Original Article

TMEM43 promotes the development of hepatocellular carcinoma by activating VDAC1 through USP7 deubiquitination

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Background: Transmembrane protein 43 (*TMEM43*), a member of the TMEM subfamily, is encoded by a highly conserved gene and widely expressed in most species from bacteria to humans. In previous studies, TMEM43 has been found to play an important role in a variety of tumors. However, the role of TMEM43 in cancer remains unclear.

Methods: We utilized the RNA sequencing (RNA-seq) and The Cancer Genome Atlas (TGCA) databases to explore and identify genes that may play an important role in the occurrence and development of hepatocellular carcinoma (HCC), such as *TMEM43*. The role of *TMEM43* in HCC was explored through Cell Counting Kit-8 (CCK-8) cloning, flow cytometry, and Transwell experiments. The regulatory relationship between *TMEM43* and voltage-dependent anion channel 1 (*VDAC1*) was investigated through coimmunoprecipitation (co-IP) and western blot (WB) experiments. WB was used to study the deubiquitination effect of ubiquitin-specific protease 7 (*USP7*) on *TMEM43*.

Results: In this study, we utilized the RNA-seq and TGCA databases to mine data and found that *TMEM43* is highly expressed in HCC. The absence of *TMEM43* in cancer cells was shown to inhibit tumor development. Further research detected an important regulatory relationship between *TMEM43* and *VDAC1*. In addition, we found that *USP7* affected the progression of HCC by regulating the ubiquitination level of *TMEM43* through deubiquitination.

Conclusions: Our study demonstrated that USP7 participates in the growth of HCC tumors through *TMEM43/VDAC1*.Our results suggest that *USP7/TMEM43/VDAC1* may have predictive value and represent a new treatment strategy for HCC.

Keywords: Transmembrane protein 43 (*TMEM43*); hepatocellular carcinoma (HCC); voltage-dependent anion channel 1 (*VDAC1*); metastasis

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Introduction

On a global scale, hepatocellular carcinoma (HCC) is a common and deadly malignant tumor. In the United States, it ranks fifth in terms of mortality rate. Patients are usually diagnosed at an advanced stage, leading to poor prognosis (1-3). At present, surgical resection and liver transplantation are the most effective treatment methods, and the prognosis of cancer is poor. Only 5–15% of early-stage patients are eligible for surgical resection (4,5). Although there have been many studies on epigenetic changes, the molecular mechanism of HCC has not yet been fully understood (6,7). Therefore, it is necessary to further explore the potential molecular mechanisms of HCC and discover new potential targets to inhibit the development of HCC.

Transmembrane protein 43 (*TMEM43*), a member of the TMEM subfamily, is encoded by a highly conserved gene and is widely expressed in most species from bacteria to humans (8). Systematic genetic analysis has shown that *TMEM43* is involved in metabolic related pathways (9). A previous study showed that *TMEM43* regulates ferroptosis by reducing the expression of P53 (10). *TMEM43* also plays an important role in tumors. A study has shown that the expression of *TMEM43* is closely related to the clinical pathological characteristics and adverse survival outcomes of pancreatic cancer. In addition, they also found that inhibition of *TMEM43* can inhibit the growth, migration, and invasion of pancreatic cancer *in vitro* and *in vivo* (11). However, the role of *TMEM43* in cancer is not yet clear.

In this study, we first mined *TMEM43* through the RNA sequencing (RNA-seq) and The Cancer Genome Atlas (TGCA) databases. Further research found that *TMEM43* is highly expressed in HCC, and *TMEM43* regulates the proliferation, cell cycle, apoptosis, and invasion of HCC through voltage-dependent anion channel 1 (*VDAC1*). Ubiquitin modification is an important post-translational modification process that regulates intracellular protein levels by adding ubiquitin molecules. This modification involves various cellular processes, including cell proliferation, apoptosis, invasion, migration, and DNA damage repair, which play crucial roles in tumor development and cancer progression (12). In addition, we found that ubiquitin-specific protease 7 (*USP7*) affected the progression of HCC by regulating the ubiquitination level of *TMEM43* through deubiquitination. We present this article in accordance with the MDAR reporting checklist (available at https://tgh.amegroups.com/article/view/10.21037/tgh-23-108/rc).

Highlight box

Key findings

- In this study, we found for the first time that transmembrane protein 43 (TMEM43) is highly expressed in hepatocellular carcinoma (HCC). TMEM43 plays an important role in the occurrence and development of HCC. Further research has found an important regulatory relationship between TMEM43 and voltage-dependent anion channel 1 (VDAC1). In addition, we also found that ubiquitin-specific protease 7 (USP7) regulates the ubiquitination level of TMEM43 through ubiquitination, thereby affecting cancer progression.

What is known and what is new?

- *TMEM43* is highly expressed in HCC and can regulate the proliferation, differentiation, apoptosis, and migration functions of HCC.
- Our research suggests that the *USP7/TMEM43/VDAC1* signaling axis plays an important role in inhibiting cancer progression.

What is the implication, and what should change now?

- *USP7/TMEM43/VDAC1* may serve as a regulatory molecular axis for HCC treatment. These findings may provide a new therapeutic target for HCC.

Methods

Clinical samples

Tumor tissue samples were taken from 96 patients with primary HCC who underwent surgery in the Affiliated Hospital of Nantong University. This study was approved by the Institutional Ethics Committee of the Affiliated Hospital of Nantong University (ID: 2022-L062). Informed consent was provided by each participant. The study was conducted in accordance with the Declaration of Helsinki (revised in 2013).

Cell culture and transfection

HCC cell lines (Huh7, HCCLM3) were purchased from American Type Culture Collection (ATCC; Manassas, VA, USA). HCCLM3 cells are a type of hepatoma cells line with high metastatic potential (13,14). Hepatoma cells were cultured in Dulbecco's modified Eagle medium (DMEM) medium containing 10% fetal bovine serum (FBS). Plasmids were transfected into cells using Lipofectamine 3000 reagent (Invitrogen, Carlsbad, CA, USA) and transfection efficiency was evaluated using western blot (WB). The sequences of the short hairpin (sh) RNAs are as follows:

sh-*TMEM43*#1, "CCTCAACCTTATGACACGGAT"; sh-*TMEM43*#2, "CCTTTGTCCAAATTGGCAGGT"; sh-*TMEM43*#3, "GAGATGTACCAATGGGTAGAA"; sh-*USP7*, "CGTGGTGTCAAGGTGTACTAA".

Quantitative real-time polymerase chain reaction (qRT-PCR)

Total RNA was extracted from cells using Trizol reagent (Invitrogen) and complementary DNA (cDNA) was obtained using Prime Script RT kit (Takara Bio, Shiga, Japan). Using DNA as a template, SYBR Select Master Mix kit (Invitrogen) and ABI Prism 7900 detection system (Applied Biosystems, Carlsbad, CA, USA) were used for qRT-PCR reaction. The primers for MiR-7 were designed and synthesized by RiboBio (Shanghai, China). The upstream primer article number of MiR-7 was SSD809232326 and the downstream primer article number was SSD089261711.

Cell Counting Kit-8 (CCK-8) assay

Cell proliferation was detected using the CCK-8 method. After inoculating the cell suspension in a 96 well plate, 10 μL of CCK-8 solution was added to each well. The culture plate was placed into the microplate reader within the specified time. Then the absorbance value of the optical density (OD) 450 nm pore was measured. Each group had 3 sets of auxiliary holes.

