

# Expression of transmembrane protein 41A is associated with metastasis via the modulation of E-cadherin in radically resected gastric cancer

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Received November 18, 2017; Accepted April 27, 2018

DOI: 10.3892/mmr.2018.9241

**Abstract.** Gastric cancer (GC) is one of the most commonly occurring malignancies worldwide, and metastasis is one of the key processes affecting the prognosis of GC. TMEM41A, which belongs to a group of transmembrane proteins that participate in signaling pathways and tumor development, is a 264-amino acid protein encoded by a gene mapped to human chromosome 3. The exact role of TMEM41A in GC has not been determined to date. In the present study, the expression of TMEM41A in 147 cases of GC was analyzed with immunohistochemistry and the prognoses of these patients were analyzed. It was revealed that TMEM41A was highly expressed in GC tissues, and may be associated with the progression of GC and poor prognosis. The expression of TMEM41A was observed to be correlated with lymph node metastasis, distant metastasis and advanced tumor, node and metastasis stages. Knockdown of TMEM41A *in vitro* and *in vivo* decreased the GC cell migration ability by regulating epithelial-to-mesenchymal transition and cell autophagy, via the upregulation of E-cadherin and downregulating N-cadherin expression in GC cells by reverse transcription-quantitative polymerase chain reaction (PCR), semi-PCR and western blotting. Furthermore, TMEM41A upregulation was associated with the upregulation of p62 and altered the conversion of light chain (LC)3-1 into LC3-2 by western blotting. Knockdown of TMEM41A was also observed to affect tumor metastasis in nude mice. Therefore,

TMEM41A may be considered as a novel therapeutic target for the treatment of GC-associated metastasis.

## Introduction

Recent advances in surgical techniques and developments in anticancer agents with improved therapeutic efficacies have been made; however, gastric cancer (GC) remains to be the most prevalent type of malignancy and is the second most common cause of cancer-associated mortality worldwide (1,2). Metastasis is one of the key molecular steps affecting the prognosis of GC, but as a complex process, further investigation is required (3). Identifying novel diagnostic or prognostic biomarkers of GC may be considered a major objective for the treatment of GC.

Epithelial-to-mesenchymal transition (EMT) is one of the key molecular steps in the process associated with distant metastasis (4), during which tumor cells exhibiting epithelial characteristics acquire mesenchymal characteristics by modulating cellular polarity and adhesion, and via the loss of cell-cell junctions (5). Throughout the process of EMT, cancer cells lose the expression of cellular adhesion proteins, such as E-cadherin, which has been considered as a hallmark of EMT; cells also acquire the expression of mesenchymal markers, including vimentin (5,6). EMT permits the invasion and migration in a variety of cancer types, including gastric and colon cancer (5).

The transmembrane (TMEM) protein superfamily is a group of transmembrane proteins that participate in particular signaling pathways and tumor development associated with established oncogenes, particularly TMEM16a (7,8), TMEM17a and TMEM17b (9). The associated proteins serve roles in colorectal, ovarian and bladder cancer. TMEM41A, a member of the TMEM41 family, is a 264-amino acid protein encoded by a gene mapped to human chromosome 3 (10,11). Chromosome 3 consists of ~214 million bases, encoding <1,100 genes (11), and possesses various human tumor-associated loci, as well as a chemokine receptor gene cluster (12). Particular areas of the chromosome 3 short arm tend to be lost in numerous types of tumor cells (13,14).

At present, the exact roles of TMEM41A in GC have not been determined. In the present study, the expression of

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**Abbreviations:** GC, gastric cancer; TMEM41A, transmembrane protein 41A; EMT, epithelial-to-mesenchymal transition; PBS, phosphate-buffered saline

**Key words:** gastric cancer, transmembrane protein 41A, epithelial-to-mesenchymal transition, cell autophagy, metastasis

TMEM41A and the prognoses of 147 patients with GC, and the functional role of TMEM41A in GC-associated metastasis were evaluated to investigate the potential therapeutic role of TMEM41A in the treatment of GC.

## Materials and methods

**Patient specimens and cell lines.** Tumor tissues and matched adjacent normal gastric tissues were obtained from 147 patients (31-84-years-old) who underwent radical GC surgery between April 2007 and June 2010 at the Qingdao Municipal Hospital (Qingdao, China). Patients that received neoadjuvant chemotherapy or irregular therapy (standard therapy treatments, followed by 6 months of chemotherapy) were excluded from the present study. The adjacent normal tissues were laterally resected at a distance of  $\geq 5$  cm from the tumor margin. None of the subjects underwent neoadjuvant chemoradiotherapy. All the resected samples were immediately frozen in liquid nitrogen and maintained at  $-80^{\circ}\text{C}$  until use.

Written informed consent was provided by all patients prior to enrollment into the present study, and the study protocols were approved by the Ethics Committee of Qingdao University (Qingdao, China). The clinical data collected from the subjects are summarized in Table I, including patient age and sex, tumor location, size and differentiation, lymph node involvement, distant metastasis, and tumor, node and metastasis (TNM) classification. Postoperative follow-up was performed every 3 months for a minimum of 5 years of 104 patients; 43 patients did not undergo follow-up due to exclusion criteria that arose in the present study, including patients withdrawal and failure to adhere to courses of standard treatment.

The human GC cell lines MKN-45, MGC-803, NCI-N87, SNU-5, KATO III, HGC-27, BGC-823, SGC-7901 and AGS were obtained from the American Type Culture Collection (Manassas, VA, USA), and were cultured in Dulbecco's modified Eagle's medium (Gibco; Thermo Fisher Scientific, Inc., Waltham, MA, USA), McCoy's 5A complete medium or RPMI-1640 (Sigma-Aldrich; Merck KGaA, Darmstadt, Germany), supplemented with 10% heat-inactivated fetal bovine serum (FBS; Sigma-Aldrich; Merck KGaA). All cells were maintained in a humidified atmosphere with 5%  $\text{CO}_2$  at  $37^{\circ}\text{C}$ .

