Mechanism of lncRNA FOXD3-AS1/miR-338-3p in the malignant progression of nasopharyngeal carcinoma through ceRNA

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ABSTRACT

This work aimed to analyze the impact of lncRNA FOXD3-AS1 (F-A) on the regulation of miR-338-3p (M) on the proliferation (Pro), migration (Mig), and invasion (Inv) of nasopharyngeal carcinoma (NPC) cells. F-A and M levels in human normal nasopharyngeal (NNP) NP69 cells and NPC CNE1 and CNE2 cells were detected by real-time quantitative PCR. After transfection or cotransfection with F-A shRNA, miR-338-3p mimic (MM), and miR-338-3p inhibitor (MI), cell Pro, Mig, and Inv (PMI) were detected using CCK-8, scratch healing, and Transwell chamber experiments, respectively. As a result, relative to NP69 cells, F-A was upregulated in CNE1 and CNE2 cells, while M was downregulated ($P<0.01$). F-A in CNE1 and CNE2 cells was downregulated and M was upregulated relative to shF-A ($P<0.01$). Furthermore, shF-A and MM groups demonstrated drastically reduced cell proliferative activity, Mig, and Inv versus CNE1/CNE2 groups. The cell proliferative activity, Mig, and Inv number of the MI group increased substantially ($P<0.01$). The shF-A+MM group exhibited markedly reduced cell proliferative activity, Mig, and cell number of Inv relative to those of CNE1/CNE2 ($P<0.01$). Moreover, the shF-A+MI group exhibited greatly increased cell proliferative activity, Mig, and cell number of Inv versus the shF-A+MM group ($P<0.01$). In short, lncRNA F-A level was abnormally upregulated, and that of M was downregulated in NPC. Interfering with lncRNA F-A level can upregulate M expression. Silencing of lncRNA F-A can inhibit PMI of NPC cells.

Introduction

Nasopharyngeal carcinoma (NPC) is a type of squamous cell carcinoma (SCC) originating from nasopharyngeal mucosal epithelial cells, and it is prevalent in South China and Southeast Asia (1). At present, radiotherapy or radiotherapy combined with adjuvant therapy is an effective methodology for the therapy of NPC, for which the prognosis of patients with NPC has been notably enhanced, and yet the prognosis of some patients is still insufficient (2). The five-year overall survival rate of patients with locally advanced NPC is 68–80%, and distant metastasis is the major factor for failed treatment of NPC (3). Age, tumor size, and molecular markers are confirmed to be closely related to NPC patient prognosis (4,5). Hence, demonstrating the molecular mechanisms involved in the pathogenesis of NPC is very important for the prevention and therapy of NPC.

It has been confirmed that the abnormal lncRNAs and miRNAs participate in the occurrence of tumors. WTAP mediates N6-methyladenosine modification of the lncRNA DIAPH1-AS1 and participates in the growth and metastasis of NPC (6). Knocking out lncRNA PVT1 can inhibit the proliferation (Pro) of NPC cells and tumorigenesis and exert the effects of NPC radiosensitivity by activating KAT2A acetyltransferase and stabilizing HIF-1α (7). High lncRNA HCG11 level is found in NPC tissues and has a positive correlation with tumor staging, lymph node metastasis, and poor prognosis. Knockout of lncRNA HCG11 can inhibit the Pro and migration (Mig) of NPC cells (8). The disease-free survival rate of NPC patients with high lncRNA FOXP4-AS1 levels is even worse (9). Zhang et al. (10) found that knocking out lncRNA FOXD3-AS1 (F-A) in NPC cells could inhibit cell Pro and promote apoptosis. Nevertheless, the mechanism of F-A in the malignant progression of NPC needs to be further verified.

In this work, F-A and miR-338-3p (M) levels in human normal nasopharyngeal (NNP) cells and NPC cells were detected, to demonstrate the influences of F-A and M levels on the Pro, Mig, and invasion (Inv) of NPC cells and provide a reference for understanding the pathogenesis of NPC and exploring the role of F-A in malignant progression of NPC.

Materials and Methods

Experimental materials

The human NNP cell line NP69, highly differentiated SCC NPC cell line CNE1, and poorly differentiated SCC NPC cell line CNE2 were purchased from the American ATCC Center. RPMI-1640 medium, fetal bovine serum (FBS), penicillin-streptomycin, trypsin, and TRIzol rea-

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gents were purchased from Invitrogen, USA. The cDNA reverse transcription kit and real-time fluorescent quantitative PCR kit were purchased from Beijing Baori Medical Biotechnology Co., Ltd.

