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Expression of Tax-interacting protein 1 (TIP-1) facilitates angiogenesis and tumor formation of human glioblastoma cells in nude mice

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ABSTRACT

Glioblastoma is the most common and fatal type of primary brain tumors featured with hyperplastic blood vessels. Here, we performed meta-analyses of published data and established a correlation between high TIP-1 expression levels and the poor prognosis of glioblastoma patients. Next, we explored the biological relevance of TIP-1 expression in the pathogenesis of glioblastoma. By using orthotopic and heterotopic mouse models of human glioblastomas, this study has characterized TIP-1 as one contributing factor to the tumor-driven angiogenesis. *In vitro* and *in vivo* functional assays, along with biochemical analyses with microarrays and antibody arrays, have demonstrated that TIP-1 utilizes multiple pathways including modulating fibronectin gene expression and uPA protein secretion, to establish or maintain a pro-angiogenic microenvironment within human glioblastoma. In conclusion, this work supports one hypothesis that TIP-1 represents a novel prognostic biomarker and a therapeutic target of human glioblastoma.

1. Introduction

Glioblastoma (also known as glioblastoma multiforme or GBM, WHO grade IV malignant glioma) is the most common and fatal type of primary brain tumor. These tumors are featured with highly invasive growth into normal surrounding tissues and a vasculature-rich tumor mass. Even with the most aggressive treatment including surgical resection of as much of tumor as possible, concurrent or sequential chemoradiotherapy, antiangiogenic therapy and symptomatic management, newly diagnosed patients with glioblastoma typically have a survival time of about 16 months [1]. Extensive studies have identified dozens of genetic or epigenetic factors that control the aggressive tumor growth and treatment resistance [2–5].

TIP-1, also known as Tax1-binding protein 3 (Tax1bp3) and glutaminase-interacting protein (GIP), is a small (124 amino acids in human and mouse) and conserved protein containing single PDZ (PSD-95/DlgA/ZO-1) domain [6]. TIP-1 involves in a wide spectrum of biological processes through selective protein interactions, such as beta-catenin [7], brain-specific angiogenesis inhibitor 2 [8], rhotekin [9], HPV16 E6 [10], HTLV-1 Tax [11], ARHGEF16 [12], potassium channel Kir2.3 [13], and glutaminase [14]. TIP-1 has been reported to regulate gastrulation movements during zebrafish embryo development [6]. In Madin-Darby canine kidney (MDCK) cells, TIP-1 modulates trafficking of intracellular proteins and contributes to the establishment of cell polarity [13]. TIP-1 is required for the HPV16 E6 oncoprotein-induced cell transformation [10]. TIP-1 has also been shown with inhibitory function on the transcriptional activity of beta-catenin and colon cancer cell proliferation [7]. However, the biological roles of TIP-1, especially in tumorigenesis, remain largely unclear.

Our previous studies have documented the elevated TIP-1 expression levels in human invasive breast cancers [15]. It was found that TIP-1 expression in human breast cancer cells contributes to cellular adhesion to extracellular matrix, invasion and pulmonary metastasis in mouse models. In this study, we further expanded the association of TIP-1 expression with cancer progression to human glioblastoma. Meta-analyses of published data indicated a correlation between the elevated TIP-1 expression levels and the poor prognosis of patients with glioblastoma. By using orthotopic and heterotopic mouse models of human glioblastomas, we have identified a novel function of TIP-1 protein in the early stages of glioblastoma formation. The data demonstrated that TIP-1 facilitates tumor-driven angiogenesis and promotes tumor formation of human glioblastoma cell lines in nude mice.

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Biochemical analyses with microarrays and antibody arrays further suggested that TIP-1 might utilize multiple pathways including modulating fibronectin gene expression and uPA protein secretion, to establish or maintain a pro-angiogenic tumor microenvironment. This work, along with our ongoing studies, supports one hypothesis that TIP-1 represents a novel therapeutic target and prognostic biomarker of human glioblastoma.

2. Materials and methods

2.1. Antibodies and reagents

Rabbit anti-human TIP-1 was produced as described previously [16]. Antibodies against IGFBP3 and SPP1 were purchased from Santa Cruz Biotechnology (Santa Cruz, CA). Antibodies recognizing FN1 were from Epitomics (Burlingame, CA). Anti-Ki67 antibody was from Abcam (Cambridge, MA) and the TUNEL assay kit from Promega (Madison, WI). HRP- or fluorescence-labeled secondary antibodies were from Invitrogen (Carlsbad, CA). Anti-actin antibody and chemicals were purchased from Sigma (St. Louis, MO) unless otherwise stated.

