TRPM2 promotes the proliferation and invasion of pancreatic ductal adenocarcinoma

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Abstract. The aim of the present study was to investigate transient receptor potential cation channel subfamily M member 2 (TRPM2), a promising therapeutic target and biomarker for pancreatic ductal adenocarcinoma (PDAC) prognosis, in addition to determining its effects regarding tumor progression and invasion. PDAC is a fatal disease with a poor prognosis, and its associated pathogenic molecular mechanisms remain to be determined. In the present study, combined analysis using genomic and transcriptomic data from two PDAC studies was performed to discover a survival-associated biomarker of PDAC. Survival analysis for genes mutated in ≥10 patients was performed using a Kaplan-Meier curve and tested for significance using a log-rank test. Furthermore, gene-expression correlation analysis was performed to determine the genes with the strongest correlations to TRPM2. In addition, a Cell Counting Kit-8 assay, a scratch wound-healing assay and a Transwell assay were performed in the present study to investigate the proliferative, invasive and metastatic ability of PANC-1 cells in TRPM2-overexpressing and downregulated groups. The mutated TRPM2 gene had a strong negative correlation with patient survival probability compared with the normal control group ($P=1.06x10^{-4}$). Expression of TRPM2 was strongly correlated with expression of probable phospholipid-transporting ATPase IM, γ-parvin, tudor domain containing 9, Toll-like receptor 7 and Scm-like with four MBT domains protein 2 according to the criterion of a correlation coefficient >0.5. Furthermore, the results of the present study demonstrated that the TRPM2 overexpression in a PDAC cell line (PANC-1) promoted cell proliferation, invasion and metastatic ability. TRPM2 represents a potential therapeutic target and prognostic marker for patients with PDAC. TRPM2 regulates cell proliferation, invasion and migration; however, the underlying mechanism requires further investigation in future studies.

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Introduction

Pancreatic ductal adenocarcinoma (PDAC) is a disease with a poor prognosis. With increasing incidence and mortality, PDAC is the 6th most common cause of cancer-associated death in China (1). Despite advances in surgical and oncological treatment strategies, PDAC exhibits an extremely poor prognosis, with a 6-month median survival period and ≤1-2% 5-year survival rate (2,3).

The exact molecular mechanism of PDAC remains undetermined. Pancreatic intraepithelial neoplasia (PanIN) in the pancreatic duct has been proposed to be the primordial precursor of PDAC (4). As PanIN progresses to carcinoma, accumulated mutations may result in the loss of cyclin-dependent kinase inhibitor 2A/p16 and/or the inactivation of cellular tumor antigen 53 and mothers against decapentaplegic homolog 4, in addition to the activation of the KRAS proto-oncogene (5). Unlike other adenocarcinomas, the usual composition of pancreatic cancer tumor is a large mass of fibroinflammatory tissue, which is interspersed with islands of neoplastic epithelia (6,7). Previous studies have suggested that pancreatic tumor stroma directly affect the progression and outcome of disease via the release of factors, including insulin-like growth factor and platelet-derived growth factor, into the tumor microenvironment that aid tumor growth and invasiveness (8-10). However, the association between differentially expressed genes and tumor cells remains poorly understood, which has limited the development of effective treatments for patients with PDAC.

The Cancer Genome Atlas (https://cancergenome.nih.gov/) may further the understanding of the molecular basis of cancer via genome analysis, including high-throughput genome sequencing. In the present study, combined analysis using genomic and transcriptomic data from two PDAC studies was performed and a survival-associated biomarker was revealed, transient receptor potential cation channel subfamily M member 2 (TRPM2). Furthermore, the role of TRPM2 in

pancreatic adenocarcinoma cell lines was investigated using an *in vitro* gene silencing method. The present study provides a comprehensive investigation of TRPM2, a promising biomarker for PDAC prognosis, and investigates how TRPM2 affects tumor progression and invasion.