Cell culture

The NP69, CNE1, and CNE2 cell lines were cultured in RPMI-1640 medium (10%FBS and 1%penicillin–streptomycin) at 37°C, 5% CO_2, and saturated humidity. It was routinely digested with 0.25% trypsin for subsequent generations. Cells in the logarithmic phase were subjected to subsequent experiments.

Vector construction and cell transfection

The design and synthesis of F-A shRNA and shRNA negative controls, shF-A: 5'-GGAAATATAGTGAATA-3', were performed by Thermo Fisher Scientific, USA. Shanghai Sangon Biotech designed and synthesized the negative controls formiR-338-3p mimic (MM), miR-338-3p inhibitor (MI), and M. Subsequently, Lipofectamine 2000 reagent was applied to transfect shF-A, shNC, MM, MI, and miR-NC and the cotransfection of shF-A, MM, MI, and miR-NC. The cells were collected after transfection for subsequent experiments.

Real-time fluorescent quantitative PCR

The total RNA in the cells was extracted with TRIzol reagent, which was reverse transcribed into cDNA regarding the Prime Script™ RT reagent kit (Perfect Real Time). The cDNA was taken as the template to quantitatively detect the target gene level by referring to the instructions of TB Green® Premix EX Taq™ II (Tli RNase H Plus). F-A: (upstream) 5'-GGAGTGCAAGGCCGCCTAGT-3', (downstream) 5'-CTCGGAACTGGTCCCCTCGT-3'. M: (upstream) 5'-GCCGATCCAGCATCAGTG-3', (downstream) 5'-CAGTGCAGGGTCCGAGGT-3'. M: (upstream) 5'-GCACCGTCAAGGCTGAGAAC-3', (downstream) 5'-GCACCGTCAAGGCTGAGAAC-3'. In this work, GAPDH was taken as the endogenous reference gene, and the results of real-time fluorescent quantitative PCR were normalized. The relative level of the target gene was calculated using $2^{-\Delta\Delta Ct}$. All experiments were repeated thrice to generate the mean value.

CCK-8 test

Cells in the logarithmic phase were subjected to the experiment. Cells at 48 h after transfection were digested with 0.25% trypsin. After the cells were counted, a 2×10^4cells/mL suspension was concocted, and 100 μL per well was inoculated into 96-well plates. After 12 h, 24 h, 48 h, and 96 h of cell culture, 10 μL CCK-8 reagent was applied in each well and incubated in a cell incubator for 1 h. Subsequently, the absorbance of each well was detected at a wavelength of 450 nm employing a microplate reader. Experiments were repeated thrice to obtain the mean value.

Scratch healing experiment

The cells were digested at 48 h after transfection with 0.25% trypsin and inoculated into 6-well plates at 1×10^6 per well. After overnight culture, the medium was changed, and when cells grew to 90% confluence by adherent growth, 10 μL of the sterile spear was utilized to score the cells in a 6-well plate. The floating cells were gently washed away with phosphate buffer and photographed after 0 h and 48 h to observe the gapped giant deer and calculate the scratch healing rate. All experiments were repeated thrice to generate the mean value.

Transwell Inv cell experiment

The Matrigel matrix gel hydrated overnight was diluted at a ratio of 1:4, and 40 μL of diluted Matrigel matrix gel was supplemented into a precooled Transwell chamber and incubated for 30 min. Cells transfected for 48 h were digested with 0.25% trypsin and configured into a 5×10^5/mL cell suspension using a serum-free medium. Then, 200 μL suspension was added into the upper chamber of the Transwell, and 700 μL of RPMI-1640 medium with 20%FBS was supplemented into the lower chamber for routine culture for 24 h. Cells below the membrane were mixed with 1% paraformaldehyde and subsequently stained with 0.2% crystal violet for 15 min. Ten fields randomly selected by the inverted microscope were counted to calculate the cells under the membrane. All experiments were repeated thrice to obtain the mean value.

Statistical analysis

Employing SPSS 22.0, all data were recorded as the mean±standard deviation. *T*-test was adopted for comparisons between two groups, and one-way ANOVA for comparisons among multiple groups. $P<0.05$ implied that the difference was statistically considerable. *$P<0.05$, **$P<0.01$, ***$P<0.001$.

