**βIII-Tubulin Regulates Breast Cancer Metastases to the Brain**

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**Abstract**

Brain metastases occur in about 10% to 30% of breast cancer patients, which culminates in a poor prognosis. It is, therefore, critical to understand the molecular mechanisms underlying brain metastatic processes to identify relevant targets. We hypothesized that breast cancer cells must express brain-associated markers that would enable their invasion and survival in the brain microenvironment. We assessed a panel of brain-predominant markers and found an elevation of several neuronal markers (βIII-tubulin, Nestin, and AchE) in brain metastatic breast cancer cells. Among these neuronal predominant markers, in silico analysis revealed overexpression of βIII-tubulin (TUBB3) in breast cancer brain metastases (BCBM) and its expression was significantly associated with distant metastases. TUBB3 knockdown studies were conducted in breast cancer models (MDA-BR, GLIM2, and MDA-MB-468), which revealed significant reduction in their invasive capabilities. MDA-BR cells with suppressed TUBB3 also demonstrated loss of key signaling molecules such as β3 integrin, pFAK, and pSrc in vitro. Furthermore, TUBB3 knockdown in a brain metastatic breast cancer cell line compromised its metastatic ability in vivo, and significantly improved survival in a brain metastasis model. These results implicate a critical role of TUBB3 in conferring brain metastatic potential to breast cancer cells. _Mol Cancer Ther; 14(5); 1152–61. ©2015 AACR._

**Introduction**

Brain metastases occur in about 10% to 30% of breast cancer patients (1, 2). The median survival of patients with brain metastases is extremely poor (<10 months), and at present there is a lack of targeted therapy. To identify appropriate treatment regimens, it is critical to understand the biology of breast cancer brain metastases (BCBM). To comprehend the brain metastatic process, researchers developed paired models systems of brain metastasis. MDA-MD-231/MDA-MB-231BrM2 and CN34/CN34BrM2 are such paired model systems in which MDA-MB-231BrM2/CN34BrM2 cells exhibit a high propensity to metastasize to the brain after intracardiac administration in athymic nude mice.

Recent literature using this model system combined with BCBM tissues documents that brain metastatic cells overexpress neuronal markers such as ST6GALNAC5 (ST6; ref. 3), Nestin, and AchE in brain metastatic breast cancer cells. Among these neuronal predominant markers, in silico analysis revealed overexpression of βIII-tubulin (TUBB3) in breast cancer brain metastases (BCBM) and its expression was significantly associated with distant metastases. TUBB3 knockdown studies were conducted in breast cancer models (MDA-BR, GLIM2, and MDA-MB-468), which revealed significant reduction in their invasive capabilities. MDA-BR cells with suppressed TUBB3 also demonstrated loss of key signaling molecules such as β3 integrin, pFAK, and pSrc in vitro. Furthermore, TUBB3 knockdown in a brain metastatic breast cancer cell line compromised its metastatic ability in vivo, and significantly improved survival in a brain metastasis model. These results implicate a critical role of TUBB3 in conferring brain metastatic potential to breast cancer cells. _Mol Cancer Ther; 14(5); 1152–61. ©2015 AACR._

**Note:** Supplementary data for this article are available at Molecular Cancer Therapeutics Online (http://mct.aacrjournals.org/).

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Materials and Methods

Cell culture and reagents
MDA-MB-231/MDA-MB-231BrM2 (will be referred to as MDA-231/MDA-Br) cells were obtained from Dr. Joan Massague (MSKCC) and were maintained in DMEM (Corning) with 10% FBS (Hyclone). MDA-MB-468 cells (courtesy of Dr. Suzanne Conzen, University of Chicago) were maintained in RPMI-1640 (Corning) medium with 10% FBS (Hyclone) whereas GLM2 cells (obtained from Dr. Vincent Cryns, University of Wisconsin) were maintained in DMEM F/12 medium with 10% FBS and insulin/transferrin/sodium selenite mix (Invitrogen). CN34/34-Br cells were obtained from Dr. Joan Massague (MSKCC) and were maintained in M199 medium supplemented with 2.5% FBS, 10 μg/mL insulin, 0.5 μg/mL hydrocortisone, 20 ng/mL EGF, and 100 ng/mL cholera toxin (Sigma). No cell line authentication was done by the authors. Penicillin–streptomycin (100 U/mL) was added to all the cell culture media.

