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Effect of RRS1 gene knockdown on BT549 cell line proliferation and apoptosis in breast cancer

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The RRS1 regulator of ribosome synthesis has recently been reported a new target gene linked to cancer development. This study therefore investigates RRS1effectsb on BT549 cell proliferation and apoptosis in breast cancer. Western blot (WB) and real - time quantitative PCR (qPCR) were used to detect the relative expression of RRS1 in breast cancer cells BT-549 and the normal HMEC mammary gland epithelial cells. BT-549 cells were cultured and infected with retroviruses and RRS1 expression was detected by qPCR and WB. The MTT assay, Caspase-3/7 and flow cytometry (FCM) then detected growth and apoptosis in the BT549 breast cancer BT cell. WB detected the expression of Bcl-2 and Bax genes related to apoptosis at the protein level, and MTT assay confirmed that RRS1 knockdown significantly decreased cell viability ($p<0.05$) and induced apoptosis which was rescued by shRNA-RRS1 expression. The amount of caspase-3 increased significantly and apoptosis was obvious. The apoptotic cells amount analyzed by FCM was significantly increased and RRS1 knockdown also decreased the expression of apoptosis related protein bcl-2 and simultaneously increased the expression of Bax ($p<0.05$). Finally, the RRS1 gene was highly expressed in breast cancer cell line BT549 and its knockdown significantly reduced proliferation and apoptosis in BT549 cell. These results suggest that RRS1 is a novel gene related to breast cancer and has an important role in breast cancer proliferation and apoptosis.

Key words: RRS1, BT549, apoptosis, bcl-2

Breast cancer is one of the most common malignant tumors in women. Although its incidence in China is lower than that in the West, with 3% morbidity increase a year in developed cities, the onset age tends to be younger and this is a serious threat to women's health [1]. Triple-negative breast cancer (TNBC) is a class of special ER, PR and HER-2 negatives type of breast cancer. Reports show that approximately 1 million new cases of breast cancer occur globally each year; with almost 170,000 (12–20%) cases involving the TNBC phenotype [2]. Compared with other types of breast cancer, TNBC is more common in young women and has a higher degree of clinical invasion and a worse prognosis [3, 4]. Although it is sensitive to chemotherapy, TNBC is prone to early metastasis and both endocrine and Her-2 targeted therapies are ineffective. Due to the heterogeneity of the disease itself and the complexity of the TNBC molecule expression characteristics, TNBC lacks corresponding targeted therapies. Exploration for new breast cancer targets has therefore become a hot spot in current research.

The regulator of ribosome synthesis 1 (RRS1) is a nuclear protein consisting of 203 amino acids, it is critical for ribosome synthesis and it can regulate ribosome synthesis speed according to the cell state and then maintain cell homeostasis [5]. The abnormal expression of RRS1 has negative influence on the synthesis of ribosomes, leading to abnormal ribosome physiological function It has also been reported that the incidence of cancer is closely related to the abnormal expression of RRS1 [6–8]. Gambe et al. used RNA interference technology to knockdown the RRS1 gene expression level, and they found that the number of cells in the tetraploid period increased and cell division time was significantly longer. This shows that cervical cancer cells RRS1 gene expression level is related to cell proliferation [9]. Wang et al. also found that the RRS1 gene expression level

in hepatocellular carcinoma tissue was significantly higher than in para-cancer tissues, and that the RRS1 gene is associated with proliferation, apoptosis, invasion and metastasis in hepatocellular carcinoma SMMC-7721 cells [10]. Recent studies have indicated that RRS1 is over-expressed in liver and colon cancer and is associated with tumor proliferation [10, 11].

In this work, BT549 cells were cultivated in vitro because BT549 is classified as TNBC. The expression of RRS1 gene was knocked down by lentivirus, and the possible mechanism was investigated by detecting the expression of bcl-2 and bax which is the regulatory gene of apoptosis. It is therefore possible to consider the RRS1 gene a new potential target in breast cancer treatment.

Materials and methods

Cell line and cell culture. The BT-549 human breast cancer cell line was obtained from the library of affiliated hospital of Qingdao University, and the normal breast HMEC cell was obtained from Gennio Biological Technology Company. The cells were maintained in DMEM medium supplemented with 10% heat-inactivated fetal calf serum at 37 °C in a tissue culture incubator with 5% CO_2 and 98% relative humidity. When 70% of the plate was covered by cells, lentivirus was added to infect the cells. Following a 48 h incubation period, the cells were harvested and used for apoptosis, mRNA and protein analysis.

