

Original Article

TMEM16A as a Potential Biomarker in the Diagnosis and Prognosis of Lung Cancer

Cong Hu¹, Ronggui Zhang², Depeng Jiang^{3*}

¹Department of Intensive Care, Zhuhai People's Hospital, The Third Affiliated Hospital, Jinan University, Zhuhai, 519000, China

²Department of Urology, The Second Affiliated Hospital, Chongqing Medical University, Chongqing 400010, China

³Department of Respiratory Medicine, The Second Affiliated Hospital, Chongqing Medical University, Chongqing 400010, China

Abstract

Background: Transmembrane protein 16A (TMEM16A), also known as ANO1 (anoctamin-1), was reported to be vital in the growth and invasion of several malignancies. However, role of TMEM16A in lung cancer remained unclear. The aim of this study was to evaluate the expression of TMEM16A and its significance in lung cancer.

Methods: qRT-PCR and Western blots were performed to evaluate the TMEM16A mRNA and protein expression. Proliferation and invasion of H1299 cancer cells were evaluated by CCK-8 and transwell assays. Tumor volumes in nude mice implanted with H1299 cells were assessed once every week for 5 weeks by measuring 2 perpendicular dimensions. Immunofluorescent staining revealed expression of TMEM16A in nude mice cancer tissues.

Results: Our findings provided compelling evidence that TMEM16A production in H1299 cells is 2.1 times higher than observations in HBE16 cells. We showed that overexpression of TMEM16A contributed to the proliferation of H1299 cells. Moreover, T16Ainh-A01, a specific TMEM16A inhibitor or shRNA targeting TMEM16A somewhat inhibited lung tumor cell growth and invasion as evident from in vitro studies and from in vivo xenograft-tumor growth. Inhibition of TMEM16A strongly suppressed EGFR phosphorylation and growth of lung cancer cells. Furthermore, a reduction of p-RAS and p-ERK1/2 was also observed.

Conclusion: TMEM16A promoted growth and invasion in lung cancer cells via an EGFR/ MAPK-dependent signaling pathway. So we infer TMEM16A membrane protein may have potential to serve as a biomarker in lung cancer.

Keywords: Invasion, Lung cancer, Metastasis, Proliferation, TMEM16A

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Introduction

Lung cancer (OMIM accession number: 211980) is a common lethal neoplasm accounting to one in every 5 deaths from cancer worldwide.^{1,2} To date, about 75% of patients have late stage lung cancer despite treatments.³ In spite of significant improvements in diagnosis and treatment modalities, the long-term survival rate remains poor for patients in advanced stage mainly due to local recurrence, distant metastasis and lack of any diagnostic biomarkers in the early stages of lung cancer.⁴⁻⁶ Increasing evidence suggests the significant role of transmembrane protein 16A (TMEM16A) (OMIM accession number:610108) in the pathogenesis and progression of cancer.⁷⁻⁹ Until now, the functions of TMEM16A in lung cancer cells have not been illuminated.

Calcium-activated chloride channels (CACCs) are widely expressed in several epithelial and non-epithelial-tissues like the secretory epithelia and sensory neurons.¹⁰ CACCs are transmembrane proteins that regulate different physiological activities like excitability and contraction of muscle.¹¹ CACCs are also known to be involved in tumorigenesis besides their physiological significance. Recently, accumulated evidence identified involvement of ion channels in regulation of

tumor growth and metastasis. Cancer metastasis has recently been proven to be related to CACCs by maintaining Ca²⁺ concentration and tissue homeostasis by regulation of cell volume.¹⁰⁻¹² Chloride channels belong to the transmembrane family that get activated due to stimulation by osmotic perturbations and stress. They are significant in regulating cell volume regulation by contributing to transport in the epithelial cells.¹³ Therefore, CACCs act fundamentally in metastasis.

TMEM16A belongs to the large CACCs family.¹⁴⁻¹⁷ Several studies have shown that TMEM16A has 8 transmembrane segments and is overexpressed in cancer metastasis in patients with advanced cancer.^{18,19} The TMEM16A gene, located in the 11q13 region, was regularly enhanced in several poor prognosis tumors including esophageal cancer, gastrointestinal stromal tumors, and SCCHN, breast and prostate cancer, human colorectal cancer, and also contributed to the metastasis of gastric and gallbladder carcinoma.²⁰⁻²² However, there is no clear understanding about whether TMEM16A contributes to lung cancer metastasis.

