

miR-539 suppresses the proliferation, migration, invasion and epithelial mesenchymal transition of pancreatic cancer cells through targeting SP1

LIANG XUE*, YAN SHEN*, ZHENGLONG ZHAI and SHUSEN ZHENG

Department of Hepatobiliary and Pancreatic Surgery, The First Affiliated Hospital, Zhejiang University, Hangzhou, Zhejiang 310003, P.R. China

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Abstract. MicroRNA (miR)-539 has inhibitory effects on certain types of cancer, but its role in pancreatic cancer (PCa) remains unclear. The present study investigated the effects of miR-539 on PCa, and aimed to determine possible therapeutic targets for the treatment of PCa. The expression of miR-539 in PCa tissues, paired normal adjacent tissues and PCa cell lines (CAPAN-2, BxPC3, CFPAC1, SW1990 and PANC1), and human non-cancerous pancreatic cells (hTRET-HPNE) was determined and compared. The effects of upregulation and downregulation of miR-539 on proliferation, apoptosis, cell cycle, invasion, migration and epithelial-mesenchymal transition (EMT) of PCa cells were investigated. Additionally, the target gene of miR-539 was predicted and its effects on PCa cells were further investigated. The results revealed low expression of miR-539 in PCa tissues and cell lines. Additionally, increasing miR-539 expression inhibited the proliferation, migration, invasion and EMT of PCa cells and induced apoptosis by blocking G1 phase of the cell cycle, while reducing miR-539 expression had the opposite results. Furthermore, specificity protein 1 (SP1) was found to be the target gene of miR-539. SP1 promoted the proliferation, migration, invasion and EMT transformation of PCa cells, but these effects were

reversed by high expression of miR-539. Additionally, miR-539 suppressed the proliferation, metastasis, invasion and EMT transformation of PCa cells through targeting SP1. Therefore, miR-539 overexpression may contribute toward development of novel therapeutic strategies for PCa in the future.

Introduction

Pancreatic cancer (PCa) has a high degree of malignancy (1). Early diagnosis of the disease is often difficult due to the physiological location and biological characteristics of the pancreas; furthermore, PCa can rapidly progress into later stages and has poor prognosis and high mortality rate in advanced stages (2,3). At present, surgery is the main therapeutic option for treating PCa. However, as PCa does not show specific symptoms in the early stages, patients are often diagnosed with the disease at later stages and therefore lose the optimal chance for surgery (4). Furthermore, patients with PCa develop multi-drug resistance to chemotherapy drugs, which results in poor outcomes for radiotherapy and chemotherapy (5,6). In recent years, previously unrecognized miRNAs and mRNAs have been detected and investigated as effective methods for the clinical diagnosis and treatment of PCa (7-9).

As a class of single-stranded nucleotides, miRNAs participate in the coding and synthesis of proteins and serve critical roles in the physiological and pathological processes of cancer (10). miRNAs can inhibit the expression of target proteins by binding to the 3'-untranslated region (3'-UTR) of the targeted genes, thereby restraining or degrading the translation and expression of mRNAs (11). Previous studies have demonstrated that miRNAs are associated with the migration and deterioration of tumors (12-14). Previous studies also demonstrated that miR-615-5p (15), miR-153 (16) and miR-206 (17) inhibited the metastasis of PCa, while miR-10b (18), miR-212 (19) and miR-367 (20) promoted the migration of PCa.

miR-539 was revealed to act as a tumor suppressor gene in different cancer types, including gastric cancer, prostate cancer and lung cancer (21-24). Low expression of miR-539 has been reported in gastric cancer tissues and cell lines, and this was correlated with the differentiation, clinical stage, poor survival outcome and lymph node metastasis of the

Correspondence to: Dr Shusen Zheng, Department of Hepatobiliary and Pancreatic Surgery, The First Affiliated Hospital, Zhejiang University, 79 Qingchun Road, Hangzhou, Zhejiang 310003, P.R. China
E-mail: shusenzheng@zju.edu.cn

*Contributed equally

Abbreviations: miR, microRNA; PCa, pancreatic cancer; EMT, epithelial-mesenchymal transition; miRNAs, microRNAs; MC, mimic control; IC, inhibitor control; NC, negative control; CCK-8, Cell Counting Kit-8; OD, optical density; FITC, fluorescein isothiocyanate; WB, western blotting

Key words: miR-539, target gene, pancreatic cancer, development, progression

disease (25). Although previous studies have reported that the role of miR-539 in cancer is mainly acting as a tumor suppressor, only few studies have investigated the specific mechanisms of miR-539 in PCa. Specificity protein 1 (Sp1) was one of the earliest transcription factors identified, and it belongs to the Sp1/Krüppel-like factor transcription factor family of sequence-specific DNA binding proteins (26,27). High expression of Sp1 has been detected in gastric cancer and is associated with a poor prognosis of the disease (28). In addition, abnormal Sp1 activation may improve the growth, metastasis and dedifferentiation of PCa and breast cancer (29,30). The results of these studies indicated that Sp1 may serve an important role in cancer development.

In order to further investigate the role of miRNAs in PCa, the present study focused on the effects of miR-539 on PCa. The roles of miR-539 in the proliferation, apoptosis, migration and invasion of PCa cells were analyzed by upregulating and inhibiting the expression of miR-539 in several PCa cell lines. Furthermore, the target gene of miR-539 and the mechanism were studied. The results of the present study provide a molecular mechanism and alternative treating strategy to PCa.

Materials and methods

Tissue samples. PCa tissues and paired normal adjacent tissues were collected from patients with PCa (36 males and 20 females; age range, 29-70 years; median age, 61 years) who received surgical resection in The First Affiliated Hospital, Zhejiang University from January to December 2018. Those who had received radiotherapy, chemotherapy, traditional Chinese medicine or immunotherapy prior to surgery were excluded, and patients enrolled in the study received routine preoperative auxiliary tests. Carcinoma tissues and their paired normal adjacent tissues (2-3 cm from the margin of the carcinoma tissue) were separated from surgical specimens within 30 min, temporarily frozen in liquid nitrogen and stored in a refrigerator at -80°C for expression detection. The present study was approved by the Ethics Committee of the First Affiliated Hospital (approval no. ZJ2018121017), Zhejiang University and written informed consent was obtained from all participants.

Cell culture. Non-cancerous pancreatic cells (hTRET-HPNE) and PCa cell lines (CAPAN-2, BxPC3, CFPAC1, SW1990 and PANC1) were purchased from American Type Culture Collection. All cells were cultured in Dulbecco's modified Eagle's medium (DMEM; Gibco; Thermo Fisher Scientific, Inc., Waltham, MA, USA), containing 10% fetal bovine serum (FBS; HyClone; GE Healthcare Life Sciences), 100 µg/ml streptomycin, 100 units/ml penicillin and 2 mmol/l glutamine (Gibco; Thermo Fisher Scientific, Inc.) in a humid environment at 37°C with 5% CO₂.

