Regulation of brain metastatic colonization of triple-negative breast cancer by novel genes

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Background of research

Breast cancer is the most common cancer affecting women. Despite recent improvement in mortality of breast cancer, this disease remains the second most common cause of death of women worldwide. In 2020, it is estimated that more than 117,000 women in China and Hong Kong die from breast cancer (WHO Global Cancer Observatory). Triple-negative breast cancer (TNBC), which lacks expression of estrogen receptor and progesterone receptor as well as HER2 amplification, accounts for 15-20% of breast cancer and is more aggressive with poorer prognosis compared to other subtypes [1, 2]. Given the lack of effective targeted therapy [3], there is an urgent clinical need to identify novel therapeutic targets for TNBC.

Brain metastasis occurs in ~20-40% of advanced stage cancers [4]. Despite recent advances in radiotherapy and neurosurgery, >50% of patients die from their brain metastases. Unlike other cancers where brain metastases appear in more advanced stages, TNBC usually spreads to the brain at an earlier stage [5]. Study of brain metastasis has been hampered by the paucity of robust preclinical models. Nevertheless, recent work has begun to shed light on molecular mechanisms of brain metastasis colonization. After traversing blood brain barrier (BBB), tumor cells enter parenchyma where they interact with various stromal cells including microglia, neurons, endothelial cells and astrocytes [6]. Glial fibrillary acidic protein (GFAP)-positive reactive astrocytes have been shown to serve as the first defense by producing plasmin. Interestingly, tumor cells can secrete serpins, inhibitors of plasminogen activator, to allow their survival [7]. In contrast to the anti-tumor function, emerging evidence has shown cancer-promoting roles for astrocytes. For example, astrocytes can induce PTEN loss in metastatic TNBC cells via exosomal microRNA, resulting in brain metastasis outgrowth [8]. In addition, a subpopulation of STAT3+ reactive astrocytes was reported to drive a pro-metastatic environment. In fact, promising results were observed in a clinical trial where blocking STAT3 signaling significantly inhibited brain metastasis [9]. In another recent study, 3-phosphoglycerate dehydrogenase, which catalyzes glucose-derived serine synthesis, was demonstrated to be critical for the outgrowth of breast and melanoma metastases in the nutrient-limited brain environment [10]. These studies highlight the potential of therapeutic targeting the metastatic vulnerabilities, but also inform us that a deep understanding of the interplay between tumor cells and the microenvironment is far from complete. Based on the compelling data that breast cancer cells can adopt brain-like properties, such as GABAergic phenotype in neurons, upon metastasizing to the brain [11], *we aim at identifying novel genes that enable TNBC cells to adapt the brain niche*. In recent years, we have utilized bioinformatics approaches to successfully identified multiple novel TNBC-specific oncogenes [12]. In this study, we applied bioinformatics analysis to search for TNBC driver genes that play critical roles in primary tumors as well as brain metastasis. Using clinically annotated gene expression datasets (n = 310; [13]), we found that TUBB2B mRNA is upregulated in 29% of TNBC cases, compared to 3% in luminal breast tumors (**Fig. 1A**). In breast cancer patients, TUBB2B is found to be upregulated in brain metastases, compared to lung or bone metastases, (**Fig. 1B**; GSE14017). Xenograft studies also showed a higher expression of TUBB2B in TNBC brain metastases than in primary tumors [8]. Importantly, TUBB2B overexpression is a predictor of poor distant metastasis-free survival in breast cancer patients (**Fig. 1C**). These clinical data highlight the potential of TUBB2B's oncogenic function in TNBC.

TUBB2B is a β-tubulin isoform which is primarily expressed in the brain in embryonic stage and plays a key role in axon guidance during development [14]. Loss-of-function mutations in TUBB2B result in congenital neuronal disorders [15]. In the context of cancer, recent studies reported overexpression of TUBB2B in hepatocellular carcinoma (HCC), neuroblastoma and Hodgkin lymphoma [16]. Recently, TUBB2B was shown to promote HCC growth by regulating cholesterol metabolism [17]. The role of TUBB2B in the pathogenesis of breast cancer or metastasis, however, is yet to be studied. To explore the function of TUBB2B in TNBC, we generated a panel of breast cancer lines with TUBB2B knockdown or knockout. **Fig. 2A** shows significant depletion of TUBB2B mRNA with 2 distinct shRNAs in TNBC BT549 line. TUBB2B