Adhesion assays

Matrigel invasion assay was conducted using BD Biocoat Matrigel invasion chambers according to the manufacturer’s instructions. Briefly, cells were seeded in serum-free medium, in the top inserts and complete medium was used as a chemoattractant at the bottom chamber. After 22 hours, the noninvading cells were removed and the inserts were fixed and stained with crystal violet. Five different fields of each chamber were photographed and cells were counted manually. Graph was plotted as relative invasion to cells to the vector control cells. The experiments were repeated twice in triplicates.

Patient samples and immunohistochemistry

The tissues were collected in accordance with a protocol approved by the Institutional Review Board at the University of Chicago. Sections (5μm-thick) of breast cancer and BCBM were deparaffinized in xylene and then rehydrated. After deparaffinization and rehydration, tissue sections were treated with antigen retrieval buffer (S1699; DAKO) in a steamer for 20 minutes. For immunohistochemical staining, antigen retrieval and rehydration, tissue sections were treated with antigen retrieval buffer (S1699; DAKO) in a steamer for 20 minutes. The tissues were then deparaffinized, rehydrated, and incubated in 3% H2O2 to block endogenous peroxidase activity.

Statistical analysis

All statistical analyses were performed using GraphPad Prism 4 (GraphPad Software Inc.). The sample size for each group was ≥3 and numerical data were reported as mean ± SEM. The RNA levels of brain predominant markers were assessed using the one-sample Student t test, and the differences on protein level were assessed using the two-sample Student t test. Comparisons on cell migration, adhesion, and invasion were conducted using one-way or two-way ANOVA with Bonferroni or Dunnett post hoc test as appropriate. For animal survival experiments, Kaplan-Meier
survival curve was generated and log-rank test was applied to compare survival distributions. Cox proportional-hazard regression was used to investigate the association between TUBB3 and breast cancer metastasis on patient data (22), and distant metastasis-free survival curve was generated for patients grouped as high vs. low expression by one SD from mean. All reported P values were two-sided and were considered to be statistically significant at *, P < 0.05; **, P < 0.01; ***, P < 0.001.

Results

Overexpression of predominant brain markers in BCBM

Given the ectopic overexpression of neuronal markers in BCBM (3–6), we hypothesized that there may be other neuronal markers that are involved in breast cancer metastases to the brain. To evaluate our hypothesis, we used two different paired model systems, MDA-231/MDA-Br and CN34/CN34-Br. MDA-Br/CN34-Br cells are the brain metastatic derivatives of the parental MDA-231/CN34 cells (respectively), which were isolated after two rounds of intracardiac injection with isolation of transplanted cells from brain tissue (3).

We conducted qRT-PCR to assess the RNA levels of a panel of brain predominant markers (TUBB3, Nestin, AchE, LMO3, Grin1, Grin2b, GFAP, PIN1, NSE2, NFL, NGFR, and NCAM1) and found upregulation of TUBB3, Nestin, and AchE by 1.5-, 2-, and 1.6-fold, respectively, in MDA-Br cells versus their corresponding parental cells (Fig. 1A). These results were further confirmed in CN34-Br cells in which TUBB3, Nestin, and AchE were upregulated by 3.6-, 1.46-, and 2.1-fold, respectively (Fig. 1B). However, there was no significant difference in other brain predominant markers that were evaluated (data not shown). We further confirmed the alterations in RNA levels of TUBB3 and Nestin at the protein level using Western blotting (βIII-tubulin; Fig. 1C) and flow cytometry (Nestin; Fig. 1E). Densitometric analysis revealed a...
2.5/4.9-fold increase of TUBB3 protein in MDA-Br/CN34-Br cells, respectively (Fig. 1D), whereas nestin protein levels were elevated by 1.4/2-fold in MDA-Br/CN34-Br cells as compared with parental cells, respectively (Fig. 1E and F).

