Biochemical and Biophysical Research Communications 500 (2018) 810–816

Contents lists available at ScienceDirect

Biochemical and Biophysical Research Communications

journal homepage: www.elsevier.com/locate/ybbrc

Down-regulation of TRAF4 targeting RSK4 inhibits proliferation, invasion and metastasis in breast cancer xenografts

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ARTICLE INFO

Article history:
Received 12 April 2018
Accepted 19 April 2018
Available online 24 April 2018

Keywords:
TRAF4
RSK4
Lentivirus
Breast cancer
AKT
NF-κB

ABSTRACT

Ribosomal S6 protein kinase 4 (RSK4) was known as a novel tumor suppressor gene, and the tumor necrosis factor receptor-associated factor 4 (TRAF4) was linked to carcinogenesis. The purpose of this study is to further investigate the effect of the TRAF4 gene on cell proliferation, invasion and metastasis in vivo and explore whether there is an interaction between TRAF4 and RSK4 in breast cancer. MDA-MB-231 cells were transfected with lentivirus TRAF4-shRNA to specifically block the expression of TRAF4, or transfected with lentivirus negative-shRNA as a negative control. Four-six weeks female BALB/c nude mice were randomly assigned to three groups (n = 14): TRAF4-shRNA, negative and control, and then inoculated subcutaneously with the corresponding cells. In-vivo metastasis model was constructed by injecting above cells into tail vein. Tumor proliferation was assessed in terms of the tumor growth curve, tumor size and weight. Invasion and metastasis were evaluated by the histopathologic examination in lung or/and liver tissues. Measurement of TRAF4 and RSK4 expression and their correlation factors (AKT, NF-κB, TGF-β1, TNF-α, MMP2 and MMP9) were performed by immunohistochemistry, western blot or fluorescence quantitative RT-PCR. We found that the size and weight of tumors in TRAF4-shRNA group was significantly smaller than the negative and blank group, and the number of the lung and liver metastases lesions was also fewer ($P < 0.05$). And TRAF4 and its correlation factors (P-AKT, P-NF-κB, TGF-β1, TNF-α, MMP2 and MMP9) in the TRAF4-shRNA group were significantly decreased compared with the negative and blank group. However, the expression of RSK4 mRNA and protein in TRAF4-shRNA group were significantly increased. Collectively, TRAF4 knockdown significantly inhibited proliferation, invasion and metastasis in the xenograft nude mouse model, possibly involving in the interaction with RSK4 through down-regulation of AKT signaling pathway and then inactivating NF-κB pathway.

1. Introduction

Cancer Statistics in 2015 showed that the mortality of breast cancer was declining, but the incidence still ranked first in female malignancy [1], which are worries for women's health. Therefore, there remains an urgent and unmet research need to explore novel ways to gain insight into its complex pathophysiology to effectively control the occurrence and development of breast cancer.

RSK4, belonging to the ribosomal S6 kinase (RSK) family, is a serine-threonine kinase [2], which is an important downstream effector of extracellular signal-regulated kinase (ERK). Its N-terminal kinase domain (NTKD) and the C-terminal kinase domain (CTKD) are linked by a conserved region, and NTKD phosphorylated the substrate by autophosphorylation of CTKD resulting in proliferation, survival and other biological activities [3]. In addition, recent reports shown that the regulatory role of RSK4 was mediated by the AKT/ERK pathway on the breast cancer development [4,5]. And RSK is involved in the activation of the downstream NF-κB pathway by phosphorylating the N-terminus of IκBα, which enables the signal to be transmitted into the nucleus and activates transcription of multiple target genes [6]. Thus, RSK plays an important role as a juncture between AKT pathway and NF-κB pathway. According to previous researches, RSK4 was regarded as a tumor suppressor gene in breast cancer [7], and it is most probably a breakthrough for us to further explore the mechanism of breast cancer.

Protein Arginine Methyltransferase 5 (PRMTs) (PRMT1-9) regulates transcription by the methylation of histone tail ends or suppressor proteins. PRMT5 is involved in the regulation of multiple cancer pathways and presents a significant over-expression,

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https://doi.org/10.1016/j.bbrc.2018.04.164
0006-291X/

which are related to the imbalance between methylation and demethylation [8]. In previous research, we found that RSK4 and PRMT5 were interacting protein [9]. Besides, PRMT5 binds to TRAF4 through the zinc finger structure [10]. It is well-known that overexpression of TRAF4 in cancer cells can promote the membrane recruitment of AKT and activate the AKT pathway [11]. In addition, TRAF4 is mediated by glucocorticoid-induced TNFR (GITR) to activate NF-κB pathway [12]. Thus, we hypothesized TRAF4 may regulate the expression of RSK4 by the AKT pathway and then affect the NF-κB pathway.

In our present study, we used lentiviral vector to block the expression of TRAF4 in MDA-MB-231 cells and constructed breast cancer xenograft model to explore whether RSK4 is regulated by TRAF4 and the mechanism by which TRAF4 affected tumor development.

