Original Article

LncRNA Sox2ot overexpression serves as a poor prognostic biomarker in gastric cancer

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Abstract: Sox2 overlapping transcript (Sox2ot) serves as an unfavorable prognostic factor in lung cancer and hepatocellular carcinoma, but the clinical significance and biological function of Sox2ot in gastric cancer is still unknown. The objective of our research is to explore the significance of Sox2ot in gastric cancer. Quantitative real-time polymerase chain reaction was performed to determine Sox2ot in gastric cancer tissues and cell lines. Furthermore, the Sox2ot status in one hundred and thirty two gastric cancer samples was detected to analyzed correlations between Sox2ot and clinicopathological features. The biological function of Sox2ot on tumor cell growth and mobility were explored through MTT, colony formation, Transwell migration assay and invasion assay in vitro. In our results, the expression of Sox2ot was overexpressed in gastric cancer tissues and cell lines. High levels of Sox2ot were correlated with malignant status and poor prognosis. Silencing Sox2ot expression effectively inhibited gastric cancer cell growth and motility in vitro. In conclusion, Sox2ot is a potential therapeutic target and a new biomarker for gastric cancer patients.

Keywords: Sox2ot, gastric cancer, lncRNA, biomarker

Introduction

Gastric cancer is still the second leading cause of cancer death worldwide [1]. A total of estimated 10,720 gastric cancer deaths and 24,590 new gastric cancer patients occur based on 2015 American Cancer Statistics [2]. Meanwhile, the mortality of gastric cancer ranked third with the proportion of 14.33% each year, and the morbidity reached to second with 3,621,000 new gastric cancer patients in China [3]. Over half of gastric cancer patients have a relapse after surgical treatment, and most patients at confirmed diagnosis already have advanced stage [4]. In order to increase curative effect and improve prognosis for gastric cancer patients, it is necessary to distinguish high risk group of gastric cancer patients [5]. Up to now, there is still no accurate biomarker to provide early diagnosis and prognosis prediction. Thus, it is very interest to discovery biomarkers which correlate to tumorigensis and progression in gastric cancer.

Long non-coding RNAs (lncRNAs) are a group of non-protein-coding RNAs that regulate gene expression at the transcriptional or posttranscriptional level [6, 7]. Recently, more and more new lncRNAs are discovered and identified as oncogene or anti-oncogene in gastric cancer, such as H19 [8-11], MEG3 [12, 13], PVT1 [14, 15], BANCR [16] and FER1L4 [17]. Sox2 overlapping transcript (Sox2ot) is mapped to human chromosome 3q26.3 [18, 19] and transcribed in the same orientation as Sox2, which is embedded in anintron of the Sox2ot gene [20]. Sox2ot has been suggested overexpressed in undifferentiated and stem cells and down-regulated in differentiated and mature cells [21]. Meanwhile, the deregulation of Sox2ot expression has been found in a variety of human cancers, such as breast cancer, esophageal cancer, and lung cancer [22]. Up to present, the biological function and clinical significance of Sox2ot in gastric cancer is unclear. In order to identify the expression level and bio-
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The role of Sox2ot in gastric cancer, the expression of Sox2ot in 132 gastric cancer samples were measured and loss-of-function studies of Sox2ot in gastric cancer cell lines were performed.

Materials and methods

Ethics statement

The Research Ethics Committees of Huai'an First People's Hospital and Fifth Hospital of Wuhan approved this protocol and written informed consents were obtained from each patient. The entire study was performed based on the Declaration of Helsinki.

Patients and samples

One hundred and thirty two fresh gastric cancer samples and twenty paired adjacent normal gastric tissue samples were collected, and the pathological information (gender, age, histological type, clinical stage, tumor depth, lymph node metastasis, and distant metastasis) was retrieved from the archives of the Pathology Department of Huai'an First People's Hospital and Fifth Hospital of Wuhan. Tissue samples were immediately frozen in liquid nitrogen and kept at -80°C. The histopathological diagnosis of all samples was respectively diagnosed by two pathologists. The clinical staging was based on the 7th edition of the AJCC Cancer Staging Manual. The system treatments were performed according to NCCN guideline.