Reverse transcription-quantitative polymerase chain reaction (RT-qPCR). Total RNA was isolated from tissues and cells using TRIzol reagent (Invitrogen; Thermo Fisher Scientific, Inc.). The purity and concentration of the extracted total RNAs were determined by NanoDrop-2000c ultramicro spectrophotometer (Thermo Fisher Scientific, Inc.) and 1% agarose modified gel electrophoresis. Taqman microRNA RT

kit (Applied Biosystems; Thermo Fisher Scientific, Inc.) and corresponding primers were used to reverse transcribe the total RNAs into cDNAs, the reverse transcription condition as follows: At 42°C for 40 min, at 85°C for 5 min. SYBR-Green I mix reagent (Toyobo Life Science) and ABI VII A7 fluorescence quantitative PCR reactor were used for amplifying the reaction under the following conditions: Pre-degeneration at 95°C for 30 sec, denaturation at 95°C for 5 sec, annealing at 60°C for 34 sec for a total of 40 cycles. Primer sequences were synthesized by Shanghai Gene Pharma Co., Ltd. (Shanghai, China) and are presented in Table I. Quantitative analysis was conducted using the 2^{-ΔΔC_q} method (31), and the expression of U6 served as the internal reference.

Cell transfection. SW1990 and BxPC3 cells in the logarithmic growth stage were inoculated into 6-well plates at a concentration of 1x10⁵ cells/well. SW1990 cells were respectively transfected with blank, miR-539 mimic (5'-GGAGAAAUU AUCCUUGGUGUGU-3'; cat. no. 4464066; Ambion; Thermo Fisher Scientific, Inc.) and mimic control (MC; 5'-UUU GUACUACACAAAAGUACUG-3'), while BxPC3 cells were respectively transfected with blank, miR-539 inhibitor (5'-ACACACCAAGGAUAAUUCUCC-3'; cat. no. 4464084; Ambion; Thermo Fisher Scientific, Inc.) and inhibitor control (IC; 5'-CAGUACUUUUGUGUAGUACAA-3'). In addition, the SW1990 cells were also co-transfected with MC and negative control (NC), MC and SP1, mimic and NC (pcDNA3.1 empty plasmid), and mimic and SP1 (pcDNA3.1-SP1 plasmid; Ambion; Thermo Fisher Scientific, Inc.). Similarly, the BxPC3 cells were co-transfected with IC and siNC (5'-UUCUCCGAA CGUGUCACGU-3'), IC and siSP1 (5'-CAGAUACCAGAC CUCUUCU-3'; cat. no. 4392420; Ambion; Thermo Fisher Scientific, Inc.), inhibitor and siNC, and inhibitor and siSP1. A total of 5 µg/well Lipofectamine 2000 reagent (Invitrogen; Thermo Fisher Scientific, Inc.) was used for cell transfection. All the cells were cultured in the medium (DMEM; Gibco; Thermo Fisher Scientific, Inc.) containing 10% FBS, 50 U/ml penicillin and 50 µg/ml streptomycin at 37°C with 5% CO₂.

Cell activity. Cell Counting kit-8 (CCK-8; Dojindo Molecular Technologies, Inc.) was mixed with the culture medium at a ratio of 1:10 to detect the proliferation abilities of SW1990 and BxPC3 cells following the manufacturer's protocol. The cells were transferred into the 96-well plates at a density of 5x10⁴/well, and 110 µl mixed medium was added. The plates were maintained in the dark at 37°C with 5% CO₂, and optical density (OD) was recorded using a microplate reader (BioTek Instruments, Inc.) at 450 nm after 24, 48 and 72 h of cultivation.

Cell apoptosis. Annexin V-FITC Apoptosis Detection kit (BD Biosciences) was used to determine the apoptotic rates of SW1990 and BxPC3 cells. The culture medium was removed 48 h after transfection. The cells were washed with pre-cooled phosphate-buffered saline (PBS) at 4°C and digested by 0.25% trypsin. Next, the supernatant was discarded (800 x g) by centrifugation, and sediments were washed twice with PBS. According to the manufacturer's protocol, 5 µl fluorescein isothiocyanate (FITC) and 5 µl propidium iodide (PI) were added into the cells and incubated together at

Table I. Primer base sequence.

Gene	Forward (5'-3')	Reverse (5'-3')
miR-539	GAAGAGGCTAACGTGAGGTTG	CACCATGACCAAGCCACGTAG
U6	GCTTCGGCAGCACATATACTAAAT	CGCTTCACGAATTTGCGTGTTCAT

room temperature in the dark for 15 min. Cell apoptosis was analyzed by Beckman CoulterFC500 (Beckman Coulter, Inc.).

Cell cycle. The transfected cells (SW1990 and BxPC3; at 1×10^5) were collected, washed with PBS and fixed using 70% ice ethanol overnight at 4°C. Next, cell DNA was stained with 1 mg/ml RNase A and 50 mg/ml PI at room temperature for 30 min. Cell Lab Quanta SC flow cytometry (Beckman Coulter, Inc.) was used to analyze the cell cycles in G1, S and G2 phases.

Cell migration. The cells (SW1990 and BxPC3) were inoculated at 1×10^5 /ml into 6-well plates and cultured at 37°C with 5% CO₂ for 24 h. After the cells have been covered with monolayer, scratches were created in the center of the 6-well plates using a 1,000 μ l spear head. Images were captured using an inverted light microscope (Eclipse TS-100; Nikon Corporation) at x100 magnification at 0 and 48 h.

Cell invasion. Transwell invasion assays (Costar; Corning, Inc.) were performed to detect cell invasion abilities. A total of 50 μ l Matrigel (Costar; Corning, Inc.) at a dilution ratio of 1:8 was inserted into the upper chamber of the Transwell and solidified at 37°C for 30 min. Cell suspension (serum-free medium) at a density of 1×10^5 /ml was added to the upper chamber, while the lower chamber was covered with DMEM containing 20% FBS. The chambers were cultured at 37°C with 5% CO₂ for 48 h. After the cells had been fixed with 70% ethanol for 30 min at room temperature and stained with 0.1% crystal violet for 20 min at room temperature, the number of cells was counted under a light microscope (magnification, x200; Zeiss AG).

Western blot analysis. Western blotting (WB) was performed to determine the expression levels of proteins associated with epithelial-mesenchymal transition (EMT). The total intracellular proteins were extracted using radioimmunoprecipitation assay lysis buffer (Beyotime Institute of Biotechnology, Haimen, China), and the protein concentration was determined using bicinchoninic acid (Pierce; Thermo Fisher Scientific, Inc.). A total of 50 μ g protein was separated on 10-12% SDS-PAGE (Beyotime Institute of Biotechnology) and transferred to polyvinylidene difluoride membranes (GE Healthcare) and then blocked with 5% non-fat milk at room temperature for 2 h. Primary antibodies: E-cadherin (E-cad; cat. no. 14472; Cell Signaling Technology, Inc.), N-cadherin (N-cad; cat. no. 14215; Cell Signaling Technology, Inc.), Snail (cat. no. ab53519; Abcam), SP1 (cat. no. ab13370; Abcam) and GAPDH (cat. no. ab8245; Abcam) at a dilution of 1:1,000 at 4°C were used to incubate the membranes overnight. GAPDH served as internal reference. Next, a horseradish

peroxidase-labeled secondary antibody (dilution, 1:1,000; cat. no. ab6728; Abcam) was added to incubate the membranes for another hour at room temperature. An enhanced chemiluminescence kit (Thermo Fisher Scientific, Inc.) was used for color development.

