MicroRNA-19a promotes nasopharyngeal carcinoma by targeting transforming growth factor β receptor 2

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Received November 16, 2016; Accepted May 5, 2017

DOI: 10.3892/etm.2017.4655

Abstract. MicroRNA (miR), a class of small non-coding RNA, functions as key regulators in gene expression through directly binding to the 3' untranslated region of their target mRNA, which further leads to translational repression or mRNA degradation. miR-19a, a member of miR-17-92 cluster, has an oncogenic role in a variety of malignant tumors. However, the exact role of miR-19a in nasopharyngeal carcinoma (NPC) has not previously been studied. The present study aimed to investigate the function and mechanism of miR-19a in regulating the viability and invasion of NPC cells. Reverse transcription-quantitative polymerase chain reaction (RT-qPCR) data indicated that the expression levels of miR-17-92 cluster members (miR-17, miR-18a, miR-19a and miR-20a) were frequently increased in NPC tissues compared to the normal tissues. It was also demonstrated that miR-19a was significantly upregulated in NPC C666-1 cells compared to NP69 cells (P<0.01). Knockdown of miR-19a led to a significant decrease in the viability and invasion of NPC C666-1 cells (P<0.01), and induced increased protein expression levels of transforming growth factor β receptor 2 (TGFβR2), which was further identified as a direct target gene of miR-19a by using a luciferase reporter assay. Overexpression of TGFβR2 also suppressed the viability and invasion of C666-1 cells, similar to the effects of miR-19a inhibition. Furthermore, knockdown of TGFβR2 reversed the suppressive effects of miR-19a inhibition on C666-1 cell viability and invasion, suggesting that the role of miR-19a in mediating cell viability and invasion is through directly targeting TGFβR2 in NPC cells. In addition, RT-qPCR data demonstrated that the mRNA expression level of TGFβR2 was markedly reduced in NPC tissues and C666-1 cells. In summary, the present study demonstrated an oncogenic role of miR-19a in NPC via mediation of TGFβR2.

Therefore, miR-19a may be a potential therapeutic target for NPC.

Introduction

Nasopharyngeal carcinoma (NPC) is a head and neck cancer that is most common in Southern China and Southeast Asia, and the annual incidence rate is ~20 cases per 100,000 people in endemic areas (1). The pathogenesis of NPC predominantly includes Epstein-Barr virus (EBV) infection and genetic susceptibility (2,3). However, the molecular mechanism underlying the tumorigenesis and malignant progression of NPC is poorly understood. Therefore, understanding the molecular mechanism may help for the improvement of NPC diagnosis and treatment.

MicroRNA (miR) are a kind of small non-coding RNA that are usually composed of 22-25 nucleotides (4). miR may inhibit gene expression at the post-transcriptional level by binding to the 3' untranslated region (UTR) of their target mRNA, resulting in translation inhibition or mRNA degradation (5). miR have been demonstrated to participate in a variety of cellular processes, including cell proliferation, apoptosis, differentiation, metabolism and motility (6). Furthermore, miR are closely associated with tumorigenesis and cancer development (7), and some have been reported to have key roles in NPC (8,9). miR-19a is a member of the miR-17-92 cluster, and generally has an oncogenic role in multiple types of human cancer, such as lung cancer (10), breast cancer (11), colorectal cancer (12), renal cell carcinoma (13), pancreatic cancer (14), gastric cancer (15), laryngeal squamous cell carcinoma (16) and bladder cancer (17). However, the function and regulatory mechanisms of miR-19a in NPC have not previously been studied.

Transforming growth factor β receptor 2 (TGFβR2), a member of the Ser/Thr protein kinase family and the TGFβ receptor subfamily, is a transmembrane protein that has a protein kinase domain and forms a heterodimeric complex with another receptor protein (18). TGFβR2 binds to TGFβ, which may further phosphorylate proteins, and then enters the nucleus to regulate the gene transcription (18). Mutations in TGFβR2 have been implicated in human cancer (19,20). A study by Zhang et al (21) reported that mRNA and protein expression levels of TGFβR2 were significantly lower in NPC tissues compared with the non-cancerous tissues, and

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Key words: nasopharyngeal carcinoma, microRNA, transforming growth factor β receptor 2, proliferation, invasion
EBV encoded small RNA hybridization signals in NPC demonstrated significant associations with TGFβR2 (21). Furthermore, the lower expression of TGFβR2 was demonstrated to be an independent contributor to NPC, and could predicate its prognosis (21). However, the reason for the down-regulation of TGFβR2 in NPC remains unknown.

