A Unique Role Of TRPV1 Ion Channels In The Suppression Of Gastric Cancer Development

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Research

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Abstract

Background: Although the aberrant expression and function of most Ca\(^{2+}\)-permeable channels are known to promote gastrointestinal tumors, the association of transient receptor potential vanilloid receptor 1 (TRPV1) channels and gastric cancer (GC) has not been explored so far. We sought to determine their role in the development of GC and to elucidate the underlying molecular mechanisms.

Methods: The expression of TRPV1 in GC cells and tissues was detected by qPCR, immunohistochemistry, western blot analysis and immunofluorescence. CCK8 and flow cytometry were used to detect the proliferation and cell cycle, while transwell assay was used to detect migration and invasion. The role of TRPV1 in GC development in vivo was tested using tumor xenograft and peritoneal dissemination assays in nude mice.

Results: The decreased expression of TRPV1 protein in primary human GC tissues was closely correlated with poor prognosis of GC patients. TRPV1 protein was predominately expressed on the plasma membrane of several GC cell lines. TRPV1 overexpression attenuated proliferation, migration and invasion of GC cells in vitro, but TRPV1 knockdown increased them. Moreover, TRPV1 significantly reduced gastric tumor sizes, numbers and peritoneal dissemination in vivo. Mechanistically, TRPV1 overexpression increased \([Ca^{2+}]_i\), activated CaMKK\(\beta\) and AMPK phosphorylation, and decreased expression of cyclin D1 and MMP2, but TRPV1 knockdown caused the opposite effects.

Conclusions: TRPV1 uniquely suppresses GC through a novel Ca\(^{2+}\)/CaMKK\(\beta\)/AMPK pathway and its downregulation is correlated with poor survival in human GC. TRPV1 upregulation and its downstream signaling may be a promising strategy for GC prevention and therapy.

Introduction

Gastric cancer (GC) is the second most common human cancers worldwide and is difficult to be diagnosed in its early stage[1]. GC is extremely hard to be cured once it develops into metastasis stage [2, 3]. Although the occurrence and progression of cancer are complex, numerous evidences indicate that aberrant intracellular Ca\(^{2+}\) ([Ca\(^{2+}\)]\(_i\)) signaling is involved in the development of several types of gastrointestinal (GI) cancers, including GC and colon cancer [4]. Since plasma membrane Ca\(^{2+}\)-permeable channels play important roles in the regulation of [Ca\(^{2+}\)]\(_i\), their aberrant expression and function are documented to be positively associated with the occurrence and development of GI tumors[5, 6]. Consistently, we revealed that activation of the G protein-coupled receptors (GPCRs), such as Ca\(^{2+}\) sensing receptors (CaSR) and vasoactive intestinal polypeptide (VIP) receptors promoted GC progression via transient receptor potential vanilloid receptor 4 (TRPV4) channels and Ca\(^{2+}\) signaling pathway[7, 8]. Therefore, the Ca\(^{2+}\) permeable TRPV channels are worth for further intensive investigation since they could be novel potential drug targets for GI tumor therapy[9].
TRPV1 channel belongs to the Ca\(^{2+}\) permeable TRPV channel family, responding to noxious heat (>43\(^\circ\)C), low pH value (<5), capsaicin and so on [10-12]. TRPV1 channel plays an important role in several physiological and pathological processes, such as nerve conduction, visceral pain sensing process [9, 13, 14], and activation of immunity[15]. Furthermore, a few studies shown previously that TRPV1 was likely involved in tumor progression [16], and its activation reduced cell proliferation, migration and invasion in breast cancer[17], urothelial cancer[18] and papillary thyroid carcinoma[19]. However, so far little is known about the role of TRPV1 channel in GI tumorigenesis, except Amaya G. et al. reported that TRPV1 regulated neurogenic inflammation in the colon to presumably protect mice from colon cancer[20]. We also revealed that TRPV1 channel inhibited EGFR-induced epithelial cell proliferation to prevent mice from colon polyps[21]. Although the expression of TRPV1 channel has been identified in rat gastric epithelial cells[22], almost nothing is known about its functional role in the upper GI epithelial cells, let alone its potential involvement in the pathogenesis of gastric disease. Importantly, the role of TRPV1 channel in gastric tumorigenesis has not been explored so far.

Aberrant [Ca\(^{2+}\)], signaling contributes to multiple aspects of tumor progression such as cell proliferation, migration, invasion, apoptosis and autophagy [23, 24], and calmodulin (CaM) is one of the key proteins that triggers various signaling events in response to an increase in [Ca\(^{2+}\)]. Upon binding with Ca\(^{2+}\), CaM activates downstream calcium/calmodulin-dependent protein kinase kinases (CaMKK) that include CaMKKa and CaMKKβ to further regulates adenosine mono phosphate activated protein kinase (AMPK). AMPK, a heterotrimeric Ser/Thr kinase, is well known to involve in tumor progression [25]. Thr-172, as one of the important sites for AMPK activation, can be phosphorylated by CaMKKβ [25]. Several studies reported previously that AMPK inhibited proliferation and induced apoptosis of GC cells [26-28]. Although CaMKK is a well-established connection between Ca\(^{2+}\) signaling and cancer pathogenesis [29, 30], the role of aberrant Ca\(^{2+}\)/CaMKKβ/AMPK pathway in GC progression and the underlying molecular mechanisms remain unexplored.

In the present study, we focused on the role of TRPV1 channel in GC progression and the underlying molecular mechanisms. We demonstrate for the first time that TRPV1 could increase AMPK phosphorylation through Ca\(^{2+}\)/CaMKKβ to downregulate the expression of cyclin D1 and matrix metalloproteinase-2 (MMP2), leading to the inhibition of GC cell proliferation, migration and invasion. Our results not only reveal a unique role of TRPV1 channel in GC suppression, but provide a novel insight to GC prevention and treatment.

