Original Article
Down-regulation of TMEM16A inhibited invasion and migration in rectal carcinoma

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Abstract: TMEM16A plays an important role in cell proliferation in various cancers. However, less was known about the expression and role of TMEM16A in rectal carcinoma. We screened the expression of TMEM16A in patients' hepatocellular carcinoma tissues, and also analyzed the biological function of hepatocellular carcinoma cells by RNA-interference of TMEM16A, as well as the expression of PI3K/AKT signaling proteins, including AKT, p-AKT, PI3K, p-PI3K, cell cycle regulatory protein cyclin D1 and metastasis-related proteins in TMEM16A siRNA-transfected SW116 cells by western blot. Our results showed that TMEM16A was overexpressed in rectal carcinoma tissues. Inhibition of TMEM16A suppressed the cell proliferation, migration, and invasion, and cell cycle progression. TMEM16A siRNA-suppressed cancer cell proliferation, invasion and migration were accompanied by a reduction of PI3K and AKT activation and cyclin D1 induction. TMEM16A is overexpressed in rectal carcinoma, and that inhibition of TMEM16A suppressed PI3K/AKT signaling in rectal carcinoma cells. TMEM16A could be a potentially novel therapeutic target for human cancers, including rectal carcinoma.

Keywords: Rectal carcinoma, TMEM16A, invasion, migration, PI3K/AKT

Introduction

Rectal carcinoma is one of the most common tumors threatening human health, as the world's third largest cancer. According to statistics, about 12 million of people are diagnosed with rectal carcinoma around the world each year, and more than 600000 people die from the disease [1]. Previous studies have shown that modifiable lifestyle related factors, including low levels of physical activity and obesity, are associated with survival after diagnosis in rectal carcinoma patients [2, 3]. Gene expression changes which disrupt the mechanisms that normally control the growth of colonic epithelial cells have been investigated in rectal carcinoma [4, 5]. However, the molecular mechanisms underlying the formation and development of rectal carcinoma are still poorly understood.

TMEM16A (Anoctamin 1) is a member of the Ano family. Recently, it has been shown to be a calcium-activated chloride channel regulating cell proliferation [6]. It is widely expressed in various tissues, such as smooth muscles, secretory epithelial cells, and sensory neurons. The upregulation of TMEM16A has also been shown in several cancers, such as breast and prostate cancer [7, 8], head and neck squamous cell carcinoma (HNSCC) [9], esophageal squamous cell cancer, and human colorectal cancer [10]. It has been shown that TMEM16A activated the PI3K/AKT signaling proteins, and promoted the tumorigenesis and invasion of various cancers, such as HNSCC, breast cancer and gastrointestinal stromal tumors [11, 12]. However, the expression and role of TMEM16A in rectal carcinoma have not been evaluated to date.

To understand the effect of TMEM16A in the development of rectal carcinoma and the underlying mechanism, we screened the expression of TMEM16A in patients' tissues. We also analyzed the biological function of rectal carcinoma cells by knockdown of TMEM16A, as well as the expression of PI3K/AKT signaling proteins, including AKT, p-AKT, PI3K, p-PI3K and cell cycle regulatory protein cyclin D1 in TMEM16A siRNA-transfected SMMC-7721 cells.

Material and methods

Patients and tissue samples

Rectal carcinoma tissues and adjacent non-tumorous liver tissue counterparts used for qRT-PCR and Western blot were collected from 53 rectal carcinoma patients who underwent surgical treatment between Oct 2010 and Feb 2012 at Shanghai Seventh People's Hospital (Shanghai, China). Tissue samples were snap frozen in liquid nitrogen immediately after surgical resection and stored at -80°C. Tumor stage was determined according to the TNM classification. This study did not include the patients who had received radiotherapy and/or immunotherapy before or after surgical treatment. The mean age of the patients was 62 years (ranging from 30 to 77 years), 54.9% of whom are men. The study protocol conformed to the ethical guidelines of the 1975 Declaration of Helsinki, and was approved by the Institutional Ethical Review Committee of Shanghai Seventh People's Hospital. All patients enrolled in the study gave written informed consents.

Cell culture

Rectal carcinoma cells (LOVO, SW116, HCT116, HT29 and RKO) were purchased from the Shanghai Cell Bank, Chinese Academy of Sciences (Shanghai, China). Cultured in DMEM (St. Louis, MO, USA) containing 10% FBS and 1% penicillin/streptomycin at 37°C in a humidified atmosphere of 5% CO_2.

siRNA transfection

For siRNA transfection, SW116 cells, which were selected for transfection due to TMEM-16A high-expression confirmed by means of RT-PCR and western blot, were seeded onto 12-well tissue culture plates at a density of $6×10^4$ cells/well. The TMEM16A siRNA or non-specific scramble siRNA sequence, both purchased from Genepharma (Shanghai, China), were then transfected into cells at 60% confluency by using Lipofectamine™ 2000 (Invitrogen, Shanghai, China) following the manufacturer's protocol. After 48 h, the transfected cells were collected and processed for the subsequent experiments.

