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TPM4 aggravates the malignant progression of hepatocellular carcinoma through negatively regulating SUSD2

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Abstract. – OBJECTIVE: The aim of this study was to elucidate the role of TPM4 in the progression of hepatocellular carcinoma (HCC), and to explore the potential underlying mechanism by interacting with SUSD2.

PATIENTS AND METHODS: TPM4 expression levels in 41 HCC tissues and paracancerous tissues were detected by quantitative Real Time-Polymerase Chain Reaction (qRT-PCR). The relationship between TPM4 level with the pathological indexes and overall survival of HCC patients was analyzed. TPM4 overexpression and knockdown models were constructed in Bel-7402 and Hep3B cells, respectively. Subsequently, Cell Counting Kit-8 (CCK-8) and transwell assay were conducted to assess the effects of TPM4 on the proliferative and migratory abilities of HCC cells. Dual-Luciferase reporter gene assay was performed to verify the binding relationship between TPM4 and SUSD2. In addition, the xenograft model was conducted in HCC-bearing mice administrated with Hep3B cells in vivo. Finally, the effect of TPM4 on the growth of HCC was explored.

RESULTS: TPM4 was significantly upregulated in HCC tissues and cell lines. Higher rates of lymphatic and distant metastasis, as well as worse prognosis, were observed in HCC patients with higher expression level of TPM4. The overexpression of TPM4 significantly enhanced the viability and migration abilities of Bel-7402 cells. However, opposite results were observed after the knockdown of TPM4 in Hep3B cells. SUSD2 was verified to be the target of TPM4 and was negatively regulated by TPM4. SUSD2 was lowly expressed in HCC tissues and cell lines. Meanwhile, SUSD2 was considered to be responsible for TPM4-regulated progression of HCC. In mice administrated with Hep3B, the cells transfected with sh-TPM4, the tumor volume and weight of HCC were markedly reduced when compared with the controls.

CONCLUSIONS: TPM4 level is correlated with high rates of lymphatic and distant metastasis, as well as poor prognosis of HCC patients. By negatively targeting SUSD2, TPM4 aggravates the progression of HCC by accelerating the proliferative and migratory abilities of HCC cells.

Key Words:
TPM4, SUSD2, Hepatocellular carcinoma (HCC), Malignancy.

Introduction

Hepatocellular carcinoma (HCC) is the six most prevalent tumor globally, which ranks third among tumor-related deaths[1,2]. The number of newly onset HCC in China accounts for 54% of global HCC cases[3,4]. Improvement of surgical procedures has largely enhanced the survival rate of HCC patients. Nevertheless, recurrence may occur in over than 41% HCC patients within 5 years postoperatively[5,6]. In recent years, the development on genomics researches and microarray analyses has broadened the HCC research on genetic levels. Based on tumor-associated genes, the individualized therapy has been established to effectively enhance the therapeutic efficacy of HCC patients[7-9]. Therefore, searching for hallmarks targeting HCC and novel drug targets are urgently required[9,10].

TPM4 belongs to the tropomyosin superfamily, which is also known as the δ gene[11,12]. TPM4 has been found to bind to myofibrillar proteins and inhibit cell contraction[12]. It was first isolated from the cDNA library of human fibroblasts. TPM4 is located on 19p13.1 and consists of 8 exons encoding a 20-kb mRNA[13,14]. Current studies[14-16] have proposed the therapeutic potential of TPM4 in HCC by in-

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teracting with the corresponding genes. In this paper, the bioinformatics predicted that SUSD2 was the potential target of TPM4. The aim of our study was to uncover the role of TPM4/SUSD2 regulatory loop in regulating the malignant phenotypes of HCC *in vitro* and *in vivo*.

Patients Methods

Patients and HCC Samples

Matched HCC tissues and paracancerous tissues were surgically resected from 41 HCC patients. None of these patients received preoperative anti-tumor treatment. The clinical data and follow-up data of enrolled patients were recorded. Tumor staging was assessed based on the guideline proposed by UICC. Informed consent was obtained from patients and their families. This study was approved by Ethics Committee of the Weinan Central Hospital. The Declaration of Helsinki was respected.

Cell Culture

Normal hepatocytes L02 and liver cancer cells (Hep3B, Huh7, SMMC-7221, MHCC88H, HepG2, and Bel-7402) were provided by American Type Culture Collection (ATCC; Manassas, VA, USA). All cells were cultured in Dulbecco's Modified Eagle's Medium (DMEM; Thermo Fisher Scientific, Waltham, MA, USA) containing 10% fetal bovine serum (FBS; Gibco, Rockville, MD, USA) and maintained in a 5% CO_2 incubator at 37°C. Cell passage was conducted at 80-90% of confluence.