2.2. Cell culture, RNA interference and cell transfection

Human glioma cell lines D54 or U87 were obtained from Dr. Yancie Gillespie (University of Alabama—Birmingham, Birmingham, AL) and ATCC (Manassas, VA), respectively. All the cell lines were genetically and morphologically verified by the provider before the experiments. Constructs expressing validated small hairpin RNA (shRNA) were purchased from Sigma, including two TIP-1-specific shRNAs (TIP-1 shRNA #1: 5′-GGCTAACAGCTGATCCCAA-3′, TIP-1 shRNA #2: 5′-GCAAAGA GTTGAAATTCACAA-3′) matching different regions of the human TIP-1 mRNA transcript. A non-targeting sequence was used as a negative control. Two validated small interfering RNAs (siRNAs) for FN1 (FN1 siRNA-1: 5′-CAAUUACACUGAUUGCA CU-3′ and FN1 siRNA-2: 5′-CACUUAUGAGCGUCCUAAA-3′) were purchased from Sigma. RNAi MAX (Invitrogen) was used for siRNA transfection by following the manufacturer's instructions. The RNAi-tranfected cells were used for experiments 48–72 h after the transfection. Transfection of the cells with the recombinant plasmids and formation of stable clones were achieved by standard protocols [17]. Protein expression level was detected by western blot analysis of whole cell lysates with the specific antibodies.

2.3. Tumor growth studies in mouse models

Tumor formation and growth were studied with intracranially and subcutaneously implanted xenograft models. In the intracranially implanted xenograft model, human glioblastoma cell lines (D54 or U87 with or without TIP-1 knockdown) were genetically modified with a luciferase-expressing construct (Addgene, Cambridge, MA). A total of 2×10^4 cells in 20 μl of phosphate-buffered saline (PBS) were injected 3 mm deep (from the skull surface) into the right burr hole of FoxN1-null nude mice (Harlan Laboratories, Prattville, AL). Tumor formation and growth were monitored every 3 days by bioluminescence imaging (BLI) [18] using an IVIS 200 imaging station (Caliper Life Sciences, Hopkinton, MA). The bioluminescence signal was acquired 12 min after intraperitoneal injection of luciferin (D-luciferin potassium salt, 150 mg/kg, Caliper Life Sciences). In the subcutaneous xenograft model, 5×10^5 D54 cells, with or without TIP-1 knockdown, were resuspended in 200 μl of PBS for subcutaneous injection into the flanks of FoxN1-null mice. Tumor sizes were measured with calipers every other day until the tumor volume reached 700 mm^3. Tumor tissues were resected for immunohistochemical (IHC) staining to determine cell death, cell proliferation and blood vessel formation. All the animal studies were approved by the Institutional Animal Care and Use Committee (IACUC) at Vanderbilt University.

2.4. Matrigel-plug assay

The impact of TIP-1 and fibronectin on D54 tumor angiogenesis were studied with matrigel-plug assays. Briefly, 5×10^5 D54 cells were resuspended in 150 μl PBS and mixed with 350 μl ice-cold matrigel (BD Biosciences, Rockville, MD). The cell/matrigel mixture was subcutaneously injected into the hind limbs of FoxN1-null mice. In order to minimize the variations between animals, cells with a specific gene manipulation and those with vector control were implanted in the same animal, respectively. Roles of fibronectin in TIP-1 regulated tumor angiogenesis were also studied by adding the purified recombinant fibronectin protein (25 μg/ml in PBS, Sigma) to the matrigel mixture with TIP-1 knockdown cells. To visualize the functional blood vessels within the matrigel-plugs, 150 μl of FITC-dextran (25 mg/ml, 2000 kD, Sigma) were injected through tail veins 15 min prior to sacrificing animals. The matrigel-plugs were removed for examination with a confocal fluorescence microscope, and FITC signals within the matrigel plugs were quantified with ImageJ software (http://rsbweb.nih.gov/ij/index.html). The matrigel-plugs were also used to extract tumor cells for quantitative measurement of angiogenic factors with antibody array or fixed with formaldehyde and embedded in paraffin for IHC staining [19].

