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ORIGINAL ARTICLE

NTF4 stimulates the progression of gastric cancer via regulating FOXL1

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Summary

Purpose: *To elucidate the diagnostic and prognostic potentials of NTF4 in gastric cancer (GC), as well as its regulatory effects on biological functions of GC cells.*

Methods: *Fifty-two GC patients treated by surgical resection were retrospectively analyzed and their cancer and adjacent tissues were collected. NTF4 levels in GC tissues were detected by quantitative real-time polymerase chain reaction (qRT-PCR). The relationship between NTF4 and clinical features of GC was analyzed. After knockdown of NTF4, the proliferative and migratory abilities of MKN45 and BGC-823 cells, and growth rate of GC in nude mice were examined. In addition, the target gene of NTF4, FOXL1 was confirmed by dual-luciferase reporter assay, and co-regulation on GC process was determined by rescue experiments.*

Results: *NTF4 was upregulated in GC tissues than in normal ones. High level of NTF4 predicted malignant progression, poor overall survival and progression-free survival in GC patients. Knockdown of NTF4 attenuated the in vitro proliferative and migratory abilities of GC cells, as well as in vivo tumorigenicity of GC in nude mice. FOXL1 was the target gene of NTF4, which was lowly expressed in GC. Knockdown of FOXL1 was able to reverse the influence of silenced NTF4 on the biological functions of GC cells.*

Conclusions: *NTF4 is an effectively diagnostic biomarker for early-stage GC, and it stimulates the proliferative and migratory potentials in GC by negatively regulating FOXL1.*

Key words: *NTF4, FOXL1, gastric cancer, malignant progression*

Introduction

Gastric cancer (GC) is a common digestive system malignant tumor, with a relatively poor prognosis [1-3]. According to the statistical data released in 2018, there were about 1 million new cases of GC globally, accounting for 5.7% of total cancer cases [3,4]. GC-induced deaths cover about 8.2% of total cancer deaths, ranking third in cancer mortality [4,5]. In recent years, the incidence and mortality of GC have declined in China owing to the health management of Helicobacter pylori infection, reduced salt intake and increased intake of fresh vegetables and fruits, and popularization of GC screening [6-8]. However, health control of GC is still a huge task. Developing targeted therapy based on effective biomarkers for GC has been well concerned [9,10].

Neurotrophic factors (NTFs) are a kind of proteins produced by nerve-dominated tissues (such as muscles) and astrocytes, which are necessary for the growth and survival of neurons [11-13]. NTFs can not only promote the growth and development of nerve cells, but also protect neurons from degeneration and promote regeneration [13]. NTF4 is a vital member of NTFs family [14-16], and it is

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involved in the nerve-immune-endocrine regulatory network [16]. Recent studies have shown an oncogenic role of NTF4 in some types of human cancers [14,17-19]. The potential function of NTF4 in GC process, however, remains largely unclear.

The newly proposed ceRNA theory suggests an interaction between RNA transcripts and mRNAs responsible for regulating cell functions [20]. Here, FOXL1 was predicted as the target of NTF4 by bioinformatic analysis. Our study aimed to uncover the potential influence of NTF4-FOXL1 axis on the process of GC.

Methods

GC samples

Fifty-two GC patients with surgical resection were retrospectively analyzed and their cancer tissues and adjacent ones were collected. Recruited patients did not have preoperative chemotherapy or radiotherapy. This study was approved by the research Ethics Committee of Fudan University Shanghai Cancer Center and complied with the Helsinki Declaration. Informed consent was obtained from all patients.

Cell lines and reagents

GC cell lines (AGS, BGC-823, SGC-7901, MKN28 and MKN45) and epithelial cells of gastric mucosa (GES-1) were purchased from American Type Culture Collection (ATCC) (Manassas, VA, USA). Cells were cultured in Roswell Park Memorial Institute 1640 medium (RPMI 1640) (HyClone, South Logan, UT, USA) containing 10% fetal bovine serum (FBS) (HyClone, South Logan, UT, USA) at 37°C with 5% CO_2. The medium was replaced every 2-3 days. Cell passage was conducted at 90% confluence, and those in the logarithmic growth phase were collected for experiments.

Transfection

Transfection plasmids were synthesized by GenePharma (Shanghai, China). Cells were cultured to 40-60% density in 6-well plates, and transfected using Lipofectamine 2000 (Invitrogen, Carlsbad, CA, USA). After 48-h cell transfection, cells were collected for verifying transfection efficacy and functional experiments.

