MiR-18a-5p Promotes NPC Cell Proliferation, Invasion, Migration, and EMT by Targeting SMAD2

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Primary research

Keywords: miR-18a-5p, NPC, Proliferation, Invasion, SMAD2, Migration

Posted Date: June 29th, 2020

DOI: <https://doi.org/10.21203/rs.3.rs-36707/v1>

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Abstract

Background: Nasopharyngeal carcinoma (NPC) is the most common malignant tumor in the head and neck that is characterized by high local malignant invasion and distant metastasis. miR-18a-5p reportedly plays an important role in tumorigenesis and development. However, little is known about the mechanism underlying miR-18a-5p's role in NPC.

Methods: Quantitative real-time PCR was used to detect the expression of miR-18a-5p in NPC tissues and cell lines. MTT assay and plate clone formation assay were used to detect the effect of miR-18a-5p on NPC cell proliferation. Woundhealing assays and Transwell assays were used to detect the effect of miR-18a-5p on NPC cell invasion and migration. The expressions of epithelial-mesenchymal transition (EMT)-related proteins N-cadherin, Vimentin, and E-cadherin were detected by Western blot. Bioinformatics and dual-luciferase reporter assay were used to detect the targeting interaction between miR-18a-5p and SMAD2. Xenotransplantation and metastasis model were used to detect the effect of miR-18a-5p on NPC growth and metastasis in vivo.

Results: miR-18a-5p was highly expressed in NPC tissues and cell lines. Overexpression of miR-18a-5p promoted NPC cell proliferation, invasion, migration, and EMT process, whereas inhibition of miR-18a-5p expression led to the opposite results. Results of dual-luciferase reporter assay showed that SMAD2 was the target gene of miR-18a-5p, and SMAD2 could reverse the effect of miR-18a-5p on NPC cell line. Xenotransplantation and metastasis model experiments in nude mice showed that miR-18a-5p promotes NPC growth and metastasis in vivo.

Conclusions: Targeting SMAD2 downregulated miR-18a-5p expression, thereby promoting NPC cell proliferation, invasion, migration, and EMT.

Background

Nasopharyngeal carcinoma (NPC) is a malignant tumor originating from the top and lateral wall of the nasopharyngeal cavity; its incidence is ranked first among malignant tumors of the head and neck. NPC incidence shows evident geographical, age, and gender differences, primarily in Southeast Asia, North Africa, and Guangdong in southern China. The peak age is between 50 and 60 years old, and the incidence rate in men is thrice higher than that in women [1, 2]. At present, radiotherapy and adjuvant chemotherapy are the primary NPC treatment methods. With the progress of treatment, most NPCs are well controlled, but the high metastatic tendency and high drug resistance of NPC patients remain a challenge to treatment [2, 3]. Therefore, an in-depth study of the invasion and migration mechanism of NPC needs to be the focus of research to look for new molecular targets, improve the effect of clinical treatment, and improve the prognosis of patients.

microRNA (miRNA) is a non-coding RNA with a single strand of 20–24 nt that exists widely in eukaryotes, and miRNA is highly conservative in evolution. miRNA can reportedly bind to the 3'-untranslated region (3'-UTR) of the target gene, inhibit the translation of mRNA, or promote the degradation of mRNA, thereby

regulating cell proliferation, differentiation, and apoptosis[4-7]. miRNA can play a vital role in the occurrence and development of NPC. miR-506-3p can downregulate the expression of LHX2 and inhibit the signal transduction of Wnt/ β -catenin, thereby inhibiting NPC growth and metastasis[8]. miR-203a-3p inhibits the growth and metastasis of NPC by targeting LASP1, and miR-129-5p inhibits lymphangiogenesis and lymph node metastasis of NPC by reducing ZIC2 expression, thereby possibly inhibiting the Hedgehog signal pathway[9]. These studies provide a theoretical basis for the study of the molecular mechanism of miRNA in NPC.

As an important miRNA, miR-18a-5p plays an important regulatory role in a variety of cancers. Certain studies have found that the expression of miR-18a-5p is upregulated in renal cell carcinoma tissues and cell lines, and the upregulation of miR-18a-5p can promote the proliferation, migration, and invasion and inhibit apoptosis of renal cancer cells[10]. The expression of miR-18a-5p is remarkably upregulated in non-small cell lung cancer (NSCLC) tissues and cell lines and can directly target interferon regulatory factor 2 (IRF2) to promote tumorigenesis in NSCLC[11]. In addition, other studies have found that miR-18a-5p plays an inhibitory role in breast cancer[12] and prostate cancer[13]. Moreover, miR-18a-5p is upregulated in NPC, which has an important clinical value in the diagnosis of NPC[14]. These results indicate that miR-18a-5p plays an important role in NPC proliferation, occurrence, and development, but the biological function of miR-18a-5p in NPC has not been reported.

