miRNA-7515 suppresses pancreatic cancer cell proliferation, migration and invasion via downregulating IGF-1 expression

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Abstract. Pancreatic cancer (PC) is a lethal malignancy of the gastrointestinal tract. Previous studies have reported that microRNAs (miRNAs/miRs) are involved in the tumorigenesis of PC. Therefore, the present study aimed to determine the effects of miR-7515 on PC cell proliferation, invasion and migration *in vitro* and *in vivo*, and investigate its underlying molecular mechanism using bioinformatics, double luciferase assay and western blotting. The results revealed that the expression levels of miR-7515 were downregulated in PC, which predicted a poor clinical outcome. The overexpression of miR-7515 significantly decreased the proliferation, invasive and migratory abilities of PC cells *in vitro* and *in vivo*, while the knockdown of miR-7515 exerted the opposite effects. miR-7515 was identified to directly bind to insulin-like growth factor 1 (IGF-1) and downregulate its expression, which subsequently downregulated the Ras/Raf/MEK/ERK signalling pathway. The overexpression of IGF-1 reversed the inhibitory effects of miR-7515 overexpression on PC cells. In conclusion, the findings of the present study indicated that miR-7515 may act as a tumor suppressor in PC, as it repressed PC cell proliferation invasion and migration via downregulating the expression of IGF-1 and the activity of the Ras/Raf/MEK/ERK signalling pathways.

Introduction

Pancreatic cancer (PC) is an aggressive malignant tumor with a 5-year survival rate of <5% (1,2). Although considerable efforts have been made in improving the effectiveness of surgical resection and chemotherapy for PC, the remission and survival rates remain poor (3,4). The most significant challenge for PC therapy is that the underlying molecular mechanism of its pathogenesis is largely unknown. Therefore, it remains an urgent priority to determine the molecular mechanisms underlying the development of PC.

MicroRNAs (miRNAs/miRs) are a class of non-coding RNAs consisting of 20-22 nucleotides in length, which regulate gene expression via directly binding to target mRNAs (5,6). Previous studies have reported the role of numerous miRNAs in the tumorigenesis of PC (7-10). For example, a previous study demonstrated that miR-221 induced autophagy and promoted PC cell apoptosis via downregulating the expression of histone deacetylase 1 (11). In another study, the expression levels of miR-661 were revealed to be upregulated in PC tissues, which predicted a poor clinical outcome in patients with PC (12). By inhibiting Beclin-1-induced autophagy, miR-216a also increased the radiosensitivity of PC cells (13). Moreover, miR-148a played a tumor-suppressive role in PC via suppressing the Wnt/β-catenin signaling pathway and inhibiting epithelial-mesenchymal transition (14). Insulin-like growth factor (IGF)-1 was revealed to be abundantly expressed in PC tissues, and could activate insulin/IGF-related signalling pathways to regulate the proliferation, migration and invasion of PC cells (15). Numerous previous studies have indicated that IGF-1 may function as an oncogene in PC and represent an effective target for treatment (16-18).

The present study aimed to investigate the expression levels of miR-7515 in PC tissues and cell lines, and to determine whether its effects on the proliferation, migration and invasion of PC cells were mediated via regulation of IGF-1. The results of the present study may provide novel insights into whether miR-7515 and its target gene, IGF-1, may represent novel biomarkers for the diagnosis and clinical treatment of PC.

Materials and methods

Patients. PC and adjacent normal tissues were obtained from 82 patients with PC (age range, 35-73 years; 42 males and 40 females) at the Department of Hepatobiliary Surgery, Guizhou Medical University (Guiyang, China) between March 2017 and April 2020. None of patients enrolled in the present study had received neoadjuvant chemotherapy,

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radiotherapy or immunotherapy prior to surgery. Written informed consent was obtained from all patients prior to participation and the experimental protocol was approved (approval no. 31) by the Human Research Ethics Review Committee of Guizhou Medical University (Guiyang, China).

Cell lines and culture. The human PC cell lines, AsPC-1, BXPC-3, SW1990 and PANC-1, and a normal pancreatic epithelial cell line, HPDE, were obtained from the American Type Culture Collection. The cells were cultured in DMEM (Gibco; Thermo Fisher Scientific, Inc.) supplemented with 10% FBS (Gibco; Thermo Fisher Scientific, Inc.), and maintained in an environment with 5% CO_2 at 37˚C. PD98059, a MEK inhibitor, was purchased from PharMingen; BD Biosciences. A solution of PD98059 (50 μM) in dimethyl sulfoxide (DMSO; Sigma-Aldrich; Merck KGaA) was prepared and used after diluting with medium for each assay. DMSO was also used as a blank control.

Cell transfection. The miR-7515 inhibitor (GCCUGUUAG CAUGAAAAAA) and miR-negative control (NC) inhibitor (CGGAACAGAUCAUACUUGCCUU) were obtained from Shanghai GeneChem Co., Ltd. A 2nd-generation lentiviral system was used, miR-7515 expression lentivirus was generated by subcloning the PCR amplified full length human miR-7515 cDNA into the pMSCV retrovirus plasmid by transient transfection of 293T (GeneCopoeia, Inc.). Empty pMSCV retrovirus plasmid was used as a negative control for miR-7515 expression lentivirus. AsPC-1 and BXPC-3 cells were seeded into 6-well plates at a density of $1x10^5$ cells/well. After the cells had adhered, a lentivirus was added according to manufacturer's protocol (2 μg/ml; MOI=5 for BXPC-3; and MOI=10 for AsPC-1). To obtain stably overexpressing miR-7515 cells or stable knockdown cells, cells were selected for 14 days using 1 g/ml puromycin 48 h after transfection. miR-7515 overexpression lentivirus and the lentivirus miRNA NC (LV-miR-NC) were obtained from GeneCopoeia, Inc. The IGF-1 overexpression and NC plasmids (5 μg/ml) were obtained from Sangon Biotech Co., Ltd. Cells were transfected 48 h after transfection at 37˚C with the oligonucleotides and plasmids using Lipofectamine® 2000 (Invitrogen; Thermo Fisher Scientific, Inc.), according to the manufacturer's protocol. After 48 h of transfection, the cells were collected for further use in the following experiments.

Reverse transcription-quantitative (RT-q)PCR. Total RNA was extracted from PC tissues and cell lines using TRIzol reagent (Invitrogen; Thermo Fisher Scientific, Inc.). mRNA was reverse-transcribed into cDNA using the SuperScript IV Reverse Transcriptase kit (Thermo Fisher Scientific, Inc.), while miRNA was reverse transcribed into cDNA using TaqMan™ MicroRNA Reverse Transcription kit (Thermo Fisher Scientific, Inc.). qPCR was subsequently performed using a PowerUp™ SYBR™ Green Master Mix (Thermo Fisher Scientific, Inc.). All the kits were used according to the manufacturer's instructions. GAPDH was used as the reference gene for IGF-1 expression, while U6 was used as the reference gene for miR-7515 expression. The following primer sequences were used for the qPCR: miR-7515, 5'-AGAAGG GAAGATGGTGAC-3'; IGF-1 forward, 5'-GCTCTTCAGTTC GTGTGTGGA-3' and reverse, 5'-GCCTCCTTAGATCAC AGCTCC-3'; GAPDH forward, 5'-GGAGCGAGATCCCTC CAAAAT-3' and reverse, 5'-GGCTGTTGTCATACTTCT CATGG-3'; U6 forward, 5'-GCTTCGGCAGCACATATA CTAAAAT-3' and reverse, 5'-CGCTTCACGAATTTGCGT GTCAT-3'. The reaction conditions were as follows: 95˚C for 10 min, followed by 40 cycles at 95˚C for 1 sec and 60˚C for 60 sec. Relative expression levels of the target mRNA or miRNA were determined using the $2^{-\Delta\Delta Cq}$ method (19).

Cell Counting Kit-8 (CCK-8) assay. PC cells were seeded into 96-well plates at a density of $2x10^3$ cells/well and incubated at 37˚C for 6, 24, 48, 72 or 96 h. Following incubation, the medium was removed and 100 μl fresh DMEM containing 10 μl CCK-8 reagent (Wuhan Boster Biological Technology, Ltd.) was added to each well. The cells were subsequently incubated with 5% CO_2 at 37˚C for 2 h. The absorbance of each well was measured at a wavelength of 450 nm using a microplate spectrophotometer (Bio-Rad Laboratories, Inc.).

Colony formation assay. Following transfection, PC cells were plated into 6-well plates at a density of $1x10^3$ cells/well. Following 14 days of incubation at 37˚C, the medium was removed and the cell colonies were fixed with 4% paraformaldehyde for 15 min at room temperature (Wuhan Servicebio Technology Co., Ltd.) and stained with 0.5% crystal violet for 30 min at room temperature (Wuhan Servicebio Technology Co., Ltd.). The cells were washed with PBS to remove the excess crystal violet and images of the colonies were captured and the number of colonies (≥10 mm^2) was counted under the naked eye.

Cell cycle distribution analysis. Cell cycle distribution was determined using flow cytometric analysis. Briefly, PC cells were harvested, washed with cold PBS and fixed with 70% ethyl alcohol at 4˚C for 24 h. The PC cells were subsequently collected by centrifugation at 4˚C, 300 x g for 30 min and resuspended in staining solution containing propidium iodide (Invitrogen; Thermo Fisher Scientific, Inc.). and 1X binding buffer at room temperature for 30 min. Following a 30-min incubation in the dark, the samples were analyzed using a flow cytometer (BD Biosciences). The results of the cell cycle distribution were analyzed using FlowJo software version 7.4.1 (FlowJo LLC).

