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**Authors**

Kanojia, Deepak

Morshed, Ramin

Zhang, Lingjiao

et al.

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# βIII-Tubulin Regulates Breast Cancer Metastases to the Brain

**Deepak Kanojia**[1], **Ramin A. Morshed**[1], **Lingjiao Zhang**[1], **Jason M. Miska**[1], **Jian Qiao**[1], **Julius W. Kim**[1], **Peter Pytel**[2], **Irina V. Balyasnikova**[1], **Maciej S. Lesniak**[1,*], and **Atique U. Ahmed**[1]

[1]The Brain Tumor Center, The University of Chicago, Chicago, IL 60637, USA

[2]Department of Pathology, University of Chicago Medical Center, Chicago, IL, 60637, USA

## Abstract

Brain metastases occur in about 10–30% 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.

### Keywords

βIII-tubulin; breast cancer; brain metastasis; integrin β3; invasion; neuronal gene expression

## Introduction

Brain metastases occur in about 10–30% 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. In order 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

*Corresponding author: Dr. Maciej Lesniak, M.D., The Brain Tumor Center, Department of Neurosurgery, 5841 South Maryland Ave, M/C 3026, Chicago, IL 60637; Tel (773) 834-4757; Fax (773) 834-2608 mlesniak@surgery.bsd.uchicago.edu.

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paired model systems where 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) (3), Nestin/CD133 (4), GABA receptor (5) and neuroserpin (6). This overexpression of neuronal markers by brain metastatic cells is believed to be an adaptation to survive in the brain microenvironment. However, the active role of these predominant brain markers in conferring BCBM has not been evaluated. Studies on molecular mechanisms of brain metastasis have also led to the identification of hyperactivated signaling pathways including Src (7), STAT3(8), Notch (9), IGFBP3 (10) and EGFR (11). The inhibition of these signaling mediators either decreased the formation of brain metastases (9–11) or improved survival in murine models of pre-existing brain metastases (7).

The unique cytoskeletal characteristics of a cancer cell are also critical mediators of its invasive behavior. Actin microfilaments are essential components for conferring metastatic potential through formation of invasive structures (12), whereas the role of microtubules is still under investigation (12). Microtubules are heterodimers of α and β subunits which are expressed in a tissue-specific manner (13). One such isotype is βIII-tubulin (*TUBB3*) which is specifically expressed by neurons (14). *TUBB3* is absent in normal mammary epithelia (15) however, it is ectopically expressed in breast, lung, ovarian, and colon cancers and has been found to confer paclitaxel resistance (16–19). Moreover, *TUBB3* overexpression is strongly associated with poor prognosis of epithelial malignancies (16–20).

In an attempt to understand the role of neuronal predominant markers in brain metastasis of breast cancer, we screened a panel of neuronal markers and found ectopic overexpression of TUBB3, Nestin and AchE in brain metastatic cells as compared to parental cells. Among these neuronal predominant markers, *in silico* analysis revealed overexpression of *TUBB3* in BCBM patients and its expression was significantly associated with distant metastases of breast cancer. Therefore, we focused our study on defining the role of *TUBB3* in brain metastases. Through *in vitro* and *in vivo* analysis, our results uncover a vital role of the cytoskeletal protein βIII-tubulin in conferring brain metastatic potential to breast cancer cells. Our findings, along with availability of clinically approved anti-microtubule agents, warrants investigation of *TUBB3* as a target for prevention of brain metastases in patients with breast cancer.

## Material 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, USA) with 10% FBS (Hyclone, Utah, USA). MDA-MB-468 cells (courtesy of Dr. Suzanne Conzen, University of Chicago) were maintained in RPMI-1640 (Corning, USA) medium with 10% FBS (Hyclone, Utah, USA) whereas GLIM2 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, USA). CN34/CN-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, MO, USA). No cell line authentication was done by the authors. 100 units/mL penicillin/streptomycin was added to all the cell culture media. Cells were routinely screened and found to be free of mycoplasma. For lentiviral transduction, non-target control shRNA and 2 different shRNA specific to *TUBB3* were procured (Sigma, MO, USA). Lentiviral particles were generated by transfection of shRNA and packaging vectors in HEK293 cells (Invitrogen, NY, USA). The primary antibodies employed were βIII-tubulin, phosphor-FAK, Src, phosphor-Src, STAT3, phosphor-STAT3 and actin (Cell signaling technologies, MA, USA), βIV tubulin (Sigma, MO), L1CAM (Thermo Fisher, IL), Integrin β3 (BioLegend, San Diego, CA) and Nestin (BD Pharmingen, San Jose, CA). Densitometric analysis was conducted using Image J software. Flow cytometry was conducted as described before (21).

