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MiR-30e-5p inhibits proliferation and metastasis of nasopharyngeal carcinoma cells by target-ing USP22

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Abstract. – OBJECTIVE: To investigate the effects of miR-30e-5p on the proliferation, invasion and migration of nasopharyngeal carcinoma (NPC) cells, as well as its underlying mechanism.

PATIENTS AND METHODS: We detected the expressions of miR-30e-5p in NPC tissues, adjacent normal tissues, NPC cells (5-8F cells) and control cells (293T cells) by quantitative reverse transcriptase-polymerase chain reaction (qRT-PCR). The target gene of miR-30e-5p was predicted by online software and ubiquitin-specific peptidase 22 (USP22) was screened out. Luciferase reporter gene assay was performed after NPC cells were co-transfected miR-30e-5p mimics or miR-30e-5p inhibitor and mutant-type or wild-type USP22, respectively. Expressions of miR-30e-5p and USP22 in 5-8F cells were detected by qRT-PCR and Western blotting. The proliferation of 5-8F cells was measured by MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide) assay, and the invasion and migration abilities were detected by transwell assay. The activation of the epithelial-mesenchymal transition (EMT) was analyzed by detecting expressions of EMT-associated proteins (E-cadherin and Vimentin) in NPC cells.

RESULTS: Expression level of miR-30e-5p was remarkably reduced, while USP22 expression was elevated in NPC tissues and cells compared with the controls. Molecular mechanism analysis con-firmed that miR-30e-5p could negatively regulate mRNA and protein levels of USP22 by binding to it specific sequence of 3'UTR. Subsequent experiments showed that USP22 knockdown resulting from up-regulation of miR-30e-5p could inhibit proliferation, invasion, migration, and EMT in 5-8F cells.

CONCLUSIONS: MiR-30e-5p was lowly expressed in NPC by targeting USP22, suggesting that miR-30e-5p could be used as a potential therapeutic target for NPC.

Key Words:
MiR-30e-5p, Nasopharyngeal carcinoma, Ubiquitin-specific peptidase 22, Epithelial-mesenchymal transition.

Introduction

Nasopharyngeal carcinoma (NPC) is a common malignancy in China, which is originated from nasopharyngeal epithelium. Its onset is closely associated with environmental factors, genetic susceptibility and EB virus infection. NPC is characterized by early distant metastasis and frequent local metastasis[1-5]. With the advanced cancer treatment methods, radiotherapy and chemotherapy had improved the remission rates of NPC to a higher degree. However, the 5-year survival rate of NPC was still hovering around 60%[6,7]. Besides, since the symptoms of NPC were dormant, approximately 30-40% of NPC patients were in the middle and advanced stages when diagnosed. A large majority of NPC patients suffer from distant metastasis and recurrence within several years after the comprehensive treatment, remarkably restricting the therapeutic effect and prognosis[8,9]. Hence, searching for an effective biomarker was of crucial significance in the diagnosis and treatment of NPC.

Micro-ribonucleic acids (miRNAs) are a type of endogenous non-coding RNA with a length of about 22 nucleotides, which exert key regulatory effects throughout the life. MiRNAs bind to specific sequences of target gene messenger RNAs (mRNAs) *via* base complementation, thus suppressing the translation of mRNAs so as to negatively regulate the gene expression[10].

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MiRNAs can also impede the gene expression *via* directly degrading target mRNAs. On the basis of predictions of computer software, a miRNA can play a regulatory role in several downstream target genes[11], indicating the potential role in transcriptional regulation and tumor treatment[12].

MiR-30e-5p is associated with a variety of malignant biological behaviors of tumor cells[13-15]. However, few reports focused on the function of miR-30e-5p in the occurrence and development of NPC and its related molecular mechanism. Therefore, this study aims at investigating the effects of miR-30e-5p on the proliferation and metastasis of NPC cells *in vitro* and explore its underlying mechanism.