silencing resulted in potent inhibition of TNBC viability in 2D cultures (**Fig. 2C**). We are particularly excited about our preliminary data that TUBB2B depletion did not affect viability of normal human mammary epithelial cells (HMEC) (**Fig. 2C**), which indicates the potential of targeting TUBB2B for therapeutic purposes. To examine the role of TUBB2B in TNBC brain metastatic growth, the tet-on doxycycline (dox)-inducible Crispr/Cas9 knockout approach was utilized [18]. Upon dox administration, TUBB2B was depleted significantly with two distinct gRNAs (**Fig. 2B**). We have recently set up an *in vivo* model for brain metastasis using an improved intracarotid injection method (**Fig. 3A-C**) [19]. Brain metastases were generated in different regions of the brain, including striatum, hippocampus, cortex and thalamus (**Fig. 3D, 3E**). Our preliminary data demonstrated that depletion of TUBB2B in TNBC cells potently inhibited brain metastasis colonization in xenograft models (**Fig. 3F**). Importantly, upon co-culturing TNBC spheroids with astrocytes, TNBC growth was enhanced significantly, and the growth-promoting effect of astrocytes on TNBC cells was abrogated by TUBB2B depletion in tumor cells (**Fig. 3G**). Based on these data, we hypothesized that there is a positive feedback loop between TUBB2B in TNBC cells and astrocytes. Indeed, TUBB2B expression in TNBC cells were increased potently when treated with astrocyte-conditioned medium (ACM; **Fig. 3H**). Furthermore, astrocytes were activated by TNBC-conditioned medium (CM), where the effect was diminished upon TUBB2B knockdown (**Fig. 3I**). These observations prompt us to investigate the interplay between TNBC cells and astrocytes as well as the role of TUBB2B in the brain microenvironment in this proposed study.

Small interfering RNA (siRNA) has emerged as a powerful technology in silencing specific gene by degrading its mRNA, and is widely explored as therapeutic approach for difference diseases. FDA has recently approved four siRNA medications, and many siRNA-based therapeutics have entered clinical investigations [20]. siRNA has also been applied as a novel cancer therapy, particularly for genes that encode "undruggable" targets. The vulnerability of siRNA to degradation inside the body, however, remains a major issue in delivery. Gold nanoparticles (AuNPs) have been proven to not only enhance siRNA stability and circulation half-life, but also cross BBB effectively to deliver siRNAs to intracranial tumors. In particular, an siRNA-based AuNP therapeutic agent (drug moniker: NU-0129) is currently being tested for treating glioblastoma (GBM) in phase 0 first-in-human trial (NCT03020017) [21]. Notably, no significant toxicity was observed in patients. Based on the unique advantages of AuNPs, in our project, we will apply the same strategy by using AuNP cores covalently conjugated with siTUBB2B, and we anticipate that it will potently reduce tumor-associated TUBB2B expression and suppress brain metastasis colonization. In this project, we will collaborate with **Gigi Lo, PhD** (City University of Hong Kong), who has expertise in developing nanomedicine for cancer therapy, to examine the effect of intravenous administration of siTUBB2B/AuNPs on TNBC brain metastatic growth. The principal goal of this project is, therefore, to explore the TUBB2B molecular networks for therapeutic intervention of TNBC brain metastasis colonization. With the *in vitro* and *in vivo* models in place, together with my expertise in dissecting molecular mechanisms of TNBC, I feel that I have both the tools and a sound rationale to conduct the proposed studies.

