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RESEARCH ARTICLE

CMTM3 inhibits cell growth and migration and predicts favorable survival in oral squamous cell carcinoma

Heyu Zhang[1] · Jianyun Zhang[2] · Xu Nan[3] · Xuefen Li[1] · Jiafei Qu[2] · Yingying Hong[2] · Lisha Sun[1] · Yan Chen[2] · Tiejun Li[2]

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Abstract Downregulation of CKLF-like MARVEL transmembrane domain-containing member 3 (*CMTM3*) has been reported in a number of human tumors. However, the role of *CMTM3* in oral squamous cell carcinoma (OSCC) remains largely unknown. In this study, we showed that the expression of CMTM3 was significantly reduced in OSCC cell lines and primary tumor specimens ($P<0.001$). Methylation-specific PCR showed hypermethylation in CMTM3 promoter in a significant proportion of tumor tissues (61 %). The expression of CMTM3 was associated with T stage, lymph node metastasis, tumor node metastasis (TNM) stage, and recurrence of OSCC patients ($P<0.05$, $n=201$). More importantly, CMTM3 expression was associated with the prognosis of OSCC patients ($P<0.001$) and was an independent prognostic factor (hazard ratio=0.593, 95 % confidence interval, 0.272–1.292; $P=0.039$). Overexpression of CMTM3 inhibited the growth and migration of OSCC cells. In vivo experiments also showed that the growth of OSCC xenografts in nude mice was significantly inhibited by CMTM3 overexpression. These findings indicate that downregulation of CMTM3 due to promoter hypermethylation contributed to the proliferation and migration of OSCC cells and suggest that CMTM3 is an independent prognostic factor for the evaluation of the survival of OSCC patients.

Keywords *CMTM3* · Promoter methylation · Tumor suppressor gene · Oral squamous cell carcinoma

Introduction

Oral squamous cell carcinoma (OSCC) is one of the most common and lethal cancers worldwide due to its high rates of recurrence and metastasis [1]. The molecular mechanisms underlying its clinical characteristics have yet to be fully elucidated. The process of carcinogenesis consists of multiple steps in which several genetic and epigenetic changes are progressively accumulated [2, 3]. Epigenetic changes are inheritable and reversible, leading to changes in the phenotype without changing the sequence of DNA bases [4]. Epigenetic events linked to the tumor suppressor gene (TSG) inactivation through promoter methylation often lead to alterations in gene function and pathway deregulation in human cancer and may drive tumorigenic initiation and progression [5]. Analyses carried out on DNA methylation patterns show that this process can be used as a biomarker for early diagnosis as well as for classification, prognosis, and therapy of human cancers [6]. Previous studies have identified the methylation of the promoter regions of over 40 tumor suppressor genes in oral squamous cell carcinoma and precancerous lesions, such as CDKN2A, MGMT, DAPK1, RUNX3, and $p14^{ARF}$ [7–13].

CKLF-like MARVEL transmembrane domain containing (CMTM) proteins are a novel protein family associated with chemokines and the transmembrane-4 superfamily (TM4SF) and play important roles in the immune system, male reproductive system, and tumorigenesis (14–22). In humans, the CMTM family consists of nine genes, CKLF and CMTM1-

✉ Tiejun Li
litiejun22@vip.sina.com

[1] Central Laboratory, Peking University School and Hospital of Stomatology, Beijing, China

[2] Department of Oral Pathology, Peking University School and Hospital of Stomatology, 22 South Zhongguancun Avenue, Haidian District Beijing 100081, China

[3] Center for Human Disease Genomics, School of Basic Medical Sciences, Peking University, Beijing 100191, China

8 [14]. CKLF-like MARVEL transmembrane domain-containing member 3 (CMTM3) serves as a scaffold for signaling proteins in the endoplasmic reticulum and the nuclear membrane and may activate extracellular signal-regulated kinases (ERK) by transducing signals from cell-surface receptors [15]. It has been reported that CMTM3 inhibited the transcriptional activity of androgen receptors and inhibited the expression of PSA in LNCaP cells [16]. Promoter CpG methylation in CMTM3 gene has been observed in numerous carcinomas such as renal cell carcinoma, gastric cancer, and testicular cancer tissues, suggesting the tumor suppressive role of CMTM3 [17–20]. In addition, it has been reported that restoration of CMTM3 inhibited cell growth and migration and induced apoptosis through activating caspase-3 [17]. However, the expression and function of CMTM3 in OSCC have not been explored.

In the present study, we evaluated the expression and methylation status of CMTM3 in OSCC cell lines and clinical specimens. Our results demonstrated that CMTM3 was downregulated in OSCC and CMTM3 expression was associated with a number of clinicopathological parameters and favorable prognosis. We also found that CMTM3 inhibited the proliferation and migration of OSCC cells in vitro and in vivo.

Materials and methods

Patients and specimens

Two hundred and one OSCC patients who received surgical resection of tumors at the Peking University School and Hospital of Stomatology between April 2008 and March 2014 were enrolled in the present study. No patients received preoperative radiotherapy or chemotherapy. All patients were followed up until November, 2014. The clinicopathological features of the OSCC patients were shown in Table 1. Forty-one matched pairs of fresh OSCC specimens including tumor tissues and normal tissues at least 1.5 cm away from the tumor margins were snap-frozen in liquid nitrogen immediately after resection and stored in −80 °C for follow-up experiments. The tissues were collected with patient's consent and the approval of Institutional Review Board of Peking University School of Stomatology.

