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# LncRNA SBF2-AS1 promotes hepatocellular carcinoma metastasis by regulating EMT and predicts unfavorable prognosis

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**Abstract. – OBJECTIVE: Recent studies have furthered our understanding of the function of long noncoding RNAs (lncRNAs) in numerous biological processes, including cancer. The present study aimed to investigate the expression of lncRNA SBF2-AS1 (SBF2-AS1) in patients with hepatocellular carcinoma (HCC) and to investigate its effect on HCC cells.**

**PATIENTS AND METHODS: Using quantitative reverse transcription-polymerase chain reaction, we detected SBF2-AS1 expression in HCC cell lines and primary tumor tissues. The associations between SBF2-AS1 expression and the clinicopathological factors and outcome of HCC patients were statistically analyzed. MTT assay and transwell assay were performed to determine the proliferation, migration and invasion, respectively. In addition, we evaluated the activation of Mesenchymal-epithelial transition (EMT) pathway by Western blot.**

**RESULTS: We found that SBF2-AS1 expression levels were significantly up-regulated in HCC tissues and cell lines compared with the corresponding noncancerous liver tissues and normal hepatic cell line. In addition, high SBF2-AS1 expression levels were correlated with vein invasion ($p = 0.008$) and TNM stage ($p = 0.013$). Furthermore, Kaplan-Meier survival analysis indicated that high expressions of SBF2-AS1 were correlated with shorter overall survival of HCC patients. Univariate and multivariate analysis identified high SBF2-AS1 expression as an unfavorable prognostic factor for overall survival. Further functional analysis demonstrated that knockdown of SBF2-AS1 significantly inhibited HCC cells proliferation, migration and invasion. Mechanistically, we found that SBF2-AS1 could promote the activation of EMT pathway, which was demonstrated by measuring the expression levels of EMT-related markers.**

**CONCLUSIONS: SBF2-AS1 might be considered as a novel molecule involved in HCC development, which provides a potential therapeutic target for HCC.**

*Key Words:*
LncRNA, SBF2-AS1, Prognosis, Proliferation, Migration, Invasion, EMT.

## Introduction

HCC is the most common aggressive hepatic disease, accounting for nearly half a million deaths worldwide[1]. Approximately 700,000 people die of HCC each year. More importantly, the global incidence is still rising[2]. HCC usually occurs in the context of chronic liver diseases and cirrhosis[3]. Despite the improvement of surgical techniques and perioperative management as well as the development of non-surgical treatments, the overall prognosis is still poor, and high metastasis and recurrence rates are the main factors affecting the prognosis of HCC patients[4-6]. Therefore, further revealing the molecular mechanisms that contribute to the development and progression of HCC is urgent for developing effective therapy. Long noncoding RNAs (lncRNAs) are a class of transcriptional products of the genome without protein-coding potential and composed of more than 200 nucleotides in length, which can regulate gene expression at the transcriptional

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or posttranscriptional level[7]. Researches[8,9] have indicated lncRNAs are involved in the regulation of various cellular processes, including but not limited to cell growth, cell cycle, apoptosis and motility. Mechanistically, several findings have suggested that lncRNAs serve as competing endogenous RNAs (ceRNAs) to sponge micro (mi) RNAs, thereby regulating gene expression[10,11]. In recent years, lncRNAs have been shown to be involved in carcinogenesis and cancer progression[12]. Recently, more and more lncRNAs, such as lncRNA HOTTIP[13], lncRNA DBH-AS1[14], linc-ITGB1[15], have been reported to have tumor-suppressive or oncogenic roles in regulating cells proliferation and metastasis in HCC. However, the function of most lncRNAs remains largely unknown in HCC. SET-binding factor 2 (SBF2) antisense RNA1 (SBF2-AS1), located at the 11p15.1 locus, is a 2,708-nt antisense RNA to SBF2. Previous studies have shown that SBF2-AS1 expression was significantly up-regulated in several tumors, such as esophageal squamous cell carcinoma[16], glioblastoma[17], non-small cell lung cancer[18]. However, to our best knowledge, few studies have been reported regarding the expression and functions of SBF2-AS1 in HCC. In the present study, we firstly found that SBF2-AS1 was highly expressed in HCC and its inhibition could suppress HCC cells proliferation, migration and invasion by modulating EMT. In addition, SBF2-AS1 served as an independent predictor for overall survival in HCC. Our findings contribute to our understanding of the mechanism underlying HCC progression and enable the development of new therapeutic strategies for HCC.