Cell lines and cell cultures

Two gastric cancer cell lines (SGC-7901 and AGS) and a normal gastric epithelial cell line (GES-1) were obtained from Chinese Type Culture Collection, Chinese Academy of Sciences, and were cultured in DMEM (Gibco) supplemented with 10% Fetal Bovine Serum (Hyclone) at 37°C in a humidified CO₂ (5%) atmosphere.

Real-time PCR

Total RNA was extracted from tissues and cell lines using RNAiso Plus (Takara) according to the manufacturer's instructions. The isolated total RNA was reverse transcribed using the PrimeScript RT Master Mix (Takara), according to manufacturer instructions. The sequence-specific forward and reverse primers sequences for Sox2ot were 5’-GCTCGTTGCTTAGGA-GATTG-3’ and 5’-CTGCAAGCATGAGGA-CT-3’, respectively. Forward and reverse primers sequences for GAPDH mRNA were 5’-ATGGGA- GAAGGTAAGGTGCG-3’ and 5’-GGGGTCATTGAT-GGCAACAATA-3’, respectively. The reactions were performed using SYBR Premix Ex TaqTM II (Takara) on a LightCycler (Roche). Relative expression was calculated via the comparative cycle threshold method and was normalized to the expression of GAPDH. Relative quantification was calculated by using the 2⁻ΔΔCt method. All reactions were run in triplicate.

Cell transfection

Sox2ot siRNA (si-Sox2ot) and non-targeting siRNA (si-NC) were purchased from Gene Pharma and used at 20 mM Opti-MEM transfection media (Invitrogen) and Lipofectamine 3000 reagent (Invitrogen) were used to transfect the cells once they reached 60% confluence. Knockdown was assessed by Real-time PCR after 48 hours of transfection.

Cell proliferation analysis

Cell proliferation was analyzed using MTT assay. Briefly, 1×10⁵ cells were seeded into a 96-well plate with quadruplicate repeat for each condition. The cells were incubated for 1, 2, 3, and 4 days. Twenty microliters of MTT (5 mg/ml) (Sigma) was added to each well and incubated for 4 h. At the end of incubation, the supernatants were removed and 150 μl of DMSO (Sigma) was added to each well. The absorbance value (OD) of each well was measured at 490 nm. Experiments were performed three times.

Colony formation assay

Briefly, Cells (0.5×10³) were plated into six well plates and cultured for ten days. Colonies were then fixed for 5 min with 10% formaldehyde and stained with 1.0% crystal violet for 30 s. The number of colonies containing ≥50 cells was counted under a microscope. Experiments were performed three times.

Cell migration and invasion assays

Briefly, 1×10⁵ cells were seeded on a fibronectin-coated polycarbonate membrane insert in a
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A

Transwell apparatus (Corning). After the cells were incubated for 12 h, Giemsa-stained cells adhering to the lower surface were counted under a microscope in five predetermined fields (100×). For the cell invasion assay, the procedure was similar to the cell migration assay, except that the transwell membranes were pre-coated with 24 mg/ml Matrigel (Corning). Experiments were performed three times.

Statistical analysis

All data were analyzed for statistical significance using SPSS 17.0 software. The chi-square test was applied to the examination of correlation between Sox2ot expression and clinicopathological characteristics. Survival curves were plotted using the Kaplan-Meier method and the log-rank test. Cox regression was used for univariate analysis. The significance of survival variables (P<0.05) in univariate analysis were included into the final multivariable Cox proportional hazards model. Two-tailed Student’s t test was used for comparisons of two independent groups. One-way ANOVA was used to determine the differences between groups or all in vitro analyses. The data are shown as the mean ± SD from at least three independent experiments. A P-value of less than 0.05 was considered statistically significant.

Results

The expression of Sox2ot was overexpressed in gastric cancer tissues and cell lines

In order to assess the role of Sox2ot in gastric cancer, we performed real-time PCR to measure the expression of Sox2ot in twenty paired...
gastrointestinal tissues and adjacent normal tissues, and two gastric cancer cell lines (SGC-7901 and AGS) and a normal gastric epithelial cell line (GES-1). Compared with adjacent normal tissues, gastric cancer tissues showed higher expression levels of Sox2ot (P<0.001, Figure 1A). Moreover, Sox2ot was significantly overexpressed in gastric cancer cell lines compared with normal gastric epithelial cell line (P=0.363, Table 1). Sox2ot overexpression was a poor independent prognostic factor in gastric cancer patients.

