

MicroRNA-153 affects nasopharyngeal cancer cell viability by targeting TGF- β_2

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Received September 1, 2016; Accepted July 20, 2018

DOI: 10.3892/ol.2018.9570

Abstract. The aim of the present study was to determine the function of microRNA-153 (miR-153) in the viability of nasopharyngeal cancer (NPC) cells and determine the underlying molecular mechanism. The expression of miR-153 in patients with NPC was markedly decreased compared with that in paracarcinoma tissue. miR-153 upregulation observably decreased cell viability, induced apoptosis, increased caspase-3 and -9 activity, and increased the B-cell lymphoma 2 (Bcl-2)-associated X protein/Bcl-2 protein expression ratio in 13-9B cells. miR-153 upregulation also suppressed transforming growth factor- β_2 (TGF- β_2) and Smad2 protein expression in 13-9B cells. TGF- β_2 inhibitor enhanced the effect of miR-153 upregulation on the inhibition of cell viability, induction of apoptosis, increase in caspase-3 and -9 activity, and increase in Bax/Bcl-2 protein expression ratio in 13-9B cells. The results of the present study indicate that miR-153 affects the progression of NPC by targeting the TGF- β_2 /Smad2 signaling pathway.

Introduction

Nasopharyngeal carcinoma (NPC), also known as 'Guangdong tumor', is a malignant tumor that commonly occurs in southern China, particularly in Guangdong Province (1). Intensity-modulated radiation therapy-based comprehensive treatment is effective in the treatment of early NPC (2). However, as diagnosis of NPC is difficult at early stages with a marked potential for metastasis, ~75% of patients with NPC are diagnosed with late-stage NPC on their initial presentation to the doctor, with local lymph node and/or distant metastasis (3). The early diagnosis of this disease is a major clinical problem;

there is a poor prognosis due to recurrence or metastasis following treatment, accounting for the majority of cases of failed NPC treatment and the low survival rate (4). Therefore, screening NPC tumor markers for early detection, reasonable treatment, prognosis prediction and recurrence monitoring may be of marked importance to the clinical diagnosis and treatment of NPC (4).

As microRNA (miRNA) serves an important function in the incidence and development of tumors, it has become a hotspot in cancer research (5). Previous studies have identified that circulating miRNA expression level dysregulation is common in hematological tumors, and its expression in lung cancer, liver cancer, and head and neck cancer, as well as in other solid tumors, also differs markedly, and is associated with the clinical features and prognosis of tumors (6,7). Research into the association between circulating miRNA and NPC is at a preliminary stage (5). It has been identified that determining serum miRNA levels offered marked potential in the early diagnosis and prediction of NPC (7). Although the results of the previous study differed, they all confirmed that certain circulating miRNAs are expressed specifically in NPC (7).

Various cytokines, including hypoxia-inducible factor, insulin-like growth factor, epidermal growth factor, hepatocyte growth factor, fibroblast growth factor, vascular endothelial growth factor and transforming growth factor (TGF), induce epithelial-mesenchymal transition (EMT) and promote the metastasis of tumor cells (8). TGF- β_1 , one of the most important TGF family members, is a 'double-edged sword', as it is able to inhibit cell proliferation and induce cell apoptosis in the early stages of primary tumors, but also promote the invasion and metastasis of cancer cells at later stages (9). In addition, a number of studies have indicated that TGF- β_2 is the primary factor inducing EMT, regulating the incidence and development of EMT through Smad and non-Smad signaling pathways (10). In addition, it is involved in normal embryonic development, and also associated with organ fibrosis and a variety of malignant tumors, including lung, breast, extrahepatic bile duct and skin cancer; however, it has not yet been identified in NPC.

Chen *et al* (11) identified that the expression of miR-153 was decreased in patients with non-small cell lung cancer relative to the adjacent tissues. The aim of the present study was to investigate the molecular mechanism underlying the

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Key words: microRNA-153, nasopharyngeal cancer cell, transforming growth factor β_2 , Smad2

effect of microRNA-153 (miR-153) on the growth of NPC and experimental validation.

Materials and methods

Culture of NPC. The human NPC 13-9B cell line was purchased from the Chinese Academy of Sciences (Shanghai, China) and was cultured in Dulbecco's modified Eagle's medium (HyClone; GE Healthcare, Logan, UT, USA) supplemented with 10% fetal bovine serum (Gibco; Thermo Fisher Scientific, Inc., Waltham, MA, USA) at 37°C in a humidified atmosphere containing 5% CO₂.