Therefore, the present study investigated the mechanism of miR-18a-5p regulating the proliferation, metastasis, and invasion of NPC cells in vivo and in vitro to understand the pathogenesis of NPC and provide new ideas for future clinical diagnosis and treatment.

Materials And Methods

Cell lines and patient

NPC cell line (SUNE1, 5-8F, 6-10B, CNE1, CNE2, and HNE1) and human normal nasopharyngeal cell line NP69 were purchased from Shanghai Institute of Biochemistry and Cell Biology, Chinese Academy of Sciences. Cell lines were cultured in RPMI1640 (Gibco, USA) medium containing 10% FBS (Gibco, USA) at 37 °C and 5%CO₂.

Patients: Thirty patients with NPC and their adjacent tissues were treated in the first affiliated Hospital of Zhengzhou University. All patients were pathologically diagnosed as NPC after operation. Samples were collected immediately after operation and stored at -80 °C.

Cell transfection

miR-18a-5p inhibitor, inhibitorNC, miR-18a-5p mimics, mimicsNC, agomiR-NC, and agomiR-18a-5p were purchased from Guangzhou RiboBio Co., Ltd. Small interference RNA (siRNA) synthesized by targeted SMAD2 was synthesized by Shanghai Shenggong Bioengineering Co., Ltd. On the basis of the reference

manual, Lipofectamine® 3000 (Invitrogen, USA) was used to transfect inhibitor, mimics, siRNA, and their respective controls into NPC cells for gene or miRNA overexpression or silencing.

Quantitative real-time PCR assays [qRT-PCR]

Total RNA was extracted from NPC tissues and cells. CDNA was synthesized using reverse transcription system kit (TransGene Biotech, Beijing, China). Quantitative real-time polymerase chain reaction (qRT-PCR) was achieved using the ABI7500 instrument (7500 bot ABI Co.). SMAD2 was normalized with GAPDH as internal reference and miR-18a-5p with U6 as internal reference. The difference of relative expression of target gene mRNA between the control group and the experimental group was compared with the value of $2^{-\Delta\Delta C_t}$, and the experiment was repeated thrice. The primers used in the experiment are shown in Table 1.

Western Blot

At 48 h after transfection, total proteins were extracted with RIPA cleavage buffer (Beyotime Biotechnology, Shanghai, China). After adding 10 μ L of the sample buffer to boil for 10 min at 95 °C, SDS-PAGE was achieved at 100 V. After electrophoresis, the protein was transferred to NC membrane at 100 mA for 120 min, sealed with 5% skimmed milk powder for 60 min, and incubated overnight at 4 °C. After the first antibody was incubated, the membrane was shaken and washed with 1 $\times$ TBST solution (Solarbio, Beijing, China) at room temperature, 5min $\times$ 3 times, and hybridized with horseradish peroxidase-labeled goat anti-rabbit IgG, at room temperature for 120 min. At 20min intervals, the photoluminescence reaction was performed using the ECL kit (Solarbio, Beijing, China), and protein imprinting was observed. Antibodies are shown in Table 2. The experiment was repeated thrice.

Cell proliferation and plate clone formation

Cell proliferation was tested by the MTT method, and the cells in the treatment group (5×10^3 per 100 μ L) were inoculated into a 96-well plate. Each process included three repeats. After culturing for 0, 12, 24, 48, and 72 h, cell proliferation was evaluated with aseptic MTT solution (Beyotime) according to the instruction method. A spectrophotometer (Molecular Devices, Sunnyvale, CA, USA) was used to measure the absorbance of the 490nm wavelength.

Plate clone formation experiment: Treated cells were cultured in a Petri dish (1.5×10^3 /plate). The cells were stirred gently and incubated with 5% CO₂ at 37 °C for 14 days; the culture medium was removed, and the cells were washed twice with phosphate-buffered saline (PBS). Next, the cells were dyed with 0.5% crystal violet at room temperature for 15 min. Then, such cells were washed with water. Colonies of more than 50 cells were counted under a light microscope.

Wound-healing assays

Treated cells (5×10^6) were inoculated in a 6-well plate. When the cell coverage reached 80%, the tip of the 200 μ L pipette was used to gently scratch the monolayer through the center of the hole. Pores were washed twice with the medium to remove isolated cells. After adding fresh medium, the cells were cultured for 24 h, and cell migration was observed and photographed for 0 and 24 h under a microscope.