## Patients and Methods

### Patients and Specimens

We retrospectively investigated 134 patients diagnosed with HCC who underwent routine curative surgery between July 2010 and January 2013 at The Xianyang No.1 People's Hospital. None of these patients received neoadjuvant or adjuvant chemotherapy before operation. All samples were immediately frozen in liquid nitrogen and stored at -80°C until use. The histopathologic diagnoses of these tissue samples were confirmed by two experienced pathologists. All clinical and pathological information were acquired from patients' medical records and pathological reports. The patients' characteristics are summarized in Table II. Informed consent was obtained from each patient and the study was approved by the Ethics Committee of The Xianyang No.1 People's Hospital, China.

### Cell Culture and RNA Interference

Four human HCC cell lines, including HCCLM3, Huh7, SK-Hep1, HepG2, and one normal liver cell line L02, were used in this study. All cell lines were purchased from the Committee on Type Culture Collection of the Chinese Academy of Sciences (Pudong, Shanghai, China). Cells were cultured in RPMI-1640 medium (Cambrex, Walkersville, MD, USA) supplemented with 10% fetal bovine serum (FBS; Taixin Technology, Xicheng, Beijing, China) at 37°C and 5% $CO_2$. SBF2-AS1 small interfering RNA (si-SBF2-AS1) and nontargeting small interfering RNA (siRNA) (si-NC) were purchased from Sigma-Aldrich (St. Louis, MO, USA). Transfections were performed using the Lipofectamine 3000 Transfection Reagent (Invitrogen, Carlsbad, CA, USA) according to the manufacturer's protocol. After infection of 48 h, the infected cells were harvested for extraction of total RNA.

### RNA Extraction and Quantitative Real-time PCR

Total RNA was extracted from tissues or cultured cells with TRIzol reagent (Invitrogen, Grand Island, NY, USA) according to the manufacturer's protocol. Reverse transcription was conducted with Revertra Ace aPCR RT master mix with gDNA remover (Toyobo, Pudong, Shanghai, China). Quantitative Real-time PCR was performed on a 7300 Real-Time PCR System (Thermo Fisher Scientific, Waltham, MA, USA) using SYBR green agent (Applied Biosystem, Foster City, CA, USA). The glyceraldehyde-3-phosphate dehydrogenase (GAPDH) level was used as a normalizing control. All qPCR reactions were performed in triplicate and the relative expression of SBF2-AS1 was calculated using the comparative cycle threshold (CT) ($2^{-\Delta\Delta Ct}$) method. Primers used for qRT-PCR assay were shown in Table I.

**Table I.** Primers for qRT-PCR.

| Primers | Sequence |
|---|---|
| SBF2-AS1 | F 5'-CACGACCCAGAAGGAGTCTAC-3'<br>R 5'-CCCGGTACCTTCCTGTCATA-3' |
| GAPDH | F 5'-GCACCGTCAAGGCTGAGAAC-3'<br>R 5'-TGGTGAAGACGCCAGTGGA-3' |

### Cell Proliferation Assays

Cell proliferation was measured using the MTT [3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl-2H-tetrazolium bromide] assay. After 24 h transfection, cells were seeded into 96-well plate at 5.0 × 103 cells/ml and continue cultured for 24, 48, 72, and 96 h, respectively. After that, the culture medium was discarded and replaced with 180 μl RPMI-1640 medium and 20 μl MTT solution (5 g/l; Shangshui Technology, Shijingshan, Beijing, China). The cells were then dissolved with 200 μ l of dimethylsulphoxide and the absorbance of the cells was measured by a MicroplateReader (Bio-Rad, Hercules, CA, USA) at 490 nm.

