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MicroRNA-296-5p Promotes Cell Invasion and Drug Resistance by Targeting Bcl2-Related Ovarian Killer, Leading to a Poor Prognosis in Pancreatic Cancer

Jun Okazaki Toshihito Tanahashi Yasushi Sato Jinsei Miyoshi Tadahiko Nakagawa Tetsuo Kimura Hiroshi Miyamoto Yasuteru Fujino Fumika Nakamura Masanori Takehara Beibei Ma Masahiro Bando Shinji Kitamura Koichi Okamoto Naoki Muguruma Masahiro Sogabe Tetsuji Takayama

Department of Gastroenterology and Oncology, Institute of Biomedical Sciences, Tokushima University Graduate School, Tokushima, Japan

Keywords

Apoptosis · Biomarker · Epithelial-mesenchymal transition · MicroRNA · Pancreatic cancer

Abstract

Background/Aims: Pancreatic ductal adenocarcinoma (PDAC) is characterized by aggressive invasion, early metastasis, and resistance to chemotherapy, leading to a poor prognosis. To clarify the molecular mechanism of these malignant characteristics, we performed a genome-wide microRNA (miRNA) array analysis utilizing micro-cancer tissues from patients with unresectable PDAC (stage IV), obtained by endoscopic ultrasound-fine needle aspiration (EUS-FNA). ***Methods:*** The expression profiles of 2,042 miRNAs were determined using micro-cancer tissues from 13 patients with unresectable PDAC obtained by EUS-FNA. The relationship between individual miRNA levels and overall survival (OS) was analyzed. Possible target genes for miRNAs were bioinformatically analyzed using the online database miRDB. Pancreatic cancer cell lines PANC-1, MIA PaCa-2, and PK-8 were transfected with miRNA mimic or small interfering RNA, and cell invasion, epithelial-mesenchymal transition (EMT), and apoptosis markers were examined. miRNA and mRNA expressions were examined by quantitative polymerase chain reaction. ***Results:*** Of 2,042 miRNAs, the 10 that exhibited the lowest correlation coefficient ($p \leq 0.005$) between miRNA expression level and OS among the patients were identified. The miRDB and expression analysis in cancer cell lines for the 10 miRNAs identified miR-296-5p and miR-1207-5p as biomarkers predictive of shorter survival ($p < 0.0005$). Bioinformative target gene analysis and transfection experiments with miRNA mimics showed that *Bcl2-related ovarian killer* (*BOK*), a pro-apoptotic gene, is a target for miR296-5p in pancreatic cancer cells; transfection of miR-296-5p mimic into PANC-1, MIA PaCa-2, and PK-8 cells resulted in significant suppression of *BOK* mRNA and protein expression. These transfectants showed significantly higher invasion capability compared with control cells, and knock down of *BOK* in pancreatic cancer cells similarly enhanced invasion capability. Transfectants of miR-296-5p mimic also exhibited aberrant expression of EMT markers, including vimentin and N-cadherin. Moreover, these transfectants showed a significantly lower apoptosis rate in response to 5-fluorouracil and

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Tetsuji Takayama
Department of Gastroenterology and Oncology
Institute of Biomedical Sciences, Tokushima University Graduate School
3-18-15, Kuramoto-cho, Tokushima 770-8503 (Japan)
E-Mail takayama@tokushima-u.ac.jp

gemcitabine with a decrease of *BOK* expression, suggesting a role of miR-296-5p in drug resistance. ***Conclusion:*** These results suggest that miR-296-5p is a useful biomarker for a poor prognosis in patients with PDAC, and that the miR-296-5p/BOK signaling axis plays an important role in cell invasion, drug resistance, and EMT in PDACs.

Introduction

Despite recent improvements in diagnostic and therapeutic strategies, pancreatic cancer remains a major cause of cancer-related death worldwide [1]. The current 5-year survival rate for pancreatic cancer patients is <10% [2]. Pancreatic ductal adenocarcinoma (PDAC), a common type of pancreatic cancer, is characterized by aggressive local invasion, early metastasis, and resistance to chemotherapy. Moreover, it has recently been reported that epithelial-mesenchymal transition (EMT) of cancer cells is associated with acquisition of invasion ability, drug resistance, and enhanced cell proliferation. To improve the prognosis of PDAC, it is essential to clarify the molecular mechanisms that contribute to the underlying malignancy and to find molecular targets for the development of therapeutic and diagnostic strategies.

MicroRNAs (miRNAs) are endogenous small noncoding RNA molecules of 20–25 nucleotides that regulate the expression of target genes through transcription and/or translation [3, 4]. miRNAs play an important role in carcinogenesis, metastasis, and invasion in various types of cancers including pancreatic cancer [5, 6]. Moreover, recent advances in microarray technologies have made it possible to measure the global expression of >1,000 miRNAs simultaneously [7]. Using this method, several studies have detected specific miRNAs related to the pathogenesis of pancreatic cancer [8–13]. However, most of these studies have used surgically-operated PDAC tissues for analyses of miRNA and/or mRNAs because inoperative metastatic PDAC tissues were not available. That is, only operable PDAC tissues, which show no metastasis, drug resistance, or EMT, have been examined thus far in efforts to identify useful molecular biomarkers and therapeutic targets.

Recently, endoscopic ultrasonography (EUS) and EUS-guided fine needle aspiration (EUS-FNA) sampling of pancreatic tumors have become popular procedures worldwide [14, 15]. In clinical practice, EUS-FNA is safely and easily performed for pathological diagnosis of not only early pancreatic cancer but also of inoperable metastatic pancreatic cancer. Although the amount of cancer tissue obtained by EUS-FNA is very small, we confirmed in our preliminary experiments that the amount of cancer tissue obtained by EUS-FNA was sufficient for miRNA array analysis. Therefore, in this study, we first performed a genome-wide miRNA array analysis using micro-tissues obtained by EUS-FNA to find useful prognostic biomarkers for unresectable advanced PDAC. We ultimately identified miR-296-5p as a specific prognostic marker for PDAC and examined its target genes and potential role in invasion, EMT, anti-apoptotic resistance, and proliferation in PDAC.

Patients and Methods

Human Tissue Specimens

This study was approved by the Ethics Committee of Tokushima University Hospital (Approval number; 1845). Thirteen patients with pancreatic tumors, suspected as advanced pancreatic cancer based on CT, were recruited at Tokushima University Hospital. After obtaining written informed consent, 3 tumor specimens per patient were obtained by EUS-FNA technique with a 22- or 25-gauge needle; 2 were fixed for pathological diagnosis and 1 was frozen at –80 °C for RNA analysis. All the touch smears were stained using Diff-Quick (Mercedes Medical, Sarasota, FL, USA) to evaluate tumor cell morphology. Hematoxylin and eosin staining was performed to confirm pathological diagnosis of pancreatic cancer. All patients were definitely diagnosed as having unresectable PDAC and were enrolled in the study. Baseline characteristics of the patients are shown in Table 1. All patients were at clinical stage IV, had not undergone surgical resection, and had received chemotherapy after the diagnosis of PDAC. The disease response to treatment was defined according to the Response Evaluation Criteria in Solid Tumors version 1.1 [16].

