





Correlations of miR-1290 and miR-191-5p with laboratory parameters as a useful test to differentiate pancreatic cancer from chronic pancreatitis: preliminary study

Korelacje miR-1290 i miR-191-5p z parametrami laboratoryjnymi jako przydany test do różnicowania raka trzustki i przewlekłego zapalenia trzustki: badanie wstępne

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Słowa kluczowe: diagnostyka różnicowa, miRNAs, zapalenie trzustki, gruczolakorak przewodowy trzustki.

Abstract

Introduction: Accurate differential diagnosis between chronic pancreatitis (CP) and pancreatic ductal adenocarcinoma (PDAC) is still problematic. Difficulties arise from the fact that the imaging techniques, clinical symptoms, and laboratory parameters of these 2 diseases are similar. Therefore, non-invasive markers to clearly distinguish between these 2 disorders are urgently needed, and microRNAs (miRNAs) seem to be the most promising biomarkers, because of their tissue specificity, stability in different biofluids, and easy detection.

Aim of the research: To evaluate the usefulness of the measurement of selected miRNAs and basic clinical parameters as a test for differentiating PDAC from CP.

Material and methods: The expression of miR-21-5p, miR-23a-3p, miR-155-5p, miR-191-5p, miR-196a-5p, miR-205-5p, and miR-1290 was assessed in 74 serum samples (PDAC $n = 26$, CP $n = 34$, and the control group $n = 14$) by reverse transcription-quantitative PCR (RT-qPCR). Correlations between the levels of miRNAs and pancreatic lipase, amylase, alanine aminotransferase (ALT), aspartate aminotransferase (AST), alkaline phosphatase (ALP), γ -glutamyltranspeptidase (GGTP), haemoglobin (Hb), bilirubin, C-reactive protein (CRP), and CA19-9 were analysed in 3 groups.

Results: We found that 2 combinations of miRNAs with blood parameters had diagnostic value. In PDAC the expression of miR-1290 positively correlated with GGTP activity ($p = 0.029$, $r_s = 0.487$) and CA 19-9 level ($p = 0.026$, $r_s = 0.464$), while in CP patients miR-191-5p expression was negatively correlated with amylase ($p = 0.018$, $r_s = -0.408$) and CRP ($p = 0.023$, $r_s = -0.394$). No statistically significant correlations were noticed between miRNAs and clinical parameters in the control group.

Conclusions: The combination of miR-191-5p with CRP and amylase characterized CP, while miR-1290 with GGTP and CA 19-9 was specific for PDAC. These non-invasive tools can help to differentiate PDAC from CP.

Streszczenie

Wprowadzenie: Różnicowanie raka trzustki (RT) i przewlekłego zapalenia trzustki (PZT) w rutynowej praktyce klinicznej jest nadal problematyczne. Wątpliwości diagnostyczne wynikają z faktu, że obie choroby są podobne pod względem objawów, badań obrazowych oraz laboratoryjnych. Stąd też stale poszukiwane są nieinwazyjne markery, wśród których obiecującą grupę stanowią mikroRNA (miRNA) ze względu na ich stabilność w różnych płynach biologicznych, specyficzność tkankową i łatwość wykrywania.

Cel pracy: Ocena ekspresji surowiczych miRNA skorelowanych z podstawowymi parametrami biochemicznymi jako przydatnego testu różnicującego RT od PZT.

Materiał i metody: Oceniono względną ekspresję miR-21-5p, miR-23a-3p, miR-155-5p, miR-191-5p, miR-196a-5p, miR-205-5p, miR-1290 w 74 próbkach surowicy (RT $n = 26$, PZT $n = 34$, grupa kontrolna $n = 14$) z wykorzystaniem techniki PCR z odwrotną transkrypcją w czasie rzeczywistym (RT-qPCR). Wykonano analizę korelacji między poziomami miRNA a lipazą trzustkową, amylazą, aminotransferazą alaninową (ALT), aminotransferazą asparaginianową (AST), fosfatazą alkaliczną (ALP), γ -glutamylotranspeptydazą (GGTP), hemoglobina (Hb), bilirubina, białkiem C-reaktywnym (CRP) i CA-19-9 w każdej z trzech grup.

