Methylation of the miR-29b-3p promoter contributes to angiogenesis, invasion, and migration in pancreatic cancer

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Abstract. The aim of the present study was to investigate the effects of microRNA (miR)-29b-3p gene promoter methylation on angiogenesis, invasion, and migration in human pancreatic cancer. Prediction of promoter methylation of miR-29b-3p was performed through the MethPrimer tool. Then the methylation levels of miR-29b-3p in human pancreatic cancer tissues and cell lines were detected by pyrosequencing, and the relative expression of miR-29b-3p was assessed in pancreatic cancer tissues by qPCR. The results were analyzed by linear regression. Western blot analysis was used to detect expression of DNA methyltransferases (DNMTs) in pancreatic cancer tissues and adjacent tissues. The Transwell assay was used to detect the ability of cell migration and invasion. Cells were co-cultured with human umbilical vein endothelial cells (HUVECs) to detect the ability of angiogenesis. The results revealed that DNMT1 expression in pancreatic cancer tissues was higher than that in adjacent tissues. Further results showed that expression of miR-29b was negatively correlated with the methylation level of the miR-29b promoter. Bxpc3 and Capan-2 cells had higher methylation levels, and the expression level of miR-29b-3p in Bxpc3 and Capan-2 cells was found to be lower than that of other cell lines. Expression of zonula occludens-1 (ZO-1) and occludin was significantly increased, and the migration of cancer cells was decreased after cells were treated with siRNA DNMT1. Further results showed that miR-29b reversed the promotive effect of DNMT1 overexpression on tumor cell malignant properties. Methylation of the miR-29b-3p promoter contributes to angiogenesis, invasion, and migration in pancreatic cancer. This study indicated that the alteration of methylation of mR-19b may be a potential approach for inhibiting the progression of pancreatic cancer.

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Introduction

Pancreatic cancer is a highly malignant tumor of the digestive tract and the fourth leading cause of cancer-related death worldwide (1,2), In recent years, its morbidity and mortality have exhibited an upward trend worldwide, due to difficulties in detection and lack of effective treatment, with a ≤5% 5-year survival rate (2). In order to effectively diagnose, prevent, and treat this disease, further study of the molecular mechanism of pancreatic cancer is needed.

DNA methylation is a form of epigenetic modification and an important mechanism of gene expression regulation (3,4). The occurrence and development of tumors are closely related to DNA methylation abnormalities, which are mainly manifested as a decrease in the overall genomic methylation level in tumor cells and an increase in the methylation level of the promoter region of specific genes (5-8). Previous studies have shown that methylation abnormalities in multiple promoter regions are closely related to the development of pancreatic cancer (5,6).

As a member of the DNA methyltransferases (DNMTs), DNMT1 plays an important role in mediating gene expression and chromatin structure, by preserving existing DNA methylation during DNA replication (7). DNMT1 was found to be upregulated in various types of cancer, including pancreatic cancer (8). A study of pancreatic cancer demonstrated that lower expression of DNMT1 reversed the resistance to 5-azadeoxycytidine (9). In addition, siRNA targeting DNMT1 led to a reduction in cell viability and induced cell apoptosis in pancreatic cancer cells (10). Abnormal DNA methylation in tumors includes overall hypomethylation of the genome and hypermethylation of certain gene promoter regions. Abnormally elevated methylation of the promoter region may lead to transcriptional silencing of important regulatory genes such as cell cycle regulatory genes, tumor-suppressor genes, and apoptotic genes, resulting in decreased expression or loss of expression of related genes, and thereby promoting tumor formation (11). This hypermethylation is another mechanism leading to the inactivation of tumor-suppressor genes.

MicroRNAs (miRNAs) are a class of small non-coding RNAs with about 21-25 nucleotides, which can regulate the expression of post-transcriptional target genes (12,13). A large number of researches also confirmed that miRNAs

have the dual role of oncogene or tumor suppressor gene, and its expression changes are closely related to tumor formation (14-16). miR-29b-3p is a member of the miR-29 family and is closely related to the behavior of various tumors (17-21). In this study, we investigated the effects of the miR-29b-3p promoter methylation status on angiogenesis, invasion, and migration of pancreatic cancer cells. This study provides information concerning the role of the methylation of miR-29b in pancreatic cancer, and this may be a target for pancreatic cancer therapy.

Materials and methods

Patients and tissue collection. A total of 18 pairs of tissues from pancreatic cancer patients (mean age, 68.65±14.23 years ranging from 39 to 91; 8 female patients and 10 male patients) were collected at the Yantai Yuhuangding Hospital of Qingdao University from March to November 2019. Patients who had received chemotherapy or radiotherapy were excluded in this study. The tissues were collected and transferred into liquid nitrogen and then were stored at -80˚C. This study was approved by the Ethics Committee of the Yantai Yuhuangding Hospital of Qingdao University, and the ethics approval number is QDU-201902-3. All patients have provided written informed consent to participate in the study.

