Overexpression of microRNA-5100 decreases the aggressive phenotype of pancreatic cancer cells by targeting *PODXL*

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Received December 9, 2015; Accepted January 21, 2016

DOI: 10.3892/ijo.2016.3389

Abstract. Metastasis is the main cause of cancer-associated death, and metastasis of pancreatic cancer remains difficult to treat because of its aggressiveness. MicroRNAs (miRNAs) play crucial roles in the regulation of various human transcripts, and many miRNAs have been reported to correlate with cancer metastasis. We identified an anti-metastatic miRNA, miR-5100, by investigating differences in miRNA profiling between highly metastatic pancreatic cancer cells and their parental cells. Overexpression of miR-5100 inhibited colony formation (P<0.05), cell migration (P<0.0001) and invasion (P<0.0001) of pancreatic cancer cells. In addition, we identified a possible target of miR-5100, podocalyxin-like 1 *(PODXL)*, and demonstrated miR-5100 directly binds to the 3' untranslated region of *PODXL* and post-transcriptionally regulates its expression in pancreatic cancer cells. Silencing PODXL resulted in diminished cell migration (P<0.0001) and invasion (P<0.05). We also clarified the close relationship between expression of PODXL in human pancreatic cancer specimens and liver metastasis (P=0.0003), and determined that post-operative survival was longer in the low-PODXL expression group than in the high-PODXL expression group (P<0.05). These results indicate that miR-5100 and PODXL have considerable therapeutic potential for anti-metastatic therapy and could be potential indicators for cancer metastases in patients with pancreatic cancer.

Introduction

Pancreatic cancer is the fourth leading cause of cancer mortality in the United States (1,2). Although efforts to reduce risk factors such as smoking, obesity, and high meat consumption and to improve early detection have been made, pancreatic cancer is still formidable because of its aggressive metastatic ability (3-6). Pancreatic cancer mainly metastasizes to the lymph nodes, liver, lung, peritoneum and bone. Although the liver is the most common target of pancreatic cancer with the exception of the lymph nodes, effective methods for prediction and treatment of liver metastasis remain unestablished (7,8). Identification of prognostic markers of metastasis would be useful for the management of post-operative patients with pancreatic cancer (9-11).

MicroRNAs (miRNAs) are small noncoding RNAs of approximately 22 nucleotides that are predicted to regulate as many as 30% of human transcripts (12,13). Several recent investigations have identified some miRNAs as potential critical regulators to inhibit the malignant characteristics of tumors (14-18). miRNAs suppress expression of many target genes at the post-transcriptional level by binding to their 3' untranslated regions (UTR), which leads to inhibition of translation or degradation of messenger RNAs (mRNAs) (12,19). The miR-200 family and miR-205 were reported to regulate epithelial to mesenchymal transition of breast cancer cells by targeting *ZEB1* and *SIP1* (20), and miR-34a was reported to inhibit prostate cancer stem cells and metastasis by directly repressing *CD44* (21). Regarding pancreatic cancer, *ZEB1* was reported to promote tumorigenicity of pancreatic cancer by repressing the miR-200 family (22), and miR-10a was reported to promote metastatic behavior of pancreatic tumor cells (23). Several miRNAs that may correlate with liver metastasis of pancreatic cancer were also reported (24), but the mechanism is still unclear. We performed miRNA expression profiling with a microarray using newly-established pancreatic cancer cell lines with high potential for liver metastasis, and identified miR-5100 as a candidate gene related to liver metastasis of pancreatic cancer. In addition, we focused on podocalyxin-like 1 *(PODXL)*, which was predicted as a target of miR-5100 using online target-predicting algorithms of miRNAs based on the global mRNA expression profiling with the microarray.

PODXL was initially identified in podocytes of renal glomeruli that are instrumental in kidney development (25,26).

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Key words: pancreatic cancer, miR-5100, *PODXL*, liver metastasis, distant metastasis, prognostic marker

Expression of PODXL was identified in podocytes, hematopoietic progenitors, vascular endothelia and embryonic stem cells (27-30) and it was reported to promote anti-adhesive and migratory characteristics of various cancer cells except for pancreatic cancer (31-35). Recent studies showed that increased expression of PODXL is correlated with poor prognoses in many types of cancer (36-41). Although the expression of PODXL has been reported to promote anti-adhesion and migration, how PODXL correlates with tumor metastases remains unclear, especially in pancreatic cancer (33).

In this study, we identify an anti-metastatic miRNA, miR-5100, that decreases the metastatic ability of pancreatic cancer partially by suppressing expression of *PODXL*.

Materials and methods

Cell culture. The following eleven pancreatic cancer cell lines and HPDE cells were used in this study: Panc-1 (Riken Cell Bank, Tsukuba, Japan), KP-2, KP-3, SUIT-2 and MIA PaCa-2 (Japanese Cancer Resource Bank, Osaka, Japan), Capan-1, Capan-2, Aspc-1, SW1990, HS766T, CFPAC-1 (American Type Culture Collection, Manassas, VA, USA), and HPDE (Dr M.-S. Tsao, University of Toronto, Toronto, Canada). All cancer cell lines were maintained in Dulbecco's modified Eagle's medium (DMEM; Life Technology, Grand Island, NY, USA) supplemented with 10% fetal bovine serum (FBS; Invitrogen, Carlsbad, CA, USA), streptomycin (100 mg/ml) and penicillin (100 mg/ml) and cultured at 37˚C in a humidified atmosphere containing 10% CO_2.

Establishment of metastatic SUIT-2 cells and metastatic PANC-1 cells. We bred BALB/c nu/nu mice (Kyudo Co., Saga, Japan) and used them at the age of 4 weeks in accordance with institutional guidelines. The parental SUIT-2 cells ($1x10^6$ cells) were orthotopically transplanted. The mice were sacrificed at 5-7 weeks after implantation of cancer cells. Liver metastases were harvested and minced. We performed primary culture using minced tissue with collagenase. The cell culture was then orthotopically transplanted. This process was repeated five times to establish metastatic SUIT-2 (MS) cells. Metastatic PANC-1 (MP) cells were established by the same process.

Total RNA extraction. Total RNA was extracted from cultured cells using a High Pure RNA Isolation kit (Roche Diagnostics, Mannheim, Germany) and DNase I (Roche Diagnostics) treatment according to the manufacturer's instructions.

Microarray analyses. We carried out microarray analyses using the parental SUIT-2 and MS cells and parental PANC-1 and MP cells. We used the 3D-Gene miRNA microarray platform (Toray, Kamakura, Japan) for these analyses.

Data analysis and filter criteria. Raw signal intensities of two samples were normalized by a quantile algorithm with the 'lumi' (42) and 'preprocess Core' library package (43) on Bioconductor software (44). We selected probes that called the 'Detection P-value <0.05' flag in at least one sample. To identify up- or down-regulated genes, we calculated intensity-based Z-scores (45) and ratios (non-log scaled fold-change) from the normalized signal intensities of each probe for comparison between control and experiment samples. Then we established criteria for regulated genes: (upregulated genes) Z-score ≥2.0 and ratio ≥1.5-fold, (downregulated genes) Z-score ≤-2.0 and ratio ≤0.7.

