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Review

Molecular Sequence of Events and Signaling Pathways in Cerebral Metastases

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Abstract. *Brain metastases are the leading cause of morbidity and mortality among cancer patients, and are reported to occur in about 40% of cancer patients with metastatic disease in the United States of America. Primary tumor cells appear to detach from the parent tumor site, migrate, survive and pass through the blood brain barrier in order to establish cerebral metastases. This complex process involves distinct molecular and genetic mechanisms that mediate metastasis from these primary organs to the brain. Furthermore, an interaction between the invading cells and cerebral milieu is shown to promote this process as well. Here, we review the mechanisms by which primary cancer cells metastasize to the brain via a mechanism called epithelial-to-mesenchymal transition, as well as the involvement of certain microRNA and genetic aberrations implicated in cerebral metastases from the lung, breast, skin, kidney and colon. While the mechanisms governing the development of brain metastases remain a major hindrance in treatment, understanding and identification of the aforementioned molecular pathways may allow for improved management and discovery of novel therapeutic targets.*

Brain metastases are a leading cause of morbidity and mortality among cancer patients, and are reported to occur in about 40% of cancer patients in the United States of America (USA), with an incidence approaching 170,000/year in the USA (1, 2). Primary organ tumors that have the greatest propensity to metastasize to the brain include lung (50-60%), breast (15-20%), skin (5-10%), kidney (7%) and colon cancers (4-6%) (3, 4). In general, the median survival following a diagnosis of cerebral metastases is between 2 and 25 months, depending on the origin of the primary tumor and time of diagnosis (5, 6). The diversity of these primary sites suggests the possibility of a common mechanism by which these tumors metastasize to the brain. Moreover, a complex interaction exists with the cerebral microenvironment that results in a propensity for these tumors to disseminate to the central nervous system (CNS).

For many cancer patients, the diagnosis of metastasis to the brain can be devastating. In some cases, only supportive care is recommended. However, several studies show that there is a survival benefit to combined treatment of surgical resection and radiation therapy. For example, a review of cerebral metastasis from gastroesophageal cancer showed a survival advantage in patients treated with resection of the metastatic lesion followed by radiation, which included whole-brain radiation therapy or stereotactic radiosurgery (7). They specifically reported a patient with a cerebellar metastasis diagnosed five months after treatment for his primary disease who had no recurrence five years after undergoing resection of the brain metastasis followed by stereotactic radiosurgery. Furthermore, Karagkiouzis *et al.*, reported that in patients with solitary extrapulmonary metastasis from NSCLC who underwent surgical resection of the primary tumor as well as the solitary metastasis had improved survival, especially if the metastasis was not present within less than six months from diagnosis (8). Despite current advances in treatment for metastatic lesions, including surgical resection, chemotherapy and radiation, there is limited benefit in the form of prolonged survival. As such, improvement in therapeutic options for metastatic brain lesions remains an unmet necessity.

Tumor cells bypass multiple checkpoints in order to establish metastasis to the brain. A complex mechanism

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termed epithelial-to-mesenchymal transition (EMT), which is governed by a sequence of multiple signaling pathways, appears to govern this process. In addition, the presence of cancer stem cells (CSC) within the tumor may contribute to this process and aid in evading current chemotherapeutics. Moreover, a distinctive genetic signature is associated with a propensity for cerebral metastases unique to discrete primary sites. In this review, we discuss the molecular process and genetic markers associated with the development of cerebral metastases, with particular focus on the five primary tumor sites, namely lung, breast, skin, colon and kidney, having the most propensity for metastasizing to the brain.

Epithelial-to-mesenchymal Transition in Cerebral Metastasis

In general, commencement of metastasis begins with detachment of primary cancer cells from the tumor mass. This is followed by invasion through the basement membrane and intravasation into the systemic hematologic and lymphatic circulation. The circulating tumor cells then extravasate through gaps in endothelial cells at a distant site, where they form a secondary lesion. This complex sequence of metastasis requires a sophisticated process, referred to as EMT. The process of EMT is well recognized as it plays an important role in embryogenesis and later in severe wound healing. EMT is characterized by the loss of cellular apical-basal polarity, resulting in the formation of a cell with mesenchymal properties, allowing for dissociation of cell-cell interactions and migratory potential. Upon reaching the secondary site, the cells appear to undergo a reversal process termed mesenchymal-to-epithelial transition (MET) by which the tumor cells regain phenotypic and genotypic properties of the primary tissue. This process is analogous in the development of metastatic tumors. Recent studies have shown that the process of EMT can propel cancer cells into a CSC-like state allowing them to acquire a mesenchymal phenotype, suggesting a functional link between CSCs and the metastatic process (9). Tam and Weinberg have attempted to elucidate a detailed and complex transcriptional process that governs the steps of EMT and its reversal mechanism MET (10). The canonical EMT/MET processes are characterized by complex genetic alteration that allows epithelial or mesenchymal cells to be distinguished by expression of a number of classical markers (11). Well-recognized epithelial markers include cadherins and tight junction proteins such as E-cadherin. Mesenchymal markers include the extracellular matrix component fibronectin and the intermediate filament protein vimentin. During embryogenesis, EMT is governed by a number of diverse growth factors including fibroblast growth factor (FGF), platelet derived growth factor (PDGF), epidermal growth factor (EGF) and transforming growth factor beta (TGFβ), leading to activation of various receptor tyrosine kinases (RTKs) (12). These mechanisms are shown to reappear during the metastatic process *via* EMT, where TGFβ plays a pivotal role.