Antibodies, reagents, plasmids, miRNA, and siRNA. Antibodies against DNMT1 (dilution 1:1,000, cat. no. ab13537), DNMT2 (dilution 1:1,000, cat. no. ab272620), DNMT3a (dilution 1:1,000, cat. no. ab228691), DNMT3b (dilution 1:1,000, cat. no. ab122932), zonula occludens-1 (ZO-1) (dilution 1:1,000, cat. no. ab191143), occludin (dilution 1:1,000, cat. no. ab242202), claudin-5 (dilution 1:1,000, cat. no. ab15106), GAPDH (dilution 1:1,000, cat. no. ab9485) were from Abcam. HRP-labeled secondary antibodies (dilution 1:10,000, cat. no. sc-2370 or sc-2371) were from Santa Cruz Biotechnology, Inc. Fetal bovine serum (FBS, Gibco; Thermo Fisher Scientific, Inc.), Dulbecco's modified Eagle's medium (DMEM, Sigma; Merck KGaA, cat. no. 5796), Lipofectamine 3000 (Invitrogen; Thermo Fisher Scientific, Inc.), and NuPAGE 4-12% Bis-Tris Gels were purchased from Thermo Fisher Scientific, Inc. Hydroquinone and sodium bisulfite were obtained from Sigma-Aldrich (Merck KGaA). Wizard DNA purification resin was obtained from Promega Corp. The CpGenome DNA Modification Kit was purchased from Intergen. The Vector and pcDNA3.1-DNMT1 were all designed and purchased from Invitrogen; Thermo Fisher Scientific, Inc. miR-29b-3p mimic, negative control mimics and all siRNA oligonucleotides were synthesized by GenePharma. All mimics and plasmids were transfected into cells using Lipofectamine 3000 (Invitrogen; Thermo Fisher Scientific, Inc). At 48-h post transfection, the transfected cells were collected for the next analysis.

Cell culture. Normal human pancreatic duct epithelial cells (HPDE6-C7) and 5 pancreatic cancer cell lines (BxPC3, PANC1, CFPAC, Capan-2, and AsPC-3) were purchased from Clontech. HUVECs were obtained from the American Type Culture Collection (ATCC). All cells were maintained and propagated in DMEM with 10% FBS and 1% penicillin streptomycin in 5% CO_2 at 37˚C.

Western blotting. Cytoplasmic and nuclear protein fractions were extracted with the NE-PER Reagent Kit (Pierce; Thermo Fisher Scientific, Inc.) according to the manufacturer's instructions. Cell or tumor tissue lysates were separated by NuPAGE 4-12% Bis-Tris Gels, under 60 V electrophoresis for 30 min, followed by 120 V electrophoresis for 120 min. After electrophoresis, proteins were transferred to PVDF membranes (Millipore), under 300 mA for 30 min. The membrane was then blocked with 5% defatted milk powder for 60 min at room temperature. Mouse anti-human antibodies against DNMT1, DNMT2, DNMT3a, DNMT3b, ZO-1, occludin, claudin-5 and GAPDH (all diluted at 1:1,000, Santa Cruz Biotechnology, Inc.) were added at 4˚C room temperature incubation overnight. The membrane was then washed with phosphate-buffered solution Tween (PBST) for 30 min, followed by incubation with horseradish peroxidase (HRP)-conjugated secondary antibody for 60 min (dilution, 1:5,000, Santa Cruz Biotechnology, Inc.). After the membrane was washed three times with PBST, chemiluminescence detection reagent was used to develop the film. Gel image system was used to analyzed the band density (Bio-Rad Laboratories, Inc.).

Methylation-specific PCR. Genomic DNA was treated with bisulfite, and all cytosines that were not methylated were converted to uracil, while methylated cytosines were unchanged. Subsequently, the primers were designed for PCR at both ends of the CPG island to purify the target product. Primer pairs for PCR amplification were purchased from Thermo Fisher Scientific, Inc. After TA cloning, each clone was selected for positive clone sequencing, and finally the sequence was compared with the original sequence, the methylation site and number were counted, and the degree of methylation was analyzed.

Transient expression of DNMT1 in Bxpc3 and Capan-2 cells. The empty plasmid vector pcDNA3.1 (Invitrogen; Thermo Fisher Scientific, Inc.) or the plasmid vector containing DNMT1 cDNA was transfected into Bxpc3 and Capan-2 cells using Lipofectamine 3000 for subsequent experiments.

Transient transfection for functional analysis of miR-29b-3p and DNMT1. The cells were seeded in 6-well plates at $1x\ 10^5$ cells/well followed by culturing for 24 h and then transfected with 30 nM of the miR-29b-3p mimic and the negative control mimics (NC) (GenePharma) using Lipofectamine 3000. The miR-29b-3p mimic and negative control mimic sequences were designed and synthesized by Gene pharma. The DNMT1 gene was knocked down by DNMT1 interfering small RNA (siRNA) obtained from Generay and transfected into the Bxpc3 and Capan-2 cells by Lipofectamine 3000. The siRNA target sequence was as follows: DNMT1, 5'-TGTTAAGCTGTCTCTTTCCAA-3' and negative control, 5'-TAGATACTATGAATTCGTCCA A-3'. Medium was replaced with fresh medium after transfection for 6 h, and the cells were cultured for another 48 h before further analysis.

