OncoTargets and Therapy

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ORIGINAL RESEARCH

Foxj2 overexpression is associated with poor prognosis, progression, and metastasis in nasopharyngeal carcinoma

This article was published in the following Dove Press journal:
OncoTargets and Therapy
24 July 2017
Number of times this article has been viewed

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Abstract: *Foxj2*, a novel member of Forkhead box family, has been reported to play an important role in tumorigenesis, progression, and metastasis of certain cancers. However, the expression status and effects of *Foxj2* on nasopharyngeal carcinoma (NPC) progression and metastasis remain debated. In this study, we first examined the expression of *Foxj2* in NPC by immunohistochemistry and Western blotting analysis. We confirmed significantly elevated expression of *Foxj2* in NPC tissues and cell lines. Next, the relationships between *Foxj2* expression levels and the clinicopathological factors were investigated. Its expression level correlated with T-classification (P=0.026), distant metastasis (P=0.004), and clinical stage (P=0.029). In addition, high expression of *Foxj2* was associated with poor prognosis in NPC patients. The effects of *Foxj2* on cell proliferation and migration were explored by RNA interference (RNAi) with CCK-8 assay, cell cycle analyses, wound healing, and transwell assay. In conclusion, our data indicate that *Foxj2* upregulation promotes the progression and migration of NPC. It makes *Foxj2* serve as a potential therapeutic target for the treatment of NPC.

Keywords: *Foxj2*, nasopharyngeal carcinoma, prognosis, progression, migration

Introduction

Nasopharyngeal carcinoma (NPC) is the most common head and neck cancer with dramatically different geographical and ethnic distribution patterns.[1] It has high rates of local recurrence and distant metastasis, and 90% of patients show cervical lymph node metastasis at the time of diagnosis.[2] Therefore, understanding the mechanisms associated with NPC progression, metastasis, and prognosis is crucial.

Forkhead box (Fox) family, which includes more than 80 members of transcription factors, share a common forkhead DNA-binding domain, termed the "fork-head" or "winged-helix" motif.[3] Most of the members of the Fox family have been reported to be widely present in different organs of different species ranging from yeast to humans and play extraordinarily diverse roles in embryonic development by regulating the expression of different sets of target genes.[4] The role played by these transcription factors of the Fox family is important for a wide spectrum of biological processes such as signal transduction, metabolism, development, cell differentiation, proliferation, apoptosis, migration, invasion, and even longevity.[5–11] Currently, mounting evidence suggests that Fox family members are abnormally expressed in many cancers, including NPC, and are involved in a variety of cellular processes that are abnormal in cancer cells, such as proliferation, differentiation, adhesion, migration, and invasion.[12–17] Forkhead box J2 (*Foxj2*), a member of this family formerly named as FHX, has dual DNA-binding

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http://dx.doi.org/10.2147/OTT.S134915

specificity.[18] Previous studies have shown its biological importance in mouse embryogenesis and tumorigenesis.[19,20] The expression patterns of *Foxj2* have been reported to be aberrant in a variety of cancers, such as breast cancer, extrahepatic cholangiocarcinoma, glioma, non-small cell lung cancer.[21–24] In addition, it is reported that *Foxj2* appears to be involved in regulating the progression and metastasis of the cancers, as well as aiding in tumorigenesis.[21,24] However, its possible roles in NPC are still unknown.

The purpose of this article was demonstrating the expression levels and roles of *Foxj2* in the prognosis, progression, and metastasis of NPC. We first determined the expression of *Foxj2* in NPC. Then, its associations with clinical and pathologic factors were evaluated. Furthermore, we explored the biological functions, such as proliferation and migration of *Foxj2* in NPC, by silencing its expression. All the data suggested that *Foxj2* might be a positive regulator in NPC progression.

Materials and methods

Tissue specimens

A total of 57 NPC sections from patients who underwent biopsy at the Affiliated Hospital of Nantong University, China, were fixed in formalin and embedded in paraffin for histopathologic diagnosis and immunohistochemistry (IHC). Noncancerous nasopharyngeal tissues were collected from patients with clinical symptoms suggestive of NPC but ruled out by biopsy. Before biopsy, none of the patients with newly diagnosed NPC had received any antitumor therapy. The main clinical and pathologic characteristics are listed in Table 1. The fresh tissues were frozen in liquid nitrogen immediately after biopsy and stored at −80°C until use. This study was approved by the ethics committee of the Affiliated Hospital of Nantong University and all participants gave informed consent.