Cell Transfection

The cells were first inoculated into 6-well plates. Cell transfection was conducted at 70% of confluence according to the instructions of Lipofectamine 3000 (Invitrogen, Carlsbad, CA, USA). 48 h later, the transfected cells were harvested for functional experiments. The transfection plasmids were constructed by GenePharma (Shanghai, China).

Cell Counting Kit (CCK-8) Assay

The cells were first inoculated into 96-well plates at a density of 2×10^3 cells/well. At day 1, 2, 3 and 4, absorbance value at 450 nm was recorded using CCK-8 kit (Dojindo Laboratories, Kumamoto, Japan), respectively. Finally, the viability curve was plotted.

Transwell Assay

Transfected cells were inoculated in 24-well plates at a density of 5.0×10^5/ml. 200 μL of cell suspension was applied in the upper side of the transwell chamber (Millipore, Billerica, MA, USA) and inserted in a 24-well plate. Meanwhile, 500 μL of medium containing 10% FBS was added to the lower side. After 48 h of incubation, the penetrated cells in the bottom side were fixed in methanol for 15 min and dyed with crystal violet for 20 min. The penetrated cells were observed using a microscope (magnification 20×), and the number of penetrating cells was counted. 5 fields were randomly selected for each sample.

Quantitative Real Time-Polymerase Chain Reaction (qRT-PCR)

Cellular or tissue RNA was isolated using TRIzol reagent (Invitrogen, Carlsbad, CA, USA). Extracted RNAs were purified by DNase I treatment, and reversely transcribed into cDNA using PrimeScript RT Reagent (TaKaRa, Komatsu, Japan). Subsequently, the obtained cDNA was used for qRT-PCR using SYBR®Premix Ex Taq™ (TaKaRa, Komatsu, Japan). β-actin and U6 were used as internal references for mRNA and miRNA, respectively. QRT-PCR reaction conditions were as follows: 94°C for 30 s, 55°C for 30 s, and 72°C for 90 s, for a total of 40 cycles. Each sample was performed in triplicate, and relative level was calculated by the $2^{-\Delta\Delta Ct}$ method. The primer sequences used in this study were as follows: TPM4, F: 5'-GGCACAAGTCAACCTGCGTC-3', R: 5'-CGGTAGGAATGCCAGAGAGCCA-3'; SUSD2, F: 5'-GAATGCACAGCCGCGACGG-3', R: 5'-AGGTTACATGTCCGGATTCGAA-3'; U6: F: 5'-CTCGCTTCGGCAGCACA-3', R: 5'-AACGCTTCACGAATTTGCGT-3'; β-actin: F: 5'-CCTGGCACCCAGCACAAT-3', R: 5'-GCTGATCCACATCTGCTGGAA-3'.

Western Blot

Cellular protein was separated by sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE) and transferred onto polyvinylidene difluoride (PVDF) membranes (Millipore, Billerica, MA, USA). After blocking in 5% skimmed milk for 1 h, the membranes were incubated with primary antibodies overnight at 4°C. On the next day, the membranes were incubated with corresponding secondary antibody for 2 h at room temperature, followed by washing with Tris Buffered Saline-Tween (TBST) washing for 1 min. Immuno-reactive

bands were finally exposed by the enhanced chemiluminescent substrate kit (Pierce, Rockville, MD, USA).

Dual Luciferase Reporter Gene Assay

Based on the predicted sequences between SUSD2 and TPM4, the wild-type and mutant-type TPM4 vectors were first constructed. The cells were co-transfected with NC/pcDNA-SUSD2 and TPM4-WT/TPM4-MUT, respectively. After 48 h, the transfected cells were lysed for determining relative Luciferase activity (Promega, Madison, WI, USA).

In Vivo Xenograft Model

Animal experiments were approved by the Animal Ethics Committee of Weinan Central Hospital. Twelve male nude mice with 8 weeks old were randomly assigned into two groups. Hep3B cells transfected with sh-NC or sh-TPM4 were subcutaneously injected into the mouse armpit, respectively. The tumor volume was recorded every 5 days. Five weeks later, mice were sacrificed, and tumor tissues were collected. Tumor volume = (width^2 × length)/2.

Immunohistochemistry (IHC)

After paraffin-embedded tissues were dewaxed and hydrated, 50 μl of anti-rat TPM4 (1:100) was added, followed by incubation at room temperature for 1 h. After washing with PBS for 3 times, 40-50 μl of corresponding secondary antibody was added and incubated at room temperature for another 1 h. Diaminobenzidine (DAB; Solarbio, Beijing, China) was added for 5-10 min of color development. Residual DBA was rinsed with deionized water. Finally, the tissue was dehydrated, sealed and observed under a microscope.