**RNA extraction and reverse transcription-quantitative polymerase chain reaction (RT-qPCR) analysis.** TRIzol reagent (Invitrogen; Thermo Fisher Scientific, Inc.) was employed to isolate total RNA from cell lines according to the manufacturer's protocol. cDNA was obtained from 2  $\mu\text{g}$  RNA using the PrimeScript RT reagent kit (Takara Biotechnology Co., Ltd., Dalian, China). The mRNA expression levels of TMEM41A were determined by semi-qPCR with GoTaq polymerase (Promega Corporation, Madison, WI, USA) following the manufacturer's protocols. qPCR was followed by amplification with SYBR<sup>®</sup> Premix Ex Taq (Takara Biotechnology Co., Ltd.) using an ABI 7500HT system (ABI; Thermo Fisher Scientific, Inc.). The thermocycling program was set as follows: Initial denaturation for 5 min at  $95^{\circ}\text{C}$ ; 40 cycles at  $95^{\circ}\text{C}$  for 15 sec and  $60^{\circ}\text{C}$  for 30 sec. The primers used were as follows: TMEM41A forward, 5'-CTGCTGTGCTGTGTGTTGAC-3' and reverse, 5'-GTGTCCCAGGAGAAAAGAGCA-3';

Table I. Association between TMEM41A expression and clinicopathologic features.

Variable	Low TMEM41A expression	High TMEM41A expression	P-value
Total	39	65	
Sex			0.545
Male	21	35	
Female	18	30	
Age (years)			0.491
$\leq 50$	15	26	
$> 50$	24	39	
Tumor size (cm)			0.566
$\leq 5$	25	40	
$> 5$	14	25	
Histology			0.488
Adenocarcinoma	34	57	
Mucinous	5	8	
Local tumor invasion			0.584
Early stage	36	61	
Late stage	3	4	
Lymph node metastasis			0.0026
Negative	25	22	
Positive	14	43	
Distant metastasis			0.0179
Negative	34	43	
Positive	5	22	
TNM stage			0.00238
I-II	27	25	
III-IV	12	40	

TMEM41A, transmembrane protein 41A; TNM, tumor, node and metastasis.

and  $\beta$ -actin forward, 5'-AGTGTGACGTGGACATCCGCAAG-3' and reverse, 5'-ATCCACATCTGCTGGAAGGTGAC-3'. The  $2^{-\Delta\Delta\text{Ct}}$  method was used to quantify the expression levels of RNA (15). For semi-qPCR, the DNA products were run on a 1.5% agarose gel containing ethidium bromide. The bands were quantified using a UV illuminating instrument (Bio-Rad Laboratories, Inc., Hercules, CA, MA, USA).

**Cell transfection.** The small interfering (si)-negative control (NC; cat. no. CON077) and si-TMEM41A (cat. no. 38422-2) oligonucleotides were synthesized by Shanghai GenePharma Co., Ltd., (Shanghai, China) and transfected at 20 nM. the oligonucleotides sequence of the siRNA-TMEM41A (5'-3') is: Ccgg-(stem)gcGGAAGTAGCTTGCCCTCACTCTC GAG (loop)-AGTGAGGCAAGCTACTTCCGC(stem)-TTT TTg. The control (pCMV6-NC) or TMEM41A overexpression (pCMV6-TMEM41A) vectors were constructed by OriGene Technologies, Inc. (Rockville, MD, USA). Oligonucleotide transfection was performed using 0.5-1.0  $\mu\text{l}$  siRNA/vector with

Lipofectamine® 2000 (Invitrogen; Thermo Fisher Scientific, Inc.), according to the manufacturer's protocol; successful transfection was confirmed via western blotting as described below. Cells were collected at 48 h following transfection.

**Immunohistochemistry.** Immunohistochemistry with the gastric tumor and adjacent normal tissues was performed as previously described (16). A TMEM41A rabbit polyclonal antibody (1:50; sc-103285; Santa Cruz Biotechnology, Inc., Dallas, TX, USA) was utilized as the primary antibody (overnight, 4°C); a secondary antibody (1:1,000; GK600705; Gene Tech Biotechnology Co., Ltd. (Shanghai, China) was applied for 1 h at room temperature. Subsequently, sections were counterstained with hematoxylin at room temperature for 40 min and analyzed under a light microscope (magnification, x10). The sections were scored semi-quantitatively by two observers independently, in a blinded manner and analyzed under a light microscope. The scoring system was as follows: 0, 0% immunoreactive cells; 1, <5% immunoreactive cells; 2, 5-50% immunoreactive cells and 3, >50% immunoreactive cells. Scores of 0 or 1 were considered as low expression levels, and scores of 2 or 3 were considered as high expression levels.

**Cell proliferation analysis.** Cell proliferation assays of transfected cells were performed using a Cell Counting kit-8 (Dojindo Molecular Technologies, Inc., Kumamoto, Japan) according to the manufacturer's protocols; absorbance measurements at 450 nm were conducted to determine the cell proliferative ability in each well. The cells were seeded in a dish at a density of  $1 \times 10^5$  cells/dish and cultured for 24 h at 37°C prior to transfection; detection was performed at 24, 48 and 72 h post-transfection.

**Wound-healing assay.** A wound-healing assay was employed to assess the migration capacity of transfected cells in a defective monolayer. In brief, cells were seeded in 6-well plates and a 100- $\mu$ l pipette tip was used to conduct scratches. The medium was discarded, the cells were washed with PBS and fresh medium (RPMI-1640/DMEM and 10% FBS) was added, followed by the analysis of scratch closure under a light microscope at 18 h. Calculations were performed using the following formula:  $(S_{x \text{ time } 0} - S_{x \text{ time point}} / S_{x \text{ time } 0}) \times 100$ , where S indicated the distance in  $\mu$ m.

**Cell migration assays.** A cell migration assay was performed using Costar Transwell inserts (Thermo Fisher Scientific, Inc.) with a diameter of 6.5 mm and a pore size of 8.0  $\mu$ m. Briefly, cells ( $1 \times 10^4$ ) resuspended in 300  $\mu$ l serum-free medium were seeded into the upper Transwell chamber, and the bottom chamber was supplemented with 10% FBS. The cells were incubated for 24 h. Subsequently, the upper chamber was stained with Diff-Quik stain (Polysciences, Inc., Warrington, PA, USA) according to the manufacturer's protocol. Cell numbers were counted in 10 fields of view under an inverted microscope (magnification, x40; DMI600B; Leica Microsystems GmbH, Wetzlar, Germany), and the proportion of migrating cells was determined after normalization to control cells.