IHC staining

IHC analysis was carried out as previously reported.[25,26] The primary antibody used was ab22857 against *Foxj2* (1:50; Santa Cruz Biotechnology Inc, Dallas, TX, USA) and slides were incubated overnight at 4°C. Negative control slides were processed in parallel with nonspecific immunoglobulin G (IgG) (Sigma Chemical Co, St Louis, MO, USA) at the same concentration as the primary antibody. Sections were then washed and treated with horseradish peroxidase-conjugated goat anti-rabbit antibody (DakoCytomation, Carpinteria, CA, USA) for 15 min. For assessment of *Foxj2*, 2 blinded pathologists evaluated the immunostaining (including the staining intensity and percentage of positive cells). The intensity was estimated as follows: strong staining (3), moderate staining (2), weak staining (1), or negative staining (0). The scale of positive cells was graded as follows: 0% of positive cells (0), 1%–10% of positive cells (1), 10%–50% of positive cells (2), 51%–75% of positive cells (3), 76%–100% of positive cells (4). The results of intensity and extent were added as described.[24] The samples with a final score of 0 were considered to represent negative expression; 2–3 indicated weak expression; 4–5 was considered moderate expression; and 6–7 was indicative of strong expression. For statistical analysis, scores of 0–3 were regarded as *Foxj2* negative or weak expression and 4–7 were determined as overexpression.

Table 1 Sequences of siRNAs targeting *Foxj2*

siRNA names	Sequences (5′-3′)
*Foxj2*_siR1	Sense: CGAACAACUACUACAUGUAdTdT
	Antisense: UACAUGUAGUAGUUGUUCGdTdT
*Foxj2*_siR2	Sense: CCUUCUGACUGGUGCUCUAdTdT
	Antisense: UAGAGCACCAGUCAGAAGGdTdT
*Foxj2*_siR3	Sense: GGCACAACCUUUCUCUCAAdTdT
	Antisense: UUGAGAGAAAGGUUGUGCCdTdT
*Foxj2*_siR4	Sense: GGUCCUUCCGCAACCUCUAdTdT
	Antisense: UAGAGGUUGCGGAAGGACCdTdT

Abbreviations: siRNA, small interfering RNA; siR, siRNA.

Western blot analysis

Western blot analysis was performed as described previously.[25,26] The primary antibodies used for Western blot analysis were as follows: *Foxj2* polyclonal antibody (1:1,000; Santa Cruz Biotechnology) and β-actin polyclonal antibody (1:2,000; Santa Cruz Biotechnology).

Cell cultures

The process for obtaining the conditioned medium from the cells was the same as that in our previous study.[27] In brief, NPC cell lines (CNE-1, CNE-2, 5-8F, and 6-10B) were maintained in RPMI 1640 (Gibco BRL, Grand Island, NY, USA) containing additionally 10% fetal bovine serum (FBS; Gibco BRL), while NP69 (normal nasopharyngeal epithelial cell line) was cultured in Keratinocyte-SFM (Invitrogen, Carlsbad, CA, USA). All the cells were incubated at 37°C in 5% CO_2 incubator.

Small interfering RNA (siRNA) transfection

The *Foxj2* siRNA and negative control siRNA were designed and obtained from Biomics Biotechnologies Co, Ltd (Nantong, China). The sequences of siRNAs are shown in Table 1. Before transfection, cells were plated and allowed to grow to 30%–50% confluence in 6-well plates. According to

the manufacturer's suggestion, the siRNAs were transfected with Lipofectamine 2000 (Invitrogen).

Cell proliferation assay

The cell proliferation was assessed using the cell counting kit-8 (CCK-8) assay. Cells transfected with *Foxj2* or control siRNA were seeded in 96-well plates (20,000 cells per well) and grown overnight. At time points of 0, 6, 12, 24, 36, and 48 h, 10 μL per well CCK-8 solution was added and incubated for 2 h at 37°C. The absorbance was measured at 450 nm using a microplate reader.

Cell cycle analyses

For cell cycle analysis, cells transfected with *Foxj2* or control siRNA were fixed and incubated with RNase A. Subsequently, the cells were stained with Cell Cycle Detection Kit (KeyGene, Wageningen, the Netherlands) according to the manufacturer's instructions. Cells were analyzed using a BD FACScan flow cytometer (Becton Dickinson, San Jose, CA, USA) and Cell Quest software. All samples were assayed in triplicate.

Transwell assays

Cell migration was measured using cell culture inserts (24-well type, 8 μm pore size; Corning Inc, Corning, NY, USA). Subsequently, 1×10^5 cells transfected with *Foxj2* or control siRNA were added into the upper chambers with the serum-free RPMI 1640 medium, while the lower chambers were filled with 500 μL complete RPMI 1640. After 16 h of incubation, the cells that had migrated to the undersurface of the membrane were fixed in 100% methanol and stained with crystal violet. Cells were visualized and 10 random fields were counted under a microscope.

Wound-healing assay

Cells transfected with *Foxj2* or control siRNA were plated in 6-well plates. After the cells reached 80% confluence, scratches were made using a 100 μL pipette tip. Wound healing was observed and the migration distance was imaged at different time points.

Calculation and statistical analysis

All the quantified data from 3 independent experiments are expressed as the mean values ± SDs and analyzed using SPSS17.0 statistical software. The χ^2 test was used to determine the significance of *Foxj2* expression and the clinicopathological features of NPC. Survival data were calculated by the Kaplan–Meier method and compared by the log-rank test. Statistical significance was determined by the 2-tailed Student's *t*-test for 2 groups and 1-way analysis of variance (ANOVA) for more than 2 groups. The value of $P<0.05$ was considered statistically significant.