We next analyzed the correlation between the expression of Sox2ot and clinicopathological characteristics of gastric cancer. Gastric cancer tissue samples were classified into the low expression group (n=66) and the high expression group (n=66) according to the median expression level of all gastric cancer samples. This classification was based on published study [16]. As summarized in Table 1, the levels of Sox2ot expression was associated significantly with clinical stage (I-II vs. III-IV; P=0.001), tumor depth (T1-T2 vs. T3-T4; P=0.015), lymph node metastasis (N0-N1 vs. N2-N3; P=0.002), and distant metastasis (M0 vs. M1; P=0.005). However, there was no significant correlation between Sox2ot expression and gender (P=0.363), age (P=0.723), and histological type (P=0.288).

Table 1: Correlations between lncRNA Sox2ot expression and clinicopathological characteristics in gastric cancer

<table>
<thead>
<tr>
<th>Characteristics</th>
<th>n</th>
<th>High expression (%)</th>
<th>Low expression (%)</th>
<th>P</th>
</tr>
</thead>
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<tr>
<td>Gender</td>
<td></td>
<td></td>
<td></td>
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</tr>
<tr>
<td>Female</td>
<td>47</td>
<td>26 (55.3)</td>
<td>21 (44.7)</td>
<td>0.363</td>
</tr>
<tr>
<td>Male</td>
<td>85</td>
<td>40 (47.1)</td>
<td>45 (52.9)</td>
<td></td>
</tr>
<tr>
<td>Age (y)</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>&lt;50</td>
<td>54</td>
<td>26 (48.1)</td>
<td>28 (51.9)</td>
<td>0.723</td>
</tr>
<tr>
<td>≥50</td>
<td>78</td>
<td>40 (51.3)</td>
<td>38 (48.7)</td>
<td></td>
</tr>
<tr>
<td>Histological type</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Differentiated</td>
<td>78</td>
<td>36 (46.2)</td>
<td>42 (53.8)</td>
<td>0.002</td>
</tr>
<tr>
<td>Undifferentiated</td>
<td>54</td>
<td>30 (55.6)</td>
<td>24 (44.4)</td>
<td></td>
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<tr>
<td>Clinical stage</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>I-II</td>
<td>61</td>
<td>21 (34.4)</td>
<td>40 (65.6)</td>
<td>0.001</td>
</tr>
<tr>
<td>III-IV</td>
<td>71</td>
<td>45 (63.4)</td>
<td>26 (36.6)</td>
<td></td>
</tr>
<tr>
<td>Tumor depth</td>
<td></td>
<td></td>
<td></td>
<td></td>
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<tr>
<td>T1-T2</td>
<td>68</td>
<td>27 (39.7)</td>
<td>41 (60.3)</td>
<td>0.015</td>
</tr>
<tr>
<td>T3-T4</td>
<td>64</td>
<td>39 (60.9)</td>
<td>25 (39.1)</td>
<td></td>
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<tr>
<td>Lymph node metastasis</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>N0-N1</td>
<td>68</td>
<td>25 (36.8)</td>
<td>43 (63.2)</td>
<td>0.002</td>
</tr>
<tr>
<td>N2-N3</td>
<td>64</td>
<td>41 (64.1)</td>
<td>23 (35.9)</td>
<td></td>
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<tr>
<td>Distant metastasis</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>M0</td>
<td>121</td>
<td>56 (46.3)</td>
<td>65 (53.7)</td>
<td>0.005</td>
</tr>
<tr>
<td>M1</td>
<td>11</td>
<td>10 (90.9)</td>
<td>1 (9.1)</td>
<td></td>
</tr>
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</table>

**Correlation between Sox2ot and clinicopathological features in gastric cancer patients**

We next analyzed the correlation between the expression of Sox2ot and clinicopathological characteristics of gastric cancer. Gastric cancer tissue samples were classified into the low expression group (n=66) and the high expression group (n=66) according to the median expression level of all gastric cancer samples. This classification was based on published study [16]. As summarized in Table 1, the levels of Sox2ot expression was associated significantly with clinical stage (I-II vs. III-IV; P=0.001), tumor depth (T1-T2 vs. T3-T4; P=0.015), lymph node metastasis (N0-N1 vs. N2-N3; P=0.002), and distant metastasis (M0 vs. M1; P=0.005). However, there was no significant correlation between Sox2ot expression and gender (P=0.363), age (P=0.723), and histological type (P=0.288).

