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SOX7 inhibits tumor progression of glioblastoma and is regulated by miRNA-24

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Abstract: Objective: Sex-determining region Y-box 7 (SOX7) is a putative tumor suppressor in various types of human cancers. In the present study, the expression and function of SOX7 was investigated in human glioblastoma (GBM) cells. Methods: Real-time PCR and western blot were carried out to reveal the expression of SOX7 in GBM specimens and cultured cell lines. A short interfering RNA (siRNA) targeting SOX7 was synthesized and transfected into U87 cells. 3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay was performed to valuate the cell proliferation ability in U87 cells. Bioinformatics analysis further predicted its regulation by microRNA-24 (miR-24). Luciferase reporter assay was performed to prove this regulation. Results: SOX7 was downregulated in GBM specimens and cell lines. Inhibition of SOX7 in cultured U87 cells resulted in a slower growth rate. Mechanically, SOX7 was a target of miR-24, demonstrated by reporter assay. Conclusion: SOX7 was a strong tumor suppressor regulated by miR-24 in human GBM cells.

Keywords: SOX7, glioma, miR-24, proliferation, invasion

1 Introduction

SOX proteins are a family of transcription factors, which have high-mobility-group DNA-binding domain (HMG box), and they have been reported to play vital roles in embryogenesis [1-3]. Some members of the SOX family are negative regulators of the WNT-β-catenin-TCF signaling pathway [4]. SOX7, a member of subgroup F along with SOX17 and SOX18, has been reported to regulate hematopoiesis and cardiogenesis originally [5-7]. Recently, accompanied with the increasing evidence, SOX7 in particular has also been revealed to be a tumor suppressor in a number of human cancers. In detail, for example, its downregulation was observed in tumors from the ovarian [8], gastric [9] and lung [10]. Functionally, studies have proved that overexpression of SOX7 could inhibit hepatocellular carcinoma cell growth, with G1 to S phase arrest [11]. ShRNA-mediated SOX7 silencing in non- tumorigenic breast cells increased proliferation, migration, and invasion. Conversely, ectopic SOX7 expression inhibits proliferation, migration, and invasion of breast cancer cells in vitro and tumor growth in vivo [12]. Although its role is finely interpreted in multiple human cancers, the expression, function and regulation have not been addressed in GBM yet.

Glioma constitutes more than 70% of all primary neoplasms that develop in the CNS [13]. GBM, the highest-grade glioma, is the most common and aggressive type of primary brain tumor in human [14,15]. Despite improved understanding of the molecular and physiological features of GBM, there are no effective treatments for this type of brain cancer. The average prognosis is still uniformly poor, and the median life expectancy after diagnosis is 15 months [16]. Hence, there is an urgent need for novel targets, concepts, and approaches to treat this disease.

Recent studies suggest that SOX7 acts as a key tumor suppressor in many cancers, but its actual expression and regulation in GBM remain unclear. In the present study, we demonstrated that SOX7 targeted by miR-24 was significantly decreased in GBM specimens and cell lines, and it could suppress the proliferation ability of U87 cells.

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2 Materials and methods

2.1 Cell Lines and Tissues

Human astrocytes were obtained from Gibco (Life Technologies). Human glioblastoma cell lines U251, U343, U87, LN229 and TJ905 were obtained from the Cell Bank of the Chinese Academy of Sciences or ATCC. Cells were cultured in DMEM medium with 10 % fetal bovine serum. All cells were maintained in a humidified incubator at 37°C and 5% CO_2.

Tumor tissues with the corresponding paired normal tissues were obtained from glioblastoma patients at the Tianjin Nankai hospital between 2013 and 2015. Informed written consent was obtained from all patients.

2.2 qRT-PCR

Expression levels of total mRNAs were quantified by two-step quantitative real-time PCR. All qRT-PCR reactions were performed in triplicate on ABI PRISM 7900HT Real-Time PCR System. Data were analysed with the RQ Manager 1.2.1 software, using the $2^{-\Delta\Delta Ct}$ method with a relative quantification RQmin/RQmax confidence set at 95%.

2.3 Cell viability assay

Cells were plated in 96-well plates at 2×10^3 per well 24h post-transfection. MTT (20μl, 5mg/ml) was added to each well and cells were incubated for another 4h at 37°C. The reaction was stopped by addition of 150μl DMSO and optical density at 590nm was determined on a microplate reader.

