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Six2 is negatively correlated with prognosis and facilitates epithelial-mesenchymal transition via TGF-β/Smad signal pathway in hepatocellular carcinoma

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ABSTRACT

Background: Increasing evidence indicates that Six2 contributes to tumorigenesis in various tumor including hepatocellular carcinoma (HCC). This study aimed to determine the role of Six2 in HCC and to elucidate the association of Six2 with clinical pathological characteristics.
Methods: The expressions of Six2 in HCC tumor, para-tumor tissue and portal vein tumor thrombus (PVTT) were detected by tissue microarray technique, immunohistochemistry, real-time RT-PCR and Western blotting. Chi-square and Kaplan-Meier analysis were used to analyze the correlation between Six2 expression and prognosis of HCC patients. Lentivirus mediated Six2 knockdown, spheroid formation assay, proliferation assay and subcutaneous tumor implantation were performed to determine the function of Six2.
Results: In 274 HCC samples, Six2 was strongly expressed. Kaplan-Meier analysis revealed that high expression of Six2 was correlated with a shorter overall survival (OS) and disease-free survival (DFS). Moreover, Six2 expression was associated with sex, alpha-fetoprotein, tumor size and portal vein invasion. Six2 was highly expressed in PVTT. Six2 knockdown inhibited HCC cell lines proliferation, migration, and self-renewal *in vitro* and *in vivo*. In addition, low-expression of Six2 weakened TGF-β induced Smad4 activation and epithelial-mesenchymal transition in HCC cell lines.
Conclusions: Elevated Six2 expression in HCC tumor patients was associated with negative prognosis. Upregulated Six2 promoted tumor growth and facilitated HCC metastasis via TGF-β/Smad signal pathway.

Introduction

Hepatocellular carcinoma is one of the most common cancers diagnosed and causes of cancer death worldwide [1]. It is reported that the incidence of HCC is increasing due to the large number of patients with HBV infection and non-alcoholic steatohepatitis (NASH) [2]. The number of newly diagnosed HCC patients in China accounts for more than 50% of the total number in the world [3]. Liver resection and liver transplantation are the main effective curable methods, but only 10%–37% of HCC patients are suitable for surgery [4]. Therefore, it is urgent to find out the molecular mechanism of HCC development and to find new therapeutic targets and molecular indicators.

The homeobox gene is a class of evolutionarily highly conserved DNA sequences and the encoded proteins of which all consist of a conserved domain with 61 amino acid residues, *i.e.* homeo-domain (HD) [5]. According to the difference of HD and extra sequence, it can be divided into Hox, Msx, Pax, Six and other families, which play an important regulatory role in biological development [6–8]. Among them, the Six family encodes 6 Six proteins, which are composed of HD, Six domain (SD), conserved N-terminal and acidic C-terminal structure [9]. The HD-domain of Six protein specifically bound to the DNA sequence of the target genes thus regulating

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their expression [10,11]. In addition, the SD-domain can also play a transcriptional regulatory role [12].

Many studies have reported the role of Six protein in tumors: Six1 participates in the process of invasion and proliferation in non-small cell lung cancer [13]; Six4 promotes lymph node metastasis through activation of the PI3K-AKT pathway in colorectal cancer [14]; however Six3 was reported to inhibit carcinogenesis in breast cancer cells and suppress metastasis in breast cancer [15]. In previous study, we analyzed the expression profiles of seven groups of HCC tumors, tumor thrombus, paracancerous and normal liver tissues. We found that *Six2* gene is highly expressed in HCC, especially in tumor thrombus tissues, suggesting that Six2 may play an important role in HCC. Six2 is a member of the Six family, consisting of 291 amino acids and it was first discovered to regulate the limb or eye development [16,17]. The present study aimed to determine the expression of Six2 in HCC tissues and the relationship between Six2 and patient prognosis, and to explore the biological function of Six2 in HCC cells.

Methods

Cell lines and tissue samples

SK-HEP-1, Huh7, MHCC-97 L, MHCC-97H, MHCC-LM3, L02, SMMC-7721 (Cell Bank of the Chinese Academy of Sciences, Shanghai, China) were cultured in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% (v/v) fetal bovine serum (Biological Industries, Kibbutz Beit Haemek, Israel) and 1% Penicillin-Streptomycin. All these cells were incubated at 37 °C and 5% CO_2 with humidified air. Transforming growth factor-β (TGF-β) was purchased from Sino Biological (10,804-HNAC, Beijing, China).

HCC tissue specimens and patients' clinical data were obtained from the Eastern Hepatobiliary Surgery Hospital, Second Military Medical University, Shanghai, China. Informed consent was obtained from all patients before subsequent use of their resected tissues. The cohort consisted of patients with HCC confirmed by pathological analysis. Primary tumor samples, the corresponding non-tumorous tissues, and portal vein tumor thrombus (PVTT) were stored in liquid nitrogen once collected.