Cell Lines and Culture Condition

Five human pancreatic cancer cell lines were used. PANC-1 and MIA PaCa-2 cell lines were purchased from the American Type Culture Collection (Manassas, VA, USA). PK-1, PK-8, and PK-45H cell lines were purchased from RIKEN Cell Bank (Kobe, Japan). PANC-1 cells were cultured in Dulbecco's Modified Eagle's medium (DMEM) containing 10% fetal bovine serum (FBS). MIA PaCa-2 cells were cultured in DMEM containing 10% FBS and 2.5% horse serum. PK-1, PK-8, and PK-45H were cultured in RPMI-1640 medium containing 10% FBS.

RNA Extraction and miRNA Microarray

Total RNA including the miRNA fraction was extracted from EUS-FNA samples and pancreatic cancer cell lines using a miRNeasy Mini Kit (Qiagen, Hilden, Germany). The concentration of RNA was measured with a NanoDrop 1000 spectrophotometer (NanoDrop Technologies; Thermo Scientific), and the quality of the purified RNA was confirmed by measuring RNA integrity with an Agilent 2100 Bioanalyzer and RNA 6000 LabChip Kit (Agilent Technologies, Palo Alto, CA, USA).

Table 1. Characteristics of 13 patients of unresectable PDAC

No.	Age, years	Gender	Histology	Location	Treatment	cStage	Survival period, months	Prognosis
1	62	Male	Adenocarcinoma	Body, tail	S-1	IVa	22	Alive
2	60	Female	Adenocarcinoma	Body, tail	SOXIRI	IVa	20	Dead
3	62	Male	Adenocarcinoma	Body, tail	SOXIRI	IVb	19	Dead
4	80	Female	Adenocarcinoma	Head	S-1	IVa	16	Alive
5	51	Female	Adenocarcinoma	Body, tail	GEM	IVb	12	Dead
6	43	Female	Adenocarcinoma	Head	SOXIRI	IVa	9	Dead
7	66	Male	Adenocarcinoma	Body, tail	GEM, nab-PTX	IVb	9	Dead
8	72	Female	Adenocarcinoma	Tail	SOXIRI	IVb	8	Dead
9	86	Male	Adenocarcinoma	Tail	S-1	IVb	8	Dead
10	67	Female	Adenocarcinoma	Body	GEM, nab-PTX	IVb	6	Dead
11	68	Male	Adenocarcinoma	Body	S-1	IVb	6	Dead
12	80	Female	Adenocarcinoma	Tail	GEM	IVb	5	Dead
13	67	Male	Adenocarcinoma	Body	GEM, S-1	IVb	4	Dead

PDAC, pancreatic ductal adenocarcinoma; SOXIRI, S-1, oxaliplatin, and irinotecan; GEM, gemcitabine; nab-PTX, nab-paclitaxel.

A human miRNA microarray (GPL18402; based on Sanger miRbase release 19.0; Agilent Technologies) was used for measuring global miRNA expression in PDCA tissues. Total RNA was labeled with cyanine 3-cytidine bisphosphate using T4 ligase and hybridized to a SurePrint G3 human miRNA microarray using miRNA complete labeling reagent and a hybridization kit (Agilent Technologies). Subsequently, each sample was scanned using a Microarray Scanner (G2505C; Agilent Technologies), and fluorescence signals were analyzed using Feature Extraction Software (version 10.7.3.1). Raw intensity miRNA data were analyzed using GeneSpring GX 12 software (Agilent Technologies). The complete datasets were deposited in the Gene Expression Omnibus database with an accession number of GSE109918.

In Silico Identification of miRNA Target Genes

To bioinformatically predict target genes based on the miRNA seed sequence, we used miRDB (http://mirdb.org/), an online database for miRNA target prediction and functional annotations [17].

Quantitative Real-Time Polymerase Chain Reaction

miRNA expression was measured by using TaqMan miRNA Assay (Applied Biosystems, Foster City, CA, USA). RNU48 snRNA was used as an internal quantity control for normalization. The assay numbers of the TaqMan miRNA reagents were as follows: hsa-miR-296-5p (#000527), hsa-miR-516a-5p (#002416), hsa-miR-557 (#001525), hsa-miR-1207-5p (#241060), hsa-miR-4740-5p (#462027), and RNU48 (#001006). Relative expression levels of miRNA in each sample were measured by using the delta-delta Ct method.

The mRNA levels of 4 predicted target genes (*Bcl2-related ovarian killer* [*BOK*], *NUMBL*, *CD276*, and *ATP11A*) were determined using Power SYBR green polymerase chain reaction (PCR) Master Mix (Applied Biosystems) and an ABI 7,500 real-time PCR system (Applied Biosystems). The primer sequences used are shown in online supplementary Table S1 (for all online suppl. material, see www.karger.com/doi/10.1159/000503225). Glyceraldehyde 3-phosphate dehydrogenase mRNA was used as an endogenous quantitative control, and the relative expression levels of mRNA in each sample were measured by using the delta-delta Ct method.

Transfection of miRNA Mimics

miR-296-5p and miR-1207-5p mimic (mirVana miRNA mimic; Life Technologies) were used for transfection into pancreatic cancer cell lines. PANC-1, MIA PaCa-2, and PK-8 cells (1.0×10^4) plated in 6-well plates were transfected with miR-296-5p or miR-1207-5p mimic (mirVana miRNA mimic; Life Technologies) at a final concentration of 30 nM using Lipofectamine RNAiMAX (Invitrogen, Carlsbad, CA, USA) in Opti-MEM medium (Gibco, Life Technologies, Gaithersburg, MD, USA). mirVana miRNA Mimic, Negative Control #1 (Life Technologies) was used as a negative control miRNA.

Gene Knockdown Using Small Interfering RNA

Specific double-stranded, small interfering RNA (siRNA) targeting *BOK* (Silencer Select s194313; Ambion, Austin, TX, USA; sense, 5′-GCAAGGUGGUGUCCCUGUAtt-3′; antisense, 5′-UACAGGGACACCACCUUGCcc-3′) was transfected into PANC-1 cells using Lipofectamine RNAiMAX (Invitrogen) according to the manufacturer's instructions. Silencer Negative Control #1 siRNA (AM4611; Ambion) was used as a negative control.

Cell Proliferation and Invasion Assays

For the proliferation assay, PANC-1 and MIA PaCa-2 cells were transfected with an miRNA mimic or negative control and then plated in a 96-well plate for incubation in DMEM medium with 10% FBS. Viable cells were counted at 6, 24, 48, 72, and 96 h using a Cell Counting Kit-8 (Dojindo Laboratories, Kumamoto, Japan).