2.4 Western blot analysis

Total proteins were extracted using RIPA buffer (Beyotime, Jiangsu, China). Then total proteins were separated by SDS-PAGE gel and transferred to nitrocellulose membranes. The membranes were probed with anti-SOX7 and anti-GAPDH antibodies (Santa Cruz). Proteins were visualized by a HRP-conjugated secondary antibody.

2.5 Vector Construction and Transfection

The 3'-UTR of SOX7 containing the putative miR-24 binding site was amplified and cloned into the pGL3basic vector (Promega, Madison, WI). Transfection was performed with Lipofectamine™ 2000 (Invitrogen) according to the manufacturer's protocol.

2.6 Luciferase activity assay

Luciferase assays were carried out as described below. Briefly, cells were co-transfected with luciferase reporter constructs, miRNA and Renila using Lipofectamine™2000 (Invitrogen), and Firefly and Renilla luciferase activities measured after 48 h with a Dual-Luciferase_Reporter Assay System (Promega) using a luminometer.

2.7 MicroRNA Mimics, inhibitors and SOX7 siRNAs

The miR-24 mimics and inhibitors and SOX7 siRNAs were purchased by GenePharma (Shanghai, China). The cells were transfected with mimics or inhibitors using Lipofectamine™ 2000.

2.8 Statistical analysis

All results were expressed as mean ± S.D. Data analysis was performed by SPSS 13.0 (SPSS Inc., Chicago, IL, USA). Statistical significance was analyzed using Student' s t-test. Differences with $P < 0.05$ were considered statistically significant.

3 Results

3.1 SOX7 was downregulated in primary GBM tumor samples and cell lines

To determine its expression level in GBM, we firstly evaluated the mRNA levels in primary GBM tumor tissues by using quantitative real-time PCR (qRT-PCR). We found that SOX7 was downregulated in more than half of GBM tissues compared with the matched non-tumor brain tissues (Figure 1A). We next examined its expression in five GBM cell lines (U251, U343, U87, LN229, TJ905) compared with

that of in normal human astrocytes. The results showed that the SOX7 mRNA was frequently downregulated in GBM cell lines, especially in U251 and U343 cells (Figure 1B). These findings were in line with previous studies characterizing decreased SOX7 expression in other malignancies [8-12], which further indicated a tumor suppressive role of SOX7 in GBM carcinogenesis.

3.2 Silencing of intrinsic SOX7 inhibited cell growth rate of GBM cell line U87

To address the functional effects of downregulated SOX7 in GBM cells, we transfected U87 cells with small interfering RNAs (siRNAs) that targeted 3'-UTR region of SOX7 gene. The knockdown efficiency was validated by qRT-PCR and western blot (Figure 2A and 2B). To test the ability of SOX7 silencing in inhibiting cell proliferation, MTT assay was performed and as the result a significant elevation of cell growth rate after SOX7 silencing was observed (Figure 2C). These findings strongly supported a tumor suppressive function of SOX7 in GBM, which had not been reported ever since.

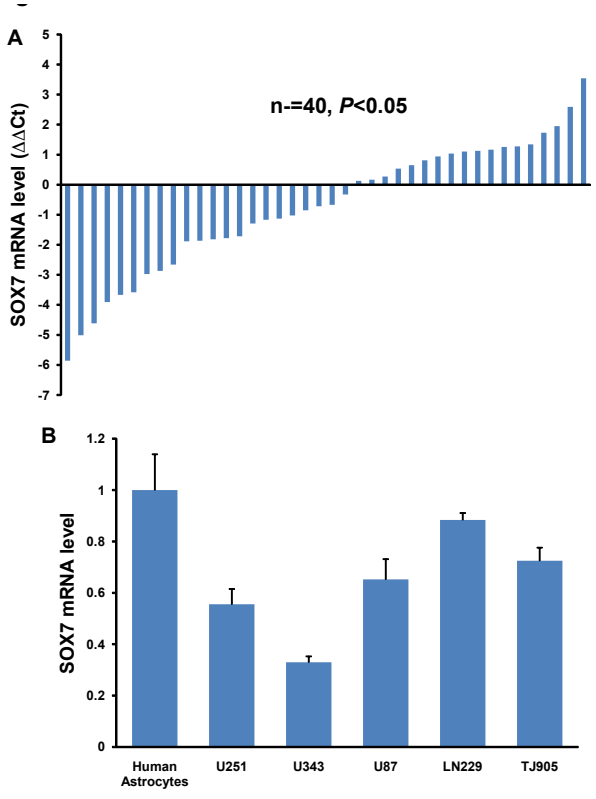

Figure 1: SOX7 was downregulated in GBM tissues and cell lines. (A) Real-time PCR was performed to test the expression level of SOX7 in paired GBM tissues. **(B)** Real-time PCR was performed to test the expression level of SOX7 in human astrocytes and human GBM cell lines U251, U343, U87, LN229 and TJ905. β-actin was used as an internal control.