Histology and immunohistochemistry

Mice and patient tumor tissue specimens were fixed in formalin for 12 h and dehydrated. After embedded in paraffin wax, the tissues were sliced into 4 μm microsection. After the antigen being retrieved, tissues were incubated with the specific antibodies (Six2 Rabbit Polyclonal antibody, Proteintech Group, Rosemont, IL, USA, 11,562–1-AP). Slides were stained with DAB (3,3′-diaminobenzidine, Takara, Beijing, China) after incubating with secondary antibodies. High resolution images of slides were captured and quantitatively analyzed by Digital Pathology Scanner (Leica, Aperio AT Turbo, Wetzlar, Germany). The slides were blindly evaluated by two independent observers. The scores were multiplied by the staining intensity (range 0–3) and the percentage of positive cells [range 0–4; 0, (0–10%), 1 (11%–25%), 2 (26%–50%), 3 (51%–75%), and 4 (76%–100%)]. Slides with scores of 8 or higher were classified as overexpression and slides with scores lower than 8 as low expression.

Western blotting assay

Whole-cell extracts were lysed by RIPA buffer (50 mmol/L Tris pH 7.4, 150 mmol/L NaCl, 1% NP-40, 0.1% SDS and 0.5% sodium deoxycholate) supplemented with protease inhibitor (Roche, Basel, Switzerland) and phosphatase inhibitor cocktails (Sigma-Aldrich, St. Louis, Missouri, USA) and centrifuged at 12,000 rpm, 4 °C, 15 min. BCA Protein Assay kit (Thermo Fisher, Waltham, Massachusetts, USA) was used to measure the proteins concentration. Same amount of proteins were separated by SDS-PAGE in TG buffer, transferred to nitrocellulose membrane (NC membrane, MILLIPORE, Burlington, Massachusetts, USA) and detected by the specific antibodies (Six2 Rabbit Polyclonal antibody, Proteintech Group, 11,562–1-AP; GAPDH Monoclonal Antibody, Abcolonal, AC033, Phospho-Smad2 Antibody, CST, 3104; Smad2 Rabbit mAb, CST, 3122; E-cadherin Rabbit Polyclonal antibody, Proteintech Group, 20,874–1-AP; Vimentin Rabbit Polyclonal antibody, Proteintech Group,10,366–1-AP; IRDye® 800CW Goat anti-Mouse IgG, LI-COR, 925–32,210; IRDye® 800CW Goat anti-Rabbit IgG, LI-COR, 925–32,211). Immune complex was detected by fluorescein-conjugated secondary antibody and then canned by Odyssey CLx imaging system (LI-COR).

Real-time RT-PCR

According to the instructions, RNAs were extracted by trizol (Invitrogen, Waltham, Massachusetts, USA) from cells and tissue samples. cDNAs were reversely transcripted from RNAs using random hexamers (Roche) and SuperScriptII reverse transcription (Invitrogen). Assessments of the genes expressions were quantified by LightCycler480 (Roche). Primer sequences were listed as follow: Six2, 5′-CCTGCGAGCACCTTCACAA-3′ and 5′-CTC GAT GTA GTG TGC CTT GAG-3′; 18S, 5′-GCC CTA CCC ACA AAG CCT CAG-3′ and 5′-GTG GCT TTC TGT TGC TGT TCA-3′.

Lentivirus infection

The lentivirus-shSix2 and negative control were constructed by Genechem Co., Ltd, Shanghai, China. MHCC-LM3 and SMMC-7721 cell lines were infected with lentivirus at a multiplicity of infection (MOI) of 20 accompanying with ploybrene (6 μg/mL) for 8 h.

Cell proliferation, migration and spheroid formation assay

3×10^3 cells per well were seeded into 96-well plate and cultured in 200 μL DMEM medium. Cell proliferation was assessed by CCK8 (Dojindo, Shanghai, China, CK04). Ten μL CCK8 were mixed with 90 μL serum-free DMEM and added into each well. After incubating for 1 h, the OD value under the weave length of 450 nm was read by Microplate Spectrophotometer. Each cell was repeated for 3 times. 1×10^4 cells were planted in the inner chamber of transwell (Corning, New York, USA, 3421), mixed with 500 μL serum-free DMEM and localized to the 24 well plate filled with 700 μL 10% DMEM. After 24–36 h, cells were fixed by 10% formaldehyde and stained with crystal violet. Images were captured to analyze. Each cell was repeated for 3 times. A total of 3000 cells were planted in the low adhesion 6 well plate with 2 mL DMEM and images were captured 2 weeks later.

