TUSC3 as a potential biomarker for prognosis in clear cell renal cell carcinoma

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Abstract. The aim of the present study was to explore the expression levels of tumor suppressor candidate 3 (TUSC3) in human clear cell renal cell carcinoma (ccRCC) and its clinical value. Immunohistochemical staining, western blotting and reverse transcription-quantitative polymerase chain reaction were used to detect TUSC3 expression in paracancerous normal tissues and ccRCC tissues. The tissues were derived from the pathological specimens of 54 patients with ccRCC. Additionally, associations among TUSC3 expression and histological grade and clinicopathological staging of ccRCC were investigated. The results of these comparisons revealed that TUSC3 expression in ccRCC tissues was significantly lower than that in paracancerous tissues ($P<0.05$). TUSC3 expression in the high differentiation group was higher than that in the median and low differentiation groups ($P<0.05$). Expression levels of TUSC3 in stage I and II tissues were higher than those in stage III and IV tissues ($P<0.05$). The expression levels of TUSC3 in the lymph node metastasis group were lower than those in the non-lymph node metastasis group ($P<0.05$). In conclusion, the expression levels of TUSC3 in human ccRCC tissues were downregulated compared with those found in normal human renal tissue, and TUSC3 may inhibit the progression of ccRCC. Furthermore, the TUSC3 gene may be used as a promising tumor marker for the early diagnosis and prognosis of ccRCC.

Introduction

Clear cell renal cell carcinoma (ccRCC) is the third most common adult genitourinary cancer, accounting for ~3% of all malignancies, with ~209,000 newly diagnosed cases and 14,970 cases of ccRCC-associated mortality worldwide in 2017 (1-3). ccRCC exhibits radiotherapy and chemotherapy resistance, but surgical tumor resection is an effective treatment strategy for ccRCC at present (4-6). However, there are certain limitations to the surgical resection treatments (7,8). Following surgery, 25-30% of patients with ccRCC exhibit distant metastasis occurrence and 40% of patients with ccRCC exhibit local recurrence (2,9). The prognosis of ccRCC is unfavorable and hard to predict due to the complicated biological behavior and unclear molecular mechanism of ccRCC (10). In summary, a novel method for early detection and advanced treatment strategies for ccRCC are urgently required.

Tumor suppressor candidate 3 (TUSC3) is considered to be a promising anti-oncogene (11). TUSC3 expression has been demonstrated to be downregulated in various malignant tumors with a poor prognosis, including prostate cancer, colorectal cancer and pancreatic carcinoma (12-15). TUSC3 is a homolog of the yeast Ost3p subunit of the oligosaccharyltransferase (OST) complex, which promotes N-linked glycation of proteins in the endoplasmic reticulum (16). Peng *et al* (17) reported that the autophagy of human non-small cell lung cancer cells may be induced by TUSC3 via the activation of the Wnt/β-catenin signaling pathway. An experiment conducted by Liu *et al* (18) suggested that the proliferation and migration of breast cancer cells were inhibited by the upregulation of TUSC3 expression. Although TUSC3 has been identified to be crucial for tumorigenesis, TUSC3 expression in ccRCC and its clinical significance remain unclear. The purpose of the present study was to compare the expression levels of TUSC3 in ccRCC and normal renal tissues, and to assess the prognostic value of TUSC3 expression in patients with ccRCC.

Materials and methods

Clinical specimens. Surgical specimens were obtained from 54 patients with ccRCC during a nephrectomy at Jingzhou Central Hospital (Jingzhou, China) between September 2014 and January 2017. The mean age of the 54 patients was 61.5±6.2 years (range, 48-76 years). A total of 20 patients were female and 34 were male. The present study was approved by the Institutional Ethics Committee of Jingzhou Central Hospital. All patients provided relevant clinical information

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and written informed consent. Half of each specimen was preserved by fixation using 4% paraformaldehyde at room temperature for 1-2 weeks, followed by routine paraffin embedding. The remaining half of each sample was preserved in liquid nitrogen at -196˚C. All specimens were identified to be ccRCC tissues or normal tissues by histological identification, and tumor stage and grade were evaluated according to the American Joint Committee on Cancer guidelines (19).