Materials and methods

Data analysis. Clinical information, somatic mutation and gene expression data of patients with pancreatic cancer were obtained from two previously published cancer genome studies (11,12). The somatic mutation and clinical information from 483 patients with pancreatic cancer was obtained from data published by Bailey *et al* (11) and Waddell *et al* (12). The survival time of 159 patients of the total 483 patients was available, with survival status indicated as 'alive', 'deceased' or 'loss' at the final follow-up. Gene expression data of 96 patients were obtained from the Gene Expression Omnibus (13) (dataset, GSE36924) (11), while 96 patients have survival information. The samples with follow-up information were used for survival analysis.

Survival analysis of genes mutated in ≥10 (out of 159) patients was performed. Patients were divided into two groups according to the mutation status of each gene. The difference in overall survival between the two groups was estimated using a Kaplan-Meier curve and tested for significance using a log-rank test. A gene was considered to be associated with patient clinical outcomes when a $P<0.05$ value was obtained. Following this, patients were additionally divided into two groups, based on mutation and gene expression levels, according to the median expression values of each gene. Survival analysis was performed using gene-expression data obtained from the Gene Expression Omnibus (dataset, GSE36924) (11). It was identified that somatic mutation and gene expression of TRPM2 were significantly associated with the patient's overall survival (Tables I and II). Thus, further experimental assays were performed to investigate the role of TRPM2 in pancreatic cancer.

It was hypothesized that genes involved in a similar biological process may exhibit similar expression patterns. Gene expression correlation analysis was performed to reveal genes functionally associated with TRPM2 in patients with pancreatic cancer. Pearson's correlation coefficient between TRPM2 and each of the genes of interest was calculated. Following this, the top genes with a correlation coefficient of >0.50 were retained (Table III).

TRPM2 knockdown and overexpression. PANC-1 cells (American Type Culture Collection, Manassas, VA, USA) were seeded in 6-well plates until 80% confluency was reached following 24 h of culture at 37˚C. Hilymax-Trpm2 siRNA complex (20 pmol/l), Hilymax-Trpm2 OverExp vector (4 μg/well), empty vector and scramble siRNA (Generay Biotech Co., Ltd., Shanghai, China) were added to the cell culture, using the transfection agent Hilymax (Dojindo Molecular Technologies, Inc., Kumamoto, Japan) well as per the manufacturer's instructions, and incubated at 37˚C in a 5% CO_2 incubator for 4 h. The medium was subsequently replaced with fresh medium (RPMI 1640; 10% fetal bovine serum, Gibco; Thermo Fisher Scientific, Inc., Waltham, MA, USA) and incubated for a further 48 h. The untransfected group was subjected to normal culture without transfection reagent, the empty vector represented the control for TRPM2 overexpression, and scramble siRNA represented the control for TRPM2 silencing. Cells were prepared for the subsequent reverse transcription-quantitative polymerase chain reaction (RT-qPCR) and Transwell assays. The Trpm2 siRNA and Trpm2 OverExp plasmid were designed and synthesized by Generay Biotech Co., Ltd. (Shanghai, China; Table IV).

RNA extraction and RT-qPCR. Total RNA from cells was extracted using Cell Culture and Tissue Total RNA Extraction and Preparation Mini kit (Thermo Fisher Scientific, Inc.) according to the manufacturer's instructions. The quantity and quality of RNA were confirmed using a NanoDrop 1000. The primers were designed using Primer Premier 5.0 software (Premier Biosoft International, Palo Alto, CA, USA) and synthesized by Generay Biotech Co., Ltd. (Table IV). RT-qPCR was performed using the KAPA SYBR Green Supermix PCR kit with the AriaMx Real-Time PCR System (both Agilent Technologies, Inc., Santa Clara, CA, USA). RT was performed at 50˚C for 30 min. qPCR conditions: Denaturing at 95˚C, 10 min, then 40 cycles of denaturing at 95˚C for 15 sec, annealing at 60˚C for 1 min, and elongation at 95˚C for 15 sec and 60˚C for 15 sec. The relative expression levels among the different genes were determined using the $2^{-\Delta\Delta Cq}$ method (14).