Wound healing assay. PC cells were cultured in DMEM supplemented with 10% FBS at 37˚C in 6-well plates until they reached 95% confluence. Then, artificial wounds were produced by scratching the cell monolayer with a 200-μl pipette tip. After removing the nonadherent cells with PBS, the cells were cultured in the serum-free DMEM and cultured for 24 h. The wound area was visualized at 0 and 24 h under a phase-contrast microscope at a magnification of x100.

Transwell invasion assay. PC cells ($1x10^5$) were suspended in 200 μl serum-free DMEM and plated into the upper chambers of a Transwell plate (8 μm pore size; Corning, Inc.), which was precoated at 37˚C with 100 μl Matrigel (BD Biosciences). A total of 700 μl DMEM supplemented with 10% FBS was plated into the lower chambers of the Transwell plate to act

as a chemoattractant. Following incubation at 37˚C for 24 h, the non-invasive cells were removed with a cotton swab, and the invasive cells were fixed for 15 min at room temperature with 4% paraformaldehyde and stained for 30 min at room temperature with 0.5% crystal violet solution. Invasive cells were visualized (magnification, x40) in five randomly selected fields of view and the number of invasive cells/field was calculated under a phase-contrast microscope.

Animal studies. A total of 10 male BALB/c nude mice (weight, 16-18 g; 4-6 weeks old) were purchased from the Experimental Animal Centre of Guizhou Medical University (Guiyang, China). All nude mice were allowed free access to food and water and maintained on a 12-h light/dark cycle, with controlled temperature (22.5±2˚C) and humidity (45±5%). To determine tumor cell proliferation, $1x10^6$ with 100 μl of PANC-1 cell suspension overexpressing miR-7515 or transfected with LV-miR-NC were subcutaneously injected into the right axilla of the BALB/c nude mice. Tumor growth was assessed once a week, and after 5 weeks, the mice were anesthetized via an intraperitoneal injection of 50 mg/kg sodium pentobarbital and euthanized by cervical dislocation. Tumor tissues were collected for subsequent analysis; the maximum tumor volume observed in the present study was 400 mm^3.

For live metastasis detection, $1x10^7$ PANC-1 cells overexpressing miR-7515 or transfected with LV-miR-NC were resuspended in 100 μl PBS and injected into the spleen of the mice (n=5 mice/group). The health of nude mice was observed once a day, and after 12 weeks, mice were anesthetized via an intraperitoneal injection of 50 mg/kg sodium pentobarbital solution and euthanized by cervical dislocation. The liver tissues were separated and the metastatic foci were counted. Furthermore, metastasis foci in the liver tissues were also detected using hematoxylin and eosin (H&E) staining. Paraffin-embedded specimens were cut into serial sections of 5-μm thickness with a microtome. Sections were deparaffinized in xylene, rehydrated through a graded ethanol series and stained for 30 min at room temperature automatically with H&E. Then images were captured using an upright light microscope (magnification, x200; Olympus Corporation). All animal experiments were performed in strict accordance with the Guide for the Care and Use of Laboratory Animals (20). The animal experimental protocols were approved (approval no. 2000025) by the Ethics Committee of Guizhou Medical University (Guiyang, China).

Immunohistochemical analysis. Tissues were fixed in 4% paraformaldehyde for 30 min at room temperature in formalin, embedded in paraffin and cut into 2-μm sections. The sections were incubated at 65˚C for 30 min, deparaffinized with xylene and rehydrated using a gradient series of ethanol. Antigen retrieval was performed using sodium citrate (Wuhan Servicebio Technology Co., Ltd.) then endogenous peroxidase activity was blocked for 15 min at room temperature with 3% H_2O_2 (Wuhan Servicebio Technology Co., Ltd.). The sections were subsequently blocked for non-specific binding with 5% BSA for 15 min at room temperature (Wuhan Servicebio Technology Co., Ltd.), and incubated overnight at 4˚C with anti-Ki67 (1:200; cat. no. 27309-1-AP; ProteinTech Group, Inc.) or anti-proliferating cell nuclear antigen (PCNA; 1:200; cat. no. 10205-2-AP; ProteinTech Group, Inc.) primary antibodies. Following the incubation, the sections were washed with PBS three times for 5 min and incubated with a goat anti-mouse/rabbit poly-HRP secondary antibody (1:500; cat. no. PR30009; ProteinTech Group, Inc.) for 2 h. A diaminobenzidine (DAB) kit (Wuhan Boster Biological Technology, Ltd.) was used to visualize the antibody-antigen binding. Images were captured using an upright metallurgical microscope (x200; Olympus Corporation).

Bioinformatics analysis. The expression of miR-7515 in pancreatic cancer tissues and adjacent tissues was first assessed by The Cancer Genome Atlas (TCGA) (https://www.cancer.gov/types) and Genotype-Tissue Expression (GTex) (http://commonfund.nih.gov/GTEx/) databases. The parameters were as follows: LogFC >1 and a P-value <0.05. The online database, TargetScan 7.2 (http://www.targetscan.org/vert_72/), was used to predict the target genes of miR-7515. The Database for Annotation, Visualization and Integrated Discovery (https://david.ncifcrf.gov/) was used to determine the significantly enriched Kyoto Encyclopedia of Genes and Genomes signaling pathway enrichment of miR-7515 target genes. Finally, RStudio software (version: 8.1.2; IBM Corp.) was used to visualize the pathway enrichment. Target genes of miR-7515 in the most significantly enriched pathway were further analyzed.

Dual-luciferase reporter assay. SW1990 and PANC-1 cells were plated into 24-well plates and cultured with 5% CO_2 at 37˚C until they reached 70% confluence. Then, the psiCHECK-2/IGF-1 3'-untranslated region (UTR) wild-type (WT) and psiCHECK-2/IGF-1 3'-UTR mutant type (Mut) reporter plasmids (Shanghai GeneChem Co., Ltd.) were transfected into $5x10^5$ SW1990 and PANC-1 cells overexpressing miR-7515 or transfected with the LV-miR-NC using Lipofectamine 2000 (Invitrogen; Thermo Fisher Scientific, Inc.). Following 24 h of transfection, SW1990 and PANC-1 cells were collected, and the fluorescence activity was detected using a Dual Luciferase Reporter assay system (Promega Corporation). Subsequently, the luciferase activity was normalized to the firefly luciferase internal control.

Western blotting. Total protein was extracted from PC cells using RIPA lysis buffer (Wuhan Servicebio Technology Co., Ltd.) supplemented with 1% PMSF (Wuhan Servicebio Technology Co., Ltd.). Total protein was quantified using a BCA method and separated via 10% SDS-PAGE (Melone pharmaceutical Co., Ltd). The separated proteins (30 μg per lane) were subsequently transferred onto PVDF membranes (BD Biosciences) and blocked with 5% BSA for 2 h at room temperature. The membranes were then incubated with the following primary antibodies overnight at 4˚C: Anti-IGF1 (1:1,000; cat. no. A12305; ABclonal Biotech Co., Ltd.), p-c-Raf (1:1,000; product code ab150365; Abcam), anti-c-Raf (1:1,000; product no. 53745; Cell Signaling Technology, Inc.), p-ribosomal protein S6 kinase α-1 (p-p90Rsk; 1:1,000; product no. 12032; Cell Signaling Technology, Inc.), anti-p90Rsk (1:1,000; product code ab32114; Abcam), anti-p-MEK1/2 (1:1,000; product no. 2338; Cell Signaling Technology, Inc.), anti-MEK1/2 (1:1,000; product no. 4694; Cell Signaling