**Materials And Methods**

**Ethics statement and human tissue samples**

Twenty pairs of GC and adjacent tissues for real-time quantitative PCR were from the surgical patients in Xinqiao Hospital of Third Military Medical University (during 2016 and 2017) and all resected specimens were confirmed by pathological examination. The informed consent was obtained for all patients. All clinical studies were approved by the Clinical Research Ethics Committee of Third Military Medical
University, and were performed in accordance with the approved guidelines. Eighty pairs of GC and adjacent tissue microarray for immunostaining were purchased from SHANGHAI OUTDO BIOTECH CO., LTD (Shanghai, China).

Cell culture

MKN45, SGC7901, AGS, MGC803, BGC823 human gastric cancer cell lines and human gastric normal epithelial mucosa cell line (GES-1) were purchased from ATCC. All cells were cultured in RPMI-1640 or DMEM-HIGH GLUCOSE medium (Hyclone, USA) and supplemented with 10% fetal bovine serum (Hyclone, USA), 100IU/mL penicillin and 100µg/mL streptomycin (Invitrogen, USA). All cells were grown in a 37°C humidified atmosphere containing 95% air and 5% CO₂.

Preparation and infection of lentiviruses

Lentiviruses were purchased from Genechem Co., Ltd (Shanghai, China). Lentivirus which containing the full-length coding sequence (CDS) of TRPV1 (NM_080704) was designed to increase its expression in BGC823. Lentivirus-based shRNA was used to silence the expression of TRPV1 in MKN45. Sequences for TRPV1 shRNA and control were as follows: shRNA-1 (5’-GCATCTTCTACTTCAACTTCC-3’), shRNA-2 (5’-GGCCGACAACACGAAGTTTGT-3’) and control (5’-GTCTTCTCGACGTGTCACGT-3’). All the shRNA group that didn’t mark the number used shRNA-1. Cells were infected with lentiviruses according to the protocol of the manufacturer. Briefly, cells were attached in 24-well plates with amount of 1×10⁵ cells/hole, lentiviruses were added into culture medium separately (the volume of lentiviruses was calculated with MOI 20), the medium were refreshed after 8 hours. Purinomycin was used to screen the stable cells after 72 hours of lentivirus infection.

Preparation and transfection of plasmids

The full length CDS of AMPK (NM_006251) was cloned into pcDNA3.1 to prepare overexpression plasmids; AMPK-siRNA sequence 5’-CTGCTTGATGCACACATGAAT-3’; CaMKKβ-siRNA sequence 5’-GTCAAGTTGGCCTACAATG-3’ were cloned into GV102 vector separately to prepare siRNA-knocked down plasmids. Transfection of plasmids into cells according to the protocol of FuGENE@ HD Transfection Reagent (Cat. No. E2311, Promega, USA). In brief, 1×10⁵ cells were cultured in a hole of 24-well plates, 1.5 µL of transfection reagent and 0.5 µg of plasmids were mixed and added to 500 µL culture medium. The cells were incubated with the mixture for 72 hours.

RNA extraction and real-time quantitative PCR (qPCR)

Total RNA was extracted from each group by RNAiso Plus reagent (Cat. No. 9109, Takara, Japan). cDNA was synthesized using PrimeScript® RT-polymerase (Cat. No. R050A, Takara, Japan). Then, 50 ng of each cDNA was amplificated as a template, and qPCR was performed using a SteponePlus device (Art. No. 272008342, Life Technologies, USA) with a SYBR® Premix Ex TaqTM II kit (Cat. No. RR820A, Takara, Japan). All samples were run in triplicate, and β-actin was used as an internal control. Data were
quantified using the $2^{-\Delta\Delta Ct}$ relative quantitative method, normalized based on β-actin expression, and expressed as the ratio of TRPV1 to β-actin mRNA levels. The primers were designed as follows:

TRPV1: 5’-TGGTATTCTCCTGGCCTTG-3’ (forward)
5’- CTTCCCGTCTTCAATCAGCG-3’ (reverse)

AMPK: 5’-TGGTAGGAAAAATCCGCAGA-3’ (forward)
5’-CGACTTTCTTTTTTCATCCAGC-3’ (reverse)

β-actin: 5’-GGCATCCACGAAACTACCTT-3’ (forward)
5’-CGGACTCGTCATACTCTGTGCT-3’ (reverse)

**Immunohistochemistry**

Tissue samples were paraffin embedded and cut into 5mm slices. After the treatment of dewaxing and rehydration, tissue samples were incubated with anti-TRPV1 (dilution 1:500, Cat. No. ab3487, Abcam, UK) overnight at 4°C after blocked. TRPV1 was detected with HRP-conjugated anti-rabbit secondary antibody (dilution 1:1000, Cat. No. ZB-2301, ZSGB-BIO, China) and visualized with DAB. The negative control contained secondary antibody only. The staining results were observed by light microscopy, and IPP (Image Pro Plus 6.0) software was used to quantitatively score the tissue sections. Briefly, images of all tissue cores were acquired at the same time with a constant set of imaging parameters on the microscope and imaging software. The images were then subjected to optical density analysis by IPP software. The intensity range selection was based on histograms, with the intensity (I), saturation (S), and maximum hue (H) set at a range in which most of the brown diaminobenzidine color could be quantified. After defining the area of interest (AOI), the mean optical density of the selected area [integrated optical density (IOD)/unit area] was determined by the software and used to represent the immunoreactivity of the candidate protein within the tumor tissue. The acquired score of the optical density was normalized and subjected to IPP for analysis.

**Immunofluorescence assay**

Cells of each group were attached to coverslips in 35 mm dishes and fixed in 4% polyformaldehyde for 15 minutes at room temperature. The coverslips were washed in PBS three times for 5 minutes. Cells were blocked in goat serum for 1 hour at room temperature, and then incubated with anti-TRPV1 antibody overnight at 4°C. After three washes with PBS, cells were incubated with Cy3 labeled anti-rabbit (diluted 1:1000, Cat. No. A0516, Beyotime, China) secondary antibody for 1 hour at room temperature. Finally, the nucleuses were stained with DAPI for 10 minutes. Images were captured on a confocal microscope (Leica SP5, Germany).