In the present study, we investigated pharmacological inhibition of TMEM16A in human metastatic lung

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 cancer cells H1299 cells. We found that treatment with T16Ainh-A01 inhibited cell proliferation and migration or invasion. Meanwhile, our results also provided the mechanism of *TMEM16A* amplification and overexpression in lung cancer cells via the EGFR/MAPK-dependent signaling pathway.

Materials and Methods

Cell Line Cultures

HBE-16 and H1299 cells were purchased from Chinese National Infrastructure of Cell Line Resource (Beijing, China). HBE-16 and H1299 cells were cultured in RPMI-1640 (Gibco-Invitrogen, Carlsbad, CA, USA), with 10% fetal bovine serum (Gibco) and 1% weight per gram of penicillin/streptomycin (Gibco) in the culture medium. The cell cultures were subjected to 37°C and 5% CO₂, humidified atmosphere. The medium was changed until the cells were 90% confluent. Transfection with plasmids was carried out using Lipofectamine™ 2000 reagent (Invitrogen, Carlsbad, USA) according to the manufacturer's instructions. Cells with >75% transfection efficiency were used for further experiments. All experiments are repeated at least 3 times.

ShRNA Preparation and Plasmid Construction

TMEM16A shRNA (short hairpin RNA) plasmid was constructed by GeneChem Co. Ltd. (Shanghai, China) (Table 1). BLAST was performed to ensure that these shRNAs did not have significant sequence homology with other genes. The integrity of expression plasmids was confirmed by DNA sequencing analysis.

RNA Extraction and q-PCR

We used TRIzol reagent (Invitrogen, Carlsbad, CA) to extract RNA followed by standard reverse transcriptase using TaKaRa First Strand Synthesis kit (Dalian, China). qRT-PCR was performed on a CFX96 Touch™ Real-Time PCR Detection system (BIO-RAD, Shanghai, China). The cDNA products were used as templates (10 µL), and primers of *TMEM16A* and *GAPDH* were used (Table 2). The PCR conditions are 95°C for 5 minutes, followed by 30 cycles of 95°C for 30 seconds; 60°C for 30 seconds; and 72°C for 60 seconds. Gene expression was normalized to *GAPDH*. That

Table 1. Three Sites for shRNA Targeting of the Human *TMEM16A* Gene

Number	Sites for shRNA targeting of the human <i>TMEM16A</i> gene
1	CGTGACAAAGGCCAAGTA (1077–1095 nt)
2	GCATCTATTTGACTTGTCT(991–1009 nt)
3	CGAAGAAGATGTACCACAT (837–855 nt)

Table 2. Primers of *GAPDH* and *TMEM16A*

Name	Sequence (5'-3')	Sequence (5'-3')
<i>GAPDH</i> primer	F:AGCCTCAAGATCATCAGCAATGCC	R:TGTGGTCATGAGTCCTTCCACGAT
<i>TMEM16A</i> primer	F:TCTGCTGGATGAAGTTTACGG	R:AGGCGACATAGAAGATGGGAG

was used as an internal control. Data analysis was performed by CFX Data and analyzed by Manager™ software version 3.1 (BIO-RAD, Shanghai, China).

Western Blotting

H1299 and HBE16 cells were ground and lysed with RIPA lysis (Beyotime, Shanghai, China) supplemented with phenylmethanesulfonyl fluoride (PMSF) (Beyotime, Shanghai, China) on ice as per manufacturer's protocols. The protein concentrations were determined with the BCA protein assay kit (Beyotime, Shanghai, China). After that, protein samples were separated using 10% sodium-dodecyl sulfate polyacrylamide gels electrophoresis (SDS-PAGE) (Beyotime, Shanghai, China) and transferred onto the polyvinylidene difluoride (PVDF) membranes (Millipore, Billerica, MA, USA). The membranes were blocked with 5% BSA in TBST for 2 hours at room temperature and incubated with anti-*TMEM16A* polyclonal antibody (ab53213, Abcam), p-ERK1/2 (ab214362, Abcam), p-EGFR(ab40815, Abcam) and anti-*GAPDH* antibody (ab8245, Abcam), respectively, at a dilution of 1:1000 overnight at 4°C. After washing 3 times with TBST, the blots were exposed to the goat secondary antibody conjugated to horseradish peroxidase at a dilution of 1:5000 (Sangon, Shanghai, China) for 2 hours at room temperature and visualized using an enhanced chemiluminescent (ECL) kit (Beyotime, Shanghai, China) on a ChemiDocMP imaging System (BIO-RAD, Shanghai, China).

