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MicroRNA-196a and -196b as Potential Biomarkers for the Early Detection of Familial Pancreatic Cancer

Emily P. Slater[*], Konstantin Strauch[†,‡], Susanne Rospleszcz[†,‡], Annette Ramaswamy[§], Irene Esposito[¶], Günter Klöppel[¶], Elvira Matthäi[*], Kristin Heeger[*], Volker Fendrich[*], Peter Langer[*] and Detlef K. Bartsch[*]

[*]Department of Surgery, Philipps University of Marburg, Marburg, Germany; [†]Institute of Medical Informatics, Biometry and Epidemiology, Chair of Genetic Epidemiology, Ludwig-Maximilians-Universität, Munich, Germany; [‡]Institute of Genetic Epidemiology, Helmholtz Zentrum München – German Research Center for Environmental Health, Neuherberg, Germany; [§]Department of Pathology, Philipps University of Marburg, Marburg, Germany; [¶]Institute of Pathology, Technical University of Munich, Munich, Germany

Abstract

Screening programs are recommended for individuals at risk (IAR) from families with familial pancreatic cancer (FPC). However, reliable imaging methods or biomarkers for early diagnosis of pancreatic ductal adenocarcinoma (PC) or its precursor lesions are still lacking. The ability of circulating microRNAs (miRNAs) to discriminate multifocal high-grade precursor lesions or PC from normal was examined. The presence of miRNA-21, -155, -196a, -196b and -210 was analyzed in the serum of transgenic KPC mice to test their ability to distinguish mice with different grades of pancreatic intraepithelial neoplasia (mPanIN1–3) or PC from control mice. Serum levels of miR-196a and -196b were significantly higher in mice with PanIN2/3 lesions ($n = 10$) or PC ($n = 8$) as compared to control mice ($n = 10$) or mice with PanIN1 lesions ($n = 10$; $P = .01$). In humans, miR-196a and -196b were also diagnostic. Patients with PC, sporadic ($n = 9$) or hereditary ($n = 10$), and IAR with multifocal PanIN2/3 lesions ($n = 5$) had significantly higher serum levels than patients with neuroendocrine pancreatic tumors ($n = 10$) or chronic pancreatitis ($n = 10$), IAR with PanIN1 or no PanIN lesions ($n = 5$), and healthy controls ($n = 10$). The combination of both miR-196a and -196b reached a sensitivity of 1 and specificity of 0.9 (area under the curve = 0.99) to diagnose PC or high-grade PanIN lesions. In addition, preoperative elevated serum levels of miR-196a and -196b in patients with PC or multifocal PanIN2/3 lesions dropped to normal after potential curative resection. The combination of miR-196a and -196b may be a promising biomarker test for the screening of IAR for FPC.

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Introduction

Pancreatic ductal adenocarcinoma (PC) is a highly malignant tumor that has a poor prognosis because of the lack of early symptoms. Familial pancreatic cancer (FPC) accounts for about 3% of all PC cases [1,2]. Families with at least two first-degree relatives with confirmed PC that do not fulfill the criteria of other inherited tumor syndromes with an increased risk for the development of PC, such as Peutz–Jeghers syndrome or hereditary pancreatitis, are defined as FPC [3,4]. The major underlying gene defect(s) of FPC has not yet been identified, but causative *BRCA2*, *PALB2*, *CDKN2a*, and *ATM* germline mutations were identified in about 10% to 15% of the FPC families [4–9]. It has been recommended by a recent consensus conference that individuals at risk (IAR) of FPC families should undergo PC screening under research protocol conditions [3]. Individuals with at least a 5- to 10-fold increased

Address all Correspondence to: Emily P. Slater, PhD, Department of Surgery, Philipps University of Marburg, Baldingerstrasse, D-35043 Marburg, Germany.
E-mail: slater@med.uni-marburg.de
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risk of PC, such as members of FPC families with two or more affected first-degree relatives, are considered to be candidates for screening. Most experts currently consider magnetic resonance imaging (MRI) and endoscopic ultrasonography to be the best imaging modalities for FPC screening [4]. Unfortunately, these imaging tools are not able to reliably visualize early PC or, even more important, its high-grade precursor lesions, i.e., pancreatic intraepithelial neoplasia grade 3 (PanIN3). Thus, there is a definite need for biomarkers to facilitate screening of IAR in the setting of FPC to identify those individuals with high-grade PanINs before the development of invasive carcinoma that could allow for a curative resection.

Familial as well as sporadic PCs are characterized by a progression from low-grade PanINs (PanIN1) over carcinoma *in situ* (PanIN3) to invasive cancer. The majority of pancreatic specimens of resected FPC individuals reveal multifocal PanIN disease in addition to small intraductal papillary mucinous neoplasms (IPMNs) of branch duct/gastric type [10–13]. Branch-duct IPMNs might be a surrogate marker for the presence of high-grade PanIN lesions in other locations of the gland in the FPC setting [14]. The stepwise progression from PanIN to invasive PC comprises activating mutations of the *Kras* oncogene and inactivation of the ARF-p53 tumor suppressor pathway in the great majority of cases [15]. Nowadays, genetically engineered mouse models of PC that closely recapitulate the histopathogenesis and progression of the human disease are available. These include the *LSL-Kras*$^{G12D/+}$;*Pdx1-Cre* (KC) mice that progress up to PanIN3 lesions and the *LSL-Kras*$^{G12D/+}$;*LSL-Trp53*$^{R172H/+}$;*Pdx1-Cre* (KPC) mice that develop PanIN lesions and ultimately invasive carcinoma at 5 to 10 months [16–18]. These mouse models are considered an adequate tool for the study of biomarkers [16,17], especially given the lack of FPC patients with preoperative, well-defined high-grade PanIN lesions.

MicroRNAs (miRNAs) are small non-coding molecules, which have an important function in regulating RNA stability and gene expression. The deregulation of miRNAs has been linked to cancer development and tumor progression [19]. Recently, it has been reported that serum contains sufficiently stable miRNA species that might deem useful as non-invasive biomarkers for several cancers, including pancreatic cancer [20–22]. Thus, the aim of the present study was to evaluate a panel of miRNAs as potential biomarkers for PC screening in IAR of FPC families.

Materials and Methods

miRNA Selection

miRNAs overexpressed in serum samples or specimens of human or murine PC were compiled by searching the PubMed and MEDLINE databases for articles published from 1 January 1990 to 31 July 2011. The search terms "miRNA," "microRNA," "pancreatic cancer" or "familial pancreatic cancer" and "protein markers" or "biomarker," or "early detection," or "diagnostic test" were used. A second-level manual search included the reference list of the articles considered to be of interest. The literature search and study selection were performed by two authors (D.K.B. and E.P.S.).

