Inhibition of Dishevelled-2 suppresses the biological behavior of pancreatic cancer by downregulating Wnt/β-catenin signaling

WEI HU[1,2*], MINGXU LI[2*], JUNYI WU[3*], HONG CHEN[1,2], TING ZHAO[1,2], CHUNJIE ZHANG[4] and ZHONG WANG[1,2]

[1]Department of Hepatobiliary Surgery, The First Affiliated Hospital of Kangda College of Nanjing Medical University; [2]Department of Hepatobiliary Surgery, Affiliated Lianyungang Hospital of Xuzhou Medical University, Lianyungang, Jiangsu 222001; [3]Department of Hepatobiliary and Pancreatic Surgery, Fujian Provincial Hospital, Fuzhou, Fujian 350001; [4]Department of Pathology, The Second People's Hospital of Lianyungang, Lianyungang, Jiangsu 222001, P.R. China

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Abstract. Dishevelled-2 (DVL2) has been proven to be involved in the tumorigenesis of several human cancers, such as colorectal cancer, lung cancer, prostate cancer, etc. However, its role in pancreatic ductal adenocarcinoma (PDAC) remains unclear. The present study investigated the effects of aberrantly expressed DVL2 on PDAC. A total of 97 pancreatic cancer (PC) samples and 85 adjacent normal samples were obtained from patients who were histopathologically diagnosed with primary PDAC. The present study demonstrated that DVL2 expression was upregulated in PDAC tissues and was positively associated with advanced clinical stage and lymph node metastasis in patients with PDAC. In addition, patients with high expression of DVL2 had a shorter overall survival rate compared with those with low expression. To elucidate the role of DVL2 in PDAC, lentivirus-mediated short hairpin RNA was used to silence DVL2 and its physiological function was analyzed in CFPAC-1 and PANC-1 cells. The results indicated that DVL2 downregulation significantly impaired its oncogenic functions including cell proliferation, migration, invasion and epithelial-mesenchymal transition. Furthermore, DVL2 knockdown inhibits the proliferation and invasion of PC cells *in vivo*. In addition, co-immunoprecipitation assays revealed that DVL2 interacted with β-catenin; knockdown of DVL2 reduced the expression level of β-catenin and inhibited β-catenin translocation into the nucleus. In conclusion the findings of the present study suggested that DVL2 may be a potential therapeutic target in the treatment of PDAC.

Correspondence to: Dr Chunjie Zhang, Department of Pathology, The Second People's Hospital of Lianyungang, 41 Hailian Road, Lianyungang, Jiangsu 222001, P.R. China
E-mail: 329357693@qq.com

Dr Zhong Wang, Department of Hepatobiliary Surgery, The First Affiliated Hospital of Kangda College of Nanjing Medical University, 6 Zhenhua Road, Lianyungang, Jiangsu 222001, P.R. China
E-mail: 18961326366@189.cn

*Contributed equally

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Introduction

PDAC remains one of the most invasive malignancies worldwide, and had the highest cancer-associated mortality rate of 12.5% between 2009 and 2017 (1,2). Although great improvements have been made in preventing and treating PDAC, including neoadjuvant chemotherapy, 2D-conformal radiotherapy etc. (3,4), the increase in survival rate is still slow with a 5-year survival rate of just 4-6% (5). In addition, the molecular mechanisms underlying the metastatic process of PDAC are complex and remain undetermined (6). Hence, there is an urgent need to explore the underlying mechanisms to identify novel potential therapeutic targets for the development of new therapeutic strategies and improve prognosis for patients with PDAC.

Wnt signaling includes 3 distinct pathways: the canonical Wnt/β-catenin transcription pathway, the planar cell polarity pathway and the Wnt/Ca^{2+} pathway (7). Wnt signaling regulation of gene transcription needs dynamic multiprotein complexes that contain axin, adenomatous polyposis coli (APC), Dishevelled (DVL) and other proteins, such as casein kinase1α (CK1α), glycogen synthase kinase 3 (GSK-3) (7,8). As a cytoplasm-nucleus shuttling protein, DVL2 has been reported as a hub of Wnt signaling (9). DVL2 protein upregulation has been found in numerous tumors including prostate cancer (10), hepatocellular carcinoma (11), ovarian cancer (12) and esophageal squamous cell carcinoma (13). Our previous study demonstrated that knockdown of DVL2 in PDAC cells CFPAC-1 and SW1990 inhibited epithelial-mesenchymal transition (EMT) induced by IQ motif-containing GTPase-activating protein 1 (IQGAP1) overexpression (14). However, the involvement of DVL2 in the progression and EMT of PDAC including

the relationship between DVL2 expression and the clinicopathologic features of PDAC remain unclear.

The present study investigated the biological function and mechanism of DVL2 in PDAC. The expression level of DVL2 in PDAC tissues and in pancreatic cancer cell lines were detected and the association between expression level and PDAC clinicopathological features were analyzed. In addition, the roles and mechanisms of DVL2 in PDAC were explored. DVL2 may have the potential to be a predictive biomarker for patients with PDAC and a therapeutic target in the treatment of PDAC.

Materials and methods

Gene datasets. RNA array datasets [GSE15471 (15) and GSE16515 (16)], which consisted of 36 pairs of tumor and normal tissue samples and 36 tumor samples and 16 normal samples, were downloaded from the Genome Expression Omnibus database (www.ncbi.nlm.nih.gov/gds). The data were used to investigate the expression level of DVL2 in PDAC tissues and normal tissue samples.

Patients. A total of 97 pancreatic cancer samples and 85 adjacent normal samples (≥1 cm distance from the tumor edge) were obtained from patients who were histopathologically diagnosed with primary PDAC at The First Affiliated Hospital of Kangda College of Nanjing Medical University (Lianyungang, China) between January 2010 and January 2019. These patients were not treated with anticancer therapy, including chemotherapy and radiotherapy prior to surgery. The tissues were surgically resected. A few adjacent normal samples were difficult to obtain as they were too close to tumor tissue. The tissues were routinely fixed at room temperature overnight in 10% neutral formalin, embedded in paraffin and stored at room temperature until use. Clinicopathological information included age, sex, pathological grade, tumor location and size, perineural invasion, lymph node metastasis, tumor stage and the follow-up data (17). The classification systems for stage and grade used in the present study were from the AJCC Cancer Staging Manual 8th edition (18). Among the 97 patients, there were 59 males and 38 females with an age range of 37-92 years and a median age of 63 years. Patients were followed-up via the telephone every 3 months in the first year and every 6 months in the second and third year until patient death. The last date of follow-up was December 1, 2019. The overall survival (OS) time was defined as the interval between surgery and death or the last follow-up. Approval from ethics committees of The First Affiliated Hospital of Kangda College of Nanjing Medical University (Lianyungang, China) (approval no. KY20190924002) were obtained before enrollment into the study. Written informed consent was also obtained from the participants prior to enrollment in the study. The study was performed in accordance with government policies and the Declaration of Helsinki.

