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The PDZ protein TIP-1 facilitates cell migration and pulmonary metastasis of human invasive breast cancer cells in athymic mice

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ABSTRACT

Tax-interacting protein 1 (TIP-1, also known as Tax1bp3) inhibited proliferation of colon cancer cells through antagonizing the transcriptional activity of beta-catenin. However, in this study, elevated TIP-1 expression levels were detected in human invasive breast cancers. Studies with two human invasive breast cancer cell lines indicated that RNAi-mediated TIP-1 knockdown suppressed the cell adhesion, proliferation, migration and invasion *in vitro*, and inhibited tumor growth in mammary fat pads and pulmonary metastasis in athymic mice. Biochemical studies showed that TIP-1 knockdown had moderate and differential effects on the beta-catenin-regulated gene expression, but remarkably down regulated the genes for cell adhesion and motility in breast cancer cells. The decreased expression of integrins and paxillin was accompanied with reduced cell adhesion and focal adhesion formation on fibronectin-coated surface. In conclusion, this study revealed a novel oncogenic function of TIP-1 suggesting that TIP-1 holds potential as a prognostic biomarker and a therapeutic target in the treatment of human invasive breast cancers.

1. Introduction

Beta-catenin is a multifunctional protein that contributes fundamentally to embryonic development and many physiological or pathophysiological processes [1]. Nuclear and cytosolic accumulation of beta-catenin or hyperactivation of beta-catenin signaling cascades has been implicated in many cancers including human breast cancer and associated with poor prognosis [2–4].

TIP-1 is a highly conserved protein across species [5]. One PSD-95/DlgA/ZO-1 (PDZ) domain (89 amino acids) is the only structural and functional unit in the small protein (total of 124 amino acids in human and mouse), distinguishing TIP-1 from other PDZ proteins that usually contain multiple structural and functional domains and serve as scaffolds in assembling large protein complex [6]. TIP-1 functions in a wide variety of biological events through selective interaction with intracellular proteins. TIP-1 interacts with rhotekin, a RhoA effector protein, to regulate cellular response to serum starvation [7]. Through selective association with glutaminase L [8], potassium channel Kir2.3 [9], or NMDA receptor [10], TIP-1 establishes and maintains cell polarity, or mediates neurotoxicity, respectively. TIP-1 is associated with several viral proteins including the Tax protein of human T-lymphotropic virus type I (HTLV-1) [11], E6 oncoprotein of human papillomavirus (HPV) [12] and NS1 protein of avian influenza [13], although more studies are needed to determine the biological relevance and respective mechanisms of such protein interactions. Interaction with TIP-1 antagonizes the transcriptional activity of beta-catenin and inhibits proliferation of colon cancer cells [14]. In contrast, TIP-1 knockdown in zebrafish embryo induced defects of cell filopodia growth and gastrulation movements [5], suggesting that TIP-1 might function as a tumor suppressor through modulating the functionality of beta-catenin.

In this study, we reported elevated TIP-1 expression levels in human invasive breast cancers. Using a combination of *in vitro* and *in vivo* model systems, it was demonstrated that TIP-1 knockdown suppressed cell proliferation, adhesion, migration and invasion of human breast cancer cells *in vitro*, and inhibited the tumor growth in mammary fat pads and pulmonary metastasis in athymic mice. With biochemical studies, this study revealed a novel oncogenic function of TIP-1 suggesting that TIP-1 holds potential as a prognostic biomarker and a therapeutic target in the treatment of human invasive breast cancers.

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2. Materials and methods

2.1. Antibodies and reagents

TIP-1 polyclonal (rabbit) antibodies were produced in our lab and characterized as described [15]. Antibodies against integrin alpha-5 and paxillin were purchased from BD Biosciences (Rockville, MD). Anti-Ki67 antibody was obtained from Abcam (Cambridge, MA). Anti-beta-actin antibody was purchased from Sigma (St. Louis, MO). Anti-phospho-paxillin (Tyr118) antibody was purchased from Cell Signaling Technology (Danvers, MA). Alexa Fluor 594-labeled phallotoxins and all other Alexa Fluor dye-labeled secondary antibodies were obtained from Invitrogen (Grand Island, NY). Laminin was purchased from BD Biosciences, collagen IV from Millipore (Hayward, CA) and fibronectin was from Sigma.

2.2. Cell culture and stable shRNA transfection

Human breast cancer cell line BT549 was a gift from Dr. Jennifer Pietenpol's lab at Vanderbilt University, cell line MDA-MB-231 was purchased from ATCC (Manassa, VA). Both lines were maintained in RPMI1640 media (Invitrogen). Constructs for the validated TIP-1 targeted shRNA (clone TRCN0000159034 and TRCN0000162886) and a non-targeting negative control were purchased from Sigma. Preparation of recombinant lentivirus, transfection, and selection of stable clones were conducted by following the manufacturer's instructions. TIP-1 knockdown was assessed by Western blot as described previously [15].

