

Original Article

TSPAN8 promotes gastric cancer growth and metastasis via ERK MAPK pathway

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Abstract: Aims: This study was designed to investigate the effects of Tetraspanin 8 (TSPAN8) overexpression and TSPAN8 suppression on gastric cancer cell proliferation and invasion. Furthermore, whether extracellular-signal regulated kinase (ERK) mitogen-activated protein kinase (MAPK) pathway was involved in TSPAN8's function on gastric cancer cells was examined. Methods: The expression of TSPAN8 in human gastric cancer tissues and gastric cancer cell lines was detected using real-time PCR and western blot analysis. TSPAN8-pcDNA3.1 plasmid or TSPAN8 siRNA was transfected into the gastric cancer cell lines to overexpress or suppress TSPAN8. Cells were treated with UO126 to inhibit ERK MAPK pathway. Cell proliferation and invasion were assessed by MTT and transwell-matrigel assay. Results: TSPAN8 was overexpressed in human gastric cancer tissues and gastric cancer cell lines compared with the normal. TSPAN8 overexpression promoted cell proliferation and invasion, while TSPAN8 suppression inhibited cell proliferation and invasion. TSPAN8 could activate the ERK MAPK pathway in gastric cancer cells, and MEK-ERK inhibition reversed the effects of TSPAN8 overexpression on cell proliferation and invasion. Conclusion: This study firstly demonstrated that TSPAN8 promotes gastric cancer cell growth and metastasis at least partially through the activation of ERK MAPK pathway. These findings provided a novel molecular basis for the understanding and treatment of gastric cancer.

Keywords: TSPAN8, ERK MAPK pathway, gastric cancer, growth, metastasis

Introduction

Gastric cancer is the second leading cause of cancer-related death worldwide [1], it is quite common in East Asia [2-4]. Most gastric cancer patients have advanced or metastatic diseases at the time of diagnosis [5], and the median survival time is often less than 1 year for these patients [6]. Therefore, it is important to understand the molecular mechanisms behind gastric cancer metastasis.

Tetraspanin 8 (TSPAN8) is a cell surface protein that belongs to the tetraspanin family. It has been implicated as a human tumor-associated antigen in certain types of cancers [7-11], such as hepatocellular carcinoma, pancreatic cancer, and colon carcinoma. Matsumura et al detected molecular markers of circulating gastric cancer cells using genome-wide microarray analysis, and found that TSPAN8 is not expressed in peripheral blood mononuclear

cells (PBMCs) of healthy volunteers, however, it is significantly upregulated in gastric cancer tissues and circulating cancer cells in the peripheral blood of patients with gastric cancer [12]. Recently, it is reported that TSPAN8 may mediate the effects of EGF on gastric cancer cells [13]. All these findings indicate that TSPAN8 may be involved in the development of gastric cancer. However, the basic mechanisms underlying TSPAN8's effects on gastric cancer cell growth and metastasis are still unclear and deserve investigation.

In the present study, we investigated the effects of TSPAN8 overexpression and TSPAN8 suppression on gastric cancer cell proliferation and invasion. Furthermore, whether extracellular-signal regulated kinase (ERK) mitogen-activated protein kinase (MAPK) pathway was involved in TSPAN8's function on gastric cancer cells was examined.

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Materials and methods

Tissues samples

The malignant tissues and matched normal tissues were collected from 15 patients with gastric cancer. All these patients received no chemotherapy or radiotherapy at the time of diagnosis. The study was approved by the Ethics Committee of Huaihe Hospital of Henan University, and all patients provided written informed consent prior to beginning this study.

Cell culture and cell transfection

Human gastric cancer cell lines (MGC-803, AGS, MKN-28, BGC-823) and human gastric epithelial cell line GES-1 were purchased from ScienCell Research Laboratories (San Diego, CA, USA). The cells were cultured in Dulbecco's modified Eagle's medium (DMEM; Gibco, Grand Island, NY, USA) supplemented with 10% fetal bovine serum (Gibco) in a humidified atmosphere of 5% CO₂ at 37°C. U0126 (Selleck Chemicals, Houston, TX, USA) was dissolved in DMSO to a final concentration of 10 µM and treated cells for 24 h. Transfection of pcDNA3.1, TSPAN8-pcDNA3.1, control siRNA and TSPAN8 siRNA was performed in MKN-28 and BGC-823 cells by using Lipofectamine 2000 transfection reagent (Invitrogen, Carlsbad, CA, USA) according to the manufacturer's instructions.

