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Medulloblastoma—Biology and Microenvironment: *A Review*

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Abstract

Medulloblastoma (MB) is a cancer of the cerebellum and the most common primary pediatric malignancy of the central nervous system. Classified as a primitive neural ectoderm tumor; it is thought to arise from granule cell precursors in the cerebellum. The standard of care consists of surgery, chemotherapy and age-dependent radiation therapy. Despite aggressive multimodality therapy; approximately 30% of MB patients remain incurable. Moreover, for long-term survivors, the treatment related sequelae are often debilitating. Side effects include cerebellar mutism, sterility, neurocognitive deficits, and a substantial risk of developing secondary cancers. In a quest for more effective and targeted therapies, scientists have begun to investigate the biological events that not only initiate but also sustain the malignant phenotype in MB. Of particular interest is, the role of the tumor microenvironment in tumor pathogenesis. This review seeks to highlight several key processes observed in cancer biology, particularly the involvement of the tumor microenvironment, with relevant examples from MB.

Keywords

brain; medulloblastoma; microenvironment; review; therapy

BIOLOGY

TUMORIGENESIS

Tumorigenesis refers to the steps involved in the initiation and formation of a tumor. It is commonly accepted that cancer is a multistep process, yet the steps resulting in tumorigenesis may vary from one tumor to another. Ultimately, these events culminate in the inability of the cells to maintain a balance between proliferation and cell death. Often this imbalance is achieved via activation of an oncogene or loss of a tumor suppressor gene.

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Declaration of Interest

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ONCOGENE ACTIVATION

Proto-oncogenes frequently code for proteins involved in cellular proliferation and differentiation. Three common mechanisms of oncogene activation are: (A) chromosomal abnormalities, (B) viruses, and (C) mutations in the DNA sequence of these genes.

Chromosomal Abnormalities—Amplification of proto-oncogenes can result in an increased amount of protein within a cell, thereby converting a proto-oncogene into an oncogene. Examples observed in human cancers that have been shown to result in consequent protein overexpression include amplification of HER2, MYC, MYL1, MYCN, AKT2, and REL15 [1].

MYC and Medulloblastoma (MB): Myc family oncogenes have been implicated in the development of several human tumors and are the most commonly detected amplifications in MB [2–4]. Perhaps, the most remarkable observation of the Myc proteins is their pleiotropic nature. Amplification and subsequent overexpression of Myc family proteins have been attributed to a host of cellular processes involved in tumorigenesis, including the ability to promote cellular proliferation, growth, and inhibition of cellular differentiation [5]. More recently, the role of Myc proteins has extended beyond tumor initiation to tumor progression and an invasive phenotype, with aberrant expression resulting in angiogenesis [6], increased invasiveness [7], and genomic instability [5, 8]. In a large study of pediatric, MB patients (n=292), both MYCC and MYCN amplification were independently and significantly associated with a poor prognosis [2].

Isochromosome 17q and MB: The loss of chromosome arm 17p and subsequent gain of 17q, resulting in isochromosome 17q is the most common chromosomal abnormality in MB, occurring in approximately 40 percent of cases [9–11]. Presence of isochromosome 17q serves as a negative prognosis indicator [12]. The mechanism by which this abnormality plays a role in tumorigenesis has not been clearly elucidated. Chromosome arm17p contains several tumor supressor genes, and thus it is postulated that deletion of this gene region may be the culprit. One of these genes is the tumor suppressor p53 [9, 12–14].

Other copy number abnormalities known to be observed in MB include losses on 6q, 8p, 9q, 10q, 11q, 20, X, and Y and gains on 1q, 2p, 4q, 6q, 9p, 13q, and 14q [15].

Viral oncogenesis—Viruses are yet another source of oncogene activation. While viruses in of themselves are relatively inert, viruses are capable of hijacking the host cellular machinery and inducing damage in the host organism. One of the earliest links between viruses and cancer was Peyton Rous' discovery that a virus could induce tumor in chickens [16–18]. Howard Temin and Harry Rubin would later expound upon Rous' discovery demonstrating that cultured cells upon infection with the Rous Sarcoma Virus were transformed into tumor cells [19, 20]. This transformation would be discovered to be the result of a viral version of the src protein, *v-src*, acting as an oncogene [21]. For those viruses that do not contain oncogenes, oncogenic activation may also occur via insertional mutagenesis of the viral genome adjacent to a proto-oncogene in which the proto-oncogene is placed under the control of the transcription promoter.