Statistical Analysis

Statistical Product and Service Solutions (SPSS) 22.0 (IBM Corp., Armonk, NY, USA) was used for all statistical analyses. Experimental data were expressed as mean ± standard deviation. The differences between the two groups were analyzed by *t*-test. Kaplan-Meier curves were introduced for survival analysis. Comparison between multiple groups was done using One-way ANOVA test followed by Post-Hoc Test (Least Significant Difference). Spearman correlation test was performed to assess the relationship between the expression levels of TPM4 and SUSD2 in HCC tissues. $p<0.05$ was considered statistically significant.

Results

Upregulated TPM4 Indicated Poor Prognosis of HCC Patients

In comparison with paracancerous tissues, TPM4 was significantly upregulated in HCC tissues (Figure 1A). Similarly, TPM4 was highly expressed in liver cancer cells when compared with

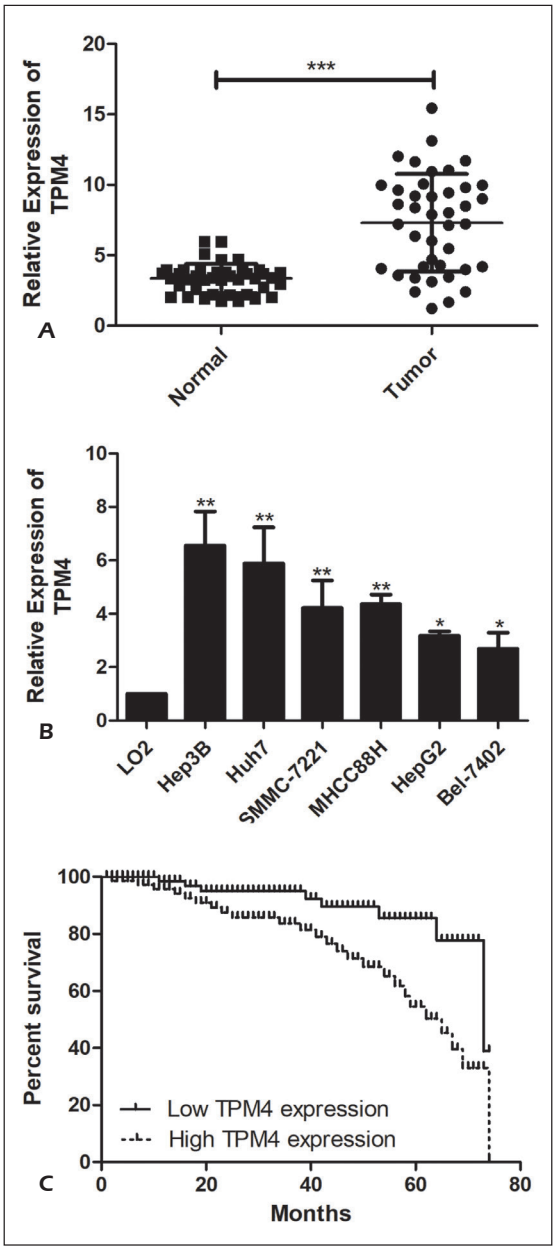

Figure 1. Upregulated TPM4 indicated poor prognosis of HCC. **A**, TPM4 levels in HCC tissues and paracancerous tissues. **B**, TPM4 levels in normal hepatocytes L02 and liver cancer cells (Hep3B, Huh7, SMMC-7221, MHCC88H, HepG2 and Bel-7402). **C**, Survival of HCC patients expressing high or low level of TPM4.

normal hepatocytes (Figure 1B). Kaplan-Meier curves uncovered that remarkably worse survival was observed in HCC patients with high expression of TPM4 (Figure 1C).

Subsequently, the pathological indexes of the enrolled HCC patients were analyzed. The results demonstrated that TPM4 level was positively correlated with the rates of distant metastasis and lymphatic metastasis. However, it was not associated with age, gender, and tumor staging of HCC patients (Table I). Therefore, TPM4 might be utilized as a hallmark for predicting the malignant progression of HCC.

Upregulation of TPM4 Promoted Proliferation and Migration of HCC Cells

To elucidate the biological function of TPM4 in HCC, the overexpression and knockdown models of TPM4 were constructed in Bel-7402 and Hep3B cells, respectively (Figure 2A). Next, the regulatory effects of TPM4 on viability and migration of HCC cells were determined. In Bel-7402 cells overexpressing TPM4, the viability and migratory abilities were markedly enhanced when compared with those of the controls (Figure 2B, 2C, left). Conversely, the transfection of sh-TPM4 in Hep3B cells exhibited the opposite results (Figure 2B, 2C, right).