MTT assay

MTT assay was used to determine cell proliferation ability. The cells were seeded into 96-well plates at 1×10⁴ cells/well. After culturing at 37°C in a 5% CO₂ humidified atmosphere for indicated times, 50 µl MTT (KeyGEN BioTECH, Nanjing, Jiangsu, China) was added to each well and incubated at 37°C for 4 hours. The media was then removed and 150 µl DMSO was added to solubilize the formazan dye. Absorbance was measured at 570 nm using a microplate reader (Multiskan Ascent 354; Thermo Labsystems, Waltham, MA, USA).

Cell invasion assay

The matrigel was diluted with serum-free DMEM and pre-coated the Transwell chamber (Corning Inc., Corning, NY, USA) at 37°C for 30 min. After solidification, 1 ml cell suspension without serum at the density of 5×10⁴ cells/ml

was added to the upper chamber. The lower chamber filled with 1 ml DMEM containing 10% fetal bovine serum was used as a chemoattractant. After incubation at 37°C for 12 h, the non-invaded cells in the upper chamber were removed using a cotton swab, the invaded cells were fixed in 95% ethanol. Fixed cells were then stained with hematoxylin for 10 min and counted under the microscope (Olympus, Tokyo, Japan).

Reverse transcription-quantitative polymerase chain reaction (RT-qPCR) analysis.

Total RNA was extracted from the human gastric cancer cell lines and human gastric epithelial cell line using TRIzol reagent (Invitrogen). 3 µg of RNA was reversely transcribed to single-stranded cDNAs using Superscript II Reverse Transcriptase (Invitrogen). For the qPCR, 2 µl cDNA, 300 nM primers and SYBRGreen (Applied Biosystems) were mixed to a final volume of 20 µl. The primers used were as follows: TSPAN8 (sense: ctgtctctgatcctgctcc, antisense: ttatttcccaatcagcagc), GAPDH (sense: aagaaggtggtgaagcaggc, antisense: tccaccaccagttgctgta). The PCR reaction was followed by 38 cycles at 95°C for 30 sec, 60°C for 30 sec and 72°C for 45 sec, in the ABI Prism 7000 Sequence Detection System (Perkin-Elmer Applied Biosystems, Foster City, CA, USA). The expression of TSPAN8 was calculated and normalized to GAPDH using the 2^(-ΔΔCt) method.

Western blot

Cells (1×10⁵) and tissues (0.5 mg) were lysed in 1 ml ice-cold RIPA buffer (Beyotime, Shanghai, China) contained 2 mM phenylmethylsulfonyl fluoride, complete protease and phosphatase inhibitor cocktail (Roche, Mannheim, Germany). The lysates were resolved on 10% SDS-PAGE and transferred onto a PVDF membrane (Millipore, Billerica, MA, USA). After blocking with 5% non-fat milk at 37°C for 2 h, immunoblotting was done by the incubation of the primary antibodies (rabbit polyclonal to TSPAN8, Abcam, Cambridge, MA, USA; rabbit monoclonal to p-MEK1/2(S217/221), mouse monoclonal to MEK1/2, Cell Signaling Technology, Inc., Beverly, MA, USA; goat polyclonal to p-ERK1/2(T202/Y204), mouse monoclonal to ERK1/2, Santa Cruz Biotechnology, Inc., Santa Cruz, CA, USA; Mouse monoclonal to GAPDH, Boster, Wuhan, Hubei, China) at 37°C for 2 h,