Colony formation assay

The density of 2,000 cells was evenly inoculated into a 6-well plate, and after 14 days of culture in the incubator sink, they were fixed with 4% paraformaldehyde for 30 minutes. The cells were then dyed with crystal violet for 20 minutes. After rinsing, photos were taken and the cell colonies were counted.

Flow cytometric analysis

Flow cytometry was used to detect cell apoptosis and cycle. At 48 hours after transfection, double staining with an Annexin V-fluorescein isothiocyanate (FITC)/propidium iodide (PI) Apoptosis Kit (Invitrogen) was performed, and the cell percentage was stained using a PI-based cell cycle assay kit (Invitrogen).

Transwell assay

After digesting the cells, serum-free culture medium was used to blow them evenly and approximately 10,000 cells were added to the Transwell's upper chamber. DMEM culture medium was added to the lower chamber. The cells were then incubated for 24 hours. After being fixed with 4% paraformaldehyde for 30 minutes, the cells were dyed with crystal violet for 20 minutes.

WB analysis

After loading the sample, the protein was completely separated by constant current electrophoresis. After the protein was transferred to the polyvinylidene fluoride (PVDF) membrane, it was sealed for 1 hour in sealing solution, and finally the antibody was added to continue incubation overnight. On the second day, after rinsing in tris-buffered saline with Tween 20 (TBST), horseradish peroxidase (HRP)-labeled secondary antibodies were added and incubated at room temperature for 1 hour. Photos were taken with an automatic chemiluminescence imaging system. Primary antibodies were generated against TMEM43 (diluted 1:500, #ab15379, Abcam, Cambridge, UK), VDAC1 (1/5,000, #55259-1-AP, Proteintech, Rosemont, IL, USA), USP7 (1/10,000, #66514-1-Ig, Proteintech), p-PI3K (1/1,000, #AF3242, Affinity, Cincinnati, OH, USA), and PI3K (1/1,000, #MA1-74183, Thermo Fisher, Waltham, MA, USA), and β-actin (1:10,000, #66009-1-Ig, Proteintech) was used as an internal control.

Cellular immunofluorescence

HCC cells were fixed with methanol and washed with phosphate-buffered saline (PBS) for 3 days ×5 minutes. After the first antibody had been added, they were sealed with goat serum overnight. After being washed in PBS, the cells continued to incubate with the second antibody for 2 hours. After cleaning, 4',6-diamidino-2-phenylindole (DAPI) was added and microscopic examination was performed.

Coimmunoprecipitation (co-IP)

The total cell lysate antibody was mixed and incubated overnight. Then, 5 μL agarose (A+G) (Absin, Shanghai, China) was added and incubated at room temperature for a

further 2 hours. The composite was rinsed 3 times with PBS. Sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) and WB were used to detect proteins.

Statistical analysis

The software SPSS 26.0 (IBM Corp., Armonk, NY, USA) was used for statistical analysis, and the data were calculated as mean ± standard deviation. Independent sample *t*-test was used to compare the two groups of data, and $P<0.05$ was considered statistically significant.

Results

Exploring genes regulating HCC through RNA-seq

MiR-7 has been described as an important factor in cancer development as it acts as a tumor suppressor and regulates a large number of genes involved in cancer development and progression (15). To explore potential genes that regulate HCC progression, we first transfected Huh7 cells with empty lentivirus (NC) and miR-7 overexpressed lentivirus (OE), respectively. Detection by qRT-PCR showed a significant increase in expression in the OE group (*Figure 1A*). Then, we performed RNA-seq on the cells and found that compared to the miR-7 overexpression group (E8321-1, E8321-2, E8321-3), the miR-7 control group (E8320-1, E8320-2, E8320-3) had a total of 342 upregulated genes and 369 downregulated genes. The difference between the two groups was visualized through a volcanic map (*Figure 1B*).

We conducted cluster analysis on long non-coding RNA (lncRNA) and messenger RNA (mRNA) in differentially expressed genes (DEGs) through bioinformatics analysis. We found that compared to the OE group, lncRNA with increased expression in the NC group included lncRNAs ARID1B-2:1, C15orf48-2:1, OTTHUMT000000473305, ENST000000421513.1, ENST000000472821.1, ENST000000624858, and MEF2C-3:1 (*Figure 1C*). The mRNA with increased expression in the NC group included DPPA3, NPHS1, DGKG, GABRR3, POM121L2, GPR15, TRIM43, FAM183A, DLL1, CCDC110, TMEM132C, NOX4, A4GNT, and TMEM43, among others (*Figure 1D*). Then, we further analyzed the distribution of DEGs on chromosomes (*Figure 1E*).

Predicting high expression of TMEM43 gene in HCC

Through RNA-seq, we found that *TMEM43* may play an important role in HCC. Therefore, we used TGCA to explore the expression of *TMEM43* in 33 cancers. We found that *TMEM43* is highly expressed in various tumors, including HCC (*Figure 2A*). We further confirmed through the Gene Expression Profiling Interactive Analysis (GEPIA) database that *TMEM43* is highly expressed in cancer (*Figure 2B*). Then, we further explored the prognostic impact of *TMEM43* in 33 cancers through TGCA and found that *TMEM43* affects the prognosis of multiple tumors (*Figure 2C*). The GEPIA database further confirmed that *TMEM43* is closely related to the poor prognosis of HCC (*Figure 2D*).

Expression levels of TMEM43 gene in HCC tissues and HCC cell lines

In order to determine the expression of *TMEM43* in HCC tissue, we used WB to analyze the expression of *TMEM43* in HCC tissue. The results showed that compared with adjacent tissues, the expression of *TMEM43* in HCC tissue was significantly increased (*Figure 3A*). We conducted a tissue microarray study on the expression of *TMEM43* in 96 HCC tissues, and found that it was highly expressed in 68 tissues. *TMEM43* was mainly brownish brown in the cytoplasm (*Figure 3B*). Then, we investigated the expression and localization of *TMEM43* in Huh7 and HCCLM3 cells through cellular immunofluorescence. The results showed that *TMEM43* was mainly expressed in the cytoplasm of HCC cells (*Figure 3C*).

WB was used to detect the transfection effect of TMEM43 gene in HCC cell lines

To verify the impact of *TMTM43* on the biological behavior of HCC cell lines, we used Lipofectamine™ 3000 for transfection. We transfected sh-*TMEM43*#1, sh-*TMEM43*#2, and sh-*TMEM43*#3 into the HCC cell lines Huh7 and HCCLM3. WB was used to detect the expression of *TMEM43*. The results showed that compared with the control group, Huh7 and HCCLM3 cells transfected with sh-*TMEM43*#1, sh-*TMEM43*#2, and sh-*TMEM43*#3 showed significant downregulation of *TMEM43* expression. Then, we selected sh-*TMEM43*#1 and sh-*TMEM43*#2 with better transfection effects for subsequent experiments (*Figure 4A*).

The effect of TMEM43 gene on the growth of HCC cells

The colony formation assay and CCK-8 assay were used to detect the effect of sh-*TMEM43* on growth, respectively.

