Biochemical and Biophysical Research Communications xxx (2018) 1–7

Contents lists available at ScienceDirect

Biochemical and Biophysical Research Communications

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

HIF-1α induces immune escape of prostate cancer by regulating NCR1/NKp46 signaling through miR-224

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ARTICLE INFO

Article history:
Received 24 May 2018
Accepted 5 June 2018
Available online xxx

Keywords:
Prostate cancer
HIF-1α
miR-224
NCR1/NKp46
Natural killer cells

ABSTRACT

Background: Metastasis of prostate cancer (PCa) is largely affected by natural killer (NK) cells. This study aimed to clarify the mechanisms underlying tumor cells escaping from NK cells mediated by HIF-1α.
Methods: MiR-224 expression in lymphocytes and HIF-1α protein level in NK cells were determined by qRT-PCR and western blot, respectively. The amount of NKp46^{+} NK cells was detected with flow cytometry. The IFN-γ level was examined by enzyme linked immunosorbent assay (ELISA). NK cells were tested for cytolytic activity with a Non-Radioactive Cytotoxicity Assay, and treated with oxygenglucose deprivation (OGD) for hypoxia simulation. Interaction between miR-224 and NCR1 was evaluated with dual luciferase reporter assay.
Results: MiR-224 was down-regulated in lymphocytes isolated from prostate cancer tissues (n = 10). Overexpression of miR-224 protected prostate cancer from NK cells. HIF-1α increased miR-224 to inhibit the killing capability of NK cells on prostate cancer. MiR-224 controlled the expression of NCR1. Overexpression of miR-224 protected prostate cancer from NK cells through NCR1/NKp46 signaling. Suppression of HIF-1α enhanced the cytotoxicity of NK cells on prostate cancer via miR-224/NCR1 pathway.
Conclusion: HIF-1α inhibits NCR1/NKp46 pathway through up-regulating miR-224, which affects the killing capability of NK cells on prostate cancer, thus inducing immune escape of tumor cells.

1. Introduction

Prostate cancer (PCa) is the most prevalent solid malignant tumor in men, with increasing morbidity and mortality in developed western countries and in Asian countries [1,2]. Worse still, it is unachievable for cure of metastatic prostate cancer, which is the leading cause of mortality of this malignancy. It has been reported that the metastasis of prostate cancer is largely affected by natural killer (NK) cells [3], and highly effective NK cells are related to good prognosis in patients with metastatic prostate cancer [4]. NK cells are emerging as a promising tool for adoptive immunotherapy of prostate cancer.

NK cells represent a lymphoid subpopulation characterized by the ability to raise a potent cytotoxic activity against tumor or virally infected cells, whose function is regulated by lots of surface receptors [5]. NKp46 is one of the natural cytotoxicity receptors, and it is a main activating receptor of NK cells, with a mouse ortholog, named NCR1 [6]. NK receptor NKp46/NCR1 played a key role in controlling tumors metastasis [7]. It has been demonstrated that NKp46 was reduced in patients with prostate cancer and prostate cancer cells down-regulated the expression of NKp46, thus preventing their recognition of tumor cells [8]. In response to hypoxia, NK cells rapidly accumulated the hypoxia-inducible factor 1α (HIF-1α), but hypoxia down-regulated NKp46 and impaired the capability of NK cells of killing tumor target cells [9]. Hypoxia is a common feature of prostate tumors, leading to increased tumor aggressiveness [10], but the interaction between HIF-1α and NKp46 and their role in hypoxia-influenced NK cells killing capability have not been clarified in prostate cancer.

MicroRNAs (miRNAs) are a class of small noncoding RNAs that negatively regulate gene expression at the posttranscriptional level.

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https://doi.org/10.1016/j.bbrc.2018.06.007
0006-291X/

Altered expression of miRNAs has been identified in prostate cancer tissues and they have been considered as prognostic and predictive markers in treating this disease [11]. Aberrant expression of miR-224 plays a vital role in tumor biology in various types of human cancer. A previous study has revealed that miR-224 was down-regulated in human prostate cancer tissues when comparing with adjacent benign tissues, and enhanced expression of miR-224 repressed cell proliferation, invasion and migration, and promoted cell apoptosis in prostate cancer [12]. Interestingly, it has been recently reported that miR-224 was up-regulated by hypoxia and HIF-1α in gastric cancer cells [13]. With bioinformatics method, we discovered the potential binding site between miR-224 and NCR1, prompting interaction between them. Therefore, it can be deduced that increased HIF-1α may inhibit NCR1/NKp46 signaling through up-regulating miR-224 expression, which affects the killing capability of NK cells on prostate cancer. We initiated the current study to shed light on this hypothesis, aiming to offer theoretical basis for developing effective immunotherapy for metastatic prostate cancer.

2. Materials and methods

2.1. Isolation of lymphocytes from prostate tissues

This study was approved by the ethics committee of the Second Affiliated Hospital and Yuying Children's Hospital of Wenzhou Medical University and performed in accord with the Helsinki Declaration. And all the patients agreed and signed the documented informed consent for tissue donation for study before samples collection. The prostate cancer tissues (n = 10) and normal prostate tissues (n = 10) were taken from patients with prostate cancer for lymphocytes isolation. Fresh prostate tissues were immediately minced using scalpels in RPMI medium, and the obtained cell suspension was filtered successively through 70- and 30-mm cell strainers (Miltenyi Biotec). The supernatant was harvested and centrifuged at a high speed to get the dissociation supernatant, which was frozen at 20 °C for further gene expression detection. Cell suspension was washed and used directly for flow cytometry analysis.

