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EphA3 promotes the proliferation of NPC cells through negatively regulating the ability of FOG2

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Abstract. – OBJECTIVE: The purpose of this study was to investigate the expression level of EphA3 in nasopharyngeal carcinoma (NPC) and its effect on the proliferative capacity of NPC. Meanwhile, the underlying mechanism by which EphA3 prompts NPC malignant progression was further explored.

PATIENTS AND METHODS: In this study, the expression of EphA3 in 42 pairs of tumor tissue specimens and paracancerous ones collected from NPC patients was detected by quantitative Real Time-Polymerase Chain Reaction (qRT-PCR), and the interplay between EphA3 expression and clinical indicators, as well as prognosis of NPC patients was analyzed. Meanwhile, qRT-PCR was also applied to further verify EphA expression in NPC cell lines. In addition, EphA knockdown model was constructed in NPC cell lines, CNE2, and 6-10B, and the impacts of EphA on NPC cell functions was assessed through Cell Counting Kit-8 (CCK-8), cell colony formation, as well as 5-Ethynyl-2'- deoxyuridine (EdU) assays. Finally, a potential interplay between EphA3 and FOG2 was also investigated.

RESULTS: In this study, qRT-PCR results revealed that EphA3 expression levels in tumor tissues of patients with NPC were markedly higher than those in adjacent tissues. Compared with patients with low expression of EphA3, those with highly expressed EphA3 had a more advanced pathological stage. In addition, *in vitro* experiments showed that knocking down EphA3 notably attenuated the proliferation capacity of NPC cells. Subsequently, it was found that the expression of FOG2 in NPC cells was remarkably decreased both in NPC cell lines and tissues, which had a negative correlation with EphA3. Finally, cell recovery experiment revealed a mutual regulation between EphA3 and FOG2, which then together affected the malignant progression of NPC.

CONCLUSIONS: EphA3 is significantly relevant to pathological staging and poor prognosis of patients with NPC and may enhance the proliferation ability of NPC cells by modulating FOG2.

Key Words:
EphA3, FOG2, NPC, Proliferation.

Introduction

Nasopharyngeal carcinoma (NPC) is a nasopharyngeal epithelial malignant tumor that occurs mostly in Southeast Asia, among which, China's NPC incidence and mortality rates rank the highest in the world[1-4]. Most NPC cases are sensitive to radiotherapy, so it becomes the main method for NPC treatment, with supplementation of chemotherapy, molecular targeted therapy, and other means[5,6]. Despite the continuous progress in chemoradiotherapy technology of NPC, the 5-year survival rate of NPC is still around 50%[4,5]. The main reasons that affect the curative effect of NPC are as follows: firstly, the lesion site of NPC is hidden, making the disease not easy to be detected and diagnosed early. Secondly, NPCS are prone to cervical lymph node metastasis and distant proliferation, which affect prognosis[7,8]. The malignant proliferation rate of NPC ranks first among head and neck tumors, and the 5-year survival rate of patients with malignant proliferation is less than 20%[9,10]. In addition, some NPC patients are not sensitive or even resistant to radiotherapy and chemotherapy, resulting in the poor prognosis[9,10]. Therefore, looking for molecular targets closely related to NPC development and

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chemoradiotherapy sensitivity, further clarifying the exact mechanism of NPC development, and exploring new methods to improve the efficacy of NPC radiotherapy and chemotherapy are still important directions of NPC research[10].

EphA3 is the first Eph receptor family molecule to be cloned. At the first time, Andrew Boyd laboratory used monoclonal antibody IIIA4 to isolate a surface antigen from pre-b lymphoblastic leukemia cell line through affinity, and it was subsequently discovered from tumor cells of melanoma patients[11]. EphA3 is also overexpressed in lung cancer, kidney cancer, breast cancer, lung cancer, and colon cancer. High expression of EphA3 is also associated with proliferation capacity of HCC cells[12-14]. In colon cancer, EphA3 expression is positively correlated with tumor cell size and malignant proliferation ability, and it changes with the development of tumor. In breast cancer, EphA3 is found to be highly expressed in lymph node metastatic cells but not in primary tumor. EphA3 is expressed in microtubules and tumor stromal cells of prostate cancer tumors, but not in tumor cells[15-17]. Under such conditions, EphA3 function is kinase activity independent, while ligand activated EphA3 inhibits tumor growth.