Quantitative real-time PCR (qPCR). The total RNA was isolated from cells using TRIzol reagent (Thermo Fisher Scientific, Inc.) according to the manufacturer's instructions. RNA (1 μg)

was converted into cDNA using the RevertAid™ First Strand cDNA Synthesis Kit (Fermentas; Thermo Fisher Scientific, Inc.). After 10-fold dilution, 4 μl of cDNA was subjected to PCR amplification using SYBR Premix Ex Taq™ II (Takara) according to the manufacturer's protocol in a StepOnePlus™ Real-Time PCR System (ABI; Thermo Fisher Scientific, Inc.). The following thermocycling conditions were used for qPCR: 95˚C for 10 sec, then 40 cycles with 95˚C for 5 sec, 60˚C for 34 sec. β-actin served as the internal control. The primer sequences were as follows: DNMT1 forward, 5'-CCTAGCCCC AGGATTACAAGG-3' and reverse, 5'-ACTCATCCGATT TGGCTCTTTC-3'; miR-29b-3p forward, 5'-ACACTCCAG CTGGGTAGCACCATTTGAAATCA-3', reverse, 5'-CTC AACTGGTGTCGTGGA-3' and reverse transcription, 5'-CTC AACTGGTGTCGTGGAGTCGGCAATTCAGTTGAGAAC ACTGA-3'; β-actin forward, 5'-TGTTCGTCATGGGTGTGA AC-3' and reverse, 5'-ATGGCATGGACTGTGGTCAT-3'; U6 forward, 5'-CTCGCTTCGGCAGCACA-3' and U6 reverse, 5'-AACGCTTCACGAATTTGCGT-3'. β-actin and U6 were used as internal references for measuring relative expression of DNMT1 and miR-29b-3p, respectively. The expressions of genes were quantified using the $2^{-\Delta\Delta Cq}$ method (22).

Cell migration and invasion assays (Transwell). Uncoated or Matrigel-coated chambers Transwells (BD Biosciences) containing 8-μm pores were used for the assays. Cells (200 μl) ($1x10^5$ cells/ml) were seeded into the upper chamber in serum-free DMEM medium. A total of 600 μl conditioned DMEM media from target cells containing 10% FBS was added to the lower chamber. Cells were fixed in 100% methanol 72 h later and stained with a 1:5 dilution of Giemsa (Sigma-Aldrich; Merck KGaA) for 40 min at room temperature. Cells remaining on the upper side of the filter were removed with a cotton swab. The filters were then mounted onto slides and images were captured under a microscope (Wetzlar, Germany, cat. no. DMI 1, Leica) at x200 magnification. From these images, the number of migratory or invasive cells was counted.

In vitro angiogenesis experiment of target cells co-cultured with HUVECs. The target cells ($1x10^5$ cells/ml) were inoculated in a cell culture flask at the same density. After 6 h of culture, the culture medium was discarded and replaced with DMEM. After further culturing for 8 h, the culture solution was collected and centrifuged at 1,000 x g for 10 min to collect the cell supernatant culture solution. Then 50 μl of Matrigel was added to each well of a 96-well plate and incubated for 1 h at 37˚C. HUVECs were then added to the upper layer of Matrigel at $5x10^3$ cells per well, and then incubated with the collected tumor cell culture supernatant. After 12-18 h, the formation of blood vessel-like structures of the HUVECs was observed and photographed under a fluorescence microscope (Keyence, cat. no. BZ-9000).

Statistics. All the quantitative data are represented as mean ± SEM of at least three independent experiments. The difference between two groups was evaluated with the 2-tailed Student's t-test. One-way ANOVA and Tukey post hoc test were used to evaluate differences of multiple comparisons. All statistical analyses were conducted using GraphPad Prism software (version 7; GraphPad Software, Inc.). Differences were considered significant at $P<0.05$.

Results

The miR-29b-3p gene promoter region methylation levels are increased, the DNMT1 expression levels are increased, and miR-29b-3p expression levels are decreased in pancreatic cancer. We identified one CpG-rich region for each genomic locus of the miR-29b-3p promoter using MethPrimer (http://www.urogene.org/cgi-bin/methprimer/methprimer.cgi) and designed primer sets to analyze the CpG-rich regions. Based on a database comparison, we predicted that the methylation level of the miR-29b-3p gene promoter region (-3,000 bp) is increased in pancreatic cancer tissues (Fig. S1).

