TUSC3 inhibits cell proliferation and invasion in cervical squamous cell carcinoma via suppression of the AKT signaling pathway

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Abstract

Background

The expression of tumor suppressor candidate 3 (TUSC3) is associated with proliferation in several types of cancer, leading to an unfavorable prognosis. The present study aimed to assess the cellular and molecular function of TUSC3 in patients with cervical squamous cell carcinoma (CSCC).

Methods

Levels of mRNA expressions of TUSC3 were analyzed in CSCC tissues and six cell lines using qRT-PCR. Immunohistochemistry(IHC) was used to evaluate the protein expression level of TUSC3 in four paired specimens, 220 paraffin-embedded CSCC specimens and 60 cases of normal cervical tissues(NCTs), respectively. Short hairpin RNA interference was employed for TUSC3 knockdown. Cell proliferation, migration and invasion was evaluated using growth curve, MTT assay, wound healing and transwell assay respectively.

Results

The results demonstrated that TUSC3 mRNA and protein expression levels were down-regulated in CSCC samples. Multivariate and univariate analyses indicated that TUSC3 was an independent prognostic factor for patients with CSCC. Decreased TUSC3 expression levels were significantly associated with proliferation and an aggressive phenotype of cervical cancer cells. Moreover, the knockdown of TUSC3 promoted migration and invasion of cancer cells, while the increased expression of TUSC3 exhibited the opposite effects. The down-regulation of TUSC3 facilitated proliferation and invasion of CSCC cells through the activation of the AKT signaling pathway.

Conclusions

Our data demonstrated that the down-regulation of TUSC3 promoted CSCC cell metastasis via the AKT signaling pathway. Therefore, TUSC3 may serve as a novel prognostic marker and potential target for CSCC.

Background

Cervical carcinoma is the second most prevalent type of female cancer in developing countries and the main cause of death related to malignancy. Approximately 529,800 new cases and 275,100 deaths occur annually worldwide from cervical carcinoma [1]. In recent years, the wide implementation of the Pap smear screening program has caused a significant decrease in the incidence of cervical cancer. However, it is still a major public health problem in developing countries [1, 2]. Generally, the major therapeutic modality for cervical squamous cell carcinoma (CSCC) (FIGO stage IA2–IIA) is radical hysterectomy and pelvic lymphadenectomy [2–4]. The aggressiveness of tumor cells is closely associated with the prognosis of patients with cervical cancer [2, 5]. Considerable efforts have been made to elucidate the molecular mechanisms of cell migration and invasion in tumor metastasis and specific methods have been utilized to decrease patient mortality, yet still with limitations [5, 6]. Therefore, the identification of tumor-specific markers for the diagnosis of CSCC and the evaluation of its aggressiveness are critical for high-risk patients who require more personalized and urgent clinical intervention.

Tumor suppressor candidate 3 (TUSC3) is encoded by the TUSC3 gene, which is mapped to chromosome 8p22 and contains three prototypical different transcripts [7]. In individual tissues and various stages of embryonic development, the expression of TUSC3 is completely different [7–13]. More importantly, TUSC3 expression levels correlate with tumor-suppressive or oncogenic function in specific cancer types. Previous studies have revealed that the downregulation of TUSC3 is associated with the incidence of several human cancers, including hepatocellular carcinoma [10], breast cancer [11], pancreatic cancer [12] and ovarian cancer [13]. Moreover, it exhibits a positive association with the proliferation, migration and invasion of tumor cells. In contrast to these observations, it was also reported that TUSC3 was overexpressed in several cancer types, such as colon cancer [14] and non-small cell lung cancer [15]. Therefore, it has been suggested that TUSC3 plays intricate and important roles in different types of cancer. However, the expression pattern of TUSC3 in CSCC and its value for predicting patient survival remain unclear. In the present study, the expression pattern of TUSC3 was investigated in patients with CSCC. The association of TUSC3 with patient prognosis was analyzed, as well as its cellular and molecular function in cervical cancer cell migration and invasion.