Cell viability assay

Cell proliferation assay SW116 cell survival was evaluated using MTT (3-(4,5-dimethyl-thiazol-2-y1)-2,5-diphenyl tetrazolium bromide; Sigma, St Louis, MO, USA) colorimetric assay. After transfection, cells were seeded in 96-well tissue culture plates at $2×10^4$ cells per well for 0, 24, 48, 72, and 96 h. Then, SW116 cells were washed with phosphate-buffered saline (PBS) and incubated in 100 µL of 5 mg/mL MTT solution (Invitrogen Inc.) for 3 h. MTT is converted into purple-colored formazen in living cells which were then solubilized with dimethylsulfoxide (Invitrogen Inc.). Absorbance of this solution was measured at 450 nm using the microplate reader (Rayto Life and Analytical Science Co. Ltd, Shenzhen, People's Republic of China).

Cell cycle analysis by flow cytometry

Flow cytometry was utilized for the analysis of the cell cycle. After 48 h transfection, cells were harvest and then fixed in ice-cold 70% ethanol (stored at -20°C) overnight. Afterwards, cells were washed with PBS prior resuspending in DNA staining solution (40 µg/ml propidium iodide, 250 µg/ml RNase in PBS with 2 mM EDTA) for 30 min at 37°C. Cell cycle distribution was analyzed using flow cytometer (FACSCalibur, BD Biosciences).

Cell invasion and migration assay

Invasion and migration activity of SW116 cells were measured by a 24-well transwell chamber coated with or without Matrigel (BD Biosciences) on the upper surface of the membrane with a pore size of 8 µm (Sigma). In brief, the transfected SW116 cells ($1×10^4$ cells/well) were suspended in culture media (100 µL, serum free) and then placed in the upper transwell chamber. The lower chamber was filled with medium containing 10% FBS. After 24 h incubation, the cells that had invaded or migrated through the membrane to the lower surface were fixed, stained and counted visually under a microscope (OLYMPUS).

Quantitative reverse transcription polymerase chain reaction

Total RNA was extracted from transfected cells, mock cells and non-transfected cells using TRIzol (Invitrogen). Then 2 µg of RNA was used for cDNA synthesis with a first strand cDNA kit (Sigma, Munich, Germany), according to the protocol provided by the manufacturer. PCR amplification was executed in ABI 7300 Thermocycler (Applied Biosystems, Foster City,
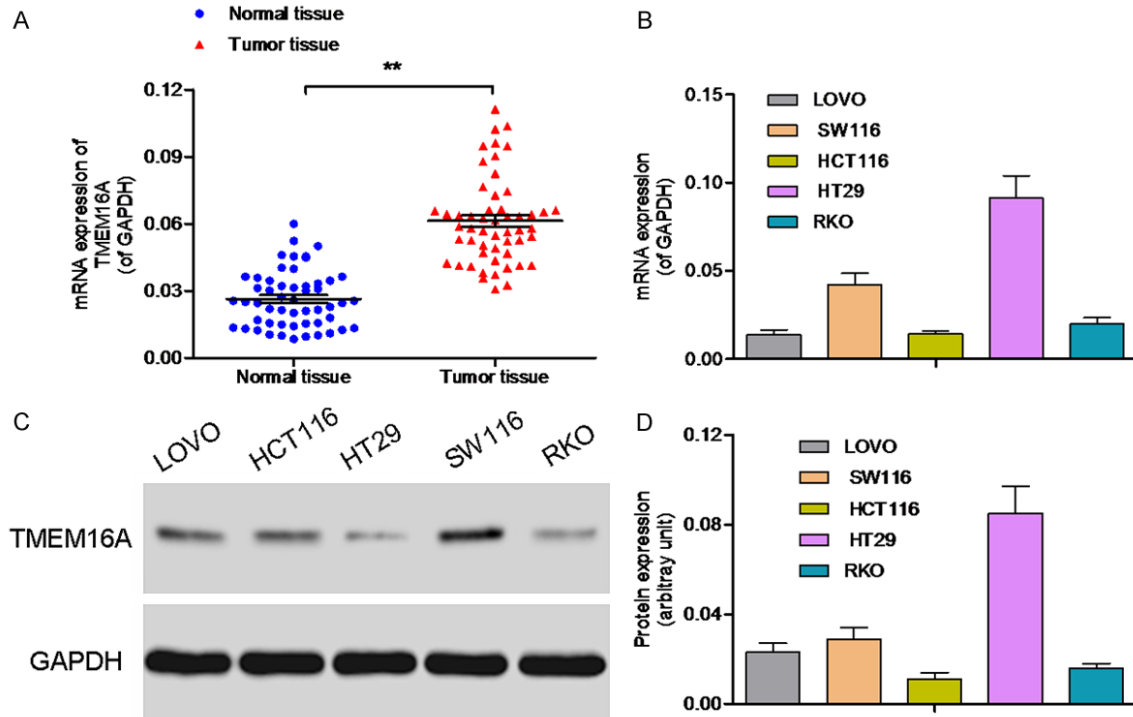
Figure 1. TMEM16A expression in human CRC tissues and CRC cell lines. A. 53 CRC tissues and the adjacent normal tissues were collected and mRNA expression of TMEM16A mRNA expression was identified by RT-PCR. **P < 0.01 compared with the normal tissues; data are expressed as the mean ± SD, n = 53. B. mRNA expression of TMEM16A in LOVO, SW116, HCT116, HT29 and RKO cell lines was identified by RT-PCR. C and D. Protein level of TMEM16A in LOVO, SW116, HCT116, HT29 and RKO cell lines was identified by western blot.

