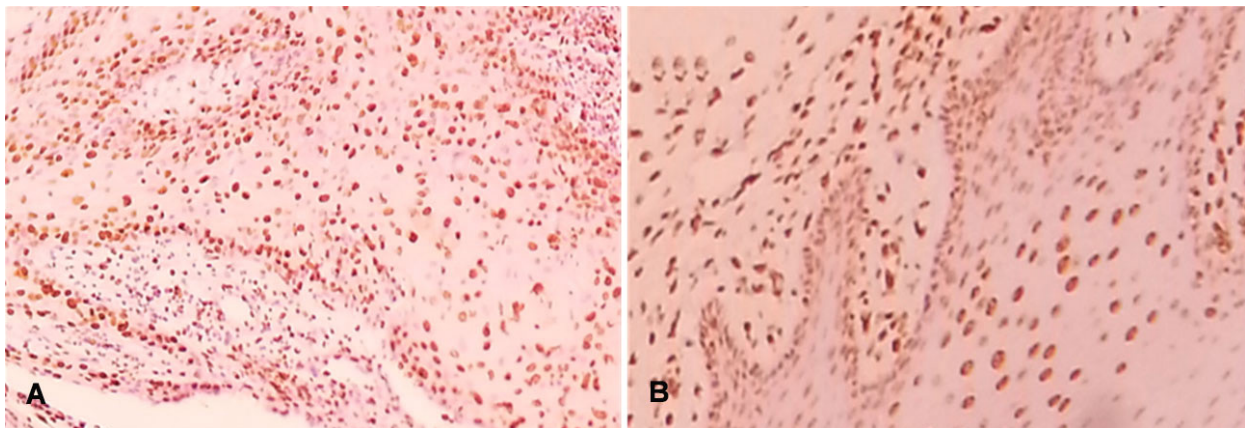


Figure 1: Nibrin protein expression (IHC, $\times 10$). (A) Nuclear protein expression of Nibrin in primary tumor of OSCC; (B) nuclear protein expression of Nibrin in adjacent normal tissue of primary OSCC tumor tissue. OSCC: oral squamous cell carcinoma

Table 1: Relation of Nibrin immunoreactivity with clinical and pathological parameters in OSCC tissue

Variables	Nibrin expression in tumor (Median: 210)			Correlation (r)	P
	n	Weak, n (%)	Strong, n (%)		
Age (year): median 45					
< 45	47	22 (47)	25 (53)	-0.004	0.972
≥ 45	53	25 (47)	28 (53)		
Gender					
Male	75	33 (44)	42 (56)	+0.104	0.303
Female	25	14 (56)	11 (44)		
Anatomic site					
Tongue	61	30 (49)	31 (51)	+0.055	0.589
Buccal mucosa	39	17 (44)	22 (56)		
Tobacco habit					
Absent	14	9 (64)	5 (36)	+0.140	0.166
Present	86	38 (44)	48 (56)		
Disease status (n = 78)					
No recurrence	46	23 (50)	23 (50)	+0.031	0.789
Recurrence	32	15 (47)	17 (53)		
Disease outcome (n = 90)					
Alive	51	23 (45)	28 (55)	-0.036	0.737
Dead	39	19 (49)	20 (51)		
Tumor size					
T1 - T2	71	28 (39)	43 (61)	-0.237	0.018
T3 - T4	29	19 (66)	10 (34)		
Tumor stage					
I	20	8 (40)	12 (60)	-0.194	0.039
II	22	8 (36)	14 (64)		
III	18	6 (33)	12 (67)		
IV	40	25 (62)	15 (38)		
Nodal status					
Negative	59	25 (42)	34 (58)	-0.111	0.271
Positive	41	22 (54)	19 (46)		
Tumor differentiation					
Well	50	29 (58)	21 (42)	+0.220	0.028
Moderately/poorly	50	18 (36)	32 (64)		
Keratin					
Absent	79	34 (43)	45 (57)	-0.154	0.126
Present	21	13 (62)	8 (38)		
Lymphatic permeation					
Absent	91	43 (47)	48 (53)	+0.095	0.874
Present	9	4 (44)	5 (56)		
Vascular permeation					
Absent	99	47 (47)	52 (53)	+0.095	0.349
Present	1	0 (0)	1 (100)		
Perineural invasion					
Absent	82	40 (49)	42 (51)	+0.076	0.451
Present	18	7 (39)	11 (61)		
Lymphocytic stromal response					
Absent	46	21 (46)	25 (54)	-0.025	0.806
Present	54	26 (48)	28 (52)		

OSCC: oral squamous cell carcinoma

and weak Nibrin expression (45%, 19/42). In relation to relapse free survival also we were unable to find any significant incidence of disease relapse in patients with strong (42%, 17/40, log-rank = 0.006, df = 1, $P = 0.937$) and weak Nibrin expression (39%, 15/38). Although we were unable to obtain any significant findings in total patients, we further sub grouped patients into early and advanced stage disease and surprisingly, we observed that in patients with early stage disease, a significant high incidence of disease relapse was observed in patients with strong Nibrin expression (43%, 10/23, log-rank = 3.884, df = 1, $P = 0.049$) as compared to patients with weak Nibrin expression (8%, 1/12) [Table 2 and Figure 2]. No such significant difference was noted for overall survival in this subgroup of patients. On the other hand in patients with advanced disease, Nibrin expression failed to discriminate such high and low risk sub group patients for survival.

DISCUSSION

Nibrin (p95, NBN, NBS1, NBS) is a 754-amino acid polypeptide which is involved in the recognition and the repair of DSBs.^[15,27-29] It interacts with Mre11 and RAD50 to form the MRN complex and is required for translocation of this complex to sites of DSBs.^[29] Although, in advanced head and neck SCC the prognostic significance of over expression of Nibrin by immunohistochemistry has been identified.^[30] However, data on the correlation of Nibrin with the clinicopathological prognosticators are limited. So, the current study evaluated correlation between Nibrin expression with clinicopathological parameters in total 100 patients with SCC of tongue and buccal mucosa.

In the present study, we observed nuclear expression of Nibrin in OSCC tissues and its corresponding adjacent normal tissues. However, there was no significant

Table 2: Univariate survival analysis (Kaplan-Meier survival function) of Nibrin expression

Variable	n	Patients relapsed or died, n (%)	Log-rank	df	P
Relapse free survival					
Nibrin (total patients, n = 78)					
Weak	38	15 (39)*	0.006	1	0.937
Strong	40	17 (42)*			
Nibrin (early stage patients, n = 35)					
Weak	12	1 (8)*	3.884	1	0.049
Strong	23	10 (43)*			
Nibrin (advanced stage patients, n = 43)					
Weak	26	14 (54)*	0.593	1	0.441
Strong	17	7 (41)*			
Overall survival					
Nibrin (total patients, n = 90)					
Weak	42	19 (45)#	0.112	1	0.737
Strong	48	20 (42)#			
Nibrin (early stage patients, n = 38)					
Weak	13	2 (15)#	0.659	1	0.417
Strong	25	7 (28)#			
Nibrin (advanced stage patients, n = 52)					
Weak	29	17 (59)#	0.010	1	0.920
Strong	23	13 (56)#			

*: patients relapsed; #: patients died

difference in Nibrin expression between OSCC tissues and their corresponding adjacent normal tissues ($t = -0.455$, $df = 99$, $P = 0.657$). Along with that in OSCC tissues, Nibrin expression was significantly positively correlated with tumor differentiation and significantly inversely correlated with tumor size and tumor stage, suggesting that up-regulation of Nibrin may be an early event in OSCC development. In accordance with our results, Ali-Fehmi *et al.*^[31] also showed that NBS1 does not show markedly higher expression in all ovarian cancer patients compared to women with serous cyst adenoma and those with normal ovaries.

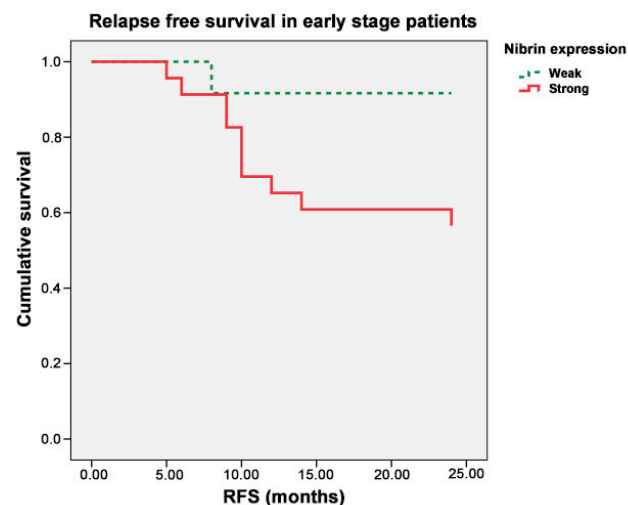


Figure 2: Kaplan-Meier univariate survival analysis of patients with early stage disease indicating significant high incidence of disease relapse in patients with strong Nibrin expression ($P = 0.049$). RFS: relapse-free survival

Plisiecka-Halasa *et al.*^[25] also showed that in human ovarian tumor tissues Nibrin expression was marked as strong nuclear staining which was present in both tumors and normal tissues. Further, Nibrin expression is up-regulated in adjacent normal tissues of OSCC tissue which is compatible with the hypothesis that Nibrin is a tumor suppressor gene.^[32] In contrast with our findings, Hsu *et al.*^[30] showed that Nibrin over expression was significantly correlated with high tumor size and metastatic diseases in OSCC patients which may be because of the inclusion of more number of patients with locally advanced diseases. Ehlers *et al.*^[33] also showed that Nibrin was associated with strong tumor severity and metastatic death marker in uveal melanoma. However, similar expression of NBS1 in class 1 tumors and normal uveal melanocytes suggests that up-regulation of NBS1 may be a late event in melanoma progression.

Kaplan-Meier univariate survival analysis showed that in patients with early stage disease high number of patients relapsed with strong Nibrin expression. However, our findings not only observed increased expression pattern of Nibrin in early stage patients but also found a strong correlation between increased Nibrin expressions in the onset of the disease with higher probability of recurrence. This could be attributed to the fact that since Nibrin acts as a sensor molecule of MRN complex which further activates the other DNA repair molecules, it might have a plausible role in constitutively activating these downstream molecules eventually leading to disease relapse in patients. While Hsu *et al.*^[30] found that in OSCC

patients strong Nibrin expression was associated significantly with shorter overall survival compared with weak expression. Ehlers *et al.*^[33] have also found that in uveal melanoma, the 6-year survival was 100% for the low NBS1 group and 22% for the high NBS1 group ($P = 0.01$). In the breast carcinoma, patients with NBS1-aberrant tumors seemed to have poorer survival than the patients with NBS1 normal tumors. This indicates that the NBS1 deficiency predicts poor survival of the breast carcinoma patients.^[34]

In conclusion, our study discovered that a Nibrin protein expression is significant in lower tumor size and early stage disease in OSCC indicating its role in early event of disease progression. Further, high incidence of disease relapse was found to be present in early stage patients with strong Nibrin expression. Thus, it could be used as a favorable prognostic factor in developing disease recurrence in patients with early stage disease. Further, among various cancers, the different patterns of the Nibrin expression have observed which indicates that the expression of Nibrin is important in cancer development and progression with cancer cell type specificity, although the mechanism behind it is unclear.

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There are no conflicts of interest.

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Squamous cell carcinoma of tongue 18 years after renal transplantation: a case report

Jyoti Poddar, Ashutosh Das Sharma, Ubrangala Suryanarayana Kunikullaya

Department of Radiotherapy, the Gujarat Cancer and Research Institute, Asarwa, Ahmedabad 380016, India.

Correspondence to: Dr. Jyoti Poddar, Department of Radiotherapy, the Gujarat Cancer and Research Institute, Asarwa, Ahmedabad 380016, India.
E-mail: poddarjyo@gmail.com

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ABSTRACT

Solid organ transplant recipients are at increased risk of developing malignancies, even decades after transplant, due to the prolonged use of immunosuppressant drugs. A 35-year-old male underwent renal transplant for end stage renal disease 18 years previously and was on immunosuppressive drugs since that time and was on regular follow up. In 2016, he developed a squamous cell carcinoma of tongue, which was operated and adjuvant radiation therapy was given. The patient is currently on follow up and asymptomatic. Though squamous cell carcinoma of tongue is a relatively common malignancy in the general population, it is very rare in transplant recipients. Hence, such patients require longer follow-up, active surveillance, and screening for early diagnosis and prompt treatment of premalignant and malignant conditions.

INTRODUCTION

Renal transplant, which is usually the treatment of choice for end stage renal failure, predisposes the recipient to an increased risk of developing malignancies and this risk increases with increasing duration of immunosuppression.^[1] This risk is largely attributable to the immunosuppressive drugs used to combat graft rejection (with an incidence of up to approximately 5% to 6%). The malignancies commonly encountered are skin cancers and those of the lymphatic system.^[2] Of these, squamous cell carcinomas make up the bulk of epithelial carcinomas, involving most commonly skin, lips, cervix, and, rarely, lung. However, malignancies

involving the tongue are an uncommon occurrence in this context, whereas it is a common intra-oral malignancy in the general population.^[3] We report such an unusual case occurring eighteen years after renal transplantation.

The purpose is to draw attention towards the need of extended follow up and active surveillance to look for malignancies in unusual locations and for early diagnosis and better management.