Cell transfection. miR-5100 mimics and negative control mimics were synthesized by TaqMan (Life Technologies, Tokyo, Japan) and transfected into cells to a final oligonucleotide concentration of 3-30 nmol/l. Transfection was performed using Lipofectamine 2000 Reagent (Invitrogen) following the manufacturer's protocol. Cells were trypsinized, counted and seeded in plates on the day before transfection to ensure suitable cell confluence.

PODXL knockdown by small-interfering RNA (siRNA). SiRNA targeting *PODXL* and non-targeting siRNA control were purchased from Sigma-Aldrich Japan (Hokkaido, Japan). Transfection was performed according to the manufacturer's reverse-transfection protocol using Lipofectamine RNAiMAX (Life Technology). In brief, siRNAs and Lipofectamine (5 μl) were diluted in 500 μl Opti-MEM (Life Technology, Tokyo, Japan) without serum, and incubated for 15 min at room temperature. Cancer cells ($2x10^5$) were resuspended in 2.5 ml of DMEM supplemented with 10% FBS without antibiotics. The siRNA and Lipofectamine mixture was added to the diluted cells (3 ml final volume, final siRNA concentration 30 nM and seeded in 6-well plates ($2x10^5$ cells/well). After 24 h incubation, plates were washed and cells were incubated in complete growth medium (DMEM with 10% FBS and antibiotics) for various time points. Cancer cells were used in subsequent experiments at 48-h post-transfection.

Blocking specific binding site of miR-5100 by protector. Protector (miScript Target Protector) was designed and purchased from Qiagen (Tokyo, Japan) to block the binding site of miR-5100. Transfection was performed according to the manufacturer's protocol using lipofectamine RNAiMAX (Life Technology) as described above.

Quantitative real-time reverse-transcription polymerase chain reaction for analysis of miRNA expression. Cultured cells were analyzed by quantitative (q)RT-PCR using SuperTaq Polymerase (Ambion) and a mirVana RT-PCR miRNA Detection kit (Ambion) according to the manufacturer's instructions. All reactions were performed in triplicate. The miRNA expression levels in each sample were normalized by the expression levels of U6 snRNA.

Quantitative assessment of mRNA levels by one-step qRT-PCR. qRT-PCR was performed using a Quantitect SYBR Green Reverse-Transcription PCR kit (Qiagen) and CFX96 Touch Real-Time PCR Detection System (Bio-Rad Laboratories, Hercules, CA, USA). Primers for *PODXL* (Forward: GCT GCAAACACAGCATGGAG; Reverse: CAGTTCCTGGGC AAACTGTTGA) and *GAPDH* (Forward: GCACCGTCAAG GCTGAGAAC; Reverse: TGGTGAAGACGCCAGTGGA) were purchased from Takara Bio Inc. (Tokyo, Japan). We used an endogenous control, *GAPDH*, to normalize expression of mRNA. All reactions were performed in triplicate.

Western blot analysis. Cultured pancreatic cancer cells were lysed in PRO-PREP protein extraction solution (iNtRON Biotechnology, Seongnam, Korea) according to the manufacturer's instructions. A total of 20 μg protein was separated by sodium dodecyl sulfate polyacrylamide gel electrophoresis and transferred to polyvinylidene difluoride membranes (Bio-Rad Laboratories). The membranes were blocked with 5% dry skimmed milk and incubated with anti-PODXL rabbit monoclonal antibody (EPR9518, 1/1000 dilution; Abcam, Cambridge, UK) and anti-actin antibody (1/5000 dilution; Abcam). Membranes were then incubated with anti-rabbit IgG (1/2000 dilution, Cell Signaling Technology, Danvers, MA, USA). Immunoreactive signals were detected using ECL Prime (GE Healthcare, Buckinghamshire, UK), and images were acquired using a ChemiDoc XRS (Bio-Rad Laboratories).

Patients. Tissue samples were obtained from primary pancreatic tumors at the time of surgery at Kyushu University Hospital (Fukuoka, Japan) from 2010 to 2011. No adjuvant therapy was performed in six patients because of poor performance status, whereas 64 patients received adjuvant therapy based on 5-fluorouracil and/or gemcitabine. Neoadjuvant therapy was performed in one patient. This study was approved by the Ethics Committee of Kyushu University and conducted according to the Ethical Guidelines for Human Genome/Gene Research enacted by the Japanese Government and the Declaration of Helsinki.

Immunohistochemical procedures and evaluation of sections. Primary antibody used for immunohistochemical analysis was as follows: PODXL (rabbit monoclonal, EPR9518, 1/250 dilution; Abcam). Antibody was diluted in 5% dry skimmed milk in phosphate-buffered saline. Sections were cut at 4 μm thickness from paraffin-embedded material, deparaffinized in xylene and dehydrated through a graded ethanol series. Endogenous peroxidase activity was blocked by incubation in methanol containing 3% H_2O_2 for 30 min. Antigen retrieval was achieved by boiling slides in a microwave in 10 mM citrate buffer (pH 6.0) for 20 min. The slides were then incubated with an anti-PODXL rabbit monoclonal antibody (EPR9518, 1/250 dilution; Abcam) at 4˚C overnight, and the Envision plus system (Dako, Glostrup, Denmark) was used to visualize the immunostaining. Counterstaining was performed with hematoxylin. Appropriate positive and negative controls were performed for all antibodies. Non-specific staining was not observed in any negative-control sections.

The distribution of stained PODXL was evaluated as the percentage of stained cells, which was scored as follows: 1, ≤10%; 2, 11-50%; 3, 51-80%; and 4, >81%. The distribution of stained PODXL was also evaluated as staining intensity, which was scored as follows: 1, no or weak staining; 2, moderate; and 3, strong. When the multiplication product of the 2 scores was ≥4, PODXL was considered highly stained, and vascular endothelial cells were compared as the positive control. All slides were evaluated without any knowledge of the background of each case.

Invasion and migration assays. Cell invasion was evaluated by counting the number of cells that invaded Matrigel-coated Transwell chambers with 8-μm pores (BD Biosciences, Franklin Lakes, NJ, USA). Briefly, Transwell inserts were coated with 20 μg/well Matrigel (BD Biosciences). Each lower well of a 24-well plate was seeded with 750 μl of DMEM supplemented with 10% FBS. Cancer cells ($5.0x10^4$/well) in 250 μl of DMEM supplemented with 10% FBS were seeded into each upper well. After 48-72 h of incubation, cells on the lower surface of the Matrigel-coated membrane were fixed with 70% ethanol, stained with hematoxylin and eosin (H&E), and counted in five randomly selected fields at x100 magnification under a light microscope. The mobility of pancreatic cancer cells was assessed using uncoated Transwell inserts after 16-36 h of incubation. The results are expressed as the mean number of invaded and migrated cells per field. Each experiment was carried out in triplicate wells and repeated at least three times.