Mice and in vivo study

Four-week-old male nude mice were purchased from SLAC Co., Ltd, Shanghai, China and housed in standard condition abiding by the requirements of the Second Military Medical University Animal Care Facility and the National Institutes of Health guidelines. All animal procedures were approved by the Institutional Animal Care and Use Committee of the Second Military Medical University, Shanghai, China. Cell lines with differential Six2 expression were subcutaneously injected into the bilateral lower flank region in 100 μL normal saline with 2×10^6 cells, respectively. One week after injection, the tumor volume was measured and calculated as width^2 (cm^2) × length (cm) × 0.5 weekly.

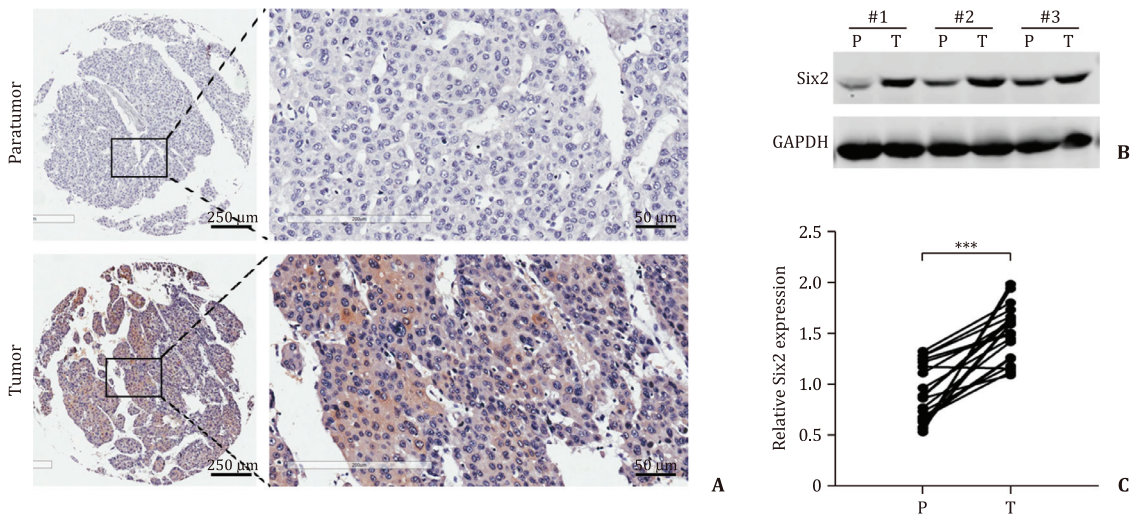

Fig. 1. Expression of Six2 in HCC tumor and paracancerous tissues. **A:** Representative images of tumor and corresponding adjacent tissues in tissue microarray stained with Six2. Right panel was the magnification of the orthogon in the left panel; **B:** Six2 expression was detected by Western blotting in three groups of HCC tissues. **C:** The mRNA expression of Six2 in 20 pairs of paracancerous and HCC tumor tissues was detected by RT-PCR. P: Paracancerous tissues; T: tumors. ***: $P < 0.001$.

Statistical analysis

SPSS 21.0 (SPSS Inc., Armonk, New York, USA) and GraphPad PRISM 5.01 (GraphPad Software, San Diego, USA) software were applied for statistical analysis. Kaplan-Meier analysis was used to determine the disease-free survival and overall survival. Qualitative variables were analyzed by Pearson Chi-square test or Fisher's exact test. Unpaired, two-tailed Student's *t*-test or Mann-Whitney test was employed in the comparison of two groups. Every group in comparison consisted of at least three independent experiments and the data were presented as the mean ± SEM. A *P* value of < 0.05 was considered statistically significant.

Results

Six2 is highly expressed in HCC tumor tissues

A total of 274 pairs of HCC tumor and paracancerous tissues were stained, and Six2 staining in the HCC tissue chip revealed that the Six2 protein was differentially expressed in tumor and paracancerous tissues (Fig. 1A). Six2 protein is located in the cytoplasm and nucleus. There are few positive staining areas in the corresponding adjacent tissues. Immune blots suggested that the Six2 protein level was higher in tumor tissue comparing with paracancerous tissues (Fig. 1B). RT-PCR results of 20 pairs of HCC and paracancerous tissues indicated that Six2 was transcriptionally overexpressed in tumor tissues than that of the adjacent tissues (Fig. 1C).