CASE REPORT

A 5-year-old child (1985) developed off and on reeling



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of head, generalized itching, low urine output, and generalized edema for one month. Upon investigation, he was found to have chronic kidney disease involving mainly the left kidney. It was advised that the patient undergo renal transplant but this was refused by the parents due to financial constraints. The child was kept on supportive care and follow up. At the age of 18, he finally underwent transplant due to worsening of renal function (1998). He was then put on azathioprine, prednisolone and cyclosporine regimen for immunosuppression following transplantation. Cyclosporine was discontinued after two years and the patient was continued on azathioprine and prednisolone. He was on regular follow-up since after, with no major complaints. In 2010, he developed hepatitis B and C co-infection and was placed on Tenofovir. In 2016, 18 years after transplantation and being on immunosuppressive therapy, he developed an ulcer on the tongue. On biopsy, it was found to be a squamous cell carcinoma. Computed tomography scan (22/04/2016) showed ill defined, hyperdense lesion, 16 mm × 6 mm in size, involving the anterior aspect of left lateral border of the tongue, not crossing midline or involving the base of tongue or vallecula. There were few subcentimeter lymph nodes on the left, level Ib and II. On history, the patient had no history of chewing tobacco, smoking, or use of alcohol. Also, there was no history suggestive of factors, which could have led to chronic irritation and subsequently to malignancy. On 22/03/2016, he underwent surgical treatment (left partial glossectomy and left modified neck dissection). Histopathology report was squamous cell carcinoma [Figure 1], size 1 cm × 0.6 cm × 0.2 cm. Two of 29 lymph nodes were positive, with perinodal extension. Invasion of deep muscles, lymphovascular invasion, and perineural infiltration were seen and resection margins were free of tumor. On polymerase chain reaction for human papilloma virus (HPV)-DNA, the patient was found to be HPV 16-positive. He then received postoperative radiotherapy of 60 Gray in 30 fractions, on a 6-MV linear accelerator using parallel opposed portals, with concurrent chemotherapy. The patient is currently free of disease and on follow-up.

DISCUSSION

Solid-organ transplant recipients are three to five times more vulnerable to develop malignancy.^[4] Immunosuppressive drugs predispose to malignancy by impairing immune reactions against viral infections, the most common being HPV.^[5]

These viruses inhibit the *p53* gene and its tumor suppressive action, and initiate a cascade of reactions, inducing malignant changes. Also, hepatitis remains a

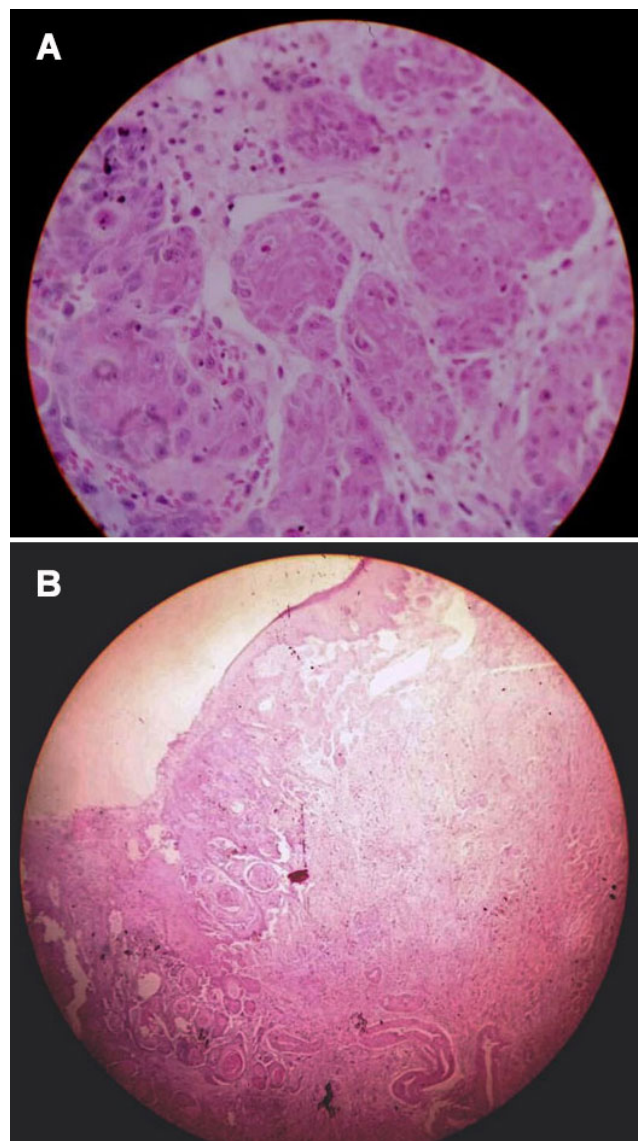


Figure 1: Power histopathology slide showing squamous cell carcinoma (A: HE, ×100; B: HE, ×40)

relevant clinical problem in patients of renal transplant.^[6] Although its incidence has decreased, nonetheless, it remains a significant problem in endemic areas and developing countries. The antiviral therapy used for hepatitis has not reported to be carcinogenic. Solid organ transplant recipients commonly suffer from fungal infections, which can even be fatal. Overall, Candidiasis tops the list and accounts for 50-60% of all fungal infections in transplant recipients. Aspergillosis is typically common in lung transplant patients.^[7] The incidence of fungal infection is around 8.6% in lung, 4.7% in liver, 3.4% in pancreas, and 1.3% in renal transplant patients. Moreover, the median time of onset of this infection ranges from several weeks to months in lung and liver transplant recipients to over two years in kidney transplants.^[8]

The incidence of malignancy increases gradually for ten years after the transplant and becomes as high as 13.8 fold higher in such patients as compared to the normal population. The cumulative frequency increases with increase in duration of follow-up. This patient developed his cancer 18 years after transplant.^[9] Other risk factors attributable to development of malignancy in these patients is advancing age, viral infections, cigarette smoking, and transmission of malignancy from donor cells.^[10] Thus, strict follow-up and vigilance for signs and symptoms of malignancy should be followed in patients with organ transplantation on immunosuppressive drugs, even after decades of transplant.

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There are no conflicts of interest.

Patient consent

Patient consent was obtained from the patient.

Ethics approval

Ethics approval was obtained from the institutional ethical committee for preparation and publication of this paper.

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Insights into mechanisms of tumor dissemination from circulating tumor cell lines of small cell lung cancer

Gerhard Hamilton, Barbara Rath

Society for Research on Biology and Treatment of Cancer, A-1160 Vienna, Austria.

Correspondence to: Dr. Gerhard Hamilton, Society for Research on Biology and Treatment of Cancer, A-1160 Vienna, Austria.
E-mail: hamilton.srbtc@gmx.org; gerhard.hamilton@meduniwien.ac.at

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ABSTRACT

Despite the fact that the majority of cancer patients succumb to metastatic disease, most aspects of tumor metastasis are not understood in detail at present. Cell biologic steps of dissemination are difficult to characterize in human tumors and research is in large part confined to cell line and experimental animal studies. Epithelial-mesenchymal transition (EMT), intravasation of malignant cells, dissemination as circulating tumor cells (CTCs) and eventually mesenchymal-epithelial transition (MET) at distal sites are steps believed to be involved in metastasis. Small cell lung cancer (SCLC) is distinguished by early dissemination and excessive numbers of CTCs, which allowed for the *ex vivo* expansion of six permanent CTC lines taken from relapsed patients. Cells exhibit an epithelial phenotype with partial EMT traits and are chemoresistant due to formation of large tumorospheres. Since cells may have invaded without undergoing EMT, the role of MET is uncertain. These SCLC CTC cell lines seem to represent the metastasis-inducing cancer cells; these are the minute subpopulation of CTCs capable of surviving in the circulation and transitioning to metastases, leading in turn to resistance and failure of therapy. Full characterization of these lines is expected to provide the markers to find the relevant CTCs among the highly heterogeneous population observable in the context of tumor recurrence.

INTRODUCTION

Early detection, precise diagnosis and monitoring of the course of disease during therapy are the objective of individualized care in current oncology. For most tumors, a tumor biopsy is costly, painful, or potentially hazardous for the patient and is not performed during

the further development of the malignancy. Especially in tumor types showing a high frequency of systemic disease, such as small cell lung cancer (SCLC), a small needle biopsy is procured first to confirm the diagnosis, and chemotherapy is started without any further invasive procedure.^[1] Thus, the opportunity to obtain essential information from blood samples, as so-called



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liquid biopsies, would offer significant advantages. Non-invasive detection and monitoring of patient tumors, employing cell-free circulating tumor DNA (ctDNA) or circulating tumor cells (CTCs), have been employed for investigations into multiple tumor types.^[2-4] CTCs are described as cells, shed by primary or secondary tumors into vasculature, that keep circulating in the bloodstream of cancer patients.^[5] Reports indicate that patients with lower counts of CTCs survive longer than the patients with higher CTCs counts. For example, lower numbers of CTCs were observed for 21 patients with limited SCLC (median = 6, range 0-220) compared with 38 patients with extensive stage (median = 63, range 0-14,040) and the absence of measurable CTCs in 27% of patients was correlated with prolonged survival (hazard ratio: 3.4; $P \leq 0.001$).^[6]

Furthermore, CTC counts have been proposed as a surrogate marker for assessment of responses to therapy in cancer patients to facilitate more rapid drug evaluation. The Food and Drug Administration approved CellSearch® system (Veridex, Raritan, NJ, USA) enumerates intact CTCs for a prognosis of overall survival for breast, prostate and colon cancer.^[7] For example, CTC count is a robust independent prognostic factor for progression-free recovery and overall survival in patients with early and metastatic breast cancer.^[8] Additionally, CTCs have been detected in patients with chronic obstructive pulmonary disease before the actual occurrence of malign lesions, thus allowing for early diagnosis of lung cancer.^[9] However, an advantage of the analysis of ctDNA in liquid biopsy is the detection of molecular changes which are occurring within the tumors in real time, especially during development of drug resistance to targeted agents.^[2,3] Numerous techniques for the enrichment of CTCs have been developed relying on immunological markers, size, rigidity or dielectric properties. These techniques have been used for genetic characterization, marker analysis and short-term cultures for investigation of their cell biology.^[10,11] Despite a host of studies dealing with CTCs, many questions regarding their generation, shedding, survival in the circulation, chemosensitivity and mechanisms of induction of secondary lesion remain to be fully resolved.

Research on tumor dissemination and CTCs has been hampered by the scarcity or heterogeneity of the enriched cells, as well as the inability to define the characteristics of the actual metastasis-inducing CTCs that are expected to be most relevant for the prognosis of the patients. In this study, we have established for the first time six CTC cell lines from a variety of patients with extended metastatic SCLC and present here the implications of the phenotype of these cell

lines for tumor dissemination in this highly aggressive malignancy.

DETECTION AND ENRICHMENT OF CTCs

In most tumor types, CTCs are rare events, with a frequency of approximately one CTC among 1-10 million mononuclear blood cells. Therefore, these cancer cells have to be enriched by various methods for further analysis. The probability to detect CTCs in a limited volume of blood has been reviewed by Gkountela *et al.*^[10] and the reported probability of collecting ≥ 1 CTCs in one aliquot of 7.5 mL blood from a patient with 500 CTCs is 50%. Therefore, 20 mL of whole blood would have to be assessed if the cell event were to be detected for a lower frequency at one CTC in 10^7 leukocytes. The frequency of the CTC population measured in an aliquot may not be a statistical representative of the entire sample.

There are a number of questions remaining for the detection of CTCs and their relationship to their parent bulk tumors. CTCs seem to stem from the frontier of the tumor and it is generally known that the tumor margin is different from the main tumor mass. Most of the CTCs populations analyzed exhibit heterogeneity, pointing to release of different cell populations from distinct regions of the tumor or of metastatic lesions.^[11] CTCs can also form clusters including immune cells in the circulation, or be engulfed by the platelets before eventually adhering to the walls of the capillaries and initiating extravasation.^[12]

The role of CTCs as prognostic marker and as possible surrogate indicators for response to therapy is discussed in numerous reviews.^[10,13,14] CTCs which are shed from tumors are mediators of metastatic dissemination and form micrometastasis at distant organs.^[15] 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.^[10] The CellSearch® system is by far the most common system for extraction and enumeration of CTCs for clinical investigations.^[7] CTCs which have downregulated EpCAM remain undetected throughout this process.^[16,17] By alternative methods, cell size-based sorting is accomplished using microfluidic technology, as with the Parsortix system (Angle, Guildford, UK).^[18,19] Isolation by Size of Tumor cells (ISET®) is another filter-based established method which is used for such cell size-based sorting.^[20]

CTCs derived from breast cancer patients are among the most extensively studied for diagnosis

and treatment.^[21] The presence of CTCs, despite ongoing treatment, has proven to be an indicator of worse overall survival; therefore, in one group of HER2⁻ breast cancer patients, HER2⁺ CTCs were identified and trastuzumab-based therapy applied to these patients.^[17,22,23] Most of the CTCs isolated from breast cancer patients show the presence of epithelial-mesenchymal transition (EMT) markers such as ETV5, NOTCH1, SNAIL, TGFB1, ZEB1, and ZEB2.^[24] Breast cancer patients who showed remaining CTCs after first cycles of chemotherapy progressed rapidly to metastatic disease.^[25] In prostate cancer, CTCs have been proposed to act as intermediate or surrogate endpoints for survival and to shorten timelines for drug approval.^[26] Patients with lower levels of CTCs have shown slower disease progression in comparison to those having higher levels of CTCs.^[27] In colon cancer, CTCs were found as individual cells or as clusters (CTMs) by a CK-based, immunomagnetic cell separation method.^[28] CTMs are of particular interest as they are considered to be markers of increased metastatic potential.^[29] In lung cancer, CellSearch[®] and ISET kits indicated a higher number of CTCs in SCLC than NSCLC, reported to be associated with larger tumor size and bone metastasis.^[30,31] The high numbers of CTCs in SCLC allowed for their enrichment and initiation of xenografts which seem to resemble the tumor characteristics of the respective patients.^[32] Furthermore, a trial to establish *ex vivo* expanded CTC cell lines was successful and has resulted in the availability of six lines from relapsed patients so far.^[33] Characteristics of the first CTC cell lines and their interaction with macrophages have been published.^[34]

SHEDDING OF CTCs FROM TUMORS

Release of CTCs into the circulation is frequently termed shedding, a designation for a process for which the details are not known. CTCs are reported to be shed from solid tumors at a daily rate of 3.2 to 4.1×10^6 per gram of tissue, based on a single artificial rat model.^[35] In one study, the rate of tumor cell shedding into efferent blood was measured in both growing and regressing MTW9 rat mammary carcinomas. Cell shedding rates of growing versus regressing tumors were not significantly different over a tumor size range of 2-4 g. Half of these CTCs perished within 2.4 h, although longer half-lives were reported in a clinical setting.^[28,36] Tumor cells are rapidly cleared from circulating blood and a 2-g MTW9 carcinoma reportedly released enough cells into the circulation to transplant the tumor every 24 h, although the majority of the cells were reported to be apoptotic/necrotic.^[35] The cell loss via blood comprised about 10% of the tumor weight and resulted in a CTC count

of approximately 20,000 CTCs/mL blood.

