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Cetyltrimethylammonium Bromide Attenuates the Mesenchymal Characteristics of Hypopharyngeal Squamous Cell Carcinoma Through Inhibiting the EGFR/PI3K/AKT Signaling Pathway

TSAI-KUN WU[1,2*], YEN-CHUAN OU[3*], YI-PING CHEN[4], FU-MEI HUANG[5], YING-RU PAN[6] and CHIA-JEN LEE[6,7]

[1]*Division of Renal Medicine, Tungs' Taichung Metroharbor Hospital, Taichung, Taiwan, R.O.C.;*
[2]*Department of Nursing, Jen-Teh Junior College of Medicine, Nursing and Management, Miaoli, Taiwan, R.O.C.;*
[3]*Division of Urology, Department of Surgery, Tungs' Taichung Metroharbor Hospital, Taichung, Taiwan, R.O.C.;*
[4]*Division of Anatomical Pathology, Tungs' Taichung Metroharbor Hospital, Taichung, Taiwan, R.O.C.;*
[5]*Operating Theatre, Chung Shan Medical University Hospital, Taichung, Taiwan, R.O.C.;*
[6]*Department of Medical Research, Tungs' Taichung Metroharbor Hospital, Taichung, Taiwan, R.O.C.;*
[7]*Department of Rehabilitation, Jen-Teh Junior College of Medicine, Nursing and Management, Miaoli, Taiwan, R.O.C.*

Abstract. *Background/Aim: Cetyltrimethylammonium bromide (CTAB), a quaternary ammonium surfactant, was shown to have antitumor effects in a cellular mode of head and neck squamous cell carcinoma (HNSCC), modulating apoptotic and cytotoxic processes. However, the mechanisms by which CTAB exerts its effects against the epithelial–mesenchymal transition in HNSCC remain poorly understood. In the present study, we investigated whether CTAB inhibits cellular mobility and invasiveness of hypopharyngeal squamous cell carcinoma (HPSCC) cells. Materials and Methods: WST-1, cell-cycle phase distribution, and wound healing, as well as transwell assays were conducted. Changes in protein expression patterns and related signaling pathways involved in effects of CTAB on HPSCC cell lines were evaluated by western blotting. Results: Treatment of human HPSCC cell lines with CTAB significantly altered their morphology from spindle-like to cobblestone-like by diminishing mesenchymal-like phenotypic characteristics. CTAB also hindered cell functional properties, including migration and invasion, independently of cell viability. In addition, western blot results demonstrated that treatment with CTAB reduced expression of mesenchymal markers. Further investigation showed that CTAB treatment suppressed the phosphorylation of extracellular-regulated kinase 1/2, mechanistic target of rapamycin kinase and AKT serine/threonine kinase 1. CTAB also repressed the expression and phosphorylation levels of epidermal growth factor receptor (EGFR) and phosphatidylinositol-4,5-bisphosphate 3-kinase (PI3K), and the partial restoration of mesenchymal phenotype by EGF addition confirmed that CTAB inhibited migration and invasion in HPSCC cells by blocking the EGFR signaling pathway. Conclusion: Our results suggest that CTAB is involved in the suppression of EGFR-mediated mesenchymal phenotype and the molecular mechanism by which CTAB obstructs HPSCC cell metastasis may represent a promising strategy for use in HPSCC treatment.*

*These Authors contributed equally to this study.

Correspondence to: Chia-Jen Lee, Department of Medical Research, Tungs' Taichung MetroHarbor Hospital, No.11, Lane 194, Wenhua Street, Wuqi District, Taichung 43549, Taiwan, R.O.C. Tel: +886 426581919 #59004, e-mail: chiajenlee54@gmail.com; Ying-Ru Pan, Department of Medical Research, Tungs' Taichung Metroharbor Hospital, No.699, Sec. 8, Taiwan Blvd., Taichung 435, Taiwan, R.O.C. Tel: +886 426581919 #59043, e-mail: ivy52071@gmail.com

Key Words: Cetyltrimethylammonium bromide, CTAB, hypopharyngeal squamous cell carcinoma, HPSCC, epithelial-to-mesenchymal transition, EMT, epidermal growth factor, EGF.

Hypopharyngeal squamous cell carcinoma (HPSCC) originates in the mucosal epithelium of the pharynx, which suggests that HPSCC should be categorized as head and neck squamous cell carcinoma (HNSCC). HNSCCs represented about 4.9% of all new cancer cases worldwide in 2020 (1). In spite of the fact that approximately 90% of HNSCCs develop from the squamous cell lining of the head and neck region, the disease characteristics of HNSCCs are remarkably heterogeneous (2). This may be attributed to the complicated anatomical structures and the variety of causes of HNSCCs, such as tobacco smoking, alcohol abuse and

chewing betel quid (3, 4). Early-stage (I or II stage) HPSCC is relatively rare because of its nonspecific symptoms. However, the vast majority of patients with HPSCC with symptoms including dysphagia and odynophagia are usually diagnosed at an advanced stage (stage III or IV) (5). Metastasis of HPSCC commonly occurs in the regional lymph node basin of the neck because of the rich lymphatics in this region (6). HPSCC portends poor prognosis among the worst of all HNSCCs because of higher rates of local recurrence and metastasis (5, 7). Studies of clinical cases have indicated that the overall 5-year relative survival rate for patients with advanced-stage HPSCC has been reported to be less than 30%, and the leading causes of HPSCC-related mortality are due to invasion and metastasis (7-9).