Results

LncRNA F-A and M in human NNP cells and NPC cells

F-A and M in NP69, CNE1, and CNE2 cells were compared (Figure 1). Relative to those in the human NNP cell line NP69, F-A in the NPC cells CNE1 and CNE2 increased considerably ($P<0.01$), and M in NPC cells CNE1 and CNE2 were drastically downregulated versus those of human NNP cell NP69 ($P<0.01$).

Transfection efficiency detection of lncRNA F-A and M

The impact of shF-A on F-A and M in CNE1 and CNE2 cells was verified (Figures 2A and 2B). In contrast to those in the shNC group, F-A in CNE1 and CNE2 cells in the shF-A group was downregulated substantially ($P<0.01$), and M in CNE1 and CNE2 cells in the shF-A group was markedly increased ($P<0.01$).
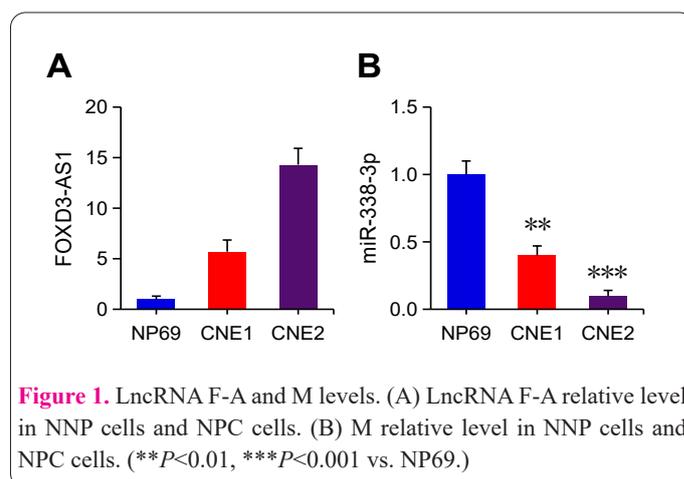

Figure 1. LncRNA F-A and M levels. (A) LncRNA F-A relative level in NNP cells and NPC cells. (B) M relative level in NNP cells and NPC cells. (**$P<0.01$, ***$P<0.001$ vs. NP69.)
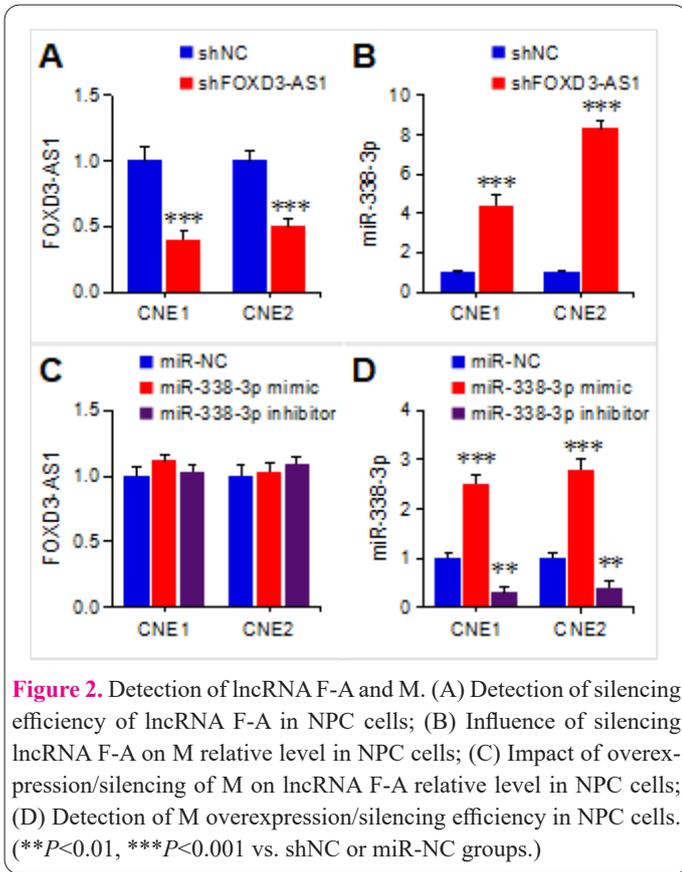
Figure 2. Detection of lncRNA F-A and M. (A) Detection of silencing efficiency of lncRNA F-A in NPC cells; (B) Influence of silencing lncRNA F-A on M relative level in NPC cells; (C) Impact of overexpression/silencing of M on lncRNA F-A relative level in NPC cells; (D) Detection of M overexpression/silencing efficiency in NPC cells. (**$P<0.01$, ***$P<0.001$ vs. shNC or miR-NC groups.)