Transwell invasion and migration assay

A 24-well Transwell chamber (8 μ m aperture, BD Biosciences) was used for Transwell invasion detection. Approximately 2×10^4 cells were added to the superior chamber, and the parietal chamber was coated with Matrigel matrix. The DMEM containing 10% FBS (Thermo fisher, USA) was filled into the lower chamber. After culturing at 37 °C for 48 h, the Transwell was washed twice with PBS, and 5% glutaraldehyde was fixed at 4 °C for 0.5 h. The cells on the upper surface were wiped with a cotton ball, observed and photographed under a microscope, and counted. No matrix was added to the apical chamber in the Transwell migration experiment, and the other processes were the same as those in the Transwell invasion experiment.

Dual-luciferase reporter assay

To identify the probability of binding between miR-18a-5p and SMAD2 3'UTR, a psiCHECK luciferase reporter vector (SangonCo., Ltd., Shanghai, China) was constructed by inserting wild-type (wt) and mutant (mut) 3'UTR of SMAD2. Next, CNE1 and 6Mel 10B cells were inoculated in 48-well plates and cultured for 24 h. Afterward, miR-18a-5p/NC and psiCHECKwt/mut plasmids were co-transfected into the cells. Finally, luciferase activity was measured by luciferase assay reagent (Promega, Fitchburg, WI, USA).

Tumour xenograft model

Fifteen male nude mice (6 weeks) were purchased from Southern Medical University (Guangdong, China) and raised under aseptic conditions (12 h black and white photoperiod, 25 °C, 60% UV, 70% humidity). CNE1 cells were inoculated into the feet of male nude mice. When the tumor volume reached 60mm³, antagomiR-18a-5p (10nmol/50mL), antagomiR-NC, and PBS were injected twice a week as negative control, and intratumoral therapy was performed eight times. Tumor volume measurement method: volume (cm³) = (length $\times$ width²)/2. After 4 weeks, the nude mice were euthanized, and the tumor weight was measured and photographed. The nursing and experimental procedures of the mice were approved by the Experimental Animal Ethics Committee.

Immunohistochemistry

The transplanted tumor tissue of mice was placed in 4% paraformaldehyde fixation solution and fixed in a refrigerator at 4 °C. After gradient dehydration of different concentrations of ethanol, xylene transparent treatment, paraffin embedding, and slicing, immunochemical staining was performed on the transplanted tumor tissue, which was finally sealed with xylene transparent and neutral gum, covered with glass slides, and dried naturally for follow-up observation. Antibodies are shown in Table 2.

Statistical analysis

Data were processed by SPSS22.0 statistical software, and the measurement data were expressed in the form of mean $\pm$ standard deviation. The comparison between the two groups was obtained using t-test. In the current paper, *, # indicated $P < 0.05$.

Results

miR-18a-5p is up-regulated in NPC tissues and cell lines.

The expression of miR-18a-5p in cancer and adjacent tissues of 30 patients with NPC was detected by qRT-PCR. miR-18a-5p was highly expressed in cancer tissues compared with adjacent tissues (Fig. 1A). In addition, the expression of miR-18a-5p was detected in human nasopharyngeal epithelial cell line NP69 and NPC cell lines (SUNE1, 5-8F, 6-10B, CNE1, CNE2, and HNE1) by qRT-PCR. The expression level of miR-18a-5p in the NPC cell line was considerably higher than that in the NP69 cell line, and the expression level of CNE1 in 6-10B cell line was the highest (Fig. 1B). Therefore, these two cell lines were chosen for further study. The miR-18a-5p was upregulated in NPC tissues and cell lines and might play an important role in the occurrence and development of NPC.

miR-18a-5p promotes proliferation, migration, invasion and EMT in the NPC cell lines