Tumor metastasis, a principal feature of cancer malignancy, is an unsolved clinical challenge in cancer treatment. It has been recognized that suppression of tumor metastasis is a crucial approach in therapeutics of cancer (10). For this reason, evaluation of the absence or presence of metastases in patients diagnosed with HPSCC is indispensable. Investigating the initiation of metastasis for tumor progression, previous research has demonstrated that there must be changes in the physiological state of the cells, including an increase of cellular motility, the disruption of cellular interactions among adjacent cells, the attenuation of cell–extracellular matrix (ECM) adhesion, and the degradation of the ECM (11, 12). In addition to these, epithelial–mesenchymal transition (EMT) is thought to be involved in cancer metastasis (11). Epithelial cells undergoing EMT obtain mesenchymal characteristics leading to rearrangement cytoskeletal organization, reduction of epithelial cell attachment, enhancement of migratory activity and gain of mesenchymal potential, resulting in increased motility and invasive abilities (12). The characteristics of the EMT procedures involve the reduction of expression of epithelial-associated markers such as E-cadherin, and the increased expression of mesenchymal markers and those transcriptional repressors such as snail family transcriptional repressor 2 (SNAI2, formerly SLUG) and twist family bHLH transcription factor 1 (TWIST) (13, 14). EMT is postulated to play a critical role in migration and invasion of cancer cells. Thus, the inhibition of EMT is important for prevention of tumor metastasis.

Cetyltrimethylammonium bromide (CTAB, C19H42BrN) also known as cetrimonium bromide, a derivative of quaternary ammonium bromide, is primarily used as an antiseptic agent against bacteria and fungi (15). CTAB possesses positively charged components which allows it to bind readily to the negatively charged surface of organisms (15). CTAB has been demonstrated to exhibit cytotoxic and anticancer properties against cancerous cells through restraining cellular proliferation, increasing the level of cytosolic Ca^{2+}, causing loss of mitochondrial membrane potential, and eventually resulting in cell death (16, 17). Mitochondria-mediated apoptotic effects of CTAB on chondrosarcoma and HNSCC have also been described (18, 19). Additionally, our previous study on CTAB found that it significantly inhibited migratory and invasive capacities of hepatocellular carcinoma cells in vitro through the reducing the expression of EMT-associated proteins (20, 21). The results of these studies suggest that the potent chemopreventive activity of CTAB may be partly derived from cytotoxic processes or mesenchymal–epithelial transition. Although HPSCC accounts for only 3-5% of all HNSCCs, patients with HPSCC have the worst prognosis among the different types of HNSCC (22, 23). Despite the documented anticancer properties of CTAB, there is no relevant literature regarding its influence on HPSCC metastasis and EMT.

The present study aimed to investigate the effects and mechanism of action of CTAB on HPSCC FaDu and D562 cells in regard to migration, invasion and their associated changes.

Materials and Methods

Cell culture and agents. Human HPSCC FaDu and D562 (also known as Detroit 562) cell lines were obtained from Bioresource Collection and Research Center (BCRC number: 60214 and 60119, respectively; Hsinchu, Taiwan, ROC) were maintained in Eagle's minimum essential medium (EMEM; 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 containing 5% CO_2 at 37˚C.

CTAB was purchased from Sigma Chemicals (St. Louis, MO, USA). Epidermal growth factor (EGF) was purchased from PeproTech (Rocky Hill, NJ, USA). The receptor tyrosine kinase (RTK) inhibitor genistein, mesenchymal-epithelial transition factor (MET) tyrosine kinase inhibitor (TKI) SU11274, fibroblast growth factor receptor (FGFR) TKI (PD166866), platelet-derived growth factor receptor (PDGFR) TKI (SU16f), and EGFR TKIs AG-528 and lapatinib were purchased from Cayman Chemical (Ann Arbor, MI, USA). All chemicals were dissolved in phosphate-buffered saline (PBS; Invitrogen) or dimethyl sulfoxide (Sigma) to prepare stock solutions.

Cell viability. WST-1 colorimetric assay was utilized to analyze cell viability by measuring the activity of mitochondrial malate dehydrogenase (24). Cell proliferation reagent WST-1 was 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 that underwent a 24-h incubation with 1, 2.5 and 5 μM 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 signal at 450 nm with an enzyme-linked immunosorbent assay microplate reader. 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. Cells at approximately 50% confluence were treated with 1, 2.5 and 5 μM of CTAB or vehicle control for 24 h at 37˚C in plates precoated with collagen type I (EMD Millipore, Billerica, MA, USA) for 1 h at 37˚C (17). After 6 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, 2.5 and 5 μM of 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), 100 μg/ml of RNase A (Sigma), and 0.1% Triton X-100 (Sigma) 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 an Ibidi 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. 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 EMEM containing 20% fetal bovine serum. Cells underwent 48-h incubation with 1, 2.5 and 5 μM of CTAB in the upper chamber containing serum-free EMEM. The experimental procedures were identical to the migration assay procedures (25). 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 a microplate reader (26).