Real-time quantitative PCR. Total RNA was extracted from the BT-549 cells (10^7) cultured in the presence or absence of shRNA-RRS1 and HMEC cells using a Trizol RNA isolation kit (Life Technologies, Gaithersburg, MD, USA) and detected by real-time quantitative PCR(qPCR), according to the manufacturer's protocol. Primers for GAPDH were: 5'-AGAAGGCTGGGGCTCATTTG-3', 5'-AGGGGCCATCCACAGTCTTC-3'. The primer of RRS1 is 5'-CCCTACCGGACACCAGAGTAA-3', 5'-CCGAAAAGGGGTTGAAACTTCC-3'. Each qPCR assay was performed in triplicate. A reverse transcription kit (Prime-Script qPCR Kit; Transgen, Beijing, China) was used to construct the template cDNA for qPCR with TransStart Probe qPCRSuperMix (Transgen, Beijing, China). The data was gathered on a Bio-Rad One-Step Plus system. (California, USA).

Lentivirus transfection. The cells were passed in a specific density and after 24 h the cell density reached 70~80%. The amount of lentivirus was accurately calculated according to the number of cells (The number of virus = the number of cells*MOI, the titer of the virus is 3×10^8 TU/ml, the virus titer of the control group is 5×10^8 TU/ml, and the MOI value of the pre-experiment is 20). The solution was changed after transfection for 8 h, and its efficiency was observed under fluorescence microscope after transfection for 48–72 h. Experimental groups; the BT549 cells infected by Lentiviral with and without RNA interference form the shRNA-RRS1 group and negative control, respectively.

Cell viability assay. The viability of cells was measured by MTT assay. Cells were infected by lentivirus with shRNA-RRS1 or control, and then seeded independently in a 96-well plate with a final volume of 100 μl of complete culture medium containing 3000 cells/well (six wells for each concentration). Cells were cultured for 1 to 5days at 37 °C. After treatment, 10 μl MTT (stock 5 mg/ml) was added to each well and incubated at 37 °C for 4 h. The absorbance of the samples was measured at 490 nm by Microplate Reader (Synergy H1; BioTek, Vermont, USA). Each independent experiment was run three times.

Apoptosis analysis by caspase-3,7 assay. Caspase activity/apoptotic activity was determined using the caspase-3/7 Active Apoptosis Assay kit (Sangon biotech, China): 3,000 cells per well were seeded and grown for 3 days. Concentrations of 10 μM (effective dose close to the IC50 of the cell viability experiments) and higher doses were tested. Caspase-3/7 activity was assessed following the manufacturer's instructions.

Apoptosis analysis by FCM. The shRNA-RRS1 group and control group cells were plated in 6-well plates at a density of 3×10^5 cells per well. The cells were incubated until they were approximately 80% confluent, and assay medium was added. Apoptosis rates were determined by the APC Annexin V Apoptosis Detection Kit I (BD Biosciences Pharmingen) and analysis by flow cytometry was performed within 1 h.

Western blot. BT-549 cells were lysed in RIPA buffer (Solabio, Beijing, China) with protease inhibitor cocktail (Sigma) on ice for 1 h and then centrifuged for 20 min at 13,000 rpm. The supernatant was collected and protein concentration was measured with BCA assay (Beyotime, Jiangsu, China). After being resolved on 10% SDS-PAGE gels, proteins were transferred to a PVDF membrane and blocked with 5% BSA in TBST for 2 h at room temperature. The membranes were subsequently incubated overnight at 4 °C with the corresponding antibody GAPDH, Bax and Bcl-2 from Abcam (California, USA). After being washed with TBST three times, the blots were incubated with 1:2,000 dilution of the HRP-conjugated secondary antibody (Boster, Hubei, China), and washed again three times with TBST. The transferred proteins were visualised by ECL (Transgen, Beijing, China).

Statistical analysis. The data was analysed by statistical software SPSS17.0 for Windows. The differences between groups were tested by t-test of independent samples, and experimental data was expressed in terms of x±s. The $p<0.05$ was considered statistically significant.