Furthermore, the influence of MM and MI on F-A and M in CNE1 and CNE2 cells was analyzed (Figures 2C and 2D). It turned out that relative to the miR-NC group, F-A in CNE1 and CNE2 cells in MM and MI groups demonstrated inconsiderable differences ($P>0.05$), while M in CNE1 and CNE2 cells of the MM group increased dramatically ($P<0.001$). Nevertheless, in the MI group, the M in CNE1 and CNE2 cells were considerably downregulated ($P<0.01$).

Impact of lncRNA F-A/M on the Pro of NPC cells

The impact of shF-A, MM, and MI on the Pro of CNE1 and CNE2 cells was detected (Figure 3). With prolonged culture time, the Pro activities of CNE1 and CNE2 cells in each group gradually increased. Relative to CNE1 and CNE2 groups, the cell proliferative activity suggested no great differences between shNC and miR-NC groups ($P>0.05$). In addition, the cell proliferative activities of shF-A and MM groups were reduced versus those of CNE1 and CNE2 groups ($P<0.001$), and the cell proliferative activity of the MI group was markedly increased ($P<0.001$).
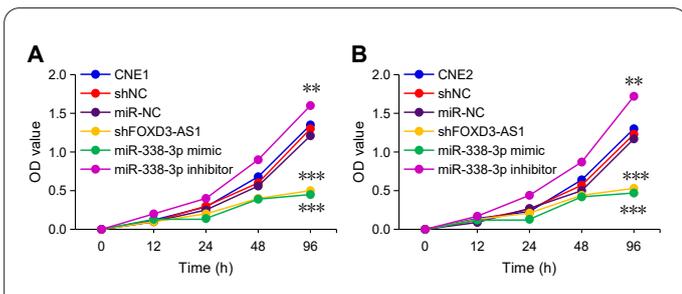
Afterward, the influence of lncRNA F-A/M on the Pro

Figure 3. Impacts of lncRNA F-A and M on the Proactivity of NPC cells. (A) Detection of Proactivity of CNE 1 NPC cells; (B) CNE2 NPC cell proliferative activity test. (**$P<0.01$, ***$P<0.001$ vs. CNE1 or CNE2 groups.)

of CNE1 and CNE2 cells through ceRNA was detected (Figure 4). It turned out that the cell proliferative activities in the shF-A+miR-NC and shF-A+MM groups were drastically reduced relative to CNE1 and CNE2 groups ($P<0.001$). In addition, in contrast to the CNE1 and CNE2 groups, the cell proliferative activity of the shF-A+MI group was substantially reduced ($P<0.05$). However, the cell proliferative activity in the shF-A+MI group was superior to that in shF-A+miR-NC and shF-A+MM groups.

Influence of lncRNA F-A/M on NPC cell Mig

The impacts of shF-A, MM, and MI on the Mig of CNE1 and CNE2 cells were detected (Figure 5). The cell Mig between the CNE1/CNE2 groups and the shNC and miR-NC groups indicated slight differences ($P>0.05$). Moreover, the cell Mig of the shF-A group, MM group, and MI group was reduced in contrast to CNE1 and CNE2 groups ($P<0.001$). In comparison with shF-A and the MM groups, the cell Mig of the MI group was greatly reduced ($P<0.001$).

Further, the influence of lncRNA F-A/M on the cell Mig of CNE1 and CNE2 cells through ceRNA was illustrated in Figure 6. In comparison with the CNE1 and CNE2 groups, the cell Mig of the shF-A+miR-NC and shF-A+MM groups was greatly reduced ($P<0.001$). In comparison with the shF-A+miR-NC group, the cell Mig of the shF-A+MM group was reduced ($P<0.01$). Additionally, the cell Mig of the shF-A+MI group was markedly increased in contrast to shF-A+miR-NC and shF-A+MM groups ($P<0.001$).
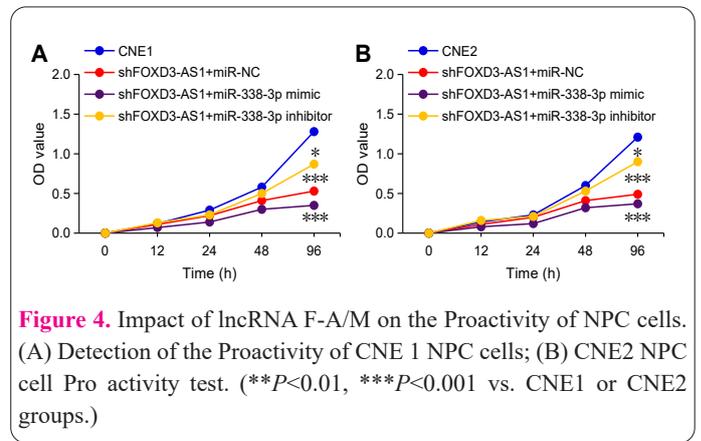