Clearly, this estimation of the release of CTCs reported from an experimental animal model cannot be extrapolated to human tumors. No form of the shedding of CTCs causes a human tumor to lose 10% of its size in one day, nor is a CTC count of 20,000 cells/mL observed in most cancer patients. A threshold of 5 CTCs/7.5 mL blood has been defined by the Cellsearch[®] system for breast and prostate cancer, and a lower threshold of 3 CTCs/7.5 mL blood has been defined for colon cancer patients. These figures are for favorable or poor prognosis, respectively.^[7,37] Consequently, the attrition rate in the circulation based on this artificial animal model seems to be a considerable overestimation. The specific mechanisms of tumor cell shedding are not known at present. CTCs seem to origin as specialized cell types, different from the bulk of the tumor cells, from the borders of the tumor. CTCs leave the particular microenvironmental milieu characterized by inflammation, acidosis and hypoxia through the interaction of a host of participating cell types. Therefore, CTCs are not expected to represent the bulk of tumor cells and are not typical of the cell biologic behavior and chemoresistance of the main body of the tumor. SCLC extended disease responds well to the first cycles of platinum-based chemotherapy but recurs within approximately one year as tumors which exhibit universal chemoradioresistance.^[38] Contrary to expectations, the first two SCLC CTC cell lines proved to be chemosensitive to the second-line chemotherapeutics topotecan and epirubicin although some tumor cells must have survived the initial successful treatment and eventually give rise to chemoresistant relapses.^[39] Therefore, the use of CTCs as surrogate markers for the bulk tumor is questionable.

EMT IN TUMOR CELL SHEDDING

A general assumption supposes that tumor cells invade through a process termed EMT.^[40] Accordingly, epithelial tumor cell downregulate epithelial markers, such as E-cadherin, EpCAM and cytokeratins, eventually expressing mesenchymal markers such as vimentin, neural cell adhesion molecule (NCAM) and others. In this way, cells gain mobility and migrate to intravasate and reach distant sites to establish secondary lesions. EMT is regulated by a number of specific transcription factors belonging to the SNAIL, TWIST and ZEB families and is modulated by microenvironmental conditions, inflammatory cytokines and chemotherapy.^[41] Since it has proven difficult to demonstrate tumor cells with EMT traits in patients, incomplete EMT or transitional EMT has been proposed as a model. This model

presupposes no complete switch in phenotype, but rather a type of transition which may be as minimal as a slight downregulation of epithelial features. EMT phenotypes have been reported among heterogeneous CTC populations and increased fractions of cells with such mesenchymal features have been demonstrated to correlate with a poorer prognosis in breast cancer patients.^[24]

It may still be possible that tumor cells with epithelial characteristics enter the bloodstream without undergoing EMT. An alternative model, termed “cooperative migration”, posits that EMT-type cells help epithelial cells to gain access to the circulation, but to reside at the bulk tumor. Excessive numbers of CTCs have been observed in SCLC, which is frequently associated with local inflammation and in inflammatory breast cancer. Thus, immune cells and inflammation may promote release of tumor cells into the circulation possibly without EMT. The SCLC CTC cell lines have been found to recruit macrophages and to lack a phenotypic switch with full expression of mesenchymal traits.^[34,42] In conclusion, complete or partial EMT is not proven to be a prerequisite to disseminate tumor cells, and therapeutic options to inhibit such a transition need to be considered cautiously.^[43]

SURVIVAL CTCs IN THE CIRCULATION

The great majority of CTCs seem to be short-lived and to perish in the circulation. Of the several forms of CTCs shed by the primary tumor, only about 0.1% survive in the circulation and only about 0.01% is responsible for metastasis.^[8,10] This attrition has been attributed to shear stress and an unfavorable microenvironment too different from the local tumor conditions. CTCs in the hematogenous circulation must survive a variety of stresses, and epithelial cells may undergo anoikis in the absence of cellular attachment.^[44] The vast majority of CTCs are likely to become trapped in various capillary beds. They are destroyed by hemodynamic shear forces and predation by cells of the innate immune system – specifically natural killer cells. Consistent with this view, a great deal of CTC-associated material is detectable in CTC-positive tumor patients.^[10] Reported half-lives have ranged from several hours, according to experimental animal models, to long-term persistence. The SCLC CTC lines show continuing disposal of microparticles and cellular fragments, thus generating CTC associated materials under optimal conditions in tissue culture in the absence of shear stress.^[42] Partial disintegration of CTC tumorspheres may function as a source of decoy material to protect other CTCs and the bulk tumor from attacks by both the immune system and chemotherapeutics.

INDUCTION OF EXTRAVASATION AND MESENCHYMAL-EPITHELIAL TRANSITION

In general, the secondary lesions induced by CTCs show an epithelial phenotype similar to the originating primary tumor.^[40] Provided that the metastases were established by cells which underwent EMT, at some point during tumor spread and extravasation this transition has to be reversed through a process termed mesenchymal-epithelial transition (MET). This process has not been observed directly, but has been inferred from the mesenchymal traits of disseminated/CTCs and the histology of secondary lesions.^[45] The factors causing this supposedly phenotypic switch, along with their possible derivation from the cancer cells themselves (seed) or the metastatic site (soil) are largely unknown. In the case of SCLC CTC lines, the cells are positive for EpCAM, E-cadherin and proteins involved in cell junctions and form spontaneously typical large spheroids with diameters of up to 1-2 mm in regular tissue culture medium without any factors preventing adhesion to cell culture flasks.^[43] Although expression of vimentin and NCAM is observed, the formation of these organized and large spheres is a typical epithelial feature not observed in SCLC tumor cell lines *in vitro*. Since these tumorspheres develop from CTCs of relapsed SCLC patients within a short time, and are found in cell suspension derived from xenografts induced by such CTCs, they seem not to stem from an *in vitro* transition in tissue culture but to present the original *in vivo* phenotype. Thus, these metastasis-inducing CTCs seem to be present as cancer cells exhibiting an epithelial phenotype and organization and may be trapped in capillaries or reside in protected sites, possibly in a dormant state. Tumorspheres exceeding diameters of 2 mm during their development tend to disintegrate and may be the source of non-proliferating cell clusters observed in the blood of metastatic cancer patients.^[10]

METASTASIS AND DRUG RESISTANCE

Metastases not only damage secondary organs and exacerbate the deleterious effects of malignancies in general but frequently exhibit chemoresistance to reinitiation of primary or second-line chemotherapy agents. Especially in SCLC, excellent response rates to initial chemotherapy can be followed by relapses within approximately one year, which exhibit broad chemoresistance and result in failure of treatment.^[1,38]

Attempts have been made to characterize the chemosensitivity of CTCs in short term cultures in various tumor entities.^[46] However, CTCs are specialized cells different from the tumor bulk and most likely also

from the developing metastases. It was universally assumed that CTCs survive as chemoresistant cells and may predict the responsiveness of resulting secondary lesions. Surprisingly, the SCLC CTC lines were found to be highly chemosensitive to agents of common use in this tumor entity, except the CTC line established from a patient refractory to initial therapy with cisplatin.^[39] Thus, some tumor cells have survived the initial cycles of chemotherapy, possibly in an inflammatory environment, and seem to develop a kind of chemoresistance not accomplished at the cellular level.^[34] Tumorospheres are known to be comprised of a small region of proliferating cells and layers of quiescent cells surrounding an inner hypoxic core which provides protection against irradiation due to the lack of reactive oxygen species [Figure 1].^[47,48]

Therapy of SCLC has not been improved for the last several decades despite the clinical testing of a host of chemotherapeutics covering the widest range of clinical targets available.^[1,49] More than 600 trials exploring therapeutic interventions in SCLC are currently in the U.S. clinical trials registry, National Institutes of Health.^[50] Since it is not reasonable to assume that SCLC tumors express a host of individual molecular mechanisms, tumorospheres provide an alternative explanation for chemoresistance, in which potential chemotherapeutic drugs are prevented from reaching their cellular target (in responsive cells) in sufficient quantities.^[51] Whether the validity of this kind of chemoresistance, in the form of tumor spheroids, is

confined to SCLC or is common to other tumor types, remains to be investigated.

CONCLUSION

Mechanisms of tumor metastasis have been investigated using numerous cell culture studies and research employing experimental animal models as well as analyses of clinical specimens. Models have been proposed for the discrete steps of tumor metastasis, but the actual dissemination of malignancies in patients is difficult to assess. Clearly, CTCs are instrumental in translocation of tumor cells to distal sites and in the induction of secondary lesions. Although CTC counts have shown prognostic significance, their limited accessibility and marked heterogeneity have limited their usefulness.

A panel of permanent CTC lines from SCLC patients revealed absence of full EMT, presence of chemosensitivity, and an epithelial phenotype with formation of tumorospheres as physical barrier against chemoradiotherapy. Unfortunately, there are currently no means available to target these spheroid structures in a clinical setting, and further investigation is needed to study the cell biology of CTC aggregates in detail, and to study overcoming resistance using targeted agents involving enzymes, cell junctions opener, nanomaterials and other mechanisms.

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Conflicts of interest

There are no conflicts of interest.

Patient consent

Informed patient consent was obtained throughout the study.

Ethics approval

The respective ethics protocol (Approval of the Medical University of Vienna Nr. 366/2003 and amendments obtained prior to the commencement of the study) was followed throughout the study.

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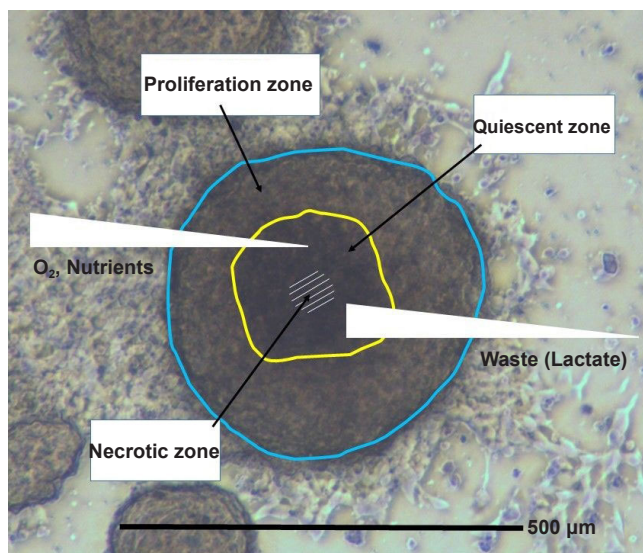


Figure 1: Cell physiologic conditions in an SCLC CTC tumorosphere. This figure shows a microscopic image of a SCLC CTC BHGc10 tumorosphere with a schematic overlay indicating regions of proliferating, quiescent and hypoxic cells. These spheroid structures grow up to diameters of 1-2 millimeters and develop gradients of nutrient and oxygen supply as well as accumulation of metabolic waste products. SCLC: small cell lung cancer; CTC: circulating tumor cell

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The significance and clinical utility of the detection of primary malignant circulating prostate cells: a review of the evidence

Nigel P. Murray^{1,2}

¹Hospital Carabineros of Chile, Nunoa, 7770199 Santiago, Chile.

²Faculty of Medicine, University Finis Terrae, Providencia, 7501015 Santiago, Chile.

Correspondence to: Dr. Nigel P. Murray, Faculty of Medicine, University Finis Terrae, Providencia, 7501015 Santiago, Chile.
E-mail: nigelpetermurray@gmail.com

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Dr. Nigel P. Murray is currently a Professor in Hematology and Internal Medicine in the University Finis Terrae, Santiago, Chile where he heads the Circulating Tumor Cell unit and is the Head of Hematology, Hospital de Carabineros de Chile, Santiago, Chile. His research interests included the use of primary and secondary circulating prostate cells in the diagnosis and management of prostate cancer, with over 30 publications in PubMed.

ABSTRACT

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Primary malignant circulating prostate cells (CPCs) are those detected in blood before definitive treatment for prostate cancer. CPCs can be detected in men with benign prostate disease; however, some methods to distinguish between benign and malignant prostate cells have to be validated. This study presents a review of the subject, including theoretical considerations for the selection of markers to detect them, the different methods used, and the utility of their detection in identifying men with prostate cancer and as a prognostic factor.

INTRODUCTION

Prostate cancer is the most common tumor diagnosed in men in the Western world. With demographic

changes and the aging population, the number of men with this cancer has steadily increased. The natural history of untreated prostate cancer is one of evolution to a metastatic disease, especially disseminating to



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bone, over a variable time period.