**Western blot analysis**
Whole-cell lysates were separated by SDS-PAGE on denaturing 10% or 12% gels and transferred to polyvinylidene fluoride membranes (Cat. No. ISEQ00010, Millipore, USA). The blots were blocked in 5% milk for 1 hour at room temperature and then separately incubated at 4°C overnight with the following specific primary antibodies: anti-TRPV1, anti-Ki67 (Cat. No. ab15580, Abcam, UK), anti-AMPK (Cat. No.5832, Cell Signaling Technology, USA), anti-phospho-AMPK (Cat. No.2535, Cell Signaling Technology, USA), anti-CaMKKβ (Cat. No. ab168818, Abcam, UK), anti-cyclin D1 (Cat. No. ab16663, Abcam, UK), anti-MMP2 (Cat. No.87809S, Cell Signaling Technology, USA), anti-β-catenin (Cat. No.8480, Cell Signaling Technology, USA), anti-phospho-β-catenin (Cat. No.9567, Cell Signaling Technology, USA), anti-AKT (Cat. No.9272, Cell Signaling Technology, USA), anti-phospho- AKT (Cat. No.4060, Cell Signaling Technology, USA), anti-ERK1/2 (Cat. No. ab184699, Abcam, UK), anti-phospho-ERK1/2 (Cat. No. ab214362, Abcam, UK), and anti-GAPDH (Cat. No. TA-08, ZSGB-BIO, China); all primary antibodies were diluted 1:1000. After rinsing, the blots were incubated in HRP-conjugated anti-rabbit or anti-mouse (diluted 1:1000, Cat. No. A0239 and A0216, Beyotime, China) secondary antibodies for 1 hour at room temperature. Enhanced chemiluminescence (Cat. No. 34094, Thermo, USA) was used to detect the immunoreactive bands. Human phospho-kinase array was used to detect changes in tumor-related signaling pathways (Cat. No. ARY003B, R&D Systems, USA). 2µM BAPTA-AM (Cas No. 126150-97-8, MedChemExpress, USA) was used to chelate intracellular calcium, cells were treated with BAPTA-AM for 2 hours. Each experiment was performed in triplicate and repeated three times. The gray value of the band measured by imageJ software for statistics.

**Cell proliferation assay**

Cell viability and proliferation were detected by CCK8 assay (Cat. No.C0038, Beyotime Biotechnology, China). Cells after cultured for 0, 24, 48 or 72 hours were added to 96-well plates (3000 cells/well) in triplicate and incubated in 100 µl of medium/CCK8 mixture (medium: CCK8, 9:1) at 1-2 hours before the endpoint of incubation. A Multiskan EX plate reader (Thermo Fisher Scientific, Germany) was used to quantify the viable cells by measuring the absorbance at 450 nm which can estimate relative cell numbers instead of counting cells. Experiments were repeated at least three times.

**Cell cycle analysis**

Cells were digested, centrifuged and washed twice with cold PBS, discarded the supernatant, and pre-cooled 70% ethanol was slowly added to the pellet, then cells were resuspended at 4 °C overnight. After centrifugation the next day, removed the ethanol, washed once with PBS, cells were incubated in a solution containing 0.2% Tween 20, 100 U/mL RNase, and 50 µg/mL propidium iodide for 20 minutes at 37°C. Cell cycle analysis was performed using flow cytometry in which samples were gated on live cells with an excitation wavelength of 488 nm and an emission wavelength of 620 nm. LMD files were further analyzed using ModFit LT (Verity Software House, Topsham, ME). Each experiment was performed in triplicate and repeated three times.

**Fat plate clone formation test**
Long-term survival of cells was assessed by the ability to form colonies. Cells were attached on 6-well plates with 3mL culture medium, each group of cells were calculated as 500 cells/hole. After 10-12 days, the cell culture medium was removed and cell clones were washed with PBS, then fixed with 4% polyformaldehyde. Counting the clone numbers after stained with crystal violet. (Relative clonogenicity = Clone numbers /Average clone numbers of control group). All experiments were repeated at least three times.

**Transwell migration and invasion assays**

Twenty-four-well transwell chambers (Corning, USA) were used for this assay. 5×10⁴ cells were placed in each the upper chambers with 8-µm pores and cultured in 200 µL serum-free RPMI-1640. The lower chambers were filled with 500 µL completed RPMI-1640 medium. After 24 hours of incubation, cells that had migrated onto the lower surface were stained with crystal violet, and counted under a microscope (Olympus Corporation, Japan). The average value of three randomly selected fields was recorded as the number of migrated cells. Then, the upper surface of the polycarbonate filter was coated with 10% matrigel (Collaborative Biomedical, USA), and 1×10⁵ cells were added to detect cell invasion. The other conditions were the same as those in the migration assay.

**Calcium measurement**

Attaching 1×10⁴ cells on coverslips, cells were loaded with 5 µM Fura-2 AM (Cat. No. F1221, Invitrogen, USA) in physiological salt solution (PSS) at 37°C for 60 minutes and then washed in PSS or PSS with the inhibitor of TRPV1 SB-705498 50µM (Cas No.501951-42-4, MCE, USA). Then, cells on coverslips were mounted in a standard perfusion chamber on a Nikon microscope stage. The ratio of Fura-2 fluorescence with excitation at 340 or 380 nm (F340/380) was followed over time and captured with an intensified CCD camera (ICCD200) and a MetaFluor Imaging System (Universal Imaging, Downingtown, PA). PSS used in Ca²⁺ measurement contained the following: 140 mM Na⁺, 5 mM K⁺, 2 mM Ca²⁺, 147 mM Cl⁻, 10 mM HEPES, and 10 mM glucose, pH 7.4.

**Tumor xenograft and peritoneal dissemination assay in nude mice**

The animal use protocol was approved by the Third Military Medical University Committee on Investigations Involving Animal Subjects. All the animal care and experimental studies were conducted in accordance with the guidelines of the Animal Ethical Committee of Third Military Medical University and the Guide for the Care and Use of Laboratory Animals published by the US National Institutes of Health (NIH Publication No.8023, revised 1978). Animal studies were reported in compliance with the ARRIVE guidelines. 1×10⁶ TRPV1-overexpression BGC823 cells and the negative control (NC) cells were injected into the armpits of seven 4-weeks old male nude mice respectively for tumor xenograft assay. TRPV1-overexpression BGC823 cells were injected into the right armpit and the NC cells were injected into the left side. The volumes of GC xenografts were assessed every 5 days. After 30 days of implantation, the mice were sacrificed and the tumor volume in mm³ was calculated by the formula1/2 (length× width²). For the
peritoneal dissemination assay, $1 \times 10^6$ TRPV1-overexpression BGC823 cells and the NC cells were injected into the abdominal cavity of nude mice. Five weeks later, the mice were sacrificed, and the nodules were observed and counted.