Tissue samples. The present study was approved by the Institute Research Ethics Committee of Beijing Army General Hospital (Beijing, China). Written informed consent was provided by all enrolled patients (n=48, 56-78 years age) at June 2014 to December 2014. The number of patients were 48, number of male patients were 35, number of female patients were 13; mean age of patients were 56-78 years age. All cancer tissue samples and para-carcinoma tissue were collected by surgical resection and were stored at -80°C until subsequent experimentation.

Reverse transcription-quantitative polymerase chain reaction (RT-qPCR) analysis of miR-153 expression. Total RNA from NPC tissue samples was extracted using TRIzol® reagent (Invitrogen; Thermo Fisher Scientific, Inc.), according to the manufacturer's protocol. cDNA synthesis was performed using an Oligo-dT kit (Applied Biosystems; Thermo Fisher Scientific, Inc.), according to the manufacturer's protocol. miR-153 expression level analysis was performed using a Power SYBR Green PCR Master mix (Applied Biosystems; Thermo Fisher Scientific, Inc.), according to the manufacturer's protocol. miR-153: Forward, 5'-TTGCATAGTCACAAAAGTGAT-3', Reverse, 5'-CAGTGCCTGTCGTGGAGT-3'; U6: Forward, 5'-CTCGCTTCGGCAGCACATATACT-3', Reverse, 5'-ACGCTTCACGAATTTGCGTGTC-3'. Fold changes in mRNA expression were quantified using the 2^{-ΔΔC_q} relative quantification method. PCR conditions included an initial holding period at 95°C for 15 sec and 60°C for 30 sec for 40 cycles [Livak, 2001 #5460].

miR-153 overexpression and TGF-β₂ inhibitor. Human miR-153 mimic (5'-UUGCAUAGUCACAAAAGUGAUC-3' and 5'-UUCUCCGAACGUGUCACGUTT-3') and negative control mimic (5'-CCCCCCCCCCCCCCCCCCCC-3' and 5'-AAAAAAAAAAAAAAAAAAAA-3') were obtained from Shanghai GenePharma Co., Ltd. (Shanghai, China). 13-9B cells were cultured in a 6- or 96-well plate and transiently transfected with the mimics using Lipofectamine® 2000 reagent (Invitrogen; Thermo Fisher Scientific, Inc.), according to the manufacturer's protocol. In addition, 1 μM pirfenidone (Sigma-Aldrich; Merck KGaA, Darmstadt, Germany), a TGF-β₂ inhibitor, was added to transfection of 13-9B cells with miR-153 for 48 h.

MTT assay of cell viability. 13-9B cells were transfected with miR-153 with or without TGF-β₂ inhibitor treatment, and were cultured in a 96-well plate. The cell viability was determined using an MTT (Beyotime Institute of Biotechnology, Haimen,

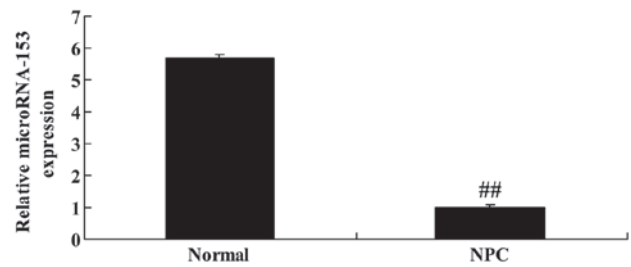


Figure 1. Expression of microRNA-153 in patients with NPC. ^{##}P<0.01 vs. normal paracarcinoma tissue. NPC, nasopharyngeal cancer.

China) assay at 0, 24 and 48 h. MTT (0.5 mg/ml) was added for 4 h and 150 μl/well DMSO was added to dissolve the formazan crystals that formed. The optical density at 492 nm was determined using a colorimetric microplate reader (Bio-Rad Laboratories, Inc., Hercules, CA, USA), according to the manufacturer's protocol.

Flow cytometric analysis of apoptosis. 13-9B cells were transfected with miR-153 with or without TGF-β₂ inhibitor treatment, and were cultured following transfection for 48 h in a 6-well plate. Cells were stained with annexin V (1 μM) and propidium iodide (5 μM) (Nanjing KeyGen Biotech Co., Ltd., Nanjing, China) for 30 min in darkness. The rate of apoptosis was determined using flow cytometry (BD FACScan; BD Biosciences, Franklin Lakes, NJ, USA).