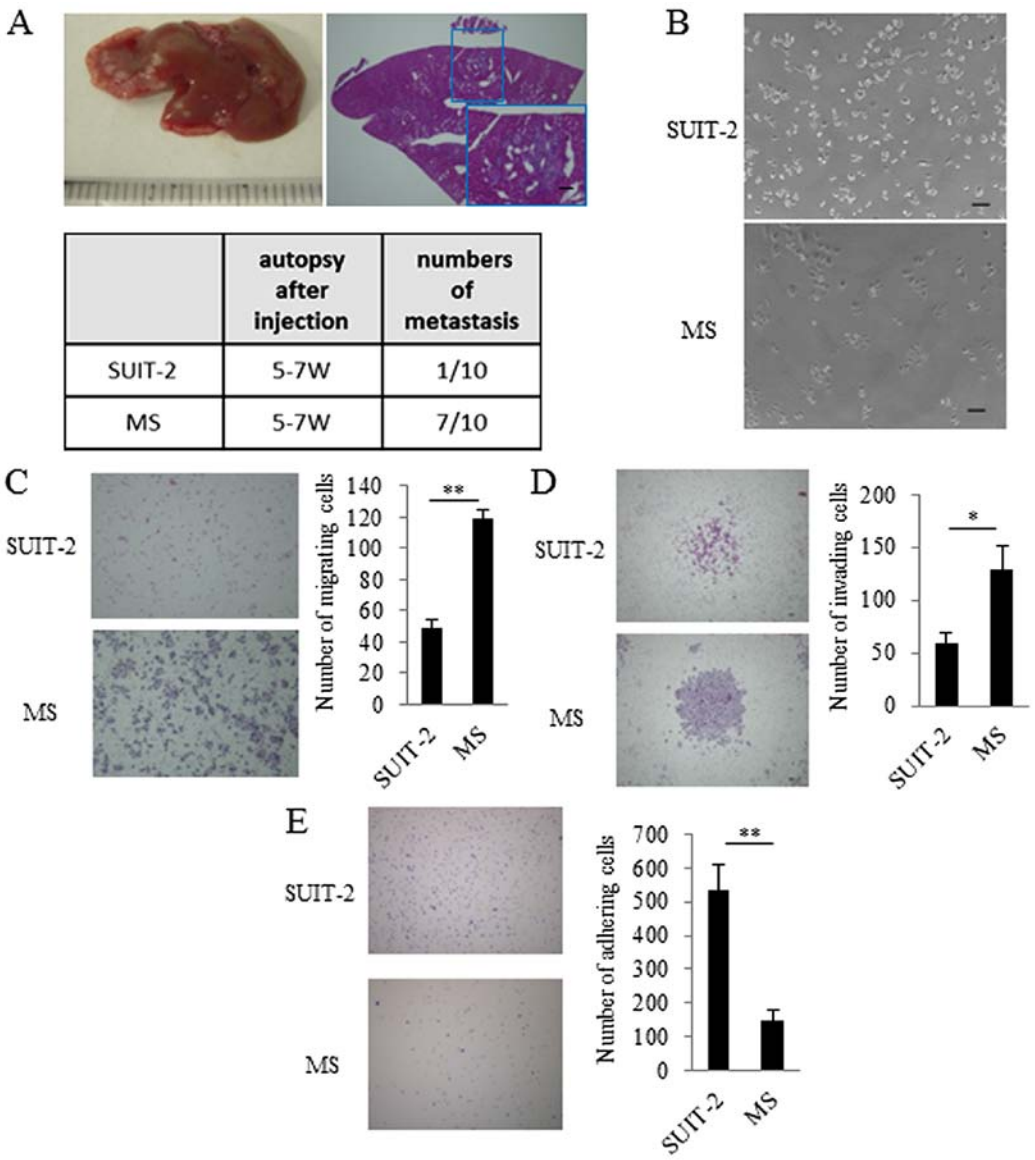
Adhesion and colony formation assays. Cell adhesion was evaluated by counting the number of cells that adhered to 96-well tissue culture plates (Becton Dickinson Labware, Franklin Lakes, NJ, USA) after seeding cells ($1x10^3$/well) for 15 min. Anchorage-independent growth was evaluated by colony formation in soft agar. Cells ($1x10^3$/well) were diluted in DMEM with 10% FBS and 0.35% Bacto-Agar (Difco, Detroit, MI, USA), and seeded in 6-well plates on top of a 0.7% agar bottom layer without cells. Cells were incubated for 14 days, and growth medium (DMEM with 10% FBS) was replaced biweekly. Adhered cells and colonies were stained with crystal violet (0.005%) for 20 min and counted under a light microscope.

In vivo experiments. To analyze the metastatic ability of MS cells *in vivo*, SUIT-2 cells ($1x10^6$) and MS cells ($1x10^6$) suspended in 100 ml DMEM were orthotopically transplanted into the 4-week-old female BALB/c nu/nu mice. At 5-7 weeks after implantation, we sacrificed the mice and all orthotopic tumors and livers were investigated. The presence of liver metastasis was evaluated by counting the number of nodules >1 mm in size on the surface of the liver. All mouse experiments were approved by the Ethics Committee of Kyushu University.

Statistical analysis. All calculations were performed with JMP 11 software (SAS Institute, Cary, NC, USA). Differences in expression levels were analyzed with Student's t-test. For qRT-PCR data, each sample was analyzed twice or in triplicate. Any sample showing a deviation in value of >10% was tested a third time. Data were analyzed by the Mann-Whitney U-test when normal distribution was not obtained. A Chi-square test was used to analyze the association between PODXL expression and clinicopathological characteristics observed by immunohistochemistry. Survival analysis was undertaken using Kaplan-Meier analysis, and survival functions were compared using the log-rank test. To evaluate independent prognostic factors associated with survival, a multivariate Cox proportional hazards regression analysis was performed. All differences were considered to be statistically significant if the P-value was <0.05 ($P<0.05$; $P<0.0001$).

Results

Establishment and characterization of a highly metastatic pancreatic cancer cell line. After five consecutive rounds of



	autopsy after injection	numbers of metastasis
SUIT-2	5-7W	1/10
MS	5-7W	7/10

Figure 1. Highly metastatic cell line representing an aggressive phenotype with high cell motility and cellular infiltration. (A) Mice injected with MS cells exhibited a high occurrence of liver metastases (stain: H&E; original magnification, x40). (B) MS cells had spindle-shaped morphology compared with parental SUIT-2 cells (original magnification, x100; scale bar, 100 μm). (C and D) Migration and invasion in MS cells were increased compared with those in parental SUIT-2 cells (16 and 48 h) (stain: H&E; original magnification, x100 and x40; scale bar, 100 μm). (E) Adhesion in MS cells was decreased compared with those in parental SUIT-2 cells (30 min) (stain: crystal violet; original magnification, x40; scale bar, 100 μm) (*P<0.05; **P<0.0001).

in vivo selection of liver metastasis, metastatic lesions were harvested to establish metastatic SUIT-2 (MS) cells. After we confirmed that MS cells occurred in liver metastases more frequently than the parental SUIT-2 cells (Fig. 1A), we investigated the *in vitro* characteristics of MS cells. The MS cells had spindle-shaped morphology compared with their parental SUIT-2 cells (Fig. 1B). To evaluate migration, invasion, and adhesion, we performed a migration assay, an invasion assay, and an adhesion assay. In these assays, we found that migration and invasion of MS cells were increased and adhesion of MS cells was decreased compared with that of the parental SUIT-2 cells (Fig. 1C, D and E).

