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Tripartite motif 16 inhibits epithelial–mesenchymal transition and metastasis by down-regulating sonic hedgehog pathway in non-small cell lung cancer cells

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ABSTRACT

The present study was to examine the effect of Tripartite motif 16 (TRIM16) on epithelial–mesenchymal transition (EMT) and metastasis in non-small cell lung cancer (NSCLC) cells, and its clinical significance in NSCLC. The correlation of TRIM16 expression and clinical features of NSCLC was analyzed in paraffin-embedded archived normal lung tissues and NSCLC tissues by immunohistochemical analysis. The effect of TRIM16 on EMT and metastasis was examined both in vitro and in vivo. The expression of TRIM16 was markedly decreased in NSCLC and correlated with tumor metastasis. Upregulation of TRIM16 significantly inhibited EMT and metastasis of NSCLC cells. In contrast, silencing TRIM16 expression significantly promoted the EMT and metastasis of NSCLC cells both in vitro and in vivo. Moreover, we demonstrated that downregulation of TRIM16 activated the sonic hedgehog pathway, and that inhibition of the sonic hedgehog pathway by cyclopamine abrogated the effect of TRIM16-downregulation induced EMT and metastasis on NSCLC cells. Our results suggest that TRIM16 is a potential pharmacologic target for the treatment of NSCLC and promotion TRIM16 expression might represent a novel strategy to NSCLC metastasis.

1. Introduction

Lung cancer is the most common cause of cancer deaths worldwide, ~1.2 million deaths people die from it per year [1]. It is the leading type of new cancer cases leading to death. Non-small cell lung carcinoma (NSCLC) is the most frequent subtype, ~85% of all cases [1]. Most of NSCLC patients have locally advanced or distant metastatic disease from onset of symptoms. It is strongly associated with poor prognosis and has a 5-year survival rate of <10 and 5% in male and female patients, respectively [1,2]. While NSCLC usually reveals better responsiveness to chemotherapy and radiation, NSCLC is relatively insensitive to both therapeutic modalities [2–4]. Hence, identification of novel targets for more effective anti-NSCLC strategies with minimal toxicity is urgent.

The TRIM16, also called the estrogen-responsive B box protein, which is a member of the RING-B box-coiled-coil/TRIM protein family [5]. It is associated with many different kinds of cancers [6–8]. Previous studies have identified TRIM16 as a DNA binding protein with histone acetyltransferase activity, which is necessary for the retinoic acid receptor β2 transcriptional response in cancer cells [9]. Overexpressed TRIM16 reduced neuroblastoma cell growth, enhanced retinoidinduced differentiation, and decreased tumourigenicity in vivo [6]. Recently studies revealed that TRIM16 acts as a tumor suppressor, promoting neuritic differentiation, cell migration, and replication through interactions with cytoplasmic vimentin and nuclear E2F1 in neuroblastoma cells [6,10]. Therefore, TRIM16 may act as a tumor suppressor, but whether TRIM16 has a function in NSCLC development, EMT, and metastasis remains unknown.

This study showed that TRIM16 expression was decreased in NSCLC tissues and NSCLC cell lines. Upregulation of TRIM16 significantly inhibited EMT and metastasis of NSCLC cells. In contrast, silencing TRIM16 expression significantly promoted the EMT and metastasis of NSCLC cells both in vitro and in vivo. In addition, we also found that TRIM16 had a function in NSCLC cell EMT and invasion could be partly associated with sonic hedgehog

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pathway. These results indicate the pivotal function of TRIM16 in NSCLC cell EMT and invasion.

2. Material and methods

2.1. Chemicals and antibodies

Lipofectamine 2000 transfection and TRIZOL LS Reagents were purchased from Invitrogen (Grand Island, NY, USA). Antibodies against TRIM16, Shh, Smo, Ptc, and Gli-1 were purchased from Abcam (Cambridge, MA, USA). E-cadherin, N-cadherin, vimentin, and β-actin antibodies were from Cell Signaling technology (Danvers, MA, USA). Anti-α-catenin antibody was from BD (Franklin Lakes, NJ, USA). Cyclopamine was from Sigma (Sigma–Aldrich, St. Louis, MO, USA). Unless otherwise noted, all other chemicals were from Sigma.

2.2. Patients and specimens

Paraffin-embedded, archived tissue samples were obtained from 51 normal lung tissues and 121 patients diagnosed with NSCLC between 2009 and 2013 at the Department of Pathology, First Affiliated Hospital, Xi'an Jiaotong University. The histologic characterization and clinicopathologic staging of the samples were determined according to the WHO criteria and current International Union against Cancer TNM (tumor-node-metastasis) Classification. Detailed clinical information of all patients is summarized in Supplementary Table 1.