Cell Proliferation

Cell viability was evaluated using the CCK-8 assay (Beyotime, Shanghai, China). After transfection with *TMEM16A* shRNA or treating with T16Ainh-A01, the H1299 cells were seeded in a 96-well culture plates at 10⁴ cells per well in 100 µL culture medium and further incubated for 1, 12, 24, 48, and 72 hours. Subsequently, 10 µL of the CCK-8 mixture solution was added to each well, mixed gently, and incubated for 4 hours at 37°C in CO₂ incubator. After that, the number of viable cells was quantified using optical density (OD) at a wavelength of 450 nm with a microplate reader (BIO-RAD, Shanghai, China).

Migration and Invasion Assays

Experiments were carried out in transwell 24-insert plate chambers (Corning, NY, USA). After pretreatment with shRNA or T16inh-A01 for 24 hours, the H1299 cells were trypsinized and counted. The cell migration experiment was performed by seeding 2×10⁴ cells in 200 µL of medium with 1% FBS (Gibco-Invitrogen, Carlsbad, CA, USA) into the

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upper compartment of the chamber with a polycarbonate filter (6.5-mm diameter, 8-mm pores; Corning Costar, Corning, NY, USA). The lower chambers contained 600 μ L of medium containing 10% FBS (Gibco) to serve as chemo-attractant. The plates were incubated in a humidified atmosphere of 5% CO₂ for 48 or 72 hours at 37°C. For the cell invasion experiment, transwells were coated with Matrigel (BD Biosciences, Franklin Lakes, NJ, USA). Noninvasive cells were wiped from the upper surface of the transwell membrane by swabbing, while the migrated or invaded cells in the lower surface of the filters were fixed with methanol, stained with 4,6-diamidino-2-phenylindole (Sigma, NY, USA) and counted under a light microscope (Olympus Corp, Tokyo, Japan) in 5 randomly selected visual fields at magnification of 100 \times . The cell number represented migration activity.

Xenograft Tumor Growth Studies

For BALB/c nude mice (4–6 weeks old) tumor implantation, H1299 cells were seeded in RPMI 1640 medium supplemented with 10% FBS in 10 cm plastic dishes. The cells were then washed 3 times with PBS and resuspended in 0.1 mL saline solution. Each mouse was injected with 10⁷ cells to form a tumor xenograft. When the tumor volume reached about 80–100 mm³, the tumor-bearing mice were randomly divided into 3 experimental groups (n = 6 mice/group): (1) control; (2) T16Ainh-A01; and (3) *TMEM16A* shRNA. *TMEM16A* shRNA or T16Ainh-A01 diluted in saline was directly injected into the tumor xenograft at a dose of 40 μ g every week. Tumor volumes were assessed once every week for a total period of 5 weeks by measuring 2 perpendicular dimensions with a caliper according to the formula. The volume of tumor was calculated using the following equation: $V = (\text{length} \times \text{width}^2)/2$. This study was approved by the Ethics Committee of Chongqing Medical University and the Second Affiliated Hospital of Chongqing Medical University.

Immunofluorescence Staining

Tissue sections were placed in EDTA antigen repair buffer (pH 8.0) for antigen repair (Beyotime, Shanghai, China). The slides were washed with PBS and placed in the spontaneous fluorescence quenching agent for 5 min and incubated with BSA (Beyotime, Shanghai, China). Tissue sections reacted overnight at 4°C with primary rabbit polyclonal antibody and *TMEM16A* (Ab. Dilution 1:100, ab53213, Abcam). Subsequently, the slides were washed with PBS and incubated with CY3 for 50 minutes. DAPI dye (Beyotime, Shanghai, China) was added and incubated at room temperature for 10 minutes. The slides were washed with PBS and observed under the fluorescence microscope (Olympus, Shanghai, China).