SUSD2 Was Downregulated in HCC

Interestingly, the protein expression level of SUSD2 was downregulated in Bel-7402 cells overexpressing TPM4. However, it was upregulated after the knockdown of TPM4 in Hep3B cells (Figure 3A). Compared with paracancerous tissues, SUSD2 was significantly downregulated in HCC tissues (Figure 3B). Meanwhile, a negative correlation was observed between the expression levels of TPM4 and SUSD2 in HCC tissues (Figure 3C). Dual-Luciferase reporter gene assay indicated that the Luciferase activity significantly decreased after the co-transfection of pcDNA-SUSD2 and TPM4-WT, verifying their binding relationship (Figure 3D).

TPM4 Influenced HCC Phenotypes by Negatively Regulating SUSD2 Level

Based on the above findings, SUSD2 might be involved in TPM4-regulated progression of HCC. The transfection efficacies of pcDNA-SUSD2 and si-SUSD2 in Bel-7402 cells and Hep3B cells were confirmed by qRT-PCR, respectively (Figure 4A). Notably, the overexpression of SUSD2 in Bel-7402 cells attenuated the migratory abilities, which were partially reversed by the overexpression of TPM4. Nevertheless, the silence of SUSD2 stimulated the migration of Hep3B cells, which was reversed by the knockdown of TPM4 (Figure 4B). As a result, SUSD2 was responsible for TPM4-induced aggravation of HCC.

Silence of TPM4 Suppressed the Growth of HCC In Vivo

Next, the *in vivo* function of TPM4 in HCC was assessed by administration of Hep3B cells transfected with sh-NC or sh-TPM4 into nude mice. HCC tumors were harvested after sacrifice. The results indicated that the average tumor volume

Table I. Association of TPM4 expression with clinicopathologic characteristics of hepatocellular carcinoma.

Parameters	No. of cases	TPM4 expression		*p*-value
		Low (%)	High (%)	
Age (years)				0.633
< 60	15	8	5	
≥ 60	26	16	10	
Gender				0.205
Male	19	14	5	
Female	22	12	10	
T stage				0.154
T1-T2	25	18	7	
T3-T4	16	8	8	
Lymph node metastasis				**0.001**
No	27	22	5	
Yes	14	4	10	
Distance metastasis				**0.001**
No	31	24	7	
Yes	10	2	8	