**Statistical analysis**

Prism 8.0 software (GraphPad, San Diego, USA) was used to analyze the data. All data are shown as MEANS ± SD, the statistical significance between two groups was determined by Student’s t-test. The one-way ANOVA was used to compare three or more groups. Significant difference was expressed in the figures or figure legends as $* P < 0.05$. All the experiments were biologically repeated three times.

**Results**

**Downregulation of TRPV1 expression was correlated with poor survival in human GC**

Since there are no reports on TRPV1 expression in human GC, we first predicted its expression from the oncomine tumor database and found that TRPV1 was generally low expression in GC (data not shown). Second, we collected human primary GC tissues and corresponding adjacent normal tissues to detect mRNA expression of TRPV1. Our qPCR result showed that TRPV1 expression was lower at transcriptional level in human GC tissues than that in normal tissues (Figure 1a). Third, immunohistochemical staining was used to examine protein expression of TRPV1 in tissue microarray containing 80 pairs of GC and adjacent normal tissues (Figure 1b). No staining was observed in the negative control without primary antibody, indicating its specific staining to TRPV1 proteins. The lower expression of TRPV1 in GC than in normal tissues was further confirmed at protein level (Figure 1b&c). Therefore, TRPV1 expression was decreased in human primary GC, which is consistent with the prediction from the oncomine tumor database.

The analysis on survival curve revealed that the GC patients with high expression level of TRPV1 had a better prognosis, but those with low expression level had a poor prognosis (Figure 1d). Furthermore, TRPV1 expression level was negatively correlated with tumor size (Figure 1e), but was not correlated with gender and tumor stage (Figure 1f&g). These data suggest that TRPV1 might be a tumor suppressor in human GC.

Afterwards, we compared TRPV1 expression level among 5 GC cell lines (MKN45, SGC7901, AGS, MGC803 and BGC823) and a normal gastric mucosal cell line GES-1. QPCR and immunoblotting analysis showed that TRPV1 was expressed at varying degrees at the levels of transcripts and proteins in aforementioned all cell lines (Figure 1h&i). Compared with normal GES-1 cells, MKN45 cells had the highest expression of TRPV1 but BGC823 cells had the lowest expression, which was further confirmed by immunocytochemistry (Figure 1j). TRPV1 proteins were predominately expressed on the plasma membrane, but also in the cytoplasm at a small amount, consistently with the previous report that TRPV1 also existed in the endoplasmic reticulum[31]. Therefore, both MKN45 and BGC823 cells were selected for further study in the following experiments.
TRPV1 and capsaicin inhibited GC cell proliferation in separate ways

We tested the role of TRPV1 in the regulation of GC cell proliferation since high proliferation is a major characteristic of tumor cells. To this end, genetic manipulation of TRPV1 was performed in BGC823 and MKN45 cells. We applied lentiviruses infection to make TRPV1-overexpressed BGC823 cells and TRPV1-knockeddown MKN45 cells. As shown in Figure 2a as representative image and Figure 2b&c as summary data (n=3 for each column), after successful overexpression or knockdown of TRPV1 in BGC823 or MKN45 cells at mRNA level (Figure 2d) and protein level (Figure 2a&b), respectively; proliferation marker Ki67 was decreased in TRPV1-overexpressed BGC823 cells but increased in TRPV1-knockeddown MKN45 cells compared with their corresponding negative controls (NC) (Figure 2a&c). Consistently, CCK8 assay showed that TRPV1 overexpression could decrease cell viability while TRPV1 knockdown increased it (Figure 2e). Moreover, plate cloning experiments confirmed the inhibitory role of TRPV1 on clonogenicity in GC cells (Figure 2i&j) but not in normal GES-1 cells with lower TRPV1 expression (Figure 2h).

We further examined the effect of capsaicin (CAP), a well-known TRPV1 activator, on GC cell proliferation. As shown in Figure 2f, although either CAP (50 µM) alone or TRPV1 overexpression inhibited GC cell proliferation in a similar potency, a combination of them could not result in any superimposed inhibition. Moreover, CAP still inhibited proliferation of TRPV1-knockeddown MKN45 cells (Figure 2g). Therefore, CAP suppression of GC cell proliferation was TRPV1-independent, as reported previously [32, 33]. Since TRPV1 and capsaicin inhibited GC cell proliferation in separate ways, we focused on the role of TRPV1 rather than CAP in GC.

TRPV1 inhibited GC growth via blockade of cell cycle at G1 phase

To test the role of TRPV1 in GC growth in vivo, xenografted gastric cancer model of nude mice was further applied. In this model, the overexpression of TRPV1 in BGC823 cells markedly suppressed growth ability of GC cells after their implantation, leading to the significant decreases both in tumor weights by about 50% and tumor volume by about 30% compared with their NC groups (Figure 3a&b). Therefore, genetic manipulation of TRPV1 expression could not only modulate GC cell proliferation in vitro but also tumor growth in vivo, confirming our early speculation that TRPV1 is a tumor suppressor in GC.