Statistical Analysis

All analyzed data are expressed as the mean \pm standard error,

as calculated using SPSS 19.0 (Chicago, IL, USA). *T* tests were performed to evaluate inter-group differences. The *t* test assumptions were checked. All figures were generated using GraphPad Prism 5 (GraphPad Software, Inc., La Jolla, CA, USA). *P* < 0.05 was considered statistically significant.

Results

Overexpression of *TMEM16A* in H1299 Cells

We discovered the expression of *TMEM16A* in H1299 cells—a cell line from metastatic lymph nodes and a controlled normal bronchial epithelium derived cell line, and in subjects of 2 groups measured by qRT-PCR (Figure 1a). Quantitative analysis indicated that the *TMEM16A* mRNA in H1299 cells was 2.1 times higher than that in HBE16 cells, indicating that *TMEM16A* mRNA transcripts were overexpressed in H1299 cells. Meanwhile, the relative protein level of *TMEM16A* was analyzed by western blotting (Figure 1b and 1c). We noticed a corresponding upregulation of the *TMEM16A* protein, which was found

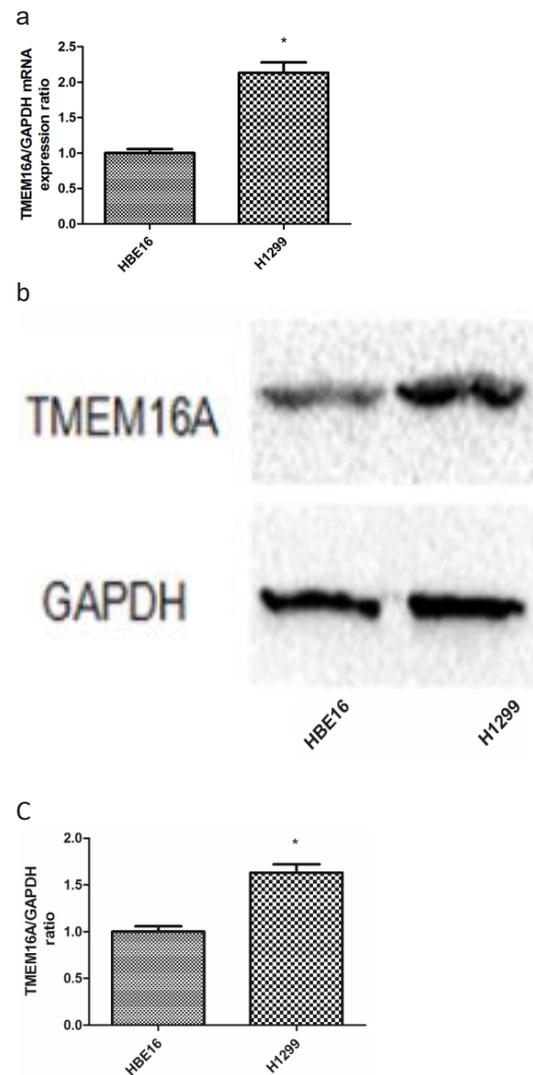


Figure 1. *TMEM16A* Expression in H1299 Cells and HBE16 (control). (a) Relative *TMEM16A* mRNA levels in H1299 and HBE16. (b) *TMEM16A* protein levels in HBE16 and H1299. (c) *TMEM16A* protein level was standardized with GAPDH. *P* < 0.05 compared with NC (n = 6).

expressed 1.6-fold higher in H1299 cells compared to normal control cells (HBE16).

TMEM16A Expression Is Critical for Cell Proliferation in H1299 Cell Proliferation

To investigate the functional role of *TMEM16A* in lung cancer cells, we used shRNA-targeting *TMEM16A* to examine the effect of *TMEM16A* on the proliferation of H1299 cells via the CCK-8 assay. As depicted in Figure 2a, knockdown of *TMEM16A* by specific shRNA resulted in a notable suppression of proliferation in a time-dependent manner. Furthermore, we treated H1299 cells with T16Ainh-A01, a specific *TMEM16A* inhibitor, and in consistence with the shRNA knockdown, a significant inhibition of cell proliferation was observed (Figure 2b). Therefore, the results indicate that *TMEM16A* expression is critical for cell growth in the H1299 cells.

