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Original Article

TMSB10 promotes migration and invasion of cancer cells and is a novel prognostic marker for renal cell carcinoma

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Abstract: Objective: The study aims to examine the effect of thymosin β10 (TMSB10) on renal cell carcinoma (RCC) progression and metastasis. Methods: Real-time PCR and immunohistochemistry analysis were used to evaluate TMSB10 expression in RCC tissue samples and renal cancer cells. Statistical analyses were applied to investigate the association between TMSB10 expression and the clinicopathological characteristics and prognosis of RCC patients. In vitro migration and invasion assays were performed in 786-0 and ACHN cells. Results: The expression of TMSB10 was significantly higher in renal cancer cells and tissues compared with normal kidney cells and tissues. TMSB10 expression was significantly related to tumor stage (P=0.002), lymph node metastasis (P=0.034), and distant metastasis (P=0.039). Kaplan-Meier analysis suggested that high TMSB10 expression was significantly associated with unfavorable overall (P=0.004) and recurrent-free survival (P=0.025) of RCC patients. Furthermore, TMSB10 knockdown inhibited the migration and invasion abilities of renal cancer cells in vitro. Conclusion: TMSB10 is overexpressed in RCC and regulates malignant cell metastasis by inducing epithelial-mesenchymal transition, which makes TMSB10 a candidate therapeutic target for RCC.

Keywords: TMSB10, RCC, migration, invasion, epithelial-mesenchymal transition

Introduction

Renal cell carcinoma (RCC) is one of the most common types of malignant kidney cancer [1]. At present, surgery is the main strategy for long-term survival of patients with RCC. Despite improvement in diagnostic modalities, 25-30% of patients present with metastasis [2], and the 5-year survival rate of patients with distant metastasis is less than 10% [3]. The molecular mechanism of RCC metastasis remains to be elucidated; therefore, identification of novel biomarkers of metastasis is of great significance to the clinical diagnosis and treatment of RCC.

Thymosin β10 (TMSB10), a member of the beta-thymosin family, was initially identified as the main intracellular G-actin-sequestering protein [4]. The TMSB10 gene is located in chromosome 2p11 and encodes transcriptional factor to play an important role in the organization of the cytoskeleton. For example, it can bind to and sequester actin monomers, which in turn inhibit actin polymerization [5]. Using immunocytochemistry, Salhab et al. demonstrated that TMSB10 was upregulated and colocalised with F-actin stress fibers in bovine cumulus cells, indicating that TMSB10 may be involved in cumulus modifications during in vitro maturation [6]. Recent studies have shown that TMSB10 was overexpressed and involved in the development of several human cancers. Alldinger et al. found that TMSB10 expression was upregulated in both human pancreatic carcinoma tissues and cell lines, suggesting a role for TMSB10 in the carcinogenesis of pancreatic carcinoma [7]. Likewise, Lee et al. reported that TMSB10 overexpression was frequently observed in non-small cell lung cancer [8]. Zhang et al. revealed that TMSB10 expression correlates with lymph node metastases of papillary thyroid carcinoma [9]. These investigations suggest that TMSB10 has a close relationship with human cancers. However, the biological

function of TMSB10 in renal carcinoma has not been investigated.

In the present study, we performed a systematic analysis of the TMSB10 for its role in RCC development and its prognostic role in patients with RCC. We found that TMSB10 was overexpressed in RCC tissues and renal cancer cells. TMSB10 overexpression was associated with advanced tumor stage, lymph node metastasis, and poor clinical prognosis of RCC patients. Furthermore, we showed that knockdown of TMSB10 inhibited migration, invasion, and epithelial-mesenchymal transition (EMT) of kidney cancer cells in vitro. Our study indicates that TMSB10 might be helpful for clinic treatments of patients with RCC.

Materials and methods

Patients and specimens

115 cases of primary RCC samples were collected, and the pathological information was retrieved from the archives of The Second Affiliated Hospital of Nanchang University between January 2005 and December 2010. The histopathological diagnosis of all samples was respectively diagnosed by two pathologists. None of the patients enrolled in this study suffered from other cancers. In addition, 20 paired fresh kidney cancer samples and adjacent normal kidney tissue samples were collected. For the use of these clinical materials for research purposes, informed consent from all patients and approval from the Institute Research Ethics Committee were obtained in accordance with our institutional guidelines. The entire study was performed based on the Declaration of Helsinki.

