Overexpression of SULT2B1b Promotes Angiogenesis in Human Gastric Cancer

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Abstract

Background/Aims: Overexpression of cytosolic sulfotransferase 2B1b (SULT2B1b) has been commonly found in colorectal and hepatocellular carcinoma, suggesting that SULT2B1b might act as a potential oncogenic protein. However, its clinical significance and biological role in gastric cancer progression remain largely unknown.

Methods: Expressions of SULT2B1b in clinical gastric cancer (GC) samples were examined using qRT-PCR and Western blot. Results: SULT2B1b was markedly overexpressed in human GC samples, and positively correlated with vessel density and associated with poor clinical features. We also demonstrated that overexpression of SULT2B1b resulted in increased tumor angiogenesis and tumor growth in mouse GC models. In addition, ablation of SULT2B1b in human GC cell lines BGC823 and MKN45 decreased the capability of the cells to recruit endothelial cells. Moreover, depletion of SULT2B1b in GC cells reduced VEGF-A expression by downregulating SP1 and AP2.

Conclusion: Our results suggested that the SULT2B1b-mediated angiogenic pathway could serve as biomarkers for GC diagnosis and prognosis, and suppressing SULT2B1b-mediated angiogenic signaling might be a promising strategy for developing novel GC treatment.

Introduction

Gastric cancer (GC) is the fourth most prevalent malignancy worldwide, and the second most frequent cancer death [1]. Despite significant achievements in the treatment of early stage GC, the long-term survival rate for advanced GC remains quite low [2]. The five-year survival rate for advanced or metastatic gastric cancer is only 5-20%, with a median overall...
survival of less than 1 year [3, 4]. Little is known about the molecular events critical to GC angiogenesis, although it is an important aspect of tumor growth. Therefore, unraveling the factors driving this process is important for future therapeutic interventions and for providing novel biomarkers for prognosis and treatment prediction [5, 6].

Cytosolic sulfotransferase 2B1b (SULT2B1b), a member of the SULT2 family, is expressed in multiple normal tissues including prostate, skin, breast, intestine, lung, and placenta [7, 8]. Previous studies have demonstrated that SULT2B1b played an important role in cholesterol and oxysterol sulfate synthesis [9, 10]. Recently, it is reported that overexpression of SULT2B1b promoted hepatocyte proliferation by inactivating oxysterol/LXR signaling, indicating its involvement in liver proliferation [11]. To date, aberrant expression of SULT2B1b has been documented in several human malignancies including breast, endometrial, prostate and hepatocellular carcinomas [12-16]. However, its expression pattern and clinical relevance in GC has not been investigated to date.

In the current study, we aimed to investigate the clinical significance and biological role of SULT2B1b in gastric cancer progression. Our results indicate that SULT2B1b potentially has a critical oncogenic role in GC progression, and provide new insights into the mechanisms that lead to upregulation of VEGF-A in GC.

**Materials and Methods**

**Cell culture**

Human GC cell lines BGC823, MKN45 cells and human umbilical vein endothelial cells (HUVECs) were purchased from the Shanghai Institute of Biochemistry and Cell Biology, Chinese Academy of Sciences (Shanghai, China). Cells were cultured in Roswell Park Memorial Institute (RPMI) 1640 medium supplemented with 100 U/ml penicillin, 100 μg/ml streptomycin and 10% fetal bovine serum. The cells were grown at 37°C in the presence of 5% CO₂ in a humidified incubator. Cycloheximide and MG132 were purchased from Sigma-Aldrich (St Louis, MO).

**Clinical samples**

Paired tumor specimens of 72 gastric cancer patients were obtained from archives of the Department of Pathology of the 2nd Affiliated Hospital of Fujian Medical University, China. The patients included 43 males and 29 females, ranging in age from 29 to 82 years. Tumor histological types and differentiation were determined based on World Health Organization for GC criteria [17]. Tumor staging was defined according to the tumor-node-metastasis (TNM) classification system from International Union Against Cancer (UICC) recommendation [18]. All patients received chemotherapy (fluorouracil, mitomycin, and adriamycin) after gastrectomy and were followed up. All human tissues were obtained with informed consent and approval from the Committees for Ethical Review of Research in the 2nd Affiliated Hospital of Fujian Medical University.