Two large questions have yet to be answered: (1) what is the role of prostate cancer screening? (2) what treatment is appropriate for men diagnosed with prostate cancer? An ideal prostate cancer screening test would not detect all prostate cancers, but only those prostate cancers which have the potential to cause harm to the patient. At present, the only widely used screening test is serum total prostate-specific antigen (PSA), which in a range of 4-10 ng/mL is associated with a positive biopsy rate for all cancers of approximately 30%.^[1] of which it has been estimated that 23-42% of screen detected prostate cancers are over treated.^[2] Men with clinically insignificant prostate cancers who were never destined to have symptoms or altered life expectancy may not benefit from knowing that they have the "disease." The detection of clinically insignificant prostate cancer may be considered an adverse effect of the prostate biopsy.

Screening for prostate cancer remains controversial. The two large studies published in the United States and Europe produced different results;^[3,4] as a consequence, the American Urology Association guidelines do not recommend screening in men over 70 years or in those with less than 10 years' life expectancy.^[5] However, they recognize that some elderly men who are healthy may benefit from screening. Why the controversy? Presently, a new diagnosis of prostate cancer is nearly always in men with an elevated screening serum total PSA who have been referred for a prostate biopsy. Serum total PSA is prostate specific. However, it is also increased in benign diseases such as hyperplasia and prostatitis.^[4,5] In fact, 10-20% of men aged 50 years and 70 years will have a raised PSA, but only 25% of those with a serum total PSA of 4-10 ng/mL will be found to have a biopsy positive for cancer.^[6] Moreover, the frequency of men with an elevated PSA and benign biopsy is country dependent^[7] and may be significantly different between rural and metropolitan populations in the same country.^[8]

To complicate matters further, not all prostate cancers need treatment. It has been estimated that 23-42% of screen-detected prostate cancers are over treated.^[2] For every 100 men with an elevated PSA between 4 ng/mL and 10 ng/mL, only about 14 will have a clinically significant prostate cancer detected. Eighty-six will undergo a biopsy, with its associated risks, for what is found to be a benign disease. Infection and hemorrhage are the main potentially serious side effects of prostate biopsy, with a 30-day complication rate of 3.7%, especially in older patients.^[9] Therefore,

avoiding unnecessary biopsies is a worthwhile aim if it does not prejudice the number of clinically significant cancers detected.

Active surveillance is a recognized initial treatment option for men with early stage low-grade prostate cancer. The option to delay or avoid definitive therapy avoids or minimizes patient morbidity without compromising long-term outcomes in appropriately selected patients.^[10,11] According to the Prostate Cancer Intervention Versus Observation Trial,^[12] men with low risk disease (defined as a PSA \leq 10 ng/mL, a Gleason score \leq 6, and T stage 1 or 2a) had no difference in all-cause mortality and prostate cancer-specific mortality, or in rate of progression to bony metastasis, when assigned to radical prostatectomy or to active observation. The criteria for active observation (AO) according to Epstein *et al.*^[13] are a diagnosis of prostate cancer, with three or fewer of the 12 prostate biopsy cores positive for cancer. That no single biopsy core with $> 50\%$ infiltration and a PSA density < 0.15 ng/mL. Using these criteria to select patients with "insignificant disease" has a positive predictive value of 95% and a negative predictive value of 66%.^[14] These men are actively followed up with repeat annual biopsies. The timing of intervention after the initial diagnosis is based on variables such as PSA kinetics, Gleason grade progression, patient preference, and clinical or radiologic evidence of disease progression.^[10,15] An increase in the Gleason score at repeat biopsy is predictive of the time to active treatment and correlates with patient outcome.^[16] It has been reported that Gleason score progression occurs in approximately 20% of men, with more than 50% of cases occurring within two years of the initial diagnosis.^[17] However, a similar increase is seen in men subjected to immediate repeat biopsy when entering an AO program.^[18] This short time interval, when compared with the long natural history of prostate cancer, suggests that sampling error rather than tumor progression is probably the primary source of tumor upgrading in these men.

The use of other biomarkers, such as circulating prostate cells (CPCs), could be useful in re-categorizing the patients who could be more adequately treated by active surveillance. One such biomarker could be circulating tumor cells, or, in the case of prostate cancer, CPCs. We review the literature on circulating tumor cells both to try to answer the question of whether they could be clinically useful to detect prostate cancer and as a guide to initial treatment, observation, or active treatment. We review the process of cancer cell dissemination from the primary tumor and how this may affect cell markers, and thus determine the criteria for detecting or identifying circulating tumor cells.

Methods of enrichment and detection of these cells are considered in how the method may affect what is being detected or not. Finally, we consider the clinical utility of these tests and how in day-to-day clinical practice they may help in decisions to proceed to prostate biopsy and treatment decisions of detected cancer.

A search for articles between the years 2000 and 2016, evaluating the detection of circulating tumor cells and CPCs was carried out using PubMed, Web of Science, and Cochrane Library. Case reports, review articles, non human models, and series involving fewer than 10 patients were excluded.

THE DISSEMINATION OF CANCER CELLS FROM THE PRIMARY TUMOR

The metastatic process by which tumor cells leave the primary tumor and implant, survive, and growth in distant sites is multistage and complex. Several steps are needed for the cancer cells to escape from the primary tumor and intra-vasation, towards extravagation and successful implantation in distant tissues. With the advent of prostate cancer screening and the use of total serum PSA, there has been a shift towards a diagnosis of localized cancers.^[19] However, despite being considered as localized by currently accepted staging methods, approximately 20-30% of patients suffer primary treatment failure,^[20] suggesting that cancer cells have disseminated prior to treatment. Using polymerase chain reaction amplification of PSA mRNA, it has been reported that prostate cancer cells disseminate early in the metastatic process into the circulation.^[21] These have been defined as primary circulating tumor cells, those detected before initial curative therapy.

Tumor cells may enter the circulation actively or passively;^[22] passive entry into the circulation is a result of vessel leakage by the growing tumor and external forces such as surgical manipulation at the time of biopsy;^[23] in these cases the circulating tumor cells do not require specific phenotypic characteristics. Active entry of tumor cells requires specific abilities which permit the cell to detach from the surrounding cells, survive free of them, and migrate towards blood vessels where they cross the capillary endothelium, enter the circulation, and disseminate. Thus, primary CPCs consist of a heterogeneous population ranging from metastatic initiating cells with specific cell properties^[24] to non-aggressive cells without any specific survival ability.

In order to escape from the primary tumor, cancer cells exhibit a decreased expression in anchor proteins

such as E-cadherin^[25-27] and beta-catenin^[25,27] and a loss of cytokeratins 8, 18, and 19, which increases tumor cell plasticity.^[28,29] These changes occur in a coordinated fashion; they are higher in higher grade and less differentiated tumors.^[28] There is increased expression of matrix metalloproteinases; these zinc-containing endopeptidases are activated in situ from their latent form and degrade the extracellular matrix. As such, they permit the cancers to disseminate to the circulation, implant, and form metastases.^[29,30] Increased expression of metalloproteinase-2 (MMP-2) has been demonstrated^[31-33] and is associated with increasing Gleason score, pathological stage, and as a prognostic factor.^[33,34] Primary CPCs detected before prostate biopsy express MMP-2, whereas one hour post-biopsy there are a mixture of MMP-2 positive and negative CPCs, inferring that MMP-2 is important in CPC dissemination from the primary tumor.^[35]

Epithelial to mesenchymal transition plays an important role in cancer dissemination. There is a change in the phenotypic expression of epithelial and mesenchymal markers, with increased expression of mesenchymal markers such as vimentin, N-cadherin, or O-cadherin.^[36,37] These patterns of expression are heterogeneous with a global decrease in epithelial cell marker expression.^[38] However, CPCs that express only mesenchymal markers be may easily able to escape from the primary tumor, but for the same reason they have limited ability to implant in distant tissues.^[39-42] Intermediate states have been reported, with circulating tumor cells expressing both epithelial and mesenchymal markers. This increased state of cell plasticity may be advantageous to implantation at distant sites and the future formation of metastasis. This plasticity is the hallmark of cancer stem cells,^[43-47] and CPCs from prostate cancer patients have been reported to express CD133^[48] or ALDH1^[49] both markers of cell stem-ness.

One important epithelial marker that has relevance in the detection of CPCs is the epithelial cell adhesion molecule (EpCAM) (CD326). This is a 40 kD glycoprotein that was originally identified as a marker for carcinoma, with an increased expression being identified in rapidly proliferating epithelial tumors.

EpCAM was initially thought to be important in cellular adhesion. However, more recent reports indicate that it plays a role in cell to cell signaling, in migration and proliferation of cancer cells, and possibly in the prevention of cell-cell adhesion. In normal cells there is a variable expression of EpCAM, but it is reported to be lower than that found in primary tumors.^[50]

Thus, the specific phenotypic characteristics of

cancer cells will determine their ability to disseminate into the circulation and may not reflect the general characteristics of the primary tumor due to the heterogeneous nature of individual cancer cells within the general tumor cell population.

In order to implant in distant sites CPCs must survive in the circulation. Only a few of the millions of tumor cells that are shed into the circulation are able to reach a distant site, implant, survive, evade the immune system, and eventually form a metastasis. It has been suggested that only 0.01% of circulating tumor cells can produce a single bony metastasis.^[51,52] CPCs obtained from men with castrate-resistant prostate cancer failed to produce metastasis when implanted in immune-compromised mice.^[53]

Firstly, circulating tumor cells have to resist anchorage dependent cell death; over-expression of anti-apoptotic proteins such as Bcl-2 overexpression^[54] or activation of specific pathways such as tropomyosin-related kinase B (TrkB)^[55] have been reported. Secondly, they have to evade the host's immune systems. Circulating tumor cells from patients with colorectal cancer CD47 expression were increased. This marker is considered to be an anti-phagocytic signal expressed on cancer cells to prevent macrophages and dendritic cells from attacking them. The counterpart of this anti-phagocytotic mechanism, the expression of pro-phagocytic calreticulin, was significantly decreased.^[56]

Circulating tumor cells escape immune surveillance by shielding themselves from the immune cell population. It has been proposed that myeloid-derived suppressor cells facilitate the survival of cancer cells by creating a defensive shield. These myeloid-derived suppressors adhere to some of the circulating cancer cells, conferring a survival advantage.^[57] Circulating tumors cells are rapidly coated by platelets. This may cause transfer of major histocompatibility complex (MHC) class I antigens on the tumor cell surface resulting in a high level of platelet-derived normal MHC class I. This coating of phenotypic normality disrupts the normal recognition of tumor cells by natural killer cells and T cell mediated immunity, thus permitting tumor cell survival.^[58]

METHODS TO DETECT AND CHARACTERIZE CIRCULATING TUMOR CELLS

All methods of detecting circulating tumor cells are based first on enrichment of circulating tumor cells from venous blood and then on detection. The Food and Drug Administration (FDA) defines a validated biomarker assay as a system of analysis

with established performance characteristics for which there is scientific evidence that elucidates the clinical significance of the results obtained. The stability, accuracy, and reproducibility of the assay are fundamental. Pre-analytical, analytical, and post-analytical variables all have to be controlled during the assay process. Parkinson *et al.*^[59] have extensively reviewed this topic as have Panteleakou *et al.*^[60] Pre-analytical factors include the type of collection tube (including anticoagulant, storage, and transport conditions of the analytical variables), the type of enrichment and enumeration methods used, the sensitivity and specificity of the assay, the reproducibility of the assay between laboratories, and assay-specific controls. Other factors include the disease characteristics, how often the target cells are detectable in the study population or in other diseases or normal people, the positive and negative predictive values, and establishing cutoff values for a positive or negative test.

Enrichment of circulating tumor cells from blood

Methods for circulating tumor cell enrichment fall into three basic categories: density gradient centrifugation, cell filtration based on size or microfluidics, and immune-magnetic isolation, often anti-EpCAM antibodies; or a combination of methods.

Density gradient centrifugation is a simple, fast, and cheap process, separating cells based on their differing densities. Circulating tumor cells separate with the mononuclear blood cells (density < 1.077 g/mL), forming an opaque layer which can be removed and further analyzed. Red blood cells and granulocytes (density > 1.077 g/mL), being denser, settle towards the bottom of the tube. The method has poor sensitivity, as tumor cells may be lost when cells sediment to the granulocyte layer, or, if present as cell clusters, when they aggregate to the bottom of the tube. This may be important because circulating tumor cell clusters have been reported in patients with metastatic prostate cancer^[61] and have been correlated with a worse outcome in breast cancer.^[62]

Furthermore, if the centrifugation is performed immediately, whole blood may be mixed with the gradient solution, causing contamination. The OncoQuick® system uses a porous barrier to prevent such contamination. It has been reported that this system improves the depletion of mononuclear cells resulting in higher relative tumor cell enrichment as compared with standard gel separation. However, using cell-spiked blood samples there was a similar tumor cell recovery rate of between 70% and 90%.^[63,64]

Circulating tumor cells are larger than circulating blood cells; filtration methods are based on the physical properties of these cells and allow enrichment by size. Isolation of circulating tumor cells was first reported in 1964.^[65] The filters use pores measuring between 7.5-8.0 μm in diameter, thus capturing 85-100% of circulating tumor cells while retaining only 0.1% of circulating blood cells.^[66] Three commercially available filters are available: Screencell[®]Cyto, ISET[®], and Metacell[®]. After filtration the filter membrane is removed and circulating tumor cells are identified by immunocytochemistry. Isolation of tumor cells by size is fast, simple, and reliable and does not require high-cost instrumentation. One drawback, though, is the need to process samples within four hours. The system does not detect the rare cells that are smaller than 8 μm ; however, it will detect tumor cell clusters. The ISET[®] system detects one tumor cell in 1 mL of peripheral blood and permits the evaluation of tumor cells based on morphological criteria. False positivity occurs due to the lack of specificity of the enrichment technique. Normal epithelial or endothelial cells may be present due to coring by the sampling needle, and circulating cells have been described in samples taken from patients with benign conditions.^[67,68]

Immunomagnetic selection methods use the specificity of antibody-antigen interactions combined with the physical properties of magnetic beads to separate tumor cells from blood cells due to the different expression of surface antigens in the differing cell populations. This is the basis of enrichment in the CellSearch[®] system, the only FDA-approved method of detecting circulating tumor cells. In the CellSearch[®] system, iron particles are coated with the epithelial cell surface marker EpCAM, an epithelial marker that is overexpressed in some cancers but not in normal blood cells.^[69] However, EpCAM positive cells have been reported in patients with benign colon disease,^[70] and in the original report of Allard *et al.*,^[69] women without evidence of breast cancer had “circulating tumor cells” detected in between 5 and 7% of cases, 1 cell/7.5 mL blood sample. In addition, the epithelial phenotype of circulating tumor cells changes, as a result of the epithelial to mesenchyme transition the expression of EpCAM decreases and thus there may be failure of enrichment and as a result circulating tumor cells are not detected. This applies also to microchip devices that incorporate microposts labeled with anti-EpCAM (CTC Chip), using EpCAM coated beads (Dynabeads[®] Epithelial enriched)(MACS/auto MACS[®])(AdnaTest[®]) or using microvortices in a herringbone pattern to increase the number of interactions between the EpCAM-coated chip surface and circulating tumor cells.^[71] The same can be said for cytokeratin-based

enrichment methods.^[72]

Negative enrichment methods that deplete normal blood cells using the pan-leukocyte antigen CD45 after red cell lysis have also been used.^[73]

Detection of circulating tumor cells

For the detection of enriched circulating tumor cells, two methods have been used: immunocytochemistry and reverse transcriptase-polymerase chain reaction (RT-PCR).