Cell proliferation assay. PANC-1 cell proliferation was investigated using the Cell Counting Kit-8 (CCK-8; Dojindo Molecular Technologies, Inc.). PANC-1 cells were seeded onto a 96-well microplate at a density of $3x10^4$ cells/well, and the cells were transfected with either Hilymax-Trpm2 OverExp vector (0.2 μg/well), Hilymax-Trpm2 siRNA (20 pmol/l), empty vector (0.2 μg/well) and scramble siRNA (20 pmol/l). The cells were subsequently cultured for 0, 24 and 48 h. Following this, 5 μl CCK-8 solution was added to each well and incubated at 37˚C for a further 2 h. Optical density was determined at 450 nm using a microplate spectrophotometer (BioTek Instruments, Inc., Winooski, VT, USA).

Scratch wound-healing assay. PANC-1 cells were seeded in 24-well plates at a density of $1x10^5$ cells per well and incubated overnight at 37˚C. Following this, the cells were transfected with Hilymax-Trpm2 OverExp vector (1 μg/well), Hilymax-Trpm2 siRNA (20 pmol/l), empty vector and scramble siRNA. The cells were subsequently cultured for 24 h. The cells at the bottom of wells were then scratched using a 10 μl tip and the floating cells were gently washed twice using RPMI 1640 medium. Images of each well were captured using a microscope (magnification, x100) at 0 and 24 h time intervals post-injury (Olympus X51; Olympus Corporation, Tokyo, Japan).

Transwell assay. PANC-1 cells were transfected with TRPM2 Vector, TRPM2 siRNA, empty vector and scramble siRNA. A total of 24 h post-transfection, the cells were seeded into Matrigel-plated upper wells with RPMI 1640 medium without serum ($5x10^4$ cells/well), and the lower wells contained 500 μl complete medium (RPMI 1640 and 10% fetal bovine serum), following a routine procedure. Following incubation for 48 h at

Table I. Survival analysis of genes associated with the patient's overall survival.

Gene	Mutation P-value	Expression P-value
TRPM2	0.0104	0.0111
COL18A1	0.0384	0.0138
THSD7B	0.144	0.166
FRAS1	0.0259	0.395
PTPRT	0.00367	0.426
SCN5A	0.102	0.886
PXDN	0.0301	0.903

37˚C, cells that did not migrate through the pores were gently removed with a cotton swab. Cells on the lower side of the insert filter were fixed by 5% glutaraldehyde for 10 min and stained with 1% crystal violet in 2% ethanol at room temperature for 20 min. Numbers of cells on the underside of the filter from five randomly selected microscopic views (magnification, x100) were counted.

Statistical analysis. The difference of overall survival between the two groups was estimated using Kaplan-Meier curve and tested for significance using log-rank test by SPSS 17.0 software (SPSS, Inc., Chicago, IL, USA). GraphPad Prism 5 (GraphPad Software, USA) was used to perform statistical analysis for the results of RT-qPCR, CCK-8, wound healing and Transwell assays. Significance between groups was evaluated by one way analysis of variance followed by a Bonferroni post hoc test. P<0.05 was considered statistically significant.

Results

TRPM2 is significantly correlated with patient survival. Following the data analysis of genes using the Kaplan-Meier curve and the log-rank test, TRPM2 was selected as a potential candidate marker of prognosis. The survival analysis revealed that in 159 patients, the mutation status of TRPM2 was significantly correlated with patient survival ($P=1.0416x10^{-2}$; Table I). The somatic mutations of TRPM2 in this cohort are detailed in Table II. Furthermore, the enhanced expression level affected survival among 91 patients ($P=4.2253x10^{-2}$; Fig. 1). Gene expression correlation analysis revealed that TRPM2 was strongly correlated with the expression of probable phospholipid-transporting ATPase IM (ATP8B4), γ-parvin (PARVG), tudor domain containing 9 (TDRD9), Toll-like receptor (TLR7) and Scm-like with four MBT domains protein 2 (SFMBT2) (Table III).