Furthermore, we conducted gene-expression analysis using gene set enrichment (GSE) datasets, where nestin and AchE overexpression did not correlate with brain metastases (data not shown). However, we found significant upregulation in TUBB3 RNA levels in BCBM samples as compared with the primary breast tumors using GSE43837 dataset (Fig. 2A). To evaluate whether TUBB3 confers metastatic potential to breast cancer cells, we conducted gene-expression analysis on a large dataset containing 683 breast cancer patients (22) and found that overexpression of TUBB3 significantly correlates with distant metastasis (P = 0.008; Fig. 2B). Moreover using TCGA database, we found that breast cancer patients with high TUBB3 expression (22) exhibited poor survival as compared with patients with low TUBB3 expression (P = 0.027 for Fig. 2C; ref. 23) and (P = 0.007 for Fig. 2D; ref. 22).

To substantiate our in vitro and in silico results, we performed immunohistochemistry on five human primary breast cancers and unmatched brain metastasis tissues (Fig. 2E). Four samples of BCBM demonstrated overexpression of βIII-tubulin as compared with primary breast cancer tissues. Therefore, these results demonstrate that βIII-tubulin levels are elevated in brain metastatic breast cancer cells and associated with poor prognosis.

Knockdown of TUBB3 in breast cancer cells decreases invasion in vitro

To understand the active role of TUBB3 in breast cancer cells, we silenced TUBB3 expression via lentiviral transduction using two different shRNA specific to TUBB3 (shRNA1 and shRNA2) in widely used breast cancer cells, namely MDA-Br, GLIM2, and MDA-MB-468 cells. Densitometric analysis of Western blots revealed 95% and 88% downregulation of βIII-tubulin in shRNA1 and shRNA2, respectively, as compared with scrambled vector control cells (Supplementary Fig. S1A–S1C). TUBB3 knockdown cells exhibited gross morphologic alterations like elongation of cells and loss of cellular structures (Supplementary Fig. S2A) as compared with the vector control cells. This observation prompted us to evaluate whether there are alterations in migration of TUBB3 suppressed MDA-Br cells by a scratch wound migration assay. MDA-Br cells with downregulated TUBB3 migrated at significantly slower rate (n = 6; P < 0.05) as shown by the images captured after 18 hours (Fig. 3A) in comparison with vector control cells. In addition, when complete closure of

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the wound was noted in vector control cells, the knockdown cells were unable to close the wound even at the end of 28 hours (Fig. 3A). These results indicate reduced migratory potential of TUBB3 knockdown MDA-Br cells.

It is well known that augmented invasion is essential for metastasis of cancer cells. Hence, to evaluate whether TUBB3 knockdown cells possess the ability to invade the basement membrane components, an invasion assay was performed using Matrigel invasion chambers. MDA-Br cells with suppressed TUBB3 demonstrated 2.7- and 2.3-fold reduction in invasion for shRNA1 and shRNA2, respectively (Fig. 3B), as compared with vector control cells. To substantiate the role of TUBB3 in invasion, we conducted invasion assay in GLIM2 cells, which were previously shown to have intrinsic brain metastasis capability (24). We noted 8-(shRNA1) and 2.1 (shRNA2)-fold reduction in invasion of TUBB3-suppressed MDA-MB-468 cells as compared with vector control cells (Fig. 3C). Moreover, TUBB3-suppressed GLIM2 cells demonstrated 9.8-(shRNA1) and 4.5 (shRNA2)-fold decrease in invasion (Fig. 3D). These results demonstrate that ectopic expression of TUBB3 regulates invasive migration of breast cancer and brain metastatic cells.

TUBB3 knockdown in brain metastatic cells decreases cell adhesion to ECM accompanied by decreased levels of β3 integrins

Adhesion to extracellular matrix (ECM) components assists the cancer cells in the metastatic cascade. Laminin, fibronectin, and collagen are abundant ECM proteins in the brain endothelial cells. Hence, we conducted adhesion assays using laminin-, fibronectin-, and collagen-coated dishes. TUBB3 knockdown cells exhibited a significantly decreased ability to attach to laminin and fibronectin (Fig. 4A–C). These results depict that βIII-tubulin modulates the adhesion of cells to the ECM components.

