Silencing of *CMTM6* Inhibits the Proliferation and Migration of SiHa Cervical Cancer Cells by Inhibiting MAPK Signaling

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Research

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Abstract

Background: CKLF like MARVEL transmembrane domain containing 6 (CMTM6) is an important programmed cell death 1 ligand 1 (PD-L1) regulator. CMTM6 was identified as an immune checkpoint inhibitor that regulates tumor T cell activity *in vivo* and *in vitro*. The function of CMTM6 in cervical cancer, however, remains unclear. In addition, whether CMTM6 participates in ovarian cancer epithelial-mesenchymal transition (EMT) is unknown.

Methods: Immunohistochemistry was used to detect CMTM6 expression in cervical cancer tissues and non-cancerous adjacent tissues. CMTM6 was knocked down and overexpressed, respectively, in the human SiHa cell line. Transwell and wound healing assays were used to assess cell migration and cell invasion. Western blotting was used to detect phosphorylated (p)-C-Jun N-terminal kinase (JNK)1/2, total (t)-JNK1/2, p-p38, t-p38, and EMT-related protein levels in SiHa cells. *In vivo* lung transplantation tumor models were used to determine if CMTM6 upregulation in ovarian cancer cells could promote metastasis.

Results: CMTM6 showed higher expression in human ovarian cancer tissues than in adjacent non-cancerous tissues. *In vitro* assays showed that CMTM6 promoted ovarian cancer cell invasion, migration, proliferation, and EMT via MAPK JNK/p38 signaling pathway activation. Stimulating CMTM6-overexpressing cells with MAPK JNK/p38 signaling antagonists reduced SiHa cell metastasis, invasion, proliferation and EMT. In a mouse model of lung metastasis, CMTM6 increased the metastasis of ovarian cancer.

Conclusion: CMTM6 activates MAPK JNK/p38 signaling to enhance the EMT and metastasis of ovarian cancer cells. These results suggest CMTM6 as a molecular target for the prevention and treatment of ovarian cancer metastasis.

Background

Cervical cancer (CC) is the second most frequent cause of cancer death in women worldwide^[1]. In developing countries, the incidence of CC is high^[2]. In these countries, it is difficult to obtain high-quality medical services^[3]. High-quality medical services can aid timely screening and promote the early diagnosis of CC^[4]. In addition, CC has a low 5year overall survival rate^[5]. Hysterectomy is a widely used treatment for early CC, together with simultaneous radiotherapy and chemotherapy^[6]. However, most treatment options for CC will lead to a poor prognosis for patients, especially in patients undergoing hysterectomy. The detailed molecular basis of CC is poorly understood. Determining the molecular mechanism of CC carcinogenesis and development could promote the discovery of new molecular targets for treatment.

CMTM6 belongs to the CKLF-like MARVEL transmembrane domain family (CMTM1–8), and is expressed widely on the plasma membrane of a variety of cells. However, the function of CMTM6 is mostly unknown^[7,8]. Two recent reports confirmed that CMTM6 increases the expression of programmed cell

death 1 ligand 1 (PD-L1) protein, thereby impairing T cell function^{[9] [10]}. In addition, it has been reported that elevated CMTM6 in glioma and head and neck squamous cell carcinoma is associated with a poor prognosis, and it is also a potential therapeutic target for in triple-negative breast cancer^[11–14]. These studies suggested CMTM6 as a potential therapeutic target. CMTM6 overexpression was shown to induce epithelial-mesenchymal transition (EMT) of oral squamous cell carcinoma cells^[15]. The transition from an epithelial to a mesenchymal phenotype promotes primary tumor transformation into metastatic and invasive types^[16]. However, the involvement of CMTM6 in human CC EMT is unclear.

Thus, the present study aimed to assess CMTM6 protein levels in cervical cancer tissues and cells in comparison with its level in adjacent non-tumor tissues and the CC cell line SiHa. We also aimed to determine whether *CMTM6* silencing or overexpression affected the invasion, migration, and proliferation of SiHa cells and the levels of related proteins *in vitro*. In addition, the *in vivo* effect of CMTM6 on cervical cancer tumor growth was determined.