Immunocytochemistry

The advantage of methods using immunocytochemistry is the morphological analysis of the detected cells. The International Society of Hematotherapy and Graft Engineering criteria^[74] for circulating tumor cell identification are an object with the appearance of cell with a nucleus. Most methods use a combination of markers; the CellSearch[®] system defines a circulating tumor cell as one positive for cytokeratin, negative for the pan-leukocyte antigen CD45, and expressing DAPI (4', 6-diamidino-2-phenylindole) nuclear staining. The ISET[®] and Metacell[®] systems use anti-cytokeratin staining, while the CTC membrane micro-filter, Rosettesep[®] and Nanovelcro CTC Chip[®], use immunofluorescence with a cocktail of anti-EpCAM, anti-cytokeratin, and CD45. All these methods in essence detect circulating epithelial cells and are not tissue specific. Using basic cell density methods, some authors have attempted to use more specific markers to detect circulating tumor cells, anti-PSA for prostate cancer,^[75] anti-mammoglobin for breast cancer.^[76] As such, these methods are not able to differentiate between benign and malignant circulating “epithelial” cells. In patients with benign colonic diseases, up to 29% of patients were positive for the Episot[®] assay, and up to 19% of patients were positive for the CellSearch[®] assay.^[70] One group has used the combination anti-PSA and anti-P504S to address this problem. The expression of P504S has been used to differentiate between benign and malignant prostate tissues in biopsy samples. P504S is expressed in prostate cancer cells and those of prostate intra-epithelial neoplasia, but not in benign prostatic tissue.^[77,78] The authors report that PSA positive cells can be detected in men with benign prostatic disease, especially prostatitis, but these cells are P504S negative, whereas men with prostate cancer had PSA positive cells which also expressed P504S.^[79]

In reference to circulating cell clusters, the identification of CTC clusters (defined as ≥ 2 CTCs) has been related to a poor outcome in stage III-IV breast cancer using the CellSearch system,^[80] whereas Paoletti *et al.*^[81] defined CTC clusters as ≥ 3 CTCs in the CellSearch gallery

and their presence was associated with a worse prognosis. However, there is no consensus regarding the morphologic characteristics necessary to define cell clusters using the CellSearch system.

RT-PCR detection of circulating tumor cells

RT-PCR is a more sensitive method than immunocytochemistry to detect circulating tumor cells. However, it has its limitations in that; (1) there may be amplification of nonspecific gene products; (2) it lacks thoroughly validated protocols for sample processing, RNA-preparation, cDNA synthesis, and PCR conditions; (3) it lacks rigorous quality control measures on a per-sample basis (the lack of a validated method increases the possibility of variations in sensitivity, specificity, and the potential of nonspecific amplification products being detected); and (4) there is no morphological confirmation of tumor cells.

The number of articles describing single or multiple markers to characterize CTCs using RT-qPCR in the blood of cancer patients has increased greatly in recent years, especially in breast cancer.^[81-85] The Adnatest® PC CTC platform consists of the ProstateCancerSelect® and ProstateCancerDetect® system. The ProstateCancerSelect® system allows for an enrichment of tumor cells by an antibody-mix (anti-EpCAM, anti-Her2) linked to magnetic particles and mRNA isolated from the selected cells. The ProstateCancerDetect® System transcribes the isolated mRNA into cDNA, and a multiplex PCR is performed for the analysis of tumor-associated gene expression (PSA, PSMA, EGFR). The use of multiplex systems permits an increased characterization of circulating tumor cells.

Cell clusters cannot be detected using methods of RT-PCR. Enumeration systems are normally imaged based, using immunocytochemistry or laser scanning techniques. Table 1 shows a summary of each commercial CTC detection kit.

CLINICAL USE OF THE DETECTION OF PRIMARY CPCs

In the detection of prostate cancer

There are few reported studies of the use of circulating tumor cells to detect prostate cancer. Early studies using different detection methods compared the presence of these cells in healthy controls, men with localized cancer, and men with metastatic prostate cancer. Circulating tumor cells appear to be less frequently detected in men with localized prostate cancer than those patients with advanced or metastatic cancer. In men with an increased PSA, there was a

detection rate of 20% in men with cancer and in 21% of men with a benign prostatic disease.^[86] Using the same CellSearch® system Thalgott *et al.*^[87] failed to detect a difference between men with localized prostate cancer and healthy controls. Using RT-PCR, only 8% of men with localized prostate cancer were positive for circulating tumor cells, and the results were concordant with the use of the CellSearch® system.^[88] In men with high risk non-metastatic prostate cancer and prior to any therapy, 14% of men had circulating tumor cells detected.^[89]

In contrast, using the MetaCell® system, circulating tumor cells were identified in 52% of men with localized prostate cancer,^[90] while Stott *et al.*^[91] using a CTC chip platform detected circulating tumor cells with a cut-off value of ≥ 14 to determine a positive test found 42% of men with localized prostate cancer to be positive. However, using a telomerase-based method Fizazi *et al.*^[92] detected tumor cells in 79% of men with localized prostate cancer. Using a combination of PSA and P504S immunocytochemistry, a study of over 1,000 men undergoing prostate biopsy for an elevated PSA reported that 35% of men were CPC positive; used as a sequential test after PSA screening, it showed a sensitivity of 81%, specificity of 89%, and a negative predictive value of 90%.^[93] The same group compared this method of CPC detection with PSA kinetics, age-defined PSA cut-off values, and the Montreal nomogram, and reported that CPC detection was superior in predicting prostate cancer at first biopsy.^[94-96] They also concluded that men with low-grade small volume tumors, those complying with the criteria for active observation, were CPC negative.^[97] Men with benign prostatic disease, especially

Table 1: Enrichment and detection systems of commercially available kits

System	Enrichment	Detection
CellSearch	IC EpCAM	IF CK, CD45, DAPI
Epispat	IC non-EpCAM	Secretion of proteins CK19, MUC1, PSA
Metacell	Cell size	ICC for CK
CTC membrane	Cell size	IF for CK
RosetteSep	ID CD45	IF for CK EpCAM CD45
Nanovelcro chip	Microfluids and IC	IF for CK EpCAM CD45
Adnatest	IC EpCAM	qRT-PCR
Ficoll-Paque	Cell density	ICC PSA and P504S

IC: immune-capture; IF: immunofluorescence; CK: cytokeratin; ICC: immunocytochemistry; ID: immune-depletion; PSA: prostate-specific antigen

Table 2: Methods reported in the detection and pretreatment prognosis of prostate cancer

	Diagnosis	Prognosis
CellSearch	Not useful	Not useful
Rt-PCR	Not useful	Possibly useful
Ficol-Paque	Possibly useful	Possibly useful

Rt-PCR: reverse transcriptase-polymerase chain reaction

prostatitis, may have PSA-positive circulating tumor cells detected but they were P504S negative.^[79] Validation in multicenter prospective clinical trials is therefore essential to assess its potential usefulness [Table 2].

As a prognostic marker to guide in the decision to treat or to observe

As a prognostic factor, primary CPCs do not appear to have a definitive use. This is because the majority of these cells will be eliminated by the primary treatment, be destroyed by the host's defense mechanisms, or not have the phenotypic characteristics to be able to implant and survive. In men with early stage prostate cancer, the detection of circulating tumor cells using RT-PCR was associated with a worse prognosis.^[98] Using PSA and PSMA genes to identify circulating tumor cells in men prior to radical prostatectomy, men negative for the test had significantly better outcomes.^[99] Using a positive/negative cutoff value, men negative for circulating tumor cells have a significantly better 10-year biochemical free failure survival after radical prostatectomy than men positive for CPCs.^[100]

When used as a predictive prognostic factor and compared with predictive nomograms, using the CellSearch® system^[101] or the PSA/P504S combined immunocytochemical assay,^[102] there was little if any improvement in predicting the prognosis of men pretreatment [Table 2].

Thus, the possibility of identifying circulating tumor cells in early stage prostate cancer seems to be achievable. However, the methods need to be clinically validated in multicenter studies. The use of primary CPCs as a sequential test to detect prostate cancer and as a guide to treatment seems a very fascinating area of research that warrants further studies.

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Patient consent

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Ethics approval

This article does not contain any studies with human participants or animals.

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Targeting Toll-like receptors against cancer

Bing Du^{1,2*}, Qiu-Li Jiang^{3*}, Joseph Cleveland¹, Bing-Rong Liu², Dekai Zhang¹

¹Center for Infectious and Inflammatory Diseases, Texas A&M University, Houston, TX 77030, USA.

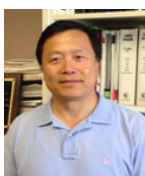
²Department of Gastroenterology and Hepatology, the Second Affiliation Hospital, Harbin Medical University, Harbin 150086, Heilongjiang, China.

³Department of Immunology, Harbin Medical University, Harbin 150086, Heilongjiang, China.

*The first two authors contributed equally to this paper.

Correspondence to: Dr. Dekai Zhang, Center for Infectious and Inflammatory Diseases, Texas A&M University, 2121 W. Holcombe Blvd., Houston, TX 77030, USA. E-mail: dzhang@ibt.tamhsc.edu; Dr. Bing-Rong Liu, Department of Gastroenterology and Hepatology, the Second Affiliation Hospital, Harbin Medical University, 246 Xuefu Rd., Nangang District, Harbin 150086, China. E-mail: bingrongliu@qq.com

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Dr. Dekai Zhang, Center for Infectious and Inflammatory Diseases, Texas A&M University. His main interests are: innate immunity; infectious and inflammatory diseases in GI tract; cancer immunotherapy.



Dr. Bing-Rong Liu, Department of Gastroenterology and Hepatology, the 2nd Affiliated Hospital of Harbin Medical University. His main interests are: endoscopic new techniques; infectious and inflammatory diseases in GI tract.

ABSTRACT

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The discovery of Toll-like receptors (TLRs) about 20 years ago was a remarkable achievement not only in the field of immunology but also in the field of medicine. The TLRs are a family of pattern recognition receptors which play an important role in immune responses by recognizing pathogen-associated molecular patterns. The TLRs also recognize danger-associated molecular patterns, which are associated with some diseases such as cancer. Recent evidence shows that TLRs are expressed not only in immune cells but also in tumor cells. The TLRs appear to play a role in tumor progression and treatment. Most likely, TLR activation has an impact on the initiation, development and treatment of tumors by modulating the inflammatory microenvironment. However, the activation of TLRs contributes to both inhibition and promotion of various tumors, with unclear underlying mechanisms. In this review article, the authors elucidate their current understanding about the role of TLRs in tumor progression, as well as the recent progress in utilizing TLR agonists as potential therapeutic agents in cancer treatment.



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INTRODUCTION

The discovery of Toll-like receptors (TLRs) approximately twenty years ago is a revolutionary event in life science and medical research, and helps improve our understanding about the role of innate immunity in both the physiology and pathology of human health. The medical community came to realize that innate immunity is essential and critical in immune responses to pathogen infections and in connection with the activation of adaptive immunity.^[1-3] Therefore, the 2011 Nobel Prize in Physiology or Medicine was awarded to scientists who made significant contributions to the discoveries concerning TLRs and their role in innate immunity. These discoveries mean that researchers now understand TLR biology much better [Figure 1].^[4-6] In this article, we summarize the role of TLRs in the immune system and focus on the expression of TLRs in cancer cells and their role in cancer progression. Finally, we discuss the current status of research in utilizing TLR agonists as potential therapeutic agents in cancer treatment.