ELISA. 13-9B cells were transfected with miR-153 with or without TGF-β₂ inhibitor treatment, and were cultured in a 6-well plate. Caspase-3/9 activity of the cells was determined using ELISA kits (catalog nos. C1115 and C1158; Beyotime Institute of Biotechnology). Cells were incubated with *N*-acetyl-Asp-Glu-Val-Asp-p-nitroanilide (caspase-3 substrate) and *N*-acetyl-Leu-Glu-His-Asp-p-nitroanilide (caspase-9 substrate) at 37°C for 2 h. The optical density at 405 nm was determined using a colorimetric microplate reader.

Western blotting. 13-9B cells were transfected with miR-153 with or without TGF-β₂ inhibitor treatment, and were cultured following transfection for 48 h in a 6-well plate. Subsequently, cells were lysed with lysis buffer (Beyotime Institute of Biotechnology) at 4°C for 30 min. Proteins were quantified using the bicinchoninic acid method (Beyotime Institute of Biotechnology) and 50 μg protein was separated by SDS/PAGE (8-12% gel) and transferred onto polyvinylidene fluoride membranes (GE Healthcare, Chicago, IL, USA). Membranes were blocked with 5% skimmed milk powder in Tris-buffered saline containing 0.1% Tween-20 followed by incubation with the following primary antibodies: Anti-B-cell lymphoma 2 (Bcl-2, sc-23960, 1:1,000, Santa Cruz Biotechnology, Inc.), anti-Bcl-2-associated X protein (Bax, sc-6236, 1:1,000, Santa Cruz Biotechnology, Inc.), anti-TGF-β₂ (sc-374658, 1:1,000, Santa Cruz Biotechnology, Inc.), anti-phospho-Smad2 (ab53100, 1:1,000, Abcam) and anti-GAPDH (sc-51631, 1:50,000, Santa Cruz Biotechnology, Inc.) at 4°C overnight. Membranes were incubated with horseradish peroxidase conjugated anti-rabbit immunoglobulin G secondary antibody (sc-2004, 1:5,000, Santa Cruz Biotechnology, Inc., Dallas, TX, USA) at 37°C for

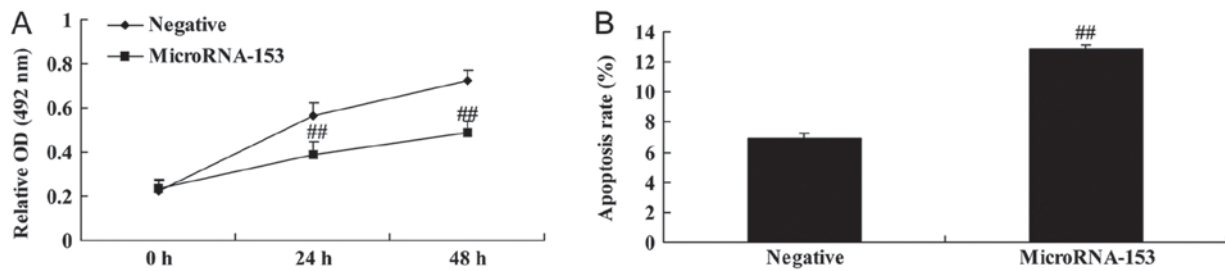


Figure 2. Upregulation of microRNA-153 decreases cell viability and induces apoptosis of 13-9B cells. Upregulation of microRNA-153 (A) decreased cell viability and (B) induced apoptosis of 13-9B cells. ^{##}P<0.01 vs. compared with negative control. OD, optical density.

1 h and SuperSignal West Pico Enhanced Chemiluminescent Substrate (Beyotime institute of Biotechnology). The intensity of each band was quantified using ImageJ software (version 3.0; National Institutes of Health, Bethesda, MD, USA).

Statistical analysis. Results are presented as the mean \pm standard deviation. Differences between groups were analyzed using Student's t-test or one-way analysis of variance with Tukey's post-hoc test. P<0.05 was considered to indicate a statistically significant difference.

Results

Expression of miR-153 in patients with NPC. To identify the expression of miR-153 in patients with NPC, RT-qPCR was performed. It was identified that miR-153 expression in patients with NPC was significantly decreased compared with that of paracarcinoma tissue (Fig. 1).

