Long non-coding RNA TUSC7 acts a molecular sponge for miR-10a and suppresses EMT in hepatocellular carcinoma

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Abstract Despite advances in the roles of long non-coding RNA (lncRNA) tumor suppressor candidate 7 (TUSC7) in cancer biology, which has been identified as a tumor suppressor by regulating cell proliferation, apoptosis, migration, invasion, cell cycle, and tumor growth, the function of TUSC7 in hepatocellular carcinoma (HCC) remains unknown. In this study, we observed that the expression of TUSC7 was immensely decreased in HCC. Clinically, the lower expression of TUSC7 predicted poorer survival and may be an independent risk factor for HCC patients. Moreover, TUSC7 inhibited cell metastasis, invasion, and epithelial-to-mesenchymal transformation (EMT) through competitively binding miR-10a. Furthermore, we found that TUSC7 could decrease the expression of Eph tyrosine kinase receptor A4 (EphA4), a downstream target of miR-10a as well as an EMT suppressor, through TUSC7-miR-10a-EphA4 axis. Taken together, we demonstrate that TUSC7 suppresses EMT through the TUSC7-miR-10a-EphA4 axis, which may be a potential target for therapeutic intervention in HCC.

Keywords TUSC7 · miR-10a · EphA4 · EMT · HCC

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Introduction

Hepatocellular carcinoma (HCC) is the most common type of primary liver cancer and the second leading cause of death from cancer worldwide [1]. Currently, the prognosis for HCC patients remains poor, with a 5-year survival rate of approximately 30% after liver resection, which is considered to be the best therapeutic strategy to treat HCC [2]. Local and systemic metastases are the main reasons for the unsatisfactory prognosis of HCC patients [3]. Elucidating the underlying molecular mechanisms for HCC metastasis is critical for identifying novel therapeutic targets of HCC.

Epithelial-to-mesenchymal transformation (EMT) has been widely accepted as a key mechanism underlying the metastatic process of HCC [4]. During the development of EMT, the expressions of epithelial markers such as E-cadherin, zonula occludens-1, and claudin decrease while the expressions of mesenchymal markers such as vimentin, N-cadherin, and fibronectin increase [5]. The EMT process in HCC cells can be regulated by various factors, including hypoxia [6], cytokines [7], long non-coding RNAs (lncRNAs) [8], microRNAs [9], and so on, and targeting the EMT process has been found to be an attractive and promising strategy to prevent the metastasis of HCC [7].

lncRNAs are RNA molecules over 200 nucleotides in length with little protein-coding potential [10]. Previous studies have shown that aberrant lncRNA expression is observed in human cancers, including those in the liver [11], breast [12], colon [13], ovary [14], pancreas [15], and bladder [16]. lncRNAs have been identified with oncogenic properties (KRASP, HULC, HOTAIR, MALAT1, HOTTIP, ANRIL, and RICTOR) or oncosuppressive properties (MEG3, GAS5, LocRNA-p21, PTENP1, TERRA, CCND1/CyclinD1, and TUG1) or both (CCAT1 and XIST) [17, 18]. Tumor suppressor candidate 7 (TUSC7), also called LOC285194 or LSAMP antisense RNA3, is an lncRNA consisting of four exons of more than 2 kb in length and is located at 3q13.31 [19]. Recent studies indicated that lncRNA TUSC7 is downregulated in cancers including gastric cancer [20], osteosarcoma [21], colorectal cancer (CRC) [22], esophageal squamous cell carcinoma (ESCC) [23], and so on. In gastric cancer, TUSC7 is a p53-regulated tumor suppressor that acts in part by repressing miR-23b to suppress tumor cell growth in vitro and in vivo [20]. In osteosarcoma, depleting TUSC7 promoted proliferation of normal osteoblasts by regulating apoptotic and cell cycle transcripts as well as the vascular endothelial growth factor (VEGF) receptor 1 [21]. In human pancreatic ductal adenocarcinoma (PDAC) and CRC, by analyzing the association of TUSC7 expression with clinicopathologic features, it was found that low TUSC7 expression was closely correlated with lymph node metastasis, liver metastasis, and more distant metastases [19, 22]. These data validated that TUSC7 is a tumor suppressor by regulating cell proliferation, apoptosis, migration, invasion, cell cycle, and tumor growth. However, the exact role of TUSC7 in HCC progression and the underlying mechanisms remain unknown.