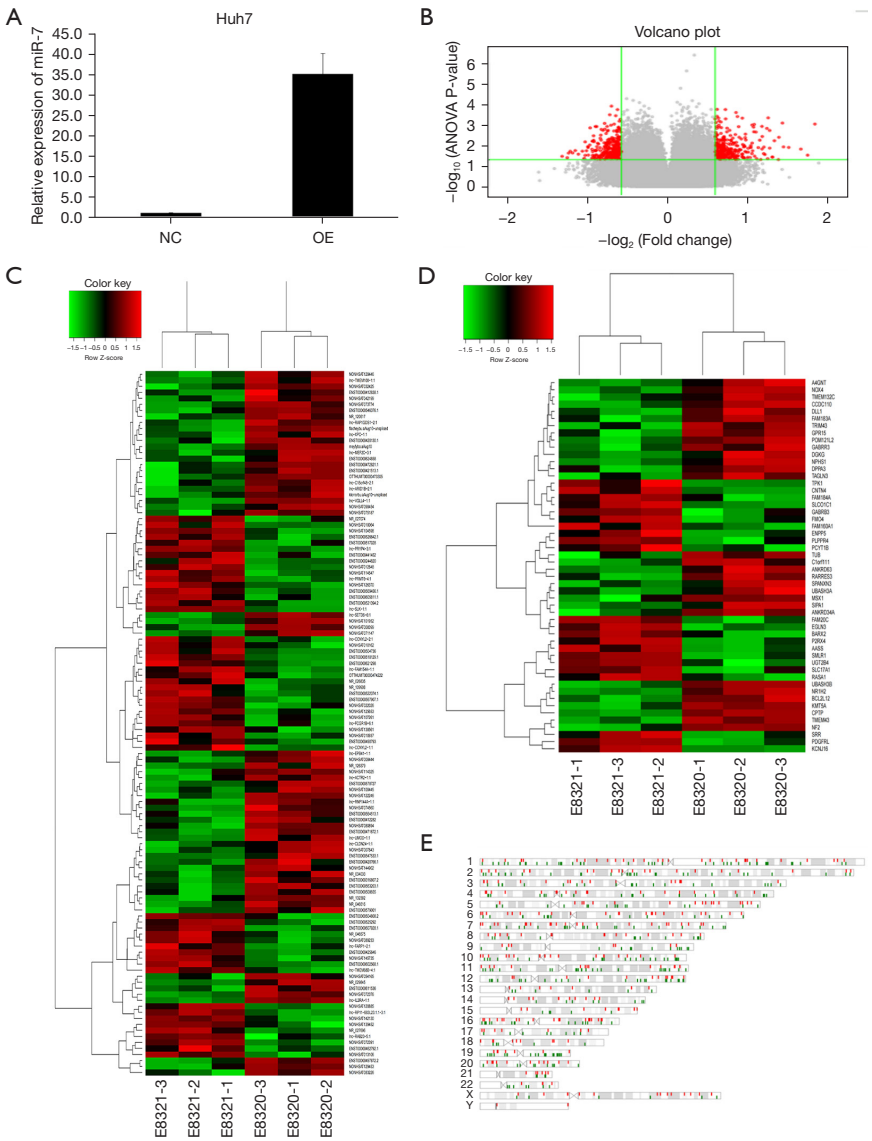

Figure 1 Exploring genes regulating hepatocellular carcinoma through RNA-seq. (A) After transfecting empty lentivirus (NC) and miR-7 overexpressed lentivirus (OE) into Huh7 cells, the expression of miR-7 was detected by qRT-PCR. (B) Displaying differential genes between the miR-7 overexpression group (E8321-1, E8321-2, E8321-3) and the miR-7 control group (E8320-1, E8320-2, E8320-3) through volcanic maps. The horizontal axis represents the multiple of differences (logarithmic transformation with a base of 2), the vertical axis represents the significance P value of differences (logarithmic transformation with a base of 10), the red dot represents the significant difference genes screened based on |fold change| ≥1.5 and P value <0.05 as the standard, and the gray dot represents other genes without significant differences. (C) LncRNA in differential genes. Red indicates a relative upregulation of gene expression, green indicates a relative downregulation of gene expression, black indicates no significant change in gene expression, and gray indicates that the signal intensity of the gene has not been detected. (D) mRNA in differential genes. Red indicates a relative upregulation of gene expression, green indicates a relative downregulation of gene expression, black indicates no significant change in gene expression, and gray indicates that the signal intensity of the gene has not been detected. (E) The distribution map of differentially expressed genes on chromosomes shows the distribution of differentially expressed genes. This figure shows the basic structure of 24 human chromosomes and the specific positions of differential genes on each chromosome. The red color in the figure represents the distribution of upregulated genes on chromosomes; the green color represents the distribution of downregulated genes on chromosomes. ANOVA, analysis of variance; RNA-seq, RNA-sequencing; qRT-PCR, quantitative real-time polymerase chain reaction; lncRNA, long non-coding RNA; mRNA, messenger RNA.

