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*Original Article*

# Up-regulation of long non-coding RNA Sox2ot promotes hepatocellular carcinoma cell metastasis and correlates with poor prognosis

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**Abstract:** Background: Long non-coding RNAs (lncRNAs) have been shown to have important regulatory roles in cancer biology, and the lncRNA Sox2ot is up-regulated in some tumors. However, the contributions of Sox2ot to hepatocellular carcinoma (HCC) remain largely unknown. Methods: In the present study, expression of lncRNA Sox2ot was evaluated by quantitative real-time PCR in tumor tissues and adjacent non-tumor tissues in 84 HCC patients. The association of lncRNA Sox2ot expression with clinicopathological features and the prognosis of HCC patients were also analyzed. Survival analysis was performed using the Kaplan-Meier method and Cox's proportional hazards model. Small interfering RNA assay was used to explore the function of lncRNA Sox2ot on HCC cell migration and invasion. Results: lncRNA Sox2ot expression level was significantly higher in HCC tissues compared with adjacent non-tumor tissues ($P<0.05$). High expression of lncRNA Sox2ot was associated with histological grade, TNM stage, and vein invasion. The 5-year overall survival of high lncRNA Sox2ot expression group was significantly shorter than that of low lncRNA Sox2ot expression group ($P<0.05$). The multivariate Cox regression analysis indicated that lncRNA Sox2ot expression was an independent prognostic factor for overall survival. In addition, the metastasis ability of HCC cells was significantly decreased by knocking down lncRNA Sox2ot expression. Conclusions: The results suggested that lncRNA Sox2ot played crucial roles in promoting HCC cell migration and invasion, and might represent a novel prognostic biomarker for HCC.

**Keywords:** lncRNA Sox2ot, hepatocellular carcinoma, prognosis, migration, invasion

## Introduction

Hepatocellular carcinoma (HCC) is one of the most frequent human malignancies worldwide. Especially in China, it has become a major cause of cancer-related death [1]. Hepatocarcinogenesis is a complex process and the occurrence is the result of coactions of multifactors, such as physical condition, aflatoxin, hepatitis virus infection, cirrhosis, and genetic susceptibility and epigenetic changes [2]. Surgical resection is still considered to be the mainly curative treatment for HCC, with about 50-70% 5-year overall survival after curative hepatectomy. However, the postoperative recurrence rate remains as high as 70% [3, 4]. Therefore, it is urgent and necessary to identify prognostic factors which could predict recurrence, metastasis and prognosis for patients with HCC to improve the individual treatment.

Recently, high-through put transcriptome analysis has revealed that 98% of the human genome can be transcripted into non-coding RNA [5]. Among them, the long non-coding RNAs (lncRNAs) are RNA molecular that is longer than 200 nucleotides in length with limited or no protein-coding capacity [6]. LncRNAs are often expressed in a disease-, tissue- or developmental stage-specific manner making these molecules attractive therapeutic targets and pointing toward specific functions for lncRNAs in development and diseases, in particular human cancer [7-9]. More and more evidences revealed the contribution of lncRNAs as having oncogenic and tumor suppressor roles in tumorigenesis [10]. For example, Gupta et al showed HOTAIR was increased in expression in primary breast tumors and metastases. Enforced expression of HOTAIR in epithelial cancer cells induced genome-wide re-targeting of Polycomb repres-

**Table 1.** Association of lncRNA Sox2ot expression with clinicopathological features in HCC patients

| Parameters | Group | Total | lncRNA Sox2ot | | *P* value |
|---|---|---|---|---|---|
| | | | Low | High | |
| Gender | Male | 53 | 25 | 28 | 0.498 |
| | Female | 31 | 17 | 14 | |
| Age (years) | <50 | 35 | 15 | 20 | 0.268 |
| | ≥50 | 49 | 27 | 22 | |
| Tumor size (cm) | <5 cm | 29 | 14 | 15 | 0.818 |
| | ≥5 cm | 55 | 28 | 27 | |
| Histologic grade | Low | 26 | 23 | 3 | 0.000 |
| | High | 58 | 19 | 39 | |
| TNM stage | I-II | 32 | 22 | 10 | 0.007 |
| | III-IV | 52 | 20 | 32 | |
| Vein invasion | Absence | 28 | 24 | 4 | 0.000 |
| | Presence | 56 | 18 | 38 | |
| Cirrhosis | Negative | 63 | 31 | 32 | 0.801 |
| | Positive | 21 | 11 | 10 | |
| Hepatitis B | Negative | 25 | 13 | 12 | 0.811 |
| | Positive | 59 | 29 | 30 | |