2.3. Migration and invasion assays

Cell migration and invasion assays were performed using 8-μm porous Boyden chambers (Corning Life Science, Lowell, MA) coated with or without Matrigel (BD Biosciences) according to the manufacturer's recommendations. Briefly, cells were starved in serum-free media overnight prior to the migration and invasion assays. 20,000 (for migration) or 40,000 (for invasion) disaggregated cells were seeded into the upper chambers with serum-free media, while complete media with 10% serum were added to the bottom chamber. Twelve hours later, cells stayed on top of the membrane were removed with cotton swabs, and cells migrated through the porous membrane were stained with DAPI for examination under fluorescence microscope.

2.4. Cell adhesion assay

96-Well culture plates (Corning) were coated with 50 μl of 10 μg/ml of fibronectin, collagen IV or laminin at room temperature for 2 h (with cover open), followed by three washes with 100 μl of 0.2% (w/v) bovine serum albumin (BSA, from Sigma) solution in phosphate-buffered saline (PBS). 1×10^5 Disaggregated cells resuspended in 50 μl of complete media were added into each well. After incubation at 37 °C for 40 min, unattached cells were removed by shaking at 2000 rpm for 10–15 s followed by washing with 0.2% BSA/PBS for three times. The cells adherent to the coated wells were fixed in 4% paraformaldehyde and stained with 0.05% (w/v) Crystal Violet. After washing with 200 μl of PBS for three times, the cell-associated dye was solubilized in 100 μl of 2% (w/v) of sodium dodecyl sulfate (SDS) solution in PBS, and quantified upon spectrometer absorbance measurement (at 560 nm).

2.5. Immunofluorescence staining and imaging

As in the cell adhesion assay, cells were seeded onto the fibronectin-coated surface for incubation at 37 °C for 40 min before fixation in 4% (w/v) formaldehyde solution in PBS. Anti-paxillin, anti-actin and fluorescence dye-labeled secondary antibodies were used for immunofluorescence staining by following the manufacturer's instructions. Images were acquired using a Zeiss LSM 510 inverted confocal microscope. Focal adhesion clusters were characterized as paxillin-positive and quantified upon digital images using the Image J software. At least 50 cells were measured in each of three independent experiments for statistic analysis.

2.6. Models of human breast cancer in athymic nude mice

Orthotopic model of human breast cancer in athymic mice was developed by injecting 5×10^5 MDA-MB-231 cells (in 50 μl of PBS) into mammary fat pads of female FoxN1-null nude mice (Harlan Laboratories, Prattville, AL) of 5-week old. Tumor size was measured with caliper in every 3 days and calculated as described [16]. At the end of the tumor growth study, lung was resected to check pulmonary metastasis. Pulmonary metastasis model was developed by injecting 2×10^5 MDA-MB-231 cells (in 100 μl of PBS) into tail veins of FoxN1-null nude mice. Tumor progression was tracked through daily monitoring animal hypomotility, difficulty breathing, and weight loss, and verified with histological assessment of the resected tissue. All the animal studies were conducted as approved by the Institutional Animal Care and Use Committee (IACUC) at Vanderbilt University.

2.7. Gene expression analysis

RNA transcripts from MDA-MB-231 cells (with or without TIP-1 knockdown) 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 microgram of total RNA was used for cDNA synthesis with a QuantiTect reverse Transcription Kit (QIAGEN, Valencia, CA). Affymetrix Exon/gene arrays were used. Hybridization, scanning and image processing were performed as recommended by the manufacturer' instructions and conducted at the Vanderbilt Functional Genomics Shared Resource (FGSR). Genes down- or up-regulated by more than 1.25-fold by TIP-1 knockdown were subjected to GoMiner analyses (http://discover.nci.nih.gov/gominer/index.jsp), the genes were classified into biological coherent categories and the significantly affected categories were identified. Clustering was based on Euclidean distance using the "average" cluster method. To determine the prognostic value of the TIP-1 protein expression in patients with invasive breast cancers, we reanalyzed published microarray datasets [17,18] that contains clinically annotated human specimens including benign cystic lesions, invasive breast carcinomas, and adjacent normal mammary glands. Dataset from Minn et al. [19] was used to determine the prognostic value of TIP-1 expression in human invasive breast cancers. Patients with the top 40% of TIP-1 expression levels were defined as "high TIP-1 level" group, and those in the bottom 40% were defined as "low TIP-1 level" group.

2.8. Statistics

All numerical data are expressed as mean ± SEM (standard error of the mean). Statistical analysis was performed by ANOVA. For survival assays, Kaplan–Meier survival analysis was used, and log rank test was performed using MedCalc to determine differences between survival curves. For all analyses, a p value less than 0.05 was considered significant.