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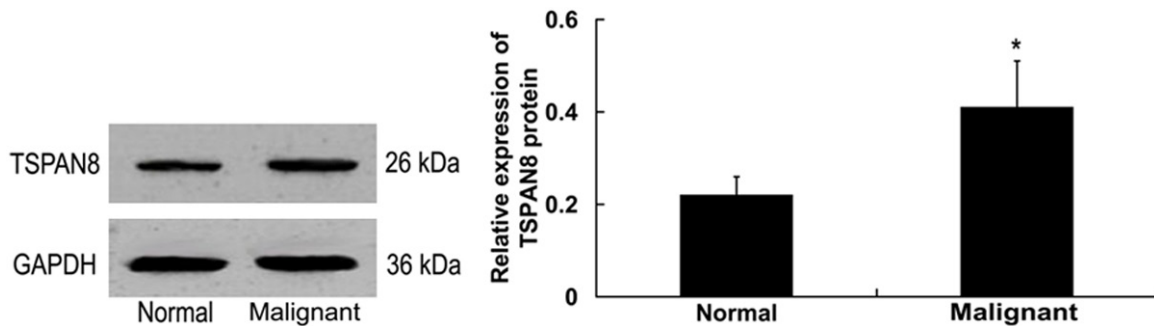


Figure 1. Expression of TSPAN8 protein in malignant tissues and matched normal tissues from patients with gastric cancer. * $P < 0.05$.

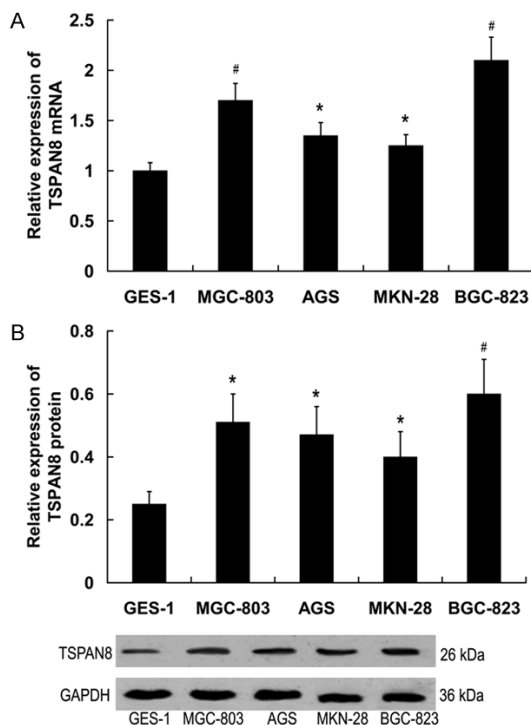


Figure 2. Expression of TSPAN8 in human gastric cancer cell lines (MGC-803, AGS, MKN-28, BGC-823) and human gastric epithelial cell line (GES-1). A. Expression of TSPAN8 mRNA. B. Expression of TSPAN8 protein. # $P < 0.01$, * $P < 0.05$, compared with GES-1 cells.

followed by incubation with the horseradish peroxidase labeled secondary antibody at 37°C for 1 h. GAPDH antibody was used to verify equal protein loading. Blots were developed with the enhanced chemiluminescence kit (Pierce Biotechnology, Inc., Rockford, IL, USA).

Statistical analysis

The data are presented as the mean \pm SD of three independent experiments. Significance of

differences was calculated by the two tailed Student's t test. A P value less than 0.05 was considered statistically significant.

Results

Expression of TSPAN8 in human gastric cancer tissues and gastric cancer cell lines

We investigated the expression of TSPAN8 in malignant tissues and matched normal tissues from patients with gastric cancer using western blot analysis. We found that TSPAN8 protein expression was higher in the malignant tissues compared with the matched normal tissues (Figure 1).

Next, the expression of TSPAN8 was examined in human gastric cancer cell lines (MGC-803, AGS, MKN-28, BGC-823) and human gastric epithelial cell line (GES-1). RT-qPCR results showed that compared with the GES-1 cells, TSPAN8 mRNA was increased in human gastric cancer cells. Among these gastric cancer cell lines, MKN-28 cells showed the lowest TSPAN8 mRNA expression, while BGC-823 cells showed the highest TSPAN8 mRNA expression (Figure 2A). The results of TSPAN8 protein expression from western blot analysis were similar to the results from RT-qPCR analysis (Figure 2B).