Cell lines and cell culture. The human renal proximal tubular epithelial HKC8 cell line, human ccRCC A498, 786-O and OS-RC-2 cell lines, and the papillary renal cell carcinoma ACHN cell line (Type Culture Collection of the Chinese Academy of Sciences, Shanghai, China; cat. nos. GNHu12, TCHu158, TCHu186, TCHu40 and TCHu199) were cultured at 37˚C with 5% CO_2 in Dulbecco's modified Eagle's medium (Thermo Fisher Scientific, Inc., Waltham, MA, USA), supplemented with 10% fetal bovine serum (ScienCell Research Laboratories, Inc., San Diego, CA, USA), 0.1 mg/ml streptomycin and 100 U/ml penicillin. The medium was replaced every 24 h.

Immunohistochemistry. Immunohistochemical staining was used to evaluate the expression levels of TUSC3. The tissues were cut into 4-μm thick sections. Endogenous peroxidase activity was inhibited with 3% hydrogen peroxide at 37˚C for 10 min. Subsequently, the sections were incubated in normal horse serum (1:50) in Tris-buffered saline (TBS) for 30 min at 37˚C. Next, rabbit polyclonal anti-TUSC3 antibody (1:1,000; cat. no. ab77600; Abcam, Cambridge, MA, USA) was applied, followed by overnight incubation at 4˚C. The sections were washed with PBS three times. Subsequently, the sections were incubated with horseradish peroxidase-conjugated secondary antibody (1:2,000; cat. no. SA00001-9; ProteinTech Group, Inc., Chicago, IL, USA) for 30 min at 20˚C. The sections were incubated with the color reagent 3,3'-diaminobenzidine for 2 min at 20˚C. Tissues were observed under a light microscope (magnification, x400) and images were captured. The integrated optical density (IOD) was calculated from five random fields of view per slide using Image-Pro Plus software version 5.0 (Media Cybernetics, Inc., Rockville, MD, USA), and the IOD was presented as the mean value of three detections for each sample.

Reverse transcription-quantitative polymerase chain reaction (RT-qPCR). Extraction of total RNA from clinical specimens and cancer cells was performed using the TRIzol® RNA Reagent kit (Takara Bio, Inc., Otsu, Japan). RT of RNA into cDNA was conducted using the Applied Biosystems SYBR Green mix kit (cat. no. 163795-75-3; Shanghai Aladdin Biochemical Company, Shanghai, China), according to the manufacturer's protocol. The primer sequences for TUSC3 and GAPDH are shown in Table I. A total of 5 μl DNA Marker was used, and 1.5% agarose gel electrophoresis was performed using 5 μl RT-PCR product. GAPDH was used as an endogenous reference gene to analyze the relative gene expression levels. The thermocycling conditions were as follows: One cycle of 95˚C for 3 min, followed by 35 cycles of 95˚C for 5 sec, 58˚C for 30 sec and 72˚C for 30 sec. The expression levels were analyzed according to the $2^{-\Delta\Delta Cq}$ method (20). All experiments were performed in triplicate. The appearance of a single peak in the melting curve implicated the specificity of the PCR products.

Western blotting. HKC8, A498, 786-O, OS-RC-2 and ACHN cells were lysed using a Total Protein Extraction kit (Wuhan Goodbio Technology Co., Ltd., Wuhan, China), according to the manufacturer's protocol. Protein concentrations were assessed using a Bicinchoninic Acid assay prior to loading the samples. Briefly, 40 μg/lane total protein from each sample was separated by 10% SDS-PAGE and transferred to a nitrocellulose membrane. The membranes were blocked for 2 h at room temperature with 5% milk dissolved in TBS containing 0.05% Tween-20 (TBST)), and incubated with the primary polyclonal antibodies anti-TUSC3 (1:500; cat. no. ab77600; Abcam) and anti-β-actin (1:500; cat. no. SA00001-9; ProteinTech Group Inc.), at 4˚C overnight. Subsequently, the membranes were washed three times with TBST, and incubated with horseradish peroxidase-conjugated secondary antibodies (1:1,000; cat. no. SA00001-9; ProteinTech Group, Inc.) in 5% non-fat milk at room temperature for 1 h. Following three washes with TBST, the membranes were developed with enhanced chemiluminescence western blotting detection kit (EMD Millipore, Billerica, MA, USA), Expression of TUSC3 protein in each group was semi-quantified using ImageJ software (National Institutes of Health, Bethesda, MD) and Image Pro Plus v6.0 software (Media Cybernetics, Inc.).