The present study aimed to identify the exact role and regulatory mechanism of miR-19 in NPC. The results suggested that miR-19a has an oncogenic role in NPC cell viability and invasion via directly targeting TGFβR2.

Materials and methods

Clinical tissue collection. The present study was approved by the Ethics Committee of the Second Xiangya Hospital, Central South University (Changsha, China). A total of 31 primary NPC tissues and 12 chronic pharyngitis nasal tissues were obtained from the Department of Pathology, Second Xiangya Hospital, between June 2013 and June 2014. The NPC patients included 22 males and 9 females, from 33 to 71 years old (mean, 52.2 years old). The 12 chronic pharyngitis nasal tissues were obtained from 12 additional patients, including 8 males and 4 females, from 49 to 63 years old (mean, 55.6 years old). Written informed consent was obtained from all participants in the present study. All patients had not received radiation therapy or chemotherapy prior to the surgery. Tissues were snap-frozen in liquid nitrogen and stored at -70°C before use.

Cell culture and transfection. NPC C666-1 cells and a normal nasopharyngeal epithelial cell line, NP69, were obtained from the Cell Bank of Central South University. Cells were cultured in RPMI-1640 medium (Thermo Fisher Scientific, Inc., Waltham, MA, USA) supplemented with 10% fetal bovine serum (Gibco; Thermo Fisher Scientific, Inc.), 100 IU/ml penicillin and 100 IU/ml streptomycin in a 37°C humidified atmosphere of 5% CO2. For transfection, C666-1 cells were grown to 70% confluence and transfected with 100 nM of pcDNA3.1-TGFβR2 open reading frame (ORF) plasmid (Amspring), miR-19a inhibitor (GeneCopoeia, Inc., Rockville, MD, USA) or negative control inhibitor (GeneCopoeia, Inc.) using Lipofectamine® 2000 (Thermo Fisher Scientific, Inc.), according to the manufacturer’s recommendations.

RNA extraction and reverse transcription-quantitative polymerase chain reaction (RT-qPCR). Total RNA was extracted from cells and tissues using TRIzol reagent (Thermo Fisher Scientific, Inc.), according to the manufacturer’s instructions. For miR expression analysis, RT-qPCR was performed using a PrimeScript® miRNA RT-PCR kit (Takara Biotechnology Co., Ltd., Dalian, China), according to the manufacturer’s instructions. U6 small nuclear RNA was used as the internal reference. Primer sequences for miRs and U6 were supplied by Fulgene (Guangzhou, China). For mRNA expression analysis, RT-qPCR was conducted using a standard SYBR-Green RT-PCR kit (Takara Biotechnology Co., Ltd.), in accordance with the manufacturer’s instructions. GAPDH was used as the internal reference. The specific primers used were as follows: TGFβR2, forward 5'-AAGATGCACCTTCAGCAAGCGAC-3' and reverse 5'-CTTATAGACCTCAGGAACGCGAC-3'; and GAPDH, forward 5'-CTGGGCTACACTGACCC-3' and reverse 5'-AAGTGGCTGCTGAGGCAATG-3'. The reaction conditions were 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. Relative expression was analyzed using the 2-ΔΔCq method (22). The experiments were repeated three times.

MTT assay. C666-1 cells (5x104 cells/well) were plated into a 96-well plate and cultured in DMEM with 10% FBS at 37°C with 5% CO2 for 24, 48, 72 or 96 h, respectively. Subsequently, 20 µl MTT (5 mg/ml; Thermo Fisher Scientific, Inc.) was added. Following incubation at 37°C for 4 h, 150 µl dimethyl sulfoxide was added. After incubation at room temperature for 10 min, the formazan production was detected by determining the optical density at 490 nm using a Multiskan FC enzyme immunoassay analyzer (Thermo Fisher Scientific, Inc.).