Methods

Cell culture

Hela, CaSki and C33A cells were obtained from The Cell Bank of Type Culture Collection of the Chinese Academy of Sciences. SiHa cells were purchased from China Center for Type Culture Collection. C4-1 and HCC94 cells were purchased from OTWO. The cell lines were cultured in high glucose medium DMEM (Gibco; Thermo Fisher Scientific, Inc.) supplemented with 1% non-essential amino acids (Thermo Fisher Scientific, Inc.), 1% antibiotics (100 U/ml penicillin and 100 µg/ml streptomycin) and 10% fetal bovine serum (Gibco; Thermo Fisher Scientific, Inc.) in a 5% CO₂ humidified atmosphere at 37°C.

Tissue specimens and patient information

In this retrospective study, patients were recruited from The First Affiliated Hospital of Hainan Medical University and Nanfang Hospital, Southern Medical University from January 1 2009 to December 31 2014. The present study was approved by the Institutional Research Ethics Committee of Nanfang Hospital and Hainan Medical University. All participants had signed written informed consent prior to the investigation. The patients who underwent chemotherapy prior to primary surgical treatment were excluded from this study. All pathological diagnoses of CSCC were confirmed by two independent pathologists. Fresh tumor specimens were timely stored in liquid nitrogen following extraction from the patients. A total of 220 paraffin-embedded specimens were obtained from patients with CSCC and 60 NCTs from benign uterine tumor patients during hysterectomy. Patients were followed up until December 31 2018. The detailed clinical data are summarized in Table 1.

Plasmids

The full-length human TUSC3 gene was subcloned into the pSin-EF1α-puro (donation from GuangZhou Institutes of Biomedicine and Health, Chinese Academy of Sciences) lentiviral vector using the restriction enzymes *EcoRI* and *BamHI*. Short harpin RNAs (shRNAs) targeting TUSC3 were subcloned into the GV248-EGFP-puromycin (GIDE77111, GENE) lentiviral vector using the restriction enzymes *AgeI* and *EcoRI*. Transfection was performed using

Lipofectamine® 2000 reagent (Invitrogen, Thermo Fisher Scientific, Inc.) according to the manufacturer's instructions. Following 48 h of cell culture, the cells were selected with 6 µg/ml puromycin for 7 days. The shRNA sequence used was as follows: shTUSC3 5'-CCTCGAAACTATTCCATGATT-3', which was designed according to the sequence corresponding to the GenBank accession number NM_006765.

Reverse transcription-quantitative PCR (RT-qPCR)

Total RNA was extracted from primary tumor tissues and cultured cells using TRIzol® reagent (Gibco; Thermo Fisher Scientific, Inc.). cDNA was synthesized from 1 µg RNA from each sample using the iScript™ cDNA Synthesis kit (Promega Corporation) according to the manufacturer's instructions. The RT-qPCR thermocycling conditions were the following: initial denaturation at 50 °C for 2 min, 95 °C for 10 min, and 40 cycles of 95 °C for 15 s, 56 °C for 45 s. The primers for TUSC3 were as follows: Forward, 5'-TGGATTGCTGACAGAACGGA-3' and reverse, 5'-CAGAGACACCATGGCCCAAC-3'. The primers for GAPDH were as follows: forward, 5'-CGAGATCCC TCCAAAATCAA-3' and reverse, 5'-TGTGGTCATGAGTCCTTCCA-3'. TUSC3 expression data were normalized to GAPDH and all experiments were performed in triplicate.

Cell growth curve

The cells were plated in 6-well plates (1×10^5 cells) and cultured for 6 days. The cells were enzymatic disaggregated and counted every day by using a hemocytometer for the determination of their growth curve. Each cell line experiment was performed in triplicates.