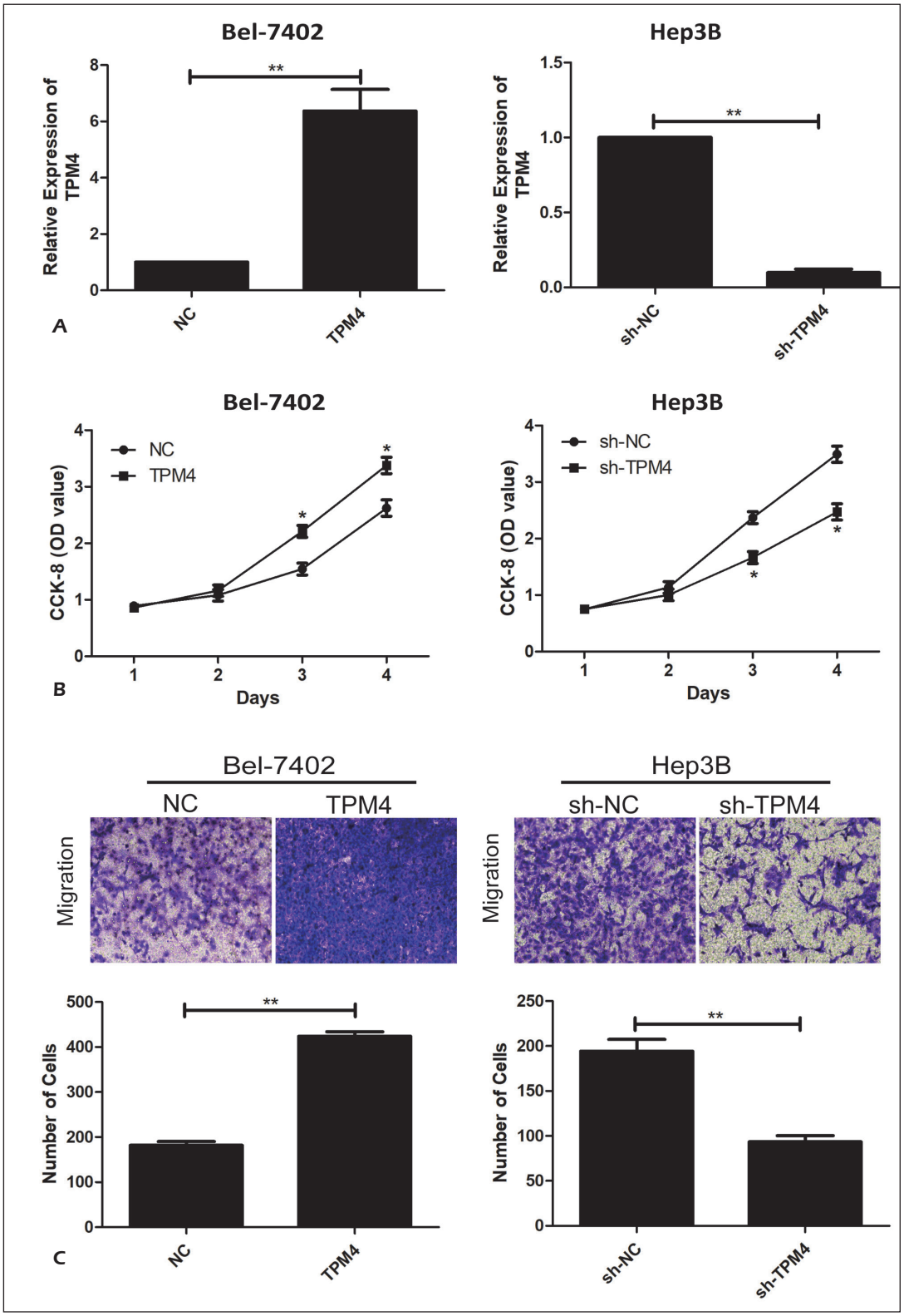

Figure 2. Upregulation of TPM4 promoted proliferation and migration of HCC cells. **A**, TPM4 levels in Bel-7402 cells transfected with NC or pcDNA-TPM4, and in Hep3B cells transfected with sh-NC or sh-TPM4. **B**, Viabilities of Bel-7402 cells transfected with NC or pcDNA-TPM4, and of Hep3B cells transfected with sh-NC or sh-TPM4. **C**, Migration of Bel-7402 cells transfected with NC or pcDNA-TPM4, and of Hep3B cells transfected with sh-NC or sh-TPM4 (magnification 40×).

