



CMTM5 exhibits tumor suppressor activity through promoter methylation in oral squamous cell carcinoma



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ABSTRACT

Oral squamous cell carcinoma (OSCC) is one of the most common types of malignancies in the head and neck region. CKLF-like MARVEL transmembrane domain-containing member 5 (*CMTM5*) has been recently implicated as a tumor suppressor gene in several cancer types. Herein, we examined the expression and function of *CMTM5* in oral squamous cell carcinoma. *CMTM5* was down-regulated in oral squamous cell lines and tumor samples from patients with promoter methylation. Treatment with the demethylating agent 5-aza-2'-deoxycytidine restored *CMTM5* expression. In the OSCC cell lines CAL27 and GNM, the ectopic expression of *CMTM5*-v1 strongly inhibited cell proliferation and migration and induced apoptosis. In addition, *CMTM5*-v1 inhibited tumor formation *in vivo*. Therefore, *CMTM5* might act as a putative tumor suppressor gene through promoter methylation in oral squamous cell carcinoma.

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1. Introduction

Oral squamous cell carcinoma (OSCC) is a common, highly lethal cancer worldwide that is due to its recurrence and metastasis [1]. Although continuous improvements in the treatment of OSCC have been observed, the survival of patients has not markedly increased. To improve the survival rate of oral cancer patients, it is important to understand the molecular mechanisms underlying oral tumorigenesis. Increasing evidence suggests that inactivation of tumor suppressor genes (TSG) through epigenetic silencing plays a major role in human carcinogenesis [2]. DNA methylation primarily induces gene silencing in carcinogenesis, and epigenetic changes are suggested to occur during the early stages of tumor formation [3,4].

Recent studies have identified the putative tumor suppressor gene *CMTM5* (CKLF-like MARVEL transmembrane domain-containing member 5). CKLF-like MARVEL transmembrane domain containing (CMTM) proteins are a novel family of proteins linking chemokines and the transmembrane-4 superfamily (TM4SF). In humans, the CMTM family comprises nine genes, *CKLF* and *CMTM1–8* [5,6]. *CMTM5* has at least six alternatively spliced forms,

CMTM5-v1-v6, and *CMTM5-v1* is the main form. *CMTM5-v1* is secreted through a vesicle-mediated secretory pathway [7]. Previous studies have demonstrated that *CMTM5* exerts tumor suppressor functions in many tumors through promoter methylation. *CMTM5* is downregulated in ovarian cancer [8], pancreatic cancer [9] and myeloid leukemia [10]. The restoration of *CMTM5-v1* strongly suppresses tumor cell growth and migration [10,11]. *CMTM5-v1* also induces apoptosis in cancer cell lines [9,12]. These results suggest an important role for *CMTM5* in carcinogenesis. However, the expression and function of *CMTM5* has not been explored in oral squamous cell carcinoma.

In the present study, we detected the expression and methylation status of *CMTM5* in OSCC cell lines and clinical samples, and provided the first evidence that *CMTM5* was downregulated in oral squamous cell lines and tissues through promoter methylation. The restoration of *CMTM5-v1* inhibited the growth and migration of oral squamous cells and induced cell apoptosis *in vitro*. Furthermore, *CMTM5-v1* inhibited tumor formation *in vivo*.

2. Materials and methods

2.1. Cell lines and tissue samples

The oral squamous cell lines (SCC-15, CAL27, Tca-83, WSU-HN6 and GNM cells) were obtained from the American Type Culture

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Collection and the Peking University School and Hospital of Stomatology. All cells were grown in Dulbecco's modified Eagle's medium (DMEM, GibcoBRL, Life Technologies, Grand Island, NY, USA), supplemented with 4 mM L-glutamine and 10% fetal bovine serum (FBS). The cells were incubated at 37 °C in a humidified 5% (v/v) CO₂ atmosphere and used for assays at the exponential growth phase. Some cell lines 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. The cells were transfected using Lipofectamine 2000 (Invitrogen) according to the manufacturer's instructions.

Fifty-six OSCC patients diagnosed and surgically treated at the Peking University School of Stomatology from 2011 to 2013 were enrolled in this study. The paired OSCC tumors and adjacent non-tumor tissues were collected with patient's consent and the approval of Institutional Review Board of Peking University School of Stomatology. None of the subjects received radiation therapy or chemotherapy before surgery. The mean age of the patients, including 27 men and 29 women, was 61 ± 10.0 years.