Effect of TSPAN8 overexpression on cell proliferation and invasion

As MKN-28 cells showed the lowest expression of TSPAN8, TSPAN8-pcDNA3.1 plasmid was then transfected into the MKN-28 cells to over-express TSPAN8. It was demonstrated in Figure 3A, the expression of TSPAN8 protein was increased about 4.8 fold in MKN-28 cells following transfection.

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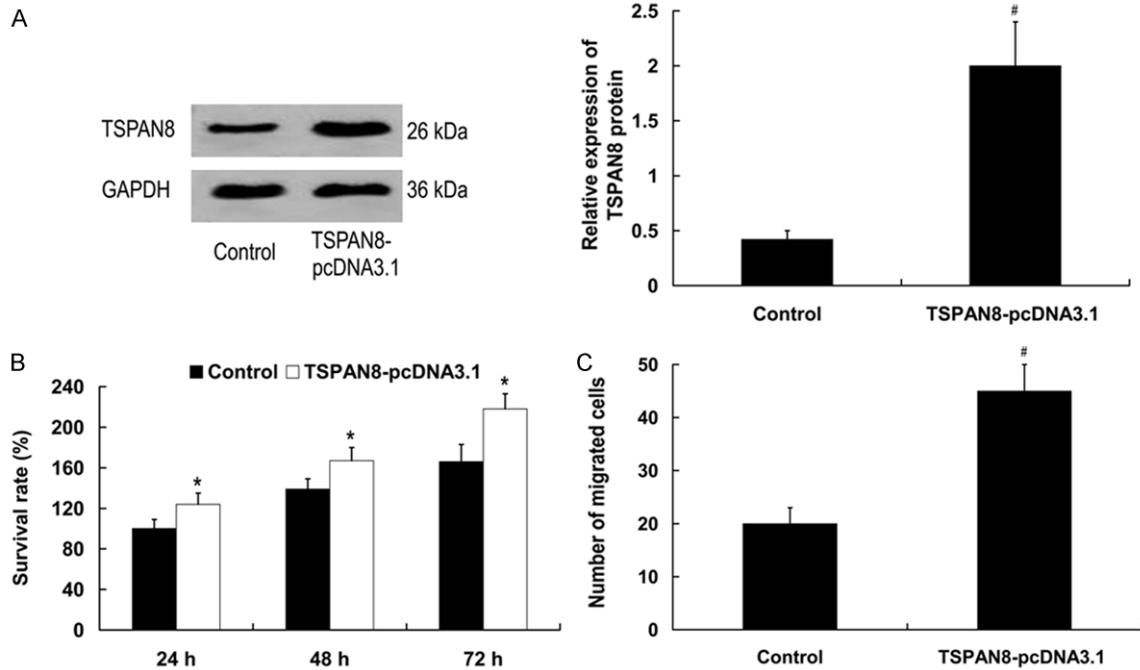


Figure 3. Effects of TSPAN8 overexpression on MKN-28 cell proliferation and invasion. A. Expression of TSPAN8 protein in MKN-28 cells following transfection with control vector and TSPAN8-pcDNA3.1 plasmid. B. MKN-28 cell proliferation detected by the MTT assay. C. MKN-28 cell invasion ability determined by the transwell-matrigel assay. # $P < 0.01$, * $P < 0.05$, compared with the cells transfected with the control vector.