MicroRNAs (miRNAs) are an abundant group of endogenous non-coding single-strand RNAs, and it is known that aberrant miRNA expression profiles are causally connected to tumor progression [24]. Recently, the competing endogenous RNA (ceRNA) hypothesis proposed that a large number of non-coding RNAs might function as molecular sponges for miRNAs and, hence, functionally liberate other RNA transcripts targeted by the aforementioned active miRNAs [25]. For example, IncRNA-UCA1 has been reported to play an oncogenic role in breast cancer through directly interacting with miR-143 to lower its expression and affect its downstream regulation [26]. miR-222 could be downregulated by IncRNA-Gas5 in glioma, thereby suppressing the tumor malignancy [27, 28], and has been reported to play critical roles in the development of a variety of human cancers [29–31], including HCC [28, 32]. In HCC, acting as a tumor promoter, the expression of miR-10a has been shown to be upregulated, which accelerates the cell migration, invasion, and EMT [32, 33]. Additionally, Eph tyrosine kinase receptor A4 (EphA4), a member of the Eph receptor tyrosine kinase family, has been identified as an EMT suppressor in cancers [34–36]. It is reported that miR-10a could regulate the EMT process in HCC through directly binding the 3’-untranslated region (UTR) of the EphA4 transcript [32]. However, limited knowledge is available concerning whether TUSC7 could act as a sponge for miR-10a to affect the biological processes of HCC and the potential primary mechanism among TUSC7, miR-10a, and EphA4 in HCC progression remains unknown.

In this study, we found that the expression of TUSC7 was decreased in HCC and that TUSC7 may be a promising prognostic or progression marker for HCC. Additionally, TUSC7 suppressed cell migration, invasion, and EMT of HCC cells. Moreover, mechanistic analysis revealed that TUSC7 may function as a ceRNA for miR-10a to regulate the expression of EphA4 to suppress EMT in HCC, thus playing an oncosuppressive role in HCC pathogenesis. Here, we provide the first evidence for the TUSC7-miR-10a-EphA4 axis, shedding new light on the mechanism of HCC.

Materials and methods

Clinical samples

HCC samples were collected from 75 patients including 51 males and 24 females, who underwent resection of their primary HCC in the Department of Hepatobiliary Surgery at the First Affiliated Hospital of Xi’an Jiaotong University during January 2009 to December 2011. Patients did not receive any preoperative chemotherapy or embolization.
Patients’ demographic and clinicopathologic data were obtained through a review of hospital records. And disease recurrence and survival information was updated at each follow-up visit. The time between the surgery date and first disease recurrence date was calculated as disease-free survival (DFS). The time between the diagnostic biopsy and surgery date to death or last follow-up was determined as overall survival (OS) duration.

Cell culture

The human immortalized normal hepatocyte cell line (LO2) and six HCC cell lines (HepG2, MHCC97L, Hep3B, SMMC-7721, MHCC97H, and Huh7) were obtained from the Institute of Biochemistry and Cell Biology, Chinese Academy of Sciences, Shanghai, China. All cells were cultured in complete Dulbecco’s modified Eagle’s medium (DMEM; Gibco, Grand Island, NY, USA) containing 10% fetal bovine serum (FBS; Gibco) with 100 units/mL penicillin and 100 μg/mL streptomycin (Sigma, St. Louis, MO, USA) in a humidified incubator containing 5% CO2 at 37 °C.