To investigate the effect of miR-18a-5p on the growth of NPC cell lines in vitro, miR-18a-5p mimics and miR-18a-5p inhibitors were used to transfect 6-10B and CNE1 cell lines, respectively, and the effect of miR-18a-5p on the proliferation, migration, and metastasis of 6-10B and CNE1 cell lines was observed. After miR-18a-5p mimicked transfection into 6-10B cell line, the expression of miR-18a-5p in the NPC cell line was remarkably increased (Fig. 2A), whereas the expression of miR-18a-5p in the NPC cell line was remarkably decreased after transfection with miR-18a-5p inhibitor (Fig. 2B). The results of colony formation assay and MTT assay showed that miR-18a-5p overexpression remarkably promoted the proliferation of NPC cell line, whereas inhibition of miR-18a-5p expression decreased the proliferation ability of NPC cell line (Fig. 2C D). The results of wound healing assays showed that miR-18a-5p overexpression could promote NPC cell migration, whereas inhibition of miR-18a-5p expression led to the opposite result (Fig. 2E). In addition, through Transwell experiments, miR-18a-5p overexpression enhanced NPC cell migration and invasion, whereas its inhibition decreased NPC's invasive ability (Fig. 2F, G). Moreover, the expression of epithelial-mesenchymal transition (EMT) related proteins was detected in two NPC cell lines CNE1 and 6-10B. N-cadherin and Vimentin protein expressions increased, and the expression of E-cadherin protein decreased after miR-18a-5p overexpression, thereby confirming that miR-18a-5p overexpression could promote the progression of EMT in NPC cell line, whereas inhibition of miR-18a-5p led to the opposite result (Fig. 2H). Based on the abovementioned experimental results, miR-18a-5p overexpression could promote NPC cell proliferation, migration, invasion, and EMT process, whereas inhibition of miR-18a-5p expression led to the opposite results.

miR-18a-5p targets and down-regulates the expression of SMAD2

To explore the molecular mechanism of miR-18a-5p promoting NPC cell proliferation, migration, invasion, and EMT process, the function of miR-18a-5p was obtained through starbase and TargetScan websites. Consequently, miR-18a-p could target the 3'-UTR region binding to SMAD2 (Fig. 3A). The difference of SMAD2 expression between human nasopharyngeal epithelial cell line NP69 and NPC cell line (SUNE1, 5-8F, 6-10B, CNE1, CNE2, and HNE1) was detected by qRT-PCR and Western blot. SMAD2 expression was remarkably downregulated in NPC cell line (Fig. 3B C). In addition, qRT-PCR results showed that overexpression of miR-18a-5p inhibited the expression of SMAD2, whereas inhibition of miR-18a-5p expression could upregulate the expression of SMAD2 (Fig. 3D). Similarly, the trend of Western blot results was consistent with that of qRT-PCR (Fig. 3E). To prove that miR-18a-5p could downregulate SMAD2 expression, the results of double-luciferase assay showed that miR-18a-5p overexpression could inhibit the luciferase activity of wild-type SMAD2 3'-UTR, but it had no effect on the luciferase activity of mutant SMAD2 3'-UTR (Fig. 3F). miR-18a-5p could directly target SMAD2 3'-UTR and inhibit SMAD2 expression.

SMAD2 inhibits the proliferation, migration, invasion and EMT of NPC cells

SMAD2 was a possible tumor suppressor and played an important role in transforming growth factor- β signal transduction. In lung cancer, miR-27A might play the role of oncogenes by targeting Smad2 and Smad4. However, the role of SMAD2 in NPC has not been reported. SMAD2 expression was inhibited in two NPC cell lines CNE1 and 6-10B (Fig. 4A). The results of clone formation and MTT assay showed that the downregulated expression of SMAD2 promoted the NPC cell proliferation (Fig. 4B C). Woundhealing assays showed that the downregulated expression of SMAD2 could promote NPC cell migration ability (Fig. 4D). In addition, the results of Transwell assay showed that NPC cell migration and invasion ability increased remarkably after the downregulation of SMAD2 expression (Fig. 4E F). The protein expression of N-cadherin, E-cadherin, and Vimentin was detected by Western blot. The downregulated expression of SMAD2 increased the expression of N-cadherin protein and Vimentin protein and decreased the expression of E-cadherin protein (Fig. 4G), which promoted the process of EMT. The downregulated expression of SMAD2 could promote NPC cell proliferation, migration, invasion, and EMT.

Rescue experiment verifies that miR-18a-5p promotes the malignant behavior of NPC through SMAD2