Transgenic KPC Mouse Samples

Conditional *LSL-Trp53*$^{R172H/+}$;*LSL-Kras*$^{G12D/+}$ and *Pdx1-Cre* [17] strains were interbred to obtain *LSL-Kras*$^{G12D/+}$;*LSL-Trp53*$^{R172H/+}$;*Pdx1-Cre* (KPC) triple mutant animals on a mixed 129/SvJae/C57Bl/6 background as described previously by our group [18]. The time span for the development of different PanINs is well established in these mice. KPC mice develop PanIN2/3 lesions after 3 to 4 months and invasive cancer after 5 months. The generation of *RIP1-Tag2* mice as a model of pancreatic islet cell carcinogenesis has been previously reported [23]. All experiments were approved by the local committee for animal care and use. Animals were maintained in a climate-controlled room kept at 22°C, exposed to a 12:12-hour light-dark cycle, fed standard laboratory chow, and given water *ad libitum*.

For genotyping, genomic DNA was extracted from tail cuttings using the REDExtract-N-Amp Tissue PCR Kit (Sigma-Aldrich, St Louis, MO). Three polymerase chain reactions (PCRs) were carried out for each animal to test for the presence of the oncogenic Kras (using LoxP) primers, p53, and Pdx1-Cre transgene constructs (using Cre-specific primers), respectively. *SV40-Tag* specific primers were used for the genotyping of the *RIP1-Tag2* mice.

Mice were killed, blood was collected from the thoracic cavity for serum, and the pancreas was removed and inspected for grossly visible tumors and preserved in 10% formalin solution (Sigma-Aldrich) for histology. Formalin-fixed, paraffin-embedded tissues were sectioned (4 μm) and stained with hematoxylin and eosin. Six sections (100 μm apart) of pancreatic tissues were histologically evaluated by an experienced pathologist (A.R.) blinded to the experimental groups. mPanIN lesions were classified according to histopathologic criteria as recommended previously [18].

Human Samples

Preoperative serum samples of patients with histologically proven sporadic PC, familial PC, chronic pancreatitis (CP), and pancreatic neuroendocrine neoplasms (pNENs) were obtained from the tissue bank of the Department of Surgery, Philipps University of Marburg (Marburg, Germany) and analyzed for the presence and expression level of miR-196a and -196b. All tumors were histologically staged according to the Union Internationale Contre le Cancer/Tumor, Node, Metastasis (UICC-TNM) classification 2009 [24]. Serum and blood samples of 10 voluntary healthy individuals, 3 males and 7 females, 25 to 50 years of age, served as controls. In addition, preoperative and early postoperative serum samples of patients with potentially curative resected PC and IAR of FPC families who either underwent total pancreatectomy or partial pancreatic resection for suspicious imaging lesions were also analyzed for miR-196a and -196b. In IAR who underwent pancreatectomy, the entire resection specimen was cut into 5-mm sections and analyzed for the presence of PanINs, IPMNs, and invasive cancer by experienced pathologists (I.E. and G.K.). Informed written consent was obtained from every individual who participated in the study according to the ethics committee vote of the Philipps University of Marburg (No. 36/1997; Amendment 5/2009).

RNA Isolation and Real-Time PCR

Total RNA was extracted from mouse serum using mirVana PARIS kit (Ambion 1556; 100 μl) according to the manufacturer's instructions. The PAXgene system (Becton Dickinson, Heidelberg, Germany) was used to isolate total RNA, including miRNA from human blood samples using the miRNeasy kit again according to the manufacturer's instructions.

Real-time PCR was performed in triplicate. miRNAs were amplified after specific reverse transcription using TaqMan microRNA assays and TaqMan MicroRNA Reverse Transcription Kit (Applied Biosystems, Darmstadt, Germany) according to the manufacturer's instructions

(Applied Biosystems, Darmstadt, Germany) and normalized against miR-24 as previously described [25]. These authors recently confirmed the validity of using miR-24 as it is ubiquitously expressed in normal and pancreatic tissues [26]. Relative expression was determined using the $\Delta\Delta C_t$ method and a C_t value > 35 indicated negative amplification.

Statistical Analysis

To assess whether the levels of the tested miRNAs in the murine PanIN and carcinoma samples were significantly different from the levels in the control samples, a Wilcoxon signed rank test was used. A P value < .05 was considered statistically significant. A logistic regression model was set up to determine the effect of the respective miRNAs on the affection status of a subject. Additionally, a model including the combination of miRNAs was calculated. To evaluate the ability of an miRNA to distinguish pairwise between PanIN, carcinoma, and control samples, true-positive rates (sensitivity) and true-negative rates (specificity) were determined by the calculation of a receiver operating characteristic (ROC) curve. The area under the curve (AUC) served as an additional performance index.

For analysis of miR-196a and -196b expression in human samples, the Wilcoxon signed rank test as well as logistic regression modeling was applied. The resulting predicted probabilities of being affected were analyzed again by the calculation of an ROC curve and the determination of sensitivity, specificity, and AUC. All steps were conducted with R version 2.13.1. A cutoff value for a range of fold change that indicates the presence of high-grade PanIN (2 or 3) and/or PC was calculated.

Results

The literature search revealed a potential association between miRNAs (miR-21, -155, -196a, -196b, and -210) and pancreatic cancer or high-grade PanIN lesions [27–33]; thus, these miRNAs were evaluated. Although all five miRNAs could be detected in the serum of the analyzed KPC mice, miR-21, -155, and -210 did not discriminate between controls, PanINs, and PC (data not shown). miR-21 levels were already increased in mice with low-grade PanIN1 and there was no greater than a two-fold increase in expression levels of miR-155 and miR-210 in the KPC mice with PC as compared to controls (data not shown). Thus, these miRNAs were excluded from further analysis. Using miR-24 as a reference and wild-type mice (n = 10) as control, we were able to consistently measure significantly increased levels of miR-196a and -196b in the serum of mice with multifocal PanIN2/3 lesions (n = 10) and mice with invasive PC (n = 8) (Figure 1 and Table 1).

The levels of miR-196a were similar between control mice (n = 10) and KPC mice with PanIN1 lesions (n = 10) or endocrine tumors (n = 4). In contrast, mice with PanIN2/3 lesions had a median fold change of 2.7 above control/PanIN1 and mice with PC revealed a median fold change of 3.0 compared to controls and mice with PanIN1 lesions, which were both statistically significant (P = .03 and P < .01, Table 1). miR-196a had a sensitivity and a specificity of 0.9 and 0.78 for the discrimination between normal and PanIN2/3 and 0.9 and 1 for the discrimination between normal and PC, respectively.