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 matrix metalloproteinase-9 (MMP9) (product #13667), MMP2 (#87809), vimentin (#5741), N-cadherin (#13116), snail family transcriptional repressor 2 (SNAI2; #9585), TWIST (#69366), β-actin (#4967), phospho (p-)phosphatidylinositol-4,5-*bis*phosphate 3-kinase (PI3K) p85 (Tyr458, #4228), PI3K p85 (#4257), phospho-AKT serine/threonine kinase 1 (AKT) (Ser473, #4060), phospho-AKT (Thr308; #13038), AKT (#4691), phospho-mechanistic target of rapamycin kinase (mTOR, Ser2448; #5536), mTOR (#2983), phospho-extracellular-regulated kinase 1/2 (ERK1/2, Thr202/Tyr204; #4370), ERK1/2 (#4695), phospho-EGFR (Tyr1068; #2234) and EGFR (#4267) 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 (25).

Statistical analysis. The values are presented as the mean± standard error 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

Morphological changes modulated by CTAB. To explore possible differences in cell morphology with CTAB treatment, we exposed two HPSCC cell lines, FaDu and D562, to increasing concentrations of CTAB for 24 h. As shown in Figure 1A, administration of 5.0 μM of CTAB significantly altered the morphology of FaDu and D562 cells from a fibroblast-like to a cobblestone-like pattern.

CTAB impeded cell adhesion of FaDu and D562 cells. Reduced adherence was observed after the treatment of FaDu and D562 cells with CTAB. The effect of CTAB on cells in suspension compared to the adherent state was quantified and the results are shown in Figure 1B. CTAB inhibited the cell adhesion of FaDu and D562 cells in a dose-dependent manner, with 40.7% and 50.1% inhibition, respectively, at 5.0 μM after incubation for 24 h.

Cytotoxicity of CTAB to FaDu and D562 cells. To clarify the role of CTAB in HPSCC proliferation, human HPSCC FaDu and D562 cell lines were analyzed to detect cell viability through WST-1 assay. After a 24-hour treatment with CTAB up to 5.0 μM, the cell viability of FaDu and D562 cells was not significantly affected by CTAB as compared to the untreated control (Figure 1C). The effect of CTAB on apoptosis was determined by flow cytometry. There were no significant increases of the sub-G_1 hypodiploid population in CTAB-treated FaDu and D562 cells compared to those treated with vehicle alone (Figure 1D). These results indicated that CTAB was not toxic to FaDu and D562 cells at these concentrations.

Effect of CTAB on cell-cycle distribution. In order to elucidate the role of CTAB on the cell-cycle distribution, the relative ratios of DNA content at respective phases were analyzed by flow cytometry. After 24 h of treatment, there were no significant differences in the cell-cycle distribution of CTAB-treated FaDu and D562 cells compared to the vehicle control (Figure 1D). Overall, CTAB had no influence on growth and survival through cell-cycle arrest and apoptosis (sub-G_1 phase) in FaDu and D562 cell lines.

CTAB suppressed cell migratory and invasive behaviors of FaDu and D562 cells. The visible changes in the cell morphology of FaDu and D562 cells treated with CTAB were reminiscent of cells that have undergone mesenchymal to epithelial transition (Figure 1A). Additionally, accumulating evidence indicates that tumor-cell migration and invasion contribute to metastatic transmission (24). Therefore, wound-healing and transwell assays were performed to identify the effect of CTAB on cellular migration of FaDu and D562 cells. Compared to untreated cells, CTAB caused a significant

reduction of the recolonization of the scratched area in the presence of 5.0 μM CTAB after incubation for 24 h in both cell lines (Figure 2A). In addition, administration of 5.0 μM of CTAB also notably inhibited the ability of FaDu and D562 cells to migrate through the membrane of a transwell insert (Figure 2B). Further, the potential of CTAB to interfere with invasion of FaDu and D562 cells were examined using the transwell Matrigel invasion assay. As show in Figure 2B, CTAB obviously suppressed the invasion of these two HPSCC cell lines, with 87.6% and 91.3% inhibition, respectively, in cell movement to the lower side of the chamber at 5.0 μM after 48 h. These results indicated that CTAB may have potential as an anti-metastatic agent in human HPSCC.

CTAB mediated down-regulation of MMPs. MMP2 and MMP9 are known to play essential roles in the generation of tumor metastasis through the degradation of collagen IV in the basement membrane and gelatin in the ECM (25). In order to assess the possible anti-metastatic impacts of CTAB, we examined the effects of treating cells with increasing concentrations of CTAB (0-5.0 μM) on the expression of MMP2 and MMP9 in FaDu and D562 cells by immunoblot analysis. In the absence of CTAB treatment, FaDu and D562 cells constitutively expressed relatively high levels of MMP2 and MMP9 protein. After 24 h of treatment, CTAB inhibited the protein expression of both MMP2 and MMP9 in a concentration-dependent manner (Figure 3).

Reversal of EMT phenotype upon treatment with CTAB. To further characterize how CTAB modulates EMT, immunoblotting was used to measured the expression levels of EMT-associated proteins in FaDu and D562 cell lines following treatment with CTAB. Compared with the controls, CTAB-treated cells displayed an obvious reduction in mesenchymal marker proteins, SNAI2, vimentin, N-cadherin and TWIST (Figure 3). We thus assumed that the influence of CTAB on the migration and invasion of FaDu and D562 cells may be associated with the reversal of the mesenchymal phenotypic characteristics.