Cell transfection

Three TUSC7-specific small interfering RNAs (siRNAs), the TUSC7-siControl (Table 1), pcDNA3.1-TUSC7 (pcDNA/TUSC7), and pcDNA3.1-Control (pcDNA/Control), were purchased from Invitrogen (Carlsbad, CA, USA). Four miRNA vectors, including anti-miR-10a, anti-Control, miR-10a, and miR-10a-Control, were purchased from GeneCopoeia (Guangzhou, China). All cell transfections were performed according to the manufacturer’s protocol.

Luciferase reporter assay

To search for the miR-10a binding site of TUSC7, we used a number of bioinformatics tools (MicroRNA, Mircode, Starbase v2.0, and RNAhybrid). The putative miR-10a target binding sequence in TUSC7 and its binding site mutant were synthesized and cloned downstream of the luciferase gene in the pmirGLO luciferase vector (Promega, Madison, WI, USA). Hep3B cells were co-transfected with wild-type or mutated pmirGLO-miR-10a reporter plasmid and pcDNA/Control or pcDNA/TUSC7 using Lipofectamine 2000 (Invitrogen). After 48 h, the cells were harvested and luciferase activity was measured using the dual-luciferase reporter assay system (Promega, Madison, WI, USA). Firefly luciferase activity was normalized to the Renilla luciferase activity. Results were obtained from three independent experiments performed in triplicate.

RNA extraction and quantitative real-time PCR

Total RNA was extracted from HCC tissues and cell lines using TRIzol (Invitrogen) following the manufacturer’s instructions. The RNA levels of TUSC7 and EphA4 were determined by quantitative real-time PCR (qRT-PCR) and calculated using the 2^−ΔΔCt method, with the Ct values normalized using GAPDH as an internal control. The primers are listed in Table 2. miRNAs were obtained using the mirVana MiRNA Isolation Kit (Ambion, Austin, TX, USA). Mature miR-10a and U6 snRNA were reversely transcribed using Stem-loop RT Primer with miScript II RT Kit (Qiagen, Valencia, CA, USA). qRT-PCR was performed using SYBR Green PCR Master Mix (Qiagen) in an ABI 7500 system (Applied Biosystems, USA).

Western blot

Western blot analysis was performed using standard techniques. The following antibodies were used: E-cadherin (3195S, Cell Signaling, Beverly, MA, USA), vimentin (sc-6260, Santa Cruz Biotechnology, Santa Cruz, CA, USA), EphA4 (SRP00347b, Saierbio, Tianjin, China), and β-actin (sc-47778, Santa Cruz Biotechnology, Santa Cruz, CA, USA).

Table 1: TUSC7-siRNAs and TUSC7-siControl sequences

| siRNA          | Sequence                        |
|---------------|---------------------------------|
| TUSC7-siRNA1  | Sense: 5'-GGCCAACCACCUACUAGAAUtt' |
|               | Antisense: 5'-AUUCAUUGAGGUUGCCGttg' |
| TUSC7-siRNA2  | Sense: 5'-GCGCAUUUUCUCUAAAACTT' |
|               | Antisense: 5'-UUUGUUAAGAGAAGCCGTT' |
| TUSC7-siRNA3  | Sense: 5'-CUGCCUCUCAUUCUAUCATT' |
|               | Antisense: 5'-UAAGAUAAGAGGAGGCGTT' |
| TUSC7-siRNA4  | Sense: 5'-GGAGAGAGAUAGCUAGUTT' |
|               | Antisense: 5'-ACUUAGCAUAUCUCUCGTT' |
| TUSC7-siControl| Sense: 5'-UUCUCCGAGAGUUCAGUGTT' |
|               | Antisense: 5'-ACGGUGACAGGUUCGGAAAT' |