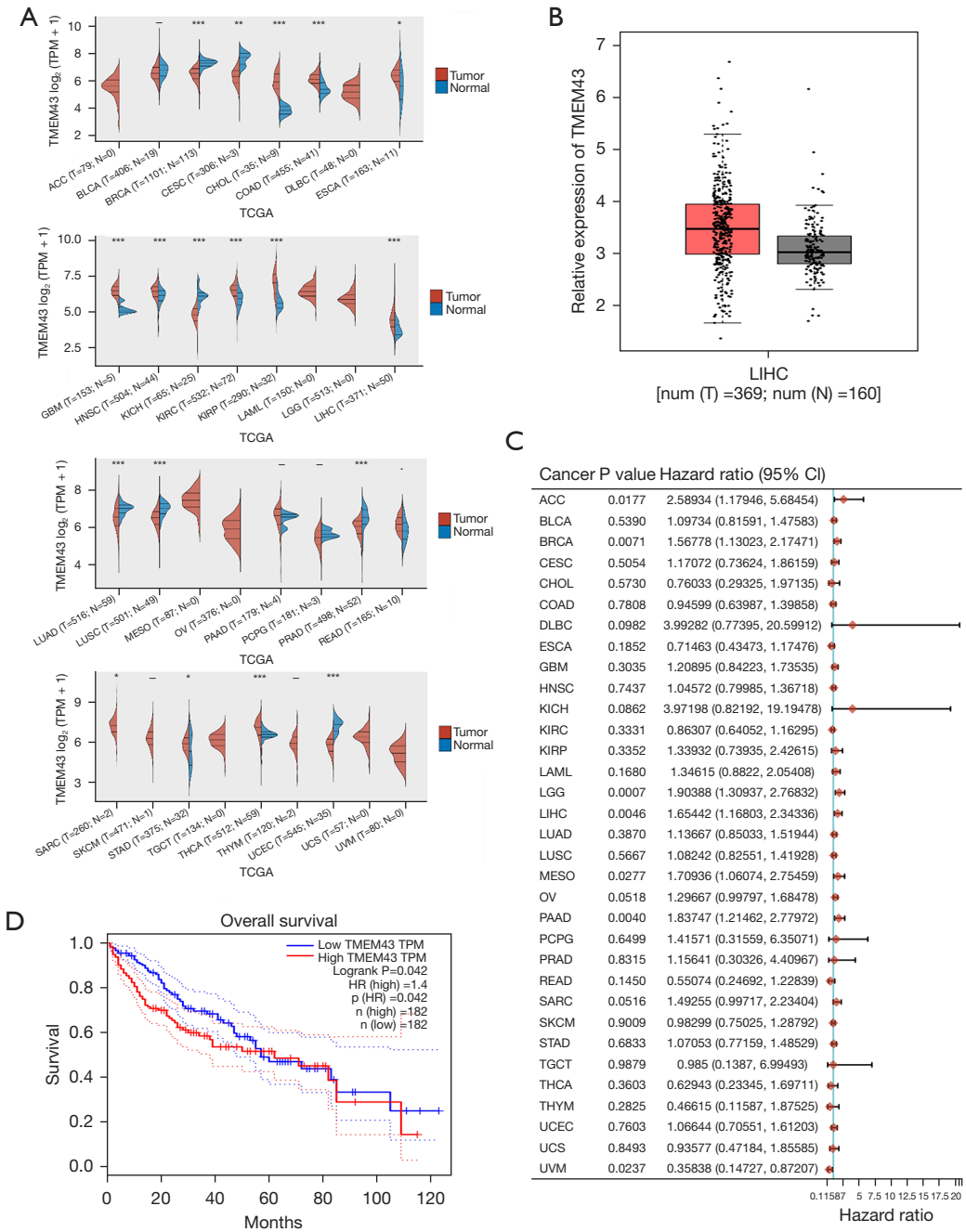

Figure 2 Predicting high expression of *TMEM43* in hepatocellular carcinoma. (A) The expression of *TMEM43* in 33 types of tumors was analyzed through the TCGA database. (B) The expression of TMEM43 in hepatocellular carcinoma was analyzed through the GEPIA database. (C) The prognosis of *TMEM43* in 33 types of tumors was analyzed through the TCGA database (D) Prognosis of molecular *TMEM43* in hepatocellular carcinoma through TCGA database. –, P>0.05; *, P<0.05; **, P<0.01; ***, P<0.001. TMEM43, transmembrane protein 43; TPM, transcripts per million; ACC, adrenocortical carcinoma; BLCA, bladder urothelial carcinoma; BRCA, breast invasive carcinoma; CESC, cervical squamous cell carcinoma and endocervical adenocarcinoma; CHOL, cholangiocarcinoma; COAD, colon adenocarcinoma; DLBC, lymphoid neoplasm diffuse large B-cell lymphoma; ESCA, esophageal carcinoma; GBM, glioblastoma multiforme; HNSC, head and neck squamous cell carcinoma; KICH, kidney chromophobe; KIRC, kidney renal clear cell carcinoma; KIRP, kidney renal papillary cell carcinoma; LAML, acute myeloid leukemia; LGG, brain lower grade glioma; LIHC, liver hepatocellular carcinoma;

LUAD, lung adenocarcinoma; LUSC, lung squamous cell carcinoma; MESO, mesothelioma; OV, ovarian serous cystadenocarcinoma; PAAD, pancreatic adenocarcinoma; PCPG, pheochromocytoma and paraganglioma; PRAD, prostate adenocarcinoma; READ, rectum adenocarcinoma; SARC, sarcoma; SKCM, skin cutaneous melanoma; STAD, stomach adenocarcinoma; TGCT, testicular germ cell tumors; THCA, thyroid carcinoma; THYM, thymoma; UCEC, uterine corpus endometrial carcinoma; UCS, uterine carcinosarcoma; UVM, uveal melanoma; TMEM43, transmembrane protein 43; T, tumor; N, normal; CI, confidence interval; HR, hazard ratio; TCGA, The Cancer Genome Atlas; GEPIA, Gene Expression Profiling Interactive Analysis.

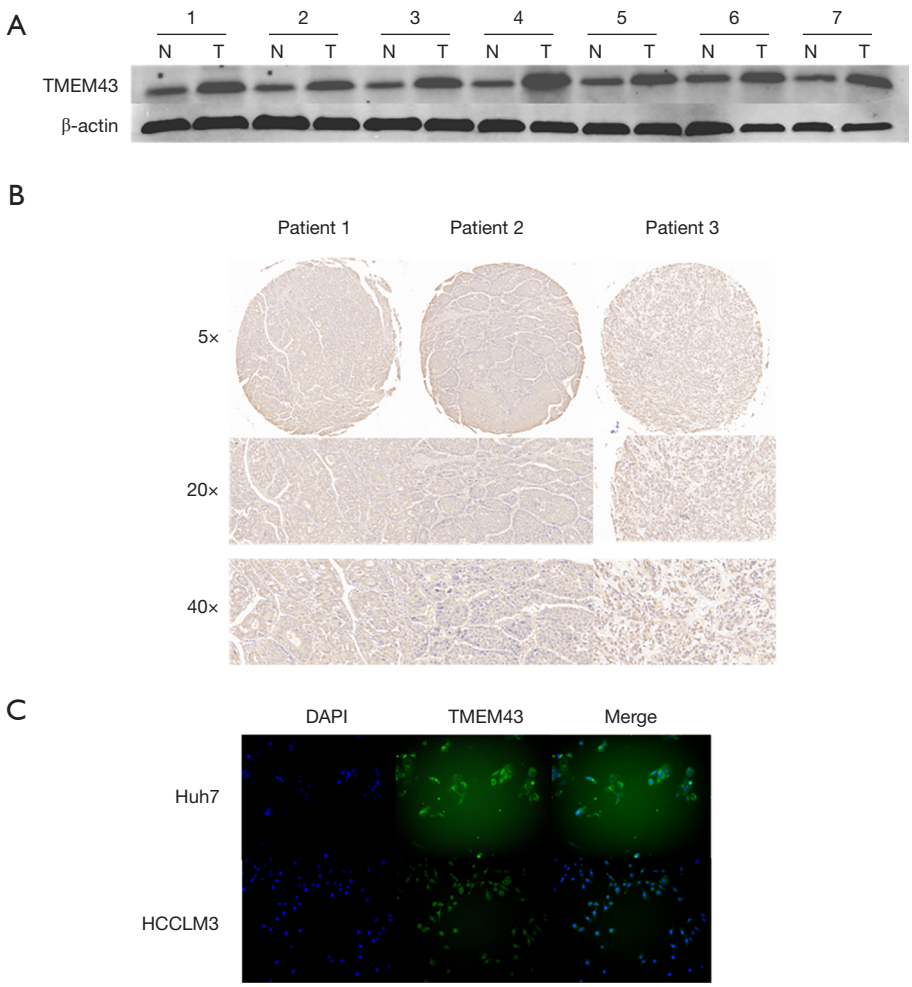

Figure 3 Expression levels of *TMEM43* in hepatocellular carcinoma tissues and hepatocellular carcinoma cell lines. (A) Detection of *TMEM43* expression in seven pairs of hepatocellular carcinoma tissues and adjacent tissues by western blot. (B) Detection of *TMEM43* expression in hepatocellular carcinoma tissue by immunohistochemistry (the original magnification is respectively 5×, 20×, 40×). (C) Localization of *TMEM43* expression in Huh 7 and HCCLM3 cells through cellular immunofluorescence assay (original magnification, ×100). TMEM43, transmembrane protein 43; N, normal; T, tumor; DAPI, 4',6-diamidino-2-phenylindole.

The colony formation experiment showed that the cell colony of *TMEM43* knockdown group was smaller than that of the control group (*Figure 4B*). The CCK-8 experiment showed that compared with the control group, *TMEM43* knockdown significantly inhibited the proliferation of Huh7 and HCCLM3 cells (*Figure 4C*). Through CCK-8 and clone formation experiments, it was revealed that knocking down *TMEM43* inhibits the growth of HCC cells.