Down-regulation of PI3K/Akt/mTOR and ERK1/2 signaling pathway by CTAB. Overall, the data from our present study suggested that CTAB interfered with migration and invasion of HPSCC cells by diminishing MMPs, cell motility, and mesenchymal features. The plausible signaling pathways for CTAB-mediated loss of mesenchymal characteristics were therefore further investigated. Several signaling pathways are known to participate in the process of EMT, include RTKs, transforming growth factor-β, NOTCH, WNT, Hedgehog and other signaling pathways (27, 28). In order to identify the signaling pathways involved in suppression of expression of proteins associated with cell migration and invasion changed by CTAB, we used a western blot-based screening platform. We found that CTAB significantly attenuated the phosphorylation of PI3K, AKT, mTOR, and ERK1/2 in FaDu and D562 cells treated with increasing CTAB concentrations (Figure 4). By contrast, the total levels of the cognate proteins PI3K, AKT, mTOR, and ERK1/2 were not significantly different (Figure 4). Taken together, these findings indicated that the inhibitory effect of CTAB on FaDu and D562 pharyngeal carcinoma cells is associated with reducing of the activities of PI3K/AKT/mTOR and other signaling proteins.

CTAB suppression of mesenchymal phenotype is mediated by the EGFR signaling pathway. To characterize which cell surface receptors are involved in inactivation of the PI3K/AKT/mTOR-associated upstream signaling pathways of CTAB-reduced mesenchymal phenotype, we treated FaDu and D562 cells with small-molecule inhibitors of upstream signaling proteins. In addition to the changes in cell morphology (data not shown), we found that the migratory capacity was reduced by 65% ($p<0.05$) and 72% ($p<0.05$), respectively, when FaDu and D562 cells were treated with the RTK inhibitor genistein (Figure 5A).

Next, to further investigate which cell surface receptors endowed with protein tyrosine kinase activity play an important role in HPSCC cell motility, we analyzed the effects of inhibitors AG-528 and lapatinib (EGFR kinase), PD166866 (FGFR1 kinase), SU16f (PDGFR kinase), and SU11274 (MET kinase) on the inhibition of cell migration. The results revealed that the EGFR TKIs AG-528 and lapatinib clearly attenuated cell migration, whereas other RTK inhibitors, including those of MET, FGFR and PDGFR, had no significant impact (Figure 5A). These findings suggest that the inhibitory effect of CTAB might be through the EGFR-dependent signaling pathway in FaDu and D562 cells.

EGF rescued CTAB-mediated suppression of the mesenchymal phenotype through EGFR/PI3K/AKT/mTOR signaling. Indeed, the role of RTKs during EMT has been illustrated in many studies. Current evidence indicates that

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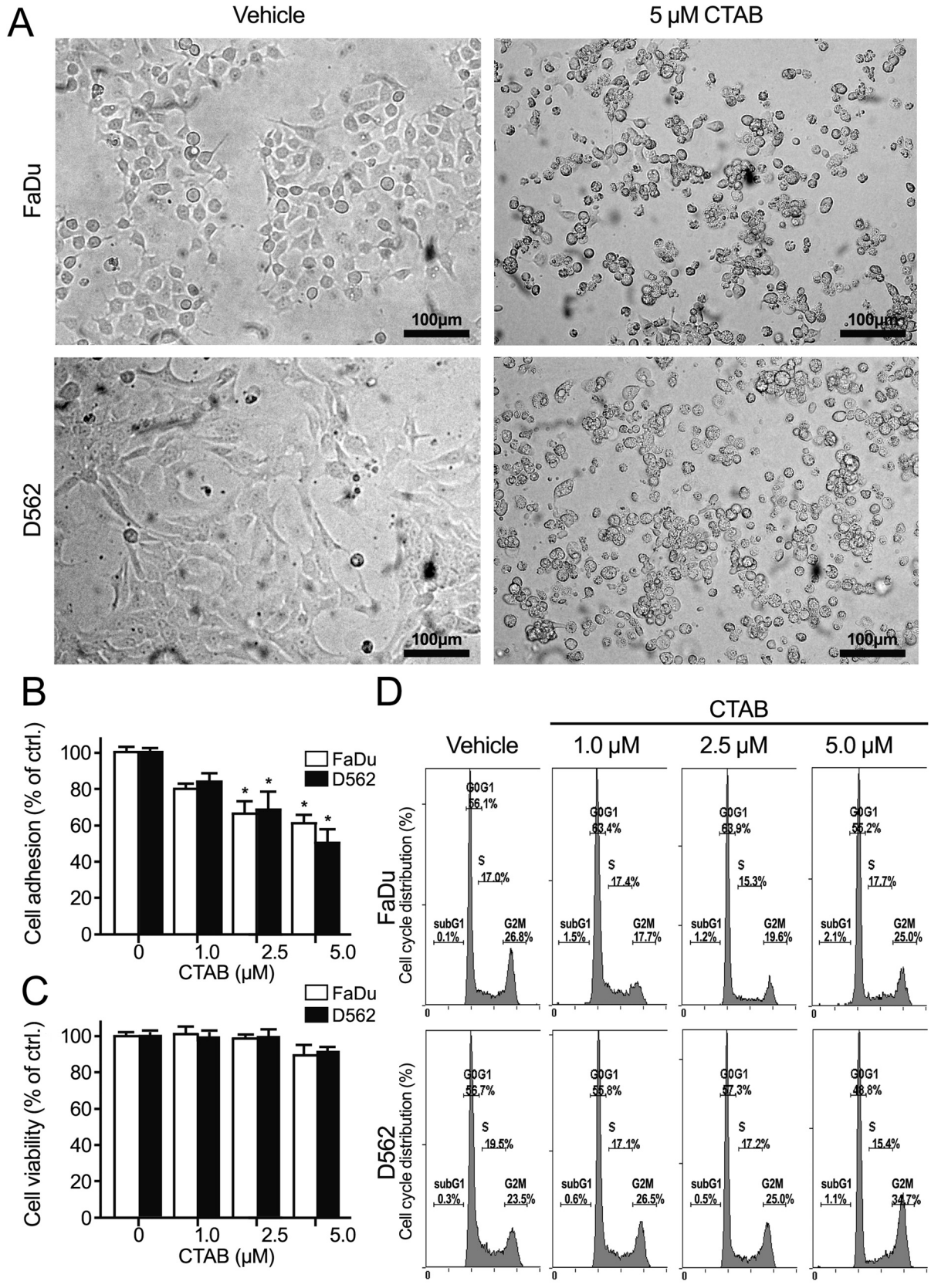
Figure 1. *Cetyltrimethylammonium bromide (CTAB) influences adhesion and morphology of FaDu and D562 cells. A: Morphological alterations in CTAB-treated FaDu and D562 cells were compared with light microscopy. B: To quantify the effect of CTAB on FaDu or D562 cell attachment, a cell adhesion assay was performed. Absorbance values obtained from FaDu and D562 cells treated with vehicle were taken as 100%. C: Cells were treated with the indicated concentrations of CTAB for 24 h and then cell viability was examined with the WST-1 assay. D: Effect of CTAB on the distribution of cells in sub-G_1, G_0/G_1, S, and G_2/M phases of the cell cycle under different treatment conditions. 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.*