**RNA isolation, reverse transcription and qRT-PCR**

Total RNA was extracted from the GC tissues and adjacent nontumourous GC tissues by Trizol Reagent (Invitrogen, Carlsbad, CA, USA). For SULT2B1b detection, reverse-transcribed complementary DNA was synthesized with the PrimeScript RT reagent Kit (TaKaRa, Dalian, China), and quantitative real-time-PCR (qRT-PCR) was performed with SYBR Premix ExTaq (TaKaRa, Dalian, China) with the Stratagene Mx3000P real-time PCR system (Agilent Technologies, Inc., Santa Clara, CA, USA). The relative expression ratio of SULT2B1b in each paired tumour and nontumourous tissue was calculated by the 2^−ΔΔCT method. For mRNA analyses, cDNA was synthesized using Moloney murine leukaemia virus reverse transcriptase (Promega, Madison, WI, USA). qRT-PCR was performed with SYBR Premix ExTaq with the Stratagene Mx3000P real-time PCR system. β-actin was validated before acting as internal controls for mRNA quantification. The average fold change of β-actin (using ribosomal 18S RNA as internal standard) in GC cell lines BGC823, MKN45 or GC tumor tissues is < 2. According to our selection criteria of < 2-fold, β-actin is the suitable gene for a reference gene. PCR reactions for each gene were repeated three times. Independent experiments were done in triplicate. The primers used in this study are as follows: SULT2B1b 5'-GCT TGT GGG ACA CCT
IHC staining using an anti-CD31 (Cell Signaling Technology), anti-VEGF-A, anti-SP1 or anti-AP2 antibodies with calipers and tumor volumes were calculated using the equation 

\[ \text{Volume} = \frac{1}{2} \times \text{length} \times \text{width}^2 \]

Tumors were examined twice weekly; length and width measurements were obtained subcutaneously with BGC823/Vector cells (5 × 10^6) in the right dorsal flank. Another group was inoculated subcutaneously with BGC823/SULT2B1b cells (5 × 10^6) in the left dorsal flank and with BGC823/SULT2B1b-RNAi cells (5 × 10^6) in the right dorsal flank. Tumors were examined twice weekly; length and width measurements were obtained with calipers and tumor volumes were calculated using the equation 

\[ \text{Volume} = \frac{1}{2} \times \text{length} \times \text{width}^2 \]

After infection. The cells without DNA and transfection reagent are used as the negative control.

**Vectors, retroviral infection and transfection**

pMSCV/SULT2B1b-overexpressing human SULT2B1b was generated by subcloning the PCR-amplified human SULT2B1b coding sequence into pMSCV vector (Clontech, Mountain View, CA, USA). To silence endogenous SULT2B1b, small interfering RNA oligonucleotides were cloned to generate pSuper-retro-SULT2B1b-RNAi. The following siRNA specific for SULT2B1b was used: 5'-CAG AUC UUC ACC AAG GCC UUC UUCA-3' (Invitrogen). BGC823 GC cells stably expressing SULT2B1b-complementary DNA or SULT2B1b-RNA interference (RNAi) were established. Transfection of small interfering RNA or plasmids was performed using the Lipofectamine 2000 reagent (Invitrogen) according to the manufacturer's instruction. Stable cell lines expressing SULT2B1b or SULT2B1b-RNAi were selected for 10 days with 0.5 μg/ml puromycin 48 h after infection. The cells without DNA and transfection reagent are used as the negative control.

