Knocking down of lncRNA SBF2-AS1 inhibits proliferation, invasion and induces apoptosis of colorectal cancer through regulating PTEN

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INTRODUCTION

Colorectal cancer (CRC) is the third-prevalent cancer throughout the world, affecting 1.36 million people in 2012 (1). The prognosis of CRC is closely related to the tumor stage at the initial diagnosis. The 5-year survival of early-stage CRC is up to 90%, which is less than 10% in metastatic CRC cases (2). It is of significance to uncover the molecular mechanism of CRC development, so as to improve the diagnostic and therapeutic efficacies of CRC.

LncRNAs are non-coding RNAs ranging from 200 nt to 100,000 nt long, which regulate gene expressions at the transcriptional and post-transcriptional levels (3,4). Recently, lncRNAs have been identified to participate in diverse physiological and pathological processes (5). Increasing evidences have proved that dysregulated lncRNAs in tumor may influence the occurrence and progression of tumors (6,7). It is reported that abnormally expressed lncRNA in CRC is capable of mediating the development of CRC (8). As a newly discovered lncRNA, SBF2-AS1 is able to stimulate the metastasis of hepatocellular carcinoma through regulating EMT (9). The specific function of SBF2-AS1 in CRC, however, remains unclear.

PTEN (phosphatase and tensin homolog deleted on chromosome ten) is a tumor-suppressor gene located on chromosome 10q23 (10). PTEN is determined as a bispecific phosphatase, and its substrate can be lipids or proteins (11). Functional incapacitation of somatic PTEN would lead to tumorigenesis (12). A recent study reported the vital function of PTEN deficiency in the progression of CRC (13).

This study aimed to elucidate whether SBF2-AS1 (SBF2 antisense RNA 1) could regulate the progression of CRC through regulating PTEN. We hope to provide new ideas for the clinical treatment of CRC.

MATERIALS AND METHODS

SUBJECTS AND CLINICAL SAMPLES

Tumor tissues and matched adjacent tissues 3 cm away from the tumor edge were harvested from CRC patients undergoing surgery in our hospital. Resected samples were placed into liquid nitrogen until analyses. Enrolled CRC patients were pathologically diagnosed and denied a medical history of other malignancies. None of the enrolled patients received preoperative treatments. This study was approved by the Medical Ethics Committee of Tongde Hospital of Zhejiang Province and informed consent from each subject. The patient consent was written informed consent, and this was conducted in accordance with the

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Declaration of Helsinki.

Cell culture and transfection

CRC cell lines (SW480, DLD1 and Lovo) and colorectal epithelial cell line (NCM640) were obtained from Cell Bank, Shanghai. Cell culture was conducted in Roswell Park Memorial Institute 1640 (RPMI 1640) containing 10% fetal bovine serum (FBS) (Gibco, Rockville, MD, USA) and 1% penicillin-streptomycin in a 5% CO₂ incubator at 37°C. For cell transfection, cells were seeded in a 6-well plate with 1×10⁴ cells per well. Until 60% confluence, cell transfection was conducted using Lipofectamine™ 2000. The complete medium was replaced 6 hours later.

RNA extraction and Quantitative real-time polymerase chain reaction (qRT-PCR)

RNA extraction was performed using the TRIzol (Invitrogen, Carlsbad, CA, USA) method. The extracted RNA was quantified and reversely transcribed into cDNA, followed by PCR using the SYBR Green method. Primer sequences are listed in Table 1.

Cell Counting Kit (CCK-8) assay

Cells were seeded in the 96-well plate with 2×10⁴ cells per well. Absorbance (A) at 450 nm was recorded at the appointed time points using the CCK-8 kit (Dojindo Laboratories, Kumamoto, Japan) to depict the viability curve.

5-Ethynyl-2′-deoxyuridine (EdU) assay

Cells were seeded in the 24-well plate with 2×10⁴ cells per well. Cells were labeled with 50 μmol/L EdU at 37°C for 2 h, fixed in 4% paraformaldehyde for 30 min and incubated in PBS containing 0.5% Triton-100 for 20 min. After washing with phosphate-buffered saline (PBS) containing 3% bovine serum albumin (BSA), 100 μl of dying solution was applied per well for 1 h incubation in the dark and cells were counter-stained with 4′,6-diamidino-2-phenylindole (DAPI) for 30 min. The ratio of EdU-positive cells was calculated.