Invasion assay. A Transwell assay was performed to evaluate cell invasion capacity using Transwell chambers (BD Biosciences, Franklin Lakes, NJ, USA) pre-coated with Matrigel (BD Biosciences). A C666-1 cell suspension (1x105 cells/ml) was prepared in serum-free DMEM, 300 µl of which was added into the upper chamber, and 300 µl of DMEM with 10% FBS was added into the lower chamber. After 24 h of culture at 37°C, cells that did not invade through the membrane in the filter were wiped out using a cotton-tipped swab. The filter was subsequently fixed in 90% alcohol at room temperature for 10 min. Cells were stained with 0.1% crystal violet at room temperature for 30 min (Sigma-Aldrich; Merck KGaA, Darmstadt, Germany). The invading cells was observed under a light microscope (CX22; Olympus Corporation, Tokyo, Japan) and images were captured (magnification, x40).

Bioinformatics prediction. TargetScan (www.targetscan.org) was used to predict the potential targets of miR-19a, according to the manufacturer’s instructions.

Luciferase reporter assay. The fragment of TGFβR2 3'UTR containing the putative binding sites of miR-19a was amplified by PCR, which was then subcloned into the psiCHECK-2 vector (Promega Corp., Madison, WI, USA) downstream of the luciferase gene sequence, named TGFβR2 3'UTR wild type (WT). The 3'UTR of TGFβR2 containing mutant binding sites of miR-19a was generated using a Quick-Change Site-Directed Mutagenesis kit (Stratagene; Agilent Technologies, Inc., Santa Clara, CA, USA), in accordance with the manufacturer’s protocol. This sequence was also subcloned into the psiCHECK-2 vector downstream of the luciferase gene sequence, named TGFβR2 3'UTR mutant (MUT). C666-1 cells were co-transfected with 100 ng TGFβR2 3'UTR WT or TGFβR2 3'UTR MUT with 50 nM miR-19a mimic or scramble miR (miR-SCR) using Lipofectamine® 2000. Following transfection for 48 h, a dual-luciferase reporter assay system (Promega Corp.) was used to determine the activities of Renilla luciferase and firefly luciferase. Renilla luciferase activity was normalized to the firefly luciferase activity.

Western blotting. C666-1 cells were lysed in cold radiomunoprecipitation assay buffer (Thermo Fisher Scientific, Inc.). Protein concentration was determined using a BCA protein
assay kit (Pierce; Thermo Fisher Scientific, Inc.). Protein (50 µg) was separated by 10% SDS-PAGE and transferred to a polyvinylidene fluoride (PVDF) membrane (Thermo Fisher Scientific, Inc.). Subsequently, the membrane was blocked in 5% non-fat dried milk in Dulbecco's phosphate-buffered saline (DPBS; Thermo Fisher Scientific, Inc.) for 3 h at room temperature. The PVDF membrane was then incubated with rabbit anti-TGFβR2 monoclonal antibody (1:500; ab184948; Abcam, Cambridge, MA, USA), or rabbit anti-GAPDH monoclonal antibody (1:250; ab181602; Abcam) as an internal reference, for 3 h at room temperature. Subsequently, the membrane was washed with DPBS for 10 min and then incubated with mouse anti-rabbit secondary antibody (1:5,000; ab99697; Abcam) for 1 h at room temperature. Following washing with DPBS for 15 min, the immune complexes on the PVDF membrane were detected using an enhanced chemiluminescence western blotting kit (Pierce; Thermo Fisher Scientific, Inc.). Image-Pro Plus v. 6.0 software (Media Cybernetics, Inc., Rockville, MD, USA) was used to analyze relative protein expression levels, represented as the density ratio vs. GAPDH.

Statistical analysis. Data were presented as the mean ± standard deviation of at least three independent experiments. SPSS v. 17.0 software (SPSS, Inc., Chicago, IL, USA) was used to perform statistical analyses. Data was analyzed using a Student's t-test for comparisons between two groups and one-way analysis of variance with Tukey's post hoc test for comparisons between multiple groups. P<0.05 was considered to indicate a statistically significant difference.

