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# miR-20a inhibits the killing effect of natural killer cells to cervical cancer cells by downregulating RUNX1

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ABSTRACT

*Background:* NK cells are presented in tumor microenvironments and acts as an essential defense line against multiple malignancies. Recently, miRNAs are reported to involve in the development of natural killer (NK) cells via negatively regulating gene expression. Here, we aim to explore the function and mechanism underlying how miR-20a modulated the killing effect of NK cells to cervical cancer cells.
*Methods:* Abundances of miR-20a and runt-related transcription factor 1 (RUNX1) in NK cells from cervical cancer patients and healthy donors were detected by qRT-PCR and western blot. The releases of IFN-γ and TNF-α were determined by ELISA. The cytotoxicity of NK cells against cervical cancer cells was measured by CytoTox 96 non-radioactive cytotoxicity assay. Luciferase reporter, western blot, and RNA immunoprecipitation (RIP) assays were performed to assess the interaction between miR-20a and RUNX1.
*Result:* miR-20a was upregulated while RUNX1 was downregulated in NK cells from cervical cancer patients compared to healthy donors. IL-2 stimulated the releases of IFN-γ and TNF-α, and the killing effect of NK cells to cervical cancer cells, which was overturned by miR-20a introduction. RUNX1 was identified to be a target of miR-20a. Restoration of RUNX1 abolished the inhibitory effects of miR-20a on the secretions of IFN-γ and TNF-α, as well as the killing effect of NK cells to colorectal cancer cells.
*Conclusion:* miR-20a attenuated the killing effect of NK cells to cervical cancer cells by directly targeting RUNX1.



## 1. Introduction

Cervical cancer is one of the most common malignancies in women, with almost 98900 new cases and 30500 deaths in China just in 2015 [1]. As a major obstacle for public health, cervical cancer can be triggered by a range of risk factors, like smoking, and immune dysfunction, and human papillomavirus infection [2]. Although standard surgery and chemoradiotherapy have improved the survival of cervical cancer patients, treatment for patients with advanced stage remains controversial. A growing number of evidences have indicated that immune disorder was responsible for the development of multiple cancers [3]. Natural killer (NK) cells, a common type of immune cells stimulated by IL-2, can involve in the innate immune defense of human body against cancers through releases of cytokines, such as interferon gamma (IFN-γ) and tumor necrosis factor alpha (TNF-α) [4]. IFN-γ and TNF-α produced by NK cells promote the apoptosis of tumor cells [5]. Therefore, it is urgent to identify a novel molecular mechanism that regulates the killing effect of NK cells to cervical cancer cells.

miRNAs are reported to play a key role in the modulation of cell progression and metastasis via base-pairing with the 3′-UTR of target mRNA, leading to the degradation or translation inhibition of this mRNA [6,7]. On the basis of previous researches, miRNAs can function as a major oncogene or tumor suppressor of various cancers by reprogramming NK cell-mediated cytotoxicity to tumor cells by different mechanisms [8,9]. miR-20a is a wildly known oncomiR that is associated with the development of several cancers, including osteosarcoma [10], prostate cancer [11], and cervical cancer [12]. However, the regulatory role of miR-20a in NK cell

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cytotoxicity against cervical cancer cells remains unclear.

Runt-related transcription factor 1 (RUNX1), located on chromosome 21 (21q22.12) with 260 kilobases (kb) in length, is closely associated with numerous tumor processes, containing cell survival, cycle arrest, apoptosis, and metabolism. Tremendous studies have described that RUNX1 was frequently downregulated and exerted tumor-suppressive role in various cancers. Mendler et al. confirmed that the mutation of RUNX1 was negatively influenced the outcome of patients with primary cytogenetically normal acute myeloid leukemia (CN-AML) in younger and older groups [13]. Sakakura et al. observed a 62% RUNX1 decrease in resected gastric cancer specimens compared to surrounding mucosa. Moreover, the percentage of downregulated RUNX1 increased with the cancer stage progressed [14]. In addition, RUNX1 was reported to play a key role in the regulation of NK cell differentiation [15]. Hence, we conjectured that RUNX1 might be implicated with the killing effects of NK cells to cancer cells.

Here, we investigated the roles of miR-20a and RUNX1 in the cytotoxicity of NK cells to cervical cancer cells. Alterations of miR-20a and RUNX1 were observed in NK cells from patients with cervical cancer. Moreover, RUNX1 was identified to be a direct target of miR-20a. Addition of miR-20a inhibited the killing effect of NK cells to cervical cancer cells by negatively regulating RUNX1 expression.

## 2. Materials and methods

### 2.1. NK cell isolation

Peripheral venous blood was collected from 25 cervical cancer patients and healthy donors recruited at Affiliated Jiangyin Hospital of South-East University, and peripheral blood mononuclear cells (PBMCs) were isolated using Human PBMCs separation kit (Solarbio, Beijing, China). Then NK cells were isolated from PBMCs using MagCellect Human NK Cell Isolation Kit (R&D Systems, Minneapolis, MN, USA) in accordance to the instruction provided by manufacturer. Written informed consents were signed by all participants with the approval of the Research Ethics Committee of Affiliated Jiangyin Hospital of South-East University prior to this study.