Upregulation of miR-153 decreases cell viability and induces apoptosis of 13-9B cells. miR-153 mimic and negative control mimic were transfected into 13-9B cells in order to determine the effect of miR-153. Upregulation of miR-153 significantly decreased cell viability and significantly induced apoptosis of 13-9B cells, compared with control negative mimic (Fig. 2).

Upregulation of miR-153 induces caspase-3 and -9 activity of 13-9B cells. To determine whether the upregulation of miR-153 induced caspase-3 and -9 activity in 13-9B cells, caspase activity of 13-9B cells was determined using ELISA. 13-9B cells transfected with miR-153 mimic exhibited significantly increased caspase-3 and -9 activity, compared with 13-9B cells transfected with negative control mimic (Fig. 3).

Upregulation of miR-153 increases the Bax/Bcl-2 protein expression ratio, and suppresses TGF- β_2 and p-Smad2 protein expression in 13-9B cells. To further determine the effect of upregulating miR-153 on the Bax/Bcl-2 protein expression ratio and TGF- β_2 and Smad2 expression in 13-9B cells, western blotting was used. The Bax/Bcl-2 protein expression ratio was significantly increased, and TGF- β_2 and p-Smad2 protein expression was suppressed in 13-9B cells following miR-153 upregulation, compared with cells transfected with negative control mimic (Fig. 4).

TGF- β_2 inhibitor enhances the effect of miR-153 upregulation on the Bax/Bcl-2 protein expression ratio, and TGF- β_2 and

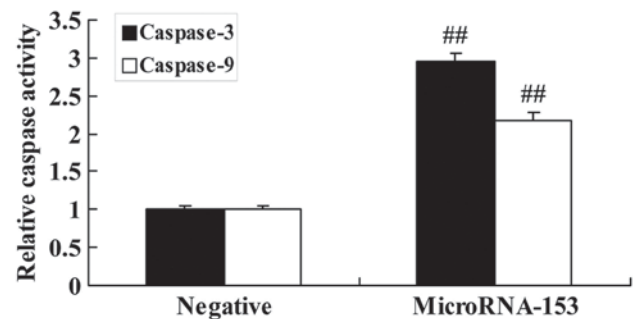


Figure 3. Upregulation of microRNA-153 induces caspase-3 and -9 activity of 13-9B cells. ^{##}P<0.01 vs. negative control.

p-Smad2 protein expression of 13-9B cells. To investigate whether TGF- β_2 is involved in the effect of miR-153 in 13-9B cells, 1 μ M pirfenidone, a TGF- β_2 inhibitor, was added to cells transfected with miR-153 mimic. In miR-153-transfected 13-9B cells, pirfenidone was able to further suppress TGF- β_2 and p-Smad2 protein expression, and induced Bax/Bcl-2 ratio caused by miR-153 upregulation (Fig. 5).

Effect of TGF- β_2 inhibitor on cell viability and apoptosis of 13-9B cells following miR-153 upregulation. The effect of TGF- β_2 inhibitor on the viability of miR-153-upregulated 13-9B cells. Following transfection with miR-153 mimic, 13-9B cells exhibited significantly decreased cell viability and significantly increased apoptosis which was enhanced by the inhibition of TGF- β_2 (Fig. 6).

Effect of TGF- β_2 inhibitor on caspase-3 and -9 activity of 13-9B cells following miR-153 upregulation. To investigate the effect of TGF- β_2 inhibitor on apoptosis of miR-153-upregulated 13-9B cells, caspase-3 and -9 activity levels were determined using ELISA. The inhibition of TGF- β_2 significantly enhanced the increase in caspase-3 and -9 activity of 13-9B cells caused by miR-153 upregulation (Fig. 7).