CA, USA), using a SYBR Green PCR kit (Thermo). The PCR cycles were 95°C for 10 minutes, followed by 40 cycles at 95°C for 15 s, annealing/extension at 60°C for 45 s. The primers used for the amplification of the indicated genes were designed using the Primer Express Software (Applied Biosystems, Foster City, CA, USA). The primers were the following: for TMEM16A, 5'-ATTTCACCAATCTTGTCTCCA-TCA-3' (forward) and 5'-TGATAACTCCAAGAAC-GATTGCA-3' (reverse); for GAPDH, 5'-ACACC-CACTCCTCCACCTTT-3' (forward) and 5'-TTAC-TCCTTGGAGGCCATGT-3' (reverse); for cycline D1, 5'-ATGCCAACCTCCTCAACGACC-3' (forward) and 5'-TGGCACAGAGGGCAACGAAGG-3' (reverse); for RhoC, 5'-TGCCTCCTCATCGTCTTCA-3' (forward) and 5'-GCCCTTAATGTCACGCACGAT-TTC-3' (reverse); for MTA1, 5'-AACAAGCCAAA-TCCGAAC-3' (forward) and 5'-GGGACCCCAAG-AATACCA-3' (reverse); for MMP2, 5'-GCTGGA-GACAAATTCTGGAGATACA-3' (forward) and 5'-GTATCGAAGGCAGTGGAGAGGA-3' (reverse); for MMP9, 5'-GCTGGCAGAGGAATACCTGTAC-3' (forward) and 5'-CAGGGACAGTTGCTTCTGGA-3' (reverse).

ABI 7300 system (Applied Biosystem, Foster City, CA, USA) was programmed to initially incubate the samples at 95°C for 10 min, and then denature at 95°C for 10 min, followed by 40 cycles of 95°C for 15 s and 60°C for 45 s. Relative expression levels were calculated using the $2^{-\Delta\Delta CT}$ method. All experiments were performed in triplicate.

Western blot analysis

Transfected cells were washed and then lysed on ice for 10 minutes in 50 mM Tris-HCl (pH 7.5), 10% glycerol, 2% sodium dodecyl sulfate, 0.1 M dithiothreitol, and 10 mM phenylmethyl-sulfonyl fluoride. Proteins were separated on 10% sodium dodecyl sulfate-polyacrylamide gels and electroblotted onto a nitrocellulose membrane in 25 mM Tris base and 190 mM glycine at 50 V for 3 hours at 4°C. To detect the expression of PI3K/AKT signaling proteins (PI3K, p-PI3K, AKT and p-AKT), cell cycle regulatory protein cyclin D1, RhoC, MTA1 and MMP2 in TMEM16A siRNA-transfected SW116

cells, blots were incubated in 1:1,000 monoclonal antibodies against PI3K, p-PI3K, AKT, p-AKT, cyclin D1, RhoC, MTA1 and MMP2 respectively. All antibodies were purchased from Santa Cruz Biotechnology. Proteins were detected by enhanced chemiluminescence as described by the manufacturer (Beyotime, Haimen, People's Republic of China).