Western blotting

The cells were washed twice with ice-cold PBS and lysed on ice in RIPA buffer (Cell Signaling Technology, Inc.) supplemented with a complete protease inhibitor cocktail (Roche Applied Science). Fresh tissue specimens were grounded to powder in liquid nitrogen and lysed with SDS-PAGE sample buffer. All protein samples (30 µg) were separated with 10% SDS-PAGE gels, transferred to PVDF membranes (Thermo Fisher Scientific, Inc.) and blocked with 5% skimmed milk in Tris-buffered saline with 0.1% Tween-20 (TBST) for 2 h at room temperature (RT). The membranes were incubated with anti-TUSC3 antibody (1:500; cat. no. ab230520; Abcam) at 4 °C overnight, rinsed with TBST and further incubated with goat anti-rabbit IgG conjugated to horseradish peroxidase (Abcam) for 1 h at RT. The expression levels of TUSC3 were detected with enhanced chemiluminescence prime western blotting detection reagents (EMD Millipore). GAPDH (1:20000; cat. no. ab8229; Abcam) was used as a loading control.

Immunohistochemistry (IHC)

IHC was performed as previously described [15,16]. TUSC3 primary antibody was purchased from Abcam (cat. no. ab230520). TUSC3 staining was scored by two different pathologists who acted independently with regard to the evaluation of the intensity of staining and the proportion of positive staining. The scores were averaged. The intensity of staining was graded as follows: 3 (strong staining, ~brown), 2 (moderate staining, ~yellow brown), 1

(weak staining, ~light yellow) and 0 (no staining). The proportion of cancer cells was scored as follows: 4 (>75% positive tumor cells), 3 (51-75% positive tumor cells), 2 (26-50% positive tumor cells), 1 (6-25% positive tumor cells) and 0 (<5% positive tumor cells). The staining index for TUSC3 expression in CSCC was calculated by multiplying the two scores of the staining intensity and the proportion of positive cells. The median of all scores was used as a cut-off value for TUSC3. An optimal cut-off value was used as follows: a score of ≥ 6 was used to define tumors with high TUSC3 expression and a score of ≤ 4 indicated low TUSC3 expression. Positive control staining was performed in human colon cancer tissues and negative control staining was performed without primary antibody.

EdU assay

The EdU assay was performed as determined by the product specifications (C00031*, Guangzhou RiboBio Co., Ltd).

MTT assay

The cells were seeded into 96-well plates (2×10^3 cells/well). At different time points, MTT solution (0.5 mg/ml, Merck & Co., Inc.) was added to each well and cultured for 4 h at 37°C. Finally, the cell culture medium was removed and 100 μ l dimethyl sulphoxide (Merck & Co., Inc.) was added to each well. The absorbance was measured at 570 nm and 655 nm was the reference wavelength. Each experiment was conducted in triplicate.

Wound healing assay

Transfected cells were grown to 80-90% confluence in 6-well plates. The cellular layer was wounded using a sterilized tip (1 ml). Following 48 h of cell culture without serum, cell migration was monitored and microscopically photographed. The migratory ability was assessed by measuring the changes in the sizes of the wounded areas of the six fields.

Transwell assay

The Transwell assay (pore size 8 μ m; BD Biosciences) was performed to assess the invasive ability. The membranes of the filters were coated with Matrigel (50 μ l per filter; BD Biosciences). The cells were added to the upper chamber in 1% FBS medium, while the lower chamber was filled with medium containing 10% FBS. Following 24 h of cell culture, the cells on the upper surface of the membrane were removed by a cotton swab and the invaded cells on the lower membrane were stained with Giemsa. Five fields were randomly selected for cell counting on the membranes. The experiments were performed in triplicate.

Colony formation assay

The cells were resuspended in DMEM supplemented with 10% FBS and plated at a density of 5×10^2 cells/well in 6-well plates. Following 10 days of cell culture, the colonies were stained with 1% crystal violet (Sigma-Aldrich; Merck

KGaA) for 30 sec following fixation with 4% aldehyde for 5 min. The colonies were counted and photographed.