Materials And Methods

Cell culture and transfection

iCell Bioscience Inc., Shanghai, China provided the CC epithelial cell line SiHa, which was verified using short tandem repeat (STR) determination. SiHa cells were cultured in Roswell Park Memorial Institute (RPMI)-1640 medium (Biochrom, Berlin, Germany) containing 10% fetal bovine serum (FBS) and 1% penicillin and streptomycin. The cells were cultured at 37 °C in a humid environment containing 5% CO₂. Lipofectamine 3000 (Invitrogen, Carlsbad, CA, USA) was used to transfect cells following to the manufacturer's guidelines.

Plasmids and antibodies

The full-length coding region of the *CMTM6* cDNA was inserted into vector pcDNA 3.1 with a myc-tag for *CMTM6* overexpression (oe-CMTM6), and was constructed by GeneCopoeia (Guangzhou, China). The short hairpin RNA (shRNA) targeting sequence specific for human *CMTM6* was as follow: 5¢-TGGAGAACGGAGCGG-TGTACA-3¢;

Rabbit anti-CMTM6 antibodies were purchased from Sigma (Sigma, St. Louis, MO, USA). Rabbit anti-E-cadherin, anti-Vimentin, anti-p38, phosphorylated (p)-p38, anti-ERK, and anti-C-Jun N-terminal kinase (JNK) were purchased from Proteintech (Chicago, IL, USA); Cell Signaling Technology (Danvers, MA, USA) provided the rabbit anti-p-extracellular regulated kinase (ERK), anti-p-JNK, and mouse anti-glyceraldehyde-3-phosphate dehydrogenase (GAPDH).

Western blotting

Different samples of cultured cells were collected by centrifugation and rinsed two times using twice ice-cold phosphate-buffered saline (PBS). The pelleted cells were suspended and the proteins extracted by

vortexing in Radioimmunoprecipitation assay buffer (Beyotime, Jiangsu, China), supplemented with a protease inhibitor cocktail (Thermo, Waltham, MA, USA), and phenylmethylsulfonyl fluoride (Thermo). Proteins (20 µg/lane) were subjected to sodium dodecyl sulfate polyacrylamide gel electrophoresis, followed by electrotransfer to a polyvinylidene fluoride membrane (Millipore, Boston, MA, USA). Membranes were incubated with primary antibody for 3 h. Tris-buffered saline-Tween 20 (TBST) with 5% skim milk was used to block the membrane overnight at 4 °C. The membrane was washed four times using TBST buffer, and then incubated for 2 h at room temperature with the horseradish peroxidase-conjugated secondary antibody. Finally, an enhanced chemiluminescence detection reagent (Millipore, Boston, MA, USA) was used to visualize the immunoreactive bands. A GS-800 scanner as used to scan the film, and then the visualized protein bands were analyzed using Quantum One software (Bio-Rad, Hercules, CA, USA).

Cell counting kit (CCK-8) assay

SiHa cells were seeded into 96-well plates at 2×10^3 cells/well and transfected with various constructs. Beginning at 24 h after transfection, CCK-8 solution (10 µL per well) was added to the wells at 0, 24, 48, 72, and 96 h, and incubated for another 2 h at 37 °C. A microplate reader (GloMax-multi microplate reader, Promega, Madison, WI, USA) was used to measure the absorbance at 450 nm.

Colony formation

The colony formation test was used to determine the ability of the cells to form colonies. At 24 h after transfection, the cells were harvested, resuspended, and seeded into a six-well plate at a density of 1000 cells per well. The cells were incubated at 37 °C for 12 days in a humidified incubator. On the 13th day, the colony formation assay was performed, and the surviving colonies were fixed with 4% paraformaldehyde and stained using methyl violet (Sigma-Aldrich; Merck KGaA, Darmstadt, Germany) before being counted (each colony > 50 cells). The experiments were performed at least in triplicate.