TLRs: THE KEY SENSORS IN INNATE IMMUNE RESPONSE

The TLRs are a family of evolutionarily conserved pattern recognition receptors which play a vital role in immune responses against infection.^[1-3,7] There

are ten TLRs in humans, classified as two subgroups based on their cellular localization:^[8] TLR 1, 2, 4, 5, 6 and 10 are located on the cell surface and respond primarily to pathogen-associated molecular patterns (PAMPs) such as lipids and bacterial proteins. In contrast, TLR 3, 7, 8 and 9 are located intracellularly in the endosomes, responding primarily to nucleic acids from both viruses and bacteria.^[9] The TLRs are a class of type I transmembrane proteins comprised of an extracellular domain, transmembrane region and intracellular domain.^[10] The ectodomain contains leucine-rich repeats and two to four evolutionarily conserved cysteine structures which recognize and bind to evolutionarily conserved molecular motifs in PAMPs. The intracellular domain is highly homologous among the TLRs and contains a toll/interleukin-1 receptor (TIR) domain, which is crucial to the intracellular activation of signaling cascades leading to the induction of pro-inflammatory cytokines and chemokines.^[11]

The TLRs recognize PAMPs from micro-organisms or danger-associated molecular patterns (DAMPs) from damaged tissues to activate innate and adaptive immune responses. A variety of ligands corresponding to distinct TLRs have been identified so far [Figure 1].^[12-14] For example, TLR2 recognizes peptidoglycan and lipoteichoic acid from bacterial cell wall;^[15] TLR3 recognizes double-stranded

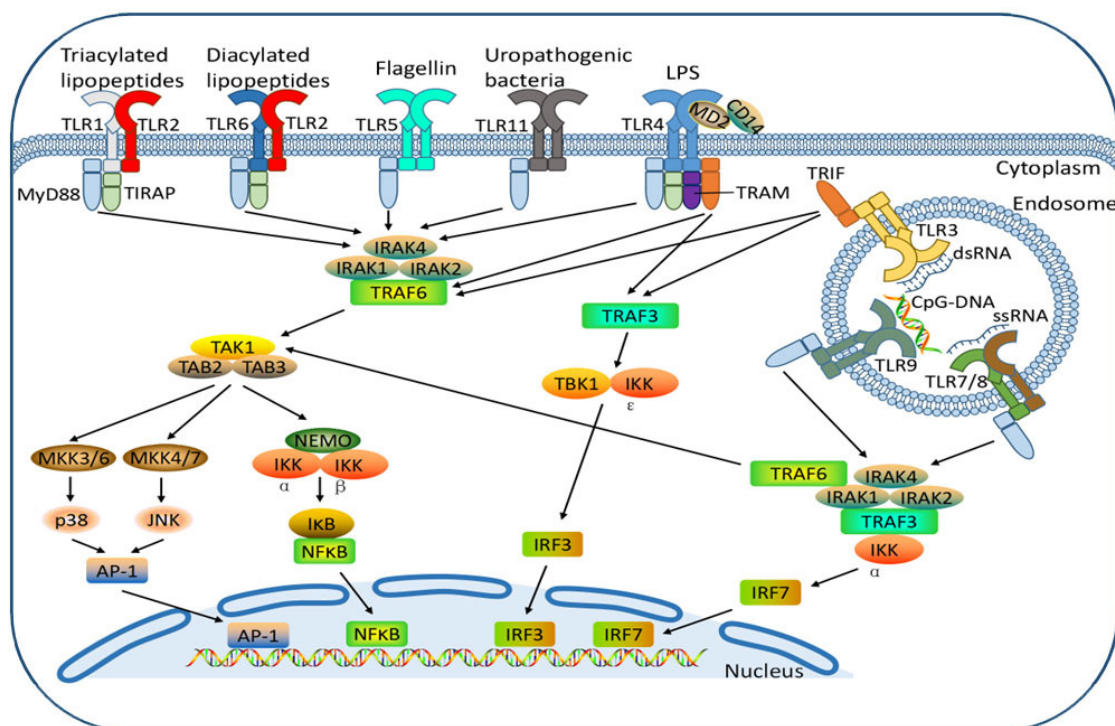


Figure 1: TLR ligands and TLR signaling pathways. Cell surface TLRs, including TLR-1, -2, -4, -5, -6, -10, and -11, and intracellular TLRs, including TLR-3, -7, -8, and -9, recognize their specific PAMPs to activate TLR signaling cascades.^[70] TLR: Toll-like receptor; PAMPs: pathogen associated molecular patterns

RNA (dsRNA)^[16] which constitutes the genome of RNA viruses; TLR4 is well known as a sensor of lipopolysaccharide (LPS) from bacteria;^[17] TLR5 is responsible for the recognition of flagellin;^[18] single-stranded RNA is identified as the ligands of TLR7 and TLR8;^[19] and intracellular TLR9 senses unmethylated CpG oligonucleotide (ODN).^[20] The specific ligand for TLR10 has not yet been defined. TLR11 in mouse macrophages is known to recognize uropathogenic *Escherichia coli*, but TLR11 is not expressed in humans.^[21]

Signaling for TLR is initiated by recognition of PAMPs and the ligand-induced dimerization of TLRs [Figure 1]. Upon activation, TLRs recruit TIR-domain-containing adaptor proteins for the subsequent activation of downstream signaling. The adaptors include myeloid differentiation factor-88 (MyD88), Toll/IL-1 receptor domain adaptor protein, TIR-domain-containing adapter-inducing interferon- β (TRIF), TRIF-related adaptor molecule and sterile- α and armadillo motif-containing protein. These adaptors provide receptor sites for relevant proteins and initiate various signaling events, which results in a variety of inflammatory cytokines transcription by mediating the phosphorylation of I κ B α to active NF- κ B. The multiple signaling pathways contribute to the rapid response of the innate immune system to the pathogens.^[22] In addition, the recognition of PAMPs by TLRs gives rise to the activation and maturation of dendritic cells, and pro-inflammatory cytokines and chemokines are produced to induce the proliferation and differentiation of Th1 and Th2, which establishes and regulates adaptive immunity.

TLRS ARE ALSO EXPRESSED IN TUMOR CELLS

The TLRs, a family of receptors in the innate immune system, are expressed and activated in innate immune cells such as macrophages and dendritic cells. In recent years, however, some studies have shown that TLRs are also highly expressed in various tumor cells.^[23-27] For example, over-expression of TLR2, TLR3 and TLR4 has been detected in majority of colonic cancer cells,^[28,29] and TLR2, TLR3, TLR4 and TLR5 are highly expressed in ovarian cancer cells.^[30,31]

Therefore, the study of TLR expression and function in cancer has become a focus for researchers. In the colon mucosa of polyposis patients, high mRNA copy numbers of TLR3 have been observed, and strong TLR3 expression has been demonstrated and associated with colorectal cancer stages.^[32] Mice deficient in TLR4 and MyD88 have shown significant

decreases in the size, incidence and number of chemical-induced liver cancer neoplasms, indicating an important contribution of TLR signaling to hepatocarcinogenesis.^[33,34] Two TLRs, TLR5 and TLR9, are considered to be associated with cervical cancer; the expression of these two receptors increases significantly in higher grades of cervical cancer while this expression is rarely detected in normal cervical squamous epithelial cells.^[35] The expression of TLR9 promotes angiogenesis and is associated with lower lung cancer survival rates.^[36] Moreover, TLR9 promotes the proliferation of prostate cancer cells in time- and dose-dependent manners confirmed by the expression level of NF- κ B and downstream c-Myc.^[37]

Although many TLRs promote the occurrence and development of tumors via a variety of mechanisms, some TLRs might have an antitumor effect. Therefore, the activation of TLRs in cancer cells can play a complex role. The upregulation of TLR1 and TLR2 in bladder cancer promotes the nuclear translocation of NF- κ B and the activation of the c-JNK signaling pathway, which increases the secretion of IL-1, IL-6 and IL-8.

Moreover, TLR3 is considered to promote the death of tumor cells in various cancers. A study by Paone *et al.*^[38] indicated that the TLR3 agonist poly I:C inhibits the proliferation, and promotes the apoptosis, of prostate cancer cells by activating protein kinases. The combination of poly I:C and 5-fluorouracil (5-FU) or IFN- α effectively induced apoptosis in human colon cancer cells.^[39] Increased expression of TLR3 in human melanoma can inhibit the proliferation and induce the death of tumor cells with pretreatment of type I IFN.^[40] In addition, our study showed that the TLR5-activated signaling pathway in breast cancer inhibits the proliferation of tumor cells by down-regulating cyclin B1, cyclin D1 and cyclin E2.^[41] In human head and neck cancers, TLR5 activated by flagellin also reduces tumor cell proliferation and promotes tumor cell apoptosis. When treated with CpG oligodeoxynucleotides (CpG ODN) 107 and irradiation, the TLR9 signaling pathway in human glioma arrests the cell cycle and reduces the proliferation of tumor cells by activating downstream NF- κ B and NO pathways.^[42] A TLR9 agonist inhibits proliferation and promotes caspase-dependent apoptosis of neuroblastoma cells. In addition, it has shown antitumor and anti-angiogenesis effects in renal cell carcinoma.^[43]

The regulation of TLRs and their signal transduction is complicated, and understanding of the mechanism is limited. Recently, the role of autophagy in immune response has drawn special attention because

TLRs can stimulate autophagy, which conversely regulates TLRs and their signal transduction, with the mechanism unknown. We recently reported that microtubule-associated protein 1S (MAP1S), an intracellular autophagy-related molecule, can regulate TLRs and their signaling.^[44] MAP1S plays an important role in cell cycle arrest induced by the flagellin/TLR5 signaling pathway in human breast cancer cell MCF-7, and it is also involved in the inhibition of cell migration of MCF-7 by flagellin. To sum up, given the increasing evidence of mutuality between TLRs and tumors, more attention has been given to innate immunity in tumor cells, especially regarding TLRs expression and signaling pathway, both of which play a significant role in the development of cancer.

TLRs PLAY A CRITICAL ROLE IN TUMOR DEVELOPMENT

TLR signaling inhibits tumor growth

The TLRs play an immune surveillance role mainly by inducing the production of multiple cytokines and the activation of immune cells. Cytokines such as type I interferon (IFN-I) and interleukin 12 (IL-12) promote the activation of NK cells and enhance the scavenging capacity of the host with tumor cells.^[45] Other cytokines such as IL-2 and IFN- γ can enhance the ability of tumor-specific cytotoxic T lymphocyte (CTL) in the host to recognize and scavenge tumor cells. Intriguingly, some TLRs agonists were found capable of inhibiting tumor growth.^[46-49] It has been reported that the combination of TLR agonists, chemotherapy drugs and tumor vaccine could improve the efficacy of eliminating tumor cells, an effect mainly based on the activation of antigen-presenting cells and the enhancement of T-cell immune response by TLRs.^[50] The increased expression of MHCII, CD88 and CCR7 in the activated antigen presenting cells of the TLRs signaling pathway significantly enhances recognition and presenting to tumor antigen. Also, TLR1/2 acting on CD8⁺ CTLs increases the secretion of IFN- γ , TNF- α and IL-2 to promote the secretion of granzyme B and perforin by CD8⁺ T cells, which play a key role in elimination of tumor cells.^[51]

In addition, TLRs also act directly on tumor cells; TLR3 is thought to be effective in promoting tumor cell apoptosis in a variety of tumors. When activated by dsRNA, an agonist of TLR3, breast cancer cells generate autocrine type I IFN, which mediates TLR3 dependent cell apoptosis.^[52,53] In type I and II lung cancer cells, the engagement of TLR3 by dsRNA induces an atypical caspase-8-containing complex, which activates apoptotic pathways leading to tumor cell death.^[53] In the development of tumors, vigorous

metabolism leads to metabolic disorders and local hypoxia, through which large amounts of tissue cell debris and proteins are released. The debris and proteins are recognized by TLRs as DAMPs, which are considered signals of danger, and this recognition consequently influences the various biological behaviors of tumor cells. It has been reported that HMGB1, an endogenous ligand of TLR2 which binds to TLR2 and activates TLR2 signaling pathways in glioblastoma, mediates antitumor immune response by inducing the activation of DCs and their migration into the brain tumor.^[54]

TLR signaling promotes tumor growth

The activation of TLRs can also promote tumor growth in many situations. Recent studies have found that the combination of highly expressed TLRs and DAMPs in tumors changes the homeostasis of the immune system, which leads to the suppression of immune function. HMGB1 has been identified as a cause of tumors of the skin, liver and pancreas. Furthermore, TLR4 recognizes and combines with HMGB1 released by necrotic cells, and this recognition may eventually cause immune tolerance by activating the downstream pro-inflammatory signaling pathway. At the same time, HMGB1 aggregates in the cell membrane and promotes the invasion and growth of tumor cells.^[23]

Although the specific mechanisms of TLR-mediated immune escape are still unknown, the high expression of TLRs in tumors often leads to immunosuppression while enhancing the invasiveness of tumors. Studies have found that the activation of the TLRs signaling pathway may lead to increased secretion of IL-10 and TGF- β , both of which are major immune suppressors *in vivo*.^[55] In addition, the activation of TLRs is also accompanied by the expression of PD-L1, HLA-G and other inhibitory costimulatory molecules.^[56] In a mouse model of colon cancer, TLR4 has prolonged the survival time of tumor cells by up-regulating programmed death ligand 1 (PD-L1/B7-H1), inducible costimulator ligand (B7-H2) and down-regulating the expression level of Fos.^[57] Supernatants generated from murine colon cancer cells stimulated with LPS were found to play a significant role in the inhibition of T cell proliferation and NK cell cytotoxicity. The effect can be reversed after the TLR4 signaling pathway is blocked, which may explain the pathway's immunosuppressive effect.^[58] In addition to the inhibiting role, TLRs also promote the proliferation of tumor cells and enhances tumor invasion, promoting immune escape, while TLR2 in human gastric cancer cell lines promotes tumor progression through the induction of COX-2, PGE-2 and IL-8.^[57]

The TLR signaling can lead macrophage polarization change, from M1 (inhibiting tumor) to M2 (promoting tumor), which might explain, at least partially, why TLR signaling promotes tumor growth. The M1/M2 polarization model has been reported in many cancer research studies in recent years. The M1 of tumor-associate macrophages (TAM) express high levels of IL-12 and IL-23, and function as inducers of Th1 responses. During tumor progression, TAM polarizes toward M2 TAM, an alternatively activated macrophage, with a tumor growth-promoting phenotype. However, this M1/M2 polarization has only been well established *in vitro*, not *in vivo*. Therefore, the role of TLR signaling in M1/Me polarization calls for further investigation.