Activation of the TGFβ pathway results in translocation of Smad transcription factor proteins into the nucleus where they interact with other transcription factors to activate or repress genes involved in EMT (13). TGFβ has a direct effect on EMT by down-regulation of epithelial and up-regulation of mesenchymal markers, through activation of a number of transcription factors including zinc finger SNAI1 (Snail), zinc finger SNAI2 (Slug), zinc finger E-box binding homeobox 1 (ZEB1), zinc finger E-box homeobox 2 (ZEB2), and twist family bHLH transcription factor 1 (TWIST), that are recognized as master regulators of this process (14). Under the influence of TGFβ, transcriptional repression of the transmembrane adhesion protein E-cadherin occurs, which emerged as a fundamental regulator in the process of tumor progression and EMT (12, 15, 16). Notably, transcription factors Snail, Slug and ZEB1/2 are recognized as key regulators in E-cadherin repression, in addition to their roles in induction of mesenchymal genes (12, 16). GATA1, a known repressor of E-cadherin, was found to be up-regulated in samples of lung-derived brain metastases, suggesting a role for E-cadherin modulation in the process of cerebral dissemination (17). Commonly, overexpression of the *HER2* gene in breast cancer is shown to be associated with TGFβ signaling, leading to the activation of Snail, Slug and ZEB1 (18). Suppression of this signaling by cucurbitacin B in mouse models led to reversal of the EMT process and reduction of brain metastases (18). Moreover, a link between TWIST and other regulators of EMT such as Snail, Slug and ZEB has been seen (16). Induction of ZEB1 by TGFβ was dependent on cooperation between Snail and TWIST in mammary epithelial cells undergoing EMT (19). TWIST, a member of the helix-loop-helix family of factors, is predominantly expressed during embryogenesis in neural crest cells. It is believed that TWIST maintains roles in suppressing expression of E-cadherin, occludins and claudin-7, and induction of pro-invasive and mesenchymal genes (16, 20-22). High TWIST expression was observed in metastatic melanoma and was considered as an independent marker of poor prognosis in these patients (23). Similar findings are reported in highly invasive ductal carcinoma, prostate cancer, esophageal squamous cell carcinoma and hepatocarcinomas (24-29). A recent study described an increased expression of EMT markers Snail and TWIST present in samples of brain metastases from lung, breast, colon and renal primary tumors (17). Similar to the TGFβ signaling pathway, downstream effectors of EGFR, namely signal transducer and activator of transcription 3 (STAT3), is linked to the activation of TWIST and subsequent promotion of EMT. STAT3 is a member of a family of latent transcription factors that are activated by cytokines and growth factors (30) and is constitutively

activated in many cancers. The link between STAT3 and TWIST has been suggested in invasive breast (31), hepatocellular (32) and gastric cancers (33). Increased STAT3 activity was also evident in models of melanoma-derived brain metastases, which display increased activity of STAT3, relative to primary melanoma cells (34). Furthermore, an inhibition of STAT3 may suppress the cerebral metastases as shown in an animal model of malignant melanoma (34).

TGFβ also elicits signaling responses *via* non-Smad pathways, which are thought to complement Smad signaling in producing an effective EMT response. Activation of non-Smad signaling pathways relies on direct interactions between effector molecules and TGFβRI and/or TGFβRII receptors (13). The intersection of TGFβ-mediated EMT activation and non-Smad signaling is thought to occur under the influence of other signaling pathways associated with Erk MAP kinases, Rho GTPases and the PI3 Kinase/AKT pathway (13, 35, 36).

MAP kinase pathways appeared to play a significant role in TGFβ-mediated EMT, as studies have revealed down-regulation of E-cadherin and up-regulation of N-cadherin and matrix metalloproteinase expression in response to MEK/Erk MAP kinase activation (13, 37-41). Interestingly, the MAPK pathway is also known to be activated by mutations in the oncogene $BRAF^{V600E}$, commonly associated with melanoma, with a higher tendency for metastasizing to the brain (42). In fact, a recent study demonstrated inhibition of melanoma brain metastasis cell lines harboring a $BRAF^{V600E}$ mutation, using the MAPK inhibitor vemurafenib (42). Furthermore, down-regulation of the PI3K/Akt pathway using temsirolimus, a mechanistic target of rapamycin (mTOR) inhibitor, reduced proliferation of melanoma-derived brain metastases harboring mutations in the *PTEN* tumor suppressor gene (42). Additionally, TGFβ is shown to activate PI3K, which results in a subsequent activation of the Akt kinase *via* integrins (43-47). In fact, αv integrin levels in cancer cell are shown to be positively correlated with the number of brain metastasis as well with the rate of occurrence. A recent study points to the role of αv integrin in promoting brain metastases in cancer cells and may be involved in early steps in the metastatic process, such as adhesion to brain vasculature and motility. Therefore, targeting αv integrin with intetumumab could provide clinical benefit in treating cancer patients with brain metastases (48). Specific genetic variations in the genes for PI3K, PTEN, AKT and mTOR have been identified as predictors of brain metastases in a model of NSCLC (49). Importantly, the PI3K/Akt pathway facilitates downstream signaling that is involved in the promotion of EMT, cell migration and cell survival (13, 43, 50). As such, inhibitors of this pathway have been found to hinder TGFβ-mediated E-cadherin down-regulation, thereby halting the process of EMT (43, 51, 52). Specifically, two multiprotein complexes of mTOR, mTORC1 and mTORC2 have been implicated in coordinating various cellular functions associated with EMT (53, 54). Consistent with these findings, increased mTOR signaling has been linked to TGFβ activity *via* mTORC1 and phosphorylation of p70S6K and 4E-BP1, which subsequently result in increased protein synthesis and cell size (51). Studies of metastatic liver (55) and colorectal cancer (56) have highlighted mTOR as an emerging target of interest in regulating tumorigenesis and metastasis. With respect to cerebral metastases, components of the mTOR signaling pathway are shown to be up-regulated in models of metastatic breast cancer and suppression of these markers resulted in significantly diminished metastatic potential (57, 58). Moreover, silencing of mTOR pathway components suppresses E-cadherin expression and enhances expression of the mesenchymal marker vimentin, suggesting an important regulatory role for mTOR in the processes of both EMT and MET (57).

