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Cetyltrimethylammonium Bromide Disrupts Mesenchymal Characteristics of Human Tongue Squamous Cell Carcinoma SCC4 Cells Through Modulating Canonical TGF-β/Smad/miR-181b/TIMP3 Signaling Pathway

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Abstract. *Background/Aim: This study investigated the anti-metastatic effects of cetyltrimethylammonium bromide (CTAB) on tongue squamous cell carcinoma (TSCC) SCC4 cells. Materials and Methods: Cell morphology, viability, cell cycle distribution, adhesion, migration, invasion and the expression levels of associated proteins were examined using microscopy, WST-1, wound-healing, Boyden chamber assays, and western blotting, respectively. Results: CTAB significantly affected SCC4 cell morphology from spindle-shaped to cobblestone-shaped and resulted in loss of adherence. CTAB significantly inhibited cell adhesion, migration, and invasion of SCC4 cells, independent of cell viability. CTAB reduced expression of matrix metalloproteinases (MMPs) such as MMP3, MMP7, and MMP14 in a concentration-dependent manner, while it increased expression of tissue inhibitors of metalloproteinase 3 (TIMP3). In addition, CTAB reduced the phosphorylation of mothers against decapentaplegic homolog 2/3 (Smad2/3) proteins, which mediated CTAB-inhibited migration and invasion in SCC4 cells. These effects were reversed by TGF-β1. Conclusion: CTAB attenuates the mesenchymal characteristics through upregulation of TIMP3 by inhibiting the canonical TGF-β/Smad/miR-181b/TIMP3 signaling involved in extracellular matrix remodeling in SCC4 cells and might be a promising anti-metastatic therapeutic agent for TSCC.*

Tongue squamous cell carcinoma (TSCC) represents the most prevalent and aggressive malignancy of oral cancer (1). Approximately 30% of newly diagnosed head and neck squamous cell carcinoma (HNSCC) patients are classified as TSCC (2). More than half of HNSCC patients with locoregionally advanced stage are confirmed at the first diagnosis because of the absence of obvious clinical symptoms in the earlier stages (3). Despite improvements in therapies including surgical excision, chemotherapy and radiotherapy, the prognosis of TSCC patients is still poor. The reasons for this may be its propensity for regional recurrence and lymph node metastasis, therefore leading to high rates of morbidity and mortality (4). Therefore, developing new medicines without adverse effects for treating TSCC is very important.

Investigating the potential molecular mechanisms of TSCC metastasis, studies have shown that increased motility and invasion of metastatic tumor cells, the destruction of cellular interactions, and remodeling of the extracellular

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matrix (ECM) facilitate tumor progression (5). The first step of the metastatic process is invasion. Remodeling of the ECM by extracellular proteinases plays a crucial step in tumor cell invasion (6). Matrix metalloproteinases (MMPs) and their tissue inhibitors (tissue inhibitors of MMPs, TIMPs) contribute to degradation of ECM components and remodeling of collagen (7). In addition, it has been reported that not only MMP2 and MMP9 but also MMP3, MMP7, MMP10, MMP11 and MMP14 (also known as membrane type 1 MMP, MT1-MMP) can degrade the ECM and allow TSCC metastatic cells to migrate and invade the target organ, contributing to tumor metastasis (8, 9).

Cetyltrimethylammonium bromide (CTAB, $[(C_{16}H_{33})N(CH_3)_3]Br$), a substance of quaternary ammonium compounds (Quats), is widely used as an antiseptic against fungi and bacteria (10). Quats are reputed to have anticancer properties by restraining cell proliferation, causing loss of mitochondrial membrane potential, elevating the levels of cytosolic Ca^{2+}, and finally leading to cell death (11). More recent research has shown that CTAB is cytotoxic against HNSCC cell lines via the mitochondria-mediated apoptosis pathway (12). These studies revealed that the potent chemopreventive effect of CTAB may be partly derived from apoptotic or cytotoxic processes. However, so far, there are no studies concerning how CTAB affects migration and/or invasion of human TSCC cell lines.

The present study aimed to investigate the anti-metastatic effects of CTAB on human TSCC SCC4 cells in regard to migration and invasion through epithelial-mesenchymal transition (EMT)-associated regulatory pathways in SCC4 cells. The role of CTAB in cell proliferation as well as cell cycle regulation, adhesion, migration and invasion were examined, and the molecular mechanism behind CTAB treatment in SCC4 cells was identified. The results demonstrated that CTAB treatment considerably diminished invasive motility by suppressing TGF-β signaling, down-regulating the Smad2/3/4 pathway and therefore inhibited EMT in SCC4 cells.

Materials and Methods

Cell culture and agents. Human TSCC SCC4 cell line was obtained from Bioresource Collection and Research Center (BCRC number: 60142; Hsinchu, Taiwan, ROC) and maintained in Dulbecco's modified Eagle's medium/Ham's F-12 nutrient mixture (DMEM/F12, 1:1 mixture; Gibco BRL, Grand Island, NY, USA) supplemented with 10% fetal bovine serum (Hyclone Laboratories, Logan, UT, USA), 100 μM non-essential amino acids (Gibco BRL), 100 units/ml penicillin, and 100 μg/ml streptomycin (Invitrogen, Carlsbad, CA, USA) in a humidified atmosphere of 5% CO_2 at 37˚C.

