FoxQ1 Promotes Glioma Cells Proliferation and Migration by Regulating NRXN3 Expression

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Abstract

Background: Forkhead box Q1 (FoxQ1) is a member of the forkhead transcription factor family, and it has recently been found to participate in cancer development. However, whether FoxQ1 expression contributes to glioma development and progression is not known. We investigate FoxQ1 expression in gliomas and the role of FoxQ1 during tumorigenesis.

Methods: Reverse transcription quantitative real-time PCR (RT-qPCR) and Western blot were used to determine the FoxQ1 and Neurexins 3 (NRXN3) expression in gliomas. Chromatin immunoprecipitation (ChIP) and luciferase assays were used to determine the regulation between FoxQ1 and NRXN3. We established depleted FoxQ1 stable clones in U-87MG cells and overexpressed FoxQ1 stable clones in SW1088 cells. MTT and transwell were used to evaluate the ability of proliferation and migration, respectively.

Results: FoxQ1 mRNA and protein were up-regulated in gliomas and negatively related to the NRXN3 expression ($r = -0.373, P = 0.042$). FoxQ1 directly binds to NRXN3 promoter region and suppresses the promoter activity. Furthermore, the ability of proliferation and migration is reduced in depleted FoxQ1 cells.

Conclusion: FoxQ1 promotes glioma cell proliferation and migration by down-regulation of NRXN3 expression.

Introduction

Gliomas are the most common tumors of the central nervous system in adults. Patients with glioblastoma routinely undergo surgery followed by adjuvant radiation therapy and chemotherapy. Although diagnosis and therapeutic strategies have been progressed, the median survival times still less than 1 year in most cases. The 5-year survival rate in patients with glioma is among the lowest for all cancers [1,2]. The understandings of the biology and molecular mechanisms of glioma development and progression are not well known.

The forkhead box (Fox) gene family is a large and diverse group of transcription factor and plays important roles in biological processes, including development, metabolism, immunity, and senescence [3,4]. Loss or gain of Fox function can alter cell fate and promote tumorigenesis as well as cancer progression [5]. Thus, Fox proteins are potential targets for therapeutic intervention, as well as biomarkers for predicting and monitoring treatment responses.

Forkhead box Q1 (FoxQ1, also known as HFH1) is a member of the Fox transcription factor family, which is a predominant regulator of the cell cycle [6–9]. The biological function of FoxQ1 has been clearly identified in hair follicle differentiation [10,11]. Previous studies have found that FoxQ1 is widely expressed at the mRNA level in murine tissues, with particularly high expression levels in the bladder and stomach [12]. Recent studies have been reported that FoxQ1 is markedly overexpressed in colorectal cancer and enhances tumorigenicity and tumor growth in vivo [13]. Furthermore, FoxQ1 is also involved in epithelial-mesenchymal transition regulation by suppressing E-cadherin transcription and is associated with aggressive cancer phenotype [14,15]. However, whether FoxQ1 expression contributes to glioma development and progression is not known.

Neurexins belong to a family of highly polymorphic neuronal-specific cell surface proteins, whose structure suggests a role in cell adhesion and recognition [16–18]. The three neurexin genes, NRXN1 (2q16.3), NRXN2 (11q13.2) and NRXN3 (14q31.1), each display two promoters for a longer transcript, α-neurexins and a shorter transcript, β-neurexins were identified [19]. Differential promoters produce six primary transcripts, which are then subjected to alternative splicing at five positions [19,20]. Several of these isoforms bind to ligands that include membrane-bound endogenous neuroligns and dystroglycans, soluble endogenous neuropsilins and α-latrotoxin spider neurotoxins [10,18,21]. Polymorphic site of NRXN3 gene (rs10146997) was significantly associated with higher risk of breast cancer development [22]. In addition, NRXN3 polymorphisms are associated...
with alcohol dependence [23]. However, no data regarding cellular and biological functions of human NRXN3, especially in cancer cells, are available.

In the present study, we showed that FoxQ1 expression was higher in glioma specimens than the normal tissues, whereas NRXN3 expression was lower in glioma specimens. Furthermore, FoxQ1 expression negatively related to NRXN3 expression in human glioma tissues. Thus, we hypothesized that FoxQ1 promotes glioma development by downregulation of NRXN3 expression.

