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Tripartite motif-containing protein 3 plays a role of tumor inhibitor in cervical cancer

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ABSTRACT

Many studies have been reported that tripartite motif-containing (TRIM) proteins play important roles in various cellular processes and involved in many diseases. TRIM3, a member of TRIM family, has been proved that it plays important roles in various cancers. Nevertheless, its effects on cervical cancer reminded unknown. This study aimed to explore TRIM3 function in cervical cancer cells. The results of quantitative real-time RT-PCR and western blotting showed that the TRIM3 expression was very low in five cervical cancer cell lines. The TRIM3 overexpression weakened cell viability, and promote apoptosis of C-33A and SiHa cells *in vitro*, and inhibit tumor growth *in vivo*, which suggested that TRIM3 could reduce proliferation of cervical cancer cells. Moreover, TRIM3 up-regulation enhanced caspase-3 activity and increased the expressions of cleaved caspase-3 and p53, at the same time decreased p38 phosphorylation level. In addition, TRIM3 down-regulation had opposite effects on cell proliferation and expressions of the three proteins, which could be suppressed by p38-specific inhibitor (SB203580). In conclusion, TRIM3 had the ability to suppress cell proliferation by inactivating p38 signaling pathway, which indicated that it might act as a tumor inhibitor and an underlying therapeutic target for cervical cancer.

1. Introduction

Cervical cancer is the third commonest diagnosed cancer and the fourth uppermost cause of tumor-related mortality among females in the word, with approximately 527,600 increased new cervical cancer patients and 265,700 cervical cancer-related deaths annually [1,2]. Although the incidence or mortality ratios of cervical cancer are steadily declining among women through decades of work, the patients who were diagnosed at late clinical stages of cervical cancer still underwent poor prognosis and bad 5-year survival rates [3–5]. Cervical cancer occurs with a complex process, including viral infection, immunologic factors, and gene mutation of proto-oncogenes and tumor suppressor genes [6–9]. In the past years, some outcomes have been made in pathogenesis and progression of cancers, which might provide new treatment strategies for the patients with cancers [10]. Unfortunately, the knowledge of molecular mechanisms and biomarkers was still very poor for cervical cancer in the field of progression and prognosis. Thus, it is essential to research cervical cancer-related underlying mechanisms and find new biomarkers for improving the management of patients.

The proteins of tripartite motif-containing (TRIM) consist of more than 70 members, which were mainly composed of a RING domain in the N-terminal end, one or two B-boxes, a coiled-coil domain, and C-terminal regions with different structures [11]. Because most of TRIM proteins have E3 ubiquitin ligase activities, they play critical roles in a variety of cellular processes, such as hereditaty immunity, intracellular signaling, carcinomatous change, cell proliferation and apoptosis, which makes TRIM have close relationships with multiple diseases, especially cancers [12]. TRIM3, a member of TRIM proteins, is localized to chromosome 11p15.5 where includes many cancer-related genes [13]. Current

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studies have proved that TRIM3 had close relationships with cell proliferation and pathogesis of various cancers, like glioblastomas, liver cancer and colorectal cancer [14–16]. Many researchers considered TRIM3 as a candidate tumor suppressor gene, because its overexpression could inhibit cancer cell proliferation. In addition, TRIM3 expression was also related with an unfavorable prognosis [17]. Nevertheless, the TRIM3 function is still unclear in cervical cancer.

In this work, we firstly examined the TRIM3 expression in cervical cancer cells via quantitative real-time RT-PCR (q RT-PCR) and western blotting. Then lentiviral and siRNA transduction were respectively used to steadily up-regulate and down-regulate the expression of TRIM3 in cervical cancer cells. We explored the effects of TRIM3 on cervical cancer cell proliferation *in vitro* and tumor growth *in vivo*, and analyzed possible mechanism.

2. Materials and methods

2.1. Cervical cancer cell lines

Human cervical cancer cell lines (C-33A, HeLa, SiHa, CaSki, and C4-1) were purchased from American Type Culture Collection (ATCC, Manassas, VA, USA). All cells were cultured in Dulbecco's modified Eagle's medium (DMEM; Hyclone, USA) containing 10% fetal bovine serum (FBS; Gibco, USA) and 1% penicillin/streptomycin (Sigma, USA) at 37 °C with 5% CO_2 in a humidified atmosphere.