Patients and Methods

Esophageal Squamous Cell Carcinoma (NPC) Tissues and Cells

50 NPC patients undergoing a surgical procedure at the First Affiliated Hospital of Anhui Medical University were enrolled. All patients were pathologically diagnosed as NPC and did not receive any preoperative chemotherapy or radiotherapy treatment. NPC tissues and adjacent normal tissues were kept in -80ºC refrigerator after surgical resection immediately. The adjacent normal tissues were concerned by biological biopsy not containing NPC cells. After all, Declaration of Helsinki should be mentioned and respected. This study was approved by the Ethics Committee of the First Affiliated Hospital of Anhui Medical University. Signed written informed consents were obtained from all participants before the study.

The human NPC cell lines (5-8F) and human embryonic kidney cell line (HEK-293T) were purchased from Guangzhou Longlong Biotech Co., Ltd., (Guangzhou, China). All the cells were resuscitated in 37°C water and then seeded in a cell culture flask. Cells were incubated with Roswell Park Memorial Institute 1640 (RPMI 1640) medium (HyClone, South Logan, UT, USA) complemented with 10% fetal bovine serum (FBS, Gibco, Rockville, MD, USA) in 5% CO_2 cell culture incubator at 37°C.

Luciferase Reporter Gene Assays

Bioinformatics websites (TargetScan, miRDB and microRNA) indicated that USP22 is a target gene for miR-30e-5p. The binding site sequences (wild-type and mutant-type sequences) were inserted into the pGL3-basic luciferase reporter vector (Promega, Madison, WI, USA). Cell co-transfection was performed according to the Dual-Luciferase Reporter Assay System (Promega, Madison, WI, USA). In brief, 5-8F cells were co-transfected with Luc-USP22-3'UTR-WT or Luc-USP22-3' UTR-Mut and miR-30e-5p mimics or negative control (NC). Results were normalized to Renilla luciferase activity. The experiment was repeated three times.

Transfection

MiR-30e-5p mimics and si-USP22 were synthesized and transfected to NPC cell lines to analyze the biological function of miR-30e-5p. Three groups were established to study the potential relevance between miR-30e-5p and 5-8F cells, namely NC group (negative control), miR-30e-5p mimics (5-8F cell transfected by miR-30e-5p mimics) and mimics + USP22 (5-8F cell transfected by miR-30e-5p mimics and si-USP22). All the regents and plasmids were purchased from RiboBio (Guangzhou, China). Cell transfection was performed using Lipofectamine RNAiMAX (Life Technologies, Gaithersburg, MD, USA) according to the manufacturer's instructions.

Quantitative Reverse Transcriptase-Polymerase Chain Reaction (qRT-PCR) Analysis

Total RNA was procured by TRIzol Reagent (Invitrogen, Carlsbad, CA, USA) in accordance with the manufacturer's protocol. SYBR green qPCR assay was used to measure USP22 expression which endogenous controlled by GAPDH (glyceraldehyde 3-phosphate dehydrogenase). TaKaRa Reverse Transcription Kit (PrimeScriptTM RT reagent Kit, Tokyo, Japan) was used to measure the level of miR-30e-5p expression, with U6 as the internal reference. The obtained threshold cycle (Ct) value was used to perform relative quantification of gene expression using the $2^{-\Delta\Delta Ct}$ method. Primer sequences used in this study were as follows: USP22, F: 5'-GGCCAATTGGCCTTGGAAATCTGCCCCTTAT-3', R: 5'- CGGTTAGGATTACGGTTAACTTGTTCA-3'; microRNA-30e-5p, F: 5'- GGCGTGTAAACATCCTTGACTG -3', R: 5'- GTGCAGGGTCCGAGGT -3'; U6: F: 5'-GCTTCGGCAGCACATATACTAAAAT-3', R: 5'-CGCTTCAGAATTTGCGTGTCAT-3'; GAPDH: F: 5'-CGCTCTCTGCTCCTCCTGTTC-3', R: 5'-ATCCGTTGACTCCGACCTTCAC-3'.

