Expression of transient receptor potential cation channel subfamily M member 8 in gastric cancer and its clinical significance

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Abstract. Transient receptor potential cation channel subfamily M member (TRPM8) is abnormally expressed in many malignant tumors, such as breast cancer and pancreatic cancer, but its expression in gastric cancer (GC) has remained unclear. The present study aimed to detect TRPM8 expression and to explore its clinical significance in GC. Western blotting and immunohistochemistry were used to detect the protein expression of TRPM8 in 134 pairs of GC and adjacent healthy tissues. The association of TRMP8 with the 5-year overall survival rate of patients with GC was assessed using a Cox regression model. TRPM8 protein expression was significantly elevated ($P<0.05$) in gastric tumor cells (SUN-1, AGS, SNU-5 and NCI-N87) and was significantly associated with tumor diameter ($P=0.003$), Tumor-Node-Metastasis stage ($P=0.003$), lymph node metastasis ($P=0.001$) and cancer cell remote metastasis ($P=0.010$) in patients with GC. The expression of TRPM8 protein was significantly higher in GC patients with a tumor diameter of ≥2.5 cm. Additionally, TRPM8 protein expression in patients with metastases was significantly higher compared with patients without metastasis. Cox regression analysis revealed that TRPM8 protein expression was an independent risk factor for prognosis (odds ratio, 1.625; 95% CI=0.552-3.128) in patients with GC. In addition, the 5-year overall survival rate of patients with high expression of TRPM8 protein (64.44%) in GC was significantly lower compared with patients with low expression (12.36%). TRPM8 was highly expressed in GC tissues and may promote GC cell proliferation and metastasis *in vivo*.

Introduction

Gastric cancer (GC) is a malignant tumor originating from the gastric mucosa and is one of the malignant tumors of the digestive tract that seriously endanger human health (1). Globally in 2012, >70% of new cases of GC occur in developing countries and ~50% of cases occur in eastern Asia of which the majority is seen in China (1). According to data released by the China Cancer Center in 2015, the number of new cases of patients with GC in China was 679,000 per year, second only to lung cancer (733,000 per year) (2). Notably, the detection rate of early GC is only 10% due to a lack of specific early clinical symptoms and diagnostic indicators (3,4). Therefore, most patients with GC are diagnosed at an advanced stage and the 5-year survival rate is poor, with a 5-year overall survival rate of 20% for those treated only by surgery and 30-50% for those treated with surgery and adjuvant therapy (5). Therefore, the exploration of specific diagnostic and therapeutic targets is of considerable significance to develop targeted drugs and improve the survival of patients with GC.

The transient receptor potential cation channel subfamily M member 8 (TRPM8) is a vital subtype of the transient receptor potential (TRP) and was first detected in prostate tissue (6). The human *TRPM8* gene, which is located on chromosome 2q37.1, is composed of 25 exons, encodes a protein containing 1,104 amino acids, is mainly expressed on the cell membrane as a transmembrane protein and is known to be involved in perceiving temperature and pain (7,8). As a cold-sensitive channel protein, TRPM8 can be activated by low temperatures (27˚C) (9). Previous studies have demonstrated that TRPM8 activation can cause Ca^{2+} influx into cells and trigger a series of biological effects, such as cell proliferation, differentiation and migration (10,11). TRPM8 was later confirmed to be abnormally expressed in a number of cancers, including breast (12), prostate (13) and pancreatic cancer (14). Additionally, TRPM8 is not only involved in regulating the biological characteristics of tumor cells (15,16) but also serves as a potential tumor treatment target (17). In addition, TRPM8 has also been found to be a receptor for menthol which is a widely used drug, including for antitumor treatments (9,18). Li *et al* (19) found that menthol increased the Ca^{2+} concentration in a human bladder cancer cell line by activating its receptor TRPM8 which reduced the mitochondrial membrane potential, caused mitochondrial depolarization, induced cell apoptosis and exerted antitumor effects.

However, the expression of TRPM8 protein and its clinical significance in GC remains unclear. The present study aimed to detect TRPM8 expression and to explore its

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clinical significance in GC. The findings revealed that TRPM8 protein was highly expressed in GC tissues and affected the proliferation and metastasis of GC cells. The present study demonstrates that TRPM8 may be a potential target for the treatment of GC.