Results

Members of the miR-17-92 cluster are upregulated in NPC tissues. Members of the miR-17-92 cluster have been demonstrated to have key roles in various types of human cancer (10-17). In the present study, RT-qPCR was conducted to determine the expression profiles of miR-17, -18a, -19a, -20a and -92a in NPC. A total of 31 primary NPC tissues and 12 chronic pharyngitis nasal (non-tumor) tissues were used. Results demonstrated that miR-17, -18a, -19a and -20a were significantly upregulated in NPC compared with non-tumor tissues (P=0.0018, 0.0009, 0.0058 and 0.0027, respectively), although no significant difference was observed in miR-92a expression (Fig. 1A-E).

miR-19a promotes C666-1 cell viability and invasion. The exact role of miR-19a in NPC is largely unclear. Therefore, the present study examined its expression in NPC C666-1 cells using RT-qPCR. A normal nasopharyngeal epithelial cell line, NP69, was used as a control. Results demonstrated that miR-19a was significantly upregulated in C666-1 cells compared with NP69 cells (P=0.0018, 0.0009, 0.0058 and 0.0027, respectively), although no significant difference was observed in miR-92a expression (Fig. 1A-E). To knockdown the miR-19a level, miR-19a inhibitor was used to transfect C666-1 cells. Following transfection with the miR-19a inhibitor, RT-qPCR results indicated that the miR-19a expression level was significantly reduced compared with the control (P<0.01; Fig. 2A). However, transfection with negative control inhibitor demonstrated no significant effect on the miR-19a level compared with the control group (Fig. 2B). Furthermore, MTT and Transwell assays were performed to
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Figure 2. Expression of miR-19a in NPC. (A) RT-qPCR was used to examine the expression of miR-19a in NPC C666-1 cells and nasopharyngeal epithelial cell line, NP69. (B) RT-qPCR was used to examine the expression of miR-19a in C666-1 cells transfected with NC inhibitor or miR-19a inhibitor, respectively. miR expression levels were calculated relative to U6. Following this, (C) MTT and (D) Transwell assays were used to determine cell viability and invasion capabilities, respectively. Non-transfected cells were used as the Control. Data are presented as the mean ± standard deviation. *P<0.01 vs. NP69; **P<0.01 vs. the Control. miR, microRNA; NPC, nasopharyngeal carcinoma; RT-qPCR, reverse transcription-quantitative polymerase chain reaction; NC, negative control; OD, optical density.

Examine cell viability and invasion abilities, respectively, of C666-1 cells with or without knockdown of miR-19a. As indicated in Fig. 2C, downregulation of miR-19a led to a significant decrease in cell viability compared with the control at 48, 72 and 96 h after transfection (P<0.01). Furthermore, invasion of C666-1 cells significantly decreased following transfection with miR-19a inhibitor compared with the control (P<0.01; Fig. 2D). Therefore, these findings suggested that miR-19a has a promoting role in NPC cell viability and invasion.

**TGFβR2 is a direct target of miR-19a.** TargetScan was used to investigate the putative target genes of miR-19a, and bioinformatics analysis demonstrated a complementary match between the miR-19a seed sequence and the 3'UTR of TGFβR2 (Fig. 3A). It was also demonstrated that knockdown of miR-19a led to a significant increase in the protein expression of TGFβR2, as well as a decrease in cell viability and invasion of C666-1 cells, it was speculated that TGFβR2 may be involved in these effects of miR-19a on C666-1 cells. To clarify this speculation, C666-1 cells were transfected with TGFβR2 ORF plasmid. Following transfection, the protein expression level of TGFβR2 was significantly increased in C666-1 cells compared with the control group (P<0.01; Fig. 4A). However, transfection with blank vector demonstrated no significant effect on the protein expression level of TGFβR2 (Fig. 4A). Furthermore, it was demonstrated that overexpression of TGFβR2 also significantly suppressed the viability (at 72 and 96 h after transfection) and invasion of C666-1 cells compared with the control, similar to the effects of miR-19a inhibition (P<0.01; Fig. 4B and C). A reversal experiment (co-transfection) was conducted to elucidate whether the suppressive effect of miR-19a knockdown on C666-1 cell viability and invasion was through upregulation of TGFβR2. Results demonstrated that transfection with TGFβR2 small interfering (si)RNA and miR-19a inhibitor significantly reversed the promoting effect of miR-19a knockdown on TGFβR2 protein expression in C666-1 cells (P<0.01; Fig. 5A). Furthermore, cell viability (at 48, 72 and 96 h after transfection) and invasion were significantly increased in the miR-19a inhibitor + TGFβR2 siRNA group compared with those in the miR-19a inhibitor only group (P<0.01; Fig. 5B and C). Taking these findings together, miR-19a may affect luciferase activity (Fig. 3E). These findings indicated that TGFβR2 is a target gene of miR-19a in C666-1 cells.
have a promoting role in NPC cell viability and invasion via inhibition of TGFβR2 expression.