Statistical analysis

Statistical analysis was performed using SPSS software (version 24.0; IBM Corp.). The association between TUSC3 levels and clinicopathological parameters was analyzed by the Pearson's χ^2 and the Fisher's exact tests. The analysis methods included the log-rank test, Spearman-rank correlation test and the Student's t-test. Kaplan-Meier curves were plotted to assess the effects of TUSC3 downregulation on PFS and OS. The multivariate Cox regression model was employed for analyzing clinicopathological variables associated with survival. $P < 0.05$ was considered to indicate a statistically significant difference. The data are expressed as the mean $\pm$ SD of three independent experiments.

Results

TUSC3 expression is downregulated in CSCC

The mRNA levels of TUSC3 were downregulated in both CSCC tissues and cell lines, as determined by RT-qPCR (Fig. 1A-C). Furthermore, the protein levels of TUSC3 were significantly decreased in CSCC tissues (T) compared with normal cervical tissues (NCTs), as evaluated by IHC and western blotting analyses (Fig. 1D and E).

TUSC3 regulates CSCC cell proliferation and invasion

To investigate the potential function of TUSC3, stable TUSC3 overexpressing and knockdown Hela and SiHa cells (Hela/SiHa-TUSC3 and Hela/SiHa-shTUSC3) were established (Fig. 2A and B). Hela and SiHa cells are the most usually used cervical cancer cell lines. Hela is cervical adenocarcinoma cell line and infected HPV18. SiHa is cervical squamous cell carcinoma cell line and infected HPV16. The experimental design includes the most common type of pathology and the predominant type [18]. The growth curve assay indicated that overexpression of TUSC3 inhibited significantly the proliferation of Hela and SiHa cells (Fig. 2C and D). The results from the colony-formation and the EdU assays revealed that the downregulation of TUSC3 increased Hela and SiHa cell proliferation *in vitro* (Fig. 2E-H).

The wound healing assay indicated that Hela and SiHa cells with lower TUSC3 levels exhibited a significantly more extensive wound closure ability compared with that of the control sample (Fig. 3A and B). The results of the Transwell assay revealed a significant increase in the invasion rate in cells with downregulated TUSC3 levels compared with the negative control group (Fig. 3C and D). Therefore, it was concluded that TUSC3 may play a role in regulating CSCC cell proliferation and invasion *in vitro*.

Downregulation of TUSC3 levels is associated with CSCC clinical features

The present study explored the expression profile of TUSC3 in 220 paraffin-embedded archived CSCC specimens by IHC, including one stage IA, 135 stage IB, 55 stage IIA and 29 stage IIB samples. The median age was 46 years (range, 26-69 years). TUSC3 expression was downregulated in 172 (78.2%) out of 220 patients, while it was normally or overexpressed in 52 patients (52/60, 88.0%) with normal cervical tissues. Statistical analysis indicated significant associations between TUSC3 downregulation and clinicopathological characteristics of patients with CSCC,

including FIGO stage ($P<0.001$), type of tumor growth ($P=0.002$), tumor size ($P=0.002$), differentiation grade ($P<0.001$), stromal invasion ($P=0.024$) and pelvic lymph node metastasis ($P=0.004$). TUSC3 expression was not shown to be associated with age, squamous cell carcinoma antigen (SCC) levels and other clinicopathological characteristics (Table 1).

Association between downregulation of TUSC3 expression and survival of patients with CSCC

A total of 48 of 220 patients were deceased, while 172 survived, according to the last follow-up in 2018. The 5-year PFS and OS rates of the TUSC3 downregulation groups were 72.7% and 75.3%, respectively, whereas the rates in the normal and overexpression groups were 95.7% and 90.2%, respectively. Kaplan-Meier analysis was used to explore the association between TUSC3 downregulation and survival. A positive association was noted between the downregulation of TUSC3 expression and the concomitant OS ($P<0.001$) and PFS ($P=0.001$) of patients with CSCC (Fig. 4A and B).