Results

Foxj2 was overexpressed in NPC

In order to characterize the role of *Foxj2* in NPC, we first detected the expression and subcellular localization of *Foxj2* in NPC tissues by IHC analysis. We found that *Foxj2* had higher expression in NPC (34/57, 59.65%) than in non-tumor tissues (4/26, 15.38%) (Figure 1A). A combination of nuclear and cytoplasmic positive staining was observed in most of the NPC samples (Figure 1A). The results of IHC staining are summarized in Table 2. Western blotting was further performed to assess the expression level of *Foxj2* (Figure 1B and C). In agreement with these data, *Foxj2* was found to be markedly overexpressed in NPC tissues (Figure 1B and C). These data raised the possibility that high levels of *Foxj2* might be a potential biomarker for NPC.

Correlation between *Foxj2* expression and clinicopathological parameters

We further explored the association of *Foxj2* expression with clinicopathologic variables in NPC. The clinicopathological data of *Foxj2* expression are summarized in Table 2. As shown, high *Foxj2* expression was significantly associated with T-classification ($P=0.026$), distant metastasis ($P=0.004$), and clinical stage ($P=0.029$).

Overexpression of *Foxj2* was indicative of poor survival for NPC patients

To know whether the overexpression of *Foxj2* was correlated with survival, Kaplan–Meier analysis was performed to study the prognostic significance of *Foxj2*. The survival curves indicated that patients with *Foxj2* overexpression had lower overall survival rate than those with negative expression (Figure 2) ($P<0.001$).

Moreover, univariate and multivariate analyses showed that NPC patients' overall survival rate were significantly related to *Foxj2* expression and that *Foxj2* expression ($P=0.031$) was an independent prognostic factor in NPC patients (Tables 3 and 4). These results strengthen our conjecture that there may be a correlation between the overexpression of *Foxj2* and clinical survival in NPC patients.

Expression of *Foxj2* in NPC cells

To further assess the biological role of *Foxj2* in NPC, we carried out cell-based experiments. First, the expression of *Foxj2* in NPC cell lines was investigated. As shown