John Cunningham (JC) Virus and MB: JC virus is a neurotropic polyomavirus and the causative agent of progressive multi-focal leukoencephalopathy (PML) [22]. JC virus is thought to promote tumorigenesis via the inactivation of tumor suppressors (pRb, p107 and p53) and deregulation of signaling pathways, such as the Wnt signaling pathway by the viral early proteins, particularly T antigen [23–29]. Within the past 35 years a body of literature has developed surrounding the JC virus and MB [30, 31]. Inoculation of hamsters with JC virus, greatly increased their propensity to develop cerebellar tumors [32, 33]. In a transgenic model, using JC virus T antigen, mice developed cerebellar tumors mimicking MB [34]. Despite compelling data from animal model research, human detection of JC virus in MB is controversial [35, 36]. Studies have reported detection of JC virus in MB patients [37, 38]. However, a few groups have challenged this claim indicating that JC virus detection was minimal to absent in large cohorts of MB patients [35, 36].

Human cytomegalovirus (HCMV) and MB: HCMV is a member of the herpes virus family. It is estimated that approximately 50–80% of the population has been exposed to the virus [39]. In most individuals, this virus remains latent, with no clinical signs of infection. Animal model research has implicated HCMV in tumorigenesis, capable of promoting cellular differentiation, proliferation, migration, and angiogenesis while inducing inflammation yet employing immune evasion strategies [40, 41]. Studies have shown that transgenic mice expressing the HCMV protein, US28 in epithelial cells, resulted in hyperplastic intestinal epithelium and tumor development [42]. The HCMV protein, IE72 has been demonstrated to induce telomerase activity, an event associated with tumor transformation. HCMV DNA and protein have been detected in several tumors, including glioblastoma, prostate, breast, and colon cancer [43]. Recently, it was discovered that both primary MB and MB cell lines contain detectable amounts of HCMV DNA and protein [44].

Mutations in Proto-oncogenes—Mutations in the DNA sequence of a proto-oncogene resulting in a gain of function mutation, such as increased enzymatic activity, could result in deregulated proliferation and/or differentiation, thus increasing the propensity for cancer to develop. A classic example observed in human cancers is the activating BRAF mutation in which valine is substituted for glutamate at the 599th position. Under normal conditions, BRAF kinase is controlled via phosphorylation at neighboring threonine or serine residues (Thr598 and Ser601) [45]. The activated form of BRAF kinase is able to override this checkpoint and renders the enzyme constitutively active [45]. This results in the phosphorylation of downstream signaling events and ultimately abnormal growth [45]. Based on the literature, mutations in proto-oncogenes resulting in activating mutations, do not appear to be highly implicated in the pathogenesis of MB. However, in sporadic cases of MB, oncogenic mutations in the wingless (Wnt) family proteins, axin and beta-catenin have been reported [46].

TUMOR SUPPRESSOR LOSS

To ensure that these processes are regulated the body is equipped with genes whose functions counteract proto-oncogene function. These genes are referred to as tumor suppressor genes. The proteins encoded by tumor suppressor genes serve to repress the cell

cycle, promote apoptosis, or aid in DNA repair. Many tumor suppressors carry out these functions by acting as transcription factors.

Unlike oncogenes, tumor suppressors are often recessive, loss-of-function mutations, following Knudson's "two-hit hypothesis" of tumorigenesis, in which two mutant alleles are required to display a cancerous phenotype. A classic example of this phenomenon is observed in hereditary retinoblastoma, in which the child inherits one mutated copy of the pRb gene [47]. A second mutation or inactivation of the second functional copy, an event known as loss of heterozygosity most always results in the development of retinoblastoma in the child.

TP53 and MB—Not all tumor suppressors adhere to the "two hit hypothesis"; mutations in the p53 gene can result in a dominant negative/antimorphmutant have been observed in several human cancers. Tumor protein 53 (TP53) is a tumor suppressor gene, involved in cell cycle regulation. TP53 is perhaps one of the most notorious tumor suppressor genes. According to a report published in 2002 by the International Agency for Research on Cancer (IACR), TP53 is mutated in 30–50% of common human cancers. A majority of these mutations are missense, resulting in an amino acid substitution versus nonsense mutations. Li-Fraumeni syndrome, in which patients have a predisposition to develop a variety of tumors, including MB, more than half have mutations in TP53 [48–52].