Based on the abovementioned experimental results, Rescue experiment was used to verify whether miR-18a-5p promoted the malignant behavior of NPC by targeting and downregulating SMAD2 expression. In addition, miR-18a-5p inhibitor and siSMAD2 were co-transfected into CNE1 and 6-10B cell lines. Colony formation assay and MTT assay showed that reducing the expression of miR-18a-5p could inhibit NPC cell proliferation, whereas co-transfection of siSMAD2 could restore NPC cell proliferation ability to a certain extent (Fig. 5 A B). Moreover, Transwell assay results revealed that decreased miR-18a-5p expression inhibited NPC cell invasion and migration, whereas simultaneous transfection of siSMAD2 could restore the invasion and metastasis ability of NPC cells to certain extent (Fig. 5 C D). Finally, the results of Western blot assay showed that downregulation of miR-18a-5p expression inhibited NPC cell EMT progression, whereas simultaneous transfection of siSMAD2 could reverse the effect of the inhibition of miR-18a-5p expression on NPC cell EMT to a certain extent (Fig. 5E). The abovementioned

results indicated that miR-18a-5p promoted the proliferation, invasion, and migration of NPC cells through SMAD2 and accelerated the process of EMT, thereby promoting NPC's malignant progression.

MiR-18a-5p promotes the growth and Metastasis of NPC in vivo

On the basis of in vitro experiments, CNE1 cells were inoculated into the feet of male nude mice to confirm the effect of miR-18a-5p on NPC and establish the transplanted tumor model of NPC and spontaneous lymph node metastasis (Fig.6A). PBS (negative control), agomiR-18a-5p or agomiR-NC, was injected into the xenograft model mice twice a week. After 4 weeks, the tumor size and the number of mice with lymph node metastasis were observed. The results of in vivo experiment showed that the tumor volume of agomiR-18a-5p mice was remarkably larger than that of the negative control and agomiR-NC groups, and the number of mice with fossa lymph node metastasis in the agomiR-18a-5p group was higher than in the negative control and agomiR-NC groups (Fig.6B). In addition, the expression of EMT-related genes was detected by the immunohistochemical method. Compared with the control group, the expression of N-cadherin protein and Vimentin protein increased, and the expression of E-cadherin protein decreased in the agomiR-18a-5p group (Fig.6C). Therefore, miR-18a-5p could promote the proliferation and metastasis of NPC in vivo.

Discussion

EMT refers to the biological process by which epithelial cells are transformed into mesenchymal phenotypic cells through specific procedures. EMT is an important biological process for malignant tumor cells derived from epithelial cells to acquire the ability of migration and invasion and is an important stage of tumor metastasis[15]. EMT is reportedly closely related to the metastasis of pancreatic cancer[16, 17], endometrial cancer[18], breast cancer[19], NPC[20] and other cancers. Moreover, miRNA can regulate the malignant progression of tumors by regulating EMT in many kinds of cancers. For example, in human hepatocellular carcinoma cell line HepG2, miR-32 can inhibit the expression of SRCIN1 and promote the proliferation and EMT of human hepatocellular carcinoma cells[21]. miR448 specifically regulates the expression of ZEB1/2 by binding to the 3'-UTR of ZEB1/2 in breast cancer cells and inhibits cell migration, invasion, and EMT[22]; miR-328 directly targets CD44-bound 3'UTR to inhibit NPC cell migration and EMT process[23]; miRNA-34a directly targets Smad4 to inhibit NPC cell invasion, migration, and EMT induction by transforming growth factor- β [24]. However, the effect of miR-18a-5p on EMT in NPC has not been studied. Therefore, the present study has explored the effect of miR-18a-5p on NPC EMT. The results have shown that overexpression of miR-18a-5p could promote NPC cell proliferation, invasion, migration, and EMT, whereas inhibiting the expression of miR-18a-5p led to the opposite results. Furthermore, our in vivo experiments have confirmed that overexpression of miR-18a-5p can promote the growth and metastasis of NPC and the progression of EMT.

TGF- β is the most classical cytokine that induces EMT in cells[25]. TGF- β can bind and activate TGF- β type I receptor (T β R1, ALK5) and then activate downstream signal pathways. The most classical downstream signal pathway is the SMAD signal pathway, in which the activated ALK5 recruits and activates the receptor SMAD (R-SMAD), which is primarily characterized by the C-terminal

phosphorylation of SMAD2 (Ser465/467) and SMAD3 (Ser423/425). These activated receptor SMAD forms a heterocomplex with the common pathway SMAD (Co-SMAD, SMAD4), and then enters the nucleus to regulate the transcription of the target gene[26, 27]. Some studies have found that miR-132 inhibits the migration, invasion, and EMT of bladder cancer through the TGFβ1/SMAD2 signal pathway[28]. miR-503-3p expression is upregulated in breast cancer cells and promotes EMT in breast cancer by directly targeting SMAD2 and E-cadherin[29]. In the present study, miR-18a-5p specifically reduces the expression of SMAD2, and downregulation of SMAD2 expression can promote NPC cell proliferation, invasion, migration, and EMT through bioinformatics prediction and dual-luciferase reporter assay. By simultaneously downregulating the expression of miR-18a-5p and SMAD2, SMAD2 can partially reverse the regulatory effect of miR-18a-5p on NPC cells. miR-18a-5p regulates the proliferation, invasion, migration, and EMT of NPC cells by targeting SMAD2.