**Histological and immunostaining**

GC tissues and adjacent nontumorous GC tissues were fixed, dehydrated, embedded and sectioned according to standard procedures. Antigens were retrieved by boiling in the citrate buffer (10 mM) for 20 min or as suggested by manufacturers. All sections were incubated with primary antibodies diluted in phosphate-buffered saline at 4 °C overnight. The rabbit anti SULT2B1b and rabbit anti-CD31 antibodies were purchased from Abcam. Specifically bound antibodies were detected with alkaline phosphatase staining or fluorescein isothiocyanate-conjugated secondary antibodies (Life Technologies, Grand Island, NY, USA). Immunochimical staining of SULT2B1b (dilution 1:2,000) in the gastric cancer tissues and matched adjacent normal tissues was performed. To validate the specificity of the SULT2B1b antibody used in immunostaining, SULT2A1 was used as an appropriate antibody isotype control. Immunostaining for SULT2B1b in human paraffin embedded tonsil tissue and immunostaining in tumor tissue with isotype control acted as positive and negative controls, respectively. For CD31 staining, nuclei were counterstained with To-Pro3 (TP3) iodide (Invitrogen, Carlsbad, CA). Two experienced pathologists independently scored the results without any information about the samples. Only epithelial staining was counted. The scores were compared, and discrepant scores were subjected to re-examining by both individuals to achieve a consensus score. Tumor cell proportions were scored as follows: 0, no positive tumor cells; 1, <10% positive tumor cells; 2, 10 - 35% positive tumor cells; 3, 35-75% positive tumor cells; 4, >75% positive tumor cells. Staining intensity was graded according to the following standard: 1, no staining; 2, weak staining (light yellow); 3, moderate staining (dark yellow/light brown); 4, strong staining (dark brown). The staining index (SI) was calculated as the product of the staining intensity score and the proportion of positive tumor cells. Using this method of assessment, we evaluated SULT2B1b protein expression in GC tissues and adjacent nontumorous GC tissues by determining the SI. Samples with a SI ≧8 were determined as high expression and samples with a SI <8 were determined as low expression.

**Xenografted tumor model and IHC staining**

All procedures and experiments involving animals in this study were performed in accordance with the National Institutes of Health Guide for Care and Use of Laboratory Animals. The protocol was approved by the Committee on the Ethics of Animal Experiments of the 2nd Affiliated Hospital of Fujian Medical University. All surgery was performed under sodium pentobarbital anesthesia, and all efforts were made to minimize suffering. Female BALB/c-nu/nu mice of 4-5 weeks of age were purchased from Shanghai SLAC Laboratory Animal Co., Ltd. (Shanghai, China) and were housed in the Animal Resource Facility. The BALB/c nude mice were randomly divided into two groups (n = 6 per group). One group of mice was inoculated subcutaneously with BGC823/Vector cells (5 × 10^6) in the left dorsal flank and with BGC823/SULT2B1b cells (5 × 10^6) in the right dorsal flank per mouse. Another group was inoculated subcutaneously with BGC823/RNAi-vector cells (5 × 10^6) in the left dorsal flank and with BGC823/SULT2B1b-RNAi cells (5 × 10^6) in the right dorsal flank. Tumors were examined twice weekly; length and width measurements were obtained with calipers and tumor volumes were calculated using the equation 

\[ \text{Volume} = \frac{1}{2} \times \text{length} \times \text{width}^2 \]

After 6 weeks, animals were killed, tumors were excised and paraffin embedded. Serial 4.0 μm sections were cut and subjected to IHC staining using an anti-CD31 (Cell Signaling Technology), anti-VEGF-A, anti-SP1 or anti-AP2 antibodies
(Abcam). The images were captured using the AxioVision Rel.4.6 computerized image analysis system (Carl Zeiss). The images were quantified by counting the number of positive cells, in 10 random fields at ×200 magnification for each tumor.

**Endothelial recruitment**

BGC823 cells (2 × 10^4/well) seeded in 24-well plates and were transfected with the indicated siRNAs. After being cultured in 37°C for 48 h, the cells were washed with phosphate-buffered saline and the culture media were replaced with 0.2% FBS Endothelial Growth Medium. Each well was then inserted a chamber with an 8.0 μm pore size membrane (BD Falcon, San Jose, CA, USA) containing 1 × 10^5 serum-starved HUVECs in 0.5 ml 0.2% FBS- Early Cleavage medium. After the co-culture for 32 h at 37°C, HUVEC cells were fixed with 4% paraformaldehyde and stained with hematoxylin. Three randomly picked areas in each insert were imaged and numbers of migrated HUVEC cells in each imaged area were counted. Data are mean ± SD of three replicates.

**Scratch wound healing**

HUVECs (1 × 10^5 cells/well) were seeded in six-well plates pre-coated with 0.1% gelatin. The cells were grown to confluence, followed by serum-starvation overnight and mitomycin treatment to stop cell proliferation. A scratch in each well was made with a pipette tip. The cells were then cultured in the Endothelial Growth Medium containing 0.5% FBS with or without supplemented with BGC823-conditioned media. The number of HUVECs that migrated was counted using a phase-contrast microscope.