Wound healing assay

Following trypsinization, the cells were resuspended in serum-free medium, transferred to a 6-well plate, grown overnight to form a cell monolayer. Next day, a pipette tip was used to score a linear wound in the cell monolayer. The cells were washed gently once to remove debris and smoothen the wound edges. The wound was photographed at $\times 40$ magnification under a Leica optical microscope (Leica DM 500, Wetzlar, Germany) after 0.24 and 48 h of incubation. The extent of wound healing (%) was calculated as follows: Percentage migration rate = migration number(Treated) / migration number(Control).

Cell invasion and migration assays

The migration and invasion capabilities of cells were measured using Corning Transwell insert chambers (pore size 8 mm; Corning Inc, Corning, NY, USA) and BD BioCoat Matrigel invasion chambers (Corning Life Sciences, Tewksbury, MA). FBS was used as the chemoattractant. After transfection, the cells were

collected and resuspended in serum-free medium. Approximately 3×10^4 (migration assay) or 1×10^5 (invasion assay) of cells were placed the chamber and incubated for 24 h at 37 °C. Migrated or invaded cells in the bottom part of the chamber were fixed using 20% methanol, and stained using 0.1% crystal violet (KeyGen, Guangzhou, China) for imaging and counting.

***In vivo* assays for tumor growth and metastasis**

The Southern Medical University provided 4-week-old male nude mice. Exponentially growing SiHa cells with *CMTM6* knockdown (*CMTM6*-shRNA) or the corresponding empty vector (shNC) were suspended in Matrigel (50% in cold PBS) and injected into the right flanks of the mice subcutaneously at 4×10^5 Cells/mouse. From one week after injection, the length (a) and width (b) of the tumor were determined using calipers every week. We calculated the tumor volume as follows: volume (mm^3) = $a \times b^2 / 2$. At 5 weeks after tumor cell injection, the mice were sacrificed. Then the excised tumors were weighed and photographed.

Further 4-week-old male nude mice were purchased from the Southern Medical University. To establish a lung metastasis model, transfected cells (1×10^6 per mouse) were injected into the tail vein. Three weeks later, the mice were sacrificed and the number of tumor nodules formed in their lungs was counted.

Statistics

The SPSS statistical software package (IBM Corp., Armonk, NY, USA) was used for the statistical analysis. Data from repeated experiments are shown as the mean $\pm$ SD. A t test was used to compare the average between groups group. A p-value of less than 0.05 from a two-tailed test was considered significant.

Results

CMTM6 is abnormally expressed in CC and is associated with poor prognosis

The GEPIA database contains expression data for 306 cervical squamous cell carcinoma and endocervical adenocarcinoma (CESC) tissues and 13 normal cervical tissues. Analysis of the GEPIA data revealed that *CMTM6* is overexpressed in CESC compared with that normal cervical tissues (box plot, Fig. 1A, $P < 0.05$). The survival rate of patients with low *CMTM6* expression was higher than that of patients with high *CMTM6* expression ($P < 0.05$, log rank test; Fig. 1B). In addition, IHC confirmed the overexpression of *CMTM6* in clinical samples of cervical cancer (Fig. 1C).

Upregulation of CMTM6 promotes SiHa cell proliferation, migration, and invasion

Treating SiHa cells with oe-*CMTM6* and sh-*CMTM6* markedly increased and decreased, respectively, the cellular levels of *CMTM6*, as detected using western blotting analysis (Fig. 2A-B).

Overexpression of CMTM6 increased SiHa cell proliferation significantly according to the results of CCK-8 analysis (Fig. 2C). Next, colony formation experiments showed that for cells transfected with sh-CMTM6, the number of colonies formed was significantly reduced compared with those formed by cells transfected with sh-NC (Fig. 2D). By contrast, cells overexpressing *CMTM6* formed significantly more colonies compared with those formed by the control cells. These results showed that increased CMTM6 expression in SiHa cells leads to increased cell proliferation.