TRPM2 is successfully overexpressed in the overexpression group and suppressed in the siRNA group. RT-qPCR analysis demonstrated that compared with the empty vector group, TRPM2 was successfully overexpressed in the Hilymax-TRPM2 OverExp group (P<0.05; Fig. 2). In addition, the expression level of TRPM2 was successfully knocked down in the Hilymax-TRPM2 siRNA group compared with the scramble siRNA group (P<0.05; Fig. 2).

Overexpression of TRPM2 enhances the proliferation of PANC-1 cells. The results of the CCK-8 assay demonstrated that TRPM2 successfully enhanced PANC-1 cell proliferation in the Hilymax-Trpm2 OverExp group compared with the empty vector group (P<0.05; Fig. 3) at the 48-h time interval, whereas the Hilymax-Trpm2 siRNA group exhibited an inhibitory effect compared with the scramble siRNA group (P<0.05; Fig. 3). There were no significant differences between the vector group and the empty vector group at the 24 h time interval, however there was a significant difference between the TRPM2 siRNA group compared with the scramble siRNA group (Fig. 3).

Overexpression of TRPM2 enhances the migratory ability of PANC-1 cells. The results of the scratch wound-healing assay revealed that the migratory ability of PANC-1 cells was significantly enhanced following overexpression of TRPM2 (P<0.01; Fig. 4), however, this was significantly suppressed in the RNA interfering group (P<0.001; Fig. 4).

Overexpression of TRPM2 enhances the invasive ability of PANC-1 cells. Invasive ability was determined via Transwell assays using Matrigel, and the results demonstrated a significantly enhanced invasive ability in the OverExp group compared with the empty vector group (P<0.01; Fig. 5). The siRNA group exhibited fewer invaded cells in the lower chamber of the microwell (P<0.01; Fig. 5).

Discussion

The TRPM2 gene encodes a tetrameric cation channel that is permeable to sodium, calcium and potassium, and is activated by free intracellular adenosine 5'-diphosphate (ADP)-ribose (15,16). The encoded protein, also activated by oxidative stress, may induce susceptibility to cell death (17,18). According to previous studies, TRPM2 may induce apoptosis in different types of cancer (19,20). However, Huang *et al* (21) reported opposite effects of TRPM2 in pancreatic cancers.

According to the data analysis performed in the present study, it was revealed that the mutated TRPM2 gene exhibits a marked negative correlation with patient survival rate compared with the normal control group. The higher TRPM2 is expressed in cancerous tissue, the shorter the survival time exhibited by PDAC patients. The results of the *in vitro* analyses in the present study revealed that the overexpression of TRPM2 enhanced cell proliferation and invasive ability, and these findings are consistent with the results of the data analysis. Therefore, it may be suggested that TRPM2 expression levels are markedly associated with proliferation, invasive ability and poor prognosis in patients with PDAC. Thus, TRPM2 represents a potential therapeutic target and prognostic marker for the treatment of patients with PDAC.

The results of the gene expression correlation analysis demonstrated that TRPM2 is strongly correlated with the expression levels of ATP8B4, PARVG, TDRD9, TLR7 and SFMBT2. Among these genes, TLR7 has previously been suggested to be associated with the progression of pancreatic cancer (22-25). TLRs are expressed in pancreatic cancer cell lines and numerous human cancer cell lines; however,

Table II. Somatic mutations of transient receptor potential cation channel subfamily M member 2 in patients.

Hugo symbol	Chromosome	Start position	End position	Variant classification	Reference allele	Tumor seq allele1	Tumor seq allele2	Tumor sample barcode	Canonical base change
TRPM2	21	45820197	45820197	Missense mutation	G	A	G	ICGC_0021	2477G/A
TRPM2	21	45833788	45833788	Missense mutation	C	A	C	ICGC_0054	3190C>A
TRPM2	21	45845642	45845642	Missense mutation	C	C	T	ICGC_0114	3940C>T
TRPM2	21	45789057	45789057	Splice site	C	C	T	ICGC_0121	NA
TRPM2	21	45810792	45810792	Missense mutation	C	C	T	ICGC_0242	1537C>T
TRPM2	21	45811433	45811433	Silent	G	A	G	ICGC_0315	1932G>A
TRPM2	21	45811283	45811283	Silent	C	C	T	ICGC_0326	1782C>T

Seq, sequence; TRPM2, transient receptor potential cation channel subfamily M member 2; C, cytosine; G, guanine; T, thymine; A, adenosine.