Cell lines

The OSCC cell lines (SCC-15, SCC-25, and CAL27) and immortalized human keratinocyte cell line (HaCaT) were obtained from American Type Culture Collection (ATCC, Manassas, VA). The OSCC cell line Tca-83 and WSU-HN6 cells were from the Cell Center of Peking University School and Hospital of Stomatology. The SCC-15 and SCC-25 cells were grown in DMEM/F-12 (Dulbecco's Modified Eagle Medium: Nutrient Mixture F-12) medium containing 400 ng/mL hydrocortisone and 10 % fetal bovine serum (FBS). The CAL27, Tca-83, WSU-HN6, and HaCaT cells were grown in DMEM medium supplemented with 10 % fetal bovine serum. The cells were incubated at 37 °C in a humidified 5 % (*v*/*v*) CO_2 atmosphere and cells at the exponential growth phase were used for assays. Tca-83 and SCC-25 cells were treated with 10 μM of 5-aza-2′-deoxycytidine (Aza) (Sigma) for 3 days, with or without further treatment with 100 nM trichostatin A (TSA) (Sigma) for 16 h.

Table 1 Relationship between CMTM3 expression and clinicopathological features of OSCC patients

Parameters	Number	CMTM3		*P* value
		Negative (%)	Positive (%)	
Gender				
Male	126	84 (66.67)	42 (33.33)	0.185
Female	75	43 (57.33)	32 (42.67)	
Age				
≤60	124	76 (61.29)	48 (38.71)	0.480
>60	77	51 (66.23)	26 (33.77)	
T stage				
T1	42	18 (42.86)	24 (57.14)	<0.001
T2	60	32 (53.33)	28 (46.67)	
T3	46	36 (78.26)	10 (21.74)	
T4	53	41 (77.36)	12 (22.64)	
Lymph node metastasis				
N0	123	66 (53.66)	57 (46.34)	0.002
N1	35	28 (80)	7 (20)	
N2	43	33 (76.74)	10 (23.26)	
Distant metastasis				
M0	182	113 (62.09)	69 (37.91)	0.319
M1	19	14 (73.68)	5 (26.32)	
TNM stage				
I	44	19 (43.18)	25 (56.82)	<0.001
II	60	32 (53.33)	28 (46.67)	
III	46	36 (78.26)	10 (21.74)	
IV	51	40 (78.43)	11 (21.57)	
Pathologic grade				
Well	109	62 (56.88)	47 (43.12)	0.119
Moderately	71	51 (71.83)	20 (28.17)	
Poorly	21	14 (66.67)	7 (33.33)	
Recurrence				
No	173	104 (60.12)	69 (39.88)	0.025
Yes	28	23 (82.14)	5 (17.86)	

Adenovirus construction and cell transfection

Construction, generation, and purification of ad5-null (vector containing adenovirus, defined as Mock) and ad5-CMTM3 were conducted according to the previous study [16]. Cells were transfected at a multiplicity of infection (MOI) of 60.

Reverse transcriptase PCR and real-time RT-PCR

Reverse transcriptase (RT)-PCR and real-time RT-PCR were carried out as previously described [17].

Methylation-specific PCR and bisulfate genomic sequencing

Methylation-specific PCR (MSP) and bisulfate genomic sequencing (BGS) analysis were conducted as described previously [17]. Amplified BGS PCR products were cloned into the pGEM-T Easy vector (Promega), and six random clones from each sample were sequenced.

Western blot analysis

Western blot was conducted as previously described [17] using anti-phospho-Akt (Ser473) antibody (#4060, Cell Signaling Technology), anti-Akt antibody (#4691, Cell Signaling Technology), anti-phospho-p44/42 mitogen-activated protein kinase (MAPK) (Erk1/2) antibody (#4370, Cell Signaling Technology), anti-p44/42 MAPK (Erk1/2) antibody (#4695, Cell Signaling Technology), and anti-CMTM3 antibody which was prepared and purified in our laboratory [16]. The immunoreactive bands were quantified using the LAS-3000 system (Fuji Film, Japan).

Immunohistochemistry analysis

Paraffin tissue sections were dewaxed, rehydrated and placed in 10 mmol/L citrate buffer (pH 6.0), and heated twice in a microwave oven for 5 min each. Sections were incubated with 3 % H_2O_2 for 10 min, washed with PBS, blocked with 10 % normal goat serum for 30 min, and then incubated with 4 mg/L purified anti-CMTM3 antibody or normal rabbit IgG (#2729, Cell Signaling Technology) as a control at 4 °C overnight [16]. After washing, the sections were stained with the catalyzed signed amplification system kit (DAKO code k5007) and visualized with a Nikon E800 with photos taken. The staining results were scored by two pathologists blinded to the clinical data.

Cell proliferation assay

The cells were seeded in 96-well plates and transfected with ad5-null or ad5-CMTM3. Cell proliferation was measured using the CCK-8 kit (Cell Counting Kit-8, Dojindo, Kumamoto, Japan) according to the manufacturer's instructions. Briefly, 10 μL of CCK-8 solution was added to each well, and the samples were incubated for the indicated times (2–4 h) and the absorbance was subsequently measured at 450 nm.

Colony formation assay

The cells were transfected with Ad5-CMTM3 or Ad5-null (Mock) and 500 cells were plated into each well of a six-well plate 24 h after transfection. The cells were cultured in complete media for 2 weeks and surviving colonies (50 cells per colony) were fixed with methanol, stained with crystal violet, counted, and photographed. Each experiment was conducted in triplicate.

