

Serum miR-210-3p can be used to differentiate between patients with pancreatic ductal adenocarcinoma and chronic pancreatitis

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Abstract. Patients with chronic pancreatitis (CP) are at risk of developing pancreatic ductal adenocarcinoma (PDAC). To the best of our knowledge, there are no suitable non-invasive biomarkers for differentiation between CP and PDAC; however, potential molecular candidates include circulating miRNAs due to ease of extraction, their stability and tissue specificity. Therefore, the aim of the present study was to identify potential serum marker(s) that may be used for differentiating between CP and PDAC. In total, 77 patients were enrolled in the present study; 34 patients with CP, 26 patients with PDAC and a control group of 17 healthy individuals. Expression of miR-10b-5p, miR-106b-5p, miR-210-3p and miR-216a-5p in serum was determined by reverse transcription-quantitative PCR. Serum miRNA expression levels in patients with CP, PDAC and in the control group were compared. Routine biochemical blood parameters were determined and correlation analysis of these parameters with miRNA expression was performed. Expression of miR-210-3p was increased in the sera of patients with PDAC compared with the CP patients ($P=0.015$) and with the control group ($P<0.001$). MiR-106b-5p ($P=0.056$) and miR-10b-5p ($P=0.080$) were not significantly upregulated in patients with PDAC compared with those with CP. Analysis of miRNA expression in relation to laboratory blood parameters showed positive correlations between miR-210-3p with alkaline phosphatase ($r=0.605$; $P=0.022$) and with γ -glutamyltranspeptidase ($r=0.529$; $P=0.029$) in PDAC. The novel finding of the present study was that miR-10b-5p was positively correlated with C-reactive protein ($r=0.429$; $P=0.047$) in patients with PDAC and with carbohydrate antigen 19-9 ($r=0.483$; $P=0.005$) in CP. Based on the preliminary data

obtained in the present study, it was concluded that miR-210-3p may be used as a non-invasive biomarker that can be used to distinguish between patients with PDAC and CP.

Introduction

Chronic pancreatitis (CP) is a type of pathologic fibro-inflammatory syndrome of the exocrine pancreas, with genetic, environmental and/or other risk factors, and patients develop persistent responses to parenchymal injury and stress (1). In ~70% of tumors of the pancreas, the tumor is primarily located within the head of the pancreas (2), and ~5% of patients with CP develop pancreatic cancer over the following 20 years (3). Pancreatic ductal adenocarcinoma (PDAC) is a common type of cancer of the pancreas with a high mortality rate, but does not have clearly defined symptoms (4). In >50% of pancreatic cancer cases, patients are diagnosed with advanced stage cancer, which has the lowest 5-year relative survival rate (9% for all stages) amongst all types of cancer (5). In ~90% of patients with PDAC, the development of the cancer is sporadic, whereas in the other 10% of cases, there is a hereditary element (6). Only 15% of patients with PDAC are diagnosed at an early stage with an indication for surgical treatment, and at present, this is the only established curative means.

The majority of studies focused on differentiating between CP and PDAC primarily include evaluation of basic parameters, such as imaging, and clinical and biochemical markers. Carbohydrate antigen 19-9 (CA 19-9) is the most widely used biomarker for the diagnosis of pancreatic cancer in symptomatic patients (7). Unfortunately, it is not specific for this pathology, as CA 19-9 concentration in the blood may increase in other diseases, such as cirrhosis of the liver, cholangitis, CP and other tumors of the gastrointestinal tract (8). In addition, 5-10% of the population with a rare Lewis antigen system do not synthesize CA 19-9 (7). Patients with elevated levels of CA 19-9 are subjected to abdominal ultrasonography (US), computed tomography (CT), magnetic resonance imaging (MRI) or endoscopic ultrasound-guided fine-needle aspiration biopsy. The research is performed with the hope that it will allow for distinguishing between benign and neoplastic lesions, but these techniques do not always precisely define the type of pathology and are most often performed too late,

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and thus only confirm the fatal diagnosis (9). In addition, the symptoms of PDAC and CP are similar: Pain in the upper abdomen, weight loss, nausea and occasionally, jaundice. Both groups of patients often have a history of smoking and alcohol abuse. Novel diagnostic tests are necessary to differentiate PDAC from CP from which surgical and radical interventions, including pancreatoduodenectomy, can be avoided if not needed, as such treatments are associated with a large number of severe complications (10,11).