**Cell immunofluorescence for cytoskeleton stain and autophagy.** Cells were seeded on coverslips when a single

monolayer was achieved from culture, fixed in 4% paraformaldehyde for 10 min at room temperature, permeabilized in 0.1% Triton X-100 in PBS for 4 min. Subsequently, cells were blocked with 1% BSA for 20 min at room temperature, and then incubated at room temperature for 30 min with rhodamine-conjugated phalloidin (Sigma-Aldrich; Merck KGaA) at 1:200 in blocking solution (1% BSA in 0.1% Triton X-100 in PBS). The nuclei were counterstained with DAPI for 20 min at room temperature. Images were captured with a fluorescence microscope.

Autophagosome formation was detected by red fluorescent protein (RFP)-LC3 puncta incorporation into autophagic vacuoles. Sterile coverslips were seeded with  $5 \times 10^5$  cells into 6-well plates. Following adherence and washing twice with PBS, cells were cultured in serum-free medium for 24 h. Subsequently, the cells were transiently transfected with RFP-LC3B-expressing GFP-vector (OriGene Technologies, Inc.) for 32 h with Lipofectamine® 2000, followed by washing with cold-ice PBS and fixation with 4% formaldehyde in PBS at room temperature for 20 min. The nuclei were counterstained with DAPI for 20 min at room temperature. Images were captured with a fluorescence microscope (magnification, x100).

**Tumor formation in nude mice.** Stably siRNA-transfected BGC-823 cells selected via incubation with 1-3  $\mu$ g/l puromycin and were then suspended in 0.2 ml PBS and injected into the caudal vein of 12 5-week-old female BALB/c nude mice (20-22 g) ( $5 \times 10^6$  cells/mouse). The mice housed under a 12 h light/dark cycle with free access to food and water at 20-24°C, 50% humidity; mice were monitored for 70 days and then sacrificed. The tumor size was recorded and tumor volume was calculated using the formula:  $(\text{length} \times \text{width}^2)/2$ . Tumors formed *in vivo* were collected, sectioned and stained with hematoxylin and eosin (H&E) at room temperature for 40 min and analyzed under a light microscope.

**Western blot analysis.** Briefly, for western blotting, after washing with cold-ice PBS, total protein of transfected cells was isolated by incubation in radioimmunoprecipitation assay buffer (Cell Signaling Technology, Inc., Danvers, MA, USA) containing complete protease inhibitor cocktail (Roche Diagnostics, Basel, Switzerland) for 1 h on ice. Following centrifugation ( $15,000 \times g$  at 4°C for 20 min), the supernatants were collected. Protein concentration was determined using a bicinchoninic acid protein assay kit (Thermo Fisher Scientific, Inc.). Samples (2-10 mg) were subjected to 8-12% SDS-PAGE according to protein mass loaded and transferred to polyvinylidene difluoride membranes activated with 100% methanol. After blocking in 5% fat-free milk for 1 h, the membranes were incubated with a primary antibody against TMEM41A (dilution, 1:200; Santa Cruz Biotechnology, Inc.), E-cadherin (ab15148), N-cadherin (ab18203), p62 (ab91526), LC3-I/II (ab51520), and  $\beta$ -actin (ab8227) (dilution, 1:1,000; Abcam, Cambridge, MA, USA) overnight on ice and incubated with the indicated secondary antibody (in-house; 1:1,000) for 2 h at room temperature. The membrane was then washed with tris-buffered saline with 1:1,000 Tween-20 in v/v. prior to treatment with an enhanced chemiluminescent reagent and placed in a dark room to allow the reaction to run to completion.  $\beta$ -actin was used as a positive control.



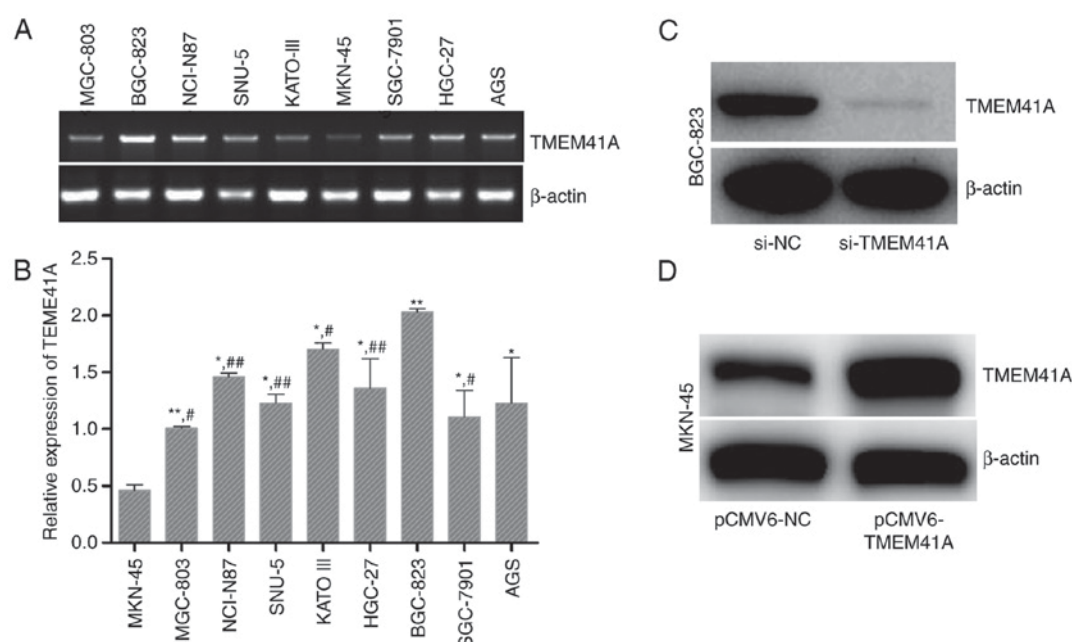


Figure 1. Expression of TMEM41A in GC cells. (A) Differential expression levels of TMEM41A mRNA in GC cells as determined by semi-qPCR. (B) Differential expression levels of TMEM41A mRNA in GC cells, as determined with RT-qPCR. (C) Effect of the knockdown of TMEM41A with siRNA in BGC-823 cells. (D) Effect of the overexpression of TMEM41A with pCMV-TMEM41A in MKN-45 cells. \* $P < 0.05$  and \*\* $P < 0.01$  vs. MKN-45; # $P < 0.05$  and ## $P < 0.01$  vs. BGC-823. GC, gastric cancer; RT-qPCR, reverse transcription-quantitative polymerase chain reaction; si-NC, small interfering RNA-negative control; TMEM41A, transmembrane protein 41A; pCMV6-TMEM41A, TMEM41A overexpression plasmid.