Wound healing assay

The transfected CAL27 cells were cultured in a six-well plate until confluent. The cell layer was carefully wounded using a 200-μL pipette tip, washed twice with PBS, and incubated in medium containing 2 % FBS. After incubation for 36 h, the cells were photographed at low magnification. To analyze the wound healing capability of the cells, three pictures per well were taken. The gap size of the wound was measured with Image-Pro Plus 6.0 software, and the percentage of coverage of the wound was evaluated. Complete coverage was defined as 100 %.

Cell migration assay

The transfected Tca-83 cells were seeded into the upper chamber of a Transwell (8-μm pore size, Corning Life Sciences). The bottom chamber contained medium supplemented with 10 % FBS. After 16-h incubation, cells adherent to the upper surface of the filter were removed using a cotton swab and those attached to the bottom of the membranes were stained with crystal violet following fixation with methanol. Cell number was counted in five randomly chosen fields.

In vivo tumor formation assay

All animal experiments were performed with the approval and under the supervision of Peking University Biomedical Ethic Committee. CAL27 cells were transfected with ad5-null (mock) or ad5-CMTM3. After 36 h of transfection, 2×10^6 cells suspended in 100 μL of sterile phosphate-buffered saline (PBS) were subcutaneously injected into the right flank of 5-week-old female BALB/c nu/nu nude mice. Tumor growth was monitored every 4 days using a linear caliper, and the tumor volume was calculated using the formula $V=(a\ b^2)/2$, where a is the larger dimension and b is the perpendicular diameter. The mice were sacrificed after the xenografts were seeded for 31 days.

Statistical analyses

All statistical analyses were performed using SPSS software version 17.0 (SPSS Inc., Chicago, IL, USA). Student's t test

was used to analyze the results expressed as the means±SD. Comparisons of CMTM3 expression in mRNA level between paired tumor and adjacent non-tumor tissue samples were conducted using the Wilcoxon signed-rank test. Chi-square χ^2 analysis was carried out to identify the association between CMTM3 expression and clinicopathological features of OSCC patients. Overall survival was plotted and calculated using the Kaplan-Meier method, and differences between groups were analyzed using the log-rank test. The Cox proportional hazard model was used for univariate and multivariate analysis. All tests were two-sided, and a *P* value less than 0.05 was considered statistically significant.

Results

Downregulation and promoter methylation of CMTM3 in OSCC cell lines

Previous studies have shown that CMTM3 was silenced or downregulated in many cancer cell lines due to the methylation of its promoter region. Herein, quantitative real-time RT-PCR assay and Western blot showed that CMTM3 was silenced or downregulated in SCC-25 and Tca-83 cells (Fig. 1a, b). To analyze the methylation status of CMTM3, methylation-specific PCR was carried out in these cells. We found that the promoter of CMTM3 gene was methylated in SCC-25 and Tca-83 cells (Fig. 1c). To show that CpG methylation is functionally associated with CMTM3 silence, we treated Tca-83 and SCC-25 cells with demethylating agent Aza (A) alone or combined with histone deacetylase inhibitor trichostatin A (T). The demethylation reagents restored CMTM3 expression through demethylating its promoter region confirmed by MSP and BGS analysis (Fig. 1d–f). These results suggest that CMTM3 expression was significantly reduced in OSCC cell lines due to methylation of its promoter region.

Downregulation and promoter methylation of CMTM3 in OSCC tissues

We next analyzed the expression of CMTM3 in 41 matched pairs of OSCC tumor tissues and adjacent non-tumor tissues. Both the mRNA and protein levels of CMTM3 were dramatically downregulated in tumor tissues compared with those in corresponding non-tumor tissues (Fig. 2a, b). Specifically, 31 of 41 tumor specimens showed more than twofold reduced expression of CMTM3 in comparison with paired non-tumor tissues. Among them, 13 showed more than tenfold reduced expression. We next investigated whether promoter methylation also contributed to the attenuated or lost expression of CMTM3 in primary OSCC