Finally, the expression of TGFβR2 in NPC tissues from patients was examined. Results demonstrated that TGFβR2 was significantly downregulated in NPC tissues compared with non-tumor tissues (P=0.0011; Fig. 6A). Furthermore, the expression of TGFβR2 was also significantly decreased in C666-1 cells compared with NP69 cells (P<0.01; Fig. 6B). Therefore, the downregulation of TGFβR2 may be due to the upregulation of miR-19a in NPC.

Discussion

The miR-17-92 gene cluster has been demonstrated to have an oncogenic role in various types of human cancer by promoting cell cycle progression and tumorigenesis. For instance, miR-20a encoded by the miR-17-92 cluster increases the metastatic potential of osteosarcoma cells by targeting Fas (23). miR-17-5p, a member of the miR-17-92 cluster, promotes human breast cancer cell migration and invasion through suppression of HMG-box transcription factor 1 (24). A study by Chen et al (25) examined the expression of 270 human miR in 13 NPC samples and 9 adjacent normal tissues by using a stem-loop RT-qPCR method. They identified that the miR-17-92 cluster was among the 35 miR that were upregulated in NPC (21). In the present study, it was demonstrated that the majority of members of the miR-17-92 cluster that were investigated were upregulated in NPC samples, including miR-19a, -18a, -19a and -20a, suggesting that these members of the miR-17-92 cluster may have a promoting role in the development and progression of NPC.

Indeed, miR-18a, a member of the miR-17-92 cluster, is significantly upregulated in NPC tissues and cells, and the increased miR-18a level was correlated with the advanced stage of NPC, lymph node metastasis, EBV infection and a higher mortality rate from NPC (26). Furthermore, miR-18a

Figure 3. TGFβR2 is a target of miR-19a. (A) TargetScan software demonstrated that TGFβR2 was a putative target of miR-19a, and their targeting relationship was evolutionally conserved. (B) Western blotting and (C) reverse transcription-quantitative polymerase chain reaction were used to examine the protein and mRNA expression levels of TGFβR2 in C666-1 cells transfected with NC inhibitor or miR-19a inhibitor, respectively, relative to GAPDH. Non-transfected cells were used as the Control. (D) The fragment of TGFβR2 3'UTR containing the putative binding sites of miR-19a was amplified and subcloned into the psiCHECK-2 vector downstream of the luciferase gene sequence, named TGFβR2 3'UTR WT. The 3'UTR of TGFβR2 containing MUT binding sites of miR-19a was generated and also subcloned into the psiCHECK-2 vector downstream of the luciferase gene sequence, named TGFβR2 3'UTR MUT. (E) Luciferase activity was measured in C666-1 cells co-transfected with TGFβR2 3'UTR WT or TGFβR2 3'UTR MUT with miR-19a mimic or NC miR. Data are presented as the mean ± standard deviation. **P<0.01 vs. the Control. TGFβR2, transforming growth factor β receptor 2; miR, microRNA; NC, negative control; UTR, untranslated region; WT, wild type; MUT, mutant.
promotes the growth, migration and invasion of NPC cells in vitro and in vivo by directly targeting Dicer1, which caused the downregulation of the tumor-suppressive miR-200 family and miR-143 (26). Additionally, miR-18a may enhance the downregulation of the epithelial mesenchymal transition (EMT) marker, E-cadherin, and upregulate the oncogene, K-Ras, in NPC cells (26). In the present study, miR-19a was also significantly upregulated in NPC cells, consistent with the tissue data. C666-1 cells were then selected to further examine the exact function of miR-19a in NPC in vitro, and knockdown of miR-19a significantly inhibited the viability and invasion of NPC cells. These findings suggested that knockdown of miR-19a may have suppressive effects on NPC growth and metastasis. Except for NPC, miR-19a has been implicated in various types of human cancer, generally having an oncogenic role. For instance, miR-19a was demonstrated to be significantly upregulated in bladder cancer tissues, and high miR-19a level was correlated with more aggressive phenotypes of bladder cancer (17). Furthermore, miR-19a may promote cell growth of bladder cancer cells by targeting phosphatase and tensin homolog (17). A study by Huang et al (12) indicated that miR-19a was upregulated in colorectal cancer tissues and high expression of miR-19a was significantly associated with lymph node metastasis, most likely through promoting cancer cell invasion and EMT. On the contrary, however, there are also studies reporting that miR-19a may function as a tumor suppressor. For example, a study by Hao et al (27) demonstrated that the serum level of miR-19a was markedly reduced in multiple myeloma, and low miR-19a level was positively correlated with international staging system advancement, del (13q14) and 1q21 amplification, as well as decreased progression-free survival and overall survival. Additionally, a study by Yu et al (28) reported that miR-19a suppressed colon cancer cell migration and invasion. Accordingly, the dual role of miR-19a is tumor-specific.