Statistical analysis. Experiments were repeated at least three times. All data are presented as the mean ± standard deviation and were analyzed using SPSS v11.0 (SPSS, Inc., Chicago, IL, USA). Differences in values and percentages among groups were compared using a paired t-test, χ^2 test, Fisher's exact test or one-way analysis of variance followed by Dunnett's multiple comparison test, respectively. Survival length was calculated from the date of surgery to the date of mortality or last follow-up. Survival curves and univariate analysis were estimated using the Kaplan-Meier method and the log-rank test. Parameters that demonstrated a statistically significant effect on overall survival in the univariate analysis were included in a Cox multivariate proportional hazards regression model. $P<0.05$ was considered to indicate a statistically significant difference.

Results

TUSC3 expression in clinical specimens. To clarify whether TUSC3 expression is associated with ccRCC progression, carcinoma tissues and adjacent non-neoplastic parenchyma were analyzed using RT-qPCR and immunohistochemical staining. The RT-qPCR data revealed that the relative expression of TUSC3 in adjacent normal tissues was 0.657±0.101 and the relative expression of TUSC3 in ccRCC tissues was 0.512±0.087. Therefore, compared with that in adjacent normal tissues, TUSC3 expression was significantly reduced in ccRCC tissues (Fig. 1). The mean (0.5845) of the relative expression of TUSC3 in tissues of all paired samples was used as a cut-off to determine high and low expression groups. In 54 paired specimens from patients with ccRCC, 35.2% of tissues exhibited high expression of TUSC3, and 64.8% of

Table I. Primers used for reverse transcription-quantitative polymerase chain reaction analysis of mRNA levels.

Gene	Primer sequence (5'-3')
TUSC3	F: GGCTCAGTTTGTGGCAGAATC
	R: CATCGCCTTTCGAAGTTGCT
GAPDH	F: CTCGCTTCGGCAGCACA
	R: AACGCTTCACGAATTTGCGT

TUSC3, tumor suppressor candidate 3.

Table II. Expression levels of TUSC3 in ccRCC and adjacent tissues.

		TUSC3 expression	
Tissues	n	Low, n (%)	High, n (%)
ccRCC	54	35 (64.8)	19 (35.2)
Adjacent normal tissues	54	21 (38.9)	33 (61.1)
χ^2			7.269
P-value			0.007[a]

P-value was calculated using a χ^2 test. [a]Statistically significant. ccRCC, clear cell renal cell carcinoma; TUSC3, tumor suppressor candidate 3.

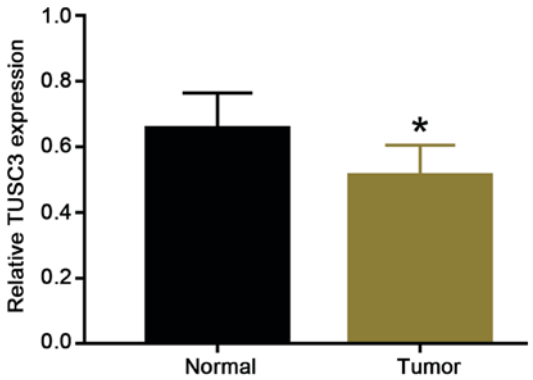

Figure 1. Reverse transcription-quantitative polymerase chain reaction analysis of TUSC3 expression in human clear cell renal cell carcinoma tissues from 54 patients. *P<0.05 vs. normal. TUSC3, tumor suppressor candidate 3.

tissues exhibited low expression of TUSC3 (Table II). TUSC3 expression in adjacent normal tissues was distinctly higher than that in ccRCC tissues (P=0.007; Table II). As shown in Fig. 2, TUSC3 expression levels were decreased in tumor tissues compared with those in adjacent normal tissues. In tumor tissues, TUSC3 was expressed in the cytoplasm and nucleus of renal epithelial cells of proximal and distal tubules (Fig. 2). Furthermore, tumor stage and grade were classified according to the Tumor-Node-Metastasis (TNM) staging system of the American Joint Committee on Cancer guidelines (19) (Table III). TUSC3 levels were significantly associated with