**Sox2ot overexpression was a poor independent prognostic factor in gastric cancer patients**

To explore the prognostic value of Sox2ot expression in gastric cancer, we measured the association between the levels of Sox2ot expression and patients’ survival using Kaplan-Meier analysis with the log-rank test. In 132 gastric cancer patients with prognosis information, we found that the level of Sox2ot expression was significantly associated with the overall survival of gastric cancer patients, as patients with lower levels of Sox2ot expression had better survival than those with higher levels of Sox2ot expression (P<0.001, Figure 1C). Furthermore, we also found that overexpression of Sox2ot showed poor prognosis in gastric cancer patients (P<0.001, Table 2), regardless of clinical stage, tumor depth, lymph node metastasis, and distant metastasis. Multivariate analysis showed that high expression of Sox2ot was a poor independent prognostic factor for patients with gastric cancer (P=0.003, Table 2).

**Knockdown of Sox2ot expression suppressed gastric cancer cells growth**

The efficiency of si-Sox2ot was confirmed by RT-PCR in gastric cancer cells (Figure 1D). Subsequently, we examined the effect of decreased Sox2ot expression on gastric cancer cells growth in vitro. The growth curves determined by MTT assay showed that si-Sox2ot gastric cancer cells had an decreased growth rate to si-NC cells over a four day period (Figure 2A, P<0.05). The results of MTT assay were
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Table 2. Univariate and multivariate Cox regression analyses of overall survival in gastric cancer

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Univariate analysis</th>
<th>Multivariate analysis</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>HR</td>
<td>95% CI</td>
</tr>
<tr>
<td>Gender (Female vs. Male)</td>
<td>0.768</td>
<td>0.613-1.434</td>
</tr>
<tr>
<td>Age (&lt;50 vs. ≥50)</td>
<td>1.131</td>
<td>0.749-1.708</td>
</tr>
<tr>
<td>Histological grade (Differentiated vs. Undifferentiated)</td>
<td>0.960</td>
<td>0.631-1.463</td>
</tr>
<tr>
<td>Clinical stage (I-II vs. III-IV)</td>
<td>4.044</td>
<td>2.469-6.626</td>
</tr>
<tr>
<td>Tumor depth (T1-T2 vs. T3-T4)</td>
<td>2.050</td>
<td>1.352-3.107</td>
</tr>
<tr>
<td>Lymph node metastasis (N0-N1 vs. N2-N3)</td>
<td>3.829</td>
<td>2.368-6.191</td>
</tr>
<tr>
<td>Distant metastasis (M0 vs. M1)</td>
<td>10.827</td>
<td>5.137-22.815</td>
</tr>
<tr>
<td>lncRNA Sox2ot (Low vs. High)</td>
<td>3.119</td>
<td>2.033-4.787</td>
</tr>
</tbody>
</table>

HR, hazard ratio; 95% CI, 95% confidence interval.

Figure 2. Knock-down of Sox2ot expression suppressed cell proliferation and plate clone formation. A: In vitro viabilities of SGC-7901 and AGS cells were decreased in Sox2ot-suppressed cells by MTT assay (both P<0.05). B: In vitro proliferative ability of SGC-7901 and AGS cells were decreased in Sox2ot-suppressed cells by colony formation assay (both P<0.001).

also consistent with clonogenicity tests as si-Sox2ot cells formed a decreased number of colonies compared to si-NC cells over a fourteen days period (Figure 2B, P<0.001). This suggested that knockdown of Sox2ot dramatically suppress gastric cancer cells growth.
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Knockdown of Sox2ot inhibits gastric cancer cells migration and invasion

To examine the effect of Sox2ot on cell migration, after 24 hours of transfection, the number of migrated cells in both si-Sox2ot gastric cancer cell groups was significantly less than that in the si-NC gastric cancer cells (for both P<0.001, Figure 3A). Using a boyden chamber coated with matrigel, we determined changes in cell invasiveness after 24 hours of transfection. Compared with the si-NC gastric cancer cells, si-Sox2ot gastric cancer cells showed significantly decreased invasiveness (for both P<0.001, Figure 3B).