In this study, all the molecules that have been reported to regulate EphA3 were searched through bioinformatics, and their protein sequences were then obtained and compared, respectively. FOG2 in these molecules attracted our attention. FOG2 is a transcriptional regulator that can bind to the GATA sequence in the gene promoter and regulate the expression of GATA family proteins, playing an important regulatory role in the occurrence and development of tumors[18,19]. The purpose of this work was to explore the relationship between EphA3 and malignant proliferation of NPC cells, and to reveal the underlying mechanism by which EphA3 may regulate the malignant proliferation of NPC cells. The findings of this study may achieve an in-depth understanding of the molecular mechanism of the occurrence and development of NPC, so as to provide clues to explore the targeted treatment and personalized treatment of EphA3 in NPC.

Patients and Methods

Patients and NPC Samples

Tumor tissue specimens and paracancerous ones of 42 patients with NPC were collected. All patients did not receive any radiotherapy or chemotherapy before surgery. The pathological classification and staging criteria of NPC were performed according to the international association of cancer (UICC) staging criteria for NPC. Patients and their families had been fully informed in this study which was approved by the Ethics Oversight Committee of Qingdao Central Hospital.

Cell Lines and Reagents

Human NPC cell lines (HNE1, SUNE2, HONE1, CNE2, and 6-10B) and human nasopharyngeal immortalized epithelial cell line (NP460) purchased from American Type Culture Collection (ATCC; Manassas, VA, USA) were cultured in Roswell Park Memorial Institute-1640 (RPMI-1640) [containing 5% fetal bovine serum (FBS)] medium (HyClone, South Logan, UT, USA). Thereafter, the cells were cultured in Dulbecco's Modified Eagle's Medium (DMEM; Life Technologies, Gaithersburg, MD, USA) containing 10% FBS (Life Technologies, Gaithersburg, MD, USA) in a 37°C, 5% CO_2 incubator.

Transfection

The control group (sh-NC) and the lentivirus containing the EphA3 knockdown sequence (sh-EphA3) were purchased from Shanghai GenePharma Company (Shanghai, China). Cells were plated in 6-well plates and grew to a cell density of 40%, and then, lentiviral transfection was performed according to the manufacturer's instructions. After 48 h, the cells were harvested for quantitative Real Tim-Polymerase Chain Reaction (qPCR), analysis, and cell functional experiments.

Cell Proliferation Assay

The transfected cells were collected and plated into 96-well plates at 2000 cells per well. After cultured for 24 h, 48 h, 72 h, and 96 h, respectively, 10 μL of Cell Counting Kit-8 (CCK-8) solution (Dojindo Molecular Technologies, Kumamoto, Japan) was added per well for incubation for 1 hours, and then, the optical density (OD) value of each well was measured in the microplate reader at 450 nm absorption wavelength. The wells contained the corresponding amount of cell culture medium and CCK-8 solution, but no cells were considered as blank control.

Colony Formation Assay

After the cell density reached 70-80%, a single cell suspension was prepared by digesting with 0.25% trypsin. Subsequently, the cell suspension was repeatedly mixed using a pipette tip, and the number of viable cells was counted by the cell

counting plate after trypan blue staining. Then, 100 cells were seeded in a six-well plate, and 2 mL of complete medium was added, and the plate was shaken for 5 min. Three parallel samples were set for each concentration, and the cells were cultured for 7 days in a 37°C, 5% CO_2 incubator to calculate the colony formation rate.

5-Ethynyl-2'-Deoxyuridine (EdU) Assay

EDU proliferation assay (RiboBio, Nanjing, China) was performed according to the manufacturer's requirements. After transfection for 24 h, the cells were incubated with 50 μM EDU for 2 h, and then, stained with ApoLo and 4',6-diamidino-2-phenylindole (DAPI). Next, the number of EDU-positive cells was detected by fluorescence microscopy. The display rate of EDU positive was shown as the ratio of the number of EDU positive cells to the total DAPI chromogenic cells (blue cells).