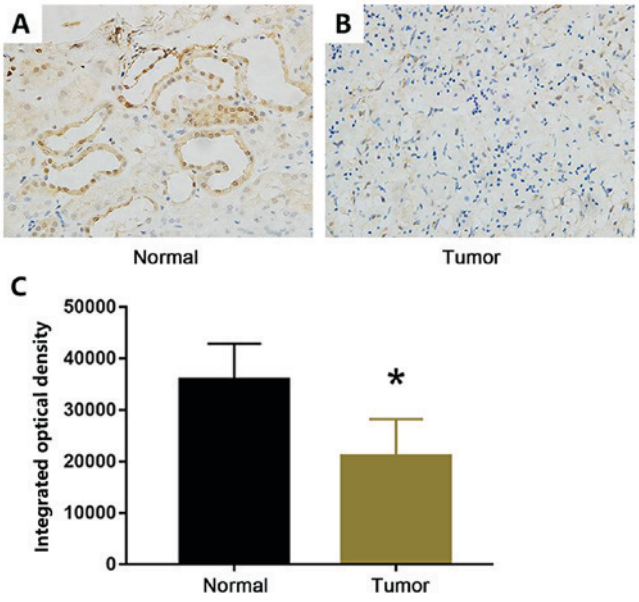

Figure 2. Immunohistochemical analysis of TUSC3 expression. (A and B) Immunohistochemical staining of TUSC3 in normal kidney tissues and clear cell renal cell carcinoma tissues (magnification, x400). (C) Quantitative analysis of immunohistochemical images. *P<0.01 vs. normal. TUSC3, tumor suppressor candidate 3.

primary tumor size (P=0.030), tumor thrombus (P=0.044), clinical tumor stage (P=0.039), regional lymph node involvement (P=0.040), distant metastasis (P=0.044), TNM stage (P=0.039) and nuclear grade (P=0.021). TUSC3 expression in tumor tissues was significantly decreased in higher TNM stages. These results indicated that TUSC3 expression was closely associated with ccRCC progression.

Association between TUSC3 expression and survival rate in patients. To the best of our knowledge, no study has demonstrated that the level of TUSC3 expression is a good predictor of survival in patients with ccRCC. In the present study, to determine the prognostic value of TUSC3, overall survival rates of 54 patients with ccRCC were explored using Kaplan-Meier survival curves. Postoperatively, patients with a high expression level of TUSC3 exhibited a higher overall survival rate compared with that of patients with a high expression level of TUSC3 (P<0.01; Fig. 3). This indicated that TUSC3 expression was a prognostic factor in patients with ccRCC. Cox univariate analysis revealed that primary tumor size (P=0.016), tumor thrombus (P=0.020), primary tumor classification (P=0.022), regional lymph node involvement (P=0.019), distant metastasis (P=0.026), TNM stage group (P=0.020), nuclear grade (P=0.015) and TUSC3 expression (P<0.001) were all significantly associated with overall survival (Table IV). Furthermore, Cox multivariate regression analysis suggested that primary tumor size (P=0.032), tumor thrombus (P=0.018), primary tumor classification (P=0.036), regional lymph node involvement (P=0.039), distant metastasis (P=0.044), TNM stage group (P=0.037), nuclear grade (P=0.012) and TUSC3 expression (P=0.015) were all independent prognostic factors for patients with ccRCC. These data indicated that low intratumoral TUSC3 expression may be used as a novel marker for ccRCC progression and a poor prognosis.

Table III. Clinicopathological characteristics of 54 patients with clear cell renal cell carcinoma and their association with TUSC3 expression.