To the best of our knowledge, there are no studies which have identified non-invasive methods for distinguishing CP from PDAC. Promising molecular candidates include short, non-coding RNAs, such as microRNAs (miRs/miRNAs), which are involved in regulation of key processes in all types of living cells, including cellular differentiation, apoptosis and growth (9). MiRNA expression profiles are tissue-specific, and aberrant miRNA expression can be indicative of pathologies such as inflammation or cancer (12). Based on the literature search performed for this study, four miRNAs: miR-10b-5p, miR-106b-5p, miR-210-3p and miR-216a-5p were selected, and their expression in the blood collected from patients with suspected CP and PDAC were compared to allow distinguishment between these two types of pathologies (13-17).

Materials and methods

Patients. A total of 77 patients were enrolled in the present study at the Department and Clinic of Gastroenterology with Endoscopic Unit, Medical University of Lublin. The study conformed with the principles outlined in the Declaration of Helsinki (18) and was approved by the Research Ethics Committee of the Medical University of Lublin (approval no. KE 0254-/54/2015). All participants provided written informed consent prior to enrolment in the study. The patients were divided into three groups: Group I consisted of 26 patients (17 males and 9 females; age range, 47-89, median age 58 years old) who were diagnosed with PDAC without a history of CP; Group II consisted of 34 patients with CP (27 males and 7 females; age range, 21-78, median age 39.5 years old); and Group III which consisted of 17 healthy patients (13 males, 4 females; age range, 22-50, median age 34 years old) and served as a control group according to imaging tests (CT, US) that excluded the presence of PDAC and CP, as well as any other acute and chronic inflammation illnesses, verified by serum C-reactive protein (CRP) concentration measurement. The patients' characteristics are presented in Table I.

Blood sample collection. For routine blood sample analysis, ~5 ml of venous blood was collected into biochemical tubes without anticoagulant. After the blood had clotted, it was centrifuged at 2,500 x g for 10 min at 4°C twice to remove insoluble residues, then the supernatant was aliquoted in RNase-free tubes and stored at -80°C until required for RNA isolation. The activity of lipase, amylase, alanine aminotransferase, aspartate aminotransferase, alkaline phosphatase (ALP), γ -glutamyltranspeptidase (GGTP), as well as hemoglobin (Hb), bilirubin, CRP and CA19-9 levels were determined by routine laboratory methods in The Central Laboratory (Independent Public Clinical Hospital No. 4, Lublin, Poland).

RNA isolation and cDNA synthesis. Total RNA, including miRNAs was isolated from serum using a miRCURY RNA Isolation kit according to the manufacturer's protocol (Biofluids; Exiqon; Qiagen, AB). Spike-ins UniSp 2, UniSp 4 and UniSp 5 were mixed with MS2 bacteriophage RNA (Roche Diagnostics) and were added to each sample to monitor RNA isolation. After optimization, the cDNA was synthesized from 4 μ l isolated RNA using a Universal cDNA Synthesis kit II according to the manufacturer's protocol (Exiqon; Qiagen, AB). Moreover, UniSp6 was used to monitor the quality of the reverse transcription reaction. For the negative controls, three reactions were prepared using the following reaction mixtures-without reverse transcriptase, without RNA template and using MS2 bacteriophage RNA as template.

Quantitative (q)PCR. Each cDNA sample was diluted with nuclease-free water and 4 μ l cDNA was mixed with 5 μ l SYBR-Green MasterMix (Exiqon; Qiagen, AB), and 1 μ l LNATM primers (Exiqon; Qiagen, AB). The final volume of the reaction mixture was 10 μ l and each reaction was carried out in triplicate. Amplification with real-time fluorescence detection was performed using a LightCycler[®] 480 II instrument (Roche Applied Science) as follows; Initial denaturation, 10 min at 95°C; followed by 45 cycles of 10 sec at 95°C and 1 min at 60°C. The degree of hemolysis of serum samples was evaluated using primers for miR-23a-3p and miR-451a. Differences between the miR-23a-3p and miR-451a Cp values <5 were considered hemolysis-free and such serum samples were further analyzed.

In accordance with recommendations from Exiqon, miR-30c, miR-103a-3p, miR-124-3p, miR-191-5p and miR-423-3p were considered as potential reference genes, amongst which, miR-103a-3p was selected for further analysis as it had the lowest degree of variation between analyzed groups ($P>0.05$). The relative expression of miRNAs was calculated using efficiency method with the LightCycler[®] 480 SW version 1.5 software (Roche Diagnostics) according to Roche Operator's manual.