Table III. Gene expression correlation analysis with transient receptor potential cation channel subfamily M member 2.

Ensembl	Gene	Correlation coefficient
ENSG00000104043	ATP8B4	0.574108
ENSG00000156414	TDRD9	0.526303
ENSG00000198879	SFMBT2	0.520375
ENSG00000138964	PARVG	0.511799
ENSG00000196664	TLR7	0.503315

ATP8B4, probable phospholipid-transporting ATPase IM; TDRD9, tudor domain containing 9; SFMBT2, Scm-like with four MBT domains protein 2; PARVG, γ-parvin; TLR7, Toll-like receptor 7.

TLRs are not expressed in the healthy pancreas (26). The expression levels of phosphorylated-extracellular signal-regulated kinase (12) 1/2 are upregulated following TLR7 activation (20), suggesting that the activation of TLR7 is at least partially associated with the mitogen-activated protein kinase-ERK1/2 pathway in BxPC-3 pancreatic cancer cells (22). Furthermore, Grimmig *et al* (23) revealed that chronic inflammation-mediated TLR7/TLR8 signaling may result in pancreatic cancer cell growth and chemoresistance.

The ATP8B4 gene can encode a P4-ATPase flippase complex, a member of the cation transport ATPase (P-type) family and type IV subfamily (27). Ni *et al* (27) suggested that ATP8B4 may be a potential prognostic marker and a therapeutic target in the treatment of patients with multiple myeloma who are B-Cell chronic lymphocytic lymphoma/small lymphocytic Lymphoma (BCL)-1/JHt(11;14)(q13;q32) translocation positive.

PARVG, a member of the parvin protein family, is an actin-binding protein associated with focal adhesion (28). Chen *et al* (29) revealed that four DNA methylation signatures (phospholipase C β2, Rac family small GTPase 2, vav guanine nucleotide exchange factor 1 and PARVG) are strongly associated with the prognosis of renal clear cell carcinoma.

TDRD9, a protein coding gene, is associated with pathways including mitotic prophase and PIWI-interacting RNA biogenesis (30-32). At present, there is no evidence to suggest that TDRD9 has an association with cancer.

Mammalian SFMBTs have been suggested to be polycomb group repressors (33). Lee *et al* (33) demonstrated that transcriptional repression was associated with human SFMBT2, which binds preferentially to methylated histone H3 and H4. In addition, it has been revealed that SFMBT2 regulates cell proliferation via epigenetic regulation of homeobox B13 gene expression in the DU145 prostate cancer cell line (34).

Further research is required to investigate the association between TRPM2 and these five genes in the mechanism of promoting pancreatic cancer.

In addition, Li *et al* (35) demonstrated that H_2O_2-mediated activation of the TRPM2 pathway enhanced the migratory ability of HeLa and prostate cancer cells by inducing filopodia formation, inducing the degradation of actin fibers and inducing the decomposition of focal adhesions. Bauer *et al* (36) reported

Table IV. Primer and siRNA information.

Primer	Sequence (5'-3')	Amplicon length, base pairs
Homo-TRPM2-F	ATTGTGAAGCGGATGATGAAGGA	158
Homo-TRPM2-R	ATGGTGAGGTAGGAGTGGTAGAC	158
Homo-GAPDH-F	TGGACCTGACCTGCCGTCTA	149
Homo-GAPDH-R	GGAGTGGGTGTCGCTGTTGA	149
TRPM2-siRNA sense	GAAAGAAUGCGUGUAUUUUTT	N/A
TRPM2-siRNA antisense	AAAAUACACGCAUUCUUUCTT	N/A
Scramble siRNA sense	UUCUUCGAACGUGUCACGUTT	N/A
Scramble siRNA antisense	ACGUGACACGUUCGGAGAATT	N/A

F, forward; R, reverse; TRPM2, Transient receptor potential cation channel subfamily M member 2; si, small interfering.