A
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B
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G0G1
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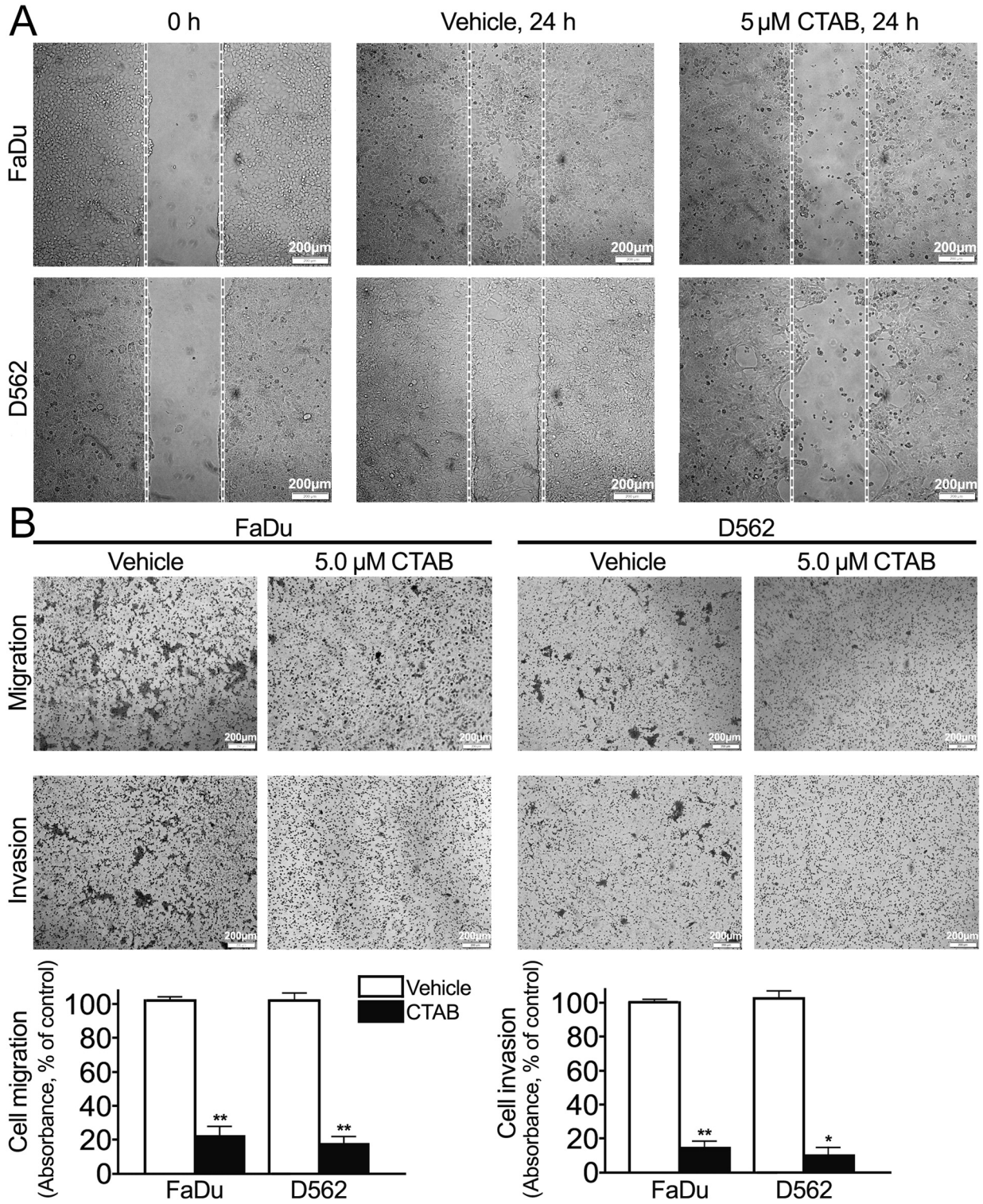