sive complex 2 (PRC2) to an occupancy pattern more resembling embryonic fibroblasts, leading to altered histone H3 lysine 27 methylation, gene expression, and increased cancer invasiveness and metastasis in a manner dependent on PRC2 [11]. Zhang et al found that lncRNA SPRY4-IT1 was increased in ccRCC tissues and ccRCC patients with higher SPRY4-IT1 expression had an advanced clinical stage and poorer prognosis than those with lower SPRY4-IT1 expression. In addition, they indicated that decrease expression of SPRY4-IT1 reduced renal cancer cell proliferation, migration, and invasion [12]. Sun et al revealed that lncRNA GAS5 expression was markedly downregulated in gastric cancer tissues and associated with larger tumor size and advanced pathologic stage, ectopic expression of GAS5 was demonstrated to decrease gastric cancer cell proliferation and induce apoptosis in vitro and in vivo [13]. Ma et al demonstrated that lncRNA LET was down-regulated in gallbladder cancer and patients with low expression of lncRNA LET have significantly poorer prognosis than those with high expression, ectopic expression of LncRNA LET could suppress gallbladder tumor growth in vivo [14]. However, to our knowledge, the expression level and biological role of lncRNA Sox2ot in HCC remains unclear.

In the present study, we investigated the expression level of lncRNA Sox2ot in human HCC tissues and explored the association between lncRNA Sox2ot expression and clinicopathological features. lncRNA Sox2ot could be served as an independent predictor for overall survival in HCC. Moreover, Down-regulation of lncRNA Sox2ot expression could inhibit the HCC cell migration and invasion in vitro. Our results suggested that lncRNA Sox2ot might represent a novel indicator of poor prognosis in HCC and could be a potential therapeutic target for diagnosis and gene therapy.

## Materials and methods

### *Patients and specimens*

A total of 84 patients with primary HCC who underwent a curative hepatectomy at the Liver Surgery Department, Shanghai Changzheng Hospital, were included in this retrospective study. These patients were diagnosed as HCC between 2006 and 2008. None of the patients recruited in this study had chemotherapy or radiotherapy before the surgery. HCC diagnosis was based on World Health Organization (WHO) criteria. Tumor differentiation was defined according to the Edmondson grading system. Tumor staging was determined according to the sixth edition of the tumor-node-metastasis (TNM) classification of the International Union against Cancer. This study was approved by the Research Ethics Committee of Shanghai Changzheng Hospital. Informed consent was obtained from all of the patients. The clinicopathological features of 84 patients are summarized in **Table 1**.

### *Cell culture and transfection*

Two HCC cell lines (HepG2 and SMMC-7721) were purchased from the Institute of Biochemistry and Cell Biology of the Chinese Academy of Sciences (Shanghai, China). Cells were cultured in DMEM (Gibco) medium supplemented with 10% fetal bovine serum (10% FBS), 100 U/ml penicillin, and 100 mg/ml streptomycin in humidified air at 37°C with 5% $CO_2$.