The levels of miR-196b were also similar between control mice (n = 10) and KPC mice with PanIN1 lesions (n = 10) or endocrine tumors (n = 4). The mice with multifocal PanIN2/3 lesions (n = 10) and invasive carcinoma (n = 8) had a median fold change in the serum levels of miR-196b of 4.2-fold and 3.6-fold compared to normal controls and mice with PanIN1 lesions (Figure 1 and Table 1). The calculated sensitivity and specificity for miR-196b was 0.86 and 1 for the discrimination between control and PanIN2/3 lesions and 0.86 and 0.86 for the discrimination between control and invasive cancer.

The combination of both miR-196a and miR-196b attained a perfect discrimination between control and PanIN2/3 with a sensitivity and a specificity of 1. Two of the 15 samples with PanIN2/3 lesions did not have elevated miR-196a levels (cycle threshold difference values: 0.022, 1.2), but both samples revealed raised miR-196b levels (cycle threshold difference values: – 2.02, – 1.2; Figure 1, *D* and *E*). For the discrimination between normal control and invasive PC, a sensitivity of 0.86 and a specificity of 1 were calculated.

Since the levels of miR-196a and miR-196b are potential diagnostic serum markers for high-grade PanIN lesions and invasive PC, we next evaluated the presence of miR-196a and -196b in human blood samples. These included samples from patients with sporadic PC (n = 9, 2 stage I, 6 stage II, and 1 stage IV), familial PC (n = 10, 1 stage II, 9 stage IV), CP (n = 10), pNENs (n = 10), IAR with histologically proven multifocal PanIN2/3 lesions (n = 5), IAR with PanIN1 or no PanIN lesions upon histopathology (n = 5), and healthy subjects (n = 10) as controls. Again, the serum levels of miR-196a and miR-196b were significantly higher in patients with sporadic and familial PC and IAR with multifocal PanIN2/3 lesions than in patients with pNENs, CP, and PanIN1 lesions and healthy controls (Figure 2 and Table 2). miRNA levels were highest (up to 46-fold) in patients with metastasized PC stage IV (n = 10). miR-196a had a sensitivity of 1 and a specificity of 0.6 (AUC = 0.64) for the discrimination between normal and PanIN2/3 (Figure 3), as well as 0.9 and 0.89 (AUC = 0.97) for the discrimination between normal and PC, respectively. miR-196b had a sensitivity and a specificity of 1 each (AUC = 1.0) for the discrimination between normal and PanIN2/3 (Figure 3) and a sensitivity of 1 and a specificity of 0.78 (AUC = 0.86) for the discrimination between normal and PC. The combination of both miR-196a and miR-196b attained the best discrimination between control and either multifocal PanIN2/3 (a sensitivity of 1 and a specificity of 1) or sporadic invasive PC (a sensitivity of 1 and a specificity of 1). The results of miR-196a and -196b ROC curves are presented in Table 2. A ΔC_t value of 7.51 for miR-196a and a ΔC_t value of 6.35 for miR-196b were calculated as cutoff values that indicate the presence of high-grade PanIN2/3 lesions or PC.

Interestingly, in nine PC patients with available preoperative and early postoperative serum samples, the preoperative elevated miR-196a and miR-196b dropped to the normal range after potential curative resection. The same was true for the five IAR with multifocal PanIN2/3 lesions (Figure 4, *A* and *B*).

Discussion

Consensus statements recommend screening IAR of FPC families with endoscopic ultrasonography and MRI, as these are considered to be the best imaging modalities [12,34]. However, these tools often fail to reliably detect high-grade lesions (PanIN3) and early PC. In addition, up to 40% of IAR show small cystic lesions on imaging that might represent small branch-duct type IPMNs [34]. It was suggested that these lesions are a surrogate for the presence of non-visible, high-grade PanIN lesions somewhere else in the pancreas of the IAR [14]. Thus, biomarkers that reliably indicate the presence of high-grade PanIN or early PC lesions would be of great value for the screening of IAR in the setting of FPC and could lead to curative resection. Several