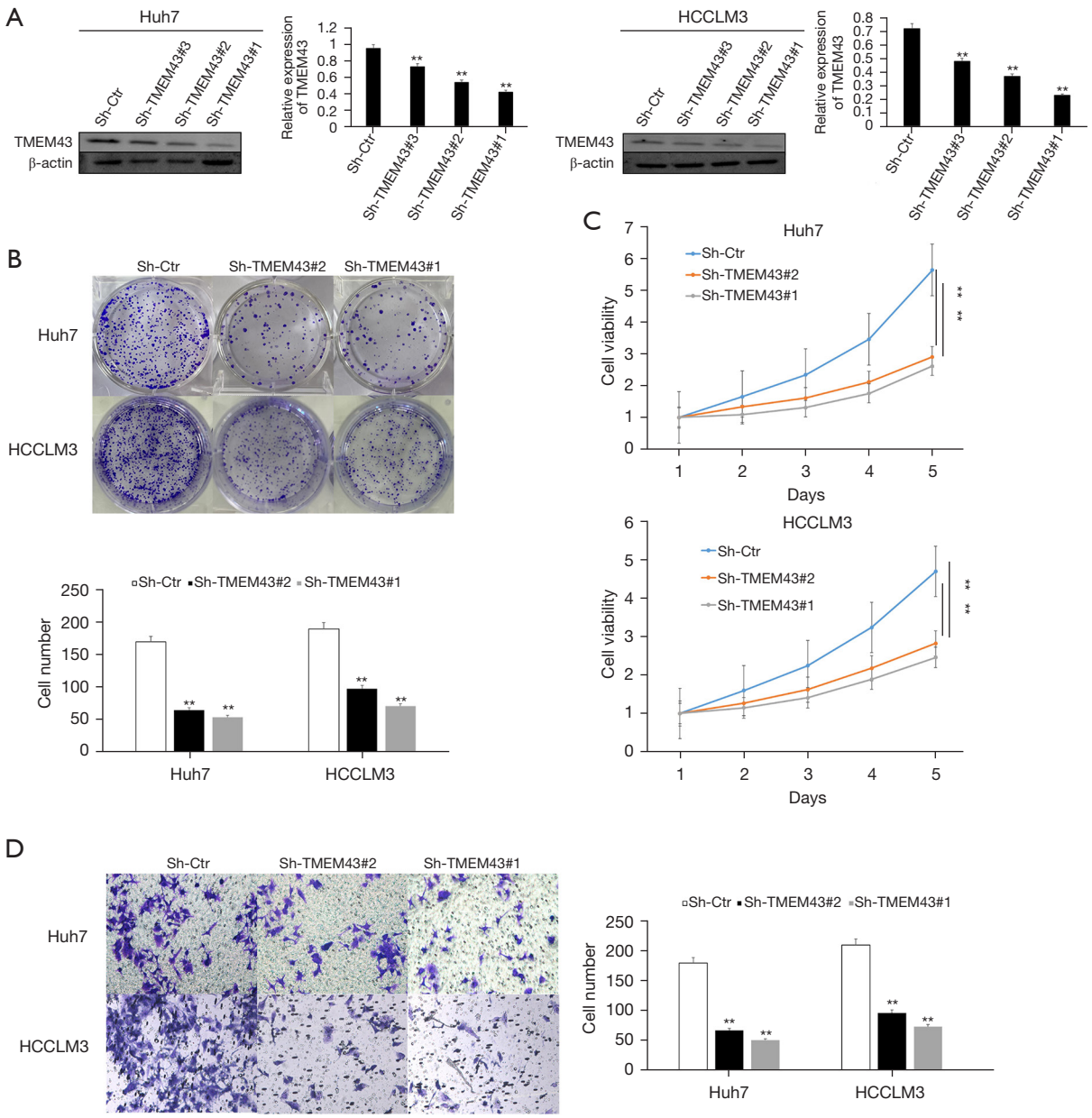

Figure 4 The effect of *TMEM43* on the growth and invasion of hepatocellular carcinoma cells. (A) After transfecting sh-*TMEM43*#1, sh-*TMEM43*#2, and sh-*TMEM43*#3 into Huh7 and HCCLM3 cells, the expression of TMEM43 in the cells was detected using western blotting. Clone formation experiment (B) (stained with crystal violet, taking photos with a camera) and CCK-8 assay (C) were used to detect the effect of sh-*TMEM43*#1 and sh-*TMEM43*#2 on growth, respectively. (D) The effect of *TMEM43* on the migration of hepatocellular carcinoma cell invasion was detected by Transwell (stained with crystal violet; original magnification, ×200). **, P<0.01. *TMEM43*, transmembrane protein 43; Ctr, control; CCK-8, Cell Counting Kit-8.

The effect of TMEM43 gene on the invasion of HCC cells

We used Transwell experiments to detect the effect of *TMEM43* on the invasion ability of HCC cells. The Transwell experiment results showed that knocking down *TMEM43* significantly inhibited the invasion of Huh7 and HCCLM3 cells (*Figure 4D*).

The effect of TMEM43 gene on cell cycle and apoptosis of HCC cells

Flow cytometry was used to detect the effects of *TMEM43* on cell cycle and apoptosis, respectively. Flow cytometry experiments showed that the apoptosis rate of Huh7 and HCCLM3 cells was significantly higher after knocking down *TMEM43* compared to the control group (*Figure 5A*). Flow cytometry experiments showed that compared with the control group, the G1 phase cells of Huh7 and HCCLM3 cells were significantly increased after knocking down *TMEM43*, whereas the S phase cells were significantly reduced (*Figure 5B*). Through cell cycle and apoptosis experiments, it was found that knocking down *TMEM43* inhibits the growth of HCC cells and promotes cell apoptosis.

The protein interaction relationship between TMEM43 gene and VDAC1 gene

In Huh7 and HCCLM3 cells, knockdown of *TMEM43* resulted in a decrease in the protein level of *VDAC1*, whereas overexpression of *VDAC1* partially restored the knockdown effect of sh-*TMEM43* (*Figure 6A*). Meanwhile, cellular immunofluorescence revealed that *TMEM43* and *VDAC1* were co localized in the cytoplasm (*Figure 6B*). The results of co-IP indicated the interaction between *TMEM43* and *VDAC1* at the protein level (*Figure 6C*). These results indicate that *TMEM43* affects the progression of HCC through *VDAC1*.

TMEM43 gene affects the proliferation and invasion of HCC cells through VDAC1 gene

To determine whether *TMEM43* promotes the proliferation and invasion of Huh7 and HCCLM3 cells through *VDAC1*, we restored the expression of *VDAC1* inhibited by sh-*TMEM43* through a recovery experiment. Through cloning (*Figure 7A*), CCK-8 (*Figure 7B*), and Transwell experiments (*Figure 7C*), it was found that overexpression of *VDAC1* partially restored the inhibitory effect of sh-*TMEM43* on cell proliferation and invasion. The above results indicate that *TMEM43* promotes the proliferation and invasion of Huh7 and HCCLM3 cells by targeting *VDAC1*.

To further investigate the mechanism of *TMEM43* inhibiting the proliferation and invasion of HCC cells through *VDAC1*, we investigated the effect of *TMEM43* on the PI3K signaling pathway through WB. We found that sh-*TMEM43* inhibited the PI3K signaling pathway in HCC cells, whereas *VDAC1* partially restored the inhibitory effect of sh-*TMEM43* on HCC cells (*Figure 7D*). The above results indicate that *TMEM43* promotes the proliferation and invasion of Huh7 and HCCLM3 cells by targeting *VDAC1*.

USP7 gene promotes the progression of HCC by regulating the level of TMEM43 gene through deubiquitination

In Huh7 and HCCLM3 cells, *USP7* knockdown resulted in a decrease in the protein level of *TMEM43* (*Figure 8A*). The interaction between *TMEM43* and *USP7* at the protein level was found through immunoprecipitation experiments (*Figure 8B*). Further, immunoprecipitation revealed that after *USP7* knockdown, the level of *TMEM43* increased (*Figure 8C*). The above results indicate that *USP7* affects the progression of HCC by regulating the ubiquitination level of *TMEM43* through deubiquitination (*Figure 8D)*.