Materials and methods

Patient ethics and characteristics. Tumor and adjacent healthy tissues (>5 cm from tumor tissue) were collected by surgery from 134 patients with GC between January and December 2013 in the Beijing Jishuitan Hospital (Beijing, China). The average age of patients was 60.65±12.38 years and other clinical data are presented in Table I. Inclusion criteria for the present study were: i) Patients with GC without any treatment before surgery; ii) patients with complete information, such as age, sex, imaging examination, tumor-node-metastasis (TNM) stage; iii) patients with a complete 5-year follow-up record; and iv) patients without any other malignant tumor or other chronic infectious diseases (such as HIV, HBV, HCV). The exclusion criteria for the present study were: i) Loss to follow-up (Telephone interviews every quarter, for a period of 5 years) or death from another illness or accident; ii) pregnant or lactating women, as well as drug users; and iii) withdrawn from the study halfway. All GC patients who provided tissues were informed of the content of this study and signed informed consent. All protocols related to human tissue samples were reviewed and monitored by the Ethics Committee at the Beijing Jishuitan Hospital (Beijing, China).

Cell lines and culture. GES-1, SNU-1, AGS, SNU-5, and NCI-N87 were purchased from the American type culture collection (ATCC) and were cultured in DMEM medium (cat. no. 11965092; Gibco; Thermo Fisher Scientific Inc.) supplemented with 10% fetal bovine serum (cat. no. 16140071; Gibco; Thermo Fisher Scientific Inc.) at 37˚C with 5% CO_2. GES-1 is a normal gastric epithelial cell line and all others are GC cell lines.

Western blotting. Total protein was extracted from cells using a RIPA lysate buffer (cat. no. R0010; Beijing Solarbio Science & Technology Co., Ltd.). Tissues were first broken apart in liquid nitrogen in a twist bowl and then total protein was extracted from the cells. The concentration of total protein was detected using a BCA kit (cat. no. C503021; Sangon Biotech Co. Ltd.). Subsequently, 50 μg of protein per lane was separated by 10% SDS-PAGE under a 90 V constant voltage. The proteins were transferred from the SDS-PAGE gel to PVDF membranes. Following blocking with 5% skimmed milk at room temperature for 1 h at room temperature, the membranes were incubated with primary antibody against TRPM8 (1:500; cat. no. ab3243; Abcam) overnight at 4˚C. Then Goat Anti-Rabbit IgG H&L (HRP; 1:2,000; cat. no. ab6721; Abcam) was added at room temperature for 2 h. After washing 3 times with phosphate-buffered saline-Tween-20, ECL solution (cat. no. WBKLS0100; Beijing Xinjingke Biotechnologies Co., Ltd.) was added for detection. β-actin was used as the loading control. ImageJ v1.8.0 (National Institute of Health) was used to analyze protein grey value.

Immunohistochemistry (IHC). TRPM8 protein expression was detected and scored by IHC as described in a previous study (20). In the present study, the primary antibody used was an anti-TRPM8 antibody (1:100; cat. no. ab3243; Abcam) and PBS buffer was used as a negative control. The primary antibody was incubated overnight at 4˚C and Goat Anti-Rabbit IgG H&L (HRP; 1:1,000, cat. no. ab6721; Abcam) as the secondary antibody was incubated at room temperature for 1 h. The number of positive cells were counted using Leica TCS SP5 microscope (magnification, x200; Leica Microsystems, Inc.).

Statistical analysis. SPSS 20.0 (IBM Corp.) was used to analyze the data in the present study. Data in the current study was presented as the mean ± standard deviation, and three repeats were used for the same measurement. Both paired and unpaired Student's t-tests and the χ^2 test were used to compare the differences between 2 groups and one-way ANOVA with the post hoc Tukey's test was used to compare the differences between multiple groups. The log-rank (Mantel-Cox) test was used to compare the survival of patients with high and low TRPM8 expression. A Cox regression model was used to analyze factors affecting the survival of patients with GC. $P<0.05$ was considered to indicate a statistically significant difference.

Results

Upregulation of TRPM8 protein expression in GC cells and tissues. Firstly, the expression of TRPM8 protein in human gastric mucosal epithelial cell (GES-1) and human GC cells (SNU-1, AGS, SNU-5, and NCI-N87) was assessed. The expression of TRPM8 protein in human GC cells was significantly higher compared with that in normal human gastric mucosal epithelial cells and the level in the NCI-N87 cell line was highest (Fig. 1A and B). Then, tumor and adjacent healthy tissues from a 134 patients with GC were measured by western blotting and IHC. The expression of TRPM8 protein in GC tissues was significantly higher compared with that noted in the normal adjacent healthy tissues (Fig. 1C and D). In addition, IHC demonstrated that TRPM8 protein was located at the cell membrane (Fig. 1E) and the IHC score of TRPM8 protein expression in GC tissues was significantly higher compared with that observed in the adjacent tissues (Fig. 1F).