Integrins are cell-surface proteins that mediate the process cell adhesion with ECM proteins. Previous reports documented elevated levels of αVβ3, αVβ6, and αvβ8 integrins in human BCBM (25). Therefore, we evaluated the transcript levels of these integrins in our brain metastasis model. Using the parental and brain

![Figure 3](image-url)
metastatic derivative of MDA-231 cells, qRT-PCR analysis revealed a 3-fold increase in integrin β3 transcripts in brain metastatic cells (MDA-Br) as compared with parental cells (MDA-231; Fig. 4D). This prompted us to evaluate whether there are any alterations in integrin β3 levels when TUBB3 was suppressed in brain metastatic cells. Flow-cytometry analysis of TUBB3 knockdown cells demonstrated 2.2- and 3.2-fold down-regulation of cell surface β3 integrin levels in shRNA1 and shRNA2, respectively (Fig. 4E), as compared with the vector control cells. These results demonstrate that βIII-tubulin regulates adhesion ability of brain metastatic cells possibly through integrin β3. Integrin downstream signaling is known to promote metastasis in a number of epithelial malignancies; therefore, it was of interest to evaluate whether the alteration in the integrin β3 surface expression in knockdown cells was accompanied by alterations in integrin downstream signaling.

**Knockdown of TUBB3 alters integrin–Src and STAT3 signaling**

Integrins mediate signaling through phosphorylation of focal adhesion kinase (FAK), which is activated when autophosphorylated at Y397 residues. To ascertain whether decreased integrin β3 in TUBB3 knockdown cells leads to decrease in FAK phosphorylation, we performed Western blotting in vector control and TUBB3 knockdown cells, which revealed reduced FAK phosphorylation (Fig. 5A). Furthermore, reduced FAK activation also resulted in blunted phosphorylation of Src at Y416 residues (Fig. 5A).

Integrin-mediated adhesion and signaling regulates activation of STAT3 (26). To evaluate whether abrogated integrin–FAK–Src signaling leads to alterations in STAT3 signaling, we conducted Western blotting in MDA-Br vector control and TUBB3 knockdown cells. We found attenuated STAT3 phosphorylation at Y705, which is responsible for its transcriptional activation (Fig. 5A). These results demonstrate an important role of TUBB3 in maintaining the integrin–Src–STAT3 axis in the brain metastatic cells (Fig. 5A).

Recently, it was shown that L1 cell adhesion molecule (L1CAM) is important for the colonization of metastatic cells in the brain (6). To address the role of TUBB3 in regulation of L1CAM levels, we conducted Western blotting using MDA-Br vector control and TUBB3 knockdown cells. We found dramatic reduction in L1CAM protein levels in of L1CAM in TUBB3 knockdown cells (Fig. 5B). These results underscore the role of βIII-tubulin in regulating the key signaling molecules that are involved in the process of BCBM.

**Knockdown of TUBB3 decreases the brain metastatic capabilities of breast cancer cells and increases survival of mice without altering primary tumor growth**

TUBB3 knockdown alters in vitro migratory and invasive properties. However, our in vitro results demonstrated a marginal statistical difference in the proliferative ability of TUBB3 knockdown cells (Supplementary Fig. 2S–2D). Therefore, it was of interest to understand whether there are any differences in the ability of vector control MDA-Br and TUBB3 knockdown cells to grow in orthotopic mammary gland. Vector control MDA-Br, TUBB3 shRNA1, and TUBB3 shRNA2 cells were injected in the third and fourth mammary gland of nude mice and tumor volume was monitored for a period of 15 weeks. There were no significant differences in the growth of vector control and TUBB3 knockdown cells (Fig. 6A).

To evaluate the in vivo functional consequences of TUBB3 knockdown on metastatic behavior of cells, a brain metastasis model was used. Vector control and TUBB3 knockdown cells were injected through an intracardiac route, and after 28 days, whole-brain tissues were sectioned to determine the number of metastatic lesions. One hundred sections (10 μm) were isolated from each mouse followed by hematoxylin and eosin staining. Ten
sections of each brain were analyzed for the presence of number of micrometastasis. Within each group an average of 9 mice were considered for calculating the number of micrometastasis. Mice injected with TUBB3 knockdown cells exhibited significant reduction in the formation of micrometastasis (Fig. 6B and C).