Characteristics	n	TUSC3 expression		P-value
		Low, n (%)	High, n (%)	
Age at surgery, years				0.999
<65	27	18 (66.6)	9 (33.3)	
≥65	27	17 (63.0)	10 (37.0)	
Sex				0.572
Male	34	21 (61.8)	13 (38.2)	
Female	20	14 (70.0)	6 (30.0)	
Primary tumor size, cm				0.030[a]
<7	38	21 (60.5)	17 (39.5)	
≥7	16	14 (87.5)	2 (12.5)	
Tumor thrombus				0.044[a]
None	47	28 (59.6)	19 (40.4)	
Level 0-IV	7	7 (100.0)	0 (0.0)	
Primary tumor classification				0.039[a]
T1+T2	42	24 (57.1)	18 (42.9)	
T3+T4	12	11 (91.7)	1 (8.3)	
Regional lymph node involvement				0.040[a]
N0	43	26 (60.5)	17 (39.5)	
N1+N2	11	11 (100.0)	0 (0.0)	
Distant metastasis				0.044[a]
M0	47	28 (59.6)	19 (40.4)	
M1	7	7 (100.0)	0 (0.0)	
TNM stage				0.039[a]
I+II	42	24 (57.1)	18 (42.9)	
III+IV	12	11 (91.7)	1 (8.3)	
Nuclear grade				0.021[a]
1+2	41	23 (56.1)	18 (43.9)	
3+4	13	12 (92.3)	1 (7.7)	

P-values were calculated using Fisher's exact test. [a]Considered statistically significant. TNM, Tumor-Node-Metastasis; TUSC3, tumor suppressor candidate 3.

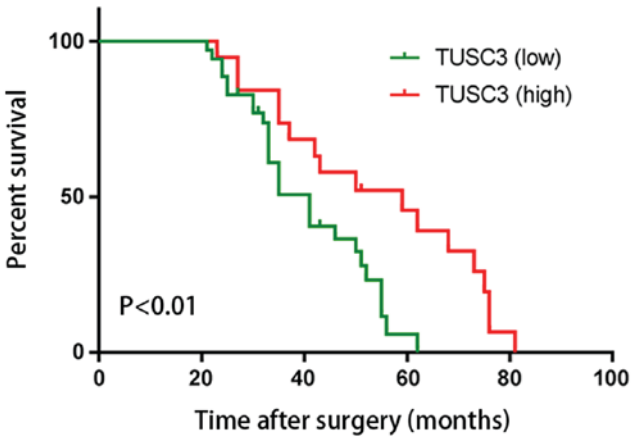

Figure 3. Kaplan-Meier survival analysis of patients with clear cell renal cell carcinoma with high (n=19) and low (n=35) TUSC3 expression. TUSC3, tumor suppressor candidate 3.

TUSC3 expression in ccRCC cells. To explore the influence of TUSC3 on RCC cell lines (A498, 786-O, OS-RC-2 and ACHN), expression levels of TUSC3 were detected by western blotting. Compared with the control group (HKC8 cells), A498, 786-O, OS-RC-2 and ACHN cells exhibited lower protein expression levels of TUSC3 (P<0.05; Fig. 4). This supported our *in vivo* data demonstrating that TUSC3 expression inhibits ccRCC tumorigenesis.

Discussion

ccRCC accounts for 2-3% of adult tumors, representing 90% of renal malignancies (21,22). Despite the development of diagnostic techniques in recent years, ~30% of patients with ccRCC are diagnosed with metastases at the first diagnosis, and 30-40% of patients exhibit localized ccRCC recurrence and metastasis following surgical resection (23,24). Surgery

Table IV. Univariate and multivariate analyses of overall survival of patients with clear cell renal cell carcinoma.