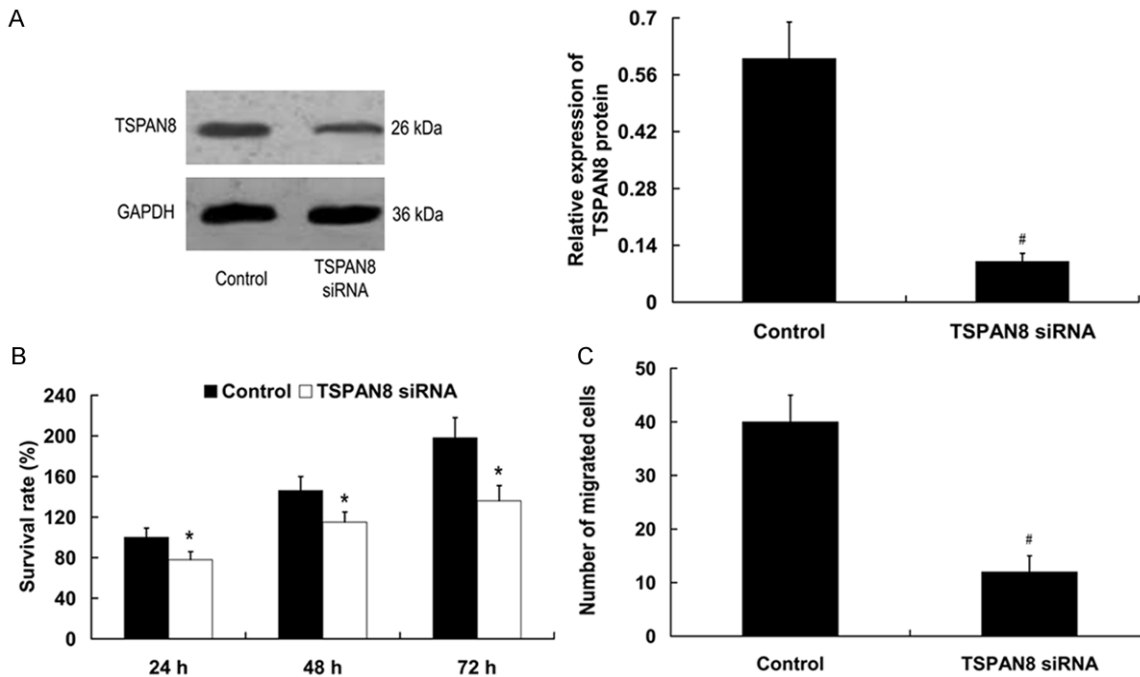


Figure 4. Effects of TSPAN8 inhibition on BGC-823 cell proliferation and invasion. A. Expression of TSPAN8 protein in BGC-823 cells following transfection with control siRNA and TSPAN8 siRNA. B. BGC-823 cell proliferation detected by the MTT assay. C. BGC-823 cell invasion ability determined by the transwell-matrigel assay. # $P < 0.01$, * $P < 0.05$, compared with the cells transfected with the control siRNA.