### Migration and Invasion Assay

For migration assay, $2 \times 10^4$ cells were seeded in fetal bovine serum-free Dulbecco's Modified Eagle Medium (DMEM) in the upper chamber of a 24-well transwell (Corning, Corning, NY, USA). For invasion assay, $2 \times 10^4$ cells were seeded in fetal bovine serum (FBS)-free Dulbecco's Modified Eagle Medium (DMEM) in the upper chamber of a 24-well Matrigel transwell invasion insert (BD Bioscience, Franklin Lakes, NJ, USA). 10% FBS medium was added to the lower chamber of the transwell. After 24 h, the noninvading cells were removed with cotton wool, invasive cells located on the lower surface of the chamber were stained with May-Grunwald-Giemsa stain (Sigma-Aldrich, St. Louis, MO, USA) and counted in at least 5 random fields using a microscope (Olympus, Tokyo, Japan). The assays were conducted three independent times.

### Western Blot Assay

Protein was isolated from cell lysis using Mammalian Protein Extraction Reagent (Thermo Fisher Scientific, Waltham, MA, USA). Protein samples were separated on 10-12% gel by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and were transferred to polyvinylidene difluoride (PVDF) membranes. The membranes were blocked with 5% non-fat milk, followed by incubation with antibodies against Fibronectin, Vimentin and E-cadherin, β-actin (Bailingke, Haidian, Beijing, China). β-actin expression was used as a standard for the normalization of the measurement. Membranes were further washed and incubated with appropriate horseradish peroxidase (HRP)-conjugated secondary antibodies for 2 h at room temperature. The protein bands were quantitated by densitometry using the gel analysis software ImageJ (National Institutes of Health, Bethesda, MA, USA).

### Statistical Analysis

All statistical analyses were carried out by SPSS13.0 software (SPSS Inc., Chicago, IL, USA). Differences between the SBF2-AS1 in tumor tissues and adjacent nontumor tissues were analyzed by the Wilcoxon matched pairs test. Association between expression level of SBF2-AS1 and each clinicopathologic parameter was evaluated using Pearson's $x^2$-test. Kaplan-Meier method and log-rank test were used to evaluate and compare the prognosis of HCC patients. A Cox proportional hazard regression analysis was used for univariate and multivariate analyses of prognostic values. A $p < 0.05$ was considered significant.

## Results

### Increased Expression of SBF2-AS1 in HCC Tissues and Cell Lines

To examine whether SBF2-AS1 expression was dysregulated in HCC patients, we firstly analyzed the RNA-Seq data of lncRNAs of HCC (n = 24) and normal hepatocellular tissue (n = 40) from the cancer genome atlas (TCGA) database by a bioinformatics tool "lncRNAtor". We found that the $p$-value = $1.09 \times 10^{-12}$ ($p < 0.001$) in HCC (Figure 1). Then, we performed qPCR to detect the expression of SBF2-AS1 in a cohort of 134 pairs HCC tissues and adjacent normal tissues. As shown in Figure 2A, we found that SBF2-AS1 expression level was significantly higher in HCC tissues compared with non-cancerous liver tissues ($p < 0.01$). RT-qPCR assays were further developed to quantify SBF2-AS1 levels in HCC cell lines, including SMMC- HCCLM3, Huh7, SK-Hep1 and HepG2 cells, and the normal hepatic cell line L02. As shown in Figure 2B, our results showed that the expression of SBF2-AS1 was higher in all four HCC cell lines compared with the levels observed in L02 cells and normal hepatocytes. HCCLM3 and SK-Hep1 cells were then selected for further studies.