QRT-PCR

The expressions of FOG2, β-actin, EphA3, and U6 mRNAs in NPC tissues and cells were examined by qRT-PCR. Total RNA was extracted in one step by TRIzol reagent (Invitrogen, Carlsbad, CA, USA), and then, reversely transcribed into the first strand of complementary deoxyribose nucleic acid (cDNA) using PrimeScript RT Reagent (TaKaRa, Otsu, Shiga, Japan) reverse transcription kit, and the primers were designed using Primer 5.0 software. The qRT-PCR reaction was performed using SYBR® Premix Ex Taq™ (TaKaRa, Otsu, Shiga, Japan) and StepOne Plus Real Time-PCR System (Applied Biosystems, Foster City, CA, USA). The following primers were used for qRT-PCR reaction: EphA3: forward: 5'-CCGCAAGGCCATCAAACCTT-3', reverse: 5'-ACTGTACTCCACACCCGTGA-3'; FOG2: forward: 5'CGCCGGATTTCAACCAATCG-3', reverse: 5'-CGGACTGCCAATCTGCTGTA-3'; β-actin: forward: 5'-CCTGGCACCCAGCACAAT-3', reverse: 5'-GCTGATCCACATCTGCTGGAA-3'. Three replicate wells were set for each sample and the assay was repeated twice. The Bio-Rad PCR instrument was used to analyze and process the data (Bio-Rad, Hercules, CA, USA). Finally, with the β-actin gene as an internal reference, and the gene expression was calculated by the $2^{-\Delta\Delta Ct}$ method.

Western Blot

The total proteins of tissue or cells were extracted by protein lysate, and the protein concentration was determined by the Bradford method. Then, the extracted protein samples were separated using a 10% sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE) gel and subsequently transferred to a polyvinylidene difluoride (PDVF) membrane (Millipore, Billerica, MA, USA). Western blot analysis was performed according to standard procedures. The primary antibodies against EphA3 (1:1 500) and FOG2 (1:l 500), and the secondary antibody were added sequentially. Then, the protein bands were detected by enhanced chemiluminescence (ECL) assay.

Statistical Analysis

Statistical analysis was performed using Statistical Product and Service Solutions (SPSS) 22.0 statistical software (IBM Corp., Armonk, NY, USA). Student's *t*-test was used to compare the measurement data, and the categorical variables were analyzed by χ^2-test or Fisher's exact probability method. Survival analysis was performed using the Kaplan-Meier method and survival curves were plotted. Data were expressed as mean ± standard deviation, and $p<0.05$ was considered to be statistically significant.

Results

EphA3 was Highly Expressed in NPC Tissues and Cell Lines

It was found that NPC tumor tissue samples had a higher EphA3 expression than paracancerous normal tissues (Figure 1A and 1B). Similarly, EphA3 was also highly expressed in NPC cell lines than in normal cells, especially in CNE2 and 6-10B cell lines, which were therefore selected for subsequent experiments (Figure 1C). These results suggested that EphA3 may serve as an oncogene in NPC.

EphA3 Expression was Correlated with Pathological Staging and Prognosis in Nasopharyngeal

As shown in Table I, high expression of EphA3 was found positively correlated with pathological stage of patients with NPC, but not with age, gender, and lymph node metastasis. In addition, in order to explore the interplay between EphA3 and NPC patients' prognosis, relevant follow-up data were collected, and Kaplan-Meier survival curves were depicted, which revealed that high expression of EphA3 was remarkably relevant to poor prognosis of NPC ($p<0.05$, Figure 1D). In sum, EphA3 expression was correlated with pathological staging and prognosis in nasopharyngeal.