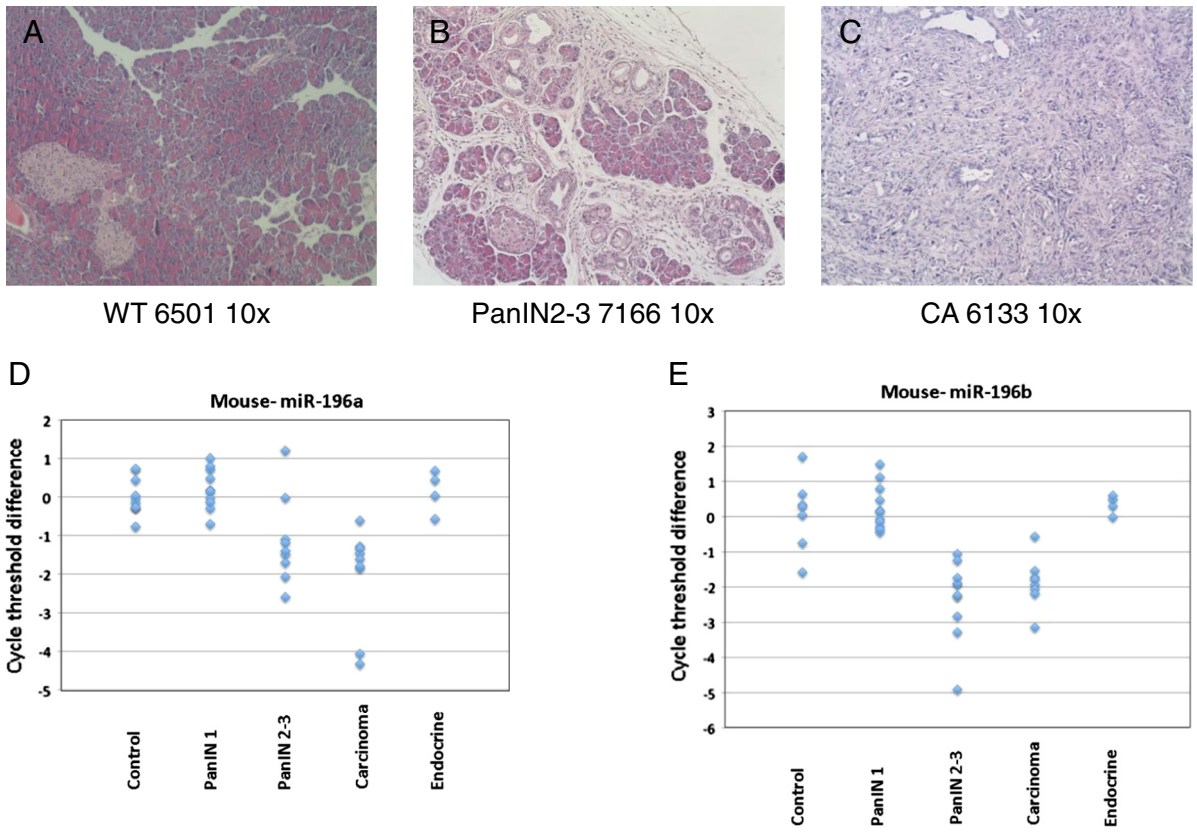

Figure 1. Histologic sections stained with hematoxylin and eosin from wild-type (A) and KPC mice with PanIN2/3 lesions (B) and invasive carcinoma (C). Scatter plot showing the results from TaqMan analyses of miR-196a (D) and miR-196b (E) in mouse serum. Results are expressed as the cycle threshold difference between miR-196a, or miR-196b, and miR-24 in the experimental animal compared to that of wild type. Control represents samples from wild-type mice, PanIN are the KPC mice with histologically proven PanIN2/3 lesions, and carcinoma are KPC mice presenting with invasive carcinoma. The values obtained with PanIN1 and mice with pancreatic endocrine tumors did not vary from control values.

miRNAs can potentially serve as such biomarkers as these are reported to be upregulated in PC [27], PanINs [35], and IPMNs [28]. A recent meta-analysis of nine studies, including four with serum analysis, evaluating 20 miRNAs in 941 patients with PC calculated a pooled sensitivity of 0.89, a specificity of 0.93, and an AUC of 0.97 for the diagnosis of PC [36]. However, high-grade precursor PanIN lesions, which are the main targets of pancreatic cancer screening in IAR of FPC families, were not analyzed in this study. Thus, the present study focused on the identification of miRNAs that allows the detection of high-grade PanINs and early PC (T1 tumors) with high sensitivity and specificity. The optimal miRNA assay for routine clinical use in FPC screening should ideally consist of a small set of miRNAs that provides quick and reproducible results. Therefore, the presented study was focused on a small panel of five miRNAs (miR-21, -155, -196a, -196b, and -210).

To ensure the investigation of properly characterized PanIN stages, the KPC mouse model mimicking the progression of PC was first used to test the five miRNAs for their diagnostic potential. All five tested miRNAs could be reproducibly detected in the serum of these animals. The important new finding of the present study is that only serum miR-196a and -196b proved to be promising in the ability to distinguish mice with high-grade PanIN lesions or PC from wild-type mice and KPC mice with no or low-grade PanIN lesions. The combination of both miRNAs reached a sensitivity and a specificity of 1 for the discrimination between control/PanIN1 and PanIN2/3 and a sensitivity of 0.86 and a specificity of 1 for the discrimination between control/PanIN1 and invasive PC. The diagnostic value also held true in human serum samples, because serum miR-196a and -196b expression revealed remarkable similarities between murine

Table 1. Results of TaqMan Analyses in Mice

	N	Median Fold Change	Range	*P versus* Control	
				T test	Wilcoxon Test
miR-196a					
Mouse					
Control	10				
PanIN1	10	1.0	1-1.2	1.0	.85
PanIN2/3	10	2.4	1-6	.03*	.05
Carcinoma	8	2.7	1.5-20	≤.01*	<.01*
Endocrine tumor	4	1.2	1-1.5	1.0	.85
miR-196b					
Mouse					
Control	10				
PanIN1	10	1.0	1-1.3	1.0	1.0
PanIN2/3	10	4.3	2.1-30	≤.01*	≤.01*
Carcinoma	8	3.6	1.5-8.8	≤.01*	.02*
Endocrine tumor	4	1.3	1-1.5	1.0	1.0

*Statistically significant as compared to control.