In the present study, we detected expression of DNMTs in pancreatic cancer tissues and adjacent tissues by western blot analysis. It was found that the expression level of DNMT1 in pancreatic cancer tissues was markedly higher than that in the adjacent tissues (Fig. 1A). DNMT1 expression was significant downregulated in adjacent tissues compared with tumor tissues, and this was used for later experiments. qPCR was used to detect expression of miR-29b-3p in the pancreatic cancer tissues, and the methylation levels of promoter regions were detected by pyrosequencing in pancreatic cancer tissues and adjacent tissues. It was found that the expression level of miR-29b-3p was decreased and this was negatively correlated with the methylation level of the miR-29b-3p promoter. ($R^2=0.2162$, 1/slope=37.96) (Fig. 1B).

Six pancreatic cancer cell lines: HPDE6-C, BxPC3, PANC1, CFPAC, Capan-2, and AsPC-1 were cultured, and qPCR was used to detect the miR-29b-3p expression levels. The expression level of miR-29b-3p in BxPC3 and Capan-2 was found to be significantly lower than that of the other cell lines ($P<0.01$) (Fig. 1C). The methylation level of the miR-29b-3p gene promoter in these six pancreatic cancer cells was detected by BSP sequencing. It was found that BxPC3 and Capan-2 had more methylation sites and higher methylation levels (Fig. 1D).

Interference with expression of DNMT1 in Bxpc3 and Capan-2 cells in order to detect angiogenesis, invasion, and migration of pancreatic cancer cells. siRNA was utilized to interfere with DNMT1 in Bxpc3 and Capan-2 cells. qPCR revealed that the expression level of the miR-29b-3p gene in the siRNA DNMT1 group was significantly increased ($P<0.001$ and $P<0.01$) (Fig. 2A). Western blot analysis revealed that expression of DNMT1 was decreased in the DNMT1 siRNA-transfected Bxpc3 and Capan-2 cells relative to that in the NC transfected Bxpc3 and Capan-2 cells, indicating that the interference effect was obvious (Fig. 2B).

Transwell assay showed that the migration and invasion abilities of pancreatic cancer cells in the siRNA DNMT1 group in Bxpc3 and Capan-2 cells were weakened, and the difference was statistically significant ($P<0.01$) (Fig. 2C). Co-culture with HUVECs revealed that the angiogenic ability of the HUVECs was markedly attenuated after siRNA interference of DNMT1 expression (Fig. 2D). Western blotting found that the expression levels of ZO-1 and occludin were increased, and claudin-5 expression was decreased in