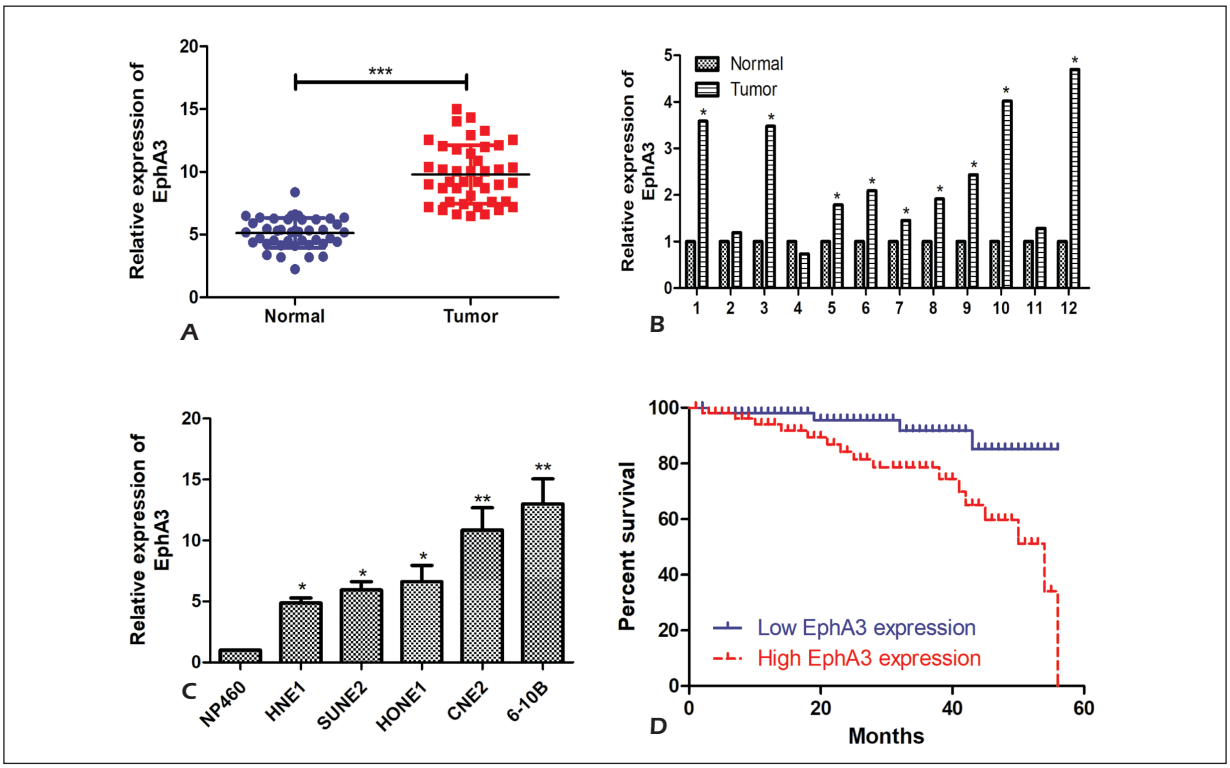

Figure 1. EphA3 is highly expressed in NPC tissues and cell lines. **A-B**, qRT-PCR was used to detect the difference in expression of EphA3 in tumor tissues and adjacent tissues of patients with NPC. **C**, qRT-PCR was used to detect the expression level of EphA3 in NPC cell lines. **D**, Kaplan-Meier survival curve of patients with NPC based on EphA3 expression is plotted. The prognosis of patients with high expression of EphA3 is significantly worse than those in low expression group. Data are shown as mean ± SD, *p<0.05, **p<0.01, ***p<0.001.

Knockdown of EphA3 Inhibited Cell Proliferation in NPC

To explore the influence of EphA3 on the NPC cell functions, EphA3 knockdown model was constructed and the transfection efficiency was verified by Western blot and qRT-PCR (Figure 2A). Cell proliferation detection experiments were subsequently performed in CNE2 and 6-10B cell lines, respectively. The results of CCK-8 assay indicated that EphA3 knockdown significantly repressed the proliferation ability of NPC cells compared to that of sh-NC group (Figure 2B). In addition, cell colony formation and EdU assays demonstrated that

Table I. Association of EphA3 expression with clinicopathologic characteristics of nasopharyngeal carcinoma.

Parameters	Number of cases	EphA3 expression		*p*-value
		Low (%)	High (%)	
Age (years)				0.361
<50	22	13	9	
≥50	20	9	11	
Gender				0.768
Male	20	10	10	
Female	22	12	10	
T stage				0.013
T1-T2	27	18	9	
T3-T4	15	4	11	
Lymph node metastasis				0.808
No	26	14	12	
Yes	16	8	8	