Discussion

NPC, also known as 'Guangdong tumor', is a malignant tumor commonly occurring in southern China and southeast Asia, particularly in Guangdong Province. It has been identified that NPC is associated with genetic factors, Epstein-Barr virus infection and environmental factors (12). Early diagnosis and early treatment is the most effective means to prolong the lives

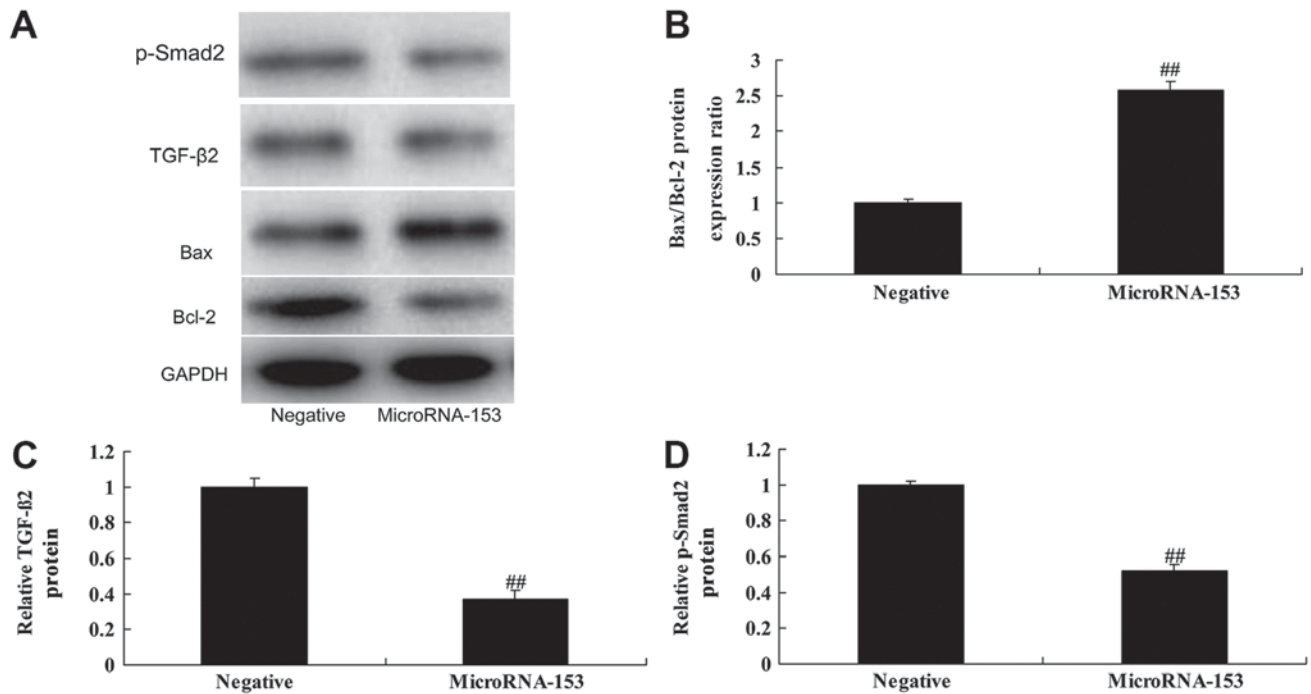


Figure 4. Upregulation of microRNA-153 increases the Bax/Bcl-2 protein expression ratio, and suppresses TGF- β_2 and Smad2 protein expression of 13-9B cells. (A) Western blot assay of p-Smad2, TGF- β_2 , Bax and Bcl-2 in 13-9B cells following upregulation of microRNA-153. Quantification of (B) Bax/Bcl-2 protein expression ratio, (C) TGF- β_2 and (D) p-Smad2 levels. ^{##}P<0.01 vs. negative control. Bcl-2, B-cell lymphoma 2; Bax, Bcl-2-associated X protein; TGF- β_2 , transforming growth factor β_2 .