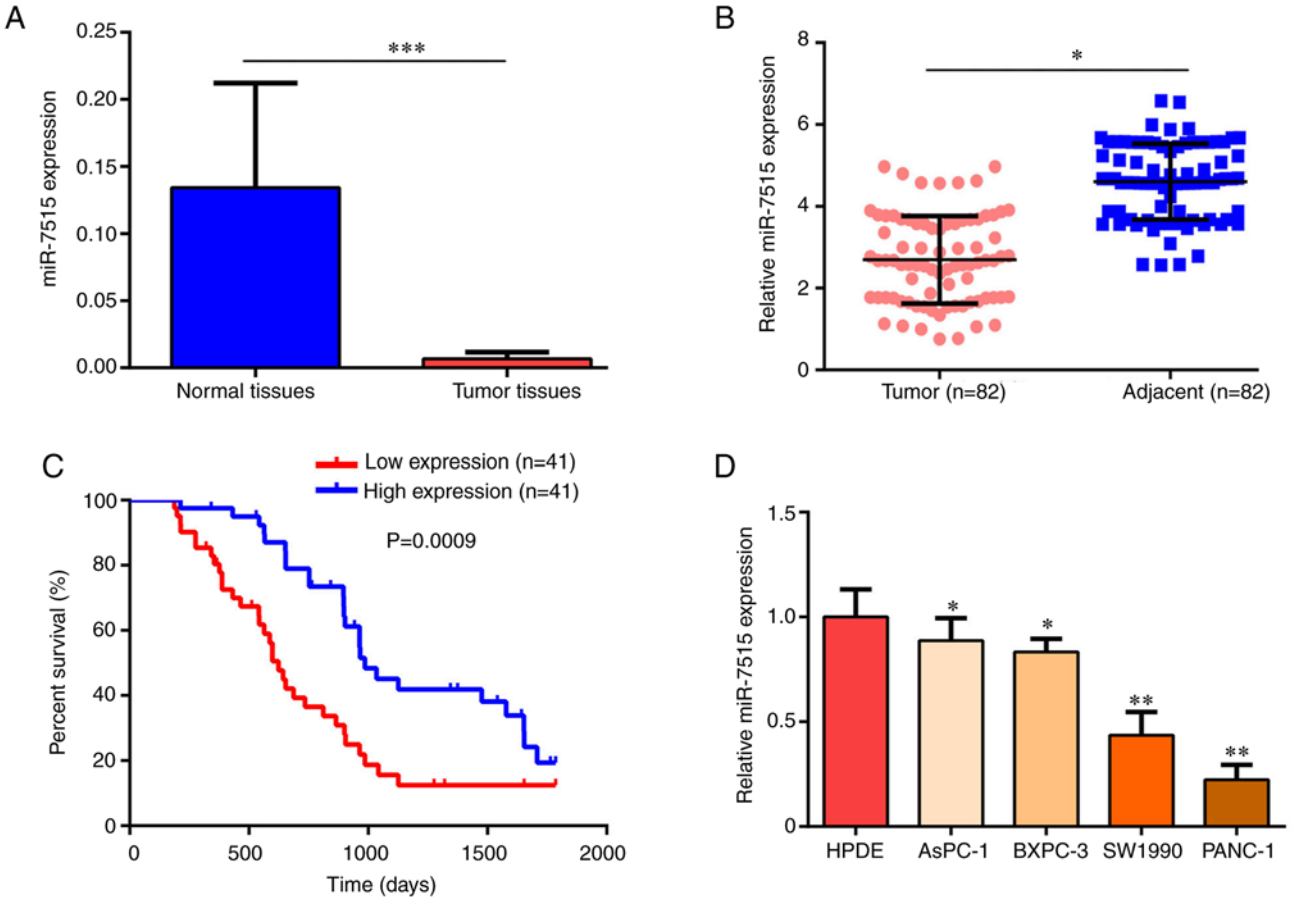

Figure 1. miR-7515 expression levels are downregulated in PC. (A) The Cancer Genome Atlas and Genotype-Tissue Expression databases were used to analyze miR-7515 expression in PC. (B) RT-qPCR was used to analyze the expression levels of miR-7515 in PC tissues (n=82) and adjacent tissues (n=82). (C) Kaplan-Meier survival analysis of the overall survival rate of patients with PC with high or low miR-7515 expression. (D) RT-qPCR was used to determine the expression levels of miR-7515 in human PC cell lines AsPC-1, BXPC-3, SW1990 and PANC-1 and the normal pancreatic epithelial cell line, HPDE. *P<0.05, **P<0.01 and ***P<0.001. miR, microRNA; PC, pancreatic cancer; RT-qPCR, reverse transcription-quantitative PCR.

Technology, Inc.), anti-p-ERK1/2 (1:1,000; product no. 8544; Cell Signaling Technology, Inc.), anti-ERK1/2 (1:1,000; cat. no. 16443-1-AP; ProteinTech Group, Inc.), anti-cyclin D1 (1:1,000; cat. no. 26939-1-AP; ProteinTech Group, Inc.), anti-CDK2 (1:1,000; cat. no. 10122-1-AP; ProteinTech Group, Inc.), anti-MMP2 (1:1,000; cat. no. 10373-2-AP; ProteinTech Group, Inc.), anti-MMP9 (1:1,000; cat. no. 10375-2-AP; ProteinTech Group, Inc.) and anti-GAPDH (1:1,000; cat. no. 60004-1-Ig; ProteinTech Group, Inc.). Following the primary antibody incubation, the membranes were washed with TBST (TBS with 0.1% Tween-20) thrice and incubated with secondary antibodies at room temperature (1:5,000; cat. nos. BA1038 and BA1039; Wuhan Boster Biological Technology, Ltd.) for 2 h. Protein bands were visualized using ECL reagent (Wuhan Boster Biological Technology, Ltd.) on a Gel imager (Bio-Rad Laboratories, Inc.). The total c-Raf, p90Rsk, MEK and ERK1/2 antibodies were used on the same blots as the respective phosphorylated proteins after stripping (Bio-Rad Laboratories, Inc.). Image-Pro Plus 5.1 software was used to analyze the expression of protein, while GAPDH was used as a loading control.

Statistical analysis. All experiments were repeated 3 times and the data was averaged. The data are presented as the mean ± standard deviation (SD). Statistical analysis was performed using SPSS 20.0 software (IBM Corp.). The association between miR-7515 expression and clinicopathological characteristics of patients with PC was analyzed using a χ^2 test. The correlation between miR-7515 and IGF-1 expression levels was analyzed using Pearson's correlation analysis. Comparisons were performed using a one-way ANOVA combined with LSD-t-test or a paired Student's t-test. P<0.05 was considered to indicate a statistically significant difference.

Results

miR-7515 expression levels are downregulated in PC. TCGA and GTex databases were first used to analyze the expression levels of miR-7515 in human normal and PC tissues. The results revealed that miR-7515 expression levels were significantly downregulated in PC tissues (Fig. 1A). Next, the expression of miR-7515 in 82 PC and adjacent normal tissues was determined. RT-qPCR analysis demonstrated that the expression levels of miR-7515 were also downregulated in PC tissues compared with adjacent normal tissues (Fig. 1B). To determine the prognostic value of miR-7515, patients were divided into two groups (high and low expression levels of miR-7515) using the median expression value of 2.7; tissues

Table I. Association between miR-7515 expression levels and the clinicopathological characteristics of patients with PC.

Features	n	miR-7515 expression		χ^2	P-value
		Low	High		
All cases	82	41	41		
Age (years)				0.44	0.507
<60	39	21	18		
≥60	43	20	23		
Sex				0.195	0.695
Males	42	22	20		
Females	40	19	21		
Tumor size (cm)				7.13	0.008
<2	36	12	24		
≥2	46	29	17		
Lymph node metastasis				10.63	0.001
Negative	28	7	21		
Positive	54	34	20		
TNM stage				12.424	<0.001
I and II	27	6	21		
III and IV	55	35	20		
Distant metastasis				4.481	0.027
Negative	40	15	25		
Positive	42	26	16		
Perineural invasion				14.233	<0.001
Negative	45	14	31		
Positive	37	27	10		
Blood vessel invasion				8.264	0.004
Negative	39	13	26		
Positive	43	28	15		

miR, microRNA.

with miR-7515 expression of >2.7 were defined as high expression, while tissues with miR-7515 expression of <2.7 were defined as low expression. The results revealed that low expression levels of miR-7515 predicted a lower survival rate (Fig. 1C). The expression levels of miR-7515 were also revealed to be negatively associated with tumor size, lymph node metastasis, TNM stage, distant metastasis, perineural invasion and blood vessel invasion (Table I). In addition, the expression levels of miR-7515 were significantly downregulated in PC cells compared with the normal pancreatic epithelial cell line, HPDE (Fig. 1D).

miR-7515 regulates PC cell proliferation in vitro. To determine the effects of miR-7515 on PC cell proliferation, miR-7515 overexpression lentivirus and miR-7515 inhibitor were used to construct miR-7515-overexpressing and -knockdown cells (Fig. 2A). CCK-8 assays were performed, and the results demonstrated that the overexpression of miR-7515 significantly decreased the proliferation rate of SW1990 and PANC-1 cells, while miR-7515 knockdown significantly increased the proliferation rate of AsPC-1 and BXPC-3 cells (Fig. 2B). Similarly, the results of the colony formation assays revealed that overexpression of miR-7515 decreased the colony formation of SW1990 and PANC-1 cells, while the knockdown of miR-7515 exerted the opposite effects on AsPC-1 and BXPC-3 cells (Fig. 2C). Furthermore, the overexpression of miR-7515 induced SW1990 and PANC-1 cell cycle arrest in the G_1 phase, while miR-7515 knockdown promoted the progression from the G_1 phase to the S phase in AsPC-1 and BXPC-3 cells (Fig. 2D). These results indicated that miR-7515 may regulate the proliferation of PC cells *in vitro*.

miR-7515 regulates PC cell migration and invasion in vitro. The effects of miR-7515 on PC cell migration and invasion were subsequently investigated. The results of the wound healing assay revealed that the migration rate was significantly decreased in the cells overexpressing miR-7515, while the knockdown of miR-7515 increased the cell migration rate (Fig. 3A). Moreover, the results of the Transwell assays demonstrated that the invasive abilities of SW1990 and PANC-1 cells overexpressing miR-7515 were decreased, while the invasive abilities of AsPC-1 and BXPC-3 cells were increased