Six2 expression was associated with negative prognosis in HCC patients

Based on immunohistochemistry (IHC) staining of Six2, we scored and graded 274 HCC tumor samples (Fig. 2A). Six2 was highly expressed in 146 cases and the rest was of low expression. We used Kaplan-Meier analysis to analyze whether there was a difference in the prognosis of HCC patients between high and low expression of Six2. The results showed that the median overall survival time of patients with high Six2 expression was 6.167±0.554 months and the counter part was 14.233 ± 1.607 mon (Fig. 2B). The median disease-free survival time with high expression of Six2 was 2.967 ± 0.508 mon, which was significantly shorter than those with Six2 low expression (6.000 ± 1.252 mon) (Fig. 2C). These indicated that patients with high expression of Six2 had shorter survival time and worse prognosis than patients with low Six2 expression. Then we compared the correlation of Six2 with other clinical data by Pearson Chi-square test. The results showed that the expression level of Six2 was higher in male samples than in females. Higher expression of Six2 correlated with serum alpha fetoprotein (AFP) level and promoted tumor growth and portal vein invasion, indicating poor prognosis of patients (Table 1).

Table 1
Association between Six2 protein expression with clinical pathological characteristics in HCC patients ($n = 274$).

Characteristics	Six2 expression level		*P* value
	Low ($n = 128$)	High ($n = 146$)	
Sex			**0.030**
Male	79 (61.7%)	108 (74.0%)	
Female	49 (38.3%)	38 (26.0%)	
Age (yr)			0.090
≥ 60	75 (58.6%)	70 (47.9%)	
< 60	53 (41.4%)	76 (52.1%)	
HBsAg			0.873
Negative	9 (7.0%)	11 (7.5%)	
Positive	119 (93.0%)	135 (92.5%)	
AFP (μg/μL)			**<0.001**
≤ 20	66 (51.6%)	56 (38.4%)	
> 20	62 (48.4%)	90 (61.6%)	
Tumor size (cm)			**<0.001**
≤ 5	76 (59.4%)	54 (37.0%)	
> 5	52 (40.6%)	92 (63.0%)	
Tumor nodules			0.745
Single	118 (92.2%)	133 (91.1%)	
Multiple	10 (7.8%)	13 (8.9%)	
PVTT			**0.013**
Negative	70 (54.7%)	58 (39.7%)	
Positive	58 (45.3%)	88 (60.3%)	
BCLC			0.075
0-A	16 (12.5%)	30 (20.5%)	
B-C	112 (87.5%)	116 (79.5%)	
TNM			0.074
I-II	32 (25.0%)	51 (34.9%)	
III-IV	96 (75.0%)	95 (65.1%)	

HCC: hepatocellular carcinoma; HBsAg: hepatitis B surface antigen; AFP: alpha-fetoprotein; PVTT: portal vein tumor thrombus; BCLC: Barcelona Clinic Liver Cancer; TNM: Tumor node metastasis.