CTAB was purchased from Sigma Chemicals (St. Louis, MO, USA). Recombinant human transforming growth factor-β1 (TGF-β1) was purchased from PeproTech (Rocky Hill, NJ, USA). The Smad2/3 pharmacological inhibitors such as PD169316(#10006727) and SB203580 (#13067), SB-431542 (specifically blocks Smad2 signaling, #13031), and SIS3 (Smad3 Inhibitor, #15945) were purchased from Cayman Chemical (Ann Arbor, MI, USA) (13, 14). All chemicals were dissolved in phosphate-buffered saline (PBS; Invitrogen) or dimethyl sulfoxide (DMSO; Sigma Chemicals) to prepare stock solutions.

Cell viability. The WST-1 colorimetric assay was utilized to analyze cell viability by measuring the activity of mitochondrial malate dehydrogenase (15). Cell proliferation reagent WST-1was purchased from BioVision (Milpitas, CA, USA). The day before drug treatment, the cells were seeded to obtain ~50% confluency at the time of drug treatment.WST-1 reagent was added to cells following a 24-h incubation with 1, 2.5, and 5.0 μM concentrations of CTAB and cells were incubated for a further 3 h. The amount of formazan, which was proportional to the number of viable cells, was calculated by measuring the absorbance at 450 nm with the Epoch 2 microplate spectrophotometer from Biotek Instruments (Winooski, VT, USA). The ratio of the absorbance of treated cells compared to vehicle-treated control cells (defined as 100%) was employed to evaluate the effect of CTAB on cell viability.

Adhesion assay. Approximately 50% confluent cells were pretreated with 1.0, 2.5, and 5.0 μM CTAB or vehicle control for 16 h at 37˚C, and then seeded in plates precoated with collagen type I (EMD Millipore, Billerica, MA, USA) for 1 h at 37˚C (16). After 8 h of incubation, the supernatant with unattached cells was discarded, and attached cells were quantified using the colorimetric WST-1 method.

Cell-cycle analysis. After treatment with 1.0, 2.5, and 5.0 μM CTAB for 24 h, cells were washed twice with ice-cold PBS and then fixed in 70% ethanol overnight at −20˚C. Fixed cells were stained with a solution containing 10 μg/ml of propidium iodide (Sigma Chemicals), 100 μg/ml of RNase A (Sigma Chemicals), and 0.1% Triton X-100 (Sigma Chemicals) for 30 min in the dark at 37˚C. The propidium iodide-labeled cells were subjected to FACScan (BD Biosciences, Bedford, MA, USA) flow cytometry to measure the nuclear DNA content and quantify the cell-cycle distribution.

In vitro migration and invasion assay. Cell migration assay was performed using anIbidi Culture-Insert (Ibidi GmbH, Munich, Germany) and Millicell 24-well hanging inserts with 8 μm pores of polyethylene terephthalate membrane (EMD Millipore). The Ibidi Culture-Insert was mainly developed to replace classical scratch assays. Briefly, the inserts consist of two chambers separated by a 500 μm cell free gap. The cells were left to adhere to 90% confluence and then exposed to 0 and 5.0 μM CTAB within the chambers for 8 h, at which time the cells reached 100% confluency, as required for the migration assay. Transwell inserts were precoated with 50 μl/insert of Matrigel (1 mg/ml, BD Biosciences) for the invasion assay. Cells treated with CTAB were seeded onto the Matrigel-treated filter, and the lower compartment was filled with DMEM/F12 containing 20% fetal bovine serum. Cells underwent 48-h incubation with 1, 2.5, and 5.0 μM CTAB in the upper chamber containing serum-free DMEM/F12. The experimental procedures were identical to the migration assay procedures (17). Migrated and invasive cells were fixed and stained with crystal violet, and then eluted by adding 33% acetic acid. The absorbance at 595 nm was measured with the Epoch 2 microplate spectrophotometer (Biotek Instruments) (18).

Western blot analysis. Cells were washed with cold PBS and then resuspended in radioimmunoprecipitation assay lysis buffer (RIPA

lysis buffer; EMD Millipore) containing protease and phosphatase inhibitors (Roche, Mannheim, Germany). The primary antibodies against human MMP2 (product #87809), MMP9 (#13667), MMP3 (#14351), MMP7 (#71031), MMP13 (#94808), MMP14 (#13130), TIMP1 (#8946), TIMP2 (#5738), TIMP3 (#5673), β-Actin (#4967), phospho-Smad2 (#3108), Smad2 (#5339), phospho-Smad3 (#9520), Smad3 (#9523), and Smad4 (#38454) were purchased from Cell Signaling Technology (Beverly, CA, USA). Total cellular extracts were quantified for protein concentration and equal amounts (20 μg) were subjected to immunoblot following methods described previously (17).

MicroRNA isolation and assessment by real-time quantitative polymerase chain reaction (RT-qPCR). For the detection of miR-181b-5p expression, total miRNA isolation from SCC4 cells treated with 1.0, 2.5 and 5.0 μM CTAB for 16 h was performed using the miRNeasy Mini kit (Qiagen, Hilden, Germany) according to the manufacturer's procedures. Total miRNA was reverse transcribed by using the TaqMan advanced miRNA cDNA synthesis kit (Applied Biosystems, Carlsbad, CA, USA) including the universal RT primer. The expression levels of miR-181b-5p were quantitated with TaqMan advanced microRNA assays (Applied Biosystems) according to the protocol of the manufacturer. The following TaqMan probes were used: hsa-miR-181b-5p (assay#478583_mir) and miRNA control assay U6 small nuclear RNA (assay#001973) (19, 20). Quantitative real-time PCR analysis was performed by the StepOnePlus Real-Time PCR system (Applied Biosystems) according to standard protocol. U6 snRNA was used as internal control for miR-181b. Each cDNA sample was analysed using real-time quantitative PCR in triplicate in each assay. Results were exported and then analyzed with StepOnePlus software. Briefly, the relative expression levels of miR-181b-5p were calculated using the $2^{-\Delta\Delta Ct}$ method by the formula: $2^{-(\text{sample } \Delta Ct - \text{control } \Delta Ct)}$, where the equation ΔCt ($Ct_{miR\text{-}181b\text{-}5p} - Ct_{U6}$) is the difference between the amplification fluorescent thresholds of the gene of interest (miR-181b-5p) and the internal reference gene (U6) used for normalization.