2.2. RNA isolation, RT-PCR and real-time quantitative RT-PCR

RNA was extracted using TRIzol reagent (Invitrogen) according to the manufacturer's instructions. RT-PCR and real-time quantitative RT-PCR analyses were performed as previously described [13]. CMTM5 was amplified using the following primers: forward primer: 5'-ATC TGC TTC ACG GCC TCC-3'; and reverse primer: 5'-GTG CCA TCT CAG TCC GGT AG-3'. The annealing temperature was 60 °C for 35 cycles.

2.3. DNA bisulfite treatment and promoter methylation analysis

Genomic DNA was isolated from cell lines and tissues using a DNA extraction kit and chemically modified using a EZ DNA Methylation-Gold kit (Zymo Research, Orange, CA, USA) according to the manufacturer's instructions [11]. The bisulfite-treated DNA was amplified using the methylation-specific primer set CMTM5m1: 5'-CGT GGT GTT TGA ATA TTT CGC and CMTM5m2: 5'-TCC AAC ATA CYR AA AAC GCG or the unmethylation-specific primer set CMTM5u1: 5'-ATT TGT GGT GTT TGA ATA TTT TGT and CMTM5u2: 5'-TCT CCA ACA TAC YAA AAA CACA. For bisulfite genomic sequencing (BGS), the bisulfite-treated DNA was amplified using the primers BGS1: 5'-AAT GGT TTA ATT TGT ATT TGG TATT and BGS2: 5'-CTC TAA CCT TAA CCC TCT TAT TTAA, and the PCR products were cloned into the pGEM-T Easy vector (Promega, Madison, WI, USA). A total of 3–5 clones were randomly selected and sequenced.

2.4. Protein extraction and Western blot analysis

The cells were harvested and lysed, and the proteins (25 μg) were subjected to Western blot analysis as previously described [12].

2.5. Immunohistochemistry analysis

Human tissue slides were deparaffinized and rehydrated. Antigen retrieval was performed after heating a solution in 0.01 M citrate buffer (pH 6.0) twice for 5 min. The tissue slides were incubated with 3% (v/v) H₂O₂ at room temperature for 10 min, rinsed twice and blocked in 10% normal goat serum for 30 min. The slides were subsequently incubated at 37 °C for 1 h with anti-CMTM5 antibody (10 mg/l). After thorough washing, Envision™ System HRP (DakoCytomation) was applied for 30 min. After rinsing in PBS, all slides were visualized using 0.05% (w/v) 3,3'-diaminobenzidine and subsequently

counter-stained with hematoxylin. Rabbit IgG was used as a negative control.

2.6. Cell proliferation assays

Cell proliferation was evaluated using a CCK-8 assay and cell counting. The cells were transfected with pCDB or pCDB-CMTM5-v1 plasmids. After 12 h, the transfected cells (1000 cells/well) were seeded in 96-well plates and tested using the CCK-8 (Cell Counting kit-8) kit according to the manufacturer's instructions. Briefly, 10 μl of CCK-8 solution (Dojindo, Kumamoto, Japan) 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. For cell counting, the transfected cells (5 × 10³/well) were seeded onto 12-well plates and counted using trypan blue exclusion at the indicated time points using a Coulter counter (Vi-Cell™ XR Cell Viability Analyzer, Beckman, Fullerton, CA, USA). The results are presented as the means ± standard deviation (SD).

2.7. In vivo tumor formation assay

All animal studies were performed with the approval of Peking University Biomedical Ethic Committee. CAL27 cells were infected with ad5-null (mock) or ad5-CMTM5-v1 at an MOI of 60 pfu/cell [12]. After 36 h, 2 × 10⁶ cells suspended in 100 μl of sterile phosphate-buffered saline 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 volume was estimated using the formula $V = (a \times b^2)/2$, where a is the larger dimension and b is the perpendicular diameter. The mice were sacrificed after the xenografts were seeded for approximately 31 days.

2.8. Cell apoptosis assay

The cells were transfected with pCDB or pCDB-CMTM5-v1 plasmids. After 48 h, the transfected cells (5 × 10⁵) were trypsinized, washed twice with PBS and resuspended in 200 μl of binding buffer (10 mM HEPES, pH 7.4; 140 mM NaCl; 1 mM MgCl₂; 5 mM KCl; and 2.5 mM CaCl₂). FITC-conjugated Annexin V was added at a final concentration of 0.5 μg/ml, and the cells were further incubated for 20 min at room temperature in the dark. After washing two times, the samples were treated with PI and analyzed on a FACSCalibur flow cytometer (Becton Dickinson, San Jose, CA).