The role of TLRs in cancer progression: a double-edged sword

Overall, as discussed above, the activation of TLRs can both promote and inhibit tumor growth and cancer progression, and the underlying mechanism remains elusive. Current knowledge shows that different TLRs share similar signaling pathways, but this cannot explain why the activation of different TLRs in cancers has opposite effects on tumor growth. Also, TLR agonists themselves might have direct pro- or anti-tumor effects, but current evidence shows that these effects, at least in majority of cases, are very minor. Another potential mechanism is that different TLRs might trigger different signaling pathways in cancer cells. We recently found that activation of TLRs in cancer cells may induce cancer cells to secrete various soluble factors, which might play distinct roles in cancer development. The role of TLRs in cancer progression needs to be further investigated, and understanding the underlying mechanism is essential for the further development of TLR agonists as therapeutic agents.

TLRS IN CANCER TREATMENT

Since the first TLR was discovered in 1997,^[59] studies of the characteristics and prospects of TLRs have become prominent in research. However, the clinical application of TLRs is just beginning.^[60] To date, only a few TLR agonists have been approved by the Food and Drug Administration for clinical trials involving cancer patients involving Bacillus Calmette-Guérin (BCG), Imiquimod and monophosphoryl lipid A (MPL). Originally used as a vaccine against tuberculosis, BCG is approved for the treatment of bladder cancer; it potently activates TLR2 and TLR4 signaling.^[61] Meanwhile, the BCG vaccine is sometimes used to help treat stage III melanoma.^[60,62] Imiquimod, a TLR7 agonist which has been in Phase II clinical trials, is efficacious in treatment of various skin tumors and epidermal metastasis,^[63] and MPL, a derivative of LPS,

is in phase I clinical trials for testing antitumor activity in colorectal cancer patients. At this time MPL has been approved as an adjuvant of Cervarix, a cancer vaccine against HPV-associated cervical cancer.^[64]

Since the anti-tumor effect of a single TLR agonist remains to be verified and the side effects need to be considered, it may be premature to apply a single TLR agonist to the clinical treatment of tumors. For example, the two-way effects of TLR3 make it a potentially risky therapeutic drug. Although the TLR3 agonist poly A:U is considered to be therapeutically effective in patients with various types of cancers, the risk of metastasis relapse is significantly decreased in TLR3-positive, not in TLR3-negative breast cancers.^[65] The function of TLRs in tumors varies with the origin and type of the tumors, which indicates that the therapeutic use of TLR agonists requires much more clinical evidence.

While most of the TLR4 studies reported a tumor-promoting effect,^[34,66-69] one study found a protective effect against lung cancer in the lung epithelium.^[70] This indicates the need for further study. Nevertheless, the anti-tumor effect of TLRs agonists is still worth exploring.^[70-72] The members of the TLR family are different in many aspects such as expression distribution, subcellular localization, adaptive molecules for inducing signal transduction, recognized PAMPs and the types of the induced immune response. Treatment using TLRs ligands has to be based on the identification of the TLRs signals with corresponding diseases, as well as identifying the types of vaccines with significant enhancement that can be used safely and effectively in clinical practice. Future studies should pursue many avenues of research.

CONCLUSION

The TLRs play a critical role in tumor immunity, and the antitumor effect is also a notable focus for future studies on cancer therapy. The perspective approach for future cancer treatment may be that the combination of some specific TLR agonists or antagonists with traditional cancer treatments might improve treatment efficacy. The role of TLRs in both promoting and inhibiting tumor growth and metastasis has been confirmed in various studies. However, the specific mechanism of action is still unclear; at the same time, cancer is a multifactorial disease, and the research of TLRs on tumor immunity is just beginning. Further studies may help us better understand TLRs and tumor immunity, and the clarification of the roles of TLRs in tumorigenesis and tumor metastasis will provide new strategies and prospects for more effective cancer treatment.

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Conflicts of interest

There are no conflicts of interest.

Patient consent

There is no patient involved.

Ethics approval

This article does not contain any studies with human participants or animals.

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ERCC1 expression in patients with colorectal cancer: a pilot study

Kinjal K. Gajjar¹, Deep Kumari Yadav¹, Toral P. Kobawala¹, Trupti I. Trivedi¹, Hemangini H. Vora², Nandita R. Ghosh¹

¹Cancer Biology Department, the Gujarat Cancer and Research Institute, NCH Compound, Asarwa, Ahmedabad 380016, Gujarat, India.

²Immunohistochemistry and Flow Cytometry Division, Cancer Biology Department, The Gujarat Cancer and Research Institute, NCH Compound, Asarwa, Ahmedabad 380016, Gujarat, India.

Correspondence to: Dr. Nandita R. Ghosh, Cancer Biology Department, The Gujarat Cancer and Research Institute, NCH Compound, Asarwa, Ahmedabad 380016, Gujarat, India. E-mail: nandita.ghosh@gcriindia.org

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ABSTRACT

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Key words:

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Aim: Excision repair cross complementation group 1 (ERCC1) has a key role in enhanced DNA damage repair caused by oxaliplatin-based therapy and may lead to resistance of these platinum drugs in colorectal cancer (CRC) patients. Hence, the present preliminary study aimed to explore the role of ERCC1 C/T polymorphism at codon 118 as well as its immunoreactivity in patients with primary CRC. **Methods:** ERCC1 polymorphism was studied using PCR-RFLP and ERCC1 protein expression was examined by immunohistochemistry in 50 CRC patients. **Results:** ERCC1 codon 118 C/T polymorphism analysis reported the predominance of C/T (52%) genotype as compared to C/C (38%) and T/T (10%) genotypes. Furthermore, 72% of patients showed positive ERCC1 protein expression. Significant correlation was not observed between clinicopathological parameters and ERCC1 polymorphism, while ERCC1 protein expression significantly correlated only with tumor site (colon vs. rectum) ($P = 0.046$). Further, the present study failed to demonstrate the role of ERCC1 C118T polymorphism or protein expression as useful prognostic markers in CRC patients. **Conclusion:** ERCC1-positive protein expression may be a useful marker for rectal cancer patients. However, further evaluation in a larger set of CRC patients is required to better understand the role of ERCC1.

INTRODUCTION

The antimetabolite, 5-Fluorouracil (5-FU), was introduced in 1957 by Heidelberger *et al.*^[1] and today it is the cornerstone of chemotherapeutic regimens in treatment of colorectal cancer (CRC). However, the

overall response rate is only 10-15% for advanced CRC when treated with 5-FU alone.^[2] In recent years, the outcome of patients with CRC has been improved significantly because of the use of oxaliplatin-based combination therapy with 5-FU (FOLFOX). Oxaliplatin, in combination with leucovorin and 5-FU, is quite



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effective in the treatment of CRC, in both the adjuvant and metastatic settings.^[3]

Oxaliplatin is a cytotoxic platinum compound which exerts its effects through development of DNA adducts.^[4] DNA repair, especially nucleotide excision repair (NER) pathway, plays an important role in platinum-based chemotherapeutic efficacy by repairing drug-produced DNA damage. Excision repair cross complementation group 1 (ERCC1) is a chief component of the NER pathway^[5,6] and a highly conserved protein, essential for elimination of DNA adducts caused by the platinum compound. It plays a major role in better repair and tolerance of DNA damage, leading to resistance of platinum drugs.^[7,8]

A common C/T single nucleotide polymorphism (SNP) at codon 118 of ERCC1 has been identified. This SNP may contribute to inter-individual variability in DNA repair capability and has been documented as a predictor for outcome in CRC patients who have been treated with platinum-based chemotherapy.^[9] This polymorphism results in the same amino acid, asparagine, but a tendency towards elevated ERCC1 mRNA and protein levels observed as the number of T alleles increases. Moreover, clinical and preclinical studies indicate that overexpression of ERCC1 protein is associated with resistance to platinum-based chemotherapy in various types of cancers.^[10,11]

Therefore, the present study aimed to evaluate the prevalence of ERCC1 C118T SNP polymorphism and the expression of ERCC1 protein in patients with primary CRC and further to evaluate their role in the clinical outcome of CRC patients.

METHODS

Patients

A total of 50 CRC patients who underwent surgical resection at the Gujarat Cancer and Research Institute, Ahmedabad, between 2013 and 2014 were included in this study. Inclusion criterion was an untreated CRC patient with histopathologically confirmed adenocarcinoma without any prior history of anticancer treatment. Patients were followed for a minimum of 15 months or until death within that period.

Sample collection

The study was approved by Institutional Scientific and Ethical Committees and informed consent was required from all patients prior to sample collection. To examine ERCC1 polymorphism, colorectal tumor was collected from the histopathology department immediately after surgery. The tumor portion was

selected by the pathologist, snap frozen in liquid nitrogen, and immediately stored at -80 °C until analysis. For the study of ERCC1 protein expression, paraffin embedded tumor tissue blocks were retrieved from histopathology department.

DNA extraction and ERCC1 polymorphism study by PCR-RFLP

DNA extraction from tumor was performed by Phenol: Chloroform method. PCR of the SNP C/T at codon 118 of ERCC1 gene was performed using QIAGEN Taq PCR kit and following primers sequences: forward primer: 5'-GCAGAGCTCACCTGAGGAAC-3'; reverse primer: 5'-GAGGTGCAAGAAGAGGTGGA-3' (Sigma-Aldrich). After initial denaturation at 94 °C for 3 min, the following PCR protocol was performed for 35 cycles: Denaturation at 95 °C for 1 min, annealing at 55.7 °C for 45 s, and extension at 72 °C extensions for 1 min. Digestion of these PCR products was performed with BsrD1 restriction enzyme (New England BioLabs, USA) at 65 °C for 4 h and separated on 2% agarose gel.

ERCC1 protein expression by immunohistochemistry

Immunohistochemistry for ERCC1 was performed on 4 µm thick formalin-fixed, paraffin embedded tissue sections. FFPE tumor tissue sections were stained using mouse- and rabbit- specific HRP/DAB (ABC) Detection IHC kit according to manufacturer's protocol. The primary antibody used was mouse monoclonal anti-ERCC1 (clone 4F9: Dako, USA, dilution: 1:50). Sections known to exhibit high expression of protein were used as positive controls, while negative controls were obtained by omission of primary antibody. A semiquantitative score was used starting from negative (no staining or, 10% of cells stained) to 3+ (1+ staining for 11-30% of cells: weak, 2+ staining for 31-50% of cells: moderate, and 3+ staining for > 50% of cells: intense).

Statistical analysis

The statistical data analysis was performed by Statistical Package for the Social Sciences software version 17. Two tailed chi (χ^2) test was used to determine the association between clinicopathological variables with ERCC1 polymorphism and ERCC1 protein expression. Correlation between two parameters was calculated according to Spearman's correlation coefficient (r) method. Relapse-free survival (RFS) and overall survival (OS) was calculated using Log rank test. P value ≤ 0.05 was considered significant.

RESULTS

Patient characteristics

Patient and tumor characteristics of the 50 patients

are outlined in Table 1. The median age was 55 years. There was a considerable proportion (54%) of elderly and dominance of male patients (60%). The incidence of patients having colon cancer was higher (56%) as compared to rectal cancer (44%). Higher occurrence of early stage patients (60%) was observed. Complete follow-up details were obtained in 80% (40/50) and were included for OS analysis. Amongst these 40, 4 died due to disease and hence were not included for the RFS analysis. Therefore, 36/40 CRC patients were considered for RFS analysis from which 2 patients developed recurrence [Table 1].

Incidence of ERCC1 codon 118 C/T polymorphism

Three types of genotypes have been identified for ERCC1 C118T SNP: C/C genotype at 208 bp, C/T genotype at 208, 128 and 80 bp and T/T genotype at 128 and 80 bp [Figure 1].

Thirty-eight percent ($n = 19$) showed the C/C genotype, 52% ($n = 26$) showed the C/T genotype, and 10% ($n = 5$) showed the T/T genotype. Thus, prevalence of C/T

Table 1: Patient and tumor characteristics ($n = 50$)

Characteristics	n (%)
Age (year)	
< 55	23 (46)
≥ 55	27 (54)
Gender	
Female	20 (40)
Male	30 (60)
Habit	
No	29 (58)
Yes	21 (42)
Tumor site	
Colon	28 (56)
Rectum	22 (44)
Tumor size	
T2	5 (10)
T3	43 (86)
T4	2 (4)
TNM stage	
Early stage (I + II)	30 (60)
Advanced stage (III + IV)	20 (40)
Dukes' stage	
B	30 (60)
C	20 (40)
Histological type	
Adenocarcinoma	35 (70)
Mucin adenocarcinoma	14 (28)
Signet ring cell carcinoma	1 (2)
Histological grade	
Well differentiated	9 (18)
Moderately differentiated	33 (66)
Poorly differentiated	8 (16)
CEA (ng/mL) ($n = 45$)	
< 5.0	19 (42)
≥ 5.0	26 (58)
Treatment	
Surgery alone	9 (18)
Surgery + chemotherapy	25 (50)
Surgery + chemotherapy + radiotherapy	12 (24)
Surgery + radiotherapy	4 (8)
Recurrence/metastasis ($n = 36$)	
Absent	34 (94)
Present	2 (6)
Disease outcome ($n = 40$)	
Alive	35 (88)
Dead	5 (12)

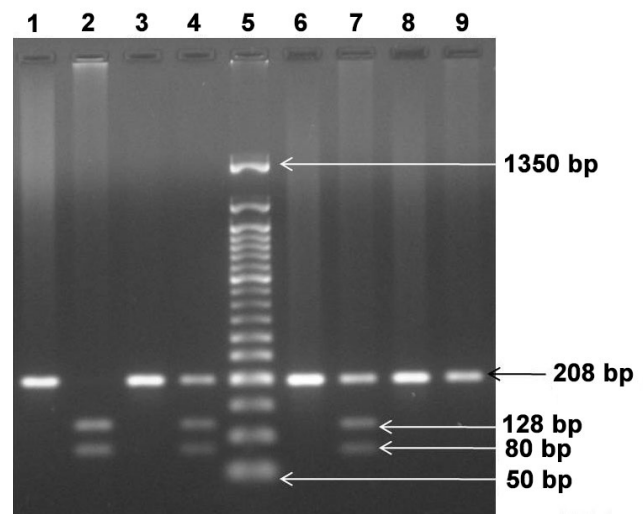


Figure 1: Representative pattern of ERCC1 codon 118 genotypes separated on 2% agarose gel. Lane 1, 3, 6 and 8: undigested PCR products; Lane 2: T/T homozygous genotype at 128, 80 bp; Lane 4 and Lane 7: C/T heterozygous genotype at 208, 128 and 80 bp; Lane 9: C/C wild type genotype at 208 bp; Lane 5: 50 bp ladder. ERCC1: excision repair cross complementation group 1; PCR: polymerase chain reaction

heterozygous genotype was observed in this group of CRC patients. The distribution of ERCC1 codon 118 polymorphism genotypes was consistent with the Hardy-Weinberg equilibrium among patients ($\chi^2 = 0.82$, $P = 0.36$).