Comparison of miRNA expression between MS cells and parental SUIT-2 cells. We next used MS cells and parental SUIT-2 cells for microarray analyses and investigated their differences by miRNA profiling. Microarray analyses showed that 13 miRNAs were downregulated and 15 miRNAs were upregulated in MS cells compared with parental SUIT-2 cells (Table I). Of these candidates, we focused on miR-5100 because it was also downregulated in metastatic PANC-1 (MP) cells established in the same manner (Table II).

To validate the accuracy of microarray analyses, we investigated the expression levels of miR-5100 in cultures of 13 different pancreatic cancer cell lines and Human Pancreatic Duct Epithelial Cell (HPDE) using quantitative real-time reverse-transcription polymerase chain reaction (RT-PCR). HPDE cells showed relatively high expression of miR-5100, and most pancreatic cancer cell lines showed lower miR-5100 expression compared with that of HPDE cells (Fig. 2A). MS cells showed extremely low expression of miR-5100 and KP2 cells showed similar levels. To explore the

Table I. Microarray analysis of miRNAs in MS cells compared with parental SUIT-2 cells.

Name	SUIT-2	MS	Ratio	Name	SUIT-2	MS	Ratio
hsa-miR-192-5p	238.3	63.6	0.27	hsa-miR-4706	33.9	257.4	7.59
hsa-miR-194-5p	430.1	126.6	0.29	hsa-miR-4324	61.2	138.5	2.26
hsa-miR-21-5p	982.8	309.1	0.31	hsa-miR-125b-5p	378.2	825.6	2.18
hsa-miR-27b-3p	168.2	69.4	0.41	hsa-miR-1246	69.1	138.3	2.00
hsa-miR-4755-3p	156.5	65.0	0.42	hsa-miR-1260a	355.5	681.3	1.92
hsa-miR-224-5p	128.7	60.6	0.47	hsa-miR-1260b	1753.5	3167.3	1.81
hsa-miR-1247-5p	101.5	48.0	0.47	hsa-miR-3178	189.2	333.0	1.76
hsa-miR-16-5p	291.2	143.9	0.49	hsa-miR-1273g-3p	754.1	1322.5	1.75
hsa-miR-5100	**683.6**	**344.0**	**0.50**	hsa-miR-1915-3p	65.3	114.0	1.75
hsa-miR-26a-5p	473.4	259.9	0.55	hsa-miR-1181	70.2	121.3	1.73
hsa-miR-1972	126.6	75.1	0.59	hsa-miR-614	64.2	106.6	1.66
hsa-miR-652-5p	168.4	111.3	0.66	hsa-miR-1285-3p	112.9	180.9	1.60
hsa-miR-4454	15681.7	10780.5	0.69	hsa-miR-3940-5p	72.5	116.2	1.60
-	-	-	-	hsa-miR-1233-1-5p	320.8	506.6	1.58
-	-	-	-	hsa-miR-4530	200.9	304.7	1.52

MS, metastatic SUIT-2. Bold text indicates the genes that the present study focuses on.

Table II. Microarray analysis of miRNAs in MP cells compared with parental PANC-1 cells.

Name	PANC-1	MP	Ratio	Name	PANC-1	MP	Ratio
hsa-miR-377-5p	126.3	17.8	0.14	hsa-miR-2964a-5p	34.2	118.0	3.45
hsa-miR-5100	**327.9**	**156.5**	**0.48**	hsa-miR-3135b	30.0	102.9	3.43
hsa-miR-4454	10154.7	5258.0	0.52	hsa-miR-1247-3p	58.6	114.3	1.95
hsa-miR-151a-5p	106.1	64.0	0.60	hsa-miR-365a/b-3p	93.5	175.7	1.88
hsa-let-7i-5p	188.4	115.3	0.61	hsa-miR-4299	58.3	105.2	1.80
hsa-miR-93-5p	220.6	137.3	0.62	hsa-miR-134	58.4	104.9	1.80
hsa-miR-1260a	1462.2	953.1	0.65	hsa-miR-494	221.9	368.9	1.66
-	-	-	-	hsa-miR-6087	94.5	151.6	1.60
-	-	-	-	hsa-miR-652-5p	101.0	159.8	1.58
-	-	-	-	hsa-miR-4667-5p	83.8	130.7	1.56
-	-	-	-	hsa-miR-370	84.4	130.8	1.55
-	-	-	-	hsa-miR-4745-5p	110.2	167.4	1.52

MP, metastatic PANC-1. Bold text indicates the genes that the present study focuses on.

role of miR-5100 in pancreatic cancer, MS and KP2 cells were transfected with miR-5100 mimics with high levels of transfection efficiency (Fig. 2B, upper panel). The morphology of miR-5100-transfected MS cells was not remarkably changed compared with control miRNA-transfected MS cells (Fig. 2B, lower panel). Colony formation assays revealed that cell population growth was significantly decreased in miR-5100-transfected cells compared with control miRNA-transfected cells (Fig. 2C). miR-5100 also inhibited cell migration and invasion in MS and KP2 cells (Fig. 2D and E). In contrast, adhesion of miR-5100-transfected MS cells was increased compared with control miRNA-transfected cells, while miR-5100-transfected KP2 cells showed no significant change in adhesion compared with control miRNA-transfected cells (Fig. 2F). These results indicate that miR-5100 decreases the aggressiveness of pancreatic cancer in MS and KP2 cells.

Identification of possible target genes of miR-5100. miRNAs exert biological functions through negatively regulating their target genes. We performed microarray analyses for global mRNA expression profiling and used online target-predicting algorithms, Target Miner, Target Scan and Mir Database, to predict the possible target genes sharing a complementary