Cell proliferation assay

Cells were inoculated in 96-well plates with 2×10^3 cells/well. At 24, 48, 72 and 96 h, optical density at 450 nm of each sample was recorded using the cell counting kit-8 (CCK-8) kit (Dojindo Laboratories, Kumamoto, Japan) for plotting the viability curves.

Transwell migration assay

Cell suspension was prepared at 5×10^5 cells/mL. 200 μL of suspension and 700 μL of medium containing 20% FBS were respectively added on the top and bottom of a transwell insert, and cultured for 48 h. Migratory cells on the bottom were induced with methanol for 15 min, 0.2% crystal violet (Solarbio, Beijing, China) for 20 min and captured using a microscope. Five random fields per sample were selected for capturing and counting migratory cells.

Quantitative real-time polymerase chain reaction (qRT-PCR)

Cells were lysed using TRIzol reagent (Invitrogen, Carlsbad, CA, USA) for isolating RNAs. Qualified RNAs were reversely transcribed into complementary DNAs (cDNAs) using AMV reverse transcription kit (TaKaRa, Otsu, Japan), followed by qRT-PCR using SYBR®Premix Ex Taq™ (TaKaRa, Otsu, Japan). Glyceraldheyde 3-phosphate dehydrogenase (GAPDH) was the internal reference. Each sample was performed in triplicate, and relative level was calculated by $2^{-\Delta\Delta Ct}$. NTF4: forward: 5'-ACAGGAGGCACTGGGTATCT-3', reverse: 5'-TTTTCCTGGGCATGGGTCTC-3'; FOXL1: forward: 5'-TTCAACGCTTCCCTGATGCT-3', reverse: 5'-GAACCGTGCCATTGTTTGCT-3'; GAPDH: forward: 5'-CCTGGCACCCAGCACAAT-3', reverse: 5'-TGCCGTAGGTGTCCCTTTG-3'.

Western blot

Cells were lysed by radioimmunoprecipitation assay (RIPA) (Beyotime, Shanghai, China) on ice for 15 min, and the mixture was centrifuged at 14000×g, 4°C for 15 min. The concentration of cellular protein was determined by bicinchoninic acid (BCA) method (Beyotime, Shanghai, China). Protein samples with the adjusted same concentration were separated by sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE), and loaded on polyvinylidene fluoride (PVDF) membrane (Millipore, Billerica, MA, USA). Later, the membrane was cut into small pieces according to the molecular size and blocked in 5% skim milk for 2 h. They were incubated with primary and secondary antibodies, followed by band exposure and grey value analyses.

Dual-luciferase reporter assay

According to the predicted binding sites in the seed sequences of NTF4 and FOXL1, wild-type and mutant-type NTF4 vectors were synthesized. They were co-transfected in cells with either pcDNA-NC or pcDNA-FOXL1 for 48 h. Luciferase activity was finally measured in a standard method.

In vivo xenograft model

This study was approved by the Animal Ethics Committee of the Animal Center of Fudan University Shanghai Cancer Center. Male nude mice aged 8 weeks old were randomly divided into two groups, and administered with MKN45 cells transfected with sh-NC (n=5) or sh-NTF4 (n=5) in the armpit. Tumor width and length were recorded every 5 days. Mice were sacrificed at 30 days for collecting tumor tissues. Tumor volume was calculated using the formula: Tumor width^2 × tumor length / 2.

Statistics

GraphPad Prism 5 V5.01 (La Jolla, CA, USA) was used for statistical analyses and data were expressed as

mean±standard deviation. Differences between groups were compared by the t-test. Clinical significance of NTF4 in GC pathology was analyzed by x^2 test. Kaplan-Meier survival curves were depicted, followed by log-rank test for comparing differences between curves. P<0.05 was considered as statistically significant.

Results

NTF4 was highly expressed in GC

QRT-PCR data revealed a higher level of NTF4 in GC tissues than normal ones (Figure 1A). Meanwhile, its level was upregulated in GC cell lines in comparison to epithelial cells of gastric mucosa (Figure 1B). In addition, Kaplan-Meier survival curves revealed worse overall survival and progression-free survival in GC patients overexpressing NTF4 than those expressing low level of NTF4 (p<0.05). In particular, MKN45 and BGC-823 cells expressed the highest abundance of NTF4 among the five tested cell lines, and they were selected for constructing NTF4 knockdown models by transfection of sh-NTF4 (Figure 1C). By analyzing clinical data of recruited GC patients, it was found that NTF4 level was positively linked to malignant progression in GC patients.