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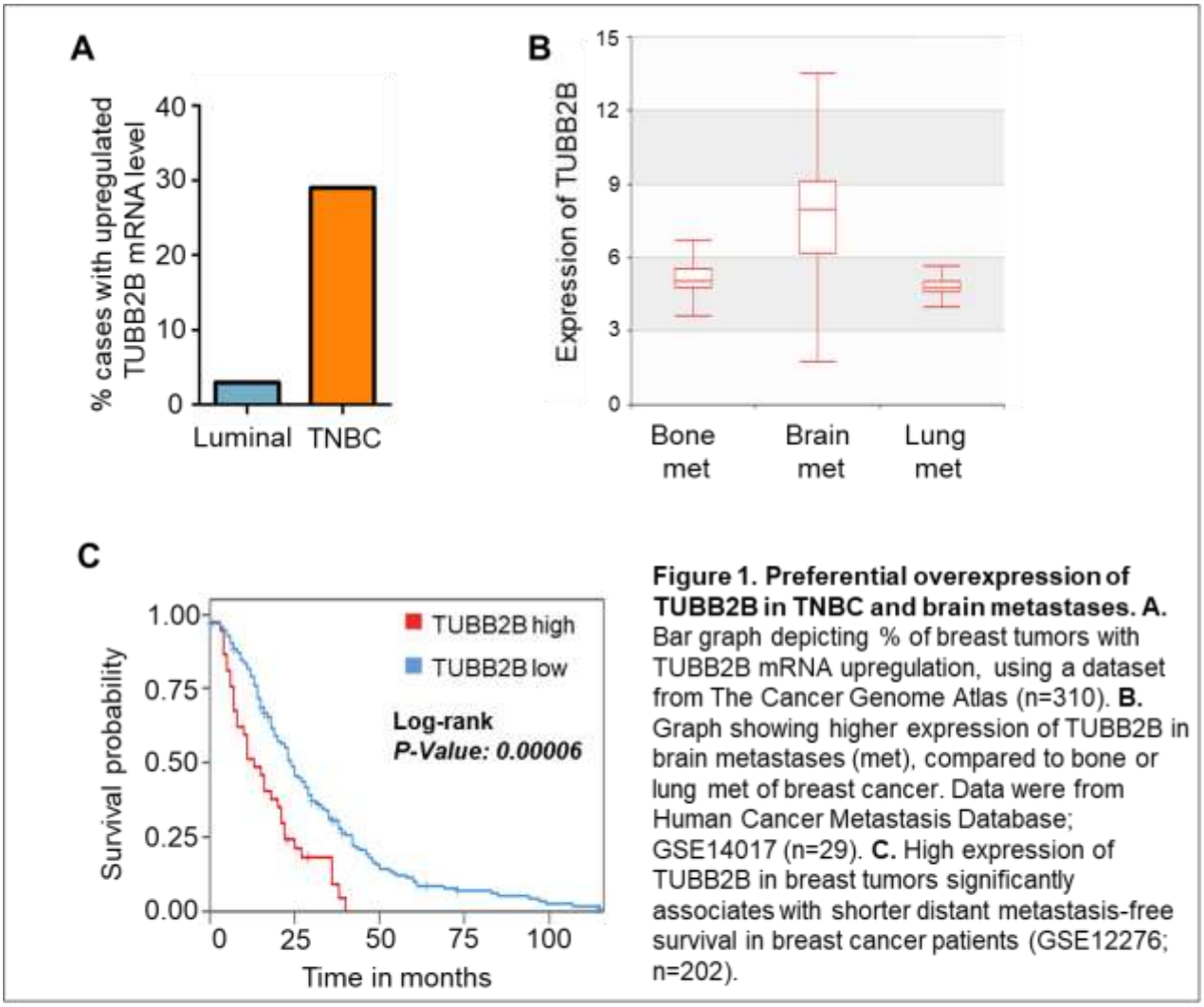

Figure 1. Preferential overexpression of TUBB2B in TNBC and brain metastases. A. Bar graph depicting % of breast tumors with TUBB2B mRNA upregulation, using a dataset from The Cancer Genome Atlas (n=310). **B.** Graph showing higher expression of TUBB2B in brain metastases (met), compared to bone or lung met of breast cancer. Data were from Human Cancer Metastasis Database; GSE14017 (n=29). **C.** High expression of TUBB2B in breast tumors significantly associates with shorter distant metastasis-free survival in breast cancer patients (GSE12276; n=202).