Cell invasion assays were performed using a CytoSelect 24-well cell invasion assay kit (Cell BioLab, San Diego, CA, USA) according to the manufacturer's instructions. PANC-1 and MIA PaCa-2 cells transfected with miRNA mimic or control mimic

were re-suspended in culture medium without FBS and placed in the upper chamber in triplicate. The upper surface of the insert membrane is coated with a uniform layer of dried basement membrane matrix solution. This basement membrane layer serves as a barrier to discriminate invasive cells from non-invasive cells. The non-invasive cells were removed from the top of the membrane and the invasive cells were stained and quantified. After 48 h incubation at 37 °C, cells migrating through the membrane were stained, lysed, and quantified by measuring the optical density at 560 nm.

Western Blotting

Western blotting was performed, as previously described [18]. Briefly, cells were lysed in RIPA buffer (50 mM Tris pH 8.0, 150 mM NaCl, 1% NP40, 0.5% sodium deoxycholate, and 0.1% sodium dodecyl sulfate) containing protease inhibitors (Sigma-Aldrich St. Louis, MO, USA), and the protein concentration was measured. Cell lysates were resolved by sodium dodecyl sulfate-polyamide gel electrophoresis and transferred to polyvinylidene fluoride membranes. Blots were blocked with 5% fat-free dry milk in Tris-buffered saline-0.1% Tween and incubated with primary antibodies. The primary antibodies used were as follows: rabbit anti-human BOK monoclonal antibody (Abcam; ab186745), rabbit anti-human caspase-9 polyclonal antibody (Cell Signaling; #9502), rabbit anti-human E-cadherin polyclonal antibody (Cell Signaling; #3195), rabbit anti-human N-cadherin monoclonal antibody (Cell Signaling; #13116), rabbit anti-human vimentin monoclonal antibody (Cell Signaling; #5741), and mouse anti-human-β-actin monoclonal antibody (Sigma-Aldrich; A5441).

The membranes were then incubated with secondary horseradish-conjugated goat anti-rabbit antibody (GE Healthcare, UK Ltd., Buckinghamshire, UK), and the proteins were visualized by standard procedures including an ECL detection system (GE Healthcare UK Ltd.). β-actin was used as an internal control.

Statistical Analysis

Experimental data were analyzed using SPSS version 12.0 (SPSS, Inc., Chicago, IL, USA). Correlations between microarray raw signals and the overall survival (OS) for each patient were assessed using Pearson's correlation test. Data from the invasion assay and proliferation assay were analyzed using Student *t* test. Survival analysis was performed with the Kaplan-Meier method, and the log-rank test was used to compare OS between high and low expression groups of miRNAs. GraphPad Prism version 8.0 (GraphPad Software, Inc., La Jolla, CA, USA) was used for this analysis.

Results

Correlation between Individual miRNA Levels and OS in Patients with PDAC

In this study, we obtained small cancer tissues by EUS-FNA from 13 patients with PDAC and extracted total RNAs (online suppl. Table S2). The clinical characteristics of the patients are described in Table 1. Total RNAs were obtained in appreciable amounts and were of sufficient purity for miRNA array analysis (online suppl. Fig. S1).

Table 2. Top 10 miRNAs related to the prognosis in PDAC patients

miRNA	Correlation coefficient	*p* value
hsa-miR-3620-3p	−0.790	0.001
hsa-miR-296-5p	−0.749	0.003
hsa-miR-6500-5p	−0.735	0.004
hsa-miR-516a-5p	−0.735	0.004
hsa-miR-557	−0.733	0.004
hsa-miR-1207-5p	−0.731	0.005
hsa-miR-6088	−0.729	0.005
hsa-miR-887	−0.728	0.005
hsa-miR-4740-5p	−0.721	0.005
hsa-miR-1249	−0.721	0.005

Pearson's correlation coefficients were calculated from the values between the microarray data and the OS periods.

Bold faces are representative 5 miRNAs for the further analysis.

miRNA, microRNA; PDAC, pancreatic ductal adenocarcinoma; OS, overall survival.

Expression profiles for 2,042 miRNAs from the 13 PDAC specimens were successfully obtained. Correlations between individual miRNA expression levels and the OS (months) were then analyzed for 13 patients. Among the 2,042 miRNAs, the 10 miRNAs that showed the lowest correlation coefficients (ranging from −0.790 to −0.721) and the lowest *p* values (under 0.005; Table 2) were selected. Representative plots for 5 miRNAs (miR-296-5p, miR-516a-5p, miR-557, miR-1207-5p, and miR-4740-5p) that showed correlations between miRNA expression and survival time are shown in Figure 1.

Predicted Target Genes of Candidate miRNAs

Possible target genes for the 10 selected miRNAs were examined using the miRDB. Unexpectedly, 4 of these miRNAs (miR-6500-5p, miR-6088, miR-887, and miR-1249) did not show the predicted target gene in the miRDB. Moreover, the sequence for miR-3620 was not suitable for the design of TaqMan PCR probes in the following series of experiments. Therefore, we present candidate target genes of the top 5 miRNAs (miR-296-5p, miR-516a-5p, miR-557, miR-1207-5p, and miR-4740-5p) identified by miRDB analysis in Table 3. Notably, the scores for the target genes of miR-296-5p, miR-557, miR-1207-5p, and miR-4740-5p were >95.

