# Epithelial-mesenchymal transition in glioblastoma progression (Review)

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Received March 6, 2015; Accepted December 10, 2015

DOI: 10.3892/ol.2016.4113

**Abstract.** Epithelial-mesenchymal transition (EMT) is a reversible biological process that occurs in epithelial cells. EMT ultimately leads to the acquisition of a mesenchymal phenotype, characterized by increased cell motility and resistance to genotoxic agents. These processes mostly overlap with the acquirement of stem cell properties in differentiated tumor cells. With regard to gliomas, the clinical picture is heterogeneous, even within the same grades and histological categories of the disease. Furthermore, the areas of invasion and responses to radiochemotherapy are markedly different among cases, and occasionally even in the same patient. Such phenotypic diversity in glioma tissues may be caused by various microenvironmental factors, as well as intrinsic genetic alterations. The current review focuses on the EMT-inducing factors that are present in gliomas; these typically vary from those observed in epithelial cancers, as no basement membrane is present. Furthermore, the most important cell-cell contact factor, E-cadherin, is rarely expressed in gliomas. The microenvironment that induces EMT in gliomas is characterized by hypoxia and the enrichment of myeloid cells following stimulation by transforming growth factor-β. Anti-vascular endothelial growth factor therapy, including the use of bevacizumab, may be a suitable candidate to modulate the glioma microenvironment.

**Contents**



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*Key words:* glioma, microRNA, epithelial-mesenchymal transition, stem cell

## 1. Introduction

Epithelial-mesenchymal transition (EMT) is a biological process in which polarized epithelial cells are induced to undergo numerous biochemical changes; this results in a mesenchymal phenotype, defined by an enhanced migratory capacity and elevated resistance to genotoxic agents (1,2). EMT is indispensable for wound healing, embryonic development and tissue remodeling. As a pathological process, EMT also induces migratory and invasive capabilities in epithelial tumor cells without a loss in viability (1,2). The process of EMT includes the detachment of tumor cells from the basement membrane. Although the central nervous system (CNS) lacks this critical tissue component, key invasive mechanisms overlap between cancers of the CNS and other cancer types (3). The factors that induce EMT in other cancers may also activate mesenchymal features in gliomas (Fig. 1). Furthermore, EMT is an important inducer of the cancer stem cell phenotype (4). The mesenchymal subtype of glioblastoma (GBM) typically expresses neural stem cell markers and is associated with an aggressive phenotype (5-7). Glioma cells that express stem cell markers are highly invasive and resistant to chemotherapy and radiotherapy *in vitro* (8-10) and in the clinical setting (11).

Gliomas are classified according to their histopathological features; these features allow clinicians to distinguish between two cellular lineages (astrocytic and oligodendrocytic) and four grades of malignancy (grades I to IV) (12). The most malignant form of grade IV is GBM, which originates from progenitor or stem cells in the astrocytic lineage. Recent genotyping and expression profiling analyses have demonstrated that GBMs may be categorized into four subclasses dependent on their neural differentiation (5,6). The proneural subtype is associated with a positive prognosis, whilst the mesenchymal subtype is characterized by higher percentages of cycling cells and neoangiogenesis, with a highly invasive nature and poor prognosis (5,6). Furthermore, non-mesenchymal subtypes of tumors typically acquire mesenchymal features at recurrence (6). A shift towards the mesenchymal subtype appears to be a common pattern in disease progression, similar to cancer