**HUVEC tube formation**

HUVECs (2 × 10^4) were seeded in 24-well plates containing 0.5 ml solidified Matrigel (10 mg/ml) and cultured in the Endothelial Growth Medium with or without the supplementation of BGC823-conditioned medium for 8 - 12 h at 37°C. Images were acquired with a phase-contrast microscope. Average numbers of tubes were counted in three individual wells and presented as mean ± SD.

**Western blot analysis**

The procedure for the preparation of the nuclear and cytoplasmic fractions from BGC823 cells was based on previously described method [19]. Western blot was performed as previously described [20], using anti-SULT2B1b, anti-SULT2A1, anti-VEGF-A, anti-SP1, anti-AP2 or anti- anti-HDAC1 antibodies (Abcam). Blotting membranes were stripped and re-probed with anti-β-Actin antibody (Cell Signaling Technology) as a loading control.

**Luciferase assay**

Twenty thousand cells were seeded in triplicate in 48-well plates and allowed to settle for 24 h. One hundred nanograms of the control-luciferase plasmid, or VEGF-A luciferase reporter plasmid or luciferase reporter plasmids containing different fragments of VEGF-A promoter, plus 1 ng of pRL-TK renilla plasmid (Promega), were transfected into cells using the Lipofectamine 2000 reagent (Invitrogen) according to the manufacturer’s recommendations. Luciferase and renilla signals were measured 36 h after transfection using the Dual Luciferase Reporter Assay Kit (Promega) according to a protocol provided by the manufacturer.

**Sulfotransferase Assay**

The sulfotransferase activity of BGC823 cells (Chinese Academy of Sciences (Shanghai, China)), SULT2B1b overexpression BGC823 cells, GC tumor tissues or adjacent tissue (2nd Affiliated Hospital of Fujian Medical University, China) was measured in 100 µL of 25 mM Tris-Cl (pH 7.2) buffer containing 0.02 nmol of [3H]-cholesterol dissolved in 3 µL of ethanol, 100 µg of total protein, 5 mM MgCl2, 8 mM DTT, 100 µM 3′-phosphoadenosyl 5′-phosphosulfate (PAPS) at 37°C for 1 h. Tissues were prepared at 4°C, and for experiments with cell-free preparations, thawed samples (200 mg) were homogenized in 1 ml ice-cold homogenization buffer. Aliquots were removed for protein determination. Whole homogenate was used to test SULT2B1b enzyme activity. Reaction mixtures were incubated in a circulating water bath at 37°C and were stopped by placing the samples on ice and adding 200 µL of barium acetate (0.1 M). The Michaelis-Menten equation, \[ V = \frac{V_{\text{max}} \times [S]}{K_m + [S]} \], was used to analyze the kinetic data. Lipids were extracted with 3.3 volumes of chloroform-methanol (1:1, v/v). Since SULT2B1b sulfates dehydroepiandrosterone (DHEA),
this steroid also was used as a substrate for validation. The radioactivity counts in methanol-water-soluble phase and chloroform phase were determined by liquid scintillation counting. The sulfotransferase activity was calculated by the ratio of methanol-water-soluble counts to the sum of chloroform/methanol-water-soluble counts.

Statistical analysis
Using GraphPad Prism software, a One-way ANOVA Newman-Keuls Multiple Comparisons Test was used to compare the differences between three or more groups, and a two-tailed Student's test was used to compare the statistical difference between two groups. The differences were considered statistically significant when the \( P \) value was < 0.05.

**Results**

Overexpression of SULT2B1b in human GC is associated with tumor angiogenesis and poor prognosis of GC patients

SULT2B1b is found to be highly expressed in colorectal and hepatocellular carcinoma [14, 16], suggesting that SULT2B1b overexpression might contribute to the development and progression of cancer. However, whether aberrant SULT2B1b signaling is associated with and contributes to overall tumor angiogenesis in human GC has not been established. The gene for human SULT2B1 encodes two isoforms, SULT2B1a and SULT2B1b. Both the two isoforms were detected by qRT-PCR. The data showed that SULT2B1a expression was almost absent, whereas SULT2B1b was highly expressed in GC tumor tissues (Fig. 1). To validate the specificity of the SULT2B1b antibody used in the study, we compared the protein expression of SULT2B1b and one isoform SULT2A1 in GC tumor tissues. The result showed that the protein level of SULT2B1b was much higher than SULT2A1 in GC tumor tissues, suggesting the specificity of the SULT2B1b was acceptable in this study (Fig. 2).