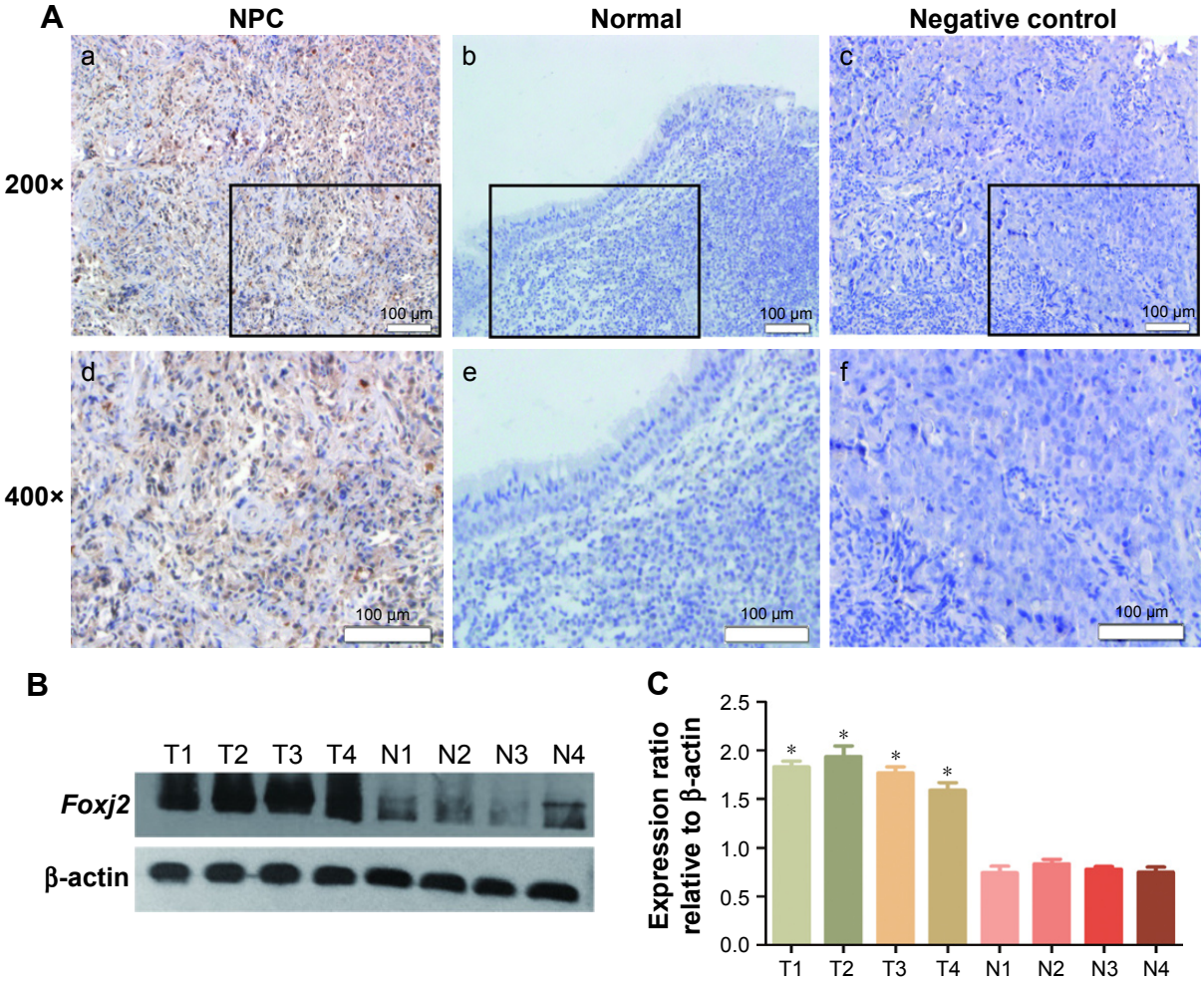


Figure 1 Expression pattern and prognosis role of *Foxj2* in NPC tissues.
Notes: (**A**) Immunohistochemistry analysis (1:50, ab22857) of *Foxj2* expression in NPC tissues. Original magnifications: ×200 (a–c); ×400 (d–f) (**B**) Protein levels of *Foxj2* in NPC (ie, tumor tissue T) and noncancerous nasopharyngeal tissues (labeled "N") by Western blotting. (**C**) Quantitative results of Western blotting. β-actin was used as a loading control. The same experiment was repeated at least 3 times. *$P<0.05$. Data are presented as mean ± SD.
Abbreviation: NPC, nasopharyngeal carcinoma.

in Figure 3A and B, *Foxj2* were overexpressed in all 4 kinds of NPC cell lines compared with the normal nasopharyngeal epithelial cell line NP69 (Figure 3A and B). To clarify the function of *Foxj2* clearly, 4 siRNAs were designed and transfected into CNE-2 cells to knock down the *Foxj2* expression. *Foxj2*-si3 showed the highest knockdown efficiency, and the expression level was inhibited by *Foxj2*-si3 up to 56.6% (Figure 3C and D).

Foxj2 siRNA inhibits cell proliferation

As IHC analysis showed overexpression of *Foxj2* in T3–T4 tissues versus T1–T2, we hypothesized that *Foxj2* was closely related to NPC proliferation (Figure 4A). Next, CCK-8 and flow cytometry assays were carried out to assess the cell proliferation function of *Foxj2* on NPC. CCK-8 assays showed that after downregulating the *Foxj2* expression, the CNE-2 cells showed a significant decrease of the cell proliferation rate (Figure 4B and C). Consistent with these results, flow cytometry analysis revealed that CNE-2 cells accumulated in the G0/G1 phase (from 42.57% to 58.78%), whereas those in the S phase decreased (from 39.01% to 30.79%) after downregulating *Foxj2* expression with *Foxj2*-si3 (Figure 4D), suggesting that *Foxj2* could promote accumulation of the cells in the S phase and thus promote the cell growth. These results suggested that *Foxj2* might play a crucial role in regulation of NPC cell proliferation.

Foxj2 siRNA inhibits cell migration

As IHC analysis showed that *Foxj2* overexpression is significantly associated with distant metastasis in NPC

Table 2 The association between *Foxj2* expression and clinicopathological features in 57 NPC patients

Clinicopathological parameters	Total	*Foxj2* expression, n		*P*-value
		Low	High	
Gender				
Male	41	17	24	1.000
Female	16	6	10	
Age, years				
<50	20	8	12	1.000
≥50	37	15	22	
T-classification				
T1–T2	37	19	18	0.026*
T3–T4	20	4	16	
Lymph node metastasis				
N0–N1	38	13	25	0.253
N2–N3	19	10	9	
Distant metastasis				
No	43	22	21	0.004*
Yes	14	1	13	
TNM clinical stage				
I–II	24	14	10	0.029*
III–IV	33	9	24	

Notes: Statistical analyses were performed by the Pearson's χ^2 test. *$P<0.05$ was considered significant.
Abbreviations: NPC, nasopharyngeal carcinoma; TNM, tumor–node–metastasis.

(Figure 5A), we investigated the cell migration effects of *Foxj2*. Wound-healing and transwell assays were carried out to examine the migration impact of *Foxj2* knockdown on CNE-2 cells. Interestingly, both the assays suggested that knockdown of *Foxj2* significantly inhibited cell migration (Figure 5B–E). These data indicated that downregulation of *Foxj2* reduced the migration of CNE-2 cells.