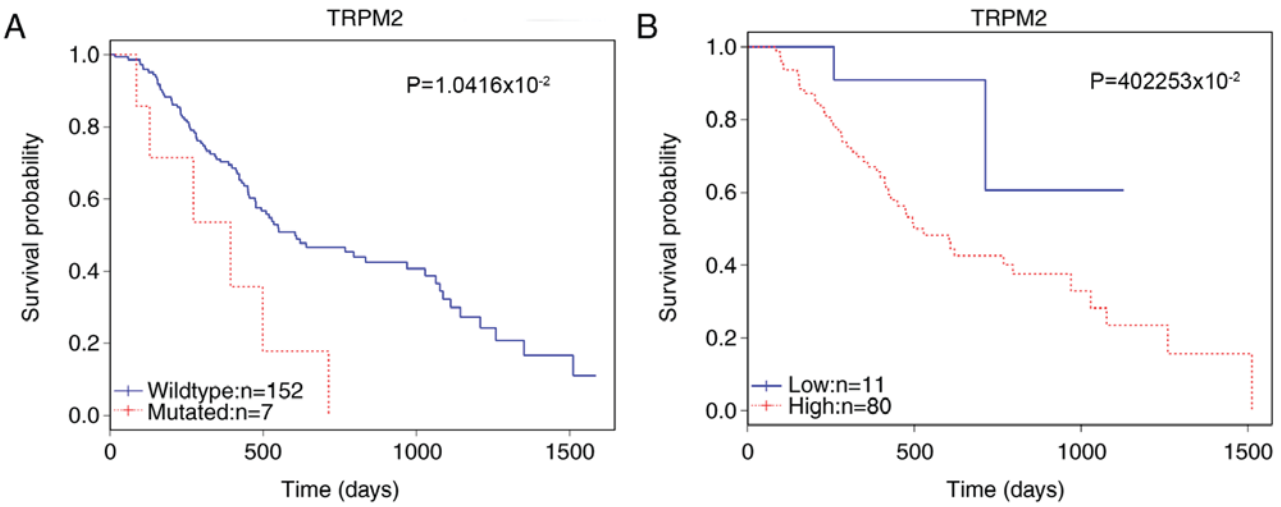

Figure 1. Survival analysis of TRPM2 mutations and gene expression. The overall survival rates of patients are presented according to (A) gene mutation status and (B) expression level. Wildtype indicates a group of patients without gene mutation. TRPM2, transient receptor potential cation channel subfamily M member 2.

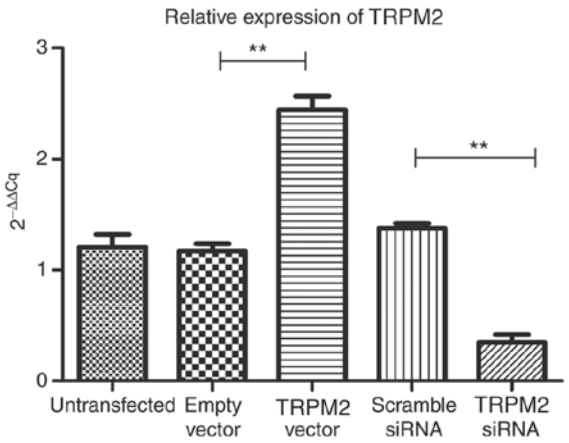

Figure 2. Reverse transcription-quantitative polymerase chain reaction analysis results in the different experimental groups. **P<0.01 vs. TRPM2 vector or Scramble siRNA vs. TRPM2 siRNA. TRPM2, transient receptor potential cation channel subfamily M member 2; si, small interfering.

