Original Article
SOX2 inhibits cell proliferation and metastasis, promotes apoptotic by downregulating CCND1 and PARP in gastric cancer

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Abstract: Inconsistent results of Sex-determining region Y-box2 (SOX2) expression have been reported in gastric cancer (GC) before. Our recent studies showed that SOX2 was significantly downregulated in GC cells compared with GES-1 at both mRNA and protein level. Transfected with pcDNA3.1-SOX2 resulted in enforced expression of SOX2 at mRNA and protein levels compared with NC group in undifferentiated cell lines including HGC27 and BGC823. MTT assay showed that exogenous expressed SOX2 suppressed cell proliferation. FC analysis revealed that SOX2-overexpressing cells exhibited cell-cycle arrest and apoptosis. Transwell assay showed the anti-metastatic effect of SOX2 in GC cells. The subsequent results suggested CCND1 and PARP were downregulated in SOX2 overexpressed GC cells, and were responsible for the SOX2-induced anticancer effects. Thus, SOX2 proved to be an expected biomarker in GC diagnosis.

Keywords: SOX2, gastric cancer, anticancer, CCND1, PARP

Introduction

Gastric cancer (GC) is the third leading cause of cancer-related death worldwide and metastasis is main reason for more than 90% death of cancer [1]. However, the understanding of the molecular mechanism that governs GC malignancy remains limited. Although tumor-node-metastasis (TNM) classification is widely used, it is not enough to evaluate and monitor the metastasis and prognosis of GC due to tumor heterogeneity [2]. Sex-determining region Y-box2 (SOX2) gene, a highly conserved transcription regulator which is essential for stem cell self-renewal and pluripotency in embryonic stem cells [3, 4] and tumor stem cells [5], plays crucial role in cell cycle, proliferation and apoptosis. SOX2 was reported to be able to promote cell growth, increase the apoptosis-resistant properties in prostate cancer [6] and stimulate cell proliferation, migration, as well as tumor metastasis in breast cancer [7]. Knocking down or silencing SOX2 resulted in cell cycle arrest, cell growth inhibition and tumorigenicity loss in pancreatic cancer [8], lung squamous cell carcinoma cells [9] and Glioblastoma tumor-initiat-
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Materials and methods

Cells and cell culture

Human GC cell lines BGC823, SGC7901, HGC27, MKN45, MGC803 and one normal gastric epithelial cell GES-1 were obtained from the Shanghai Institute of Biochemistry and Cell Biology, Chinese Academy of Sciences (Shanghai, China). Cells were grown in RPMI1640 (HyClone) supplemented with 10% fetal bovine serum (FBS, GIBCO), 2 mM L-Glutamine and penicillin-streptomycin (100 U/ml and 100 μg/ml). Cells were maintained at 37°C in a humidified atmosphere containing 5% CO2.

RNA extraction and quantitative reverse transcription PCR (qRT-PCR)

Total RNA was extracted from cell precipitation by using TaKaRa MiniBEST Universal RNA Extraction Kit according to the manufacturer’s instructions. DNA was removed with 1 μl gDNA Eraser in a reaction containing 7 μl RNA and 2 μl 5×g DNA Eraser Buffer at 42°C for 2 min. The DNA-free RNA was reverse transcribed with a PrimeScript® RT reagent Kit with gDNA Eraser (Perfect Real Time) according to the manufacturer’s protocol. Quantitative PCR (qPCR) was performed on a CFX-96 Connect system (Bio-Rad) using FastStart Essential DNA Green Master (Roche) according to the manufacturer’s protocol. Amplification was performed by denaturation at 95°C for 5 min, followed by 40 three-step cycles of 95°C for 10 s, 60°C for 15 s and 72°C for 20 s. Melt curve analysis was carried out after the amplification. Each reaction was performed in triplicate and the expression of target genes mRNA was normalized to GAPDH by using the 2ΔΔCT method. Primers used in qRT-PCR were listed in Table 1.

Western bolt

Cells used for western blot were prepared using RIPA Lysis Buffer and protein concentrations were determined using the BCA assay kit (Beyotime, Shanghai, China). Equal amount of cell total protein was separated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and transferred to NC membrane (Millipore). After blocked with 5% non-fat milk, the membrane was then incubated with primary antibody, i.e., anti-SOX2 (1:1000, CST, #3579, rabbit), anti-CCND1 (1:1000, Atagenix, ata11263, rabbit), anti-PARP1 (1:1000, Atagenix, ata17635, rabbit), anti-GAPDH (1:5000, Atagenix, ata17456, rabbit) at 4°C overnight. After washing, the membrane was incubated with the corresponding secondary antibody conjugated to HRP (1:5000) for 1 h at room temperature. GAPDH was used as an internal control.