RhoGTPases are also known regulators of cell migration, gene regulation and cytoskeleton organization (13). TGFβ regulates Rho activity in many cell types; however, the interaction between TGFβ, RhoA, and its effector kinase Rho-associated protein kinase (ROCK), at tight junctions is most significant in the process of metastasis. Signaling between TGFβ and TGFβRII results in recruitment of the E3 ubiquitin ligase Smurf1 and subsequent RhoA ubiquination and degradation at tight junctions (13, 59, 60). These observations are supported by a study utilizing a model of metastatic breast cancer, revealing a distinct role for TGFβ-mediated Par6 signaling in promoting loss of cellular polarity and morphologic transformation in mammary cells (60). Furthermore, ROCK inhibition resulted in an increased number of cells permitted to migrate through the BBB, promoting the formation of cerebral metastatic lesions (61).

Cancer Stem Cells, EMT and Cerebral Metastasis

A critical role for CSCs in cancer recurrence, maintenance and metastasis has become evident. Much like normal adult stem cells, the CSC is endowed with the capacity to self-renew and differentiate (62). Furthermore, normal stem cells are active in two phases; cycling or quiescent; and, as such, CSCs may function in the same manner, potentially explaining the dormancy phase of a tumor, prior to the development of a metastatic lesion. Similar to embryonic stem cells, the CSCs require a specific niche provided by the microenvironment consisting of components needed to maintain stemness and differentiation (63). CSCs may contribute to tumor metastasis by the process of EMT, in which TGFβ mediated pathways generate cells with stem-like properties (9, 64).

Aberrant signaling of the Notch, Hedgehog and Wnt/β-catenin pathways are crucial in the maintenance and activity of CSCs, as well as the process of EMT (13, 62). The critical role of Notch signaling in embryogenesis and development,

particularly in cellular patterning and allocation of cell types to tissues strengthens the notion of its role in control of CSCs (63). Following binding of a Notch receptor to its specific ligands (*i.e.* Delta or Jagged), a cleaved intracellular domain translocates to the nucleus, activating transcription factors and promoting transcription of downstream target genes, such as Hes and Hey (65, 66). Signaling crosstalk between the Notch and components of the EMT pathway (TGFβ, Snail, Slug and ZEB) has been shown to contribute to tumor progression (66-68). In fact, TGFβ-mediated EMT was abrogated by knockdown of Hey1 and Jagged1 or inactivation of Notch (69). Furthermore, Notch also induces EMT by stabilizing Snail under hypoxic conditions, *via* recruitment of hypoxia-inducible factor (HIF) 1a and HIF-2a (70-71). In addition, elevated levels of Notch 1-3 and Jagged1 were found to be associated with disease progression and serve as markers for poor prognosis and metastasis in a model of NSCLC (72). Constitutively active Notch1 in colorectal carcinoma cell lines has been associated with an increase in EMT and stemness-related proteins such as Slug, Smad3, Jagged1, and CD44 (73). The role of the Notch pathway in brain metastasis was initially described using an animal model of breast cancer, where cerebral metastases were associated with activation of Notch1 components, along with nuclear localization of Hey1 and Hes1 (74). Moreover, inhibition of Notch significantly reduces the incidence of brain metastases, particularly by altering the $CD44^+/CD24^-$ sub-population (75). Importantly, recent evidence suggests that jagged1 and Notch signaling is involved in establishing and promoting brain metastases from breast cancer, in a process mediated by interleukin 1b in astrocytes (63).

The Wnt/β-catenin signaling pathway appears to play a crucial role in regulation of stem cells and progression of many cancers including colon, breast and cutaneous malignancies (76-79). It is worth mentioning that Wnt also plays an important role in normal brain development, therefore activity of this pathway in metastases may suggest emulation, providing further evidence for the "seed and soil" hypothesis, as suggested by Fidler (80). The clinical significance of the canonical β-catenin-dependent pathway pertains to its involvement in cellular proliferation, differentiation and survival as well as in stem cell maintenance and reprogramming (81, 82). In addition, the Wnt signaling pathway regulates TGFβ-mediated EMT through its interaction with E-cadherin repressors including Snail, TWIST and ZEB (83, 84). E-cadherin normally exists in a complex with β-catenin at the cell membrane. Therefore, loss of E-cadherin during EMT allows β-catenin to translocate to the nucleus in order to stimulate transcription of regulatory genes involved in cellular proliferation and differentiation (82, 85). Further crosstalk between the Wnt and TGFβ signaling pathways is evidenced by the close interaction of Smad and transcription factors induced by the Wnt pathway (86, 87). In a model of lung cancer, increased levels of dishevelled-3 mRNA in pleural effusions, suggesting this as a possible marker for micrometastases (88). Expression of dishevelled-1 and dishevelled-3 has been found to be increased in lung-derived brain metastases as well (89). A recent study of triple-negative breast cancer provided evidence of up-regulated Wnt pathway activity, identifying this as a marker of poor prognosis and metastatic disease, particularly to the lung and brain (90). Furthermore, up-regulation of Wnt/β-catenin activity in brain metastases from basal-type breast carcinoma have been documented, as evidenced by gene expression analysis (91). On the other hand, down-regulation of Wnt/β-catenin signaling in the luminal B subtype of breast cancer prevents metastasis to the brain, strengthening its role in this process (91). Therefore, targeting this pathway may prove effective in inhibiting the development of brain metastases. In fact, monoclonal antibodies against Wnt ligands and associated receptors are currently being tested for their ability to inhibit tumor growth, though most trials are still in early stages (32).