### Correlation Between SBF2-AS1 Expression and Clinical Features and Prognosis of HCC Patients

To evaluate the correlation between the SBF2-AS1 expression levels and the clinicopatholog-

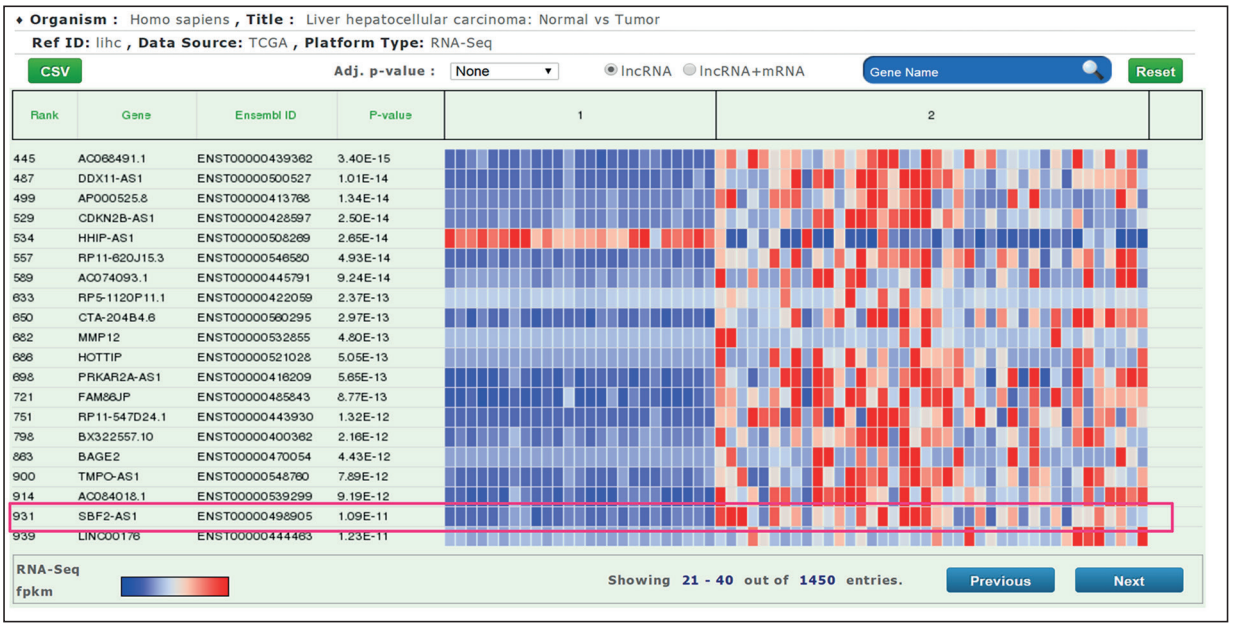


**Figure 1.** Screening out SBF2-AS1 by bioinformatics tools "lncRNAtor" RNA-Seq data from TCGA of lncRNAs of HCC were analyzed by bioinformatics tools "lncRNAtor" HCC (n = 40), normal hepatocellular tissue (n = 25).

ical characteristics, we divided the 134 HCC patients into a high expression group (n = 68) and a low expression group (n = 66), according to the median expression level of SBF2-AS1 in all HCC samples. We found that high SBF2-AS1 expression levels were correlated with vein invasion ($p$ = 0.008) and TNM stage ($p$ = 0.013) (Table II). However, there were no significant correlations between SBF2-AS1 expression and other clinicopathological factors of patients. We further evaluated the association of SBF2-AS1 expression level with survival of HCC patients. The results of survival analysis showed that the overall survival rate was significantly lower in the patients with high SBF2-AS1 level than in those with low levels ($p$ = 0.0049, Figure 2C). In addition, univariate analyses suggested that SBF2-AS1 expression, as well as vein invasion and TNM stage, were significantly correlated with overall survival of HCC patients (Table III). More importantly, SBF2-AS1 expression level ($p$ = 0.008, HR = 2.889, 95 % CI = 1.327-5.231) served as an independent prognostic factor for poor overall survival.

### *Knockdown of SBF2-AS1 Suppresses HCC Cell Proliferation, Migration and Invasion in Vitro*

Then, we assessed the biological role of SBF2-AS1 in HCCLM3 and SK-Hep1 cells. Transfection with si-SBF2-AS1 resulted in increased SBF2-AS1 level in HCCLM3 and SK-Hep1 cells (Figure 3A). Cell viability curves were drawn using the MTT method to determine the proliferation of the transfected HCCLM3 and SK-Hep1 cells. As shown in Figure 3B and 3C, we found that down-regulation of SBF2-AS1 significantly decreased cell viability of HCCLM3 and SK-Hep1 cells when compared to their corresponding controls. Subsequently, we further investigate the effect of SBF2-AS1 on cell migration and invasion ability of HCC cells. The results showed that SBF2-AS1 down-regulation significantly reduced the migration and invasion of HCCLM3 and SK-Hep1 cells (Figure 3D and 3E). Taken together, our findings indicated that SBF2-AS1 may participate in HCC cells proliferation and metastasis.