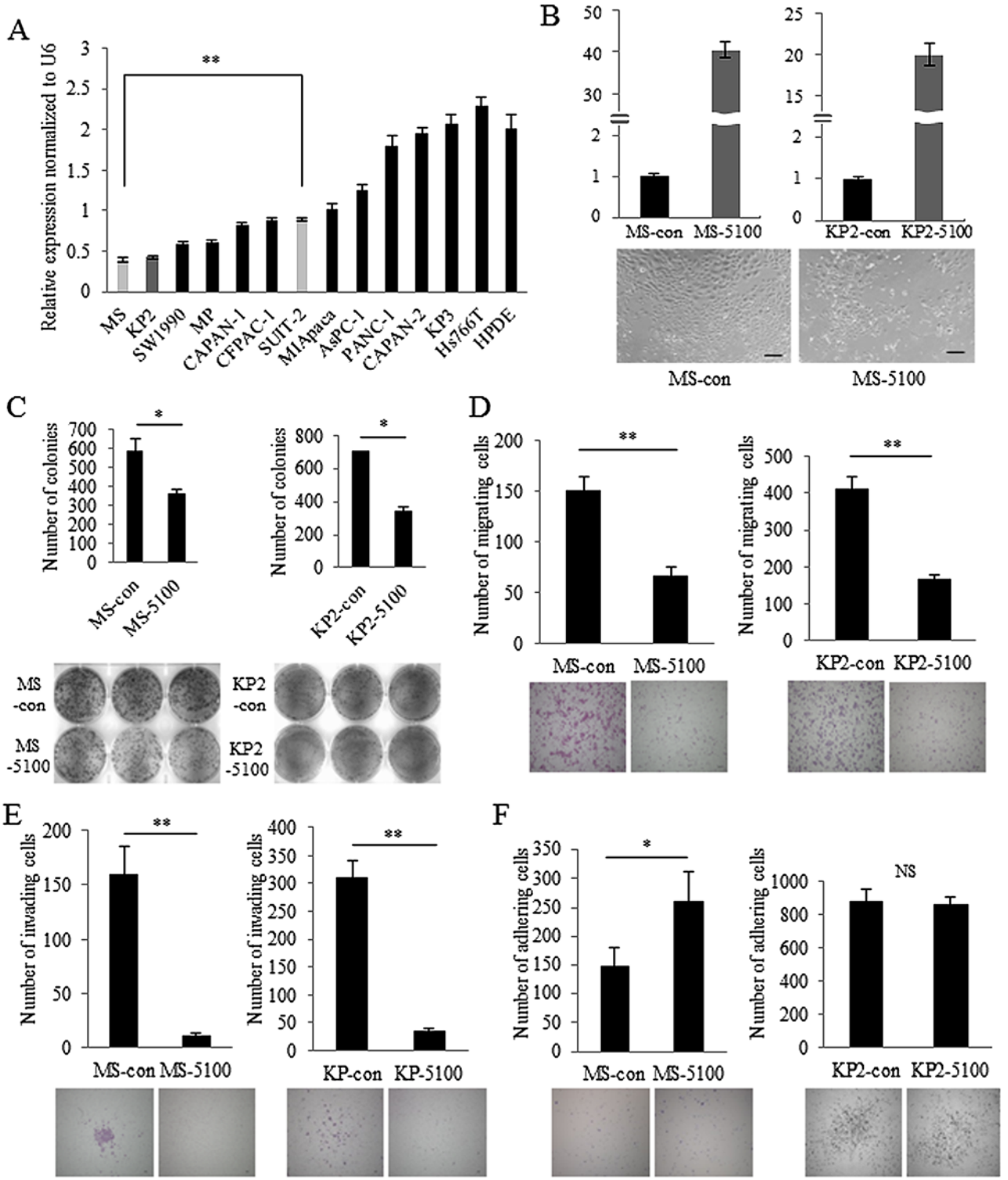


Figure 2. Validation of microarray analysis and miR-5100 expression levels in cell lines. (A) Expression of miR-5100 in pancreatic cancer cell lines. miR-5100 expression was normalized by U6 expression. (B) MS and KP2 cells were transfected with miR-5100 mimics with high levels of transfection efficiency (final oligonucleotide concentration of 3 nmol/l) (upper panel). Morphology of miR-5100 transfected cells was not remarkably changed compared with control miRNA-transfected MS cells (lower panel) (original magnification, x100; scale bar, 100 μm). (C) Colony formation in miR-5100 transfected cells was decreased compared with control miRNA-transfected cells (stain: crystal violet). (D) Migration in miR-5100 transfected cells was decreased compared with control miRNA-transfected MS cells (16 h) and KP2 cells (36 h) (stain: H&E; original magnification, x100; scale bar, 100 μm). (E) Invasion in miR-5100-transfected cells was decreased compared with control miRNA-transfected MS cells (48 h) and KP2 cells (48 h) (stain: H&E; original magnification, x40; scale bar, 100 μm). (F) While adhesion in miR-5100-transfected MS cells was increased compared with control miRNA-transfected MS cells, adhesion in miR-5100-transfected KP2 cells was not remarkably changed compared with control miRNA-transfected KP2 cells (60 min) (stain: crystal violet; original magnification, x40) (*P<0.05; **P<0.0001).

sequence with miR-5100. As shown in Table III, we found several possible candidate genes as targets of miR-5100 in MS cells. Of these genes, we focused on *PODXL* because it is a prognostic marker in many types of cancers (36-41). We

Table III. Possible target genes of miR-5100 predicted by Target Miner, Target Scan and Mir Database.

Symbol	Definition	Z score	Ratio
DCLK1	Doublecortin-like kinase 1	6.46	6.24
CLEC2D	C-type lectin domain family 2, member D	5.67	5.44
LOX	Lysyl oxidase	5.15	4.30
PODXL	**Podocalyxin-like 1(PODXL), transcript variant 1**	**4.39**	**3.71**
DCBLD2	Discoidin, CUB and LCCL domain containing 2 (DCBLD2)	3.26	3.05
ADRB2	Adrenergic, β-2-, receptor, surface (ADRB2)	3.85	2.98
TSPAN13	Tetraspanin 13 (TSPAN13)	3.37	2.74
CDK6	Cyclin-dependent kinase 6 (CDK6)	3.18	2.59
RERG	RAS-like, estrogen-regulated, growth inhibitor (RERG)	7.57	2.52
LACTB	Lactamase, β (LACTB), nuclear gene encoding mitochondrial protein, transcript variant 1	2.91	2.38

Bold text indicates the genes that the present study focuses on.