According to the Lipofectamine 2000 reagent protocol (Invitrogen), HCC cells were transfected with Sox2ot siRNA. The cells transfected with siRNA ($1\times10^5$ cells/well) were seeded into 6-well cell culture plates and allowed to continue growing for 24 h before harvesting for fur-

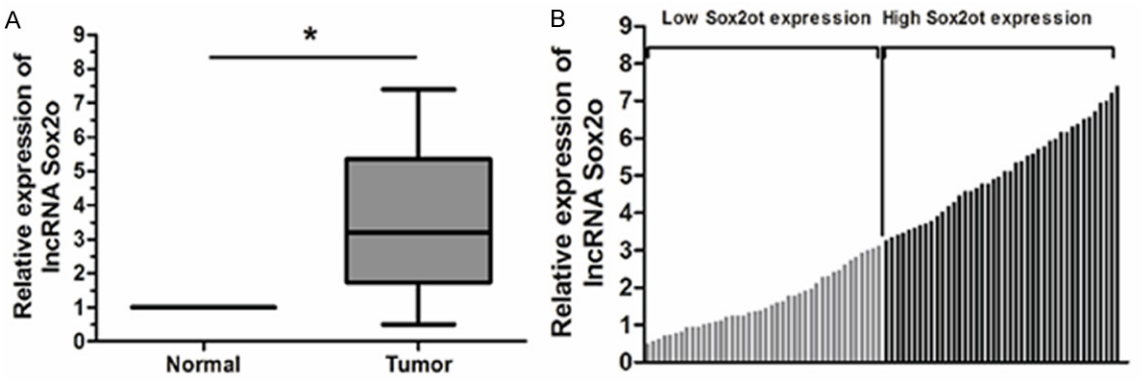


**Figure 1.** lncRNA Sox2ot expression was up-regulated in HCC patients. A. lncRNA Sox2ot expression was determined by quantitative real-time PCR and normalized against GAPDH (an endogenous control). Expression was compared between 84 pairs of HCC tissues and adjacent non-tumor tissues; B. HCC patients were divided into two groups according lncRNA Sox2ot expression. Results are expressed as mean ± SD for three replicate determination. *$P$<0.05.

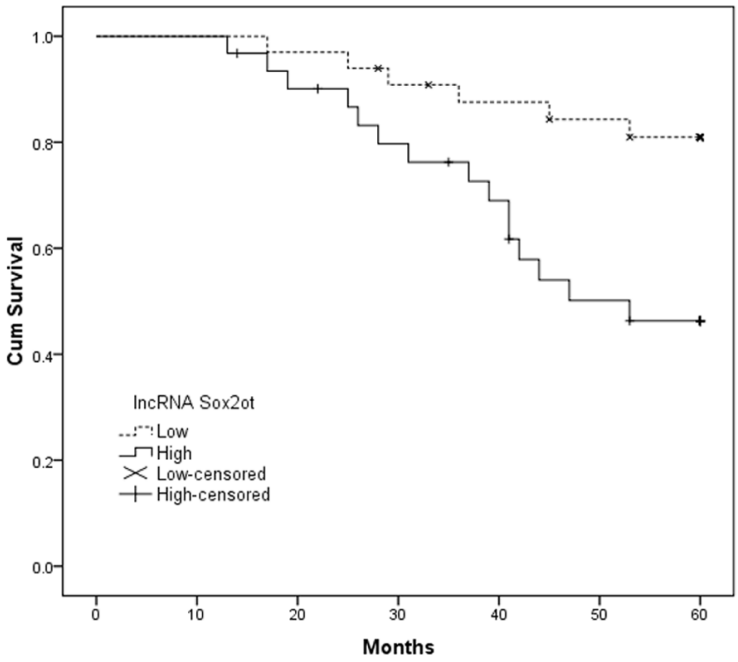


**Figure 2.** Survival analysis of 84 HCC patients by Kaplan-Meier method. Overall survival rate in patients with high lncRNA Sox2ot expression was significantly lower than that in patients with low lncRNA Sox2ot expression ($P$<0.05).

ther analysis. siRNAs for the human Sox2ot (siR-Sox2ot-1: 5'-CAAUCAACUCUGAGAUCAtt-3'; siR-Sox2ot-2: 5'-CAAAAUAGGUCAUAGCAAAtt-3') and the negative control siRNA (siR-NC: 5'-UUCUCCGAACGUGUCACGUtt-3') were puchased from Invitrogen.