### *SBF2-AS1 Affects HCC Cells Epithelial-Mesenchymal Transition (EMT)*

EMT is one of the crucial mechanisms by which tumor cells detach from their primary site and invade into surrounding tissues as well as the vascular system. In order to explore the potential mechanism by which SBF2-AS1 promoted HCC cells migration and invasion, our attention focused on EMT. Then, we performed Western blot to explore the effect of SBF2-AS1 on EMT-related proteins. Our results showed that

**Table II.** Correlation between SBF2-AS1 expression and clinicopathological features of HCC patients.

| Clinicopathological features | Number of cases | SBF2-AS1 expression | | *p*-value |
|---|---|---|---|---|
| | | Low | High | |
| Gender | | | | NS |
| Male | 78 | 38 | 40 | |
| Female | 56 | 28 | 28 | |
| Age | | | | NS |
| < 50 | 65 | 30 | 35 | |
| ≥5 0 | 69 | 36 | 33 | |
| Tumor size (cm) | | | | NS |
| < 5 | 81 | 43 | 38 | |
| ≥ 5 | 53 | 23 | 30 | |
| Serum AFP (μg/L) | | | | NS |
| < 400 | 57 | 29 | 28 | |
| ≥ 400 | 77 | 37 | 40 | |
| Tumor differentiation | | | | NS |
| Well+ Moderate | 67 | 37 | 34 | |
| Poor | 67 | 29 | 38 | |
| Tumor number | | | | NS |
| Single | 55 | 30 | 25 | |
| Multiple | 79 | 36 | 43 | |
| Live cirrhosis | | | | NS |
| No | 41 | 18 | 23 | |
| Yes | 93 | 48 | 45 | |
| Vein invasion | | | | 0.008 |
| No | 76 | 45 | 31 | |
| Yes | 58 | 21 | 37 | |
| TNM stage | | | | 0.013 |
| I-II | 79 | 46 | 33 | |
| III-IV | 55 | 20 | 35 | |

SBF2-AS1 siRNA markedly reduced N-cadherin and Vimentin expression, while slightly restored E-cadherin expression in both HCCLM3 and SK-Hep1 cells (Figure 4). Our work showed that SBF2-AS1 functioned as a promoter lncRNA by promoting EMT pathway in HCC.

## Discussion

HCC is one of the most mortal malignant tumors, and is becoming one of most lethal threat to human health and life[19]. A better understanding of the development and progression of HCC