Dual-luciferase reporter. Targetscan 7.2 (<http://www.targetscan.org/>) predicted that SP1 was possibly the target gene for miR-539, and the prediction was further verified by double-luciferase reporter gene analysis. The SP1 3'-UTR region containing the sequence of miR-539 binding site was inserted into the pMIR-reporter vector (Guangzhou RiboBio Co., Ltd.) to construct mutant carrier (SP1-MUT) and wild-type vector (SP1-WT). miR-539 mimic was transfected into SW1990 cells with SP1-MUT or SP1-WT using Lipofectamine 2000 (Invitrogen; Thermo Fisher Scientific, Inc.), while BxPC3 cells were respectively co-transfected with miR-539 inhibitor and the two plasmids. The cells were collected 48 h after the transfection, and double-luciferase reporter gene analysis system (Promega Corporation) was performed to determine the activities of firefly and *renilla* luciferase.

Statistical analysis. Statistical Package of the Social Sciences 20.0 software (IBM Corp.) was used for data analysis. The data are presented as the mean \pm standard deviation, and Student's t-test was performed for comparison in two groups, while one-way analysis of variance, followed by the Tukey's test, was conducted for comparing differences among multiple groups. The association between miR-539 expression and clinicopathological factors of PCa was analyzed using the Pearson's χ^2 test. All independent experiments *in vitro* were performed in triplicate. $P < 0.05$ was considered to indicate a statistically significant difference.

Results

miR-539 had a low expression in PCa tissues and cell lines. The expression of miR-539 was significantly reduced in cancer tissues compared with that in their paired normal adjacent tissues of PCa ($P < 0.001$; Fig. 1A). According to the median as the segmentation point, the expression of miR-539 was divided into high expression and low expression. As shown in Table II, miR-539 expression is associated with tumor size, Tumor-Node-Metastasis (TNM) stage and lymph node metastasis (LNM) of patients. In brief, patients with tumor size ≥ 2 , higher TNM stage and presenting LNM had lower miR-539 expression. In PC cell lines, the expression level of miR-539 was the lowest in SW1990 cells and the highest in BxPC3 cells ($P < 0.001$; Fig. 1B). In addition to the expression of miR-539 in different PCa cell lines, cells with fast growth rates and good growth were selected. Therefore, in subsequent experiments,

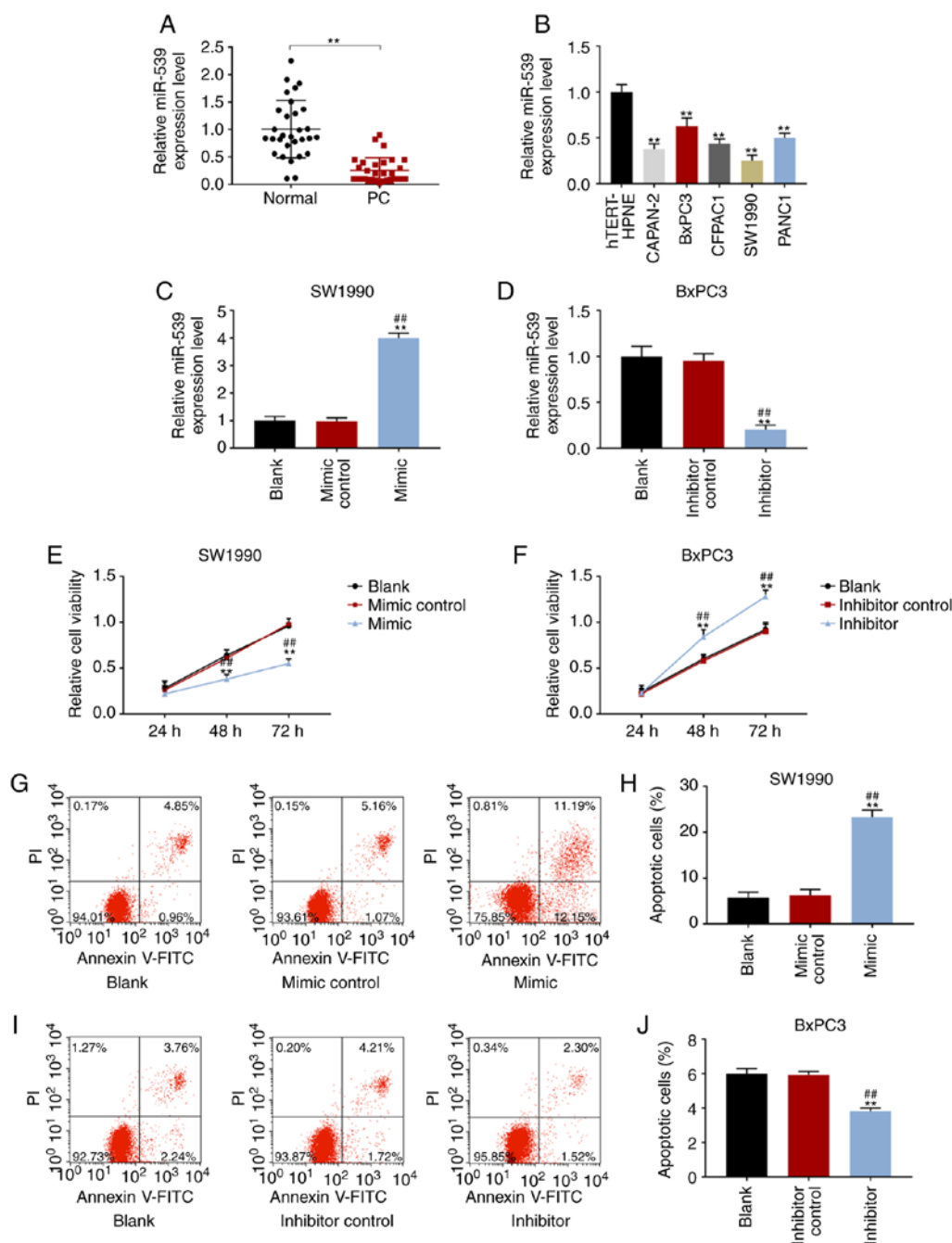


Figure 1. Overexpression of miR-539 inhibited the activities of PCa cells and promoted apoptosis. According to the results from RT-qPCR, the expression levels of miR-539 in (A) cancerous tissues and their paired normal adjacent tissues from patients with PCa (n=56) and in (B) non-cancerous pancreatic cells (hTERT-HPNE) and PCa cell lines (CAPAN-2, BxPC3, CFPAC1, SW1990 and PANC1). RT-qPCR assays showed the expression levels of miR-539 in (C) SW1990 and (D) BxPC3 cells after transfection. The activities of (E) SW1990 and (F) BxPC3 cells after transfection for 24, 48 and 72 h were detected by Cell Counting kit-8. Apoptosis figures and corresponding quantitative analyses of (G and H) SW1990 and (I and J) BxPC3 cells after transfection were tested by flow cytometry. SW1990 cells were transfected with blank, mimic control and miR-539 mimic, while BxPC3 cells were transfected with blank, inhibitor control and miR-539 inhibitor. **P<0.001, vs. normal, hTERT-HPNE, or blank; ##P<0.001, vs. mimic control or inhibitor control (n=3). PCa, pancreatic cancer; RT-qPCR, real time-quantitative polymerase chain reaction.

miR-539 was overexpressed in SW1990 cells, while BxPC3 cells were treated with an miR-539 inhibitor. The transfection results revealed that the expression of miR-539 was increased markedly in the mimic group but was decreased significantly in the inhibitor group, suggesting that the transfection was successful (P<0.001; Fig. 1C and D).