Figure 2. *Cell migration and invasion by FaDu and D562 cells are suppressed by cetyltrimethylammonium bromide (CTAB). A: The migratory ability of CTAB-treated FaDu and D562 cells was evaluated based on wound-healing assays via Ibidi Culture-Insert and compared to that of vehicle-treated cells. B: Boyden chamber assays and Matrigel invasion assays 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 and **p<0.01 versus the vehicle-treated control.*

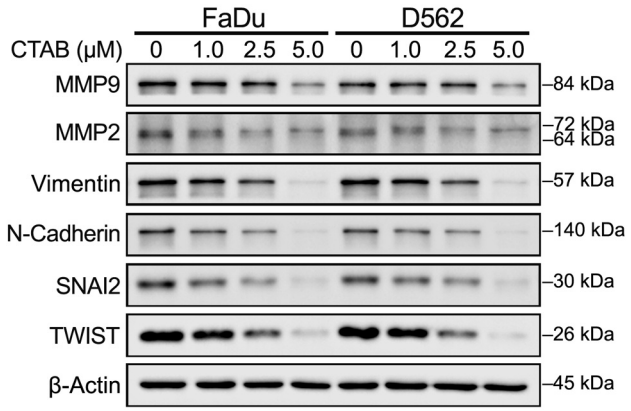

Figure 3. *Cetyltrimethylammonium bromide (CTAB) suppresses the expression of epithelial–mesenchymal transition (EMT)-associated proteins. FaDu and D562 cells were treated with vehicle or CTAB (1.0, 2.5 and 5.0 μM) for 16 h, and then whole-cell extracts were subjected to western blot analysis. Representative western blot images reflecting the protein levels of matrix metalloproteinase-2 (MMP2), MMP9, mesenchymal markers, snail family transcriptional repressor 2 (SNAI2), twist family bHLH transcription factor 1 (TWIST), and EMT-related transcription factors in CTAB-treated FaDu and D562 cells are shown. β-Actin was used as a loading control. The results are representative of three independent experiments.*

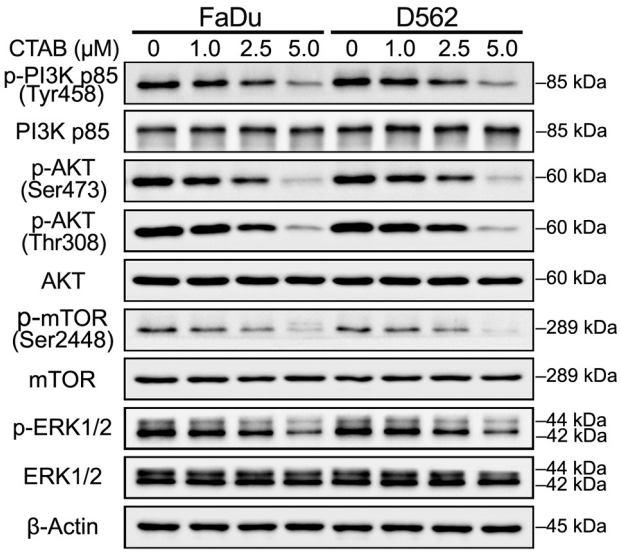

Figure 4. *Cetyltrimethylammonium bromide (CTAB) down-regulates epidermal growth factor receptor (EGFR)-dependent phosphatidylinositol 3-kinase (PI3K)/AKT serine/threonine kinase 1 (AKT) and mechanistic target of rapamycin (mTOR) signaling in FaDu and D562 cells. Western blot analysis examined the levels of the phosphorylated and total forms of EGFR, PI3K, AKT, mTOR and extracellular signal-regulated kinase 1/2 (ERK1/2) in cells treated with CTAB. The results are representative of three independent experiments. β-Actin was used as an internal control.*

EGFR and its ligand, EGF can increase the mesenchymal phenotype and affect the expression of the key group of EMT-associated proteins in HNSCC (27, 28). The next step of this study was to confirm whether CTAB exerts an inhibitory effect on EGFR and its downstream target proteins involved in EMT in its repression of FaDu and D562 cell migration and invasion. Therefore, cells pretreated with EGFR ligand (EGF) were subsequently treated with CTAB. Our results showed that administration of ≥50 ng/ml of EGF significantly restored the expression of mesenchymal markers SNAI2, TWIST, vimentin and N-cadherin (Figure 5B). In addition, EGF also rescued the expression of MMP9 when cells were pretreated with EGF and subsequently treated with CTAB. These findings show that CTAB exerted its anti-EMT effect by altering EGF signaling in FaDu and D562 cells. Results obtained show that the phosphorylation of EGFR, PI3K and downstream signaling proteins, including AKT and mTOR were dose-dependently restored following treatment with EGF (Figure 5C). Moreover, addition of EGF also partially restored cell migration and invasion (Figure 5D). Taken together, these data suggested that CTAB at a concentration of 5 μM potently restrained mesenchymal characteristics of FaDu and D562 cell lines through modulation of the EGFR/PI3K/AKT/mTOR signaling axis.

Discussion

Despite the recent advances in cancer treatment, the 5-year survival rate for patients with HNSCC have not noticeably improved for decades (29). This may be attributed to the fact that it is difficult to diagnose because most people do not show observable symptoms in early stages of HNSCC. The majority of patients with HNSCC present with advanced or terminal stage (stage III or IV) confirmed disease at initial diagnosis (27). Those with metastasis have comparatively poor prognosis and most HNSCC-related deaths are associated with metastasis. Exploring the process of metastasis, EMT is an important biological process that enables polarized epithelial cells to gain the ability for migration and invasion during tumor progression (30). Many signaling pathways and molecules that can affect cancer invasion and metastasis still remain to be investigated. Related reports regarding the molecular mechanisms of HNSCC metastasis indicate that activation of EGFR-increased tyrosine phosphorylation and induction of EMT can occur through the PI3K/AKT and ERK1/2 signaling pathways (27, 31). Atypical increase of EGFR signaling is frequently associated with a decrease in cell adhesion and an increase in tumor cell migration and invasion in HNSCC (32-35).