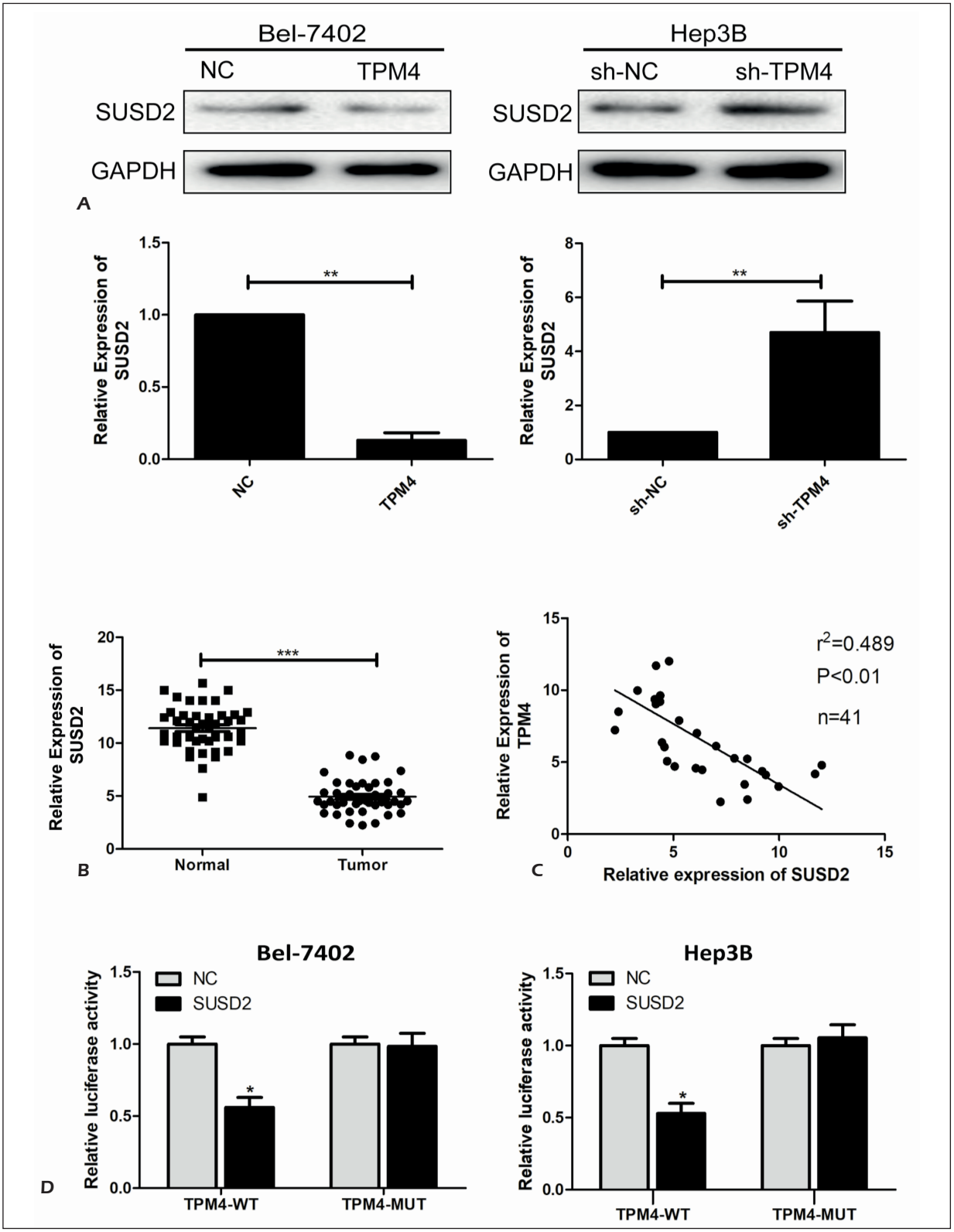

Figure 3. SUSD2 was downregulated in HCC. **A**, Protein levels of SUSD2 in Bel-7402 cells transfected with NC or pcDNA-TPM4, and in Hep3B cells transfected with sh-NC or sh-TPM4. **B**, SUSD2 levels in HCC tissues and paracancerous tissues. **C**, A negative correlation between the expression levels of TPM4 and SUSD2 in HCC tissues. **D**, Luciferase activity in Bel-7402 and Hep3B cells co-transfected with NC/pcDNA-SUSD2 and TPM4-WT/TPM4-MUT.