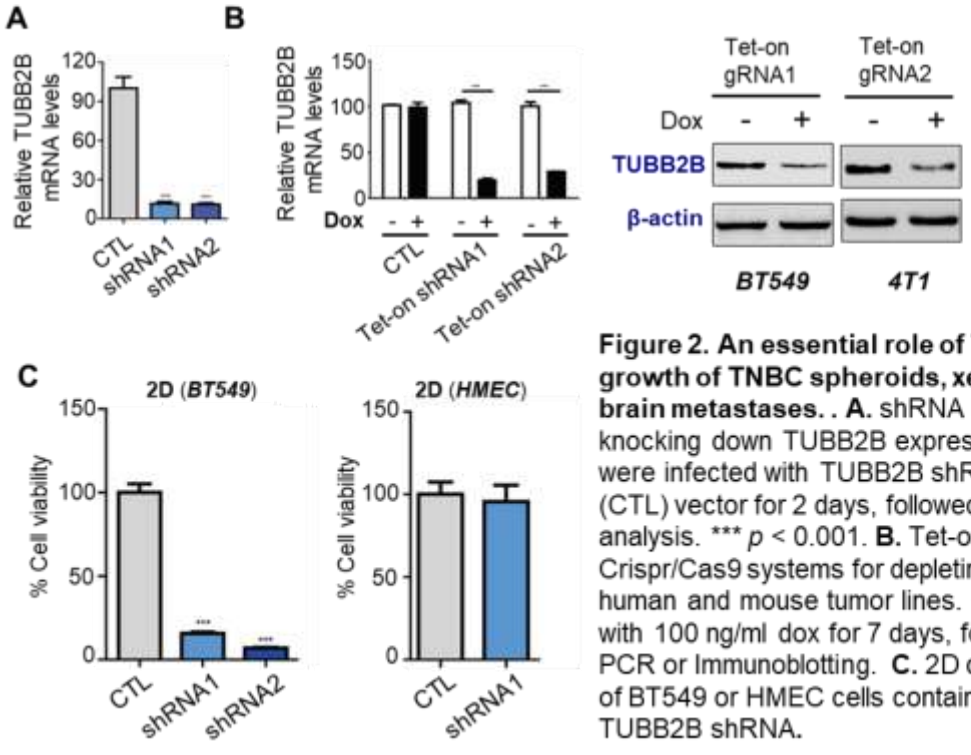

Figure 2. An essential role of TUBB2B for the growth of TNBC spheroids, xenografts and brain metastases. . A. shRNA lentiviral system for knocking down TUBB2B expression. BT549 cells were infected with TUBB2B shRNA or control (CTL) vector for 2 days, followed by RT-PCR analysis. *** $p < 0.001$. **B.** Tet-on shRNA and Crispr/Cas9 systems for depleting TUBB2B in human and mouse tumor lines. Cells were treated with 100 ng/ml dox for 7 days, followed by RT-PCR or Immunoblotting. **C.** 2D cell titer-Glo assay of BT549 or HMEC cells containing CTL or TUBB2B shRNA.