Knockdown of NTF4 attenuated proliferative and migratory abilities of GC

After knockdown of NTF4, viability in MKN45 and BGC-823 cells was markedly reduced, indicating the attenuated proliferative ability in GC (Figure 2A). In addition, Transwell assay demonstrated the weakened migratory ability in GC cells transfected with sh-NTF4 than those of controls (Figure 2B).

FOXL1 was a direct target of NTF4

Bioinformatic analysis suggested that NTF4 can directly bind to FOXL1. Further-

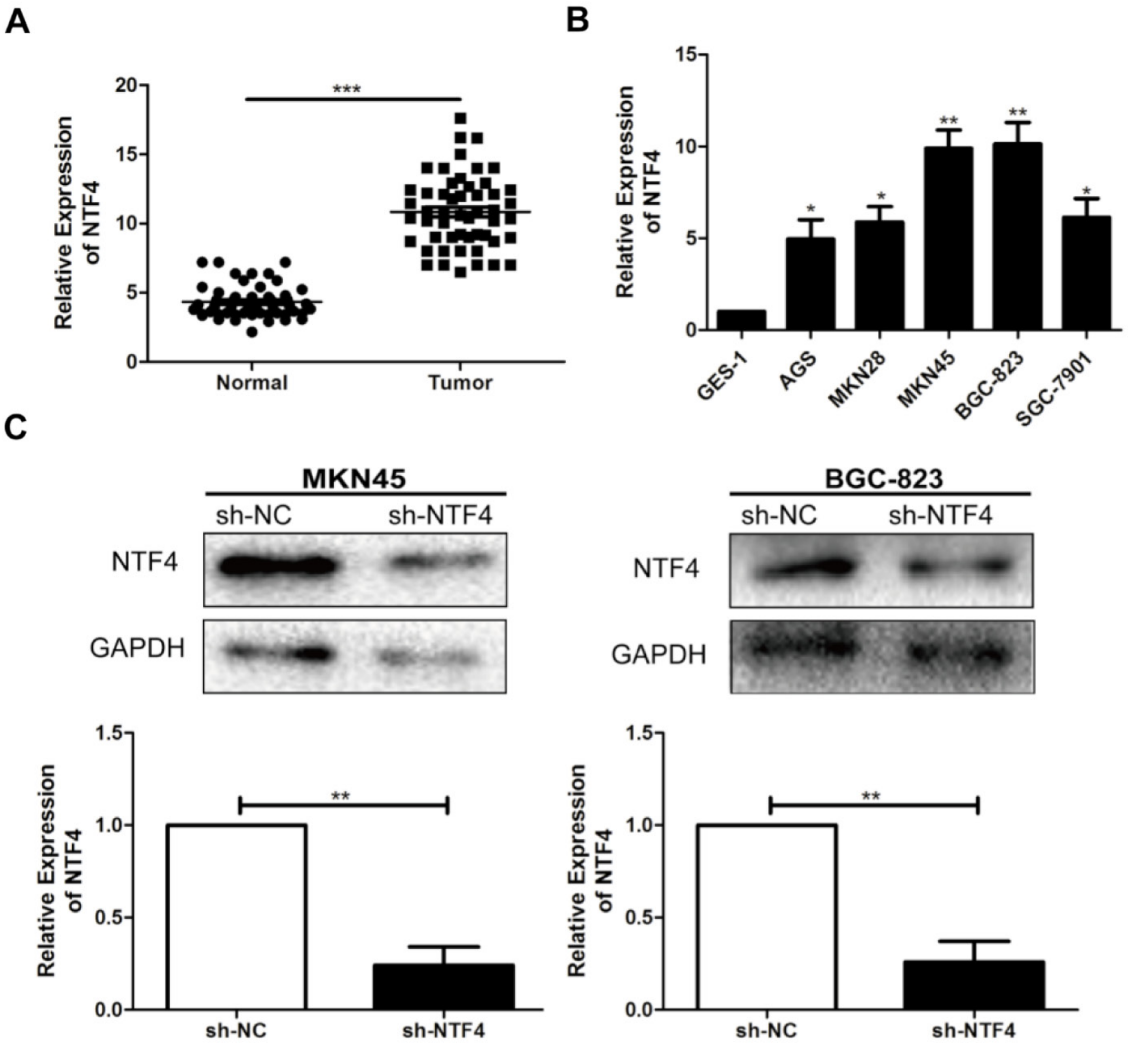

Figure 1. NTF4 was highly expressed in GC. A: NTF4 level in GC tissues and normal tissues. **B:** NTF4 level in GC cell lines. **C:** Transfection efficacy of sh-NTF4 in MKN45 and BGC-823 cells. *p<0.05, **p<0.01, ***p<0.001.