Discussion

In recent years, molecular targeted therapy for HCC has remained a research hotspot (16,17). Therefore, finding effective key therapeutic targets is of great significance for the treatment of HCC. In the occurrence and progression of HCC, multiple oncogenes and tumor suppressor genes all play a role (18,19). Compared with normal control samples and low-grade glioma samples, the expression level of *TMEM43* is upregulated in high-grade glioma malignant samples. The higher the expression of *TMEM43*, the lower the survival rate. *In vitro* and *in vivo* experiments showed that knockdown of *TMEM43* could inhibit the proliferation and metastasis of glioma (20). However, the role of *TMEM43* has not been reported in HCC. In this study, we discovered through the TCGA database that TMEM43 is highly expressed in HCC tissues. TMEM43 is closely related to the prognosis of HCC, and high expression of TMEM43 can lead to poor prognosis. We found that the expression level of *TMEM43* in HCC tissue was higher than that in adjacent tissues by WB. Through experiments such as CCK-8, clone formation assay, flow cytometry, and Transwell, we found that overexpression of *TMEM43* was associated with HCC cell viability, proliferation ability, migration, and increased apoptosis ability of HCC cells. Therefore, *TMEM43* can affect the growth, proliferation, and invasion of HCC cells, thereby affecting the occurrence and development of HCC, which undoubtedly provides

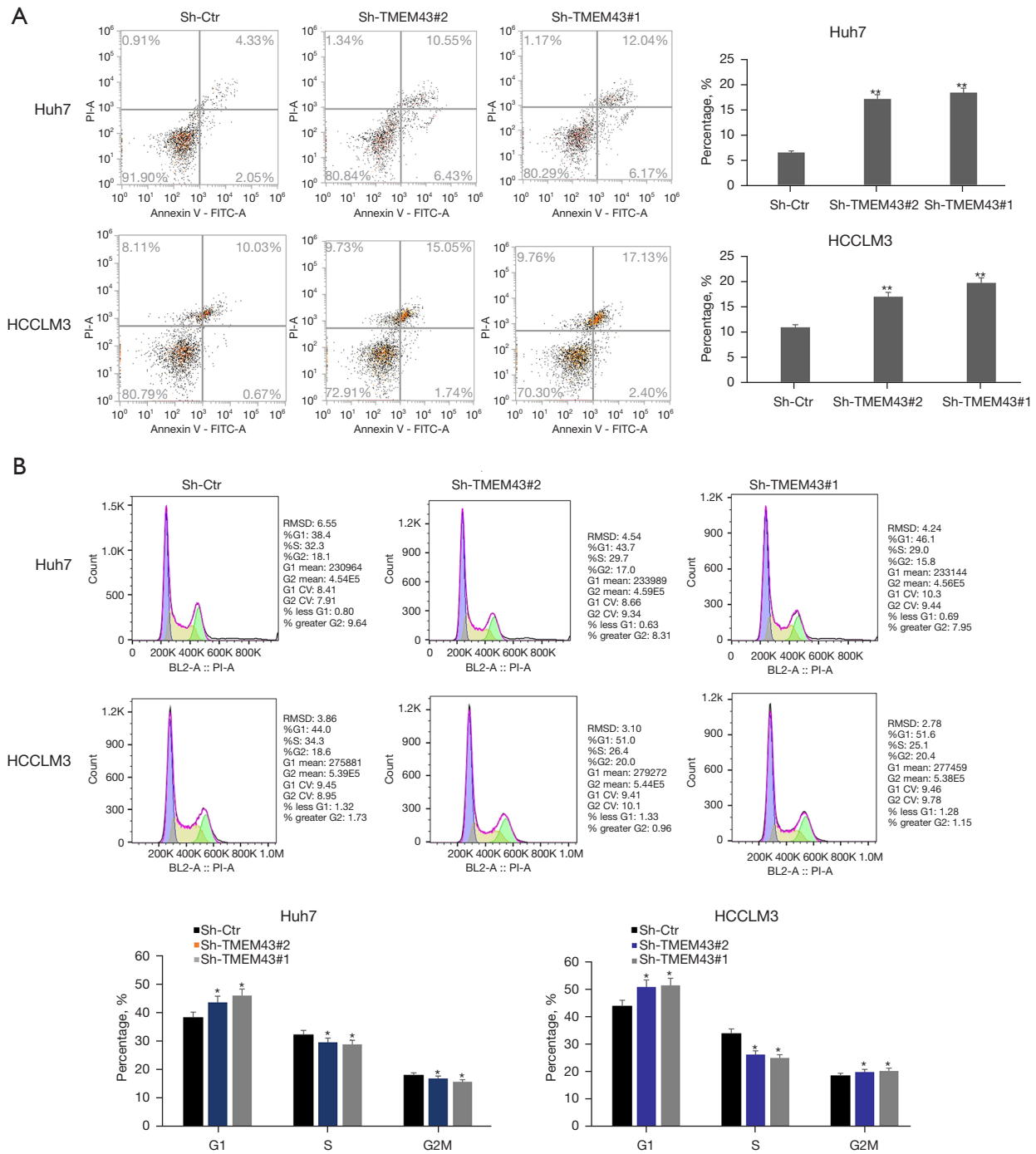

Figure 5 The effect of *TMEM43* on cell cycle and apoptosis of hepatocellular carcinoma cells. (A) Analyzing the effect of *TMEM43* gene cycle distribution on apoptosis in Huh 7 and HCCLM3 cells through flow cytometry analysis. (B) Analyzing the distribution of *TMEM43* gene cycle in Huh 7 and HCCLM3 cells through flow cytometry analysis. *, P<0.05; **, P<0.01. TMEM43, transmembrane protein 43; Ctr, control; FITC, fluorescein isothiocyanate; PI, propidium iodide; RMSD, root mean square deviation: CV, coefficient of variance.