*Cell migration and invasion assay*

The cell invasion assay were performed using a transwell insert (8 μm, Corning). 24 h after transfection, $5x10^4$ cells were first starved in 200 ml serum-free DMEM medium and then placed in the uncoated (migration assay) or Matrigel-coated (1:10 dilution; BD Biosciences) top chamber (invasion assay). The lower chamber was filled with 500 ml of complete DMEM medium. The cells were incubated for 48 h (migration assay) and 72 h (invasion assay) at 37°C, and then the cells on the top surface of the membrane were removed through wiping with a cotton swab. The cells that had migrated to or invaded the bottom surface of the filter membrane were stained with 0.5% crystal violet solution and photographed in five preset fields per insert. The results represented the average of three independent experiments.

*Quantitative real-time PCR (qRT-PCR)*

Total RNA was extracted from tissues or cultured cells using TRIzol reagent (Invitrogen). For qRT-PCR, RNA was reverse transcribed to cDNA by using a Reverse Transcription Kit (Takara). Real-time PCR analyses were performed with Power SYBR Green (Takara). Results were normalized to the expression of GAPDH. The PCR primers for lncRNA Sox2ot or GAPDH were as follows: lncRNA Sox2ot sense, 5'-GCTCGTGG-CTTAGGAGATTG-3' and reverse, 5'-CTGGCAAA-GCATGAGGAACT-3'; GAPDH sense, 5'-GTCAACG-

**Table 2.** Univariate and multivariate Cox regression analyses of overall survival in HCC patients

| Variable | Univariate analysis | | | Multivariate analysis | | |
|---|---|---|---|---|---|---|
| | Risk ratio | 95% CI | *P-value* | Risk ratio | 95% CI | *P-value* |
| Gender | 1.291 | 0.754-1.843 | 0.419 | | | |
| Male vs Female | | | | | | |
| Age (years) | 2.371 | 0.582-4.108 | 0.273 | | | |
| ≥50 vs <50 | | | | | | |
| Tumor size | 1.863 | 0.674-3.492 | 0.316 | | | |
| ≥5 cm vs <5 cm | | | | | | |
| Histologic grade | 2.794 | 1.531-4.295 | 0.008 | 2.425 | 1.386-4.178 | 0.011 |
| High vs Low | | | | | | |
| TNM stage | 3.706 | 1.656-6.072 | 0.013 | 3.312 | 1.473-5.628 | 0.006 |
| III-IV vs I-II | | | | | | |
| Vein invasion | 3.464 | 1.528-5.773 | 0.009 | 2.846 | 1.373-5.071 | 0.004 |
| Presence vs Absence | | | | | | |
| Cirrhosis | 1.082 | 0.314-2.906 | 0.722 | | | |
| Positive vs Negative | | | | | | |
| Hepatitis B | 1.195 | 0.776-2.214 | 0.317 | | | |
| Positive vs Negative | | | | | | |
| lncRNA Sox2ot | 2.913 | 1.621-6.471 | 0.011 | 2.644 | 1.473-5.845 | <0.001 |
| High vs Low | | | | | | |

GATTTGGTCTGTATT-3' and reverse, 5'-AGTCTTCTGGGTGGCAGTGAT-3'. qRT-PCR and data collection were performed on ABI 7900. The relative expression of lncRNA Sox2ot was calculated and normalized using the $2^{-\Delta\Delta Ct}$ method relative to GAPDH.

*Statistical analysis*

All statistical analyses were performed using SPSS version 18.0 software (IBM). Comparisons between two groups were done using the student's t test for continuous data and the chi-square test for categorical data. The relationships between lncRNA Sox2ot and clinicopathologic features were evaluated by a chi-square test. The effects of lncRNA Sox2ot expression on the overall survival were evaluated by Kaplan-Meier curves, and the probability values were calculated using the log rank test. The multivariate analyses were evaluated with Cox proportional hazards models. The data are shown as the mean ± SD from at least three independent experiments. Differences were considered statistically significant when *P* was less than 0.05.