Results

The expression of RRS1 in BT549 and HMEC cells. The mRNA expression of RRS1 in BT549 cells was 11.91±0.37, significantly higher than that in normal breast cell HMEC ($p<0.001$, Figure 1A). The results of WB assay showed that the expression of RRS1 protein in breast cancer BT549 cells

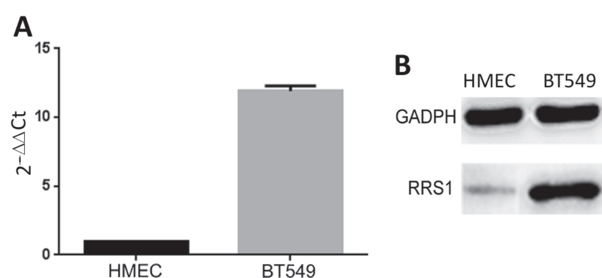

Figure 1. mRNA expression of RRS1 in BT549 cell line and HMEC normal breast cells (A). Protein expression of RRS1 in BT549 cell line and HMEC normal breast cells (B).

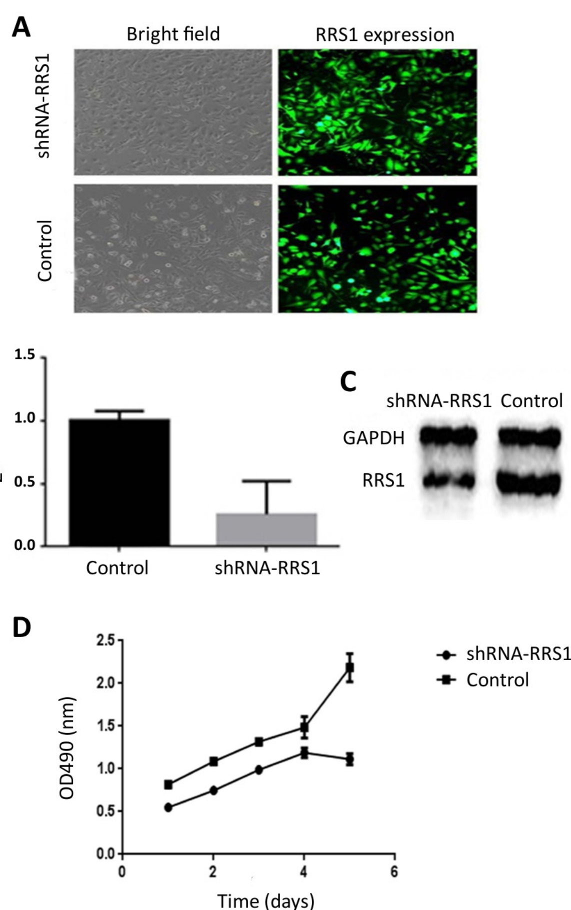

Figure 2. The lentivirus transfection efficiency of breast cancer BT549 cells was observed under a fluorescence microscope (A). The RNA expression of RRS1 gene in shRNA-RRS1 and control group of lentivirus infected breast cancer BT549 cells (B). The protein expression of RRS1 gene in shRNA-RRS1 and control group of lentivirus infected breast cancer BT549 cells (C). The proliferation of shRNA-RRS1 and control groups was detected by MTT assay (D).

was 0.38±0.04, and normal breast cells 0.09±0.01 (p=0.001, p<0.01, Figure 1B). The mRNA and protein expression of RRS1 in BT549 cells was significantly higher than that in normal breast cells HMEC, so these two results were consistent.

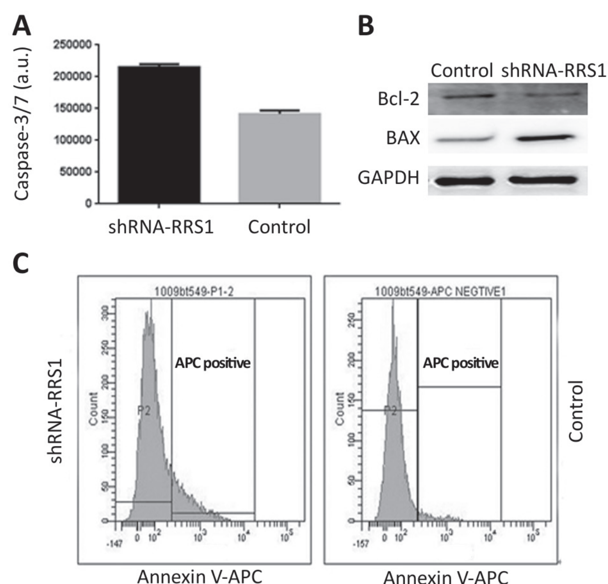

Figure 3 The proliferation of shRNA-RRS1 and control groups was detected by MTT assay. (A). BAX and Bcl-2 protein of breast cancer cell line BT549 after RRS1 gene knockdown was detected by WB (B). Apoptosis of breast cancer cell line BT549 after RRS1 gene knockdown was detected by FCM (C).