The sonic hedgehog (SHH) pathway is known to be a critical regulator of embryogenesis, body patterning and cancer progression (92). In the presence of SHH, smoothened proteins are released and phosphorylated to promote the activation of glioma-associated oncogene homologs (GLIs), which subsequently regulate the expression of a multitude of target genes (93, 94). The interaction of SHH, Wnt and TGFβ pathways is involved in regulation of the process of EMT. For example, in fibroblasts and keratinocytes, up-regulation of TGFβ signaling revealed Smad3-dependent activation of GLI1 and GLI2 (95). Activated GLI2 was associated with loss of E-cadherin and the potential to form bone metastases in a melanoma model (96). *In vitro* studies of the SHH pathway and EMT have revealed GLI1-mediated suppression of E-cadherin expression *via* induction of Snail (97). Studies have shown the SHH/GLI pathway to be a critical regulator of EMT, facilitating recurrence and metastasis, as well as chemotherapy resistance in models of squamous cell lung carcinoma and NSCLC (98-100). While there is limited evidence for the involvement of SHH in promoting brain metastases, a study conducted using samples of six metastatic brain tumors demonstrated increased expression of downstream mediators of the SHH pathway, particularly GLI1, which correlated positively with expression of Snail, and negatively with expression of E-cadherin, suggesting a role for EMT in brain metastases (101).

Role of Micro RNA in Cerebral Metastasis

miRNAs are small, non-coding, single-stranded RNAs that regulate gene expression by targeting mRNA transcripts, leading to their translational repression or degradation (102).

A single miRNA can simultaneously regulate multiple genes, resulting in complex functional outcomes (103). The identification of groups of genes targeted by the same miRNA provides insight into the cross-talk between multiple signaling networks and their role in controlling diverse biological processes. Numerous miRNAs are now being identified for their role as oncogenes or tumor suppressors (104). Furthermore, extensive research has provided evidence that several microRNA are involved in the process of EMT; many of these having a role in mediating cell-cell adhesion, cytoskeletal arrangement or oncogene expression (102, 103, 105). Recently, much of the work regarding miRNA in EMT regulation focuses on the miR-200 family, which includes miR-200a, miR-200b, miR-220c, miR-141, and miR-429 (105). This association was realized upon the finding that the cells undergoing EMT in response to TGFβ had noticeably reduced miR-200 expression (15). Specifically, members of the miR-200 family were found to exert a negative regulation on ZEB1 and SIP1 expression, suggesting that down-regulation of these miRNA is required for initiation of EMT (15). This observation was further supported by the fact that members of the miR-200 family repress EMT by silencing ZEB1 and ZEB2 in gastric and breast cancer (106-108). In addition, miR-429 expression has been associated with down-regulation of mesenchymal markers including MMP2, Snail and ZEB2 (109). Interestingly, induced expression of miR-200 correlated with increased levels of E-cadherin mRNA, indicative of the reversal process of EMT, MET (15). In metastatic NSCLC cells, the expression of miR-200 correlated with reduced gene expression, particularly related to genes involved in cell signaling, invasion and proliferation (110).

Recently, miRNA expression has become a topic of interest as it pertains to mediating pathways involved in tumorgenicity as well as CSC differentiation, self-renewal and maintenance (111). Of interest, miR-107, miR153, miR-204 and miR-218 are shown to influence the self-renewal and maintenance of glioma stem-like cells (112-115). In a model of breast cancer, Lin28-mediated repression of let-7 was associated with CSC production (116). Expression of miR-7 in metastatic breast CSCs was correlated with increased expression of the pluripotency gene KLF4, suggesting an important role for miRNA in CSC stemness and metastasis (117). In concordance with these studies, a pivotal role for microRNA in CSC maintenance has also been reported in models of colorectal, lung and hepatocellular carcinoma (118-121). Moreover, a recent review provided evidence for a 30 miRNA signature correlating with expression in the TGFβ, Notch and Wnt signaling pathways, implicating their role in regulating EMT and CSCs (103).

Recently, groups of miRNA have been identified as unique markers for metastatic tumors, suggesting a role for miRNA in developing organ-specific metastases. Importantly, a recent study using expression-based profiling of miRNA was able to accurately identify the primary tumor of origin of brain metastases in 84% of samples, suggesting an important role in diagnosis (122). Also, a group of miRNAs were described that are exclusively expressed in metastatic tumors based on the analysis of 336 cancer samples from 22 unique sites, suggesting a role in site specific metastasis (123).

While the precise mechanism by which the cerebral microenvironment interacts with tumor cells has yet to be understood, studies suggest a possible interaction between the astrocytic milieu and tumor cells, in which complex alterations of miRNA expression could take place (124). In support of this theory, a recent study demonstrated the ability of astrocytes to alter the microRNA expression patterns of lung cancer cells when co-cultured together (125). Specifically, co-cultured cells exhibited reduced expression of miR-768-3p, which was linked to increased cell viability *via* increases in K-ras (125). Additionally, a study of breast and bone metastatic models revealed down-regulated miR-7 expression in CSC derived from metastatic tumors, as well as an inverse relationship between miR-7 and the pluripotency gene KLF4 (117). Interestingly, this inverse relationship between miR-7 and KLF4 was also associated with metastasis-free survival only in brain metastases, demonstrating a site-specific interaction between microRNA, CSCs and the cerebral microenvironment, leading to prognostic implications. Global patterns of gene expression demonstrated an up-regulation of hsa-miR-17-5p in triple-negative breast cancer tissues in The Cancer Genome Atlas (TCGA). In addition, a negative correlation between hsa-miR-17-5p and overall survival as well as PTEN and BCL2 target genes was observed in TCGA breast cancer specimens (126). Other miRNAs were found to have roles in tumor cell invasion and extravasation through the BBB. Specifically, miR-1258 was shown to regulate expression of heparanase, a pro-metastatic enzyme stored in endothelial and glial cells, involved in breakdown of heparan-sulfate chains, rendering cells more capable of crossing the BBB (127, 128). In addition, miR-22 and miR-378 mediate expression of MMP-2, MMP-9 and VEGF, implicating a cross-talk between the tumor cells, extracellular matrix and vasculature in facilitating invasion and establishing secondary lesions in the brain. Interestingly, aberrant expression of miR-10b, miR-29c, miR-145, miR-146a, miR-200, miR-210, miR-199a/b and miR-768-3p were discovered in cerebral metastatic lesions from multiple primary tumors, suggesting the pivotal role of miRNA in brain metastasis, which has potential to aid in diagnosis, prognosis and discovery of therapeutic targets.