We further elucidated the underlying mechanisms how TRPV1 inhibits GC cell proliferation in vitro and tumor growth in vivo. Since cell cycle is an essential biological process that controls cell proliferation, we investigated how TRPV1 controls GC cell cycle by applying genetic manipulation of TRPV1 expression and flow cytometry analysis [34]. As shown in Figure 3c&d, comparing with NC cells, the number of TRPV1-overexpressed BGC823 cells in G1 phase was increased by around 30%, and was decreased by around 25% in S phase. In the opposite, the number of TRPV1-knockeddown MKN45 cells in G1 phase was decreased by around 40%-50%, and was increased by around 30% in S phase. However, the G2 phase of cell cycle were not significantly altered by the manipulation of TRPV1 expression in GC cells. Therefore, mechanistically TRPV1 blocked cell cycle at G1 phase to inhibit GC cell proliferation in vitro and tumor growth in vivo.
TRPV1 suppressed GC cell migration and invasion *in vitro* and *in vivo*

High migration and invasion are not only the important characteristics of GC but also the major reasons to cause the high mortality of GC[35]. Our transwell assay showed that cell migration was reduced in TRPV1-overexpressed BGC823 cells, but enhanced in TRPV1-knockeddown MKN45 cells (*Figure 4a*). Furthermore, the changes in cell migration in both groups were over 50% compared to their NC groups (*Figure 4b*). Parallely, the trend of invasion was as the same as migration compared to their NC groups (*Figure 4c*). The invasion in both groups was significantly changed at least 30% (*Figure 4d*).

Using abdominal transplantation tumor model of nude mice, we revealed that the overexpression of TRPV1 in BGC823 cells markedly suppressed GC cell metastasis after their peritoneal implantation (*Figure 4e*), leading to the decrease in tumor numbers about 40% compared to NC group (*Figure 4f*). Therefore, TRPV1 suppressed GC cell migration and invasion both *in vitro* and *in vivo* models.

Screening of TRPV1-mediated key signaling pathways in GC cells

Various signaling pathways are involved in GC tumorigenesis [36, 37]. To examine the downstream signaling pathway of TRPV1 in GC cells, we screened 40 key proteins which are involved in signaling pathways and closely related to tumorigenesis, AMPK was found to be most likely involved (*Figure 5a*). In addition, we detected if some other key signaling proteins are also likely involved, such as ERK1/2, β-catenin and AKT.

As shown in *Figure 5b*, TRPV1 overexpression attenuated but TRPV1 knockdown enhanced the phosphorylation of ERK1/2, suggesting TRPV1 suppression of ERK1/2 signaling in GC cells. However, due to the well-documented oncogenic role of ERK1/2 signaling in many cancers, including GC, we did not want to focus on it in further study. Other two signaling proteins, β-catenin and AKT were not significantly altered by genetic manipulation of TRPV1, excluding their involvements in TRPV1-mediated downstream signaling in GC cells (*Figure 5c&d*).

**AMPK phosphorylation is critical in TRPV1-mediated downstream signaling in GC cells**

We focused on AMPK signaling since it is most likely involved in TRPV1-mediated downstream pathways in GC cells. This notion was further supported by AMPK phosphorylation study. As shown in *Figure 5f&g*, the phosphorylation levels at Thr-172 site of AMPK were markedly increased or decreased after TRPV1 was overexpressed in BGC823 cells or knocked down in MKN45 cells compared with their corresponding NC; however, mRNA levels of AMPK were unchanged in both cells (*Figure 5e*). Therefore, TRPV1 stimulates AMPK phosphorylation in GC cells.

We further studied the critical role of AMPK in TRPV1 activation in GC cells after constructing AMPK overexpression and interference plasmids. As shown in *Figure 5f&g*, compared with their corresponding NC, AMPK phosphorylation was markedly decreased by AMPK interference in TRPV1-overexpressed BGC823 cells, or increased by AMPK overexpression in TRPV1-knockeddown MKN45 cells.
TRPV1 stimulates AMPK phosphorylation in GC cells. Taken together, AMPK phosphorylation is critical in TRPV1 activation in GC cells.

**TRPV1 suppressed GC development through activation of AMPK but inhibition of Cyclin D1 and MMP2**

We examined the role of AMPK in GC cell proliferation. Indeed, the reduced proliferation of TRPV1-overexpressed BGC823 cells could be recovered by siAMPK, but the enhanced proliferation of TRPV1-knockeddown MKN45 cells could be reduced by AMPK overexpression (Figure 6a). Similarly, as shown in Figure 6e&f, the reduced migration and invasion of TRPV1-overexpressed BGC823 cells were recovered by siAMPK, but the enhanced migration and invasion of TRPV1-knockeddown MKN45 cells were reduced by AMPK overexpression. Therefore, TRPV1 inhibited GC cell proliferation, migration and invasion through AMPK activation.

We further elucidated the underlying mechanisms how TRPV1-activated AMPK suppresses proliferation, migration and invasion of GC cells. Since both cyclin D1 and matrix metalloproteinase-2 (MMP2) play important roles in controlling cell cycle and metastasis[38, 39], we tested whether they are involved in TRPV1/AMPK-mediated suppression on GC cells. Western blotting analysis exhibited that compared to their corresponding NC, the expression of both cyclin D1 and MMP2 was decreased in TRPV1-overexpressed BGC823 cells, which were recovered by AMPK-siRNA (Figure 6b-d). In contrast, the expression of both cyclin D1 and MMP2 was enhanced in TRPV1-knockeddown MKN45 cells, which was attenuated by AMPK overexpression (Figure 6b-d). Therefore, TRPV1-activated AMPK signaling suppressed GC development via inhibition of both cyclin D1 and MMP2.

**TRPV1 activated AMPK phosphorylation through Ca\(^{2+}\)/CaMKKβ pathway**

We further examined how TRPV1 activates AMPK phosphorylation in GC cells. TRPV1 is a well-known plasma membrane cation channel, opening of which raises \([Ca^{2+}]_i\) level predominately [10], and CaMKKβ is a recognized downstream kinase of calmodulin (CaM), a well-known Ca\(^{2+}\) binding protein [40]. Since CaMKKβ is an upstream AMPK activator [25], we speculated that AMPK activation requires the Ca\(^{2+}\)/CaMKKβ in GC cells.