Cell lines

Human RCC cell lines (786-O and ACHN) and normal kidney cell lines (293T and HK-2) were obtained from the Chinese Academy of Science (Shanghai, China) and cultured in RPMI 1640 (Invitrogen) supplemented with 10% fetal bovine serum (Gibco) and 1% penicillin-streptomycin (Gibco) in a humidified incubator containing 5% CO_2 at 37°C.

RNA extraction and quantitative real-time PCR

Total RNA was isolated from tumor samples and cells with Trizol reagent (Invitrogen) according to the manufacturer's protocol. Relative expression was calculated via the comparative cycle threshold method and was normalized to the expression of GAPDH. The following primer sequences were used: TMSB10, forward, 5'-CTTATCGAAGCTGGCGATTT-3', and reverse, 5'-AGTGGGAGCACCAGGATCT-3'; a-catenin forward 5'-CAACCCTTGTAAACACCAAT-3' and reverse 5'-CCTTCTCCAAGAAATTCTCA-3'; Vimentin forward 5'-AGGAAATGGCTCGTCACCTTCGTGAATA-3' and reverse 5'-GGAGTGTCGGTTGTTAAGAACTAGAGCT-3'; Fibronectin forward 5'-TTATGACGACGGGAAGACCT-3' and reverse, 5'-GCTGGATGGAAAGATTACTC-3'; Snail forward 5'-ACCACTATGCCGCGCTCTT-3' and reverse, 5'-GGTCGTAGGGCTGCTGGAA-3'; Twist forward 5'-CGGGAGTCCGCAGTCTTA-3' and reverse, 5'-TGAATCTTGCTCAGCTTGTC-3'; GAPDH, forward, 5'-CGAGATCCCTCCAAAATCAA-3', and reverse, 5'-TTCACACCCATGACGAACAT-3'. Each experiment was conducted for at least three times.

Cell transfection

TMSB10 siRNA (TMSB10-Ri1 and TMSB10-Ri2) and non-targeting siRNA (Control) were purchased from RiboBio (Guangzhou, Guangdong, China). Lipofectamine 2000 reagent (Invitrogen, USA) were used to transfect the cells. Knockdown was assessed by Real-time PCR after 48 hours of transfection.

Cell migration and invasion assay

Cell motility and invasive abilities were assessed by Transwell and Matrigel invasion assays. Briefly, 1×10^5 cells were seeded on a fibronectin-coated polycarbonate membrane insert in a transwell apparatus (Costar). After the cells were incubated for 24 h, the non-invaded cells on the upper membrane surface were removed with a cotton tip, and the cells that passed through the filter were fixed with 4% paraformaldehyde and stained with hematoxylin. For the cell invasion assay, the procedure was similar to the cell migration assay, except that the transwell membranes were pre-coated with 24 mg/ml Matrigel (Corning, USA). Experiments were performed three times.

Western blot analysis

Total cellular and tissue proteins were extracted and separated in SDS PAGE gels, and Western blot analysis was performed according