Statistic analysis

For quantitative data, all results are expressed as the mean ± standard deviation. Statistical significance between groups was determined using one-way analysis of variance or an unpaired Student's t-test using SPSS 18.0 (SPSS, Chicago, IL, USA). Each experiment was repeated at least three times. $P < 0.05$ was considered statistically significant.

Results

TMEM16A expression in human colorectal tissues

At first, we investigated the role of TMEM16A in CRC. We compared the expression of TMEM16A between CRC tissues and adjacent non-tumorous liver tissue counterparts (**Figure 1A**). The mRNA expression of TMEM16A was obviously upregulated in hepatocellular carcinoma tissues compared to pericarcinous tissue ($P < 0.01$), which suggests an important role of TMEM16A in the development of human CRC.

TMEM16A expression in gastric cancer cell lines

As an obvious difference in TMEM16A expression was observed between human CRC tissues and normal tissues, the mRNA and protein expression of TMEM16A in gastric cancer cell lines including LOVO, SW116, HCT-116, HT29 and RKO cells were then detected by RT-PCR and western blot, respectively. As shown in **Figure 1B**, TMEM16A expression was significantly up-regulated in SW116 cells compared with that of other cells ($P < 0.01$). Similarly, western blot displayed that the protein levels of TMEM16A was remarkably higher than other cells (**Figure 1C** and **1D**). Then, a set of experiments was designed to detect the role of TMEM16A in the proliferation, cell cycle, cell invasion and migration in SW116 cells.

Effect of TMEM16A siRNA on cell viability and cell cycle of SW116 cell line

TMEM16A mRNA was interfered in SW116 cell line as previously described. RT-PCR and western blot were employed to identify the interference efficient. **Figure 2A** showed that TMEM16A mRNA expression in siRNA group was decreased significantly compared with the control and the mock group ($P < 0.01$). Western blot showed that protein level was declined notably in TMEM16A siRNA group in comparison with the control group and mock group ($P < 0.01$, **Figure 2B** and **2C**).

We then identified the effect of TMEM16A siRNA on cell viability of SW116 cells, which was measured by MTT assay. In **Figure 2D**, cell viability was decreased in TMEM16A siRNA group markedly after transfection 48, 72 and 96 h in comparison with that of control and mock cells ($P < 0.01$).

Down-regulation of TMEM16A can suppress cell proliferation, we further detect the effect of siRNA-TMEM16A on cell cycle of SW116 cells. After 48 h of transfection, cell cycle distribution was analyzed using flow cytometer. As shown in **Figure 2E**, compared with the control and mock groups, cells of siRNA group were arrested in G0/G1 phase. Cell population at G0/G1 phase was markedly increased, while S-phase cells were notably decreased in CRC cells transfected with TMEM16A siRNA.