To test this hypothesis, we measured \([Ca^{2+}]_i\) in GC cells. Once again, CAP (50 µM) did not affect basal \([Ca^{2+}]_i\) in GC cells (data not shown), indicating that CAP is not a useful tool in these cells, as reported previously [32, 33]. Since TRPV channels are usually stimulated by GPCR activation, we stimulated Ca-sensing receptor (CaSR) with calcium (5 mM) in GC cells [7]. Indeed, CaSR-mediated Ca\(^{2+}\) signaling was significantly increased in TRPV1-overexpressed BGC823 cells, which could be inhibited by SB-705498, a specific inhibitor of TRPV1 (Figure 7a). In contrast, CaSR-mediated Ca\(^{2+}\) signaling was significantly decreased in TRPV1-knockeddown MKN45 cells (Figure 7b). Thus, TRPV1 is a functional cation channel that causes Ca\(^{2+}\) entry in GC cells.
We then tested the effect of TRPV1 on CaMKKβ, and found that the protein expression of CaMKKβ was markedly increased in TRPV1-overexpressed BGC823 cells, but decreased in TRPV1-knockeddown MKN45 cells compared with their NC (Figure 7c). Furthermore, the increased CaMKKβ expression could be attenuated either by CaMKKβ-siRNA (Figure 7d) or by pretreatment with $[\text{Ca}^{2+}]_i$ chelator, BAPTA-AM (2 µM) (Figure 7e) in TRPV1-overexpressed BGC823 cells. Therefore, TRPV1-mediated Ca$^{2+}$ entry indeed activates CaMKKβ. We further showed that the increased AMPK phosphorylation could be attenuated by CaMKKβ-siRNA (Figure 7f) in TRPV1-overexpressed BGC823 cells. Therefore, TRPV1/Ca$^{2+}$/CaMKKβ pathway is essential for AMPK phosphorylation in GC cells.

Discussion

In the present study, we found that TRPV1 expression was significantly decreased in GC tissues compared to adjacent normal tissues, consistently with the prediction from the oncomine tumor database. The expression level of TRPV1 proteins in GC tissues was positively correlated with better prognosis and survival ratio of the patients, indicating that TRPV1 is likely a tumor suppressor. It seems a unique role for TRPV1 channel in GC suppression because the aberrant expression and function of most Ca$^{2+}$-permeable TRP channels are usually associated with GI tumor promotion [5, 6, 32]. We further revealed that the enhanced expression of TRPV1 attenuated GC cell proliferation, invasion and metastasis both in vitro and in vivo. Mechanistically, we demonstrated that TRPV1 channel uniquely exhibits an anti-cancer role in GC progression through activation of the Ca$^{2+}$/CaMKKβ/AMPK pathway.

It is well known that $[\text{Ca}^{2+}]_i$ is an important second messenger to regulate a wide range of cellular functions. The opening of TRP channels can promote Ca$^{2+}$ entry which activates downstream signaling pathways, such as Ca$^{2+}$/calmodulin kinase II (CaMKII), mitogen-activated protein kinase (MAPK), AMPK and so on to control cell proliferation, apoptosis and migration [41, 42]. Over the past two decades, several research groups including ours have identified six TRP channels (TRPC6, TRPM2, 5, 7, TRPV4, 6) that play an important role in GC development [6-8, 43-46]. However, all these six TRP channels have been suggested as oncogene and tumor promoter in GC. Intriguingly, in contrast to the enhanced expression of these six TRP channels, TRPV1 channel is down-expressed in human GC and plays a unique suppressing role in GC. Therefore, it is important to elucidate the underlying molecular mechanisms how TRPV1 as a Ca$^{2+}$ permeable channel suppresses rather than promotes GC development.

Using phosphorylation chip screening for signaling pathway, we screened 40 key molecules that are closely related to cancer development. Interestingly, we found that the phosphorylation level of AMPK was increased most significantly after overexpression of TRPV1 in GC cells, but that of ERK1/2 was decreased. Although TRPV1 regulation of AMPK is well studied in endothelial cells[47], smooth muscle cells[48], cardiomyocytes[49], and immune cells[50, 51], this has not been reported in digestive cancer cells. In the present study, we have provided sufficient evidence to demonstrate for the first time that TRPV1 as a Ca$^{2+}$ permeable channel uniquely suppresses GC development through activation of a novel CaMKKβ/AMPK pathway. The interesting unique role of TRPV1 channels in GC suppression needs further
investigation, but it is not surprising since different Ca\(^{2+}\) permeable channels mediate cellular Ca\(^{2+}\) signals with various temporal and spatial precision, which may play different roles of anti-tumor or pro-tumor.

We revealed cyclin D1 and MMP2 are the downstream molecules of AMPK activation to finally inhibit both proliferation and migration of GC cells. Cyclin D1 has been recognized as a proto-oncogene to promote cell cycle from G1 phase to S phase by activating CDK4, a cyclin-dependent kinase specific to G1 phase. Some studies reported that AMPK inhibits GC growth via cyclin D1 suppression [38, 52]. As is known, the metastasis is a crucial characteristic for the late stages of cancer[39, 53]. MMP2 belongs to zinc-dependent metalloproteinase gene family and plays a critical role in cancer metastasis[54]. AMPK, a upstream regulator of MMP2[55], could decrease the migration and invasion of colorectal cancer through inhibition of MMP2 [56].

Capsaicin has been recognized as an anti-cancer agent in variety of cancers due to its apoptotic effect and inhibitory effect on cancer cell growth, metastasis and tumor angiogenesis [57]. Although capsaicin is a commonly used TRPV1 agonist in neurons to be able to induce Ca\(^{2+}\) influx, its mechanism of action in tumorigenesis are complex. Capsaicin directly activates PI3K/AKT and PKA in a TRPV1/Ca\(^{2+}\)-independent manner [58, 59]. Moreover, capsaicin activates TRPV6 instead of TRPV1 to induce apoptosis in GC and lung cancer cells [32, 33]. Indeed, we found that capsaicin could not induce Ca\(^{2+}\) signaling in GC cells expressing functional TRPV1 channels and that capsaicin and TRPV1 inhibited GC cell proliferation in separate ways. Therefore, capsaicin is not a useful agent to study the role of TRPV1 in GI tumorigenesis as previously reported [7, 32, 33].