Conclusion

In this study, we found that miR-18a-5p is highly expressed in NPC and promotes the proliferation, invasion, migration, and EMT process of NPC cells by targeting and downregulating SMAD2 expression. This result provides a better understanding of the role of miR-18a-5p in NPC and lays a foundation to find a new targeted therapy for NPC.

Declarations

Abbreviations

NPC: Nasopharyngeal carcinoma; qRT-PCR: quantitative real-time PCR; EMT: epithelial mesenchymal transition; NPC: non-small cell lung cancer; IRF2: interferon regulatory factor 2; FBS: Foetal Bovine Serum; GAPDH: glyceraldehyde-3-phosphate dehydrogenase; RIPA: radio-immunoprecipitation assay; SDS-PAGE: sodium dodecyl sulfatepolyacrylamide gel electrophoresis; TBST: tris-buffered saline tween-20; MTT: Methyl Thiazolyl Tetrazolium; PBS: phosphate-buffered saline; DMEM: dulbecco's modified eagle medium.

Ethics approval and consent to participate

The present study was approved by the Ethics Committee of the first affiliated Hospital of Zhengzhou University. All patients who participated in the trial signed an informed written consent form.

Consent for publication

Not applicable

Availability of data and materials

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

Competing interests

The authors declare that they have no competing interests

Funding

This study was supported by the National Natural Science Foundation Youth Project 81703158

Authors' contributions

Junqi Liu and Yanzhou Zhang provided research ideas and experimental materials; Pengcheng Li and Junhui Xing participated in designing experimental schemes, conducting experiments and drafting manuscripts; Jianwu Jiang, Xinyu Tian, Xuemeng Liu, Rui Yao helped to consult the literature and analyze data; Leiming Wu and Cui Liang provided substantial contributions to conception of the manuscript, helped draft the article, and made critical revisions related to important intellectual content of the manuscript.

Acknowledgements

Not applicable

Reference

1. MLK C, JTS W, EP H, ATC C: **Nasopharyngeal carcinoma**. *Lancet (London, England)* 2016, **387**(10022):1012-1024.
2. YP C, ATC C, QT L, P B, Y S, J M: **Nasopharyngeal carcinoma**. *Lancet (London, England)* 2019, **394**(10192):64-80.
3. A Z, M T, C B, M M, C LV, G F, M L, D S, J P: **Metabolic disorders and the risk of nasopharyngeal carcinoma: a case-control study in Italy**. *European journal of cancer prevention : the official journal of the European Cancer Prevention Organisation (ECP)* 2018, **27**(2):180-183.
4. H Z, JY L, FJ S, KX C: **Advances in circulating microRNAs as diagnostic and prognostic markers for ovarian cancer**. *Cancer biology & medicine* 2013, **10**(3):123-130.
5. I S, M N, K U, A P, V Z, J H, MJ A, C B, S M, H Z: **Changes in circulating microRNAs after radiochemotherapy in head and neck cancer patients**. *Radiation oncology (London, England)* 2013, **8**:296.
6. CSDC D, BP S, MS LC, CMM M, FJB V, AB SACR, ARR F, MFR B, MARF D: **Epithelial-mesenchymal transition and microRNAs: Challenges and future perspectives in oral cancer**. *Head & neck* 2018, **40**(10):2304-2313.
7. M L, X H, CB D, Q D, X X: **MicroRNAs and their role in environmental chemical carcinogenesis**. *Environmental geochemistry and health* 2019, **41**(1):225-247.