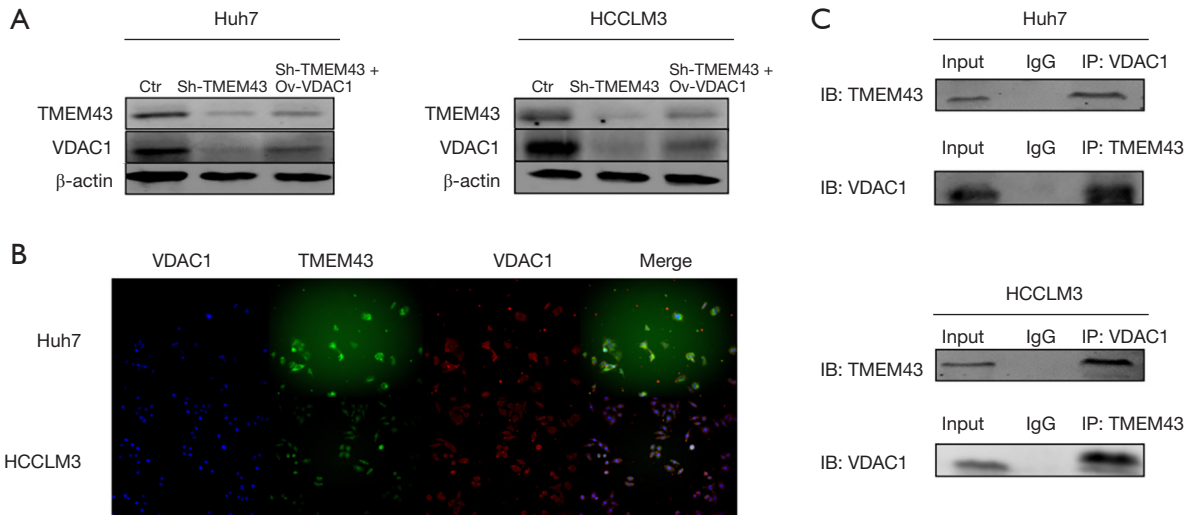

Figure 6 The protein interaction relationship between *TMEM43* and *VDAC1*. (A) After transfection of Ctr, sh-*TMEM43*#1, and Ov-VDAC1 in Huh7 and HCCLM3 cells, the protein expression effects of *TMEM43* and *VDAC1* were detected by western blotting. (B) Co-localization of *TMEM43* and *VDAC1* in hepatocellular carcinoma cells (immunofluorescence assay; original magnification, ×100). (C) Detection of the interaction between *TMEM43* and *VDAC1* at the protein level through coimmunoprecipitation. TMEM43, transmembrane protein 43; VDAC1, voltage-dependent anion channel 1; Ctr, control; sh, short hairpin; Ov, overexpression; DAPI, 4',6-diamidino-2-phenylindole; IB, immunoblot; IP, immunoprecipitation; IgG, immunoglobulin G.

new therapeutic targets for HCC treatment.

However, the mechanism of *TMEM43* in HCC cells is still unclear. In this study, we found that *TMEM43* regulates the proliferation, apoptosis, and invasion of HCC through *VDAC1*. VDAC protein is the most abundant porogen located in the outer mitochondrial membrane (OMM) of eukaryotic cells. As the gatekeeper of mitochondria, it regulates the entry and exit of metabolites, Ca^{2+}, fatty acid ions, and reactive oxygen species in OMM (21,22). VDAC protein has been identified as three isoforms encoded by three homeotic genes: *VDAC1*, *VDAC2*, and *VDAC3*. *VDAC1* has the most abundant expression level among them (23). A study has found that *VDAC1* is overexpressed in HCC and can serve as a new diagnostic biomarker. *VDAC1* is an independent factor in predicting poor prognosis (24). According to reports, overexpression of *VDAC1* is closely related to different types of cancer (25-28). *VDAC1* may serve as a new pharmacological target for anticancer therapy (29). This study found that overexpression of *VDAC1* can partially counteract the proliferation and invasion of sh-*TMEM43* on HCC cells. In addition, we found that overexpression of *VDAC1* can partially counteract the inhibitory effect of sh-*TMEM43* on the PI3K signaling pathway in HCC cells. These results further confirm the inseparable relationship between *VDAC1* and *TMEM43* in the occurrence and development of HCC.

Through the action of deubiquitinating enzymes (DUBs), deubiquitination is responsible for removing ubiquitin/polyubiquitin chains from protein substrates and reversing the function of ubiquitination (30,31). The post-translational modification of proteins known as ubiquitination is a key process that has been observed to be dysregulated in several types of cancer, including HCC (32). DUBs play an important role in tumor development by removing ubiquitin from substrate proteins through the deubiquitination process (33). Several families of DUBs exist in the human proteome, including ubiquitin C-terminal hydrolase (UCH), ubiquitin-specific proteases (USPs), ovarian tumour proteases (OTUs) and motif interacting with ubiquitin-containing novel DUB family proteases (MINDYs) (34). The diversity of these DUB families highlights the complexity of deubiquitination and its potential involvement in cancer progression. Not unlike most post-translational modification, ubiquitination is also associated with many cell functions, including cell cycle progression, apoptosis, gene transcription, DNA repair, and signal transduction, which regulate cell development and differentiation and tumorigenesis (35,36). Therefore, components in the ubiquitin pathway have been proposed as potential targets for treatment strategies for diseases and cancer (37,38). *USP7* is one of the most abundant