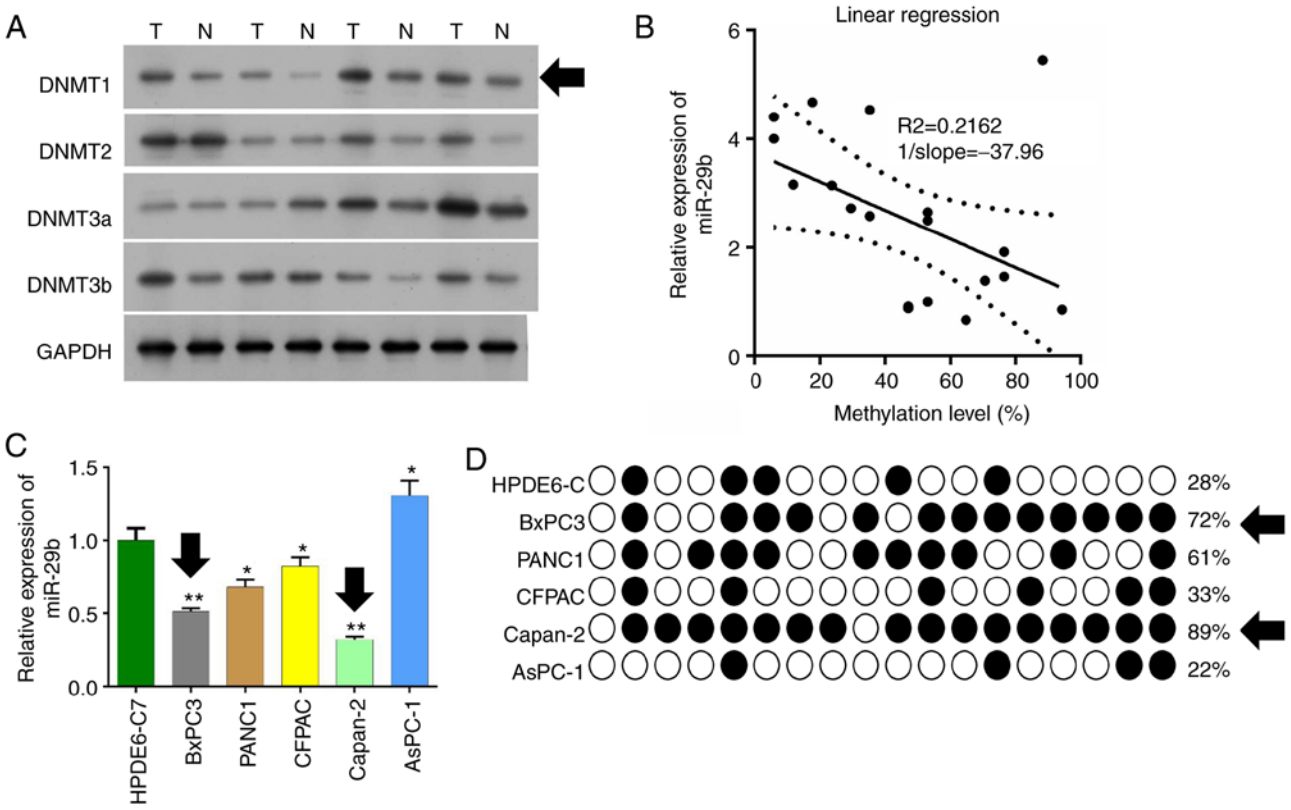

Figure 1. miR-29b-3p is negatively correlated with the methylation level of DNMT1 in pancreatic cancer. (A) Expression levels of DNMTs in pancreatic cancer tissues. (B) Correlation analysis of miR-29b-3p and the methylation level of DNMT1 in pancreatic cancer (R^2=0.2162). (C) The expression level of miR-29b-3p in normal human pancreatic duct epithelial cells (HPDE6-C7) and 5 pancreatic cancer cell lines (BxPC3, PANC1, CFPAC, Capan-2, and AsPC-3) (*P<0.05, **P<0.01 vs. HPDE6-C7 cells). (D) The methylation level of the miR-29b-3p gene promoter in pancreatic cancer cells. T, tumor tissues; N, non-tumor tissues; DNMTs, DNA methyltransferases.

the DNMT1 siRNA-transfected Bxpc3 and Capan-2 cells compared to that in the NC-transfected Bxpc3 and Capan-2 cells (Fig. 2E).

DNMT1-overexpressing Bxpc3 and Capan-2 cells were cultured, and miR-29b-3p mimic transfection was utilized in order to detect angiogenesis, invasion, and migration of pancreatic cancer cells. Results of the qPCR found that DNMT1 expression was significantly increased (P<0.001), miR-29b-3p expression was significantly decreased (P<0.05, P<0.001) in the DNMT1-overexpressed group compared with vector group; while the expression of DNMT1 was not significantly different in the DNMT1+miR-29b-3p group from that in the DNMT1 group, the expression of miR-29b-3p was significantly increased in DNMT1 and miR-29b-3p co-transfection group relative to that in DNMT1-overexpressed group (P<0.001) (Fig. 3A). Western blotting showed that DNMT1 expression in the DNMT1 group was higher than that in the NC group and the blank group, and the DNMT1+miR-29b-3p group had lower DNMT1 expression than the DNMT1 group (Fig. 3B). The results indicated that overexpression and interference were effective.

Transwell assay showed that the migration and invasive abilities of Bxpc3 and Capan-2 cells were significantly enhanced in the DNMT1 group vs. that in the vector group, while the enhancement of migration and invasion capacities mediated by DNMT1 overexpression were significantly weakened by miR-29b-3p in Capan-2 and BxPC3 cells (P<0.01) (Fig. 3C). Co-culture with HUVECs showed that the angiogenic ability of the HUVECs was enhanced in the DNMT1 group compared with that in the vector group, which also could be attenuated by miR-29b-3p addition in Capan-2 and BxPC3 cells (Fig. 3D). Western blotting analysis also discovered that ZO-1 and occludin expressions were markedly reduced, and claudin-5 expression was dramatically elevated in the DNMT1 overexpression group relative to that in the vector group, while the addition of miR-29b-3p then could prominently reverse the expression changes of ZO-1, occludin and claudin-5 in Bxpc3 and Capan-2 cells (Fig. 3E).

Discussion

DNA methylation in mammals means that methyl (-CH3) is covalently bound to the carbon atom of the cytosine (C) base of the DNA molecule under the catalysis of DNA methyltransferases (DNMTs) (23,24). This usually occurs at the 5-position carbon atom of cytosine, forming 5-methylcytosine (5Mc), which is an epigenetic covalent modification process and the main way to inhibit gene expression and loss of function (24,25).

Promoter methylation is involved in the early stage of cancer, and the degree of methylation increases with the increase in structural anomalies (26). Abnormal methylation of specific genes can be used as an indicator to judge the