3.3 SOX7 was a putative target of microRNA-24 in GBM

Previous studies have pointed out that SOX7 might be a target of microRNA-24 (miR-24) in hepatocellular carcinoma (HCC), in which forced expression of SOX7 substantially attenuated the oncogenic effects of miR-24 [17]. To justify a direct interaction between miR-24 and the 3'-untranslated region (3'-UTR) of SOX7, which contains the putative miR-24 recognition sites, we constructed a renilla luciferase reporter gene in the pGL3 vector by inserting the 3'-UTR region of human SOX7 and flanking sequences. The mimics and inhibitor of miR-24, as well as the negative control oligonucleotides were transfected into U87 cells along with the reporter gene. As shown in Figure 3A, the luciferase activity was significantly decreased in miR-24-mimics-transfected cells (compare lane 2 with lane 1, $P<0.01$), whereas the miR-24-inhibitor-transfected U87 cells exhibited no obvious change of SOX7 transcription (compare lane 3 with lane 1), which might be explained by the low endogenous expression of SOX7 in U87 cells. In addition, we further confirmed the protein expression of SOX7 upon miR-24 mimics or inhibitor transfection, which was also direct evidence, that SOX7 is regulated by miR-24 (Figure 3B). Finally, qRT-PCR was carried out to detect the miR-24 expression in GBM cell lines. The results shown in Figure 3C revealed its upregulation in GBM cell lines, which was another evidence to support the miR-24/SOX7 axis since the negative correction of these two molecules.

4 Discussion

In the present study, we observed the downregulation of SOX7 in primary GBM tumor specimens as well as cell lines. We also discovered that knockdown of SOX7 in GBM U87 cells resulted in cell growth acceleration. Mechanically, we proved that SOX7 was a potential target of miR-24 by base pairing within its 3'-UTR.

Accumulating evidence shows that miRNAs participate in the development and progression of various human cancers, including GBM [18,19]. Several reports have found that miR-24 was increased and appeared as an oncogene in some tumors, including GBM [20,21]. In this study, we also showed that miR-24 was indeed upregulated in our selected GBM cell lines, and targeting of SOX7

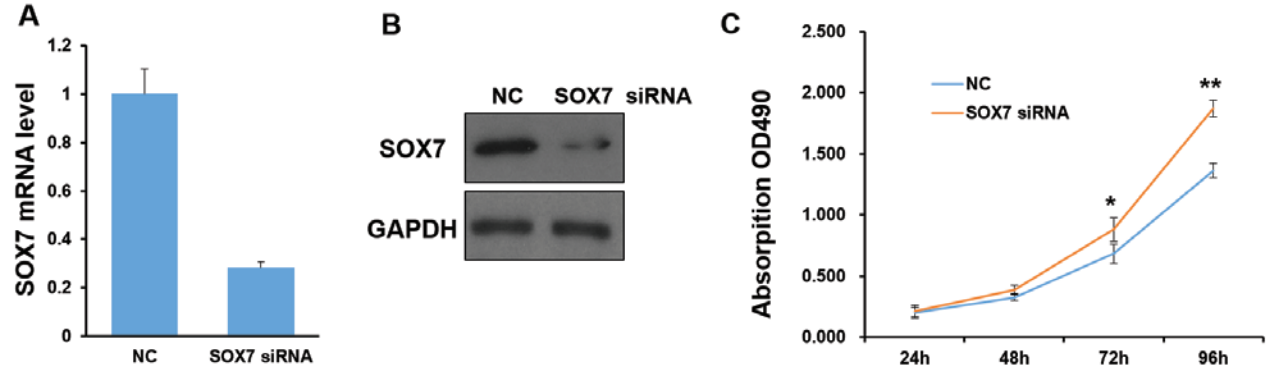

Figure 2: Knockdown of SOX7 promotes U87 cell proliferation. U87 cells were transfected with SOX7 siRNA or control siRNA. After 48h, cells were harvested for qPCR test (A) and western boltting (B). (C) U87 cells were transfected with SOX7 siRNA or control siRNA. In the indicated time periods after transfection, cell viability was evaluated using MTT assays by measuring the absorption at 590 nm.