8. TS L, YJ Z, J W, JY Z, DK Y, ZS L: **MicroRNA-506 inhibits tumor growth and metastasis in nasopharyngeal carcinoma through the inactivation of the Wnt/ β -catenin signaling pathway by down-regulating LHX2.** *Journal of experimental & clinical cancer research : CR* 2019, **38**(1):97.
9. D Y, GH H, X Z, X L, K X, D W, CB X: **MicroRNA-129-5p suppresses nasopharyngeal carcinoma lymphangiogenesis and lymph node metastasis by targeting ZIC2.** *Cellular oncology (Dordrecht)* 2019.
10. L Z, Z L, X P, Y L, J Q, L Z, J X, W X, X G, H L *et al*: **Identification of miR-18a-5p as an oncogene and prognostic biomarker in RCC.** *American journal of translational research* 2018, **10**(6):1874-1886.
11. Tsao SW, Tsang CM, Lo KW: **Epstein-Barr virus infection and nasopharyngeal carcinoma.** *Philosophical transactions of the Royal Society of London Series B, Biological sciences* 2017, **372**(1732).
12. N Z, H Z, Y L, P S, J Z, X W, M S, B C, W Z, L W *et al*: **SREBP1, targeted by miR-18a-5p, modulates epithelial-mesenchymal transition in breast cancer via forming a co-repressor complex with Snail and HDAC1/2.** *Cell death and differentiation* 2019, **26**(5):843-859.
13. G Z, G H, X Z, Q Y, Z L, Z L, J L: **Long non-coding RNA FENDRR reduces prostate cancer malignancy by competitively binding miR-18a-5p with RUNX1.** *Biomarkers : biochemical indicators of exposure, response, and susceptibility to chemicals* 2018, **23**(5):435-445.
14. H W, X W, B W, J S, W T, K Y: **Tumor-educated platelet miR-34c-3p and miR-18a-5p as potential liquid biopsy biomarkers for nasopharyngeal carcinoma diagnosis.** *Cancer management and research* 2019, **11**:3351-3360.
15. Yoshida EJ, Luu M, David JM, Kim S, Mita A, Scher K, Shiao SL, Tighiouart M, Lee NY, Ho AS *et al*: **Facility Volume and Survival in Nasopharyngeal Carcinoma.** *International journal of radiation oncology, biology, physics* 2018, **100**(2):408-417.
16. K S, S H, T S: **Involvement of epithelial to mesenchymal transition in the development of pancreatic ductal adenocarcinoma.** *Journal of gastroenterology* 2015, **50**(2):140-146.
17. MM T, SA D: **Epigenetics of epithelial-to-mesenchymal transition in pancreatic carcinoma.** *International journal of cancer* 2017, **141**(1):24-32.
18. HC C, CJ L, GT Y, AP T, MY W: **Epithelial to Mesenchymal Transition and Cell Biology of Molecular Regulation in Endometrial Carcinogenesis.** *Journal of clinical medicine* 2019, **8**(4).
19. IK G, BD A, BA W: **ER α , microRNAs, and the epithelial-mesenchymal transition in breast cancer.** *Trends in endocrinology and metabolism: TEM* 2012, **23**(2):73-82.
20. YJ Z, JY Z, TS L, P W, J W, DK Y, ZS L: **Long noncoding RNA SMAD5-AS1 acts as a microRNA-106a-5p sponge to promote epithelial mesenchymal transition in nasopharyngeal carcinoma.** *FASEB journal : official publication of the Federation of American Societies for Experimental Biology* 2019, **33**(11):12915-12928.
21. R C, JY L, J H, WL C, XJ M, XD L: **Downregulation of SRC Kinase Signaling Inhibitor 1 (SRCIN1) Expression by MicroRNA-32 Promotes Proliferation and Epithelial-Mesenchymal Transition in Human Liver Cancer Cells.** *Oncology research* 2018, **26**(4):573-579.

22. P M, K N, J K, W Z, Y F, Q M: **miR-448 inhibits the epithelial-mesenchymal transition in breast cancer cells by directly targeting the E-cadherin repressor ZEB1/2.** *Experimental biology and medicine* (Maywood, NJ) 2018, **243**(5):473-480.
23. CH L, MC C, YJ C: **MicroRNA-328 inhibits migration and epithelial-mesenchymal transition by targeting CD44 in nasopharyngeal carcinoma cells.** *OncoTargets and therapy* 2018, **11**:2375-2385.
24. G H, MY D, H Z, N Z, ZW L, LX Q, W Z, X T, X H, L Y: **MiRNA-34a reversed TGF- β -induced epithelial-mesenchymal transition via suppression of SMAD4 in NPC cells.** *Biomedicine & pharmacotherapy = Biomedecine & pharmacotherapie* 2018, **106**:217-224.
25. DM G, D M: **Signaling mechanisms of the epithelial-mesenchymal transition.** *Science signaling* 2014, **7**(344):re8.
26. J M: **TGF β signalling in context.** *Nature reviews Molecular cell biology* 2012, **13**(10):616-630.
27. Y S, J M: **Mechanisms of TGF-beta signaling from cell membrane to the nucleus.** *Cell* 2003, **113**(6):685-700.
28. XC W, ZH L: **MicroRNA-132 inhibits migration, invasion and epithelial-mesenchymal transition via TGF β 1/Smad2 signaling pathway in human bladder cancer.** *OncoTargets and therapy* 2019, **12**:5937-5945.
29. Z Z, X F, L J, Z X, L X, Q Z, Y S: **miR-503-3p promotes epithelial-mesenchymal transition in breast cancer by directly targeting SMAD2 and E-cadherin.** *Journal of genetics and genomics = Yi chuan xue bao* 2017, **44**(2):75-84.