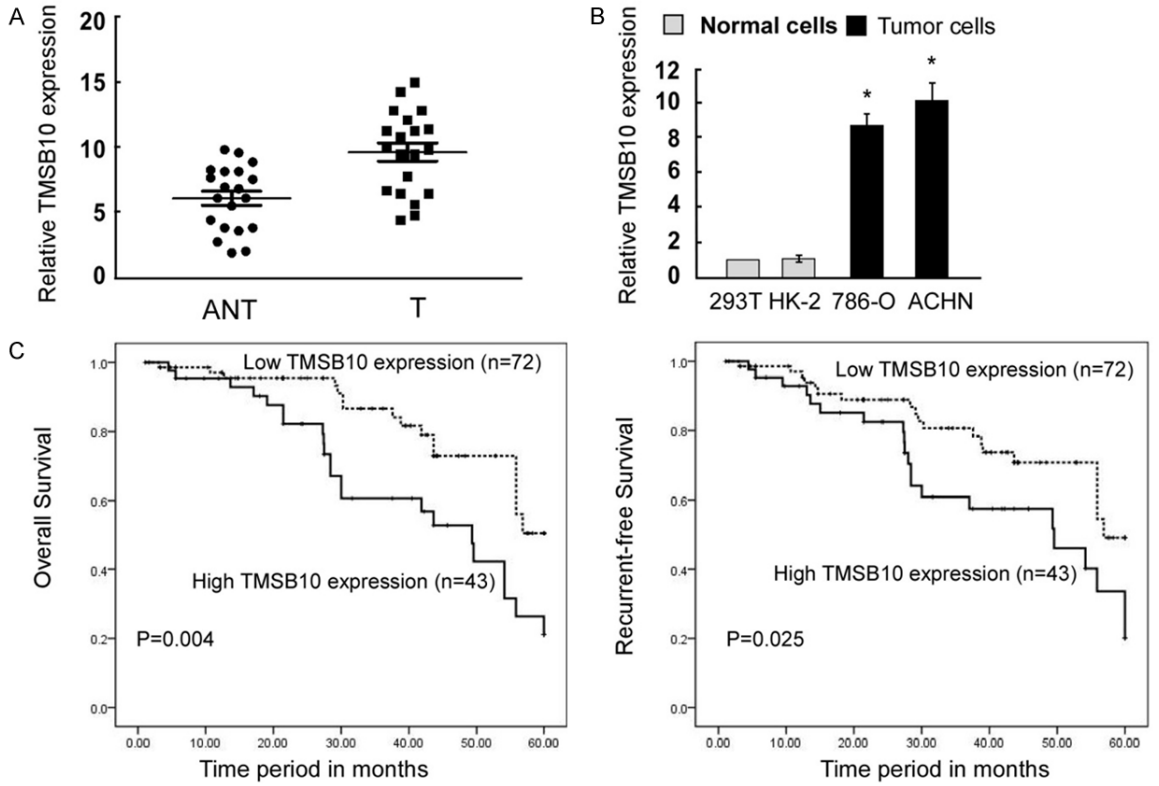

Figure 1. TMSB10 is overexpressed in primary human RCC. A. Real-time PCR analysis of TMSB10 expression in 20 paired human RCC tissues (T) and the adjacent normal kidney tissues (ANT) from the same patient. B. TMSB10 expression in two RCC cell lines (786-O and ACHN) and normal kidney cell lines (293T and HK-2). C. Kaplan-Meier overall survival curves for patients with RCC indicating the correlation of TMSB10 overexpression with worse overall survival and recurrence-free survival rates.

to standard procedures. The antibodies used in this study included: anti-TMSB10 (1:1000, Abcam) and GAPDH (1:2000, Santa Cruz Bio-technology).

Immunohistochemistry

Paraffin-embedded sections of tumor tissues were stained according to standard protocols with primary antibody against TMSB10 (1:100, Abcam) followed by staining with biotinylated secondary antibody. After washing, the sections were incubated with a buffer containing HRP-conjugated streptavidin followed by addition of substrate solution containing peroxidase. The results were combined to give a mean score for further comparative evaluations. Briefly, the IHC score was determined by combining the score for the percentage of positively-stained tumor cells with the grade of the staining intensity. The percentages of positively-stained tumor cells were scored as follows: 1 (<10% positive tumor cells); 2 (10-35% positive tumor cells); 3 (35-75% positive tumor cells), and 4 (>75% positive tumor cells). The staining intensities were graded as follows: 0 (negative), 1 (weak staining exhibited as light yellow), 2 (moderate staining exhibited as yellow brown), 3 (strong staining exhibited as brown). The IHC score≥6 was defined as high expression and SI<6 was defined as low expression.

Statistical analysis

All statistical analyses were performed using SPSS version 18.0 (SPSS Inc., Chicago, IL, USA). Correlations between TMSB10 expression and clinicopathological characteristics of the patients were analyzed using the χ^2-test. The Kaplan-Meier method was used to analyze the overall and recurrent-free survival time. Cox regression was used for multivariate analysis. Values were expressed as the mean ± SD from at least three independent experiments. $P< 0.05$ was considered significant.

