MicroRNA-663b promotes cell proliferation and epithelial mesenchymal transition by directly targeting SMAD7 in nasopharyngeal carcinoma

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Abstract. MicroRNAs (miRs) serve important roles in the development of various types of human cancer, including nasopharyngeal carcinoma (NPC). In the present study, the expression levels of miR-663b in NPC were investigated and its role and underlying mechanisms were examined. Reverse transcription-quantitative polymerase chain reaction was performed to assess miR-663b expression levels in NPC tissues and C666-1 cells. TargetScan was applied to predict the putative targets of miR-663b and the dual luciferase reporter assay was used to confirm the predictions. To investigate the role of miR-663b in NPC, the NPC C666-1 cell line was transfected with miR-663b mimics, miR-663b inhibitors or negative control. The Cell Counting kit-8 assay was performed for cell proliferation detection and western blot analysis was applied to determine the expression levels of epithelial mesenchymal transition (EMT)-associated proteins. Results indicated that when compared with the adjacent normal tissues and the normal nasopharyngeal epithelial cells, miR-663b expression levels were significantly upregulated in the NPC tissues and the NPC cells ($P<0.01$). Notably, SMAD7 is a target gene of miR-663b and may be inhibited by miR-663b. Results indicated that NPC cell proliferation was significantly promoted by miR-663b mimics and significantly inhibited by miR-663b inhibitors ($P<0.05$ and $P<0.01$). In addition, the results indicated that, when compared with the negative control group the expression levels of E-cadherin were significantly decreased, whereas the expression levels of N-cadherin, Vimentin and matrix metalloproteinase-9 were significantly increased in the cells of the miR-663b mimics group ($P<0.05$ and $P<0.01$). However, cells in the miR-663b inhibitors group exhibited the opposite effects. In conclusion, the results of the present study indicated that miR-663b functions as a tumor promoter in NPC via promoting NPC cell proliferation and EMT by directly targeting SMAD7.

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Introduction

Nasopharyngeal carcinoma (NPC), which is considered the most common malignant epithelial tumor of the head and neck in Southern China, represents a significant disease burden that seriously impacts the quality of human life (1-4). Due to the frequent metastasis and poor prognosis, NPC is considered a highly malignant tumor (5). The standard approach for the treatment of NPC is chemoradiotherapy (6). Although great progress has been made in the development of therapeutic strategies for treating NPC, the therapeutic efficacy is still unsatisfactory. Therefore, it is of great importance to identify novel effective treatment therapies for NPC.

MicroRNAs (miRs) are conserved short non-coding RNAs that are ~22 nucleotides in length. miRNAs can negatively regulate gene expression by binding to the 3'-untranslated region (3'-UTR) of the targeted mRNA (7). It has been reported that miRs are involved in various tumor cellular processes, including proliferation, differentiation, apoptosis and metastasis (8-11). Various studies have suggested that miRs participate in the initiation and development of NPC, including miR-29c, the let-7 family of miRs, miR-10b and miR-92a, and their exact roles in NPC have been elucidated (12-15). Thus, miRs may be considered as a potential target for NPC treatment.

Among the numerous identified miRs, miR-663b has been identified to be a novel cancer-associated miR. A previous study indicated that miR-663b exerts its tumor-suppressive function via targeting insulin-like growth factor 2 in pancreatic cancer (16). A further study suggested that circulating miR-663b in plasma may be a potential novel biomarker for bladder cancer (17). Notably, Zhao *et al* (18) reported the upregulation of miR-663b in osteosarcoma. Additionally, a previous study demonstrated that miR-663 was upregulated in the serum of patients with NPC, and its increased levels were associated with malignant progression and poor prognosis (19).

However, to the best of our knowledge, the expression and exact role of miR-663b in NPC remain to be fully elucidated. Thus, in the present study, the expression of miR-663b in NPC was investigated and its role in NPC and the mechanisms were further explored.

Materials and methods

Clinical specimens. A total of 30 paired human NPC tissues and matched adjacent normal tissues were collected from 30 patients (12 women and 18 men; age range, 32-61 years), who had undergone routine surgical procedures in the Central Hospital of Wuhan (Wuhan, China). The tissue samples were collected between January 2014 and August 2016. All tissue samples were immediately flash-frozen in liquid nitrogen and stored at -80˚C. The present study was approved by the Ethics Committee of the Central Hospital of Wuhan. Informed consent was obtained from every patient.

