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TRIM28, a new molecular marker predicting metastasis and survival in early-stage non-small cell lung cancer

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ABSTRACT

TRIM28 is a universal corepressor for Kruppel-associated box zinc finger proteins. In this study, we demonstrated the expression of TRIM28 gene was significantly higher in cancerous tissues than in noncancerous tissues ($P < 0.001$). TRIM28 knockdown resulted in a decrease in cell proliferation in liquid media as well as in soft agar. The proliferation rate was impaired and the cell cycle progression was inhibited after knockdown of TRIM28 in non-small cell lung cancer cell lines PAa and SK-MES-1. We used real-time polymerase chain reaction to detect circulating cancer cells in 138 non-small cell lung cancer patients. The overall positive detection rate was 30.4% (42 of 138) in peripheral blood of NSCLC patients and was 29.9% (29 of 97) in early-stage patients. In a 70-month follow-up study, 20 of 29 patients (69.0%) in TRIM28 positive group had recurrence and/or metastasis, significantly higher ($P = 0.004$) than in the TRIM28 negative group (25 of 68, 36.8%). In addition, non-small cell lung cancer patients whose circulating cancer cells expressed TRIM28 suffered shorter tumor-specific survival compared with those with absent TRIM28 expression ($P < 0.001$). Results of our study showed that TRIM28 provides a survival advantage to lung cancer cells and may be a new marker to predict metastasis and prognosis in early-stage non-small cell lung cancer patients.

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1. Introduction

Lung cancer is the leading cause of cancer deaths worldwide, and non-small cell lung cancer (NSCLC) accounts for nearly 80% of those cases [1]. The overall survival rate is only 15% at 5 years, mainly because of the recurrence of the disease [2]. In early-stage NSCLC, the prognosis after complete tumor resection is much better. However, a large portion of early-stage NSCLC patients, defined by the current staging system and available imaging modalities, still develop distant metastasis although they received surgical removal of the tumor mass. This fact suggests the existence of occult metastatic cells, which are undetectable by current methods [3]. The spread of cancer cells by the hematogenous route is important in lung cancer metastasis. Therefore, it is critical to find reliable markers capable of detecting circulating cancer cells to accurately predict metastasis and disease progression in early-stage NSCLC patients.

Recent studies have indicated that some members of the tripartite motif (TRIM) proteins function as important regulators for carcinogenesis [4–11]. TRIM28 is a nuclear corepressor with conserved domains for RING fingers, B boxes, leucine zipper α helical coiled-coil region, plant homeo domain finger, and bromo domain. TRIM28 recruits heterochromatin protein 1 (HP1) to histones through a PxVxL motif in its centrally located HP1 binding domain [12,13]. DNA double-strand break-induced chromatin decondensation is modulated by phosphorylated TRIM28 in an ATM-dependent manner [14]. TRIM28 stimulates P53–HDAC1 complex formation and inhibits P53 acetylation by interacting with MDM2 [15]. TRIM28 binds the E2F1 transcription factor in a retinoblastoma protein (pRb)-independent fashion and inhibits E2F1 activity. TRIM28 stimulates formation of E2F1–HDAC1 complex and inhibits E2F1 acetylation. Moreover, the CBF-A/TRIM28/FTS-1 complex activates FSP-1 transcription and subsequently epithelial–mesenchymal transition (EMT), a notable phenomenon in cancer metastasis (Fig. 1) [16–19].

Although a growing number of studies have demonstrated the function of TRIM28, no reports have shown the expression status of TRIM28 in lung cancer and any clinical significance associated with TRIM28 expression. We reported here the biological function of

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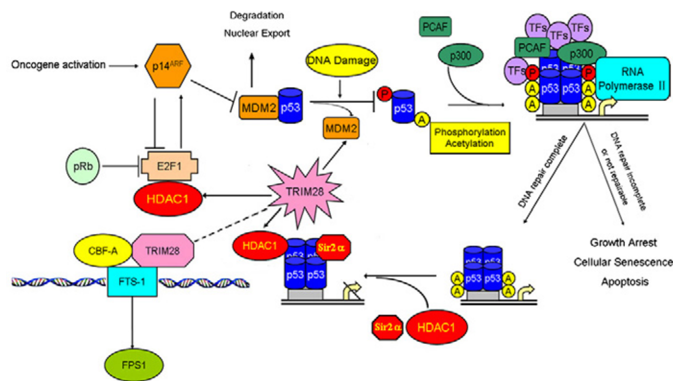

Fig. 1. Model for pathway of TRIM28 involved in tumorigenesis.

TRIM28 in lung cancer cells by using siRNA gene silencing. We also identified TRIM28 as a new marker for detection of circulating cancer cells in NSCLC patients and also provide evidence for its possible role to predict metastasis development and prognosis in early-stage NSCLC patients.

2. Materials and methods

2.1. Cell lines

The human NSCLC cell lines used in this study were as follow: adenocarcinoma represented by A427, A549, NCI-H1373, LC319, PAa; squamous cell carcinoma (SCC) NCI-H226, SK-MES-1; and large cell carcinoma (LCC) LX-1. A human bronchial epithelia cell, BEAS2B was included in the panel of the cells as control. All cells were grown in monolayer in appropriate medium supplemented with 10% FCS and were maintained at 37 °C in an atmosphere of humidified air with 5% CO_2.