2.9. Cell migration assay

The migration assay was performed in a 48-well chemotaxis chamber (Neuroprobe, Inc.), according to the manufacturer's instructions [11]. Briefly, NIH 3T3 cells were grown in serum-free medium containing 0.5 μg/ml of bovine serum albumin for 24 h and added to the bottom of the chamber. A polycarbonate filter (8-μm pore size) was used. The GNM cells were transfected with pCDB or pCDB-CMTM5-v1 plasmids. After 36 h, the cells were trypsinized and washed twice, and 50 μl of the single-cell suspension (3 × 10⁵ cells/ml in 0.1% bovine serum albumin/RPMI 1640) was added to the upper well of the chamber. The cells were incubated for 8 h at 37 °C in a humidified, 5% CO₂ atmosphere. After incubation, the non-migrated cells in the upper chamber were scraped off, and the migrated cells on the bottom part of the filter were fixed with methanol, followed by H&E staining. In each individual experiment, the cells that migrated through the filters were counted from at least three randomly selected fields. The results were obtained from at least three individual experiments and

represented as a cell migration index, i.e., the number of cells per high-power field.

2.10. Wound healing assay

The transfected cells were cultured in a 6-well plate until confluent. The cell layer was carefully wounded using sterile tips and washed twice with PBS, DMEM medium without adding FBS. After incubation for 36 h, the cells were photographed at low magnification (4 × objective). 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%.

2.11. Statistical analysis

Student's *t* test was used to analyze the results, expressed as the means ± SD. The different rates of CMTM5 expression were compared using chi-square χ^2 analysis. All tests were two-sided, with a significance level of *P* < 0.05.

3. Results

3.1. CMTM5 is downregulated in oral squamous cell lines

Previous studies have shown that CMTM5 is silenced in many cancer cell lines, including breast, nasopharyngeal, esophageal and gastric cancers. Herein, we examined CMTM5 at the mRNA level in five oral squamous cell lines (SCC-15, WSU-HN6, CAL27, Tca-83 and GNM). RT-PCR analysis showed that CMTM5 was undetectable in SCC-15, CAL27, Tca-83 and GNM and expressed moderately in WSU-HN6 cells (Fig. 1A).

To clarify whether the expression of CMTM5 was regulated through DNA hypermethylation, we examined the promoter methylation status of CMTM5 in oral squamous cell lines using methylation-specific PCR (MSP). As shown in Fig. 1B, the CMTM5 promoter was partially methylated in WSU-HN6 and fully methylated in the other four cell lines. The methylation status was correlated with the CMTM5 expression level in all oral squamous cell lines. To further examine the link between the methylation and silencing of CMTM5, we treated CAL27, GNM and Tca-83 cell lines with the methyltransferase inhibitor Aza (A) alone or combined with the histone deacetylase inhibitor trichostatin A (T). The demethylation reagents restored CMTM5 expression and the demethylation of the promoter (Fig. 1C, D). We conducted BGS to examine the detailed methylation status of individual CpG sites. The results confirmed that the CMTM5 promoter was methylated in the CAL27 cell line, and A + T treatment restored the expression of this gene (Fig. 1E). These results strongly suggest that hypermethylation is responsible for the defective expression of CMTM5 in oral squamous cell lines.

3.2. CMTM5 is downregulated in OSCC tissues

Next, we evaluated CMTM5 expression using immunohistochemistry in primary oral squamous cell carcinoma tissues and paired adjacent non-tumor tissues (*n* = 56) (Table 1). A pathologist, blinded to the experimental conditions, scored the levels of CMTM5 expression in individual samples as negative, weakly positive or strongly positive. CMTM5 expression was primarily observed in the cytoplasm (Fig. 2A). Weakly positive CMTM5 staining was observed in 22 (39.29%) normal tissue samples, while strongly positive staining was observed in 34 (60.71%) samples.