TMEM16A siRNA inhibited invasion and migration of SW116 cell line

To determine whether TMEM16A influences migration and invasion of SW116 cells, transwell assay was performed and the number of migrated and invaded cells was assessed following a 24 h-culture period. Treatment with TMEM16A siRNA for 24 h significantly suppressed the invasion ability of cells when compared with the control group of untreated cells and mock-vehicle group (36.47 ± 5.37% versus 100 ± 11.85% in the control group; 36.47 ± 5.37% versus 96.12 ± 12.56% in the mock group; all $P < 0.01$, **Figure 3A**). The effects of TMEM16A siRNA on migration of SW116 cells were also evaluated. As **Figure 3B** shows, migrated cells decreased dramatically after TMEM16A siRNA transfection treatment for 24 h (48.36 ± 6.14% versus 100 ± 10.89% in the
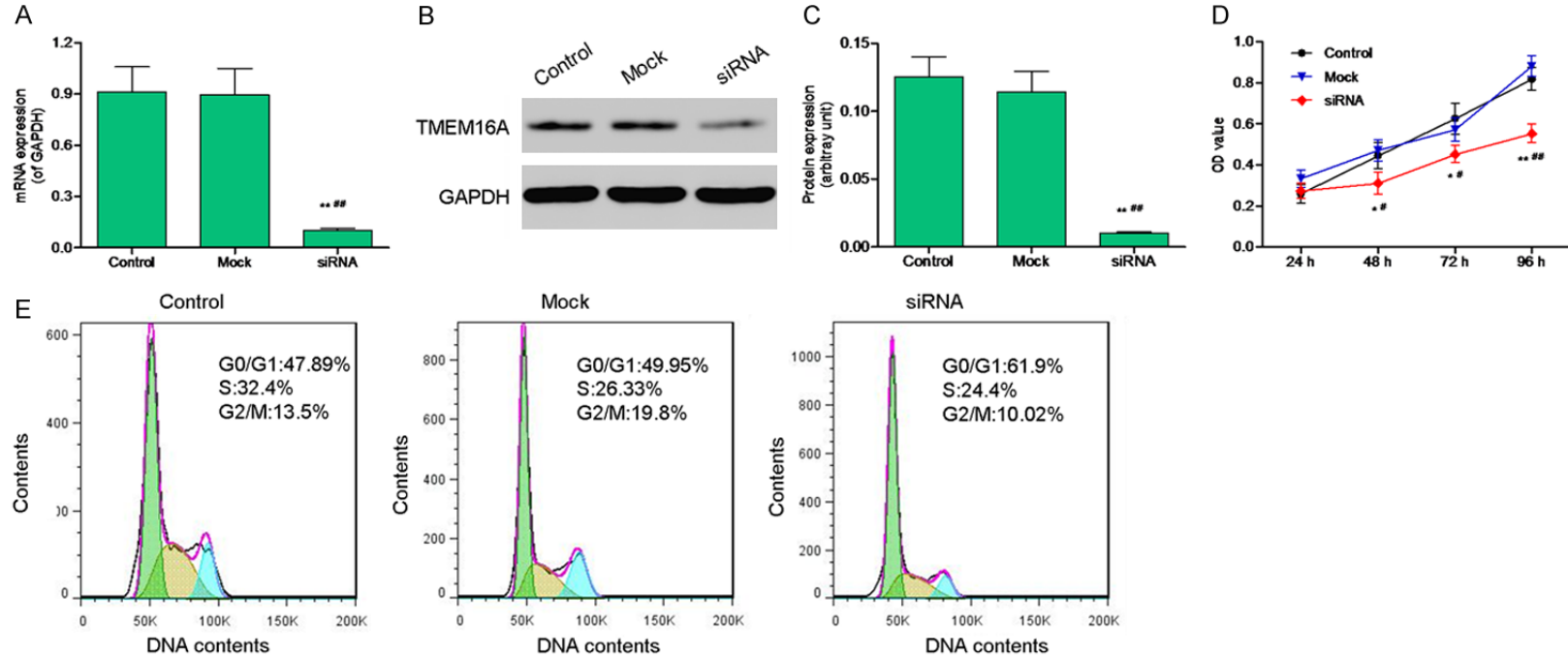
Figure 2. Down-regulation of TMEM16A suppressed cell proliferation and arrested cell cycle at G0/G1 of SW116 cells. A. mRNA expression of TMEM16A in SW116 cells after TMEM16A-siRNA transfection for 48 h was quantified by RT-PCR. B and C. Protein expression of TMEM16A in SW116 cells after TMEM16A-siRNA transfection for 48 h was quantified by western blot analysis. D. After TMEM16A-siRNA