Involvement of approximately 38 distinct miRNA associated with cerebral metastases from different primary tumors (NSCLC, breast, CRC, melanoma and renal tumors) are presented in Table I (129-165). Of these miRNA, 25 were found to have increased expression, 10 were found to

have decreased expression, and 3 were found to have variable expression, when compared to their matched primary tumor counterparts (Table I).

Genetics Associated with Cerebral Metastasis

Studies of gene expression have suggested the existence of a genetic signature present in primary tumors that defines their metastatic potential (166). While, much debate has since emerged regarding the significance of genetic signatures and their role in metastasis, Fidler and colleagues suggested that the process of metastasis requires cancer cells to acquire additional mutations in order to develop metastatic potential (167). Furthermore, it has been demonstrated that while a genetic signature for poor prognosis may exist, an additional set of genetic aberrations contributes to the site-specific dissemination of metastatic tumor cells (168). Rather than directing the process of metastasis, this study suggests that the genes corresponding to poor prognosis may instead provide tumor cells with baseline metastatic properties with a phenotype encouraging metastasis (168). Alternatively, a model of breast-derived brain metastases suggests that it is not a genetic signature that predetermines a tumors clinical course, but rather a unique interaction between the tumor and its microenvironment that contributes to disease progression (80). Given the significance of genetic markers in defining tumor progression and metastasis, it is prudent to detail genetic markers as they pertain to cerebral dissemination from the breast, lung, skin, colon and kidney (See Figure 1 for details).

Breast

Approximately 10-30% of breast cancer patients will develop cerebral metastases (169). In patients with metastatic breast cancer brain metastases were shown to have the worst prognosis (7.35 months), followed by metastases to the liver (36.7 months), bone (44.4 months) and lung (58.5 months). Brain metastases from breast cancer can be stratified based on hormone receptor status. About 25% of patients with breast cancer have an amplification in HER2 and, of these, 30-55% develop metastatic brain lesions (170-174). This risk is elevated in the setting of hormone-receptor negativity (175, 176). Median survival of patients with HER2-positive, ER-negative brain metastases has been found to be approximately 28 months (177). A study of 66 patients with HER2 breast cancer displayed good performance status, controlled extracranial disease and single brain metastases had better outcome (178). Patients with triple-negative breast cancer ($ER^-/PR^-/HER2^-$), on the other hand, are at increased risk of first recurrence of cerebral metastasis (179) with a tendency to cluster early in the patient's disease trajectory (175). These patients are at a 25-46% risk of developing CNS metastasis (180-182) with a survival time of less than six months (179, 182, 183).

In a model of breast cancer, gene expression analysis revealed 243 genes that were differentially expressed in metastatic cell lines, of those, 17 genes were highly correlated with brain metastasis (184). More importantly, these genes did not coincide with those involved in metastasis to other organs. Among the 17 genes, *COX2*, *EGFR* ligand *HBEGF* and the a2,6-sialyltransferase ST6GALNAC5 were identified as mediators of homing, cancer cell migration and passage through the BBB. Findings also indicate that in breast cancer, a long period of remission often precedes distant relapse, supporting the notion that breast cancer cells initially lack the full competence for outgrowth in distant organs but develop this under the selective pressure of different organ microenvironments (168, 184-186). Alternatively, a recent study of 18 primary and 42 breast cancer-derived brain metastases found mutations in the *TP53*, *PIK3CA*, *KIT*, *MLH1* and *RB1* genes, within which no mutations were found to be unique to cerebral metastases (187). Interestingly, in a matched pair of primary tumor and metastatic brain lesion, a mutation in p53 was discovered, however the acquisition of additional mutations might have occurred during the metastatic process (187). Mutations in TP53 were also found with a higher frequency in metastatic tumors, compared to primary breast cancer cells (187, 188). A similar study, using expression profiling of 23 matched sets of brain metastases and primary breast tumors, found DNA double-strand break repair genes *BARD1* and *RAD51* to be up-regulated in samples of metastatic lesions, suggesting a role for these genes in evading the effects of reactive oxygen species in the brain (189). Studies of epigenetic gene regulation have revealed differential methylation patterns of genes exist between brain metastases and their primary tumor counterparts (190, 191). Importantly, one study found the *BNC1* gene to be more frequently methylated in metastatic tumors than in primary breast cancer (190). BNC1 is a target of TGFβ in mediating EMT, as its expression is preserved in primary breast tumors while silent in metastatic lesions (191). This suggests its initial role in promotion of EMT, thereafter possibly contributing to MET while establishing secondary lesions (190).

Lung

The pathogenesis of lung cancer exists in two broad clinical subtypes, namely non-small cell carcinoma (NSCLC), representing approximately 75-85% of tumors, and small cell lung carcinoma (SCLC) accounting for the remaining 15-25%. Of these, approximately 30-50% are likely to develop cerebral metastases; where 25% arise from NSCLC (192, 193). The development of cerebral metastases is considered an indicator of advanced disease and poor

Table I. *microRNAs involvement in brain metastasis*.