Cell transfection. The miR-181b-5p mimic (assay #MC12442), miR-181b-5p inhibitor (also known as anti-miR-181b-5p, assay #MH12442), and negative control (miR-NC, containing non-targeting scrambled oligonucleotide) sequences were purchased from Ambion Inc. (Austin, TX, USA). Cell transfection was performed with Lipofectamine 2000 (Invitrogen) according to the manufacturer's protocol.

Statistical analysis. The values are presented as the mean±SEM of three independent experiments and were analyzed by one-way analysis of variance using GraphPad Prism software (Graph Pad Software, La Jolla, CA, USA). The significance of differences in two-group comparisons was carried out with Student's *t*-test. Values of $p<0.05$ were considered as statistically significant.

Results

Effect of CTAB on the viability of SCC4 cells. To evaluate the effect of CTAB on the viability of SCC4, we performed WST-1 assay. CTAB was not found to affect cell viability in any of the concentrations and incubation periods examined (Figure 1A). In addition, flow cytometry assay confirmed that treatment with 5.0 μM CTAB did not affect the distribution of SCC4 cells in sub-G1, G0/G1, S, and G2/M phases of the cell cycle (Figure 1B). Therefore, CTAB was used in the subsequent assays at the concentrations of 0, 1.0, 2.5, and 5.0 μM.

CTAB reduces SCC4 cell adhesion. When SCC4 cells were treated with increasing doses of CTAB up to 5.0 μM, a progressive increase in the percentage of detached SCC4 cells was observed, suggesting an effect of CTAB on cell adhesion. To further analyze this phenomenon, we performed an adhesion assay in which SCC4 cells were pretreated with the indicated concentration of CTAB for 16 h, then seeded into a plate coated with type I collagen, a component of extracellular matrix, and the number of cells attached to the plate at 8 h after seeding was quantified. As illustrated in Figure 1C, treatment with 5.0 μM CTAB for 16 h decreased adhesion by 36.4%. Therefore, CTAB regulates adhesion of SCC4 cells.

CTAB alters the morphology of SCC4 cells. Morphological changes of SCC4 cells were detected after treatment with CTAB for 24 h. SCC4 cells treated with 5.0 μM CTAB seemed to lose intercellular junctions and tended to change from the spindle-like shape to the cobblestone-like shape compared with the control group (Figure 1D).

CTAB suppressed cell migratory and invasive abilities of SCC4 cells. Alterations in cell morphology are considered as crucial characteristics of EMT for the onset of tumor progression (21). In addition, recent studies demonstrated that tumor cell migration and invasion contribute to metastasis (22). Therefore, we hypothesized that CTAB could trigger the EMT program in SCC4 cells. To examine our hypothesis, would-healing and transwell assays were performed to investigate whether CTAB modulates migration and invasion of SCC4 cells. As presented in Figure 2A, administration of 5.0 μM CTAB obviously restrained the recolonization of the scratched area compared with the control group. Consistent with the wound healing assay, 5.0 μM CTAB suppressed the ability of SCC4 cells to migrate through the membrane of a transwell insert (Figure 2B). Furthermore, CTAB distinctly inhibited the invasive ability as evidenced by the decreased number of migrated cells in the transwell Matrigel invasion assay (Figure 2C). Taken together, these data suggested that CTAB inhibits the migratory and invasive capabilities of SCC4 cells.

CTAB inhibited the expression of MMP-related proteins. MMPs and their tissue inhibitors of MMPs (TIMPs) are found to play a pivotal role in the metastatic process through the degradation of collagen IV in the basement membrane and gelatin in the ECM (23, 24). To explore whether CTAB affects tumor cell motility by altering the biological activities