Western Blot Analysis

After 48 h of transfection, total cellular protein was extracted and the protein concentration was determined quantitatively using the bicinchoninic acid (BCA) protein concentration assay kit (Pierce, Rockford, IL, USA), and stored at -20°C until use. 20 μg total protein was loaded and subjected to 10% sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) gel (Sigma-Aldrich, St. Louis, MO, USA), and then transferred onto polyvinylidene difluoride (PVDF) membranes (Roche, Basel, Switzerland). Membranes were blocked with 5% milk at room temperature for 1 h. Then, membranes were incubated with primary antibodies of USP22 and β-actin [diluted at 1:1000, Cell Signaling Technology (CST) Inc. Danvers, MA, USA] at 4°C overnight. The corresponding secondary antibodies (CST, Inc. Danvers, MA, USA) were used for incubation at room temperature for 30 min the next day. The expression level of USP22 was determined by horseradish peroxidase-ECL (enhanced chemiluminescence) method and β-actin was selected as an internal reference (Thermo Fisher Scientific, Waltham, MA, USA).

Cell Proliferation

When cells grew to the logarithmic growth phase, the transfected cells were collected and seeded into 96-well plates at a density of 5×10^3/well. Cell viability was determined *via* MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide) colorimetric assay (Sigma, St. Louis, MO, USA). At the 24 h, 48 h, 72 h, 96 h, and 120 h after incubation, 20 μL of 0.5 mg/mL MTT solution was added and incubated at 37°C for 4 h, respectively. The reaction was terminated by adding 150 μL of dimethyl sulfoxide (DMSO) (Sigma-Aldrich, St. Louis, MO, USA). An enzyme-labeled spectrophotometer was set at the wavelength of 450 nm and the optical density (OD) of each well was measured followed by zero setting using blank wells. The average value was plotted to draw the cell proliferation curve.

Cell Invasion and Migration Assays

After 48 h of transfection, cell migration and invasion abilities were measured using a transwell chamber (Corning, Corning, NY, USA) with a pore size of 8 μm. Matrigel (BD, Franklin Lakes, NJ, USA) at a concentration of 1:9 was paved in the upper chamber for detection of invasion ability. Subsequently, the upper chamber was added with 250 μL serum-free medium, while the lower chamber was added with 700 μL medium containing 10% FBS. Cells (5×10^4/well) were then added to the upper chamber and placed in an incubator for cell culture. 24 hours later, the chamber was removed, and the remaining cells in the upper chamber were gently wiped off with a cotton swab. The lower chamber cells were then fixed with paraformaldehyde and stained with crystal violet. Finally, 5 fields of view were randomly selected under an inverted microscope (magnification ×200) for counting, and the average was calculated.

Statistical Analysis

Statistical Product and Service Solutions SPSS 16.0 software (SPSS Inc., Chicago, IL, USA) was used for statistical analysis. The results were expressed by ($\bar{x} \pm s$). The *t*-test was used for the intergroup differences. $p < 0.05$ suggested that the difference was statistically significant.

Results

MiR-30e-5p Expression was Reduced both in NPC Tissues and Cells, while USP22 Expression was Increased in NPC Cells

To elucidate the effect of miR-30e-5p on NPC development, qRT-PCR assay was performed to detect miR-30e-5p expression in NPC tissues and the adjacent normal tissues. As shown in Figure 1A, we found that miR-30e-5p expression was significantly reduced in NPC tissues compared with that of adjacent tissues. Similar results were obtained in detecting cellular level of miR-30e-5p (Figure 1B). However, USP22 expression in 293T cells was significantly higher than that in the NPC cells (Figure 1C). We thought miR-30e-5p and USP22 might be involved in the progression of NPC.