Over-expression of miR-539 inhibited the activities of PCa cells and promoted apoptosis. After transfection for 48 and

72 h, the activity of SW1990 cells was significantly reduced in the mimic group, while that of BxPC3 was notably increased in the inhibitor group (P<0.001; Fig. 1E and F). Furthermore, the apoptotic rate of SW1990 cells transfected with miR-539 increased greatly, while that of BxPC3 cells transfected with an miR-539 inhibitor markedly decreased (P<0.001; Fig. 1G-J). In addition, the cell proportion in G1 phase was notably increased by elevated miR-539 expression, but decreased in S phase of SW1990 cells (P<0.001); however, no significant

Table II. Associations between miR-539 expression and clinicopathological characteristics of patients with pancreatic cancer.

Clinical factor	miR-539 expression		P-value
	Low expression (n=28)	High expression (n=28)	
Age, years			0.567
<60	18	20	
≥60	10	8	
Sex			0.577
Male	19	17	
Female	9	11	
Tumor size, cm			0.016 ^a
<2	9	18	
≥2	19	10	
Tumor differentiation			0.179
Well	10	15	
Poor	18	13	
TNM stage			0.014 ^a
I+II	7	16	
III+IV	21	12	
Lymph node metastasis			0.031 ^a
Negative	8	16	
Positive	20	12	
Distant metastasis (M) status			0.105
M0	13	19	
M1	15	9	

^aP<0.05. TNM, Tumor-Node-Metastasis.

effect of miR-539 on G2 phase was observed (Fig. 2A and B). Additionally, in BxPC3 cells, suppression of miR-539 significantly reduced cell proportion in G1 phase, but increased cell proportion in the S phase (P<0.001), and there was no notable difference in G2 phase (Fig. 2C and D).

Over-expression of miR-539 suppressed the migration and invasion abilities of PCa cells. The migration distance of SW1990 cells in the mimic group was shorter than that in the blank and mimic control groups 48 h after the transfection (P<0.001; Fig. 2E and F), while the distance of BxPC3 cells transfected with miR-539 inhibitor was significantly longer than that of the blank and inhibitor control groups (P<0.001; Fig. 2G and H). The results of Transwell assays revealed that the number of SW1990 cells in the mimic group that penetrated into the lower chamber was significantly fewer than that in the blank and control groups (P<0.001; Fig. 3A and B), while number of BxPC3 cells in the inhibitor group that penetrated into the lower chamber was markedly more than the blank and control groups (P<0.001; Fig. 3C and D). Furthermore, WB of EMT-related proteins showed that over-expression of miR-539 increased the expression of E-cad in SW1990 cells, while the expressions of N-cad and Snail were inhibited (P<0.001; Fig. 3E and F). Correspondingly, in BxPC3 cells, low expression of miR-539 suppressed the expression of E-cad,

but increased the expressions of N-cad and Snail (P<0.001; Fig. 3G and H).

SP1 was the target gene of miR-539. TargetsCan7.2 revealed that the position 3197-3204 of SP1 3'-UTR could be coupled with hsa-miR-539-5p, suggesting that SP1 may be the target gene for miR-539 (Fig. 4A). Dual-luciferase reporter gene analysis demonstrated that the luciferase activity of SW1990 cells co-transfected with SP1-WT and miR-539 mimic decreased significantly (P<0.001; Fig. 4B), while that of BxPC3 cells co-transfected with SP1-WT and miR-539 inhibitor increased significantly (P<0.001; Fig. 4C), suggesting that SP1 was the target gene for miR-539. Therefore, in the subsequent experiments, miR-539 mimic, inhibitor and SP1, and siSP1 were co-transfected to SW1990 and BxPC3 cells, respectively. The results of WB showed that the expression of SP1 in MC+SP1 group was significantly higher than that in MC+NC and mimic+SP1 groups, and that the expression of SP1 in mimic+SP1 group was significantly higher than that in mimic+NC group (P<0.001; Fig. 4D and E). In BxPC3 cells, SP1 expression was successfully inhibited in IC+siSP1 and inhibitor+siSP1 groups (P<0.001; Fig. 4F and G).

Over-expression of miR-539 inhibited the activity, migration and invasion of PCa cells and promoted cell apoptosis by