2.2. Quantitative real-time RT-PCR (q RT-PCR)

Total RNA was extracted from cervical cancer cells by Trizol reagent (Invitrogen, USA), according to the manufacturer's protocol. Then the collected RNA was used to reverse-transcribed into complementary DNA (cDNA) using a cDNA Reverse Transcription Kit (Fermentas, USA) according to the manufacturer's protocol. The expression levels of TRIM3 were quantified using real-time PCR with the SYBR® Green (Thermo, USA) dye detection method on an ABI-7300 sequence detection system (Applied Biosystems, USA). In this study, GAPDH was used as a reference gene. The primers are as follows: TRIM3, forward primer: 5′-GCAGACAGCAACAACCAGTG-3′ and reverse primer: 5′-AGAAGATGCTGACCCAACGG-3'; GAPDH, forward primer: 5′-AATCCCATCACCATCTTC-3′ and reverse primer: 5′-AGGCTGTTGTCATACTTC-3'. The threshold cycle (Ct) values of each sample were tested to character the relative mRNA levels of TRIM3, and compared with the Ct values of GAPDH. The quantity of TIRM3, relative to GAPDH, was calculated using the $2^{-\Delta Ct}$ method, where $\Delta Ct = Ct_{TIRM3} - Ct_{GAPDH}$. The comparison of TRIM3 expression was using the formula of $2^{-\Delta\Delta Ct}$, where $\Delta\Delta CT = (Ct_{TIRM3} - Ct_{GAPDH})_{experiment} - (Ct_{TIRM3} - Ct_{GAPDH})_{control}$.

2.3. Western blotting

Cervical cancer cells were lysed in RIPA buffer (Solarbio, China), and protein concentrations were measured using the BCA protein assay kit (Thermo, USA). Then collected proteins were separated by SDS-PAGE and transferred to nitrocellulose membranes (Millipore, USA) using semi-dry method. Subsequently, the membrane was blocked for 2 h using 5% skim milk (using bull serum albumin in detecting protein phosphorylation) at room temperature. After that, the membranes were incubated with primary antibodies overnight at 4 °C. A monoclonal antibody against GAPDH acted as a protein loading control. The primary antibodies against TRIM3, cleaved caspase3, p53, p38, p-p38 and GAPDH were purchased from Abcam (UK) and used according to the manufacturer's direction. The membranes were washed three times with PBS-T buffer (JRDUN Biotechnology Co. Ltd, China), and then incubated with respective HPR-conjugated secondary antibodies (Santa Cruz, USA) used at 1:1000 dilutions at 37 °C for 1 h. Finally, the membranes were scanned with Bio-Rad imaging system after washing with PBS-T buffer, and the signals were detected using enhanced chemiluminescence reagents (Millipore, USA). Quantity One Software (Bio-Rad) was used to analyze the intensity of signals.

2.4. TRIM3 overexpression

We used lentiviral transduction in order to overexpress TRIM3 in C-33A and SiHa cells. The lentiviruses overexpression vectors (pLVX-Puro-TRIM3) and empty pLVX-Puro were purchased from JRDUN Biotechnology Co. Ltd (China). C-33A and SiHa cells were transfected with pLVX-Puro-TRIM3 or empty pLVX-Puro for 24 h. The cells without transduction acted as blank control. The overexpression efficiency was measured by q RT-PCR and western blotting. In addition, the expression of cleaved caspase3, p53, p38, and p-p38 were also measured using western blotting.

2.5. TRIM3 inhibition

In order to inhibit TRIM3 expression in cervical cancer cells, human TRIM3 specific siRNA (si-TRIM3) and its negative control siRNA (si-NC) were obtained from JRDUN Biotechnology Co. Ltd (China). HeLa cells were then transfected with si-TRIM3 or si-NC for 24 h. Moreover, p38MAPK inhibitor SB203580, which was dissolved in 5% DMSO (2 μg/10 μL; Sigma, USA), applied an almost identical pattern to those cells treated with siRNA transfection. q RT-PCR and western blotting were also used to measure transfection efficiency, and the expression of cleaved caspase3, p53, p38, p-p38 were tested using western blotting.

2.6. Cell apoptosis assay

Cell apoptosis rate was detected with annexin-V-fluorescein isothiocyanate (FITC)/propidium iodide (PI) apoptosis detection kit (Blue sky biotechnology co. LTD. China). Briefly, after transfection 48 h, C-33A and SiHa cells were wished once with PBS buffer and suspended in PBS buffer. Then cells were seeded at a density of 1×10^5 cells per well in 6-well plates, and incubated with 5 μL of Annexin V-FITC (50 μg/mL) at 4 °C for 15 min in dark environment. Subsequently, 5 μL of PI (50 μg/mL) was added into each well, and the plates continued culturing 5 min at 4 °C. After that, the cells were detected with flow cytometer (Beckman Coulter, USA), and the result was analyzed by the standard Cell Quest Software. For all samples, a minimum of 10,000 events were collected and detected in three parallel experiments. Apoptotic ratio was defined as the rate of the number of apoptotic cells to the total number.

2.7. Caspase-3 activity determination

The caspase-3 enzymatic activities of C-33A and SiHa cells transfected by lentivirus overexpression vector were determined using Caspase-3 colorimetric assay kits (KeyGen, China) according to the manufacturer instructions. After transfection 48 h, the collected cells were washed twice by ice-cold PBS, and then lysed in 50 μL lysis buffer for 5 min at 4 °C. The lysate was centrifuged with 10,000 rpm at 4 °C for 1 min, and resulting supernatant was harvested. BCA assay was used to determine protein concentrations. Then 50 μL resulting supernatant supplemented with 200 μg protein was added into 96-well pate. Subsequently, 50 μL 2 × reaction buffer and 5 μL caspase-3 substrate were added into each well. After incubation 4 h at 37 °C, the optical density of the reaction