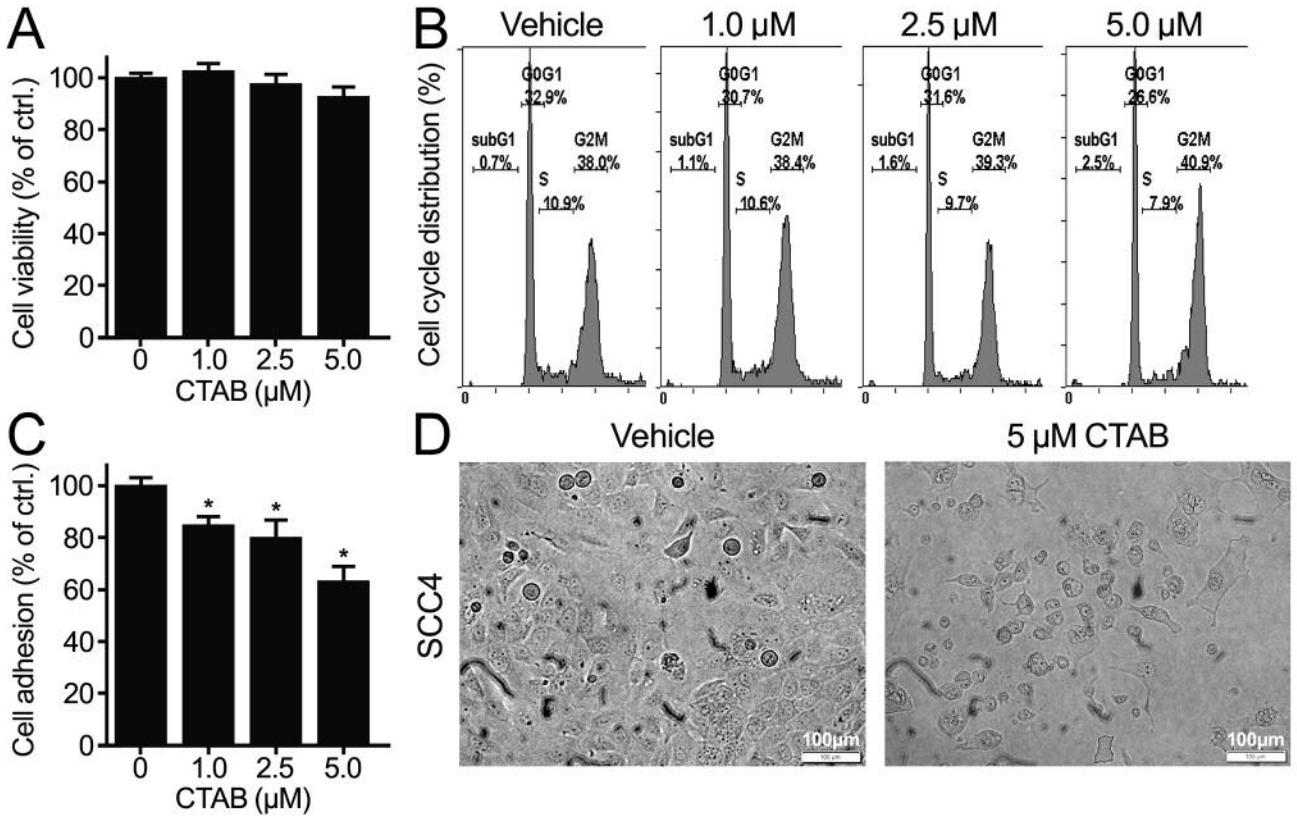

Figure 1. *Cetyltrimethylammonium bromide (CTAB) affects adhesion and morphology of SCC4 cells. A: Cells were treated with the indicated concentrations of CTAB for 24 h and then cell viability was examined with the WST-1 assay. B: Effect of CTAB on the distribution of cells in subG1, G0/G1, S, and G2/M phases of the cell cycle under different treatment conditions. C: To quantify the effect of CTAB on SCC4 cell attachment, a cell adhesion assay was performed. Absorbance values obtained from SCC4 cells treated with vehicle were considered as 100%. D: Morphological alterations in CTAB-treated SCC4 cells were observed using a light microscope. Data shown represent the mean±SEM of three independent experiments performed in duplicate. Statistically significantly different at: *p<0.05 when compared with the vehicle-treated control.*

of MMPs and TIMPs, western blot analysis was used to examine the expression levels of MMPs/TIMPs-related proteins. Our results revealed that the protein levels of particular MMPs, including MMP2, MMP9, MMP3, MMP7, MMP13 and MMP14, were down-regulated compared with the control group when SCC4 cells were treated with 1.0, 2.5, and 5.0 μM CTAB (Figure 3). Meanwhile, the levels of TIMP3 were significantly elevated on SCC4 cells in response to CTAB treatment (Figure 3). These data suggested that CTAB may regulate the expression of a number of defined MMPs and TIMP3 to control ECM remodeling during SCC4 cell migration.

CTAB modulated the levels of MMPs and TIMP3 by down-regulating Smad2/3/4 signaling. To clarify the possible mechanism associated with ECM remodeling triggered by CTAB in SCC4 cells, we further examined the expression levels of well-defined proteins on major signaling pathways involved in ECM remodeling, including the receptor tyrosine kinases (RTKs), transforming growth factor-β (TGF-β), Notch, Wnt, Hedgehog and others (25, 26). We found that CTAB significantly attenuated the phosphorylation of Smad2 and Smad3, and Smad4 protein in SCC4 cells treated with increasing concentrations of CTAB (Figure 4A). By contrast, the total levels of proteins such as Smad2 and Smad3 were not significantly affected (Figure 4A). These results suggested that Smad2, Smad3 and Smad4 might be involved in the CTAB-induced decrease in MMPs expression in SCC4 cells. All the three proteins have been classified as downstream substrates of the canonical TGF-β signaling pathway. Taken together, these findings indicated that the inhibitory effect of CTAB on SCC4 cells is associated with reducing Smad-associated signaling.

CTAB upregulated TIMP3 via down-regulating the expression of the canonical TGF-β signaling and miRNA-181b.