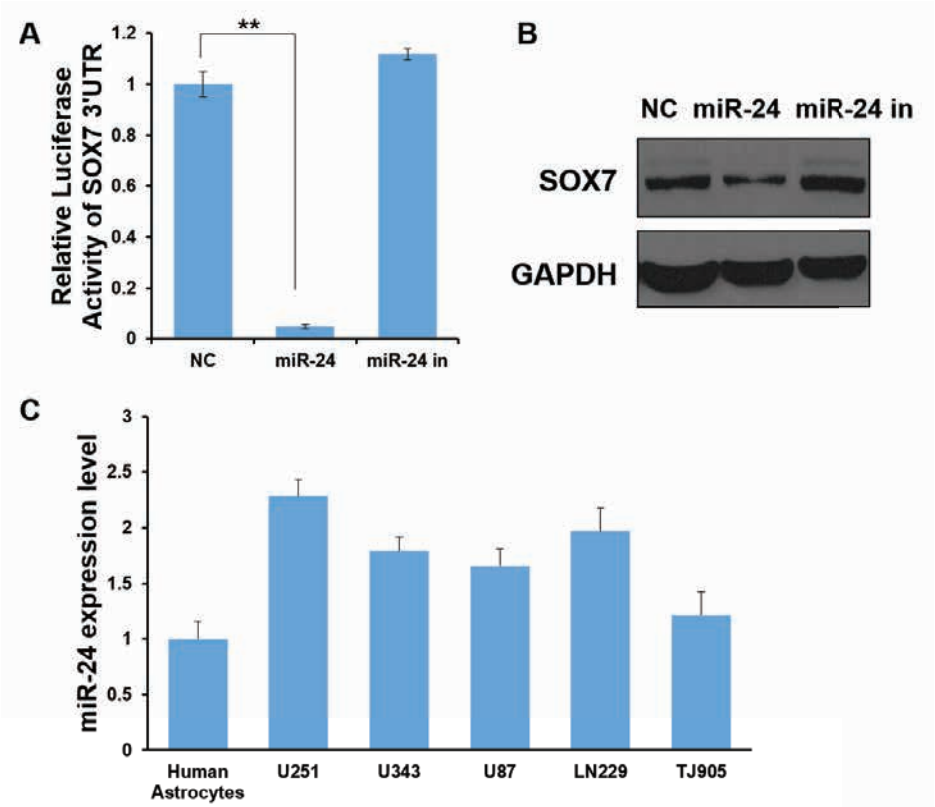

Figure 3: SOX7 might be a target of miR-24 in glioblastoma. (A) Luciferase reporter assays in U87 cells following co-transfection with 3'-UTR of SOX7 and miR-24 mimics or inhibitors, as indicated. Data are presented as means of three independent experiments. All data are shown as means ± SD. **P < 0.01. (B) U87 cells were transfected with 3'-UTR of SOX7 and miR-24 mimics or inhibitors, and SOX7 expression was measured via western blot. GAPDH was used as an internal control. (C) qPCR analysis of miR-24 in the GBM cell lines as in Figure 1B. Data were expressed as means ± SD. β-actin was used as an internal control.

provided another explanation of the oncogenic activity of miR-24 in GBM, through negatively regulating SOX7 transcription. Since this targeting had been reported in another paper discussed in HCC [17], we did not pay more attention in this regulation aspect. The result also supported the tumor suppressive role of SOX7 in GBM cells, by inhibiting cell proliferation. However, since it was known that SOX7 could also impact on cancer cell apoptosis, migration and invasion processes [22,23], further evidence demonstrating its role in these mentioned biological processes should be studied.

Data from luciferase reporter assays showed that miR-24 could directly binds to the 3'-UTR of SOX7 mRNA and repressed its expression at translation levels. Yu

and his colleagues reported that in colorectal cancer cells, decreased expression of SOX7 was partially due to the aberrant DNA methylation of the gene [24], in which SOX7 promoter was frequently hypermethylated in colorectal cancers. This finding provided important information about the regulation of intrinsic SOX7 expression. In future studies, we should also consider this epigenetic regulation mechanism governing low SOX7 expression.

Collectively, we demonstrated in this study that SOX7 mRNA was frequently downregulated in primary GBM tumor samples and established cell lines, which was at least partially due to the oncogenic miR-24 targeting. In detail, SOX7 could inhibit GBM cell proliferation. Data presented in this report represent the attempt to disclose the role of SOX7 in suppressing GBM, which may be useful in the development of new therapeutic strategies for GBM.

Conflict of interest statement: Authors state no conflict of interest.

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