Tables

Table 1
qRT-PCR primer sequences

Genesymbol	primer sequence
SMAD2	F:5'-CGTCCATCTTGCCATTCACG-3'
	R:5'-CGTCCATCTTGCCATTCACG -3'
GAPDH	F:5'-GGAGCGAGATCCCTCCAAAAT-3'
	R:5'-GGCTGTTGTCATACTTCTCATGG-3'
miR-18a-5p	F:5'-TCGGCAGGGATAGACGTGATCT-3'
	R:5'-CTCAACTGGTGTCTGGA-3'
U6	F:5'-CTCGCTTCGGGCAGCACA-3'
	R:5'-AACGCTTCACGAATTTGCGT-3'

Table 2
Antibody information used in the laboratory

Antibody	WB(IHC)	Specificity	Company
N-cadherin	1 µg/ml×1 µg/ml	Rabbit polyclonal	Abcam, China
E-cadherin	1/10000×1/500	Rabbit monoclonal	Abcam, China
VIMENTIN	1/1000×1/100	Rabbit monoclonal	Abcam, China
GAPDH	1/10000	Rabbitmonoclonal	Abcam, China

Figures

Figure 1

miR-18a-5p is up-regulated in NPC tissues and cell lines. A The relative expression of miR-18a-5p in NPC and paracancerous tissues. B The relative expression of miR-18a-5p in human nasopharyngeal epithelial cell line and NPC cell line.

Figure 2

miR-18a-5p promotes proliferation, migration, invasion and EMT in the NPC cell lines. A The expression of miR-18a-5p in CNE1 and 6-10B cells transfected with miR-18a-5p mimic was determined by qRT-PCR. B The expression of miR-18a-5p in CNE1 and 6-10B cells transfected with miR-18a-5p inhibitor was determined by qRT-PCR. C The cell clone formation ability was determined by plate clone formation assay. D Cell viability analysis by MTT assay. E Wound healing assay. F Cell migration and invasion was analysis by Transwell assay. G was analysis by Transwell assay. H The EMT-related protein expression was analysis by Western blot.

Figure 3

miR-18a-5p targets and down-regulates the expression of SMAD2. A The potential binding sites of SMAD2 and miR-18a-5p was predicted by starbase and TargetScan. B The relative expression of SMAD2 mRNA in human nasopharyngeal epithelial cell line and NPC cell line. C The relative expression of SMAD2 protein in human nasopharyngeal epithelial cell line and NPC cell line. D The expression of SMAD2 mRNA in CNE1 and 6-10B cells transfected with miR-18a-5p mimic or miR-18a-5p inhibitor was determined by qRT-PCR. E The expression of SMAD2 mRNA in CNE1 and 6-10B cells transfected with miR-18a-5p mimic or miR-18a-5p inhibitor was determined by Western blot. F Relative luciferase activity of the SMAD2 wild type and mutant SMAD2 3'-UTR.

Figure 4

SMAD2 inhibits the proliferation, migration, invasion and EMT of NPC cells. A The expression of SMAD2 mRNA in CNE1 and 6-10B cells transfected with si-SMAD2 was determined by qRT-PCR. B The cell clone formation ability was determined by plate clone formation assay. C Cell viability analysis by MTT assay. D Wound healing assay. E Cell migration and invasion was analysis by Transwell assay. F The invasion was analysis by Transwell assay. G The EMT-related protein expression was analysis by Western blot.

Figure 5

miR-18a-5p promotes the malignant behavior of NPC through SMAD2.A The cell clone formation ability was determined by plate clone formation assay.B Cell viability analysis by MTT assay.C Cell migration and invasion (D) was analysis by Transwell assay.E The EMT-related protein expression was analysis by Westernblot.

Figure 6

miR-18a-5p promotes the growth and Metastasis of NPC in vivo. A The size and body weight of transplanted tumor in nude mice. B The number of nude mice with fossa lymph node metastasis. C The E-cadherin, N-cadherin and Vimentin expression was measured by IHC in different xeograft tissues.