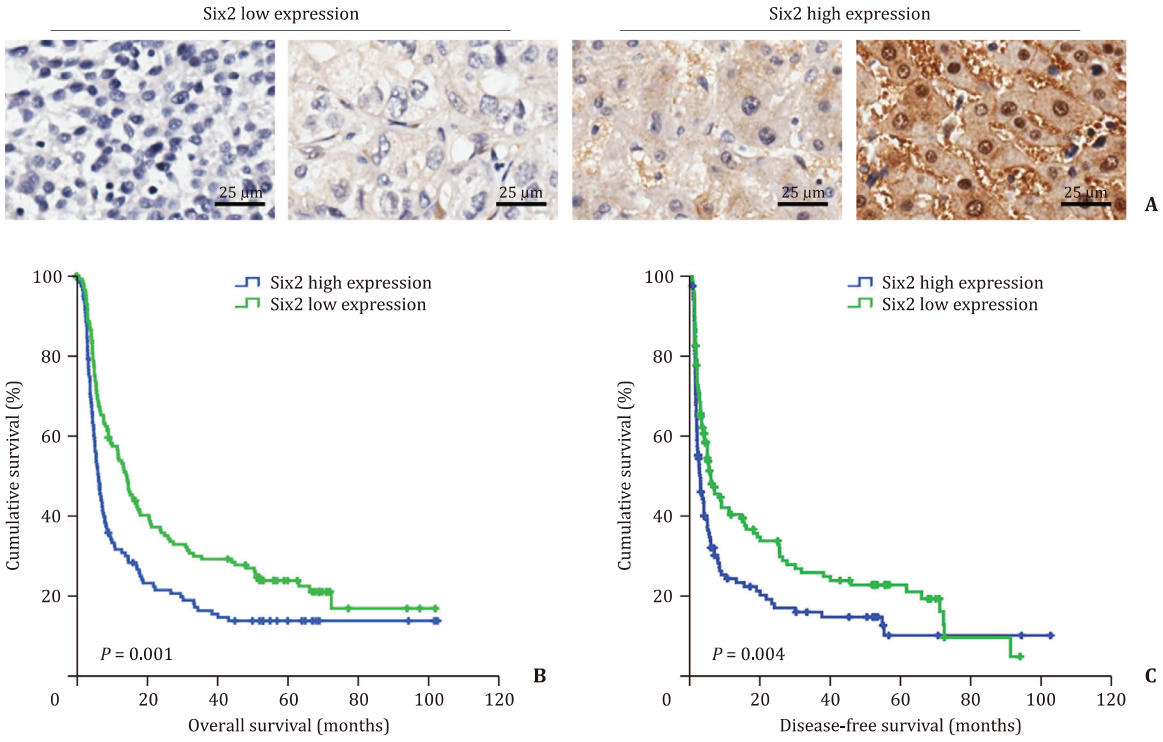

Fig. 2. Relationship between the expression of Six2 in HCC tumor tissues with survival of patients. **A:** Representative images with Six2 low and high expression; **B** and **C:** Kaplan-Meier analysis of the relationship between Six2 expression and overall survival time and tumor-free survival time.

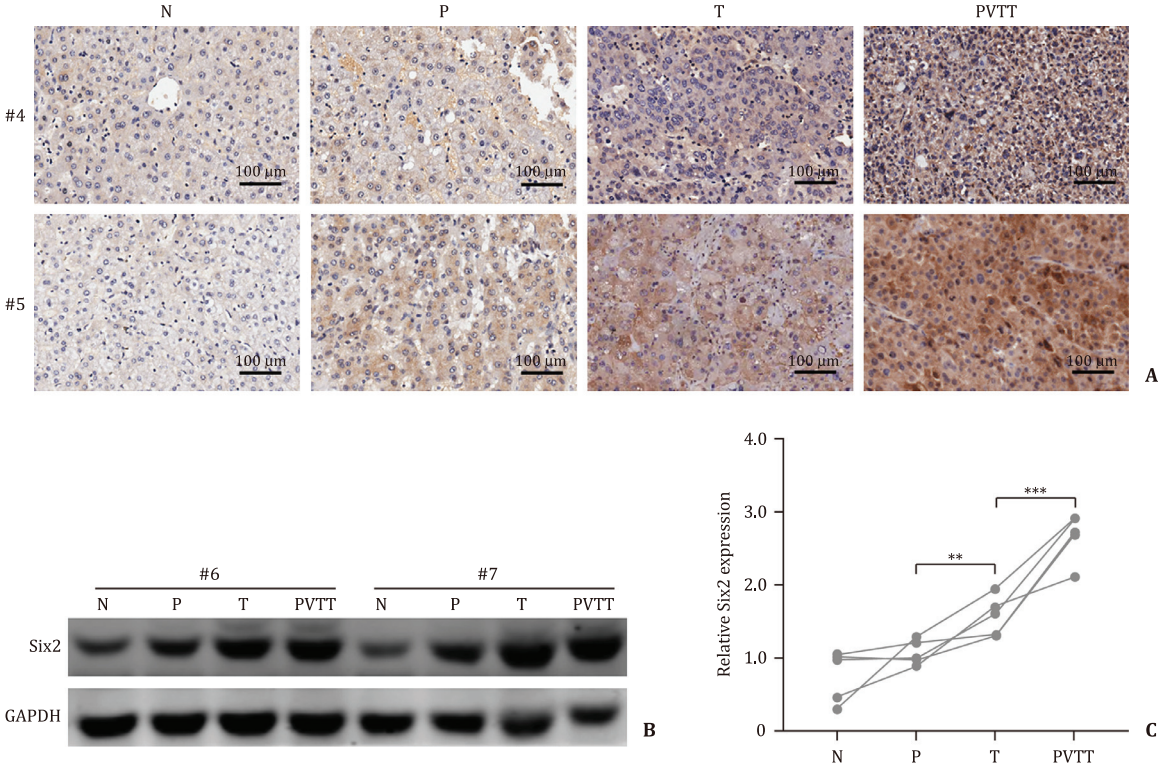

Fig. 3. Expression of Six2 in HCC tumor thrombus. **A:** Immunohistochemical staining of Six2 in normal liver (N), paracancerous (P), primary tumor (T) and tumor thrombus tissues (PVTT) of #4 and #5 HCC patients; **B:** Protein expression of Six2 in normal liver (N), paracancerous (P), primary tumor (T) and tumor thrombus tissues (PVTT) of #6 and #7 HCC patients by Western blotting; **C:** mRNA expression of Six2 in four groups of HCC primary tumor and tumor thrombus tissues by RT-PCR. **: $P < 0.01$; ***: $P < 0.001$.