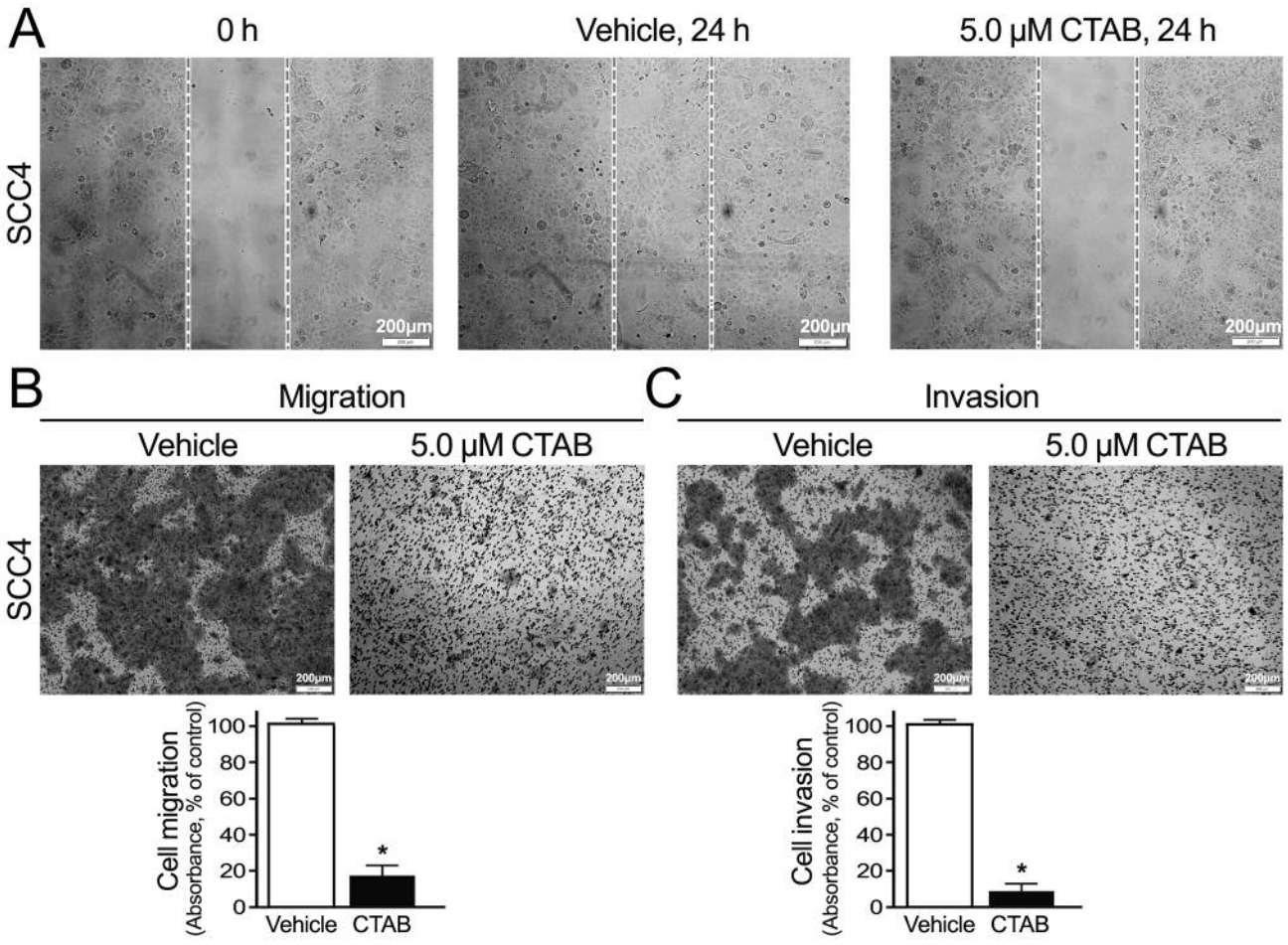

Figure 2. *Cell migration and invasion by SCC4 cells are suppressed by cetyltrimethylammonium bromide (CTAB). A: The migratory ability of CTAB-treated SCC4 cells was evaluated based on wound-healing assays using Ibidi Culture-Insert and compared to that of vehicle-treated cells. Boyden chamber assays (B) and Matrigel invasion assays (C) were utilized to examine cellular migration at the indicated concentrations of CTAB. Data are means±SEM (n=3) performed in duplicates. Scale bars: 200 μm. Statistically significantly different at: *p<0.05 versus the vehicle-treated control.*

MicroRNAs (miRNAs) have shown to influence tumor progression, including cell viability, cell cycle control, metabolism, invasion and metastasis (27). In the present study, our western blot data revealed strong effects on several members of the MMPs and TIMPs, specifically TIMP3 that was upregulated and MMP3, MMP9, MMP13 and MMP14 that were down-regulated by CTAB treatment in a concentration-dependent manner (Figure 3). Previous studies have indicated that TIMP3 is a target of miR-181b and is involved in tumorigenesis (28, 29). The present study determined whether the miR-181b/TIMP3 axis is involved in regulating the mesenchymal characteristic of SCC4 cells following treatment with CTAB. The expression levels of miR-181b were measured in SCC4 cells treated with increasing concentrations of CTAB. As shown in Figure 4B, miR-181b expression was down-regulated in SCC4 cells following CTAB treatment. In addition, to investigate the possible involvement of Smad2/3 in CTAB-attenuated miR-181b expression, SCC4 cells were treated with previously described inhibitors of Smad2 and/or Smad3 phosphorylation, including PD169316, SB203580, SB431542 and SIS3 (13, 14). The results showed that treatment with these inhibitors of Smad2/3 phosphorylation also down-regulated the expression of miR-181b (Figure 4B). These results suggested that the down-regulation of miR-181b in response to CTAB treatment might require the Smad2/3 signaling.