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Wyniki: Wykazano dwie korelacje miRNA z parametrami krwi o właściwościach diagnostycznych. W RT ekspresja miR-1290 była dodatnio skorelowana z aktywnością GGTP ($p = 0,029$, $rs = 0,487$) i poziomem CA 19-9 ($p = 0,026$, $rs = 0,464$), natomiast u pacjentów z PZT ekspresja miR-191-5p – ujemnie skorelowana z amylazą ($p = 0,018$, $rs = -0,408$) i CRP ($p = 0,023$, $rs = -0,394$). W grupie kontrolnej nie odnotowano korelacji statystycznie istotnych między analizowanymi miRNA i parametrami klinicznymi.

Wnioski: Kombinacja miR-1290 z GGTP i CA 19-9 charakteryzuje RT, natomiast kombinacja miR-191-5p z CRP i amylazą – PZT. Te dwa testy mogą być wykorzystane w diagnostyce różnicowej RT i PZT.

Introduction

Chronic pancreatitis (CP) is an inflammatory disease that is characterized by the progressive destruction of acinar cells, ductal cells, and pancreatic islets [1]. This leads to the loss of organ parenchyma and fibrosis, and consequently to the gradual impairment of endo- and exocrine pancreatic functions [2]. The aetiology of CP is diverse and multifactorial, including genetic mutations, environmental and autoimmune factors, obstructive mechanisms, and anatomic abnormalities [3]. Five years after diagnosis patients with CP have a nearly 8-fold increased risk of developing pancreatic [4, 5]. CP is associated with pancreatic cancer formation and progression, and little is known about this relationship, although some *in vivo* studies have indicated a significant role of interleukin-22 (IL-22) in the promotion of pancreatic adenocarcinoma (PDAC) development [6]. PDACs represent the vast majority of pancreatic tumours (~90% of cases), arising in exocrine glands of the organ, which have a poor prognosis: 1-year survival is 24%, and 5-year survival is 9% [7, 8]. Ongoing inflammation in the pancreas may mimic PDAC at imaging that precludes pre-operative diagnosis and may lead to unnecessary surgical intervention [9]. At imaging, CP presents as a tumour-like mass that is hard to clearly distinguish from PDAC [10]. Many patients who undergo surgical interventions for suspected PDAC suffer from inflammation, particularly CP, autoimmune pancreatitis, and their subtypes; in these cases surgical procedures are not required but can improve the quality of life by relieving pain or help to determine the nature of lesions [11]. Moreover, these 2 pathologies show similar biochemical parameters and clinical manifestations [12]. For these reasons there is an urgent need to identify non-invasive markers that help distinguish PDAC from CP, because all available tests are non-specific for these diseases. The small, non-coding RNAs (miRNAs) seem to be the most promising and valuable markers. These molecular players, 18–25 nucleotides in length, are involved in various biological processes, but aberrant expression of specific miRNAs determine a wide variety of cellular networks governing human malignancies or inflammation [13]. miRNAs are present in body fluids such as plasma or serum in stable forms, so their expression profiles are closely related to the pathological conditions inside the cells [14]. Given the great value of miRNAs, we propose the combination of miRNA

expression and biochemical parameters as a simple method for the differentiation of PDAC from CP. For this purpose, we have designed a pilot study focused on 2 miRNA panels associated with the pancreatic inflammation and carcinogenesis, the expression of which correlated with basic biochemical parameters: haemoglobin, bilirubin, CRP, CA19-9, amylase, lipase, alanine aminotransferase (ALT), aspartate aminotransferase (AST), alkaline phosphatase (ALP), and γ -glutamyltranspeptidase (GGTP). The list of selected miRNAs dysregulated in PDAC and CP was chosen by systematic literature research. The first panel, which comprised 4 miRNAs: miR-10b-5p, miR-106b-5p, miR-210-3p, and miR-216a-5p, showed promising results, which we published earlier [15].

Aim of the research

The current study includes the combination of 7 circulating miRNAs: miR-21-5p, miR-23a-3p, miR-155-5p, miR-191-5p, miR-196a-5p, miR-205-5p, and miR-1290, which correlated with the above-mentioned biochemical parameters as a potential diagnostic tool to differentiate CP and PDAC.