Six2 expression was elevated in HCC thrombus

It is reported that about 50%–80% of liver cancers are associated with PVTT, which indicates a poor prognosis [4]. As it has been proven that Six2 expression was correlated with portal vein invasion, we testified the relationship further by determining Six2 expression in PVTT. IHC staining showed that the expression of Six2 protein in PVTT and tumor tissues was higher than that in paracancerous and normal liver tissues (Fig. 3A). Moreover, Six2 expression was even higher in PVTT comparing with tumor tissues which hinted that Six2 enhanced tumor abilities to metastasis. Western blotting assay also suggested that the Six2 protein was expressed higher in PVTT and tumor tissues than in normal liver and paracancerous tissues (Fig. 3B). In addition, we compared

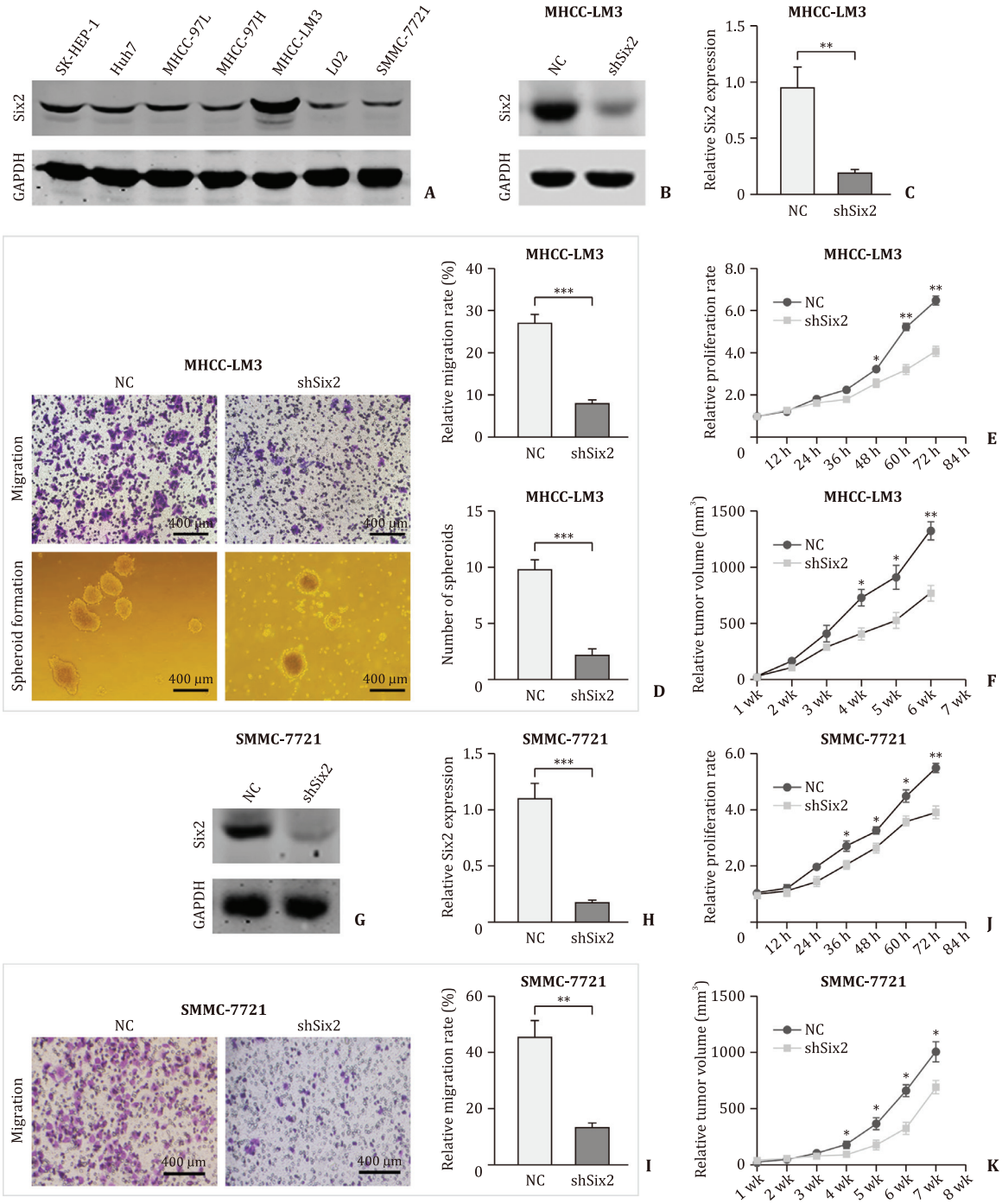

Fig. 4. Biological function studies after interfering Six2 expression in HCC cell lines. **A:** Western blotting detected the expression of Six2 in SK-HEP-1, Huh7, MHCC-97 L, MHCC-97H, MHCC-LM3, L02 and SMMC-7721 HCC cell lines. **B, C, G** and **H:** Lentivirus targeting on Six2 was transfected in to MHCC-LM3 or SMMC-7721 cell and the interference effect was determined by Western blotting and RT-PCR. **D** and **I:** Difference in abilities of migration, low adhesion spheroid formation of Six2 differentially expressed MHCC-LM3 or SMMC-7721 cell. Left panel was the representative images and right histogram was the quantification. **E** and **J**: Difference in proliferation of Six2 differentially expressed MHCC-LM3 or SMMC-7721 cell were detected by CCK8. **F** and **K:** Xenograft of Six2 differentially expressed MHCC-LM3 or SMMC-7721 cell growth curve in nude mice. *: $P < 0.05$; **: $P < 0.01$; ***: $P < 0.001$.

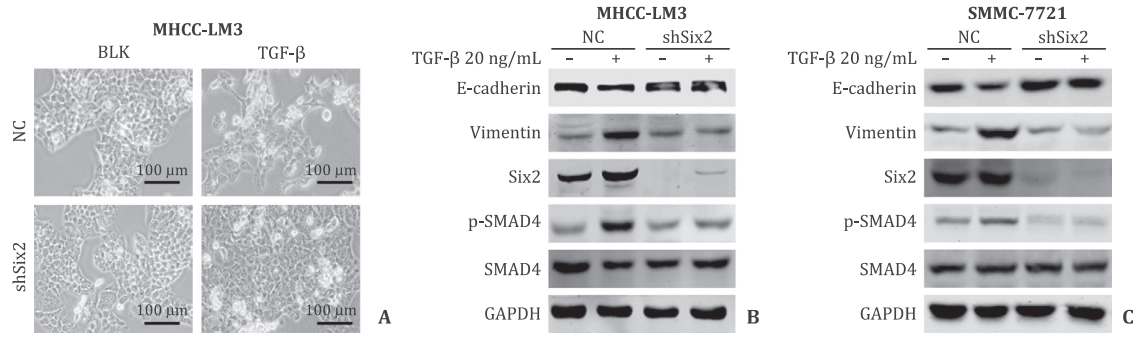