Reagents and antibodies. Primary antibodies for human DVL2 (1:1,000; cat. no. 3224), β-catenin (1:1,000; cat. no. 8480), cyclin D1 (1:1,000; cat. no. 2978), vimentin (1:1,000; cat. no. 5741), E-cadherin (1:1,000; cat. no. 3195), N-cadherin (1:1,000; cat. no. 13116), Snail (1:1,000; cat. no. 3879), and matrix metalloproteinase (MMP-9) (1:1,000; cat. no. 13667) were purchased from Cell Signaling Technology, Inc. Horseradish peroxidase (HRP)-conjugated anti-mouse IgG (1:1,000; cat. no. 7076) and anti-rabbit IgG (1:1,000; cat. no. 7074) secondary antibodies were purchased from Cell Signaling Technology, Inc. The control antibody (anti-GAPDH and anti-Histone H3) was acquired from Bioworld Technology, Inc. and Proteintech Group, Inc., respectively. Anti-human DVL2 (1:100; cat. no. sc-390303) was acquired for immunohistochemistry from Santa Cruz Biotechnology, Inc. Cell counting kit-8 (CCK-8) was purchased from Dojindo Molecular Technologies, Inc. TRIzol® reagent and PrimeScript RT Master Mix (Perfect Real Time) were obtained from Takara Bio Inc.

Haemotoxylin-eosin (H&E) staining, immunohistochemistry (IHC) and scoring. Tissue samples were fixed in 10% buffered formalin for 24 h at room temperature, then dehydrated and paraffin-embedded. Paraffin sections (4-μm-thick) were dewaxed in xylene at room temperature and rehydrated using a graded ethanol series (incubated 50% ethanol for 10 min, 70% ethanol for 10 min, 80% ethanol for 10 min, 95% ethanol for 10 min, 100% ethanol for 10 min thrice). In order to ensure that cancer tissue was collected, all slides were reviewed using H&E staining. Hematoxylin staining was performed for 4 min and eosin staining for 1 min at room temperature. DVL2 protein levels in PDAC tissues were detected by immunohistochemistry using a standard immunoperoxidase staining procedure. The slides were blocked in BSA blocking buffer (cat. no. 37520; Thermo Fisher Scientific Inc.) at room temperature for 1 h. The slides were then incubated at 4˚C overnight with anti-DVL2 (1:100; cat. no. sc-390303; Santa Cruz Biotechnology, Inc.). The slides were then incubated with horse radish peroxidase-conjugated secondary antibodies (1:1,000; cat. no. 7076; Cell Signaling Technology, Inc.) at room temperature for 2 h. The slides were then developed using diaminobenzidine (DAB) staining. These slides were observed by an inverted light microscope (magnification, x200; Olympus Corporation). All stained sections were independently viewed by 2 pathologists (Department of Human Pathology, The First Affiliated Hospital of Kangda College of Nanjing Medical University, Lianyungang, China), who were blinded to patient's clinical data. The ratio of positive tumor cells was quantified, and tumor cell proportion was evaluated as previously described (13): 0, no staining; 1+, staining <10%; 2+, staining of 10-35%; 3+, staining of 35-70%; and 4+, staining of >70%. The staining intensity was graded from 0 to 3, (0, none; 1, weak; 2, moderate; and 3, strong staining) The IHC score for each case was calculated as the ratio of positive tumor cells x staining intensity score. An optimal cutoff value was identified as follows: cases with IHC score >4 were regarded as high expression of DVL2, whereas cases with IHC score of ≤4 as low expression.

Cell culture and stable transfection. Normal human pancreatic ductal cell line hTERT-HPNE, pancreatic adenocarcinoma cell line SW1990, PDAC cell lines CFPAC-1, PANC-1 and BxPc-3 were purchased from the American Type Culture Collection. Cell culture was performed as previously

described (14). SW1990, CFPAC-1, PANC-1 and BxPc-3 were maintained in Dulbecco's Modified Eagle's Medium (DMEM; Gibco; Thermo Fisher Scientific Inc.) supplemented with penicillin (100 U/ml), 10% fetal bovine serum (FBS, Gibco; Thermo Fisher Scientific Inc.) and streptomycin (100 μg/ml) at 37˚C under air with 5% CO_2. hTERT HPNE was cultured in medium containing 3 vol of glucose-free DMEM (cat. no. D-5030; Sigma; Merck KGaA with additional 1.5 g/l sodium bicarbonate and 2 mM L-glutamine), 5% FBS (Gibco; Thermo Fisher Scientific Inc.), 750 ng/ml puromycin, 1 vol of Medium M3 Base (cat. no. M300F-500; InCell Corporation LLC), 5.5 mM D-glucose and 10 ng/ml human recombinant epidermal growth factor (cat. no. CM1013; Guangzhou Cellcook Biotech, Co., Ltd.) at 37˚C in 5% CO_2. The cell line was thawed every 2 months.

Lentiviral constructs expressing short hairpin (sh) RNA-DVL2 and scrambled shRNA (shNC) were purchased from Shanghai GenePharma Co., Ltd. The sequences of the 2 target shRNAs were as follows: DVL2-shRNA1# 5'-AGT CAACCTGTCTCTCAAT-3'; DVL2-shRNA2# 5'-TCC ACAATGTCTCTCAATA-3'; and shNC, 5'-TTCTCCGAA CGTGTCACGT-3'. The 3rd lentivirus generation system, which consisted of the GenePharma Supersilencing Vector containing the shRNA sequence and lentiviral packaging plasmids (Helper vector-I, Helper vector-II, Helper vector-III; Shanghai GenePharma Co., Ltd.) were used to produce lentiviral particles for the transduction of shRNA-mediated knockdown system. 293T cells, supplied by Shanghai GenePharma Co., Ltd., were used to produce the lentivirus. A total of 20 μg plasmids were used for transfection, and the ratio of the lentiviral plasmid, packaging vector and envelope vector was 1:4:3. Cells were cultured in DMEM medium with lentiviruses at a multiplicity of infection of 30 for 48 h at room temperature. After 48 h of infection at room temperature, PANC-1 and CFPAC-1 cells were treated with puromycin (1 μg/ml) and cultured for ~2 weeks to obtain stably transfected cells.

RNA extraction and reverse transcription-quantitative (RT-q) PCR. Total RNA of different PDAC cells (CFPAC-1, PANC-1, BxPC-3 and SW1990) and HPNE cells were extracted using TRIzol® reagent and reverse-transcribed into cDNA using PrimeScript RT Master Mix according to the manufacturer's protocol. The RT-qPCR amplification was detected by SYBR Green PCR master mix (Takara Bio Inc.). The primer sequences used were as follows: DVL2 forward, 5'-CGTCACAGATTCCACAAT GTCT-3' and reverse, 5'-TCGTTGCTCATGTTCTCAAAG T-3' and GAPDH forward, 5'-CCATGTTCGTCATGGGTG TGAACCA-3'; and reverse, 5'-GCCAGTAGAGGCAGG GATGATGTTC-3'. The thermocycling conditions were as follows: initial denaturation at 95˚C for 5 min followed by 40 cycles of denaturation at 95˚C for 15 sec, annealing at 60˚C for 15 sec and extension at 72˚C for 30 sec. Each experiment was performed at least 3 times. GAPDH was used as the internal control. Relative results of mRNA expression were analyzed using the $2^{-\Delta\Delta Cq}$ method (19).