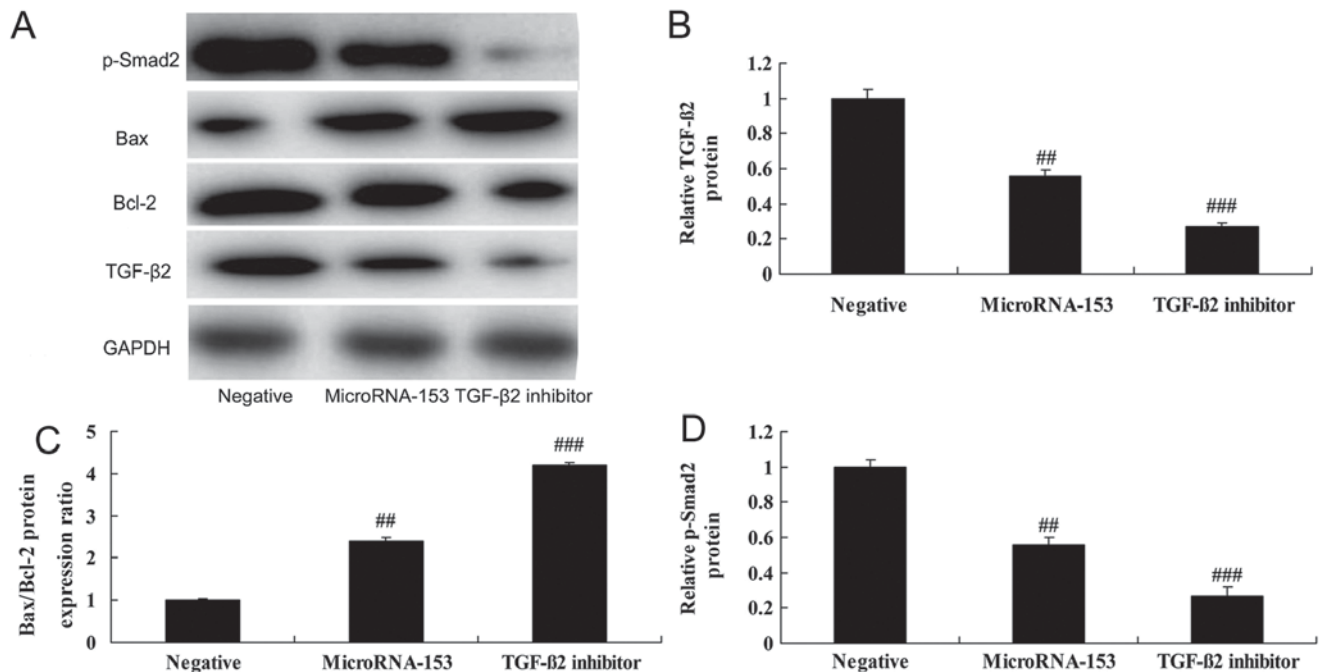


Figure 5. TGF- β_2 inhibitor enhances the effect of microRNA-153 upregulation on TGF- β_2 , Bax/Bcl-2 protein expression ratio and p-Smad2 protein expression of 13-9B cells. (A) Western blot assay of p-Smad2, Bax, Bcl-2 and TGF- β_2 in 13-9B cells following upregulation of microRNA-153 and in the presence of TGF- β_2 inhibitor. Quantification of (B) TGF- β_2 protein expression, (C) Bax/Bcl-2 protein expression ratio and (D) p-Smad2. ^{##}P<0.01 vs. negative control; ^{###}P<0.01 vs. microRNA-153.

of patients and improve their quality of life (13). Unfortunately, diagnosis of NPC is difficult in its early stage, with a high likelihood of metastasis (14). In the present study, the expression of miR-153 was identified to be suppressed in NPC tissues. Notably, it was identified that the upregulation of miR-153 significantly

decreased cell viability and induced apoptosis of 13-9B cells. Chen *et al* (11) identified that the expression of miR-153 was decreased in patients with non-small cell lung cancer relative to the adjacent tissues (14). Therefore, miR-153 may be involved in the proliferation of NPC cells and patient mortality.

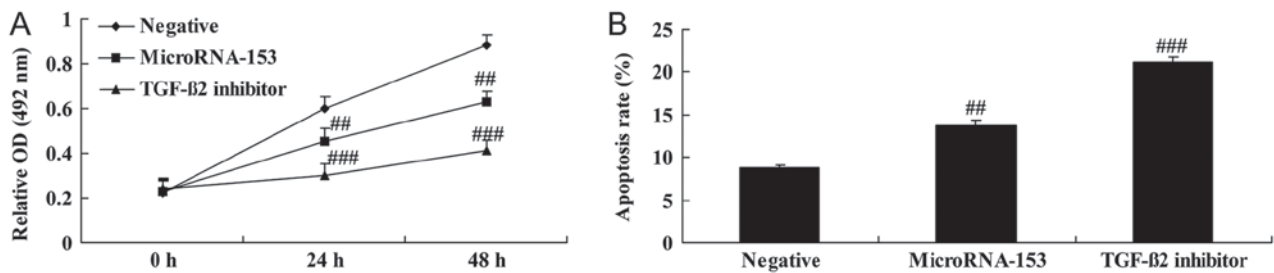


Figure 6. TGF- β_2 inhibitor enhances the effect of microRNA-153 upregulation on (A) viability and (B) apoptosis of 13-9B cells. ##P<0.01; ###P<0.01 vs. negative control. TGF- β_2 , transforming growth factor β_2 ; OD, optical density.