In the present study, we demonstrated that CTAB, a quaternary ammonium compound, diminishes expression of EGFR/PI3K/AKT signaling pathway members. In addition, treatment with CTAB altered the mesenchymal-like phenotype of FaDu and D562 cells to one with epithelial features. Our results indicate that CTAB disturbs cellular functions controlled by the EGFR/PI3K/AKT signaling axis, including cell migration and invasion. Nevertheless, our data showed that CTAB treatment had no obvious affect on cell viability or the cell-cycle distribution (Figure 1C and D).

In addition, we found that CTAB displayed anti-adhesion potential as evidenced by the suppression of the attachment of FaDu and D562 cells (Figure 1B). Non-lethal doses of CTAB led to FaDu and D562 cells to obtain nearly orbicular shape, losing mesenchymal features, indicating that CTAB induced transition from the mesenchymal to epithelial phenotype.

MMP protein family members, such as MMP2 and MMP9, have the ability to degrade and remodel the ECM (mainly collagen) and are associated with cancer metastasis and recurrence (27, 36). The up-regulation of MMP9 is correlated with shortened relapse-free and cause-specific survival, suggesting that MMP9 is critical to the highly invasive ability of malignant tumors and poor prognosis in patients with HNSCC (37). Down-regulation of MMP2 is correlated with suppression of the migration and invasive of HNSCC cells, leading to repression of malignant progression and tumor metastasis in HNSCC (38, 39). Here, CTAB inhibited the migration and invasion of FaDu and D562 cells through a dose-dependent reduction in the protein levels of MMP2 and MMP9 (Figure 3).

The activation of EGF and the EGFR signaling axis has been implicated in the transcription of repressors of E-cadherin, including the SNAI family (40). Members of the SNAI family of transcription factors contribute to cell migration and invasion via modulation of EMT (27). In addition, dysregulation of EGF/EGFR signaling has been implicated in malignant properties of HNSCC through activation of its downstream transduction pathways, such as PI3K/AKT and RAS/ERK (41). The PI3K/AKT/mTOR axis plays a key role in cell migration involved in the regulation of MMPs, including MMP2 and MMP9 caused by EGFR (27, 42). In the current study, we demonstrated that CTAB significantly attenuated activation of EGFR, PI3K and AKT, and reduced the phosphorylation of EGFR and its downstream signaling proteins, including PI3K, AKT, mTOR and ERK1/2 in FaDu and D562 cells (Figure 4).

To further validate the role for EGFR activation in EMT of FaDu and D562 cells, small-molecule inhibitors of the receptor tyrosine kinases were screened through examining changes in cell motility. Treatment with AG-528 and laptinib induced changes in cell migration, thus we believe EGFR is involved CTAB-induced changes in cellular motility (Figure 5A). Furthermore, the addition of EGF restored the down-expression of mesenchymal proteins after CTAB treatment (Figure 5B). Finally, we showed that EGF could rescue CTAB-mediated inhibition in migration, invasion, and cell shape through the reactivation of EGFR/PI3K/AKT/mTOR signaling (Figure 5C and D). Based on our findings, CTAB appears to have anticancer effects on FaDu and D562 cells.

In conclusion, we report for the first time that CTAB treatment leads to inactivation of EGFR/PI3K/AKT signaling and causes mesenchymal cell transitioning to epithelial characteristics in FaDu and D562 cells. In addition, our data also showed that EGF treatment leads to restoration of PI3K/AKT signaling and re-transition of cells to mesenchymal characteristics. Collectively, these findings provide an important basis for a further evaluation toward understanding the mechanism of action of CTAB on HPSCC cells, and indicate CTAB may be a potential therapeutic agent for patients with aberrant EGFR-expressing HPSCC.