Association of TRPM8 and clinicopathological features of patients with GC. Patients with GC (n=134) were divided into 2 groups based on the expression of TRPM8 protein detected using immunohistochemistry, a TRPM8 protein low expression group (n=45) with an IHC score of <4 and a high expression group (n=89) with an IHC score of ≥4. As shown in Table I, expression of TRPM8 protein was not significantly associated with sex (P=0.863), age (P=0.737), histological grade (P=0.769) or tumor number (P=0.672). However, it was significantly associated with tumor diameter (P=0.003), TNM stage (P=0.003), lymph node metastasis (P=0.001) and remote metastasis (P=0.010).

TRPM8 protein expression in gastric tumors of variable sizes. Based on preoperative imaging examination, the largest diameter of tumor tissue was determined (median

Table I. Association of TRMP8 protein expression and clinicopathological characteristics of patients with gastric cancer (n=134).

		TRPM8 expression			
Features	Number of patients	Low	High	χ^2	P-value
Sex				0.030	0.863
Female	94	32	62	-	-
Male	40	13	27		
Age (years)				0.113	0.737
<60	48	17	31	-	-
≥60	86	28	58		
Tumor diameter (cm)				8.945	0.003
<2.5	65	30	35	-	-
≥2.5	69	15	54		
Histological grade (43)				0.524	0.769
I	37	14	23	-	-
II	52	16	36		
III	45	16	29		
Tumor number				0.179	0.672
Single	74	26	48	-	-
Multiple	60	19	41		
TNM stage (44)				14.195	0.003
I	14	8	6	-	-
II	38	19	19		
III	49	13	36		
IV	33	5	28		
Lymph node metastasis				10.269	0.001
Yes	82	19	63		
No	52	26	26		
Cancer cell remote metastasis				6.668	0.010
Yes	33	5	28		
No	101	40	61		

TNM, tumor-node-metastases; TRMP8, transient receptor potential cation channel subfamily M member 8.

diameter of gastric tissues=2.5 cm) and patients with GC (n=134) were divided into 2 groups: Tumor diameter <2.5 cm (n=65) and tumor diameter ≥2.5 cm (n=69). As presented in Fig. 2A, the relative expression levels of TRPM8 in gastric cancer tissues with tumor diameters of 0.5, 1.5, 3.8, 4.7 and 7.1 cm are 0.12, 0.33, 0.58, 1.18 and 1.42, respectively. TRPM8 protein expression in the <2.5 cm group was significantly less compared with that in the ≥2.5 cm group using western blotting (Fig. 2A and B). In addition, the differences in TRPM8 protein expression were detected using IHC in the 2 groups divided by tumor diameter. Similarly, as indicated by Fig. 2A, the IHC score of TRPM8 in gastric cancer tissues with tumor diameters of 0.5, 1.5, 4.7 and 7.1 cm are 1, 2, 4 and 6, respectively. It was also indicated that the IHC score in the <2.5 cm group was significantly lower compared with the ≥2.5 cm group (Fig. 2C and D).

TRPM8 protein expression and GC cell metastasis. Patients with GC (n=134) were divided into 2 groups based on whether they had metastasis (lymph node or remote metastasis) or not and then TRPM8 protein expression was compared in these 2 groups. A total of 82 GC patients exhibited cancer cell metastasis (metastasis group) and 52 patients exhibited no cancer cell metastasis (non-metastasis group). TRPM8 protein expression in tissues of patients who had metastases was significantly higher compared with those without metastases as detected by western blotting (Fig. 3A and B). This was confirmed in the subsequent IHC analysis. As presented in Fig. 3C, TRPM8 protein IHC scores of patients with GC without and with lymph node metastasis were 2 and 3, respectively; TRPM8 protein IHC scores of patients with GC without and with distant cancer cell metastasis were 4 and 6, respectively. The IHC score of TRPM8 protein in patients with GC who had metastases was significantly elevated compared with those without metastases (Fig. 3C and D).