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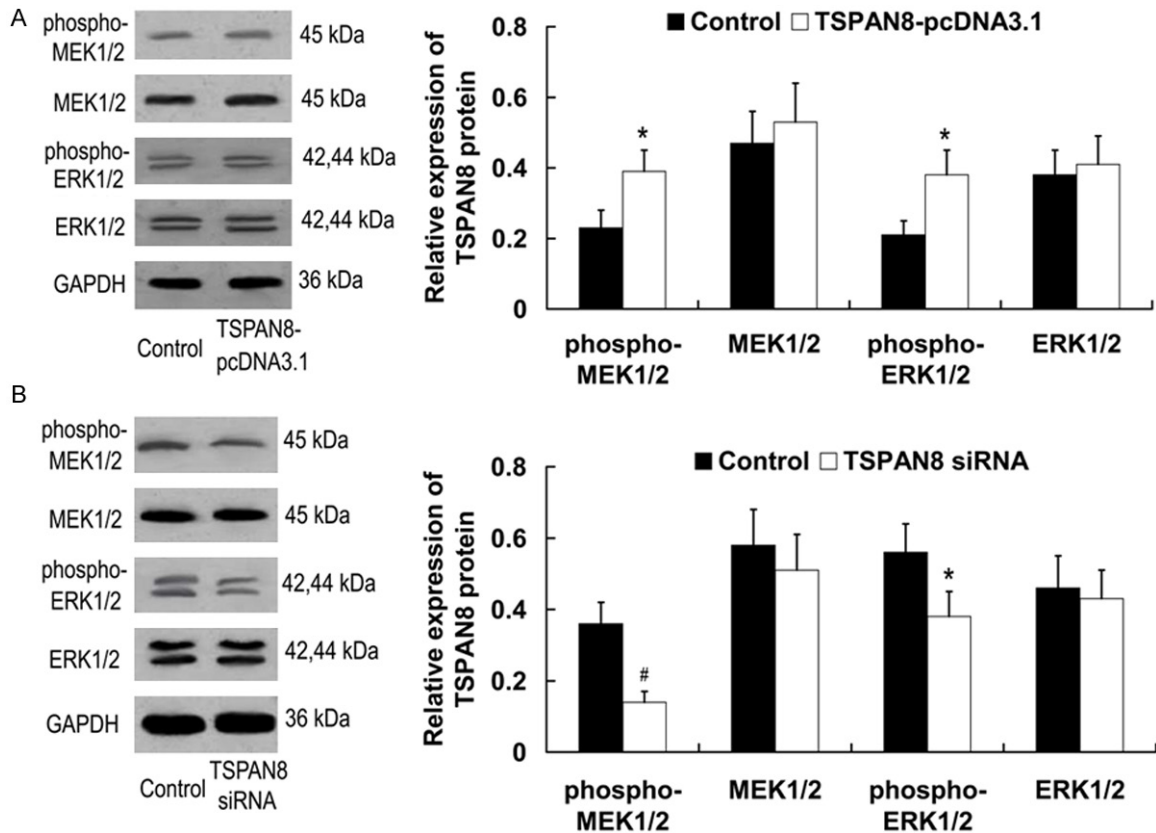


Figure 5. Effect of TSPAN8 on MEK-ERK expression in MKN-28 and BGC-823 cells. A. Effect of TSPAN8 overexpression on MEK-ERK expression in MKN-28 cells. * $P < 0.05$, compared with the cells transfected with the control vector. B. Effect of TSPAN8 inhibition on MEK-ERK expression in BGC-823 cells. # $P < 0.01$, * $P < 0.05$, compared with the cells transfected with the control siRNA.

The effect of TSPAN8 overexpression on MKN-28 cell proliferation was determined by MTT assay. It was shown that the survival rate was significantly increased in the TSPAN8 overexpression cells compared with the control cells (Figure 3B).

Transwell-matrigel assay was used to examine the effect of TSPAN8 overexpression on MKN-28 cell invasion. The migrated cell number in the control group was 20 ± 3 , however, it was increased to 45 ± 5 in the TSPAN8 overexpression group (Figure 3C).

Effect of TSPAN8 suppression on cell proliferation and invasion

TSPAN8 siRNA was transfected into the BGC-823 cells, which exhibited the highest expression of TSPAN8 among these human gastric cancer cell lines, to suppress TSPAN8 expression. Western blot analysis confirmed the significantly decreased expression of TSPAN8 pro-

tein in BGC-823 cells following transfection with the TSPAN8 siRNA (Figure 4A).

The MTT assay revealed that the survival rate of TSPAN8 suppressed-BGC-823 cells was significantly decreased compared with the control cells (Figure 4B). In addition, the migrated cell number was significantly decreased by TSPAN8 suppression in BGC-823 cells (Figure 4C).

Effect of TSPAN8 on MEK-ERK expression in MKN-28 and BGC-823 cells

To investigate the effect of TSPAN8 on MEK-ERK expression, TSPAN8-pcDNA3.1 plasmid and TSPAN8 siRNA were transfected into the MKN-28 cells and BGC-823 cells, respectively. Then, the expression levels of phospho-MEK1/2 and phospho-ERK1/2 were determined by western blot analysis, as normalized to total MEK1/2 and ERK1/2. We found phospho-MEK1/2 and phospho-ERK1/2 expression was significantly increased in the TSPAN8 overex-