USP22 was the Direct Target of miR-30e-5p

Target genes of miR-30e-5p were predicted in three publicly available algorithms, TargetScan, miRDB and microRNA. As shown in Figure 2A, USP22 was considered as a supposed target of miR-30e-5p. Thus, we established luciferase reporter vectors containing the wild or mutant-type USP22. Luciferase activity was decreased in cells co-transfected with miR-30e-5p and wide-type

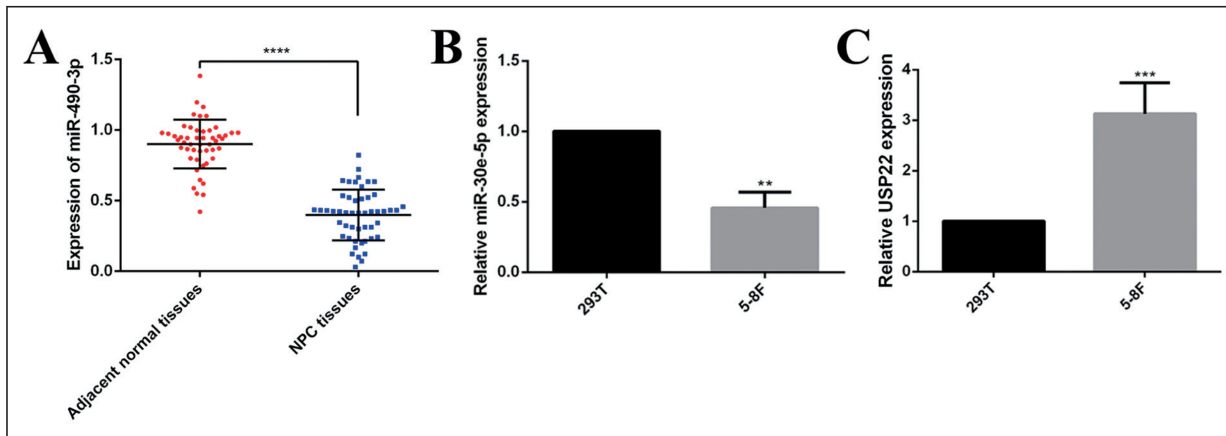

Figure 1. The expressions of miR-30e-5p and USP22 in nasopharyngeal carcinoma (NPC) tissue samples and cells comparing with corresponding adjacent normal tissues and normal human embryonic kidney cells (293T). ***A,*** Difference in the expression of miR-30e-5p between NPC tissues and corresponding adjacent normal tissues (****$p < 0.001$ compared with adjacent normal tissue). ***B,*** The expression of miR-30e-5p in human NPC cells (5-8F) and normal human embryonic kidney cells (293T) (**$p < 0.01$ compared with 293T). ***C,*** The expression of USP22 in 5-8F cells and 293Ts (***$p < 0.001$ compared to 293T).

USP22. However, luciferase activity did not alter after co-transfection of miR-30e-5p and mutant-type USP22 (Figure 2B), suggesting that USP22 could be regulated by miR-30e-5p.

MiR-30e-5p Decreased the Expression Level of USP22

Three groups were established (miR-NC group, miR-30e-5p mimics group and the mimics