In the present study, we applied genetic manipulation of TRPV1 channel to focus on its role in GC development, and for the first time clearly reveal its critical suppression action and molecular mechanisms. TRPV1 could not be activated in GC cells by capsaicin at high concentration of 50 uM (comparing the IC\(_{50}\) of 0.5 uM in many other cell types)[33]. One possibility is loss of TRPV1 channel function in GC cells. However, this is not the case since: 1) pharmacological blocker and genetic manipulation of TRPV1 could alter [Ca\(^{2+}\)]\(_i\) in GC cells, 2) genetic manipulation of TRPV1 could alter GC cell proliferation, migration and invasion both \textit{in vitro} and \textit{in vivo}, and 3) [Ca\(^{2+}\)]\(_i\), chelator BAPTA-AM efficiently prevented TRPV1-mediated CaMKK\(\beta\) activation and AMPK phosphorylation in GC cells. Another possibility is an aberrant functional alteration/mutation of TRPV1 channel in GC cells that causes a loss of TRPV1 sensitivity to capsaicin, which needs further investigation. Due to the fact that TRPV1 can be stimulated by a variety of substances[10, 11], TRPV1 channel in GC cells could be still activated by the other non-capsaicin agents, such as gastric acid and heating diets.

In conclusion, we demonstrate for the first time that TRPV1 channel uniquely suppresses GC development in vitro and in vivo. Mechanistically, TRPV1/Ca\(^{2+}\) activates CaMKK\(\beta\)/AMPK pathway to consequently decrease the expression of cyclin D1 and MMP2 (\textbf{Figure 7g}). Moreover, TRPV1 channel loses its sensitivity to capsaicin, suggesting a possible functional alteration/mutation of TRPV1 channel
in GC cells. Although TRP channels represent a relatively new field of cancer research with most studies still in their infancy, these channels hold tremendous potential that has yet to be uncovered in the hopes of achieving major clinical breakthroughs in GC therapy. Particularly, the decreased expression of TRPV1 has potentially diagnostic and prognostic significance for human GC, and upregulation/recovery of TRPV1 expression, function and its downstream signaling may be a novel promising strategy for prevention/therapy of GC.

**Abbreviations**

TRPV1: transient receptor potential vanilloid receptor 1, CaMKKβ: calcium/calmodulin-dependent protein kinase kinase β, AMPK: adenosine mono phosphate activated protein kinase, MMP2: matrix metalloproteinase-2, OE: overexpression, KD: knockdown, shRNA: Short hairpin RNA.

**Declarations**

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**Authors’ contributions**

Conceptualization, Nannan Gao, Xiaoyan Zhao and Hui Dong; Data curation, Nannan Gao, Feng Yang, Siyuan Chen, Hanxing Wan, Xiaoyan Zhao and Hui Dong; Formal analysis, Nannan Gao, Feng Yang, Hanxing Wan, Xiaoyan Zhao and Hui Dong; Funding acquisition, Hanxing Wan and Hui Dong; Investigation, Nannan Gao, Feng Yang and Siyuan Chen; Methodology, Nannan Gao, Feng Yang, Siyuan Chen, Hanxing Wan, Xiaoyan Zhao and Hui Dong; Supervision, Xiaoyan Zhao and Hui Dong; Validation, Nannan Gao, Feng Yang and Siyuan Chen; Visualization, Xiaoyan Zhao and Hui Dong; Writing – original draft, Nannan Gao, Feng Yang and Siyuan Chen; Writing – review & editing, Nannan Gao, Feng Yang, Xiaoyan Zhao and Hui Dong.

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**Availability of data and materials**

Not applicable.

**Ethics approval and consent to participate**
This study was approved by the Ethics Committee of Third Military Medical University. Informed consent was obtained from each participant.

**Consent for publication**

All authors have consented to publication of the results presented in this manuscript.

**Competing interests**

The authors have no conflicts of interest to declare.

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**Figures**
Figure 1

TRPV1 expression in gastric cancer and its correlation with clinical progression. (a) Transcript level of TRPV1 detected by qPCR in human primary GC tissues and their adjacent normal tissues (*P<0.05, n=20 patients). (b) Representative images of immunohistological staining on TRPV1 proteins in GC tissues and the adjacent normal tissues. (c) Summary data of immunohistological staining on TRPV1 proteins (**P<0.001, n=80 patients). (d) Analysis of survival ratio of GC patients with low and high TRPV1 expression levels (*P<0.05, n=100 patients). (e) Protein expression levels of TRPV1 in GC tissues from the patients with different tumor sizes (*P<0.05, n=100 patients). Correlation between TRPV1 expression and gender of GC patients (f) or GC stages (g). NS: no significant difference, n=100 patients. (h) Western blot analysis of TRPV1 protein levels in a normal human gastric epithelial cell line (GES-1) and nine GC cell lines (**P<0.01 vs. GES-1, n=3). (i) qPCR analysis of TRPV1 mRNA levels in GES-1 and nine GC cell lines (**P<0.01, ***P<0.001, ****P<0.001 vs. GES-1, n=3). (j) The images of immunofluorescence staining on
TRPV1 proteins with primary antibody in GES-1, MKN45 and BGC823 cells. Negative control was not treated with primary antibody against TRPV1. The nucleus was stained in blue with DAPI. The white scale bars on the lower right is 7.5 µm. These images are a representative of three independent experiments.
Figure 2

Genetic manipulation of TRPV1 expression and its alteration on cell proliferation. (a) Representative images of TRPV1 and Ki67 proteins in TRPV1-overexpressed BGC823 cells and TRPV1-knockeddown MKN45 cells. (b-c) The summary data of TRPV1 or Ki67 protein expression in GC cells (*P<0.05, **P<0.01, vs. NC, n=3). (d) mRNA level of TRPV1 detected by qPCR in TRPV1-overexpressed BGC823 cells and TRPV1-knockeddown MKN45 cells (***P<0.01, ****P<0.0001, vs. NC, n=3). (e) GC cells proliferation in TRPV1-overexpressed BGC823 cells (left) and in TRPV1-knockeddown MKN45 cells (right) (shRNA-2 ****P<0.0001, shRNA-1 ####P<0.0001 vs. NC, n=3). (f) Effects of TRPV1 overexpression or capsaicin (50 µM) alone and a combination of them on BGC823 cells proliferation (*P<0.05, **P<0.01, n=3). NS: no significant difference. (g) Effects of TRPV1 knockdown or capsaicin (50 µM) alone and a combination of them on MKN45 cell proliferation (*P<0.05, n=3). (h-i) Effects of TRPV1 knockdown on clonogenicity of
GES-1 and MKN45 cells (*P<0.05, **P<0.01 vs. NC, n=3). (j) Effects of TRPV1 overexpression on clonogenicity of BGC823 cells (**P<0.01 vs. NC, n=3).