Association between TRPM8 protein expression and the prognosis of patients with GC. Patients with GC were followed up for 5 years after surgery to record the time of death and to compare

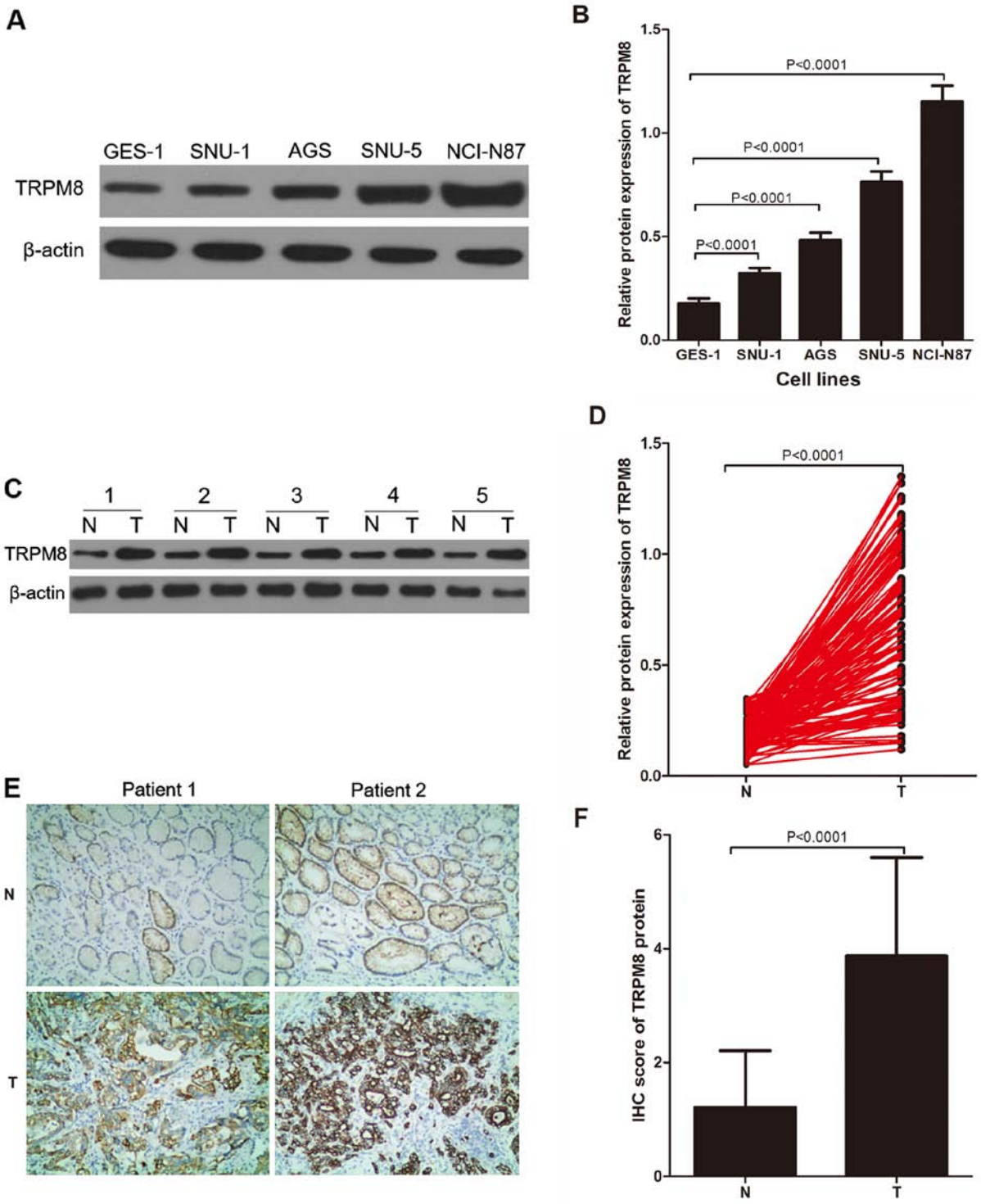

Figure 1. TRPM8 protein expression is elevated in GC cells and tissues. (A) Representative protein bands and (B) TRPM8 protein expression quantification in human gastric mucosal epithelial (GES-1) cells and human GC cells (SNU-1, AGS, SNU-5 and NCI-N87) using western blotting. (C and D) Western blotting was used to detect the expression of TRPM8 protein in normal and tumor tissues from patients with GC and (C) presents the protein bands and (D) the result obtained using a before-after connection graph. (E and F) TRPM8 IHC staining of normal and tumor tissues from patients with GC (n=134) was performed and (E) presents the staining results from 2 patients with GC (x100) and (F) comparison of TRPM8 protein IHC scores between normal and tumor tissues. P-values were calculated using one-way ANOVA with the post hoc Tukey's test (B), but were calculated using a paired Student's t-test in (D) and (F). GC, gastric cancer; N, normal tissue; T, tumor tissue; IHC, immunohistochemistry; TRMP8, transient receptor potential cation channel subfamily M member 8.