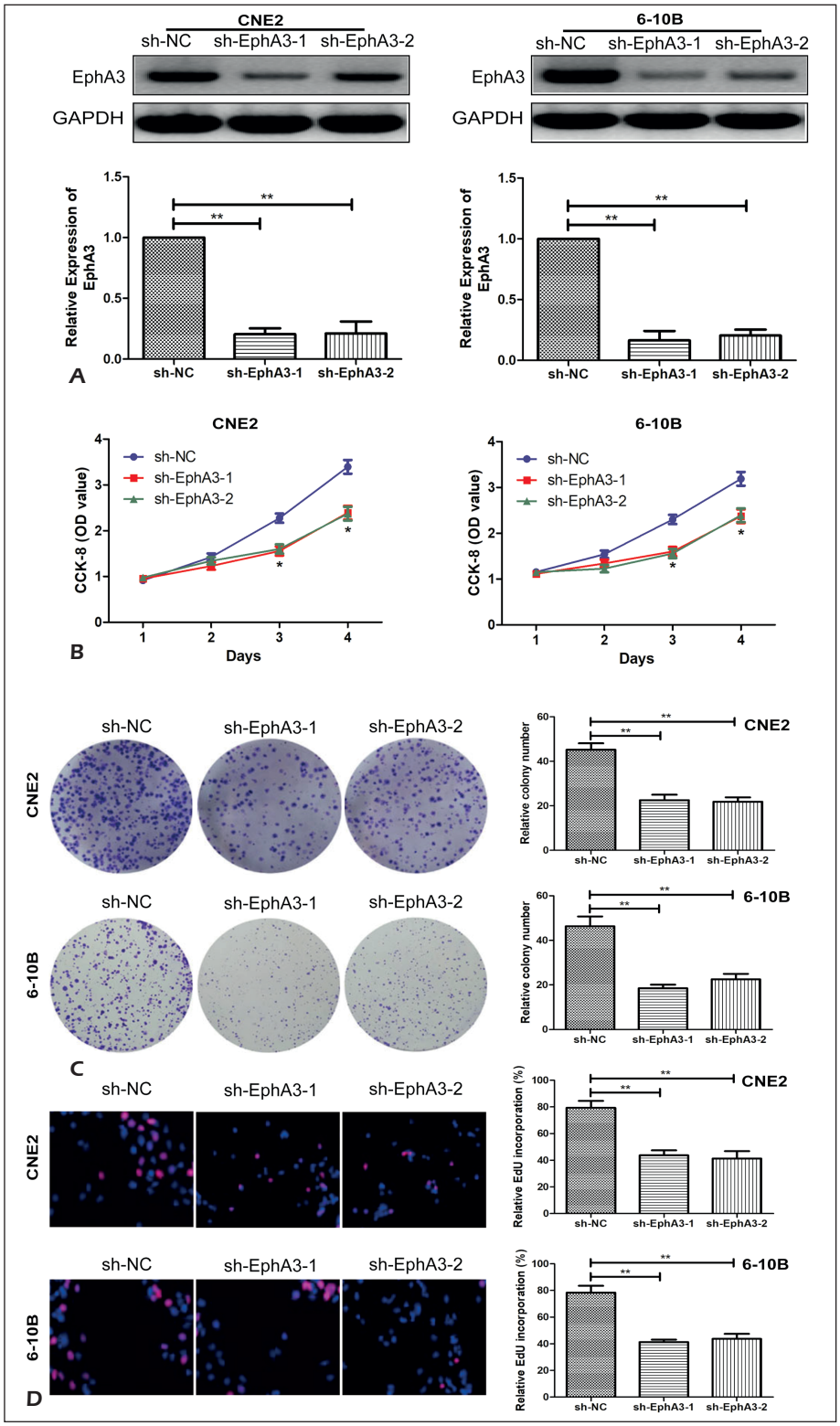

Figure 2. Silencing EphA3 inhibits the proliferation of NPC cells. **A**, Western blot and qRT-PCR verify the interference efficiency of EphA3 after transfection of EphA3 knockdown vector in NPC CNE2 and 6-10B cell lines. **B**, CCK-8 assay detects the effect of knockdown of EphA3 on proliferation ability of NPC CNE2 and 6-10B cell lines. **C**, Plate cloning experiments are performed to detect the effect of EphA3 knockdown on proliferation of NPC cells (magnification: 10×). **D**, The EdU assay detects the number of positive proliferating NPC cells after knocking down EphA3 (magnification: 40×). Data are shown as mean ± SD, *p<0.05, **p<0.01.

EphA3 knockdown reduced the number of positive NPC cells compared with the sh-NC group, indicating a consistent result with CCK-8 assay (Figure 2C and 2D). These results showed that the knockdown of EphA3 could inhibit cell proliferation in NPC.