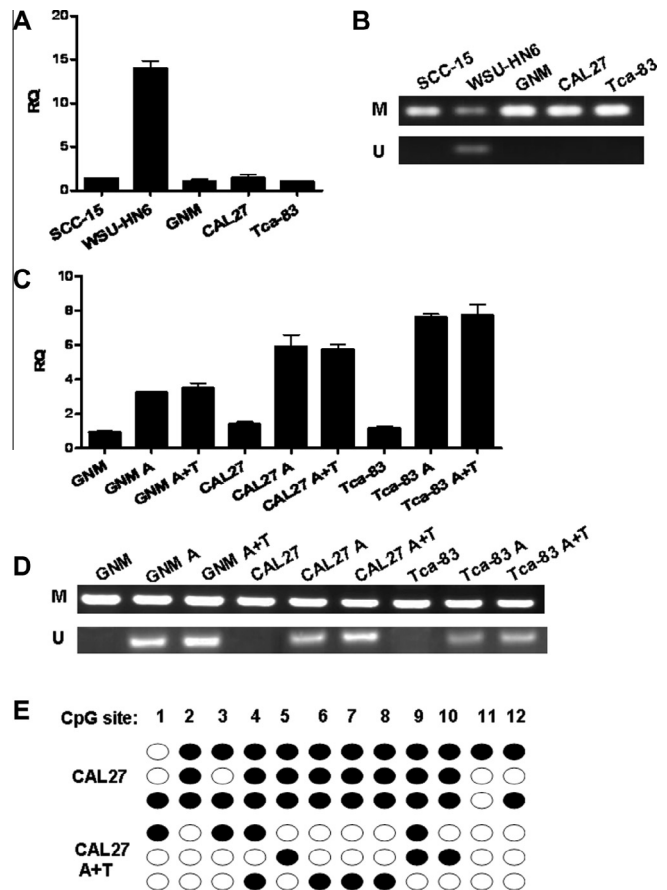


Fig. 1. CMTM5 is downregulated and methylated in oral squamous cell lines. (A) Real-time RT-PCR analysis of CMTM5 expression in oral squamous cell lines. GAPDH was used as a reference gene. (B) MSP analysis of CMTM5 in oral squamous cell lines. M: methylated and U: unmethylated. (C) Pharmacological demethylation using 5-aza-2'-deoxycytidine alone (A) or in combination with trichostatin A (A + T) restored CMTM5 expression in methylated and silenced oral squamous cell lines. (D) MSP analysis of CMTM5 in oral squamous cell lines with or without A or A + T treatment. (E) Detailed BGS analysis of the CMTM5 promoter in CAL27 cells with or without A + T treatment. Each row represents an individual randomly cloned and sequenced allele. The circles represented the analyzed CpG sites (12 sites); black circles, methylated CpG site; and white circles, unmethylated CpG site.

Table 1
Clinicopathological characteristics of OSCC patients.

| Variables | N |
|-----------------------|----|
| Gender | |
| Male | 27 |
| Female | 29 |
| Age | |
| <60 | 23 |
| ≥ 60 | 33 |
| Differentiation | |
| Well | 12 |
| Moderate | 31 |
| Poor | 13 |
| Tumor stage | |
| I | 28 |
| II | 19 |
| III | 9 |
| Lymph node metastasis | |
| Positive | 7 |
| Negative | 49 |