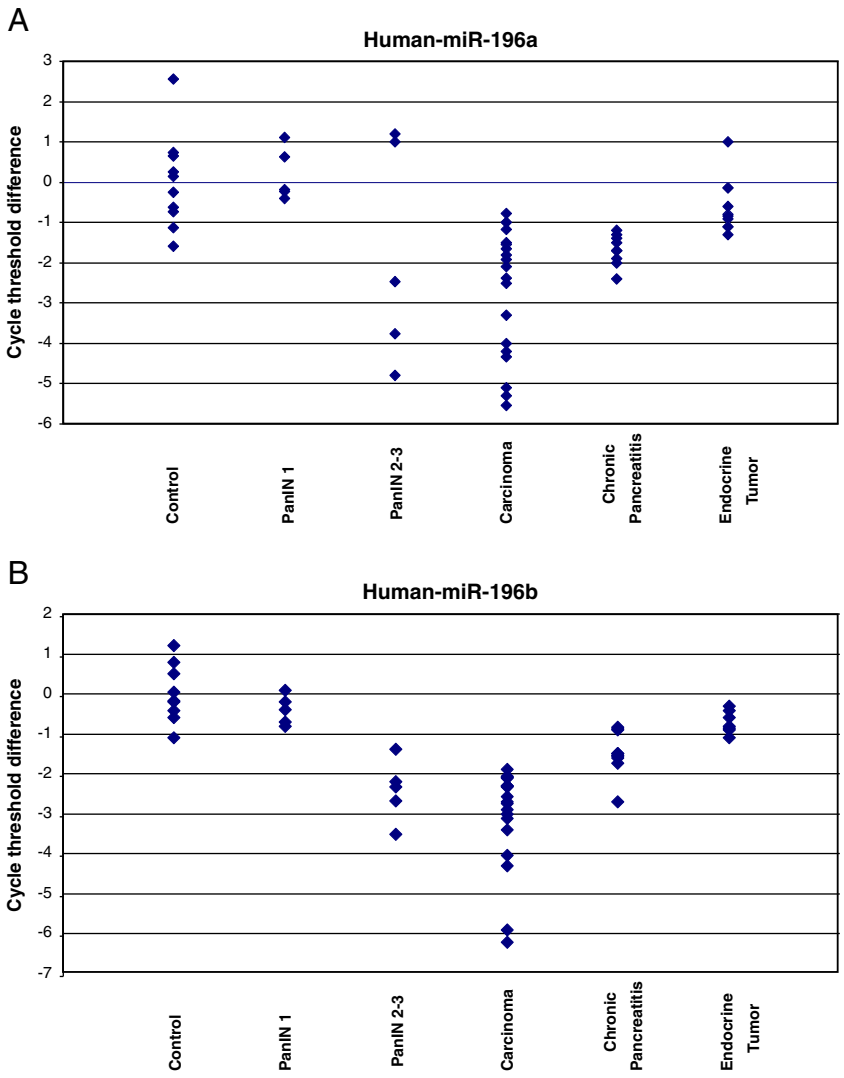

Figure 2. Scatter plot showing the results from TaqMan analyses of miR-196a (A) and miR-196b (B) in samples of patients with FPC, sporadic PC, CP, and pNENs, IAR with multifocal PanIN2/3 lesions, IAR with PanIN1 lesions, and healthy controls. Results are expressed as the cycle threshold difference between miR-196a or miR-196b and miR-24 in the patient's blood compared to that of control.

and human samples. Again, the serum levels of miR-196a and miR-196b were significantly higher in patients with PC and most importantly in IAR with multifocal PanIN2/3 lesions than in patients with pNENs and CP, IAR with none or PanIN1 lesions, and healthy controls, respectively. The combination of both miR-196a and miR-196b attained the best discrimination between control/PanIN1 and invasive PC (a sensitivity of 1 and a specificity of 1) as well as between control/PanIN1 and PanIN2/3 (a sensitivity of 1 and a specificity of 1).

The presented findings are supported by previous reports. Significantly, a study on laser-dissected human PanIN lesions revealed miR-196b as the most selectively differentially expressed miRNA in PanIN3 lesions [35]. In addition, Liu et al. reported that serum levels of miR-196a were significantly higher in PC patients than in healthy controls, although the combination of miR-16, miR-196a, and CA19-9 was most effective for the PC diagnosis [37]. However, the present study shows for the first time based on well-defined PanIN lesions in the KPC mouse model that miRNA-196a/b might also be promising serum markers to detect high-grade PanIN lesions in IAR of FPC families. This is also supported by the fact that preoperatively elevated miR-196a and -196b levels in patients with PC as well as IAR with multifocal, non-imageable PanIN2/3 lesions dropped to normal values after potentially curative pancreatic resection. This provides strong evidence for the hypothesis that the diseased organ was the true cause of the overexpressed miR-196a and -196b levels. As available imaging methods alone are not sufficient for the diagnosis of high-grade PanIN precursor lesions in IAR, they might be complemented by the results of biomarkers miRNA-196a/b to make a decision for further surveillance or surgery.

According to a large-scale microarray analysis, no single miRNA, including miR-196a and miR-196b, was able to reliably discriminate between PC and CP in serum samples [38]. In the present study, the combination of miR-196a and -196b reached a sensitivity of 0.89 and a specificity of 1.0 with an AUC of 0.96 for the discrimination between CP and multifocal PanIN2/3. However, this reduced sensitivity is of minor importance in the setting of FPC, because individuals with FPC usually do not present with the phenotype of CP.

In contrast to miR-196a and -196b, miR-21, -155, and -210 could not discriminate between mice with high-grade PanIN or PC lesions and low-grade PanIN lesions or even wild-type mice. miRNA-21

Table 2. ROC Curve Analyses of miR-196a and -196b in Human Blood Samples

Human		miR-196a	miR-196b	miR-196a/b
Con-PanIN2/3	Sensitivity	1.00	1.00	1.00
	Specificity	0.60	1.00	1.00
	AUC	0.64	**1.00**	**1.00**
	Cutoff*	7.51	6.35	7.51/6.35
Con-spPC	Sensitivity	0.90	1.00	1.00
	Specificity	0.89	0.78	1.00
	AUC	0.97	0.86	**1.00**
	Cutoff	7.96	6.35	7.51/8.01
Con-FPC	Sensitivity	0.90	1.00	1.00
	Specificity	1.00	1.00	1.00
	AUC	0.99	**1.00**	**1.00**
	Cutoff	7.96	6.35	7.96/6.35
Con-CP	Sensitivity	0.90	0.89	1.00
	Specificity	1.00	1.00	1.00
	AUC	0.96	0.96	**1.00**
	Cutoff	7.96	6.87	8.84/6.87
CP-PanIN2/3	Sensitivity	1.00	0.78	0.89
	Specificity	0.60	1.00	1.00
	AUC	0.60	0.91	0.955
	Cutoff	7.09	5.90	7.09/5.76
CP-spPC	Sensitivity	1.00	0.89	1.00
	Specificity	0.78	0.67	0.78
	AUC	0.83	0.70	0.90
	Cutoff	7.09	5.76	7.41/4.83
CP-FPC	Sensitivity	1.00	0.89	1.00
	Specificity	0.90	1.00	0.90
	AUC	0.91	0.96	0.99
	Cutoff	7.09	5.76	7.41/4.83

*Cutoff is expressed in ΔC_t for the corresponding miRNA; a perfect discrimination (AUC = 1.00) is highlighted in bold; PanIN1 and endocrine tumor values *versus* control values showed no significant differences and are not presented here.
con, control; spPC, sporadic pancreatic carcinoma.

already showed significant overexpression in low-grade murine PanIN 1 lesions, as reported previously [39,40]. In the study of LaConti et al., miR-21 levels were even higher in PanIN1 than in PanIN2/3 lesions [40]. Because the major goal of FPC screening is the identification of high-grade PanIN lesions, miR-21 was considered not to be useful for further analysis in the present study.

In the present study, there was no greater than a two-fold increase in serum levels of miR-155 in the KPC mice with PC as compared to controls and mice with PanIN1 lesions. This is in line with the study of LaConti et al. who reported an up-regulation of miR-155 in murine and human PC of at most two- to three-fold [40]. In another study of human laser-dissected PanIN lesions, miR-155 was also not significantly overexpressed in PanIN3 lesions, which is the most important lesion to identify in IAR undergoing PC screening.

Ho et al. reported in a small-scale study of 22 PC patients and 25 controls that miR-210 was reliably detected and quantified in serum samples with a statistically significant four-fold increase in expression in PC patients compared with normal controls ($P < .0001$)[31]. In the present study, however, there was no greater than a two-fold increase in expression of miR-210 in the KPC mice with PC as compared to controls and mice with PanIN1 lesions. This is in line with the results of previous miRNA microarray analyses of human blood and tissue samples [37] and microdissected PanIN lesions [35], in which no significant overexpression of miR-210 was detected. Thus, miR-210 is not useful for the FPC screening.