Poor prognosis of CC is related to metastasis. To determine the role of CMTM6 in CC metastasis, in SiHa cells, wound healing assays and Transwell invasion and migration assays, and were carried out. In the wound healing assays, cells transfected with shCMTM6 showed significantly inhibited migration. Consistently, cell migration was increased significantly in cells harboring oe-CMTM6 (Fig. 2E). The Transwell tests showed that the migration and invasiveness of CC cells transfected with sh-CMTM6 were reduced significantly, while migration and invasiveness increased in cells transfected with oe-CMTM6 (Fig. 2F-G). Together, these data suggested that CMTM6 promotes CC invasion and metastasis.

Genes and proteins co-expressed with CMTM6 are related to the MAPK pathway

Enrichment analysis of co-expressed genes performed using KEGG database analysis indicated that CMTM6 is primarily involved the mitogen-activated protein kinase (MAPK) pathway (Fig. 3A-B), which was consistent with the Biological Process analysis results.

The above cellular pathway enrichment suggested that CMTM6 can activate the MAPK signaling pathway. After transfection with oe-CMTM6 and sh-CMTM6, western blotting analysis was carried out to assess the protein levels of MAPK-related proteins. The levels of phosphorylated p38, JNK, and ERK were reduced significantly in cells transfected with sh-CMTM6 compared with those in cells transfected with shNC. Levels of phosphorylated p38, JNK, and ERK were increased in oeCMTM6transfected cells compared with those in cells transfected with oe-NC (Fig. 3C-D).

Inhibition Of Mapk Signaling Reversed Emt Induced By Cmtm6

Then, we used western blotting to study whether inhibiting the MAPK pathway in cancer cells overexpressing *CMTM6* could inhibit EMT. As shown in Fig. 4A and B, compared with those in the control group, the levels of MAPK signaling pathway proteins and vimentin in SiHa cells overexpressing *CMTM6* were significantly increased. The levels of E-cadherin, a typical molecular marker EMT, were downregulated significantly in SiHa cells overexpressing *CMTM6*. In cells treated with oe-CMTM6 and the ERK/MAPK inhibitor PD98059, reduced ERK phosphorylation was observed compared with that in cells treated with oe-CMTM6 + DMSO, meanwhile the levels of phosphorylated JNK and p38 were not significantly different. Moreover, treating cells with oe-CMTM6 and the JNK/MAPK inhibitor SP600125, resulted in reduced levels of phosphorylated JNK, while the levels of phosphorylated ERK and p38 did not differ significantly. In addition, in cells treated with oe-CMTM6 and the p38/MAPK inhibitor SB203580,

the level of phosphorylated p38 was reduced significantly; however, there were no significant changes in the levels of phosphorylated ERK and JNK (Fig. 4A-B).

Wound healing and Transwell analysis were used to assess the role of CMTM6 in CC migration and invasion. CMTM6 overexpression enhanced the cell migration and invasion abilities of SiHa cells, whereas the MAPK pathway inhibitor decreased cell migration and invasion (Fig. 4C-D; the statistics are shown in Fig. E-F). In plate clonal formation assays and CCK-8 experiments, CMTM6 could promote the proliferation of SiHa cells; however, after inhibiting the MAPK pathway, the proliferation ability of SiHa cells decreased.

Knockdown of CMTM6 inhibits cervical cancer tumor growth and metastasis *in vivo*

The tumors formed by SiHa cells transfected with shCMTM6 grew slowly and were small compared with those formed in the control mice (Fig. 6A-D), indicating that low *in vivo* expression levels of CMTM6 inhibited tumor formation and progression significantly.