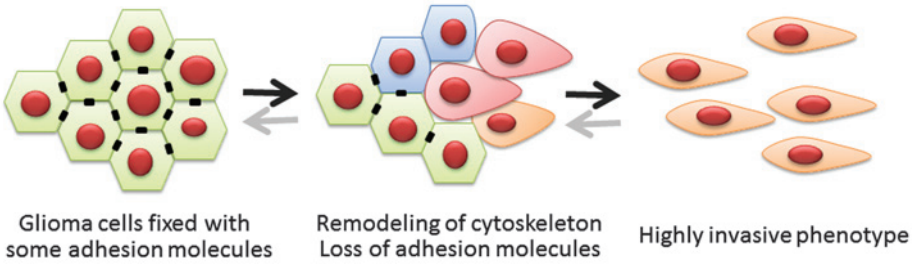


Figure 1. Scheme for the epithelial-mesenchymal transition in glioma cells. Glioma cells lose adhesion molecules and alter their cytoskeleton through a reprogramming process. It is essential that this phenotypic change is reversible, and the reverse process, termed mesenchymal-epithelial transition, is necessary for the formation of distant or disseminated tumor nodules.

cells undergoing EMT in order to acquire a more aggressive nature (13).

Paradoxically, migrating tumor cells are required to lose the mesenchymal phenotype to establish a secondary tumor at distant sites (1,2). This suggests that EMT is a reversible process, and is most likely to be mediated by epigenetic alterations that are induced by microenvironmental stimuli, rather than as a result of genetic alterations (14,15). Differentiated tumor cells change their phenotype through a dynamic reprogramming process that is affected by a repair-associated process or pathological stresses, such as hypoxic insults (16-20). Acquisition of the stem cell phenotype may be closely associated with epigenetic alterations that are induced by EMT. Although EMT may be a common pattern in glioma progression, numerous therapeutic interventions affect the occurrence and magnitude of EMT during the clinical course of GBM (21-23). The present review discusses the participation of EMT in GBM progression, and the resulting acquisition of the stem-cell phenotype.

## 2. Classification of EMT

EMT is classified into three different subtypes (1,2). EMT type 1 is an essential mechanism required for the transitioning of primitive epithelial cells in embryos into motile mesenchymal cells, which are required for the gastrulation and migration of neural crest cells (24). Certain cells generated by EMT become secondary epithelial cells in mesodermal and endodermal organs through a reverse event known as mesenchymal-epithelial transition (MET). Thus, during embryogenesis, type 1 EMT serves a critical role in generating morphologically and functionally distinct cell types, including mesenchymal and secondary epithelial cells, through the process of MET (1,2).

EMT type 2 occurs in adults and is associated with tissue regeneration, wound healing and organ fibrosis, in which fibroblasts are formed in injured tissues. Organ fibrosis is mediated by fibroblasts and inflammatory cells that secrete a number of inflammatory signals alongside the components of a complex extracellular matrix, consisting of elastin, collagens, tenacin and laminins. The transition of epithelial cells into fibroblasts occurring over a few days in culture is one line of evidence for this type of EMT, and an active diversion to MET occurs in the presence of bone morphogenic protein-7 (25). Cancer-associated fibroblasts in primary epithelial tumor nodules have recently been demonstrated to share certain genetic mutations with tumor cells, suggesting that type 2 EMT emerges prior to the full onset of tumorigenesis (26).

EMT type 3 is observed in subsets of cancer cells undergoing a phenotypic conversion to increase migration, invasion and metastasis. Certain studies have noted that transforming growth factor (TGF)-β can induce EMT in epithelial cancer cells through Smad or p38 mitogen-activated protein kinase/Ras homolog family member A pathways (27-29). Activation of EMT programs, through tumor microenvironment stimuli, has been proposed as the critical mechanism for the acquisition of highly malignant phenotypes of cancer cells (30). In type 3 EMT, certain cancer cells with a transitioning mesenchymal phenotype undergo MET to form metastatic tumor nodules at distant sites (1,2).

## 3. EMT-inducing microenvironment

The genetic and epigenetic alterations that cancer cells undergo render them sensitive to EMT-inducing signals. Highly motile mesenchyme-like cancer cells are typically observed at the invasive front, suggesting that dedifferentiating signals usually originate from the tumor microenvironment (30). As aforementioned, the reversibility of EMT suggests that epigenetic alterations, as a result of environmental signals, generate highly aggressive tumor phenotypes (31-34).