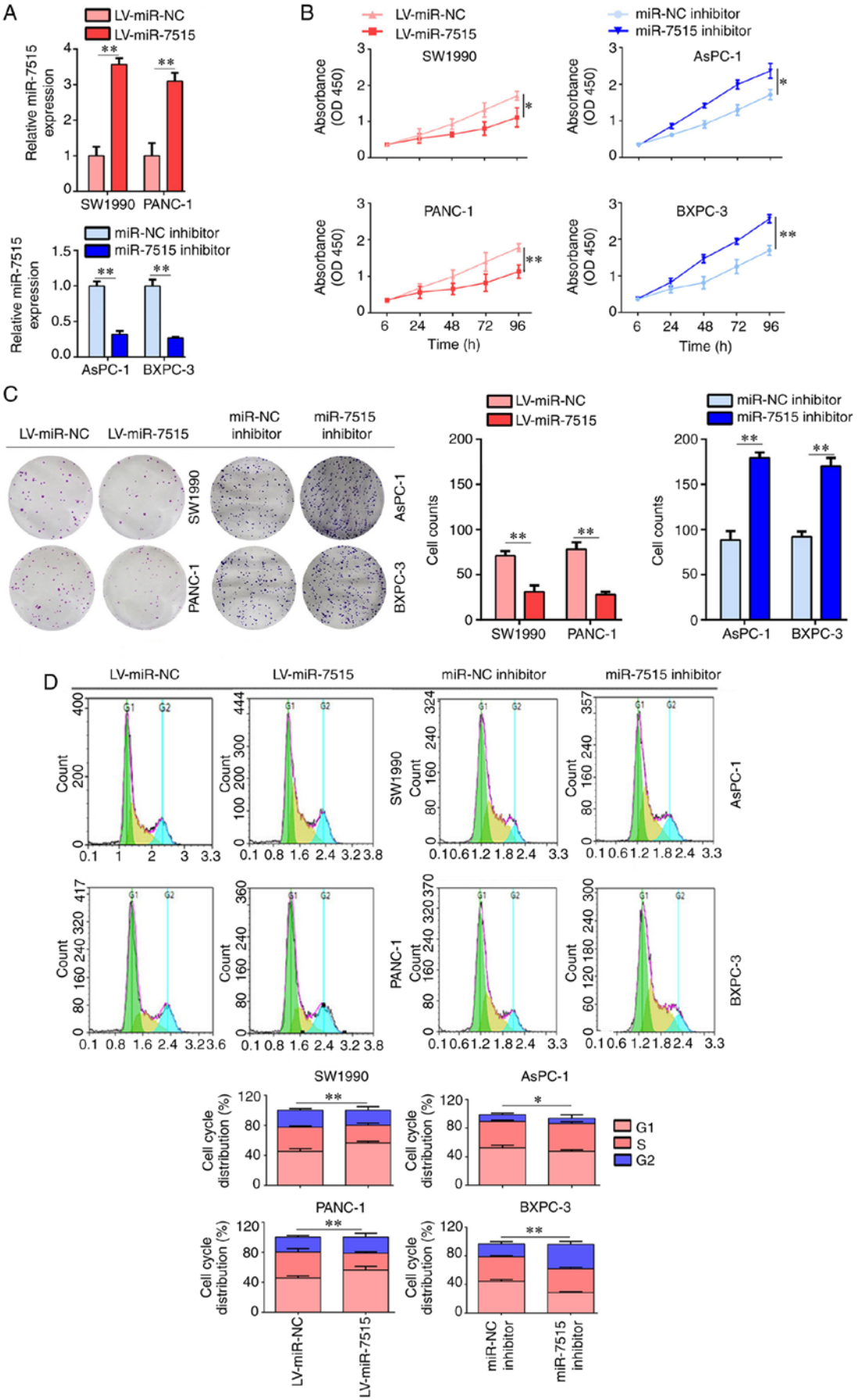

Figure 2. miR-7515 regulates PC cell proliferation *in vitro*. (A) Reverse transcription-quantitative PCR was used to determine the efficiency of the miR-7515 overexpression lentivirus and miR-7515 inhibitor. (B) Cell Counting Kit-8 and (C) colony formation assay were used to determine the effects of miR-7515 on proliferation of PC cells. (D) Cell cycle distribution of PC cells was determined in miR-7515-overexpression, -knockdown and corresponding negative control cells. *P<0.05 and **P<0.01. miR, microRNA; PC, pancreatic cancer; NC, negative control; LV, lentivirus.

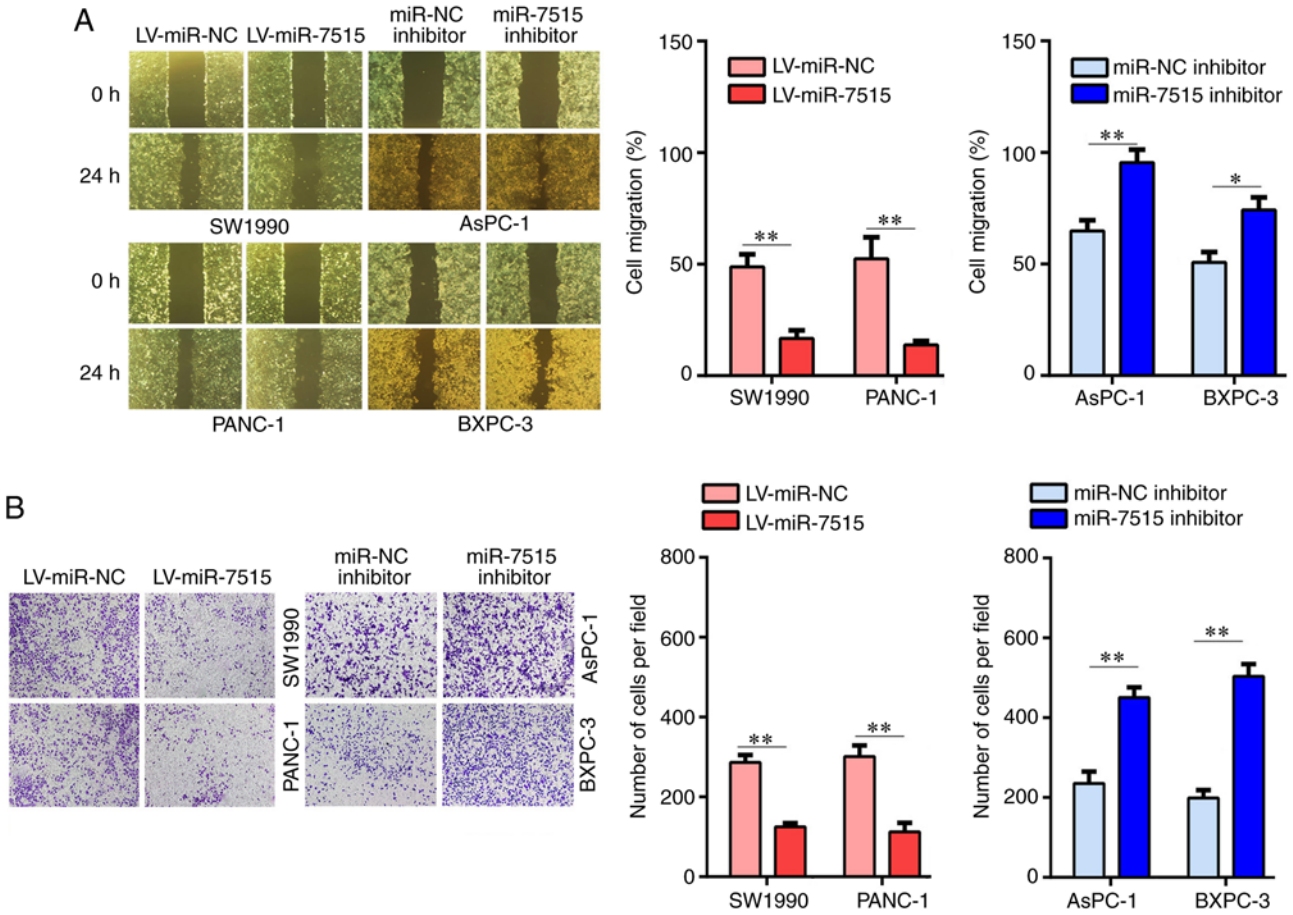

Figure 3. miR-7515 regulates PC cell migration and invasion *in vitro*. (A) Wound healing assay was used to determine the effects of miR-7515 on migration of PC cells. (B) Transwell assays were used to analyze the effects of miR-7515 on invasion of PC cells. *P<0.05 and **P<0.01. miR, microRNA; PC, pancreatic cancer; NC, negative control; LV, lentivirus.

following knockdown of miR-7515 compared with the respective NC cells (Fig. 3B). These results indicated that miR-7515 may regulate PC cell migration and invasion *in vitro*.