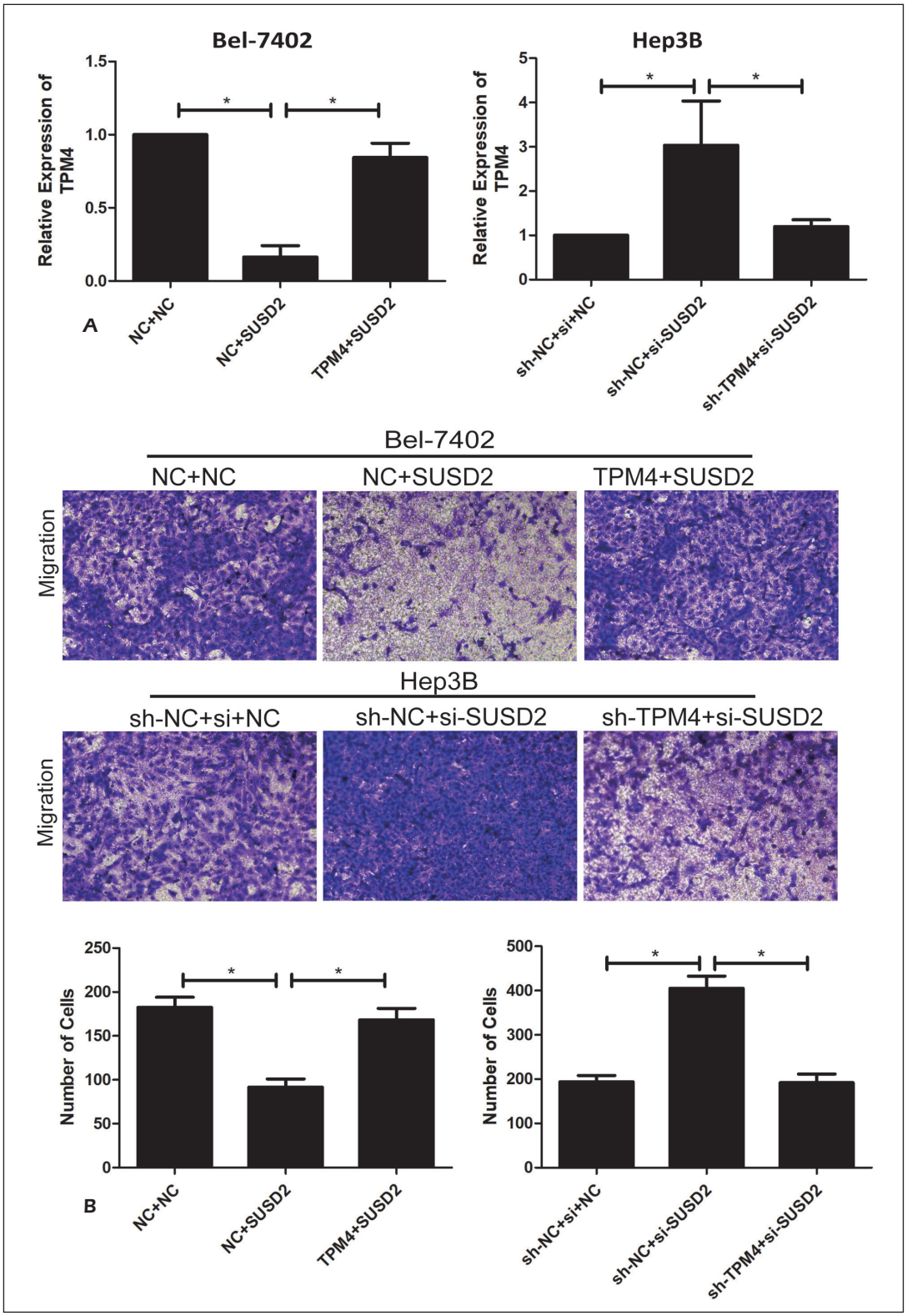

Figure 4. TPM4 influenced HCC phenotypes by negatively regulating SUSD2 level. **A**, TPM4 levels in Bel-7402 cells transfected with NC+NC, NC+pcDNA-SUSD2 or pcDNA-TPM4+pcDNA-SUSD2, and in Hep3B cells transfected with sh-NC+si-NC, sh-TPM4+si-SUSD2 or sh-TPM4+si-SUSD2. **B**, Migration of Bel-7402 cells transfected with NC+NC, NC+pcDNA-SUSD2 or pcDNA-TPM4+pcDNA-SUSD2, and of Hep3B cells transfected with sh-NC+si-NC, sh-TPM4+si-SUSD2 or sh-TPM4+si-SUSD2 (magnification 40×).

and weight were significantly lower in mice with knockdown of TPM4 *in vivo* (Figure 5A, 5B). *In vivo* abundance of TPM4 was significantly lower in mice administrated with Hep3B cells transfected with sh-TPM4 than those of the controls (Figure 5C), while SUSD2 was upregulated (Figure 5D). Furthermore, IHC showed remarkably lower positive expression of TPM4 in mice with *in vivo* knockdown of TPM4 (Figure 5E).

Discussion

HCC is one of the most common malignancies in the digestive tract system globally[1-3]. As a major subtype of primary liver cancer, HCC accounts for 70-85%[4-6]. It is estimated that the incidence and mortality of HCC rank fifth and third among all types of malignancies, respectively[1,2]. Even after surgery and postoperative chemotherapy or radiotherapy, recurrence and metastasis of HCC severely limit the clinical outcomes of affected patients. Currently, the 5-year recurrence of HCC is up to 70%, whose 5-year survival remains lower than 30%[5-7]. Therefore, searching for effective and sensitive targets predicting the recurrence or metastasis of HCC is of great significance[8-10].

TPM4, first discovered in Xenopus embryos, belongs to the low-molecular-weight group in the tropomyosin family[11-14]. Kee et al[17] have