Figure 3
Effects of TRPV1 on GC growth in nude mice and GC cell cycle. (a) Images of subcutaneously xenografted gastric tumors in 7 nude mice. TRPV1-overexpressed BGC823 cells were implanted on the right side of mouse armpits, and NC cells were implanted on the left side. (b) The summary data of tumor weight (left) and time courses of the changes in tumor volume (right) (**P<0.01, ***P<0.001 vs. NC, n=7 mice). Cell cycle analysis by flow cytometry in TRPV1-overexpressed BGC823 and NC cells (c), and summary data (d) (*P<0.05, ***P<0.001 vs. NC, n=3). Cell cycle analysis by flow cytometry in TRPV1-knockeddown MKN45 and NC cells (e), and summary data (f) (*P<0.05, **P<0.01, ***P<0.001 vs. NC, n=3).
Figure 4

Effects of TRPV1 genetic manipulation on GC cell migration, invasion and metastasis. GC cell migration presented as original images and presented as summary data in TRPV1-overexpressed BGC823 cells (a) and TRPV1-knockeddown MKN45 cells (b) (*P<0.05, **P<0.01 vs. NC, n=3). GC cell invasion presented as original images in and presented as summary data in TRPV1-overexpressed BGC823 cells (c) and TRPV1-knockeddown MKN45 cells (d) (*P<0.05, **P<0.01 vs. NC, n=3). (e) The images of nude mice injected peritoneally with NC cells (left) and TRPV1-overexpressed BGC823 cells (right). (f) Summary data of tumor numbers from each groups of abdominal transplantation mouse model (**P<0.01 vs. NC, n=7 mice).
Figure 5

The changes in tumor-related signaling molecules after genetic manipulation of TRPV1 in GC cells. (a) The screening in phosphorylation of 40 key cancer-related signaling molecules analyzed by signaling pathway phosphorylation microarray after TRPV1 overexpression in BGC823 cells (*P<0.05, n=3). (b) ERK1/2 phosphorylation in TRPV1-overexpressed BGC823 cells and TRPV1-knockeddown MKN45 cells. Representative images are shown on the left and summary data on the right (*P<0.05 vs. NC, n=3). (c-d) The phosphorylation of β-catenin and AKT in TRPV1-overexpressed BGC823 cells and TRPV1-knockeddown MKN45 cells (NS: no significant difference, vs. NC, n=3). (e) AMPK mRNA levels in TRPV1-overexpressed BGC823 or TRPV1-knockeddown MKN45 cells (NS: no significant difference, vs. NC, n=3). (f) AMPK phosphorylation after TRPV1 overexpression in BGC823 cells and AMPK knockdown in TRPV1-
overexpressed BGC823 cells (*P<0.05, n=3). (g) AMPK phosphorylation after TRPV1 knockdown in MKN45 cells and AMPK overexpression in TRPV1-knockeddown MKN45 cells (*P<0.05, n=3).

Figure 6

Effects of genetic manipulation of AMPK on GC cell proliferation, migration and invasion. (a) Effect of AMPK on cell proliferation in TRPV1-overexpressed BGC823 cells and in TRPV1-knockeddown MKN45 cells (***P<0.001, ****P<0.0001, n=3). (b) Representative images of protein expression of cyclin D1 and MMP2 in TRPV1-overexpressed BGC823 cells transfected with AMPK-siRNA or TRPV1-knockeddown MKN45 cells transfected with AMPK-overexpressed as well as their NC cells. (c-d) Summary data of cyclin D1 and MMP2 proteins in GC cells generated from the original data like in b (*P<0.05, **P<0.01, n=3). (e-f) The effect of genetic manipulation of AMPK on cell migration and invasion in TRPV1-overexpressed BGC823 cells and TRPV1-knockeddown MKN45 cells. Representative images are shown on the left and summary data on the right (*P<0.05, **P<0.01, n=3).
Figure 7

TRPV1/Ca2+-mediated GC suppression through activation of CaMKKβ/AMPK phosphorylation. (a) Representative time courses of 5 mM CaCl2-induced [Ca2+]i signaling in TRPV1-overexpressed BGC823 cells (middle) vs. NC (left) and TRPV1-overexpressed BGC823 cells treated with SB-705498 (50 µM) (right). Summary data are shown as a bar graph (**P<0.01, ****P<0.0001, n=20 cells). (b) Representative time courses of 5 mM CaCl2-induced [Ca2+]i signaling in TRPV1-knocked-down MKN45 cells (middle) vs. NC (left). Summary data are shown as a bar graph (right) (***P<0.001 vs. NC, n=20 cells). (c) The expression level of CaMKKβ proteins after TRPV1 overexpression in BGC823 cells or TRPV1 knockdown in MKN45 cells. Representative images are shown on the left and summary data on the right (*P<0.05 vs. NC, n=3). (d) Effect of CaMKKβ knockdown on CaMKKβ expression in TRPV1-overexpressed BGC823 or...
NC cells (*P<0.05, n=3). (e) Effect of BAPTA-AM (2 µM) on CaMKKβ expression in TRPV1-overexpressed BGC823 or NC cells (*P<0.05, n=3). (f) Effect of CaMKKβ knockdown on AMPK phosphorylation in TRPV1-overexpressed BGC823 cells or NC cells (*P<0.05, **P<0.01, n=3). (g) The proposed mechanisms of TRPV1-mediated GC suppression. The Ca2+ entry through TRPV1 channels cause CaMKKβ activation and AMPK phosphorylation that inhibits cyclin D1 and MMP2, leading to the suppression of GC cell proliferation, migration and invasion. OE: overexpression, KD: knockdown, CaMKKβ: calcium/calmodulin-dependent protein kinase kinase β, AMPK: adenosine mono phosphate activated protein kinase, MMP2: matrix metalloproteinase-2.