FOG2 was Lowly Expressed in NPC Tissue Samples and Cell Lines

Bioinformatics studies predicted some association between EphA3 and FOG2 in NPC. Western blot and qRT-PCR analysis showed that downregulation of EphA3 remarkably enhanced the protein and mRNA expression level of FOG2, indicating a close interplay between EphA3 and FOG2-related proteins (Figure 3A, 3B). In addition, qRT-PCR revealed a significant decrease in FOG2 expression in tumor tissues of NPC patients compared to the paracancerous one (Figure 3C), indicating a negative correlation between EphA3 and FOG2 in NPC (Figure 3D).

EphA3 Modulated FOG2 Expression in Human NPC Cells

To further specify how EphA3 and FOG2 worked together to regulate the malignant progression of NPC, EphA3, and FOG2 were simultaneously knocked down in NPC cell lines to explore their cellular functional changes, and the transfection efficiency was confirmed by qRT-PCR assay (Figure 4A). Later, CCK-8 and plate cloning experiments demonstrated that the downregulation of FOG2 significantly enhanced the proliferation of NPC cells and the number of positive proliferating cells, thereby offsetting the impact of silencing EphA3 on NPC cell proliferation capacity (Figure 4B and 4C). These results demonstrated that EphA3 could modulate FOG2 in human NPC cells.