Materials and Methods

Cell culture and human samples

The human glioma cell line HS683, SW1088, LN-229, and U-87MG were purchased from American Type Culture Collection (ATCC) and maintained in DMEM (Gibco) supplemented with 10% fetal bovine serum (FBS, Gibco) and 1% penicillin/streptomycin in a humidified atmosphere of 5% CO2 at 37 °C. Normal human astrocytes (NHA) were obtained from Gibco human astrocytes kit and cultured in astrocyte medium (Gibco).

Glioma specimens were obtained from Affiliated Hospital of Logistics College of CPAPF. This study was approved by the Institutional Review Board of the Affiliated Hospital of Logistics College of CPAPF and written consent was obtained from all participants. All tumors were from patients with a newly diagnosed glioma who had received no therapy before sample collection. After radical prostatectomy, tissues were flash-frozen in liquid nitrogen and stored at −80 °C.

Reverse transcription quantitative real-time PCR (RT-qPCR).

Total RNA was extracted with TRIZOL reagent according to the manufacturer’s instructions. 5 μg of total RNA was used to perform reverse transcription by using SuperScript II and oligo dT following the manufacturer recommendations (Invitrogen). The RT-qPCR analysis was performed using the Fast SYBR Green MasterMix System (Invitrogen) according to the manufacturer’s instructions. The targeted gene relative quantification was given following the manufacturer recommendations (Invitrogen). The PCR product was cloned into EcoR I and Xho I sites of the mammalian expression vector pcDNA3.1 (+) (Invitrogen). The FoxQ1 and NRXN3 shRNA plasmids were purchased from Santa-Cruz biotechnology. Stably transfected cell lines were isolated by neomycin (G410) selection.

Promoter reporters and luciferase assay

The NRXN3 promoter (−1500−+1) was amplified by from genomic DNA of U-87MG cells and the fragment was cloned into the Bgl II and Kpn I restriction sites in the luciferase reporter plasmids pGL3-basic vector (Promega) (pGL3-NRXN3). We generated mutant NRXN3 promoters by Fast Mutagenesis System (TransGen Biotech). For luciferase assay, 5 × 10^4 cells per well in 12-well plates were cultured without antibiotics overnight and then transfected with NRXN3 promoter reporter plasmids. After 24 hours, cells were washed with phosphate-buffered saline (PBS), subjected to lysis, and their luciferase activities measured by using a dual luciferase assay kit (Promega). The results were normalized against Renilla luciferase. All transfections were performed in triplicate.

Chromatin immunoprecipitation assay

ChIP was carried out using kit from Upstate Biotechnology according to manufacturer’s protocol. Briefly, U-87MG cells were transfected with pcDNA3.1-FoxQ1 or vector control. The putative binding sites of NRXN3 were amplified with the following primers: site 1, 5'-ATTCTTCTAAGACTTTGGAG-3' and 5'-GTTGATGTTAGAGATACACTGG-3'; site 2, 5'-GGAGGAGGATCAAAGCTATCCAG-3' and 5'-AGAAATGAGCAGCTTGTG-3'. The PCR products were resolved electrophoretically on a 2% agarose gel and visualized by ethidium bromide staining.

MTT assay

For MTT assays, 2 × 10^3 cells in 200 μl culture medium were plated into a well of 96-well plates. After culturing cell for an appropriate time, 10 μl of 5 mg/ml MTT was added into each well and cultured for 4 h. Then, the cell culture medium was replaced by 100 μl of dimethyl sulfoxide. Thirty minutes after dimethyl sulfoxide addition, the plates were placed on a microplate autoreader (Thermo). Optical density was read at 570 nm wavelength and cell growth curves were determined according to the optical density value.

Transwell assay

For transwell assay, 1 × 10^4 cells were cultured in the upper chamber with serum-free medium. The lower chamber contained complete medium (10% fetal bovine serum). After incubation for 12 hours, cells adherent to top surface of the membrane were removed with a cotton applicator, whereas cells migrated to bottom surface were fixed with 70% methanol and stained with crystal violet. The migrated cells on the bottom surface of the membrane were photographed and counted on an inverted microscope.

Xenograft assay

Glioma cells (1 × 10^6) were injected intracranially into nude mice (n = 5, per group). Mice were euthanized when they were moribund or on day 90 after glioma cell injection.