Western blotting. Total protein from PDAC cells (CFPAC-1, PANC-1, BxPC-3 and SW1990) and HPNE cells was collected and lysed on ice by using an NP40 lysis buffer containing protease and phosphatase inhibitors (Thermo Fisher Scientific Inc.). The concentrations were measured using a bicinchoninic acid (BCA) protein assay kit (Pierce; Thermo Fisher Scientific, Inc.). An equivalent amount of protein (0.6 mg/lane) from each protein lysates was separated with 10% SDS-PAGE electrophoresis and transferred to a PVDF membrane (MilliporeSigma). The membranes were incubated in 0.05% Tris-buffered saline with 5% skimmed milk for 1 h at room temperature and then incubated overnight with the appropriate primary antibodies at 4˚C, followed by HRP-conjugated secondary antibody at room temperature for 1 h. Signal detection was performed using an enhanced chemiluminescence kit (Beyotime Institute of Biotechnology) and western blotting detection system (Tanon Science & Technology Co., Ltd.). Western blotting analysis was used by standard methods.

CCK-8 assay and colony formation assay. CFPAC-1 and PANC-1 cells were plated at a density of $2.5x10^3$ cells/well in 96-well plates. The relative growth rate of transfected cells with small interfering (si)DVL2 and negative controls were measured by CCK-8 reagent (Dojindo Molecular Technologies, Inc.) at different time points (24, 48, 72, 96 and 120 h). The absorbance value was measured in a microplate reader at 450 nm. At least 3 wells were evaluated for each group.

For the colony formation assay, CFPAC-1 and PANC-1 cells were seeded at a density of 500 cells/well in 6-well plates and incubated for 10 days at room temperature. Cells were fixed with 96% ethanol for 10 min and subsequently stained with 1% crystal violet for 5 min at room temperature. The colonies were counted manually and imaged using an inverted light microscope (magnification, x40; Olympus Corporation) and a digital camera (Olympus Corporation).

Gap closure assay. CFPAC-1 and PANC-1 cells ($1x10^5$) were seeded in Culture Inserts (EMD Millipore) as previously reported (14). The culture inserts were removed when cells reached 95% confluence. Cells were incubated in serum-free DMEM and photographed at 0 and 24 h after scraping using an inverted light microscope (magnification, x200; Olympus Corporation).

Transwell migration and matrigel invasion assays. Transwell migration and invasion assays were performed using transwell chambers fixed with or without Matrigel at 37˚C for 24 h, respectively. CFPAC-1 and PANC-1 (~$2x10^4$ cells) were added into the upper 8-μm-pore chamber of transwell inserts (Merck KGaA) in a 200 μl serum-free medium. The lower chamber contained 600 μl of 10% FBS culture medium. After 48 h incubation at 37˚C, the migrated or invaded cells were fixed with paraformaldehyde at 37˚C for 20 min, stained with crystal violet at 37˚C for 20 min and then counted using an inverted light microscope (magnification, x200; Olympus Corporation).

Co-immunoprecipitation (co-IP) and immunofluorescence imaging. PANC-1 or CFPAC-1 cells were lysed in NP40 lysis buffer (Beyotime Institute of Biotechnology). Following centrifugation at 13,000 x g for 15 min at 4˚C, the 1/10 volume of supernatant was collected as input and the remaining

supernatant was incubated with 20 µl/ml protein A/G sepharose beads (Beyotime Institute of Biotechnology) at 4˚C for 1 h to remove non-specific hybrid proteins. Following centrifugation (12,000 x g at 4˚C for 5 min), half of the supernatant was incubated with 2 µg anti-DVL2 (1:100; cat. no. sc-390303, Santa Cruz Biotechnology, Inc.), half of the supernatant was incubated with 2 µg negative control rabbit IgG (cat. no. A7016 Beyotime Institute of Biotechnology), together with 20 µl protein A sepharose beads (Beyotime Institute of Biotechnology) at 4˚C overnight. The beads were washed 5 times with cell lysis buffer and collected by centrifugation for 5 min at 3,000 x g at 4˚C. The proteins were released by boiling the samples and were analyzed by western blotting as previously described.

For immunofluorescence, cells were plated on coverslips, washed 3 times with PBS, and were fixed by 4% paraformaldehyde for 15 min at room temperature, permeabilized in 0.2% Triton X-100 for 10 min, blocked in 5% bovine serum albumin for 40 min at room temperature and incubated with primary antibodies (1:100; cat. no. 8480, Cell Signaling Technology, Inc.) overnight at 4˚C. Subsequently, Alexa Fluor-conjugated secondary antibodies (1:1,000; cat. no. A31627; Thermo Fisher Scientific Inc.) were incubated for 2 h at room temperature followed by counterstaining with DAPI for 10 min at room temperature. Images were obtained by confocal laser microscopy (magnification, x20; FV3000; Olympus Corporation). The β-catenin expression levels were determined using Quantity One v.4.6.2 software (Bio-Rad Laboratories, Inc.).

Xenograft tumorigenicity assays. Male BALB/c nude mice, 4-6 weeks of age, weight 15-20 g were obtained from the Model Animal Research Center at Nanjing University (Nanjing, China). and were housed under specific pathogen-free conditions, with food and water provided *ad libitum*. The mice were randomly divided into 2 groups, 5 mice in each group. All animal studies (including the mice euthanasia procedure) were approved by the Animal Care Committee of The First Affiliated Hospital of Kangda College of Nanjing Medical University (Lianyungang, China) (approval no. KY20190924002) in compliance with the regulations and guidelines of the Institutional Animal Care.

Inhalation anesthesia was used with 1.5% isoflurane in this experiment. A total of $1x10^6$ PANC-1 cells with shDVL2 PANC-1 cells or non-target shRNA suspending in 50 µl phosphate-buffered saline were orthotopically injected (in the pancreatic tail) in the mice through a median lateral laparotomy (20,21). Three months after the laparotomy, the mice were euthanized by cervical dislocation and examined for tumor volume and suspected metastatic liver tissues.

Statistical analysis. All statistical results were calculated by SPSS 23 statistical software (IBM Corp.). Data are presented as the mean ± SD of 3 independent experiments. A Chi-square (χ^2) test and Fisher's exact test were performed to analyze the association between DVL2 expression and clinicopathological features. A Kaplan-Meier analysis and log-rank test were used to assess and compare the survival curves. Unpaired two-tailed Student's t-tests were used to assess the differences between 2 independent groups. ANOVA with the post hoc Tukey's test was performed to compare differences between multiple groups. $P<0.05$ was considered indicate a statistically significant difference.

Results

DVL2 is significantly upregulated in PDAC tissues and cells. mRNA expression and prognostic values of DVL2 in PDAC were analyzed by using the open Gene Expression Omnibus (GEO) database (https://www.ncbi.nlm.nih.gov/geo/). The GEO database was examined and the data from 2 mRNA expression profile datasets (GSE15471 and GSE16515), which consisted of 36 pairs of tumor and normal tissue samples, and 36 tumor samples and 16 normal samples, respectively were extracted. Compared with adjacent normal tissues, a significant upregulation of DVL2 in PC tissues was found after analyzing the data from the 2 datasets ($P<0.05$; Fig. 1A). In addition, 97 PDAC tissues and 85 adjacent normal samples from The First Affiliated Hospital of Kangda College of Nanjing Medical University (Lianyungang, China) were used for IHC staining to analyze the association of DVL2 with clinicopathological features. DVL2 was highly expressed in PDAC tissues (66.0%, 64/97) compared with adjacent normal tissues (20.0%, 17/85) ($P<0.001$; Fig. 1B and C).