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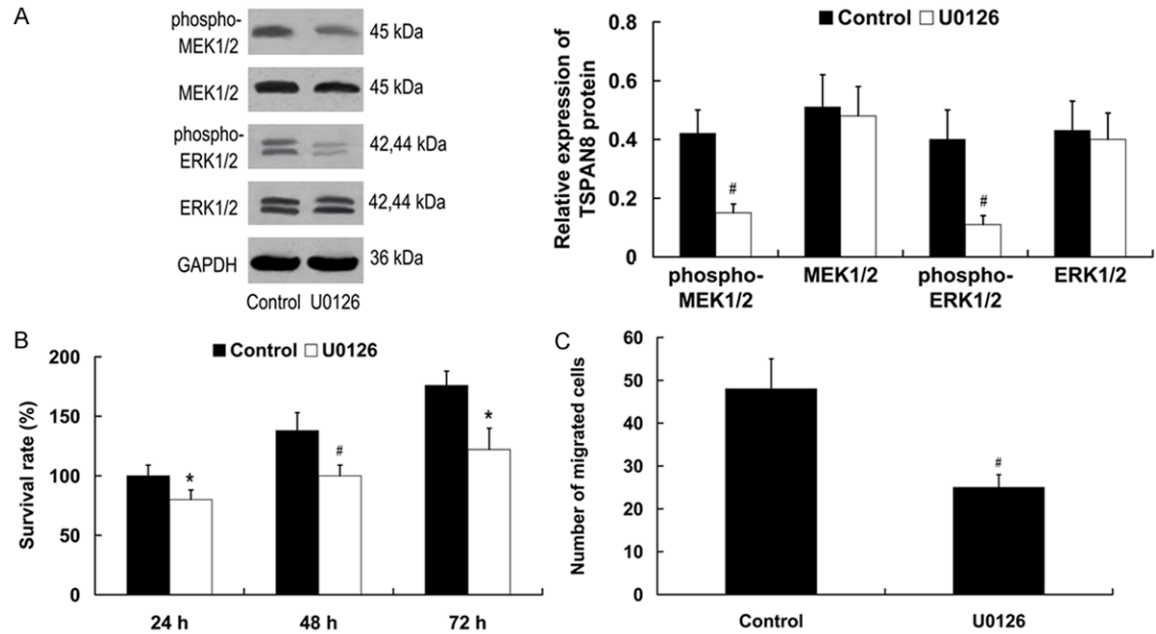


Figure 6. MEK-ERK inhibition reversed the effect of TSPAN8 overexpression on MKN-28 cell proliferation and invasion. A. Expression of MEK-ERK in MKN-28 cells following treatment with U0126. # $P < 0.01$, compared with the vehicle control. B. MKN-28 cell proliferation detected by the MTT assay. # $P < 0.01$, * $P < 0.05$, compared with the vehicle control. C. MKN-28 cell invasion ability determined by the transwell-matrigel assay. # $P < 0.01$, compared with the vehicle control.

pression cells, but decreased in the TSPAN8 suppressed cells (Figure 5A and 5B).

MEK-ERK inhibition reversed the effects of TSPAN8 overexpression on cell proliferation and invasion

To determine whether MEK-ERK was involved in the effects of TSPAN8 overexpression on cell proliferation and invasion, U0126 was used to inhibit MEK-ERK expression in TSPAN8-pcDNA3.1-transfected MKN-28 cells. As expected, western blot analysis showed that the expression of phospho-MEK1/2 and phospho-ERK1/2 was significantly decreased following U0126 treatment (Figure 6A). In the meanwhile, the increased survival rate and migrated cell number caused by TSPAN8 overexpression was significantly reduced following MEK-ERK inhibition (Figure 6B and 6C).

Discussion

In this study, we examined the expression of TSPAN8 in human gastric cancer tissues and a series of human gastric cancer cell lines. Consistent with the previous study [13], TSPAN8 was overexpressed in the malignant tissues

compared with the matched normal tissues from patients with gastric cancer. In addition, we found that TSPAN8 expression was also increased in human gastric cancer cell lines compared with the normal control cell line. Recently, it was found that TSPAN8 shows organ-specific metastasis in hepatocellular carcinoma, and TSPAN8 was differentially expressed in hepatocellular carcinoma cell lines with differing metastatic potential [14]. In the present study we detected the expression of TSPAN8 in human gastric cancer cell lines with different differentiation and metastatic potential, and it seems that among these gastric cancer cell lines, the endogenous expression of TSPAN8 was higher in low differentiation and high metastasis gastric cancer cell line BGC-823, and TSPAN8 expression was lower in MKN-28 gastric cancer cell line, which with high differentiation and low metastasis.