**Bioinformatic analysis.** Investigation with The Cancer Genome Atlas (TCGA; <https://cancergenome.nih.gov/>) dataset was conducted to analyze the expression levels of TMEM41A in normal gastric tissues; Protein Atlas ([www.proteinatlas.org](http://www.proteinatlas.org)) also revealed that the mRNA and protein expression levels of TMEM41A may be inconsistent.

**Statistical analysis.** Data were analyzed using SPSS version 18 (SPSS Inc., Chicago, IL, USA). The results are presented as the mean  $\pm$  standard error of the mean. Kaplan-Meier analysis was conducted, followed by a log-rank test. All experiments were performed in triplicate. The differences between groups were analyzed using one-way analysis of variance followed by the Least Significant Difference t-test. Categorical data was analyzed with a  $\chi^2$  test. In all cases,  $P < 0.05$  was considered to indicate a statistically significant difference and  $P < 0.01$  was considered to indicate a highly statistically significant difference.

## Results

**Expression of TMEM41A in human GC cell lines.** The expression levels of TMEM41A in the nine human GC cell lines were determined. As presented in Fig. 1A and B ( $F = 5.381 > F_{0.05}$ ;  $P < 0.05$ ), the expression levels of TMEM41A were the highest in BGC-823 cells and the lowest in MKN-45 cells (Fig. 1B). Therefore, BGC-823 cells were transfected with siRNA against TMEM41A, and MKN-45 cells were transfected with a TMEM41A-overexpression plasmid to investigate the effects of siRNA and plasmid transfection on the expression levels of TMEM41A. Western blot analysis demonstrated that si-TMEM41A markedly decreased the expression levels of TMEM41A in BGC-823 cells, and that pCMV6-TMEM41A

notably increased the expression levels of TMEM41A in MKN-45 cells (Fig. 1C and D).

**TMEM41A expression in human GC cells and its association with clinicopathological characteristics.** To investigate the potential role of TMEM41A in GC-associated metastasis, RT-qPCR was used to assess TMEM41A expression in 147 cases of GC and paired adjacent normal tissues, which revealed the overexpression of TMEM41A mRNA in the GC tissue exhibited by 123/147 (83.67%) of the pairs, with a mean fold change of 2.22 for the cancer tissues; expression levels were significantly higher within the tumor tissues compared with in that of the normal tissues (Fig. 2A). In addition, the relative mean expression levels of TMEM41A were significantly higher in subjects with lymph node involvement compared with those without ( $P < 0.01$ ; Fig. 2B). Consistently, TMEM41A expression was significantly higher in subjects with distant metastasis compared to those without ( $P < 0.01$ ; Fig. 2C). The associations of TMEM41A expression with the clinicopathological characteristics of patients with GC are summarized in Table I. TMEM41A expression was observed to be significantly associated with advanced TNM stages (III and IV), lymph node involvement and distant metastasis ( $P < 0.01$ ; Fig. 2B-D); however, TMEM41A expression was not significantly associated with other clinicopathological characteristics, including patient age, sex, tumor location, differentiation or size (all  $P > 0.05$ ).

**TMEM41A overexpression is correlated with lymph node metastasis, distant metastasis and advanced TNM stage in human GC tissues.** Subsequently, the present study determined the expression levels of TMEM41A protein in the same pathological specimens. TMEM41A protein was