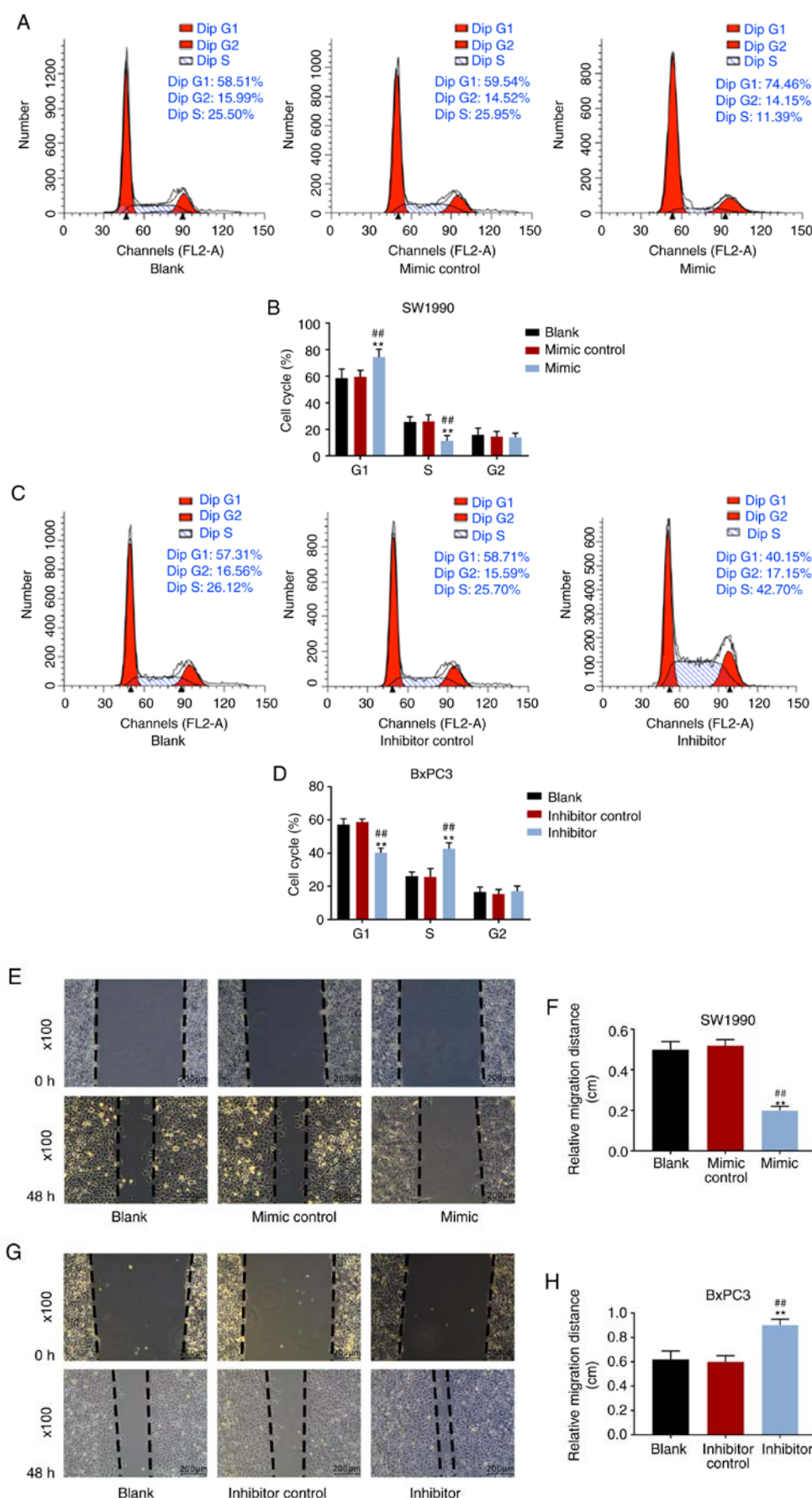


Figure 2. Over-expression of miR-539-suppressed the migration of pancreatic cancer cells. Cell cycle (G1, S and G2) diagrams and corresponding quantitative analyses of (A and B) SW1990 cells and (C and D) BxPC3 cells after transfection were measured by flow cytometry. Migration distances under the microscope and corresponding quantitative analyses of (E and F) SW1990 and (G and H) BxPC3 cells at 0 or 48 h after transfection were determined by scratch tests. SW1990 cells were transfected with blank, mimic control and miR-539 mimic, while BxPC3 cells were transfected with blank, inhibitor control and miR-539 inhibitor. ** $P < 0.001$, vs. blank; *** $P < 0.001$, vs. mimic control or inhibitor control ($n = 3$).

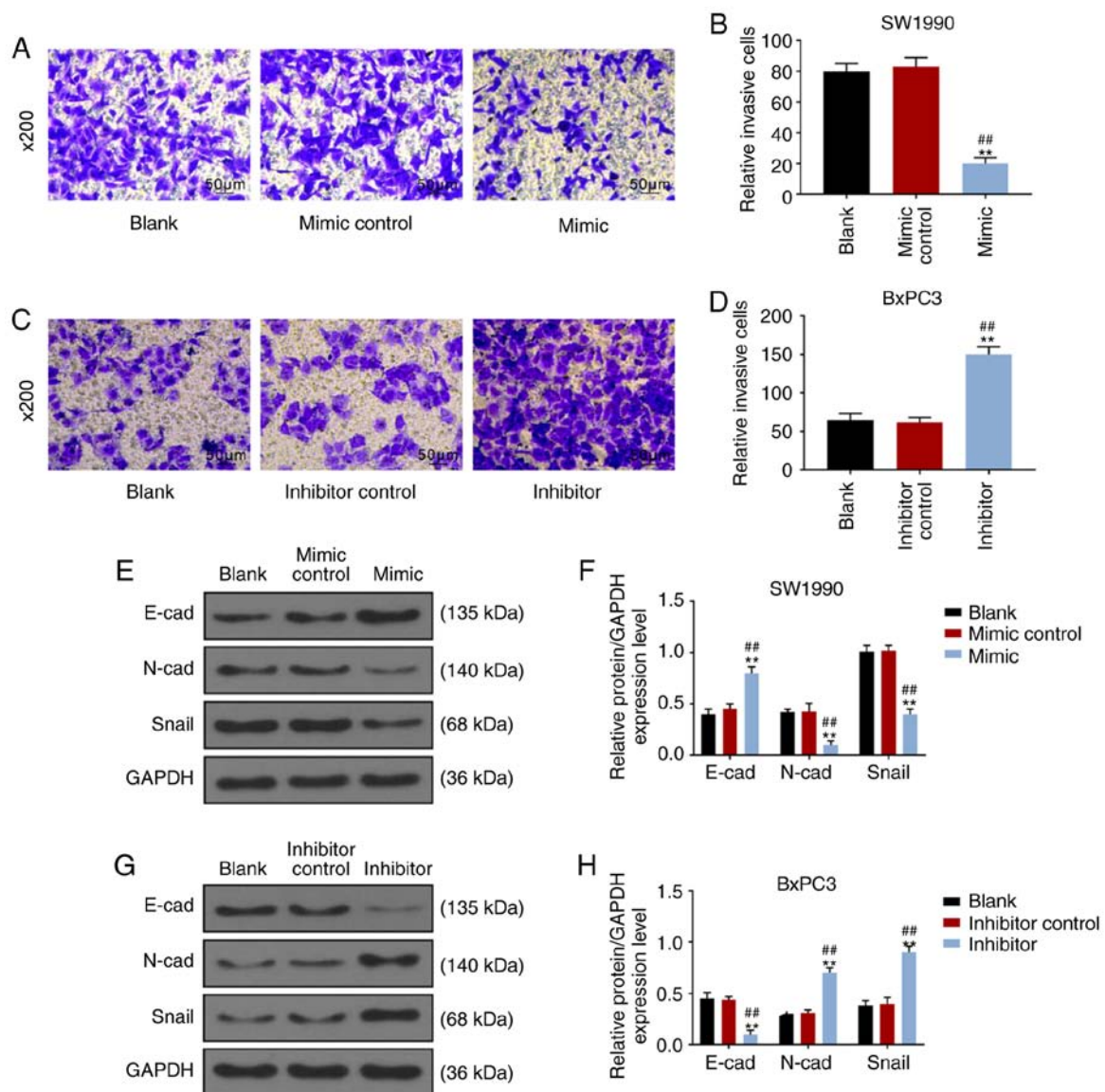


Figure 3. Overexpression of miR-539 inhibited the invasion and epithelial-mesenchymal transition of pancreatic cancer cells. The numbers of invaded cells under the microscope and corresponding quantitative analyses of (A and B) SW1990 and (C and D) BxPC3 cells based on the Transwell assays. Western blot analysis showed the expression levels of EMT-related proteins, including E-cadherin (E-cad), N-cadherin (N-cad) and Snail, in (E and F) SW1990 and (G and H) BxPC3 cells. GAPDH served as the internal reference. SW1990 cells were transfected with blank, mimic control and miR-539 mimic, while BxPC3 cells were transfected with blank, inhibitor control and miR-539 inhibitor. ** $P < 0.001$, vs. blank; ^{##} $P < 0.001$, vs. mimic control or inhibitor control (n=3).