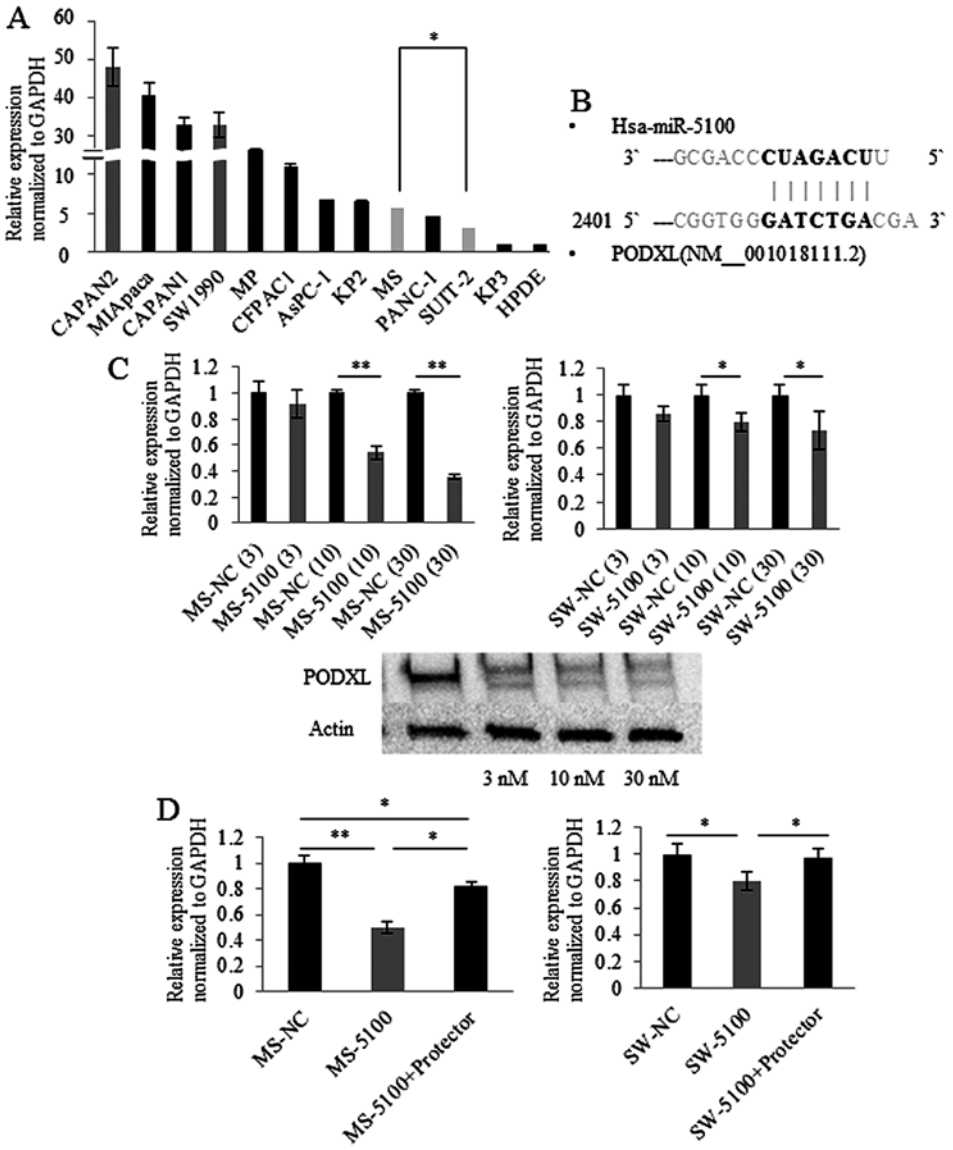


Figure 3. Validation of microarray analysis and PODXL expression levels in cell lines and miR-5100-transfected MS cells. (A) Expression of PODXL in pancreas cancer cell lines. (B) Binding site of miR-5100 to PODXL mRNA. (C) Expression of PODXL was decreased by transfecting miR-5100 mimics in a concentration-dependent manner. (D) Blocking of binding site resulted in recovery of PODXL expression ($^{*}P<0.05$; $^{**}P<0.0001$).

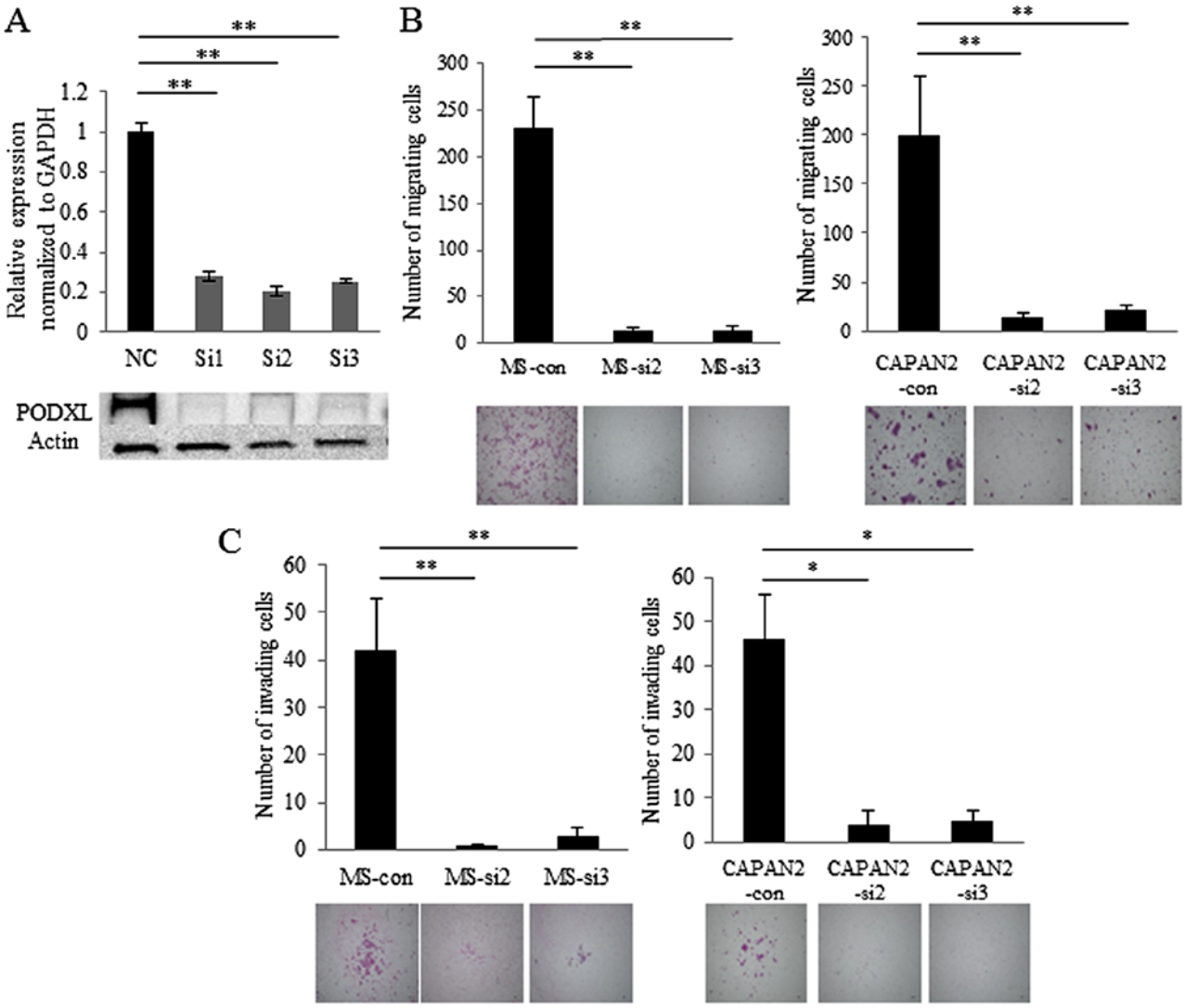


Figure 4. RNA silencing of PODXL inhibits migration and invasion of pancreatic cancer cells. (A) Expression of PODXL was decreased by siRNA. (B) Migration in siPODXL cells was decreased compared with control siRNA-transfected MS cells and CAPAN2 cells (24 or 36 h) (stain: H&E; original magnification, x100; scale bar, 100 μm). (C) Invasion in siPODXL cells was decreased compared with control siRNA-transfected MS cells and CAPAN2 cells (48 or 72 h) (stain: H&E; original magnification, x40; scale bar, 100 μm) (*P<0.05; **P<0.0001).

investigated the expression levels of *PODXL* in cultures of 12 different pancreatic cancer cell lines and HPDE cells using quantitative RT-PCR. The majority of pancreatic cancer cell lines expressed *PODXL*, while HPDE cells showed extremely low expression of *PODXL* (Fig. 3A).

miR-5100 directly regulates expression of PODXL. PODXL has seven specific nucleotides at its 3'UTR that have the ability to bind miR-5100 (Fig. 3B) and transfection of miR-5100 showed a concentration-dependent reduction in *PODXL* expression in MS and SW1990 cells (Fig. 3C). The effect of miR-5100 on translation of *PODXL* was assessed by protecting the binding site of *PODXL*. miR-5100 mimic-transfected MS cells showed approximately 50% decrease in *PODXL* expression levels and the decrease was partially relieved by blocking the binding site of *PODXL* 3'UTR (Fig. 3D, left panel). SW1990 cells showed similar results (Fig. 3D, right panel). These results indicate that miR-5100 directly binds to the 3'UTR of *PODXL* and post-transcriptionally regulates *PODXL* expression in MS and SW1990 cells.

