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miR-24 inhibited the killing effect of natural killer cells to colorectal cancer cells by downregulating Paxillin

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ABSTRACT

Objective: To identify the molecular mechanism that modulates the killing effect of natural killer (NK) cells to colorectal cancer cells.

Materials and methods: Expressions of miR-24 and Paxillin were detected by qRT-PCR and Western blot. Secretions of IFN-γ and TNF-α were measured by ELISA. The killing effect of NK cells was detected by CytoTox 96 non-radioactive cytotoxicity assay. Luciferase reporter assay was conducted to confirm the regulation of miR-24 on Paxillin.

Results: miR-24 was overexpressed in NK cells from patients with colorectal cancer than healthy volunteers. Secretions of IFN-γ and TNF-α in activated NK cells were significantly increased, indicating the enhancement of the killing effect of NK cells. Paxillin expression was overexpressed in activated NK cells. Interference of Paxillin significantly decreased Paxillin expression, secretions of IFN-γ and TNF-α, and the killing effect of NK cells to colorectal cancer cells. In addition, we confirmed that Paxillin was a direct target of miR-24, and miR-24 was negatively correlated with Paxillin. Moreover, overexpression of miR-24 inhibited secretions of IFN-γ and TNF-α, and decreased cytotoxicity by downregulating Paxillin expression. Finally, we observed that overexpression of Paxillin significantly decreased tumor volume of colorectal cancer.

Conclusion: Overexpression of miR-24 supressed the killing effect of NK cells to colorectal cancer cells by downregulating Paxillin expression.

1. Introduction

Colorectal cancer is one of the most common malignancies in digestive system and the third most common cancer of all malignancies [1,2]. It has been reported that the death of colorectal cancer was mainly caused by tumor metastasis [3]. Researchers have reported that immune system was responsible for eliminating tumor cells and infected cells [4]. And natural killer (NK) cells are the major cells that are responsible for eliminating tumor cells by contact-dependent cytotoxicity and cytokine production for immune modulation [5]. Interferon gamma (IFN-γ) and Tumor necrosis factor alpha (TNF-α) are cytokines produced by NK cells which promote the apoptosis of tumor cells and induce cytolysis of tumor cells [6]. Therefore, it is vital to identify the molecular mechanism that modulates the killing effect of NK cells to colorectal cancer cells.

MicroRNAs play a pivotal role in the cancer development, progression and metastasis by targeting the 3′-untranslated region (3′-UTR) of specific target mRNAs [7]. According to the previous researches, miRNAs can regulate the function of NK cells through different mechanisms [8–10]. It has been reported that miR-24 could be expressed in NK cells with unexplained recurrent spontaneous abortion [11], and downregulation of miR-24 promoted the secretion of IFN-γ and TNF-α [12]. Therefore, we speculate that miR-24 may also negatively regulate the secretion of IFN-γ and TNF-α in colorectal cancer.

Paxillin is a cytoskeletal protein involved in tumor progression and metastasis [13]. Researches have found that Paxillin was highly expressed in many cancers [14,15]. Qin et al. observed that Paxillin was upregulated in colorectal cancer tissue [16]. Moreover, Gismondi et al. found that Paxillin could be expressed in human NK cells [17], and phosphorylation of Paxillin was related with antibody-dependent cell-mediated cytotoxicity (ADCC) [18]. Hence, Paxillin may be related with the killing effect of NK cells in colorectal cancer. In addition, bioinformatics software predicted there were combination sites between miR-24 and Paxillin. Thus, we assumed miR-24 might regulate Paxillin to affect the killing effect of NK cells to colorectal cancer cells.

In this study, we detected the expressions of miR-24 and Paxillin in NK cells from patients with colorectal cancer. We also detected the secretion of IFN-γ and TNF-α and Paxillin expression in activated NK

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cells, and found interference of Paxillin inhibited the killing effect of NK cells to colorectal cancer cells. Meanwhile, we confirmed Paxillin was a direct target of miR-24. Moreover, we found miR-24 was overexpressed and inhibited the killing effect of NK cells to colorectal cancer cells by downregulating Paxillin expression.

2. Materials and methods

2.1. Isolation of NK cells

Human NK cells were isolated from human peripheral blood mononuclear cells (PBMCs) of 20 patients with colorectal cancer and healthy volunteers by NK cell isolation kit (Miltenyi Biotech, German) according to the manufacturer's instructions. The patients and healthy volunteers were recruited at The First Affiliated Hospital of Zhengzhou University, and the PBMCs were collected according to protocols approved by the Ethics Committee of The First Affiliated Hospital of Zhengzhou University. Non-NK cells were magnetically labeled by biotin-conjugated antibodies and the NK cell microBead mixture. Magnetically labeled cells were depleted to obtain highly pure NK cells.

2.2. Cell culture and transfection

Human NK cell line NK92 and mouse NK cell line LNK were cultured as previously reported [19]. NK92 cells were cultured in minimum essential medium Eagle's with Earle's salts and nonessential amino acids, supplemented with 12.5% fetal bovine serum (FBS; Gibco, USA), 12.5% horse serum (Gibco, USA), 100 U/ml penicillin (Sangon Biotech, China), 100 U/ml streptomycin (Sangon Biotech, China), 2 mM L-glutamine (Gibco, USA), 100 μM 2-mercaptoethanol (Sangon Biotech, China), 100 U/ml IL-2 (PeproTech, USA). LNK cells were cultured in RPMI 1640 medium supplemented with 10% FBS (Gibco, USA), 100 U/ml penicillin (Sangon Biotech, China), 100 U/ml streptomycin (Sangon Biotech, China), 2 mM L-glutamine (Gibco, USA), and 50 μm 2-mercaptoethanol (Sangon Biotech, China).