In addition, survival analysis was performed in FIGO I stage and II stage subgroups. Survival analysis revealed that TUSC3 downregulation was associated with poor OS ($P=0.001$) and PFS ($P=0.012$) in 136 patients with stage I (Fig. 4C and D), but not with OS ($P=0.132$) and PFS ($P=0.118$) in 84 patients with stage II tumors (Fig. 4E and F). Using multivariate Cox analysis, the data indicated that the downregulation of TUSC3 expression could be used as an independent prognostic factor for PFS ($P=0.005$) and OS ($P=0.002$) in patients with CSCC (Tables 2 and 3).

TUSC3 might regulate the AKT signaling pathway in CSCC cells

It was reported in previous studies that the loss of TUSC3 was associated with the activation of the AKT signaling pathway [19,20]. In order to investigate the association between TUSC3 expression and the AKT signaling pathway in CSCC, the phosphorylated levels of AKT were evaluated in HeLa cells transfected with TUSC3 plasmids. The data demonstrated that the overexpression of TUSC3 significantly inhibited AKT phosphorylation, whereas it decreased AKT activity (Fig. 5A). The AKT phosphorylation site in this study is Ser473. Subsequently, the targets involved in the AKT signaling pathway were examined, including BAD, caspase 9 and MMP9. These targets were regulated by TUSC3 in HeLa cells (Fig. 5A). To confirm whether TUSC3 regulates HeLa cell proliferation via the AKT signaling pathway, a specific inhibitor, MK-2206, was used to block this pathway. When HeLa cells were cultured in the absence of the MK-2206 inhibitor, the downregulation of TUSC3 expression enhanced cell proliferation, whereas this effect was reversed using MK-2206 (Fig. 5B).

Discussion

In the present study, the clinical significance of TUSC3 was assessed in patients with CSCC. The present study revealed that the down-regulation of TUSC3 expression increased the proliferation and invasion of CSCC cells, whereas its upregulation exhibited the opposite effects. Furthermore, TUSC3 may modulate the activity of the AKT signaling pathway to promote tumor progression and metastasis. The down-regulation of TUSC3 expression was associated with a high risk of metastasis and unfavorable survival in patients with CSCC. Therefore, TUSC3 may be regarded as a novel prognostic factor for patients with CSCC.

TUSC3 has been characterized as a candidate tumor suppressor gene, which acts as an important signaling hub that modulates a variety of cellular processes [21]. Previous studies have shown that the downregulation of TUSC3 expression is tightly associated with the incidence of several human cancer types, including hepatocellular carcinoma, breast cancer, pancreatic cancer and ovarian cancer [10–13], suggesting a functional association between the downregulation of TUSC3 expression and cancer progression. The present study revealed that the downregulation of TUSC3 expression may enhance aggressive clinical behavior in CSCC and accelerate the metastatic properties of HeLa and SiHa cells, whereas the overexpression of TUSC3 exhibited the opposite effects. The analyses of lung, pancreatic, ovarian and glioblastoma cancer indicated that the risk of proliferation and invasion was higher in subjects with downregulated TUSC3 expression [10, 12, 14, 20–23]. However, the involvement of TUSC3 in CSCC, either epigenetically or genomically, has not been previously reported. Therefore, to the best of our knowledge, the present study was the first to demonstrate the function of TUSC3 in the development of CSCC.

According to a recent study, the downregulation of TUSC3 expression may lead to lymph node or distant metastasis formation in a variety of cancer types, suggesting that the induction of cancer cell migration and invasion by TUSC3 may be the underlying pathogenic mechanism [21]. Certain *in vitro* studies have suggested that the downregulation of TUSC3 may induce the proliferation and migration of ovarian cancer cells, and increase the adhesion to the extracellular matrix [22, 23]. Similar results were reported in prostate cancer [19]. The present study demonstrated that the protein and mRNA levels of TUSC3 were both downregulated in CSCC. It was further shown that the downregulation of TUSC3 expression was significantly associated with unfavorable survival outcomes and disease progression. It is important to note that a significant association between the downregulation of TUSC3 expression and the clinicopathological characteristics of patients with CSCC was observed, including FIGO stage ($P < 0.001$), types of tumor growth ($P = 0.002$), differentiation grade ($P < 0.001$), stromal invasion ($P = 0.024$), pelvic lymph node metastasis ($P = 0.004$) and recurrence ($P = 0.03$), all of which were important factors in predicting the progression and prognosis of CSCC.