miRNA	Primary site	Pattern of expression	Proposed role in metastasis	Reference
miR-1	CRC	↑	Interaction with MACC1	129, 130
miR-7	BC	↓	Increased KLF4 expression	117
miR-9	BC; CRC	↑	Inhibition of E-cadherin	131, 132
miR-10b	BC; CRC; ccRCC	↑ BC ↑ CRC ↓ ccRCC	Modulation of HOXD10, TIAM1, MICB, TIP30, 129, 133, 134 Twist and E-cadherin expression	
miR-15b	Melanoma	↑		135
miR-16	Melanoma	↑	Inhibition of EMT *via* phosphorylation of FAK and Akt proteins	135, 136
miR-19a	BC	↓	Regulation of cyclin D1, Bim, TNFa and PTEN expression	137, 138
miR-20b	BC	↑	Suppression of PTEN	139, 140
miR-21	NSCLC	↑	Downstream mediator of STAT3	141
miR-22	CRC	↑	Modulation of TIAM1, MMP-2, MMP-9 and VEGF expression	129, 142
miR-28	CRC	↑	Targeting of CCND1 and HOXB3 expression	129, 143
miR-29c	BC; Melanoma	↓ BC ↓ Melanoma	Epigenetic regulation of tumor-related genes; Targeting of TIMP3, PDCD4 and RASA1	137, 144, 145
miR-31	CRC	↓	Downstream effector of TGFb that targets TIAM1	129, 146
mIR-95	NSCLC	↓	Suppression of cyclin D1	147
miR-125b	CRC	↑	Suppression of LIN28B	129
miR-126	CRC	↑	Inhibition of the RhoA/ROCK signaling	129, 148
miR-133a/b	CRC	↑	Downstream target of TPp63 with inhibitory effect on RhoA, E-cadherin and vimentin	129, 149
miR-143	CRC	↑	Regulation of MACC1 expression	121, 129
miR-145	NSCLC; CRC	↑ NSCLC ↓ CRC	Targeting of OCT4, EGFR, c-myc, MUC1, TPD52 and NUDT1	129, 150, 151
miR-146a	BC; CRC	↓ BC ↑ CRC	Modulation of b-catenin and hnRNPC expression	129, 152
miR-150	Melanoma	↑	Targeting of c-Myb	135, 153
miR-184	NSCLC	↑	Inhibitor of c-myc and CCND1; induction of p15 and p21	154, 155
miR-197	NSCLC	↑	Negative regulator of FUS1	154, 156
miR-199a/b	CRC; ccRCC	↑ CRC ↑ ccRCC	Regulation of HES1; inhibition of c-Met	129, 134, 157, 158
miR-200	BC; Lung	↑ BC ↑ Lung	Targeting of ZEB1 and ZEB2	159
miR-210	BC; Melanoma	↑ BC ↑ Melanoma	Targeting of MNT; Induction of angiogenesis after hypoxia	137, 160, 161
miR-328	NSCLC	↑	Up-regulation of PRKCA	162
miR-374	Melanoma	↑		135
miR-378	NSCLC	↑	Up-regulation of MMP-2, MMP-9 and VEGF	163
miR-509	BC	↓	Suppression of RhoC and TNFa	164
miR-542	Melanoma	↓	Down-regulation of PIM1	165
miR-576	CRC	↑		129
miR-768-3p	BC; Lung	↓ BC ↓ Lung	Targeting K-ras	125
miR-1258	BC	↓	Inhibition of heparanase	127
HS_170	CRC	↓		129
HS_287	CRC	↑		129

prognosis. Without treatment, median survival time ranges from only 1-2 months, expanding to 4-6 months with the addition of radiotherapy (193-195). Improved survival in patients with single brain metastases derived from NSCLC has been shown with the addition of neurosurgical resection and/or radiosurgery with mean survival times approaching 12-14 months (193).

A study comparing expression data between 16 metastatic brain tumors with 37 primary NSCLC samples revealed 244 genes with altered expression levels, corresponding to genes involved in adhesion, cell-cell communication and motility (196). A similar study, comparing brain metastases from NSCLC to non-metastatic lung tissue assessed 17,000 genes and found 1,561 to have altered expression, showing genes involved

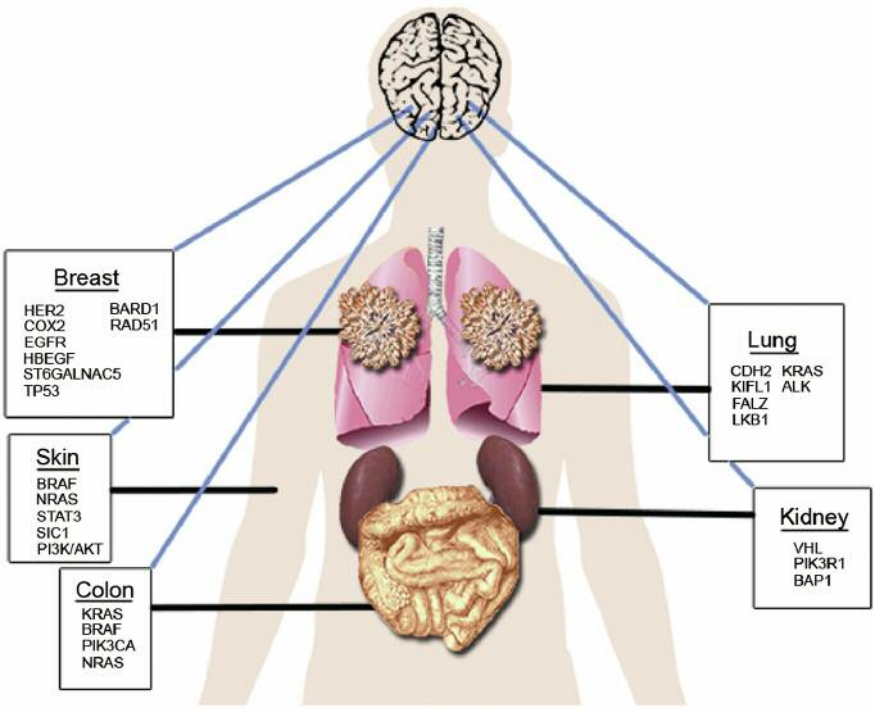

Figure 1. *Genes associated with cerebral metastasis from distinct organ sites. Figure represents the alterations in unambiguous genes linked with brain metastasis from breast, lung, kidney, colon or skin.*

in adhesion, motility and angiogenesis to be up-regulated, and genes involved in apoptosis and neuroprotection to be down-regulated (197). Furthermore, analysis of 12 candidate genes from samples of NSCLC, assessing the correlation between gene expression and occurrence of brain metastases found three genes, CDH2, KIFL1 and FALZ, to be predictive of brain metastases (4). Importantly, CDH2 is known to regulate adhesion, and is a mediator of EMT, suggesting its role in this process (4, 198, 199). Similarly, loss of LKB1 and mutation of KRAS have been shown to be predictive of brain metastases in NSCLC (200). Recent genomic analysis using comparative genome hybridization techniques has revealed numerous copy number variations (CNV) predictive of brain metastases from primary NSCLC (201, 202). An increase in the number of CNVs has been observed in secondary tumors in comparison to their primary counterpart, suggesting a degree of genetic variability that takes place during the metastatic process (201).