Expression levels of DVL2 were also detected in 4 pancreatic cancer cell lines (BxPC-3, SW1990, CFPAC-1, PANC-1) and a normal cell line (hTERT-HPNE) by western blotting and RT-qPCR. The protein and mRNA expression of DVL2 was highly upregulated in pancreatic cancer cells, especially in PANC-1 and CFPAC-1 compared with hTERT-HPNE cells ($P<0.01$; Fig. 1D and E).

In order to ascertain the prognostic value of DVL2 in PDAC, we assessed the relationship between DVL2 gene expression and OS. The Kaplan-Meier analysis demonstrated that the patients with a high level of DVL2 expression had poorer OS rate compared with those with a low level of DVL2 expression ($P<0.05$; Fig. 1F). The aberrantly high expression of DVL2 in PDAC tissues and cells, and the Kaplan-Meier analysis results indicated that DVL2 might be a prognostic predictor in the OS of PDAC patients.

DVL2 protein expression is associated with PDAC clinicopathological features. According to immunohistochemical scores, the 97 PDAC samples were divided into high (n=64) and low (n=33) expression groups. The representative samples revealed that majority of the DVL2 expression was positively immunostained in the cytoplasm of PDAC tissues (images in the middle and the right 2 columns of Fig. 1C). χ^2 and Fisher's exact test was performed to assess the association between DVL2 expression and clinicopathologic variables in PDAC tissues. The results demonstrated that DVL2 expression levels were significantly associated with tumor node metastasis (TNM) stage ($P=0.015$) and pathological node (N) stage ($P=0.046$), but not with sex, age, histological differentiation, T classification, perineural invasion, blood vessel invasion, tumor size and location (Table I). These results suggest that DVL2 expression level is associated with metastatic ability of PDAC.

Knockdown of DVL2 inhibits cellular proliferation in CFPAC-1 and PANC-1 cells. To further assess the potential role of DVL2

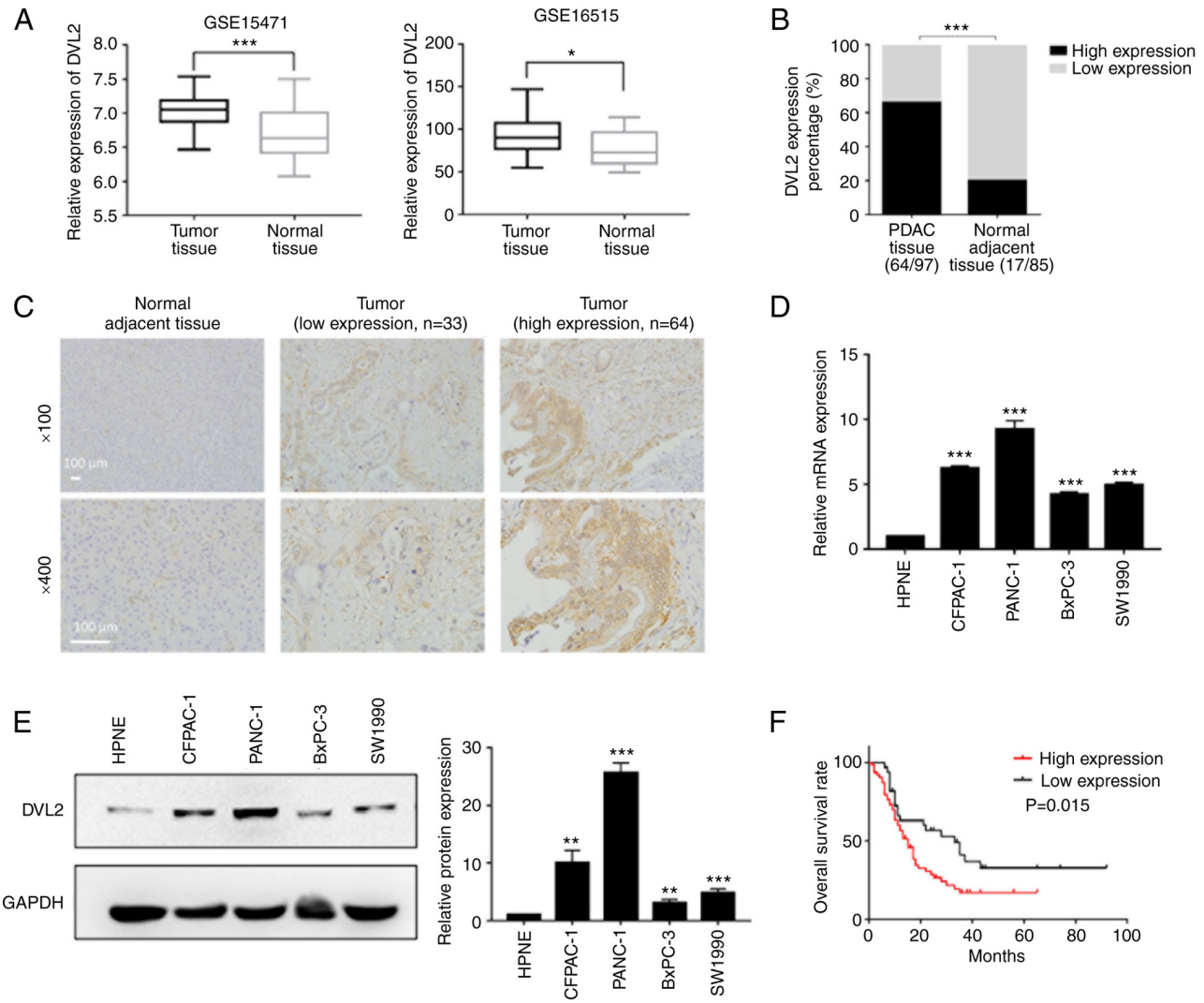

Figure 1. Expression of DVL2 in PDAC tissues and pancreatic cancer cell lines. (A) Bioinformatic analysis from the GSE15471 and GSE16515 datasets from the GEO database was used to detect the expression of DVL2 in tumor and normal tissues. (B) Expression levels of DVL2 were detected by IHC in PDAC tissues and matched adjacent normal tissues, respectively. (C) Representative images of DVL2 expression were showed by IHC. DVL2 was highly expressed in PDAC tissues (66.0%, 64/97) compared with adjacent normal tissues (20.0%, 17/ 85) (D and E) mRNA and protein expression of DVL2 were tested in different pancreatic cancer cell lines compared with normal human pancreatic ductal cell line hTERT-HPNE. (F) Kaplan-Meier analysis was used to estimate overall survival rate according to the DVL2 expression level. *P<0.05, **P<0.01 and ***P<0.001. IHC, immunohistochemistry; RT-q, reverse transcription-quantitative; DVL-2, Dishevelled-2; PDAC, pancreatic ductal adenocarcinoma; GEO, Gene Expression Omnibus.

in PDAC, 2 cell lines (PANC-1 and CFPAC-1) with relatively higher DVL2 expression levels were used for DVL2 knockdown using 2 shRNAs. The interference efficiency of the 2 shRNAs was confirmed by western blotting and RT-qPCR. Both protein and mRNA expression levels of DVL2 were decreased in shRNA#1- and shRNA#2-transfected cells compared with negative controls (P<0.001; Fig. 2A and B). ShRNA#2 (shDVL2) displayed the highest knockdown efficiency (P<0.001; Fig. 2A and B) and was selected for subsequent experimentation. In addition, the results from the CCK-8 assays demonstrated that DVL2 knockdown significantly suppressed the pancreatic cell proliferation rate compared with negative control cells (P<0.001; Fig. 2C and D). The same trend from the colony formation assays was observed in the pancreatic cell lines. DVL2 knockdown significantly suppressed the colony-forming capacity of pancreatic cell compared with control cells (P<0.01; Fig. 2E and F). Together, these results demonstrated that DVL2 knockdown inhibited the growth of PDAC cells *in vitro*.