Flow cytometry

Cells were prepared for single-cell suspension. Cells were double-stained with 5 μl of Annexin V and 5 μl of fluorescein isothiocyanate (FITC) in the dark for 10 min. The apoptotic rate was finally determined using flow cytometry.

Transwell invasion assay

50 μl of FN (100 μg/ml) was coated in the bottom of the Transwell chamber. Cell density was adjusted to 1×10⁶/ml. 100 μl of suspension was applied in the upper side of the Transwell chamber (Millipore, MA, USA) pre-coated with 100 μl of diluted Matrigel (BD bioscience, Franklin Lakes, USA). On the bottom side, 600 μl of medium containing 10% FBS was applied. After 48 h of incubation, invasive cells were fixed in methanol for 30 min and dyed with 0.1% crystal violet for 10 min. Invasive cells were captured and counted in 6 randomly selected fields per sample.

RNA immunoprecipitation (RIP)

Cells were treated according to the procedures of Millipore Magna RIP™ RNA-Binding Protein Immunoprecipitation Kit. The cell lysate was incubated with anti-EZH2, anti-SUZ12 or IgG antibodies at 4°C for 6 h. A protein-RNA complex was captured and digested with 0.5 mg/ml proteinase K containing 0.1% sodium dodecyl sulphate (SDS) to extract RNA. The magnetic beads were repeatedly washed with RIP washing buffer to remove non-specific adsorption as much as possible. Finally, the extracted RNA was subjected to qRT-PCR.

Western blot

Total protein was extracted from cells or tissues using radioimmunoprecipitation assay (RIPA) (Beyotime, Shanghai, China) and loaded for electrophoresis. After transferring on a polyvinylidene fluoride (PVDF) membrane (Millipore, Billerica, MA, USA) membrane, it was blocked in 5% skim milk for 2 hours, incubated with primary antibodies at 4°C overnight and secondary antibodies for 2 hours. Bands were exposed by electrochemiluminescence (ECL) (Pierce, Rockford, IL, USA) and analyzed by Image Software.

Determination of subcellular distribution

Cytoplasmic and nuclear RNAs were extracted using the PARIS kit (Invitrogen) and subjected to qRT-PCR. U6 was the internal reference of the nucleus and glyceraldehyde-3-phosphate dehydrogenase (GAPDH) was that of the cytoplasm.

Statistical analysis

Statistic Package for Social Science (SPSS) 16.0 (SPSS

Table 1. Primer sequences.

<table>
<thead>
<tr>
<th>Primer</th>
<th>Forward (5’-3’)</th>
<th>Reverse (5’-3’)</th>
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<tr>
<td>U6</td>
<td>F: 5’-CTCGCTTCGCGAGCGAGACA-3’</td>
<td>R: 5’-AACGGCTCAGAATTTGCGT-3’</td>
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<tr>
<td>PTEN</td>
<td>F: 5’-ACACGACGGGAAGACAAGUU-3’</td>
<td>R: 5’-UCCUCUGGUCCUGGUAAGAAG-3’</td>
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<td>EZH2</td>
<td>F: 5’-TGCACATCTCTGACTTCTGTG-3’</td>
<td>R: 5’-AAGGGCATTCACCAACTCC-3’</td>
</tr>
<tr>
<td>SBF2-AS1</td>
<td>F: 5’-CGGAGTCAACGGATTTGGTCGT-3’</td>
<td>R: 5’-UAGCAGUAGACUCCUUCUGGG-3’</td>
</tr>
<tr>
<td>GAPDH</td>
<td>F: 5’-CGGAGTCAACGGATTTGGTCGT-3’</td>
<td>R: 5’-GGGAAGGATCTGTCTCTGACC-3’</td>
</tr>
</tbody>
</table>
IBM, Armonk, NY, USA) software was used for data analyses. Data were expressed as mean ± standard deviation. Intergroup differences were analyzed by the t-test. Pearson’s correlation analysis was conducted to evaluate the expression relationship between two genes. P < 0.05 was considered statistically significant.

Results

SBF2-AS1 was upregulated in CRC

Compared with matched normal tissues, qRT-PCR data showed upregulated SBF2-AS1 in CRC tissues (Figure 1A). In particular, the SBF2-AS1 level was higher in CRC tissues with > 5 cm in size than those ≤ 5 cm (Figure 1B). Based on the tumor stage of CRC patients, SBF2-AS1 level was higher in CRC patients with stage III-IV compared with those with stage I-II (Figure 1C). It is indicated that SBF2-AS1 was closely correlated to the disease progression of CRC.