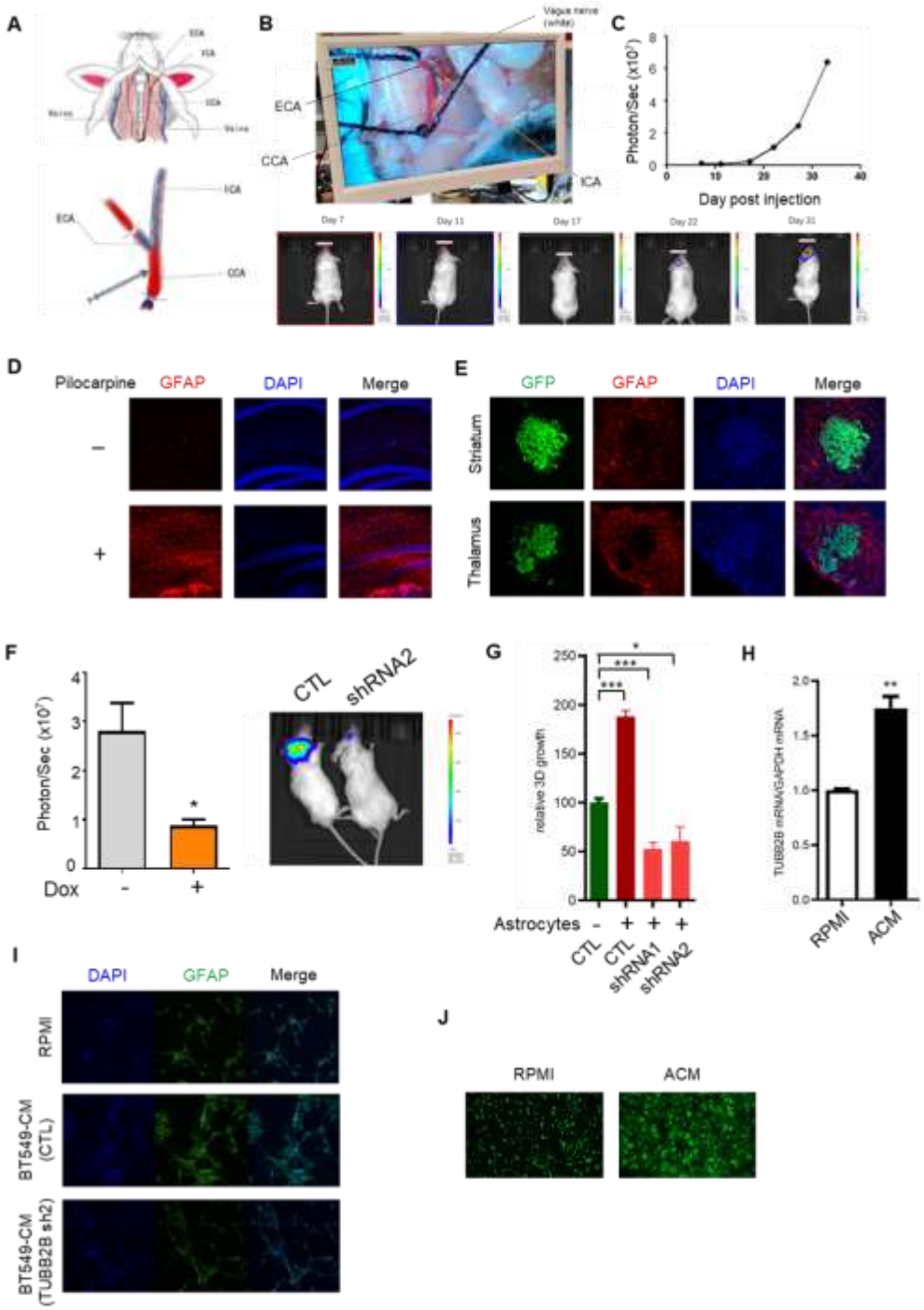

Figure 3. An essential role of TUBB2B for the outgrowth of TNBC brain metastasis. A. Establishment of brain metastasis via intracarotid injection. Schematic showing anatomical position of different carotid arteries. Injection and ligation sites are shown. External carotid artery (ECA), Internal carotid artery (ICA), Common carotid artery (CCA). **B.** Surgery done in our lab for ligating ECA and CCA. **C.** Successful establishment of brain metastasis in mice and monitoring of bioluminescence signals over time, demonstrated in the representative images and graph. **D.** Staining of activated astrocytes in hippocampus. Astrocytes were stimulated with Pilocarpine (275mg/kg), followed by immunofluorescence (IF) labelling with GFAP antibody. **E.** Activated astrocytes associated with TNBC brain metastasis in mice. IF labeling of activated astrocytes (GFAP+) and tumor cells (GFP+) in brain metastasis. Images correspond to metastases found in different regions of the brain at 35 days post-injection. **F.** MDA-MB-468 cells containing TUBB2B gRNA were treated with dox for 7 days. Cells were then subject to intracarotid injection (n = 4). Bar graphs depict tumor BLI signals on day 27 post-injection. * $p < 0.05$. Right: representative BLI images. **G.** HCC1143 spheroids containing TUBB2B shRNA or CTL shRNA were co-cultured with astrocytes (1:1 ratio) or cultured alone for 48 h, followed by 3D Cell-titer assays. **H**. Astrocyte-conditioned medium (ACM) was prepared by growing astrocytes in RPMI complete medium for 72 h. HCC1806 cells were cultured in ACM or RPMI for 48 h, followed by RT-PCR. **I**. TNBC-CM was prepared from BT549 cells containing TUBB2B shRNA or CTL shRNA. Astrocytes were cultured with RPMI or TNBC-CM for 48 h, followed by GFRAP staining. **J**. BT549 cells were treated with ACM or control medium followed by transwell migration assay for 4h.