To understand the functional consequences of TUBB3 knockdown in a metastatic model on the survival of mice, we conducted survival experiments. Here, the vector control and stable TUBB3 knockdown cells were injected via an intracardiac route in the left ventricle of nude mice, and survival outcomes were determined. The vector control group exhibited a median survival of 34 days whereas shRNA1 and shRNA2 demonstrated median survival of 45 (P = 0.0053) and 50 (P = 0.0011) days, respectively (Fig. 6D). These results demonstrate that TUBB3 knockdown increases survival of mice via decreasing brain metastatic ability of breast cancer cells.

**Discussion**

There is an urgent need to identify rational molecular targets for the treatment of brain metastases. In this report, we present the first evidence that a microtubule family member, βIII-tubulin, confers brain metastatic potential to breast cancer cells by regulating invasion and Integrin-Src signaling. Our studies support the concept that (i) TUBB3 overexpression is a malignant adaptation of breast cancer cells for invasion in the brain and (ii) suppression of TUBB3 function holds the potential for improving the survival in brain metastasis patients.

TUBB3 plays an important role in axonal guidance of neurons and perturbations in TUBB3 function lead to ocular motility disorders (14). Within the brain only a specific subset of tubulins (TUBB3, TUBB2C, TUBB, TUBB2A, and TUBB2B) are expressed (15), whereas in normal breast epithelium (which express TUBB2C, TUBB1, TUBB2A, and TUBB6) TUBB3 is virtually absent (15). Overexpression of TUBB3 is associated with poor prognosis of multiple epithelial malignancies, including lung, colorectal, and ovarian cancers (16–20). Moreover, 85% of small-cell lung cancer patients exhibit TUBB3 overexpression (27), a cohort of which shows the highest incidence of CNS metastasis (28). These reports support the notion that TUBB3 overexpression imparts metastatic potential to cancer cells. However, there are no studies demonstrating a direct correlation of TUBB3 overexpression and brain metastases. We found overexpression of TUBB3 in human BCBM tissues in silico, which is in agreement with the report that TUBB3 overexpression in primary breast cancers is associated with poor prognosis (29). Although a clear relation between overexpression of TUBB3 and prognosis exist in the literature, its role in invasion and metastasis has not been evaluated.

Invasion of cancer cells holds a central role in the process of metastasis. It is a combined interplay of various cancer hallmarks that are governed by both interactions of cancer cell with the ECM components and cell signaling through surface receptors. In this context, we have evaluated the functional role of TUBB3 in invasion by generating TUBB3 knockdown cells in different breast cancer cell lines. In all these models, knockdown of TUBB3 levels significantly decreased the invasion of cells. Moreover, we found...
significant reduction in the adhesion ability of TUBB3-suppressed brain metastatic cells to ECM components (Laminin, fibronectin and collagen). This decrease in adhesion was also associated with compromised levels of L1CAM, a critical protein involved in the process of BCBM (6).

L1CAM binds to integrin β1, β2, and β3, and heterodimerization of L1CAM to integrins results in recruitment and auto phosphorylation of FAK. Phosphorylation of FAK recruits Src, which is then autophosphorylated, leading to activation of MAPK (30). This adhesion-mediated cascade of signaling then promotes invasion and colonization of cancer cells (6, 30). Integrins are overexpressed in malignant cells, and hence promote invasion and metastasis (31). Integrin αVβ3, αVβ6 and αVβ8 are overexpressed in human BCBM tissues (25). Given the critical role of integrin signaling in invasion and metastasis, we investigated the levels of integrin and found downregulation of integrin β3 levels in TUBB3-suppressed cells. These results are in concordance with decrease in invasion in MDA-Br TUBB3 knockdown cells. Taken together, our results suggest that TUBB3 regulates L1CAM and Integrin β3 levels to regulate the invasive nature of brain metastatic cells.