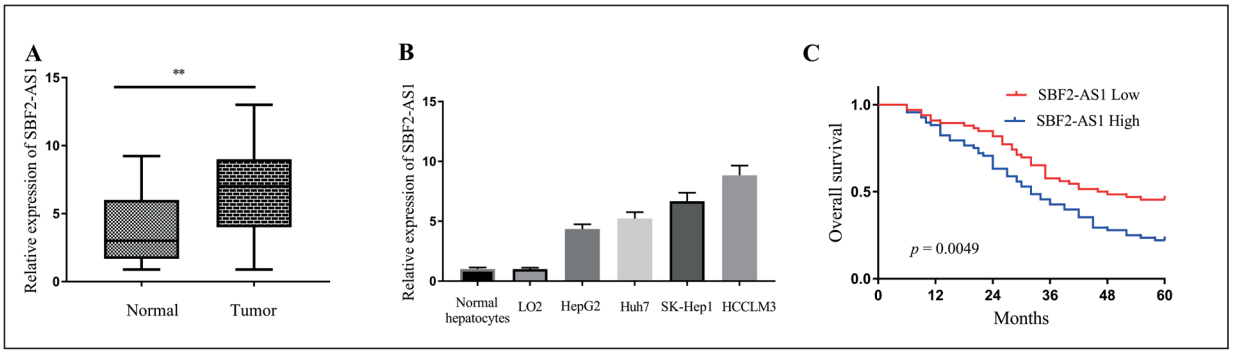


**Figure 2.** SBF2-AS1 expression is increased in HCC and correlated with poor overall survival. ***(A)*** The expression levels of SBF2-AS1 in 134 pairs of HCC tissues and adjacent non-tumor tissues were determined by qRT-PCR. ***(B)*** SBF2-AS1 expression levels in the four cell lines were analyzed by qRT-PCR. SBF2-AS1 expression levels in the HCC cell lines were normalized to that in one normal liver cell line L02. ***(C)*** Kaplan-Meier survival curves of patients with HCC based on SBF2-AS1 expression status. $^{**}p < 0.01$, $^{*}p < 0.05$.

**Table III.** Univariate and multivariate analysis of overall survival in HCC patients.

| Variables | HR | 95% CI | p |
|---|---|---|---|
| Univariate analysis | | | |
| Gender | 1.213 | 0.567-1.632 | 0.238 |
| Age | 0.934 | 0.671-1.895 | 0.488 |
| Tumor size | 1.032 | 0.522-2.138 | 0.166 |
| Serum AFP | 1.423 | 0.677-1.996 | 0.149 |
| Tumor differentiation | 1.564 | 0.788-2.215 | 0.114 |
| Tumor number | 1.788 | 0.935-2.556 | 0.093 |
| Live cirrhosis | 1.547 | 1.132-2.633 | 0.086 |
| Vein invasion | 3.554 | 1.423-5.354 | 0.005 |
| TNM stage | 2.893 | 1.147-4.455 | 0.016 |
| SBF2-AS1 expression | 3.367 | 1.547-6.667 | 0.001 |
| Multivariate analysis | | | |
| Vein invasion | 2.783 | 1.218-4.779 | 0.013 |
| TNM stage | 2.457 | 1.022-3.672 | 0.031 |
| SBF2-AS1 expression | 2.889 | 1.327-5.231 | 0.008 |