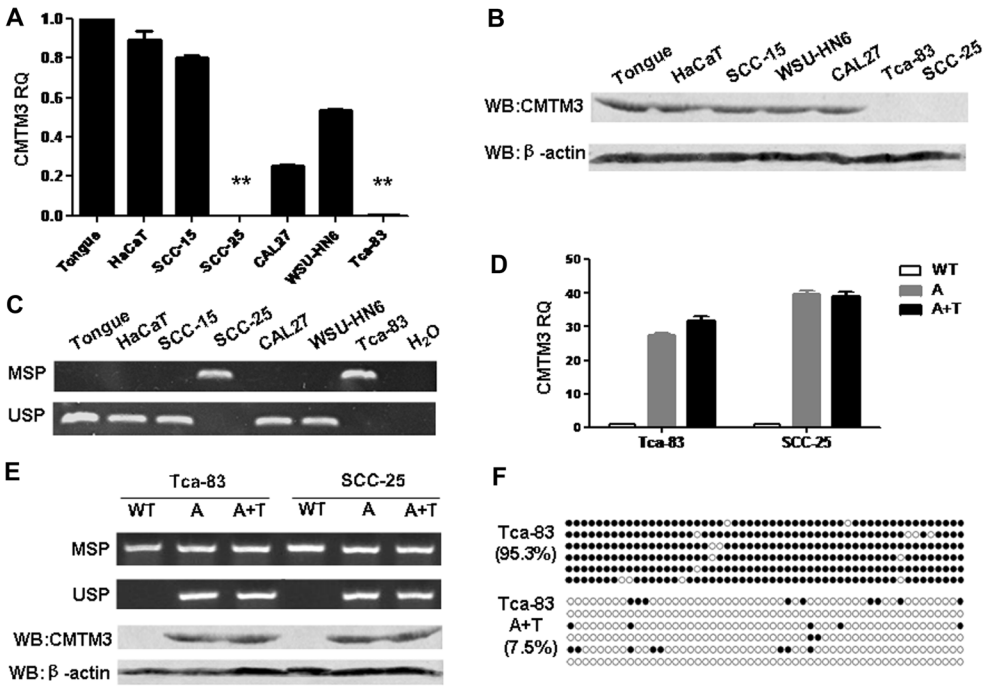


Fig. 1 Expression and methylation status of CMTM3 in OSCC cell lines. **a** The expression of CMTM3 in OSCC cell lines was analyzed using real-time RT-PCR. *RQ* relative quantity. **b** The expression of CMTM3 in OSCC cell lines was analyzed using Western blot. **c** The methylation status of CMTM3 in OSCC cell lines was analyzed using methylation-specific PCR (MSP) and unmethylation-specific PCR (USP). **d** Demethylation of CMTM3 using 5-aza-2′-deoxycytidine alone (*A*) or in combination with trichostatin A (A+T) restored CMTM3 mRNA expression in Tca-83 and SCC-25 cells. **e** The methylation status and protein expression of CMTM3 were analyzed. **f** Detailed BGS analysis of CMTM3 was demonstrated. *WT* untreated cells. Circles, CpG sites analyzed; *row of circles*, an individual promoter allele that was cloned, randomly selected, and sequenced; *filled circle*, methylated CpG site; *open circle*, unmethylated CpG site. **$P<0.001$

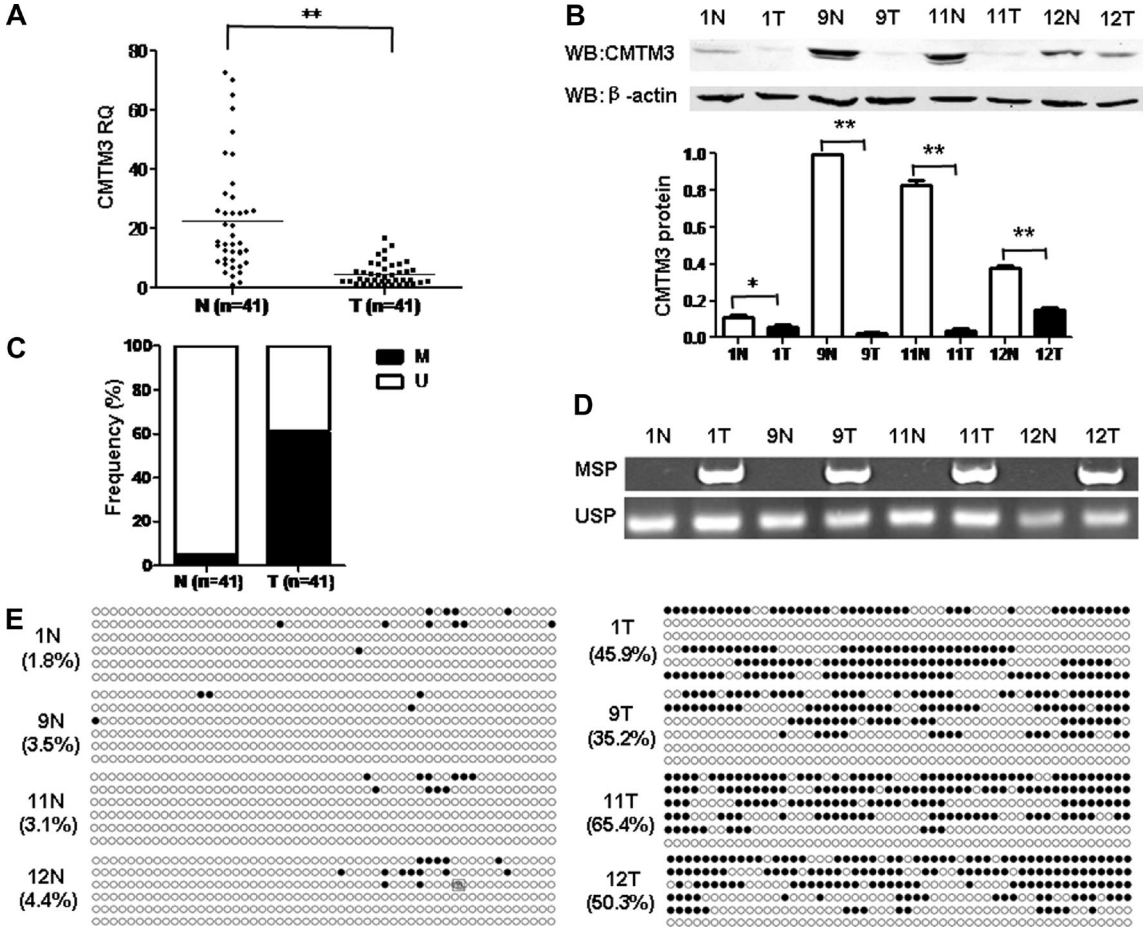


Fig. 2 Expression and methylation status of CMTM3 in primary OSCC. **a** Comparison of the relative expression levels of CMTM3 in 41 paired OSCC tumor specimens and adjacent non-tumor tissues as measured by real-time RT-PCR. **b** The protein expression of CMTM3 in four representative pairs of OSCC tumor and adjacent non-tumor tissues was analyzed using western blot. **c** Percentage of DNA methylation in CMTM3 promoter on the basis of results from MSP in 41 paired OSCC tumor and adjacent non-tumor tissues. **d** The methylation status of CMTM3 in four representative pairs of OSCC tumor and adjacent non-tumor tissues was analyzed by MSP and USP. **e** Detailed BGS analysis of CMTM3 in four representative pairs of OSCC tumor and adjacent non-tumor tissues was demonstrated. *N* adjacent non-tumor tissues, *T* OSCC tumor specimens. *$P<0.05$, **$P<0.001$