The present study has several limitations. First, the number of human samples is small, such that no definitive conclusion can be drawn. However, tissue and blood samples from IAR who underwent pancreatectomy and meticulous pathologic analysis are extremely rare. It would take years to accumulate significant numbers of samples from IAR with histologically proven PanIN2/3 lesions, even in a multicenter study. Nevertheless, the detected significance is strong underscoring the strengths of the finding. Second, neither the murine nor the human samples originated from living beings with pure PanIN2 or PanIN3 lesions, so that we could not determine, whether or to which extent miR-196a and -196b were exclusively expressed by either PanIN2 or PanIN3 lesions. Third, meanwhile other promising miRNAs such as miR-221, miR-27a-3p, miR-10b, and RNU2-1f were reported [41–45] that might also have potential value for the diagnosis of PC. However, there are no studies yet that analyzed their discriminatory potential between patients with different PanIN lesions and invasive cancer.

In summary, the present study provides first evidence that miR-196a and -196b might be promising biomarkers for the detection of multifocal high-grade PanIN lesions and PC in IAR of FPC families. These results should be validated in larger controlled trials. If confirmed, these biomarkers could supplement imaging for an adequate timing of a curative pancreatic resection in IAR of FPC families.

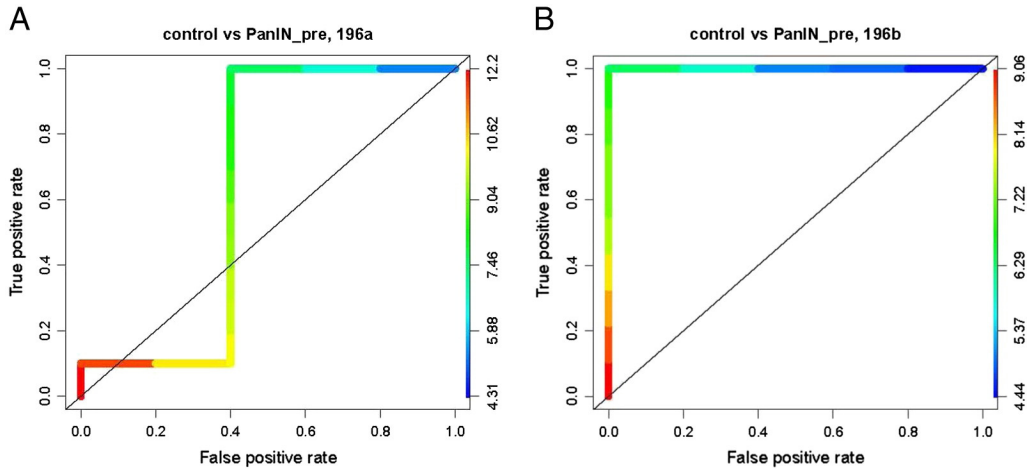

Figure 3. Serum miR-196a (A) and miR-196b (B) from preoperative IAR with multifocal PanIN2/3 lesions compared to healthy controls yielded ROC curves with AUC values of 64% and 100%, respectively.

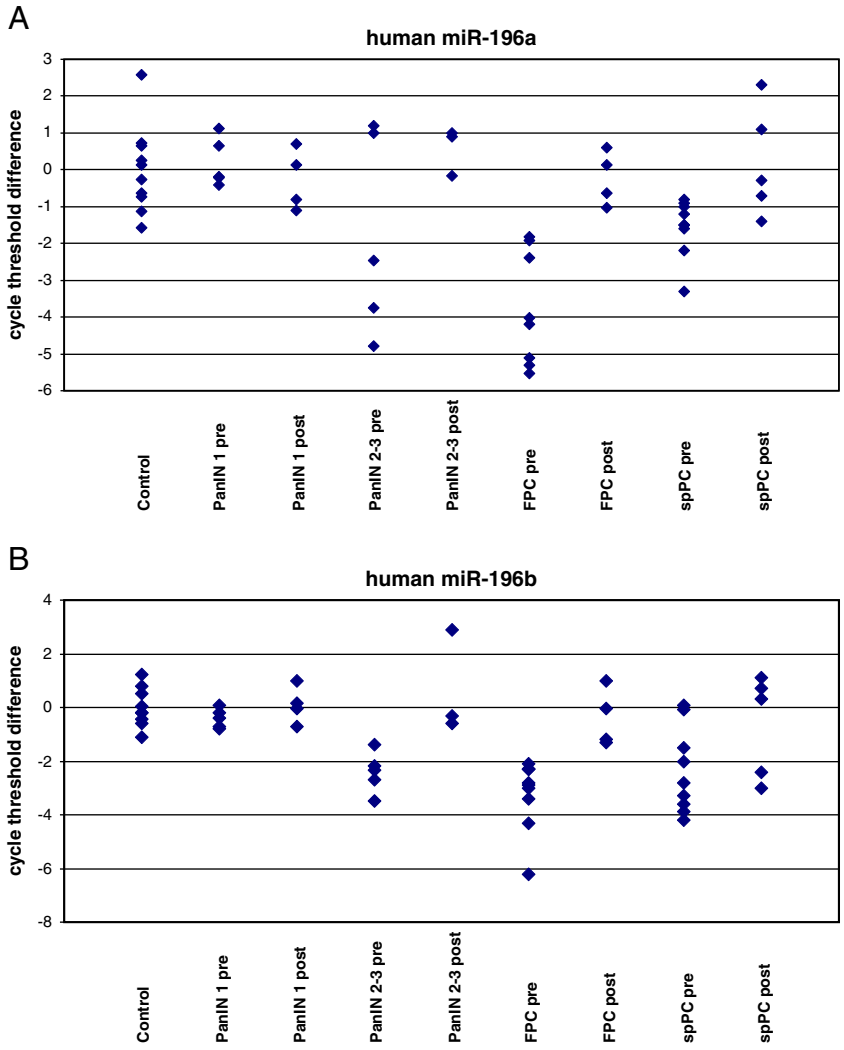

Figure 4. Scatter plot showing the results from TaqMan analyses of miR-196a (A) and miR-196b (B) in serum samples from patients both before and after resection of PC or multifocal PanIN2/3 lesions. Results are expressed as the cycle threshold difference between miR-196a or miR-196b and miR-24 in the patient's blood compared to that of control.

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References

[1] Bartsch DK, Kress R, Sina-Frey M, Grützmann R, Gerdes B, Pilarsky C, Heise JW, Schulte KM, Colombo-Benkmann M, and Schleicher C, et al (2004). Prevalence of familial pancreatic cancer in Germany. *Int J Cancer* **110**, 902–906.