RNA silencing of PODXL inhibits migration and invasion of pancreatic cancer cells. To explore the roles of *PODXL* in pancreatic cancer, we suppressed *PODXL* in MS (Fig. 4A) and CAPAN-2 cells using RNA interference. The morphology of *PODXL*-knockdown MS cells was not remarkably changed compared with control siRNA-transfected cells. Knockdown of *PODXL* in MS and CAPAN-2 cells resulted in diminished cell migration and invasion compared with control siRNA-transfected cells (Fig. 4B and C). These results indicate that *PODXL* promotes the aggressiveness of pancreatic cancer in MS and CAPAN-2 cells.

Associations between PODXL expression and clinicopathological factors in pancreatic cancer. To evaluate the correlation of PODXL expression in human specimens from pancreatic cancer patients with clinicopathological factors, we divided all pancreatic cancer patients into two groups: a high-PODXL expression group (n=16) and a low-PODXL expression group (n=54) (Fig. 5A). We compared the clinicopathological differences between the groups (Table IV).

Table IV. Relationship between PODXL expression and clinicopathological factors.

Characteristics	PODXL-low (n=54)	PODXL-high (n=16)	P-value
Age			
<65	24 (44%)	5 (31%)	0.354
≥65	30 (56%)	11 (69%)	
Lymph node metastasis			
No	7 (13%)	3 (19%)	0.568
Yes	47 (87%)	13 (81%)	
Liver metastasis			
No	43 (80%)	5 (31%)	0.0003
Yes	11 (20%)	11 (69%)	
Distant metastasis			
No	28 (52%)	2 (13%)	0.005
Yes	26 (48%)	14 (87%)	
Lymphatic invasion			
No	14 (26%)	3 (19%)	0.563
Yes	40 (74%)	13 (81%)	
Vascular invasion			
No	25 (46%)	3 (19%)	0.049
Yes	29 (54%)	13 (81%)	
Perineural invasion			
No	6 (11%)	0 (0%)	0.168
Yes	48 (89%)	16 (100%)	
UICC stage			
I/II	53 (98%)	15 (94%)	0.322
III/IV	1 (2%)	1 (7%)	
Pathologic margin			
Negative	38 (70%)	9 (56%)	0.298
Positive	16 (30%)	7 (44%)	

UICC, Union for International Cancer Control.

Table V. Univariate survival analysis of conventional prognostic factors and PODXL expression.

Characteristics	No. of cases	Three-year survival rate	P-value
PODXL			
Low	54	24 (44.4%)	0.006
High	16	4 (25.0%)	
Age			
<65	29	11 (37.9%)	0.389
≥65	41	17 (41.5)	
Lymph node metastasis			
No	10	8 (80.0%)	0.091
Yes	60	20 (33.3%)	
Liver metastasis			
No	48	25 (52.1%)	<0.0001
Yes	22	3 (13.6%)	
Distant metastasis			
No	30	17 (56.7%)	0.0002
Yes	40	11 (27.5%)	
Lymphatic invasion			
No	17	11 (64.7%)	0.009
Yes	53	17 (32.1%)	
Vascular invasion			
No	28	15 (53.6%)	0.027
Yes	42	13 (31.0%)	
Perineural invasion			
No	6	4 (66.7%)	0.631
Yes	64	24 (37.5%)	
UICC stage			
I/II	68	28 (41.2%)	0.17
III/IV	2	0 (0%)	
Pathologic margin			
Negative	47	21 (44.7%)	0.044
Positive	23	7 (30.4%)	

UICC, Union for International Cancer Control.

These included patient age (<65 vs. ≥65 years), lymph node metastasis, liver metastasis, distant metastasis (liver, lung, peritoneum and bone), lymphatic invasion, vascular invasion, perineural invasion, Union for International Cancer Control (UICC) stage (I/II vs. III/IV), and pathologic margin. There were no significant differences in age, lymph node metastasis, lymphatic invasion, perineural invasion, UICC stage and pathologic margin between the high-PODXL expression and low-PODXL expression groups. On the contrary, liver metastasis, distant metastasis and vascular invasion were observed more frequently in the high PODXL expression group than in the low PODXL expression group (P<0.05 each). These results indicate that PODXL expression is associated with metastatic rate in post-operative patients with pancreatic cancer.

Association between PODXL expression and survival in pancreatic cancer. We then investigated the association between PODXL expression and overall survival of post-operative patients with pancreatic cancer. Survival analysis showed that post-operative survival was longer in the low-PODXL expression group than in the high-PODXL expression group (Fig. 5B). In addition, the analysis of disease-free survival of post-operative patients showed similar results (Fig. 5C).

Univariate and multivariate analyses for factors correlated with metastasis and survival in post-operative patients with pancreatic cancer. To evaluate the prognostic value of PODXL expression in pancreatic cancer, we used the Cox proportional hazards model to evaluate PODXL expression and clinicopathological factors. Univariate analysis