tissues. Among 41 paired samples, 25 (61 %) tumor specimens showed methylation of the CMTM3 promoter in contrast to that 2 (4.9 %) non-tumor samples showed weak methylation by MSP analysis ($P<0.001$) (Fig. 2c, d). Furthermore, bisulfite genomic sequencing (BGS) confirmed that the CpG island within the CMTM3 promoter was markedly methylated in OSCC tumors, but not in paired non-tumor tissues (Fig. 2e). These results indicate that promoter hypermethylation may be the main contributor to the inactivation of CMTM3 in OSCC.

Reduced expression of CMTM3 in OSCC tissues and its association with OSCC progression and prognosis

To explore the expression pattern of CMTM3 as well as its association with clinicopathological features in OSCC patients, the expression of CMTM3 in 201 OSCC specimens and 54 adjacent normal specimens was analyzed using immunohistochemistry. Strong CMTM3 staining was shown in non-tumor tissues, mainly in the epithelium (Fig. 3a). Positive expression of CMTM3 in OSCC tumor specimens (74/201, 36.8 %) was significantly lower than that in adjacent normal tissues (50/54. 92.6 %) ($P<0.001$) (Fig. 3b).

The relation of CMTM3 immunohistochemical expression and clinicopathological parameters is depicted in Table 1. Statistical analyses showed that CMTM3 expression was significantly associated with T stage ($P<0.001$), lymph node metastasis ($P=0.002$), tumor node metastasis (TNM) stage ($P<0.001$), and recurrence ($P=0.025$). No significant correlation was detected between CMTM3 expression and other clinicopathological parameters including age, gender, distant metastasis, and pathologic grade ($P>0.05$). These results indicate that downregulation of CMTM3 was associated with the progression of OSCC.

The prognostic effect of CMTM3 on the overall survival of OSCC patients was analyzed through comparing the overall survival between patients with negative and positive CMTM3 expression using the Kaplan-Meier curve assessment. In the