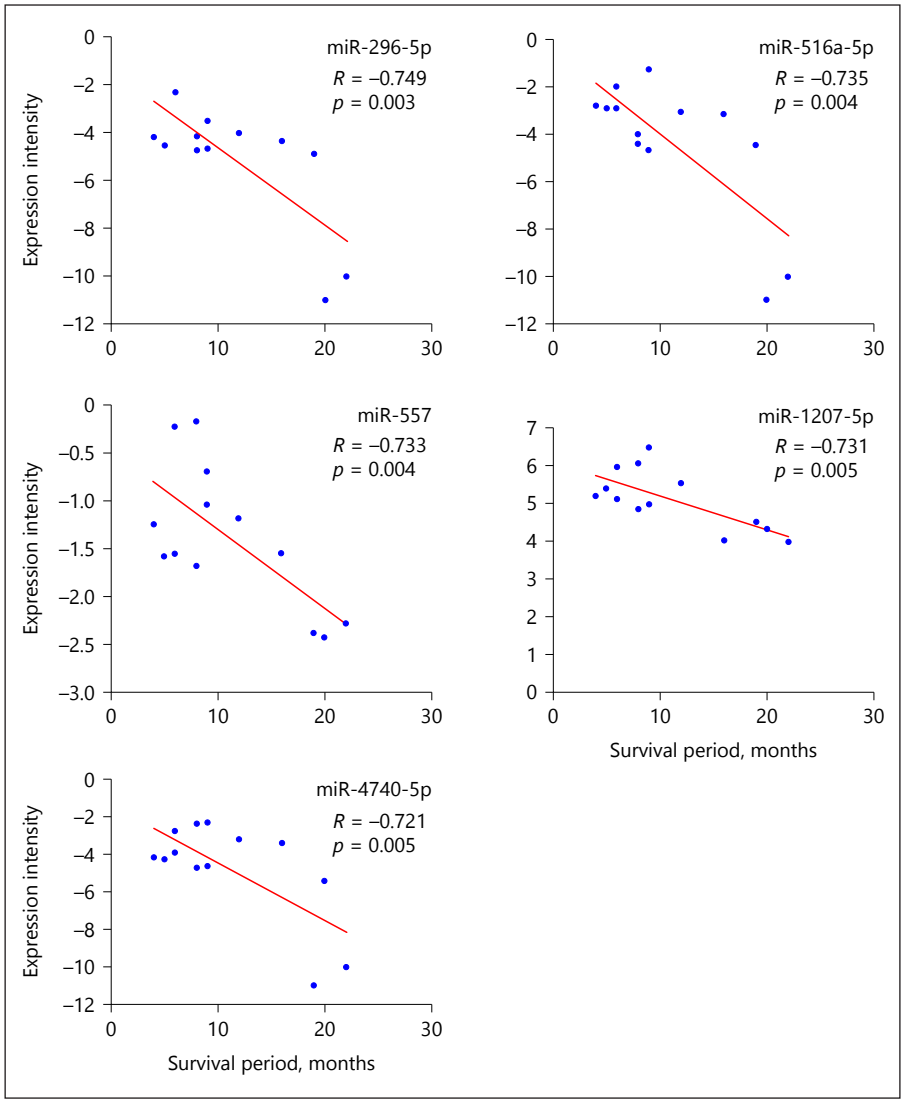

Fig. 1. Negative correlations between the expression of 5 miRNAs and survival period in 13 unresectable PDAC patients. Using genome-wide miRNA arrays, the expression of 2,042 miRNAs was analyzed in 13 micro-pancreatic cancer tissues by EUS-FNA. Correlations between miRNA expression and the OS (months) were calculated using Pearson's correlation coefficient. The results for 5 representative miRNAs (miR-296-5p, miR-516a-5p, miR-557, miR-1207-5p, and miR-4740-5p) are shown. For each of the miRNAs, *p* values were calculated using Pearson's test. *R* indicates the coefficient of determination.

Validation of miRNA Expressions in Pancreatic Cancer Cell Lines

The expression levels of the 5 miRNAs in 5 pancreatic cancer cell lines (PANC-1, MIA PaCa-2, PK-1, PK-8, and PK-45H) were examined using the TaqMan quantitative PCR (qPCR) method. The expression levels of miR-1207-5p were high in all cell lines (Fig. 2). Expression of miR-296-5p was high in MIA PaCa-2, PK-8, and PK-45H cells but not in PANC-1 and PK-1 cells. Expression of miR-516a-5p, miR-557, and miR-4740-5p was relatively low or not detected, suggesting the possibility that their cellular function was lower in the 5 cancer cell lines. Thus, we focused on miR-296-5p and miR-1207-5p in the following experiments.

Correlation of miR-296-5p and miR-1207-5p Expression with OS

We also examined correlations of miR-296-5p and miR-1207-5p expression with OS in 13 PDAC patients using the Kaplan-Meier method (online suppl. Fig. S2). Based on the expression intensity of the miR-296-5p in 13 cancer tissues (Fig. 1), we categorized 11 and 2 patients into high and low miR-296-5p groups, respectively. The OS of the low miR-296-5p group was significantly longer than that of high-miR-296-5p group ($p = 0.031$; online suppl. Fig. S2a). Similarly, we categorized 4 and 9 patients into low and high miR-1207-5p groups, respectively. The OS of the low miR-1207-5p group was significantly longer than that of the high miR-1207-5p group ($p = 0.002$; online suppl. Fig. S2b).

Table 3. Predicted target gene of miRNAs by miRDB

miRNA	Gene symbol	Description	Score
has-miR-296-5p	BOK	BOK	99
	NUMBL	NUMB like, endocytic adaptor protein	97
	AMMECR1L	AMMECR1 like	96
	ZMYM3	Zinc finger MYM-type containing 3	96
	CYB5R3	Cytochrome B5 reductase 3	96
has-miR-516a-5p	TMEM68	Transmembrane protein 68	79
	PIK3R4	Phosphoinositide-3-kinase regulatory subunit 4	74
	FAM200B	Family with sequence similarity 200 member B	72
	TXNRD3	Thioredoxin reductase 3	68
	HAUS6	HAUS augmin-like complex subunit 6	65
has-miR-557	ZER1	Zyg-11 related cell cycle regulator	100
	RBMS3	RNA binding motif single stranded interacting protein 3	100
	SCN1A	Sodium voltage-gated channel alpha subunit 1	100
	BACH2	BTB domain And CNC homolog 2	100
	CLOCK	Clock circadian regulator	99
has-miR-1207-5p	CD276	CD276 molecule	100
	ATP11A	ATPase phospholipid transporting 11A	100
	CBX6	Chromobox 6	100
	TMEM201	Transmembrane protein 201	100
	SLC6A9	Solute carrier family 6 member 9	100
has-miR-4740-5p	ACLY	ATP citrate lyase	99
	ATG12	Autophagy related 12	99
	SPTLC2	Serine palmitoyltransferase long chain base subunit 2	97
	MLEC	Malectin	96
	AGAP1	ArfGAP with GTPase domain, ankyrin repeat And PH domain 1	96

miRNA, microRNA; BOK, Bcl-2 related ovarian killer.

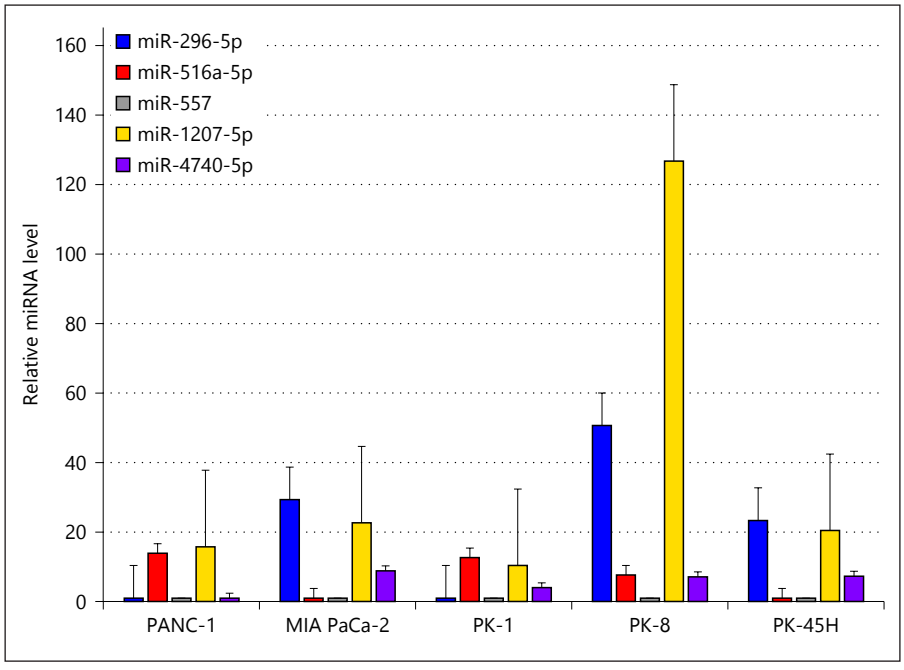

Fig. 2. Expression levels of miR-296-5p, miR-516a-5p, miR-557, miR-1207-5p, and miR-4740-5p in 5 pancreatic cancer cell lines. Expression levels of each miRNA in PANC-1, MIA PaCa-2, PK-1, PK-8, and PK-45H cells were determined by TaqMan qPCR. Relative expression levels are shown as the value normalized to RNU48. miRNA, microRNA.