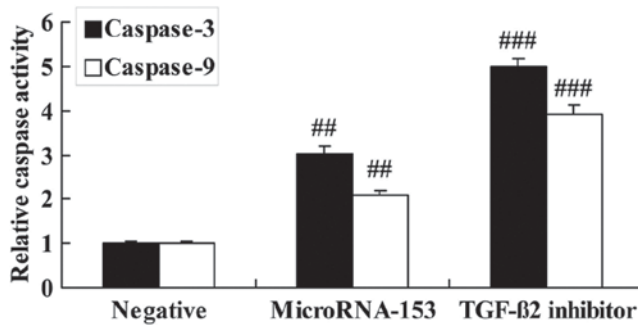


Figure 7. TGF- β_2 inhibitor enhances the effect of microRNA-153 upregulation on caspase-3 and -9 activity of 13-9B cells. ##P<0.01; ###P<0.01 vs. negative control.

In addition to tumor-associated proteins and their coding genes, non-coding genes are also associated with the incidence and development of tumors. In particular, markedly conserved miRNAs are able to pair with 3'-untranslated regions incompletely, to inhibit gene expression post-transcriptionally (7). It has been identified that >50% miRNA are located in tumor-associated genomes, and chromosomal abnormalities directly led to an alteration in miRNA gene copy number, resulting in the disordered expression of miRNAs in a variety of tumor types, promoting or inhibiting cancer-associated genes (15,16).

In the process of tumor cell apoptosis, the apoptotic signal is transmitted through the intrinsic pathway, and mitochondria serve an important function (17). Mitochondria generally transmit the apoptotic signal through the caspase cascade signaling pathway, which may be inhibited by the overexpression of Bcl-2/B-cell lymphoma extra-large (18). Apoptosis is promoted through the mitochondrial pathway to activate caspases and form DNA fragments, thus interfering with the functions of mitochondria (19). In the present study, it was identified that miR-153 upregulation significantly increased caspase-3 and -9 activity, and promoted the Bax/Bcl-2 protein expression ratio of 13-9B cell. Anaya-Ruiz *et al* (20) identified that miR-153 induces apoptosis in the MDA-MB-231 breast cancer cell line through activating caspase 3/7.

TGF- β inhibits the malignant proliferation of epithelial cells at an early stage, while promoting tumor growth and metastasis at the late stage (8). It has been identified previously that patients with increased TGF- β had a relatively poor prognosis. TGF- β exerts its biological functions mainly through the Smad protein family: TGF- β binds to its receptor to phosphorylate Smad2/3, and then the latter binds to Smad4 and enters the nucleus where

the Smad transcription complex regulates the expression of targeted genes (21). When TGF- β induces EMT through Smad proteins, Smad3 and Smad4 interact with each other, and form a transcription complex with Snail (22). Snail-Smad3/4 binds to the promoter regions of epithelial cadherin, coxsackie adenovirus receptor and occludin genes, to inhibit their transcriptional activity and thereby induce EMT (22).

In the present study, it was identified that miR-153 upregulation significantly suppressed TGF- β_2 and Smad2 protein expression of 13-9B cells. Niu *et al* (23) suggested that miR-153 inhibits osteosarcoma cell viability and invasion through targeting TGF- β_2 . Liang *et al* (24) also identified that miR-153 disturbs TGF- β_1 /p-SMAD2/3 signal transduction, acting as an anti-fibrotic element in the development of pulmonary fibrosis.

In conclusion, the results of the present study indicate that miR-153 affects NPC cell viability by targeting TGF- β_2 /Smad2. Therefore, miR-153 may be a target for the treatment of NPC.

Acknowledgements

Not applicable.

Funding

No funding was received.

Availability of data and materials

The analyzed data sets generated during the present study are available from the corresponding author on reasonable request.

Authors' contributions

XB designed the experiment; GG, YZ and LH performed the experiment; XB and GG analyzed the data; XB wrote the manuscript.

Ethics approval and consent to participate

The present study was approved by the Institute Research Ethics Committee of Beijing Army General Hospital (Beijing, China). Written informed consent was provided by all enrolled patients.

Patient consent for publication

All patients provided consented for the publication of this data and any associated images.

Competing interests

The authors declare that they have no competing interests.

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