Although elevated expression of TSPAN8 has been detected in human gastric cancer tissues and a series of human gastric cancer cell lines, the mechanisms underlying its role in gastric tumorigenesis remain unclear. Next, we performed the in vitro study to elucidate the func-

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tion of TSPAN8 on gastric cancer cell growth and metastasis. We selected MKN-28 cell line with low expression of TSPAN8 and transfected with TSPAN8-pcDNA3.1 to overexpress TSPAN8. BGC-823 cell line with high expression of TSPAN8 was selected and transfected with TSPAN8 siRNA to suppress TSPAN8. The results from MTT assay and transwell-matrigel assay demonstrated TSPAN8 overexpression promoted cell proliferation and invasion, while TSPAN8 suppression inhibited cell proliferation and invasion. Collectively, these results indicate that TSPAN8 promotes gastric tumorigenesis.

TSPAN8 is a member of the transmembrane 4 superfamily, also known as the tetraspanin family. It has been evidenced that tetraspanins have a unique ability to organize a network of molecular interactions, including other members of the tetraspanin family, signaling molecules and integrins [15, 16]. Based on this 'tetraspanin-web', tetraspanins play important roles in fundamental cellular processes such as cell proliferation, differentiation and immune [17, 18]. More importantly, they can modulate the motility and the metastatic potential of tumor cells [19,20]. It has been demonstrated in metastatic pancreatic and colorectal carcinoma cell lines, TSPAN8 associates with CD151 and integrin $\alpha6\beta4$ to promote cell migration [21]. In addition, after protein kinase C (PKC) activation [11, 22], TSPAN8 could associate with integrins $\alpha3\beta1$ and $\alpha6\beta1$. Kuhn et al reported that TSPAN8 associates with EpCAM, CD44v6 and claudin-7 to form a complex that facilitates metastasis formation [23]. As TSPAN8 are able to connect a variety of proteins involved in different signaling pathways, we investigated whether ERK MAPK pathway, which was reported to be associated with gastric tumorigenesis [24-27], was involved in TSPAN8's function on gastric cancer cells.

The mitogen-activated protein kinases (MAPKs) are widely expressed in all cell types. It has been demonstrated that MAPK pathways play a major role in controlling a variety of cellular physiological processes [28, 29]. The extracellular-signal regulated kinase (ERK, also known as p42/44 MAP kinase) is a core kinase of the MAPK family and has been implicated in the regulation of cell proliferation, apoptosis, differentiation, and progression in a variety of human

cancers [30]. ERK can be activated by a wide variety of oncogenes and extracellular stimuli, and MEK is the canonical upstream regulator of ERK. In the present study, it was revealed that the phosphorylated protein levels of MEK and ERK were significantly increased in the TSPAN8 overexpression cells, but decreased in the TSPAN8 suppressed cells. It suggests that TSPAN8 could activate the ERK MAPK pathway in gastric cancer cells. U0126 was used to inhibit ERK MAPK pathway, in the meantime, we found the increased survival rate and migrated cell number caused by TSPAN8 overexpression was also significantly reduced. These results indicate that ERK MAPK pathway is essential for the effect of TSPAN8 on gastric cancer cell proliferation and migration.

In summary, this study analyzed the expression profile of TSPAN8 in malignant tissues from patients with gastric cancer and human gastric cancer cell lines. In addition, the in vitro study firstly demonstrated that TSPAN8 promotes gastric cancer cell growth and metastasis at least partially through the activation of ERK MAPK pathway. These findings provided a novel molecular basis for the understanding and treatment of gastric cancer.

Disclosure of conflict of interest

None.

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