Moreover, to evaluate the consequences of TUBB3 knockdown on metastatic burden, we conducted an in vivo brain metastasis assay, which demonstrated a significant decrease in the number of spontaneous micrometastasis in mice injected with TUBB3-suppressed cells. Decreased metastatic load also correlated with improved survival in the preclinical model, which establishes TUBB3 as a critical mediator of brain metastasis.

Our results on proliferation of TUBB3-suppressed cells in vitro demonstrated marginal difference in proliferation (Supplementary Fig. S2B–S2D). However, tumor volume analysis of TUBB3-suppressed metastatic MDA-Br cells showed no difference. These results are in concordance with a very recent report on non–small cell lung cancer model, in which TUBB3 regulates lung cancer metastasis without altering growth of tumor cells in vivo in an anchorage-dependent manner (32). Our results are further supported by the fact that parental (MDA-231) and brain metastatic cells (MDA-Br) do not exhibit differences in proliferation (3), although MDA-Br demonstrated a 2.5-fold increase in βIII-tubulin protein levels. We therefore believe that TUBB3 in brain metastatic cells predominantly regulate the molecular players of invasion and metastasis.

Pharmacologic inhibition of invasion is believed to be a plausible approach for successful intervention of cancer

**Figure 6.**

**In vivo** brain metastatic capability of TUBB3 knockdown cells. A, orthotopic mammary gland injections of vector control and TUBB3 knockdown cells. A total of 1 × 10⁶ cells were injected in mammary fat pad and tumor growth was monitored for 15 weeks. B, in vivo brain metastasis assay. A total of 2.5 × 10⁵ cells were injected in nude mice via the intracardiac route. Mice were sacrificed 28 days and later the brains were removed for histologic analysis. Whole-brain histology image of mice injected with MDA-Br vector control, TUBB3 knockdown cells. C, evaluation of micrometastatic lesions. Average values from 10 sections per mouse brain (mean value and range; n = 9). D, Kaplan-Meier survival curve of vector control and TUBB3 knockdown cells. The log-rank test was applied to compare mice survival; (n = 9); **, P < 0.01.
progression. For example, inhibition of cathepsin B (via VBY-999; ref. 33) and COX2 (via cetuximab; ref. 3) decrease the occurrence of metastasis. These reports provide a clear evidence of preventing brain metastases before they arise. βIII-tubulin protein is known to be sensitive to vinorelbine-mediated drug therapy. In systemic lung cancer patients with TUBB3 overexpression, it has been shown that these patients benefit from vinorelbine-mediated chemotherapy with improvements seen in progression-free survival (34). Hence, it will be of interest to evaluate vinorelbine mediated prevention studies for inhibiting the occurrence of BCBM. In this context, our analysis of TCGA datasets (22, 23) implicates that, over time, the presence of high levels of TUBB3 in primary breast cancers predicts distant metastasis. Furthermore, our results in a brain metastasis model reveal that the suppression of TUBB3 in these cells decreases the incidence of BCBM. Taken together, TUBB3 in the primary/disseminated breast cancer cells regulates brain metastases of breast cancer and TUBB3 is a potential target for prevention of BCBM. Moreover, given that overexpression of TUBB3 predicts poor prognosis of lung (34, 35) and ovarian (19) cancer, this finding supports a broad role of TUBB3 in metastasis of different malignancies, which are known to metastasize to the brain (36–39). Hence, research on the role of TUBB3 in brain metastasis of other malignancies warrants further investigation.

In conclusion, our work suggests for the first time a novel role of TUBB3/βIII-tubulin in regulation of metastases and integrin-Src signaling. TUBB3/βIII-tubulin is, therefore, a possible marker for high-risk patients for development of brain metastases. Our work also demonstrates that TUBB3/βIII-tubulin regulates key mediators of brain metastases (Src and STAT3), and thus TUBB3/βIII-tubulin should be targeted to improve the prognosis of patients with metastatic brain cancer.

Disclosure of Potential Conflicts of Interest
No potential conflicts of interest were disclosed.

Authors’ Contributions
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Acquisition of data (provided animals, acquired and managed patients, provided facilities, etc.): D. Kanojia, R.A. Morshed, M.S. Lesniak
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