	Overall survival			
	Univariate analysis		Multivariate analysis	
Characteristics	HR (95% CI)	P-value	HR (95% CI)	P-value
Age at surgery (<65 years vs. ≥65 years)	1.784 (1.077-3.531)	0.062	1.580 (1.012-3.230)	0.079
Sex (male vs. female)	0.728 (0.358-1.369)	0.236	0.625 (0.310-1.158)	0.291
Primary tumor size (<7 cm vs. ≥7 cm)	4.632 (2.653-6.758)	0.016[a]	5.220 (2.793-9.365)	0.032[a]
Tumor thrombus (None vs. level 0-IV)	9.458 (4.635-21.325)	0.020[a]	8.457 (3.656-17.632)	0.018[a]
Primary tumor classification (T1+T2 vs. T3+T4)	4.320 (2.542-9.326)	0.022[a]	4.550 (2.860-11.736)	0.036[a]
Regional lymph node involvement (NX+N0 vs. N1+N2)	7.236 (4.335-26.324)	0.019[a]	6.358 (3.886-23.656)	0.039[a]
Distant metastasis (M0 vs. M1)	8.873 (2.365-24.325)	0.026[a]	5.637 (1.875-17.639)	0.044[a]
TNM stage (I+II vs. III+IV)	3.369 (2.859-9.635)	0.020[a]	3.963 (3.582-12.362)	0.037[a]
Nuclear grade (1+2 vs. 3+4)	5.238 (3.596-11.387)	0.015[a]	4.698 (3.157-9.692)	0.012[a]
TUSC3 expression (low vs. high)	0.185 (0.102-3.112)	<0.001	0.326 (1.786-5.447)	0.015[a]

[a]P<0.05. CI, confidence interval; HR, hazard ratio; TNM, Tumor-Node-Metastasis.

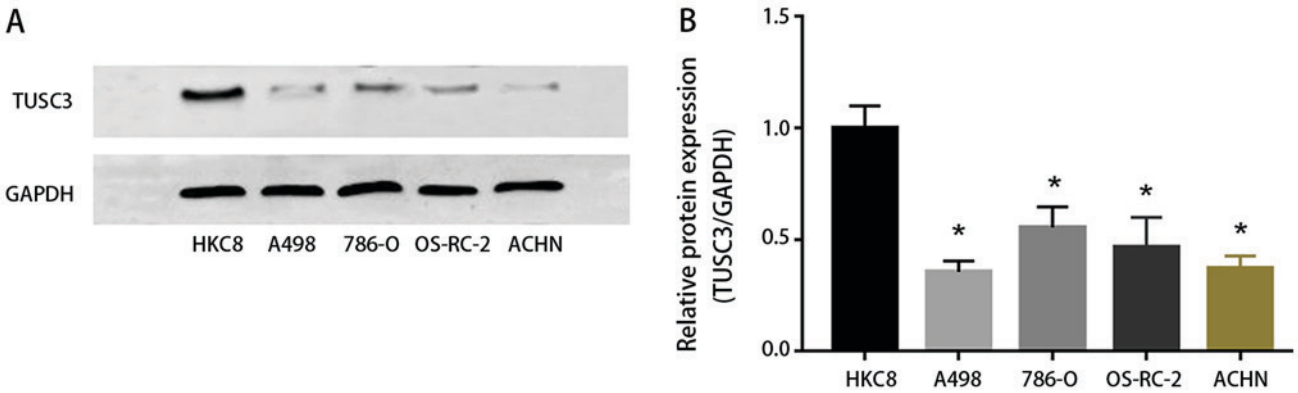

Figure 4. TUSC3 expression was downregulated in clear cell renal cell carcinoma cells. (A and B) Western blot analysis of TUSC3 relative to GAPDH for HKC8, A498, 786-O, OS-RC-2 and ACHN cells. *P<0.01 vs. HKC8. TUSC3, tumor suppressor candidate 3.

is the most common primary therapeutic method for ccRCC; however, ccRCC cannot be treated completely by radical surgery. Recent studies have focused on the possibility of combining modalities for improving the therapeutic value of existing standard therapies, including chemotherapy and radiotherapy (25,26); however, ccRCC is not sensitive to radiotherapy and chemotherapy. Patients with ccRCC continue to have extremely poor outcomes (27,28). The genesis and progression of ccRCC involve various factors, including carcinogenic substances and environmental factors (29,30). The mortality rate of patients with metastatic ccRCC is high, although novel targeted therapies have been developed. Therefore, determining prognostic markers to more accurately select patients with ccRCC with poor survival is becoming increasingly important.