2.3. Histological and immunohistochemical analysis

The normal human lung tissues and human lung tumor tissues were fixed in 4% paraformaldehyde in phosphate-buffered saline (PBS) overnight and subsequently embedded in paraffin wax. Sections cut at a thickness of 4 μm were stained with hematoxylin and eosin for histological analysis. Immunohistochemical analysis was performed for different markers in these arrays as described previously. The proportion of stained cells was semiquantitatively determined following published protocols.

2.4. Cell culture

NSCLC cell lines (ATCC, Manassas, VA, USA) were cultured under the following conditions: A549, H1299, H460, A427 and H1650 cell lines were cultured using 10% fetal bovine serum (Cat#10099-141, Invitrogen, Carlsbad, CA) in RPMI-1640 (Cat#C11875, Invitrogen). BEAS2B cell line was cultured using 10% fetal bovine serum (Invitrogen) in Dulbecco's modified Eagle medium (Cat#C11965, Invitrogen). Cell culture was according to manufacturer's protocol. All the cell lines were grown at 37 °C in a 5% CO_2/95% air atmosphere and were revived every 3–4 months.

2.5. Establishment of TRIM16 stable expression and knockdown cell lines

Retroviral construct containing human pBabe-TRIM16 cDNA, and pSuper.retro.puro with shRNA against human TRIM16 were prepared as described previously [11]. The generation of retrovirus supernatants and transfection of lung carcinoma cells were conducted as described previously [12]. The expression of TRIM16 was confirmed by qRT-PCR and Western blotting analysis.

2.6. Cell invasion and motility assay

Invasion of cells was measured in Matrigel (BD, Franklin Lakes, NJ, USA) -coated Transwell inserts (6.5 mm, Costar, Manassas, VA, USA) containing polycarbonate filters with 8-μm pores as detailed previously [13,14]. The inserts were coated with 50 μl of 1 mg/ml Matrigel matrix according to the manufacturer's recommendations. 2×10^5 cells in 200 μl of serum-free medium were plated in the upper chamber, whereas 600 μl of medium with 10% fatal bovine serum were added to lower well. After 24 h incubation, cells that migrated to the lower surface of the membrane were fixed and stained. For each membrane, five random fields were counted at ×10 magnification. Motility assays were similar to Matrigel invasion assay except that the Transwell insert was not coated with Matrigel.

2.7. Confocal immunofluorescence microscopy

Cell lines were plated on culture slides (Costar, Manassas, VA, USA). After 24 h, the cells were rinsed with PBS and fixed with 4% paraformaldehyde, and cell membrane was permeabilized using 0.5% Triton X-100. These cells were then blocked for 30 min in 10% BSA and then incubated with primary antibodies overnight at 4 °C. After three washes in PBS, the slides were incubated for 1 h in the dark with FITC-conjugated secondary antibodies (Invitrogen, Grand Island, NY, USA). After three further washes, the slides were stained with DAPI for 5 min to visualize the nuclei, and examined using a Carl Zeiss confocal imaging system (LSM 780) (Carl Zeiss, Jena, Germany).

2.8. Western blotting

Cells were lysed in lysis buffer and total protein contents were determined by the Bradford method. 30 μg of lysis were separated by reducing SDS-PAGE and probed with specific antibodies. Blots were washed and probed with respective secondary peroxidase-conjugated antibodies, and the bands visualized by chemoluminescence (Amersham Biosciences).

2.9. qRT-PCR

Total RNA was extracted using Trizol reagent and cDNA was synthesized using SuperScript II Reverse Transcriptase (Invitrogen). qRT-PCR and data collection were performed with an ABI PRISM 7900HT sequence detection system. The primers used for the amplification of the indicated genes are available upon request.

2.10. Gene expression profiling

Total RNA quality and quantity were determined using Agilent 2100 Bioanalyzer and NanoDrop ND-1000. Affymetrix HU U133 plus 2.0 arrays were used according to manufacturer's protocol. The data were initially normalized by robust multiarray average (RMA) normalization algorithms in expression console software (Affymetrix). Significantly altered genes between TRIM16 knockdown and its control cells were considered by scatter plots and the genes up- and down-regulated ≥5-fold. Clustering analysis was done using gene list by Gene Cluster v3.0 software, and heat maps were visualized using Java TreeView v1.1.4r3 software. Gene set enrichment analysis was carried out using ConceptGen (http://conceptgen.ncibi.org). Gene sets were either obtained from the ConceptGen or from published gene signatures.