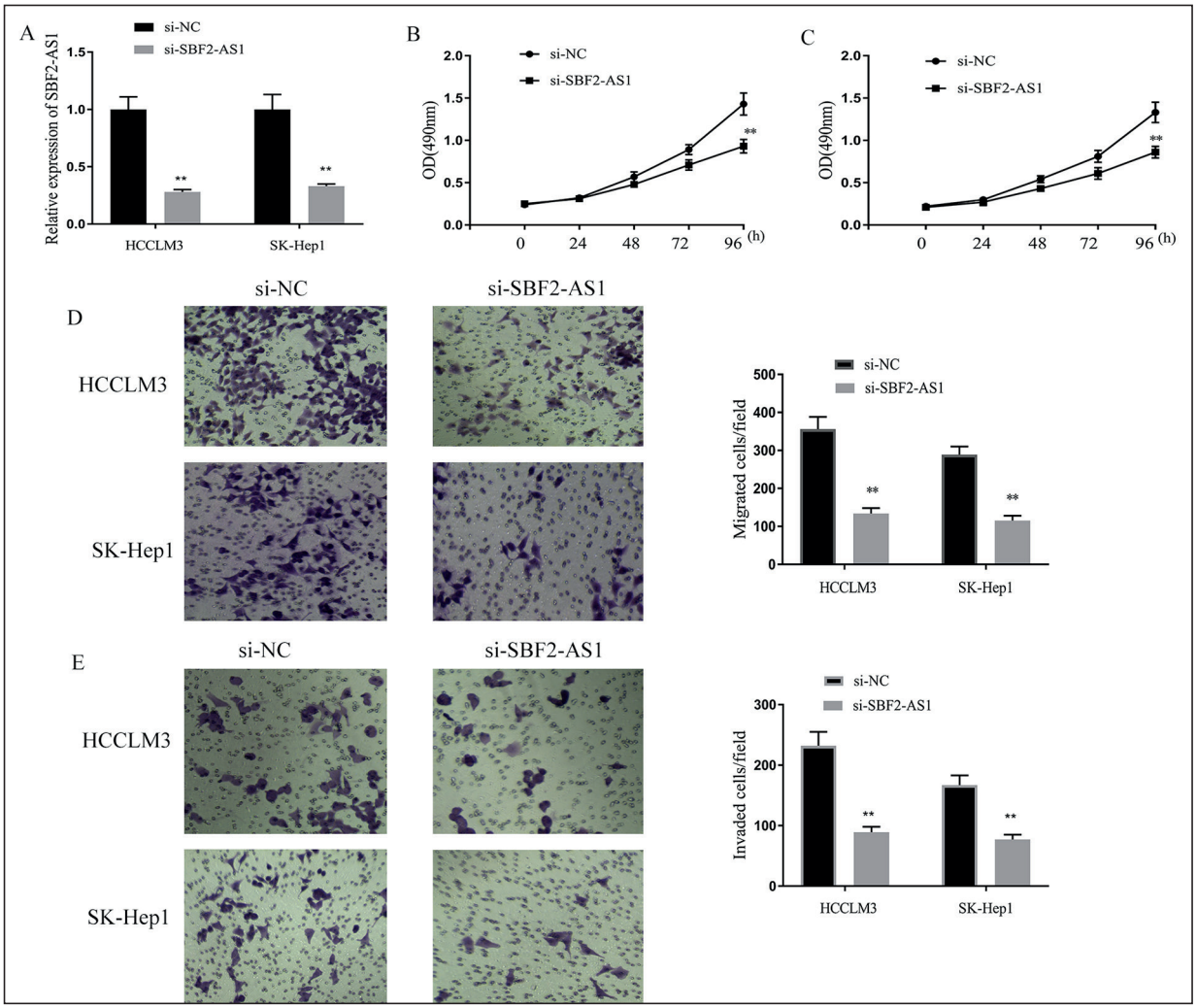


**Figure 3.** Decreased expression of SBF2-AS1 inhibited the proliferation, migration and invasion ability of HCC cells. ***(A)*** Transfection efficiency of si-SBF2-AS1 in HCC cells was indicated by qRT-PCR. ***(B, C)*** MTT assay analysis of cell proliferation of HCCLM3 and SK-Hep1 cells transfected with si-SBF2-AS1 or si-NC. ***(D, E)*** Transwell invasion and migration assays showed that the number of invaded/migrated cells was significantly lower in the si-SBF2-AS1-transfected group than in the si-NC-transfected group. $^{**}p < 0.01$, $^{*}p < 0.05$.

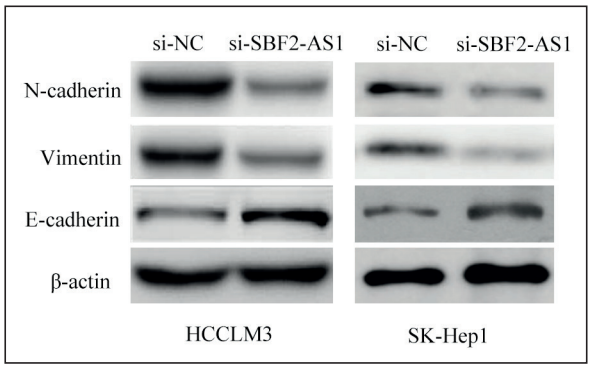


**Figure 4.** Fibronectin, Vimentin and E-cadherin protein expression levels in HCCLM3 and SK-Hep1 transfected with si-SBF2-AS1 or si-NC were analyzed by using Western-blotting.