TUSC3, also known as N33, is a gene segment with a length of ~349,435 bp that is composed of 11 exons (31). The OST complex, which is a component of the endoplasmic reticulum, promotes N-linked glycation of proteins during the protein folding process (32,33). The human OST complex is composed of seven elements, including OST complex subunit 4, dolichyl-diphosphooligosaccharide protein glycosyltransferase non-catalytic subunit, defender against cell death 1, ribophorin I, ribophorin II, STT3 oligosaccharyltransferase complex catalytic subunit A or STT3 oligosaccharyltransferase complex catalytic subunit B, TUSC3/N33 and magnesium transporter 1 (16,34). TUSC3 has been demonstrated to exert various biological functions in human learning and memory processes, and its mechanism is associated with the alteration of the magnesium ion transport system (35). Numerous studies have demonstrated that TUSC3 is mainly expressed in the epithelium of the liver, lung, placenta, prostate, ovary, colon, testis and adipose tissues (13,18,36-40). TUSC3 was identified as a potential anti-oncogene when it was first identified in the 1990s, and deletion of TUSC3 expression is associated with the malignant transformation of cells (37). A large number of studies reported that the carcinogenesis of pancreatic, gastric, ovarian and prostate cancer may be associated with the mutation or deletion of TUSC3 (11,41,42). Furthermore, the loss

of TUSC3 expression may result in an increase in cancer cell growth, migration and invasion (43,44).

In the present study, TUSC3 protein was identified to be downregulated in human ccRCC tissues, and its expression was significantly associated with clinical stage and lymph node metastasis. Patients with ccRCC with low expression of TUSC3 exhibited a higher TNM stage, and TUSC3 was a prognostic factor for the overall postoperative survival of patients. Numerous studies have demonstrated the anti-cancer properties of TUSC3 (11,13,42,44); however, the precise molecular mechanism of the function of TUSC3 in the development of tumor remains poorly understood. The sequence of the chromosomal band 8p22, where TUSC3 is located, has been revealed to be lost in human prostate cancer (45). Subsequent studies indicated that the proliferation, migration and invasion of prostate and ovarian cancer cells can be increased due to decreased TUSC3 expression (40,46). A previous study demonstrated that TUSC3 expression is downregulated in higher grades of ovarian cancer (46). Subsequently, a study explored the molecular mechanism of TUSC3 in ovarian cancer, which revealed that the hypermethylation of the TUSC3 promoter could lead to low expression of TUSC3, and the methylation of promoter is a prognostic indicator of patients with cancer (37). The present study explored TUSC3 expression in ccRCC specimens and its association with TNM staging and overall survival of patients with ccRCC, and demonstrated that immunohistochemical staining for TUSC3 served an important role in the prediction of ccRCC progression. Furthermore, the data suggested that low expression of TUSC3 in ccRCC cells was associated with the poor prognosis of patients, revealing that TUSC3 expression may be associated with the progression of ccRCC. In addition, the functions and biological mechanisms of TUSC3 are currently being elucidated (33). N-linked glycosylation of proteins serves an important role in protein synthesis, suggesting that TUSC3 may block tumor progression by transforming the glycosylation reaction in various types of carcinoma (33). Dysfunction of TUSC3 may result in improper protein glycosylation, potentially leading to disorders of cellular biological function (42). The present study revealed that TUSC3 expression may inhibit tumor progression in patients with ccRCC, and TUSC3 may serve as a biomarker to identify tumor progression and the prognosis of patients with ccRCC.

There were several limitations to the present study. One was the relatively small sample size. Second, the biomarker role of TUSC3 was only tested in ccRCC cell lines and a papillary renal cell carcinoma cell line, thus further verification in other types of RCC cell lines is required. Third, the present study did not investigate the antitumor mechanism of TUSC3 in ccRCC.

In conclusion, the present study demonstrated that TUSC3 is associated with the progression of ccRCC and the prognosis of patients with ccRCC. However, the antitumor mechanism of TUSC3 in ccRCC requires further investigation.

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Availability of data and materials

The datasets used and/or analyzed during the present study are available from the corresponding author on reasonable request.

Authors' contributions

JJZ conceived, designed and supervised the study. YJY drafted the manuscript. YJY and ZJC conducted the experiment. YXL contributed to statistical analysis. All authors have read and approved the final manuscript.

Ethics approval and consent to participate

The present study was approved by the Institutional Ethics Committee of Jingzhou Central Hospital, Jingzhou, China. All patients enrolled in the present study signed informed consent.

Consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

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