SBF2-AS1 promoted proliferative ability and suppressed apoptosis of CRC cells

Similarly, SBF2-AS1 was upregulated in CRC cell lines relative to the colonic epithelial cell line (Figure 2A). SW480 and DLD1 cells expressed high abundance of SBF2-AS1 and were selected for the following experiments. Among the three constructed shRNAs targeting SBF2-AS1, sh-SBF2-AS1 1# and sh-SBF2-AS1 2# presented an effective transfection efficacy to downregulate SBF2-AS1 level in CRC cells (Figure 2B). Cell viability was inhibited greatly after transfection of sh-SBF2-AS1 1# or sh-SBF2-AS1 2# in SW480 and DLD1 cells (Figure 2C, D). EdU assay identically showed the reduced number of EdU-positive cells after transfection of sh-SBF2-AS1 1# or sh-SBF2-AS1 2# in CRC cells (Figure 2E). The apoptotic rate was elevated by transfection of sh-SBF2-AS1 1# or sh-SBF2-AS1 2# in CRC cells (Figure 2F).

Collectively, the knockdown of SBF2-AS1 suppressed proliferative ability and enhanced apoptosis in CRC.

SBF2-AS1 stimulated the invasive ability of CRC

To evaluate the potential influence of SBF2-AS1 on the malignant phenotype of CRC cells, a Transwell assay was conducted to assess the invasive ability. After transfection of sh-SBF2-AS1 1# or sh-SBF2-AS1 2#, the invasive ability of SW480 and DLD1 cells was attenuated (Figure 3).

SBF2-AS1 mediated PTEN level via recruiting EZH2

Pearson’s correlation analysis identified a negative correlation between SBF2-AS1 and PTEN in CRC (Figure 4A). Transfection of sh-SBF2-AS1 1# or sh-SBF2-AS1 2# upregulated mRNA and protein levels of PTEN in CRC cells (Figure 4B-E). To analyze the biological function of SBF2-AS1, its subcellular distribution was assessed. SBF2-AS1 was found to be mainly distributed in the nuclear fraction (Figure 4F). Compared with control IgG, RIP assay showed higher enrichment of SBF2-AS1 in enhancer of zeste 2 polycomb repressive complex 2 subunit (EZH2) and SUZ12 polycomb repressive complex 2 subunit (SUZ12) (Figure 4G). Interestingly, transfection of sh-EZH2 markedly upregulated PTEN at both mRNA and protein levels (Figure 4H-J). To reveal the influence of SBF2-AS1 on PTEN expression, ChIP assay was conducted to analyze their interaction. As the data illustrated, the knockdown of SBF2-AS1 decreased the recruitment ability of EZH2 to PTEN (Figure 4K). To sum up, the above results suggested that SBF2-AS1 mediated PTEN level by recruiting EZH2.

SBF2-AS1 mediated the proliferative ability of CRC through regulating the PTEN level

The transfection efficacy of sh-PTEN was verified in

![Figure 1](image1.png)

**Figure 1.** SBF2-AS1 was upregulated in CRC. (A) The relative level of SBF2-AS1 in CRC tissues and matched normal tissues. (B) Relative level of SBF2-AS1 in CRC tissues > 5 cm in size and those ≤ 5 cm. (C) Relative level of SBF2-AS1 in CRC patients in stage I-II and stage III-IV.

![Figure 2](image2.png)

**Figure 2.** SBF2-AS1 promoted proliferative ability and suppressed apoptosis of CRC cells. (A) The relative level of SBF2-AS1 in CRC cell lines (DLD1, SW480 and Lovo) and colonic epithelial cell line (NCM460). (B) Transfection efficacy of sh-SBF2-AS1 1#, sh-SBF2-AS1 2# and sh-SBF2-AS1 3# in SW480 and DLD1 cells. (C) CCK-8 showed viability in SW480 cells transfected with sh-NC, sh-SBF2-AS1 1# or sh-SBF2-AS1 2#. (D) CCK-8 showed viability in DLD1 cells transfected with sh-NC, sh-SBF2-AS1 1# or sh-SBF2-AS1 2#. (E) EdU assay showed EdU-labeled cells in DLD1 and SW480 cells transfected with sh-NC, sh-SBF2-AS1 1# or sh-SBF2-AS1 2#. (F) The apoptotic rate in DLD1 and SW480 cells transfected with sh-NC, sh-SBF2-AS1 1# or sh-SBF2-AS1 2#.