### Patient samples and immunohistochemistry

The tissues were collected in accordance with a protocol approved by the Institutional Review Board (IRB) at the University of Chicago. Five micron thick sections of breast cancer and BCBM were deparaffinized in xylene and then rehydrated. After de-paraffinization and rehydration, tissue sections were treated with antigen retrieval buffer (S1699, DAKO) in a steamer for 20 minutes. βIII-tubulin rabbit monoclonal antibody (Cell Signaling Technology MA, USA) was applied on tissue sections at a dilution of 1:20 for 1-hour and incubated at room temperature in a humidity chamber. The antigen-antibody binding was detected by Bond Polymer Refine Detection (DS9800, Leica Microsystems).

### Quantitative real-time real time-polymerase chain reaction (qRT-PCR) analysis

One microgram RNA was isolated from breast cancer cells using RNeasy plus kit (Qiagen, Boston, MA) and was reverse-transcribed using iScript cDNA conversion kit (Biorad, CA, USA) according to the manufacturer's instructions. qRT-PCR was conducted using SYBR green qPCR kit (Biorad, CA, USA) using primers indicated in supplementary table S1. Data analysis was performed using the $2^{-\Delta CT}$ method for relative quantification, and all sample values were normalized to the GAPDH expression value.

### Migration assay

Cells ($1\times10^5$) were seeded in 6 well plates and grown to 95% confluency. A wound was created using a sterile 200μl pipette tip. The cells were fed with fresh medium with 1% serum and observed under inverted microscope for 28 hrs. Images were captured at various time points and migration of cells was measured using Image J software. The experiments were conducted three times in duplicates.

### Invasion assay

Matrigel invasion assay was conducted using BD Biocoat Matrigel invasion chambers according to manufacturer's instructions. Briefly, cells were seeded in serum free medium, in the top inserts and complete medium was used as a chemo-attractant at the bottom chamber. After 22hr the non-invading 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.

### Adhesion assays

Ninety-six well microplate was coated with laminin (5 μg/ml), fibronectin (2.5 μg/mL) and collagen (50 μg/ml). Plates were incubated overnight at 4°C for polymerization of ECM proteins. Unpolymerized substrates were washed with PBS and the plates were blocked with 2% BSA for 2 hr at 37°C. Vector control and *TUBB3* knockdown cells were plated ($4 \times 10^4$) and cell adhesion was monitored for 15–60 min at 37°C. Following incubation, the non-adherent cells were removed by 2 washes with PBS. The adherent cells were quantified using MTT assay (Roche) and expressed as a percentage relative of the respective total unwashed cells. The experiments were repeated twice in triplicates.

### Animal experiments

All surgical procedures were completed in accordance with NIH guidelines on the care and use of laboratory animals for research purposed. The protocols were approved by the Institutional Committee on Animal Use at the University of Chicago. Six to eight week old athymic nude female mice were obtained from Harlan laboratories and maintained in a specific pathogen free facility. Mice were anaesthetized with an intraperitoneal injection of 0.1 ml of a stock solution containing ketamine HCl (25 mg/ml), xylazine (2.5 mg/ml) and 14.25 % ethyl alcohol (diluted 1:3 IN 0.9 % NaCl) and inoculated with 250,000 MDA-Br Vector control and *TUBB3* knockdown cells in 100μl PBS via injection into the left ventricle of the heart. After 28 days or appearance of signs of morbidity, mice were sacrificed and whole brain tissues were excised immediately and embedded in OCT freezing agent. Approximately 100 sections with thickness of 10um across the horizontal plane were cut, air dried and stained with hematoxylin and eosin. For orthotopic mammary gland assay, sub-confluent MDA-Br Vector control and *TUBB3* knockdown cells were suspended in plain DMEM medium and mixed with Matrigel (Becton Dickinson) at a ratio of 1:1 in a volume of 50uL. $1X\ 10^6$ cells were injected in 3rd or 4th Mammary gland of HSd: athymic nude female mice and tumor size was monitored for 15 weeks.