The sequences of the microRNA LNATM primers (Exiqon; Qiagen, AB) used in the present study are: hsa-miR-30c-5p, UGUAAACAUCUACACUCUCAGC; hsa-miR-103a-3p, AGCAGCAUUGUACAGGGCUAUGA; hsa-miR-124-3p, UAAGGCACGCGGUGAAUGCC; hsa-miR-191-5p, CAA CGGAUCCCAAAAGCAGCUG; and hsa-miR-423-3p, AGC UCGGUCUGAGGCCCCUCAGU. For evaluation of the level of hemolysis target miRNA sequences were: miR-23a-3p, AUC ACAUUGCCAGGGGAUUUCC; and hsa-miR-451a, AAACCG UUACCAUACUGAGUU. Target miRNA sequences for potential miRNA biomarkers in differentiation of PDAC from CP were: hsa-miR-10b-5p, UACCCUGUAGAACCGAAU UUGUG; hsa-miR-106b-5p, UAAAGUGCUGACAGUGCA GAU; hsa-miR-210-3p, CUGUGCGUGUGACAGCGGCUGA; and hsa-miR-216a-5p, UAAUCUCAGCUGGCAACUGUGA. Spike-in target sequences used for RNA isolation control were: UniSp2, GUACUCGGCUUACGAUCGUAA; UniSp 4, GAU GGCAUUCGAUCAGUUCUA; and UniSp 5, GAUCUACG GUCAAUGUCUAAAG; for cDNA synthesis control, the UniSp 6 target sequence was CUAGUCCGAUCUAAAGUCUUCGA.

Statistical analysis. Normality of distribution of miRNA expression was assessed using histograms and a Kolmogorov-Smirnov

Table I. Clinicopathological characteristics of the recruited patients.

Clinicopathological characteristic	Chronic pancreatitis group	Pancreatic ductal adenocarcinoma group	Control group	P-value
Sex, n (%) ^c				
Female	7 (20.59)	9 (34.61)	4 (23.53)	0.455
Male	27 (79.41)	17 (65.38)	13 (76.47)	
Median age, years (range) ^d	39.5 (21-78)	58.0 (47-89)	34.0 (22-50)	0.001 ^b
Smoking status, n (%) ^a				
Smokers	30 (88.23)	20 (76.92)	9 (52.94)	0.019 ^a
Non-smokers	4 (11.76)	6 (23.08)	8 (47.06)	
History of alcohol abuse, n (%) ^c	31 (91.18)	17 (65.38)	5 (29.41)	0.001 ^b
Diabetes n (%) ^c	14 (41.18)	6 (23.08)	2 (11.76)	0.068
Stage, n (%)				
Early stage	-	4 (15.38)	-	-
Locally advanced stage	-	12 (46.15)	-	-
Advanced stage	-	10 (38.46)	-	-

^aP<0.05; ^bP<0.01; ^c χ^2 -test; ^dKruskal-Wallis test.

or Shapiro-Wilk tests. Since the distribution was not normal, differences in miRNA expression amongst the three groups (CP, PDAC and the control group) were analyzed using the non-parametric Kruskal-Wallis ANOVA by ranks and Mann Whitney U tests. Correlations between variables were analyzed using a Spearman-rank correlation coefficient test. Differences in frequencies were compared using a χ^2 test. P<0.05 was considered to indicate a statistically significant difference. Statistical analysis was performed using Statistica, version 13.3 (TIBCO Software Inc.).

Results

Expression of miR-10b-5p was observed in 24/26 patients with PDAC (92.3%), 22/34 patients with CP (64.7%) and in 17/17 individuals in the control group (100%). Expression of miR-106b-5p was observed in 25/26 patients with PDAC (96.1%), 31/34 patients with CP (91.7%), and in 17/17 individuals in the control group (100%). Expression of miR-210-3p was observed in 22/26 patients with PDAC (84.6%), 21/34 patients with CP (61.8%), and 16/17 patients in the control group (94.1%). Expression of miR-216a-5p was observed in only two of the groups; in 5/26 patients with PDAC (19.2%) and in 8/34 patients with CP (20.6%). The percentage of samples expressing each miRNA assessed is shown in Fig. 1.

Serum miRNA levels were compared in the patients with CP, PDAC and the control group. Significantly higher expression levels of miR-210-3p were observed in the patients with CP and PDAC compared with the control group (P=0.0002; Fig. 2).