![Fig. 1.](image1.png)

**mRNA levels of SULT2B1a and SULT2B1b in GC tumor tissues.** The mRNA expression levels of SULT2B1a and SULT2B1b in human gastric cancer tissues and corresponding adjacent non-tumor tissues were determined by qRT-PCR. Data are presented as the mean ± SD from three independent experiments. **, \( p < 0.01 \) compared with the control.

![Fig. 2.](image2.png)

**Protein levels of SULT2B1b and SULT2A1 in GC tumor tissues.** The expression of the SULT2B1b and SULT2A1 in human gastric cancer tissues and corresponding adjacent non-tumor tissues were determined by Western blot.
Next, to study the role of SULT2B1b in gastric cancer, we analyzed the mRNA and protein levels of SULT2B1b in 72 pairs of gastric cancers and matched adjacent normal gastric tissue samples. The results indicated that SULT2B1b was differentially upregulated at both
Table 1. Summary of SULT2B1b immunostaining scores from the human tumor samples

<table>
<thead>
<tr>
<th>Score of staining index (SI)</th>
<th>Tumor tissues</th>
<th>Adjacent non-tumor tissues</th>
</tr>
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<tbody>
<tr>
<td>&lt;2</td>
<td>2 (2.8%)</td>
<td>71 (98.6%)</td>
</tr>
<tr>
<td>2-4</td>
<td>3 (4.2%)</td>
<td>1 (1.4%)</td>
</tr>
<tr>
<td>4-6</td>
<td>51 (70.8%)</td>
<td>0</td>
</tr>
<tr>
<td>6-8</td>
<td>11 (15.3%)</td>
<td>0</td>
</tr>
<tr>
<td>&gt;8</td>
<td>5 (6.9%)</td>
<td>0</td>
</tr>
</tbody>
</table>

Table 2. The correlation between SULT2B1b levels and the tumor stage

<table>
<thead>
<tr>
<th>Stage</th>
<th>High</th>
<th>Low</th>
</tr>
</thead>
<tbody>
<tr>
<td>I</td>
<td>2/6</td>
<td>4/6 (66.7%)</td>
</tr>
<tr>
<td>II</td>
<td>4/11 (36.4%)</td>
<td>7/11 (63.6%)</td>
</tr>
<tr>
<td>III</td>
<td>23/31 (74.2%)</td>
<td>8/31 (25.8%)</td>
</tr>
<tr>
<td>IV</td>
<td>20/24 (83.3%)</td>
<td>4/24 (16.7%)</td>
</tr>
</tbody>
</table>

mRNA and protein levels compared with the matched adjacent non-tumor tissues (Fig. 3A, 3B and Fig. 4A, 4B), suggesting that SULT2B1b is upregulated in GC. Moreover, immunohistochemical staining for SULT2B1b was also performed using sequential sections from the same tissue. As shown in Fig. 3C, appropriate antibody isotype control (SULT2A1) sections were negative for SULT2B1b immunostaining. Although only a small group of cases had very strong staining, the majority of the GC samples had a score between 4 and 6 (Table 1). This was higher than the expression score in the adjacent non-tumor tissues, indicating that the expression level of SULT2B1b by immunohistochemistry was correlated with that by qRT-PCR and Western blot assays. In addition, expression levels of SULT2B1b were positively correlated with microvessel density determined by CD31 staining. To assess whether SULT2B1b expression correlated with the prognosis of GC patients, we divided tumor samples into two groups on the basis of SULT2B1b amounts (cut off at the median score), and studied the differences of SULT2B1b expression with clinical gastric cancer. The data showed that the high expression score of SULT2B1b in clinical gastric cancer was found to predict shorter overall survival of patients (Fig. 3D), and there was a positive correlation between SULT2B1b levels and the tumor stage (Table 2), suggesting that SULT2B1b might represent a novel and potentially useful independent biomarker for the prognosis of patients with GC.