### Statistical analysis

All statistical analyses were performed using Graphpad Prism 4 (GraphPad Software Inc., San Diego CA). The sample size for each group was   3 and numerical data was reported as Mean±SEM. The RNA levels of brain predominant markers were assessed using one sample t-test, and the differences on protein level were assessed using two sample Student's t test. Comparisons on cell migration, adhesion, and invasion were conducted using one-way or two-way ANOVA with Bonferroni or Dunnett's 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 standard deviation 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 employed 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 intra-cardiac 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 vs their corresponding parental cells (Figure 1A). These results were further confirmed in CN34-Br cells where TUBB3, Nestin and AchE were upregulated by 3.6. 1.46 and 2.1 fold, respectively (Figure 1B). However, there was no significant difference in other brain predominant markers which 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) (Figure 1C) and flow cytometry (Nestin) (Figure 1E). Densitometric analysis revealed 2.5/4.9 fold increase of *TUBB3* protein in MDA-Br/CN34-Br cells, respectively (Figure 1D), whereas nestin protein levels were elevated by 1.4/2 fold in MDA-Br/CN34-Br cells as compared to parental cells, respectively (Figure 1E, 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 to the primary breast tumors using GSE43837 dataset (Figure 2A). To evaluate if *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) (Figure 2B). Moreover using TCGA database we found that breast cancer patients with high *TUBB3* expression (22) exhibited poor survival as compared to patients with low *TUBB3* expression. (p=0.027 for Figure 2C) (23) and (p=0.007 for (Figure 2D) (22).

To substantiate our *in vitro* and *in silico* results, we performed immunohistochemistry on five human primary breast cancer and unmatched brain metastasis tissues (Figure 2E). Four samples of BCBM demonstrated overexpression of βIII-tubulin as compared to 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*

In order to understand the active role of *TUBB3* in breast cancer cells, we silenced *TUBB3* expression via lentiviral transduction using 2 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 to scrambled vector control cells (Supplementary figure S1 A, B and C). *TUBB3* knockdown cells exhibited gross morphological alterations like elongation of cells and loss of cellular structures (Supplementary Figure S2A) as compared to the vector control cells. This observation prompted us to evaluate if 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 hr (Figure 3A) in comparison to vector control cells. In addition, when complete closure of the wound was noted in vector control cells, the knockdown cells were unable to close the wound even at the end of 28hr (Figure 3A). These results indicate reduced migratory potential of MDA-Br *TUBB3* knockdown cells.

It is well known that augmented invasion is essential for metastasis of cancer cells. Hence, in order to evaluate if *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 (Figure 3B) as compared to 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 to vector control cells (Figure 3C). Moreover *TUBB3* suppressed GLIM2 cells demonstrated 9.8 (shRNA1) and 4.5 (shRNA2) fold decrease in invasion (Figure 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 (Figure 4A, B and C). These results depict that βIII-tubulin modulates the adhesion of cells to the ECM components.

Integrins are cell-surface proteins which 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. Employing the parental and brain metastatic derivative of MDA-231 cells, qRT-PCR analysis revealed 3 fold increase in integrin β3 transcripts in brain metastatic cells (MDA-Br) as compared to parental cells (MDA-231) (Figure 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 (Figure 4E) as compared to 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 if 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 auto-phosphorylated at Y397 residues. To ascertain if 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 (Figure 5A). Further, reduced FAK activation also resulted in blunted phosphorylation of Src at Y416 residues (Figure 5A).

Integrin mediated adhesion and signaling regulates activation of STAT3 (26). To evaluate if 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 (Figure 5A). These results demonstrate an important role of *TUBB3* in maintaining integrin-Src-STAT3 axis in the brain metastatic cells (Figure 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 (Figure 5B). These results underscore the role of βIII-tubulin in regulating the key signaling molecules which 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 Figure 2, B, C and D). Therefore, it was of interest to understand if 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 3rd and 4th 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 (Figure 6A).