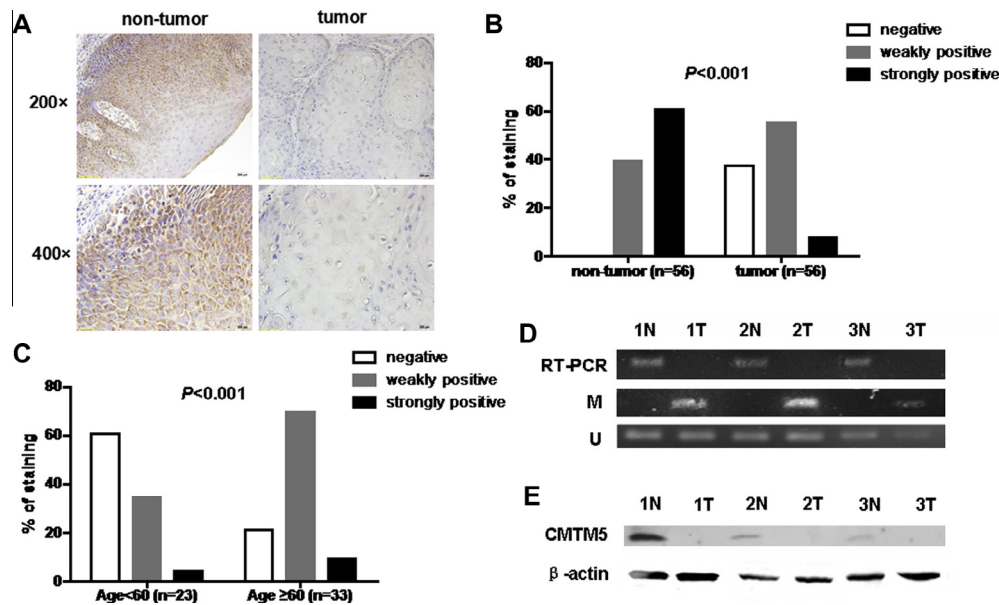


Fig. 2. *CMTM5* is downregulated in oral squamous cell carcinoma tissues. (A) *CMTM5* immunostaining in OSCC tumor tissues and adjacent non-tumor tissues. Left, adjacent non-tumor tissues; right, OSCC tumor tissues. (B) Analysis of *CMTM5* expression in OSCC tumor tissues and adjacent non-tumor tissues ($n = 56$). (C) Correlation between *CMTM5* expression and the OSCC patient age. (D) RT-PCR and MSP analysis of *CMTM5* in OSCC tumor tissues and adjacent non-tumor tissues ($n = 3$). N: adjacent non-tumor tissues and T: OSCC tumor tissues. M: methylated and U: unmethylated. (E) Western blot analysis of *CMTM5* in OSCC tumor tissues and adjacent non-tumor tissues ($n = 3$).

In cases of oral squamous cancer, negative *CMTM5* staining was observed in 21 (37.50%) samples, weakly positive staining was observed in 31 (55.36%) samples and strongly positive staining was observed in 4 (7.84%) samples (Fig. 2B). The expression of *CMTM5* protein was stronger in normal tissues than in cancer tissues ($P < 0.001$). We also observed that *CMTM5* expression is associated with the patient age ($P < 0.001$) (Fig. 2C). We did not detect a correlation between the level of *CMTM5* expression and other Clinicopathological characteristics (data not shown).

We also examined the *CMTM5* mRNA expression in primary oral squamous tumors and paired non-tumor tissues. As shown in Fig. 2D, *CMTM5* mRNA expression was detected in non-tumor tissues and undetectable in tumor tissues. *CMTM5* promoter methylation was observed in tumor tissues. Consistent with the mRNA expression, *CMTM5* was downregulated at the protein level in oral squamous tumor tissues compared with non-tumor tissues through Western blot analysis (Fig. 2E).

3.3. The restoration of *CMTM5-v1* inhibits cell growth in vitro and in vivo

Based on the transcriptional silencing of *CMTM5* endogenously maintained in CAL27 and GNM cells detected through RT-PCR, we examined the effects of the ectopic *CMTM5-v1* expression on cell growth using a CCK-8 assay and cell counting. *CMTM5-v1* strongly inhibited the growth of CAL27 (Fig. 3A, B) and GNM cells (Fig. 3C, D) compared with the control groups.

CAL27 cells are highly tumorigenic when subcutaneously injected into nude mice. To examine whether *CMTM5* inhibits the tumor formation, CAL27 cells transduced with ad5-null (control) or ad5-*CMTM5* were subcutaneously injected into the right flank of nu/nu mice. At 31 days after inoculation, the mean tumor volume in the control group was $128.18 \pm 43.65 \text{ mm}^3$ (Fig. 3E, F), and the mean tumor weight in the control group was $39.46 \pm 6.45 \text{ mg}$ (Fig. 3G). Tumor formation was completely suppressed in the animals injected with ad5-*CMTM5*-infected cells.

The restoration of *CMTM5-v1* expression in these cell lines transfected or infected 24 h later was evidenced through Western blot analysis (Fig. 3H).

3.4. The restoration of *CMTM5-v1* induces cell apoptosis and inhibits cell migration

Next, we determined whether the inhibition of tumor cell growth through *CMTM5* was associated with apoptosis. We observed that the overexpression of *CMTM5-v1* significantly increased early and late apoptosis in CAL27 (Fig. 4A, B) and GNM (Fig. 4C, D) cells compared with cells transfected with the control vector.

The effect of *CMTM5-v1* on oral squamous cell motility was investigated using wound healing and Boyden chamber migration assays. The wound-healing assay revealed that *CMTM5-v1*-transfected cells spread along wound edges remarkably slower than vector-transfected cells after 36 h, indicating that *CMTM5-v1* inhibited CAL27 cell migration ($P < 0.001$) (Fig. 4E). The restoration of *CMTM5-v1* also strongly inhibited the migration of GNM cells, evidenced through a significant decrease in the number of migrated cells in *CMTM5-v1*-expressing cells compared with controls ($P < 0.001$) (Fig. 4F).