Plasmid construction and transient transfection

Sequence encoding human SOX2 was PCR amplified from cDNA prepared by reverse transcription (RT) of RNA extracted from human normal gastric epithelial cell GES-1. pcDNA3.1-SOX2 was constructed by inserting a NheI-XbaI fragment of SOX2 encoding sequence. Primers used for plasmid construction are listed in Table 2. To overexpress SOX2, pcDNA3.1 or pcDNA3.1-SOX2 were transiently transfected into HGC27 and BGC823 respectively using Lipofectamine 2000 transfection reagent (Invitrogen, Shanghai, China) following the manufacturer’s protocol and samples were collected at indicated times.

MTT assay

Transfected cells (24 h) were reseeded in to a 96-well plate (5×10³ cells/well, five wells per group). Cells were cultured for 24-96 h and then added 20 μl MTT solution [3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide, 5 mg/mL, Sigma, St.Louis, MO, USA] to each well. The supernatant was discarded after incubated at 37°C for 4 h. Next, 150 μl DMSO (Dimethylsulfoxide, Sigma) was added to each well and the plate was gently shaken for 10 min. The optical density (OD) value was obtained at a wavelength of 490 nm. The experiment was repeated 3 times.

Cell-cycle assay

Transfected cells were harvested and washed with PBS, followed by fixation with 70% ethanol

<table>
<thead>
<tr>
<th>Gene</th>
<th>Oligonucleotide sequence</th>
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<tbody>
<tr>
<td>SOX2</td>
<td>GCCGAGTGGAACTTTTGGC</td>
</tr>
<tr>
<td>CCND1</td>
<td>GCCGAGGAGAAACAAACAGAT</td>
</tr>
<tr>
<td>PARP</td>
<td>GCCGAGATCATCAGAGAAGTATG</td>
</tr>
<tr>
<td>GAPDH</td>
<td>GGTGTGAACCATGAGAAGTATGA</td>
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Table 1. Primers used for qRT-PCR
overnight at 4°C. After washing with PBS, the cells were resuspended in PBS containing 20 μg/ml propidium iodide (Biolegend) and 200 μg/ml RNase A for 30 min at room temperature in the dark. Samples were analysed for DNA content by flow cytometry. The cell-cycle phases were analysed using CytExpert software. The experiments were performed at least three times and representative results were shown.

Apoptosis assay

Transfected cells were harvested and washed with PBS, the cells were resuspended with 200 μl Annexin Binding Buffer (DojinDo). Then, 5 μl of Annexin V-FITC and PI (Dojin-Do) were added to the cell suspension and incubated at room temperature for 15 min in the dark before flow cytometry analysis. The experiments were performed at least three times and representative results were shown.

Cell migration and invasion assays

The migration and invasion assays were conducted using 24-well plates with 8-μm polycarbonate membranes Transwells (Costar, 3422). For cell migration and invasion assays, the Transwells were coated with or without Matrigel (BD Biosciences, 356234) according to the manufacturer’s instructions. After 24 h transfection, HGC27 and BGC823 transfected with pcDNA3.1 or pcDNA3.1-SOX2 in RPMI-1640 with 1% FBS were resuspended in the upper chamber (1×10⁵/well). The lower chamber was filled with 600 μl RPMI-1640 with 20% FBS. After 48 h incubation at 37°C, cells in the upper chambers were removed with a cotton swab, then the Transwell chambers were fixed in 4% PFA for 30 min and

Table 2. Primers used for plasmid construction

<table>
<thead>
<tr>
<th>Gene</th>
<th>Oligonucleotide sequence</th>
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<tr>
<td></td>
<td>Forward (5’-3’)</td>
</tr>
<tr>
<td>SOX2</td>
<td>GGAGACCCAAGCTGGCTAGCATGTACAACATGATGGAGACGG</td>
</tr>
</tbody>
</table>
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Figure 3. SOX2 overexpressing cells exhibited cell-cycle arrest and apoptosis. A, B: Apoptosis was examined by flow cytometry via Annexin/PI staining. An obvious apoptosis rate rise was observed in SOX2 overexpressed HGC-27 and BGC-823 cells compared with NC group. C, D: Cell-cycle analysis was measured by flow cytometry via propidium iodide staining. *P < 0.05, **P < 0.01, compared to NC. Note NC, pcDNA3.1; SOX2, pcDNA3.1-SOX2.

stained with crystal violet (Beyotime, Shanghai, China) for 20 min. The cells on the bottom of the chamber were then photographed and counted in 5 random visual fields.
Results

SOX2 is downregulated in GC cell lines

The expression of SOX2 mRNA in human GC cell lines HGC27, BGC823, MGC803, SGC7901, MKN45, and one normal gastric epithelial cell GES-1 was analyzed by qRT-PCR. The SOX2 mRNA level in GC cell lines was significantly downregulated compared with GES-1 (Figure 1A). Log_{10}(2^{-\Delta\DeltaCT}) was shown. Consistently, the result of western blot indicated SOX2 protein was also low expression or barely detectable in the GC cell lines (Figure 1B).