Cell culture. Normal nasopharyngeal epithelial cells NP69 and the NPC cell line C666-1 were obtained from the Chinese Academy of Sciences Cell Bank (Shanghai, China) and cultured in RPMI-1640 medium supplemented with 10% fetal bovine serum (HyClone; GE Healthcare, Logan, UT, USA) and 1% penicillin-streptomycin solution at 37˚C in a 5% CO_2 incubator.

Cell transfection. C666-1 cells ($5x10^4$ cells per well) were transfected with 50 nM miR-663b mimics, 100 nM miR-663b inhibitors or 50 nM negative control (NC; Genepharm, Inc., Sunnyvale, CA, USA) using Lipofectamine 2000 (Invitrogen; Thermo Fisher Scientific, Inc., Waltham, MA, USA) according to the manufacturer's protocol. Following transfection for 48 h, the subsequent experiments were performed. All experiments were repeated three times.

Reverse transcription-quantitative polymerase chain reaction (RT-qPCR). Total RNA was extracted from tissues and cells using TRIzol reagent (Invitrogen; Thermo Fisher Scientific Inc.) according to the manufacturer's protocol. Following this, cDNA was generated from the RNA using a cDNA Synthesis kit (Thermo Fisher Scientific, Inc.). qPCR was performed using Maxima SYBR-Green/ROX qPCR Master Mix (Thermo Fisher Scientific, Inc.) according to the manufacturer's protocol. The thermocycling conditions were as follows: 95˚C for 5 min, followed by 40 cycles of denaturation at 95˚C for 15 sec and annealing/elongation at 60˚C for 30 sec. All reactions were performed in triplicate. U6 and GAPDH were used as an internal control for miRNA and mRNA expression, respectively. The $2^{-\Delta\Delta Cq}$ method was applied to determine the relative expression values (20). The primer sequences for RT-qPCR were as indicated in Table I.

Cell Counting kit (CCK-8) assay. C666-1 cells transfected with miR-663b mimics, miR-663b inhibitors or NC were collected at 24, 48 and 72 h following transfection. Cells were seeded in a 96-well plate at the concentration of $5x10^3$ cells per well. Subsequently, 10 μl CCK-8 assay solution (Dojindo Molecular Technologies, Inc., Kumamoto, Japan) was added to each well and cells were incubated for a further 1 h at 37˚C. Cell proliferation was assessed by measuring the absorbance at 450 nm using a microplate reader.

Western blot analysis. A total of 48 h after transfection, C666-1 cells were harvested using trypsin. Total cellular proteins were collected using the radioimmunoprecipitation assay lysis buffer (Auragene Bioscience, Changsha, China). The bicinchoninic acid kit (Pierce; Thermo Fisher Scientific, Inc.) was performed to determine the protein concentration. Protein samples (25 μg/lane) were loaded and separated by 10% SDS-PAGE and then transferred to nitrocellulose membranes (GE Healthcare Life Sciences, Little Chalfont, UK). Membranes were blocked with 5% non-fat milk in phosphate-buffered saline solution at room temperature for 1.5 h and then incubated overnight at 4˚C with the following primary antibodies: SMAD7 (cat. no. Sc-11392; 1:1,000; Santa Cruz Biotechnology, Inc., Dallas, TX, USA) E-cadherin (cat. no. 3195), N-cadherin (cat. no. 13116), Vimentin (cat. no. 5741), and matrix metalloproteinase (MMP)-9 (cat. no. 13667; all 1:1,000) and β-actin (cat. no. 4970; 1:2,000; all Cell Signaling Technology, Inc., Danvers, MA, USA). Subsequently, membranes were washed with Tris-buffered saline with Tween-20 three times and incubated with horseradish-peroxidase conjugated anti-rabbit immunoglobulin G secondary antibodies (cat. no. 7074; 1:1,000; Cell Signaling Technology, Inc.). at room temperature for 1 h. Blots were visualized with enhanced chemiluminescence reagent (EMD Millipore, Billerica, MA, USA). Protein bands were analyzed using WinMDI version 2.5 software (Purdue University Cytometry Laboratories, West Lafayette, IN, USA).