Table 2: Primers used in qRT-PCR

| Primer name | Sequence (5’–3’) | Primer name | Sequence (5’–3’) |
|-------------|------------------|-------------|------------------|
| GAPDH       | Forward: 5'-CCGGGAACGTTGGCGTGATG-3' | Reverse: 5'-AGGGTAGGAGTGGGTCGTGTT-3' |
| TUSC7       | Forward: 5'-CCTGTCCTAGTGCACA-3' | Reverse: 5'-AGAGTCCCGCAAGAAGAACA-3' |
| E-cadherin  | Forward: 5'-GCCGTTGGGCTCTTAAAGA-3' | Reverse: 5'-TGACCACGCTTCTCCCGGA-3' |
| Vimentin    | Forward: 5'-GAGAATTTGCGGTAGAAAGC-3' | Reverse: 5'-GCTTCCTCTAGTTGGCATAATC-3' |
| EphA4       | Forward: 5'-ATGGATGCTGTTGCTAC-3' | Reverse: 5'-CAGAAATCCCTCTACCCTACC-3' |
Wound healing assays

To determine cell motility, HCC cells were seeded into six-well plates and grown to 80–90 % confluence. A 200-μL sterile plastic tip was used to create a wound line across the surface of plates, and cellular debris was removed by washing with phosphate-buffered saline (PBS). Cells were cultured in DMEM in a humidified incubator with 5 % CO₂ at 37 °C for 48 h, and then images were taken with a phase-contrast microscope.

Transwell assays

The 8 μM pore-size transwell inserts (Nalge Nunc, Penfield, New York, NY, USA) were coated with Matrigel (BD Biosciences, Franklin Lakes, NJ, USA) at 1:8 dilution on the inner layer. Hep3B and MHCC97H cells were resuspended with reduced serum DMEM, and the density was adjusted to 2.5 × 10⁵/mL 48 h after transfection. A 200-μL cell suspension was added into the upper chamber, and 750 μL DMEM containing 10 % FBS was added into the lower chamber and then incubated for 24 h.

Cells were fixed in 4 % paraformaldehyde for 2 min and then permeabilized in 100 % methanol for 20 min. The cells on the inner layer were softly removed with a cotton swab, and the adherent cells on the undersurface of the insert were stained with 0.3 % crystal violet dye for 15 min. The filters were washed with PBS, and images were taken. Cells on undersurface were counted under a light microscope.

Immunohistochemistry

Immunohistochemistry staining was performed on paraformaldehyde-fixed paraffin sections. The sections were dewaxed and dehydrated. Following rehydration and antigen retrieval in citrate buffer, endogenous peroxidase activity was blocked for 10 min using 3.0 % hydrogen peroxide. The sections were blocked for 30 min using 10 % goat plasma and then separately incubated with the primary antibodies directed against E-cadherin (1:400) and vimentin (1:200) at 4 °C overnight. The primary antibody was detected using biotinylated secondary antibodies (Golden Bridge Biotechnology, Zhongshan, China) according to the manufacturer’s recommendations. The sections were visualized with diaminobenzidine and counterstained with hematoxylin and then dehydrated in alcohol and xylene and mounted onto glass slides.

Statistical analysis

Results are presented as mean ± SD. The SPSS statistical package for Windows version 13 (SPSS, Chicago, IL, USA) and GraphPad Prism 5 software (GraphPad Software, Inc., San Diego, CA, USA) were used for the Pearson chi-square
test, a two-tailed Student’s *t* test, a Kaplan-Meier plot, a log-rank test, or an ANOVA where appropriate. Differences were considered to be significant when *p* < 0.05.

**Results**

**The expression of TUSC7 was decreased in HCC**

First, we examined the lncRNA TUSC7 expression level in 75 paired HCC tissues and adjacent non-tumor tissues by qRT-PCR and normalized them to GAPDH. Our results showed that TUSC7 levels were significantly decreased in HCC tissues compared with adjacent non-tumor tissues (*p* < 0.05, Fig. 1a). HCC cases with at least one of the clinicopathological features, including intrahepatic spreading, venous infiltration, or tumor invasion, tend to be considered as aggressive HCC tissues. When compared with non-aggressive HCC tissues, TUSC7 levels were markedly downregulated in aggressive HCC tissues (*p* < 0.001, Fig. 1b). Furthermore, TUSC7 levels were notably lower in tumor tissues arising from patients with tumor recurrence than that without tumor recurrence (*p* < 0.001, Fig. 1c). Then, expression levels of TUSC7 in HCC cells were determined by qRT-PCR. Our experiments showed that TUSC7 expression was significantly downregulated in all HCC cell lines when compared with that in LO2 cells (*p* < 0.05, Fig. 1d). These data suggest that TUSC7 might be associated with migration and metastasis of HCC cells.