2.5. Immunohistochemistry

Five (5) μm of paraffin-embedded tissue sections were dewaxed in xylene and rehydrated at gradient concentrations of ethanol (100%, 95%, 90%, 80%, 70% in PBS, 5 min each solution). Antigen retrieval was performed by incubating the slides with 100 mmol/L sodium citrate solution (pH 6.0) for 20 min. Tumor blood vessels were stained with anti-vWF antibody (Vector Labs, Burlingame, CA). Cell proliferation and apoptosis were detected by staining with anti-Ki67 antibody and a TUNEL assay kit, respectively. Positively stained cells and total cells per field were counted to calculate the proliferative index (Ki67) and the apoptotic index (TUNEL).

2.6. Endothelial cell migration and tubule formation assays

Angiogenic factors secreted by D54 human glioblastoma cells were analyzed with HUVEC migration and tubule formation assays. Briefly, a monolayer culture of D54 cells (with or without TIP-1 knockdown) at 75% confluence was washed with cold PBS three times before being incubated with serum-free DMEM/F12 media for 24 h. The pre-conditioned media were filtered to remove cell debris for HUVEC migration and tubule formation assays. The HUVEC migration assay was performed using 8-μm porous Boyden chambers (Corning Life Science, Lowell, MA) according to the manufacturer's instructions. Prior to the migration assays, HUVECs were starved in serum-free medium overnight. Approximately 20,000 disaggregated HUVECs were seeded into the insert chambers with serum-free medium. Pre-conditioned media were added to the lower chambers. After 5 h, the cells that stayed on the top of the membrane were removed with cotton swabs, and the cells that migrated to the bottom of the membrane were stained with DAPI and counted under a fluorescence microscope. In the tubule formation assay, 8000 disaggregated HUVECs were seeded into each well of a 96-well plate that had been coated with 50 μl matrigel (BD Biosciences) and contained 200 μl preconditioned medium. Tubule-like structures were imaged by the use of a phase contrast microscope after incubation at 37 °C for 5 h. Images were taken from at least four microscopic fields from each well, and nodes with more than three branches were counted as tubule networks.

2.7. Antibody arrays and ELISA quantification

Human angiogenesis array kits (R&D, Minneapolis, MN) were utilized for semi-quantification of the angiogenic factors within *in vitro* and *ex vivo* samples. The *in vitro* samples were pre-conditioned media from D54 cells with or without TIP-1 knockdown as described above. *Ex vivo* samples were prepared by recovering tumor cells from matrigel-plugs after 8 days of the matrigel-plug implantation, incubating the tumor cells with serum-free DMEM/F12 medium for 24 h, and collecting the pre-conditioned media by filtration to remove the cell debris. Staining of the membranes with arrayed antibodies against human-originated angiogenic factors was conducted by following the manufacturer's instructions. Signal intensity was semi-quantified upon the histograms of scanned films. All of the data were normalized to the inner controls. uPA concentrations in the pre-conditioned media were determined by use of a uPA ELISA kit from Syd Labs (Malden, MA) according to the manufacturer's instructions.

2.8. RNA isolation, microarray profiling, and quantitative RT-PCR (qRT-PCR)

RNA transcripts from D54 cells were isolated by the use of a RNAqueous Kit (Ambion, Austin, TX) as instructed by the manufacturer. The quality of the RNA was validated by agarose gel electrophoresis. One μg of total RNA was used for cDNA synthesis with a QuantiTect reverse Transcription Kit (QIAGEN, Valencia, CA) in microarray profiling and quantitative reverse transcriptase PCR (qRT-PCR). Gene expression profiling was performed using Affymetrix Gene Chips at Vanderbilt's Functional Genomics Shared Resource (FGSR). Heatmaps of gene expression were generated using Cluster and TreeView (Michael Eisen, Stanford University). Hierarchical clustering of genes was performed using average linkage and Pearson correlation distance matrices. The functions of clustered genes were analyzed by GoMiner software [20]. The qRT-PCR was performed using a QuantiFast SYBR Green PCR Kit (QIAGEN) according to the manufacturer's instructions on a Bio-Rad CFX96 qRT-PCR system (Bio-Rad, Hercules, CA). Specific primer sets for FN1, SPP1, IGFBP3 and GAPDH were purchased from QIAGEN. The ΔC_T method was applied to calculate the relative gene expression levels [5].

2.9. Statistics

Meta-analysis was based upon published microarray data sets [5,21]. To compare TIP-1 expression levels within human malignant gliomas of various stages, one dataset [21] including 157 cases of primary human malignant gliomas of the grade II to IV, and 23 cases of non-tumor normal human brain samples was reanalyzed. To determine the prognostic value of TIP-1 expression level, 55 cases of