Dual luciferase reporter assay. TargetScan (http://www.targetscan.org/) was applied to predict the putative targets of miR-663b. To confirm whether miR-663b directly targets the 3'-UTRs of SMAD7, the pmiR-RB-Report™ dual luciferase reporter gene plasmid vector (Guangzhou RiboBio Co., Ltd., Guangzhou, China) with the wild-type (WT) and mutated (MUT) 3'UTR of SMAD7 mRNA were constructed (SMAD7-3'UTR-WT and SMAD7-3'UTR-MUT, respectively). Following this, 293T cells were co-transfected with SMAD7-3'UTR-WT or SMAD7-3'UTR-MUT and miR-663b or its NC (NC) vector using Lipofectamine 2000 according to the manufacturer's instructions. Following transfection for 48 h, the Dual-Luciferase Reporter Assay kit (Promega Corp., Madison, WI, USA) was performed to detect the luciferase activity according to the instructions of the manufacturer. Data were normalized to *Renilla* luciferase activity.

Statistical analysis. Data were expressed as mean ± standard deviation. SPSS 17.0 statistical software (IBM Corp., Armonk, NY, USA) was performed for all statistical analyses. Student's t-test and one-way analysis of variance followed by Tukey's test were applied to analyze the association between groups. $P<0.05$ was considered to indicate a statistically significant difference.

Results

miR-663b expression levels increase in NPC tissues and C666-1 cells. To determine the expression levels of miR-663b

Table I. Primer sequences for reverse transcription-quantitative polymerase chain reaction.

Gene	Direction	Sequence (5'-3')
Smad7	Forward	TCCTGCTGTGCAAAGTGTTC
Smad7	Reverse	TTGTTGTCCGAATTGAGCTG
E-cadherin	Forward	TTAAGCACAACAGCAACAG
E-cadherin	Reverse	GCATCAGCATCAGTCACT
N-cadherin	Forward	GACAATGCCCCTCAAGTGTT
N-cadherin	Reverse	CCATTAAGCCGAGTGATGGT
Vimentin	Forward	AGATGGCCCTTGACATTGAG
Vimentin	Reverse	TGGAAGAGGCAGAGAAATTC
MMP-9	Forward	CCAAAACTACTCGGAAGACTTGC
MMP-9	Reverse	GCGACACCAAACTGGATGA
miR-663b	Forward	CGCTAACAGTCTCCAGTC
miR-663b	Reverse	GCGACACCAAACTGGATGA
U6	Forward	GCTTCGGCAGCACATATACTAAAAT
U6	Reverse	CGCTTCACGAATTTGCGTGTCAT
GAPDH	Forward	CTTTGGTATCGTGGAAGGACTC
GAPDH	Reverse	GTAGAGGCAGGGATGATGTTCT

miR, microRNA; MMP, matrix metalloproteinase.

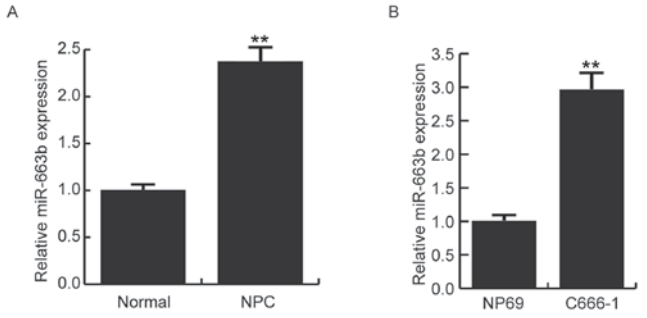

Figure 1. miR-663b expression in NPC. Relative miR-663b expression levels in NPC tissues and cells were determined by reverse transcription-quantitative polymerase chain reaction. Relative miR-663b expression levels in (A) NPC and normal tissues and (B) NPC cell line C666-1 and normal nasopharyngeal epithelial cells NP69 were indicated. Data are presented as the mean ± standard deviation. **P<0.01 vs. Normal or NP69. NPC, nasopharyngeal carcinoma; miR, microRNA.