**Clinical significance of TUSC7 expression in HCC**

To determine whether TUSC7 expression is associated with clinicopathological features in HCC patients, HCC patients were divided into two different groups according to the median level of TUSC7 expression. Further analysis showed that the expression level of TUSC7 was significantly correlated with tumor nodes (*p* < 0.001), venous infiltration (*p* = 0.017), Edmondson-Steiner grading (*p* = 0.003), and tumor-node-metastasis (TNM) tumor stage (*p* = 0.004) (Table 3). Thus, our results demonstrated that the reduced expression of TUSC7 was correlated with poor prognostic features of HCC.

Kaplan-Meier survival curves further revealed that patients with lower TUSC7 expression had a significantly reduced OS and DFS than those with high TUSC7 expression (*p* < 0.05, respectively, Fig. 2a, b). Moreover, multivariate Cox proportional hazard regression analysis indicated that venous infiltration and TUSC7 expression were independent prognostic factors for predicting both 3-year OS and DFS in HCC patients (*p* = 0.007 and 0.015, respectively, Table 4). The data implied that TUSC7 may be a promising prognostic or progression marker for HCC.

**TUSC7 inhibits the migration and invasion of HCC cells**

To explore the biological significance of TUSC7 in HCC progression, we manipulated TUSC7 levels in HCC cells and examined the alteration of the metastatic behavior of HCC cells. Firstly, we used TUSC7-siRNAs (TUSC7-siRNA1, TUSC7-siRNA2, and TUSC7-siRNA3) to downregulate the expression of TUSC7 in Hep3B cells. Additionally, pcDNA/TUSC7 vector and pcDNA/TUSC7

### Table 3  
Correlation between the clinicopathologic characteristics and expression of TUSC7 in HCC

| Characteristics | Total no. of patients (*n* = 75) | No. of patients | *p*  |
|-----------------|---------------------------------|----------------|-----|
| Age (year)      |                                 |                |     |
| <50             | 19                              | 8              | 11  | 0.362 |
| ≥50             | 56                              | 29             | 27  |      |
| Gender          |                                 |                |     |
| Male            | 51                              | 27             | 24  | 0.466 |
| Female          | 24                              | 10             | 14  |      |
| HBV             |                                 |                |     |
| Absent          | 55                              | 26             | 29  | 0.346 |
| Present         | 20                              | 11             | 19  |      |
| Serum AFP level (ng/mL) |                       |                |     |
| <400            | 18                              | 7              | 11  | 0.309 |
| ≥400            | 57                              | 30             | 27  |      |
| Tumor size (cm) |                                 |                |     |
| <5              | 43                              | 22             | 21  | 0.366 |
| ≥5              | 32                              | 13             | 19  |      |
| No. of tumor nodes |                               |                |     |
| 1               | 40                              | 28             | 12  | <0.001*** |
| ≥2              | 35                              | 9              | 26  |      |
| Cirrhosis       |                                 |                |     |
| Absent          | 55                              | 28             | 27  | 0.651 |
| Present         | 20                              | 9              | 11  |      |
| Venous infiltration |                               |                |     |
| Absent          | 54                              | 22             | 32  | 0.017* |
| Present         | 21                              | 15             | 6   |      |
| Edmondson-Steiner grading |               |                |     |
| I + II          | 49                              | 18             | 31  | 0.003** |
| III + IV        | 26                              | 19             | 7   |      |
| TNM tumor stage |                                 |                |     |
| I + II          | 43                              | 15             | 28  | 0.004** |
| III + IV        | 32                              | 22             | 10  |      |

