Original Paper

MicroRNA-135a Inhibits Nasopharyngeal Carcinoma Cell Proliferation Through Targeting Interleukin-17

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Key Words

MiR-135a • IL-17 • Proinflammatory cytokines • Nasopharyngeal carcinoma

Abstract

Background/Aims: The objective of this study was to investigate the potential role of IL-17 in the development of nasopharyngeal carcinoma (NPC) and to screen microRNAs (miRNAs) that potentially target IL-17 in NPC cells. **Methods:** Blood was collected from NPC patients and normal subjects, and plasma IL-17 concentration was quantified by enzyme-linked immunosorbent assay. An immortalized normal human nasopharyngeal epithelial cell line, NP69, was treated with or without human IL-17 (15 ng/mL) for various times, and expression of IL-1 β , IL-6, IL-12, and TNF- α mRNA was assessed by real-time reverse transcription PCR. The candidate miRNAs that potentially target IL-17 were predicted by a bioinformatics strategy. The selected miR-135a mimic was transfected into primary NPC cells, and cell proliferation was assessed by MTT assay. **Results:** The concentration of plasma IL-17 was significantly higher in the NPC patients (92.5 ± 7.3 pg/mL) than in the control subjects (56.8 ± 2.9 pg/mL). In response to IL-17 treatment, the mRNA expression of IL-1 β and IL-6 was significantly upregulated and reached a peak at 12 h, followed by a slight decrease at 24 h, while the mRNA expression of IL-12 and TNF- α was significantly upregulated at 12 h and remained high even at 48 h after exposure to IL-17. Moreover, miR-135a specifically targets IL-17 and was dramatically downregulated in NPC cells compared with NP69 cells. Transfection of exogenous miR-135a mimic resulted in significant suppression of IL-17 secretion and subsequent inhibition of NPC cell proliferation. **Conclusions:** Blood IL-17 was significantly higher in NPC patients compared with normal subjects. Expression of miR-135a in the cancer cells isolated from nasopharyngeal tumors was significantly lower than that in NP69 cells, and suppression of IL-17 by miR-135a mimic resulted in significant inhibition of NPC cell proliferation. These findings suggested that downregulation of miR-135a may contribute to the development of NPC via the mechanism of IL-17 stimulation of proinflammatory cytokine expression.

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Introduction

Nasopharyngeal carcinoma (NPC) with high malignancy is one of the common malignant tumors in Southern China and Southeast Asia. The 5-year survival rate of NPC is less than 60% [1]. Studies have shown that Epstein-Barr virus infection, chemical carcinogens, and genetic susceptibility may be implicated in pathogenesis of NPC [2]. Histopathological examination revealed massive infiltration of inflammatory cells in the microenvironment of NPC, which is believed to play a role in NPC cell proliferation [3].

NPC was initially regarded as lymphoepithelioma, and abnormal immunity is considered to play an important role in the development of NPC [4]. In this regard, IL-17, secreted by CD4⁺ T cells, has been reported to promote tumor progression by anti-apoptotic and pro-angiogenic mechanisms [5, 6]. IL-17 expression is elevated in various kinds of human tumors such as cervical cancer, hepatocellular carcinoma, ovarian cancer, esophageal cancer, breast cancer, gastric cancer, and NPC [7-10]. IL-17 was proposed to exert significant effects on tumor initiation and progression by affecting the antitumor immune response, [11, 12] and thus a novel immune-evasion mechanism utilized by the tumors through the inhibition of T-helper 17 (Th17) cell development was proposed [13]. The role of IL-17 in the development of NPC, however, remains to be investigated.

MicroRNAs (miRNAs) are highly conserved, endogenous small noncoding RNAs (17–25 nucleotides in length) that repress gene expression by binding to the 3' untranslated region (3'UTR) of their target mRNAs, inducing their degradation or translational repression. Studies have indicated that miRNAs could be used as novel diagnostic and prognostic biomarkers, or therapeutic tools for cancer treatment [14-16]. However, the function of miRNAs in the regulation of cytokines in NPC patients remains largely undefined.

In this study, we found that the level of plasma IL-17 was significantly higher in NPC patients. Furthermore, IL-17 stimulated the expression of IL-1 β , IL-6, IL-12, and TNF- α in NPC cells. Importantly, we identified IL-17 as a direct target of miR-135a and transfection of exogenous miR-135a mimic into NPC cells resulted in significant suppression of IL-17 and subsequent inhibition of NPC cell proliferation.

Materials and Methods

Patient samples

A total of 17 NPC patients (9 men and 8 women) and 7 normal healthy subjects (5 men and 2 women) were enrolled in this study. Tumor tissues were collected from patients with NPC who were treated in the Department of Otolaryngology, Head and Neck Surgery at the Second Affiliated Hospital of Xi'an Jiao Tong University. Written informed consent was obtained from each participant. The study protocol was approved by The Ethical Committee of the Second Affiliated Hospital of Xi'an Jiao Tong University Hospital.