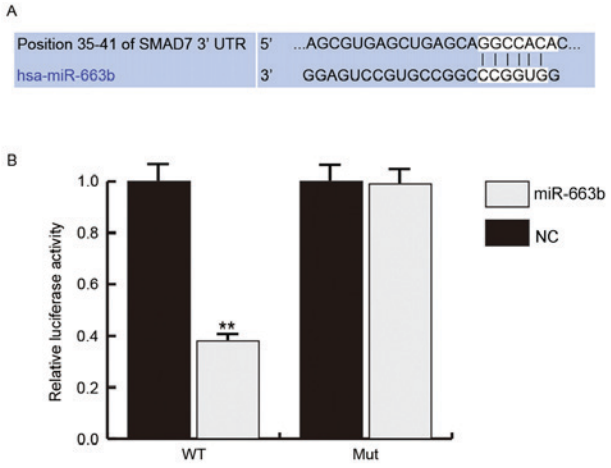

Figure 2. miR-663b directly targets Smad7. (A) Interaction between miR-663b and the 3'UTR of Smad7 was predicted using TargetScan. (B) Luciferase activity of a reporter containing a WT Smad7 3'UTR or a Mut Smad7 3'UTR were indicated. Data are presented as the mean ± standard deviation. **P<0.01 vs. NC. UTR, untranslated region; miR, microRNA; Mut, mutation; WT, wild-type; NC, negative control group.

in NPC tissues and NPC cell lines, RT-qPCR was performed. As indicated in Fig. 1A, miR-663b expression levels were significantly increased in NPC tissues when compared with those in normal tissues (P<0.01). Furthermore, the results also indicated that miR-663b expression levels were significantly upregulated in the C666-1 cells compared with the NP69 cells (P<0.01; Fig. 1B).

miR-663b directly targets Smad7. In order to investigate the role of miR-663b in NPC, the putative targets of miR-663b were predicted using TargetScan (Fig. 2A). Furthermore, the dual luciferase reporter assay was performed to verify the prediction. Results indicated that miR-663b significantly reduced the luciferase activity in 293T cells transfected with the WT 3'-UTR of SMAD7 compared with the NC groups (P<0.01), whereas the MUT SMAD7 3'-UTR abrogated the suppression by miR-663b (Fig. 2B). These findings indicated that miR-663b targets Smad7.

To further investigate the regulation of miR-663b on Smad7 expression in NPC cells, C666-1 cells were transfected with miR-663b mimics, miR-663b inhibitors or control, respectively. The transfection efficiency was confirmed by RT-qPCR (Fig. 3A). The overexpression of miR-663b significantly inhibited the mRNA expression levels of SMAD7 in C666-1 cells, whereas the miR-663b inhibitor significantly increased SMAD7 mRNA expression levels (P<0.05 and P<0.01, respectively; Fig. 3B). Similar effects

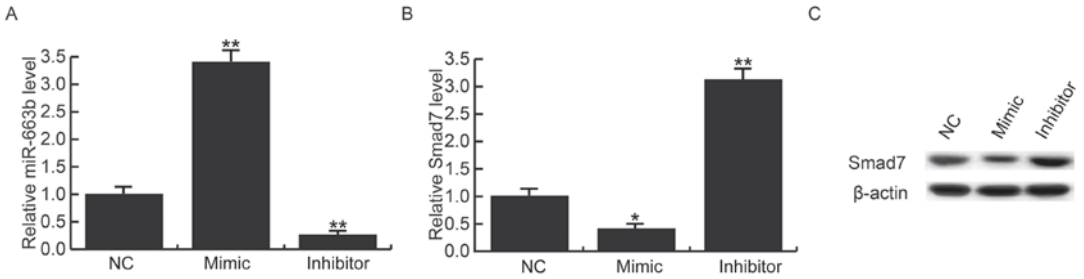

Figure 3. miR-663b and Smad7 expression in C666-1 cells. A total of 48 h following transfection, the expression levels of miR-663b and Smad7 mRNA and protein in C666-1 cells from different groups were revealed by reverse transcription-quantitative polymerase chain reaction and western blot analysis, respectively. (A) Relative miR-663b, (B) Smad7 mRNA and (C) Smad7 protein expression levels in C666-1 cells in different groups were indicated. Data are presented as the mean ± standard deviation. *P<0.05 and **P<0.01 vs. NC. miR, microRNA; NC, negative control group.

were observed regarding the protein expression levels of SMAD7 (Fig. 3C). These results indicated that SMAD7 is a target of miR-663b and that miR-663b may function in NPC via regulating SMAD7.