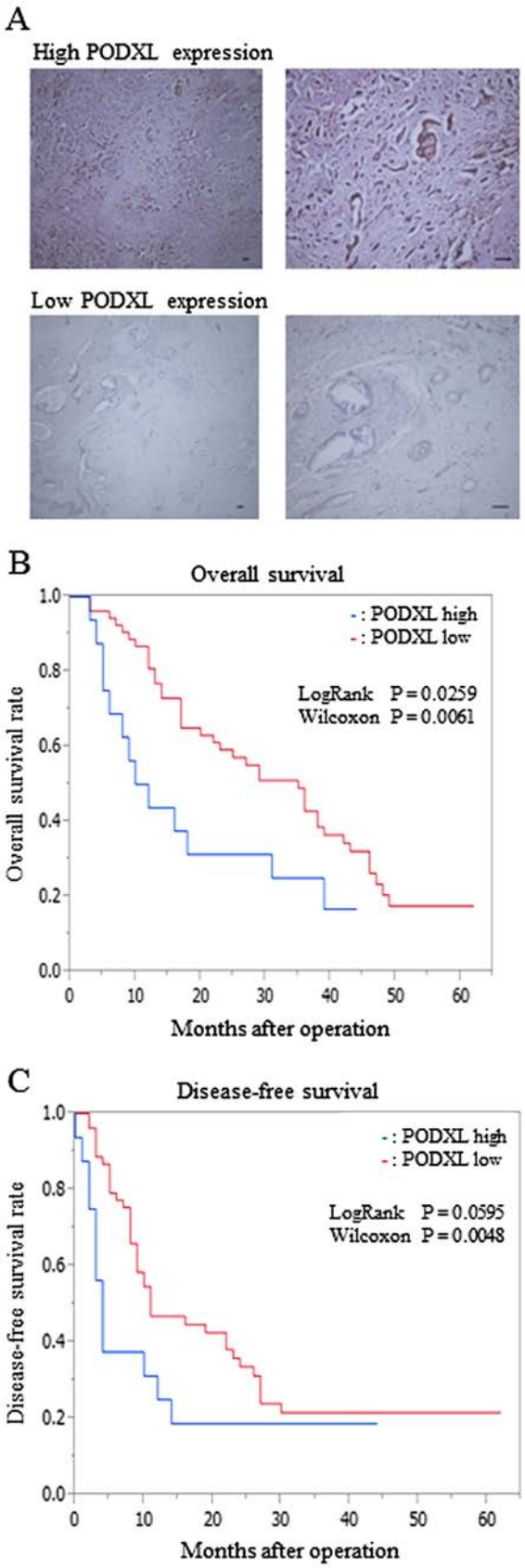


Figure 5. Kaplan-Meier survival analysis based on PODXL expression in pancreatic cancer tissues. (A) Immunohistochemical staining (original magnification, x40 and x100; scale bar, 100 μm). (B) Postoperative survival of high-PODXL expression group versus low-PODXL expression group. High PODXL expression was associated with shorter survival than low PODXL expression (log-rank test, P=0.0259; Wilcoxon test, P=0.0061). (C) Post-operative disease-free survival showed similar results (log-rank test, P=0.0595; Wilcoxon test, P=0.0048).

Table VI. Multivariate survival analysis of conventional prognostic factors and PODXL expression.

Characteristics	Relative risk	95% CI	P-value
PODXL			
Low	0.01	-0.38-0.36	0.9051
High			
Liver metastasis			
No	8.05	0.18-1.00	0.0046
Yes			
Distant metastasis			
No	0.89	-0.19-0.53	0.3445
Yes			
Lymphatic invasion			
No	4.77	0.07-1.51	0.0290
Yes			
Vascular invasion			
No	1.58	-0.21-0.99	0.2083
Yes			
Pathologic margin			
Negative	0.46	-0.84-0.39	0.4992
Positive			

showed significant prognostic values in PODXL expression (P=0.006), liver metastasis (P<0.0001), distant metastasis (P=0.0002), lymphatic invasion (P=0.009), vascular invasion (P=0.027), and pathologic margin (P=0.044) (Table V). We then performed multivariate survival analysis based on the Cox proportional hazards model for all these parameters. Multivariate analysis showed significant prognostic values in liver metastasis (P=0.0046) and lymphatic invasion (P=0.029) (Table VI).

Discussion

Recently, miRNAs have been studied in many types of cancer. Several miRNAs are already reported to correlate with pancreatic cancer in various pathways (46,47). In the present study, we performed microarray analyses and found 13 downregulated and 15 upregulated miRNAs in MS cells, and seven downregulated and 12 upregulated miRNAs in MP cells compared with their parental cells. Of these miRNAs, miR-1247, miR-16, miR-26a and let-7i were reported as tumor-suppressing miRNAs, and miR-125b was reported as tumor-promoting. High expression of miR-1247 was reported to correlate with higher overall and recurrence-free survival rates, and neuropilin, a target of miR-1247, was reported to promote extravasation and liver metastasis in pancreatic cancer and clear cell renal cell carcinoma (48-50).

miR-16 and miR-26a were reported to suppress tumor growth by regulating B-cell lymphoma 2 *(BCL-2)* and phosphorylation of P53, respectively, in pancreatic cancer (51,52). Let-7i was reported to suppress tumor growth by regulating RAS GTPase activity in pancreatic cancer (53). miR-125b

upregulation in MS cells was reported to promote a chemoresistant mesenchymal phenotype in pancreatic cancer by suppressing BCL-2 binding component 3 (BBC3) which is antagonist of BCL-2 (54). Furthermore, we also identified several possible candidate cancer-related miRNAs, miR-4755, miR-5100, miR-4454, miR-1972, miR-4706, miR-1260a, miR-1273g, miR-2964a, miR-3135b, miR-4299, miR-6087, miR-4667 and miR-4745 that have not previously been reported to be involved in cancer. Of these miRNAs, miR-5100 and miR-4454 were downregulated in both MS and MP cells compared with their parental cells. Then, we validated the data using real-time RT-PCR and found a consistent result in miR-5100 expression, but not in miR-4454 expression. Therefore, in the following experiments, we focused on miR-5100. Herein, we showed overexpression of miR-5100 suppressed cell proliferation, migration and invasion in MS and KP-2 cells and also identified PODXL as a direct target of miR-5100. The present findings suggest that miR-5100 plays an inhibitory role in tumorigenesis and metastasis of pancreatic cancer. However, miR-5100 was recently reported to promote tumor growth in lung cancer by targeting Rab6 (55), which is inconsistent with our results regarding pancreatic cancer. Therefore, further examinations will be needed to elucidate such differences in the functional roles of miR-5100 depending on cancer type.

PODXL was previously reported to enhance tumor aggressiveness in breast cancer, prostate cancer, oral squamous cell carcinoma and astrocytoma (56-59). Hsu *et al* reported PODXL-EBP50-Ezrin molecular complex enhances the metastatic potential of renal cancer through recruiting RAC1 guanine nucleotide exchange factor, ARHGEF7 (60). Lin *et al* reported that PODXL promotes invadopodia formation and metastasis through activation of RAC1-Cdc42-cortactin signaling in breast cancer cells (61) and it is thought to regulate cell adhesion through its connections to intracellular proteins and to extracellular ligands (31-35). In our study, the adhesion capability of MS cells that showed high PODXL expression was remarkably decreased compared with that of low PODXL-expressing cells, as previously reported (31-35). In addition, we showed for the first time that PODXL played an important role in pancreatic tumor aggressiveness by promoting cancer cell migration and invasion. Taken together, these data suggest that PODXL may promote cell migration and invasion by regulating cell adhesion in many types of cancers.

High immunohistochemical expression of PODXL in human specimens was reported to correlate with poor prognosis in high grade serous ovarian cancer, breast cancer and colorectal cancer (36-40). Regarding pancreatic cancer, Saukkonen *et al* reported that PODXL is an independent factor for poor prognosis (41). In the present study, our results did not show independent prognostic values for PODXL expression, possibly because of the limited number of cases, but indicated a close relationship between PODXL expression and liver metastasis of pancreatic cancer in human specimens. Although PODXL expression has previously reported to correlate with distant metastasis of colorectal cancer (39), details of metastatic target organs were not described. Our results also revealed that PODXL expression was correlated with distant metastasis of pancreatic cancer including liver metastasis. However, we did not find any significant relationships between PODXL expression and other distant metastases such as lung metastasis, peritoneal metastasis and bone metastasis, possibly because of the limited number of samples. Further study of additional case samples would hopefully clarify the relationships between PODXL expression and metastases to each organ.

In conclusion, miR-5100 directly regulates *PODXL* expression and this pathway correlates with the aggressive and metastatic characteristics of pancreatic cancer. Thus, miR-5100 and *PODXL* could be potential indicators for cancer metastases, particularly for liver metastases, and attractive anti-metastatic therapeutic targets for patients with pancreatic cancer.

Acknowledgements

This study was supported in part by Japan Society for the Promotion of Science (JSPS) Grant-in-Aid for Scientific Research (B) and (C) and Scientific Research on Innovative Areas (grant numbers: 15K10185, 26293305, 26462062, 25293285, 25462117, 26462063 and 15H04933).

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