*HBV* hepatitis B virus, *AFP* alpha-fetoprotein, *TNM* tumor-node-metastasis

*p* < 0.05; **p** < 0.01; ***p*** < 0.001
Control vector were transfected into MHCC97H cells. The result of qRT-PCR revealed that TUSC7-siRNA3 was the most effective siRNA which inhibited the expression of TUSC7 in Hep3B cells significantly \((p<0.05, \text{Fig.}\ 3a)\). Then, we found that downregulation of TUSC7 resulted in increased migration and invasion of Hep3B cells (Figs. 3b and 4a). Conversely, the pcDNA/TUSC7 vector significantly upregulated the levels of TUSC7 in MHCC97H cells \((p<0.05, \text{Fig.}\ 3a)\) and resulted in diminished migration and invasion of MHCC97H cells (Figs. 3c and 4b). These data indicated that TUSC7 can inhibit migration and invasion of HCC cells.

**TUSC7 suppresses EMT in HCC**

It is well recognized that EMT plays a critical role in HCC cell migration and invasion \([37]\). Therefore, we explored whether TUSC7 had effects on EMT of HCC. Firstly, we respectively analyzed the correlation of expression levels of TUSC7 and E-cadherin as well as TUSC7 and vimentin in 75 paired HCC tissues and adjacent non-tumor tissues by immunohistochemical staining. We found that E-cadherin expression was distinctly repressed and vimentin expression was notably increased in the low TUSC7 tissues group compared to that in the high group (Fig. 5(a–d)). Moreover, the results were confirmed by qRT-PCR in HCC tissues (Fig. 5(e, f)) and western blot in HCC cells (Fig. 5(g)). Therefore, we conclude that TUSC7 inhibited EMT in HCC.

**miR-10a is a downstream target of TUSC7**

As we have mentioned before, recent studies show that TUSC7 may function as a competing endogenous RNA (ceRNA) or a molecular sponge by modulating the biological functions and concentration of miRNAs in cancers \([20,38]\). To investigate the potential downstream miRNAs of TUSC7 and their interactions in HCC, bioinformatics tools (MicroRNA, Mircode, Starbase v2.0, and RNAhybrid) were used to analyze the potential complementary base pairing between TUSC7 and miRNAs. The result revealed that dozens of miRNA binding sites were present in TUSC7 (data not shown). We found that miR-10a contained the complementary sequence of TUSC7 (Fig. 6a). Additionally, our results have shown that TUSC7 could repress EMT progression of HCC (Fig. 5(a–g)) and miR-10a has been reported to facilitate EMT

### Table 4 Multivariate Cox regression analysis of 3-year overall and disease-free survival of 75 HCC patients

| Variables             | Overall survival | Disease-free survival |
|-----------------------|------------------|-----------------------|
|                       | HR               | 95 % CI               | \(p\)  | HR               | 95 % CI               | \(p\)  |
| No. of tumor nodules  | 0.801            | 0.347, 1.853          | 0.605  | 0.598            | 0.256, 1.400          | 0.236  |
| Venous infiltration   | 0.259            | 0.101, 0.665          | 0.005**| 0.303            | 0.121, 0.759          | 0.011* |
| TNM tumor stage       | 1.105            | 0.278, 4.399          | 0.887  | 1.089            | 0.280, 4.230          | 0.902  |
| TUSC7 expression      | 3.411            | 1.392, 8.357          | 0.007**| 2.928            | 1.227, 6.985          | 0.015* |
| Edmondson-Steiner grading | 1.905          | 0.837, 4.338          | 0.125  | 1.660            | 0.727, 3.792          | 0.229  |