Discussion

Despite of declining mortality rate of gastric cancer, gastric cancer remains the fifth most frequently occurring malignant tumor and the third leading cause of cancer-related death [23, 24]. Many patients are diagnosed at an advanced stage with lymphatic or distant metastasis because of no early specific symptoms. The value of traditional biomarkers is limited for gastric cancer patients in diagnosing and predicting prognosis [25, 26]. Novel screening strategies for gastric cancer will be of great significance to reduce the mortality and elevate the quality of life of patients.

Long noncoding RNAs (lnc-RNAs) are broadly defined as RNA longer than 200 nucleotides lacking extended open reading frames [27]. Recent studies have revealed that lncRNAs are often aberrantly expressed in gastric cancer. LINC00152 has been suggested to overexpress in gastric cancer tissues, cell lines, blood, and gastric juice [28-30]. Moreover, MALAT1 is upregulated in gastric cancer tissues and cell lines compared with normal gastric tissues and normal gastric epithelial cell lines [31, 32]. Long non-coding RNA Sox2 overlapping transcript (Sox2ot) acts as an enhancer for SOX2 transcription and localizes on human chromosome 3q26.33. The level of Sox2ot in gastric cancer is still unknown. Our study first found the expressions of Sox2ot were obviously increased in gastric cancer tissues and cell lines compared with paired adjacent normal gastric tissues and normal gastric epithelial cell line. Similarly, Shi X.M. et al showed the expression of Sox2ot was significantly higher in hepatocellular carcinoma tissues compared with adjacent non-tumor tissues [33]. In esophageal squamous cell carcinoma, Shahryari A. et al suggested that Sox2ot was upregulated in tumor samples in comparison to their apparently normal marginal tissues [34].

Recently, the clinical significance of Sox2ot has been reported in several types of human can-
cancers such as hepatocellular carcinoma [33], lung cancer [35], breast cancer [36, 37]. In hepatocellular carcinoma, overexpression of Sox2ot was associated with vein invasion, histological grade, and TNM stage [33]. Hou Z.B et al reported that the expression level of Sox2ot is markedly higher in lung squamous cell carcinomas than that in lung adenocarcinomas [35]. In breast cancer, Askarian-Amiri M.E. et al indicated that Sox2ot was overexpressed in estrogen receptor positive breast cancer cell lines, in comparison with the estrogen receptor negative breast cancer cell lines [36]. However, Iranpour M. et al showed no association was found between gene expressions and individual clinical data including PR status and Estrogen receptor status [37]. The discrepancy between Askarian-Amiri M.E. et al’s data and Iranpour M. et al’s data would be most likely due to the different samples between both studies and limited samples in Iranpour M. et al’s study. In gastric cancer, we presented the evidence that Sox2ot expression was positively associated significantly with clinical stage, tumor depth, lymph node metastasis, and distant metastasis. In addition, we further explored the biological function of Sox2ot in gastric cancer cell lines, and found that knocking down Sox2ot expression obviously suppressed gastric cancer cells proliferation, migration, and invasion abilities. Similarly, Hou Z.B et al’s report in lung cancer indicated that knocking down Sox2ot inhibited cell proliferation by inducing G2/M arrest, and reduced protein levels of Cyclin B1 and Cdc2 [35]. Meanwhile, the metastasis ability of hepatocellular carcinoma cells was markedly suppressed by knocking down Sox2ot expression [33].

In recent studies, high level of Sox2ot expression has been shown to be an independent poor prognostic factor in lung cancer and hepatocellular carcinoma. In lung cancer, high Sox2ot expression predicted poor survival for lung cancer patients, and the Risk Ratio (HR) and 95% confidence interval (95% CI) was 2.808 (1.131-6.967) [35]. According to multivariate Cox regression analysis, high expression of Sox2ot was a poor independent prognostic factor for patients with gastric cancer, and the HR (95% CI) was 2.052 (1.278-3.298).

In conclusion, Sox2ot was overexpressed in gastric cancer tissues and cell lines, and correlated with malignant status and poor prognosis of gastric cancer patients. Knockdown of Sox2ot expression suppressed gastric cancer cell proliferation, migration and invasion in vitro. Sox2ot could act as a novel biomarker and a potential therapeutic target for gastric cancer patients.

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Disclosure of conflict of interest

None.

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