the 5-year overall survival rate of patients with different TRPM8 protein expression levels. A total of 64.44% (29/45) of patients with low TRPM8 protein expression were still alive 5 years after surgery, while only 12.36% (11/89) of patients with high TRPM8 protein expression levels were still alive (Fig. 4). The difference in 5-year survival of patients with variable TRPM8 protein expression levels was significant (Fig. 4). In addition, the influencing factors for survival were assessed using the Cox regression model. According to the results of univariate analysis, histological grade (OR=2.842; 95% CI=2.342-3.210),

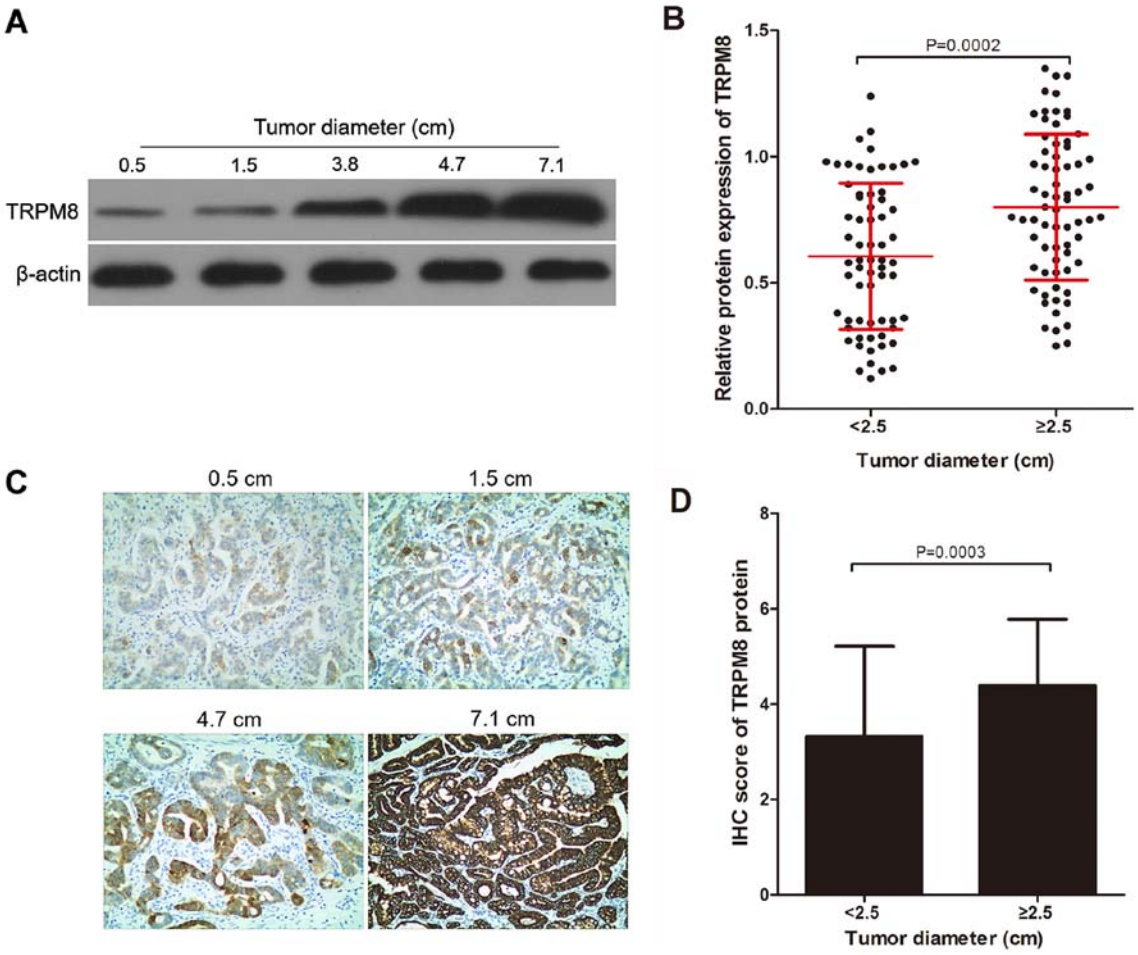

Figure 2. TRPM8 protein expression is related to tumor size of GC. (A) Representative protein bands of TRPM8 in different sizes of tumor tissues and (B) quantitative results of western blotting. (C and D) Representative Immunohistochemistry images of TRPM8 in different sizes of tumor tissues (x100) (C) and comparison of IHC score (D) in patients with GC with tumor sizes <2.5 cm (n=65) or ≥2.5 cm (n=69). P-value was calculated using the unpaired Student's t-test in (B) and (D). GC, gastric cancer; IHC, immunohistochemistry; TRMP8, transient receptor potential cation channel subfamily M member 8.

TNM stage (OR=0.854; 95% CI=0.328-1.264), lymph node metastasis (OR=1.964; 95% CI=1.405-3.218), remote metastasis (OR=3.264; 95% CI=2.501-4.448) and TRPM8 levels (OR=1.032; 95% CI=0.846-2.371). Multivariate analysis was also performed and it was indicated that TNM stage [odds ratio (OR)=2.032; 95% CI=0.625-3.102], lymph node metastasis (OR=3.516; 95% CI=1.653-4.021), remote metastasis (OR=3.237; 95% CI=1.354-4.021) and TRPM8 protein expression levels (OR=1.625; 95% CI=0.552-3.128) were independent risk factors that affected the 5-year survival of patients with GC (Table II).

Discussion