## Results

*Expression level of lncRNA Sox2ot in HCC*

We firstly examined lncRNA Sox2ot expression level in 84 paired HCC tissues and adjacent non-tumor tissues by qRT-PCR, and normalized to GAPDH. Our results showed that the lncRNA Sox2ot level was significantly increased in HCC tissues compared with adjacent non-tumor tissues ($P<0.05$, **Figure 1A**). The data indicated that abnormal lncRNA Sox2ot expression may be related to HCC pathogenesis. Furthermore, the correlation between lncRNA Sox2ot expression level and clinicopathological features was analyzed in 84 HCC patients. According to the median ratio of relative lncRNA Sox2ot expression (3.1) in tumor tissues, the 84 HCC patients were classified into two groups: relative high-Sox2ot group (Sox2ot expression ratio ≥ median ratio) and relative low-Sox2ot group (Sox2ot expression ratio < median ratio) (**Figure 1B**). The high-Sox2ot group was correlated with advanced histologic grade, higher TNM stage, and positive vein invasion ($P<0.05$) than the low-Sox2ot group. However, Sox2ot expression level was not associated with other parameters of HCC patients, such as gender, age, tumor size, cirrhosis and hepatitis B ($P>0.05$).

*Prognostic value of lncRNA Sox2ot expression in HCC*

The association between lncRNA Sox2ot expression in HCC and the survival time of HCC patients was analyzed with Kaplan-Meier survival analysis. We found that the overall survival

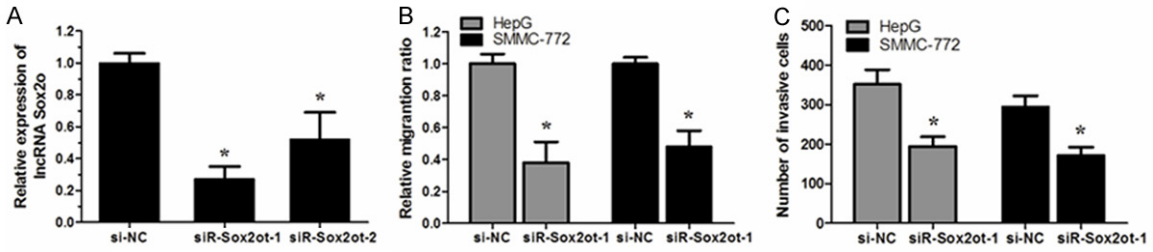


**Figure 3.** Decreased expression of lncRNA Sox2ot inhibited the migration and invasion ability of HCC cells. A. Transfection efficiency of si-Sox2ot in HCC cells was indicated by qRT-PCR; B. Wound healing assay was performed to investigate the effects of lncRNA Sox2ot on HCC cell migration; C. Transwell invasion assay was used to investigate the effects of lncRNA Sox2ot on HCC cell invasion. Results are expressed as means ± SD for three replicate determination. *$P$<0.05.

(OS) time of high lncRNA Sox2ot expression group was significantly shorter than that of low lncRNA Sox2ot expression group ($P$<0.05, **Figure 2**). The data indicated up-regulated expression of lncRNA Sox2ot in HCC was significantly correlated with patients' survival time. Next, Univariate analysis indicated that the overall survival of patients with HCC was associated with histologic grade, TNM stage, vein invasion and lncRNA Sox2ot expression. Furthermore, multivariate Cox proportional hazard regression analysis demonstrated that lncRNA Sox2ot expression, histologic grade, TNM stage and vein invasion were significantly associated with overall survival of HCC patients as independent prognostic factors (**Table 2**). Taken together, these data demonstrated that high lncRNA Sox2ot expression level is an independent risk factor for HCC patients.

*Effect of lncRNA Sox2ot on HCC cell migration and invasion in vitro*

To assess the biological roles of lncRNA Sox2ot in HCC, we investigated the effect of targeted knockdown of Sox2ot on cell migration and invasion. As shown in **Figure 3A**, 48 h after transfection of siR-Sox2ot, qRT-PCR results showed that lncRNA Sox2ot expression was significantly decreased in HepG2 cells. After transfection, cell migration and Invasion assays were conducted. Cell migration assay revealed that cell migration ability was significantly down-regulated in HepG2 and SMMC-7721 cells transfected with siR-Sox2ot compared with cells transfected with si-NC (**Figure 3B**, $P$<0.05). In addition, the results of cell invasion assay showed that cell invasion ability was decreased in siR-Sox2ot transfected HepG2 and SMMC-7721 cells (**Figure 3C**, $P$<0.05). Taken together, these results suggested that down-regulation of Sox2ot suppresses HCC cell migration and invasion in vitro.