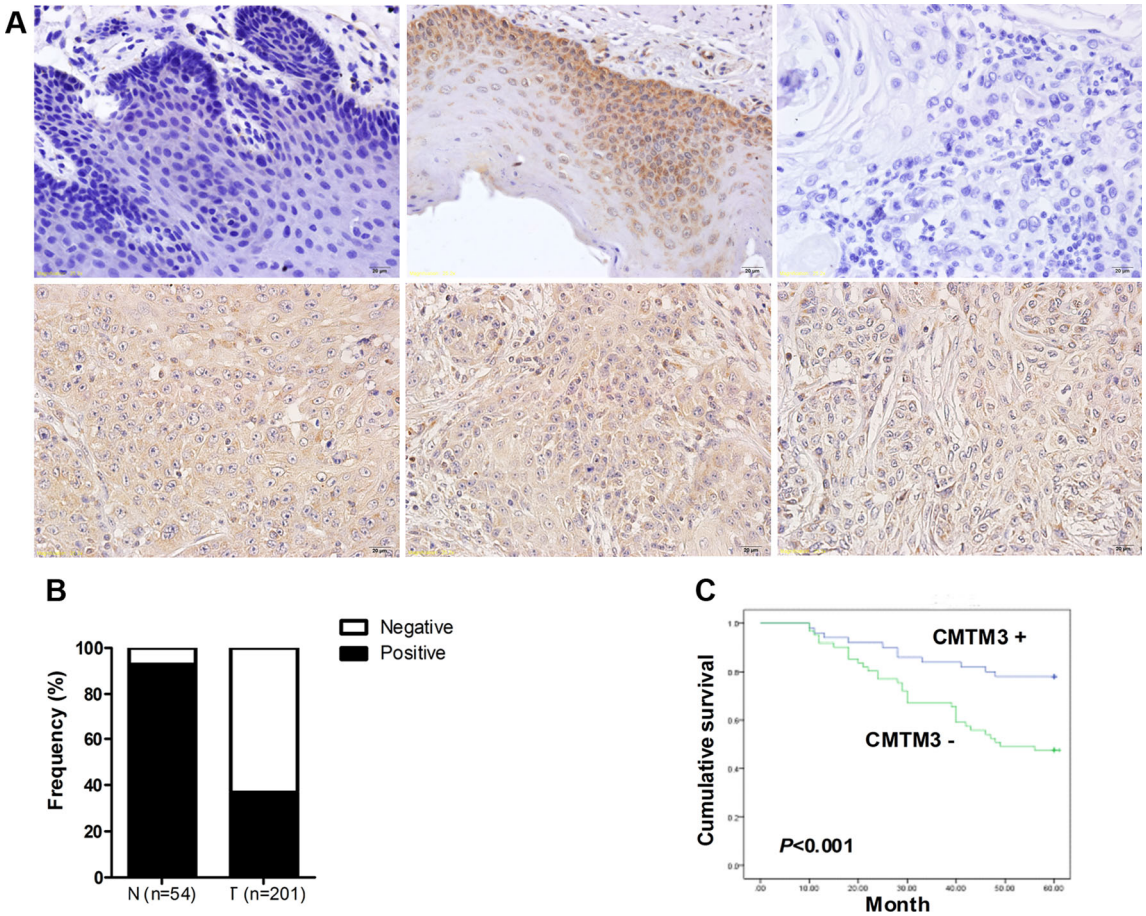


Fig. 3 Immunohistochemical staining of CMTM3 in OSCC. **a** Representative results of immunohistochemical staining for CMTM3 protein expression in OSCC specimens and adjacent non-tumor tissues (magnification, ×400). *Upper left*, negative staining of CMTM3 in adjacent non-tumor tissues. *Upper middle*, positive staining of CMTM3 in adjacent non-tumor tissues. *Upper right*, negative staining of CMTM3 in OSCC tumor tissues. *Lower left*, positive staining of CMTM3 in well-differentiated OSCC tumor specimens. *Lower-middle*, positive staining of CMTM3 in moderately differentiated OSCC tumor specimens. *Lower right*, positive staining of CMTM3 in poorly differentiated OSCC tumor specimens. **b** Comparison of the protein levels of CMTM3 in OSCC tumor tissues and adjacent non-tumor tissues as measured by IHC. *N* adjacent non-tumor tissues, *T* OSCC tumor specimens. **c** Kaplan-Meier survival curve showed the association between CMTM3 expression and patients' survival. CMTM3 +, CMTM3 positive; CMTM3 −, CMTM3 negative

111 OSCC cases with prognosis information, we observed that the overall survival in patients with positive CMTM3 expression was significantly higher than that in patients with negative expression of CMTM3 ($P<0.001$, Fig. 3c). The multivariate Cox proportional hazards model revealed that TNM stage ($P=0.029$), T stage ($P=0.035$), distant metastasis ($P<0.001$), recurrence ($P=0.002$), and CMTM3 ($P=0.039$) had independent prognostic values for OSCC patients (Table 2). Taken together, these data suggest that CMTM3 downregulation might represent a useful independent biomarker for the prognosis of patients with OSCC.

CMTM3 inhibited the growth and migration of OSCC cells both in vitro and in vivo

To understand the role of CMTM3 in the progression of OSCC, we investigated the effects of CMTM3 overexpression on the proliferation and migration of OSCC cell lines. CAL27 and Tca-83 cell lines were transfected with adenovirus. We first evaluated the effects of CMTM3 overexpression on cell proliferation using CCK-8 assay and colony formation assay. As shown in Fig. 4a, the growth of ad5-CMTM3-transfected CAL27 and Tca-83 cells was significantly inhibited compared with ad5-null-transfected cells. CMTM3 expression also significantly suppressed colony formation in CAL27 and Tca-83 cells compared with the control cells transfected with ad5-null ($P<0.05$) (Fig. 4b).

The effects of CMTM3 on the motility of OSCC cells were examined based on a wound healing assay and Transwell system. We found that the control group (CAL27 cells transfected with ad5-null) healed the wounded area in 36 h after the scratch, whereas the CMTM3 group was unable to heal in the same time period. The relative migration rates were statistically significant ($P<0.001$, Fig. 4c). The Tca-83 cells transfected with ad5-CMTM3 also exhibited significantly lower migration ability than the cells transfected with ad5-

Table 2 Univariate and multivariate Cox analysis of factors associated with survival in OSCC patients

Parameters	Univariate analysis		Multivariate analysis	
	HR (95 % CI)	*P* value	HR (95 % CI)	*P* value
Gender				
Male vs. female	1.426 (0.732–2.777)	0.297	1.051 (0.521–2.119)	0.890
Age				
≤60 vs. >60	0.685 (0.376–1.247)	0.216	0.556 (0.286–1.081)	0.084
TNM stage				
I–II vs. III–IV	0.146 (0.075–0.281)	<0.001	0.294 (0.098–0.884)	0.029
T stage				
T1–2 vs. T3–4	0.164 (0.087–0.308)	<0.001	0.343 (0.127–0.926)	0.035
Lymph mode metastasis				
Positive vs. negative	0.250 (0.137–0.456)	<0.001	1.220 (0.467–3.191)	0.685
Distant metastasis				
Positive vs. negative	0.094 (0.049–0.178)	<0.001	0.092 (0.041–0.206)	<0.001
Recurrence				
Yes vs. no	0.164 (0.086–0.314)	<0.001	0.316 (0.153–0.654)	0.002
Pathologic grade				
Well vs. moderately–poorly	1.898 (1.043–3.453)	0.036	1.296 (0.636–2.641)	0.475
CMTM3				
Positive vs. negative	0.349 (0.176–0.694)	0.003	0.593 (0.272–1.292)	0.039

HR hazard ratio, *CI* confidence interval

null, evidenced by a significantly reduced number of migrated cells expressing CMTM3 compared with the controls ($P<0.001$, Fig. 4d). These findings suggest that CMTM3 inhibited the migration of OSCC cells in vitro.