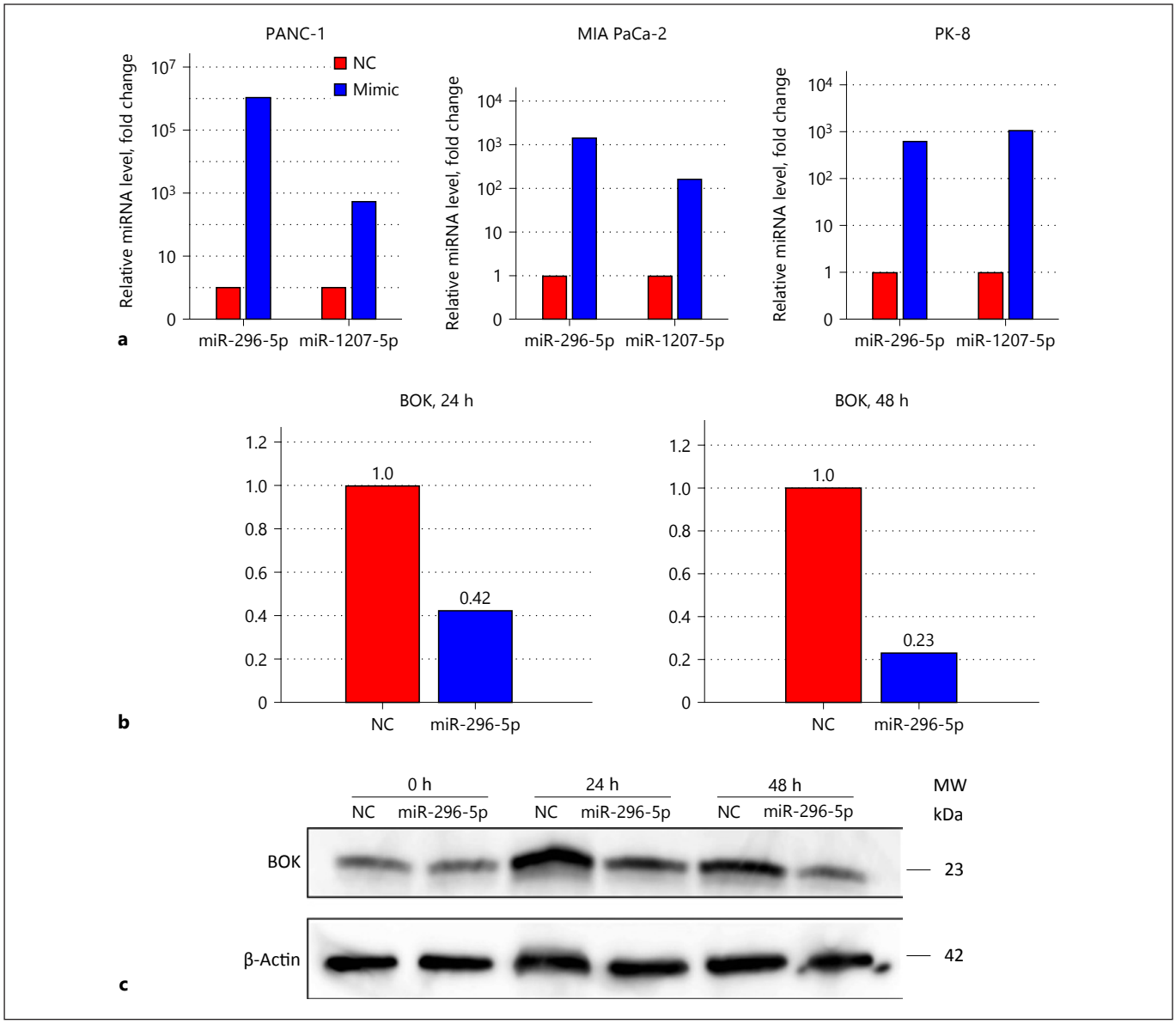

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Fig. 3. Overexpression of miR-296-5p decreased *BOK* expression. **a** The expression levels of miR-296-5p or miR-1207-5p in PANC-1, MIA PaCa-2, and PK-8 cells transected with each mimic were determined by TaqMan qPCR. **b** Levels of *BOK* mRNA in PANC-1 cells transfected with miR-296-5p or control mimic were determined by TaqMan qPCR at 24 and 48 h after transfection. **c** BOK protein levels in transfectants of miR-296-5p or control mimic were determined by Western blotting at 24 and 48 h after transfection. miRNA, microRNA; BOK, Bcl2-related ovarian killer; MW, molecular weight.

Altered Expression of Target Genes by miR-296-5p

To investigate whether miR-296-5p and miR-1207-5p regulate their candidate target genes in pancreatic cancer cells, we transfected their mimics into the cell lines and examined the target gene mRNA levels using SYBR green qPCR. Among the target genes for miR-296-5p, *BOK* and *NUMBL* (NUMB like, endocytic adaptor protein) were selected based on their known functions in cancer cells [19, 20] and predicted high scores in the miRDB (Table 3). Similarly, *CD276* and *ATP11A* (ATPase phospholipid transporting 11A) were chosen as the represented target for miR-1207-5p (Table 3).

The expression levels of miR-296-5p or miR-1207-5p were 10^2 to 10^6-fold higher in PANC-1 cells transfected with their mimics compared with those in control cells, indicating high transfection efficiency (Fig. 3a). Similarly,

miR-296-5p and miR-1207-5p expression levels in MIA PaCa-2 and PK-8 cells transfected with their mimics were 10^2 to 10^3-fold higher as compared with control cells, indicating high transfection efficiency.