TRPM8 was initially cloned as a prostate-specific protein and was found to be activated by cold stimulation and menthol in a heat-sensitive response (6). TRP family proteins are involved in the occurrence of various diseases, such as TRPM8 is the principal mediator of menthol-induced analgesia of acute and inflammatory pain (21), anti-hyperalgesic effects of a novel TRPM8 agonist in neuropathic rats (22) and cancer (23,24). Jiang *et al* (25) found that transient receptor potential cation channel subfamily V member 6 knockdown could inhibit the invasion and migration of cancer cells. Orfanelli *et al* (26) found that transient receptor potential cation channel subfamily M member 2 played an essential role in the regulation of biological functions of tumor cells, such as proliferation, invasion and migration.

In the present study, TRPM8 protein expression in human GC cells was significantly higher compared with that in human gastric mucosal epithelial cells, and was also elevated in human GC tissues compared with adjacent healthy tissues. Numerous studies have demonstrated that TRPM8 plays a vital role in the development of tumors, suggesting TRPM8 may serve as an oncogene affecting the progression of a number of types of malignant tumors. Overexpression of the cation-permeable channel TRPM8 in prostate cancers promoted cancer progression and menthol inhibited the proliferation of prostate cancer cells by inhibiting the expression of TRPM8 protein (16). Knowlton and McKemy (27) summarized the emerging role of TRPM8 in a variety of biological systems, including thermoregulation, cancer, bladder function, and asthma. Furthermore, Wang *et al* (15) found that knockdown of TRPM8 suppresses cancer malignancy and enhances epirubicin-induced apoptosis in human osteosarcoma cells. The findings of the present study revealed that TRPM8 was highly expressed in GC cells and tissues, hence it may be involved in the regulation of GC development as an oncogene.