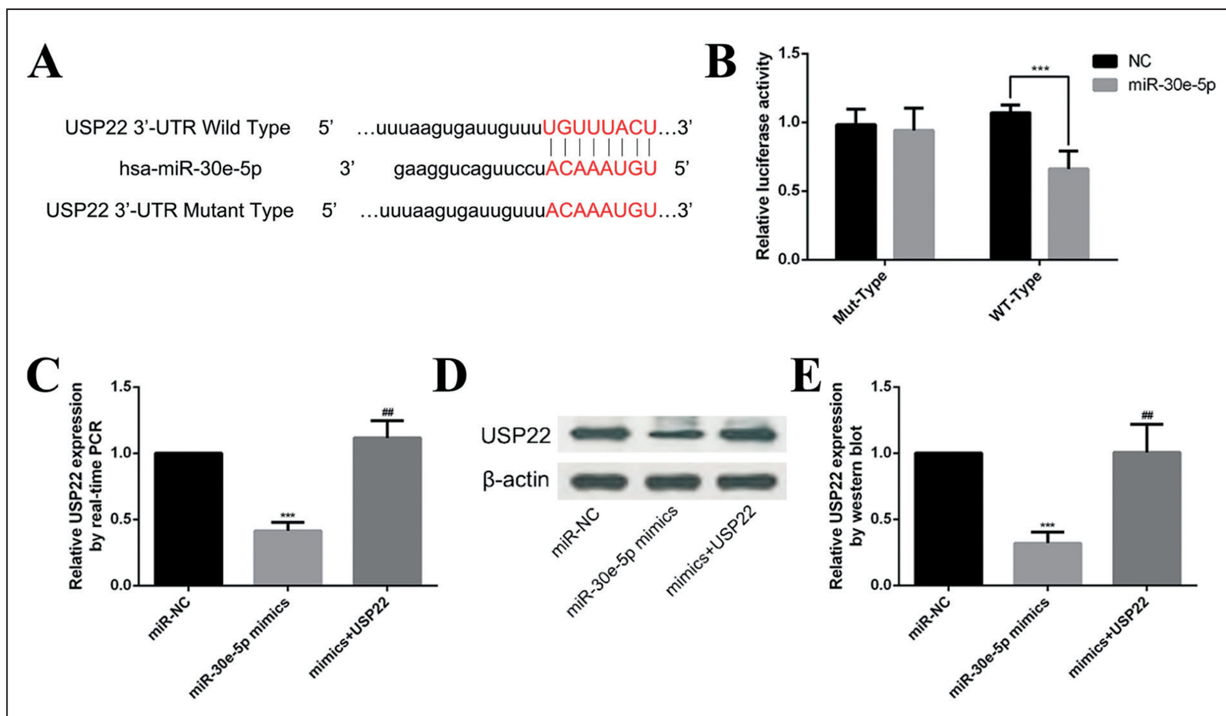

Figure 2. USP22 is a direct and functional target of miR-30e-5p. 5-8F cells were transfected with miR-30e-5p mimics or inhibitor, respectively. ***A,*** Diagram of putative miR-30e-5p binding sites of USP22. ***B,*** Relative activities of luciferase reporters (***$p < 0.001$). ***C-E,*** MiR-30e-5p decreases the expression level of USP22. Data were presented as means ± standard deviations (***$p < 0.001$ *vs.* NC group; $^{##}p < 0.01$ *vs.* mimics group).

+ USP22 group) in 5-8F cells. The transfection efficiency was verified by qRT-PCR. The results showed that miR-30e-5p expression in 5-8F cells was up-regulated by (2.74 ± 0.51) folds, confirming that transfection of miR-30e-5p mimics successfully up-regulated the miR-30e-5p expression *in vitro* (Figure 3A).

As we expected, after transfection of miR-30e-5p mimics, the expression level of USP22 was found decreased in 5-8F cells. Again, the data confirmed the regulatory effect of miR-30e-5p on USP22 expression (Figure 2C-2E).

MiR-30e-5p Suppressed Proliferation of NPC Cells

To verify the effect of miR-30e-5p on the proliferation of NPC cells, MTT assay was performed to detect cell proliferation after altering miR-30e-5p expression. The results suggested that the cell proliferation rates of 5-8F cell were reduced by miR-30e-5p mimics transfection. In contrast, cell proliferation of 5-8F cells was found increased in the mimics+ USP22 group (Figure 3B).

MiR-30e-5p Inhibited Invasion and Migration of NPC Cells

Migration and invasion are the two major factors leading to cancer cell metastasis. Transwell experiments showed that the invasion of 5-8F cells was restricted after transfection of miR-30e-5p mimics. Meanwhile, cell migration was inhibited after miR-30e-5p overexpression. Therefore, we detecteed that miR-30e-5p could inhibit migration and invasion of NPC cells (Figure 4).

MiR-30e-5p Inhibited Epithelial-Mesenchymal Transition (EMT)

EMT plays an important role in the metastasis processes of tumor cells. However, whether miR-30e-5p exerted an effect on the EMT of NPC cells has not yet been reported. Therefore, Western blotting was employed to detect the expressions of EMT-related markers in 5-8F cells. The results manifested that the expression of E-cadherin (the epithelial marker) was notably increased, while the expression of Vimentin (the mesenchymal marker) was significantly decreased after overexpression of miR-30e-5p. The above results suggested the inhibitory effect of miR-30e-5p on EMT (Figure 5).