Up to now, the regulatory mechanism of miR-19a in NPC has not previously been reported. The findings of the present study demonstrated that TGFβR2 was a direct target gene of miR-19a in NPC in vitro, and knockdown of miR-19a significantly inhibited the viability and invasion of NPC cells. These findings suggested that knockdown of miR-19a may have suppressive effects on NPC growth and metastasis. Except for NPC, miR-19a has been implicated in various types of human cancer, generally having an oncogenic role. For instance, miR-19a was demonstrated to be significantly upregulated in bladder cancer tissues, and high miR-19a level was correlated with more aggressive phenotypes of bladder cancer (17). Furthermore, miR-19a may promote cell growth of bladder cancer cells by targeting phosphatase and tensin homolog (17). A study by Huang et al (12) indicated that miR-19a was upregulated in colorectal cancer tissues and high expression of miR-19a was significantly associated with lymph node metastasis, most likely through promoting cancer cell invasion and EMT. On the contrary, however, there are also studies reporting that miR-19a may function as a tumor suppressor. For example, a study by Hao et al (27) demonstrated that the serum level of miR-19a was markedly reduced in multiple myeloma, and low miR-19a level was positively correlated with international staging system advancement, del (13q14) and 1q21 amplification, as well as decreased progression-free survival and overall survival. Additionally, a study by Yu et al (28) reported that miR-19a suppressed colon cancer cell migration and invasion. Accordingly, the dual role of miR-19a is tumor-specific.

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Figure 6. Expression of TGFβR2 in NPC. (A) RT-qPCR was used to examine the mRNA expression level of TGFβR2 in 31 primary NPC tissues and 12 chronic pharyngitis nasal tissues. Horizontal lines indicate the mean ± standard deviation. (B) RT-qPCR was used to examine the mRNA expression levels of TGFβR2 in NPC C666-1 cells and the nasopharyngeal epithelial cell line NP69. mRNA expression levels were calculated relative to GAPDH. Data are presented as the mean ± standard deviation. *P<0.01 vs. NP69. TGFβ, transforming growth factor β; NPC, nasopharyngeal carcinoma; RT-qPCR, reverse transcription-quantitative polymerase chain reaction.

Figure 5. Reversal experiment to determine the effect of miR-19a knockdown on TGFβR2. (A) Western blotting was used to examine the protein expression level of TGFβR2 in C666-1 cells transfected with miR-19a inhibitor, or co-transfected with miR-19a inhibitor and TGFβR2 siRNA, respectively, relative to GAPDH. Following this, (B) MTT and (C) Transwell assays were used to determine cell viability and invasion capabilities, respectively. Data are presented as the mean ± standard deviation. *P<0.01 vs. miR-19a inhibitor. miR, microRNA; TGFβR2, transforming growth factor β receptor 2; siRNA, small interfering RNA; OD, optical density.
NPC tissues compared with the non-tumor tissues, consistent with previous research (21). In addition, miR-93, a parologue of the miR-17-92 cluster, was also demonstrated to directly target TGFβR2 and promote cell proliferation, invasion and metastasis of NPC cells in vitro and in vivo (32). Therefore, the present study expands the understanding of the function and regulatory mechanism of the miR-17-92 cluster and its paralogues in NPC.

In conclusion, the present study demonstrated that the expression of miR-19a, as well as the other members of the miR-17-92 cluster, was significantly upregulated in NPC tissues compared with non-tumor tissues. Furthermore, the results indicated that miR-19a has a role in promoting the viability and invasion of NPC cells via directly targeting TGFβR2. Therefore, miR-19a may be a potential therapeutic target for the treatment of NPC in the future.

Acknowledgements

The present study was supported by the Project of Science and Technology Department of Hunan Province (grant no. 2011FJ6043) and the Project of Health Department of Hunan Province (grant no. B2011-017).

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