4. Discussion

Previous studies have shown that *CMTM5* exerts tumor suppressor functions with frequent epigenetic inactivation through promoter methylation in many tumors. *CMTM5* is located at 14q11.2, a locus containing multiple genes associated with various carcinomas. Studies have shown that the abnormalities in the genes on chromosome 14q11.2 cause generation or growth of various cancers, such as nasopharyngeal carcinoma [14], leukemia [15], glioblastoma [16] and meningioma [17]. Herein, we provided the first examination of the tumor suppressor effects of *CMTM5* in oral squamous cell carcinoma.

Similar to other tumor cell lines, *CMTM5* was downregulated in SCC-15, CAL27, GNM and Tca-83, and the *CMTM5* promoter was

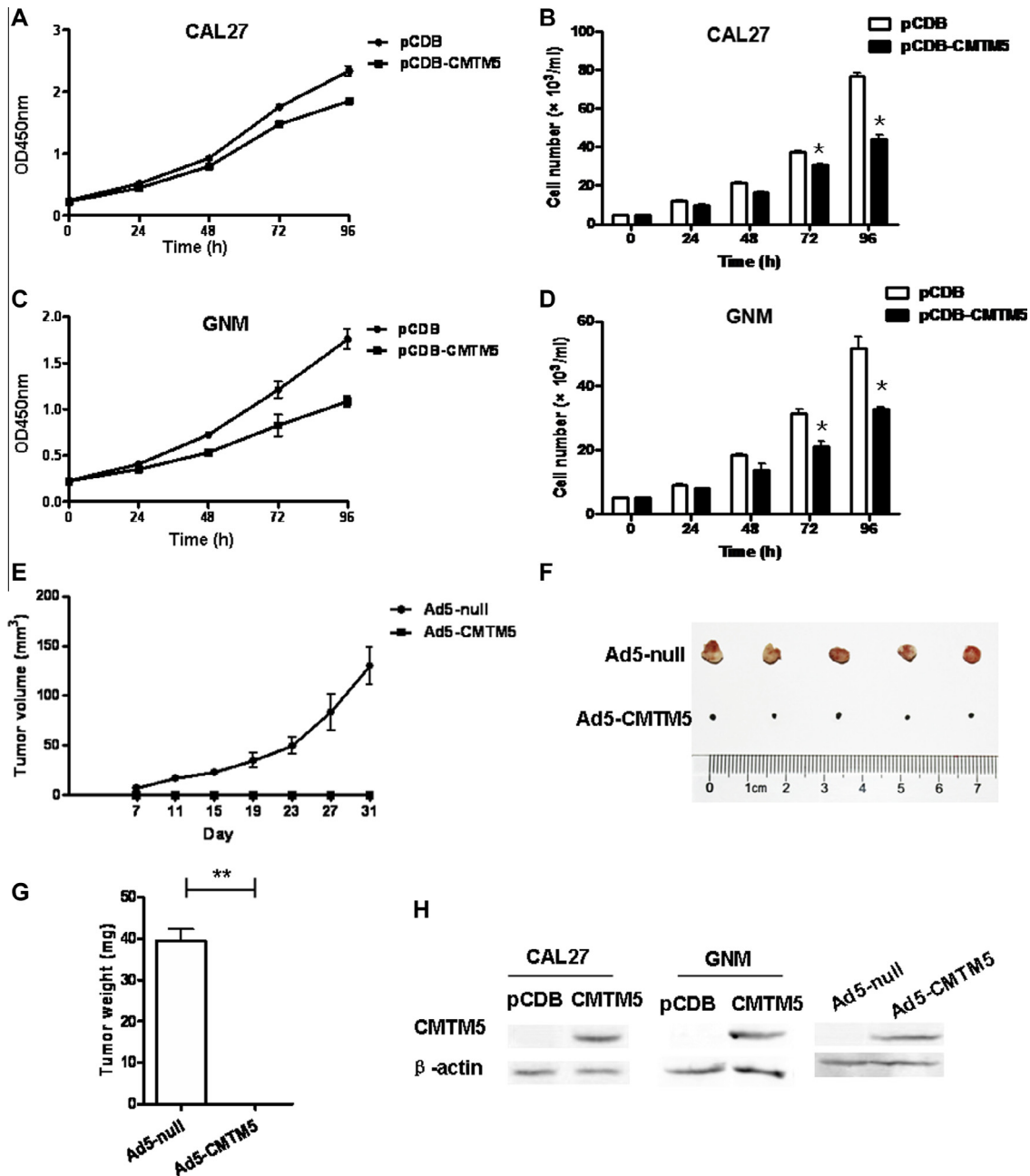


Fig. 3. Restoration of CMTM5-v1 inhibits cell growth. (A) Representative CCK-8 assay showing that CMTM5-v1 inhibited CAL27 cell growth. (B) Growth curve for transfected CAL27 cells showing that CMTM5-v1 inhibited CAL27 cell growth. (C) Representative CCK-8 assay showing that CMTM5-v1 inhibited GNM cell growth. (D) Growth curve for transfected GNM cells showing that CMTM5-v1 inhibited GNM cell growth. (E) CMTM5-v1 effectively suppressed tumorigenicity in nude mice. The average tumor volumes of CMTM5 versus mock cells are expressed as the means \pm SD in 5 inoculated sites for each group. (F) The tumor sizes of CMTM5-v1 versus mock cells are shown. (G) The tumor weights of CMTM5-v1 versus mock cells are shown. (H) CMTM5-v1 expression in cells transfected or infected 24 h later was confirmed through Western blot analysis. * $P < 0.05$. ** $P < 0.001$.