Cell culture, cell proliferation, and cytotoxicity assay

The immortalized normal human nasopharyngeal epithelial cell line NP69 was obtained from the Chinese Academy of Science (Beijing, China). The cells were cultured in Dulbecco's modified Eagle medium (DMEM, Gibco) supplemented with 10% fetal bovine serum (FBS, Gibco) and grown in a humidified atmosphere of 5% CO₂ and 95% air.

Primary NPC cells were isolated from six randomly selected tumor tissues, which were removed by surgical resection. Briefly, tumor tissues were washed with sterile phosphate buffered saline (Gibco). The tissues were then cut into 1-cm³ pieces and grinded followed by digestion with 0.25% trypsin/ethylene diamine tetraacetic acid and 0.2% collagenase (type II, *Clostridium histolyticum*) overnight at 4°C. Digested tissues were resuspended in DMEM and filtered through a 55- μ m pore size nylon mesh. Filtered cells were counted and then transferred into 6-well plates in DMEM with 10% FBS.

Cell proliferation and cytotoxicity assays were performed using colorimetric 3-[4, 5-dimethylthiazol-2-yl]-2, 5-diphenyltetrazolium bromide (MTT) following the manufacturer's instructions (Sigma Aldrich).

Analysis of IL-17 production

Blood was harvested from NPC patients and control subjects, and plasma was isolated by centrifugation. Plasma IL-17 concentration was quantified using an enzyme-linked immunosorbent assay (ELISA) kit (R&D Systems) following the manufacturer's instructions and using the Luminex 100 detection system (Life Technologies, Paisley, UK).

2.4 RNA extraction and quantitative real-time reverse transcription PCR (RT-PCR)

Total RNA was extracted from the cultured cells using TRIzol reagent (Invitrogen) following the manufacturer's instructions. RT-PCR analysis was performed using the SuperScript III First-Strand kit (Invitrogen) and TaqMan Gene Expression Assay (Thermo Fisher Scientific). The primer sequences used for amplification were as follows: IL-1 β , sense: CATTGAGCCTCATGCTCTGTT, anti-sense: CGCTGTCTGAGCGGATGAA; IL-6, sense: ACTCACCTCTTCAGAACGAATTG and antisense: CCATCTTTGGAAGGTTTCAGGTTG; IL-12, sense: ACCCTGACCATCCAAGTCAAA, antisense: TTGGCCTCGCATCTTAGAAAG; TNF- α , sense: CCTCTCTCTAATCAGCCCTCTG, antisense: GAGGACCTGGGAGTAGATGAG; glyceraldehyde 3-phosphate dehydrogenase (GAPDH), sense: TGTGGGCATCAATGGATTTGG, antisense: ACACCATGTATTCCGGGTCAAT. Experiments were performed with an ABI 7500HT System instrument (Bio-Rad Laboratories) using the following PCR protocol: one cycle at 95°C for 10 min, followed by 40 cycles at 95°C for 15 s and 60°C for 1 min with optical readings at 60°C. Gene expression was normalized to internal controls and fold changes were calculated using relative quantification ($2^{-\Delta\Delta C_t}$). U6 small nuclear RNA (U6) and GAPDH were used as endogenous controls.

Luciferase reporter assay

The mutant (MUT) or wild-type (WT) 3'UTR of IL-17 was subcloned into the pGL3 Luciferase Reporter Vector (Promega). In IL-17 MUT, the 7-bp sequence complementary to the 5' sequence of miR-135a was deleted. Cells were cotransfected with miR-135a mimic, inhibitor, or scrambled control and the vectors carrying IL-17 MUT or WT 3'UTR using Lipofectamine 2000 (Invitrogen). Activities of firefly and Renilla luciferases were measured using a dual-luciferase assay system (Promega).

Statistical analysis

Data are expressed as the mean $\pm$ SD and were analyzed with a two-tailed Student's *t* test using GraphPad Prism 5 (GraphPad Software). $P < 0.05$ was considered statistically significant.

Results

Comparison of IL-17 in NPC patients and normal subjects

We first evaluated plasma IL-17 levels between NPC patients and control subjects. The IL-17 concentration was significantly higher in the NPC patients (92.5 ± 7.3 pg/mL) compared with control subjects (56.8 ± 2.9 pg/mL, Fig. 1). Clinical data analysis suggested that a high IL-17 plasma level in NPC was correlated with pathologic stage of the tumor ($P < 0.05$), but not with age or gender ($P > 0.05$) (data not shown).