## Discussion

HCC is the leading cause of cancer-related death in the world, so finding new molecular targets for its diagnosis, prognosis and treatment has the potential to improve the clinical strategies and outcomes of this disease [15]. In recent years, studies showed that dysregulation in lncRNAs are proved to contribute in tumor development in many cancer types and can be used to develop as biomarkers and therapy target [16]. So, the relationship between lncRNAs and tumors has currently become one of the focuses of cancer studies. In the present study, our attention focused on the lncRNA Sox2ot.

The SOX2 gene family (SRY-related HMG-box) encodes a group of transcription factors that are each characterized by the presence of a highly conserved high-mobility group (HMG) domain [17]. Recently studies showed that SOX2 participated in reprogramming of adult somatic cells to a pluripotent stem cell state and implicated in tumorigenesis in various organs [18]. Long non-coding RNA Sox2 overlapping transcript (lncRNA Sox2ot) localize on human chromosome 3q26.33, which has been proposed that Sox2ot has a role in processes related to SOX2 transcription, acting as an enhancer. Breast cancer study revealed that Sox2ot over-expression could lead to increased anchorage independent cell growth and Increased the number of colonies in MDA-MB-231 cells, they indicated that Sox2ot play a key role in breast cancer progression [19].

Shahryari et al. found that lncRNA Sox2ot was significant increased in esophageal squamous cell carcinoma tissues compared to the non-tumor tissues, suppressing Sox2ot caused a profound alteration in cell cycle distribution of NT2 cells, They suggested that Sox2ot play critical roles in tumor initiation and/or progression [20]. Hou et al showed Sox2ot was up-regulated in lung cancers, and high Sox2ot expression was correlated with patients' survival time after surgery. In addition, Down-regulated Sox2ot expression inhibited cell proliferation by inducing G2/M arrest. They suggested that Sox2ot play a key role in regulating lung cancer cell proliferation, and may represent a novel prognostic indicator for the disease [21]. However, lncRNA Sox2ot expression in HCC and underlying mechanism remains unclear.

In the present study, we first detected the expression of lncRNA Sox2ot in HCC tissues, our results showed that the mean level of lncRNA Sox2ot expression in HCC tissues was significantly higher than that in the adjacent non-tumor tissues. Meanwhile, we showed that high lncRNA Sox2ot expression was closely correlated with histologic grade, TNM stage, and vein invasion. Furthermore, patients with high lncRNA Sox2ot expression showed poorer survival than those with low lncRNA Sox2ot expression. A multivariate analysis with the Cox proportional hazards showed that the status of lncRNA Sox2ot expression was an independent predictor of overall survival in HCC. Then, we analyzed the effect of lncRNA Sox2ot expression on the metastasis of HCC cells. We showed that decreased expression of lncRNA Sox2ot could inhibit migration and invasion capability of HCC cells compared with control group, suggesting that up-regulation of lncRNA Sox2ot expression promote the metastasis capability of HCC cells, indicating that lncRNA Sox2ot may play an tumor oncogenic role in HCC.

In conclusion, we demonstrated that lncRNA Sox2ot was up-regulated in human HCC and could be considered an independent prognostic factor in patients with HCC. Down-regulation lncRNA Sox2ot expression could suppress HCC cell metastasis. These results suggested that lncRNA Sox2ot could be used as a new biomarker and a potential therapeutic target for HCC.

## Acknowledgements

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## Disclosure of conflict of interest

None.

## Abbreviations

lncRNAs, Long non-coding RNAs; HCC, hepatocellular carcinoma; TNM, tumor-node-metastasis; siRNA, small interfering RNA; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; Sox2ot, Sox2 overlapping transcript.

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