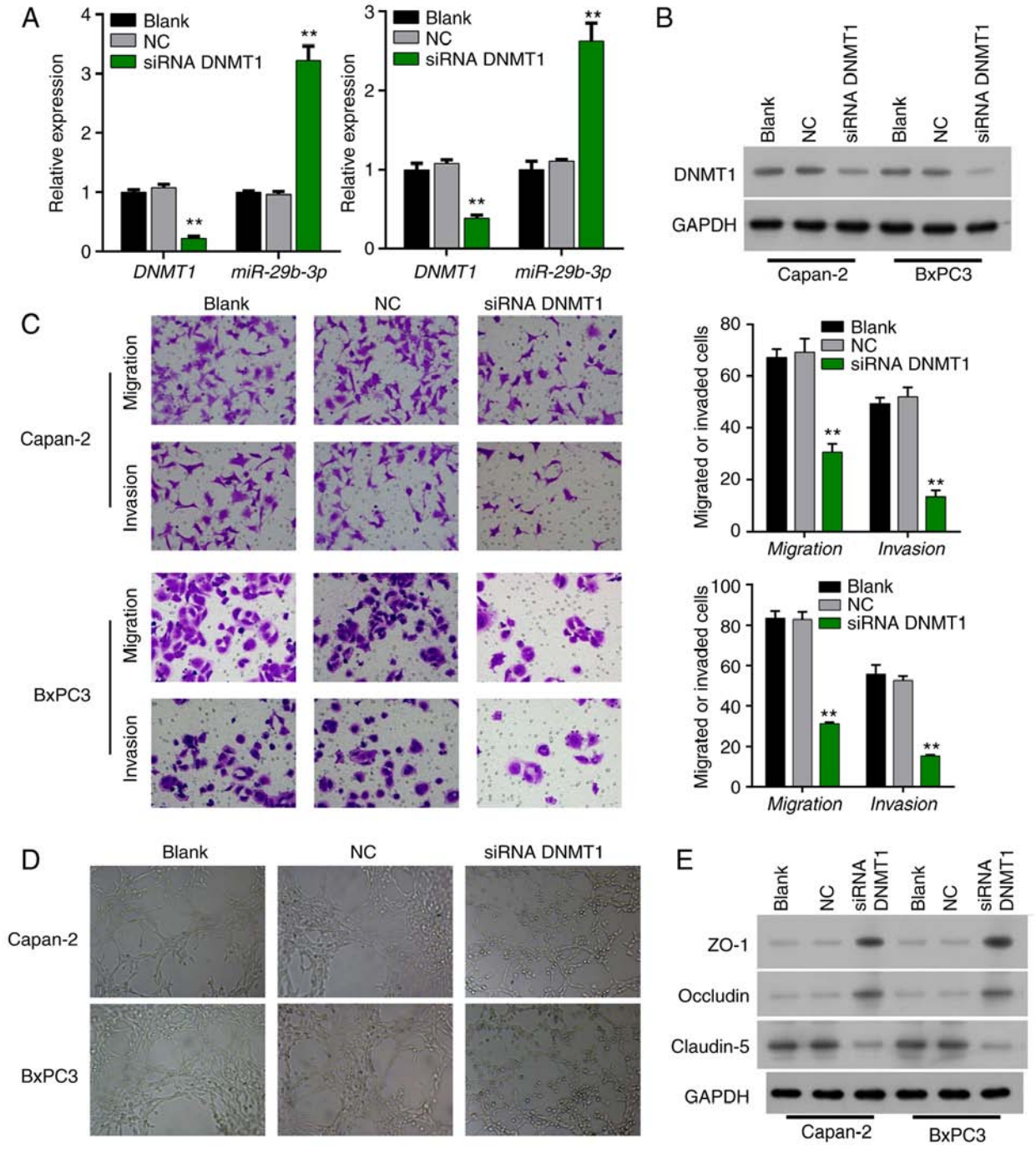

Figure 2. Knockdown of DNMT1 suppresses the methylation of miR-29b-3p and affects Capan-2 and BxPC3 cell migration and invasion. (A) Expression level of miR-29b-3p gene in cells transfected with DNMT1 siRNA. (B) Expression of DNMT1 in cells transfected with DNMT1 siRNA. (C) Migration and invasion of pancreatic cancer cells transfected with DNMT1 siRNA. (D) Angiogenic ability of HUVECs after siRNA interference of DNMT1. (E) Expression of ZO-1, claudin-5, and occludin in cells transfected with DNMT1 siRNA. $^{**}P<0.01$, vs. NC group. n=3. HUVECs, human umbilical vein endothelial cells; DNMT1, DNA methyltransferase 1; ZO-1, zonula occludens-1.

progression of pancreatic tumors (27). Numerous studies have shown that multiple gene methylation abnormalities are often detected in pancreatic cancer (28-31). It was also found that in precancerous lesions of pancreatic cancer, the methylation of the NPTX2 promoter increases with the degree of abnormal proliferation, suggesting that NPTX2 promoter regional hypermethylation is associated with early tumorigenesis in pancreatic cancer (32,33). One study also found that pENK is highly methylated in pancreatic cancer tissue samples and pancreatic juice in pancreatic cancer patients, and its methylation to some extent promotes the formation of pancreatic cancer (34). In the present study, we found that the methylation of the miR-29 promoter was involved with malignant activities of pancreatic cancer cell lines. Overexpression of DNMT1 resulted in lower expression of miR-29, which led to cell migration, invasion, and angiogenesis.