![Figure 3](image3.png)

**Figure 3.** SBF2-AS1 stimulated the invasive ability of CRC. Transwell assay showed invasion in SW480 and DLD1 cells transfected with sh-NC, sh-SBF2-AS1 1# or sh-SBF2-AS1 2#.

![Figure 4](image4.png)

**Figure 4.** SBF2-AS1 mediated PTEN level via recruiting EZH2. (A) Pearson’s correlation analysis identified a negative correlation between SBF2-AS1 and PTEN in CRC. (B) Transfection of sh-SBF2-AS1 1# or sh-SBF2-AS1 2# upregulated mRNA and protein levels of PTEN in CRC cells. (C) CCK-8 showed viability in SW480 cells transfected with sh-NC, sh-SBF2-AS1 1# or sh-SBF2-AS1 2#. (D) CCK-8 showed viability in DLD1 cells transfected with sh-NC, sh-SBF2-AS1 1# or sh-SBF2-AS1 2#. (E) EdU assay showed EdU-labeled cells in DLD1 and SW480 cells transfected with sh-NC, sh-SBF2-AS1 1# or sh-SBF2-AS1 2#. (F) The apoptotic rate in DLD1 and SW480 cells transfected with sh-NC, sh-SBF2-AS1 1# or sh-SBF2-AS1 2#.
SW480 cells (Figure 5A-B). CCK-8 assay showed that transfection of sh-PTEN remarkably enhanced the viability in SW480 cells (Figure 5C). Notably, decreased EdU-positive cell number by transfection of sh-SBF2-AS1 1# was partially reversed after co-transfection of sh-PTEN (Figure 5D). Hence, it is believed that SBF2-AS1 accelerated CRC cells to proliferate via inhibiting PTEN level.

Discussion

CRC is a major public health problem globally that leads to tumor death (14). CRC is a heterogeneous disease. Its pathogenesis involves oncogene activation and tumor suppressor inactivation. These genetic alterations are mainly resulted from epigenetic changes (15,16). This experiment investigated the correlation between SBF2-AS1 and the progression of CRC in the Chinese population. SBF2-AS1 was identified to be upregulated in CRC, especially in the advanced stage. It is speculated that SBF2-AS1 was involved in the malignant progression of CRC.

LncRNA participates in biological processes by interacting with various macromolecules such as proteins and RNAs (17,18). Certain lncRNAs are related to the survival of CRC patients, which could serve as prognostic hallmarks (19). In this paper, the knockdown of SBF2-AS1 attenuated the proliferative and invasive abilities of CRC cells. RIP assay further identified the binding between SBF2-AS1 and EZH2. As a catalytic subunit of PRC2, EZH2 catalyzes the trimethylation of H3K27me3 and subsequently silences downstream genes (20-22). A relevant study pointed out that lncRNA HOXD antisense growth-associated long non-coding RNA (HOXD-AS1) inhibits p57 level by binding to EZH2, thus accelerating the progression of osteosarcoma (23). This study consistently verified that SBF2-AS1 recruited EZH2 to further mediate the progression of CRC.

PTEN is one of the most common mutant tumor-suppressor genes in human cancers (10). PTEN exerts important cellular functions in tumor cells (24). By analyzing human samples and mice samples, it is indicated that partial deficiency of PTEN is able to stimulate malignant phenotypes of tumor cells. Moreover, downregulation of PTEN levels below 50% aggravates tumor progression (25). This study demonstrated that PTEN level was negatively regulated by SBF2-AS1 in CRC cells. Interestingly, the silence of PTEN reversed the regulatory effect of SBF2-AS1 on the proliferative ability of CRC cells. Collectively, SBF2-AS1 aggravated the progression of CRC via downregulating PTEN through EZH2 recruitment.

Conclusions

LncRNA SBF2-AS1 is upregulated in CRC, and LncRNA SBF2-AS1 is upregulated in CRC. Knocking down of lncRNA SBF2-AS1 inhibits proliferation, and invasion and induces apoptosis of colorectal cancer by interacting with EZH2 to downregulate PTEN level. SBF2-AS1 may be a promising hallmark for the diagnosis and treatment of CRC.

Conflict of Interests

The authors declared no conflict of interest.

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References