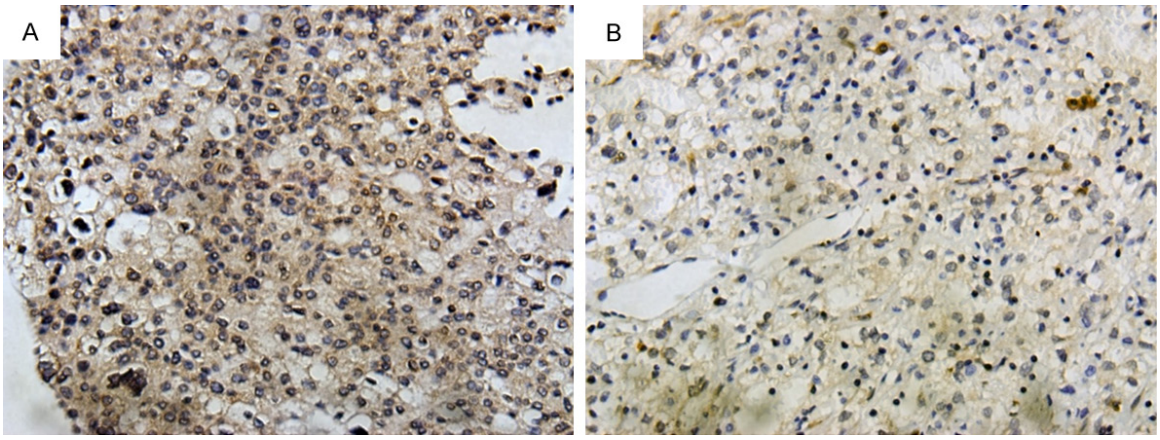

Figure 2. Representative immunostaining pictures of TMSB10 in RCC. A. High expression of TMSB10; B. Low expression of TMSB10 in RCC tissues. 400×.

Table 1. Relationship between TMSB10 expression and clinicopathological parameters of 115 RCC patients

Characteristic		N	TMSB10		*p*
			Low	High	
Age	≤55 y	44	26	18	0.539
	>55 y	71	46	25	
Sex	Male	80	53	27	0.222
	Female	35	19	16	
Tumor stage	T1-T2	67	50	17	0.002
	T3-T4	48	22	26	
LN metastasis	No	73	51	22	0.034
	Others	42	21	21	
Distal metastasis	No	94	36	31	0.039
	Yes	21	9	12	
Tumor size	≤4 cm	74	44	30	0.348
	>4 cm	41	28	13	
Fuhrman grade	I-II	69	41	28	0.387
	III-IV	46	31	15	
Total		115	72	43	

Results

TMSB10 expression is upregulated in renal cancer

To investigate whether TMSB10 is involved in human RCC progression, real-time quantitative RT-PCR was applied in twenty paired RCC tissues and adjacent normal kidney tissues, and two RCC cell lines (786-O and ACHN) and normal kidney cell lines (293T and HK-2) to assess the expression of TMSB10 in RCC. RCC tissues showed higher expression levels of TMSB10 compared with adjacent normal kidney tissues (**Figure 1A**), in addition, TMSB10 expression was significantly higher in RCC cell lines compared with normal kidney cell lines (**Figure 1B**). The representative immunostaining of TMSB10 in RCC tissues was shown in **Figure 2**.

TMSB10 expression is associated with clinicopathological features in RCC patients

To elucidate the relationship between TMSB10 and the clinicopathological features in RCC, chi-square test was performed in 115 RCC patients. As summarized in **Table 1**, high expression of TMSB10 was significantly associated with tumor stage (P=0.002), lymph node metastasis (P=0.034), and distant metastasis (P=0.039). However, there were no significant associations between TMSB10 expression and age, gender, tumor size, and Fuhrman grade. Moreover, the expression level of TMSB10 expression was significantly associated with the overall survival (P=0.004) and recurrent-free survival (P=0.025) of RCC patients, as patients with lower levels of expression had better survival than those with higher levels of TMSB10 expression. Multivariate Cox regression analysis showed that high expression of TMSB10 was a poor independent prognostic factor for RCC patients (P=0.007 and P=0.018, respectively) (**Table 2**).

Suppression of TMSB10 inhibits RCC cells migration and invasion

To examine the effect of TMSB10 on cancer cell migration, we performed Transwell and Matrigel

Table 2. Multivariate Cox regression analysis of overall survival and recurrence-free survival

Characteristic	Overall survival		Recurrence-free survival	
	Hazard Ratio (95% CI)	*P*	Hazard Ratio (95% CI)	*P*
Age (>55 y vs ≤55 y)	1.601 (0.163-2.410)	0.624	1.134 (0.267-3.135)	0.787
Sex (Male vs Female)	1.616 (0.534-6.457)	0.276	1.105 (0.723-5.822)	0.375
Tumor Stage (T3-T4 vs T1-T2)	2.437 (1.165-4.727)	0.036	2.612 (1.612-7.944)	0.067
LN metastasis (Yes vs no)	1.482(0.277-3.275)	0.156	2.267 (1.212-6.182)	0.125
Distal metastasis (Yes vs no)	2.142 (1.624-7.871)	0.097	2.276 (1.943-5.712)	0.156
Tumor size (>4 cm vs ≤4 cm)	3.576 (1.612-6.755)	0.043	2.295 (1.373-5.278)	0.087
Fuhrman grade (III-IV vs I-II)	1.621 (0.143-2.121)	0.681	1.593 (0.201-2.635)	0.805
TMSB10 expression (High vs low)	4.129(0.205-7.612)	0.007	3.735 (0.276-5.825)	0.0018