Fig. 5. Six2 promotes HCC metastasis by facilitating epithelial-messenchymal transition. **A:** Representative images of Six2 differentially expressed MHCC-LM3 cells morphology changes under 20 ng/mL TGF-β treatment for 24 h; **B** and **C:** Six2 differentially expressed MHCC-LM3 and SMMC-7721 cells were treated with 20 ng/mL TGF-β for 24 h to observe protein changes by Western blotting.

the mRNA expression of Six2 in PVTT. Same as the results of IHC, Six2 was highly expressed in HCC and PVTT (Fig. 3C). Thus Six2 may enhance tumor metastasis.

Six2 enhances migration, proliferation and self-renewal of HCC cells

After demonstrating the relationship between Six2 expression and prognosis in HCC tissues, we explored the cell biology function of Six2. First, we detected the protein expression of Six2 in a series of HCC cells and Six2 expression varied in different cells (Fig. 4A). Subsequently, we knocked down Six2 expression by lentivirus to establish Six2 differentially expressed MHCC-LM3 and SMMC-7721 cell lines. Both Western blotting and RT-PCR assays confirmed down-regulation of Six2 expression (Fig. 4B, C, G, H).

We applied these cells to study the function of Six2 *in vitro*. Knockdown of Six2 weakened MHCC-LM3 cell migration abilities confirming the relation between Six2 and metastasis (Fig. 4D). Under low adhesion conditions, shSix2 MHCC-LM3 cell line grew into less spheroids implying debilitated self-renewal abilities (Fig. 4D). The CCK8 assay evidenced that MHCC-LM3 cells proliferated slower after interfering Six2 expression (Fig. 4E). In addition, we observed Six2 function *in vivo* by subcutaneous injection in nude mice and found that Six2 knockdown xenograft grew more slowly (Fig. 4F). All these data above indicating that interfering Six2 expression attenuated the malignant biological behavior of HCC cells. And results of Six2 deletion in SMMC-7721 cell confirmed it further (Fig. 4I–K).