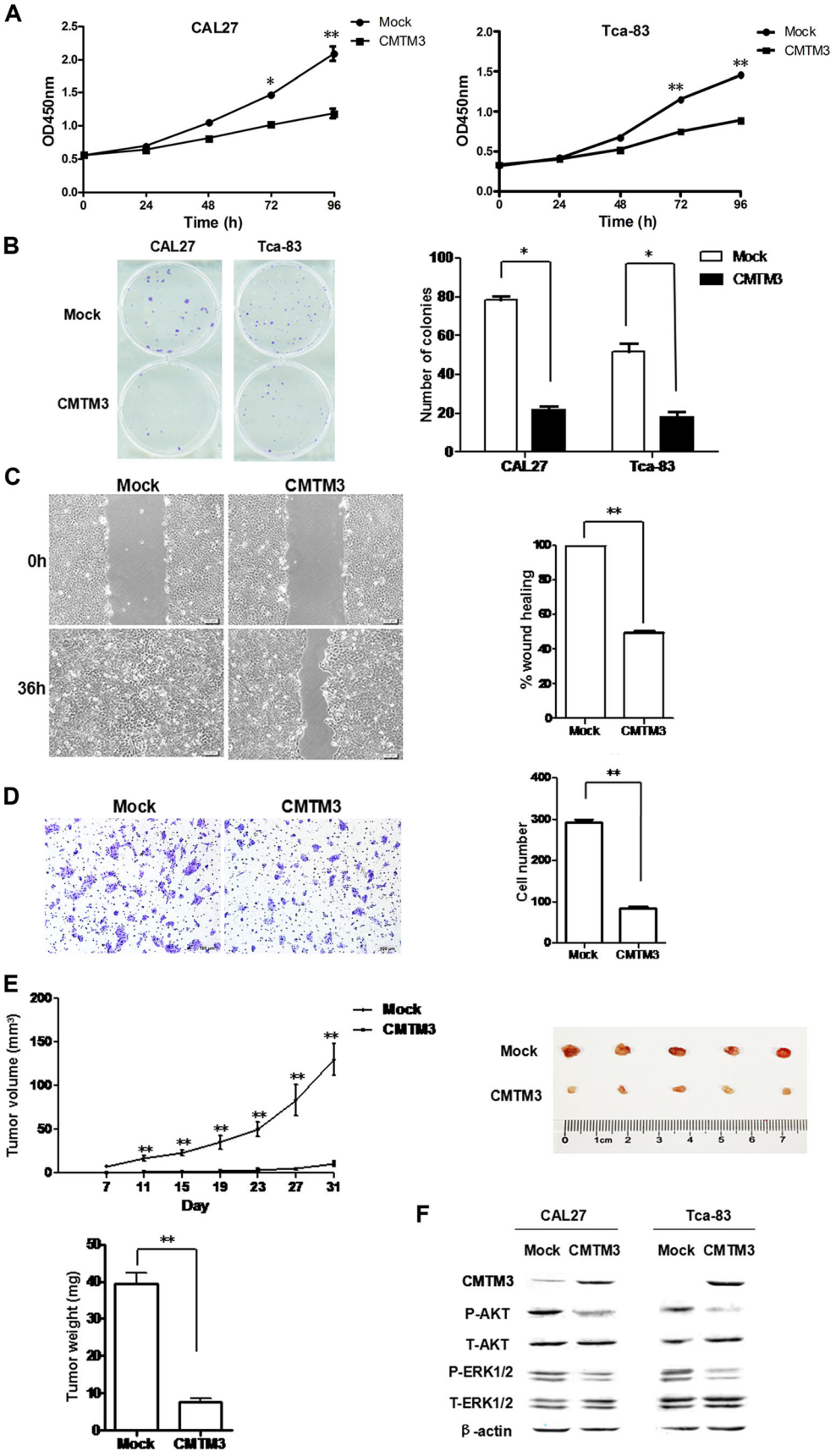
CAL27 cells are highly tumorigenic when subcutaneously injected into nude mice. To examine whether CMTM3 inhibits the tumor formation and growth in vivo, CAL27 cells transfected with ad5-null (mock) or ad5-CMTM3 were subcutaneously injected into the right flank of nu/nu mice. At the 31st day after inoculation, the mean tumor volume and weight in the control group were 129.77±41.38 mm^3 and 39.46±6.45 mg, respectively (Fig. 4e). While the mean tumor volume and weight in the ad5-CMTM3-infected group were 10.82±7.09 mm^3 and 8.28± 2.83 mg, respectively, which were significantly lower than those in the control group ($P<0.001$). The results showed that CMTM3 significantly inhibited tumor growth in vivo.

The MAPKs and PI3K/Akt pathways are two major signaling pathways responsible for regulating cell growth and proliferation, and the existence of a constitutive increase of the Raf/MEK/ERK and/or PI3K/Akt pathway activities in tumor has been reported [21, 22]. We subsequently examined the effect of CMTM3 on ERK and protein kinase B (Akt) activation (Fig. 4f). After CMTM3 expression in CAL27 and Tca-83 cells, phosphorylated ERK and phosphorylated Akt were downregulated. These results suggest that CMTM3 may inhibit cell growth and migration through the inhibition of phosphorylation of ERK and Akt.

Discussion

The CMTM3 gene is located at 16q22.1, which harbors a number of tumor suppressor genes involved in human cancers such as breast cancer [23], prostate cancer [24], renal cancer [24], hepatocellular carcinoma [25], and acute myelomonocytic leukemia [26]. CMTM3 has also been reported as a candidate of tumor suppressor gene in several tumors [18–20]. Herein, we provided evidence showing the tumor suppressor effects of CMTM3 in OSCC.