Comparative analysis showed significantly higher expression levels of miR-210-3p in the patients with PDAC compared with the patients with CP (P=0.015), whereas expression of miR-106b-5p and miR-10b-5p tended to be higher in the patients with PDAC compared with those with CP, although

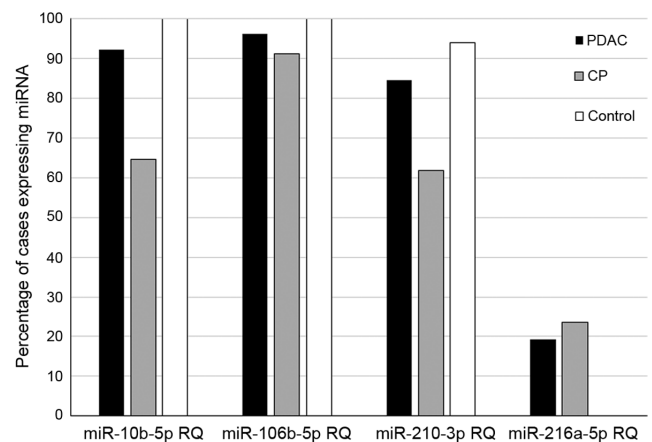


Figure 1. Graph representing the percentage of samples expressing each analyzed miRNA in patients with PDAC and CP, as well as the control group. RQ, relative expression; miRNA/miR, microRNA; CP, chronic pancreatitis; PDAC, pancreatic ductal adenocarcinoma.

the difference was not significant (P=0.056 and P=0.080, respectively). When analyzing miRNA expression in the PDAC group in comparison with the control group, only miR-210-3p expression in PDAC patients was significantly increased (P<0.001), and additionally its expression was correlated with ALP activity (r=0.605, P=0.022), as well as with GGTP activity (r=0.529, P=0.029).

Of minor clinical importance there was a statistically significant correlation between miR-10b-5p and the concentration of CRP (r=0.429, P=0.047) in the PDAC group, and with CA 19-9 content (r=0.483, P=0.005) in the CP group. All four statistically significant correlations between expression of miRNA and basic clinical parameters in PDAC and CP are shown in Table II. There were no other statistically significant correlations identified (P>0.05). Inter-correlations between selected miRNAs within analyzed patient groups were also

Table II. Correlation between the assessed miRNAs and clinical parameters in patients with PDAC and CP.

A, PDAC			
Clinical parameter	Relative expression of the miRNA	r	P-value
C-reactive protein	miR-10b-5p	0.429	0.047 ^a
Alkaline phosphatase	miR-210-3p	0.605	0.022 ^a
γ -glutamyltranspeptidase	miR-210-3p	0.529	0.029 ^a
B, CP			
Clinical parameter	Relative expression of the miRNA	r	P-value
Carbohydrate antigen 19-9	miR-10b-5p	0.483	0.005 ^b

^aP<0.05, ^bP<0.01. CP, chronic pancreatitis; PDAC, pancreatic ductal adenocarcinoma; miR/miRNA, microRNA.

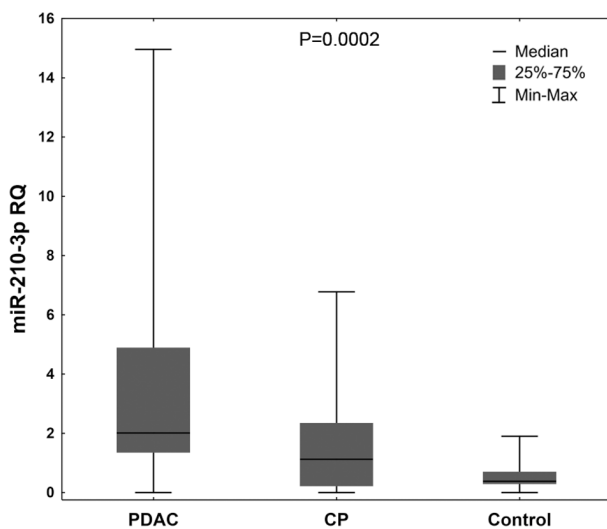


Figure 2. RQ of miR-210-3p in patients with PDAC and CP, as well as the control group. Data were analyzed using a Kruskal-Wallis test to determine whether a significant difference existed between the 3 groups. RQ, relative expression; miRNA/miR, microRNA; CP, chronic pancreatitis; PDAC, pancreatic ductal adenocarcinoma.

identified, but they were not statistically significant (data not shown).