Statistical analysis

Results of in vitro experiments were depicted as mean ± SD and student’s t-test (two-tailed) was used to compare values of test and control samples. All calculations were performed with the SPSS for
Results

FoxQ1 is overexpressed in human glioblastomas and negatively correlates with NRXN3 expression

We first determined the FoxQ1 and NRXN3 mRNA expression in 30 human glioblastoma and the paired adjacent normal brain specimens by RT-qPCR analyses. The results indicated FoxQ1 mRNA expression was up-regulated in glioma specimens. Furthermore, we observed for NRXN3 mRNA expression, we found that NRXN3 mRNA was down-regulated in tumor cells than the paired adjacent normal brain tissues (Fig. 1A). In addition, we found a significant correlation between the FoxQ1 and NRXN3 mRNA expression levels (Fig. 1B; \( r = -0.373, P = 0.042 \)). Furthermore, we performed Western blot analyses using total protein extracts in 4 matched human glioblastoma (T) and adjacent normal tissues (N). As shown in Fig. 1C, FoxQ1 protein levels were upregulated in 5 of 6 malignant tumor samples, whereas NRXN3 protein levels were downregulated in the 5 malignant tumor samples. Furthermore, NRXN3 expression negatively correlated with FoxQ1 expression in 5 of 6 paired samples. Therefore, our results suggest that NRXN3 expression negatively related to FoxQ1 expression in human glioma tissues.

FoxQ1 suppresses NRXN3 expression in glioma cells

To determine the FoxQ1 and NRXN3 expression levels in glioma cells lines and normal human astrocytes cells, we examined the FoxQ1 and NRXN3 expression in Hs683, U-87MG, SW1088, LN-229 and NHA cells by RT-qPCR and Western blot (Fig. 2A). The results showed that higher expression of FoxQ1 mRNA and protein was evident in Hs683, U-87MG, SW1088 and LN-229 glioma cells than the normal human astrocytes. Moreover, the FoxQ1 expression levels were negatively related to the NRXN3 levels. To determine the effect of increased FoxQ1 expression on NRXN3 expression, we studies SW1088, which had low levels of the FoxQ1 expression. We transfected these cells with FoxQ1 expression vector pcDNA3.1-FoxQ1 as well as their vector control. We found that the FoxQ1-transfected cells exhibited significantly decreased NRXN3 mRNA and protein expression (Fig. 2B).

Conversely, to determine the effect of decreased FoxQ1 expression on NRXN3 expression, we transfected FoxQ1 shRNA into U-87MG cells, which typically express high levels of FoxQ1 as well as their vector control. The FoxQ1 mRNA and protein expression were significantly decreased in U-87MG-RNAi than the control and parental cells (Fig. 2C); the cells exhibited significantly increased NRXN3 mRNA and protein expression (Fig. 2C). Our results indicate that suppression of FoxQ1 expression increase NRXN3 expression in glioblastoma cells.

FoxQ1 regulates NRXN3 promoter activity in glioma cells

To investigate the role of FoxQ1 in regulating NRXN3 transcription, we explored whether FoxQ1 regulates NRXN3 promoter activity. The NRXN3 promoter luciferase construct pGL3-NRXN3 was transfected into SW1088 cells with pcDNA3.1-FoxQ1 or the vector control. The luciferase activity was higher in SW1088-FoxQ1 cells than the control and parental cells (Fig. 2D). Conversely, to estimate the effect of decreased FoxQ1 expression on NRXN3 transcription, we knocked down the FoxQ1 expression by co-transferring FoxQ1 shRNA and the NRXN3 promoter into U-87MG cells. The luciferase activity was lower in U-87MG-RNAi than the control and parental cells (Fig. 2E). These results suggest that FoxQ1 inhibit the NRXN3 promoter activity in glioma cells.

Direct interaction of FoxQ1 with the NRXN3 promoter

To determine whether NRXN3 could be a direct transcriptional target of FoxQ1, we analyzed the sequence of the NRXN3 promoter by using the MAPPER [24]. We identified two putative FoxQ1 binding sites in the NRXN3 upstream promoter region (Fig. 3A). To demonstrate that FoxQ1 directly binds to endogenous NRXN3 promoter region, we performed chromatin immunoprecipitation assays with U-87MG cells. We found that endogenous FoxQ1 protein bound to both of the FoxQ1 binding sites of the NRXN3 promoter (Fig. 3B). Thus, our results indicate that FoxQ1 directly bind to NRXN3 promoter region in vivo.