PTCH1 and MB—Haplo-insufficiency is another mechanism by which tumor suppressors may be unable to maintain homeostasis, resulting in cancer formation. An example of this is the PTCH1mutation in MB [53]. PTCH1 is a member of the patched gene family. Its product, Patched (Ptch1) is a transmembrane receptor involved in the hedgehog signaling pathway. Members of the sonic hedgehog signaling pathway (SHH) function to regulate growth-inducing signal transduction for normal cerebellar development. In the absence of ligand, Ptch1 serves as an inhibitor of downstream signaling in the hedgehog pathway [54]. The association between MB and PTCH1 was uncovered through studying the biology of familial nevoid basal cell carcinoma, also known as Gorlin's syndrome. Patients with Gorlin's syndrome possess germ line inactivating mutations in the PTCH1 gene and are predisposed to developing a variety of tumors, including MB. Roughly 20% of sporadic, nonfamilial cases of MB have also been shown to contain PTCH1 or other SHH pathway activating mutations. In further studying individuals with these mutations, it was discovered that these patients also possess wildtype copies of the PTCH1 gene. In mice, knocking out PTCH1 results in an embryonic lethal phenotype. PTCH1 heterozygotes survive, yet are predisposed to developing cerebellar tumors, resembling MB, in further support of haploinsufficiency [51, 55, 56].

APC and MB—The Wingless (Wnt) signaling pathway consists of a network of proteins involved in embryogenesis via gene regulation and cell–cell control. Among the target genes of this pathway include the proto-oncogenes MYCC and MYCN. The tumor suppressor gene product, Adenomatous polyposis coli (Apc) is a member of the Wnt signaling pathway, whose function is to regulate activity of beta-catenin. Interestingly, patients with Turcot's syndrome, a syndrome characterized by intestinal polyp formation and brain tumors, such as MB, possess a germ line mutation in the APC gene [57].

EPIGENETIC MODIFICATIONS—In recent years, there has been great emphasis placed on studying heritable information other than the underlying DNA sequence capable of altering gene expression or cellular phenotype, the epigenome. Evidence of epigenetic influence on tumorigenesis has been observed in several cancers. One mechanism that is gaining increasing credibility is promoter hypermethylation in tumor suppressor gene silencing. The tumor suppressor genes RASSF1A, HIC1, and CASP8 have been shown to exhibit increased methylation when compared to normal cerebellum [58].This methylation was associated with transcriptional silencing and expression was induced upon treatment with DNA methyl transferase inhibitors [58].

MAINTENANCE OF THE CANCEROUS PHENOTYPE

The aforementioned processes serve to describe key events in the initiation of tumor formation. In order to maintain a cancerous phenotype often other mechanisms must be employed. With continuous growth comes an increased nutritional demand. A key mechanism through which tumor development is achieved is via cell surface receptor signaling.

RECEPTOR TYROSINE KINASES AND MB

Among the most notable receptors in this class are receptor tyrosine kinases. Receptor tyrosine kinases serve as cell surface receptors for growth factors, cytokines, and hormones. Upon ligand binding receptor tyrosine kinases cluster, activating the protein's cytoplasmic domains; autophosphorylation of tyrosine residues within the receptor form binding sites for Src homology 2 (SH2) and phosphotyrosine binding (PTB) domains. Phosphorylation of these proteins then results in the activation of downstream signaling pathways involved in diverse cellular responses, such as cell division, differentiation and motility. Members of this family, specifically neurotrophin-3 receptor (TrkC), human epidermal growth factor receptor 2 (HER2/erbb-2), platelet derived growth factor receptor (PDGFR), insulin-like growth factor receptor (IGFR), and nerve growth factor receptor (NGFR) have been associated with MB pathogenesis [59].

HER2—Of the erbb family members, HER2, is most highly expressed by MB and is associated with a poor prognosis. In a study of 81MB, 40% of tumors tested positive forHER2 [60]. Of these tumors, the large cell anaplastic variant, characterized by a more aggressive clinical course, expressed HER2 with the highest frequency [60]. In this study, HER2 overexpression was found to associate with a poor prognosis independent of histological variant [60].