DVL2 knockdown inhibits the migration and invasion of PC cells in vitro. Given that upregulation of DVL2 was positively associated with TNM stage and lymph node metastasis in PDAC tissues (Table I), we speculated that DVL2 may contribute to the migration and invasion of PC. Indeed, migratory and invasion abilities were markedly decreased in cells with DVL2 knockdown compared with control cells (Fig. 3). DVL2 knockdown cells exhibited significantly slower wound closure in wound healing assays compared with control cells (P<0.001; Fig. 3A and B). In addition, the results from transwell migration (without Matrigel) or invasion (with Matrigel) assays revealed that knockdown of DVL2 expression significantly reduced the migration and invasion abilities of both cell lines compared with control cells (P<0.01; Fig. 3C and D). Our previous study demonstrated that DVL2 was involved in EMT induced by IQGAP1 overexpression in PDAC (14). Hence, DVL2-mediated changes in EMT of pancreatic cells was examined. EMT markers, including E-cadherin, N-cadherin,

Table I. Association between DVL2 expression and clinicopathological variables in patients with PDAC (n=97).

Variables	Number	DVL2 expression		P-value
		Low	High	
Sex				0.363
Male	59	18	41	
Female	38	15	23	
Age[a], years				0.474
≤63	49	15	34	
>63	48	18	30	
Tumor location				0.342[b]
Pancreatic head	82	30	52	
Non-head	15	3	12	
Tumor size, cm				0.913
<4	61	21	40	
≥4	36	12	24	
Histological differentiation				0.259
I /I-II/II	66	20	46	
II-III/III	31	13	18	
TNM stage				0.015[c]
I-IIA	51	23	28	
IIB-IV	46	10	36	
T classification				0.940
T1 or T2	26	9	17	
T3 or T4	71	24	47	
N classification				0.046[c]
N0	54	23	31	
N1	43	10	33	
M classification				1.000[b]
M0	93	32	61	
M1	4	1	3	
Perineural invasion				0.451
Present	14	6	8	
Absent	83	27	56	
Blood vessel invasion				1.000[b]
Present	8	3	5	
Absent	89	30	59	

[a]Median age; [b]continuity correction; TNM, tumor node metastasis; N, node; M, metastasis; T, tumor; PDAC, pancreatic ductal adenocarcinoma; DVL2, dishevelled 2; [c]P<0.05.

vimentin and Snail (22,23), induced by DVL2 knockdown were analyzed in the PANC-1 and CFPAC-1 cell lines by western blotting. The epithelial marker E-cadherin (22,23) was significantly upregulated in DVL2 knockdown group compared with control group (Fig. 3E). By contrast, the mesenchymal markers N-cadherin and vimentin (11), as well as the EMT-activating transcription factor Snail, were significantly decreased on DVL2 knockdown in both PANC-1 and CFPAC-1 cell lines compared with control cells (P<0.05; Fig. 3E). These results demonstrated that DVL2 could promote migration and invasion of PDAC via induction of the EMT pathway.

Decreased DVL2 promotes Wnt/β-catenin signaling inactivation in PDAC. Several studies have reported that the Wnt/β-catenin signaling pathway is significantly involved in EMT, tumorigenesis and progression of PDAC (14,22,23). It has been reported that knockdown of DVL2 reduces the expression of β-catenin in esophageal cancer cells and lung cancer (13,24). In contrast, DVL2 knockdown does not affect the expression levels of β-catenin in hepatocellular carcinoma and human gliomas (11,25). Hence, the present study examined whether the expression of β-catenin could be regulated by changes of DVL2 in PDAC. The expression