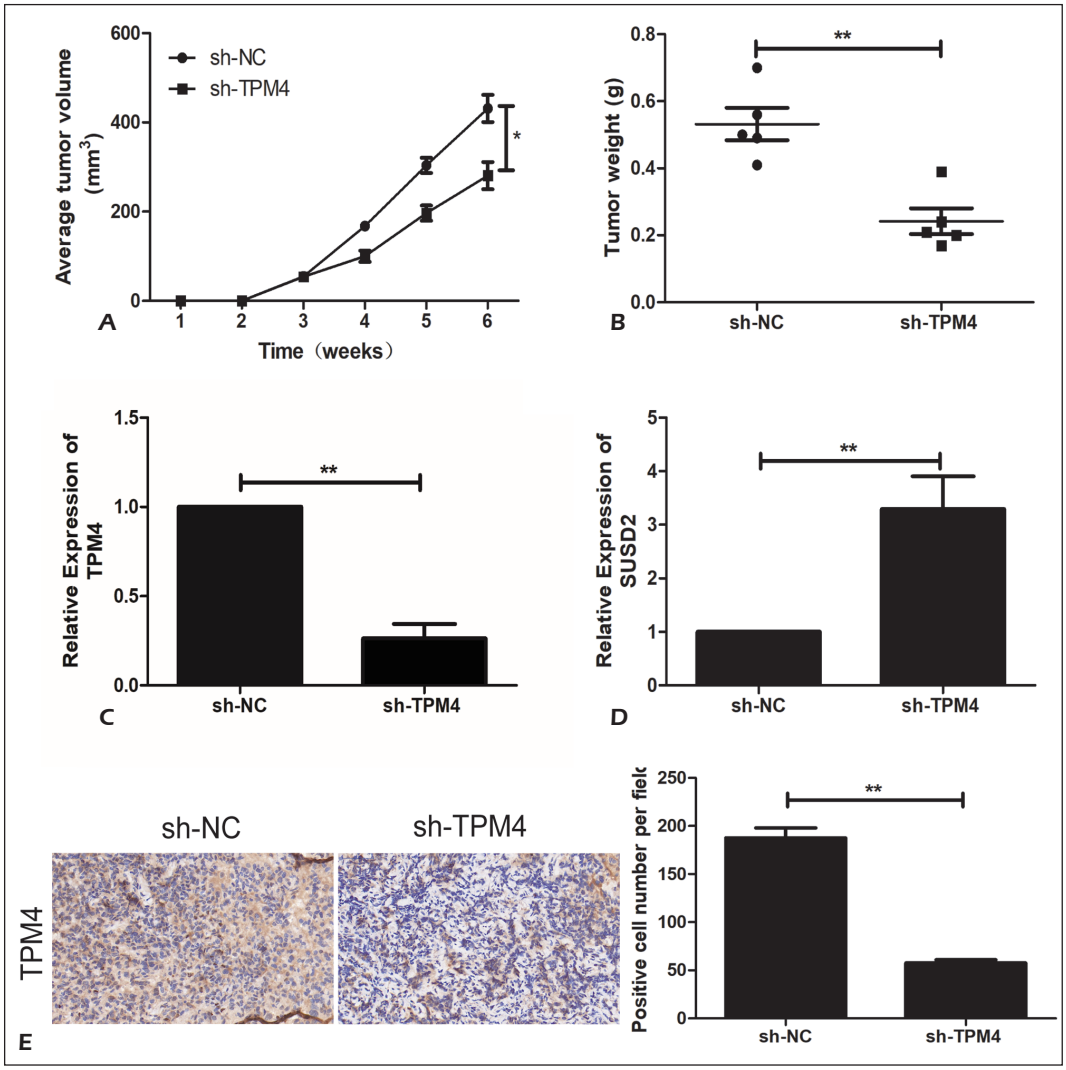

Figure 5. Silence of TPM4 suppressed the growth of HCC in vivo. A, B, Average tumor volume (**A**) and tumor weight (**B**) in mice administrated with Hep3B cells transfected with sh-NC or sh-TPM4. C, D, Relative levels of TPM4 (**C**) and SUSD2 (**D**) in mice administrated with Hep3B cells transfected with sh-NC or sh-TPM4. **E**, Positive expression of TPM4 in mice administrated with Hep3B cells transfected with sh-NC or sh-TPM4 detected by IHC (magnification 200×).

demonstrated the critical role of TPM4 in regulating adhesion structure and absorption capacity of osteoclasts. This may be explained by stabilization of pseudopod and dense band by binding myosin. During the development of zebrafish embryo, the deficiency of TPM4 can result in cardiac arrest[18]. Some researchers[14-16] believe that TPM4 cannot bind to the myofibrils of cardiac myocytes to further regulate cell contraction in the absence of other low-molecular tropomyosin. In addition, TPM4 has been identified to be involved in the progression of malignant tumors[13,19]. In our study, TPM4 level in 41 matched HCC tissues and adjacent normal tissues was first detected. TPM4 was markedly upregulated in HCC tissues. Notably, TPM4 level was positively correlated with metastatic rate (distant metastasis and lymphatic metastasis) in HCC patients. Therefore, we considered that TPM4 served as a carcinogenic role during the progression of HCC. *In vitro* experiments further verified that the overexpression of TPM4 in Bel-7402 cells enhanced viability and migratory ability, while the knockdown of TPM4 in Hep3B cells yielded the opposite results.

SUSD2 was predicted as a potential downstream gene of TPM4. QRT-PCR results indicated that SUSD2 was lowly expressed in HCC. Interestingly, SUSD2 could abolish the regulatory effect of TPM4 on cellular behaviors of HCC. As a result, SUSD2 was responsible for TPM4-induced aggravation of HCC.

Furthermore, *in vivo* experiments were conducted by administration of Hep3B cells with stably knockdown of TPM4 in mice. *In vivo* knockdown of TPM4 reduced the volume and weight of HCC tissues. Identically, IHC images confirmed significantly lower positive expression of TPM4 in HCC-bearing mice with TPM4 knockdown.

Conclusions

These data revealed that TPM4 level is correlated with high rates of lymphatic and distant metastasis, as well as poor prognosis of HCC patients. By negatively targeting SUSD2, TPM4 aggravates the progression of HCC by accelerating the proliferative and migratory abilities of HCC cells.

Conflict of Interest

The Authors declare that they have no conflict of interests.

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