In contrast to breast cancer where overexpression of the protein HER2 is associated with HER2 gene amplification; this same mechanism of overexpression has not been demonstrated in MB. In a study of 70 primary MB out of those who tested positive for HER2, expression amplification of the gene HER2 was not detected [61]. In addition to this finding, work from our group has demonstrated that when compared to HER2 expression in breast cancer, MB express HER2 at low levels [62]. Furthermore, HER2 is not expressed in the normal cerebellum [61]. Taken together, these observations implicate HER2 as a key

player in MB pathology. However, further investigation needs to be conducted in an attempt to better understand the mechanism behind HER2 overexpression in MB.

Notch Signaling—Other signaling pathways that have associated with MB pathogenesis include the Notch and CXCR4 signaling pathways. Both of these pathways, like many others listed in this review, play an integral role in embryogenesis. The receptor Notch2 has been detected in proliferating progenitor cells in MB and is associated with a poor prognosis [59, 63].

CANCER STEM CELLS

An active area of research within the past decade has been to identify the cancer stem cell populations of various tumors. Cancer “stem cells” (cscs) or cancer initiating cells describe a population of cells capable of not only self-renewal but also producing progenitor cells capable of differentiating into more terminally differentiated cell types, such as endothelial cells. In order to identify such a population, several xenograft studies have been conducted in mice in which human tumors are serially transplanted into mice and the phenotype of the cells that are able to establish a tumor are assayed. Brain cells expressing the glycoprotein CD133 are thought to be enriched for cscs. Work from our lab has demonstrated that the CD133 positive population of MB cell lines also expresses higher levels of the receptor tyrosine kinase, HER2, which is associated with a poor prognosis in MB [62]. This finding could provide a link between stemness and the HER2 pathway in MB. Although there is still a considerable amount of work to be done in the field of cscs evidence of such a population may contribute to our understanding of epithelial-mesenchymal transition and tumor invasion and metastasis.

MICROENVIRONMENT—In 1889, English surgeon, Stephen Paget proposed that the ability of tumor cells, “the seed,” to metastasize to distant organs was dependent upon a compatible environment in those organs, “the soil.” [64, 65] This theory was supported by work conducted by Paget in which he analyzed the autopsy records of over 700 women with breast cancer [64]. From his analyses he was able to conclude that metastasis to visceral organs and bone was not a random occurrence [64]. Since then there has been a wealth of literature published in support of Paget’s initial observations indicating that the ability of cancer cells to flourish is dependent on the presence of a niche that favors its survival and progression. This niche most often referred to as the “tumor microenvironment” exists as a complex of several biological systems, consisting of stromal tissue (nerves, immune cells, fibroblasts, smooth muscle cells, and blood vessels), extracellular matrix and the cancerous cells [66–68].

Most cancers are not merely a collective body of homogenous cells running the same aberrant program. Instead most often cancers are heterogeneous in nature that includes in addition to the cancer cell a myriad of different cell types that collectively constitute the tumor microenvironment. Greater than 80% of malignancies are classified as carcinomas, having derived from epithelial origin. Consequently much of what we know about the tumor microenvironment is based on research on carcinomas. In order to conduct such research, it

is crucial to understand the structure and function of normal epithelium in order to better understand the biology of cancerous epithelium.

THE TUMOR STROMA

The stroma or surrounding connective tissue of an organ serves as a support system for that organ; acting as a reservoir of cells, capable of differentiating upon insult or injury to that organ. In the case of cancer, homeostasis is lost and the tissue resembles a wound incapable of repairing, with fibrosis and chronic inflammation. A once highly structured tissue now displays a loss of membrane integrity, polarity, and contact inhibition. These changes result in an increased secretion and mixing of soluble factors and the line between the cancerous cells of the epithelium and the underlying stroma is blurred.