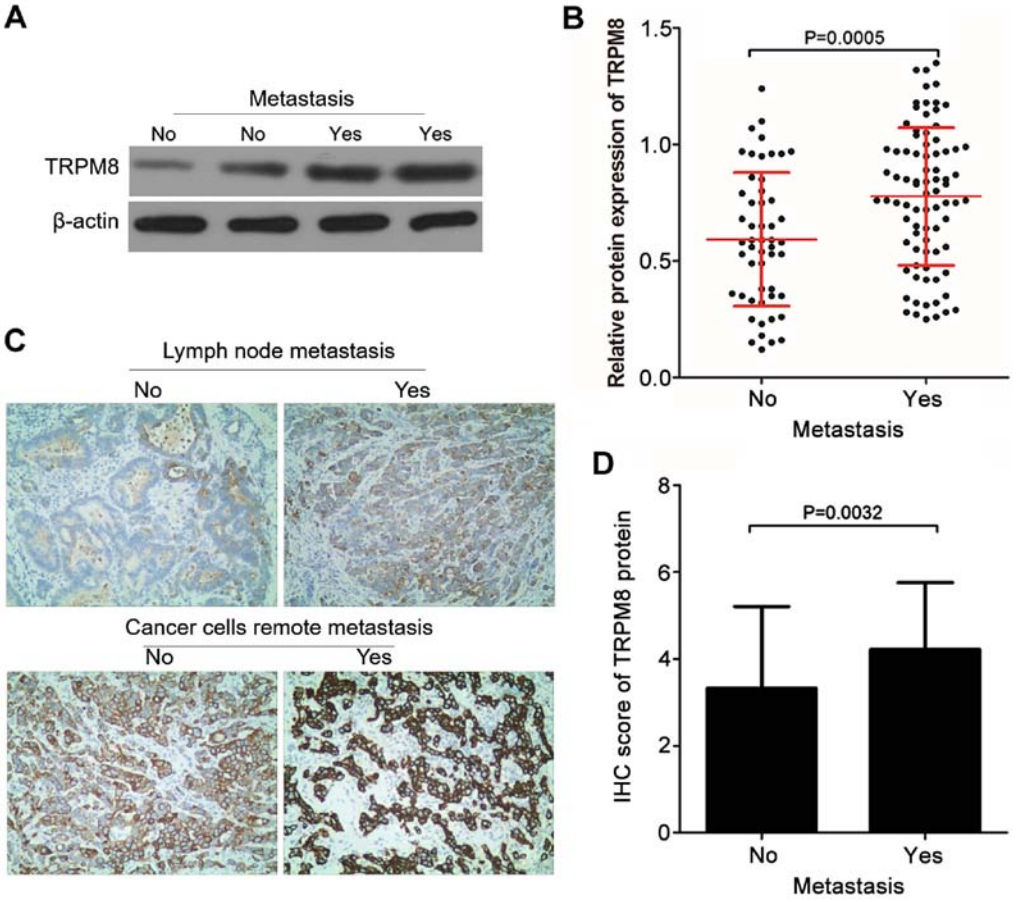

Figure 3. TRPM8 protein expression is associated with metastasis in patients with GC. Western blotting was used to detect the protein expression of TRPM8 in patients with GC with or without metastasis and (A) presents the protein bands and (B) quantitative data obtained. (C and D) Representative Immunohistochemistry pictures of TRPM8 in different sizes of tumor tissues (x100) (C) and comparison of IHC score (D) in patients with GC with (n=82) or without (n=52) metastasis. P-value was calculated by the Student's unpaired t-test in (B) and (D). GC, gastric cancer; IHC, immunohistochemistry; TRMP8, transient receptor potential cation channel subfamily M member 8.

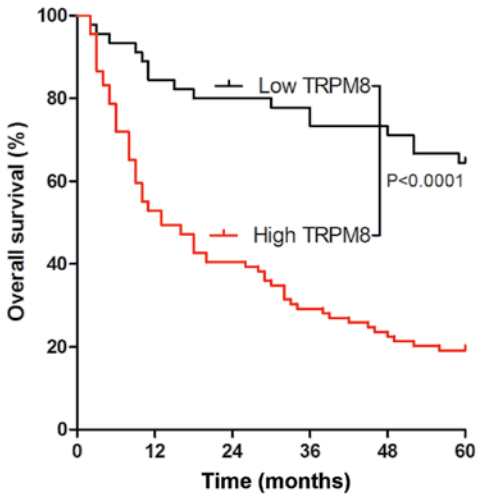

Figure 4. Effect of TRPM8 protein expression on 5-year overall survival of patients with GC. GC, gastric cancer; TRMP8, transient receptor potential cation channel subfamily M member 8.

In the present study, the association between TRPM8 protein expression and clinicopathological data from patients with GC was assessed and it was found that the expression of TRPM8 protein in GC tissues was significantly associated with the tumor diameter, TNM stage, lymph node metastasis and remote metastasis. Notably, in the present study TRPM8 protein was higher expressed in patients who had larger tumor diameters. The abnormal proliferation of tumor cells is the root cause of cancer and forms the basis for distinguishing them from normal cells (28). One of the functions of numerous antitumor drugs is the suppression of proliferation of tumor cells, including mechanisms that regulate cell cycle-related protein expression and mitochondrial toxicity (15,29). TRPM8 has been found to be involved in promoting cell proliferation. Bidaux *et al* (30) found that the epidermal TRPM8 channel controls the balance between keratinocyte proliferation and differentiation in a cold-dependent manner and Yee (31) found that overexpression of TRPM8 is necessary for pancreatic cancer cell proliferation. On the other hand, TRPM8 has also been found to inhibit the proliferation of non-cancer cells and topical application of TRPM8 agonists can reduce epidermal proliferation induced by barrier insult *in vivo* (32) and inhibit the proliferative airway smooth muscle cell phenotype (33). In conclusion, these data indicate that TRPM8 is a cell proliferation-related protein.