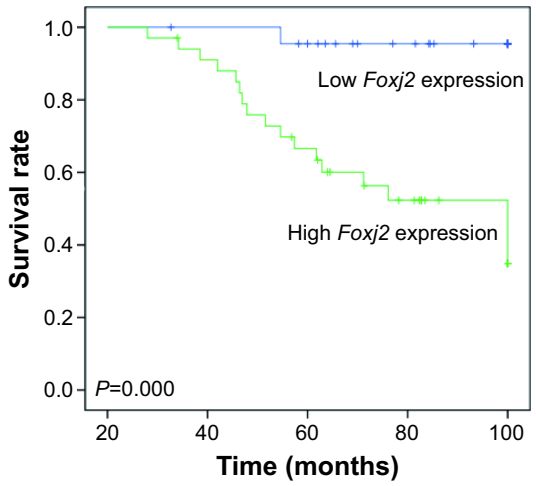


Figure 2 Prognosis role of *Foxj2* in NPC.
Notes: Kaplan–Meier survival curves of NPC patients based on *Foxj2* expression status; $P<0.05$, log-rank test.
Abbreviation: NPC, nasopharyngeal carcinoma.

Table 3 Survival status and clinicopathological parameters in 57 human NPC tissues

Clinicopathological parameters	Total	Survival status, n		*P*-value
		Alive	Dead	
Gender				
Male	41	29	12	0.545
Female	16	10	6	
Age, years				
<50	20	12	8	0.377
≥50	37	27	10	
T-classification				
T1–T2	37	29	8	0.039*
T3–T4	20	10	10	
Lymph node metastasis				
N0–N1	38	23	15	0.081
N2–N3	19	16	3	
Distant metastasis				
No	43	36	7	0.000*
Yes	14	3	11	
TNM clinical stage				
I–II	24	19	5	0.161
III–IV	33	20	13	
Foxj2 expression				
Low	23	22	1	0.000*
High	34	17	17	

Notes: Statistical analyses were performed by the Pearson's χ^2 test. *$P<0.05$ was considered significant.
Abbreviations: NPC, nasopharyngeal carcinoma; TNM, tumor–node–metastasis.

Discussion

NPC is the most common head and neck cancer and continues to be one of the leading causes of cancer-related death worldwide. It has long been acknowledged that gene changes play crucial roles in NPC development and metastasis and affect patients' outcome. Therefore, it is imperative for us to understand the gene changes that govern its progression and enable early diagnosis. This study provides the first evidence that overexpressed *Foxj2* might be a potential regulator in NPC progression and migration.

Table 4 Contribution of various potential prognostic factors to survival, determined by Cox regression analysis, in 57 human NPC tissues

Prognostic factors	Hazard ratio	*P*-value	95% confidence interval
T-classification I–II versus III–IV	2.455	0.165	0.691–8.721
Distant metastasis M0 versus M1	6.689	0.002*	2.021–22.143
TNM clinical stage I–II versus III–IV	0.275	0.123	0.053–1.420
Foxj2 expression Low versus high	0.099	0.031*	0.012–0.806

Notes: Statistical analyses were performed by Cox regression analysis. *$P<0.05$ was considered significant.
Abbreviations: NPC, nasopharyngeal carcinoma; TNM, tumor–node–metastasis.

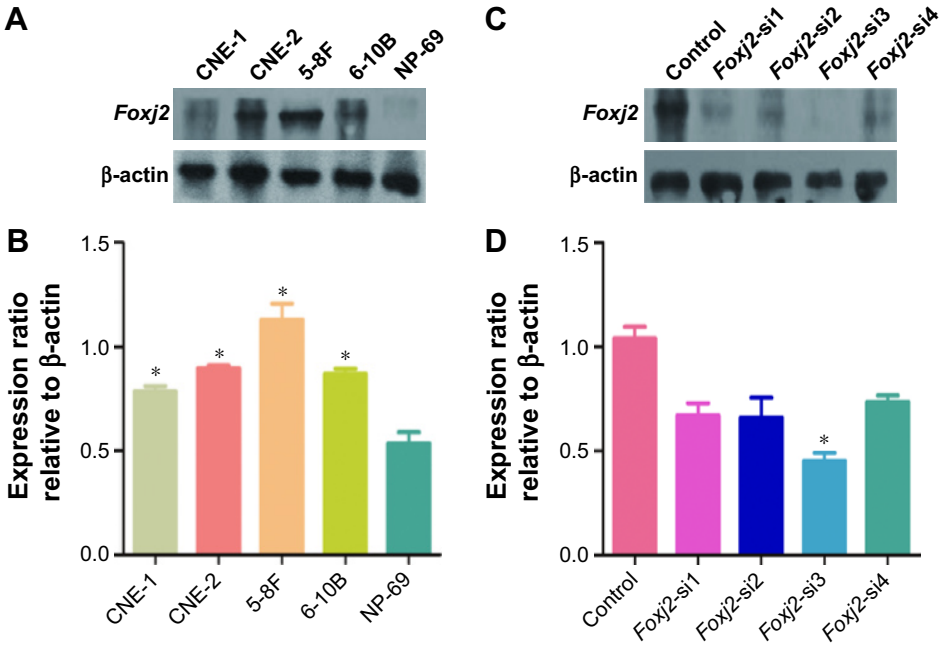


Figure 3 Expression of *Foxj2* in NPC cells.
Notes: (**A**, **B**) Western blot analysis of *Foxj2* expression in 4 kinds of NPC cell lines (CNE-1, CNE-2, 5-8F, and 6-10B) and in NP-69 cell line. (**C**, **D**) The expression of *Foxj2* was suppressed using siRNAs, and interference efficiency was detected by Western blotting. β-actin was used as control. **P*<0.05. Data are presented as mean ± SD.
Abbreviations: NPC, nasopharyngeal carcinoma; siRNA, small interfering RNA.