A hypoxic microenvironment is generally regarded as a potent inducer of EMT in various types of epithelial cancer (30,35). In gliomas, inflammatory processes, or a hypoxic microenvironment within the tumor or neighboring normal tissues, may result in the recruitment of circulating or residential myeloid cells (including macrophages or microglia) into the tumor stroma (34). These cells release a number of growth factors, including TGF-β, epidermal growth factor, platelet-derived growth factor and fibroblast growth factor-2, which trigger alterations in the levels of transcription factors required for the initiation of EMT, and also in numerous proteases that increase invasiveness into the surrounding normal brain (17,21,34,36). Thus, glioma cells that are affected by the bystander myeloid cells and such signaling molecules may undergo EMT in a hypoxic microenvironment.

## 4. EMT-inducing signals in gliomas

*Twist*. Twist is a protein with a basic helix-loop-helix structure and is transcriptionally active during cell differentiation and lineage determination (37,38). During the establishment of

cancer metastases by EMT, Twist acts independently of Snail to suppress E-cadherin and to upregulate N-cadherin and fibronectin (38). Using a brain slice culture and an orthotopic model of xenotransplantation, it has been reported that Twist is upregulated in malignant gliomas, and promotes glioma cell invasion through the mesenchymal target gene Slug and the fibroblast activation protein, independent of the cadherin switch (39,40). It has also been demonstrated that the inhibition of Twist expression results in a significant reduction in GBM stem cell sphere growth and formation. Nagaishi *et al* (41) observed that the expression of Twist is characteristic of mesenchymal areas of gliosarcomas, indicating that EMT is involved in the formation of biphasic tumor gliosarcoma.

*Snail*. Snail is a member of the SNAIL family of transcriptional activators and is a primary suppressor of E-cadherin expression (1,2,42). Snail regulates a range of other EMT phenotypes, including the decreased expression of various epithelial markers (occludins, claudins and cytokeratin) and the increased expression of mesenchymal markers (vitronectin and fibronectin) (43). The transcriptional activity of Snail is predominantly regulated by its subcellular localization. Phosphorylation of Snail results in its exportation from the nucleus to the cytoplasm, leading to inactivation of the protein as a transcription factor (42). TGF-β is secreted from mesenchymal cells following irradiation and induces the nuclear localization of Snail via Smad2/3 pathways (22).

*Slug*. Slug is another member of the SNAIL family of transcriptional activators and serves an important role in suppressing the epithelial phenotype in numerous cancer cells (1,2,44). Slug is closely associated with the increased migration and invasion of malignant gliomas (45). A multi-cancer mesenchymal transition signature of mRNA expression levels from The Cancer Genome Atlas (TCGA) data has been highlighted by strong expression of Slug and cluster of differentiation (CD)44 (5,6).

*ZEB*. The zinc finger E-box-binding homeobox (ZEB) proteins, ZEB1 and smad1-interacting protein-1 (also known as ZEB2), are another family of noteworthy transcription factors that are responsible for the mediation of EMT in numerous types of cancer and glioma (1,2,46). ZEB proteins bind to the promoter region of E-cadherin and suppress its expression, resulting in the loss of cell-cell contact and increased motility (47,48). Wang *et al* (46) observed that patients with GBM containing high levels of ZEB2 demonstrated significantly earlier recurrence with malignant transformation compared to those with low levels of ZEB2. Connective tissue growth factor also renders glioma cells highly invasive through the activation of nuclear factor-κB, which subsequently initiates ZEB1 expression (49).