2. Materials and methods

2.1. Cell culture and transfections

MDA-MB-231 cells were purchased from Shanghai Cell Biology Institute (Shanghai, China). shRNA targeting human TRAF4 gene and non-targeting shRNA (RNAi sequences: TTCTCCGAACGTGTCACGT and GCCCTGCACCTACTGCACTAA, respectively) were synthesized by GeneChem Technology Co., Ltd. (Shanghai, China), which were transfected into MDA-MB-231 cells to block the expression of TRAF4 or to be as a negative control. The cells were cultured in DMEM medium (Gibco BRL, USA) with 10% fetal bovine serum (FBS, BI, US) at 37 °C in a 5% CO2 incubator. The green fluorescence protein GFP expression was observed by fluorescence microscope (Olympus, Tokyo, Japan) 72 h after transfection. These transfected cells were evaluated by fluorescence quantitative RT-PCR and western blot.

2.2. Construction of breast cancer xenograft model

Forty-two female BALB/c nude mice (4–6 weeks, 16–20g) obtained from Guangxi Medical University Experimental Animal Center were fed with sterilized food and water and housed in SPF animal room, all of which were in accordance with the guidance on the care and use of laboratory animals approved by Committee for Animal Management and Use of the Affiliated Tumor Hospital of Guangxi Medical University. The nude mice were randomly divided to three groups (n = 14): TRAF4-shRNA group, negative group and blank group.

The well-growing cells transfected with lentivirus TRAF4-shRNA, lentivirus negative-shRNA and MDA-MB-231 in 75cm2 bottles were digested, centrifuged (5 min, 1000 rpm, 4 °C), counted and made into cell suspension containing 5 × 106 cells. Then, the suspension (200μl) were injected respectively into the nude mouse mammary fat pad. Tumor proliferation was analyzed by observing and recording the changes of the xenografts every week. After 6 weeks, the nude mice were sacrificed by cervical dislocation, and the xenograft tumors, lung and liver tissues were harvested.

The Tumor volume formula: Volume (mm3) = width2 (mm2) × length (mm)/2.

2.3. Construction of xenograft metastasis model

The cells from the three groups cultured in 25cm2 bottles were respectively made into cell suspension containing 2 × 106 cells, and then suspension (100 μl) were injected into the left or right tail vein of female BALB/c nude mice. After 6 weeks, the nude mice were dissected, and lung and liver tissues were collected.

2.4. Hematoxylin and eosin (H&E) staining

The obtained lung and liver tissues were fixed in 10% formalin, dehydrated, transparent and embedded in paraffin. Consecutive sections were stained with hematoxylin and eosin, and then evaluated by experienced pathologists who were blinded to the experimental treatment conditions.

2.5. Immunohistochemistry

The mice xenograft tumors were fixed in 10% neutral formalin and embedded in paraffin after dehydration. The paraffin sections were placed in a 60-degree thermostat oven for 2 h, and were dewaxed and hydrated routinely. And then antigen retrieval was achieved by incubating the samples with sodium citrate buffer in high pressure. Next, the sections were blocked in 3% H2O2 for 10 min and in normal goat serum for 10 min. Then they were incubated separately with rabbit anti-human RSK4 (1:150, Abcam), TRAF4 (1:800, Abcam) solution at 4 °C overnight, followed by a second incubation with goat anti-rabbit-IgG for 10 min at room temperature, then were developed with DAB (3, 3′-diaminobenzidine tetrahydrochloride) and counterstained with hematoxylin. All specimens were assessed by two pathologists who were blinded to the experiment. The genes expression should be analyzed from the aspects of positive range and intensity. Staining intensity was scored as follows: 0 (negative staining); 1 (weak staining, light yellow); 2 (moderate staining, yellowish brown); 3 (strong staining, brown). The percentage of positive staining was also converted into 4 grades: 0 (0%–10%); 1 (10%–25%); 2 (25%–50%); 3 (50%–100%). The final score was determined by multiplying the intensity scores and positive scores. By observing at least 10 high-power fields and scoring, the score ≥4 is considered as high expression.

2.6. RNA extraction and qRT-PCR

Total RNA was extracted from tumor tissues of the three groups by RNAiso Plus.

(TAKARA, Japan) and reversely transcribed into cDNA according to the instruction of real-time fluorescence quantitative reverse transcription kit. qRT-PCR was performed by mixing 10 μl SYBR® Premix Ex Taq™ II with 0.8 μl sense and 0.8 μl antisense primers, 2 μl cDNA and 6.4 μl RNase-Free H2O through predenaturating 95 °C for 30 s followed by 40 cycles of 95 °C for 5s and 60 °C for 30s. Two controls needed to be set for every sample, and glyceraldehyde-3-phosphate dehydrogenase (GAPDH) was used as the internal control. The relative expression of target genes were calculated by the following formula: 2−ΔΔCt (ΔΔCt = ΔCt of the treated group-ΔCt of the control group).

Primers for RSK4, TRAF4, MMP2, MMP9 and GAPDH were as follows:

RSK4 Forward: 5′-TTGCCAAATGATCAGCCAAAGA-3′
Reverse: 5′-CTGGGCTAAGCTTGAAGCAGCTA-3′
TRAF4 Forward: 5′-CGTCTTTGACACCATCCAGAGC-3′
Reverse: 5′-TACAGCTGTCCTTCAGATGGC-3′
GAPDH Forward: 5′-GAAGGTGAAGGTCGGAGTC-3′
Reverse: 5′-GAAGATGGTGATGGGATTTC-3′
MMP2 Forward: 5′-CCCTGTCACTCCTGAGATCTGC-3′
Reverse: 5′-CACAGTCCGCCAAATGAACC-3′
MMP9 Forward: 5′-CCACCACAACATCACCTATTGG-3′
Reverse: 5′-ACTGGATGATGACGATGTCTGCG-3′