RRS1 knockdown efficiency. After BT549 cells were transfected with lentivirus for 72h, fluorescence microscope cell counts determined that transfection efficiency was over 70.9% (Figure 2A). RNA and protein were extracted and RNA was amplified by qPCR. There was significant difference between the expression of RRS1 gene in shRNA-RRS1 and control group (0.27±0.12 and 1.00 – p<0.001 – Figure 2B). RRS1 protein expression in the shRNA-RRS1 and control group were 0.74±0.14 and 1.80±0.13 (p=0.001) and there was statistical difference between them (Figure 2C).

Cell viability was detected by MTT. The knockdown of RRS1 gene in BT549 cells significantly reduced cell proliferation ability, and the difference between two groups was significantly increased from the third day. The number of cells in the shRNA-RRS1 group began to decrease from the third day (Figure 3).

Detection of apoptosis by Caspase-3/7. After transfection with lentivirus, the fluorescence signal of shRNA-RRS1 group was obviously higher than the control group. The fluorescence signal in shRNA-RRS1 group is 214,377±5,518 while in the control group it was 141,528±5,471. There was significant difference between the two groups (p<0.001, Figure 3A).

Apoptosis detected by APC Annexin V. The number of pre-apoptotic cells in shRNA-RRS1 group was significantly increased after breast cancer BT549 cells were infected by

lentivirus for 3 days. The number of early apoptotic cells was 15.8±2.5% and 6.3±1.4% of the total cells in shRNA-RRS1 and control groups, respectively; with significant statistical difference between them. (p=0.006, Figure 3C).

Expression of BAX and Bcl-2 proteins. After RRS1 gene knockdown, the expression of BAX protein increased and the expression of bcl-2 protein decreased. BAX protein expression is 4.30±0.23 and 2.9±0.11 in shRNA-RRS1 and control group, respectively (p=0.001). Bcl-2 protein expression is 1.56±0.10 and 2.21±0.10 in both groups respectively (p=0.002). There is a statistically significant difference between these groups (Figure 3B).

Discussion

The RRS1 regulator of ribosome synthesis is a ribosomal protein closely related to ribosome function. Recent studies have shown that RRS1 is related to tumor development in cervical and liver cancer. However, there is little research on the relationship between RRS1 gene and breast cancer [9]. We found that the expression of RRS1 gene in breast cancer BT549 cells was significantly higher than in normal HMEC breast cells. The experiment also showed that silencing the RRS1 gene in BT549 breast cancer cells can inhibit cell proliferation and promote cell apoptosis. Changes in bcl-2 and bax protein expression are closely related to apoptosis, and these provide new potential targets for breast cancer treatment.

Tumor formation results from imbalanced cell regulation of proliferation and apoptosis. Therefore, neoplasia inhibition could not only inhibit cell proliferation, but also induce their apoptosis. In this experiment, the reduced RRS1 expression significantly inhibited cell proliferation, as detected by MTT. We then observed that caspase3 significant increased after RRS1 gene knockdown; so this may induce BT549 cell apoptosis in breast cancer.

Bcl-2 family proteins are closely related to apoptosis and Bcl-2 is the most studied anti-apoptotic protein with an important role in a variety of cell types [12]. Bax gene and bel-2 gene are in the bcl-2 gene family of proto-oncogene, and while bax promotes apoptosis bcl-2 inhibits it. It is generally accepted that the expression of the bax gene does not immediately lead to apoptosis, but plays an important role after the cells receive the death signal through bcl-2 role inhibition; providing subsequent apoptosis [13]. Oltrai et al [14] found that the occurrence of apoptosis was related to the value of (bcl-2:bax); the higher the ratio, the higher the cell survival rate and the lower the ratio, the higher the apoptosis rate. After knocking down the RRS1 gene in this experiment, the expression of bcl-2 protein decreased and the expression of bax protein increased compared to the negative control group. The experiment group found that RRS1 gene knockdown inhibited the proliferation of BT549 breast cancer cells and induced apoptosis. The most likely mechanism is that decreased bcl-2 protein expression and increased bax protein expression lower the ratio of bcl-2/bax, and therefore RSS1 knockdown induced BT549 cells apoptosis through the bcl-2/bax pathway.

Finally, our combined results show that the RRS1 gene is highly expressed in the BT549 breast cancer cell line and its knockdown significantly reduces cell proliferation and induces apoptosis. While study of the precise molecular mechanisms involved is required, we confirm RRS1 is a novel gene related to breast cancer, and that it has an important role in breast cancer proliferation and apoptosis.

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