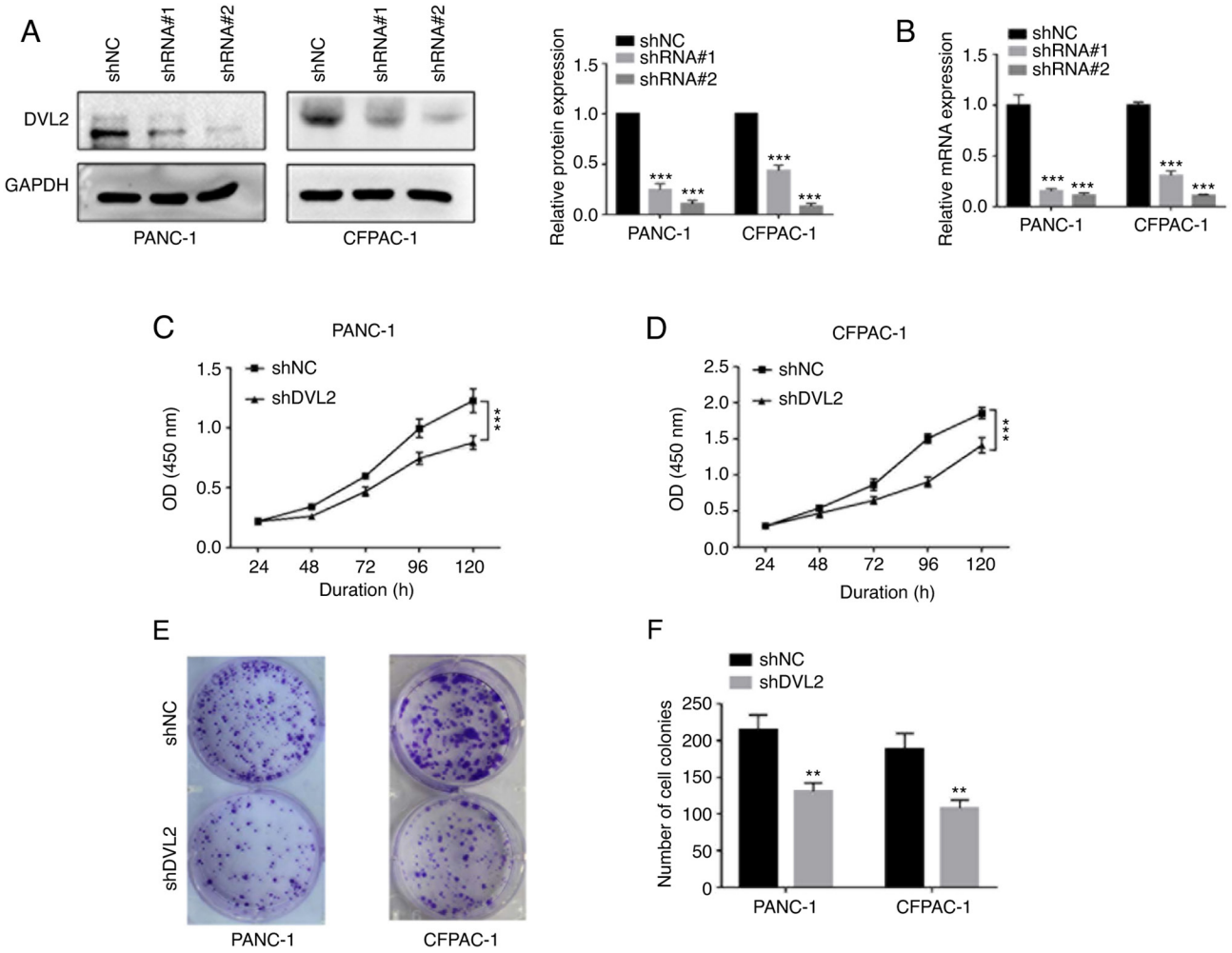

Figure 2. Interference efficiency of 2 shRNAs for DVL2 and cell proliferation assays. (A,B) Western blotting and RT-qPCR were used to examine the knockdown effects of 2 shRNAs for DVL2 in PANC-1 and CFPAC-1 cells compared with control cells. DVL2 knockdown inhibits PC cell growth, as examined by (C and D) CCK-8 and (E and F) colony formation assays. **P<0.01 and ***P<0.001. RT-q, reverse transcription-quantitative; DVL-2, Dishevelled-2; NC, negative control; sh, short hairpin; OD, optical density.

levels of β-catenin were tested by western blotting and immunofluorescence analysis. The results revealed that β-catenin expression as well as the specific Wnt target genes Cyclin D1 and matrix metalloproteinase 9 (MMP-9) (13) were downregulated in silenced DVL2 cells compared with control cells (P<0.01; Fig. 4A). In addition, it was demonstrated that DVL2 interacted with β-catenin in PDAC cells (Fig. 4B). Immunofluorescence analysis further revealed that the decreased expression levels of β-catenin and nuclear translocation of β-catenin were observed in shDVL2 cells compared with in shNC cells as the fluorescence in the nucleus decreased (P<0.001; Fig. 4C). These results suggested that DVL2 modulated both protein expression and nuclear translocation of β-catenin.

DVL2 knockdown inhibits the proliferation and invasion of PC cells in vivo. Finally, it was investigated whether DVL2 could participate in tumorigenesis of PC cells *in vivo*. Pancreatic tails of nude mice were orthotopically injected with PANC-1-shDVL2 and corresponding negative control cells. The mice were euthanized three months after the injection. Their metastatic liver nodules and orthotopic pancreatic tumors were counted. Mice with silenced DVL2 had a reduction in tumor weight compared with control mice (P<0.001; Fig. 5A). IHC staining demonstrated that tumors in the PANC-1-shDVL2 group had lower DVL2 expression compared with those in the control group (Fig. 5A). The DVL2 positive cells with brown stain almost disappeared in shDVL2 mice. The PANC-1-shDVL2 group also exhibited fewer visible liver metastatic nodules compared with the control group (P<0.001; Fig. 5B). Taking together, these *in vivo* results indicated that DVL2 silencing inhibited the proliferation and metastasis of pancreatic cells.

Discussion

The present study demonstrated that the DVL2 expression was significantly elevated in PDAC tissues compared with adjacent normal tissues. In addition, it also demonstrated that DVL2 expression was positively linked to the TNM stage and N classification in PDAC. Previous studies have reported that an elevated DVL2 expression level is significantly associated with poor prognosis in hepatocellular carcinoma and esophageal squamous cell cancer (11,13). To the best of our knowledge,

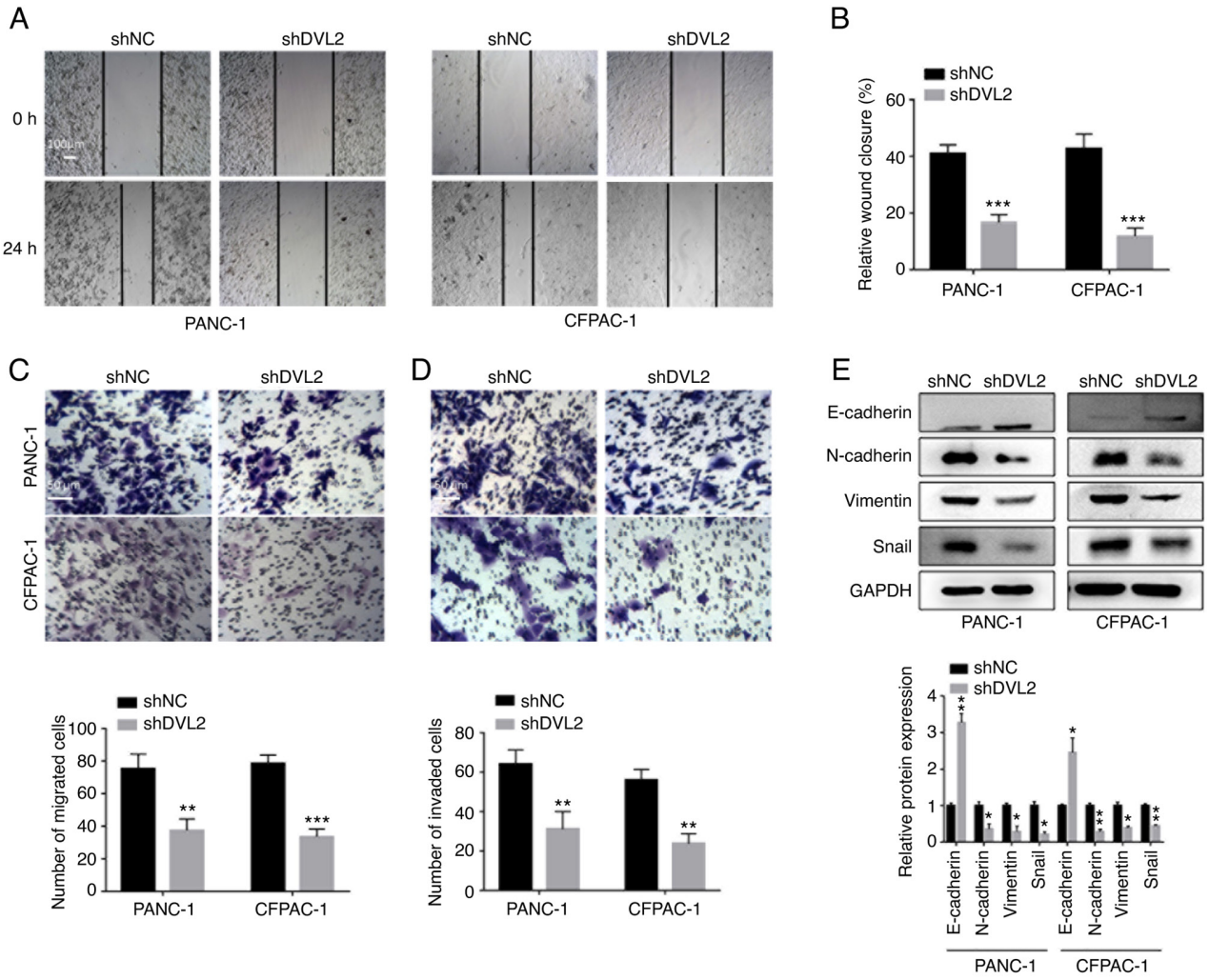

Figure 3. Effects of DVL2 knockdown on the migration and invasion of pancreatic cells in vitro. (A and B) Typical images of wound healing for the shNC and shDVL2 groups were captured at 0 and 24 h after scraping. (C and D) Cell migration and invasion assays were performed without (C) Matrigel or with (D) Matrigel. The number of migrated or invaded cells was presented as the mean ± SD from 3 independent experiments. (E) EMT marker proteins were detected in PANC-1 and CFPAC-1 cells with DVL2 knockdown by western blotting. *P<0.05, **P<0.01 and ***P<0.001. DVL-2, Dishevelled-2; NC, negative control; sh, short hairpin; EMT, epithelial mesenchymal transition.

the present study is the first study that reported that patients with PDAC with high DVL2 expression had shorter OS times compared with patients with low DVL2 expression, thus suggesting that DVL2 acts as a potential tumor promoter.