\(HR\) hazard ratio, CI confidence interval

\(*p<0.05;**p<0.01\)
in HCC [32]; we then focused on miR-10a. To further investigate whether miR-10a was a functional target of TUSC7, the dual-luciferase reporter assay was performed. We found that co-transfection of pcDNA/TUSC7 and miR-10a-WT strongly decreased the luciferase activity while co-transfection of pcDNA/Control and miR-10a-WT did not change the luciferase activity (Fig. 6b), suggesting that miR-10a was a target of TUSC7. In parallel, we constructed a reporter plasmid where the TUSC7 seed region binding site was mutated (miR-10a-Mut) to test binding specificity (Fig. 6a). Consequently, co-transfection of pcDNA/TUSC7 and miR-10a-Mut did not change luciferase activity (Fig. 6b). Thus, these results demonstrated that TUSC7 could directly bind to miR-10a at the miRNA recognition site.

To further confirm whether TUSC7 exerted its function through miR-10a, we determined the expression levels of miR-10a in Hep3B cells transfected with TUSC7-siRNA3 and in MHCC97H cells transfected with pcDNA/TUSC7. The qRT-PCR results revealed that miR-10a expression was visibly elevated in Hep3B cells transfected with TUSC7-siRNA3 and in MHCC97H cells transfected with pcDNA/TUSC7.
siRNA3 and clearly reduced in MHCC97H cells transfected with pcDNA/TUSC7 (Fig. 6c, d, respectively). It has been reported that miR-10a could promote EMT in HCC through the miR-10a/EphA4 axis [32]. Our data showed that ectopic expression of TUSC7 could affect the messenger RNA (mRNA) levels of EphA4 in HCC cells (Fig. 6e, f), which further confirmed that miR-10a is a target of TUSC7 in HCC. Taken together, these data suggest that TUSC7 might repress EMT through the TUSC7-miR-10a-EphA4 axis in HCC.

miR-10a reverses the inhibitory effects of TUSC7 in HCC cells

Although our experiments had confirmed that miR-10a was a target of TUSC7, the function of miR-10a in TUSC7-induced inhibition in HCC cells remained unclear. And in order to confirm whether TUSC7 could suppress EMT through the TUSC7-miR-10a-EphA4 axis, the further experiments were performed. Wound healing assays (Fig. 7a–d) and Transwell assays (Fig. 7f–i) showed that miR-10a could largely reverse the inhibitory effect of TUSC7 on HCC cell migration and invasion. Western blot also revealed that the inhibition of EphA4 protein expression and EMT by TUSC7 could be largely reversed by miR-10a (Fig. 7e). These results indicated that miR-10a could reverse the inhibitory effects of TUSC7 in HCC cells and TUSC7 could suppress EMT through the TUSC7-miR-10a-EphA4 axis.

Discussion

HCC patients currently have a poor prognosis, and it is without doubt that early detection and treatment could
significantly increase their chances of survival. Recently, IncRNAs have shown great therapeutic potential for human diseases, including HCC [39]. For example, studies from Yuan SX et al. have revealed that DANCR increases stemness and offers a potential prognostic marker, and a therapeutic target, for HCC [40]. Research from Chen CL et al. unveiled the molecular mechanisms of how PTENP1 repressed the tumorigenic properties of HCC cells and demonstrated the potential of the SB-BV hybrid vector for PTENP1 IncRNA modulation and HCC therapy [41]. Accordingly, TUSC7 was identified as a robust suppressor of cancer [21]. In this study, we found that TUSC7 expression in HCC was significantly downregulated. TUSC7 expression in HCC tissues was negatively associated with more tumor nodes, more venous infiltration, advanced Edmondson-Steiner grading, and advanced TNM tumor stage. Moreover, comparison of Kaplan-Meier survival curves indicated that patients with lower TUSC7 expression in HCC tissues had notably worse prognosis. TUSC7 was also confirmed to be an independent risk factor for HCC patients. Altogether, these clinical data suggest strongly that TUSC7 is critical for prognosis determination in HCC patients. Furthermore, we tested the action of TUSC7 on tumor invasion and metastasis of HCC cells by taking different approaches and found that TUSC7 inhibited cell invasion and metastasis in HCC.