A recent study has demonstrated that about 21.9% patients with NSCLC showed the expression of programmed cell death-ligand 1 (PD-L1) in brain metastases. Furthermore, PD-L1 positivity in brain metastasis samples was seen in patients with heavy smoking history as well as radio-therapeutic treatments giver prior to surgery (203).

Interestingly, a newly discovered rearrangement of anaplastic lymphoma kinase (ALK) is seen in about 2-7% of NSCLC (204) and has recently become a marker of interest for targeted chemotherapy. The propensity for ALK-rearranged NSCLC to metastasize to the brain has been a subject of contention, with several studies citing greater likelihood (205), however others show no significant increase in brain metastases (206). Crizotinib, a first-generation ALK inhibitor, is shown to be highly efficacious against ALK-positive NSCLC and is currently approved for first-line treatment (207, 208), however it is important to consider that CNS progression develops in up to 60% of patients treated with this chemotherapeutic agent (204). The pattern of CNS progression in patients receiving crizotinib was studied by Costa *et al*., who discovered poor CSF concentrations of the drug, due, in part, to both passive diffusion restriction and active efflux *via* P-glycoprotein (209, 210). Several second generation ALK-inhibitors, such as alectinib, ceritinib, and brigatinib, are currently in clinical trials, with positive results due to better CNS penetrance, however, ongoing preclinical studies are awaiting outcome (211-213). Perhaps the novel of ALK-inhibitors is the third-generation, loratinib, believed to be effective against all resistant mutants of ALK+ NSCLC (214, 215). Clinical trials for this novel chemotherapeutic agent are currently underway, with published evidence from one patient indicating a favorable treatment response and re-sensitization to crizotinib (211, 214).

Skin

Brain metastasis is common in association with melanoma, occurring in up to 75% of patients with stage IV disease (216). Greater than 50% of melanoma-related deaths are a result of brain metastases, with the median survival for these patients determined to be approximately 3 to 6 months (217, 218). Interestingly, while lung, breast and melanoma cancers are the most common sources of cerebral metastases; melanoma is by far the least prevalent in the general population, reflecting its heightened propensity to metastasize to the brain (216).

Comparisons of metastatic melanoma to non-metastatic primary cutaneous malignancies have revealed many genetic variations, as confirmed by expression array analysis (219). With respect to melanoma, the BRAF gene is crucial in the development of disease, while members of the MAPK-ERK pathway, including MEK1/2 and ERK1/2 components, are also involved in progression of disease (220, 221). There are a number of somatic mutations, including an activating mutation in the BRAF gene, which lead to subsequent activation of the MAPK pathway in melanomas that are thought to promote tumorigenic progression and subsequent invasion of cells with malignant potential (222). Interestingly, a study of paired primary melanomas with metastases from the same patients revealed BRAF/NRAS mutations of similar frequency in both sets of samples, suggesting that BRAF confers metastatic potential, however does not confer organ-specificity (223). Studies have shown that the *BRAF*^{V600K} mutation is associated with higher cerebral (75% *vs*. 36.3%) and lung (91.6% *vs*. 47.7%) metastases, relative to other BRAF mutations (224). Furthermore, patients with *BRAF*^{V600K} mutations were found to have a shorter latency to metastasis and overall survival, suggesting this mutation to be a potential therapeutic target (224). Ipilimumab, a monoclonal antibody against cytotoxic T-lymphocyte-associtated antigen 4 (CTLA4), and specific inhibitors of mutated *BRAF*^{V600E} protein, such as dabrafenib and vemurafenib, are newer therapies approved for treating metastatic melanoma. These therapeutics, however, have poor efficacy in attenuating cerebral metastases (225).

Recent studies have identified signaling molecules PSTAT3, SOC1 and PI3K/AKT to be activated in melanoma-derived brain metastases and have a significant difference from extra-cranial metastases (34, 226, 227). Molecular analysis of matched pairs of melanoma-derived brain and extra-cranial metastases revealed enhanced activation of PI3K/AKT pathway in patients with brain metastases (216). The activation of the PI3K/AKT pathway is governed by multiple upstream signaling components and, as such, it is possible that an interaction between the tumor cells and cerebral microenvironment exists and contributes to progression of disease (216). Interestingly, activation of the PI3K/AKT pathway is often correlated with treatment resistance in BRAF-mutant cell lines, implying that combined inhibition of both the PI3K/AKT and MAPK pathways in melanoma may be necessary to reduce tumor burden and disease progression (216).

Colon

Colorectal carcinoma (CRC) is one of the most commonly diagnosed cancers worldwide, and despite total resection of the primary lesion; distant metastases have been reported to occur in up to 15% of patients (228, 229). The incidence of brain metastases in patients with CRC is between 0.3-6% (3, 230, 231). Interestingly, the brain is the sole organ of metastatic disease in up to 10% of patients with metastatic CRC (229, 232, 233). While cerebral metastatic disease is more uncommon than perhaps the liver or lung, it remains a primary focus as the course is far more fatal, with median survival times reported between 3-6 months (229, 234-236).