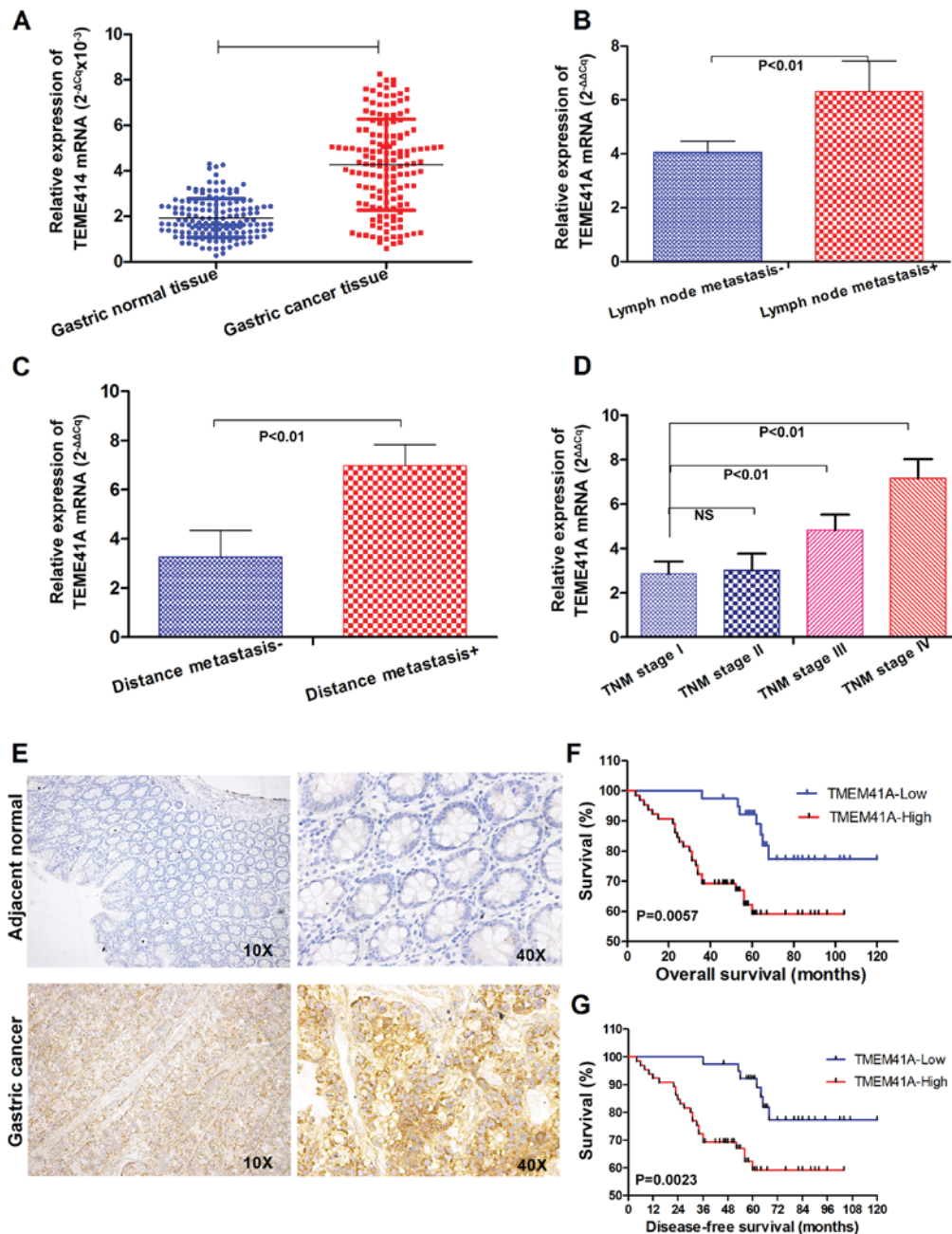


Figure 2. Expression of TMEM41A in GC tissues vs. normal gastric tissues and its correlation with prognosis and clinicopathological characteristics. (A) mRNA expression levels of TMEM41A in GC were higher compared with in adjacent normal gastric tissues. (B) Patients with GC and lymph node metastasis exhibited higher TMEM41A mRNA expression levels compared with in lymph node metastasis-negative patients with GC. (C) Patients with GC and distant metastasis exhibited higher TMEM41A mRNA expression levels compared with patients with GC, but without distance metastasis. (D) Patients with GC and more advanced TNM stages exhibited higher TMEM41A mRNA expression levels compared with in patients that exhibited early-stage disease (TNM stage I/II). (E) Expression levels of TMEM41A appeared to be higher in GC tissues compared with in normal gastric tissues (magnification, x10 or 40). (F) Patients with GC and high expression levels of TMEM41A exhibited a poorer overall survival following radical tumor resection. (G) Patients with GC and high expression levels of TMEM41A exhibited a poorer disease-free survival following radical tumor resection. GC, gastric cancer; NS, not significant; TMEM41A, transmembrane protein 41A; +, patients with GC and metastasis; -, patients with GC without metastasis.

suggested to be localized on the cell plasma membranes of GC samples (Fig. 2E). As presented in Table I, of the 147 patients with GC, 104 (selected based on follow-up, staining, and other factors, including variations in therapy) were divided into two subgroups based on the expression levels of TMEM41A. The high-expression group included 65 patients with a higher expression levels of TMEM41A in the carcinoma tissue compared with in adjacent normal gastric tissues, whereas in the low-expression group (n=39), the mean TMEM41A

staining intensity obtained during semi-quantitative analysis of carcinoma tissue samples decreased compared with the adjacent normal gastric tissues (data not shown).

Survival analyses were performed on patients with GC. Patients in the TMEM41A high-expression group exhibited poorer overall and disease-free survival compared with the low-expression group (Fig. 2F and G).

There were no significant differences in the 1-year overall survival rate between the two groups; however, the 3- and

Table II. Association between TMEM41A expression and survival rate.

Survival measurement (%)	TMEM41A density		P-value
	Low-TMEM41A	High-TMEM41A	
1-year overall survival	95.6±4.0	89.5±4.4	0.012 <sup>a</sup>
3-year overall survival	90.4±8.8	75.5±8.7	
5-year overall survival	75.6±6.0	45.8±6.8	

<sup>a</sup>P=0.012 across all survival measurements. TMEM41A, transmembrane protein 41A.

5-year overall survival rates were poorer in the TMEM41A high-expression group. The 5-year overall survival rates for the high- and low-expression groups were 75.6 and 45.8%, respectively (P=0.012; Table II). This finding suggested that high expression levels of TMEM41A in GC may be considered as a predictor of poor prognoses of GC.

*TMEM41A does not promote the proliferation of GC cells.* The present study performed Cell Counting kit-8 assays to investigate the effects of TMEM41A on GC cell proliferation. The Cell Counting kit-8 assays demonstrated that the stable knockdown of TMEM41A mediated by siRNA was not associated with variations in BGC-823 cell proliferation (P>0.0; Fig. 3A). Similar results were observed in TMEM41A-overexpressing MKN-45 cells (P>0.05; Fig. 3B).

*Association between TMEM41A expression and the migration of human GC cells.* In order to investigate the role of TMEM41A in cell migration, wound-healing and Transwell assays were performed. The results indicated that knockdown of TMEM41A significantly decreased the migration ability of BGC-823 cells (Fig. 4A; P<0.01). Conversely, a significant increase in the rate of migration was observed when TMEM41A was overexpressed in MKN-45 cells compared with in the control (Fig. 4B; P<0.01). Transwell assays indicated that the migration ability of BGC-823 cells was significantly decreased following the silencing of endogenous TMEM41A compared with in the control (Fig. 4C; P<0.01). Conversely, the overexpression of TMEM41A significantly increased the migration ability of MKN-45 cells compared with in the control (Fig. 4D; P<0.01).

*Different expression of TMEM41A leads to cytoskeletal rearrangement and inhibition of cell adhesion and spreading.* To determine the role of TMEM41A in the regulation of cytoskeletal dynamics, rhodamine-conjugated phalloidin staining was applied (Fig. 5A). Inside the cells, actin filaments were distributed in a disorderly manner, and no evident stress fiber formation was observed in TMEM41A knockdown cells, indicating that the knockdown of TMEM41A may inhibit stress fiber formation. On the cell membranes, membrane ruffling and the formation of pseudopodia, reflective of cell migration, were markedly observed in control and siTMEM41A transfected cells; in the overexpression group, opposing findings were observed. These results indicate that high expression levels of TMEM41A may promote metastatic properties of GC cells, consistent with the wound-healing assay as aforementioned.