To evaluate the *in vivo* functional consequences of *TUBB3* knockdown on metastatic behavior of cells, a brain metastasis model was employed. 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 microns) were isolated from each mouse followed by H&E staining. Ten sections of each brain were analyzed for the presence of number of micro-metastasis. 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 (Figure 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 (Figure 6D). These results demonstrate that *TUBB3* knock-down 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 (a) *TUBB3* overexpression is a malignant adaptation of breast cancer cells for invasion in the brain and (b) 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, TUBB2B) are expressed (15), whereas in normal breast epithelium (which express TUBB2C, TUBB1, TUBB2A, 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 (SCLC) 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 which are governed by both interactions of cancer cell with the ECM components and cell signaling through surface receptors. In this context, we evaluated the functional role of *TUBB3* in invasion by generating *TUBB3* knockdown cells

in different breast cancer cells. 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 L1 cell adhesion molecule (L1CAM), a critical protein involved in the process of BCBM (6).

L1CAM binds to integrin β1, β2 and β3, and hetro-dimersation of L1CAM to integrins results in recruitment and auto phosphorylation of focal adhesion kinase (FAK). Phosphorylation of FAK recruits Src, which is then auto phosphorylated 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 micro-metastasis 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 Figure S2B–D). 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, where *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 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.

Pharmacological inhibition of invasion is believed to be a plausible approach for successful intervention of cancer progression. For example, inhibition of cathepsin B (via VBY-999) (33) and COX2 (via cetuximab) (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.

## Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

## Acknowledgments

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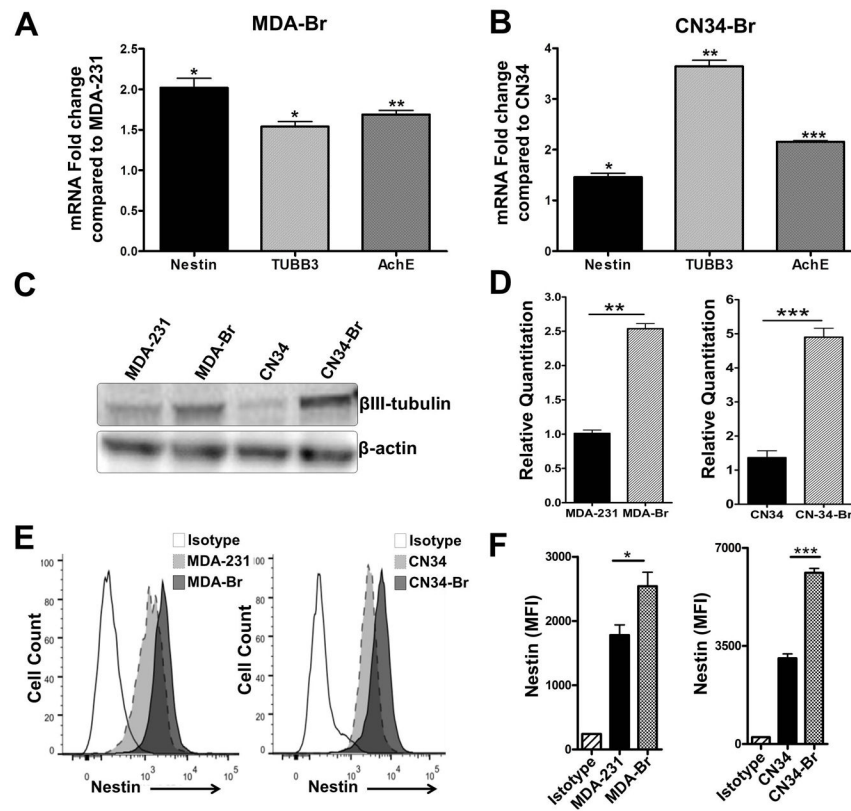
**Figure 1. Overexpression of *TUBB3* in BCBM**