*Wingless-related integration site (WNT)/β-catenin*. In multiple types of cancer, β-catenin is sequestered in the cytoplasm by E-cadherin, with the translocation of β-catenin into the nucleus following the downregulation of E-cadherin being directly correlated with acquisition of the mesenchymal phenotype (1,50,51). Although the majority of GBMs do not express E-cadherin, nuclear localization of β-catenin is primarily observed at the invasive front of the tumor (52). Furthermore, GBMs that express high levels of WNT/β-catenin are correlated with significantly shorter patient survival times (53). The WNT/β-catenin pathway is an important stem cell maintenance pathway and is involved in therapy resistance (54). GBM cells, in which the WNT/β-catenin pathway is activated, trigger the expression of a set of EMT activators, including Twist1, ZEB1, Snail and Slug (55). Furthermore, high expression levels of the WNT/β-catenin receptor, Frizzled-4, promotes the expression of Snail and the acquirement of a mesenchymal phenotype in GBM (56).

*NOTCH*. NOTCH is a cell surface receptor that serves an important function in the development of numerous types of cells and tissues (1). NOTCH signaling is a primary inducer of EMT in a number of epithelial cancers, including cancer of the lung, breast and pancreas (57). Fan *et al* (58) reported that inhibition of this signaling pathway by γ-secretase inhibitors reduces CD133-positive stem-like cells in GBMs. In addition to WNT/β-catenin, NOTCH is a major regulator of glioma stem cells within their microenvironments. NOTCH is also directly correlated with phosphoinositide-3 kinase/Akt pathway activation (59-61).

*CD44*. CD44 is a hyaluronic acid receptor that interacts with ligands such as collagens, osteopontin and matrix metalloproteases (62,63). In addition to the standard isoform of CD44 (CD44s), alternative splicing results in 11 other isoforms of CD44 variants (CD44v2-v12) (64). CD44s is a primary inducer of EMT in breast and colorectal cancer. TCGA data indicates that GBMs with high levels of mRNA expression of EMT-inducing signature molecules, including Slug and CD44, are correlated with increased resistance to therapies and tumor invasion (65). However, functional data for CD44-mediated EMT in GBM have not been fully elucidated (66).

## 5. MicroRNAs (miRs) that regulate EMT in gliomas

miRs are small, 20-23-nucleotide non-coding RNAs that serve as epigenetic regulators of gene expression through the downregulation of target genes; this occurs through the binding of miRs to regions of partial complementarity in the target gene 3'-untranslated regions (67). Each miR has hundreds of target genes, and numerous genes are targeted by multiple miRs, creating a highly complex gene expression regulatory network (68). Control of gene expression by miRs is one of the most important modulating processes in cellular differentiation during normal embryogenesis (69,70). A number of studies have demonstrated that miRs may function as negative regulators of gene expression in normal tissues and as tumor suppressors or oncogenes in various tumors (67,70,71). In several types of cancer, epigenetic regulation (involving miRs) is a core mechanism of EMT modulation, and thus, reversible modulation of the genes that mediate EMT is possible (72). The majority of miRs are negatively correlated with tumorigenesis, tumor invasion and mesenchymal changes in gliomas. Notably, the expression of miR-21, -34a, -128a, -124 and -184 is correlated with the downregulation of mesenchymal markers and decreased invasiveness. By contrast, a relatively small number of miRs are oncogenic and may function as therapeutic targets. The

inhibition of a Dicer enzyme for a specific oncogenic miR was recently indicated to block maturation of the miR and suppress tumor invasion (73). Furthermore, evidence is growing concerning the effect of miRs on the progression and maintenance of glioma stem cells (67,71).