Material and methods

Study group

A total of 74 patients were enrolled in the study at the Department and Clinic of Gastroenterology with Endoscopic Unit, Medical University of Lublin, Poland. Written informed consent was obtained from all patients, and the study protocol was approved by the institutional committee on human research (Research Ethics Committee of the Medical University of Lublin approval no. KE 0254-/54/2015), ensuring that it conformed to the ethical guidelines of the 1989 Declaration of Helsinki. The study group was divided into 3 groups: the first group consisted of 26 patients who were diagnosed with PDAC without a history of CP; the second group consisted of 34 subjects with CP; and the third group consisted of 14 healthy individuals who served as a control group according to imaging tests (abdominal computed tomography – CT, transabdominal ultrasonography – 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 study involved patients diagnosed with CP based on definite criteria (fibrosis, calcifica-

Table 1. Patients' characteristic

Parameter	CP	PDAC	Control group	P-value
Gender, <i>n</i> (%):				
Female	7 (20.59)	9 (34.62)	3 (21.43)	0.431*
Male	27 (79.41)	17 (65.38)	11 (78.57)	
Median age [min.–max.] [years]	39.5 [21–78]	58.0 [47–89]	34.5 [22–50]	< 0.001#
Smoking status, <i>n</i> (%):				
Smokers	30 (88.24)	20 (76.92)	8 (57.14)	0.058*
Non-smokers	4 (11.76)	6 (23.08)	6 (42.86)	
History of alcohol abuse, <i>n</i> (%):				
Abuse	31 (91.18)	17 (65.38)	4 (28.57)	< 0.001*
None	3 (8.82)	9 (34.62)	10 (71.43)	
Diabetes <i>n</i> (%):				
Diabetic	14 (41.18)	6 (23.08)	1 (7.14)	0.045*
Non-diabetic	20 (58.82)	20 (76.92)	13 (92.86)	
Stage of disease, <i>n</i> (%):				
Early-stage	–	4 (15.38)	–	
Locally advanced stage	–	12 (46.15)	–	NA
Advanced stage	–	10 (38.46)	–	

*Heterogeneity among the 3 groups calculated with chi-squared test; #heterogeneity among the 3 groups calculated with Kruskal-Wallis test; NA – not applicable.

tions in the pancreas) found on imaging examinations (CT, US, magnetic resonance imaging – MRI) or histopathological findings after surgical procedures due to CP. PDAC in our patients was recognized on the basis of histopathology after surgery. The characteristics of the 3 experimental groups are shown in Table 1.

Collection of serum samples

Venous blood samples (approximately 5 ml) were collected into biochemical tubes without anticoagulant from patients with CP and PDAC, and from healthy individuals. After the clot was formed, the blood was centrifuged twice (2500 g for 10 min) to remove insoluble residues, then the supernatant was aliquoted in RNase-free tubes and stored at –80°C until RNA isolation. Basic biochemical parameters: haemoglobin, bilirubin, CRP, CA19-9, amylase, lipase, AST, ALT, ALP, and GGTP, were also determined by routine laboratory methods.

miRNA isolation and analysis by quantitative PCR (qPCR)

Total RNA, including miRNA was extracted from serum samples using miRCURY RNA Isolation Kit Biofluids (Exiqon, Qiagen) according to the manufacturer's instructions. Three spike-in controls: UniSp 2, UniSp 4, and UniSp 5, were mixed with MS2 bacte-

riophage RNA (Roche Applied Science) and added to each sample for monitoring of RNA isolation. After optimization of the volume of RNA input for cDNA synthesis, 4 µl of isolated RNA was used for reverse transcription using the Universal cDNA Synthesis Kit II (Exiqon, Qiagen). Spike-in UniSp6 was used to monitor the quality of reaction with reverse transcriptase. Negative controls were also prepared: without reverse transcriptase, without RNA template, and using MS2 bacteriophage RNA as a template. Diluted cDNA was mixed with 5 µl SYBR Green Master Mix (Exiqon, Qiagen) and 1 µl of LNA™ primers (Exiqon, Qiagen). Each reaction was carried out in triplicate. Amplification with real-time fluorescence detection was performed using a LightCycler® 480 II Instrument (Roche). To ensure the quality of serum samples the difference in threshold cycles between miR-23a-3p and miR-451a (*DCp* values) lower than 5 was assumed as haemolysis-free, and such samples were further analysed. In accordance with the manufacturer's recommendations (Exiqon, Qiagen), several miRNAs were considered as potential reference genes, among which miR-103a-3p was selected because of the lowest variation between analysed groups ($p > 0.05$, *t*-test). The relative expression of miRNAs was calculated using efficiency method (E-Method) with LightCycler® 480 SW 1.5 software according to Roche instructions.