Effect of IL-17 on cytokine mRNA expression in NP69 cells

Next, the effect of IL-17 on mRNA expression of IL-1 β , IL-6, IL-12, and TNF- α by NP69 cells was assessed in vitro in cell culture. As shown in Fig. 2, mRNA expression of IL-1 β and IL-6 was significantly higher and reached a peak at 12 h following IL-17 treatment, and then slightly decreased at 24 h,

Fig. 1. Comparison of IL-17 concentration in patients with NPC and normal subjects. Plasma was harvested from NPC patients ($n = 17$) and normal subjects ($n = 7$), and IL-17 concentration was quantified by ELISA as described in the methods. Vertical axis: IL-17 concentration (pg/mL); horizontal axis: groups. * $P < 0.05$. Data presented are the average of 17 patients' samples or 7 normal control samples.

Fig. 2. Effect of IL-17 on cytokine mRNA expression by NP69 cells. NPC cells were cultured in the serum free medium with or without IL-17 for varying hours as indicated. Cells were then harvested and used for quantification of IL-6 (Panel A), IL-1 β (Panel B), TNF- α (Panel C), and IL-12 (Panel D) mRNA by real-time RT-PCR as described in the methods. Data presented are an average of three separate experiments with the NP69 cells.

Fig. 3. Suppression of IL-17 by miR-135a. Panel A: Targeting sequence of IL-17 3'UTR by miR-135a. Panel B: Relative luciferase activity of IL-17 reporter. Firefly luciferase activity was assessed as described in the methods, and normalized to renilla luciferase activity and plotted as relative luciferase activity. Vertical axis: Relative luciferase activity; horizontal axis: wild type (WT) or mutant IL-17 3'UTR (Mut); solid bar: transfected with scramble miRNA; hatched bar: transfected with miR-135a. Data presented are one representative of three separate experiments.

while mRNA expression of IL-12 and TNF- α was significantly higher at 12 h and remained high even at 48 h after exposure to IL-17.

IL-17 was targeted by miR-135a

Next, using TargetScan and PicTar, we predicted that miR-135a might directly target IL-17 (Fig. 3A). To experimentally test whether miR-135a targets IL-17, we constructed luciferase reporters by subcloning the WT or MUT 3'UTR of IL-17 downstream of the firefly luciferase cDNA in the pGL3 Luciferase Reporter Vector. We found that the relative luciferase activity of the reporter containing IL-17 WT 3'UTR was significantly decreased by approximately 70% upon miR-135a transfection as compared with the reporter containing IL-17 MUT 3'UTR in 293T cells (Fig. 3B). The cotransfection of miR-135a substantially decreased the luciferase activity of the WT reporter but barely affected that of the MUT reporter.

miR-135a was downregulated in primary NPC cells

Since miR-135a specifically targets IL-17, expression of miR-135a in primary NPC cells and normal (NP69) cells was compared by quantitative real-time PCR. As shown in Fig. 4, the expression of miR-135a was 72% lower in primary NPC cells than in NP69 cells.

Next, following the transfection of miR-135a mimic or scrambled oligonucleotide into NPC cells, IL-17 concentration in the culture was quantified by ELISA and NPC cell proliferation was assessed by MTT assay. As expected, transfection of miR-135a mimic significantly upregulated the expression of miR-135a (Fig. 5A), which resulted in a more than 47% decrease in IL-17 synthesis compared with that in the cells transfected with scrambled oligonucleotide (Fig. 5B, $P < 0.01$). Furthermore, transfection of miR-135a mimic into primary NPC cells significantly inhibited proliferation from day 4 through day 8 (Fig. 5C, $P < 0.01$).

Fig. 4. Comparison of miR-135a expression in the NPC cells and normal cells (NP69). Total RNA was extracted from the NPC cells and normal control cells (NP69). Expression of miR-135a in the NPC and normal cells were then quantified by real-time RT-PCR as described in the methods. Vertical axis: expression of miR-135a; horizontal axis: cells. * $P < 0.05$. Data presented are one representative experiment, which was performed three times using the NP69 cell line and one of the NPC cells.

Fig. 5. Effect of exogenous miR-135a mimic on IL-17 synthesis and NPC cell proliferation. Panel A: Level of miR-135a following transfection of miR-135a mimic. Exogenous miR-135a mimic or scramble mimic were transfected into the NPC cells as described in the methods. Vertical axis: relative level of miR-135a over U6; horizontal axis: cells transfected with scramble mimic or miR-135a mimic (Mimic). Panel B: Effect of miR-135a mimic on IL-17 synthesis. NPC cells were transfected with miR-135a mimic or scramble mimic. IL-17 concentration was quantified by ELISA. Vertical axis: IL-17 concentration (pg/mL); horizontal axis: cells transfected with nothing (Blank), scramble mimic, or miR-135a mimic (Mimic). Panel C: Effect of miR-135a mimic on NPC cell proliferation. NPC cells were transfected with scramble mimic or miR-135a mimic and allowed to grow for 8 days. Cell number was assessed by MTT assay as described in the methods. Vertical axis: OD value at 450nm reading; horizontal axis: time (day); solid circle: cells transfected with scramble mimic; solid square: cells transfected with miR-135a mimic (mimic). Data presented are one representative of three separate experiments using three different NPC cells.