FoxQ1 binding sites are critical for the suppression of the NRXN3 promoter in glioma cells

To assess the functional role of the FoxQ1 binding sites in NRXN3 regulation, we performed site-specific mutagenesis within the FoxQ1-binding sites of the NRXN3 promoter pGL3-NRXN3. As shown in Fig. 3C, various mutant reporters were generated from the wild-type NRXN3 promoter construct, including a FoxQ1-binding site 1 mutation only (pGL3-NRXN3-Mut1), a FoxQ1-binding site 2 mutation only (pGL3-NRXN3-Mut2), and both site 1 and site 2 mutations (pGL3-NRXN3-Mut3). We transfected these mutant luciferase reporters into U-87MG cells and compared the activity with that of wild-type NRXN3 promoter pGL3-NRXN3. Disruption of one or both of the FoxQ1-binding sites significantly increased NRXN3 promoter activity (Fig. 3D). In addition, disruption of one or both of the FoxQ1-binding sites significantly inhibited NRXN3 promoter repression by pcDNA3.1-FoxQ1 in SW1088 cells (Fig. 3E). These results suggest that the FoxQ1 binding site is critical for the NRXN3 promoter suppression in glioma cells.

Figure 1. Expression of FoxQ1 and NRXN3 in human normal brain and glioma tissues. A. NRXN3 and FoxQ1 mRNA expression by RT-qPCR. The mRNA expression was analyzed in 30 matched primary glioblastoma tissues and the adjacent normal brain tissues. B. FoxQ1 expression levels correlated negatively with NRXN3 expression levels in glioblastoma samples (Pearson’s correlation test \( r = -0.373; P = 0.042 \)). C. Expression of FoxQ1 and NRXN3 protein in primary glioblastoma tissues and the adjacent normal brain tissues. Normal (N) and tumor (T) samples were analyzed by western blot. \( \beta \)-actin used as the loading control.

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Altered FoxQ1 expression affects the proliferation and migration of glioblastoma cells in vitro

Next, we tested the function of FoxQ1/NRXN3 interaction by assessing their roles in glioma cells biological behaviors. We established two stable clones FoxQ1-shRNA-transfected U-87MG (FoxQ1-shRNA-1 and FoxQ1-shRNA-2) as well as vector control clones. RT-qPCR and Western blot analyses showed that the FoxQ1 mRNA and protein levels were decreased in FoxQ1-shRNA-1 and FoxQ1-shRNA-2, whereas the cells showed increased NRXN3 mRNA and protein levels in FoxQ1-shRNA-1 and FoxQ1-shRNA-2 (Fig. 4A). To observe the effects of FoxQ1/NRXN3 on the glioma cells, cell proliferation in FoxQ1-shRNA stable clones were evaluated by MTT assay. During 9-day observations, we found that the proliferation rate of FoxQ1-shRNA cells was apparently lower compared to the control and parental cells (Fig. 4B). Furthermore, we performed transwell assay to assess the function role of FoxQ1/NRXN3 in glioma cells migration. The rate of migrated cells was lower in FoxQ1-shRNA cells than the control and parental cells (Fig. 4C).

Conversely, to determine the effect of increased FoxQ1 expression on NRXN3 expression, we established another two stable clones pcDNA3.1-FoxQ1-transfected SW1088 cells (FoxQ1-1 and FoxQ1-2) as well as their vector control. RT-qPCR and Western blot analyses showed that the FoxQ1 mRNA and protein levels were increased in FoxQ1-1 and FoxQ1-2, whereas the cells showed decreased NRXN3 mRNA and protein levels (Fig. 4D). MTT assay showed that proliferation rate of FoxQ1-1/2 cells was apparently higher compared to the control and parental cells (Fig. 4E). Moreover, Transwell assay showed that the rate of migrated cells was higher in FoxQ1-1/2 cells than the control and parental cells (Fig. 4F).

Together, our results indicated that FoxQ1 enhances the ability of glioma cells proliferation and migration by down-regulation of NRXN3 expression in vitro.

Down-regulation of NRXN3 rescues the malignant phenotype of FoxQ1 down-regulated glioma cells in vitro and in vivo

To provide direct evidence that FoxQ1 affect the malignant phenotype by down-regulation of NRXN3 in glioma cells, we transfected NRXN3 shRNA into FoxQ1-shRNA2 to rescue the NRXN3 expression and established the stable clone (NRXN3-rescue). RT-qPCR and Western blot analyses showed that the mRNA and protein levels of NRXN3 were down-regulated in NRXN3-rescue cells (Fig. 5A). To observe the effects of FoxQ1/NRXN3 regulation on the glioma cells, cell proliferation and
migration in stable clones were evaluated by MTT and migration assay. As shown in Fig. 5B and C, FoxQ1-dependent stimulation of cell proliferation and migration was rescued by down-regulation of NRXN3 in NRXN3-rescue cells. All the results described above pointed out a possibility that FoxQ1 promote glioma progression by regulating NRXN3. To test this possibility, we intracranially injected U-87MG, U-87MG-control, FoxQ1-shRNA2, and NRXN3-rescue cells into nude mice and found that U-87MG and Control cells produced brain tumors in all of the injected mice, and NRXN3-rescue cells produced brain tumor in 4 of 5 injected mice, however, the FoxQ1-shRNA2 cells produced brain tumor in only one injected mice (Fig. 5D). Furthermore, the mice became moribund ~40 days after the injection. In contrast, the mice injected FoxQ1-shRNA2 cells have a significant increase in overall survival time (Fig. 5E; P<0.05). Our results indicate that inhibition of FoxQ1 expression significantly suppresses the tumorigenicity of human glioblastoma cells, whereas rescue the NRXN3 expression can recover the ability of tumorigenicity.