MicroRNAs (miRNAs) are a family of non-coding RNAs that are very conservative and are approximately 15 to 25 nt in length. In tumor research, according to the target gene of its downstream action, there are two major types of

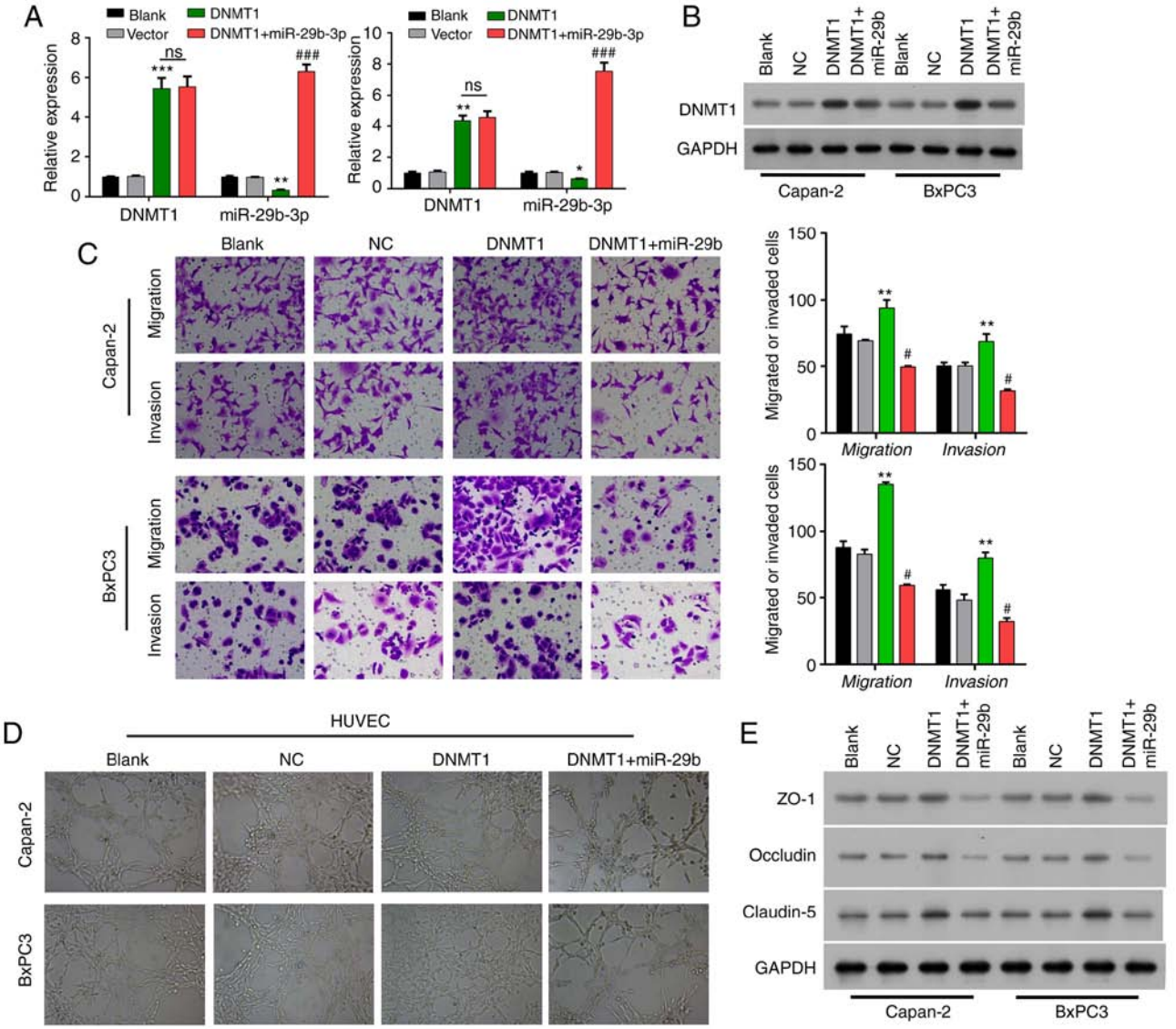

Figure 3. Methylation of the miR-29b-3p promoter contributes to angiogenesis, invasion, and migration in pancreatic cancer. (A) Expression of DNMT1 and miR-29b-3p in cells transfected with the DNMT1 expression plasmid and co-transfected with the DNMT1 expression plasmid and miR-29b-3p mimics in BxPC3 and Capan-2 cells. (B) Expression of DNMT1 in cells transfected with the DNMT1 expression plasmid and co-transfected with the DNMT1 expression plasmid and miR-29b-3p mimics. (C) The migration and invasive ability of pancreatic cancer cells in cells transfected with the DNMT1 expression plasmid and co-transfected with the DNMT1 expression plasmid and miR-29b-3p mimics. (D) The angiogenic ability of HUVECs co-cultured with pancreatic cancer cells transfected with the DNMT1 expression plasmid and co-transfected with the DNMT1 expression plasmid and miR-29b-3p mimics. (E) Expression of ZO-1, claudin-5, and occludin in cells transfected with the DNMT1 expression plasmid and co-transfected with the DNMT1 expression plasmid and miR-29b-3p mimics. HUVECs, human umbilical vein endothelial cells; DNMT1, DNA methyltransferase 1; ZO-1, zonula occludens-1. *P<0.05, **P<0.01, ***P<0.001 vs. the Vector group; $^{\#}$P<0.05, $^{\#\#\#}$P<0.001 vs. the DNMT1 group; ns, not significant.