[2] Hemminki K and Li X (2003). Familial and second primary pancreatic cancers: a nationwide epidemiologic study from Sweden. *Int J Cancer* **103**, 525–530.

[3] Brand RE, Lerch MM, Rubinstein WS, Neoptolemos JP, Whitcomb DC, Hruban RH, Brentnall TA, Lynch HT, and Canto MI (2007). Advances in counselling and surveillance of patients at risk for pancreatic cancer. *Gut* **56**, 1460–1469.

[4] Bartsch DK, Gress TM, and Langer P (2012). Familial pancreatic cancer—current knowledge. *Nat Rev Gastroenterol Hepatol* **9**, 445–453.

[5] Hahn SA, Greenhalf W, Ellis I, Sina-Frey M, Rieder H, Korte B, Gerdes B, Kress R, Ziegler A, and Raeburn JA, et al (2003). BRCA2 germline mutations in familial pancreatic carcinoma. *J Natl Cancer Inst* **95**, 214–221.

[6] Slater EP, Langer P, Fendrich V, Habbe N, Chaloupka B, Matthäi E, Sina M, Hahn SA, and Bartsch DK (2010). Prevalence of BRCA2 and CDKN2a mutations in German familial pancreatic cancer families. *Fam Cancer* **9**, 335–343.

[7] Slater EP, Langer P, Niemczyk E, Strauch K, Butler J, Habbe N, Neoptolemos JP, Greenhalf W, and Bartsch DK (2010). PALB2 mutations in European familial pancreatic cancer families. *Clin Genet* **78**, 490–494.

[8] Bartsch DK, Sina-Frey M, Lang S, Wild A, Gerdes B, Barth P, Kress R, Grützmann R, Colombo-Benkmann M, and Ziegler A, et al (2002). CDKN2A germline mutations in familial pancreatic cancer. *Ann Surg* **236**, 730–737.

[9] Roberts NJ, Jiao Y, Yu J, Kopelovich L, Petersen GM, Bondy ML, Gallinger S, Schwartz AG, Syngal S, and Cote ML, et al (2012). ATM mutations in patients with hereditary pancreatic cancer. *Cancer Discov* **2**, 41–46.

[10] Sipos B, Frank S, Gress T, Hahn S, and Klöppel G (2009). Pancreatic intraepithelial neoplasia revisited and updated. *Pancreatology* **9**, 45–54.

[11] Shi C, Klein AP, Goggins M, Maitra A, Canto M, Ali S, Schulick R, Palmisano E, and Hruban RH (2009). Increased prevalence of precursor lesions in familial pancreatic cancer patients. *Clin Cancer Res* **15**, 7737–7743.

[12] Langer P, Kann PH, Fendrich V, Habbe N, Schneider M, Sina M, Slater EP, Heverhagen JT, Gress TM, and Rothmund M, et al (2009). 5 Years of

prospective screening of high risk individuals from familial pancreatic cancer—families. *Gut* **58**, 1410–1418.

[13] Aichler M, Seiler C, Tost M, Siveke J, Mazur PK, Da Silva-Buttkus P, Bartsch DK, Langer P, Chiblak S, and Dürr A, et al (2012). Origin of pancreatic ductal adenocarcinoma from atypical flat lesions: a comparative study in transgenic mice and human tissues. *J Pathol* **226**, 723–734.

[14] Bartsch DK, Dietzel K, Bargello M, Matthaei E, Kloeppel G, Esposito I, Heverhagen JT, Gress TM, Slater EP, and Langer P (2013). Multiple small "imaging" branch-duct type intraductal papillary mucinous neoplasms (IPMNs) in familial pancreatic cancer: indicator for concomitant high grade pancreatic intraepithelial neoplasia? *Fam Cancer* **12**, 89–96.

[15] Hezel AF, Kimmelman AC, Stanger BZ, Bardeesy N, and DePinho RA (2006). Genetics and biology of pancreatic ductal adenocarcinoma. *Genes Dev* **20**, 1218–1249.

[16] Hingorani SR, Petricoin EF, Maitra A, Rajapakse V, King C, Jacobetz MA, Ross S, Conrads TP, Veenstra TD, and Hitt BA, et al (2003). Preinvasive and invasive ductal pancreatic cancer and its early detection in the mouse. *Cancer Cell* **4**, 437–450.

[17] Hingorani SR, Wang L, Multani AS, Combs C, Deramaudt TB, Hruban RH, Rustgi AK, Chang S, and Tuveson DA (2005). *Trp53*^{R172H} and *Kras*^{G12D} cooperate to promote chromosomal instability and widely metastatic pancreatic ductal adenocarcinoma in mice. *Cancer Cell* **7**, 469–483.

[18] Fendrich V, Chen NM, Neef M, Waldmann J, Buchholz M, Feldmann G, Slater EP, Maitra A, and Bartsch DK (2010). The angiotensin-I-converting enzyme inhibitor enalapril and aspirin delay progression of pancreatic intraepithelial neoplasia and cancer formation in a genetically engineered mouse model of pancreatic cancer. *Gut* **59**, 630–637.

[19] Esquela-Kerscher A and Slack FJ (2006). Oncomirs—microRNAs with a role in cancer. *Nat Rev Cancer* **6**, 259–269.

[20] Gilad S, Meiri E, Yogev Y, Benjamin S, Lebanony D, Yerushalmi N, Benjamin H, Kushnir M, Cholakh H, and Melamed N, et al (2008). Serum microRNAs Are promising novel biomarkers. *PLoS One* **3**, e3148.

[21] Song M-Y, Pan K-F, Su H-J, Zhang L, Ma J-L, Li J-Y, Yuasa Y, Kang D, Kim YS, and You W-C (2012). Identification of serum microRNAs as novel non-invasive biomarkers for early detection of gastric cancer. *PLoS One* **7**, e33608.

[22] Wang J, Chen J, Chang P, LeBlanc A, Li D, Abbruzzesse JL, Frazier ML, Killary AM, and Sen S (2009). MicroRNAs in plasma of pancreatic ductal adenocarcinoma patients as novel blood-based biomarkers of disease. *Cancer Prev Res (Phila)* **2**, 807–813.

[23] Hanahan D (1985). Heritable formation of pancreatic beta-cell tumours in transgenic mice expressing recombinant insulin/simian virus 40 oncogenes. *Nature* **315**, 115–122.