Foxj2 belongs to the human Forkhead box (Fox) family of proteins, which are implicated in carcinogenesis through their action of transcriptional regulation.[28,29] Upregulation of these genes, caused by various mechanisms such as mutation, amplification, and gene fusion, leads to congenital disorders, diabetes mellitus, or carcinogenesis.[30] Many Fox family genes, such as *FoxA*, *FoxQ*, *FoxC*, *FoxM*, and *FoxO*, have been shown to play an important role in the genesis, progression, and cell dissemination of certain cancers.[12–17,31,32] Previous evidences suggest that *Foxj2* is abnormally expressed in various malignancies and is involved in positively regulating the progression of the cell cycle or actively participating in the metastatic process.[20] The current study extended these findings by investigating

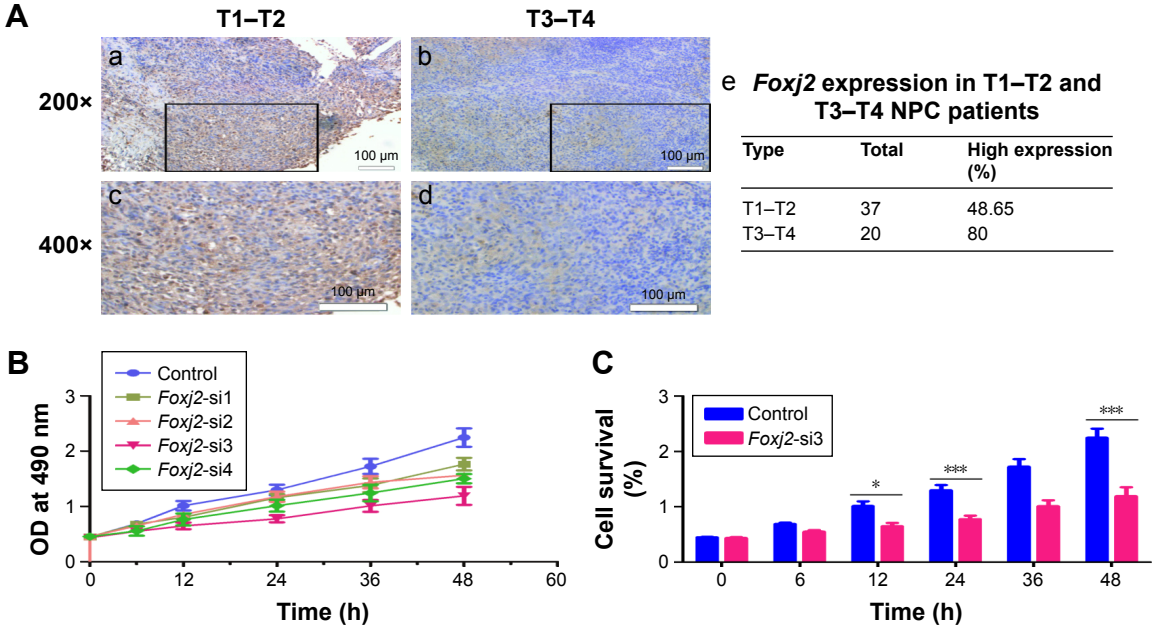


Figure 4 (*Continued*)