Discussion

Increasing evidence has revealed that differentially expressed miRNAs were related to the occurrence of various diseases, including tumors. MiRNAs exert vital effects on the pathogenetic process of multiple tumors, including NPC, which have been well concerned[16-18]. MiRNAs can regulate tumor cell proliferation, migration and survival as tumor-suppressor genes or oncogenes[10,19]. Furthermore, miRNAs

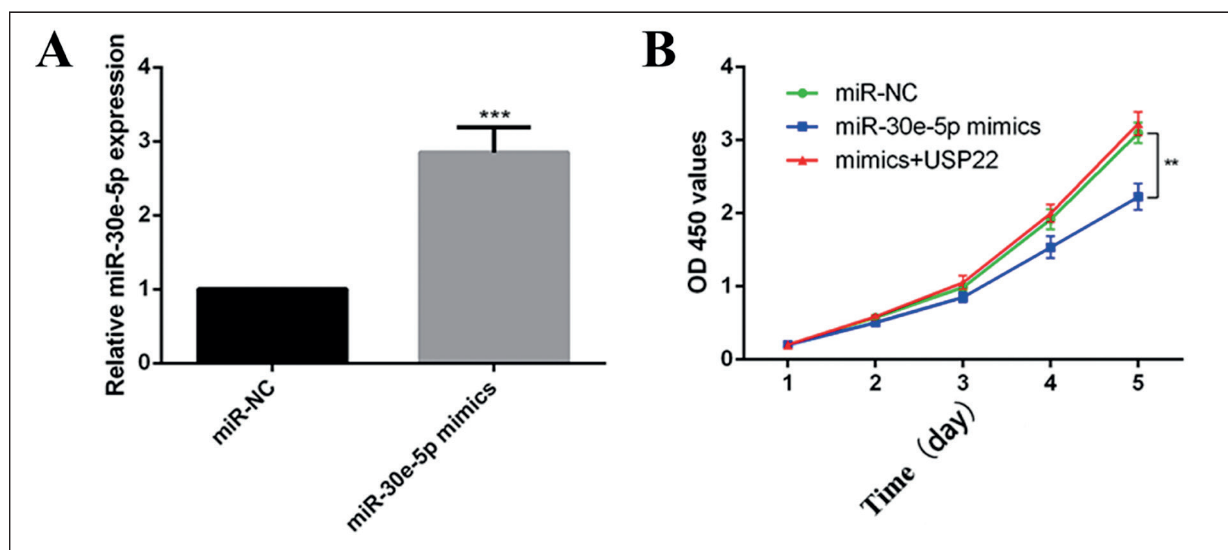

Figure 3. ***A,*** The miR-30e-5p expression after transfection of miR-30e-5p mimics (***$p < 0.001$). ***B,*** The proliferation of NPC cells transfected with miR-30e-5p mimics or inhibitor was measured by MTT assay (**$p < 0.01$).