**Overexpression of SULT2B1b promotes GC angiogenesis in vivo**

To explore the biological role of SULT2B1b in GC progression, BGC823 GC cells stably expressing SULT2B1b-complementary DNA or SULT2B1b-RNA interference (RNAi) were established (Fig. 5A) and inoculated subcutaneously in the dorsal flanks of nude mice. The

![Full size gels of the tissues probed with SULT2B1b.](image-url)

Fig. 4. Full size gels of the tissues probed with SULT2B1b. (A), Lysates of 5 pair gastric cancer tissues and matched adjacent normal tissues were analyzed by Western Blot, equivalent amounts of protein (40 μg) from each lysate were resolved on SDS-PAGE. Full size gel of the tissues was probed with SULT2B1b antibody (dilution 1:1000). (B), Lysates of 2 pair gastric cancer tissues and matched adjacent normal tissues were analyzed by Western Blot, equivalent amounts of protein (20 μg) from each lysate were resolved on SDS-PAGE. Full size gel of the tissues was probed with SULT2B1b antibody (dilution 1:2,000). (N, adjacent nontumor tissues; T, tumor tissues).
tumors formed by BGC823/SULT2B1b cells were larger in size than the control tumors. In contrast, the tumors formed by BGC823/SULT2B1b-RNAi cells were smaller than the tumors formed by RNAi-vector cells (Fig. 5B). In addition, the microvascular density was significantly increased in BGC823/SULT2B1b tumors compared with vector control tumors. Moreover, the BGC823/SULT2B1b-RNAi tumors had less CD31+ endothelial cells than RNAi-vector control tumors (Fig. 5C). Further, sulfotransferase assay showed that the sulfotransferase activity was significantly increased in BGC823/SULT2B1b cells compared with BGC823 cells, and the sulfotransferase activity of tumor tissues was higher than adjacent tissues (Fig. 6A-C). We also carried out kinetic characterization of sulfotransferase enzyme activity. As expected, the estimated Km value for PAPS was lower in BGC823/SULT2B1b cells compared with BGC823 cells (44.3 ± 5.6 µM versus 72.7 ± 9.2 µM). Together, the results indicated that overexpression of SULT2B1b resulted in increased angiogenesis and sulfotransferase activity.
Silencing SULT2B1b in GC cells reduces their ability to recruit endothelial cells via soluble factors

As recruiting endothelial cells is a critical step in tumor angiogenesis, we then tested whether SULT2B1b was essential for human GC cells to recruit endothelial cells via secretory factors. The BGC823 cells were transfected with SULT2B1b-specific small interfering RNA (siRNA) to deplete SULT2B1b (Fig. 7A). The medium conditioned by the cells was collected and its activity for recruiting HUVECs was assessed by Transwell cell culture analysis. As shown in Fig. 3B, BGC823 cells with depleted SULT2B1b in the bottom chamber had a reduced ability to promote migration of HUVECs in the upper chamber. In addition, the medium conditioned by SULT2B1b-depleted BGC823 cells also decreased HUVEC cell migration in the scrape wound-healing assay (Fig. 7C). It has been clearly illustrated in previous reports that, endothelial cells can form luminal capillary-like structures in Matrigel in vitro, which is an indispensable step for the formation of neo-vasculature [21, 22]. We further tested the activity of BGC823 cell-conditioned medium for promoting endothelial cell tube formation. As expected, the medium conditioned by SULT2B1b-depleted BGC823 cells was also less active in inducing tube formation of HUVECs compared with that of control BGC823 cells (Fig. 7D). These results indicated that SULT2B1b-mediated signals in GC cells promoted the recruitment of endothelial cells via soluble factors.

Depletion of SULT2B1b in GC cells reduces VEGF-A expression via downregulation of SP1 and AP2 expression