miR-663b promotes NPC cell proliferation. To investigate the effect of miR-663b on NPC cell proliferation, the CCK-8 assay was performed using a CCK-8 kit. The findings indicated that, when compared with the NC group the cell proliferation was significantly increased when C666-1 cells were transfected with miR-663b mimics, whereas the cell proliferation was significantly decreased in cells transfected with miR-663b inhibitors (P<0.05 and P<0.01; Fig. 4). These data suggested that miR-663b promotes NPC cell proliferation.

miR-663b promotes NPC cell epithelial mesenchymal transition (EMT). As Smad7 is an inhibitor of the transforming growth factor (TGF)-β signaling pathway that participates in the EMT of tumor cells, the effects of miR-663b on NPC cell EMT were explored in the present study. The protein expression levels of EMT-associated proteins E-cadherin, N-cadherin and Vimentin were determined following transfection with miR-663b mimics or miR-663b inhibitors in C666-1 cells, respectively. Results indicated that the protein expression levels of E-cadherin were markedly decreased in C666-1 cells transfected with miR-663b mimics compared with the NC group (Fig. 5A). Futhermore, mRNA expression of E-cadherin was significantly decreased in C666-1 cells transfected with miR-663b mimics compared with the NC group (P<0.01; Fig. 5B). Notably, the protein expression levels of N-cadherin, Vimentin and MMP-9 were markedly increased in C666-1 cells transfected with miR-663b mimics compared with the control group (Fig. 5A). As expected, the mRNA expression of N-cadherin (P<0.01), Vimentin (P<0.05) and MMP-9 (P<0.05) were significantly increased in C666-1 cells transfected with miR-663b mimics compared with the NC group (Fig. 5C-E). However, transfection of C666-1 cells with miR-663b inhibitors had the opposite effects. These data suggested that miR-663b could promote NPC cell EMT.

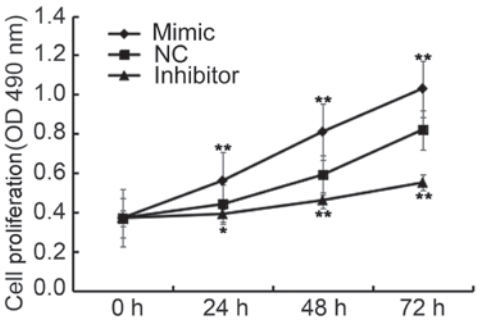

Figure 4. miR-663b affects C666-1 cell proliferation. A total of 24, 48 and 72 h following transfection, the Cell Counting kit-8 assay was performed to detect the cell proliferation of C666-1 cells in different groups. Data are presented as the mean ± standard deviation. *P<0.05 and **P<0.01 vs. NC. miR, microRNA; NC, negative control group; OD, optical density.

Discussion

miRs are a class of highly conserved non-coding single-stranded small molecule RNAs. Over 50% of the miRs are located in the tumor-associated genome region (21). Chromosomal abnormalities directly lead to changes in the number of miR gene copies, which results in abnormal expression of miRs in a variety of tumors that serves the role of oncogene or tumor suppressor (22). At present, miRs have been indicated to be disordered in NPC tissues and cells, and widely involved in various processes in NPC cells, including proliferation, invasion and metastasis (23).

Various studies concerning the role of miRs in NPC regulation have been conducted (24,25). Notably, a study reported that miR-216 was downregulated in NPC tissues and cells and inhibited the proliferation and invasion of NPC cells (26). A further study indicated that miR-200a could promote the EMT of NPC cells by regulating ZEB2 and β-catenin (27). miR-26a has been reported to be downregulated in NPC and acts as an inhibitor of the proliferation, invasion and migration of NPC cells (28,29). It has been acknowledged that miRs are majorly involved in the development of NPC and therefore have an immeasurable prospect in the basic and clinical research of NPC.

To the best of our knowledge, miR-663b, a novel cancer-associated miR, has rarely been studied in NPC. Thus, in the present study, the expression and role of miR-663b in NPC progression was investigated. Results indicated that miR-663b was significantly upregulated in NPC tissues and NPC cells. To investigate the role of miR-663b in NPC, the putative targets of miR-663b were predicted using TargetScan,