2.2. Quantitative real-time PCR (qRT-PCR)

The qRT-PCR was performed for gene expression analysis in lymphocytes. Total RNA was extracted from lymphocytes using the Trizol reagent (Invitrogen). Quantity and quality of RNA were evaluated using the Nanodrop technology (Thermo Scientific) with A260/A280 ratios between 1.9 and 2.1. Total RNA was reverse-transcribed using *M*-MLV Reverse Transcriptase Kit (Promega), which is a recombinant DNA polymerase that synthesizes a complementary DNA strand from single-stranded RNA. For qRT-PCR detection, the obtained cDNA was mixed with SYBR Select Master Mix (Thermo Fisher) and amplified on an ABI 7900-fast thermocycler (Applied Biosystems). Primers were designed and synthesized by Sangon Biotech (Shanghai, China). The relative expression was calculated using the $2^{-\Delta\Delta Ct}$ method.

2.3. Flow cytometry

For percentage analysis, lymphocytes were diluted into 1×10^6 in single-cell suspension and washed twice with phosphate-buffered saline (PBS). Then cells were stained by FITC or PE-conjugated antibodies at 4 °C for 30 min in PBS containing 1% FBS. Samples were evaluated using BD FACSCalibur cytometer (BD Biosciences) and data were analyzed via Flow Jo V10 software (Tree Star Inc.).

2.4. Cell culture and transfection

The IL-2-dependent NK cell line NK92 was cultured in α-MEM (Invitrogen) supplemented with 10% heat-inactivated fetal bovine serum (FBS), 100 U/ml penicillin, 100 μg/ml streptomycin, 2 mM L-Glutamate, and 100U/ml IL-2 (Proleukin, Chiron Corp). The cells were maintained at 37 °C in a 5% CO_2-humidified atmosphere. Cells from a mid-log phase culture were harvested. For cell transfection, miR-224 mimic and its negative control (pre-NC), miR-224 inhibitor and its negative control (NC), and the small interference RNA (siRNA) were designed by GenePharma Co. Ltd (Shanghai, China). They were transfected into NK92 cells using Lipofectamine2000 (Invitrogen) according to the manufacturer's specifications.

2.5. Enzyme-linked immuno sorbent assay (ELISA)

The IFN-γ level in NK92 cells were measured by ELISA system kits (R&D Systems). Monoclonal antibodies were coated onto standard ELISA plates in a volume of 100μl/well overnight at 4 °C. Uncoated sites were blocked with 100 μl 1% BSA in PBS for 1 h at room temperature. The supernatant of NK92 cells culture were added and incubated at 37 °C for 2 h, and followed by biotinylated anti-IFN-γ (Biosynthesis Biotechnology, China) at 37 °C for 1 h. Subsequently, streptavidin−horseradish peroxidase was added and incubated at 37 °C for 30 min. Color reaction was developed with 100 μl of tetramethylbenzidine (TMB, Sigma) substrate for 30min and the reaction was terminated with 2 M sulfuric acid (Sigma). Finally, absorbance was measured at 450 nm using a microplate ELISA reader. Each sample was detected for triplicate.

2.6. Cytotoxicity measurement

NK92 cell populations were tested for cytolytic activity with the CytoTox 96 Non-Radioactive Cytotoxicity Assay [14] (Promega). 100 μl NK 92 cells (1×10^5) and 100 μl prostate cancer cell line P3 (1×10^5) were mixed and seeded in 96-well round bottom plates. Cells were co-cultured at 37 °C and 5% CO_2 for 5 h. After performed according to the manual, absorbance was determined at 490 nm with a VersaMax microplate reader (Molecular Devices), and data were analyzed with the SOFTmax PRO 2.6.1 software (Molecular Devices).

2.7. Hypoxia treatment

The hypoxia treatment was achieved by keeping NK92 cells in an anaerobic workstation incubator flushed with a mixture of 1% O_2, 5%CO_2, and 94% N_2. Before use, the medium DMEM without glucose (Gibco) was placed in the hypoxic incubator for 2 h to equilibrate in the hypoxic environment and monitored with a portable oxygen analyzer (Oxoid Ltd., UK). After OGD completed, cells were returned to a normal incubator for reperfusion and OGD media were replaced with normal DMEM medium.

2.8. Western blot

RIPA lysis buffer (Beyotime Biotechnology) was used for lysis of cells, for extraction of soluble proteins. NK92 cells were treated with RIPA lysis buffer for 30 min on ice, and the acquired lysates were centrifuged at 12000g for 10 min at 4 °C. The supernatants were collected and concentration of proteins was measured with a BCA protein assay kit (Thermo Fisher). Then proteins were separated by SDS-PAGE with electrophoresis system and transferred into the polyvinylidene difluoride (PVDF) membrane (Bio-Rad). The membrane was then blocked with 5% skimmed milk for 1 h at RT, and then incubated with primary antibodies against HIF-1α, NCR1,

Please cite this article in press as: C.-h. Chen, et al., HIF-1α induces immune escape of prostate cancer by regulating NCR1/NKp46 signaling through miR-224, Biochemical and Biophysical Research Communications (2018), https://doi.org/10.1016/j.bbrc.2018.06.007