mircoRNAs, which are similar to the properties of oncogenes or tumor-suppressor genes (35). DNA aberrant methylation causes epigenetic silencing of some microRNAs and plays an important role in tumorigenesis and development (36). The human microRNA-29 (miRNA-29, miR-29) family is a group of small RNAs with the same seed sequence 'AGCACCA', including miR-29a, miR-29b, and miR-29c. There are miR-29 expression disorders in various tumor tissues, which are involved in expression of genes involved in tumor cell metabolism, proliferation, differentiation, and apoptosis through post-transcriptional regulation, and have the dual role of oncogene or tumor-suppressor gene (37). miR-29b-3p is a member of the miR-29 family and is involved in the development of pancreatic cancer (38,39), colorectal cancer (40), lung cancer (41), bladder cancer (19), and multiple myeloma (42). The relationship between miR-29b-3p promoter methylation and pancreatic cancer has not yet been reported. In the present study, we investigated the methylation of the miR-29b-3p promoter in pancreatic cancer and its expression level, and explored the effect of miR-29b-3p promoter methylation on angiogenesis, invasion, and migration of pancreatic cancer, thus providing a new theoretical basis for the treatment of pancreatic cancer.

It was found that the methylation level of the miR-29b-3p promoter region in pancreatic cancer tissues was significantly higher than that in adjacent tissues. In addition, the expression level of miR-29b-3p was significantly decreased, which was negatively correlated with the methylation

level of its promoter. CpG methyltransferases (DNMTs) play a key role in DNA methylation, including DNMT1, DNMT2 DNMT3a, and DNMT3b. DNMT1 is the most important catalytic enzyme in the DNMT family. DNMT1 is associated with abnormal methylation of DNA and both are closely related to the occurrence and development of tumors (43,44). The expression level of the DNMT1 protein in pancreatic cancer tissues was higher than that in adjacent tissues, suggesting that DNMT1 promotes promoter region methylation of the miR-29b-3p gene. siRNA was used to interfere with DNMT1 in Bxpc3 and Capan-2 cell lines, and expression of miR-29b-3p was significantly increased. We cultured DNMT1-overexpressing Bxpc3 and Capan-2, and expression of miR-29b-3p was significantly decreased. The above experiments proved that the methylation degree of the miR-29b-3p gene in pancreatic cancer leads to a change in its gene expression level, and the hypermethylation of the miR-29b-3p gene leads to its low expression.

Angiogenesis is the budding and subsequent stabilization of existing vascular wall cells (45). In 1973, FoIkman first discovered that tumor cells induce angiogenesis and rapid growth, and since then, more and more attention has been paid to solid tumor angiogenesis (46). The vascular endothelial growth factor (VEGF), insulin-like growth factor 1 (IGF1), and other factors can play a role in promoting tumor angiogenesis, which is the basis of malignant tumor growth and metastasis (47-49). Zhang *et al* (50) found that exogenous low expression of miR-29a/c can increase expression and release of VEGF in gastric cancer cells, and promote the growth of vascular endothelial cells. Melo and Kalluri (51) found that miR-29b can inhibit the signaling molecules involved in angiogenesis and the extracellular matrix, such as VEGF, MMP9, ANGPTL4, and lysyloxidase (LOX), thereby inhibiting tumor angiogenesis and metastasis. This study investigated the role of miR-29b-3p in angiogenesis in pancreatic cancer cells, and found that miR-29b-3p inhibits angiogenesis and pancreatic cancer cell migration and invasion, and after inhibition of miR-29b-3p, the migration and invasive ability of pancreatic cancer cells increased. In this study, we aimed to investigate the role of DNMT1 and miR-29b-3p in pancreatic cancer, on cell migration, invasion and angiogenesis. However, the effect of DNMT1/miR-29b-3p on cell apoptosis and cycle was not investigated in the present study. Based on previous research, DNMT1 siRNA induces a significant cell viability decrease, leads to a G2-phase block and cell apoptosis in pancreatic cancer (10,52), indicating that this axis may promote cell survival. Further study will focus on this aspect.

In conclusion, methylation of the miR-29b-3p promoter contributes to angiogenesis, invasion, and migration in pancreatic cancer. Its molecular mechanisms of regulating tumorigenesis and development need to be further studied.

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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

LW designed the experiments. LW and NM performed the experiments, collected the data and analyzed the data. LW drafted the manuscript, and NQ validated the data analysis and revised the manuscript. All authors read and approved the manuscript and agree to be accountable for all aspects of the research in ensuring that the accuracy or integrity of any part of the work are appropriately investigated and resolved.

Ethics approval and consent to participate

This study was approved by the Ethics Committee of Yantai Yuhuangding Hospital of Qingdao University, and the ethics approval number is QDU-201902-3. Written informed consent was obtained from each participant.

Patient consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

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