Incidence of ERCC1 protein expression

Expression of ERCC1 protein was localized in the cytoplasm of epithelial cells of colon and rectum [Figure 2]. ERCC1-positive protein expression was detected in 72% ($n = 36$) of patients whereas 28% ($n = 14$) showed ERCC1-negative protein expression.

Correlation of ERCC1 polymorphism and ERCC1 protein expression with clinicopathological parameters

ERCC1 codon 118 C/T polymorphism was not significantly associated with any of the clinicopathological parameters. On the other hand, the incidence of ERCC1 protein immunoreactivity was significantly higher in patients having rectal (86%) than with colon cancer (61%) ($\chi^2 = 4.020$, $r = +0.284$, $P = 0.046$; Figure 3). ERCC1 protein expression was not significantly associated with the other parameters.

Correlation between ERCC1 polymorphism and ERCC1 protein expression

Predominance of ERCC1-positive protein expression was observed in all three sub-groups of patients having C/C (74% 14/19), C/T (69% 18/26), and T/T genotypes (80% 4/5). However, the difference was not statistically significant ($P = 0.981$).

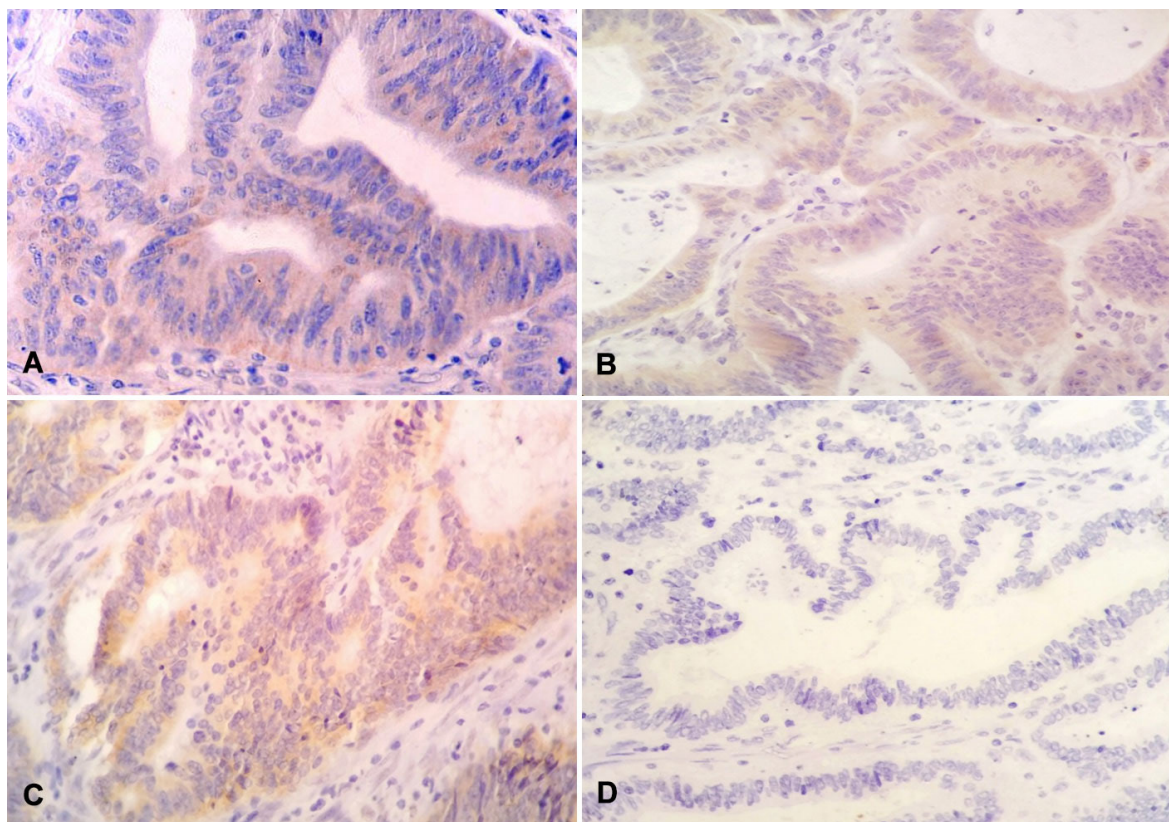


Figure 2: Representative staining of ERCC1 (×40). (A) Colon cancer; (B) colon cancer; (C) rectal cancer; (D) negative control. ERCC1: excision repair cross complementation group 1

Univariate survival analysis according to ERCC1 codon 118 C/T polymorphism and ERCC1 protein expression

When patients were stratified according to ERCC1-118 polymorphism, it was observed that both patients (100%) who had developed relapse had the C/T genotype, whereas amongst the 5 patients who died, 2 (40%) each had the C/C and C/T genotypes, and one (20%) had T/T. Further, when patients were stratified according to ERCC1 protein expression, of the 2 who developed recurrences, one (50%) was ERCC1-positive and another (50%) was ERCC1-negative. Among the patients who had died, 4 (75%) were ERCC1-positive and one (25%) was ERCC1 negative. However, the data were statistically non-significant.

DISCUSSION

Oxaliplatin, a platinum-based chemotherapeutic drug, induces DNA damage by forming DNA adducts. DNA repair proteins involved in the NER pathway, such as ERCC1, play a key role in repair of this damage, thus leading to resistance to platinum-based therapy. Several clinical studies have demonstrated that both ERCC1 polymorphism and protein expression are associated with resistance to platinum-based

chemotherapy and have the potential to be used as candidate biomarkers for CRC. Therefore, the present study explored the role of ERCC1 C118T SNP as well as ERCC1 immunoreactivity in CRC patients.

A predominance of the C/T genotype (52%) was observed as compared to C/C (38%) and T/T (10%) genotypes. In accordance with the current study, one CRC study reported a higher incidence of the C/T genotype, with 44% as compared to C/C (24%) and T/T (32%).^[12] However, another reported that the frequencies of C/C, C/T, and T/T genotypes

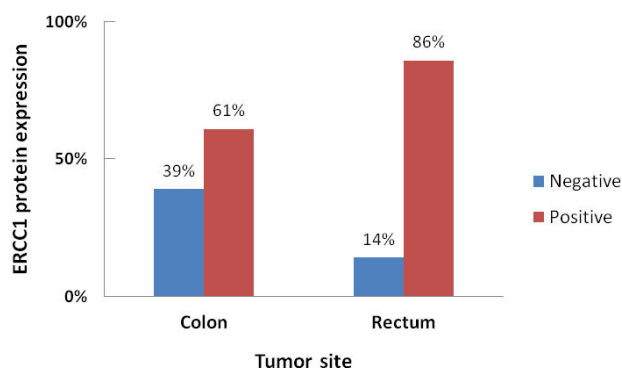


Figure 3: Significant correlation of ERCC1 protein expression with tumor site; $P = 0.046$ between the colon and rectum groups. ERCC1: excision repair cross complementation group 1

were 47.6%, 39.9% and 12.5%, respectively in Asian patients, very similar to previous reports in Caucasian populations.^[4,13]

In the present study, it was found that there was positive ERCC1 protein expression in 72% of CRC patients. Likewise Wang *et al.*^[14] reported 68.1% positive ERCC1 immunoreactivity in patients with gastric cancer. However, in 2 studies, ERCC1 positivity was observed in 45% and 55% of patients with colorectal and stage III disease, respectively.^[4,15] This discrepancy may be due to several factors including antibody used, scoring technique, used and in preparation of the paraffin embedded tissue blocks.

To date, most studies have focused more on pathologic and molecular features, but less on clinical features with molecular variables. Therefore, in the present study, correlation of ERCC1 polymorphism and protein expression with clinicopathological parameters was evaluated. However, none of the clinicopathological parameters was significantly associated with ERCC1 gene polymorphism. Similarly, another study did not find statistically significant correlations between genotype distributions and gender, tumor location, tumor invasion, lymph node metastasis, tumor stage, or histology in CRC patients.^[16] The present study showed no significant association between ERCC1 protein expression and clinicopathological parameters apart from tumor site. Still another study reported no significant differences in gender, tumor stage, nodal stage, histological differentiation, lympho-vascular invasion, neural invasion, or postoperative CEA levels between the ERCC1 positive and negative groups. However, ERCC1 positive expression was significantly associated with older age group patients ($P = 0.031$).^[17] Also it has been shown in patients with nasopharyngeal carcinoma that the expression level of ERCC1 increased significantly with higher T stage and clinical stages ($P < 0.05$). Thus, at least in that malignancy, ERCC1 seemed to be a valid biological indicator to predict prognosis.^[18] In the present study, ERCC1 positive protein expression was found to be significantly higher only in rectal cancer patients as compared to colon cancer patients ($P = 0.046$).

In one report the identical ERCC1 C/T polymorphism at codon 118 was found to influence the level of ERCC1 expression. This may be due to that, although both the AAC and AAT codons encode asparagine, the AAT codon usage is significantly reduced, thereby decreasing ERCC1 translation capability and protein level.^[19] Therefore, in the present study we correlated ERCC1-118 polymorphism and ERCC1 protein expression. However, there was no significant

correlation found between ERCC1 polymorphism and ERCC1 protein expression. Similarly, Qi *et al.*^[20] in patients with gastric cancer and Takenaka *et al.*^[21] in patients with non-small cell lung cancer also showed that ERCC1 genotypes were not correlated with ERCC1 protein expression. On the other hand, another study found increased ERCC1 protein levels in CRC patients with the C/T or T/T genotypes.^[4]

Several studies have investigated the prognostic role of ERCC1-118 SNP and ERCC1 protein expression in CRC patients and other cancers types but the results obtained were controversial. In one report ERCC1 codon 118 C/C genotype was significantly associated with higher response rates, progression-free survival, and OS in metastatic CRC.^[4] However, another supported the pharmacogenetic role of the ERCC1-118 C > T change and emphasized that the T allele was a marker of a better outcome in patients with CRC treated with OX-based schemes.^[22,23] Thus, the relationship between ERCC1 codon 118 SNP and clinical outcome in patients with CRC remains controversial although one study reported significantly shorter progression-free ($P < 0.01$) and overall ($P < 0.01$) survival in patients having positive ERCC1 IHC staining in colorectal tumor tissues.^[4] It was also observed that 5-year DFS and OS were significantly lower in combined therapy group with positive ERCC1 tumors than in the same group patients with negative ERCC1 tumors.^[15] However, in present study, due to short follow-up period of 15 months, no correlation of RFS and OS was observed with ERCC1 C118T SNP and ERCC1 protein expression. We recognize that a limitation of this study is that SNP assessment was not confirmed by Sanger sequencing. Further studies will be necessary to validate our findings.

In summary, the current study reveals that ERCC1 protein expression was significantly higher in patients with rectal cancer as compared to patients with colon cancer, which may indicate biological and functional differences between the two subsets and may emerge as an important marker for patients with rectal cancer. However, further studies with larger sample sizes and longer follow-up period are necessary for a more definite conclusion.

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Conflicts of interest

There are no conflicts of interest.

Patient consent

Patient consent was acquired from all patients prior to sample collection.

Ethics approval

The study has been approved by Institutional Scientific and Ethical Committees.

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Journal articles by individual authors	Weaver DL, Ashikaga T, Krag DN, Skelly JM, Anderson SJ, et al. Effect of occult metastases on survival in node-negative breast cancer. <i>N Engl J Med</i> 2011;364:412-21. [PMID: 21247310 DOI: 10.1056/NEJMoal008108]
Organization as author	Diabetes Prevention Program Research Group. Hypertension, insulin, and proinsulin in participants with impaired glucose tolerance. <i>Hypertension</i> 2002;40:679-86. [PMID: 12411462]
Both personal authors and organization as author	Vallancien G, Emberton M, Harving N, van Moorselaar RJ, Alf-One Study Group. Sexual dysfunction in 1,274 European men suffering from lower urinary tract symptoms. <i>J Urol</i> 2003;169:2257-61. [PMID: 12771764 DOI: 10.1097/01.ju.0000067940.76090.73]
Journal articles not in English	Zhang X, Xiong H, Ji TY, Zhang YH, Wang Y. Case report of anti-N-methyl-D-aspartate receptor encephalitis in child. <i>J Appl Clin Pediatr</i> 2012;27:1903-7. (in Chinese)
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.

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