Next, an *in vivo* metastasis assay was performed. To mimic CC tumor metastasis via the circulation, SiHa cells transfected with shCMTM6 were injected into 6weekold nude mice via their tail-vein. Six weeks later, we counted the metastatic nodules that had formed on the surface of the lungs (Fig. 6E-F). In the shCMTM6 group, the mice had fewer and smaller lung metastatic nodules compared with those in the control mice (Fig. 6G).

Discussion

Cervical cancer is considered a dangerous malignant tumor, posing a huge threat to women worldwide^[17]. Although encouraging results have been produced using certain diagnostic and treatment methods for cervical cancer, such as HPV DNA testing, pre-invasive disease management, and cervical cytology, the prognosis of patients with advanced disease is frequently poor^[18, 19].

Increasing evidence indicates that CMTM6 overexpression is a common molecular event in human malignancies^[20]. However, we do not know what role CMTM6 dysregulation plays in CC. Herein, the results showed that high expression of CMTM6 is more common in cervical cancer tissues compared with that in adjacent normal tissue. The prognosis of patients with CC is better if the disease is diagnosed early; however, patients with metastatic CC have a worse prognosis. The continuous survival analysis indicated that patients with CC with a low expression of CMTM6 had a longer OS. Therefore, the expression of CMTM6 is roughly inversely proportional to the prognosis of patients with CC.

Several upstream signaling pathways such as MAPK, PI3K/AKT, and TGF- β can regulate EMT^[21, 22]. The MAPK JNK/p38 signaling pathway plays a vital role in tumor invasion and metastasis^[23], and induces EMT, which has an impact on tumor metastasis. In addition, the MAPK JNK/p38 pathway affects the expression of E-cadherin and vimentin, which are EMT-related proteins^[24]. Taken together, previous studies indicated that in ovarian cancer, MAPK JNK/p38 signaling pathway activation might induce EMT

[25]. The results indicated that upregulation of CMTM6 leads to EMT-mediated MAPK JNK/p38 signaling pathway activation. In addition, Pang et al. described that CMTM6 promotes tumor metastasis of oral squamous cell carcinoma through the ERK1/2 signaling pathway [26]. In the future, we will explore whether CMTM6 can activate the ERK1/2 pathway, and whether inhibitors of the ERK1/2 pathway can reverse ERK1/2-induced ovarian cancer metastasis

Our analysis indicated that cervical cancer tissues have upregulated levels of CMTM6, which is associated significantly with cervical squamous cell carcinoma's aggressive behavior. We speculated that CMTM6 expression may relate to the pathological grade of the cervical cancer tissue. In the present study, IHC analysis showed that CMTM6 was overexpressed in human cervical cancer tissue samples compared with that matched non-tumor samples.

Consequently, to determine the effects of CMTM6 on human cervical cancer cell biological functions, we knocked down *CMTM6* using siRNA and overexpressed *CMTM6* from a plasmid in SiHa cells. The number of living cells defines cell viability. In addition, cell apoptosis and cell number are important indicators of cell death or survival under exposure to a variety of stimuli. SiHa cell proliferation and viability were reduced in response to *CMTM6* silencing, while overexpression of *CMTM6* significantly increased SiHa cell survival and proliferation. Moreover, *in vivo*, *CMTM6* overexpression promoted the growth of cervical cancer. Therefore, in terms of proliferation, it is possible that CMTM6 has dual functions in different human malignancies or cell lines depending on the type of tissue or cell.

In conclusion, we identified a novel biological role of CMTM6 in ovarian cancer, and revealed that CMTM6 upregulation activates the MAPK p38/JNK signaling pathway, leading to enhanced migration and invasion of ovarian cancer through EMT. Therefore, targeting CMTM6 in ovarian cancer might be a promising treatment strategy. In addition, CMTM6 might serve as a molecular biomarker to predict the metastasis and prognosis of ovarian cancer.