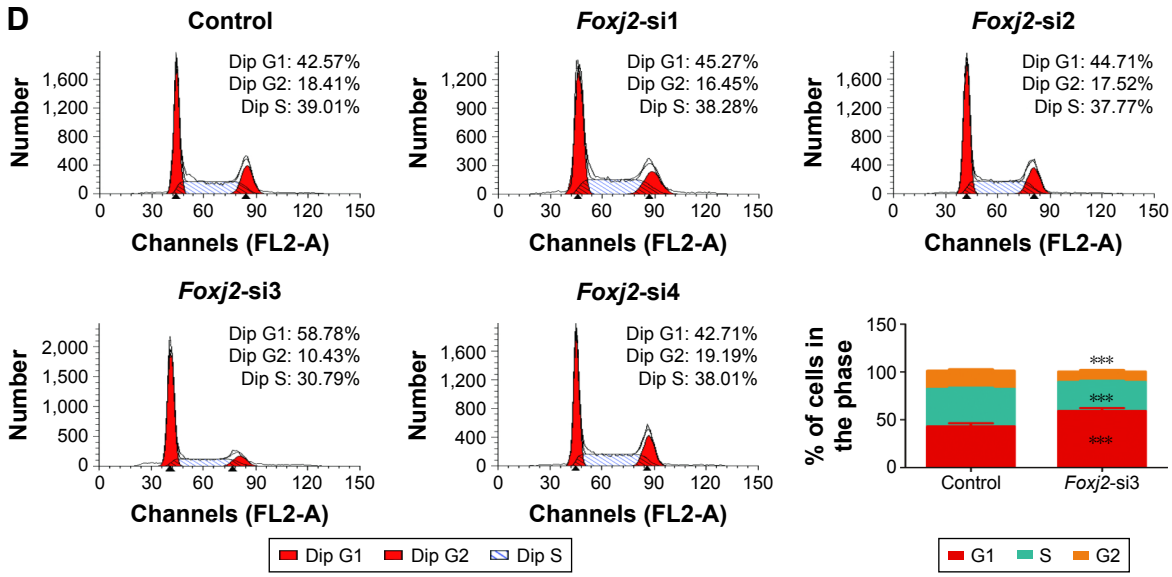
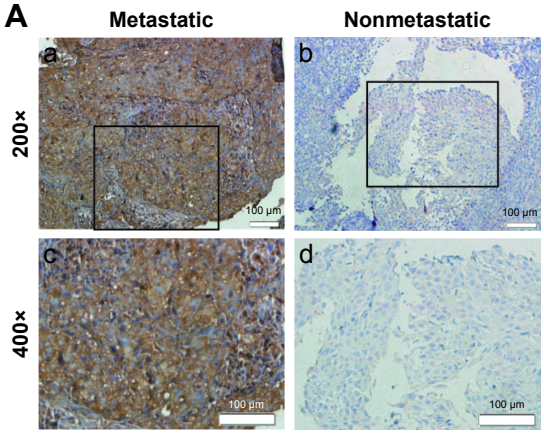


Figure 4 Knockdown of *Foxj2* reduces CNE-2 cell proliferation.
Notes: (**A**) IHC analysis (1:50, ab22857) of *Foxj2* expression in T1–T2 and T3–T4 NPC tissues. Original magnifications: ×200 (a–b); ×400 (c–d); (e) percentage expression of *Foxj2*. (**B**, **C**) CCK-8 assay was used to determine cell proliferation of CNE-2 cells treated with *Foxj2* siRNA for the indicated time. (**D**) Cell cycle distribution analyzed by flow cytometry in CNE-2 cells after *Foxj2* downregulation. The data are presented as mean value ± SEM (compared with the control). The same experiment was repeated at least 3 times. **P*<0.05; ****P*<0.001. Data are presented as mean ± SD.
Abbreviations: CCK-8, cell counting kit 8; IHC, immunohistochemistry; NPC, nasopharyngeal carcinoma; OD, optical density; siRNA, small interfering RNA.

Foxj2 expression and functions in NPC tissues and cell lines. We found that *Foxj2* was remarkably increased in NPC by IHC and Western blot. The association of *Foxj2* expression with NPC clinicopathologic variables was further explored. As shown, overexpressed *Foxj2* was significantly associated with T-classification, distant metastasis, and clinical stage. These results indicated that the overexpressed *Foxj2* might play an important role in NPC progression and migration. Several Fox family genes have been reported in the literature to influence NPC patients' prognosis. For example, Huang et al[33] recently observed that elevated expression of FoxM1 predicts poor survival in patients with NPC. Our research confirmed that patients with *Foxj2* overexpression had a shorter survival time and overexpressed *Foxj2* was a remarkable independent predictor of poor prognosis for NPC. Therefore, *Foxj2* expression in NPC might serve as a predictor for the clinical outcome.

Chen et al[34] established that *Foxj2* was colocalized with proliferating cell nuclear antigen (PCNA), which had been used as a general marker of cell proliferative activity. However, the contribution of *Foxj2* to malignant proliferation of cancers including NPC remained largely unidentified and need to be determined. In order to explore this role, a series of experiments were performed in cell lines. We



e ***Foxj2* expression in metastatic and nonmetastatic NPC patients**

Type	Total	High expression (%)
Metastatic	14	92.86
Nonmetastatic	43	48.84

Figure 5 (*Continued*)