Figure 4. Impact of lncRNA F-A/M on the Proactivity of NPC cells. (A) Detection of the Proactivity of CNE 1 NPC cells; (B) CNE2 NPC cell Pro activity test. (**$P<0.01$, ***$P<0.001$ vs. CNE1 or CNE2 groups.)
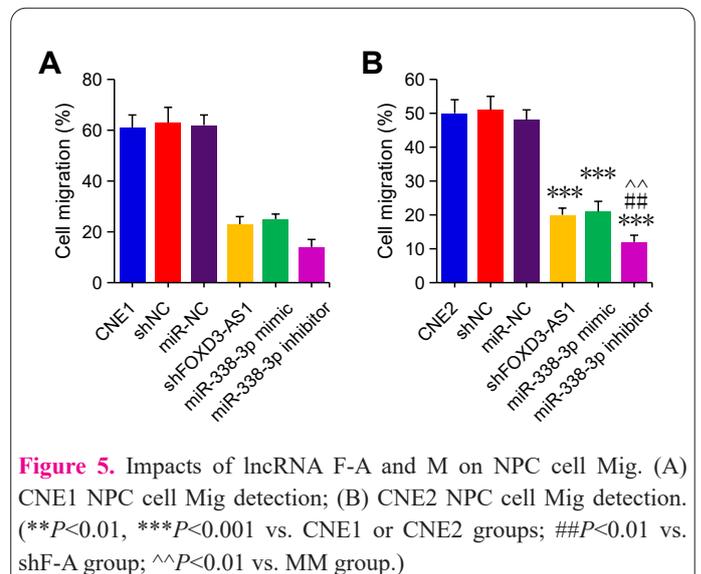

Figure 5. Impacts of lncRNA F-A and M on NPC cell Mig. (A) CNE1 NPC cell Mig detection; (B) CNE2 NPC cell Mig detection. (**$P<0.01$, ***$P<0.001$ vs. CNE1 or CNE2 groups; ##$P<0.01$ vs. shF-A group; ^^$P<0.01$ vs. MM group.)
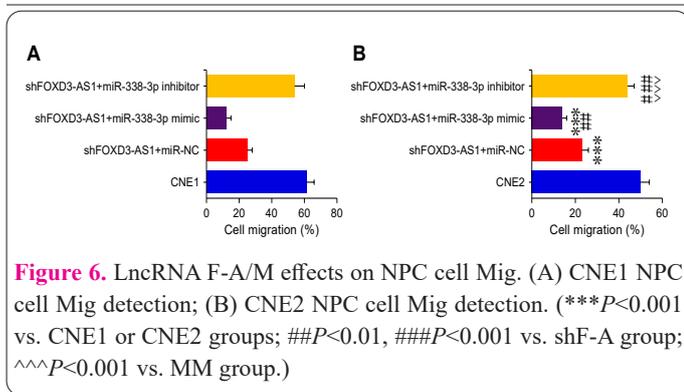
Figure 6. LncRNA F-A/M effects on NPC cell Mig. (A) CNE1 NPC cell Mig detection; (B) CNE2 NPC cell Mig detection. (***$P<0.001$ vs. CNE1 or CNE2 groups; ##$P<0.01$, ###$P<0.001$ vs. shF-A group; ^^^$P<0.001$ vs. MM group.)

Figure 7. Impacts of lncRNA F-A and M on NPC cell Inv. (A) CNE1 NPC cell Inv detection; (B) CNE2 NPC cell Inv detection. (**$P<0.01$, ***$P<0.001$ vs. CNE1 or CNE2 groups; ##$P<0.01$ vs. shF-A group; ^^$P<0.01$ vs. MM group.)