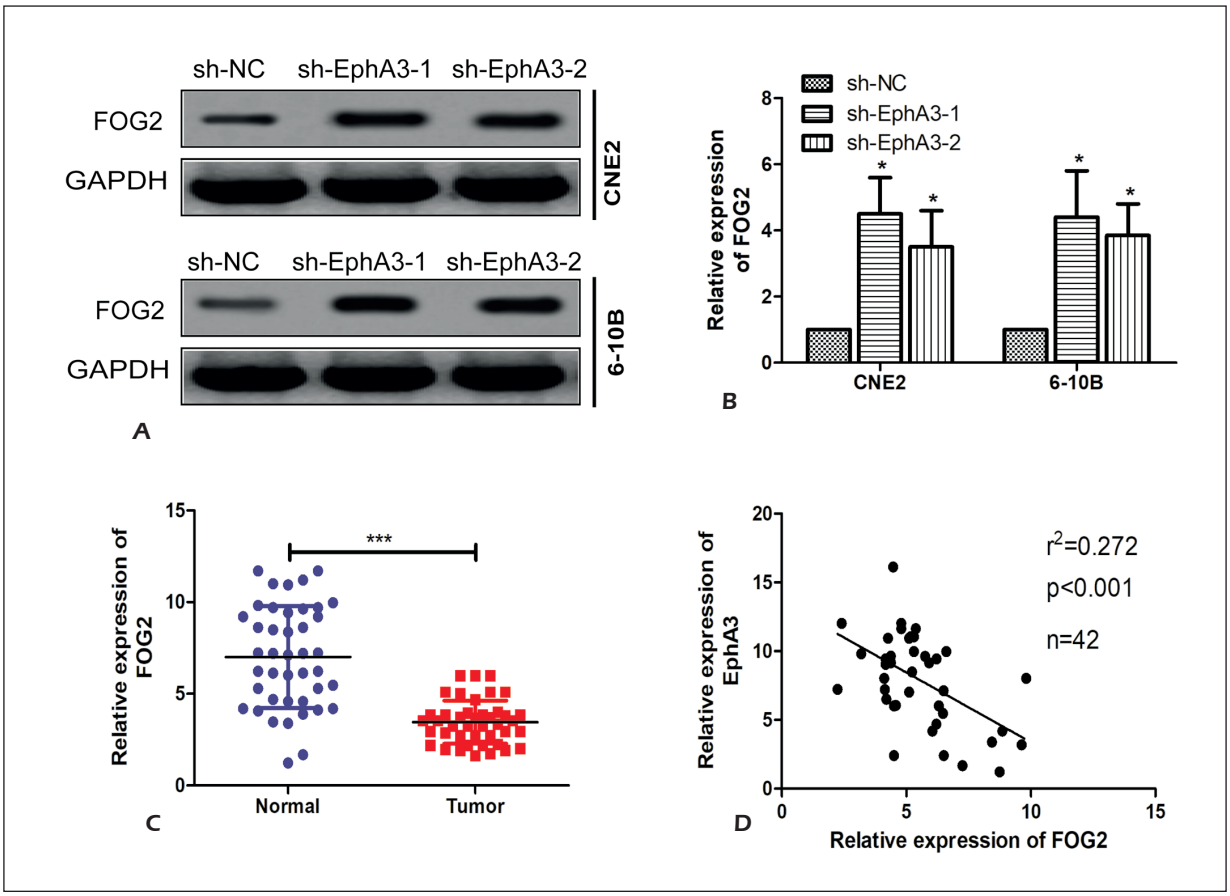

Figure 3. Low expression of FOG2 in NPC tissues and cell lines. **A**, Western blot verifies the expression level of FOG2 after transfection of EphA3 knockdown vector in NPC CNE2 and 6-10B cell lines. **B**, qRT-PCR verifies the expression level of FOG2 after transfection of EphA3 knockdown vector in NPC CNE2 and 6-10B cell lines. **C**, qRT-PCR is used to detect the difference of FOG2 expression in tumor tissues and paracancerous tissues of patients with NPC. **D**, There is a significant negative correlation between the expression levels of EphA3 and FOG2 in NPC. Data are shown as mean ± SD, *p<0.05, ***p<0.001.