In the present study, we found that CMTM3 was downregulated in OSCC specimens and cell lines. Real-time PCR results showed that the mRNA level of CMTM3 was significantly lower in 31 of 41 OSCC specimens compared with the corresponding non-tumor tissues. In addition, the intensity of CMTM3 staining in primary tumor tissues was remarkably weaker than that in non-tumor tissues based on immunochemistry staining. A number of mechanisms underlie the inactivation of tumor suppressor genes. For example, methylation of the promoter regions, histone deacetylation, mutation of allele, and loss of heterozygosity of remaining allele commonly cause downregulation or inactivation of tumor suppressor genes [9]. The CMTM3 promoter contains a typical CpG island consisting of 53 CpG sites which can be methylated by the addition of a methyl group through the action of the DNA methyltransferase enzymes (DNMTs). Promoter methylation of CMTM3 was closely associated with the


A
CAL27
Tca-83
Mock
CMTM3
OD450nm
Time (h)
B
CAL27
Tca-83
Mock
CMTM3
Number of colonies
C
Mock
CMTM3
0h
36h
% wound healing
D
Mock
CMTM3
Cell number
E
Tumor volume (mm³)
Day
Tumor weight (mg)
F
CAL27
Tca-83
Mock CMTM3 Mock CMTM3
CMTM3
P-AKT
T-AKT
P-ERK1/2
T-ERK1/2
β -actin

◀ **Fig. 4** CMTM3 overexpression inhibited the growth and migration of OSCC cells. **a** Representative CCK-8 assay showed that CMTM3 inhibited the growth of CAL27 and Tca-83 cells. **b** Overexpression of CMTM3 inhibited the colony formation of CAL27 and Tca-83 cells. **c** The effects of CMTM3 on the migration of CAL27 cells were evaluated by wound healing assay. The images were captured at 0 and 36 h after the scratch (magnification, ×100). **d** The effects of CMTM3 on the migration of Tca-83 cells were analyzed using Transwell assay (magnification, ×100). **e** CMTM3 suppressed the growth of OSCC xenografts in nude mice. The average volumes and weights of tumors established by CMTM3-overexpressing CAL27 cells versus mock cells were expressed as the means±SD in five inoculated sites. **f** The protein expression of phosphorylated and total Akt and ERK in CAL27 and Tca-83 cells were detected by Western blot. *$P<0.05$, **$P<0.001$

downregulation of CMTM3 [17, 20]. Previous studies also showed that a single CpG site located at the junction of the two Sp1/Sp3 binding sites had moderate methylation, and the methylation values were significantly inversely correlated with the expression of CMTM3 in testicular cancer [19]. In the present study, we detected the methylation of CMTM3 promoter region in OSCC cell lines. High levels of promoter methylation were observed in about 61 % of primary OSCC tissues. In addition, DNA methyltransferase inhibitor restored CMTM3 expression in OSCC cell lines. These findings suggest that DNA methylation represent an important mechanism for the downregulation of CMTM3 in OSCC. However, among 31 cases of CMTM3-downregulation samples, 6 cases did not have methylation. Other studies have reported that methylation of the promoter region of CMTM3 was not detected in renal cell carcinoma and suggested that downregulation of CMTM3 in this tumor might be associated with other epigenetic or genetic mechanisms [18]. More comprehensive studies should be conducted in the future to determine whether other mechanisms are involved in the regulation of CMTM3 expression in OSCC. In addition, DNA methylation has been proposed as a diagnostic molecular marker for OSCC and intensively studied in the past [8, 27]. In this study, our results suggest that the methylation of CMTM3 may be a specific biomarker in OSCC and has potentials for molecular diagnosis.

Several important molecular markers have been identified to reflect the biological diversity of OSCC and predict clinical outcome of OSCC patients [28–30]. Apart from T stage, CMTM3 expression was also significantly associated with gender, lymph node metastasis, TNM stage, and recurrence of OSCC patients. In addition, OSCC patients with negative CMTM3 expression had significantly lower disease-free survival rates than OSCC patients with positive CMTM3 expression. The result was confirmed by univariate and multivariate analysis using the Cox model, suggesting that reduced expression of CMTM3 is a significant predictor of poor prognosis of OSCC patients regardless of their disease status. The association of reduced expression of CMTM3 and poor prognosis is consistent with previous reports in gastric cancer [20]. These findings not only highlight the utility of CMTM3, but also expand the prognostic system for OSCC.

Based on the observation that CMTM3 was downregulated in OSCC specimens and cell lines, we speculated that CMTM3 may contribute to malignant transformation of oral squamous cells. To check this hypothesis, in vitro and in vivo studies were conducted to evaluate the effects of CMTM3 on the proliferation and migration of oral squamous cells. As expected, overexpression of CMTM3 significantly inhibited tumor cell growth and migration in vitro or in vivo. CMTM3 expression was significantly associated with T stage, which meant more positive CMTM3 expression in smaller tumor size, supporting the cellular experiment results. However, expression of CMTM3 in OSCC patients did not correlate with distant metastasis, perhaps because of the limitation of the M1 stage sample size (18 patients). These results suggest that CMTM3 acted as a tumor suppressor gene in the development of OSCC and inaction or downregulation of CMTM3 may promote tumor growth and migration.

Previous studies have showed that CKLF-like MARVEL transmembrane domain containing (CMTM) proteins were involved in several signal pathways. CMTM7 could promote epidermal growth factor receptor (EGFR) internalization, and further suppress Akt signaling pathway [31]. CMTM8 functions as a negative regulator of HGF/c-MET signaling to ERK [13]. CMTM3 might regulate MMP2 expression through the ERK1⁄2 signaling pathway [20]. In this study, we found that CMTM3 may suppress cell proliferation and migration through the inhibition of phosphorylation of ERK1⁄2 and Akt, which extends the current understanding of the roles of MARVEL proteins.

In summary, the present study identified the expression and function CMTM3 in OSCC for the first time. These results suggest that methylation of CMTM3 can be used as a relatively sensitive marker for OSCC diagnosis and CMTM3 may serve as a prognostic factor for OSCC patients.

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Conflicts of interest None

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