To elucidate the possible functional significance of DVL2 in pancreatic cancer, loss-of-function assays were performed *in vitro* using pancreatic cell lines. The present study, revealed that knockdown of DVL2 in PANC-1 and CFPAC-1 cells inhibited cell proliferation, migration, invasion and suppressed tumor growth and metastasis *in vivo*. Taken together, the results of the present study indicated that DVL2 was critical for the proliferation and invasion of PDAC. In addition, the present study also evaluated the effect of DVL2 knockdown on normal hTERT-HPNE cells to determine whether the effect is cancer-cell specific. However, hTERT-HPNE cells transfected with shDVL2 were prone to death (data not shown). DVL2 was reported to be a signal transducing protein that participates in canonical and noncanonical WNT signaling, which is involved in pancreas development, islet function, insulin production and secretion, and the attenuation of which perturbed pancreatic growth (26). The expression level of DVL2 needs to be maintained within a certain range. The aberrant high expression of DVL2 in pancreatic cells made it a potential therapeutic target, while the absence or knockdown of DVL2 in normal pancreatic cells was hazardous for cell survival (26).

There are 3 highly conserved domains in DVL (DVL1-3) protein sequences: DIX (Dis/Axinhomologous domain), DEP and PDZ (PSD-95 and ZO-1 domain) (27,28). The PDZ domain can bind DVL2 with Frizzled proteins (29). The DEP domain is required for DVL membrane recruitment to Frizzled proteins after Wnt3a treatment (30). The Wnt signalosome transduction is associated with DIX polymerization and DEP dimerization (31). Studies have suggested that the deletion of PDZ domain can reduce cytosolic β-catenin levels, decrease T cell factor-dependent transcriptional activity of β-catenin and suppress tumorigenesis of mesothelioma (32). In addition, DVL2 has been proven to enhance tumorigenesis by binding with the ataxia-telangiectasia group D complementing gene through stabilizing β-catenin (33). Downregulation of DVL reduces the binding of β-catenin to the c-myc promoter and the transcription of Wnt target genes, including c-myc, Axin2 and Fgf8 (34). The nuclear translocation of DVL is believed

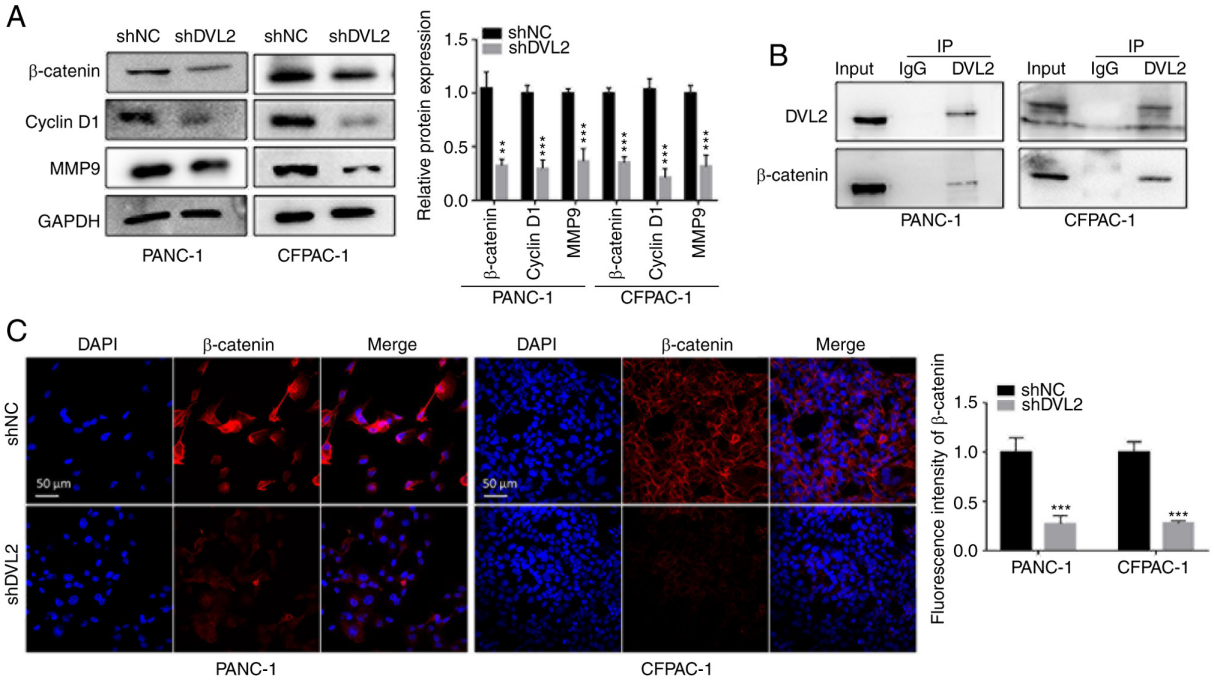

Figure 4. DVL2 functions in pancreatic cell progression though the Wnt/β-catenin signaling pathway. (A) Western blotting revealed that β-catenin as well as the specific Wnt target genes Cyclin D1 and MMP-9 expression was downregulated in silenced DVL2 cells compared with control cells. (B) Co-IP assays revealed that DVL2 interacts with β-catenin. (C) Immunofluorescence further showed that DVL2 knockdown inhibited β-catenin translocation into the nucleus. Nuclei were stained by DAPI. **P<0.01 and ***P<0.001. DVL-2, Dishevelled-2; NC, negative control; sh, short hairpin; MMP-9, matrix metalloproteinase 9; co-IP, co-immunoprecipitation.