targeting *SP1*. CCK-8 results revealed that *SP1* increased the activity of SW1990 cells and reversed the inhibitory effect produced by overexpressed miR-539 ($P < 0.001$; Fig. 4H), while downregulation of *SP1* reduced the activity of BxPC3 cells and weakened the promoted effect produced by low expression of miR-539 ($P < 0.001$; Fig. 4I). In cell apoptosis experiments, the apoptosis rate of SW1990 cells transfected with *SP1* decreased significantly ($P < 0.05$), which could be reversed by over-expression of miR-539 ($P < 0.001$; Fig. 5A and B). Additionally, the apoptotic rate of BxPC3 cells transfected with si*SP1* increased significantly ($P < 0.001$), and miR-539 inhibitor could reverse the pro-apoptosis effect produced by low expression of *SP1* ($P < 0.001$; Fig. 5C and D). Furthermore, it was revealed that the migratory distance and number of invasive SW1990 cells were increased by *SP1*. By contrast, the migratory distance became shorter and the number of invasive BxPC3 cells was reduced by inhibiting *SP1*. However, this phenomenon could

be reversed by overexpression of miR-539 or its inhibitor ($P < 0.001$; Figs. 5E-H and 6A-D). Furthermore, *SP1* inhibited the expression of E-cad and promoted the expression of N-cad and Snail, while silencing *P1* produced the opposite results ($P < 0.001$). Notably, overexpression of miR-539 attenuated the effects of *SP1* on EMT-related proteins, while miR-539 inhibitor reversed the effects of si*SP1* ($P < 0.001$; Fig. 6E-H).

Discussion

PCa is a highly invasive cancer and its 5-year survival rate is less than 5% (32). Surgical resection is an effective method in treating PCa, but ~60% of PCa patients have lost the optimal opportunities for undergoing surgery at the time of diagnosis, and only 15% of patients are suitable for taking radical surgery. Furthermore, median postoperative survival time for PCa patients is only 20-23 months even they underwent

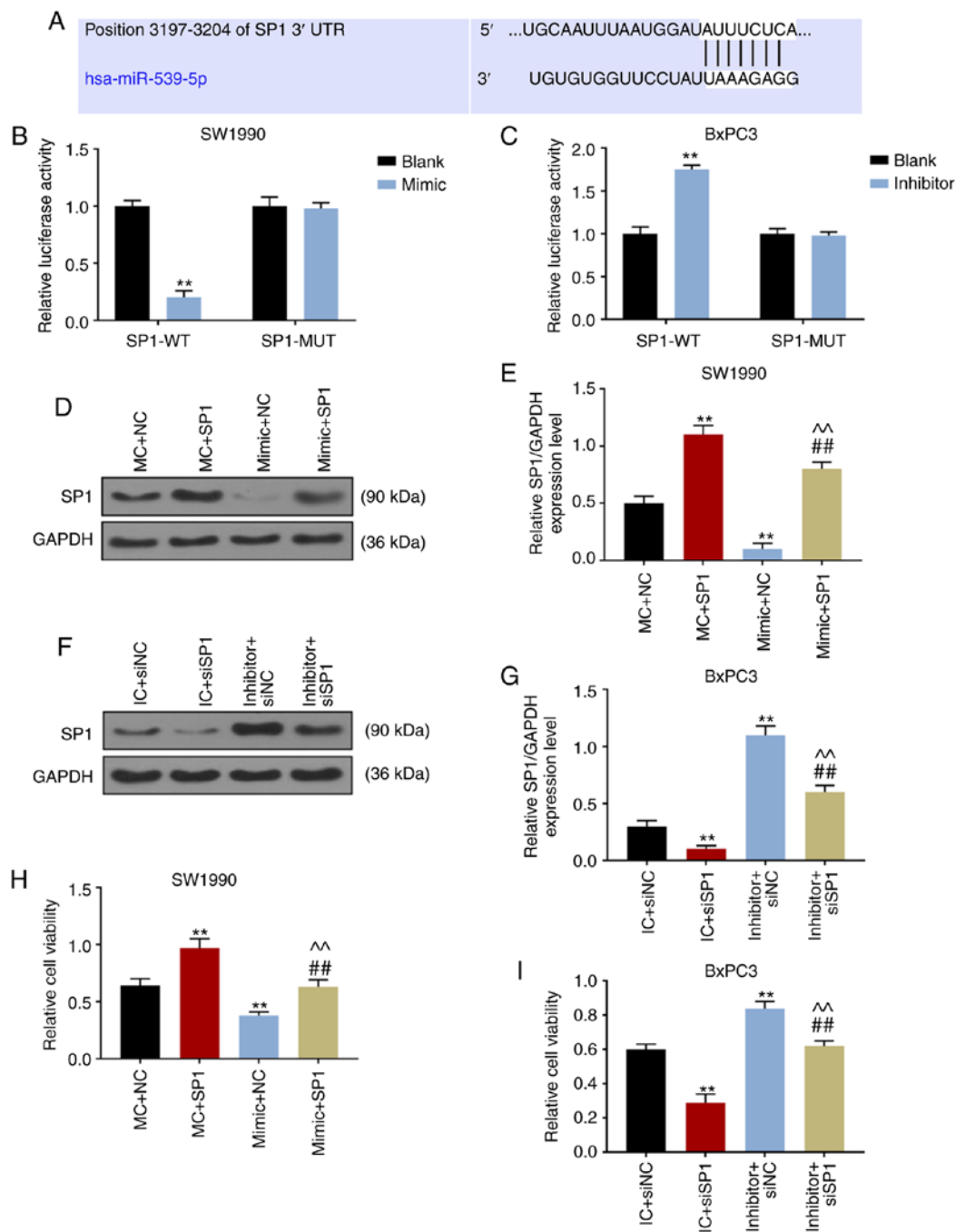


Figure 4. SP1 was the target gene of miR-539. (A) Targetscan7.2 predicted that the position 3197-3204 of SP1 3'URT could be coupled with hsa-miR-539-5p. Results of dual-luciferase reporter gene analysis in (B) SW1990 cells co-transfected miR-539 mimic with wild-type SP1 (SP1-WT) and mutant SP1 (SP1-MUT) and that in (C) BxPC3 cells co-transfected miR-539 inhibitor with SP1-WT or SP1-MUT. The expression levels of miR-539 in (D and E) SW1990 and (F and G) BxPC3 cells after transfection were determined by the western blot analysis. The activities of (H) SW1990 and (I) BxPC3 cells after 48-h transfection were detected using Cell Counting kit-8. (D-I) SW1990 cells were co-transfected with MC and negative control (NC, pcDNA3.1 empty plasmid), mimic control (MC) and SP1, mimic and NC, and mimic and SP1. Similarly, BxPC3 cells were co-transfected with inhibitor control (IC) and siNC, IC and siSP1, inhibitor and siNC, and inhibitor and siSP1. ** $P < 0.001$, vs. blank, MC + NC or IC + siNC; ## $P < 0.001$, vs. MC + SP1 or IC + siSP1; ^ $P < 0.001$, vs. mimic + NC or inhibitor + siNC (n=3).

surgery and chemotherapy (33-35). Therefore, finding effective methods for treating PCa is important. Previous studies have found abnormal expression of miRNAs in PCa, and that this correlates with the development, differentiation, invasion and metastasis of PCa (36,37). The results of the present study revealed that the expression level of miR-539 in cancerous tissues from PCa patients was markedly lower than that in adjacent tissues and PCa cell lines, suggesting that miR-539 may be associated with the occurrence and progression of PCa.