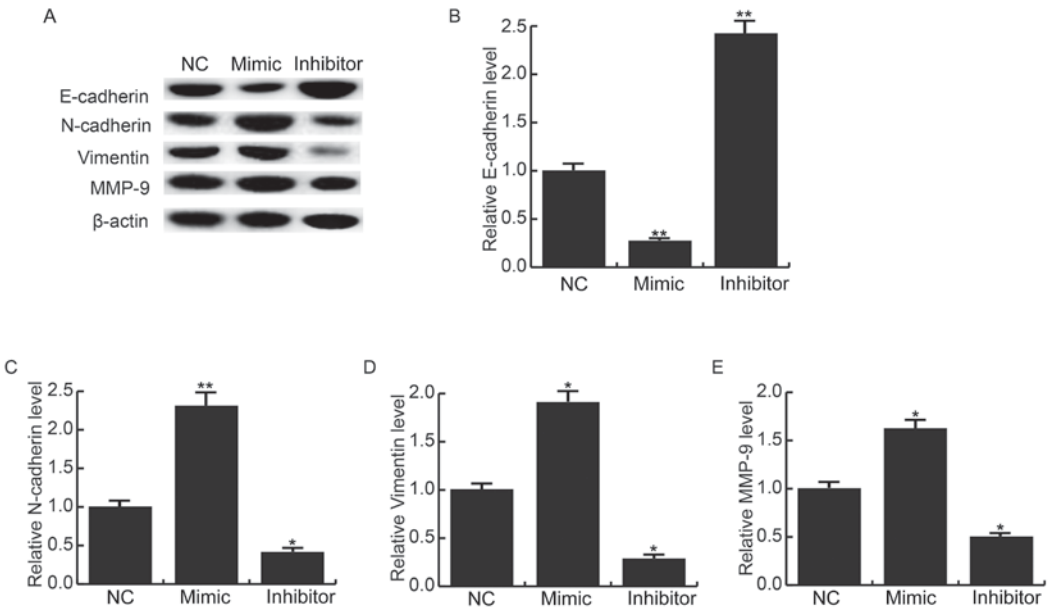

Figure 5. miR-663b affects the expression of EMT-associated proteins in C666-1 cells. A total of 48 h following transfection, the protein and mRNA expression levels of E-cadherin, N-cadherin, Vimentin and MMP-9 in C666-1 cells were detected by western blot analysis and reverse transcription-quantitative polymerase chain reaction, respectively. (A) Protein expression levels of E-cadherin, N-cadherin, Vimentin and MMP-9. mRNA expression levels of (B) E-cadherin, (C) N-cadherin, (D) Vimentin and (E) MMP-9. Data are presented as the mean ± standard deviation. *P<0.05 and **P<0.01 vs. NC. NC, negative control group; MMP, matrix metalloproteinase.

which identified hundreds of candidate targets, including cluster of differentiation 99, TP73, SCAI and Smad7. Smad7 belongs to the I-Smad family and serves an inhibitory role in the TGF-β signaling pathway (30). The TGF-β signaling pathway is one of the important signaling pathways in tumor cell EMT, which is an important cause of distant metastasis of malignant tumor cells (31). Blocking the TGF-β signaling pathway may effectively control tumor cell metastasis (32). Notably, Smad7 is one of the target genes of TGF-β, which can provide feedback to modulate the TGF-β/Smad signaling pathway and maintain the balance of the pathway (33). In the present study, it was hypothesized that miR-663b may affect NPC cell proliferation and EMT via regulating Smad7. As expected, miR-663b mimics were demonstrated to negatively regulate Smad7 expression in NPC cells and resulted in changes of the protein expression levels of EMT-associated proteins. These findings suggested the promoting role of miR-663b in NPC cell EMT and indicated that miR-663b functioned as a tumor promoter in NPC via promoting NPC cell proliferation and EMT. It was also suggested that miR-663b may exert its functions though directly targeting SMAD7.

In conclusion, to the best of our knowledge, the present study demonstrated for the first time that miR-663b was upregulated in NPC tissues and NPC cells. Furthermore, it was indicated that miR-663b could promote the proliferation and EMT of NPC cells by regulating Smad7 expression. These findings suggest miR-663b may be a novel therapeutic target for NPC.

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Availability of data and materials

The datasets used and/or analyzed during the current study are available from the corresponding author on reasonable request.

Authors' contributions

MW contributed to study design, statistical analysis, data interpretation, manuscript preparation and the literature search. MJ and KY contributed to study design, data collection and statistical analysis.

Ethics approval and consent to participate

The present study was approved by the Ethics Committee of the Central Hospital of Wuhan. Informed consent was obtained from each patient.

Consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

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