Abbreviations

PD-L1

programmed cell death 1 ligand 1; EMT [epithelial-mesenchymal transition]

CC

Cervical cancer ; CMTM [CKLF-like MARVEL transmembrane]

STR

short tandem repeat; FBS: fetal bovine serum; JNK: anti-C-Jun N-terminal kinase;

ERK

anti-p-extracellular regulated kinase ; GAPDH: anti-glyceraldehyde-3-phosphate dehydrogenase;

PBS: phosphate-buffered saline; TBST: Tris-buffered saline-Tween 20

CCK-8

Cell counting kit ; CESC [cervical squamous cell carcinoma and endocervical adenocarcinoma] MAPK [mitogen-activated protein kinase]

Declarations

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Availability of data and materials

The data sets used and/or analyzed during the current study are available from the corresponding author on reasonable request.

Authors' contributions

All authors have made substantial contributions to this study and have been involved in the interpretation of the results and writing the manuscript.

All authors have given final approval of the version to be published. MYL, YWY and JQL: conceived and designed the experiments. XTH ,WL: conceived and designed the experiments; CSL :performed the experiments; BYWanalyzed the data; GTY contributed analysis tools; JJH revised the paper.

Ethics approval and consent to participate

This study was approved by the Institutional Review Board at Medicine Center of Guangzhou Medical University Affiliated Cancer Hospital. All the participants provided written consent to participate in the study.

Consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

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Figures

Figure 1

Cervical cancer tissues overexpress CMTM6, which indicates poor prognosis. (A) Expressional boxplot of CMTM6 in CC based on GEPIA data. (B) Patient survival data from the TCGA database. (C) IHC determination of CMTM6 protein levels in six pairs of matched cervical cancer tissues. * $p < 0.05$.

Figure 2

In vitro, CMTM6 promotes cervical cancer metastasis, invasion, and proliferation. (A-B) Western blotting showing the cellular protein level of CMTM6 in each group. Data were normalized the GAPDH levels. (C). CCK-8 assay of cell viability. (D) The clone formation, migration, and invasion abilities of the indicated groups were determined using plate clone, wound healing, and Transwell assays, respectively. *, $p < 0.05$; **, $p < 0.001$; ***, $p < 0.0001$.

Figure 3

CMTM6 activates MAPK signaling to regulate EMT. (A-B) Pathway enrichment, biological process, and reactome pathways analyses. (C-D) Western blotting showing the levels of MAPK-related proteins. Data were normalized to the level of GAPDH. *, $p < 0.05$; **, $p < 0.001$; ***, $p < 0.0001$.

Figure 4

Effect of inhibited MAPK signaling on CMTM6-regulated cell invasion and migration. (A-B) Western blotting showing the levels of MAPK-related proteins in each group. Data were normalized to the level of GAPDH. (C-D) Cell metastasis and migration assessed using Transwell assays in which CMTM6 acted as an inhibitor of the MAPK signaling pathway. *, $p < 0.05$; **, $p < 0.001$; ***, $p < 0.0001$.

Figure 5

Inhibitory effect of the MAPK signaling pathway on cell proliferation is regulated by CMTM6. (A-B) Clone formation and CCK-8 assays were used to detect the proliferation ability of cells, in which CMTM6 acted as an inhibitor of the MAPK signaling pathway. *, $p < 0.05$; **, $p < 0.001$; ***, $p < 0.0001$.

Figure 6

CMTM6 promotes CC cell growth and metastasis in vivo. (A) Representative images of tumor formed in each group. (B-D) Quantitative growth curve for the tumor volume and weight in each group, expressed as the mean $\pm$ SEM. (E) Image of lung nodules with metastasis in mice injected with HCC cells via their tail vein. The arrow indicates a metastatic tumor on the surface of the lung. (F-G) At 6 weeks following HCC cell tail vein injection, the number and diameter of lung nodules were determined in the nude mice ($n = 6$ per group). SPSS and GraphPad software were used for the statistical analysis. *, $p < 0.05$; **, $p < 0.001$; ***, $p < 0.0001$.

Figure 7

Schematic model of how silencing of CMTM6 inhibits SiHa cancer cell migration and proliferation through MAPK signaling inhibition.