Figure 8. Impacts of lncRNA F-A/M on NPC cell Inv. (A) CNE1 NPC cell Inv detection; (B) CNE2 NPC cell Inv detection. (**$P<0.01$, ***$P<0.001$ vs. CNE1 or CNE2 groups; ##$P<0.01$ vs. shF-A group; ^^$P<0.01$ vs. MM group.)

Impact of lncRNA F-A/M on the Inv of NPC cells

The cell Inv effects of shF-A, MM, and MI on CNE1 and CNE2 cells were detected (Figure 7). The cell Inv between the CNE1/CNE2 groups and the shNC and miR-NC groups indicated inconsiderable differences ($P>0.05$). Relative to the CNE1 and CNE2 groups, the number of invading cells in the shF-A group, MM group, and MI group was substantially reduced ($P<0.001$). Additionally, the number of invading cells in the MI group was markedly reduced versus the shF-A and MM groups ($P<0.001$).

Then, the influence of lncRNA F-A/M on the Inv of CNE1 and CNE2 cells by ceRNA was demonstrated (Figure 8). In contrast to CNE1 and CNE2 groups, the cell Inv numbers in the shF-A+miR-NC and shF-A+MM groups were reduced ($P<0.001$). In addition, the number of invading cells in the shF-A+MM group was reduced relative to the shF-A+miR-NC group ($P<0.01$). Furthermore, the cell Inv number of the shF-A+MI group was increased markedly relative to shF-A+miR-NC and shF-A+MM groups ($P<0.001$).

Discussion

NPC is a malignant tumor at the top and lateral wall of the nasopharyngeal cavity, and the incidence of NPC is the highest among otorhinolaryngologic malignancies (11). The main clinical symptoms of NPC patients are nasal obstruction, the feeling of ear blockage, hearing loss, diplopia, and headache. Radiotherapy is the preferred treatment for NPC. Nevertheless, patients with a high differentiation level, late course of the disease, and recurrence after radiotherapy still need to receive surgical resection and adjuvant chemotherapy (12). LncRNAs are involved in chromatin remodeling, transcription, and DNA methylation (13,14). In addition, lncRNAs are involved in NPC progression and chemotherapy/radiotherapy sensitivity (15). In this work, the mechanism of action of lncRNA F-A in NPC was analyzed.

LncRNA F-A is involved in many cancer progressions and shows a trend of medium and high expression. F-A in cervical cancer tissues and cells shows a trend of high expression, while FOXOD3-AS1 is related to poor differentiation of tumor tissues, increased tumor size, and lymph node metastasis (16). High F-A level is found in breast cancer tissues, and knockout of F-A can participate in the inhibition of the Pro and metastasis of breast cancer cells by regulating the miR-127/ARF6 axis (17). F-A level in non-small cell lung cancer (NSCLC) tissues and cells is superior to that in normal tissues and cells, and silencing F-A can play a role in inhibiting the Pro and inducing apoptosis of NSCLC cells by upregulating miR-135a-5p
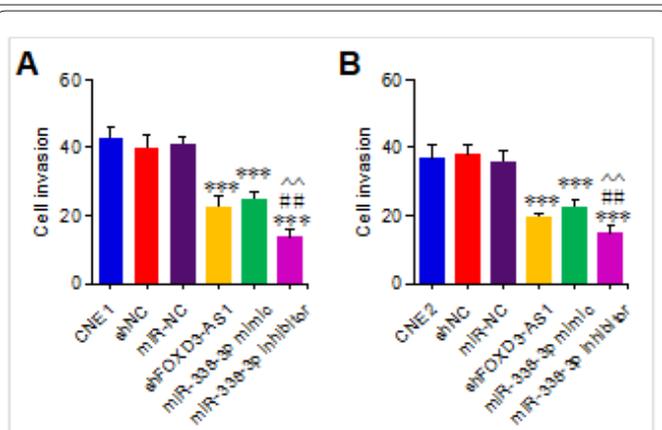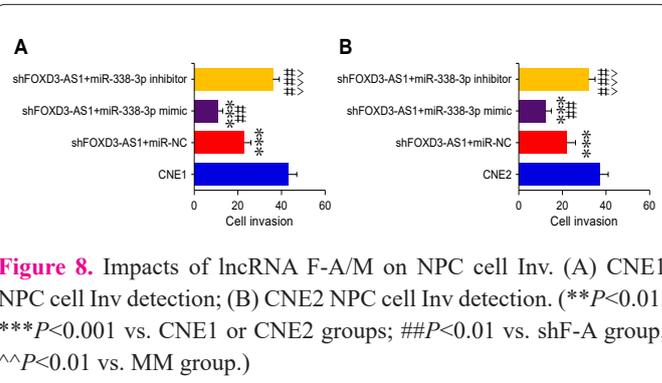
level (18). In this work, it was revealed that the F-A level in NPC cells was superior to that in NNP cells, but that in poorly differentiated SCC CNE2 cells was higher than that in well-differentiated SCC CNE1 cells. These results indicated that the abnormal level of F-A might be involved in the occurrence of NPC. Later, the impact of F-A on the Pro, Mig, and Inv (PMI) of NPC cells was examined. The results suggested that after silencing F-A expression, the PMI of NPC CNE1 and CNE2 cells were greatly inhibited, which is in line with the results of Hu et al. (19) that the F-A in NPC cells is abnormally increased, and inhibition of FODX3-AS1 can inhibit cell survival, Inv and Mig. In summary, F-A plays a role as a pro-oncogene in the progression of NPC, and silencing F-A expression can inhibit the malignant progression of NPC.