Discussion

Fox transcription factors, an evolutionarily conserved superfamily, control a wide spectrum of biological processes. Several Fox gene family members are involved in carcinogenesis and thought to act as either an oncogene or a tumor suppressor [5,25,26]. Although previous studies have suggested that FoxQ1 plays an important role in the tumorigenesis of several malignancies, including non-small cell lung cancer, breast cancer, and colorectal cancer [13–15,27], its role and molecular mechanisms in glioma are not known.

FoxQ1 belongs to the human Forkhead-Box (Fox) gene family, which consists of at least 43 members. Deregulation of the Fox family genes, caused by various mechanisms such as amplification,
Mutation, and gene fusion, leads to congenital disorders, diabetes mellitus, even carcinogenesis [28]. Many Fox family genes play key roles in vertebrate development. Specifically, FoxQ1 has been shown to be a downstream mediator of Hoxa1 in embryonic stem cells [29]. Moreover, overexpression of FoxQ1 has been reported in several cancers including lung cancer [27,30], pancreatic ductal adenocarcinomas [31], colorectal cancer [13] and breast cancer [2]. Consistent with these results, we found that the FoxQ1 expression level was higher in glioma specimens, whereas the NRXN3 expression level was higher in normal brain tissues. FoxQ1 expression negatively related to the NRXN3 expression in glioma specimens. Moreover, we found that FoxQ1 suppresses NRXN3 through direct binding to the NRXN3 gene promoter.

Inhibition of FoxQ1 in glioma cells by transfection of FoxQ1 shRNA significantly up-regulated NRXN3 expression and reduced the ability of proliferation and migration in glioma cells, whereas overexpression of a FoxQ1 expression vector did the opposite. Therefore, FoxQ1 overexpression contributes directly to NRXN3 underexpression in gliomas and seems to be critical for glioma development.

In this study, we found both clinical and causal experimental evidence that aberrant FoxQ1 expression critically regulates the tumorigenicity of human glioma cells. We sought to determine the molecular mechanism by which FoxQ1 promote glioma development by down-regulating NRXN3 expression. Our RT-qPCR analyses showed a significant association between FoxQ1 overexpression and decreased NRXN3 expression in 30 matched primary glioblastoma tissues and the adjacent normal brain tissues and Western blot further confirmed the correlation in 6 matched specimens. Our findings suggest that FoxQ1 could be a critical
FoxQ1 in Gliomas

Recently, accumulating evidence has shown that FoxQ1 to be a valuable prognostic indicator for poor outcome in patients with breast cancer and non-small cell lung cancer. Furthermore, high expression of FoxQ1 was also observed in lung cancer, gastric cancer, and colon cancer cell lines [13]. Our present results indicated that FoxQ1 was also high expression in gliomas. Furthermore, we first identified NRXN3 was down-regulation in the glioma specimens, which suggested that NRXN3 is a potential tumor suppressor. The relation between FoxQ1/NRXN3 expression and survival of patients with gliomas need to be clarified in future study.

In conclusion, we have shown that FoxQ1 was highly expressed, whereas NRXN3 was lowly expressed in gliomas. Of more importance, we found that FoxQ1 directly regulated NRXN3 expression and glioma proliferation and migration. Because of the diverse roles of FoxQ1 in cancer development, including regulation of tumor cell proliferation, invasion, angiogenesis, and anti-apoptosis, a better understanding of FoxQ1 signaling and function may help identify novel and effective targets for cancer therapy.

Author Contributions
Conceived and designed the experiments: HTS SXC SZ. Performed the experiments: HTS SXC YT XHL SZ. Analyzed the data: HTS SXC YT XHL. Contributed reagents/materials/analysis tools: YT XHL. Wrote the paper: HTS SXC SZ.

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