Cancer Associated Fibroblasts (CAF)—Fibroblasts are the most abundant cell type of the stromal compartment. Perhaps this is due to their critical role in maintaining the structural integrity of the connective tissues. Fibroblasts synthesize the components of the extracellular matrix, the formation of which is important in growth and wound healing [69]. Fibroblasts, similar to other cell types found in the stromal compartment are of mesenchymal origin. The expression of the intermediate filament vimentin serves as a marker for cells of mesenchymal origin. In cancer, fibroblasts often develop into myofibroblasts coexpressing vimentin and alpha smooth muscle actin. Cells that follow this pattern are commonly referred to as cancer-associated fibroblasts (cafs). Cafs or myofibroblasts, although helpful in wound repair under conditions of chronic inflammation, have proven deleterious resulting in aberrant fibrosis. The aptly named desmoplastic variant of MB is characterized by densely packed cells, with a collagen-rich stroma. The fibrotic nature of this variant implicates aberrant fibrosis as a causative agent in a subset of MB.

Transforming growth factor beta (TGF-β) secreted by platelets upon damage to the tissues, is a key mediator of tissue homeostasis. In cancer, TGF-beta is released not only by platelets, but is overexpressed also by both the cancer cells and surrounding stromal cells. This secretion in turn serves as a proliferative signal for both the cancer cells and its stromal compartment. TGF-β has also been shown to induce angiogenesis and dampen the immune response supporting amore invasive phenotype [70].

The chemokine interleukin-8 (IL-8) also referred to as CXCL8 functions similarly to TGF-beta to promote tumor progression. It is secreted by several cell types in the tumor microenvironment and promotes angiogenesis. Acting as a chemoattractant IL-8 is capable of recruiting cells expressing the receptors CXCR1 and CXCR2. Cells that express one or more of these receptors include neutrophils recruited to sites of inflammation.

Both TGF-beta and IL-8 have been shown to be overexpressed in primary MB and MB cell lines [59, 71].

THE TUMOR VASCULAR ENDOTHELIUM: Normal epithelium is avascular; dependent upon the vascular network of the underlying stroma for its nutrients. With an increasing demand for oxygen and nutrients by these rapidly dividing cells, angiogenesis is often a rate-limiting factor in sustaining tumor growth and promoting tumor progression.

This process is carried out in large part by endothelial cells, which make up the inner linings of arteries, veins and capillaries. This cell type can be induced to generate new vessels from pre-existing vessels in a process known as angiogenesis.

The bulk of angiogenesis occurs during embryogenesis. In adults angiogenesis is likely to occur as a repair process, as observed in wound healing. In order for angiogenesis to occur pericytes need to be removed from the branching vessel [72], after which the basement membrane of the vessel must be degraded and restructured via matrix metalloproteinases (mmps) [72, 73]. Once the matrix has been remodeled, growth signals then act to recruit endothelial cells and induce endothelial cell proliferation [72]. These cells then form tube-like structures and depending on the size of the vessel recruit either pericytes or smooth muscle cells for added support [72].

Tumors, similar to normal tissues, require an adequate supply of oxygen and nutrients as well as a mechanism to export metabolic waste. This is often provided by expanding the network of blood vessels generated during angiogenesis. Despite many similarities between normal and tumor-associated vasculature, tumor-associated vasculature is often abnormal. In contrast to normal vasculature, tumor-associated vasculature frequently lacks pericytes and is dilated, containing large fenestrations and having a tortuous morphology [74]. In some instances, the vasculaturemay be an admixture of both endothelial cells and tumor cells [72].

Vascular endothelial growth factor (VEGF) has been shown to increase endothelial cell permeability to metabolites, while also stimulating plasminogen activators and interstitial collagenase [72, 75]. Collectively, VEGF acts as a regulator of proteolytic activity required for matrix remodeling and subsequent endothelial cell tube formation [72]. Production of VEGF is mediated by local oxygen concentration. Hypoxia induces VEGF expression via binding of the hypoxia-inducible factor 1a (HIF-1alpha) to the VEGF promoter [72,76]. The tumor-associated vasculature with its leaky nature and increasing demand for nutrients as it grows and expands induces a hypoxic environment. This in turn signals an increase in VEGF production thereby promoting angiogenesis.

Functional VEGF and VEGF receptors have been detected in primary MB and MB cell lines [77]. However, whether VEGF expression is correlated with a different prognosis in MB is still under investigation. In one study seven of 32 tumors expressed VEGF; there was no detectable difference in the 5-year survival rate on the basis of VEGF expression [78].