2.2. Clinical tissues and peripheral blood samples

All samples from cancer patients were collected with written consents and were approved by the Hospital Ethics Review Committee. Clinical diagnosis was confirmed by pathological examination. Tumor stages and grades were classified according to the fifth edition of the Tumor-Node-Metastasis classification of the International Union Against Cancer.

Forty-eight samples of primary NSCLC tissues, including 19 adenocarcinomas, 16 SCCs and 13 LCCs, were obtained along with paired noncancerous tissues (5 cm away from the tumor) during surgical resection. The resected tissue samples were immediately cut into small pieces and snap frozen in liquid nitrogen until use.

Venous blood samples from 138 NSCLC patients were collected preoperatively by standard transcutaneous needles venipuncture and placed into citrate sodium containing tube. Blood was collected into two tubes, with 1 ml in the first tube and 5 ml in the second tube. The blood in the first tube, which may have been contaminated with epithelial cells picked up by the needle when it pierced the skin, was discarded, whereas the blood in the second tube was loaded on a Ficoll–Hypaque layer (Gibco BRL), and the PBMCs were collected after density gradient centrifugation. The collected cells were washed twice with sterile phosphate buffer solution. Cell pellets were then snap frozen and stored at −80 °C until RNA extraction.

2.3. RNA preparation and cDNA synthesis

Total RNA was extracted from tumor specimens and cell lines using Trizol reagent (Invitrogen, Carisbad, CA) according to the manufacturer's protocol. Total RNA was checked by agarose gel electrophoresis for ribosomal RNA integrity, and RNA quantity was measured by an ultraviolet spectrometer. Total RNA (2 μg) was primed with an oligo$(dT)_{15}$ oligonucleotide (Promega, Madison, WI) and reverse-transcribed into cDNA using advantage reverse transcriptase (Clontech Laboratories, Inc.), according to the protocol provided by the manufacturer.

2.4. RT-PCR analysis

Semiquantitative reverse transcription-PCR (RT-PCR) was carried out with synthesized gene-specific primers (Table 1). Sample cDNA (2.5 μl) was 10-fold diluted with a PCR mixture containing 0.1 μmol per primer pair, 0.2 mM deoxynucleotide triphosphate, 50 mM Tris–HCl (pH 8.3), 10 mM KCl, 5 mM $(NH4)_2SO_4$, 2 mM $MgCl_2$ and 0.75 U of *Taq* polymerase (Tianwei, Beijing, China) in a total volume of 25 μl. For cancer cell lines, RT-PCR of TRIM28 was performed with primers T2 and T4. The parameters were as follows: 35 cycles at 94 °C for 50 s, at 60 °C for 50 s, and at 72 °C for 35 s, followed by 10 min at 72 °C. Visualization of target bands on a 1.0% agarose gel with ethidium bromide staining was performed to determine the expression of mRNA transcripts. For clinical tissue samples, real-time RT-PCR was performed with primers T3 and T4. The condition was 95 °C for 10 min followed by 40 cycles at 95 °C for 15 s, 60 °C for 25 s, and then 72 °C for 35 s.

To detect a few cancer cells in the circulation, a highly sensitive nested real-time RT-PCR was necessary. The primer sets of marker genes were listed in Table 1. Taking 2.5 μl of 10-fold-diluted cDNA as template for the first round with outer primers, PCR conditions were as follows: 25 cycles at 94 °C for 50 s, at 60 °C for 50 s, and at 72 °C for 35 s, and a final extension at 72 °C for 10 min. For the second round of the nested PCR amplification, the reaction mixture contained 2 μl of the first round PCR product, 0.25 μmol/L inner primers, and SYBR Green PCR master mix (Solomonbio, shanghai, China) in a total volume of 20 μl. The real-time qPCR assays were done with an ABI prism 7000 SDS (Applied Biosystems) instrument. The real-time qPCR condition was 95 °C for 10 min followed by 40 cycles at 95 °C for 15 s, 60 °C for 25 s, and then 72 °C for 35 s. All reactions were performed in triplicate.

By using a real-time qPCR instrument, the threshold cycle (C_T), the fractional cycle number at which the SYBR green fluorescence exceeded a set level above baseline, was determined. We used GAPDH mRNA as an internal control. The relative amount of mRNA, normalized against the GAPDH mRNA, was expressed as $\Delta C_T = C_T^{(GAPDH)} - C_T^{(target\ gene)}$. If the fluorescence signal was undetected after 40 cycles, the C_T value was given the maximum cycle number of 40 for analysis convenience. The differential expression ratio of a candidate marker gene, Q, for patients versus normal controls was calculated by $Q = 2^{\Delta C_T - \text{mean of } \Delta C_T \text{ in normal}}$.

2.5. Immunohistochemistry

Immunohistochemical studies for TRIM28 were performed on formalin-fixed, paraffin-embedded surgical sections obtained from 48 NSCLC patients by using the streptavidin–peroxidase method (Beijing ZhongShan Golden Bridge Biotech CO., Ltd., China). Slides were deparaffinized and rehydrated with xylene and graded alcohol. Optimal antigen retrieval was carried out in citrate buffer (pH 6.0) for 10 min with a steam oven to enhance the immunoreactivity. Primary rabbit polyclonal antibody against TRIM28 (Proteintech, USA) was used at a dilution of 1:100.

2.6. Western blotting

Cell were lysed in lysis buffer: 50 mmol/L Tris–HCl (pH 8.0), 150 mmol/L NaCl, 0.5% NP40, 0.5% sodium deoxycholate, 0.1% SDS,

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