TMEM16A Modulates Cancer Cell Growth Via EGFR/ MAPK-Dependent Signaling Pathway

We observed a higher level of phospho-EGFR along with activation of RAS and ERK1/2 in H1299 cells relative to the control group (*TMEM16A* overexpression) (Figure 3). This led us to investigate whether *TMEM16A* promoted cancer cells growth via EGFR/MAPK signaling. We treated H1299 cells with T16Ainh-A01, a specific *TMEM16A* inhibitor, and found that EGFR phosphorylation was strongly inhibited. Consistently, a reduction in p-RAS and p-ERK1/2 was also observed (Figure 3). Altogether, our findings suggest that *TMEM16A* promoted H1299 cell growth via the EGFR/ MAPK-dependent signaling pathway.

TMEM16A Was Associated with H1299 Cell Migration and Invasion

To determine the correlation between *TMEM16A* expression and metastatic potency, we analyzed cell motility and invasion and found a significant difference between H1299 and the control group. H1299 cells indicated approximately a 1.6-fold greater cell migration and 2-fold greater cell invasion compared to the control cells ($P < 0.01$). However, when treated with T16Ainh-A01, the H1299 cell migration and invasion was significantly reduced (Figure 4a). Additionally, we conducted shRNA transfection to

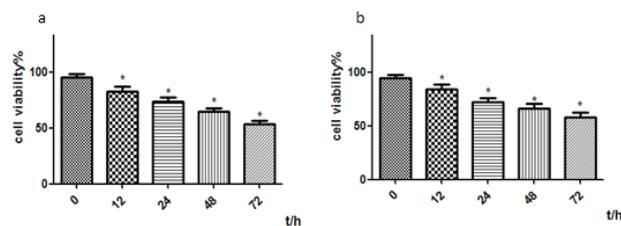


Figure 2. Effects of *TMEM16A* on Proliferation in H1299 Cells Via CCK-8 Assay. Results for cell viability after treatment with shRNA (a) and T16Ainh-A01(b) for 0, 12, 24, 48, and 72 h in H1299 cells. (b) Summary data of cell viability of H1299. $P < 0.05$ compared with control (n = 6).

down regulate *TMEM16A* expression and again observed cell migration and invasion in the H1299 cell line. We found that the migration and invasion of H1299 were reduced after the shRNA transfection (Figure 4b). Our

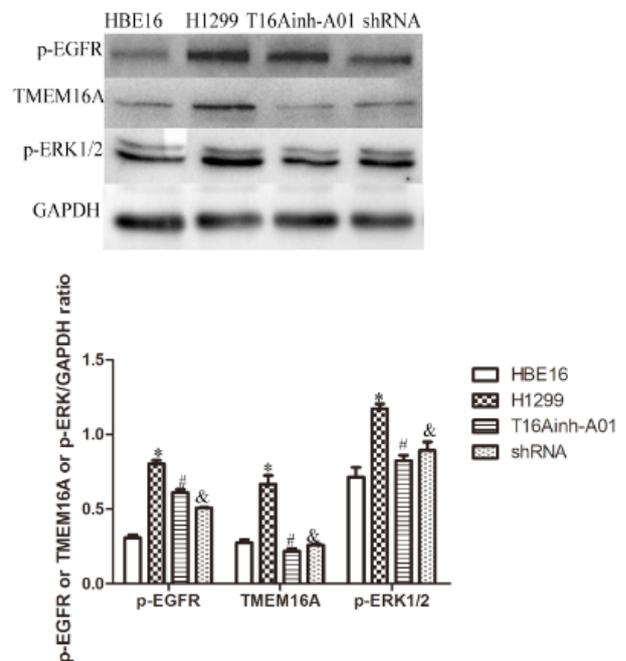


Figure 3. Expression of p-EGFR, *TMEM16A* and p-ERK1/2 After Treatment with T16Ainh-A01 and shRNA in H1299 Cells. The p-EGFR, *TMEM16A* and p-ERK1/2 levels were standardized with GAPDH. * $P < 0.05$ and # $P < 0.05$, & $P < 0.05$ compared with control (n = 6).