Six2 promoted HCC metastasis by facilitating epithelial-messenchymal transition

Epithelial-mesenchymal transition (EMT) plays an important role in the process of tumor metastasis, which refers to a series of changes in epithelial cell involving skeletal structure, attachment to surrounding cells, basement membrane and extracellular matrix due to various factors and obtaining messenchymal cell-like morphology and characteristics [18,19]. To determine the role of Six2 in the process of EMT in HCC, Six2 differentially expressed MHCC-LM3 and SMMC-7721 cells were treated with TGF-β. The morphology of cells in negative control converted from polygon to fusiform resembling mesenchymal cells while alterations was not observed in the counterpart [20] (Fig. 5A). Western blotting indicated that, under TGF-β treatment, TGF-β/Smad signal pathway was activated resulting in repressed E-cadherin and upregulated Vimentin expression in negative control (Fig. 5B, C). TGF-β/Smad signal pathway was not activated and the changes in E-cadherin and Vimentin were not induced under the context of Six2 deletion indicating that Six2 may promote HCC metastasis by facilitating TGF-β/Smad signal pathway.

Discussion

The homeobox gene family plays an important regulatory role in cell biological development and differentiation, and its spatial and temporal specificity determines the different processes of biological development and cell differentiation [21,22]. Each member of the family regulates the transcription of target genes and multiple signaling pathways [23,24]. Six1/2 is the key regulatory determinant in Ciona neuron developmental process [25] and mammalian organogenesis [5]. It has been elucidated that aberrant Six1 expression is correlated with metastasis and poor survival in esophageal squamous cell carcinoma patients depending on TGF-β signaling [26]. But whether Six2 regulates tumor development remains to be determined.

The present study found that Six2 was highly expressed in HCC thrombus and primary tumor tissue comparing with paracancerous and normal liver tissue, suggesting that Six2 may participate in the development of HCC. Subsequently, we detected the expression of Six2 in 274 pairs of HCC tissues by immunohistochemistry and found that Six2 was highly expressed in tumor tissues. Real-time quantitative RT-PCR and protein immunoblotting confirmed these findings. Combined with clinical data analysis, we found that Six2 expression was associated with poor prognosis in HCC patients. The overall survival time and disease-free survival time of patients with high expression of Six2 were significantly shorter than those with low expression. The expression of Six2 was associated with sex, tumor size, AFP and portal vein invasion suggesting that the Six2 enhanced tumor growth and invasion clinically. We also found that Six2 was highly expressed in the tumor thrombus of HCC implying that Six2 facilitated tumor metastasis. We found that interfering Six2 expression impaired the abilities of migration, proliferation, self-renewal in HCC cells, suggesting that Six2 may be involved in the malignant biological behavior of HCC.

The TGF-β family is a class of cytokines that plays an important role in embryonic development and tissue homeostasis, and participates in various physiological and pathological processes [27]. When TGF-β binds to its receptor, TβRI and TβRII constitute a heterodimer, causing serine phosphorylation, thereby triggering a series of receptor autophosphorylation to activate the downstream Smad2/3 [28]. Phosphorylated Smad2/3 binds to Smad4 to form a complex that, along with other transcription factors, activates downstream target genes, causing a series of cytological changes [28,29]. The Smad complex, in combination with Zeb1, Zeb2/SIP1, inhibits the expression of E-cadherin and initiates EMT [29,30]. TGF-β induced EMT has been associated with the maintenance of tumor stem cell and drug resistance [31,32].

We also found that Six2 deletion affected the activation of TGF-β/Smad signal pathway, thus inhibited the EMT in HCC cells. This

may be the reason why highly expressed Six2 enhanced tumor metastasis and growth abilities *in vitro* and *in vivo*. And EMT has been reported to be related with drug resistance [33], we speculate that different expression of Six2 in HCC may impact drug sensitivity. However, we did not manage to find the exact mechanism how Six2 facilitated TGF-β/Smad signal pathway thus it remains to be determined in the future.

In conclusion, Six2 plays an important role in the development of HCC. Six2 can be used as a molecular marker to predict the prognosis of patients with HCC and potential target in the treatment of patients with HCC.

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Contributors

WZH, JTY, WHY and DLW designed the study, directed the work and wrote the manuscript. WZH and JTY performed the immunohistochemistry, cell-based assays and data analysis. MYH, LYK, SYY and TYX provided clinical tissue samples and technical guidance. TYX, WHY and DLW guided this project and revised the manuscript. WZH, MYH and JTY contributed equally to this article. WHY is the guarantor.

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Ethical approval

The study was approved by Research Ethics Committee of Eastern Hepatobiliary Surgery Hospital, Second Military Medical University, Shanghai, China. All animal procedures were approved by the Institutional Animal Care and Use Committee of the Second Military Medical University, Shanghai, China.

Competing interest

No benefits in any form have been received or will be received from a commercial party related directly or indirectly to the subject of this article.

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