also methylated in these cell lines. *CMTM5* expression was significantly decreased in oral squamous cell tumor tissues and *CMTM5* promoter methylation was detected in OSCC tumor tissues. DNA methylation is one of the most common epigenetic modifications in the mammalian genome involved in the regulation of gene expression. The aberrant promoter hypermethylation of several genes, such as adenomatous polyposis coli (APC) [18], p16(INK4A) [19], p14^{ARF} [20], etc., has been demonstrated in OSCC [21]. Epigenetic alterations occur at a relatively early stage, suggesting that these genes could be used as prognostic markers in the clinic. In recent years, the ages of patients diagnosed with oral squamous cell carcinoma have become increasingly younger. We observed

that *CMTM5* expression was significantly associated with the age of OSCC patients. The decrease or absence of *CMTM5* expression is more frequent in younger patients, suggesting that *CMTM5* inactivation might induce early on-set carcinogenesis and play an important role in the pathological changes observed in young OSCC patients.

We also investigated the tumor suppressor role of *CMTM5* in OSCC *in vitro* and *in vivo*. The restoration of *CMTM5* expression in silenced CAL27 and GNM cells inhibited cell growth and migration and induced cell apoptosis. Decreased tumor growth through *CMTM5* expression was confirmed by the reduction of tumorigenesis in nude mice. These results implicated *CMTM5* as