**(A)** Quantitative RT-PCR analysis of Nestin, TUBB3 and AchE expression in MDA-Br cells as compared to parental MDA-231 cells. **(B)** Quantitative RT-PCR analysis of Nestin, TUBB3 and AchE expression in CN34-Br cells as compared to CN34. The primers used for quantitative PCR are listed in supplementary table S1. Data are representative of three independent experiments. **(C)** Western blot demonstrating overexpression of βIII-tubulin in MDA-Br and CN34-Br cells as compared to respective parental cells. **(D)** Semi-quantitative densitometry analysis was performed using Image J software. Data are representative of three independent experiments. **(E)** Flow cytometry of MDA- 231/MDA- Br and CN34/ CN34-Br cells stained with nestin antibody. **(F)** Quantitation of nestin MFI. *** indicates p<0.001; ** indicates p<0.01; * indicates p<0.05.
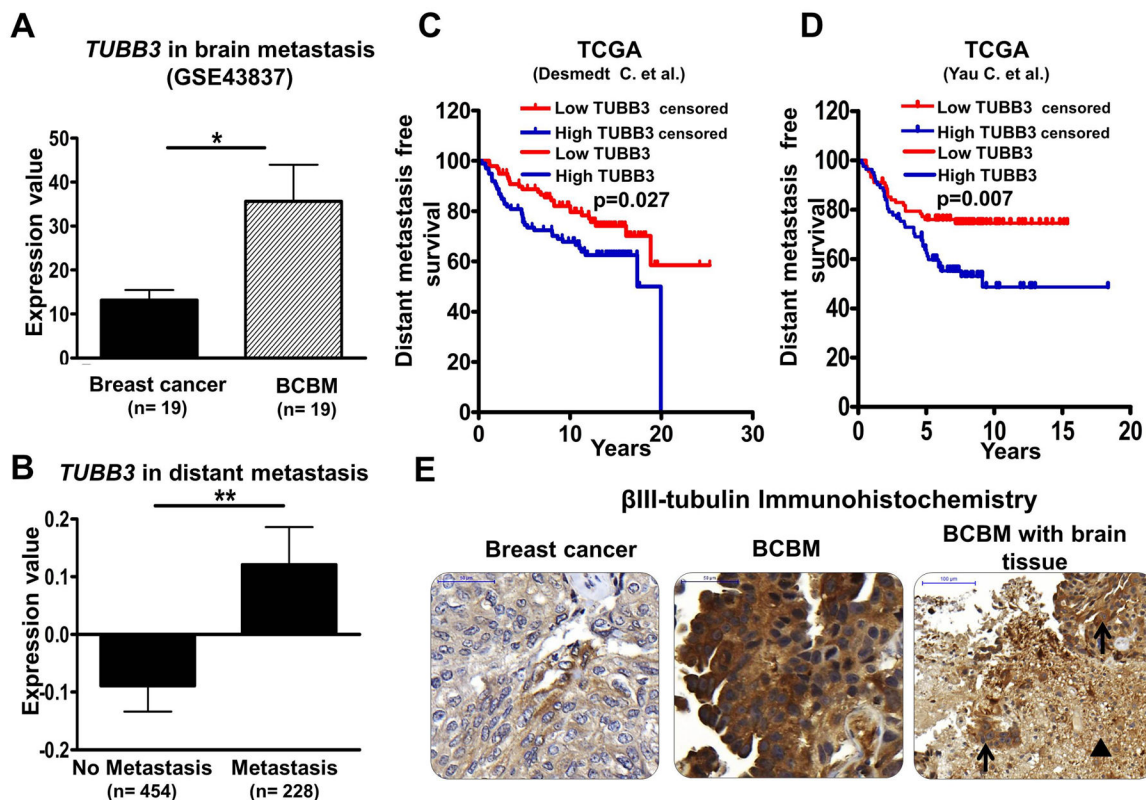
**Figure 2. Overexpression of *TUBB3* in brain metastasis and distant metastasis patients**
**(A)** Analysis of GSE43837 dataset demonstrating overexpression of *TUBB3* in brain metastasis as compared to primary breast cancer (n=38). **(B)** Analysis of microarray dataset, demonstrating a significant correlation of *TUBB3* expression with distant metastasis of breast cancer (22) (n=682), p=0.008. **(C and D)** Distant metastasis free survival analysis of breast cancer patients exhibiting high or low levels of *TUBB3* (n=171) and n=198 