Overexpression of miR-7515 inhibits PC cell proliferation and metastasis in vivo. The effects of miR-7515 on PC cell proliferation and metastasis *in vivo* were also determined. Establishment of a xenograft tumor model indicated that the tumors overexpressing miR-7515 grew slower compared with the tumors derived from cells transfected with LV-miR-NC (Fig. 4A and B), and also expressed downregulated levels of Ki67 and PCNA (Fig. 4C). The liver metastasis model revealed that the overexpression of miR-7515 inhibited the metastasis of PANC-1 cells *in vivo* compared with the tumors derived from cells transfected with LV-miR-NC (Fig. 4D-F). Furthermore, the findings indicated that mice with tumors overexpressing miR-7515 had a lower death rate compared with the mice with tumors derived from cells transfected with LV-miR-NC (Fig. 4G). These results indicated that miR-7515 may play a tumor-suppressive role in PC.

miR-7515 targets IGF-1 and regulates Ras/Raf/MEK/ERK signaling pathway. To determine the molecular mechanism underlying the role of miR-7515 in PC, bioinformatics analysis was performed. The results demonstrated that the target genes of miR-7515 were most significantly enriched in 'Ras signaling pathway' (Fig. 5A). Among the genes, IGF-1 was identified as a target gene of miR-7515 and had a high binding score for its involvement in the Ras signaling pathway (Fig. 5B). As previous studies revealed that the Ras signaling pathway played a key role in the progression of PC (21-23), it was hypothesized that miR-7515 may regulate IGF-1 expression in PC cells. Results from the dual luciferase reporter assay demonstrated that the relative luciferase activity was significantly decreased in the cells co-transfected with the psiCHECK-2/IGF-1 3'-UTR WT plasmid and miR-7515 overexpression lentivirus, while the relative luciferase activity was not altered in the SW1990 and PANC-1 cells co-transfected with the psiCHECK-2/IGF-1 3'-UTR Mut plasmid and miR-7515 overexpression lentivirus (Fig. 5C). RT-qPCR and western blot analysis demonstrated that miR-7515 overexpression significantly downregulated the expression levels of IGF-1, while the knockdown of miR-7515 upregulated the expression levels of IGF-1 in SW1990 and PANC-1 (Fig. 5D and E). Similarly, SW1990 and PANC-1cells overexpressing miR-7515 had decreased expression levels of p-c-Raf, p-p90Rsk, p-MEK and p-ERK1/2 compared with the LV-miR-NC-transfected cells, while the knockdown of miR-7515 in SW1990 and PANC-1 cells upregulated the expression levels of p-c-Raf, p-p90Rsk, p-MEK and p-ERK1/2 (Fig. 5E). Moreover, the expression levels of IGF-1 were revealed to be upregulated in PC tissues

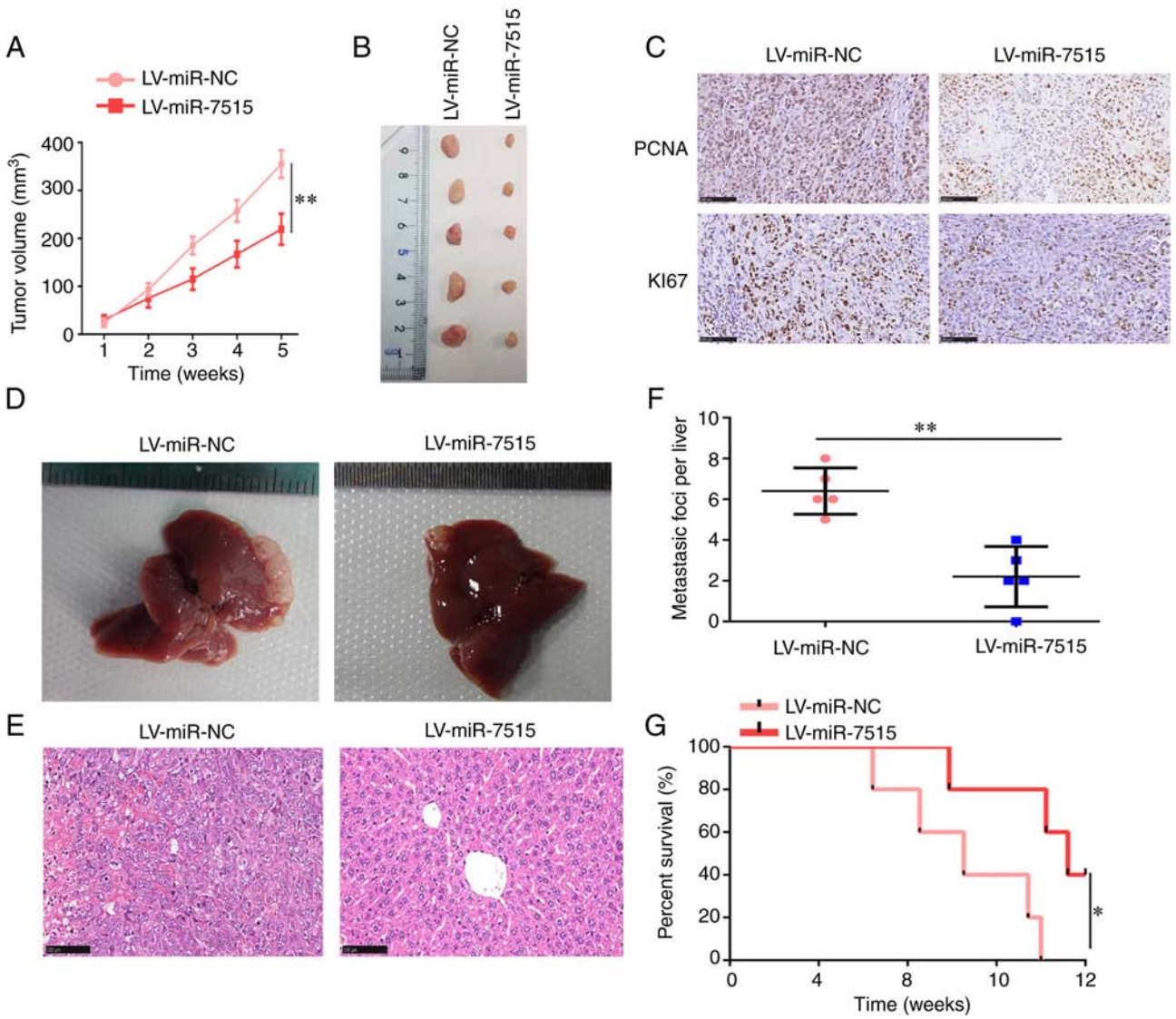

Figure 4. miR-7515 regulates PANC-1 cell proliferation and metastasis *in vivo*. (A) Proliferation rate of tumor tissues overexpressing miR-7515 and derived from LV-miR-NC-transfected cells. (B) Images of the subcutaneous xenografts. (C) Expression of Ki-67 and PCNA in tumor tissues overexpressing miR-7515 or derived from LV-miR-NC-transfected cells. (D) Images of the liver metastasis model. (E) Typical immunohistochemical staining of hematoxylin and eosin images revealing metastasis loci in liver in indicated groups. (F) Metastatic foci per liver in the mice injected with PANC-1 cells overexpressing miR-7515 or LV-miR-NC-transfected PANC-1 cells. (G) Survival rate of mice injected with PANC-1 cells overexpressing miR-7515 or LV-miR-NC-transfected PANC-1 cells. *P<0.05 and **P<0.01. miR, microRNA; LV-miR-NC, lentivirus microRNA negative control; PCNA, proliferating cell nuclear antigen.

compared with adjacent normal tissues (Fig. 6A), particularly in tumors from patients with III-IV stage disease (Fig. 6B). miR-7515 expression was also revealed to be negatively correlated with IGF-1 expression in PC tissues (Fig. 6C). Furthermore, the expression levels of IGF-1 were significantly upregulated in PC cell lines compared with the normal pancreatic epithelium cell line, HPDE (Fig. 6D and E). These results indicated that miR-7515 may target IGF-1 and the downstream Ras/Raf/MEK/ERK signaling pathway.

Overexpression of IGF-1 reverses the inhibitory effects of miR-7515 overexpression. To determine whether miR-7515 regulated the proliferation migration and invasion of PC cells via regulating IGF-1, the miR-7515 overexpression lentivirus and IGF-1 overexpression plasmid were co-transfected in SW1990 and PANC-1 cells (Fig. 7A). The results demonstrated that IGF-1 overexpression relieved the inhibitory effects of miR-7515 overexpression on the Ras/Raf/MEK/ERK signaling pathway (Fig. 7B). The results of the CCK-8 assay revealed that overexpression of IGF-1 in SW1990 and PANC-1 cells decreased the inhibitory effects of miR-7515 overexpression on cell proliferation (Fig. 8A). Similarly, the wound healing assay results demonstrated that the migratory rate of SW1990 and PANC-1 cells co-transfected with the miR-7515 overexpression lentivirus and IGF-1 overexpression plasmid was increased compared with cells only transfected with miR-7515 overexpression lentivirus (Fig. 8B). Furthermore, the overexpression of IGF-1 in miR-7515 overexpression cells upregulated the expression levels of cyclin D1 (G_1 phase marker), CDK2 (G_1 phase marker), MMP-2 (metastasis marker) and MMP-9 (metastasis marker) (Fig. 8C). These results indicated that IGF-1 may regulate the proliferation migration and invasion of PC cells via targeting the IGF-1-induced Ras/Raf/MEK/ERK signaling pathway.