Recent advances in genomic analyses have revealed abnormalities in several genes, namely KRAS, BRAF, PIK3CA and NRAS, which may play a role in patterns of metastatic CRC. A study of advanced cancers possessing the mutant BRAF gene revealed that patients with CRC exhibited a trend towards a shorter interval to metastasis, albeit this relationship failed to reach statistical significance due to the small number of patients (224). Consistent with this, another study showed trend level data linking BRAF mutations to the development of brain metastases in colorectal cancer (237). As seen in studies of metastatic melanoma, one CRC patient with cerebral metastases exhibited the V600K variant of the BRAF mutation (224). Interestingly, the BRAF mutation, found in up to 15% of metastatic CRC, is associated with induction of EMT and the development of lymph node metastases (238). These finding suggest an important functional mechanism governed by mutations in BRAF for the progression of CRC and overall risk of developing metastatic disease (239). KRAS mutations are the most common genetic aberrations associated with CRC, occurring in up to 50% of colorectal malignancies (239). While there has been no evidence to suggest the presence of a specific mechanism linking KRAS mutations to the development of cerebral metastases, a recent study has shown an increased prevalence of KRAS mutations in CRC-derived brain and lung metastases, relative to liver metastases (240). Furthermore, an association between KRAS mutations and increased recurrence of brain metastases is seen in up to 14.5%, as compared to only 2% in wild-type populations (241). Although the KRAS mutation is considered a driver event in CRC, the mechanism of its involvement in metastasis remains to be elucidated. Interestingly, another genetic aberration in the *PIK3CA* gene, which encodes a catalytic isoform of the phosphatidylinositol 3-kinase (PI3K) presents in up to 20% of CRC (239). Up to 1.4% of patients

with CRC-derived brain metastases display a mutation in the PIK3CA gene (242). It is important to note that up to 70% of patients harboring this mutation, concurrently possess mutations in KRAS, which may exert a strong contribution to the formation of cerebral metastases (239).

Kidney

Despite many advances in defining the molecular mechanisms by which cancers disseminate and form cerebral metastases, those underlying the progression metastasis of renal cell carcinoma (RCC) remain unclear. Mutations in the von Hippel Lindau (*VHL*) gene and aberrations in mTOR pathway activity have been established as mediators of cell growth and disease progression in RCC (243). Evidence from expression analysis comparing matched samples from primary and adrenal metastases of RCC revealed 166 differentially expressed genes, which included several genes related to cell adhesion and extracellular matrix proteins (244). Importantly, metastatic samples exhibited up-regulation of TGFβ-related genes, as well as markers of mesenchymal cell types, suggesting a role for EMT and MET in the metastatic process in RCC (244). Recent studies have also shown that a high degree of intratumoral heterogeneity within primary renal cell carcinoma contributes to evasion of available therapeutics, which allows for rapid progression of disease (245, 246). Interestingly, while RCC appears to be a heterogeneous tumor, a recent study demonstrated that brain metastases derived from RCC appear to be monoclonal in origin, implying a discrete cell population had metastatic potential with a propensity to disseminate to the brain (246). Additionally, a non-sense mutation in the PIK3R1 gene, encoding the inhibitory subunit of PI3K, was identified as a specific mutation unique to RCC-derived brain metastases (246). Loss of BRCA-Associated Protein 1 (BAP1), a deubiquitinating enzyme, has also been implicated as a marker of poor prognosis and aggressive behavior in RCC, representing a potential target for new therapeutics (243, 247). While these studies have shed light on the mechanisms that may be responsible for cerebral metastasis in RCC, additional studies are needed to characterize the genetic contribution to cerebral dissemination, especially given the propensity for these tumors to metastasize to the brain.

Conclusion

Brain metastases are a leading cause of morbidity and mortality among cancer patients, and are reported to occur in about 40% of cancer patients in the United States (1, 2). In general, the median survival following a diagnosis of cerebral metastases is between 2 and 25 months, depending on the origin of the primary tumor and time of diagnosis (5, 6). Most brain metastases are typically associated with peritumoral edema. A correlation of tumor and edema volumes with overall survival in patients with cerebral metastases showed that the extent of edema surrounding cerebral metastases is not linked to effect overall survival in patients with brain metastases (248). Given that metastasis is the driving force behind morbidity and mortality for most patients, it is essential to identify the characteristics of these aberrant cancer cells that allow them to spread to distant sites in the body and develop into metastatic tumors.

Primary tumors are thought to be comprised of multiple subpopulations of cells having the capacity to metastasize through activation of multiple interrelated signaling pathways and a complex interaction between the primary cell, host cellular environment (platelets, endothelium, leukocytes and astrocytes) and effector molecules. Specifically, TGFβ–mediated EMT and its reversal process MET play critical roles in the control of many aspects of cancer progression by allowing epithelial cells the capacity to extravasate into the peripheral circulation, migrate and colonize in the cerebral microenvironment. The progression of tumor cells through a CSC-like state provides a crucial link in facilitating the transition between cell phenotypes and offers insight into the mechanisms underlying chemotherapy resistance and tumor re-growth in metastatic cancers. Furthermore, the discovery that miRNAs play a significant role in the regulation of multiple genes related to both the processes of EMT and CSC maintenance provides another layer of evidence for the complex interaction between epigenetic regulation and the molecular pathways responsible for metastasis, as well as potential therapeutic targets for intervention.

With improved local control and management of primary tumors, the incidence of late diagnosed brain metastases as well as their associated morbidity and mortality remains an unmet concern (80). While the mechanisms described above provide insight into a host of potential therapeutic targets, the development of an effective and innocuous agent remains a considerable challenge. In recent years, the discovery of genetic aberrations associated with brain metastases has provided a fundamental contribution to the development of novel therapeutic agents. While much progress has been made, further characterization of these pathways and processes is necessary to allow for more efficacious therapeutic targets. The continued development of brain metastasis models and identification of molecular signatures of metastatic pathways will advance our understanding of these complex communication networks and will ultimately lead to the development of therapeutic strategies.

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