respectively. **(E)** Immunohistochemical analysis of βIII-tubulin in breast cancer and BCBM. In brain metastasis, βIII-tubulin shows strong staining in brain metastatic cells as compared to the breast cancer cells (n=5). Scale Bar = 50μm. There is some weaker staining in the adjacent brain parenchyma and in particular in gemistocytic astrocytes at the edge of the lesion. Arrow indicates BCBM and arrowhead indicates brain tissue. Scale Bar = 100μm. *** indicates p<0.001; ** indicates p<0.01; * indicates p<0.05.
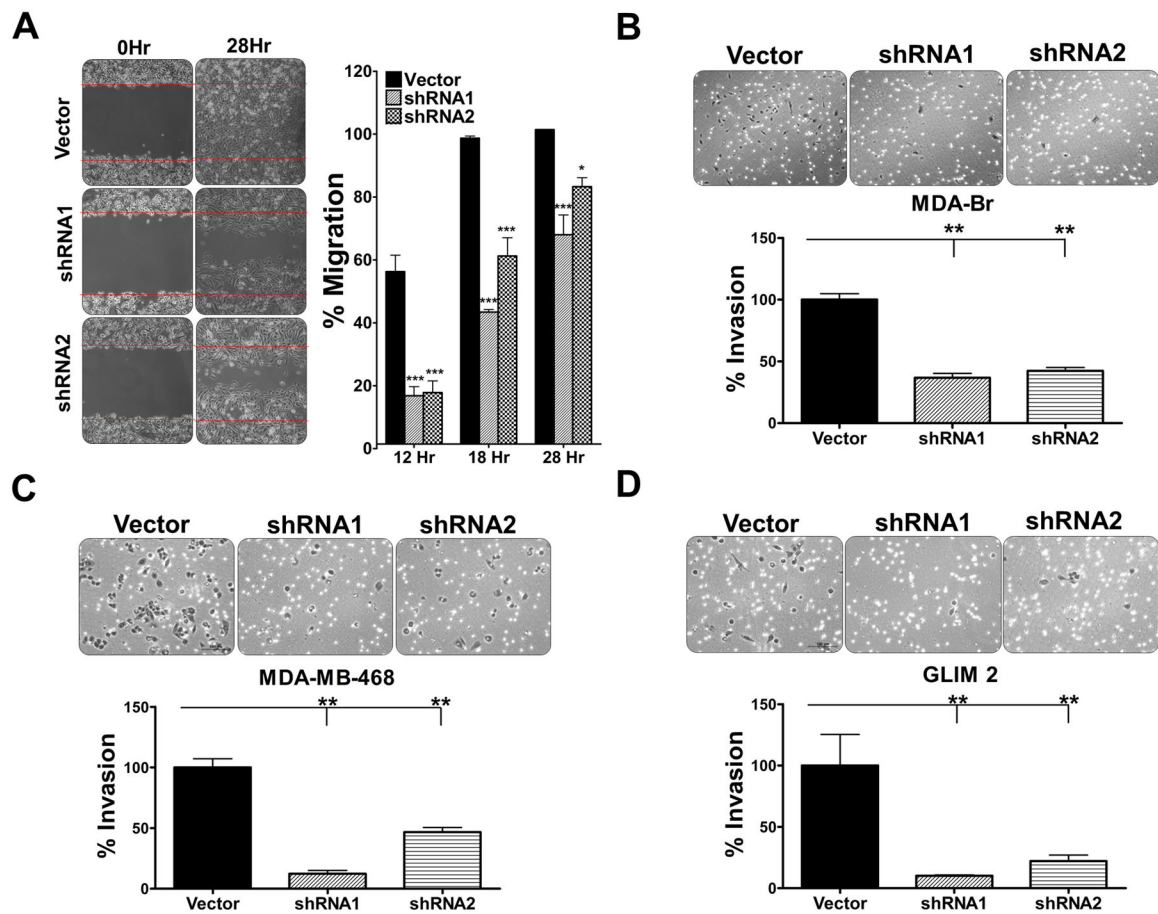
**Figure 3.** *TUBB3* **suppression decrease migration and invasion of breast cancer cells**
Scratch wound assay for migration **(A)** Images demonstrating wound closure of vector control MDA-Br and *TUBB3* knockdown cells at 0hr and 28 hr time points. Right panel depicts statistical analysis of migration using image J software. Images showing invasion of vector control and *TUBB3* knockdown cells. **(B)** MDA-Br, **(C)** MDA-MB-468 and **(D)** GLIM2 cell lines, respectively. (from left to right) vector control, shRNA1 and shRNA2. Graph indicates percentage of invasion as compared to vector control cells. The results represent means ± S.E.M. for triplicate experiments. *** indicates p<0.001; ** indicates p<0.01; * indicates p<0.05.
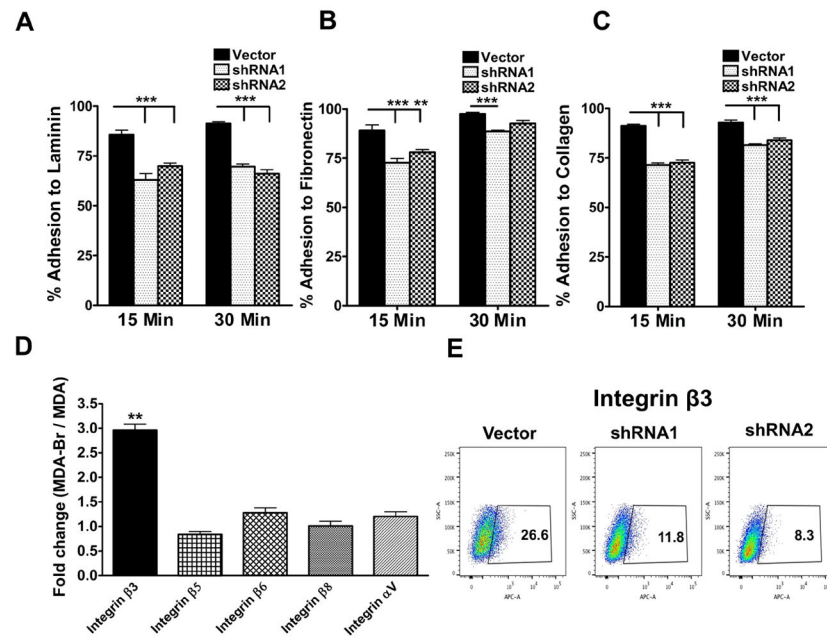
**Figure 4. Decreased adhesion of MDA-Br *TUBB3* knockdown cells to laminin, fibronectin and collagen**