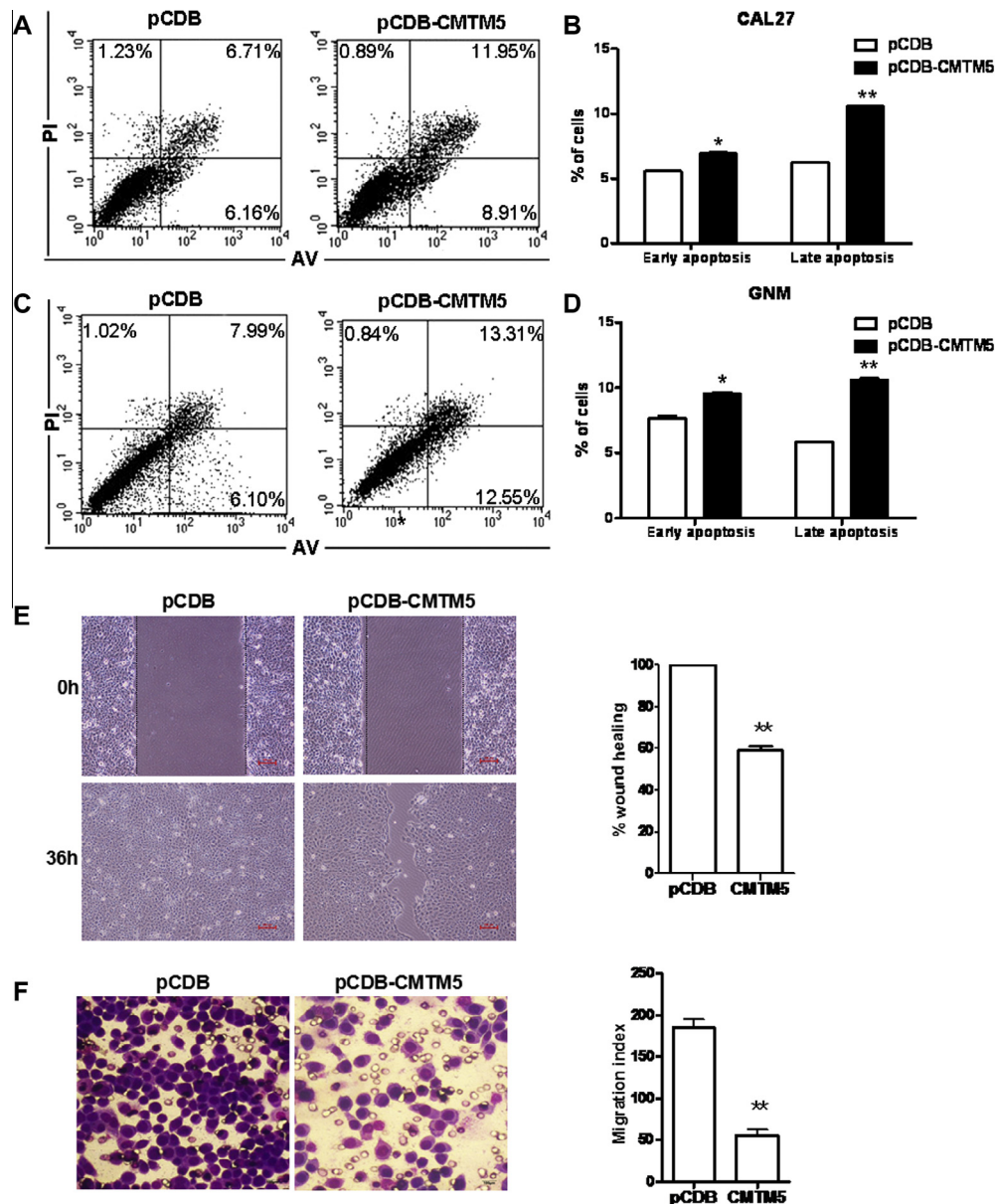


Fig. 4. Restoration of CMTM5-v1 induces cell apoptosis and inhibits cell migration. (A) Flow cytometric assays with Annexin V and PI double staining of CAL27 cells transfected with pCDB or pCDB-CMTM5-v1 plasmids after 48 h. (B) Graphic representation of apoptotic cells as a percentage of total CAL27 cells in three independent experiments. (C) Flow cytometric assays with Annexin V and PI double staining of GNM cells transfected with pCDB or pCDB-CMTM5-v1 plasmids after 48 h. (D) Graphic representation of apoptotic cells as a percentage of total GNM cells in three independent experiments. (E) Wound healing assay using CAL27 cells transfected with pCDB or pCDB-CMTM5-v1 showing that CMTM5-v1 inhibited CAL27 cell mobility. The images were captured at 0 and 36 h after the scratch. (F) Boyden migration assay using GNM cells transfected with pCDB or pCDB-CMTM5-v1 showing that CMTM5-v1 inhibited GNM cell migration after 8 h. * $P < 0.05$. ** $P < 0.001$.

a tumor suppressor in OSCC. The demethylation of tumor suppressor genes leads to re-expression, which could be utilized in cancer therapy [22]. The development of improved demethylating agents is in progress. Zebularine sensitized ovarian cancer cells to cisplatin and caused the re-expression of tumor suppressor genes, including RASSF1A, ARHI and BLU [23], whereas Aza and decitabine showed differential nonrandom DNA demethylation in colon cancer and leukemia cells [24]. Epigenetic drugs sensitize cancer cells, inhibit the formation of progenitor cells, showing potential for use in combination drug therapies with other cytotoxic drugs [22].

In conclusion, we provided the first evidence of the aberrant expression and tumor suppressive function of CMTM5 in oral squamous cell carcinoma. CMTM5 methylation might serve as a potential epigenetic biomarker for the early diagnosis of OSCC patients.

While CMTM5 might be a novel tumor suppressor gene in oral squamous cell tumors, further studies are needed to elucidate the function and underlying mechanisms of CMTM5.

Conflict of interest

None declared.

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