more, dual-luciferase reporter assay results showed that NTF4 could be targeted by FOXL1 through the predicted binding site (Figure 3A). QRT-PCR data showed that FOXL1 was down-regulated in GC tissues than normal ones (Figure 3B).

FOXL1 was responsible for NTF4-mediated GC process

We subsequently focused on the biological function of FOXL1 in GC process. Transfection efficacy of si-FOXL1 was tested in MKN45 and BGC-823 cells with NTF4 knockdown by Western

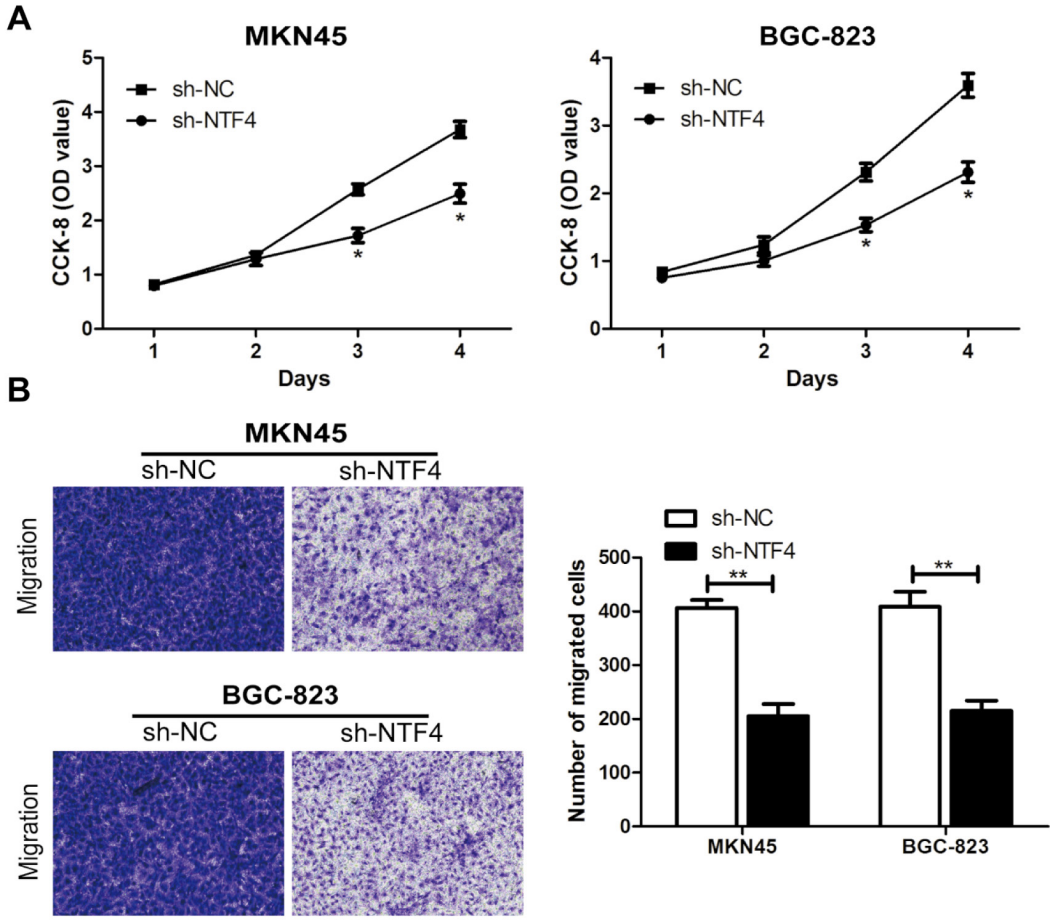

Figure 2. Knockdown of NTF4 attenuated proliferative and migratory abilities of GC. **A:** Viability in MKN45 and BGC-823 cells with NTF4 knockdown; **B:** Migration in MKN45 and BGC-823 cells with NTF4 knockdown. *p<0.05, **p<0.01.