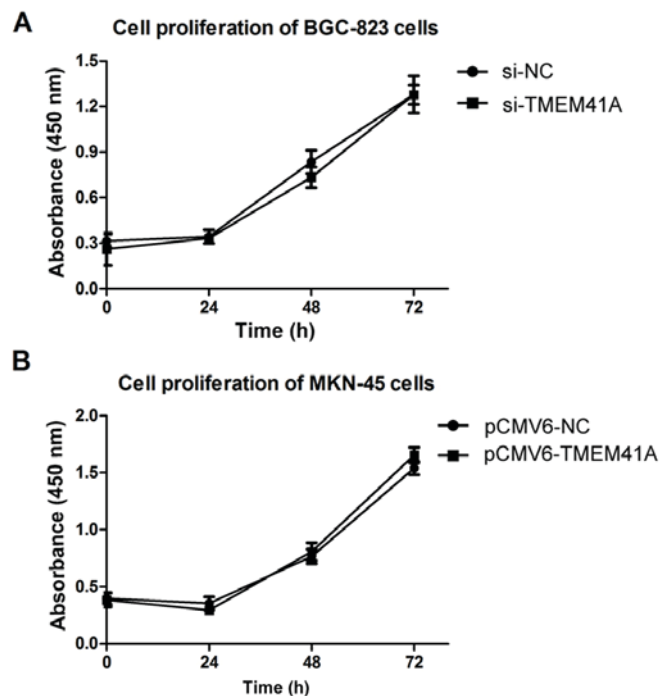


Figure 3. TMEM41A does not promote the proliferative potential of GC cell lines. (A) Cell Counting kit-8 assay demonstrated that stable knockdown of TMEM41A mediated by siRNA transfection did not lead to varying proliferation of BGC-823 cells. (B) Similar results were observed in TMEM41A-overexpressing MKN-45 cells. GC, gastric cancer; NC, negative control; si, small interfering RNA; TMEM41A, transmembrane protein 41A; pCMV6-TMEM41A, TMEM41A overexpression plasmid.

*TMEM41A may induce autophagy within GC cells.* To further verify the hypothesis of the present study, RFP-LC3B plasmids were transiently transfected into the cells. Punctate fluorescence demonstrated the induction of autophagy. As presented in Fig. 5B, no notable punctate fluorescence was observed in si-TMEM41A-treated BGC-823 cells, but not in control vector-transfected or TMEM41A-overexpressing cells. These results suggested that TMEM41A may be associated with autophagy.

*Effect of TMEM41A downregulation on E-cadherin expression and autophagy in GC cells.* EMT is crucial for the invasion and migration of tumor cells, and the development of metastasis. To investigate the mechanisms underlying the effects of TMEM41A on cell migration, the effects of TMEM41A knockdown or overexpression on



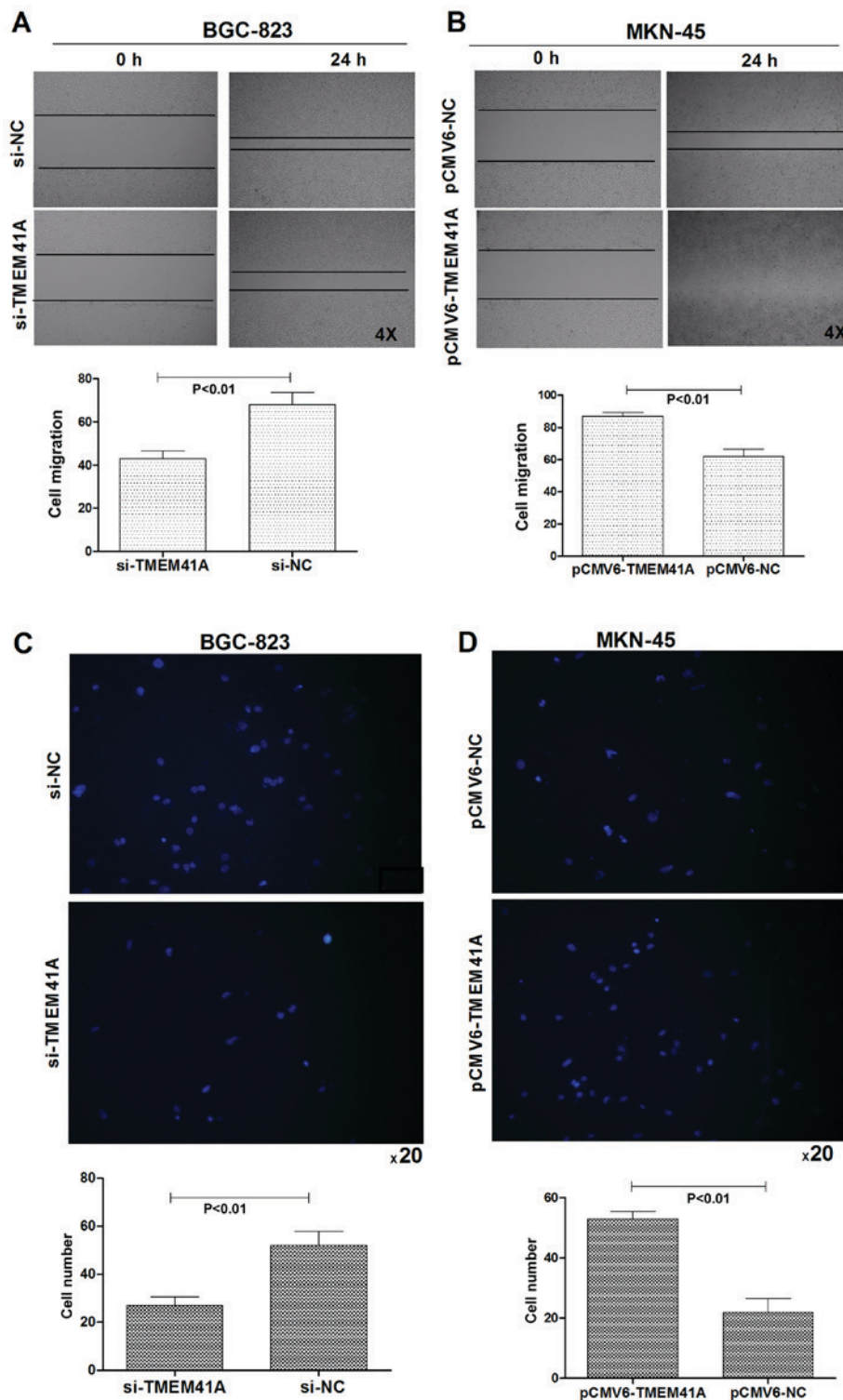


Figure 4. Up- and downregulation of TMEM41A expression affects the migration potential of GC cells. (A) Downregulation of TMEM41A expression decreased the motility of BGC-823 cells. (B) Upregulation of TMEM41A expression promoted the motility of MKN-45 cells. (C) Downregulation of TMEM41A expression decreased the motility of BGC-823 cells. (D) Upregulation of TMEM41A expression promoted the motility of MKN-45 cells. GC, gastric cancer; si, small interfering RNA; NC, negative control; TMEM41A, transmembrane protein 41A; pCMV6-TMEM41A, TMEM41A overexpression plasmid.