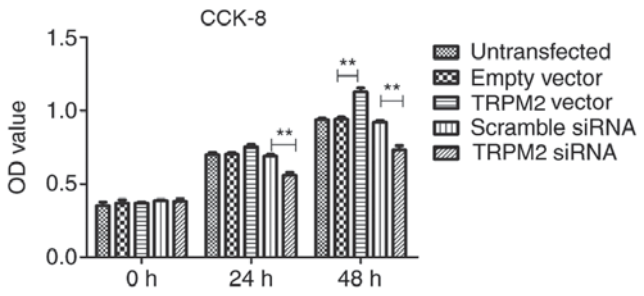

Figure 3. CCK-8 array results in the untransfected, empty vector, TRPM2 vector, scramble siRNA and TRPM2 siRNA groups at 0-, 24-, and 48-h time intervals. **P<0.01. TRPM2, transient receptor potential cation channel subfamily M member 2; si, small interfering; CCK-8, Cell Counting Kit-8; OD, optical density.

that the expression of ADP-ribose, a calcium channel activator, was increased by NAD-dependent protein deacetylase sirtuin-6 (SIRT6), which in turn enhanced the expression of interleukin 8 and tumor necrosis factor pro-inflammatory cytokines and promoted pancreatic cancer cell migration. Thus, TRPM2 may promote PDAC metastasis via the aforementioned mechanisms, however, further investigation is required to determine the underlying molecular mechanisms.