To investigate the underlying mechanism of SULT2B1b-induced GC angiogenesis, Western blot assay was performed to examine the expression levels of vascular endothelial growth factor (VEGF) in SULT2B1b-RNA interference (RNAi) GC cells. The VEGF family comprises from VEGF (VEGF-A), VEGF-B, VEGF-C, VEGF-D, and placental growth factor (PGF), which have been reported to be the key regulators of tumor-angiogenesis [23-25]. No significant alterations in VEGF-C, VEGF-B, VEGF-D and PGF expression were observed (data not shown). However, the expression of VEGF-A was significantly decreased in
BGC823/SULT2B1b-siRNA and MKN45/SULT2B1b-siRNA GC cells (Fig. 8A). Consistently, immunochemical staining showed that the expression of VEGF-A was reduced in BGC823/SULT2B1b-silenced tumors (Fig. 8B). These results suggested that VEGF-A might contribute to SULT2B1b-induced angiogenesis. To further investigate the mechanism underlying regulation of VEGF-A by SULT2B1b-mediated signals, we tested the expression of potential VEGF-A upstream regulators. As shown in Fig. 8A, the expression of SP1 and AP2 was reduced in BGC823/SULT2B1b-siRNA and MKN45/SULT2B1b-siRNA cells at the protein level. SULT2B1b protein was also found in the nuclear fractions of BGC823 cells (Fig. 9). Moreover, immunohistochemistry assay showed that the expressions of SP1 and AP2 were
Fig. 8. Depletion of SULT2B1b reduces the expression of VEGF-A in GC cells and tumors. (A) Western blot analyses of VEGF-A, SP1 and AP2 expression in BGC823 and MKN45 cells treated with control siRNA or SULT2B1b siRNA. (B) BGC823/SULT2B1b-RNAi tumors were harvested and paraffin-embedded tumor tissues were subjected to immunostaining for VEGF-A, SP1 and AP2. Images were captured by Zeiss Axioskop-2 microscope under 200× magnification. Scale bars, 100 μm.

Fig. 9. The expression of SULT2B1b in the nuclear and cytoplasmic fractions of BGC823 cells. SULT2B1b protein was measured in the nuclear and cytoplasmic fractions of BGC823 cells by Western blot.

downregulated in BGC823/SULT2B1b-silenced tumors (Fig. 8B). These results suggested that downregulation of SP1 and AP2 contributed to the reduced expression of VEGF-A in SULT2B1b-silenced cells and tumors.

We then investigated whether SP1 and AP2 were required for VEGF-A expression in human GC cells. A luciferase reporter construct driven by the VEGF-A promoter was used to determine whether depletion of these two transcription factors compromised expression of the reporter. As shown in Fig. 10A, depletion of SP1 or AP2 in BGC823 cells reduced expression of the luciferase reporter, suggesting that SP1 and AP2 were important for VEGF-A expression in BGC823 cells. To further determine whether SP1 and AP2 mediated SULT2B1b signals were required to support VEGF-A expression in BGC823 cells, expressions of SP1 or AP2 was depleted with siRNA. Western blot analysis showed that expression of VEGF-A was reduced in the cells treated with SP1 or AP2 siRNA (Fig. 10B). Moreover, the expression of SULT2B1b was significantly decreased with SP1 or AP2 siRNA treatment (Fig. 10B). Consistently, overexpression of SP1 or AP2 in SULT2B1b-depleted BGC823 cells partially restored their activity in promoting HUVEC migration (Fig. 10C). These data demonstrated that SP1 and AP2 were involved in the upregulation of VEGF-A expression by SULT2B1b-mediated signals in GC cells.
Chen et al.: ULT2B1b has a Critical Oncogenic Role in GC Progression

Discussion

In this report, we demonstrated that overexpression of SULT2B1b-mediated angiogenic signaling was associated with tumor angiogenesis and poor clinical features of human GC. We also showed that overexpression of SULT2B1b results in increased tumor angiogenesis and tumor growth in mouse GC models. Moreover, we found that ablation of SULT2B1b in human GC cells compromised the capability of the cells to recruit endothelial cells. Depletion of SULT2B1b in GC cells reduced VEGF-A expression by downregulating SP1 and AP2 expression. Our results suggest that the SULT2B1b-mediated angiogenic pathway can be used as biomarkers for GC diagnosis and prognosis. They also suggest that suppressing SULT2B1b-mediated angiogenic signaling is promising for the development into a strategy for GC treatment.

It has been reported that SULT2B1 has two isoforms, SULT2B1a and SULT2B1b. SULT2B1a and SULT2B1b message expression occurs in a number of human tissues including prostate, placenta, respiratory system and skin [7]. Previous reports have shown that analysis of the expression of the two specific SULT2B1 messages in different human tissues using RT-PCR shows variability especially in SULT2B1a expression. The levels of SULT2B1b specific message are generally several-fold greater than those for SULT2B1a and SULT2B1b message is detectable in more tissue types [26, 27]. Consistent with previous reports our results showed that mRNA level of SULT2B1b was much higher than SULT2B1a in GC tumor tissues.