Discussion

CP and PDAC are diseases with several similar symptoms. There is an inherent problem of distinguishing inflammation within the pancreas from neoplastic lesions, augmented by the lack of non-invasive diagnostic methods. Often, surgical interventions turn out to be an unnecessary risk that endangers a patient's health, therefore, discovery of blood-based markers is urgently required, which, together with determination of conventional clinical parameters, can enable a physician to distinguish between CP and PDAC. Complex networks formed by miRNAs regulate cellular development, differentiation and homeostasis (19). Alterations in the expression of miRNAs are

associated with the number of diseases, including inflammatory diseases and cancer (20). In order to search for serum miRNAs that can be used to differentiate between CP and PDAC, candidate miRNAs associated with cancer and the inflammatory process were assessed; specifically miR-10b-5p, miR-106b-5p, miR-210-3p and miR-216a-5p, all of which have been previously found to be significantly upregulated in PDAC (13,14,21,22). Amongst the selected miRNAs, miR-210-3p was deemed to be the most promising as a serum biomarker for use in differentiation between CP and PDAC, as its expression was higher in the patients with PDAC compared with those with CP, and miR-106b-5p expression tended to be higher in patients with PDAC than those with CP. Studies using larger cohorts are thus required to determine/confirm the reliability of these miRs as markers to use to differentiate between these two pathologies. In ~70% of cases of pancreatic cancer, the cancer is located within the pancreatic head and the majority of patients develop obstructive cholestasis resulting in increased ALP and GGTP serum levels (23). Significant positive correlations were identified between the expression of miR-210-3p and the activities of the cholestasis-related enzymes ALP and GGTP in patients with PDAC. This suggests that combined assessment of these parameters (miR-210-3p levels and ALP/GGTP activity) may improve the accuracy of early diagnosis and may provide a non-invasive diagnostic tool for distinguishing between PDAC CP in the future. There was a significant correlation between miR-10b-5p and CRP levels and CA 19-9 content. To date, there are no studies that have shown an association between expression of miR-10b-5p and CP, to the best of our knowledge. The role of miR-10b-5p in the course of carcinogenesis has already been described (24). Moreover, recently published data has shown that miR-10b may serve as a candidate predictive marker, as its expression is associated with significantly improved overall survival (~2 months) in PDAC patients treated with a combination of gemcitabine and Galunisertib (a transforming growth factor β receptor I inhibitor) (25,26).

The majority of published studies have focused on comparative analysis of miR-210 expression in the serum, tissue specimen or pancreatic juice of patients with PDAC in relation to individuals with non-pancreatic diseases or healthy

subjects (27-29). In the present study, significantly higher levels of miR-210-3p were observed in the sera of patients with PDAC compared with the healthy individuals, which confirms reports on the involvement of miR-210 in the regulation of expression of a number of genes associated with malignant transformation. The oncogenic effects of miR-210 have been described in pancreatic, prostate, ovary, bladder, breast and bone cancer (15,28,30-33). Development of hypoxia in the most common pancreatic cancer, adenocarcinoma, results in a poor response to radiotherapy and chemotherapy (34). A state of oxygen deficiency in tissues induces the expression of specific miRNAs, including miR-210-3p, which is termed hypoxamiR (16,35). Genes regulated by miR-210 are involved in cell division and migration, angiogenesis, mitochondrial metabolism, DNA repair or chromatin remodeling (36-38). Anaerobic environments stimulate activation of pancreatic stellate cells, angiogenesis and fibrosis (39-42). Angiogenesis underlies the repair process that is observed in the course of CP, and is a mechanism that also enables the development and growth of cancer (43). Upregulated expression of miR-210-3p expression in cells is reflected by its high levels in the blood (28); experimentally confirmed higher serum levels in patients with PDAC indicate higher expression of this miRNA in the cells where the aforementioned molecular changes may have occurred.

Limitations of the present study include the variance in sample sizes and a relatively low number of enrolled participants, and thus, the results of the present pilot study should be validated using larger cohorts.

In conclusion, the role of miRNAs in the pathogenesis of CP and PDAC highlights their potential use as non-invasive markers for detection and diagnosis of these diseases. It is hypothesized that miR-210-3p may be a useful biomarker for differentiation between these two pancreatic diseases. Additionally, the correlation between miR-210-3p with biochemical parameters, such as the activity of GGTP and ALP, enhances its prognostic value.

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Availability of data and materials

The datasets used and/or analyzed during the present study are available from the corresponding author on reasonable request.

Authors' contributions

AM, MG and JKu designed, planned and supervised the study. MG and AM wrote and revised the manuscript. MG and WJ performed the experiments. MC performed the statistical analysis. MG, WJ and MC interpreted the results.

Samples and clinical data were collected by JKo. All authors read and approved the final version of the manuscript.

Ethics approval and consent to participate

The present study was approved by the Research Ethics Committee of the Medical University of Lublin (approval no. KE 0254-/54/2015).

Patients consent for publication

All participants provided written informed consent prior to enrolment in the study.

Competing interests

The authors declare that they have no competing interests.

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