Human colorectal cancer cell line SW620 was cultured in Dulbecco's Modified Eagle's Medium (DMEM) (Invitrogen, USA), supplemented with 10% FBS (Gibco, USA), 100 U/ml penicillin and 100 U/ml streptomycin in 5% CO_2 incubator at 37 °C.

si-Paxillin, pcDNA-Paxillin, miR-24 mimic, miR-24 inhibitor and their negative controls were synthesized by Invitrogen (Shanghai, China). The siRNA transfection was performed using Lipofectamine 2000 transfection reagent (Invitrogen, USA) according to the manufacturer's instructions.

Before coculture, culture medium was removed, and NK cells were washed with PBS. 1×10^5 NK cells were seeded in each well of Transwell plate (Corning, USA). Then, 1×10^6 SW620 cells in RPMI 1640 (2 ml) were added into each well. Coculture was performed for 72 h.

2.3. Quantitative real-time PCR

Total RNA was extracted using TRIzol (Invitrogen, USA) according to manufacturer's instructions. cDNA was synthesised with Maxima H Minus First Strand cDNA Synthesis Kit (Thermo Scientific, USA). Real-time PCR reactions were done by using an ABI Prism 5700 Sequence Detection System (Applied Biosystems, USA). A total fluid volume of 25 μL and TaqMan RT-PCR Master Mix Reagents (Applied Biosystems, USA) were used for amplification. GAPDH was used as an endogenous control to normalize the difference in the amount of total RNA in each sample. Results were analyzed using ABI Prism 5700 SDS software (Applied Biosystems, USA), and the relative expression of miR-24 was calculated by the comparative method $2^{-\Delta\Delta Ct}$. miR-24 (human) forward primer 5′-CCGTGGCTCAGTTCAGCAG-3′, reverse primer 5′-CAG TGCAGGGTCCGAGGTAT-3′.

2.4. Western blot analysis

The proteins from the cells were washed for 3 times and extracted by using RIPA buffer on the ice for 30 min and centrifuged at 12,000 rpm for 15 min at 4 °C. Protein concentrations were determined by BCA Protein Assay kit (Pierce Biotechnology, USA). Samples were separated by 10% SDS-PAGE and transferred to PVDF membranes (Millipore, USA) in transfer buffer (25 mM Tris, 192 mM glycine, 10% methanol, 0.1% SDS), then blocked in 5% skim milk for 2 h. Anti-Paxillin (Invitrogen, USA) and anti-β-actin (Invitrogen, USA) were used as the first primary antibody at 1:1000 dilutions, then the corresponding horseradish peroxidase-conjugated secondary antibody (Invitrogen, USA) was added and incubated at room temperature for 1 h. Protein was detected by the enhanced chemiluminescence system (Roche, Switzerland), and β-actin was used as a control protein.

2.5. Enzyme-linked immunosorbent assay (ELISA)

The protein expressions of IFN-γ and TNF-α were quantified by human IFN-γ ELISA KIT (Signalway Antibody, USA) and human TNF-α ELISA KIT (Creative Diagnostics, USA) according to the manufacturer's instructions. The absorbance was measured at 450 nm with a microplate reader (Synergy HT; BioTek).

2.6. Cytotoxicity assays

CytoTox 96 non-radioactive cytotoxicity assay (Promega, USA) was used to detect the killing effect of NK cells according to the manufacturer's instructions. SW620 cells were washed with PBS, resuspended with fresh NK92 culture medium, and seeded in a 96-well plate (5000 cells/well). IL-2-induced NK92 cells were added at effector-to-target (E:T) ratios of 32 : 1, 16 : 1, and 8 : 1 and incubated in 5% CO_2 incubator at 37 °C for 4 h. The supernatant was obtained and analyzed by the CytoTox 96 non-radioactive cytotoxicity assay. The killing effect of NK cells against SW620 cells was calculated by the following equation: Cytotoxicity = (Experimental-Effector spontaneous-Target spontaneous)/(Target maximum − Target spontaneous) × 100%.

2.7. Dual luciferase reporter assays

Luciferase report gene vectors containing Paxilln 3′UTR Wild Type (WT) or Paxilln 3′UTR Mutant (MUT) was transfected into HEK293T cells with pRL-TK vectors (Promega, USA). miR-24 mimic or miR-24 inhibitor or negative controls were co-transfected with reporter plasmids for 48 h. Cells were obtained to measure the luciferase activity by dual Glo™ Luciferase Assay System (Promega).

2.8. Establishment of nude mice model of colorectal cancer

Twelve female BALB/c nude mice were purchased from the Animal Experimental Center of Zhengzhou University. All experiments were approved by Ethics Committee of The First Affiliated Hospital of Zhengzhou University. Human SW620 cells (6×10^6 cells) were subcutaneously injected into nude mice. IL-2-induced LNK cells or NK-92 cells (2.8×10^6 cells) transfected with pcDNA or pcDNA + Paxillin were subcutaneously injected at 2 h after SW620 implantation. Tumor volume was measured every five days and mice were sacrificed twenty days later after cell implantation.

2.9. Statistical analysis

All experiments were performed in triplicate, and data were presented as mean ± standard deviation (SD). SPSS 18.0 software was used for data analysis. The differences in miR-24 level, IFN-γ secretion, TNF-α secretion, and cytotoxicity between control and other treated groups were analyzed by *t*-test or one-way analysis of variance