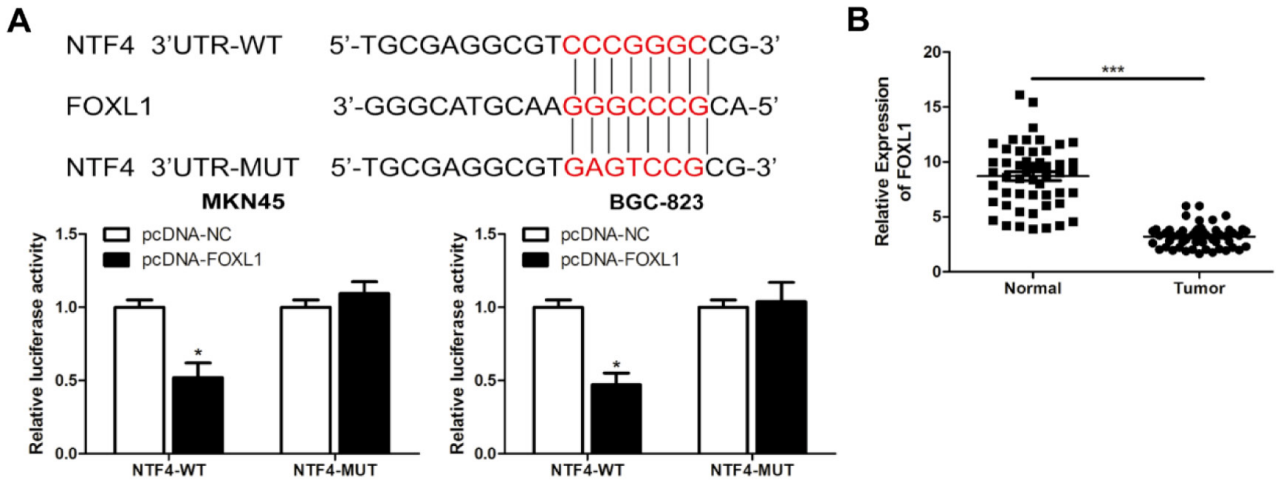

Figure 3. FOXL1 was a direct target of NTF4. **A:** Direct binding between NTF4 and FOXL1. **B:** FOXL1 levels in GC and normal tissues. *p<0.05, ***p<0.001.

blot (Figure 4A). Compared with GC cells with solely knockdown of NTF4, those with co-knockdown of NTF4 and FOXL1 had higher viability and migratory cell number (Figures 4B,4C). It is suggested that knockdown of FOLX1 reversed the role of silenced NTF4 in regulating GC cell phenotypes.

NTF4 promoted the tumorigenicity of GC in nude mice

The above data proved the *in vitro* regulation of NTF4 in GC cell phenotypes. We thereafter performed tumorigenicity assay in nude mice with xenografted GC aiming to explore the *in vivo* function of NTF4 in influencing GC growth. Mice were administered with MKN45 cells transfected with sh-NC or sh-NTF4, and they were sacrificed on day 30 for harvesting tumor tissues. Compared with controls, *in vivo* knockdown of NTF4 reduced tumor growth and tumor weight of GC (Figures 5A,5B). In mice with *in vivo* NTF4 knockdown, NTF4 was markedly downregulated in tumor sections, while FOXL1 was upregulated (Figures 5C,5D). Therefore,