LncRNAs can directly bind to target genes to play a role in activating or inhibiting the target genes and can also serve as competitive endogenous RNAs that bind to miRNAs and participate in the expression regulation of the target gene (20). MiRNAs are small noncoding RNAs with a length of 20–25 nt that can complementarily bind to the 3' noncoding region of target mRNA, degrade target mRNA, or inhibit mRNA translation, thus participating in the regulation of cell Pro, differentiation, apoptosis, and the cell cycle (21-23). M, located on chromosome 17q25, is involved in cell death, neural differentiation, and neurite elongation. In recent years, studies have confirmed that M shows a trend of low levels in cancers such as gastric cancer, ovarian cancer, and NSCLC and plays the role of a tumor suppressor gene (24-26). In this study, it was verified that M in NPC cells was lower than that in NNP cells and that in poorly differentiated SCC CNE2 cells was infe-

rior to that in well-differentiated SCC CNE1 cells. Wang et al. (27) indicated that M in NPC tissue showed a trend of low expression, and the survival rate of patients with low levels was drastically inferior to those with high levels. It indicated that M was involved in the regulation of NPC progression. Later, the influence of M on the PMI of NPC cells was examined. It was verified that MM transfection substantially inhibited the PMI of NPC CNE1 and CNE2 cells, while MI transfection notably activated the PMI of NPC CNE1 and CNE2 cells. Li et al. (28) found that inhibition of M affects the Pro, apoptosis, Mig, and radiosensitivity of NPC cells. According to the detection results of FODX3-AS1, the expression trends and effects of FODX3-AS1 and M in NPC cells were opposite. Hence, it was speculated that FODX3-AS1 can act as a ceRNA to affect M expression and further affect the progression of NPC.

A study confirmed that lncRNA can be utilized as ceRNA to affect the regulation of miRNA on target mRNA level through a competitive sponge effect and thus participate in the progression of cancer (29). CeRNA constructs a complex action network. In the normal physiological state of the ceRNA network, each molecule is in a state of equilibrium, and the destruction of equilibrium leads to the occurrence of diseases (30). MiRNAs play a central role in the ceRNA network (31-33). In this work, the effects of silenced FODX3-AS1 on the malignant progression of NPC cells after cotransfection with MM and MI were explored. It turned out that after simultaneous silencing of FODX3-AS1, M was upregulated. Silencing of FODX3-AS1 and overexpression of M inhibited the PMI of NPC CNE1 and CNE2 cells. Second, it was found that silencing of FODX3-AS1 and silencing of M could offset the inhibition of PMI of NPC CNE1 and CNE2 cells. Such results indicated that lncRNA FODX3-AS1 could be taken as a ceRNA to participate in the regulation of the occurrence of NPC.

LncRNA FODX3-AS1 played a role as an oncogene in NPC and that lncRNA FODX3-AS1 could promote the PMI of NPC by regulating M. Nevertheless, this study only analyzed the role of the lncRNA FODX3-AS1/M axis in the malignant progression of NPC and did not further identify miRNA downstream target genes. The mechanism of the lncRNA/miRNA/mRNA axis in NPC progression needs to be further elucidated in the future. In conclusion, this work provides a reference for understanding the pathogenesis of NPC.

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