It has been shown that the AKT signaling pathway exhibits a significant impact on cancer progression by regulating cell growth [19,20,24], cell apoptosis and mesenchymal transition [10, 11, 25–28]. Silencing of TUSC3-dependent AKT signaling in GBM cells may lead to a high level of proliferation [20] and a similar phenomenon could be observed in prostate cancer [19]. Therefore, the current study investigated whether the downregulation of TUSC3 may modulate the AKT signaling pathway in CSCC cells and the current analysis demonstrated an influence of TUSC3 on AKT phosphorylation and activity. Furthermore, the targets of the AKT signaling pathway, including BAD, caspase 9 and MMP9 were also regulated by TUSC3. Application of MK-2206 (AKT signaling inhibitor) to the cells completely abolished the effects of TUSC3. The results indicated the important functions of the AKT signaling pathway in tumor progression, which were in accordance with previous studies. In the present study, the overexpression of TUSC3 resulted in the downregulation of the AKT signaling pathway, which could partially explain the inhibitory effect on the proliferation and invasion of CSCC cells caused by this protein.

Currently, the main therapies used for patients with CSCC include surgery followed by adjuvant therapy [2–4]. The present study suggested that TUSC3 may act as a potential tumor suppressor gene in CSCC, which revealed a unique Janus-like character in cancer pathogenesis and development. Notably, the role of TUSC3 was examined in specific cancer subgroups based on their FIGO stage and the prognostic potential of the downregulation of its expression was investigated in FIGO I stage subjects. This result demonstrated that TUSC3 may participate in the initial phase of CSCC carcinogenesis. For this reason, the detection of TUSC3 protein in CSCC tissues could assist in evaluating prognosis and providing guidance in the patient follow-up schedule.

Conclusions

The present study demonstrated for the first time that the downregulation of TUSC3 was associated with the enhanced aggressiveness of CSCC cells and poor survival outcomes. This was achieved by using cell functional and survival analysis models. The downregulation of TUSC3 expression promoted CSCC cell metastasis. TUSC3 might regulate the CSCC cells through AKT signaling pathway. This feature may be used as an independent prognostic factor of CSCC patient survival. Therefore, TUSC3 may become a new prognostic biomarker for patients with CSCC.

List Of Abbreviations

TUSC3

Tumor Suppressor Candidate 3

CSCC

Cervical Squamous Cell Carcinoma

IHC

Immunohistochemistry

NCT

Normal Cervical Tissues

T

Tissues

RT

Room Temperature

PFS

Progression Free Survival

OS

Overall Survival

FIGO

International Federation of Gynecology and Obstetrics

Declarations

Ethics approval and consent to participate

Our study was approved both by the Institutional Research Ethics Committee of Nanfang Hospital and Hainan Medical University. All participants have signed written consent prior to the investigation.

Availability of data and materials

The datasets used during the present study are available from the corresponding author upon reasonable request.

Competing interests

The authors declare that they have no competing interests.

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Authors' contributions

M.Y., Z.J. and S.F. initiated and designed the project. M.Y. and S.F. wrote the manuscript. J.Q. and S.F. performed most experiments and analyzed result data. S.F. collected all patients, specimens and data to analyze. W.Y. and M.J. performed IHC and WB. L.P. performed the RT-qPCR. L.Q. gave suggestions about experiments. M.Y. supervises the designed structure of manuscript, reviews and helps revise this manuscript. All authors read and approved the final manuscript.

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Tables

Table 1 Clinicopathological characteristics of patients with CSCC and TUSC3 expression (N=220).