EMT were examined by analyzing the expression levels of EMT-associated factors (Fig. 6A). The knockdown or overexpression of TMEM41A affected the expression levels of E-cadherin and N-cadherin, and the autophagy-associated factors p62 and LC3-1/2, indicating that the knockdown or overexpression of TMEM41A may affect EMT and the autophagy process (Fig. 6A).

*Knockdown of TMEM41A affects tumor metastasis in nude mice.* To further determine the effect of TMEM41A on GC tumor metastasis *in vivo*, the present study employed an siRNA-mediated stable knockdown cell model. H&E staining indicated that tumors were formed *in vivo* at a lower rate in the si-TMEM41A group compared with in the si-NC group (Fig. 6B). Both groups exhibited successful tumor formation

in the lung, and tumor size of the si-TMEM41A group was significantly smaller compared with in the si-NC group (Fig. 6C;  $P < 0.01$ ). These results indicated alterations in the expression of TMEM41A and EMT-associated markers may be associated with metastatic behavior *in vivo*.

## Discussion

To retain normal organ size and specific organ morphology, cell numbers in tissues of high proliferative potential are strictly controlled by the balance between cell proliferation and apoptosis during organ development (17). This suggests that the dysregulation of genes associated with these processes may be detected in human malignancy.

Metastasis is one of the key processes affecting the prognosis of GC, but it is a complex process which requires further investigation (3). A total of ~70% of patients with GC develop metastasis (18). Advances in treatments for GC have been made; however, patients with advanced or metastatic GC exhibit poor prognosis (19). Therefore, the identification of novel diagnostic or prognostic biomarkers for GC is one of the major goals in this field.

The TMEM protein superfamily comprises >310 different members, including membrane proteins, which are extensively expressed on cell and lysosomal membranes, and the endoplasmic reticulum, are strongly associated with membrane integrity, transport and signaling pathways (20). An increasing number of studies have investigated the TMEM superfamily and tumor-associated genes, particularly the oncogenes, TMEM16a (7,8), TMEM17a and TMEM17b (9) and the tumor suppressor gene TMEM100 (21,22). Certain TMEM genes have been reported to be involved in the development and metastasis of GC, such as TMEM16a (20).

TMEM41A has been considered to be a potential cancer-associated gene, as it is located on the long arm of chromosome 3 (10,11). Various human cancer-associated gene loci are present on chromosome 3. One of the most common abnormalities in human cancers is the loss of the short arm of chromosome 3 (23). Therefore, the tumor suppressor gene(s) located on 3p may serve a key role in the development of numerous types of cancer (13,14). In addition, the amplification of chromosome 3q is associated with cancer; the amplification of the distal portion of chromosome 3q has been reported to be an important signature associated with lung squamous cell carcinoma (24). The role of TMEM41A in cancer has not been previously investigated. The present study revealed that the TMEM41A expression levels were high in patients with GC. Increased TMEM41A expression levels were positively associated with distant metastasis, advanced TNM stage and lymph node involvement, as observed in the present study. Patients with low TMEM41A expression levels exhibited improved overall survival compared with those with high TMEM41A expression levels. To the best of the authors' knowledge, the present study is the first to investigate the prognostic potential of TMEM41A in patients with GC.

An increasing number of studies (25-28) have investigated the mechanisms underlying the aberrant expression of TMEM41A; however, the regulation of TMEM41A protein expression remains unclear. It has previously been

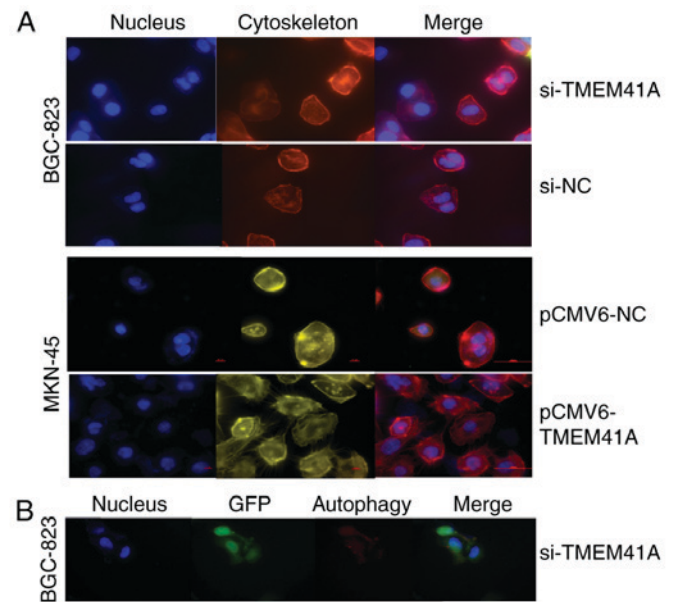


Figure 5. Alterations in the expression of TMEM41A leads to cytoskeletal rearrangement and induces autophagy in gastric cancer cells. (A) TMEM41A downregulation altered the cytoskeletal dynamics both inside and outside the cells, including an effect on membrane ruffling and pseudopodia formation. (B) TMEM41A induced autophagy in BGC-823 cells transiently transfected with RFP-LC3B plasmids in si-TMEM41A-treated BGC-823 cells. NC, negative control; RFP-LC3, red fluorescent protein-light chain 3; si, small interfering RNA; TMEM41A, transmembrane protein 41A; pCMV6-TMEM41A, TMEM41A overexpression plasmid; GFP, green-fluorescent protein.

demonstrated that its expression may be regulated by numerous mechanisms, including chromosomal abnormalities (25), polymorphisms (26), epigenetic mechanisms (27) and histone modifications (28). Further investigation in identifying the underlying mechanisms of irregular TMEM41A expression in GC is required. In particular, whether the upregulation of TMEM41A may be due to chromosomal abnormalities requires further investigation.

EMT is a series of events including the alteration of cell-cell and cell-extracellular matrix interactions, and cancer cell transformation from an epithelial to a mesenchymal phenotype (29). These alterations are often accompanied with increased cell motility. Therefore, EMT is a hallmark of cancer, and is associated with a poor clinical outcome and metastasis (30,31). During tumor progression and metastasis, EMT may be induced by autonomous oncogenic activation or inactivation of signaling molecules, in the presence or absence of external stimuli (32). In this process, markers of epithelial differentiation are downregulated, including E-cadherin; however, N-cadherin, Slug, vimentin, Twist-related protein-1 and Snail are upregulated (33). EMT is a key process in the metastasis of GC (34). The findings of present study suggested that TMEM41A may facilitate the metastasis of GC cells via EMT. Furthermore, TMEM41A may participate in the regulation of E-cadherin and N-cadherin expression; however, further experiments may be conducted in the future to elucidate the association between TMEM41A and the EMT signaling pathway.