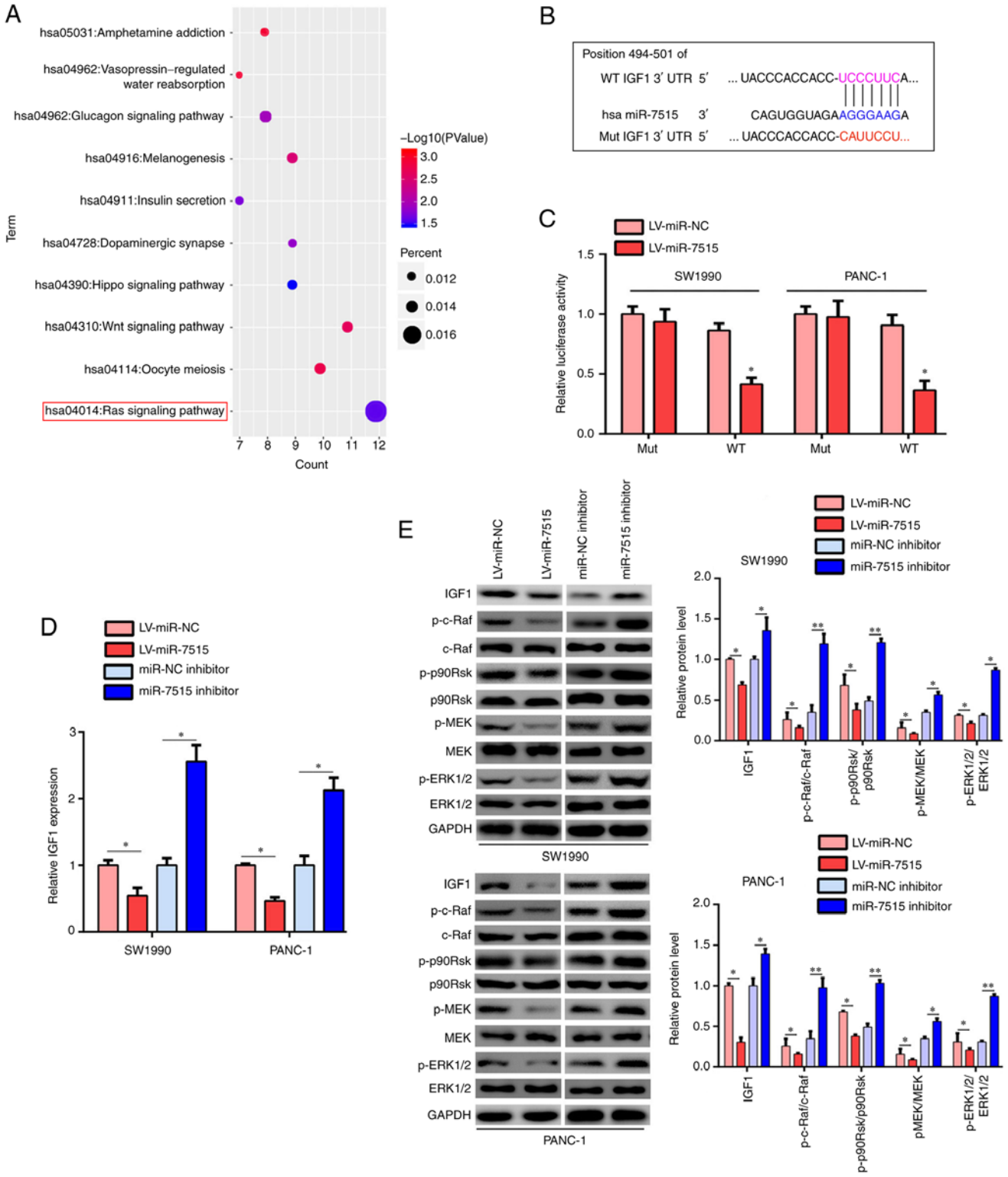

Figure 5. miR-7515 targets IGF-1 and regulates the Ras/Raf/MEK/ERK signaling pathway. (A) Kyoto Encyclopedia of Genes and Genomes signaling pathway enrichment analysis of the target genes of miR-7515. (B) Binding site between miR-7515 and IGF-1 is presented. (C) Dual luciferase reporter assays were performed to validate the binding between miR-7515 and IGF-1. (D) Reverse transcription-quantitative PCR was performed to analyze the expression levels of IGF-1 in SW1990 and PANC-1 cells transfected with the miR-7515 overexpression lentivirus and miR-7515 inhibitor. (E) Western blotting was used to analyze the expression levels of IGF-1, p-c-Raf, c-Raf, p-p90RSK, p90RSK, p-MEK, MEK, p-ERK1/2 and ERK1/2 in SW1990 and PANC-1 cells transfected with the miR-7515 overexpression lentivirus and miR-7515 inhibitor. *P<0.05 and **P<0.01. miR, microRNA; IGF-1, insulin like growth factor 1; p-, phosphorylated; p90RSK, ribosomal protein S6 kinase α-1; NC, negative control; LV, lentivirus.

MEK inhibitors block the effects of miR-7515 knockdown on proliferation invasion and migration of PC cells. To determine whether miR-7515 regulated the proliferation invasion and migration of PC cells via regulating IGF-1 expression, AsPC-1 and BXPC-3 cells were transfected with the miR-7515 inhibitor and treated with the MEK inhibitor PD98059. The

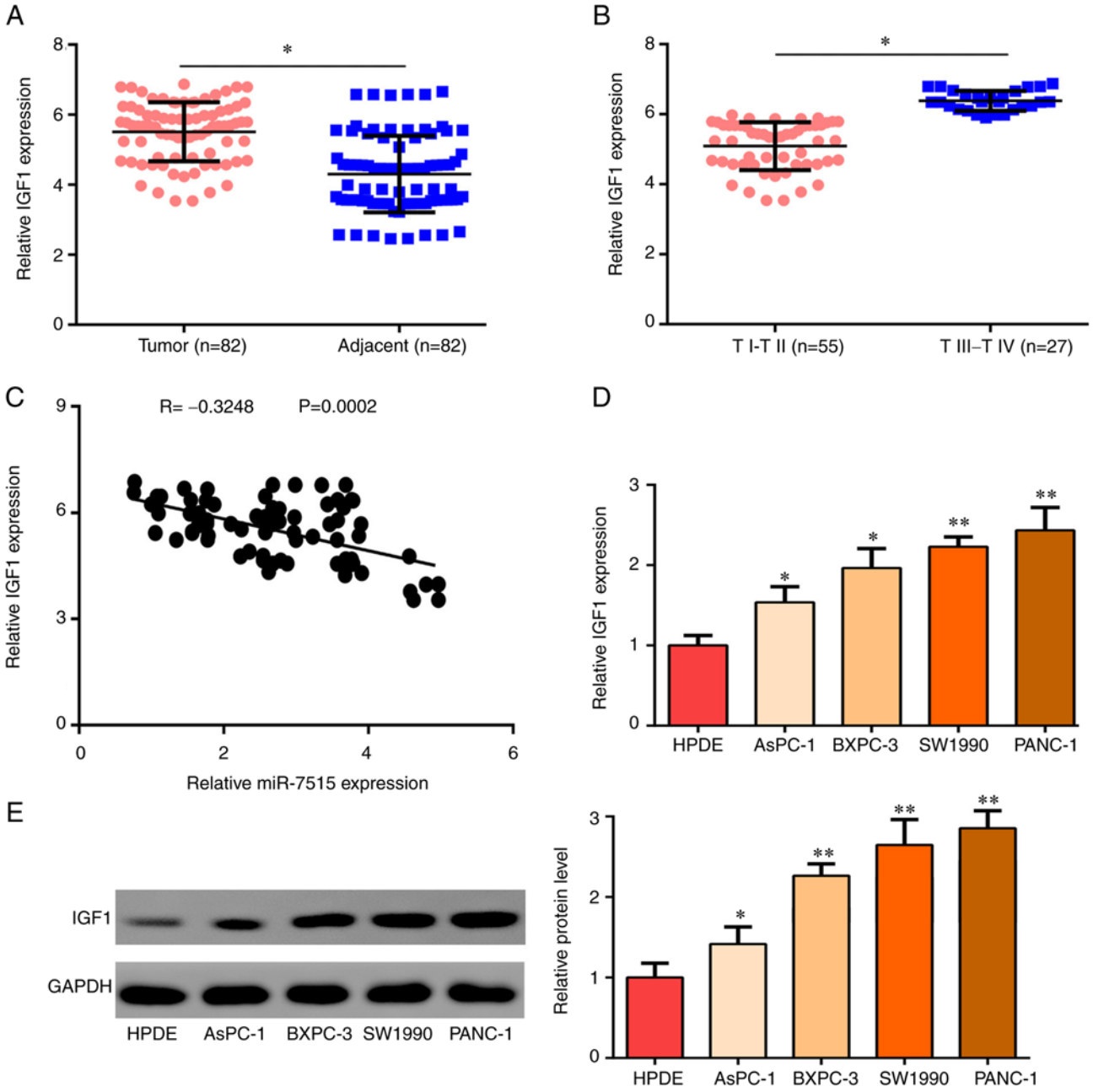

Figure 6. IGF-1 expression levels are upregulated in PC and negatively correlated with miR-7515 expression. (A) RT-qPCR was used to detect the expression levels of IGF-1 in PC tissues (n=82) and adjacent normal tissues (n=82). (B) RT-qPCR was used to analyze the expression levels of IGF-1 in PC tissues obtained from the patients with stage I-II (n=55) and stage III-IV (n=27) disease. (C) RT-qPCR was used to evaluate the co-expression between IGF-1 and miR-7515. (D) RT-qPCR and (E) western blotting were used to analyze the expression levels of IGF-1 in human PC cell lines, AsPC-1, BXPC-3, SW1990 and PANC-1, and normal pancreatic epithelial cell line, HPDE. *P<0.05 and **P<0.01. IGF-1, insulin like growth factor 1; PC, pancreatic cancer; miR, microRNA; RT-qPCR, reverse transcription-quantitative PCR.

results of the CCK-8 assays demonstrated that the treatment of AsPC-1 and BXPC-3 cells with the MEK inhibitor decreased the promoting effects of the miR-7515 inhibitor on cell proliferation (Fig. 9A). The results of the wound healing assay revealed that the migration rate in AsPC-1 and BXPC-3 cells transfected with the miR-7515 inhibitor and treated with the MEK inhibitor, PD98059, was decreased compared with cells only transfected with the miR-7515 inhibitor (Fig. 9B). Furthermore, compared to the group only transfected with the MEK inhibitor, the inhibition of MEK in miR-7515 inhibitor-transfected cells upregulated the expression levels of cyclin D1, CDK2, MMP-2 and MMP-9 (Fig. 9C). These findings indicated that IGF-1 may regulate the proliferation, migration and invasion of PC cells via targeting the IGF-1-induced Ras/Raf/MEK/ERK signaling pathway.