BOK mRNA levels in PANC-1 cells transfected with miR-296-5p mimic were decreased by 58% at 24 h and 78% at 48 h compared with control cells ($p < 0.01$, respectively; Fig. 3b). Likewise, MIA PaCa-2 and PK-8 cells transfected with miR-296-5p mimic showed decreases of 85 and 70%, respectively, compared with control cells ($p < 0.01$ respectively; online suppl. Fig. S3a). BOK protein expression was also significantly decreased in PANC-1 cells at 24 and 48 h after transfection with miR-296-5p mimic (Fig. 3c). There were no significant changes in *NUMBL* mRNA levels after transfection of cells with miR-296-5p mimic (online suppl. Fig. S3a). Moreover, the levels of *CD276* and *ATP11A* mRNA in PANC-1 cells did not change significantly after transfection with miR-1207-5p mimic (online suppl. Fig. S3b). Thus, it is evident that *BOK* could be a functional target of miR-296-5p in pancreatic cancer cells. It was also confirmed that a core 7-nucleotide sequence of mature miR-296-5p (GGGGCCC) matched 17 sites within the 3′-untranslated region of *BOK* (1732 bp) in the miRDB (online suppl. Fig. S4).

miR-296–5p Modulated Cell Invasion and EMT Properties

Since miR-296-5p was associated with poor survival in patients with PDAC, we first investigated the effect of miR296-5p on the invasion capability using transfectants of miR-296-5p mimics. The invasion capability of PANC-1 and MIA PaCa-2 cells transfected with a miR-296–5p mimic was significantly higher than that of cells transfected with a negative control mimic (Fig. 4a, $p < 0.01$, respectively). Moreover, significantly higher invasion capability was observed in PK-1 cells after transfection of miR296-5p mimics (online suppl. Fig. S5, $p < 0.01$).

Next, we examined the role of *BOK* in cell invasion by knockdown of the gene using siRNA in MIA PaCa-2 cells. The level of *BOK* mRNA in MIA PaCa-2 cells transfected with siRNA targeting *BOK* was inhibited by 85%, although the *BOK* mRNA levels in PANC-1 and PK-1 transfectants were not inhibited efficiently, probably due to the low expression of miR-296-5p in these cell lines (data not shown). MIA PaCa-2 cells in which *BOK* was knocked down showed significantly enhanced cell invasion compared with cells transfected with scrambled siRNA (Fig. 4b, $p < 0.05$). These data strongly suggest that miR-296-5p enhances cell invasion ability through inhibition of *BOK*.

Since cancer cell invasion is reportedly associated with EMT, we next investigated the expression of EMT-associated proteins including E-cadherin, N-cadherin, and vimentin by Western blotting (Fig. 4c). The expression of E-cadherin in MIA PaCa-2 cells transfected with miR-296-5p mimic was slightly lower at 24 h and significantly lower at 48 h as compared with control cells, while the expression of N-cadherin was similar or slightly increased and the expression of vimentin was significantly higher at 48 h. These results suggest that miR-296-5p overexpression induces EMT in pancreatic cancer cells.

miR-296–5p Affected Drug-Induced Apoptosis by BOK Inhibition

We investigated whether miR-296–5p affects the drug resistance of PANC-1 cells to 5-fluorouracil (5-FU) by Western blotting for caspase-9 (Fig. 5a). Addition of 5-FU to PANC-1 cells transfected with negative control mimic led to a significant increase in cleaved caspase-9 expression, suggesting 5-FU-induced apoptosis. However, cleaved caspase-9 expression in PANC-1 cells transfected with 296-5p mimic was significantly lower, while caspase-9 expression in the transfectants was higher than that of control cells. Moreover, BOK protein expression in transfectants of miR-296-5p was decreased as compared with that of control cells. Similar results were obtained for MIAPaCa-2 cells transfected with miR-296-5p mimic; cleaved caspase-9 expression and BOK expression were lower than in control cells (online suppl. Fig. S6). These results suggest that miR-296-5p induced drug resistance to 5-FU through inhibition of *BOK* expression in pancreatic cancer cells. We also examined the effect of miR-296-5p on gemcitabine (GEM) resistance in PANC-1 cells (online suppl. Fig. S7). Addition of GEM (30 μM) to PANC-1 cells transfected with negative control mimic resulted in a significant increase in cleaved caspase-9. In PANC-1 cells transfected with miR-296-5p, however, cleaved caspase-9 expression and BOK expression were lower than in control cells. Thus, our data suggest that miR-296-5p induced drug resistance to 5-FU and GEM in pancreatic cancers.

The effect of miR-296-5p on cell proliferation in PANC-1 and MIA PaCa-2 cells was investigated by cell counting (Fig. 5b). No significant difference in proliferation was observed between each transfectant with miR-296-5p mimic compared with control cells.