invasion assays in TMSB10 silenced RCC cells (**Figure 3A**). As shown in **Figure 3B** and **3C**, compared with cells that were transfected with empty vector, TMSB10 knockdown strongly restrained the motility of 786-O and ACHN cells. Moreover, the expression of EMT-related genes such as α-cadherin, Vimentin, Fibronectin, Snail, and Twist, was analyzed by real-time PCR. After knockdown of TMSB10, the expression of α-cadherin was up-regulated, while Vimentin, Fibronectin, Snail, and Twist were down-regulated in each siTMSB10 group compared with the negative control group (**Figure 3D**). These results suggested that TMSB10 plays a role in promoting cell migration in RCC.

Discussion

Despite the improvement in therapeutic methods, metastatic RCC patients have dismal prognosis. Therefore, identification of predictive biomarkers of tumor metastasis is extremely important for effective prevention and treatment of RCC. The present study investigated that TMSB10 is up-regulated in RCC tissues and cell lines. The expression level of TMSB10 was significantly associated with tumor stage, lymph nodes metastasis, and distal metastasis of RCC patients. High TMSB10 expression was a significant indicator of poor clinical prognosis for patients with RCC. Moreover, we found that downregulation of TMSB10 suppresses kidney cancer cell migration and invasion. Together with our results, TMSB10 may contribute to the progression of renal cancer progression.

TMSB10 belongs to the family of beta-thymosins, which are N-terminally acetylated peptides of about 5 kDa molecular mass and composed of about 40-44 amino acid residues [10]. As actin binding proteins, beta-thymosins interact with actin monomers to prevent actin polymerization and disrupt the formation of F-actin [11]. Beta-thymosins and their fragments have been reported to exhibit diverse physiological functions such as the induction of chemotaxis, metallo-proteinases, and inhibition of inflammation, angiogenesis, and the inhibition of bone marrow stem cell proliferation [12]. Of note, recent studies suggested that increased expression of beta-thymosins or the synthesis of a beta-thymosin may promote tumor metastasis possibly by increasing the cells mobility [13]. Cha et al. observed that B16-F10 cells infected with a TMSB4-expressing adenovirus produced more lung metastases in vivo than control groups [14]. Santelli et al. revealed that suppression of TMSB10 can significantly reduce anchorage-independent growth and improve act in filament organization in human thyroid carcinoma cells [15]. Bao et al. found that transfection of rat prostatic carcinoma cells with anti-sense TMSB15 constructs significantly reduced the migration of stimulated cells [16]. However, the roles of beta-thymosins in renal cancer remain largely unknown. In the present study, we for the first time reveal the correlation between TMSB10 overexpression and tumor stage and tumor metastasis in RCC. Additionally, we present evidence that knockdown of TMSB10 reduced migration and invasion of renal cancer cells. Together, these studies indicate that TMSB10 may play an important role in renal cancer progression.

Accumulating studies have shown that EMT constitutes an early metastatic step, and plays a crucial role in cancer invasion and development. Recently, a family member of beta-thy-