SOX2 expression inhibits GC cells proliferation

HGC27 and BGC823 were transiently transfected with pcDNA3.1 or pcDNA3.1-SOX2 using Lipofectamine 2000 transfection reagent to overexpress SOX2 respectively. qRT-PCR and western blot assays indicated that transfected with pcDNA3.1-SOX2 resulted in enforced expression of SOX2 compared with the negative control (Figure 2A and 2B). To determine the effect of SOX2 overexpression on GC cells proliferation, MTT assay was measured with pcDNA3.1 or pcDNA3.1-SOX2 transfected HGC27 or BGC823. OD490 was compared among different time points. SOX2 overexpressed HGC27 and BGC823 were greatly inhibited (P < 0.05) in a time-dependent manner at 48 h, 72 h and 96 h compared with NC group (Figure 2C and 2D).

SOX2 induces cell-cycle arrest and apoptosis in GC cell lines

To clarify the mechanisms underlying growth inhibition induced by SOX2 in HGC27 and
SOX2 overexpression inhibits GC cell migration and invasion

An *in vitro* Transwell chamber assay was performed to determine the effect of SOX2 overexpression on the migration ability of HGC27 and BGC823. The results demonstrated that the number of cells that migrated through the polycarbonate membrane to the lower surface of the membrane 72 h after transfection had significant difference between NC group and SOX2 overexpression group in both HGC27 and BGC823 cells. Exogenous SOX2 overexpression in HGC27 and BGC823 showed a strong migration resistance (Figure 4A). For HGC, NC group vs SOX2 group was 293.0±3.286 vs 240.2±3.2 (P < 0.01); for BGC, NC group vs SOX2 group was 318.4±4.32 vs 184.4±2.909 (P < 0.01).

Cell migration is a critical step in tumor invasion and metastasis. Therefore, we investigated whether the observed decreased cell migration in SOX2 overexpressed cells was associated with a decreased invasion ability in both HGC and BGC. An additional Transwell chamber assay was performed to determine the effect of exogenous SOX2 on the ability of HGC27 and BGC823 to penetrate the basement membrane. The transwell chamber result suggested that the number of cells that penetrated through the polycarbonate membrane to the lower surface of membrane in the SOX2 overexpressed HGC27 and BGC823 was significantly lower than the NC group (Figure 4B). For HGC27, NC group vs SOX2 group was 219.4±5.627 vs 135.2±4.091 (P < 0.01); for BGC823, NC group vs SOX2 group was 222.0±1.304 vs 95.2±1.985 (P < 0.01). Thus, exogenous SOX2 suppressed the invasion ability of HGC27 and BGC823.

**SOX2 mediates anticancer effect by downregulating CCND1 and PARP**

To investigate the potential molecular mechanisms of SOX2-induced anti-proliferation, cell-cycle arrest, anti-metastatic and pro-apoptotic
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In the present study, we validated SOX2 was downregulated or barely detectable in all the human GC cell lines HGC27, BGC823, MGC803, SGC7901, MKN45 compared with the normal gastric epithelial cell GES-1, which was consistent with the results of most researchers [22-25]. SOX2 was reported having an important function in lymph node metastasis [26]. Next, we firstly studied the function of exogenous SOX2 in undifferentiated cell lines HGC-27 and BGC-823 and discovered the anti-proliferation effects of SOX2. FC analysis revealed that the anti-proliferation effects were due to the cell-cycle arrest and apoptosis increase in SOX2-overexpressing cells. Subsequently, the effect of SOX2 overexpression inhibited the migration and invasion ability of HGC and BGC were confirmed by transwell chamber assay. Our research verified the results of most researchers, and enriched the data of SOX2’s role in GC cell lines.

CCND1 is an important cell-cycle-regulatory protein, whose main function is regulating the transition process from G1 phase to the DNA synthesis phase (S phase). CCND1 overexpression results in disorder of cell cycle or cell proliferation out of control, thus causing a variety of tumor diseases. PARP, a DNA repair enzyme, plays an important role in DNA damage repair and cell apoptosis. Otsubo et al [13] observed exogenous SOX2 diminished CCND1 and PARP in NUGC3 and GCIY cell lines. We also detect the two important factors after observing SOX2 induced anticancer effect and demonstrated that the reduction of CCND1 and PARP at mRNA and protein level was associated with SOX2 overexpression in both HGC and BGC. These results indicated that the two cell-cycle-regulating factors may be regulated by SOX2 at transcriptional levels. But further studies are necessary to verify the role of SOX2 in the regulation of cell cycle and cell apoptosis.

In conclusion, we revealed that SOX2 expression was downregulated in several GC cell lines. Exogenous SOX2 expression inhibited cell growth through cell cycle arrest and apoptosis in GC cell lines HGC-27 and BGC-823. Moreover, SOX2 contributed to the inhibition of GC cells metastasis and invasion, which may be due to the down-regulation of CCND1 and PARP. Our findings enriched the data of SOX2 as a specific and prognostic biomarker for metastatic GC but this should be validated in more prospective studies.

Disclosure of conflict of interest

None.

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