transfection for 24, 48, 72 and 96 h, cell viability of SW116 cells was identified by flow cytometry. E. After TMEM16A-siRNA transfection for 48 h, cell cycle distribution of SW116 cells was identified by flow cytometry. *$P < 0.05$, **$P < 0.01$ compared with the control cells; #$P < 0.05$, ##$P < 0.01$ compared with the mock cells; data are expressed as the mean ± SD, n = 6.
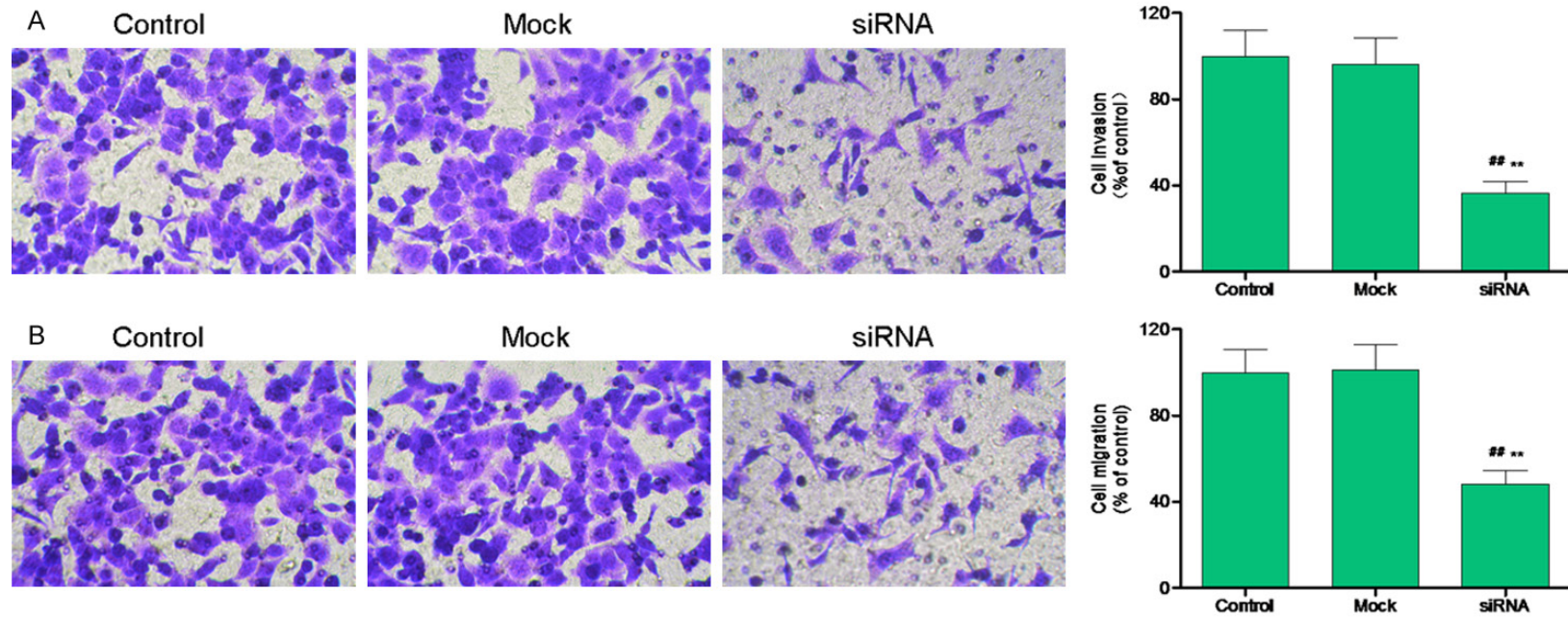
Figure 3. Down-regulation of TMEM16A suppressed cell migration and invasion of SW116 cells. A. After TMEM16A-siRNA transfection for 48 h, cell migration was identified as previously described. B. After TMEM16A-siRNA transfection for 48 h, invasive ability of SW116 cells was identified by transwell assay. $**P < 0.01$ compared with the control cells; $^{##}P < 0.01$ compared with the mock cells; data are expressed as the mean ± SD, n = 6.
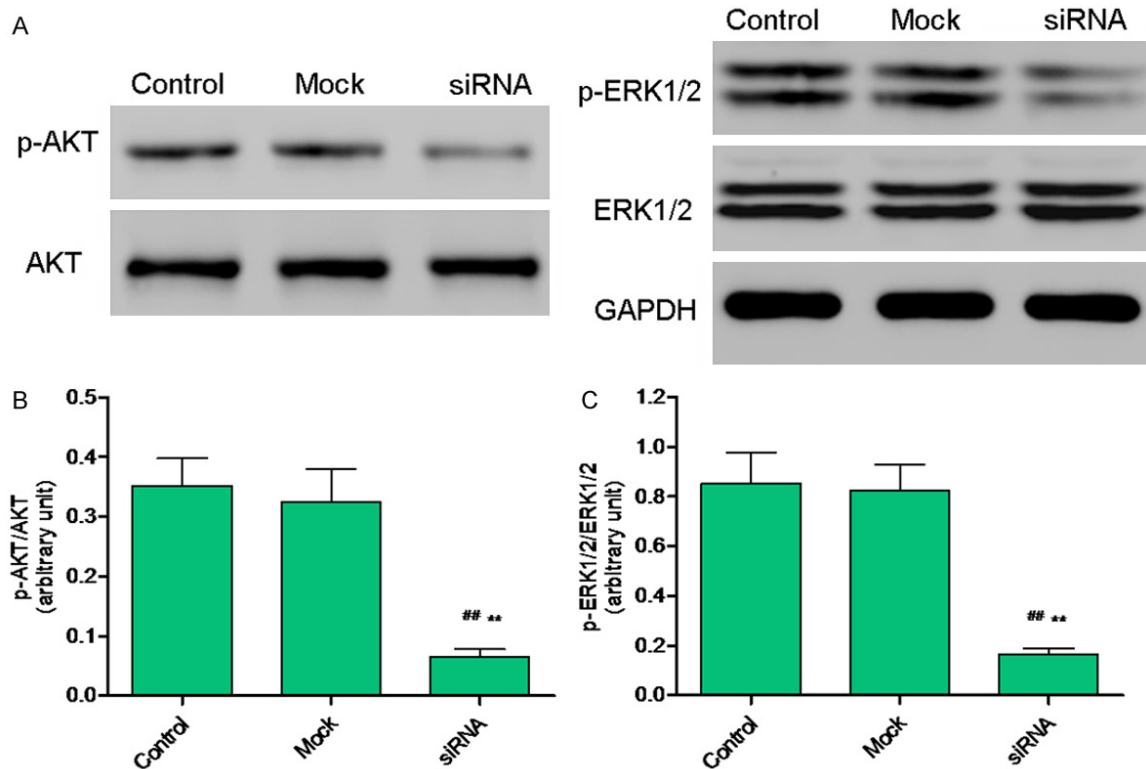
Figure 4. Effect of TMEM16A down-regulation on AKT/ERK signaling. Western blot was performed to identify the protein levels of p-AKT, AKT, p-ERK1/2 and ERK1/2, GAPDH was also detected as the control of sample loading. **P < 0.01 compared with the control cells; ##P < 0.01 compared with the mock cells; data are expressed as the mean ± SD, n = 6.