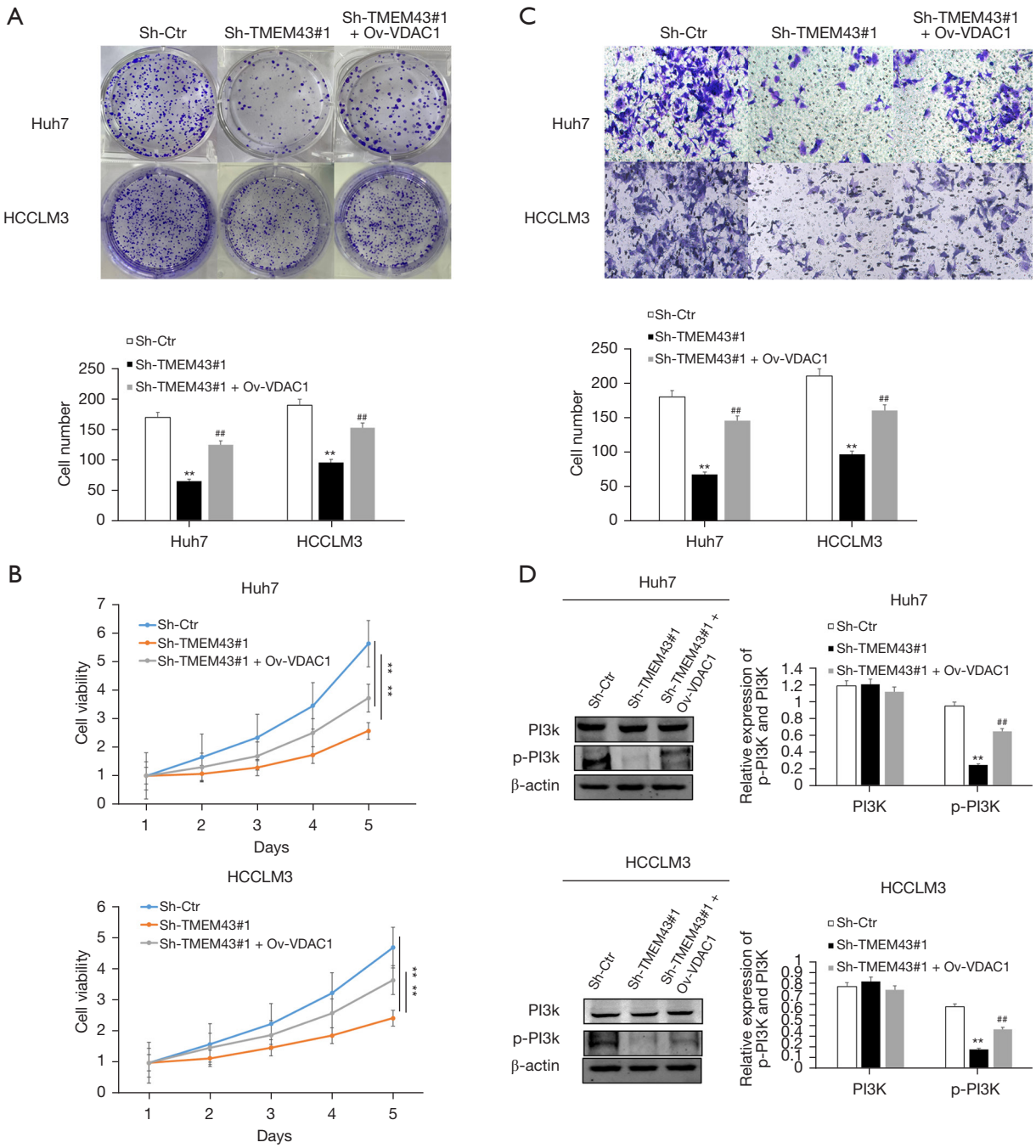

Figure 7 Effects of *TMEM43* on proliferation and invasion of hepatocellular carcinoma cell migration and PI3K signaling pathways. (A) The overexpression of *VDAC1* partially eliminated the inhibitory effects of sh-*TMEM43* on cell proliferation through clone formation experiment (stained with crystal violet, taking photos with a camera). (B) The overexpression of *VDAC1* partially eliminated the inhibitory effects of sh-*TMEM43* on cell proliferation through CCK-8. **, P<0.01. (C) The overexpression of *VDAC1* partially eliminated the inhibitory effects of sh-*TMEM43* on cell invasion through Transwell experiments (stained with crystal violet; original magnification, ×200). (D) Overexpression of *VDAC1* can partially restore the inhibitory effect of *TMEM43* on p-PI3K, as shown by WB. **, compared with sh-Ctr group, P<0.01; ##, compared with sh-*TMEM43*#1 group, P<0.01. sh, short hairpin; Ctr, control; TMEM43, transmembrane protein 43; VDAC1, voltage-dependent anion channel 1; Ov, overexpression; WB, western blot; CCK-8, Cell Counting Kit-8.

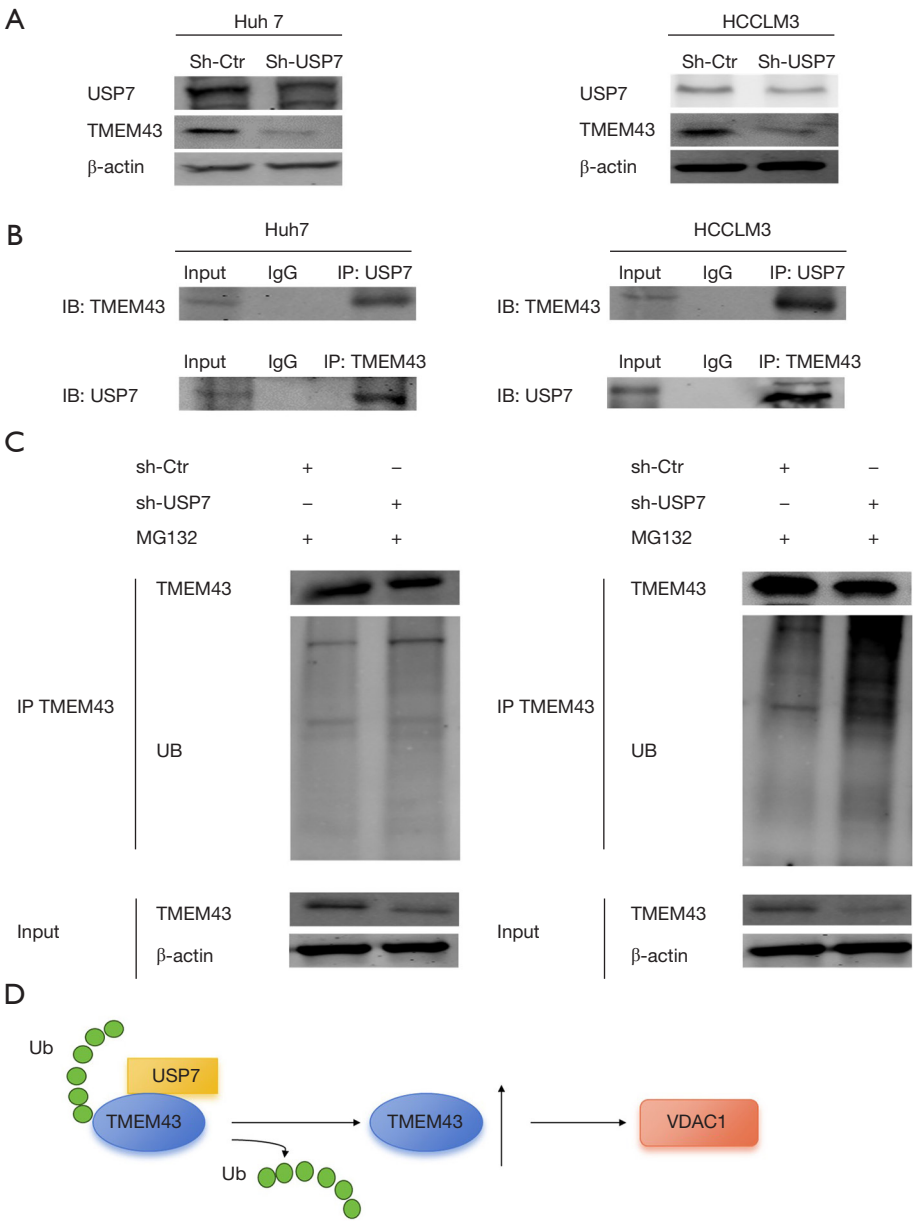

Figure 8 *USP7* promotes the progression of hepatocellular carcinoma by regulating the ubiquitination level of *TMEM43* through ubiquitin. (A) Detection of the effect of *USP7* knockdown on the protein expression of *TMEM43* in Huh7 and HCCLM3 cells by western blotting. (B) Detection of the interaction between *TMEM43* and *USP7* at the protein level through coimmunoprecipitation. (C) After knocking down *USP7*, the ubiquitination level of *TMEM43* was detected by immunoprecipitation. (D) The schematic diagram shows that *USP7* promotes tumor progression through VDAC1 by de ubiquitination of *TMEM43* in hepatocellular carcinoma. sh, short hairpin; Ctr, control; USP7, ubiquitin-specific protease 7; TMEM43, transmembrane protein 43; IgG, immunoglobulin G; IB, immunoblot; IP, immunoprecipitation; Ub, ubiquitin.

USPs and plays a multifaceted role in many cellular events, including carcinogenic pathways (39-42). A study has found that *USP7* is a promising target for cancer treatment by regulating the MDM2/MDX-p53 pathway (43). We found that *USP7* can affect the expression of *TMEM43* in HCC by deubiquitination of *TMEM43*. This study

provides experimental and theoretical basis for studying the development and mechanism of *TMEM43* in cancer, as well as for subsequent treatment of cancer.

Conclusions

TMEM43 plays a crucial role in HCC. Further analysis indicates that *TMEM43* affects the proliferation, invasion, and apoptosis of HCC cells through *VDAC1*. To our knowledge, this study is the first to explore the role of *TMEM43* in HCC. *TMEM43* can serve as a promising biomarker for HCC, laying the foundation for further understanding the mechanism of its occurrence and development. These findings provide a new understanding of the pathology and treatment of HCC, and demonstrate the potential of USP7/TMEM43/VDAC1 axis as a new biomarker for monitoring and treating the disease, laying a solid foundation for the development of targeted therapies for invasive cancer.

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Footnote

Reporting Checklist: The authors have completed the MDAR reporting checklist. Available at https://tgh.amegroups.com/article/view/10.21037/tgh-23-108/rc

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Conflicts of Interest: All authors have completed the ICMJE uniform disclosure form (available at https://tgh.amegroups.com/article/view/10.21037/tgh-23-108/coif). The authors have no conflicts of interest to declare.

Ethical Statement: The authors are accountable for all aspects of the work in ensuring that questions related to the accuracy or integrity of any part of the work are appropriately investigated and resolved. The study conformed to the provisions of the Declaration of Helsinki (as revised in 2013) and was approved by the Institutional Ethics Committee of the Affiliated Hospital of Nantong University (ID: 2022-L062). Informed consent was provided by all participants.

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