is essential for early diagnosis and treatment. Recent investigations[20-22] revealed that several lncRNAs have pivotal roles in critical cellular processes, including proliferation, differentiation, apoptosis, and senescence, indicating lncRNAs may have potential diagnostic and prognostic value for HCC patients. In the present study, by bioinformatics tools, we screened the differential expressed lncRNA and found that SBF2-AS1 expression was significantly up-regulated in HCC. Next, we detected the expression of SBF2-AS1 in HCC tissues and cell lines and found SBF2-AS1 was upregulated in HCC cell lines and tissues. These findings confirmed that SBF2-AS1 may contribute to the progression of HCC. Subsequently, we explored whether SBF2-AS1 was correlated with poor prognosis of HCC by analyzing the clinical data via indicated statistical approach. We found that high SBF2-AS1 expression levels were correlated with vein invasion and TNM stage. More importantly, we proved that SBF2-AS1 expression was significantly associated with overall survival of patients with HCC. Further multivariate survival analysis showed that SBF2-AS1 is involved in HCC and could be used as an independent potential prognostic biomarker for HCC patients. Taken together, these results supported that SBF2-AS1 may have utility as a prognostic marker of patient with HCC. Recently, several studies showed that SBF2-AS1 expression was significantly up-regulated and served as a tumor promoter in several tumors. For instance, Lv et al[18] firstly reported that high SBF2-AS1 expression was observed in non-small cell lung cancer and associated with advanced TNM stage. Lost-function assay indicated that down-regulation of SBF2-AS1 significantly suppressed decreased the proliferation and metastasis ability of non-small cell lung cancer cells. In addition, Zhao et al[23] provided evidence that SBF2-AS1 was associated with overall survival of non-small cell lung cancer patients. Chen et al[16] found that a high expression of SBF2-AS1 in esophageal squamous cell carcinoma and the association with an inferior prognosis and inhibition of SBF2-AS1 expression suppressed the proliferative and invasive ability of esophageal squamous cell carcinoma cells. Yu et al[17] revealed that SBF2-AS1 was upregulated in glioma samples and knockdown of SBF2-AS1 repressed glioblastoma cell-driven angiogenesis via enhancing the inhibitory effect of miR-338-3p. All these findings indicated SBF2-AS1 as a promoter lncRNA in several tumors. However, the function and molecular mechanism of SBF2-AS1 in HCC has not been investigated. In the present study, we regulated SBF2-AS1 expression by transfected siRNA vector into HCC cell line and observed the influence of SBF2-AS1 on HCC cells *in vitro*. We found that knockdown of SBF2-AS1 inhibited HCC cell proliferation, migration and invasion, which was consistent with clinical test that high SBF2-AS1 expression was closely associated with lymph node metastasis. These data indicated that SBF2-AS1 was important in controlling hepatocellular carcinogenesis. To date, the molecular mechanisms whereby lncRNAs promote tumor metastasis are not fully understood. In order to explore the potential mechanism by which SBF2-AS1 promoted the metastasis of HCC cells, our attention focused on EMT signaling pathway. EMT governs many developmental processes such as gastrulation, neural crest development, somite dissociation, and palate and lip fusion[24]. EMT process could confer more mesenchymal fibroblast-like and more motile on the polarized epithelial cells and leads to invasion and metastasis of cancer cells[25]. Decreased E-cadherin and elevated vimentin and snail expression are hallmarks of EMT, which is a key element in the cancer invasion[26,27]. Furthermore, several lncRNAs have been shown to modulate EMT pathway in several tumors, such as lncRNA XIST[28], lncRNA PCAT-1[29] and lncRNA FOXD2-AS1[30]. In this study, we down-regulated the levels of SBF2-AS1 and performed Western blot to observe the effect of SBF2-AS1 on EMT-related factors. As expected, down-regulated SBF2-AS1 expression

resulted in increased E-cadherin expression and decreased fibronectin and vimentin expression, suggesting that SBF2-AS1 exerted its effect on HCC via modulating EMT pathway.

## Conclusions

To the best of our knowledge, we firstly demonstrated that SBF2-AS1 was upregulated in HCC cells and clinical samples. Elevated SBF2-AS1 expression was associated with poor prognosis. Furthermore, SBF2-AS1 served as a tumor promoter via inactivation of EMT pathway in HCC. Our study enriches the underling molecular mechanisms of carcinogenesis and metastasis, and provides a novel biomarker and potential therapeutic target for HCC.

### Conflict of Interest

The Authors declare that they have no conflict of interests.

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