To fully understand the relationship between miR-181b and TIMP3, we next examined whether miR-181b could regulate TIMP3 expression on the protein level in SCC4 cells. The miR-181b-5p mimic or its inhibitor was

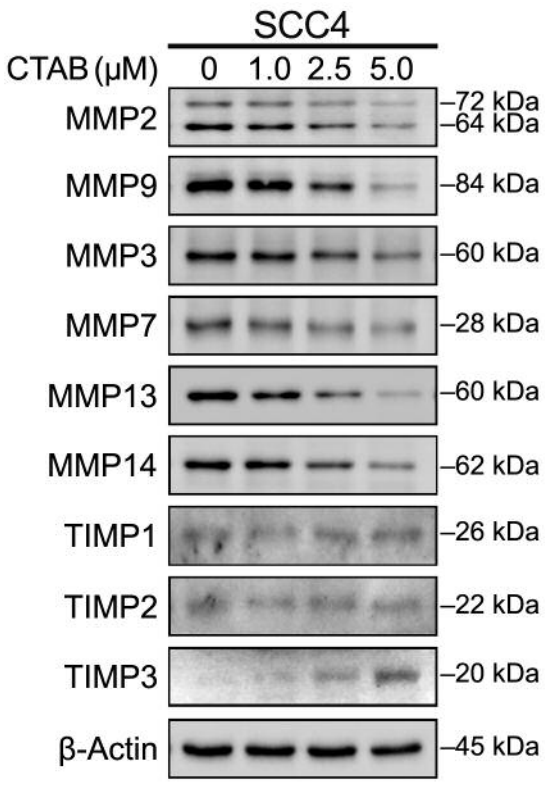

Figure 3. *Cetyltrimethylammonium bromide (CTAB) suppresses the expression of extracellular matrix (ECM)-remodeling associated proteins such as matrix metalloproteinases (MMPs) and tissue inhibitors of metalloproteinases (TIMPs). SCC4 cells were treated with vehicle or CTAB (1.0, 2.5, and 5.0 μM) for 16 h, and then whole-cell extracts were subjected to western blot analysis. Representative western blot images showing the protein levels of MMPs and TIMPs in CTAB-treated SCC4 cells. β-Actin was used as a loading control. The results are representative of three independent experiments.*

transfected into SCC4 cells, and the levels of TIMP3 protein were analyzed. SCC4 cells transfected with miR-181b-5p inhibitor showed increased expression of endogenous TIMP3 protein relative to the cells transfected with miR-NC (Figure 4C). Furthermore, the over-expression of miR-181b-5p mimic had no significant effect on TIMP3 protein expression compared with the control (Figure 4C). The data shown in Figure 4C indicate that miR-181b regulated TIMP3-mediated remodeling of the ECM components and transcription of metastasis-related genes in SCC4 cells. Taken together, these findings indicated that CTAB inhibited SCC4 cell metastasis through upregulation of MMPs inhibitor, TIMP3, via Smad-driven expression of miR-181b.

TGF-β/Smad/miR-181b/TIMP3 signaling axis might be involved in CTAB-mediated EMT. Accumulating findings suggest that EMT process is modulated through multiple signaling pathways including the TGF-β/Smad pathway. Smads are widely recognized as important downstream transcript factors in the TGF-β signaling pathway (30). To explore the detailed mechanisms of CTAB in regard to EMT, we verified the effect of CTAB on TGF-β signaling pathway in SCC4 cells. Cells pretreated with or without TGF-β1 were subsequently incubated with CTAB. Our results showed that treatment with TGF-β1 significantly restored the levels of MMP2, MMP3, MMP7, MMP9, MMP13, and MMP14 that were reduced by CTAB (Figure 5A). Furthermore, the CTAB-induced increase in TIMP3 expression was significantly attenuated by treatment of TGF-β1 (Figure 5A). As illustrated in Figure 5B, treatment of SCC4 cells with TGF-β1 and CTAB significantly restored the decreased phosphorylation of Smad2, Smad3 and Smad4 compared to cells treated with CTAB in the absence of TGF-β1. Moreover, we measured the expression of miR-181b upon treatment with TGF-β1 and CTAB. TGF-β1 treatment considerably reduced the ability of CTAB to inhibit the Smad-mediated increase in miR-181b (Figure 5C). Furthermore, addition of TGF-β1 also reversed the inhibitory effect of CTAB on migratory and invasive abilities of SCC4 cells (Figure 5D). Altogether, these data revealed the essential role of TGF-β/Smad axis in EMT progression and proposed that the canonical TGF-β signaling could be involved in the inhibitory effects of CTAB on EMT in SCC4 cells.

Discussion

This study demonstrates for the first time that CTAB inhibits the mesenchymal features in TSCC SCC4 cells. CTAB significantly induced morphological changes, and attenuated cell adhesion, migratory and invasive capacities in SCC4 cells. We also found that the inhibitory effects of CTAB on ECM remodeling may be mediated by the down-regulation of the levels of TGF-β-mediated Smads, miR-181b, TIMP3 and MMPs. In addition, CTAB down-regulated the expression levels of MMPs and increased the levels of TIMP3 protein.

CTAB has been reported to exhibit positively charged micelles, which allow its binding to the negatively charged surface of cells (31). Compared to noncancerous cells, malignant tumor cells often show increased exposure of negatively charged phosphatidylserine (PS) on the external surface (32). An increased cytotoxicity of CTAB on tumor cells compared to normal cells may be due to differences in the electrostatic attraction between the negatively charged PS of tumor cells, compared to normal cells, and the positively charged CTAB. Therefore, it can be hypothesized that CTAB would strongly bind and kill tumor cells through direct interaction with negatively charged surface-exposed PS. Previous studies have described that CTAB has the ability to induce apoptosis on chondrosarcoma and HNSCC through mitochondrial dysfunction (33, 34). EMT is characterized by