Discussion

PC is a lethal malignant tumor of the digestive system that is accompanied by high mortality rates. At present, the successful treatment for PC remains a significant challenge (2,21). Previous studies have reported the role of numerous miRNAs in the occurrence and the development of PC (22-24). However, to the best of our knowledge, the

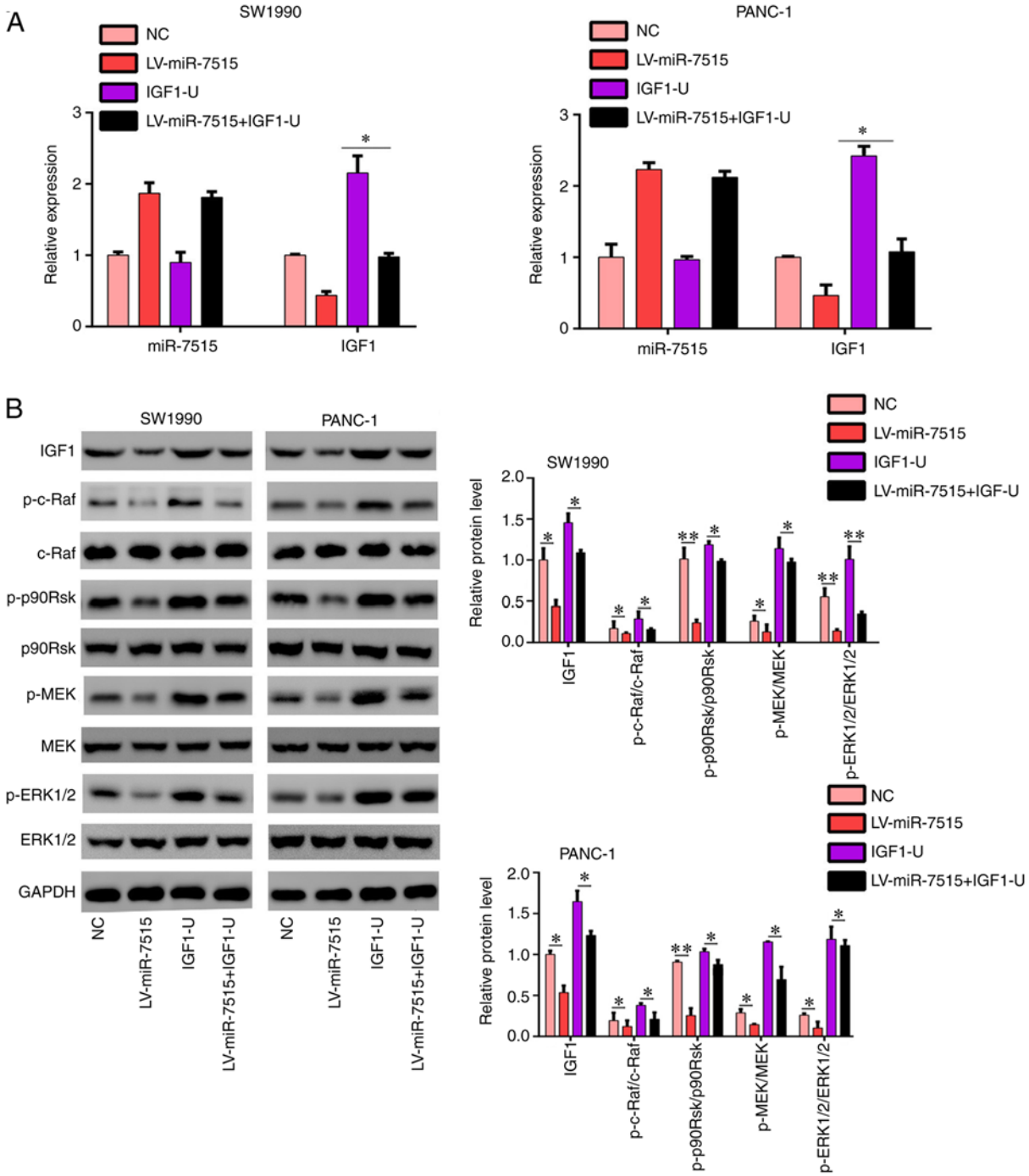

Figure 7. IGF-1 overexpression reverses the regulatory effects of miR-7515 overexpression on the Ras/Raf/MEK/ ERK signaling pathway. PANC-1 and SW1990 cells were transfected with miR-7515 overexpression lentivirus, IGF-1 overexpression plasmid, miR-7515 overexpression lentivirus + IGF-1 overexpression plasmid and corresponding negative control. (A) Reverse transcription-quantitative PCR was used to analyze the expression levels of IGF-1 and miR-7515 in each group. (B) Western blotting was used to analyze the expression levels of IGF-1, p-c-Raf, p-p90RSK, p-MEK, MEK, p-ERK1/2 and ERK1/2 in each group of cells. *P<0.05 and **P<0.01. IGF-1, insulin like growth factor 1; miR, microRNA; p-, phosphorylated; p90RSK, ribosomal protein S6 kinase α-1; NC, negative control; LV, lentivirus.

molecular mechanisms underlying the role of miRNAs in PC remain largely unknown.

miR-7515 is a novel miRNA identified by Lee *et al* in 2013 (25). A previous study demonstrated that miR-7515 expression levels were downregulated in lung cancer cells, which inhibited lung cancer cell proliferation via targeting c-Met (25). Similarly, Chong *et al* (26) revealed that the expression levels of miR-7515 were downregulated in recurrent ovarian cancer compared with primary ovarian cancer. However, to the best of our knowledge, the role of miR-7515 in PC remains unknown. The results of the present study revealed that the expression levels of miR-7515 were downregulated in PC tissues and cell lines, and the low expression of miR-7515 predicted a poor clinical outcome. The overexpression of miR-7515 in PC cells inhibited cell proliferation, migration and invasion both *in vitro* and *in vivo*, while miR-7515 knockdown increased cell

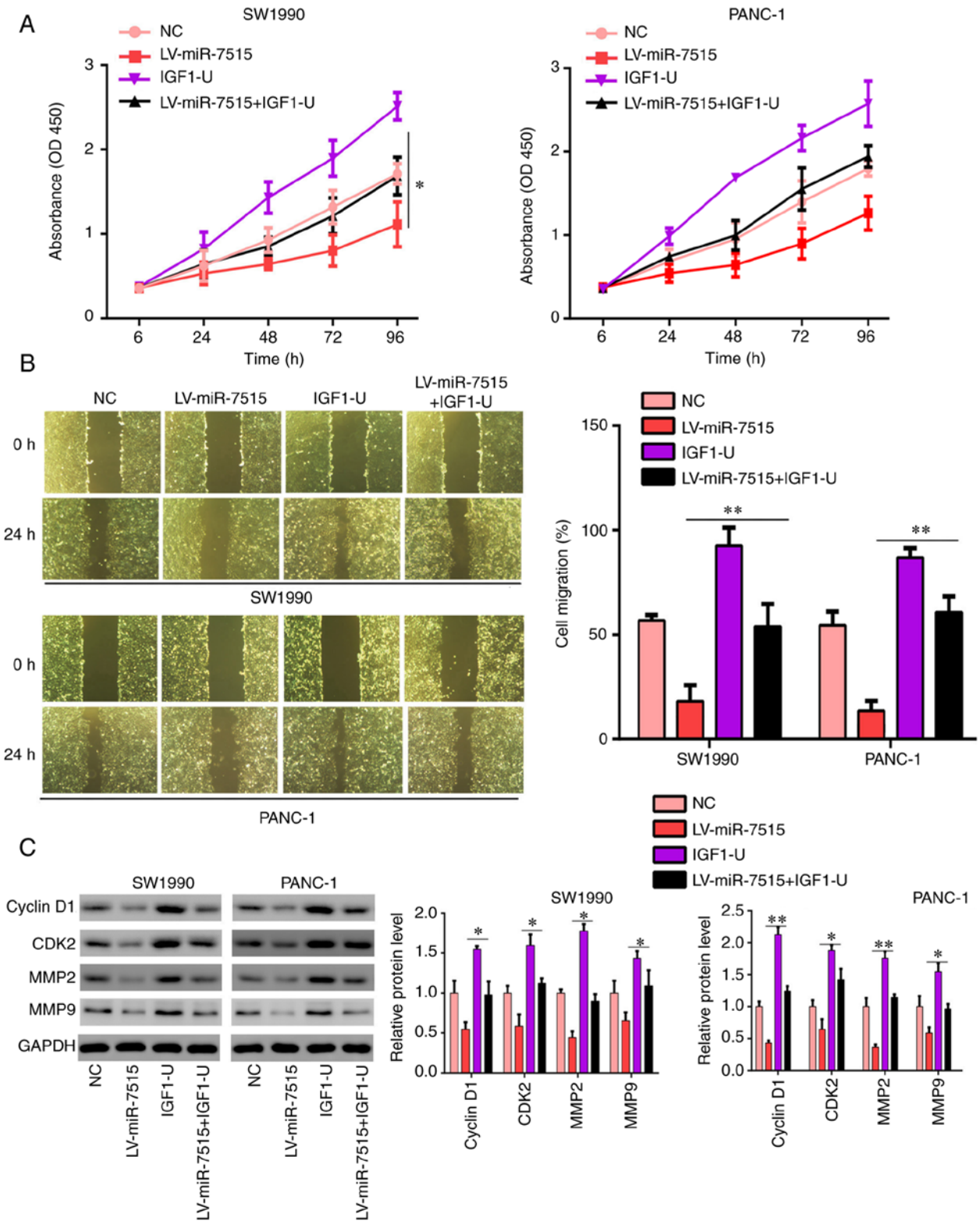

Figure 8. IGF-1 overexpression reverses the effects of miR-7515 on pancreatic cancer cell proliferation and migration. PANC-1 and SW1990 cells were transfected with miR-7515 overexpression lentivirus, IGF-1 overexpression plasmid, miR-7515 overexpression lentivirus + IGF-1 overexpression plasmid and corresponding negative controls. (A) Cell Counting Kit-8 assay was used to analyze the proliferation of each group. (B) Wound healing assay was used to detect the migration of each group. (C) Expression levels of cyclin D1, CDK2, MMP2 and MMP9 were analyzed in each group. *P<0.05 and **P<0.01. IGF-1, insulin like growth factor 1; miR, microRNA; NC, negative control; LV, lentivirus.

proliferation, invasion and migration *in vitro*. To the best of our knowledge, this is the first evidence to suggest that miR-7515 may play a tumor-suppressive role in PC by inhibiting the proliferation, migration and invasion of PC cells.