control group; 48.36 ± 6.14% versus 101 ± 12.04% in the mock group all $P < 0.01$).

TMEM16A siRNA regulated the phosphorylation of AKT and ERK1/2

AKT and ERK1/2 are associated with tumor cell migration and invasion. We examined the effect of TMEM16A siRNA on phosphorylation of AKT and ERK1/2 by western blot analysis. In **Figure 4A** and **4B**, siRNA-TMEM16A treatment for 12 h, the phosphorylation of AKT was significantly decreased compared with the control and mock group ($P < 0.01$). We can see in **Figure 4A** and **4C**, the phosphorylation of ERK1/2 was also declined notably by the treatment of siRNA-TMEM16A treatment for 12 h ($P < 0.01$).

Effect of TMEM16A siRNA on metastasis-related gene and protein expression

Cycline D1, RhoC, MTA1, MMP2 and MMP9 are considered as crucial role in tumor migration and invasion. mRNA and protein expression of

cycline D1, RhoC, MTA1, MMP2 and MMP9 are examined by RT-PCR and western blot, respectively. As shown in **Figure 5A** and **5B**, mRNA expression of MTA1, MMP2, MMP9, cycline D1 and RhoC were all down-regulated significantly in siRNA group compared with the control and the mock group (all $P < 0.01$). In **Figure 5C** and **5D**, the protein expression of MTA1, MMP2, MMP9, cycline D1 and RhoC were also inhibited after siRNA-TMEM16A treatment for 24 h.

Discussion

A leading cause of death in cancer patients is tumor metastasis, due to failure of complete removal of tumor tissue during surgery and radiotherapy. Although there are numerous chemical drugs of choice used in chemotherapy, too many side effects or uncertain actions impede their clinical applications hence it is necessary to search for novel antitumor agents. TMEM16A is a functional calcium-activated chloride channel that influences tumor growth and progression. It is widely overexpressed in various cancers, including breast and prostate
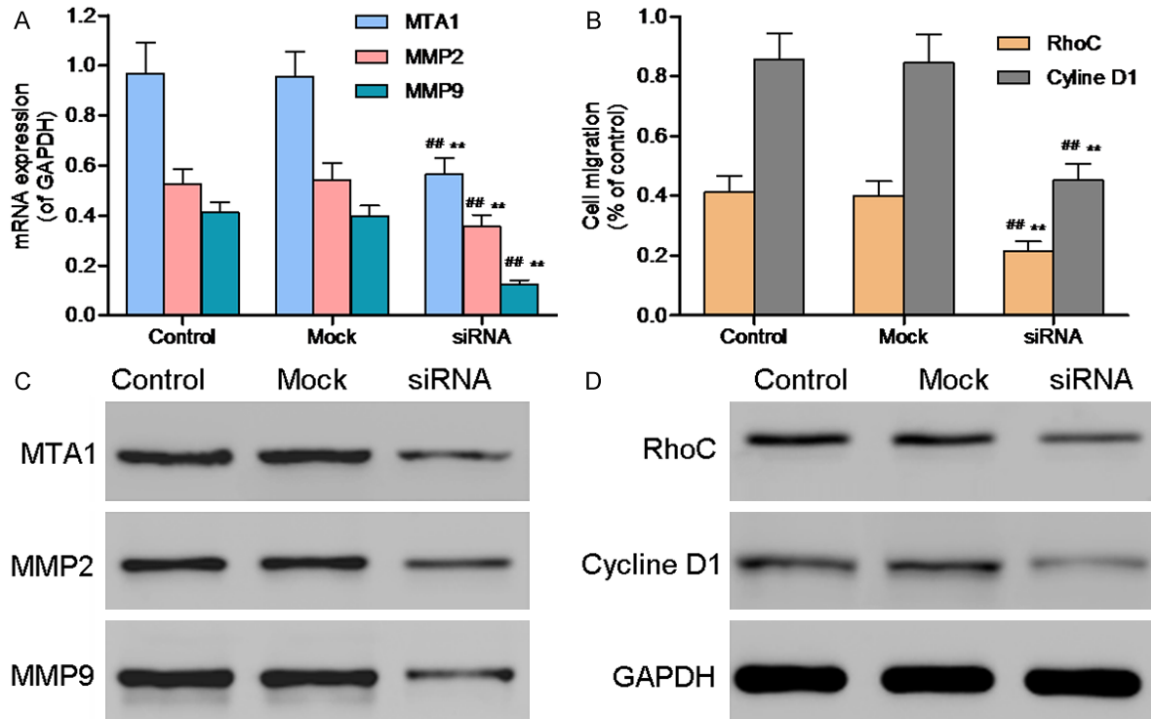
Figure 5. Effect of TMEM16A down-regulation on RhoC, cycline D1 MTA1, MMP2 and MMP9. A, B. After 24 h of TMEM16A siRNA treatment, the mRNA expression of RhoC, cycline D1 MTA1, MMP2 and MMP9 in cells was analyzed by RT-PCR. GAPDH was also detected as the control of sample loading. $**P < 0.01$ compared with the control cells; $##P < 0.01$ compared with the mock cells; data are expressed as the mean ± SD, n = 6. C, D. After 48 h of TMEM16A siRNA treatment, the protein expressions of RhoC, cycline D1 MTA1, MMP2 and MMP9 in cells were analyzed by western blot. GAPDH was also detected as the control of sample loading.