Variable	N	Percentage (%)	TUSC3 expression		p Value
			Downregulation(n=172)	Normal (n=48)	
Age					P=0.254
≥45	122	55.5	99	23	
<45	98	44.5	73	25	
GO stage					P<0.001
Ia2	1	0.5	0	1	
Ib1	76	34.5	42	34	
Ib2	59	26.8	52	7	
IIa1	22	10.0	19	3	
IIa2	33	15.0	32	1	
IIB	29	13.2	27	2	
of tumor growth					P=0.002
Ulcerative endophytic	94	42.7	63	31	
Exophytic	33	15.0	30	3	
Tumor size	93	42.3	79	14	
≥4					P=0.002
<4	110	50.0	96	14	
ICC level	110	50.0	76	34	
≥1.5					P=0.070
<1.5	101	45.9	85	16	
Differentiation grade	119	54.1	87	32	
G1					P<0.001
G2					
G3	9	4.1	1	8	
Distant invasion					
≥1/2	77	35	58	19	
<1/2	134	60.9	113	21	
Perivascular space invasion					P=0.024
Yes					P<0.001
No					
lymph node metastasis	159	72.3	131	28	
Yes					P=0.106
No	61	27.7	41	20	
Received adjuvant therapy					
Yes					P=0.007
No	13	5.9	13	0	
Recurrence	207	94.1	159	48	
Yes					P=0.007
No					
Status at follow-up					
Alive	67	30.5	60	7	
Death from					

153	69.5	112	41	<i>P=0.028</i>
129	58.6	108	21	
91	41.4	64	27	
				<i>P=0.030</i>
49	22.3	44	5	<i>P<0.001</i>
171	77.7	128	43	
172	78.2	126	46	
48	21.8	46	2	

P < 0.05 was considered statistically significant.

Table 2 Univariate analysis of prognostic factors for CSCC patients.

Outcomes	Variable	Exp(B)	P	95%CI
PFS	Age	1.321	0.346	0.741-2.356
	Tumour size	2.050	0.017	1.134-3.705
	SCC levels	2.939	0.001	1.596-5.413
	Tumour differentiation	1.082	0.755	0.659-1.776
	Stromal invasion	2.441	0.029	1.095-5.441
	LVSI	2.565	0.031	1.089-6.042
	Lymph node metastasis	3.328	0.001	1.880-5.894
	FIGO stage(I vs II)	2.204	0.006	1.248-3.891
	Types of tumor growth	1.824	0.002	1.248-2.667
	TUSC3 expression	0.129	0.005	0.031-0.534
Outcomes	Variable	Exp(B)	P	95%CI
OS	Age	1.346	0.315	0.754-2.402
	Tumour size	2.111	0.014	1.167-3.820
	SCC levels	2.837	0.001	1.540-5.227
	Tumour differentiation	1.089	0.736	0.665-1.784
	Stromal invasion	2.369	0.035	1.062-5.285
	LVSI	2.515	0.064	0.949-6.204
	Lymph node metastasis	4.457	0.035	1.068-5.919
	FIGO stage (I vs II)	2.162	0.008	1.224-3.817
	Types of tumor growth	1.853	0.002	1.263-2.719
	TUSC3 expression	0.106	0.002	0.025-0.439

Abbreviations: LVSI=lymphovascular space invasion; CI = confident interval; HR = hazard ratio; OS = overall survival; PFS = progression-free survival; *P* < **0.05** was considered statistically significant.

Table 3 Multivariate analysis of prognostic factors for C5CC patients.