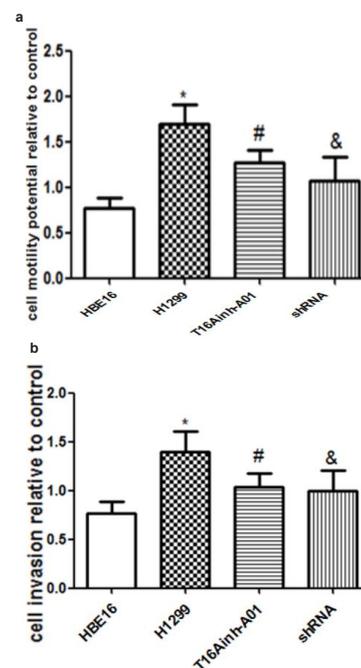


Figure 4. Cell Motility and Invasion of H1299 and HBE16. Results for cell invasion and motility and after treatment with T16Ainh-A01 and shRNA in H1299 cells. * $P < 0.01$ compared with that of control. # $P < 0.01$, & $P < 0.01$ compared with untreated (n = 6).

results showed that *TMEM16A* plays an important role in H1299 metastasis.

TMEM16A Inhibition Suppresses Xenograft Tumor Growth in Null Mice

Tumor volumes were measured once a week during the period of treatment until the animals were sacrificed. During the experiment, no animal death or signs of toxicity was observed. As shown in Figure 5a and 5b, the H1299 cell induced an average tumor volume of $580.15 \pm 97.12 \text{ mm}^3$ ($n = 6$) for the blank control. In contrast, treatment with *TMEM16A* shRNA or T16Ainh-A01 resulted in a significant reduction in tumor volume to $364.25 \pm 58.27 \text{ mm}^3$ and $314.55 \pm 48.95 \text{ mm}^3$ (Figure 5b). The results indicated that inhibition of *TMEM16A* significantly inhibits tumor growth *in vivo*.

Discussion

In various studies, *TMEM16A* overexpression has been implicated in tumor progression.²³⁻²⁷ Our findings show that when the *TMEM16A* gene was overexpressed, it contributed to migration, invasion and proliferation of H1299 cells, a lung cancer cell line from metastatic lymph nodes. Our results provided an analysis of the tumorigenic properties of *TMEM16A*. Many reports proved that *TMEM16A* could serve as biomarker for GIST.^{28,29} Silencing *TMEM16A* suppressed lung cancer cell proliferation, migration and invasion, which represented that the *TMEM16A* proteins have the potential to serve as biomarker in lung cancer.

Recently *TMEM16A* was discovered overexpressed in several cancer cell lines and was demonstrated as a regulator of tumor cell proliferation. *TMEM16A* promoted cell proliferation and tumor growth in a variety of cancers.²³⁻²⁷ Interestingly, *TMEM16A* retarded proliferation in vascular smooth muscle cells.³⁰ In addition, some studies have also shown that *TMEM16A* has no effect on cell proliferation in HNSCC and pancreatic ductal adenocarcinoma cells.³¹ These reports suggested the role of *TMEM16A* in tumor proliferation was inconsistent and the results differ with changes in the celltype and the expression levels of *TMEM16A*.³² We used shRNA and T16Ainh-A01 in targeting *TMEM16A* to examine the effect of *TMEM16A* on proliferation in H1299. Treatment with shRNA or T16Ainh-A01 resulted in a notable suppression of proliferation in a time-dependent manner. Our findings provided compelling evidence for the close correlation of *TMEM16A* overexpression in lung cancer cells with their proliferation. Hence, it is reasonable to assume that the overexpression of *TMEM16A* in lung cancer cells significantly contributes to proliferation.

Several studies have well established the relevance of ion channel currents and metastasis.²³⁻²⁶ Activation of Cl⁻ channels play an important role in cell migration and metastasis.²⁷ Cancer cell migration accompanied by cell retraction at the rear part in several cell types and subsequent cell shrinkage,