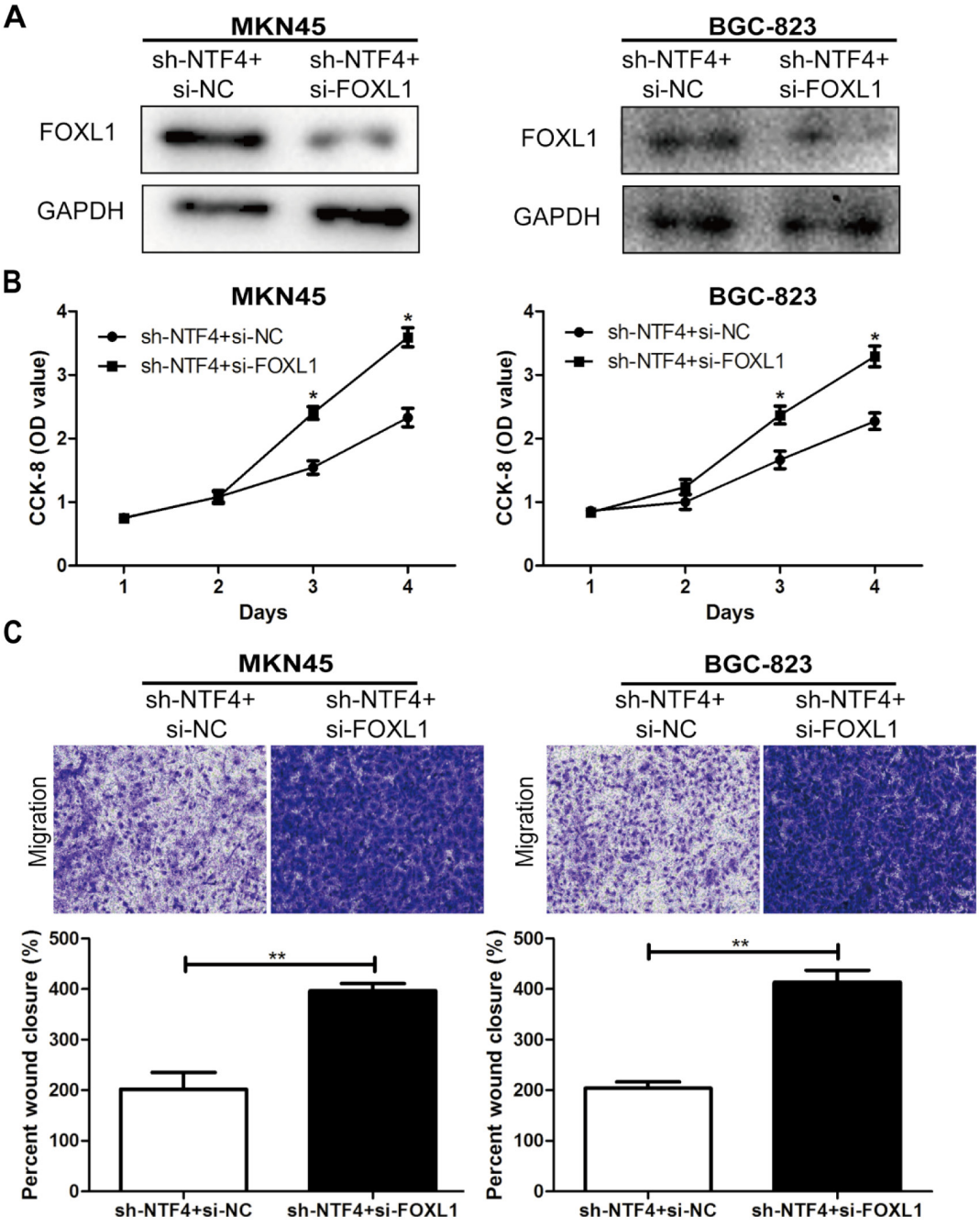

Figure 4. FOXL1 was responsible for NTF4-mediated GC process. **A:** Transfection efficacy of si-FOXL1 in MKN45 and BGC-823 cells. **B:** Viability in MKN45 and BGC-823 cells with co-knockdown of NTF4 and FOXL1; **C:** Migration in MKN45 and BGC-823 cells with co-knockdown of NTF4 and FOXL1. *p<0.05, **p<0.01.