cancer, HNSCC, esophageal squamous cell cancer and human colorectal cancer [7-9, 12]. Consistent with studies mentioned above, we found that TMEM16A was overexpressed in rectal carcinoma tissues compared with their normal tissue in all of 53 patients. However, the mechanism underlying the overexpression of TMEM16A remains unclear. The overexpression is possible due to the 11q13 amplification and signaling pathways or transcription factors during the tumorigenesis. Human cyclin D1 could be used to identify cancers that have an amplification of the 11q13. Our data show that the induction of TMEM16A is associated with the decrease of cyclin D1, indicating that the amplification of 11q13 is important for the TMEM16A overexpression.

Metastasis-associated gene 1 (MTA1), an integral part of the nucleosome remodeling and histone deacetylation (NuRD) complex, can inhibit the transcription of target genes by recruiting histone deacetylases into the promoter regions of target genes thus inducing histone

deacetylation [13]. MTA1 is up-regulated in human tumors and increases the metastatic and invasive potential of carcinoma cells [14, 15]. In this study, siRNA-TMEM16A evidently inhibited the mRNA and protein expression of MTA1.

The extracellular matrix (ECM) regulates cell attachment, motility, invasion, and metastasis. Degradation of ECM is performed by matrix metalloproteinases (MMPs), especially MMP-2 and MMP-9, which play a key role in degrading basement membranes and cancer invasion and metastasis, but are regulated by TIMPs, the endogenous inhibitors of the zinc-dependent endopeptidases of MMPs [16, 17]. In the present study, siRNA-TMEM16A suppressed the protein expression of MMP-2 and the mRNA expression of MMP-2 and MMP-9. These findings indicate that the anti-metastatic effects of siRNA-TMEM16A in tumor cells are associated with regulation of the MMP/TIMP balance. RhoA and RhoC, are GTPases and are part of the extensive Ras superfamily, have 92% amino

acid identity and interact with various regulators and effectors with modulation activity to regulate invasion, metastasis, actin cytoskeleton, cell proliferation, oncogenesis, and survival of cells [18]. RhoC mRNA is one of the targets of miRNA miR-493 and miR-138, both of which decrease the migration of tumor cells [19]. Therefore, we investigated the protein expression levels of RhoC in the cells. As a result, treatment with siRNA for 48 h remarkably depressed the expression of RhoC, indicating that siRNA-TMEM16A inhibited the invasion and migration by suppressing the RhoC expressing. Previous reports indicated that RhoC promoted tumor cell invasion and migration by PI3K/AKT signaling [20, 21]. PI3K/AKT signaling is one of the most important signaling associated with the tumor metastasis. Excessive phosphorylation of PI3K and/or AKT tends to give rise of tumor metastasis. In our study, we found that siRNA-TMEM16A effectively inhibited the phosphorylation of PI3K and AKT, which indicated that siRNA-TMEM16A suppressed the invasion, migration and RhoC expression by blocking the PI3K/AKT pathway. Thus, TMEM16A could be a potentially novel therapeutic target for human cancers, including rectum cancer.

Acknowledgements

Disclosure of conflict of interest

None.

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