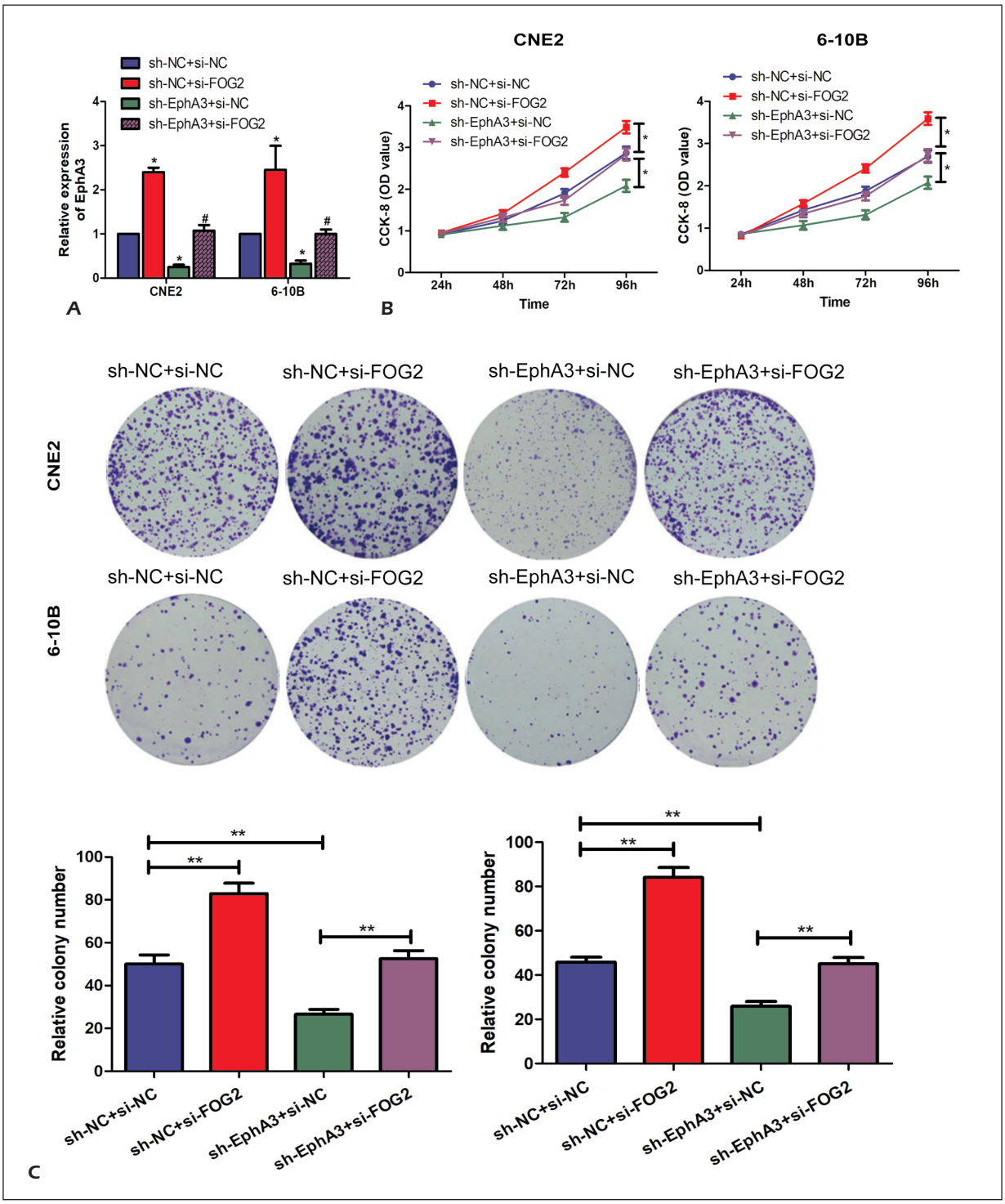

Figure 4. EphA3 regulates the expression of FOG2 in nasopharyngeal carcinoma cell lines. **A**, EphA3 expression levels in co-transfected NPC cell lines with EphA3 and FOG2 are detected by qRT-PCR. **B**, CCK-8 assay detects the effect of co-transfection of EphA3 and FOG2 on the proliferation ability of NPC cells. **C**, Plate cloning experiments are performed to examine the effect of co-transfection of EphA3 and FOG2 on the proliferation ability of NPC cells (magnification: 10×). Data are shown as mean ± SD, *$p<0.05$, **$p<0.01$.

Discussion

NPC is a common epithelial malignant tumor in south China and Southeast Asia. Currently, Epstein barr virus, chemical carcinogens, and genetic susceptibility are believed to be the main factors leading to the occurrence of NPC[1-4]. Therefore, it is of great significance to study the malignant proliferation of NPC cells[6,7]. In recent years, the research on the proliferation of many common human malignant tumors, including head and neck malignant tumors, has become more and more extensive. The study has gradually deepened from the cellular level to the discussion on the molecular mechanism and gene regulation, and some cell molecules or genes that are closely related to tumor proliferation have been identified[8-10].

EphA3 is an important member of EPH receptor family, with a molecular weight of 135kDa, and it plays a pivotal role in tumor cell proliferation, tumor angiogenesis, and disease progression[11-13]. EphA3 was first found to be associated with tumor cell adhesion and progression of malignant T cells[12,13]. In recent years, EphA3 has been proved to have abnormal expression in liver cancer, lung cancer, kidney cancer, malignant pigmentation tumor, and colorectal cancer, and is related to prognosis and angiogenesis[13-17]. When EphA3 expression in various tumors and its influence on biological behaviors became increasingly clear, some scholars also focused on whether EphA3 could be regulated by other factors[15,16]. Despite the fact that EphA3 is a factor that plays a pivotal role in tumor biological behavior, its relationship with NPC is still unclear. In this research, it was found that EphA3 was highly expressed in NPC tissues, and its expression was correlated with diagnostic prognosis. In addition, when studying the baseline characteristics of patients with NPC, it was found that the high expression of EphA3 was positively correlated with the pathological stages of patients, suggesting that EphA3 may play a vital role in the malignant proliferation of NPC cells.

In this study, lentivirus EphA3 knockout model was constructed to detect the effect of EphA3 on NPC cell proliferation and migration. The results of CCK-8, plate cloning, and EdU experiments showed that the number of positive proliferating cells of NPC cell lines CNE2 and 6-10b decreased markedly after the downregulation of EphA3 expression, suggesting that EphA3 may play an important role in the proliferation process of NPC. The above phenomenon indicates that knockdown of EphA3 can significantly reduce the proliferation capacity of NPC cell lines, thereby reducing the malignant progression capacity of NPC.

In order to specify the biological function of EphA3 in NPC, its target genes were further determined, and the role and influence of its interaction with the target genes on the occurrence and development of tumors were explored. FOG2 was found through bioinformatics prediction to be the target gene of EphA3 and the expression of FOG2 in the tumor tissues of patients with NPC was notably downregulated compared with the matched paracancerous tissues. It was further verified that EphA3 knockdown remarkably increased the mRNA and protein expression of FOG2, while simultaneous knockdown of FOG2 could counteract the effect of EphA3 on the proliferation of NPC cells.

Conclusions

In summary, EphA3 is markedly relevant to pathological staging and poor prognosis of NPC patients, which may promote proliferation of NPC *via* regulating FOG2.

Conflict of Interests

The authors declare that they have no conflict of interest.

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