The present study also found that the protein expression of TRPM8 in patients with GC who had metastases was significantly higher compared with patients without metastases. The characteristic that tumor cells are transferred from the primary site to other sites to continue to grow is not only a

Table II. Analysis of factors that influence the overall survival of patients with gastric cancer (n=134).

	Univariate analysis			Multivariate analysis		
Features	95% CI	OR	P-value	95% CI	OR	P-value
Sex	1.864-4.926	3.021	0.089	-	-	-
Age	0.792-2.657	1.325	0.328	-	-	-
Tumor diameter	1.358-4.328	2.682	0.068	-	-	-
Histological grade	1.969-3.427	3.025	<0.001	1.129-3.641	2.03	0.061
Tumor number	0.729-3.124	1.682	0.062	-	-	-
TNM stage	0.658-2.354	1.382	<0.001	0.625-3.102	2.032	0.001
Lymph node metastasis	1.223-3.512	2.003	<0.001	1.653-4.021	3.561	0.042
Remote metastasis	2.235-3.126	2.658	<0.001	1.354-4.021	3.237	<0.001
TRPM8 levels	0.635-3.012	1.324	0.012	0.552-3.128	1.625	0.028

TNM, tumor-node-metastases; TRMP8, transient receptor potential cation channel subfamily M member 8; OR, odds ratio.

criterion for distinguishing between benign and malignant tumors, but also a major cause of treatment failure and death in patients with cancer (34). Numerous previous studies have found that upregulation of TRPM8 promotes metastasis. Okamoto *et al* (35) found that TRPM8 was highly expressed in oral squamous cell carcinomas and reducing the activity of TRPM8 could significantly decrease the activity of invasion in oral squamous carcinoma cells *in vitro*. TRPM8 has also been found to promote metastasis in lung and prostate cancer (36,37). The results of the present study suggest that TRMP8 protein is a metastasis-related protein in GC.

In addition, the present study found that high expression of TRPM8 was associated with a poorer prognosis for patients with GC, this may be due to the promotion and proliferation of GC cells induced by TRPM8. Similarly, studies of TRPM8 expression in pancreatic cancer (38), urothelial carcinoma of the bladder (39) and osteosarcoma (40) also found that high expression of TRMP8 leads to poor prognosis. This indicates that TRMP8 could be used to evaluate the efficacy of treatment by comparing the expression level of TRPM8 in GC tumor tissue before and after treatment. In addition, since the TRPM8 protein expression is associated with the prognosis of patients with GC, modulating the expression of TRPM8 in patients with GC through treatment may affect the prognosis. In conclusion, TRPM8 may be a potential target for treatment or a parameter for tumor screening in GC. As TRPM8 is a menthol receptor, it has been used as a target for menthol-induced apoptosis of human bladder cancer cell line T24 (19) and human leukemia cell HL-60 (41). TRPM8 has been widely used in the development of drugs for the treatment of malignant tumors, such as menthol and cannabigerol (10,42).

The present study had several limitations, no cell proliferation and metastatic assays were performed to investigate the effects of TRPM8 expression in GC cells. The findings of this study were based on *in vivo* data, functional *in vitro* mechanistic studies are required to verify the findings of the present study. In conclusion, the present study found that the TRPM8 protein was highly expressed in GC tissues and is associated with the development of GC and may promote GC cell proliferation and metastasis as an oncogene. The current study demonstrated that TRPM8 is a potential target for the treatment of gastric cancer, and some drugs used to treat cancer that target TRPM8 can be used for the treatment of gastric cancer.

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Availability of data and materials

All data generated or analyzed during the present study are included in this published article.

Authors' contributions

WL was responsible for the conception and design of the study. QX drafted the manuscript and revised the final draft for important intellectual content; QX, NK, JZ, NB and JB performed the experiments and analyzed the data. All authors have read and approved the manuscript.

Ethics approval and consent to participate

This study was performed with the approval of the Ethics Committee of Beijing Jishuitan Hospital (Beijing, China). All aspects of the study complied with the Declaration of Helsinki. All patients provided written informed consent.

Patient consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

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