It was previously suggested that autophagy may contribute towards the regulation of the migration and invasive abilities



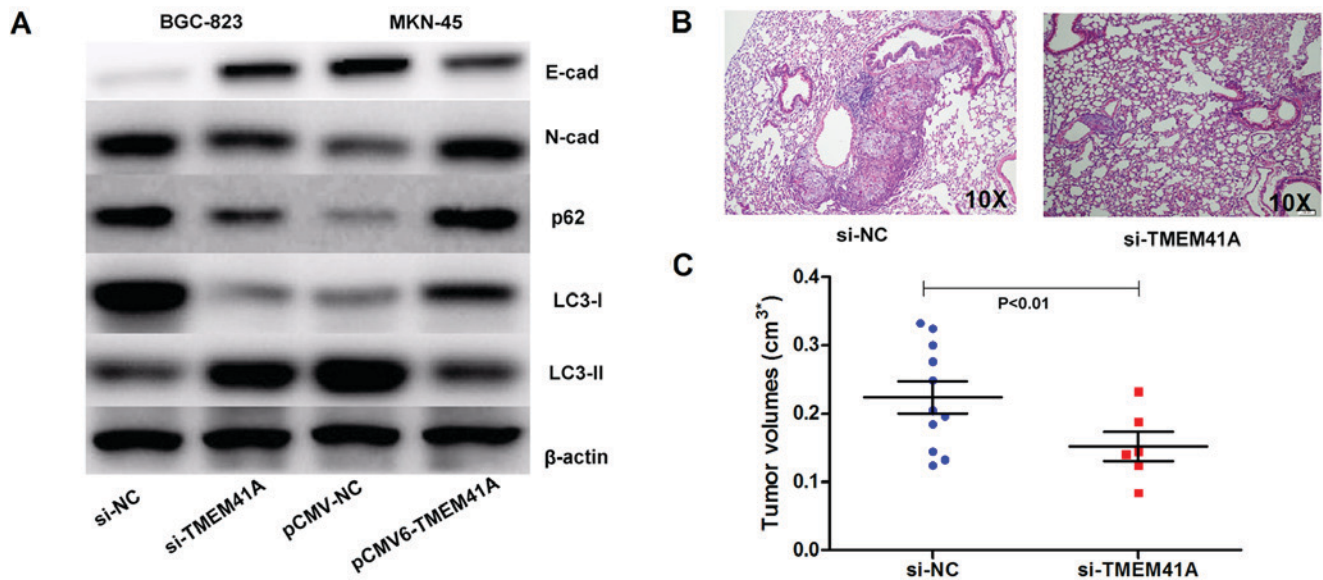


Figure 6. Knockdown of TMEM41A decreases the migration ability associated with EMT and autophagy in GC cells. (A) Western blot analysis demonstrated the effects of TMEM41A expression on EMT factors, including E-cad and N-cad expression and autophagy via the dysregulation of p62, and the conversion of LC3-I into LC3-II. (B) Knockdown of TMEM41A in BGC-823 cells impaired the formation of tumor metastases in the lungs of nude mice, as demonstrated by hematoxylin and eosin staining. (C) Knockdown of TMEM41A in the BGC-823 cells was associated with smaller tumor volume. Cad, cadherin; EMT, epithelial-mesenchymal transition; GC, gastric cancer; LC3, light chain 3; NC, negative control; si, small interfering RNA; TMEM41A, transmembrane protein 41A; E-cad, E-cadherin; N-cad, N-cadherin.

of cancer cells, and the activation of autophagy has been associated with the inhibition of EMT (35-37). It has been reported that autophagy accelerates the invasion of glioblastoma stem cells by regulating DNA damage-regulated autophagy modulator 1 and p62 (38). The present study demonstrated similar results, as the upregulation of E-cadherin was accompanied with that of p62 and the altered conversion of LC3-I to LC3-II. In addition, immunofluorescence analysis in the present study was not conducted with a control and the pCMV-TMEM41A group. Therefore, further analysis in the future is required to verify the findings of the present study. However, whether TMEM41A regulates EMT via autophagy requires further investigation.

Additionally, the data presented in The Cancer Genome Atlas dataset, revealed that TMEM41A was highly expressed in normal gastric tissues; however, immunohistochemistry analysis did not demonstrate such expression in tumor samples in the present study. Furthermore, analysis with Protein Atlas ([www.proteinatlas.org](http://www.proteinatlas.org)) also revealed that the mRNA and protein expression levels of TMEM41A may be inconsistent.

As the expression levels of TMEM41A at the gene and protein levels have not been fully investigated, contradictory findings may be reported. The present study reported that the mRNA expression levels of TMEM41A were similar at the protein level; however, the protein expression levels revealed by immunohistochemistry were contradictory. Therefore, the present study suggested that the dilution of antibody (1:50 vs. 1:400), post-translational modulation, or other unknown components may be associated with these differing results (data not shown). Conversely, the aforementioned databases have demonstrated a high correlation between TMEM41A mRNA expression and poor prognoses in endometrial, liver and pancreatic cancers, but not gastric cancer.

Therefore, further investigation is required to understand these differing expression profiles.

The present study demonstrated a reduction in TMEM41A expression levels in a pair of GC cell lines, and revealed that high TMEM41A expression levels may promote GC-associated metastasis, which may be mediated by the downregulation of E-cadherin expression. The present study aimed to highlight the potential of TMEM41A as a novel target against GC-associated metastasis and suggest that TMEM41A may be considered to be an important oncogene in GC. Further studies examining the mechanisms underlying the regulation of TMEM41A expression are required to investigate the possible association between TMEM41A with E-cadherin expression in GC.

#### Acknowledgements

Not applicable.

#### Funding

No funding was received.

#### Availability of data and materials

Not applicable.

#### Authors' contributions

BL and YX made substantial contributions to the design of the present study and wrote the manuscript. CQ made substantial contributions to the design of the present study and performed the animal experiment, XC conducted software analysis and revised the manuscript and WM directed and managed the

design of the present study. All authors read and approved the final version of the manuscript.

### Ethics approval and consent to participate

The present study was approved by the Ethics Committee of Qingdao University.

### Patient consent for publication

Not applicable.

### Competing interests

The authors declare that they have no competing interests.

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