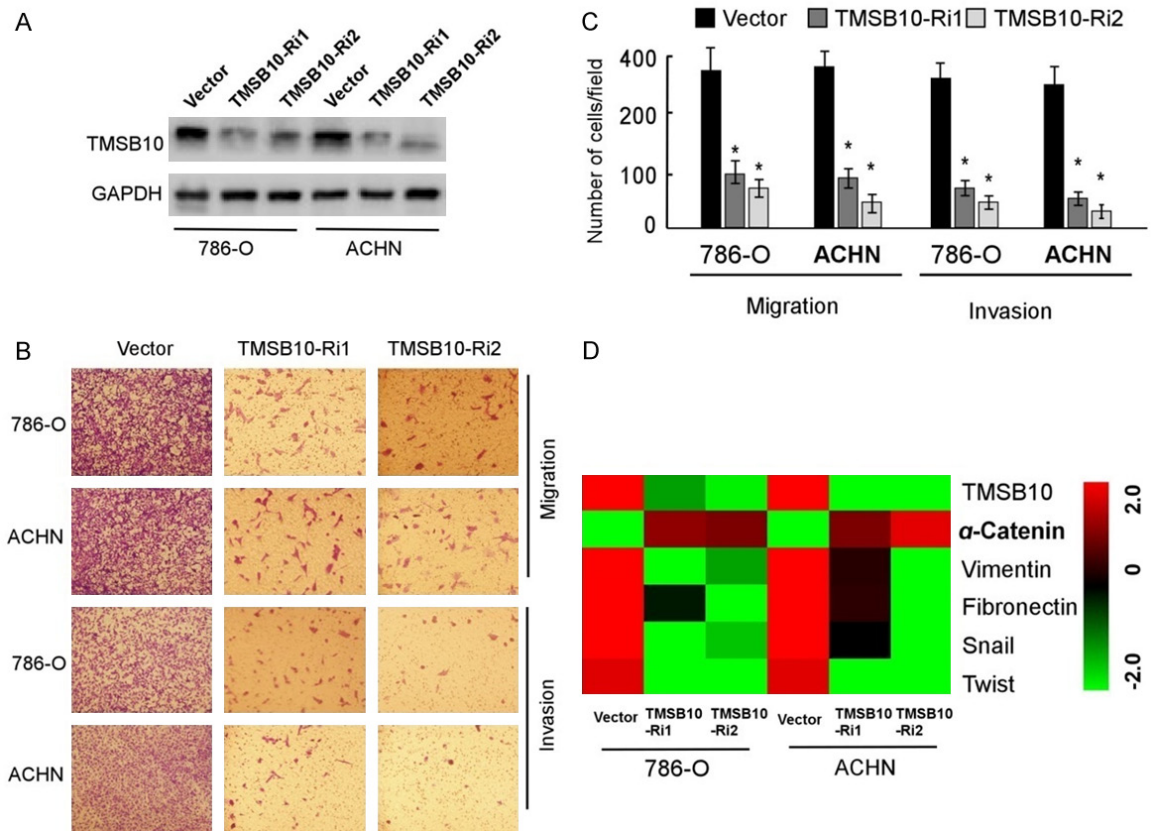

Figure 3. TMSB10 is involved in the invasion and metastasis of RCC cells. A. Western blotting analysis of TMSB10 expression in vector and TMSB10- silencing 786-O and ACHN cells; GAPDH was used as a loading control. B. Representative photos of Transwell and Matrigel invasion assays in control and TMSB10-shRNA RCC cells. C. Quantifications of migrated cells through the membrane and invaded cells through Matrigel of each cell line are shown as proportions of their vector controls. D. Real-time PCR of the expressions of TMSB10 and EMT-relevant markers a-Catenin, fibronectin and vimentin, as well as EMT regulators Snail and Twist in RNAi vector and TMSB10 shRNA 1- and 2-transduced 786-O and ACHN cells. Bars represent the mean ± SD of three independent experiments. *P<0.05.

mosins, TMSB4, was shown to be involved in EMT of cancer cells. Huang et al. reported that TMSB4 promotes colorectal carcinoma progression by inducing an EMT in tumor cells through up-regulation of ILK and activation of AKT [17]. Wang et al. demonstrated that overexpressing TMSB4 significantly increased motility, and subsequent invasiveness of bladder cancer cells [18]. Fu et al. found that the expression of TMSB4 was significantly higher in liver samples from patients with metastatic hepatoblastoma, and that TMSB4 may promote hepatoblastoma metastasis via the induction of EMT [19]. In the present study, we demonstrated that TMSB10 contributes to invasion, metastasis and progression of RCC by inducing EMT. Our results provide the preliminary molecular mechanisms underlying the role of TMSB10 in RCC metastasis.

Importantly, TMSB10 expression was shown to be associated with the clinical prognosis of human cancers. Zhang et al. showed that high TMSB10 expression significantly correlated with poor prognosis and distant metastases in patients with breast cancer [12]. Bouchal et al. suggested that TMSB10 expression was associated with relapse free survival and distant metastasis free survival of breast cancer patients [20]. In contrast, Lee and colleagues reported that TMSB10 hypomethylation was not significantly correlated with the clinical prognosis in non-small cell lung cancer patients, while they did not revealed the correlation of TMSB10 and the outcomes of the patients [8]. In the present study, we found that TMSB10 expression was significantly correlated with the over and recurrent-free survivals of RCC patients, further suggesting that TMSB10 expres-

sion was well correlated with cancer development.

In summary, we demonstrated that TMSB10 expression in RCC is significantly correlated with tumor metastasis and prognosis. We showed that downregulation of TMSB10 suppresses migration, invasion, and EMT of renal cancer cells. Thus, we provide information for the prediction of RCC prognosis and the establishment of targeted therapies.

Disclosure of conflict of interest

None.

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