Statistical analysis

Normality of distribution of miRNA expression was assessed using histograms and the Kolmogorov-Smirnov and Shapiro-Wilk tests. Because the distribution was not normal, differences among the 3 groups (CP, PDAC, and control group) in miRNA expression were analysed using non-parametric tests: Kruskal-Wallis ANOVA by ranks and Mann-Whitney *U* tests. Correlations between variables were analysed using the Spearman test. Heterogeneity among the 3 analysed groups was calculated with χ^2 or Kruskal-Wallis tests. The frequency of positive versus negative expression for each miRNA between PDAC and CP was analysed by Fisher exact test. The differences were considered significant at $p < 0.05$. Statistical calculations were performed using Statistica, version 13.3 (TIBCO Software Inc. [2017], Statistica, Tulsa, OK, USA).

Results

The expression of all selected miRNAs including miR-21-5p, miR-23a-3p, miR-155-5p, miR-191-5p, miR-196a-5p, miR-205-5p, and miR-1290 were detected in CP and PDAC, and in the control group. The percentage of samples expressing each analysed miRNA in PDAC and CP and in the control group is shown in Figure 1.

Comparative analysis between 2 groups: patients with PDAC and CP showed a trend toward increased miR-21-5p level in PDAC than in the CP group ($p = 0.093$). Expressions of 3 miRNAs in PDAC patients were significantly higher in comparison to the control group (miR-23a-3p, $p = 0.007$; miR-155-5p, $p = 0.015$; miR-196a-5p, $p = 0.031$). Comparison of CP and the control group showed significantly higher expression of miR-21-5p ($p = 0.029$), miR-155-5p ($p = 0.017$), miR-191-5p ($p = 0.008$), and miR-196a-5p ($p = 0.021$) in CP patients than in the control group.

Correlations between levels of miRNAs and basic clinical parameters: haemoglobin, bilirubin, CRP, CA19-9, amylase, lipase, AST, ALT, ALP, and GGTP, were carried out in the 3 patient groups. In the PDAC group miR-1290 was positively correlated with GGTP activity ($p = 0.029$, $r_s = 0.487$) and the CA 19-9 level ($p = 0.026$, $r_s = 0.464$). In the CP patient group miR-191-5p expression was negatively correlated with amylase ($p = 0.018$, $r_s = -0.408$) and CRP ($p = 0.023$, $r_s = -0.394$). No statistically significant correlations were noticed between miRNAs and clinical parameters in the control group. Table 2 demonstrates statistically significant correlations between miRNAs and biochemical parameters in the sera of patients with PDAC and CP, respectively.

We also analysed inter-correlations between selected miRNAs within each of the analysed patient groups. The summary of all statistically significant correlations in PDAC and CP and in the control group are shown in Table 3. Other correlations were statistically insignificant ($p > 0.05$).

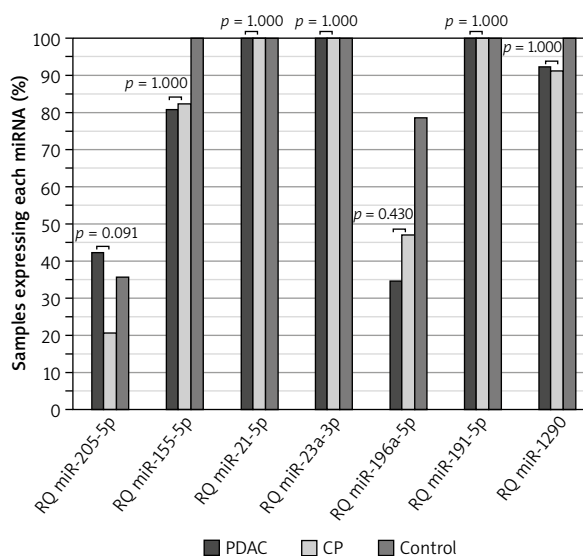


Figure 1. Graph representing the percentage of samples expressing each analyzed miRNA in patients with PDAC and CP, as well as the control group

RQ – relative expression, miRNA/miR – microRNA, CP – chronic pancreatitis, PDAC – pancreatic ductal adenocarcinoma, *p*-values PDAC vs. CP by Fisher exact test.