Conflicts of Interest

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

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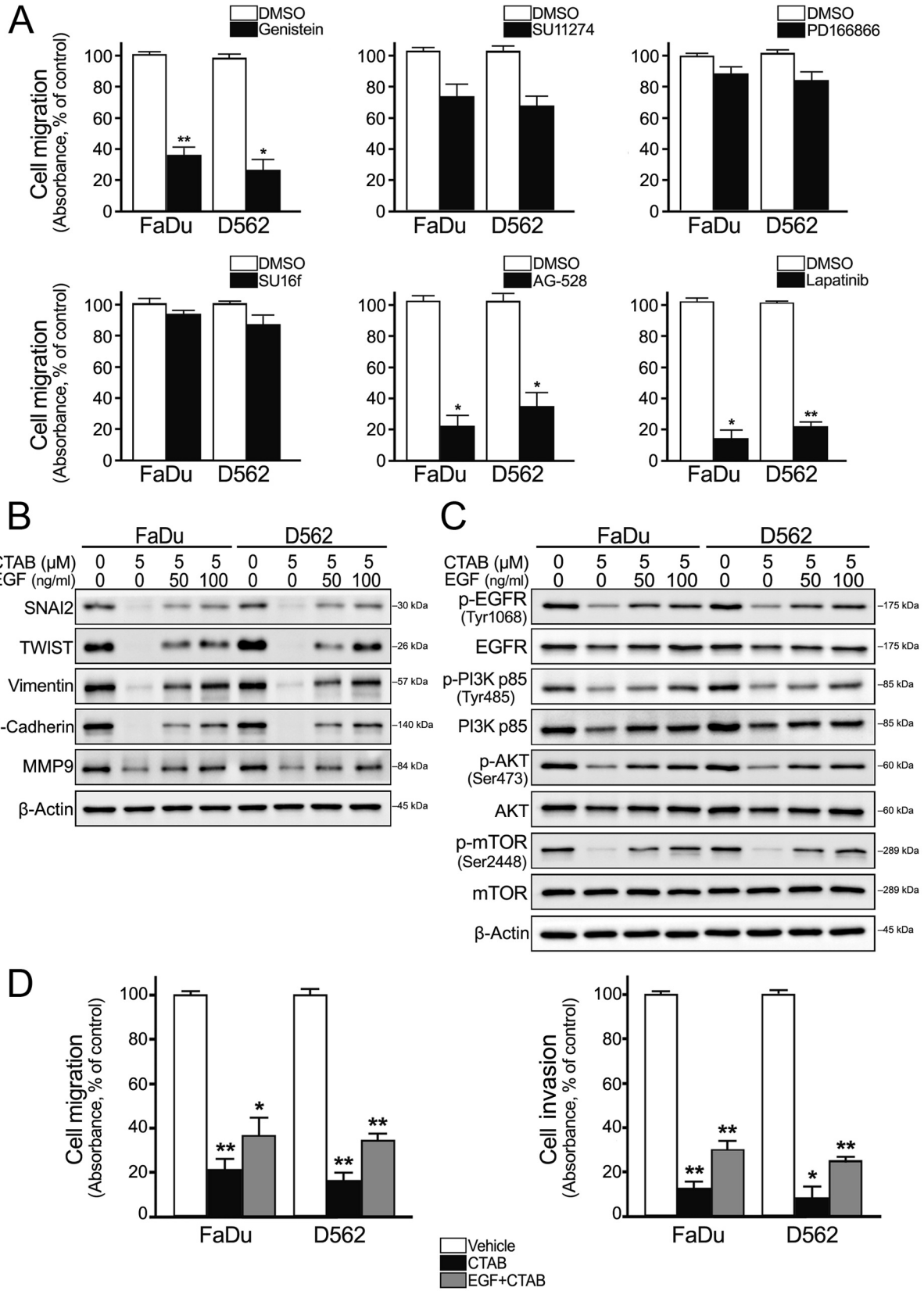
Figure 5. *Epidermal growth factor receptor (EGFR)-dependent phosphatidylinositol 3-kinase (PI3K)/AKT serine/threonine kinase 1 (AKT)/mechanistic target of rapamycin (mTOR) signaling pathway is involved in the suppressive effect of cetyltrimethylammonium bromide (CTAB) on EGF-mediated epithelial–mesenchymal transition (EMT) in both FaDu and D562 cells. A: Comparison of the migratory properties of FaDu and D562 cells in the presence of dimethyl sulfoxide (DMSO) or 20 μM receptor tyrosine kinase inhibitor genistein, 50 μM tyrosine-protein kinase Met inhibitor SU11274, 10 μM fibroblast growth factor receptor inhibitor PD166866, 0.5 μM platelet-derived growth factor receptor inhibitor SU16f, 10 μM EGFR inhibitor AG-528, and 10 μM EGFR inhibitor lapatinib using Boyden chamber assays. Cells were pretreated with vehicle or EGF, the native ligand of the EGFR tyrosine kinase receptor (50 or 100 ng/ml) for 4 h, and then co-treated with CTAB for another 8 h. B: Protein expression of matrix metalloproteinase-2 (MMP2), MMP9, mesenchymal markers, snail family transcriptional repressor 2 (SNAI2), twist family bHLH transcription factor 1 (TWIST), and EMT-related markers. C: Expression of phosphorylated and total proteins of the EGFR/PI3K/AKT/mTOR signaling axis in CTAB-treated cells with and without EGF were analyzed by western blot. β-Actin was reprobed to confirm equal loading. D: FaDu and D562 cells were precultured with EGF for 4 h prior to addition of CTAB for 16 h and then their cell migration and invasion were examined. CTAB-mediated inhibition of motility was analyzed using the transwell migration, and Matrigel invasion assays. EGF reversed the effects of CTAB 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: *$p<0.05$ and **$p<0.01$ compared with the vehicle control.*


A
DMSO
Genistein
SU11274
PD166866
SU16f
AG-528
Lapatinib
Cell migration
(Absorbance, % of control)
FaDu
D562
B
C
CTAB (μM)
EGF (ng/ml)
SNAI2
TWIST
Vimentin
N-Cadherin
MMP9
β-Actin
p-EGFR
(Tyr1068)
EGFR
p-PI3K p85
(Tyr485)
PI3K p85
p-AKT
(Ser473)
AKT
p-mTOR
(Ser2448)
mTOR
D
Cell invasion
Vehicle
CTAB
EGF+CTAB

Authors' Contributions

Conceptualization was by CJL, funding acquisition by TKW and YCO, Methodology and validation by YRP and YPC. Writing of the original draft was by FMH and CJL, reviewing and editing by TKW and YCO. All Authors read and approved the final article.

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