A previous study reported that the expression of miR-539 suppressed the proliferation, invasion and migration of PCa cell lines (38). Jin and Wang (39) also found that miR-539 could act as an inhibitor to control osteosarcoma progression. A recent study reported that miR-539 overexpression suppressed the invasion, migration and EMT of BxPC-3 and PANC-1 cells through targeting TWIST1 (40). Similarly, in this study, the overexpression of miR-539 reduced the proliferation, migration and invasion of PCa cells, whereas the inhibition

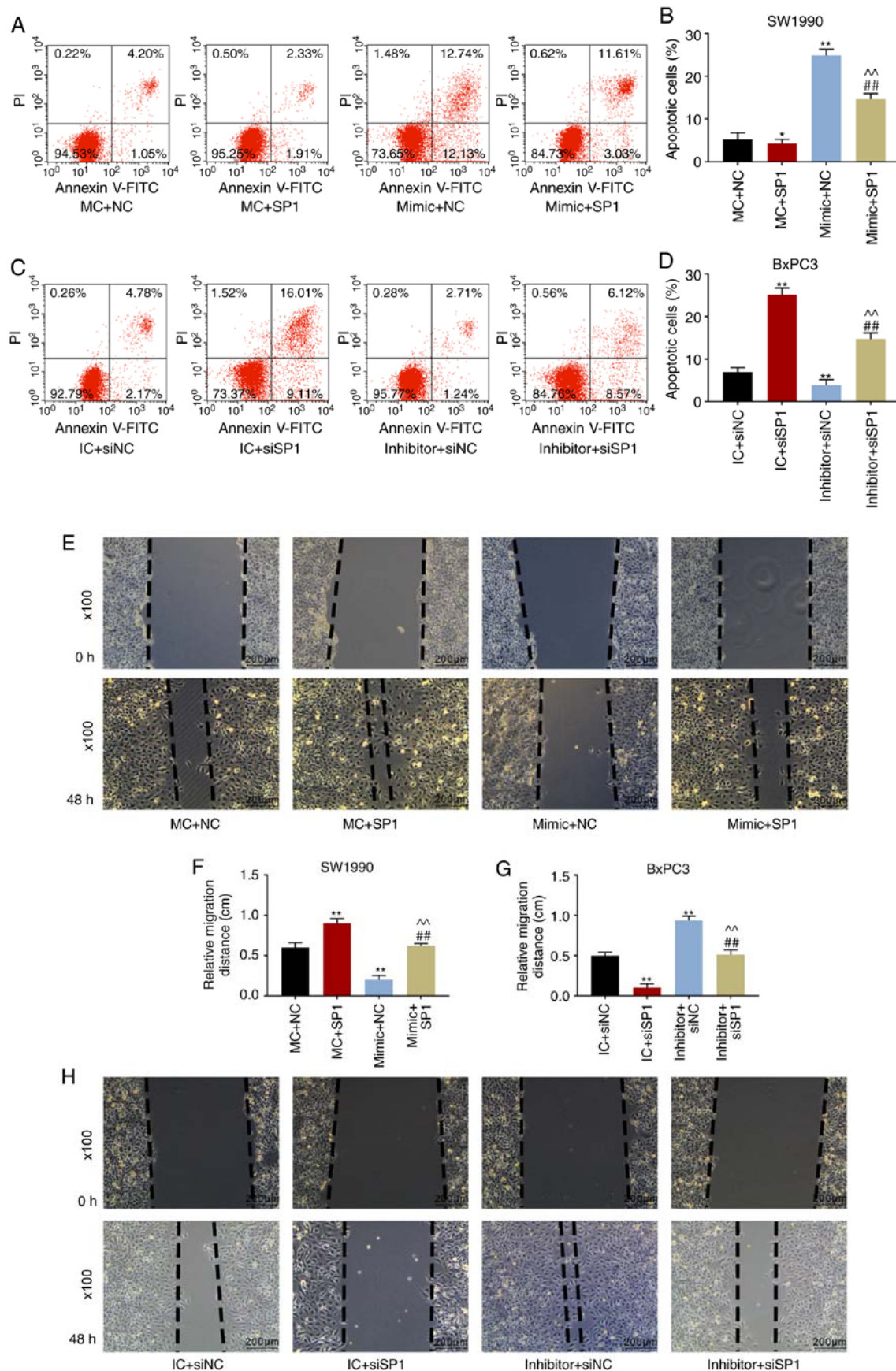


Figure 5. Over-expression of miR-539 suppressed migration of pancreatic cancer cells and promoted apoptosis. Apoptosis figures and corresponding quantitative analyses of (A and B) SW1990 and (C and D) BxPC3 cells after transfection were measured by flow cytometry. Migration distances under the microscope and corresponding quantitative analyses of (E and F) SW1990 and (G and H) BxPC3 cells at 0 or 48 h after transfection were determined by scratch tests. SW1990 cells were co-transfected with MC and negative control (NC, pcDNA3.1 empty plasmid), mimic control (MC) and SP1, mimic and NC, and mimic and SP1. Similarly, BxPC3 cells were co-transfected with inhibitor control (IC) and siNC, IC and siSP1, inhibitor and siNC, and inhibitor and siSP1. *P<0.05, **P<0.001, vs. MC + NC or IC + siNC; ##P<0.001, vs. MC + SP1 or IC + siSP1; ^^P<0.001, vs. mimic + NC or inhibitor + siNC (n=3).