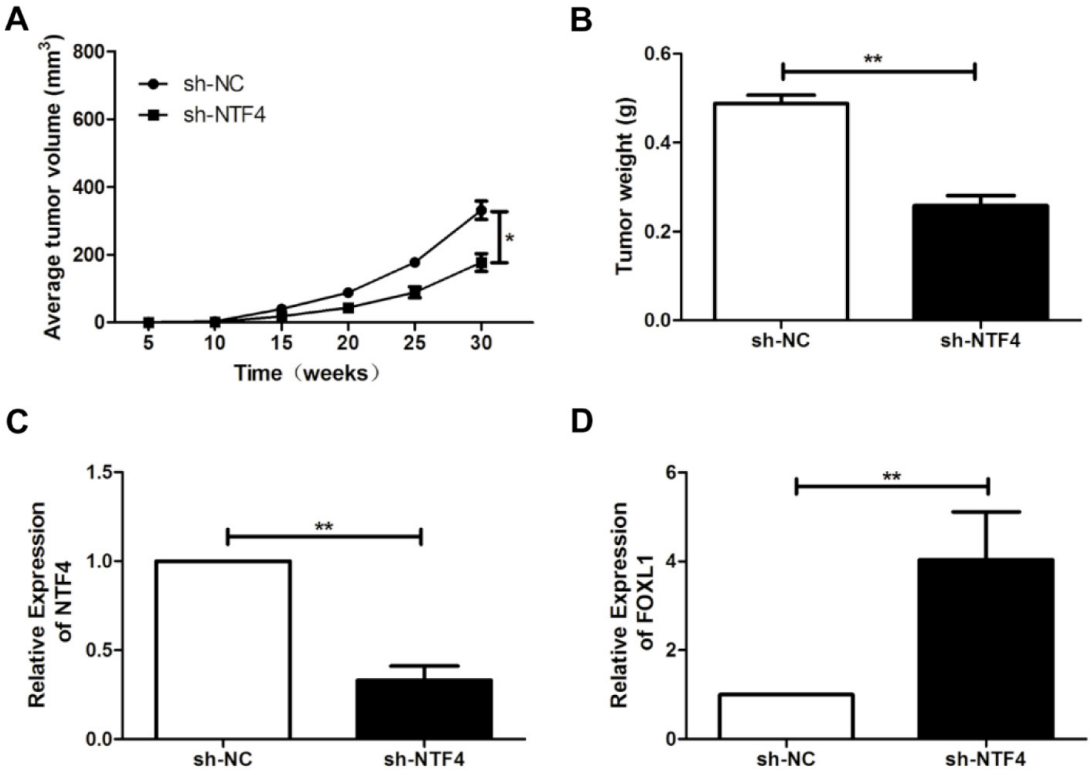

Figure 5. NTF4 promoted the tumorigenicity of GC in nude mice. **A:** Average tumor volume in nude mice with xenografted GC from day 5 to day 30. **B:** Tumor weight in nude mice with xenografted GC. **C:** NTF4 level in GC sections harvested from nude mice. **D:** FOXL1 level in GC sections harvested from nude mice. *p<0.05, **p<0.01.

NTF4 was able to stimulate *in vivo* growth of GC, which was consistent with our *in vitro* findings.

Discussion

The globally high incidence and mortality of GC severely threatens human health and even lives [1-4]. In China, new cases and death numbers of GC both account for nearly 50% of global estimates [3,4]. About 60% of progressive GC patients die of metastasis, which is a major reason for the low 5-year survival [5-8]. It is urgent to clarify the molecular mechanism underlying GC metastasis, which is conductive to improving clinical outcomes of GC patients [9,10].

Tumor invasiveness and metastasis are the essential characteristics for malignancy, and they are the most lethal causes for tumor patients [21,22]. Previous studies have demonstrated that NTF4 is an oncogene, and its high level is related to poor prognosis of tumor patients [14,17-19]. Our findings consistently revealed that NTF4 was upregulated in GC tissues, and its level was linked to the malignant progression. Kaplan-Meier survival curves verified the prognostic value of NTF4 in GC. To further elucidate the role of NTF4 in mediating GC cell phenotypes, we constructed NTF4 knockdown models in MKN45 and BGC-823 cells. Knockdown of NTF4 markedly attenuated proliferative and migratory abilities of GC cells. In addition, in nude mice with xenografted GC, *in vivo* knockdown of NTF4 obviously slowed down the growth rate of GC. Taken together, we proved the oncogenic role of NTF4 in GC.

Using online bioinformatic software, we found that FOXL1 may be a potential target of NTF4, which was further confirmed by dual-luciferase reporter assay. FOXL1 was detected to be lowly expressed in GC tissues, and presented a negative correlation to NTF4 level. Rescue experiments uncovered that knockdown of FOXL1 was able to reverse the influence of silenced NTF4 on biological functions of GC cells. To sum up, NTF4 stimulated the malignant process of GC by negatively regulating FOXL1.

Conclusions

NTF4 is an effective diagnostic biomarker for early-stage GC, and it stimulates proliferative and migratory potentials in GC by negatively regulating FOXL1.

Conflict of interests

The authors declare no conflict of interests.

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