Adhesion assay was performed by seeding equal amount of cells of different groups in 96-well tissue culture plates coated with (A) laminin, (B) fibronectin **C** and collagen. The results represent means ± S.E.M. for triplicate experiments. **(D)** Quantitative RT-PCR analysis of the expression of Integrin genes in MDA-Br cells as compared to parental MDA-MB-231 cells. **(E)** Flow cytometric analysis of β3 integrin showing *TUBB3* shRNA cells demonstrating reduction in β3 integrin levels. ** indicates $p<0.01$, *** indicates $p<0.001$.
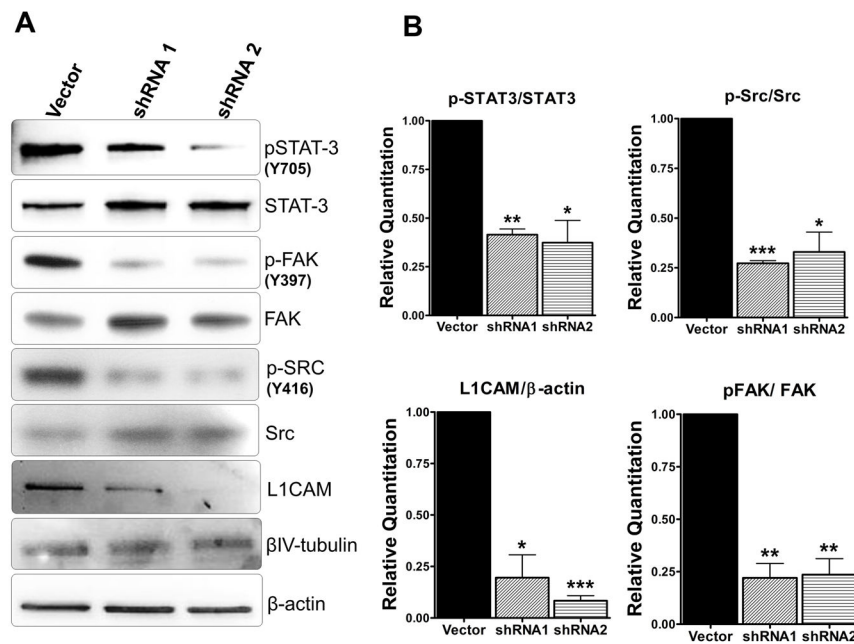
**Figure 5. *TUBB3* knockdown in brain-seeking MDA-Br cells, attenuates FAK, Src and STAT3 signaling**