IMMUNE CELLS IN THE TUMOR MICROENVIRONMENT: The mammalian immune system is a complex network of specialized cell types and factors that serve to protect against disease. To do so the immune system must be able to identify self from non-self. Through this mechanism the immune system is able to target foreign invaders, while maintaining tolerance to self-antigen. The function of the immune system is not limited to fighting infection. Like other components found in the stromal compartment it also plays an essential role in maintaining tissue homeostasis.

In the case of cancer, aberrant signaling may result in chronic inflammation thereby inducing immunosuppression and promoting tumor growth. In cancer an increased innate

immune response at the site of the tumor microenvironment is associated with angiogenesis and in many cases a poor prognosis [79].

Tumor Infiltrating Lymphocytes (TILs)—TILs have been reported in a variety of cancers [80–82]. However, whether these cells function primarily in an antitumor or protumor capacity remains controversial [80,82,83]. In this review, we focus on tumor-infiltrating T-lymphocytes. However, it would be remiss if we failed to mention, the "other" TILs [82], B cells (for more on B-TILs please refer to Nelson BH. *J Immunol* 2010).

Emerging data suggest that T cells and their impact on tumor growth may depend heavily upon the particular TIL subset. In the literature, it is the growing consensus that the majority of CD4 + T cells, with the exception of the Th1 subset favor tumor progression, while CD8 + T cells (cytotoxic T cells) favor tumor rejection [84]. However, there are several reports that counter this claim [85–87].

In 2003, Wakabayashi et al. reported that CD4+T cells in cancer stroma, not CD8+ T cells in cancer nests were associated with a favorable prognosis in human nonsmall cell lung cancers [85]. Also in 2003, Cho et al. reported that in patients with esophageal squamous carcinoma both the CD4+and the CD8+T cells work together to improve prognosis [86]. There have also been reports indicating that a high density of Tumor-Infiltrating FOXP3 T Regulatory cells were associated with improved survival in patients with colorectal cancer [87].

TILs in MB—Work from our lab has shown that T lymphocytes consistently migrate to and infiltrate primary MB [71]. Our experimental model revealed that MB cell lines interact with the tumor endothelium to recruit T cells to the MB microenvironment [71]. In particular, Macrophage Migration Inhibitory Factor (MIF) is the key chemokine molecule secreted by MB cells which induces the endothelial cells within the MB microenvironment to secrete the potent T lymphocyte attractant "Regulated upon Activation, Normal T-cell Expressed, and Secreted (RANTES)."This in turn creates a chemotactic gradient for RANTES-receptor bearing T lymphocytes [71]. Regarding the role of T lymphocytes in the MB microenvironment, in a clinical study comparing patients with MB to healthy controls, MB patients had a higher proportion of Th17 cells at the site of the tumor and in their peripheral blood [88]. In a recent report by Weigering et al., high expression of IFN-γ and tnfα in T cells in the peripheral blood of MB patients in the early post-transplant period correlated with a better prognosis [89].

CONCLUSION

Overall a substantial body of work focused on the biology of MB has been conducted. Results of this work have implicated disrupted pathways involved in embryogenesis and cellular proliferation and differentiation as key mediators of MB pathogenesis. Chromosomal abnormalities in the form of amplifications and deletions have been identified as a common culprit. Often these modifications result in the upregulation of growth factor signaling and inactivation of tumor suppressor genes to contribute to tumor formation. We also cannot forget the increasing evidence of the association of viral infection, particularly

JC virus and HCMV, and MB. All together, with an emphasis in understanding not only the biology of cancer cells, but the surrounding tumor microenvironment it is likely that our understanding of MB pathogenesis will increase. The next logical step is to apply our increasing knowledge regarding MB pathogenesis towards the development of new therapeutic options. Our lab has previously validated HER2/erbb-2 as a MB (and glioblastoma) restricted target antigen, demonstrated in preclinical models the efficacy of targeting this antigen [62, 90] the product of which is currently being used in a clinical trial for patients with Glioblastoma (ClinicalTrials. gov identifier: NCT01109095). Perhaps small molecule inhibitors in conjunction with immunotherapy targeting viral and tumor co-associated antigens may prove advantageous in accessing the brain and targeting tumors like MB.

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