## 6. Radiation-induced EMT

Radiation therapy is a major modality of cancer therapy and also serves a key role in the multimodal treatment of GBM. However, irradiation that is sublethal to malignant glioma cells consequently promotes cell migration and invasion through the expression of TGF-β, epidermal growth factor, vascular endothelial growth factor (VEGF) and the hepatocyte growth factor pathway (74-76). Glioma cells that are resistant to irradiation have a gene expression signature that is enriched in the EMT pathway, leading to highly invasive recurrence patterns (22,23,77,78). TCGA data indicates a shift from a proneural to mesenchymal phenotype at the time of tumor recurrence. Recently, Mahabir *et al* (22) observed that two different pathways are involved in the radiation-associated EMT induction in malignant gliomas; TGF-β, derived from the mesenchymal cells in the tumor environment, evokes the activation of Smad2/3, whilst reactive oxygen species activate extracellular signal-regulated kinase1/2, with each pathway leading to the nuclear localization of Snail. Such data suggests that EMT serves a crucial role in the acquisition of radiation resistance. Furthermore, emerging evidence suggests that such a role for EMT in the generation of refractory cancer cells is associated with an accumulation of stem cell markers. The NOTCH pathway and WNT/β-catenin signaling are important for stem cell maintenance and are associated with the radiation resistance of GBM (78).

A further important aspect of the biological effects of radiation therapy on GBM is the induction of hypoxia or necrosis. Tissue hypoxia directly induces EMT and recruits myeloid cells into tumor tissues (15-20). Glioma cells, under a hypoxic microenvironment, and recruited myeloid cells each secrete TGF-β, leading to the induction of hypoxia-inducible factor-1α (HIF-1α), which subsequently promotes the malignant progression of glioma cells (21,79).

## 7. Bevacizumab-induced EMT

VEGF is one of the most important factors facilitating angiogenesis and resultant tumor growth in GBM. Inhibiting the VEGF-VEGF receptor (VEGFR) signal transduction pathway with anti-VEGF therapy (including the use of bevacizumab) and VEGFR inhibitors (including sunitinib) is a promising strategy in cancer therapy (80). Bevacizumab is effective in prolonging progression-free survival (PFS) in newly diagnosed GBM patients, but is not effective in prolonging overall survival (81,82). During the early phases of bevacizumab therapy, tumor oxygenation improves through the process of vascular normalization (83). However, with prolonged treatment with bevacizumab, in a similar manner to radiation therapy, the tumor develops progressive hypoxia that directly or indirectly promotes the mesenchymal phenotype (21,79,83). Furthermore, hypoxia induces the release of HIF-1α from glioma cells and subsequently attracts myeloid cells, including macrophages and granulocytes, from bone marrow into the glioma tissues (21,23). The recruited myeloid cells release TGF-β, which then directly induces EMT in the glioma cells. Myeloid cells also secrete multiple growth factors, including interleukin (IL)-6, IL-10 and matrix metalloproteinases (15-19). TGF-β, alongside VEGF, also recruits mesenchymal stem cells into the glioma tissues, which contributes to the further malignant progression of GBMs (83). By contrast, VEGFR inhibitors lack the efficacy in PFS prolongation, due to the induction of hypoxia in the early phase, without the vascular normalization phase, or due to dose-limiting adverse events. Similarly, anti-VEGF therapy is more effective than VEGFR inhibitors in decreasing myeloid cell infiltration, which may contribute to the efficacy of bevacizumab observed during early phases.

## 8. Conclusion

Glioma cells undergoing EMT acquire the potential to initiate metastasis and invasion. This process is highly affected by the tumor microenvironment, particularly a hypoxic environment or one involving the release of proinflammatory molecules from recruited myeloid or mesenchymal stem cells. The evidence that type I and II EMT occur during the normal physiological processes of embryogenesis and wound healing in a relatively short time suggests that epigenetic mechanisms are more crucial than genetic changes. This notion is also supported by the evidence that migrating tumor cells that have undergone EMT may also undergo MET to establish metastatic tumor nodules. Therefore, tumor microenvironments are emerging as a therapeutic target, particularly when in a hypoxic state, which controls epigenetic alterations in tumor cells. The microenvironmental modifier, bevacizumab, has recently been developed; however, future clinical trials to maximize the efficacy of anti-VEGF therapy are required, with the aim that such treatment will normalize oxygen concentration and suppress the excessive recruitment of myeloid and mesenchymal stem cells.

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