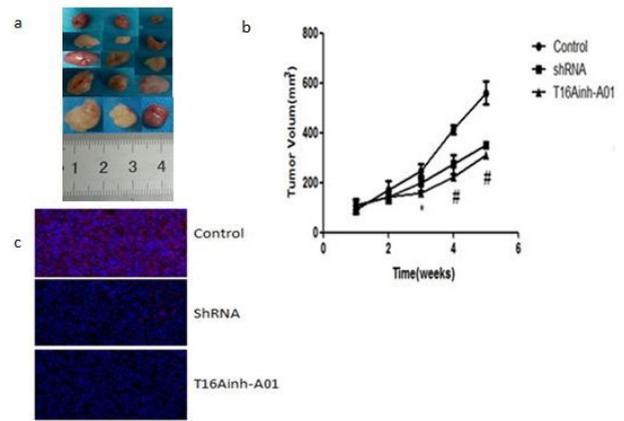


Figure 5. *In Vivo* Suppression of Tumor Cell Growth by Intratumoral Injection of *TMEM16A* shRNA and T16Ainh-A01. (a) The tumor size was measured in 5th week, and tumor growth curves were generated over the 5-week period. (b) Measurements of tumor volumes from nude mice at various time points for Control, *TMEM16A* shRNA and T16Ainh-A01. The tumor volumes in each group ($n = 6$) were measured with calipers every week. Statistical significance between groups is indicated as * $P < 0.05$, # $P < 0.01$. Data expressed as mean \pm SEM ($n = 6$). (c) Representative immunofluorescence staining (Red) of *TMEM16A* in tumor xenografts in Control, *TMEM16A* shRNA and T16Ainh-A01 groups under the fluorescence microscope (X40).

which is integral to cell migration and metastases. To date, enough evidence has accumulated showing that fluctuations in the activities of CACCs modulate the cell volume by mediating fluid secretion to control intracellular calcium.³³ Since *TMEM16A* belongs to the large CACC family, we hypothesized that volume-activated *TMEM16A* may play a particularly important role in the metastasis of lung cancer. Furthermore, we used shRNA targeting *TMEM16A* that lead to significant reduction of *TMEM16A* and resulted in suppression of cell migration and invasion. This proved a likely contribution of *TMEM16A* in the metastasis of lung cancer cells. In consistence with our results, other researchers showed that the expression and activity of *TMEM16A* were propitious to regulate HNSCC cells volume and migration, which facilitated to metastatic progression. In summary, we propose that *TMEM16A* expression level could correlated with cell volume regulation that in turn facilitated cell metastasis.

TMEM16A-induced tumor growth mechanisms are far from being clear, although evidence suggested that ion channels exhibited a correlation with EGFR signaling, intracellular calcium can stimulate EGFR phosphorylation.^{34,35} We found pharmacologic inhibition of EGFR diminishing effect of *TMEM16A*, which indicated EGFR activation in the *TMEM16A*-mediated proliferation of cancer cells. So we focused on the EGFR pathway to elucidate the molecular mechanisms underlying the promotion of lung cancer cell proliferation in response to *TMEM16A*. In addition, our data showed that knockdown of *TMEM16A* or *TMEM16A* inhibitors, CaCC inhA01, abolished activation of EGFR and subsequently inhibited ERK phosphorylation in lung cancer. Our results showed

that *TMEM16A* stimulated tumorigenesis in lung cancer and this is mediated through EGFR and ERK1/2 activation. Therefore, we identified EGFR/MAPK dependent signalling pathways as the underlying mechanism involved in the tumorigenesis of *TMEM16A*-related lung cancer.

In conclusion, we showed that *TMEM16A* overexpression in H1299 cells and activation of *TMEM16A*-mediated CaCC was a positive regulator of lung cancer cell proliferation. It promoted tumor cell migration and invasion. Knockdown of *TMEM16A* led to a reduction of EGFR signaling and a subsequent reduction of p-RAS and p-ERK1/2. These results highlighted the role of *TMEM16A* in lung cancer progression, and also the potential of *TMEM16A* as a prognostic tool in lung cancer. However, since the role of ANO1 in cell migration, invasion and metastasis is determined in *in vitro* studies, whether or not this finding is translated to the *in vivo* situation requires to be further verified.

Authors' Contribution

CH and DJ designed experiments together. We performed experiments and statistical analysis. CH wrote the manuscript, DJ revised it.

Conflict of Interest Disclosures

The authors have no conflicts of interest.

Ethical Statement

This study was approved by the Ethics Committee of Chongqing Medical University and the Second Affiliated Hospital of Chongqing Medical University, Chongqing, China.

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