Outcomes	Variable	Exp(B)	P	95%CI
PFS	Tumour size	1.364	0.333	0.728-2.556
	SCC levels	1.949	0.040	1.031-3.681
	Tumour differentiation	0.706	0.261	0.385-1.295
	Stromal invasion	1.965	0.102	0.874-4.415
	Lymph node metastasis	2.464	0.003	1.366-4.446
	FIGO stage	1.379	0.280	0.770-2.471
	Types of tumor growth	1.251	0.329	0.798-1.961
	TUSC3 expression	0.210	0.034	0.050-0.888
Outcomes	Variable	Exp(B)	P	95%CI
OS	Tumour size	1.393	0.307	0.737-2.630
	SCC levels	1.638	0.133	0.861-3.117
	Tumour differentiation	0.677	0.199	0.373-1.228
	Stromal invasion	1.753	0.175	0.779-3.947
	Lymph node metastasis	2.471	0.003	1.362-4.483
	FIGO stage	1.235	0.480	0.688-2.217
	Types of tumor growth	1.339	0.200	0.857-2.093
	TUSC3 expression	0.187	0.023	0.044-0.797

Abbreviations: LVSI=lymphovascular space invasion; CI = confident interval; HR = hazard ratio; OS = overall survival; PFS = progression-free survival; *P* < **0.05** was considered statistically significant.

Figures

Figure 1

Down-regulation of TUSC3 mRNA in CSCC. (A) Expression of TUSC3 mRNA in twelve paired CSCC tissues was examined by qRT-PCR; (B) Relative expression level of TUSC3 compared with paired normal cervical tissues (NCT); (C) Expression of TUSC3 mRNA in five cervical cancer cell lines (SiHa, HeLa, CasKi, C4-1 and HCC94) and normal cervical tissues (NCT) were examined by qRT-PCR. Expression levels were normalized to GAPDH. Error bars represent standard deviation of the mean (SD) calculated from three parallel experiments. *P < 0.05. (D) Immunohistochemical assay of TUSC3 protein expression in four pairs of matched CSCC. (E) Representative images of Western blotting analyses of TUSC3 protein expression in four matched pairs of CSCC tissues (T) and adjacent noncarcinomaous tissues (ANT). GAPDH loading control.

Figure 2

Down-regulation of TUSC3 enhanced Hela and SiHa cells proliferation. (A, B) WB was performed to confirm the over-expression and knockdown of AGK in stable cell lines. GAPDH was used as an internal control. (C, D) Growth curve assays in Hela and SiHa cells with TUSC3 knockdown and over-expression. (E, F) Colony-forming assay (G, H) all indicated that down-regulation of TUSC3 enhanced Hela and SiHa cells growth and over-expression of TUSC3 inhibited Hela and SiHa cells growth. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$ compared to vector control cells.

Figure 3

Down-regulation of TUSC3 enhanced HeLa and SiHa cells invasion. (A, B) Stable TUSC3 expression HeLa and SiHa cells were subjected to scratch wound-healing assay. The wound space was photographed at 0 and 36h. (C, D) The invasion assays showed different cell motilities in stable HeLa and SiHa cells. The depletion of TUSC3 clearly enhanced the invasion of HeLa and SiHa cells. Conversely, the ectopic expression of TUSC3 inhibited the invasion of HeLa and SiHa cells. The data are presented as the mean $\pm$ s.e.m. from three independent experiments. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$ by Student's t-test.

Figure 4

The level of TUSC3 protein expression affects the progression, free survival and overall survival of CSCC patients. Kaplan–Meier curves with univariate analysis (log-rank) for CSCC patients with low TUSC3 expression ($n = 172$) versus high or normal TUSC3 expression ($n = 48$) for OS (A) and PFS (B). OS (C) and PFS (D) rates for cases with low TUSC3 expression versus cases with high or normal TUSC3 expression in patients with FIGO I stage. OS (E) and PFS (F) rates for cases with low TUSC3 expression versus cases with high or normal TUSC3 expression in patients with FIGO II stage.

Figure 5

TUSC3 might regulate the AKT signaling pathway in CSCC. (A) Protein levels of total and phosphorylated Akt (Ser473), BAD, caspase-9 and MMP9 in different groups of Hela cells were assayed by western blotting. GAPDH was used as the loading control. (B) MTT assays were measured in different groups of Hela cells, in the presence or absence of 1 μ M MK-2206. * $p < 0.05$ compared to vector control cells in the absence of MK-2206.