Discussion

CP and PDAC are 2 related diseases of the exocrine pancreas, where CP increases risk of PDAC [16]. The first hypothesis for an association between cancer development and chronic inflammation was proposed by Rudolf Virchow based on his observations of finding white blood cells in the stroma of neoplastic tissue [17, 18]. With the development of science and new discoveries, Virchow’s hypothesis is still valid. Chronic inflammation regulates carcinogenesis on different levels starting from tumour initiation, through proliferation and progression, and finally

Table 2. Four statistically significant correlations between miRNAs and basic clinical parameters in PDAC and CP patients

Variable	Biochemical parameter	<i>r_s</i>	<i>P</i> -value
PDAC:			
RQ miR-1290	GGTP	0.487	0.029
	CA 19-9	0.464	0.026
CP:			
RQ miR-191-5p	Amylase	-0.408	0.018
	CRP	-0.394	0.023

RQ – relative quantification, *r_s* – Spearman’s correlation coefficient, *P* – Spearman’s correlation *p*-value, PDAC – pancreatic ductal adenocarcinoma, CP – chronic pancreatitis.

Table 3. Summary of statistically significant correlations between miRNA-miRNA expression in the sera of patients with PDAC and CP and in the control group

RQ	rs	P-value
PDAC:		
miR-21-5p vs. miR-23a-3p	0.761	< 0.001
miR-196a-5p vs. miR-1290	0.453	0.020
CP:		
miR-205-5p vs. miR-191-5p	0.351	0.042
miR-205-5p vs. miR-1290	0.384	0.025
miR-21-5p vs. miR-23a-3p	0.666	< 0.001
miR-23a-3p vs. miR-191-5p	0.354	0.040
miR-196a-5p vs. miR-191-5p	-0.363	0.035
miR-196a-5p vs. miR-1290	-0.357	0.038
Control group:		
miR-155-5p vs. miR-21-5p	0.590	0.026
miR-21-5p vs. miR-23a-3p	0.873	< 0.001
miR-21-5p vs. miR-196a-5p	0.673	0.008
miR-21-5p vs. miR-1290	0.692	0.006
miR-23a-3p vs. miR-1290	0.771	0.001

RQ – relative quantification, rs – Spearman's correlation coefficient, P – Spearman's correlation p-value, PDAC – pancreatic ductal adenocarcinoma, CP – chronic pancreatitis.

metastasis [19]. Inflammation activates transcription factors in the future tumour cells which regulate expression of acute phase proteins, cytokines, reactive oxygen species, prostaglandins, and enzymes. This enhances inflammatory cells to the microenvironment, followed by further stimulation of transcription factors and activated immune cells [19]. Depending on the type of tumour and immune cells involved in this process, mediators produced by inflammatory cells increase mutagenesis and activate epigenetic machinery, including histone modifications, long non-coding RNA, and miRNAs that modulate gene expression [20]. Aberrant miRNA expression profiles have been studied in many types of cancers, including PDAC and inflammation of the pancreas [21–26]. miRNAs are attractive blood-based biomarkers in clinical applications because they are stable in circulation, they are not degraded by endogenous RNases, and they are non-invasive and simple to collect [27]. Because miRNAs are mediators in carcinogenesis and inflammation, we have selected a group of miRNAs potentially implicated in these 2 pancreatic diseases (miR-21-5p, miR-23a-3p, miR-155-5p, miR-191-5p, miR-196a-5p, miR-205-5p, and miR-1290) and determined their expression together with biochemical parameters in the blood as a future approach to improve differentiation between CP and PDAC. We