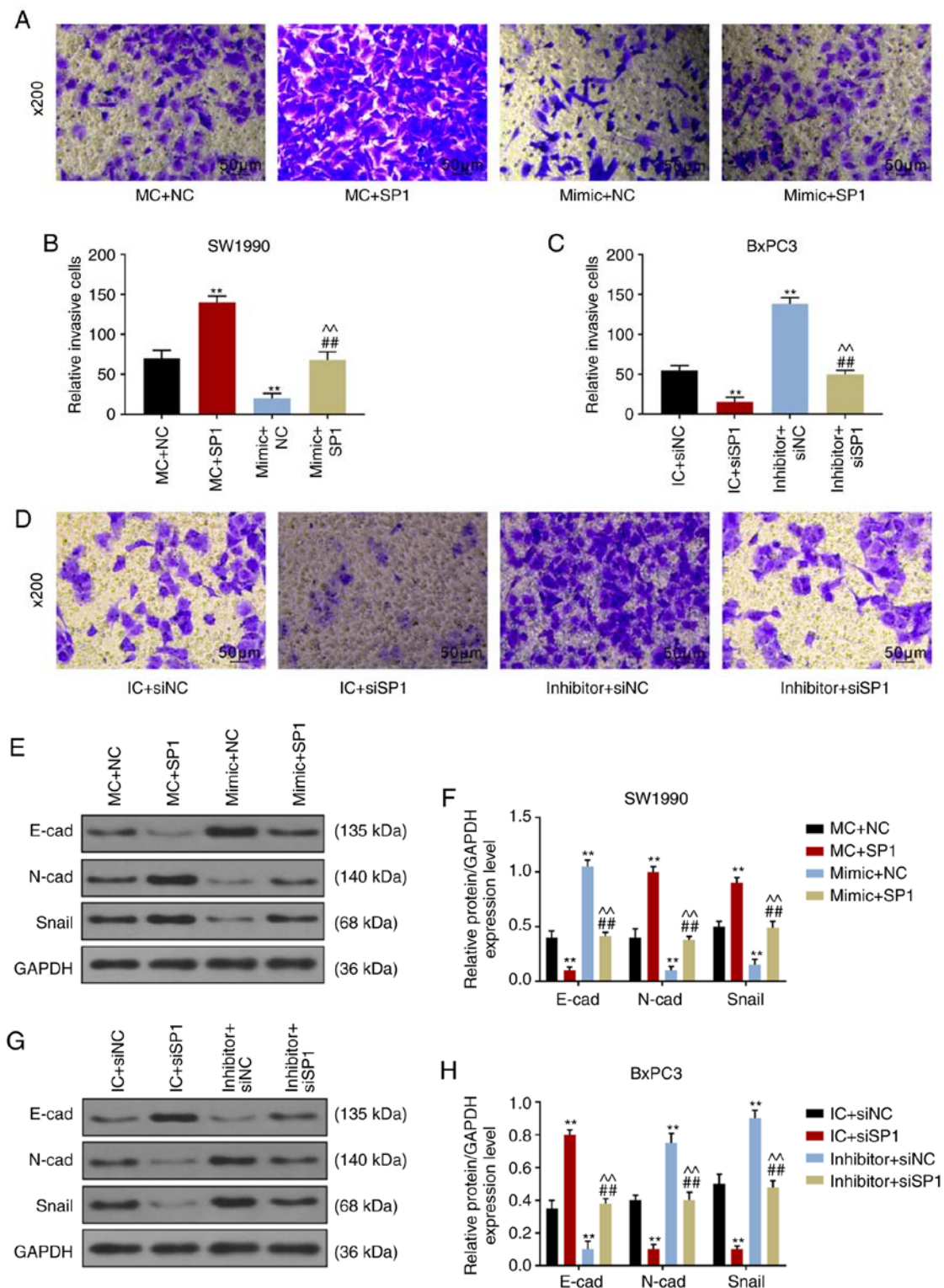


Figure 6. The numbers of invaded cells under the microscope and corresponding quantitative analyses of (A and B) SW1990 cells and (C and D) BxPC3 cells based on the Transwell assays. Experimental results of western blotting showed the expression levels of EMT-related proteins, including E-cadherin (E-cad), N-cadherin (N-cad) and Snail, in (E and F) SW1990 and (G and H) BxPC3 cells. GAPDH served as the internal reference. SW1990 cells were co-transfected with MC and negative control (NC, pcDNA3.1 empty plasmid), mimic control (MC) and SP1, mimic and NC, and mimic and SP1. Similarly, BxPC3 cells were co-transfected with inhibitor control (IC) and siNC, IC and siSP1, inhibitor and siNC, and inhibitor and siSP1. ** $P < 0.001$, vs. MC + NC or IC + siNC; ^## $P < 0.001$, vs. MC + SP1 or IC + siSP1; ^^ $P < 0.001$, vs. mimic + NC or inhibitor + siNC ($n = 3$).

of miR-539 produced the opposite effect. Furthermore, the present study revealed that miR-539 induced the apoptosis of PCa cell lines mainly through blocking the cell cycle in G1 phase, which was consistent with the results obtained by

Deng *et al* (41) that miR-539 could cause cell cycle arrest in non-small cell lung cancer cell lines. A previous study also confirmed that miR-539 regulates the growth of nasopharyngeal carcinoma cells through cell cycle arrest (42). Therefore,

it was confirmed that the upregulation of miR-539 played an active role in PCa.

EMT is the initial event indicating tumor metastasis (43). E-cad, N-cad and Snail are EMT-related proteins. E-cad is an adhesion protein, and downregulating the expression of E-cad can decrease or even eliminate adhesion between cells, thereby leading to tumor detachment, local migration or distant spread via blood vessels and lymphatic ducts from primary site (44). Notably, N-cad and Snail have completely opposite effects to that of E-cad, and the upregulation of N-cad and Snail accelerates tumor metastasis (45). A previous study revealed that certain miRNAs could inhibit the occurrence of EMT by reducing the expression of N-cad and Snail, and increasing the synthesis of E-cad, thereby preventing tumor metastasis (46). The results of the present study revealed that overexpression of miR-539 may promote the expression of E-cad, while inhibiting the expression of N-cad and Snail. Additionally, the suppression of miR-539 resulted in opposite outcomes, suggesting that miR-539 could block the metastasis and invasion of PCa cells through blocking EMT.

Furthermore, while investigating the mechanism of miR-539 in PCa, the results of the present study suggested that SP1 was the target gene for miR-539. SP1, which is an important transcription factor, is involved in cell proliferation, apoptosis and differentiation, and associated with the expression of genes related to tumor growth and metastasis (47,48). The results of the present study demonstrated that increased SP1 promoted the proliferation, migration, invasion and EMT of PCa cells and inhibited cell apoptosis, while silencing of SP1 produced the opposite effects, indicating that SP1 may facilitate the development and progression of PCa. Xia *et al* (49) reported that the downregulation of SP1 could reduce the proliferation rate of ovarian cancer cells and restrain tumor metastasis. Mei *et al* (50) revealed that certain miRNAs may suppress the multiplication, migration and EMT of esophageal cancer cells by targeting SP1. Notably, it was discovered that the overexpression of miR-539 inhibited the effects of SP1 on PCa cell lines, and that SP1-silencing reversed the effect of low expression of miR-539, showing that miR-539 was involved in the occurrence and development of PCa through targeting SP1. However, there are questions that remain to be answered from basic research to clinical application. The biological effects of miRNAs are microenvironment-dependent or cell-type-dependent, and the expression of miR-539 varies in different PCa cell lines, which may also have different effects on the proliferation, invasion, migration and EMT of different PCa cells. The expression of miR-539 differs in different PCa cells, and the effect of miR-539 on the viability of different PCa cells may also vary, which requires further investigation. However, miR-539 may still serve an anti-cancer role in different PCa cells. Although there are limitations to the present study, and *in vivo* experiments should be performed to further support the present data, the findings in the present study further elucidate the underlying mechanism of miR-539 in PCa.

In conclusion, miR-539 is involved in the development and progression of PCa through targeting SP1 to suppress the proliferation, metastasis, invasion, apoptosis and EMT of PCa cells, at least in SW1990 and BxPC3 cells. Therefore, we hypothesize that miR-539 may be investigated as a novel therapeutic strategy for PCa treatment in the future.

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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

LX and YS made substantial contributions to the conception and design. ZZ was involved in data acquisition, analysis and interpretation. LX was responsible for drafting the article or critically revising it for important intellectual content. SZ agreed to be accountable for all aspects of the work, in ensuring that questions related to the accuracy or integrity of the work are appropriately investigated and resolved. All authors gave final approval of the version to be published.

Ethics approval and consent to participate

The present study was approved by the Ethics Committee of the First Affiliated Hospital (approval no. ZJ2018121017), Zhejiang University and written informed consent was obtained from all participants. All procedures involving human participants were performed in accordance with the 1964 Declaration of Helsinki and its later amendments or comparable ethical standards.

Patient consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

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