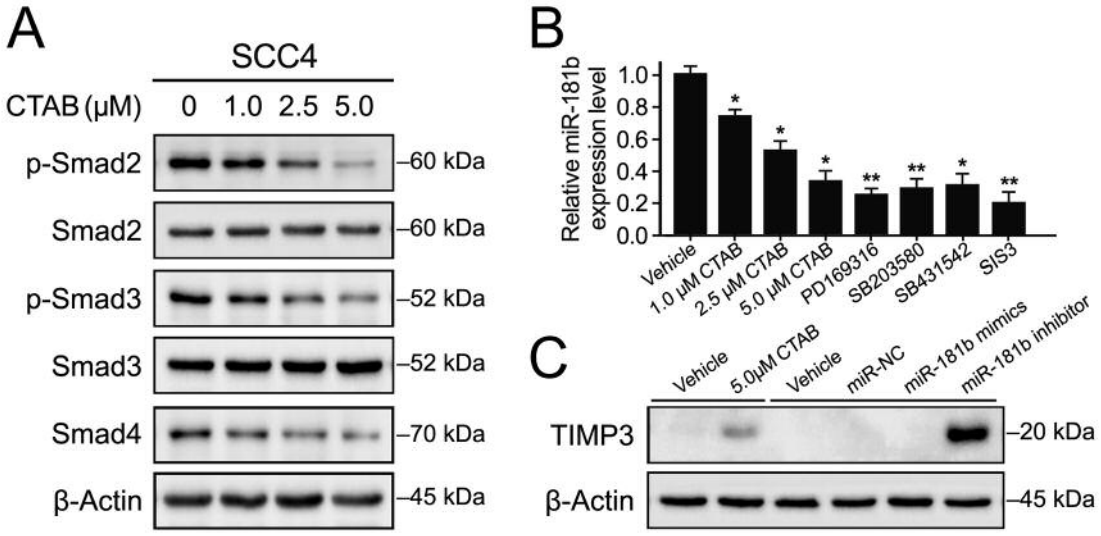

Figure 4. *Cetyltrimethylammonium bromide (CTAB) up-regulates tissue inhibitor of metalloproteinase 3 (TIMP3) via down-regulating transforming growth factor β (TGFβ)/receptor-regulated Smads (R-Smads)/miR-181b signaling axis in SCC4 cells. A: Western blot analysis of the levels of the canonical TGF-β signaling-related Smad proteins such as the phosphorylated or the total forms of Smad2, Smad3 and Smad4 in cells treated with CTAB. B: Relative expression levels of miR-181b were determined by quantitative RT–PCR in SCC4 cells treated with CTAB (1.0, 2.5 and 5.0 μM), PD169316 (10 μM), SB203580 (10 μM), SB-431542 (10 μM) and SIS3 (5 μM) for 16 h, respectively. C: The protein expression levels of TIMP3 were analyzed in SCC4 cells transfected with miR-181b-5p mimic, miR-181b-5p inhibitor or miR-NC by western blotting. β-Actin was used as a loading control. The results are representative of three independent experiments. β-Actin was used as an internal control. Statistically significantly different at: *p<0.05 and **p<0.01 versus the vehicle-treated control.*

cell morphological and biochemical changes. Currently, our attention is focused on the effects of CTAB on EMT and metastasis of HNSCC cell lines. In this study, our data suggest that CTAB is involved in the suppression of TGF-β-mediated mesenchymal features. In addition, current evidence shows that TGF-β participates in EMT, mediates tumor metastasis, destruction of cell adhesion, migration, and invasion of HNSCC cells (35). Furthermore, TGF-β signaling has been shown to be associated with tumor initiation and progression of HNSCC in mouse models (36). Each class of TGF-β ligands bind with its particular TGF-β type II receptor and then initiates downstream signaling pathways, including the canonical (Smad-dependent) and non-canonical (Smad-independent) TGF-β signaling (35). Both canonical and non-canonical TGF-β signaling pathways have been implicated in EMT. Canonical TGF-β signaling is initiated through the binding of ligands to their corresponding receptors, constituting a ligand-receptor complex that phosphorylates receptor-activated Smads (R-Smads). Phosphorylated R-Smads, including Smad2 and Smad3, bind with the common Smad (Smad4) and regulate the expression of target genes involved in EMT (37). In the present study, we observed that treatment with CTAB decreased phosphorylation of Smad2 and Smad3 and reduced protein levels of Smad4 in SCC4 cells. Thus, the results of our study suggest that CTAB inhibits TGF-β-mediated EMT in SCC4 cells by blocking the phosphorylation of both Smad2 and Smad3, and the expression levels of Smad4.

TIMP3, a secreted protein, inhibits the proteolytic activity of MMPs and is involved in the remodeling of ECM. TIMP3 has been considered to be associated with cancer invasion and metastasis. Accumulating studies have shown the tumor-suppressive role of TIMP3 in several cancer types, including brain, thyroid, lung, kidney and colon (38). In addition to being regulated in response to mitogenic stimulation, TIMP3 is also regulated by several other molecules including microRNAs (39). Recent studies have demonstrated that the expression of TIMP3 was down-regulated through modulating of microRNAs, including miR-21, miR-181b, miR-221, and miR-222 (38, 39). However, the role of miR-181b in SCC4 cells treated with CTAB remains unclear. Here, we found that the levels of miR-181b were decreased by blocking the TGF-β/Smad signaling pathway (Figure 4B). In addition, transfection of miR-181b inhibitor into SCC4 cells obviously induced TIMP3 expression compared to cells transfected with miR-NC (Figure 4C). We also observed that down-regulation of miR-181b suppressed migration and invasion of SCC4 cells, and that increasing miR-181b expression with the addition of TGF-β1 could rescue this effect (Figure 5C and D).