were the first to discover that miR-1290 was positively correlated with GGTP ($p = 0.029$, $rs = 0.487$), which may help to discriminate PDAC from CP. GGTP (EC 2.3.2.2) is a membrane-bound enzyme involved in the glutathione homeostasis, whose main source in the blood is liver [28, 29]. Because of its pro-oxidation and pro-inflammatory properties, high GGTP levels are linked to the development and progression of various types of cancers, and recently it was considered as a potential marker of overall survival in metastatic PDAC patients [30]. Also, it was suggested that GGTP, as an early marker of oxidative stress, is associated with poor prognosis in cervical cancer, renal cell carcinoma, prostate cancer, and in PDAC [31]. The second important relationship between miR-1290 and blood parameters is the positive correlation of miR-1290 and CA 19-9 ($p = 0.026$, $rs = -0.464$) that was noted in PDAC, but not for CP patients. *In vivo* studies have confirmed that miR-1290 acts as an oncogene and promotes PDAC by targeting I κ B kinase complex (IKK), a pivotal regulator of NF- κ B signalling pathway critical for immune and inflammatory response, cell survival, and proliferation [32, 33]. Nowadays CA 19-9 is the validated blood marker that is regularly measured in patients with PDAC, but it has reduced diagnostic value because of false positive and false negative results [34]. This means that the CA 19-9 level is elevated not only in PDAC but also in biliary tract, stomach colorectal, lung, or thyroid tumours, as well as in non-malignant pathologies, including pancreatitis, diabetes mellitus, pulmonary, thyroidal, and gynaecological diseases [35]. Moreover, another limitation of CA 19-9 is the fact that as a sialylated Lewis blood group antigen, CA 19-9 is not detected in people who lack the expression of fucosyltransferase, an enzyme required for the production of both CA 19-9 and Lewis antigen [36]. For these reasons special attention should be paid to the use of miR-1290, which will strengthen the diagnostic potential of CA 19-9 and may help to differentiate between PDAC and CP in the future, which is in concordance with other findings [37, 38]. Our novel data showed correlations between miR-191-5p and conventional blood parameters: amylase and CRP in CP ($p = 0.018$, $rs = -0.408$) and ($p = 0.023$, $rs = -0.394$), respectively. MiR-191 is involved in a wide range of processes: cell proliferation, differentiation, and apoptosis, by targeting transcription factors, chromatin remodellers, and genes associated with cell cycle [39]. In addition, miR-191 is aberrantly expressed in many cancers, and in the context of pancreas it is known that miR-191 promotes proliferation, invasion, and metastasis of PDAC cells by targeting ubiquitin-specific peptidase 10 (USP10), lowering stability of p53, thus activating NF- κ B signalling pathway [40, 41]. As a result, proinflammatory cytokines, including IL-6, interleukin-1 β , and TNF- α , are upregulated by miR-191 [41, 42]. In our study we confirmed that miR-191-5p was negatively corre-

lated with amylase ($p = 0.018$, $r_s = -0.408$) and CRP ($p = 0.023$, $r_s = -0.394$) in CP patients. Pancreatic α -amylase (EC 3.2.1.1), an essential component of pancreatic fluid, catalyses the initial step of starch hydrolysis [43]. The negative relationship between high expression of miR-191 and low level of serum pancreatic amylase is because during the course of the CP the secretory capacity of the pancreas is decreased due to progressive loss of acinar cells [44, 45]. To enhance the diagnostic role of miR-191 in CP, CRP determination may be significant as well. CRP is an acute-phase reactant and non-specific marker of inflammation that exhibits high levels during tissue injury, infection, and inflammation and decreases exponentially after the stimulation ends [46, 47]. In the area of inflammation, CRP is also an important regulator with an impact on the complement pathway, apoptosis, phagocytosis, NO release, and cytokine production [46]. The evaluation of CRP and amylase is useful for monitoring disease activity; after the destruction of the tissue their levels are normalized or even decrease, which may be indicated by the negative correlation with miR-191-5p [48]. The increase of miR-191 in serum may be explained by ongoing inflammation, but also its expression is induced by cigarette smoking, one of the major risk factors for CP and PDAC [49, 50]. Higher expression of miR-21 in PDAC compared to CP showed a trend toward statistical significance ($p = 0.093$). This miRNA is a well-known oncomir overexpressed in various types of cancers, which targets tumour suppressors such as programmed cell death protein 4 (PDCD4) and tissue inhibitor of matrix metalloproteinase 3 (TIMP3), promoting the development of PDAC [51, 52]. It is also a marker of impaired apoptosis in cancer through suppression of F-box protein 11 (FBXO11) or pro-apoptotic FAS-ligand (FasL) [53, 54]. High levels of circulating miR-21-5p are associated with poor prognosis in patients with PDAC independently of other clinicopathological factors like age, gender, and adjuvant therapy [55]. Despite the promising features of miRNAs in future clinical practice, our study has some limitations. As a pilot study, limitations are particularly related to the limited number of patient groups. A large-scale research project with much more precision will define the diagnostic role of the above-mentioned miRNAs.

Conclusions

Because of difficulties in differentiation of PDAC from CP there is an urgent need to discover non-invasive biological markers that allow fast and effective diagnosis. We can conclude that circulating miR-1290 and miR-191-5p have potential diagnostic properties, where parallel determination of miR-1290 with CA 19-9 and GGTP is characteristic for PDAC, while

miR-191-5p with amylase and CRP characterize CP. We also emphasize the supporting role of miR-21-5p to maximize the differential diagnosis in the future.

Conflict of interest

The authors declare no conflict of interest.

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