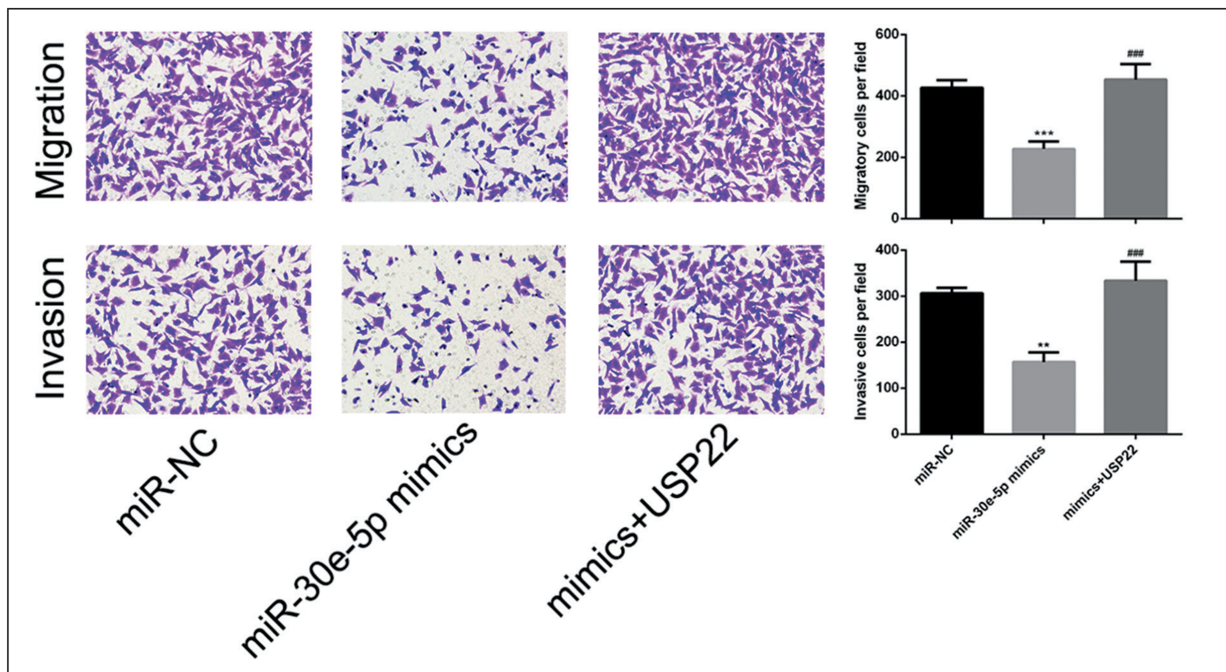

Figure 4. MiR-30e-5p/USP22 axis inhibits the invasion and migration of NPC cells. USP22 overexpression attenuated the promotive effect of miR-30e-5p on invasion and migration of NPC cells (** $p < 0.01$, *** $p < 0.001$ *vs.* NC group; ### $p < 0.001$ *vs.* mimics group).

serve as potential biomarkers for disease status assessment, treatment guidance and prognosis prediction in a variety of tumors. In this experiment, the expression level of miR-30e-5p in human NPC samples and cells was analyzed by qRT-PCR. It was discovered that miR-30e-5p was evidently lower expressed in both NPC tissues and cells compared with those in the control group.

MiRNAs primarily functioned *via* the regulation on the target gene expression. Our study found that USP22 was a potential target gene of