The present study demonstrated that TIMP3 expression was increased and that miR-181b expression was inhibited

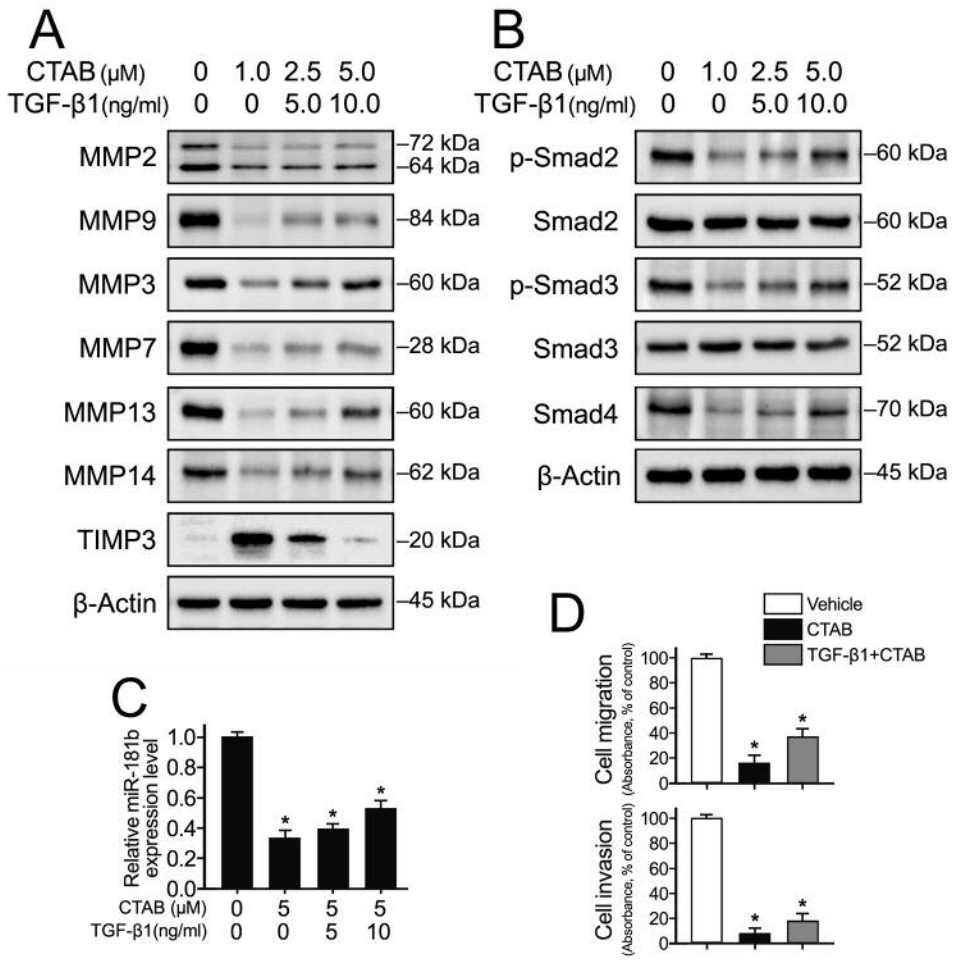

Figure 5. *Transforming growth factor β (TGF-β)-dependent receptor-regulated Smads (R-Smads)/miR-181b/tissue inhibitors of metalloproteinase 3 (TIMP3) signaling pathway is involved in the suppressive effect of cetyltrimethylammonium bromide (CTAB) on TGF-β1-mediated SCC4 cell epithelial–mesenchymal transition (EMT). A: Cells were pretreated with vehicle or TGF-β1, the ligand of the TGF-βreceptor (5 or 10 ng/ml) for 4 h, and then co-treated with CTAB for another 16 h. Expression of matrix metalloproteinases (MMPs) and their natural occurring inhibitors-tissue inhibitors of metalloproteinases (TIMPs)- required in remodeling of the ECM (A) and the phosphorylation and total protein levels of the canonical Smad signaling axis (B)in CTAB-treated cells with and without TGF-β1 were analyzed by western blot. β-Actin reprobing was used to confirm equal loading. C: SCC4 cells were preincubated with TGF-β1 for 4 h prior to addition of CTAB for12 h and then the relative expression levels of miR-181b were determined by quantitative RT–PCR. D: TGF-β1 rescued the CTAB-mediated inhibition of motility as analyzed using the transwell migration and Matrigel invasion assays. TGF-β1(10 ng/ml) reversed the effects of CTAB (5.0 μM) on cell migration and invasion as indicated by the transwell assay. The results are presented as a percentage of the control (100%). Statistically significantly different at: *p<0.05 compared with the vehicle control.*

in SCC4 cells treated with CTAB. Our findings are consistent with those of previous studies (25, 26). In addition, our findings suggested that down-regulation of miR-181b decreased the migration and invasion of SCC4. Moreover, rescue experiments showed that treatment with TGF-β1 reversed the activity of Smad/miR-181b/TIMP3/MMPs signaling axis, the mobility and invasiveness of SCC4 cells.

In conclusion, the results of this study illustrated that treatment with CTAB inhibited the mesenchymal characteristics and induced morphological changes in SCC4 cells via suppressing the canonical TGF-β/Smads/miR-181b/TIMP3/MMPs signaling axis. Thus, CTAB may be valuable as a potential therapeutic agent to inhibit EMT and metastasis of SCC4 cells.

Conflicts of Interest

All Authors declare that they have no conflicts of interest in regard to this study.

Authors' Contributions

Conceptualization: CJL; funding acquisition: CHY and CHC; methodology and validation: YRP and YPC; writing of the original draft: FMH and CJL; reviewing and editing: CHY and CHC. All Authors read and approved the final article.

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