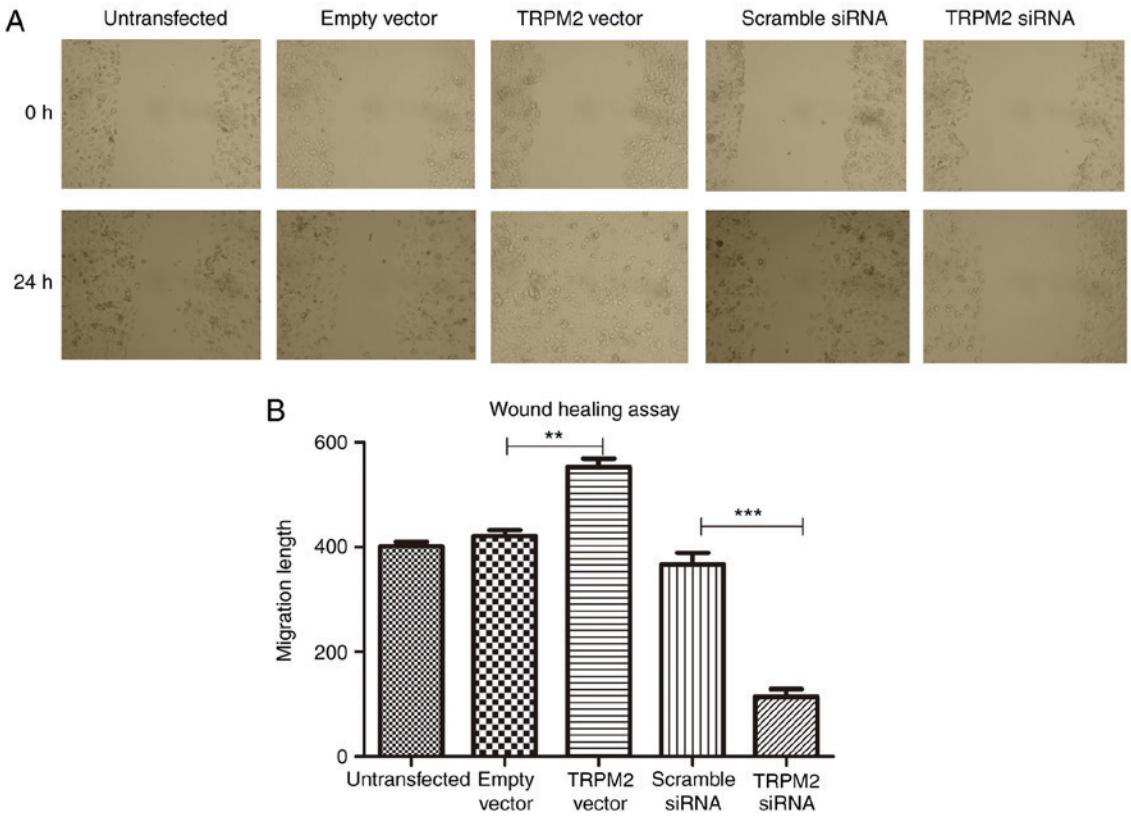

Figure 4. Scratch wound-healing assay results. (A) Images of all groups at 0- and 24-h time intervals post-injury (magnification, x100). (B) Quantitative analysis of scratch wound-healing assay results among untransfected, empty vector, TRPM2 vector, scramble siRNA and TRPM2 siRNA groups at 24 h post-injury. **P<0.01, ***P<0.001. TRPM2, transient receptor potential cation channel subfamily M member 2; si, small interfering.

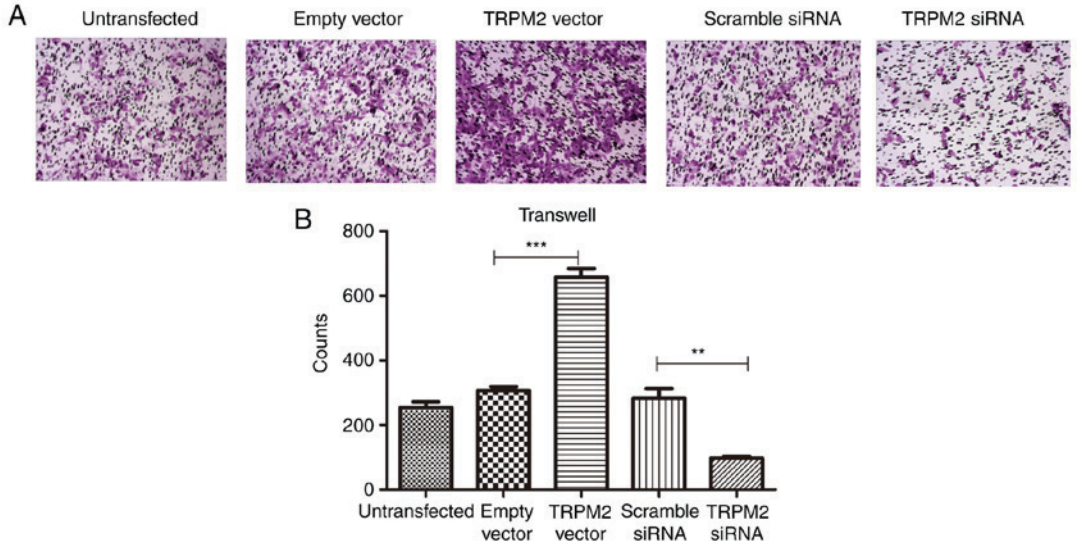

Figure 5. Transwell assay results suggest that the TRPM2 overexpression group has a stronger invasive ability and TRPM2 siRNA group has a weaker invasive ability. (A) Images under microscopy (magnification, x100). (B) Quantitative analysis of Transwell assay results among untransfected, empty vector, TRPM2 vector, scramble siRNA and TRPM2 siRNA groups. **P<0.01, ***P<0.001. TRPM2, transient receptor potential cation channel subfamily M member 2; si, small interfering.

The results of the survival analyses regarding TRPM2 mutations suggest a poor prognosis for the TRPM2 mutation group compared with in the wild type group. The results of the present study have demonstrated that overexpression of TRPM2 promotes PANC-1 cell growth, migration and invasion ability. Furthermore, the results of the present study hypothesize that TRPM2 may mediate cell proliferation via regulation of TLR7 and SFMBT2. In addition, TRPM2 may be associated with the promotion of invasion and migration via the regulation of PARVG and SIRT6, or by inducing filopodia formation, however, the mechanism underlying this process remains to be determined.

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Availability of data and materials

The datasets used and analyzed during the current study are available from the corresponding author on reasonable request.

Authors' contributions

BMS designed the research. RL and YFW conducted the research. QNC and ZYL helped collect and interpret the data. SX and BYW helped culturing cells, collecting reference articles and writing the manuscript. All authors read and approved the final manuscript.

Ethics approval and consent to participate

Not applicable.

Consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

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