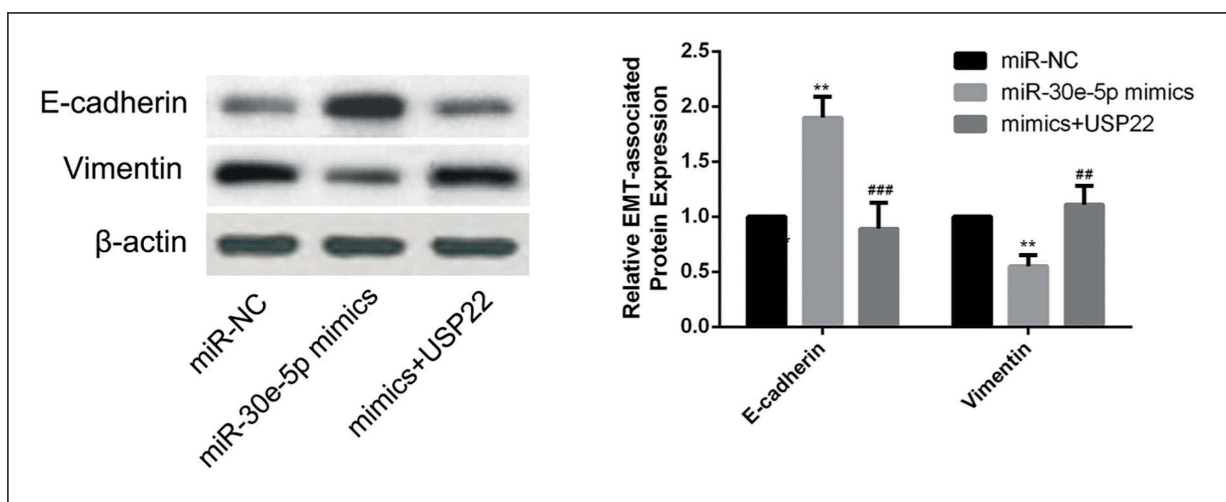

Figure 5. The decreased expression of USP22 resulting from up-regulation of miR-30e-5p could reduce the epithelial-mesenchymal transition (EMT) of NPC cells. The expression of USP22 and EMT-related markers (E-cadherin and Vimentin) after transfection with miR-30e-5p mimics or si-USP22 were detected by Western blotting. Data were presented as means ± standard deviations (** $p < 0.01$ *vs.* NC group; ## $p < 0.01$, ### $p < 0.001$ *vs.* mimics group).

miR-30e-5p predicted by online software. USP22 is a newly found member of the ubiquitin hydrolase family that can act on ubiquitinated protein substrates in the ubiquitin-proteasome system. It exerts extensive biological functions by blocking the linkage between the ubiquitin chain and the substrate protein[20,21]. Relative studies have proved that USP22 is associated with malignancy, proliferation, metastasis and prognosis of hepatocellular carcinoma[21], non-small-cell lung cancer (NSCLC)[22], papillary thyroid carcinoma[23], cervical cancer[24], and other various tumors. Meanwhile, it has been showed that USP22 stimulated EMT by regulating EMT-related markers[25,26]. In the present study, USP22 expression in 5-8F cells was down-regulated after miR-30e-5p overexpression Subsequently, the correlation between miR-30e-5p and USP22 was further verified *via* the dual-luciferase assay.

Tumorigenesis is the process in which normal cells slip the leash of the intrinsic regulatory mechanism of cells, thus finally forming an uncontrolled, persistently colonized population. Abnormal proliferation is a crucial performance in the process of cancerization. Generally speaking, malignant tumors such as NPC were mainly biologically characterized by local invasion and distant metastasis. In this work, we detected proliferation, migration, and invasion abilities after altering miR-30e-5p expression in 5-8F cells. Overexpression of miR-30e-5p remarkably inhibited invasion and metastasis of 5-8F cells. Nevertheless, overexpressed USP22 reversed the aforementioned effects of miR-30e-5p on cell invasion and metastasis, suggesting that miR-30e-5p suppresses the proliferation, invasion and metastasis of 5-8F cells *via* USP22.

EMT is a process where the connection between normal epithelial polar cells is absent, which is ultimately characterized by interstitium and intimately associated with the embryonic development, tissue remodeling, tumor metastasis, etc.[27]. EMT can induce the occurrence of invasion and metastasis of malignant tumors and exert vital effects on numerous cancer-related events[28-30]. EMT is mainly featured with the reduced expression of the cell adhesion molecule (E-cadherin) and the increased expression of vimentin[31]. E-cadherin deficiency allows tumor cells to be easily discriminated and escaped from primary foci for invasion and metastasis. The increased vimentin expression results in the cytoskeletal structure change, which is manifested as the mesenchymal phenotype, decreased adhesion ability and increased mobility, making cells more aggressive[32,33]. As expected, after miR-30e-5p was overexpressed in 5-8F cells, the expression of E-cadherin declined while vimentin expression was increased. It is indicated that the occurrence of malignant transformation in NPC was accompanied by the occurrence of EMT, thus significantly enhancing the mobility and invasion abilities of tumor cells. Correspondingly, USP22 overexpression evidently inhibited the expression of vimentin and increased the expression of E-cadherin, indicating that the tumor-suppressing effect of miR-30e-5p was closely correlated with USP22.

Conclusions

We showed that the miR-30e-5p expression was correlated with the NPC cell proliferation, migration, invasion, and malignancy. Up-regulated miR-30e-5p expression could enhance the biological functions of NPC cells through down-regulating USP22 expression. Our study showed that miR-30e-5p could alleviate NPC development, indicating that miR-30e-5p is expected to be a novel target for NPC treatment.

Conflict of Interest

The Authors declare that they have no conflict of interests.

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