To date, there are evidences linking SULT2B1b gene to tumor growth of colorectal, hepatocellular and prostate carcinoma [14-16]. However, the reported effects of SULT2B1b on tumor biology are conflicting. Hu et al. [14] provided the evidence that overexpression of SULT2B1b promoted cell growth and invasion in colorectal carcinoma. Yang et al. [16] showed that overexpression of SULT2B1b promoted the proliferation of human hepatocarcinoma cells, whereas Seo et al. [15] demonstrated that the expression of SULT2B1b was decreased in prostate cancer and depletion of SULT2B1b resulted in increased proliferation rate of prostate cancer cells. These conflicting results indicate that the role of SULT2B1b in different
cancers is varies with the type of cancer. Recently, several single-nucleotide polymorphisms (SNP) in SULT2B1 gene have been reported to be associated with risk of progression in prostate and esophageal carcinomas [28, 29]. Additionally, earlier studies demonstrated that expression of SULT2B1b was significantly increased in breast and liver cancer tissues relative to their corresponding non-tumor counterparts [7, 30, 31]. Moreover, it has been reported that increased expression of SULT2B1b protein was significantly correlated with disease progression and poor postoperative prognosis of colorectal cancer patients [14], indicating a possible relationship between SULT2B1b and clinical prognosis of cancer patients. Nevertheless, the expression pattern and clinical relevance of SULT2B1b has not been assessed in GC. In agreement with published results, we showed that the expression of SULT2B1b is significantly upregulated in GC tissues and associated with poor prognosis for patients bearing GC. These findings suggest that SULT2B1b might be a new candidate diagnostic tool or marker for GC.

It is well documented that most of GC-related deaths are due to metastatic disease [32]. Recent studies have shown that angiogenesis in GC is a key step of metastasis [33]. Besides proteins, expressions of many non-coding RNAs, such as microRNA and long non-coding RNAs, were also shown to be altered in GC metastasis [34, 35], which could potentially serve as diagnostic and prognostic biomarkers of GC [36]. A deeper understanding of the molecular mechanisms of GC angiogenesis could reveal new potential targets for the disease. Angiogenesis is a multistep process, which includes endothelial cell proliferation, migration and the formation of blood vessels [37, 38]. For instance, LAPTM4B down regulation was found to inhibit the proliferation, invasion and angiogenesis in vitro [39]. In this study, we found that overexpression of SULT2B1b correlated with upregulated CD31 expression; the HUVEC tube formation assay confirmed that SULT2B1b knockdown in GC cells resulted in decreased endothelial cell tube formation ability. Moreover, SULT2B1b expression in GC cells positively regulated the supportive vasculature in vivo. These findings suggest that SULT2B1b is a potential inducer of angiogenesis in GC. Furthermore, consistent with previous report [16], we found that SULT2B1b high expression promotes sulfotransferase activity in GC cells and tumor tissues. However, the molecular basis for SULT2B1b overexpression in GC is currently unknown and requires further investigation. VEGF-A has been reported to be overexpressed in a number of human cancers and the ability of VEGF-A to promote angiogenesis has been demonstrated in multiple cancer types, including colon adenocarcinoma and gastric cancer [40, 41]. The VEGF-A promoter region contains multiple consensus transcriptional regulatory factor-binding sites, which include SP1/SP3, AP1, AP2, Egr1, STAT3 and HIF1α [42, 43]. In the current study, we demonstrated that depletion of SP1 or AP2 decreased VEGF-A expression in GC cells, suggesting SULT2B1b could interact with transcription factors SP1 and AP2. Consistently, overexpression of SP1 or AP2 in SULT2B1b-depleted GC cells partially restored their activity in promoting HUVEC migration. The results suggest that SP1 and AP2 are involved in the SULT2B1b-mediated signals in GC cells, and upstream regulators of VEGF-A may control VEGF-A expression via these transcription factors.

In summary, we report here that SULT2B1b-mediated signals contribute to GC angiogenesis. The overexpression of SULT2B1b results in increased tumor angiogenesis and tumor growth. The ablation of SULT2B1b decreases the capability of GC cells to recruit endothelial cells. Moreover, SP1 and AP2 are involved in the SULT2B1b-mediated signals in GC cells. The results suggest that suppression of the SULT2B1b-mediated pathway may be of therapeutic value for GC therapies and that the SULT2B1b signaling pathway is a potential biomarker for GC diagnosis.

Disclosure Statement

Nothing to declare.
References