Accumulating evidence indicates that IGF-related signaling pathways promote the progression of PC to an advanced stage via stimulating the proliferation, metastasis and chemoresistance of PC cells (27). IGF-1 is a member of IGF family, and previous

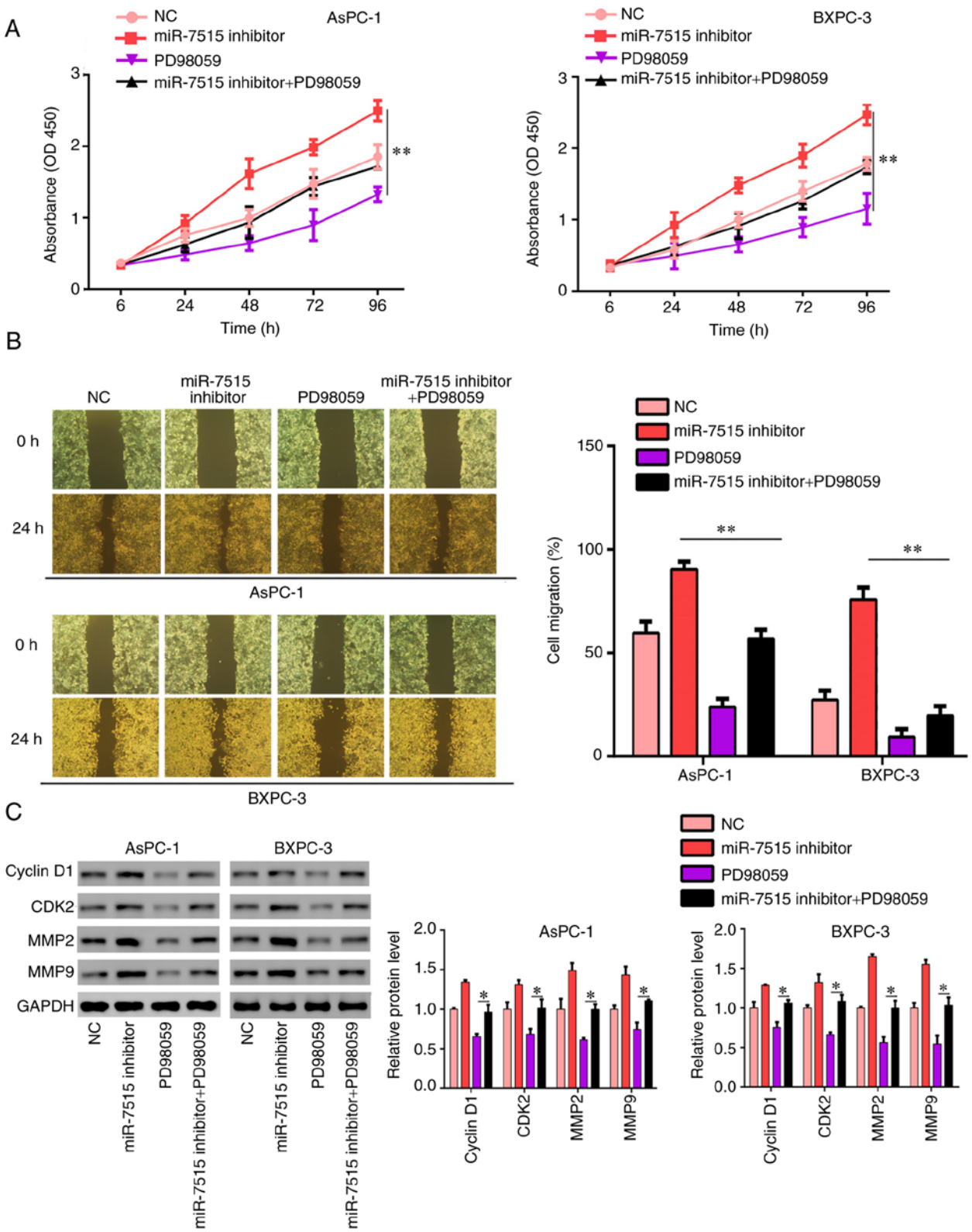

Figure 9. MEK inhibitor blocks the effects of the knockdown of miR-7515 on pancreatic cancer cell proliferation and migration. AsPC-1 and BXPC-3 cells were transfected or treated with miR-7515 inhibitor, MEK inhibitor, PD98059, miR-7515 inhibitor + PD98059 and corresponding negative control. (A) Cell Counting Kit-8 assay was used to detect the proliferation of each group. (B) Wound healing assay was used to detect the migration of each group. (C) Expression levels of cyclin D1, CDK2, MMP2 and MMP9 were analyzed in each group. *P<0.05 and **P<0.01. miR, microRNA; NC, negative control.

studies have demonstrated that the expression levels of IGF-1 were upregulated in PC tissues, which indicates its potential as a diagnostic biomarker in PC (28,29). The upregulated expression of IGF-1 also predicted a poor clinical outcome in patients with PC. Moreover, the IGF-1 receptor (IGF-1R) was revealed to have tyrosine kinase activity (30). Upon IGF-1R binding with IGF-1, the receptor is activated and increases the phosphorylation levels of AKT and MEK proteins, leading to the activation of

intracellular signaling pathways, such as the PI3K/Akt/mTOR pathway and RAS/RAF/MEK/ERK signaling pathway (31). Therefore, the knockdown of IGF-1 expression may represent a potential strategy for the treatment of PC. In the present study, the target genes of miR-7515 were demonstrated to be most enriched in the Ras signaling pathway. In particular, IGF-1 was identified as a target gene of miR-7515, and was revealed to have a high binding score and to be involved in the Ras signaling pathway. Thus, it was hypothesized that miR-7515 may play a tumor-suppressive role in PC via targeting IGF-1 expression, as well as the Ras/RAF/MEK/ERK signaling pathway. Consistent with this hypothesis, the results of the present study revealed that miR-7515 directly bound with IGF-1 and regulated its expression, in addition to regulating the Ras/Raf/MEK/ERK signaling pathway. However, the exact mechanism whereby miRNA-7515 inhibits the Ras/RAF/MEK/ERK signaling pathway requires further investigation. IGF-1 expression levels were revealed to be upregulated in PC tissues and negatively co-expressed with miR-7515 expression. In addition, the overexpression of IGF-1 blocked the inhibitory effects of miR-7515 overexpression on proliferation, migration and invasion of PC cells. Treatment with the MEK inhibitor, PD98059, also blocked the effects of knockdown of miR-7515 on proliferation, migration and invasion of PC cells. To the best of our knowledge, the present study was the first to determine the potential molecular mechanism of miR-7515 in PC. In fact, miRNAs could exhibit powerful effects on the development of malignancy due to their potential to control numerous target genes (32). However, this phenomenon is also a limitation in miRNA research, and future studies are needed to further explore whether there are other pathways involved in PC progression.

In conclusion, the findings of the present study indicated that miR-7515 may be a novel tumor suppressor in PC, and it may suppress proliferation, migration and invasion of PC cells by targeting the IGF-1-induced Ras/Raf/MEK/ERK signaling pathway. Although miRNA-based therapeutics are still under development, our results are encouraging, and suggest that miR-7515 and IGF-1 could serve as clinical targets for the treatment of PC or other tumor types in the future.

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Availability of data and materials

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

Authors' contributions

SL and ZZ confirmed the authenticity of all the raw data. WC and ZZ designed the study. WC, ZZ and ZH performed the experiments and analyzed the data. ZZ and WC wrote the manuscript and were responsible for language revisions. SL made substantial contributions to conception and design, acquisition of data, and analysis and interpretation of the data. All the authors have read and approved the final manuscript.

Ethics approval and consent to participate

Written informed consent was obtained from all patients prior to participation and the patient experimental protocol was approved by the Human Research Ethics Review Committee of Guizhou Medical University (Guiyang, China). All animal experiments were performed in strict accordance with the Guide for the Care and Use of Laboratory Animals and the Principles for the Utilization and Care of Vertebrate Animals. The animal experimental protocols were approved (approval. no. 2000025) by the Ethics Committee of Guizhou Medical University.

Patient consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

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