**(A)** Western blotting demonstrating decrease in phosphorylation of FAK/Src/STAT3 and decreased levels of L1CAM in *TUBB3* knockdown cells as compared to vector control MDA-Br cells. **(B)** Densitometric analysis of pFAK, pSrc, pSTAT3 and L1CAM. The experiments were repeated three times. *** indicates p<0.001; ** indicates p<0.01; * indicates p<0.05.

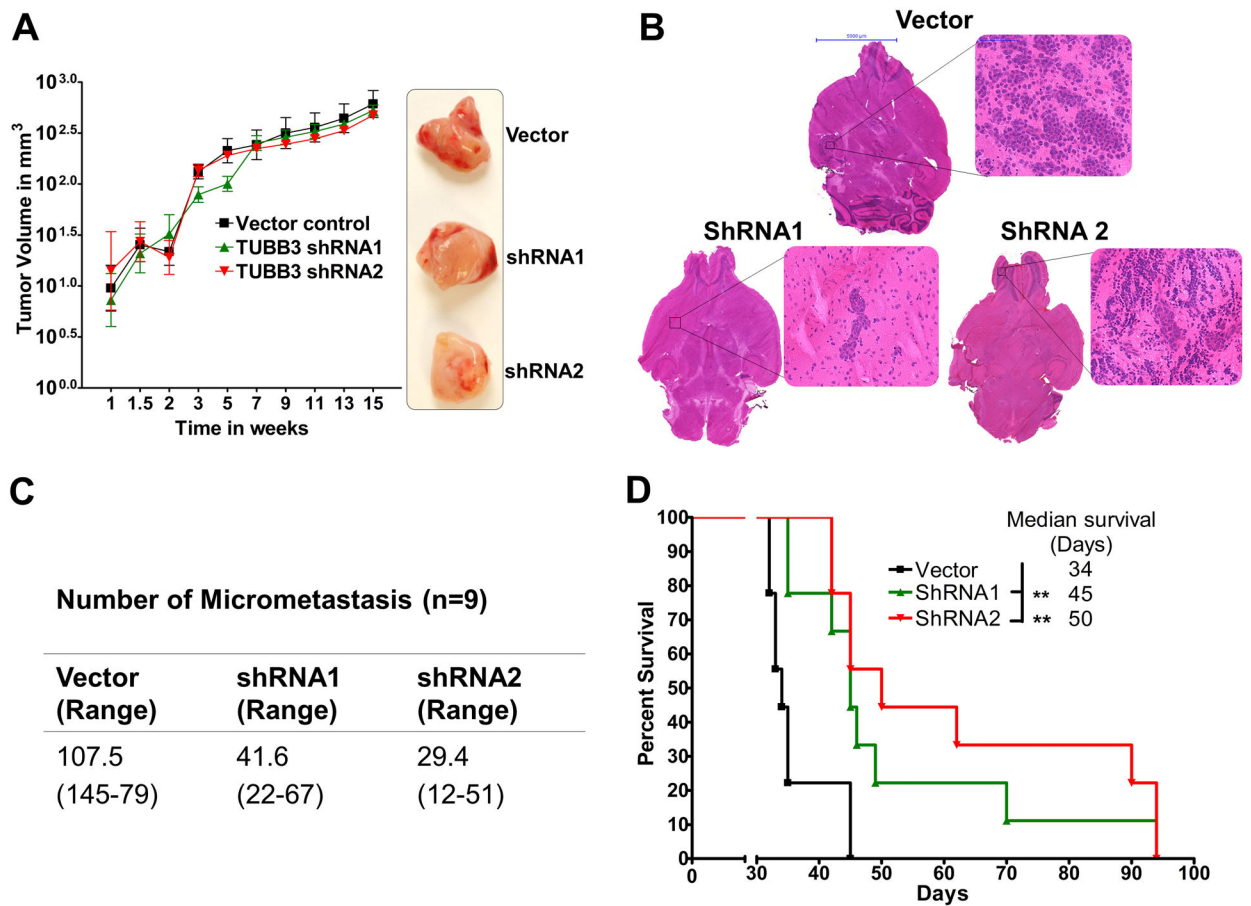**Figure 6.** *In vivo* **brain metastatic capability of** *TUBB3* **knockdown cells**
(**A**) Orthotopic mammary gland injections of vector control and *TUBB3* knockdown cells. $1\times10^6$ cells were injected in mammary fat pad and tumor growth was monitored for 15 weeks. (**B**) I*n vivo* brain metastasis assay. $2.5\times 10^5$ cells were injected in nude mice via the intracardiac route. Mice were sacrificed 28 days and later the brains were removed for histological 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. Log rank test was applied to compare mice survival. (n=9). ** indicates $p<0.01$.