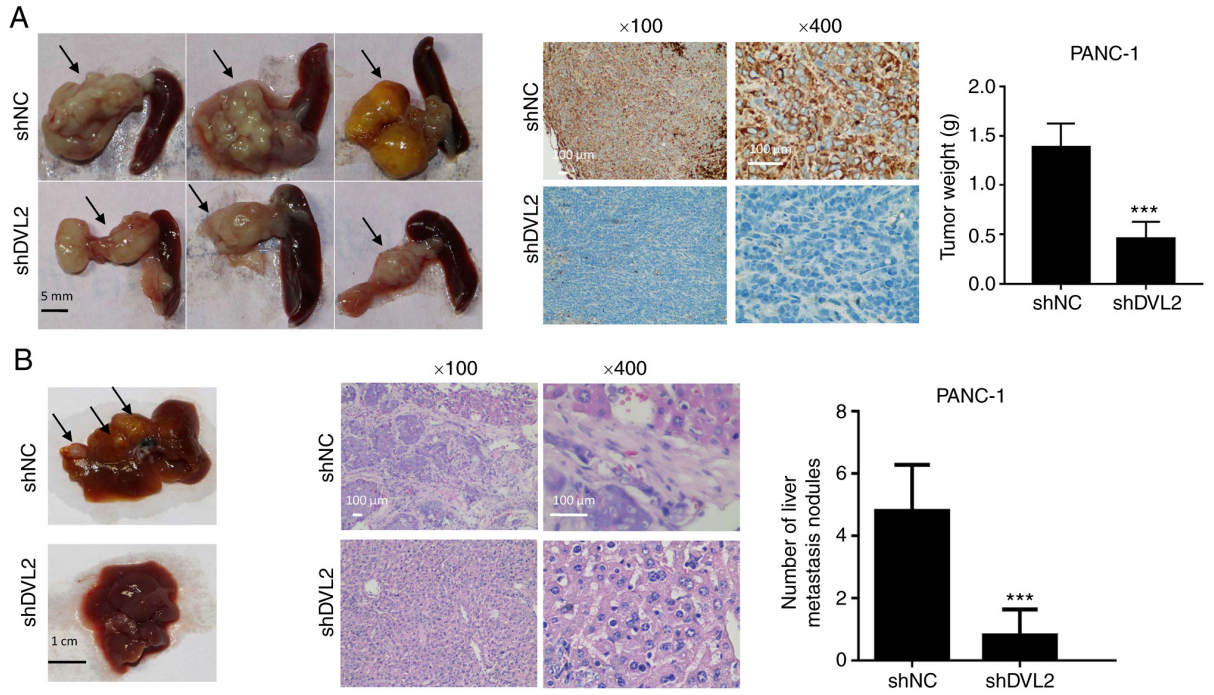

Figure 5. Effects of DVL2 knockdown on the proliferation and metastasis of PC cells in vivo. (A) Left, representative photographs of orthotopic pancreatic tumors (black arrows) from PANC-1-shDVL2 group and shNC group 3 months after injection (n=5 per group). Middle, the tumor tissues were treated with IHC staining of DVL2. Right, the weight of orthotopic pancreatic tumors. (B) Hepatic metastasis model. Left, representative liver metastatic nodules (black arrows) from DVL2 knockdown group and control group. Middle, typical images of metastatic nodules in the liver by H&E staining. Right, the numbers of metastatic liver nodules from the mice with the indicated treatments (n=5 per group). ***P<0.001. DVL-2, Dishevelled-2; NC, negative control; sh, short hairpin; IHC, immunohistochemistry.

to display functional activity in Wnt/β-catenin signaling (35). Suppression of DVL2 activates the destruction complex including APC, axin, casein kinase Iα (CKIα) and Glycogen synthase kinase 3β (GSK3β), decreases the phosphorylation

GSK-3β and reduces the accumulation of β-catenin in esophageal cancer cells and lung cancer (13,24), while DVL2 knockdown does not affect the expression levels of β-catenin in hepatocellular carcinoma and human gliomas (11,25). The transfection of DVL2 siRNA doesn't alter the total protein levels and the nuclear protein levels of β-catenin in anaplastic lymphoma kinase-positive anaplastic large cell lymphoma (36). The downregulation of DVL2 expression has less effect on Wnt/β-catenin signaling compared with the other 2 isoforms (DVL1 and DVL3) in mouse F9 teratocarcinoma cells (37). The results of the present study revealed that DVL2 forms a complex with β-catenin by co-IP assays. In addition, the findings of the present study indicated that knockdown of DVL2 in PANC-1 and CFPAC-1 cells decreased β-catenin levels, as well as that of specific Wnt target genes, including cyclin D1 and MMP9. In the present study, the knockdown of DVL2 diminished β-catenin nuclear translocation, suggesting that DVL2 serves a vital role in the aberrant activation of the canonical Wnt/β-catenin pathway. The function of this interaction may be that DVL2 promotes the binding of β-catenin to the c-myc promoter and the transcription of Wnt target genes in the nucleus, while downregulation of DVL2 activates the destruction complex, decreases the phosphorylation GSK-3β and reduces the accumulation of β-catenin (24).

EMT initiates tumor metastasis with the loss of tumor cell-cell adhesion and the gain of tumor invasion (38). DVL2 is involved in the regulation of EMT in breast cancer (39) and has been found to increase Snail transcription through recruitment of Forkhead box El (40). It has been previously demonstrated that the knockdown of DVL2 inhibited EMT induced by IQGAP1 overexpression (14). In the present study, DVL2 downregulation augmented the expression of epithelial marker E-cadherin, decreased the mesenchymal markers (N-cadherin and vimentin) and EMT-activating transcription factor Snail, further confirming its role on EMT regulation in pancreatic cancer. In addition, the present study demonstrated that DVL2 interacted with β-catenin, modulated its expression level and facilitated β-catenin translocation into the nucleus, suggesting that DVL2 was involved in the regulation of EMT mediated by Wnt/β-catenin signaling. Previous research found that DVL2 phosphorylation was essential for the activation of canonical Wnt signaling (41). However, the phosphorylation level of DVL2 in PC tissues and cells was not determined in the present study. Further work is needed to elucidate the role of DVL2 phosphorylation in Wnt/β-catenin signaling mediated EMT.

In the present study, the effects of aberrantly expressed DVL2 on PDAC were investigated. DVL2 expression was upregulated in PDAC tissues and was positively associated with advanced clinical stage and lymph node metastasis in patients with PDAC. DVL2 knockdown impaired its oncogenic functions including cell proliferation, migration, invasion and epithelial-mesenchymal transition. DVL2 interacted with β-catenin and knockdown of DVL2 reduced the expression level of β-catenin and inhibited β-catenin translocation into the nucleus. However, there were several limitations in the present study. The molecular mechanisms by which DVL2 was aberrantly regulated in PDAC cells and DVL2-knockdown induced downregulation of β-catenin were not very clear. Future mechanistic studies are required to verify the findings of the present study. Further studies are needed to further explore the precise oncogenic function and molecular mechanism of DVL2-induced carcinogenesis.

In conclusion, the findings of the present study suggested that DVL2 enhanced pancreatic tumorigenesis and development, making it a promising new therapeutic target for PDAC. Both *in vitro* and *in vivo* results of the present study improved understanding of the underlying cellular mechanism of DVL2 in the progression of EMT and metastasis of PDAC mediated by Wnt/β-catenin signaling.

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Availability of data and materials

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

Authors' contributions

WH, ML and JW designed and performed the experiments. WH, ML, HC and TZ contributed to the analysis of data and wrote the article. CZ and ZW made contributions to conception, discussed the results and revised the manuscript. WH and ML confirm the authenticity of all the raw data. All authors have read and approved the final manuscript.

Ethics approval and consent to participate

Approval from Ethics committees of the First Affiliated Hospital of Kangda College of Nanjing Medical University (Lianyungang, China) (approval no. KY20190924002) were obtained before enrollment into the study. Informed consent was obtained from all individual participants included in the study. The study was performed in accordance with government policies and the Helsinki Declaration. All animal studies were approved by the Animal Care Committee of the First Affiliated Hospital of Kangda College of Nanjing Medical University (Lianyungang, China) (approval no. KY20190924002) in compliance with the regulations and guidelines of the Institutional Animal Care.

Patient consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

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