[24] Sobin LH, Gospodarowicz MK, and Wittekind CH (2009). UICC: TNM classification of malignant tumours. . 7th edn.Oxford, England: Wiley-Blackwell; 2009 .

[25] Szafranska AE, Doleshal M, Edmunds HS, Gordon S, Luttges J, Munding JB, Barth RJ, Gutmann EJ, Suriawinata AA, and Marc Pipas J, et al (2008). Analysis of microRNAs in pancreatic fine-needle aspirates can classify benign and malignant tissues. *Clin Chem* **54**, 1716–1724.

[26] Munding JB, Adai AT, Maghnouj A, Urbanik A, Zöllner H, Liffers ST, Chromik AM, Uhl W, Szafranska-Schwarzbach AE, and Tannapfel A, et al (2012). Global microRNA expression profiling of microdissected tissues identifies miR-135b as a novel biomarker for pancreatic ductal adenocarcinoma. *Int J Cancer* **131**, E86-95.

[27] Bloomston M, Frankel WL, Petrocca F, Volinia S, Alder H, Hagan JP, Liu CG, Bhatt D, Taccioli C, and Croce CM (2007). MicroRNA expression patterns to differentiate pancreatic adenocarcinoma from normal pancreas and chronic pancreatitis. *JAMA* **297**, 1901–1908.

[28] Habbe N, Koorstra J, Mendell J, Offerhaus G, Ryu J, Feldmann G, Mullendore M, Goggins M, Hong S, and Maitra A (2009). MicroRNA miR-155 is a biomarker of early pancreatic neoplasia. *Cancer Biol Ther* **8**, 340–346.

[29] Ryu JK, Hong SM, Karikari CA, Hruban RH, Goggins MG, and Maitra A (2010). Aberrant microRNA-155 expression is an early event in the multistep progression of pancreatic adenocarcinoma. *Pancreatology* **10**, 66–73.

[30] Szafranska AE, Davison TS, John J, Cannon T, Sipos B, Maghnouj A, Labourier E, and Hahn SA (2007). MicroRNA expression alterations are linked to tumorigenesis and non-neoplastic processes in pancreatic ductal adenocarcinoma. *Oncogene* **26**, 4442–4452.

[31] Seux M, Iovanna J, Dagorn JC, and Dusetti NJ (2009). MicroRNAs in pancreatic ductal adenocarcinoma: new diagnostic and therapeutic clues. *Pancreatology* **9**, 66–72.

[32] Zhang Y, Li M, Wang H, Fisher W, Lin P, Yao Q, and Chen C (2009). Profiling of 95 microRNAs in pancreatic cancer cell lines and surgical specimens by real-time PCR analysis. *World J Surg* **33**, 698–709.

[33] Ho AS, Huang X, Cao H, Christman-Skieller C, Bennewith K, Le QT, and Koong AC (2010). Circulating miR-210 as a novel hypoxia marker in pancreatic cancer. *Transl Oncol* **3**, 109–113.

[34] Canto MI, Hruban RH, Fishman EK, Kamel IR, Schulick R, Zhang Z, Topazian M, Takahashi N, Fletcher J, and Petersen G, et al (2012). Frequent detection of pancreatic lesions in asymptomatic high-risk individuals. *Gastroenterology* **142**, 796–804.

[35] Yu J, Li A, Hong S-M, Hruban RH, and Goggins M (2012). MicroRNA alterations of pancreatic intraepithelial neoplasias. *Clin Cancer Res* **18**, 981–992.

[36] Wan C, Shen Y, Yang T, Wang T, Chen L, and Wen F (2012). Diagnostic value of microRNA for pancreatic cancer: a meta-analysis. *Arch Med Sci* **8**, 749–755.

[37] Liu J, Gao J, Du Y, Li Z, Ren Y, Gu J, Wang X, Gong Y, Wang W, and Kong X (2012). Combination of plasma microRNAs with serum CA19-9 for early detection of pancreatic cancer. *Int J Cancer* **131**, 683–691.

[38] Bauer AS, Keller A, Costello E, Greenhalf W, Bier M, Borries A, Beier M, Neoptolemos J, Büchler M, and Werner J, et al (2012). Diagnosis of pancreatic ductal adenocarcinoma and chronic pancreatitis by measurement of microRNA abundance in blood and tissue. *PLoS One* **7**, e34151.

[39] du Rieu MC, Torrisani J, Selves J, Al Saati T, Souque A, Dufresne M, Tsongalis GJ, Suriawinata AA, Carrère N, and Buscail L, et al (2010). MicroRNA-21 is induced early in pancreatic ductal adenocarcinoma precursor lesions. *Clin Chem* **56**, 603–612.

[40] LaConti JJ, Shivapurkar N, Preet A, Deslattes Mays A, Peran I, Kim SE, Marshall JL, Riegel AT, and Wellstein A (2011). Tissue and serum microRNAs in the Kras^{G12D} transgenic animal model and in patients with pancreatic cancer. *PLoS One* **6**, e20687.

[41] Costello E, Greenhalf W, and Neoptolemos JP (2012). New biomarkers and targets in pancreatic cancer and their application to treatment. *Nat Rev Gastroenterol Hepatol* **9**, 435–444.

[42] Baraniskin A, Nöpel-Dünnebacke S, Ahrens M, Jensen SG, Zöllner H, Maghnouj A, Wos A, Mayerle J, Munding J, and Kost D, et al (2013). Circulating U2 small nuclear RNA fragments as a novel diagnostic biomarker for pancreatic and colorectal adenocarcinoma. *Int J Cancer* **132**, E48-57.

[43] Kawaguchi T, Komatsu S, Ichikawa D, Morimura R, Tsujiura M, Konishi H, Takeshita H, Nagata H, Arita T, and Hirajima S, et al (2013). Clinical impact of circulating miR-221 in plasma of patients with pancreatic cancer. *Br J Cancer* **108**, 361–369.

[44] Wang WS, Liu LX, Li GP, Chen Y, Li CY, Jin DY, and Wang XL (2013). Combined serum CA19-9 and miR-27a-3p in peripheral blood mononuclear cells to diagnose pancreatic cancer. *Cancer Prev Res (Phila)* **6**, 331–338.

[45] Xue Y, Abou Tayoun AN, Abo KM, Pipas JM, Gordon SR, Gardner TB, Barth Jr RJ, Suriawinata AA, and Tsongalis GJ (2013). MicroRNAs as diagnostic markers for pancreatic ductal adenocarcinoma and its precursor, pancreatic intraepithelial neoplasm. *Cancer Genet* **206**, 217–221.