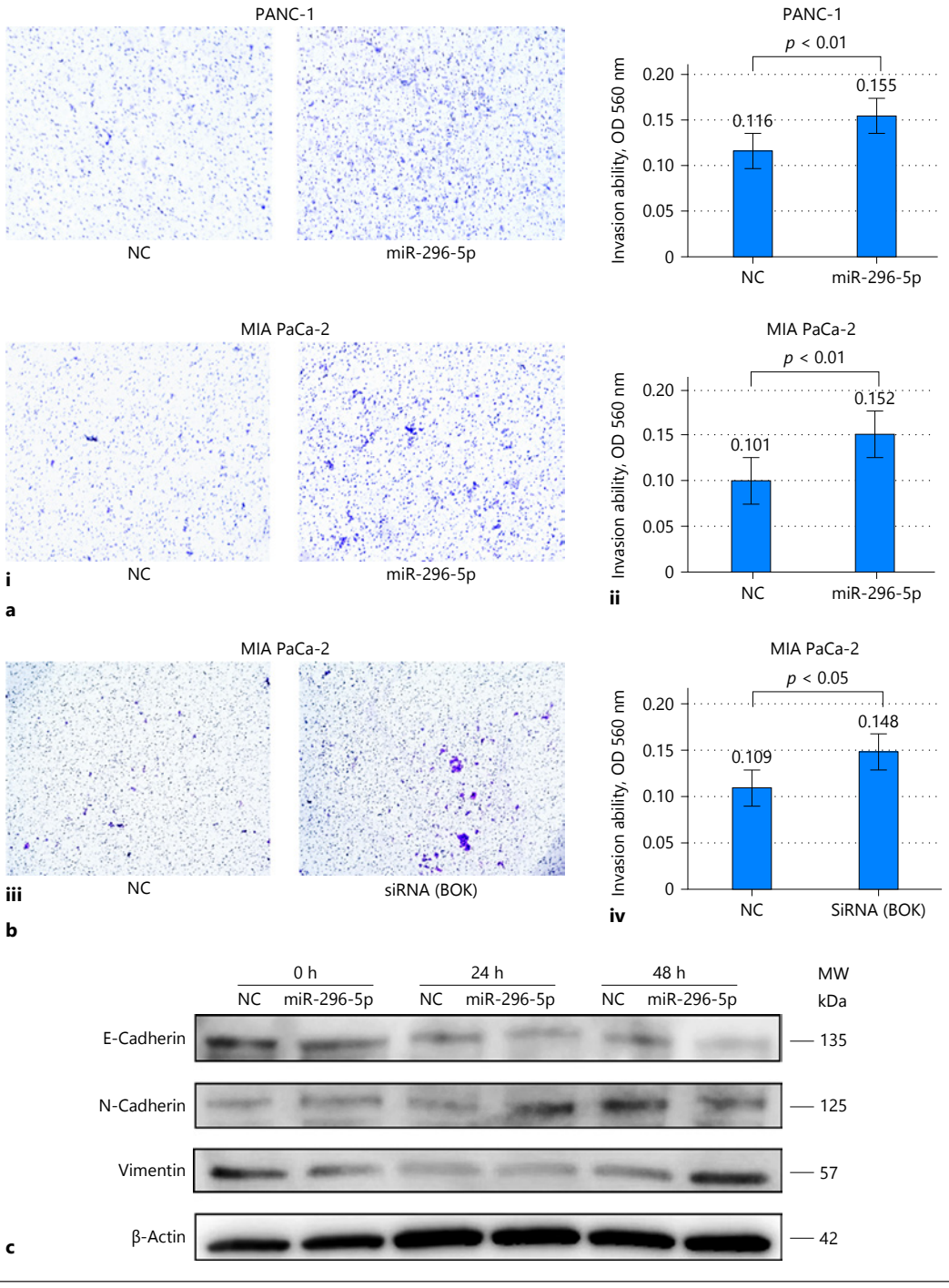

Fig. 4. Cell invasion capability and EMT properties induced by transfection of miR-296-5p and knockdown of *BOK*. **a** The invasion capability of PANC-1 or MIA PaCa-2 cells transfected with miR-296-5p or control mimic was determined using a CytoSelect 24-well cell invasion assay kit. The invading cells were stained, lysed, and quantified by measuring OD at 560 nm. **b** The invasion capability of MIA PaCa-2 cells transfected with siRNA targeting *BOK* or control siRNA was determined using a CytoSelect 24-well cell invasion assay kit. Representative photographs of invading cells are shown at low magnification (**i**, **iii**) and the quantified results after extraction are presented as mean ± SD of the OD values obtained in triplicate (**ii**, **iv**). **c** Expression of the EMT-associated proteins E-cadherin, N-cadherin, and vimentin were determined by Western blotting before and 24 and 48 h after transfection. β-actin was used as a control. OD, optical densities; BOK, Bcl2-related ovarian killer; siRNA, small interfering RNA; MW, molecular weight.

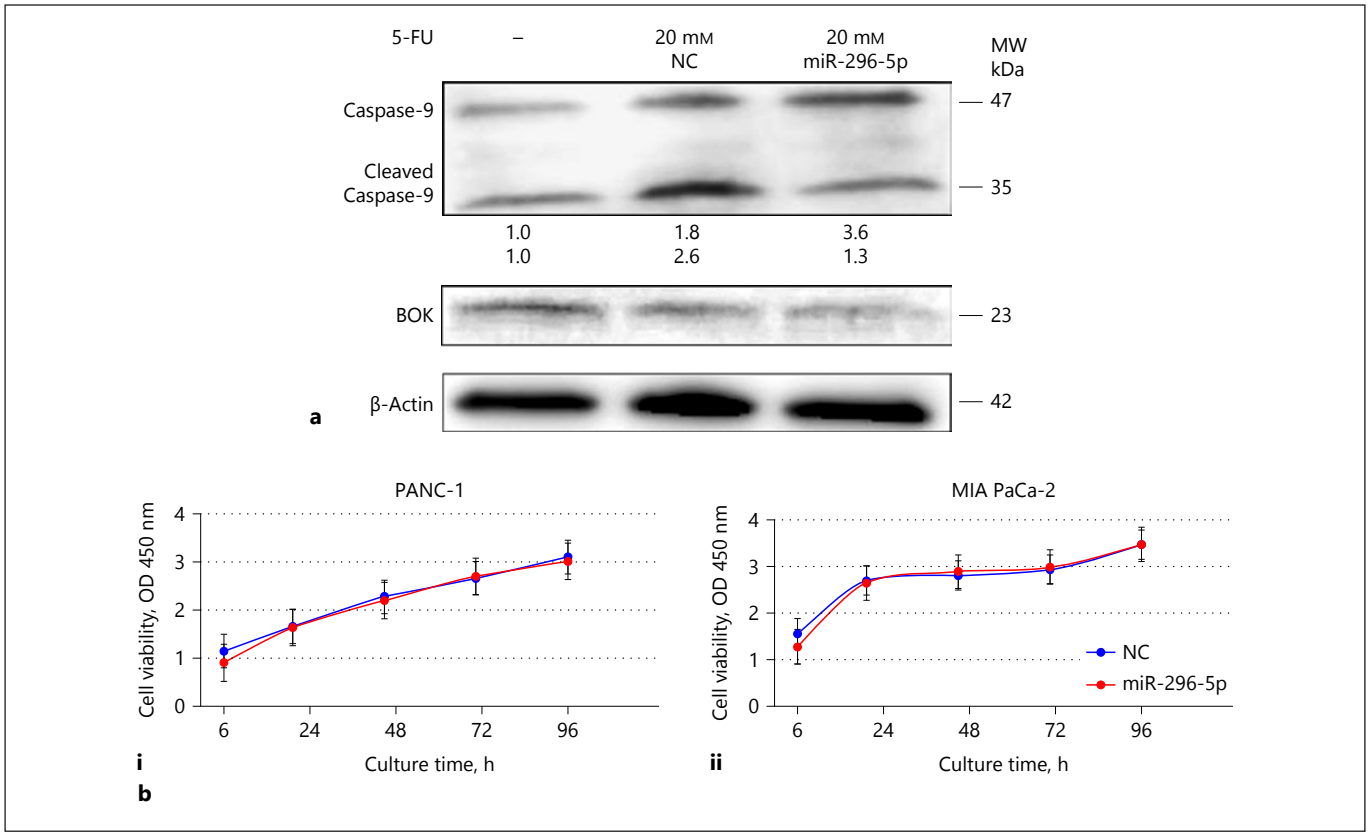

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Fig. 5. Anti-apoptosis and cell proliferation in PANC-1 cells transfected with miR-296-5p. **a** Caspase-9 and cleaved caspase-9 were detected by Western blotting in PANC-1 cells transfected with miR-296-5p or control miRNA mimic (NC) treated with 5-FU. PANC-1 cells were transfected with miR-296-5p or control mimic and incubated with 20 mM 5-FU. The cells were then lysed and Western blot analysis was performed as described in the Materials and Methods section. BOK protein was analyzed using the same samples. β-actin was used as a control. Protein expression levels were quantified using Image J software. **b** The viability of PANC-1 (**i**) and MIA PaCa-2 (**ii**) cells transfected with miR-296-5p or control mimic was determined using a Cell Counting Kit-8 at 6, 24, 48, 72, and 96 h after transfection. Results are presented as mean ± SD of the OD value obtained in triplicate. 5-FU, 5-fluorouracil; MW; molecular weight; BOK, Bcl2-related ovarian killer; OD, optical densities.