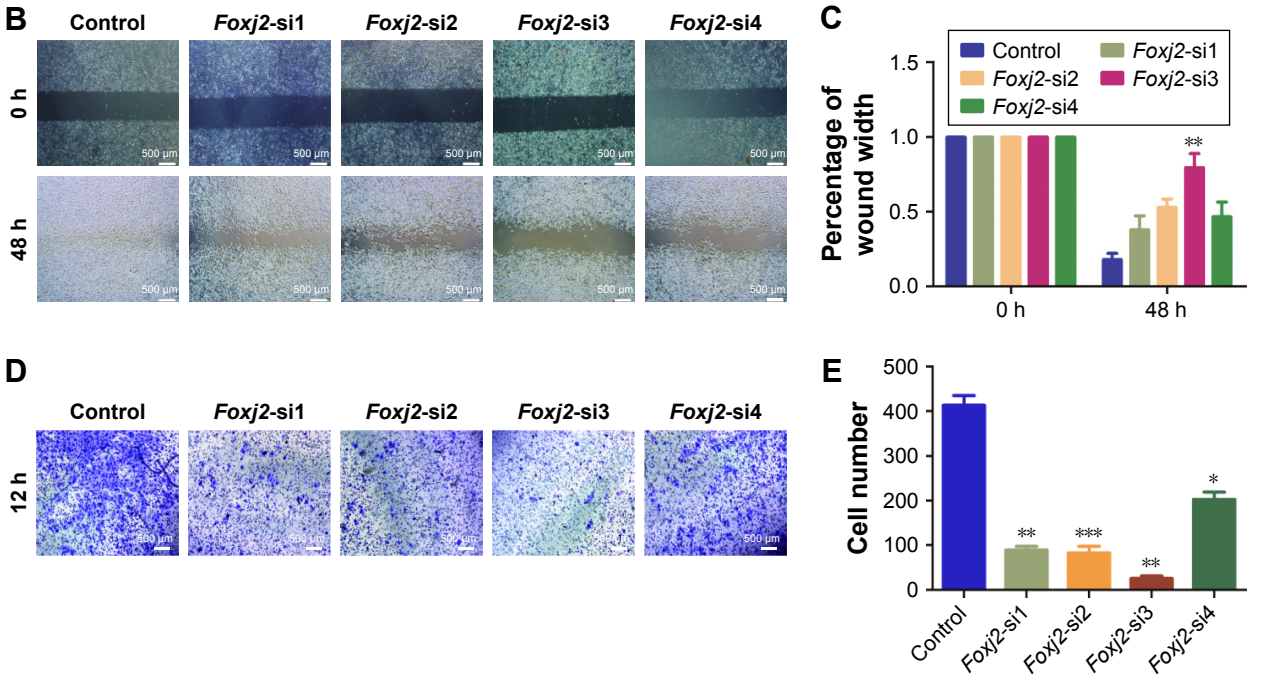


Figure 5 Knockdown of *Foxj2* decreases the migration of CNE-2 cells.
Notes: (**A**) IHC analysis (1:50, ab22857) of *Foxj2* expression in metastatic and nonmetastatic NPC tissues. Original magnifications: ×200 (a–b); ×400 (c–d); (e) percentage expression of *Foxj2*. (**B**) CNE-2 cells transfected with *Foxj2* siRNAs showed a slower migration rate than those transfected with the control siRNA. Migration of cells to the wound was visualized at 0 and 48 h with a microscope (original magnification: ×200). (**C**) The histogram shows the relative migration distance of (**B**). (**D**–**E**) Knockdown of *Foxj2* in CNE-2 cells inhibited cell migration, as determined by transwell assays. Number of cells migrating through the membrane was counted in 10 fields under ×20 objective lens. Error bars, SD. n=3; **P*<0.05, ***P*<0.01, ****P*<0.001. The same experiment was repeated at least 3 times.
Abbreviations: IHC, immunohistochemistry; NPC, nasopharyngeal carcinoma; siRNA, small interfering RNA.

used siRNA to knock down *Foxj2* expression in NPC cells. After *Foxj2* was silenced, we found that the proliferation of CNE-2 cells was significantly inhibited. Cell cycle analysis demonstrated that downregulation of *Foxj2* decreased the cell population in the S phase as well as augmented G1 cycle arrest in CNE-2 cells, leading to inability to complete cell division. As *Foxj2* participates in regulating the cell cycle, this might partly explain the mechanism by which *Foxj2* accelerated cell proliferation. Taken together, these findings indicated that *Foxj2* might contribute to carcinogenesis by regulating cell proliferation through the regulation of cell cycle distribution.

Increasing evidence suggests that *Foxj2* might actively participate in the metastatic process of various cancers, including glioma, extrahepatic cholangiocarcinoma, breast cancer, and lung cancer,[21,22,24,35] as NPC patients with metastasis had a higher rate of treatment failure and mortality.[26] As NPC has the characteristics of highly malignant local invasion and early distant metastasis and metastasis is an important character that influences NPC patients' prognosis, we tried to explore the relationship between *Foxj2* and metastasis in NPC. Wound-healing and transwell migration assays demonstrated that silencing of *Foxj2* inhibited the migration of CNE-2 cells. These data suggested that *Foxj2* overexpression promoted metastasis of NPC.

Conclusion

This study indicated that *Foxj2* functioned as a potential oncogene and played an important role in NPC progression and migration. In this research, we confirmed that *Foxj2* was upregulated in NPC and the level of *Foxj2* is associated with the clinical progression and poor prognosis. Furthermore, our data obtained by downregulation of *Foxj2* expression establish the role of *Foxj2* in modulating the biological properties of NPC cells, including proliferation and migration. We believe that precise understanding of the biological functions of *Foxj2* will provide specific targets for cancer therapeutic intervention.

Acknowledgment

This study was funded by the Chinese National Natural Science Foundation (grant numbers 81672682 and 81602385) and the Natural Science Foundation of Jiangsu Province (grant number SBK2015022581).

Disclosure

The authors report no conflicts of interest in this work.

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