Discussion

IL-17 is an important proinflammatory cytokine secreted by Th17 cells. Despite the rapid expansion in studies investigating the role of IL-17 in the pathogenesis of autoimmunity and inflammatory diseases, its activities in cancer remain largely unknown. In the current study, we have investigated a potential role of IL-17 in the pathogenesis of NPC. We found that plasma IL-17 was significantly higher in patients with NPC compared to that in normal subjects, and that IL-17 significantly stimulated proinflammatory cytokines such as IL-6, IL-1 β , TNF- α , and IL-12 in immortalized NP69 cells. In contrast, the level of miR-135a in tumor cells isolated from NPC tissues was significantly lower than that in the NP69 cell line. We further demonstrated that miR-135a targeted IL-17 and suppression of IL-17 by exogenous miR-135a mimic resulted in significant inhibition of NPC cell proliferation. These findings suggested that downregulation of miR-135a may lead to upregulation of IL-17, which contributes to the pathogenesis of NPC.

IL-17 has been reported to be involved in the process of immune regulation [17]. IL-17 binds to its receptors to trigger downstream signaling cascades, which activates the production of inflammatory cytokines/chemokines and stimulates proliferation and differentiation of lymphocytes [12, 17]. As a proinflammatory cytokine, IL-17 plays a role in host defense, immune surveillance, and regulation of tumor growth [18, 19]. Elevation of IL-17 expression has been demonstrated in a variety of malignancies, including ovarian cancer, hepatocellular carcinoma, breast cancer, prostate cancer, esophageal and gastric cancer, and non-melanoma skin cancers [9, 18, 20, 21]. However, the role of IL-17 in NPC remains

relatively unknown. In this regard, Li et al. have demonstrated that macrophage inhibitory factor can influence the survival of NPC through IL-17 [10]. Consistent with this observation, we demonstrated that the level of plasma IL-17 was significantly higher in NPC patients than in control subjects, suggesting IL-17 may be associated with NPC formation.

IL-17 could indirectly facilitate tumor progression through stimulating other cytokines/chemokines [11]. For instance, IL-17 induces the release of the pro-angiogenic factor VEGF, which acts as a survival factor for tumor cells [22]. Furthermore, IL-17 promotes tumor growth through an IL-6/STAT3 signaling pathway [5]. However, the role of the IL-17-stimulated cytokines in tumorigenesis remains controversial. In this regard, some animal studies revealed that IL-17 could protect animals from tumor outgrowth, while others revealed that IL-17 could promote tumor progression, depending on tumor origin and site [23]. The current study demonstrated that IL-17 stimulated IL-6, IL-1 β , TNF- α , and IL-12 synthesis in NP69 cells, suggesting these cytokines may play a role in mediating the tumor-promoting effect of IL-17 in NPC.

miRNAs are known to regulate the expression of genes that induce mRNA decay or suppress translation. In the IL-17 signaling pathway, several miRNAs, including miR-23a, miRNA cluster 106a-363, miR-181a-5p, miR-15b, and miR-155, have been found to target signaling intermediates [24-27]. In this study, we further demonstrated that miR-135a expression was significantly lower in the NPC cells compared to that of normal cells, and found that miR-135a potentially targets IL-17, in that the 3'UTR of IL-17 mRNA contains the binding sequence for miR-135a. We have further demonstrated that ectopic expression of miR-135a in NPC cells significantly suppressed IL-17 synthesis, which resulted in significant inhibition of NPC cell proliferation. These findings suggested that downregulation of miR-135a in NPC cells may result in upregulation of IL-17, and consequently, the higher level of IL-17 leads to proliferation of NPC cells. However, one limitation of this study was the relatively small case number of NPC subjects.

Taken together, the results of the current study demonstrated that miR-135a expression was significantly decreased in the NPC cells, while IL-17 secretion by NPC cells was significantly higher than that by normal cells. Furthermore, transfection of exogenous miR-135a mimic into the NPC cells resulted in significant downregulation of IL-17 and inhibition of NPC cell proliferation. These findings suggested that targeting IL-17 through miR-135a could potentially be used as a therapeutic strategy for the treatment of NPC.

Acknowledgements

This study was supported by the Science and Technology Bureau Pilot Research Projects of Shiyan, Hubei Province (Grant No. 17Y26)

Disclosure Statement

The authors declare that there is no conflict of interests.

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