Discussion

We used an miRNA microarray to examine the expression profile of 2,042 miRNAs in micro-tissues obtained by EUS-FNA from 13 unresectable advanced PDACs, and found significant correlations between the high expression of several miRNAs (e.g., miR-296-5p) and short survival times. Moreover, transfection of an miR-296-5p mimic into pancreatic cancer cell lines enhanced cell invasion and drug resistance with a transition to EMT, via inhibition of *BOK* as a target gene. This is the first report to analyze miRNA profiles of unresectable metastatic PDACs using EUS-FNA samples. Our results strongly suggest that miR-296-5p is a true prognostic biomarker for unresectable metastatic PDACs because we used unresectable metastatic PDAC tissue specimens for analysis in this study rather than surgically operated non-metastatic PDAC tissues as were used in the majority of previous studies. Our results raise the possibility of using miR-296-5p as a serum prognostic biomarker for liquid biopsy, and also of developing a future therapy targeting miR-295-5p and BOK for unresectable metastatic PDACs with a poor prognosis.

The development of EUS-FNA has made it possible to easily obtain micro samples from pancreatic tumors, even when the tumor is surgically unresectable. Some studies have investigated miRNA expression in PDAC specimens obtained by EUS-FNA [11, 21]; however, a limited number of miRNAs was analyzed by qPCR, and consequently very little information was provided about the miRNA expression. To overcome these limitations, we used an unbiased approach with recently developed mi-

croarrays (Sanger miRbase release 12.0) to measure global expression profiles of 2,042 miRNAs. In addition, almost all previous studies compared miRNA expression levels between PDAC specimens and normal pancreatic tissues or non-malignant pancreatic tissues such as those from patients with chronic pancreatitis. In contrast, our study focused on identifying miRNA-based prognostic biomarkers in unresectable PDACs, and found that miR-296-5p, which targets *BOK*, was correlated with shorter survival. In this context, miR-296-5p would likely be a poor prognostic biomarker in patients with PDACs characterized by metastasis, drug resistance, and EMT.

It has been reported that miR-296-5p acts as a cancer-promoting miRNA in several types of malignant tumors [22–24]. Increased expression of miR-296-5p in breast and gastric cancers reportedly promotes cell proliferation by regulating transcription of certain genes or by activating signal transduction [23, 24]. Moreover, miR-296-5p has been reported to play an important role in promoting tumor progression in several types of cancer. In contrast, miR-296-5p was shown to serve as a tumor suppressor in prostate and lung cancer cells [25, 26]. These conflicting results have been explained by differences in the target genes among different types of cancer cells. In this study, we showed that miR-296-5p enhances cell invasion, migration, and drug resistance in association with EMT probably via inhibition of *BOK*, which acts as a tumor suppressor gene.

There has been only one study reporting altered expression of miR-296-5p in pancreatic neoplasia to date [12]. However, the function of miR-296-5p in pancreatic carcinogenesis remains unclear. In the current study, we showed that overexpression of miR-296-5p plays an important role in invasion, drug resistance, and EMT through inhibition of *BOK* function. *BOK* is a Bcl-2 family protein with high homology to the pro-apoptotic proteins (Bcl-2 associated X, apoptosis regulator) and (Bcl-2/killer 1) [27]. It has been reported that transient transfection of *BOK* in CHO cells induced apoptosis via the classical apoptosis pathway involving the release of cytochrome c, activation of caspase-3, and nuclear and DNA fragmentation [28, 29]. To obtain a better model in which cancer cell types can be compared, we performed gain-of-function experiments using PANC-1 (low miR-296-5p expressing) and MIA PaCa-2 (high miR-296-5p expressing) cells. Effective transfections of miR-296-5p were performed, which resulted in significant reduction of *BOK* expression in different types of cells. Furthermore, reduction of *BOK* expression inhibited caspase-3 activation in both types of pancreatic cancer cells. Known genetic mutations, including those of *TP53*, *KRAS*, *CDKN2A*, and *SMAD4*, are important in the cascade of pancreatic carcinogenesis, but do not fully account for its aggressive behavior [30]. Thus, our data suggest that upregulation of miR-296-5p promoted PDAC progression, including metastasis and drug resistance, via inhibition of *BOK* function.

Only a few studies have reported that miRNAs promote EMT in PDACs. Zhang et al. [31] showed that overexpression of miR-15b induced EMT by inhibiting SMURF2 in the pancreatic cancer cell line BxPC-3. Moreover, Wang et al. showed that upregulation of miR-935 promoted EMT by inhibiting inositol polyphosphate 4-phosphatase type I gene in the pancreatic cancer cell line HPDE6-C7 [32]. However, these studies focused only on miR-15b and miR-935, respectively, and did not examine miRNA profiles in pancreatic cancer tissues or pancreatic cancer cell lines. More importantly, their results are not supported by clinical data to confirm that the overexpressed miRNA was correlated with survival in pancreatic cancer patients. It has also been reported that *BOK* inhibits EMT in lung cancer cell lines. In this context, it is even more plausible that miR-295-5p promotes EMT and enhances invasion capability and drug resistance via *BOK* inhibition, which was analyzed from the clinical survival data and miRNA profiles in patients with PDAC.

The tissue samples obtained by EUS-FNA in this study included non-cancerous stromal cells to some degree as well as cancer cells. That is, our miRNA data reflected the miRNA profile of not only cancer cells but also of stromal cells on some level. This is a weak point of the sample acquisition method with EUS-FNA, and is a limitation of this kind of studies. Therefore, we have to take it into consideration when interpreting our data. Moreover, the role of other miRNAs including miR-1207-5p in unresectable PDAC with a poor prognosis remains unclear. Therefore, the involvement of these miRNAs in advanced PDAC should be further investigated in future studies.

Conclusion

We comprehensively analyzed miRNA expression profiles in unresectable metastatic PDACs specimens obtained by EUS-FNA, and we identified miRNAs with aberrantly high expression that were correlated with shorter survival. Moreover, the miR-296-5p/*BOK* signaling axis was closely associated with increased cell invasion, drug resistance, and EMT in PDACs. These data suggest that miR-296-5p expression is a useful biomarker for a poor

prognosis in patients with unresectable metastatic PDACs, and that miR-296-5p may serve as a therapeutic target for the treatment of advanced metastatic PDACs in the future.

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Statement of Ethics

This study was approved by the Ethics Committee of Tokushima University Hospital (Approval number; 1845).

Disclosure Statement

The authors declare that they have no competing interests.

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Author Contributions

T. Takayama and J.O.: designed the research. J.O., T.N., T.K., and M.B.: performed the experiments. J.O. and T. Tanahashi: analyzed the data and drafted the manuscript. All authors contributed to the writing and approval of the final manuscript.

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