EMT, a dynamic and reversible cellular process, is characterized by a loss of cell polarity and intracellular junctions and acquisition of mesenchymal features, which could result in

**Fig. 5** TUSC7 inhibited EMT progression in HCC. a–d Immunohistochemistry staining of E-cadherin and vimentin in HCC tissues. In cases of high TUSC7 expression tissue group (a, b), there was strong E-cadherin and no detectable vimentin protein expression in the same tissue section. In contrast, in the cases of low TUSC7 expression tissue group (c, d), there was no detectable E-cadherin and strong vimentin protein expression. Values are depicted as mean ± SD; **p<0.001, by t test. Scale bar = 100 μm. e, f Expression of EMT mRNA markers was assessed by qRT-PCR in the low TUSC7 expression tissue group (≤0.33, n = 38) and high TUSC7 expression tissues group (>0.33, n = 37), both groups from HCC samples. g Hep3B and MHCC97H cells with different TUSC7 levels were subjected to western blot for E-cadherin and vimentin. Representative western blot showed that downregulation of TUSC7 obviously increased protein expression of vimentin and reduced E-cadherin expression in HCC cells. *P<0.05, by t test
increase HCC cell migration and invasion [42]. Recent studies showed that IncRNAs may play critical roles in the EMT progress not only in HCC but also in other cancers [43–45]. Furthermore, it has been found that some IncRNAs could promote EMT [45, 46] while some could restrain EMT [47, 48]. For example, IncRNA-AOC4P has been shown to act as an HCC tumor suppressor by enhancing vimentin degradation and suppressing EMT progress [47]. Overexpression of IncRNA-UCA1 induced EMT and increased the migratory and invasive abilities of bladder cancer cells [49]. IncRNA-ATB may also act on colon tumorigenesis by suppressing E-cadherin expression and promoting EMT [50]. In this study, we analyzed EMT biomarkers of HCC tissues by using immunohistochemistry and qRT-PCR and those of HCC cells by western blot. Then, we determined the expression of an epithelial marker (E-cadherin) and mesenchymal marker (vimentin) in HCC with either low or high TUSC7 expression. Interestingly, it was found that TUSC7 expression was positively associated with E-cadherin expression and negatively associated with vimentin expression in HCC. We concluded that TUSC7 could suppress EMT in HCC.

Growing evidence suggests that IncRNA may act as a ceRNA to regulate miRNAs in cancer progression [51]. As we have stated before, TUSC7 acts as a tumor suppressor in human cancers by interacting with miRNAs, such as miR-23b [20] and miR-211 [38]. It has been reported that miR-10a could facilitate cell migration, invasion, and EMT by directly targeting the 3′-UTR of EphA4 transcript to reduce its expression in HCC [32]. EphA4 could inhibit cell migration and invasion by regulating the EMT process through the β1-integrin signaling pathway [32]. Hence, combining our previous results and the bioinformatics analysis, we focused on miR-10a and its downstream target EphA4. Our results showed that miR-10a was indeed a downstream target of TUSC7. We found that acting as a sponge of miR-10a,
TUSC7 could therefore directly interact with miR-10a to restrain its function. Thus, when the expression level of TUSC7 was reduced, its inhibition on miR-10a would be attenuated. The expression level of miR-10a would then be increased, which could lead to decreased expression of EphA4. Therefore, we have confirmed that the downregulation of TUSC7 could enhance miR-10a expression to reduce EphA4 expression, thereby promoting migration, invasion, and EMT in HCC, at least in part.

In summary, our data indicate that TUSC7 may function as a tumor suppressor in HCC. Mechanistically, our experimental data demonstrate that targeting the TUSC7-miR-10a-EphA4 axis may represent a novel therapeutic application in HCC.

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Compliance with ethical standards This study was reviewed and approved by the Research Ethics Committee of the Xi’an Jiaotong University. All patients provided informed consent before surgery. All samples were handled according to the ethical and legal standards.

Conflicts of interest None

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