### 2.2. Cell culture and transfection

Isolated NK92 cells were maintained in an incubator of 5% $CO_2$ at 37 °C for 24 h in presence of RPMI-1640 complete medium containing 10% of fetal bovine serum (FBS; Gibco, Grand Island, NY, USA), 2 mM L-glutamine (Gibco), 100 U/ml penicillin and streptomycin (Solarbio), 100 U/ml IL-2 (PeproTech, USA). 293T cells and cervical cancer cells HeLa required for this study were obtained from the American Type Culture Collection (ATCC, Manassas, VA, USA), and incubated in RPMI-1640 medium supplemented with 10% of FBS, 1% penicillin/streptomycin.

RUNX1-overexpressed plasmid (pcDNA-RUNX1) was synthesized in Genepharma (Shanghai, China) by inserting the full-length sequences of RUNX1 into pcDNA.3.1 vector. MiR-20a mimics and negative control (NC), miR-20a inhibitor and inhibitor control (NC) were also purchased from GenePharma Co., Ltd (Shanghai, China). NK Cells ($1 \times 10^5$) were seeded into 6-well microplates, followed by cell transfection with miRNAs or plasmids using Lipofectamine 2000 reagent (Invitrogen, Carlsbad, CA, USA) in term of manufacturer's procedures. About 48 h post-transfection, cells were collected for the following studies.

### 2.3. Quantitative real-time polymerase chain reaction (qRT-PCR)

Total RNA from NK cells was extracted using Trizol reagent (Thermo Fisher Scientific, Wilmington, DE, USA). Then, cDNA for miR-20a or RUNX1 mRNA was synthesized using TaqMan™ MicroRNA Reverse Transcription kit (Thermo Fisher Scientific) or High Capacity cDNA Reverse Transcription Kit (Thermo Fisher Scientific). For the evaluation of miR-20a and RUNX1 mRNA expression, RT-PCR reaction was performed using Universal SYBR Green PCR Kit (Takara, Dalian, China) and specific primers, and ran on an ABI 7500 Real-Time PCR system (Applied Biosystems, Foster City, CA, USA). U6 snRNA or GAPDH was employed as a housekeeping gene to normalize the difference of miR-20a or RUNX1 mRNA in each sample. qPT-PCR primers were shown as below: RUNX1 (Forward, 5′- CTGCCCATCGCTTTCAAGGT-3'; Reverse, 5′-GCCGAGTAGTTTTCATCATTG CC-3′); GAPDH (Forward, 5′- TATGATGATATCAAGAGGG TAGT -3'; Reverse, 5′- TGTATCCAAACTCATTGTCATAC -3′). The primers for miR-20a and U6 were synthesized in GenePharma Co., Ltd. All experiments were carried out independently in triplicate and results were calculated using $2^{-\Delta\Delta Ct}$ method.

### 2.4. Western blot assay

The protein from NK cells were extracted by using RIPA lysis buffer (Thermo Fisher Scientific) in the presence of proteinase inhibitor (Solarbio). Proteins denatured at 98 °C for 5min were separated by SDS-PAGE gel and electrotransferred to PVDF membranes (Millipore, Billerica, MA, USA). Then, the membranes were blocked in 5% skim milk for 2 h at 37 °C and probed with rabbit anti-RUNX1 (1:1000) or anti-beta actin (1:5000) (Abcam, Cambridge, MA, USA) overnight at 4 °C. After washing, the membranes were hatched with horseradish peroxidase (HRP)-conjugated goat anti-rabbit IgG antibody (1:10000) (Abcam) for 1.5 h at 37 °C. Finally, proteins were detected by the enhanced chemiluminescence system (Roche, Basel, Switzerland).

### 2.5. ELISA assay

The levels of IFN-γ and TNF-α proteins were determined using human IFN-γ ELISA kit (Abcam) or human TNF-α ELISA kit (Abcam) according to manufacturers' instructions.

### 2.6. Cytotoxicity assay

CytoTox 96 non-radioactive cytotoxicity assay (Promega, Madison, WI, USA) was carried out to test the killing effect of NK cells to cervical cancer cells. Briefly, HeLa cells were washed with PBS, resuspended with complete NK medium, and then seeded into a 96-well microplate at a density of $5 \times 10^3$ cells/well. IL-2-induced NK cells ($5 \times 10^4$) were mixed with HeLa cells, and then incubated for 4 h at 37 °C. The supernatant derived from mixed cells was obtained by centrifugation, and the cytotoxicity of NK cells against cervical cancer cells was evaluated in terms of the standard formula: Cytotoxicity= (Experimental-Effector spontaneous-Target spontaneous)/(Target maximum - Target spontaneous) × 100%.

### 2.7. Luciferase reporter assay

The 3′-UTR fragments of RUNX1 containing putative miR-20a binding sites were cloned into psiCHECK-2 vector (Promega) to generate wild-type RUNX1 reporter (WT). Then, KOD-plus-mutagenesis kit (Toyobo, Osaka, Japan) was employed to generated mutant RUNX1 reporter (MUT) by mutating the putative binding sites of miR-20a in RUNX1 3′-UTR. Wild-type or mutant

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