Expression of selected MicroRNAs in pancreatic ductal adenocarcinoma in relation to tumor progression and patient's outcome.

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Abstract

Background: pancreatic ductal adenocarcinoma (PDAC) remains a disease with extremely poor prognosis and limited effective available treatment. Differential expression of miRNAs isolated from tumor tissue has been proposed as a marker for tumor diagnosis, progression and prognosis. Nevertheless, prognostic value of miRNAs expression in PDACs for patient outcome still remains unclear.

Methods: expression of 7 selected miRNAs, isolated from FFPE samples of 54 PDAC patients, was quantified using RT-qPCR. The relationship of miRNA expression levels with tumor histology, clinico-pathological characteristics, patient overall survival (OS) and progress-free survival (PFS), was subsequently evaluated.

Results: overexpression of miR-21, miR-155 and miR-210 was observed in PDACs (up to 72.62-fold, 232.36 and 181.38-fold respectively), in comparison with non-neoplastic tissues. On the other hand, miR-96 and miR-217 were significantly downregulated in PDACs (up to one hundred times). No differences were, however, noticed between cancer and normal tissues for the expression levels of miR-148a and miR-196a.

On the other hand, expression levels of all 7 miRNAs failed to demonstrate significant correlation with parameters of tumor progression, such as tumor stage, grade, nodal involvement, perineural and vascular invasion. Positive correlation of miR-210 levels was, however, observed with patient age (ρ=0.35). Additionally, miR-148a and miR-217 expression have shown positive association with tubular tumor growth pattern (ρ=0.39; ρ=0.28). Negative correlation of miR-148a values was also demonstrated with dissociative growth pattern and nuclear atypia (ρ=-0.30; ρ=0.27).

Background

Pancreatic ductal adenocarcinoma (PDAC) is the most common primary pancreatic neoplasm, with great potential for locoregional spread (close to 35% of cases) and, in up to 50% of patients, with metastatic disease at the time of diagnosis. Furthermore, PDAC is highly resistant to chemoradiotherapy [1–3]. Curative resection is possible only in 15–20% of the patients, with 5 year overall survival of 5–7% [4–8]. Despite dramatic progress in the management of malignancies, the outcome of PDAC patients failed to show significant improvement and by 2030, PDAC is estimated to be the second leading cause of cancer-related death [9]. Therefore, there is an intensive ongoing search for biomarkers permitting tumor detection, characterization of cancer progression and prediction of patient survival.

MicroRNAs (miRNAs) have emerged as a new class of such biomarkers [10, 11].

Dysregulation in miRNA expression profiles has been detected in a wide variety of neoplastic diseases [12–14]. They have been theorized to act as oncogenes and tumor suppressors, with aberrant miRNA

expression being already presented in neoplastic precursor lesions [15–17]. MiRNAs can be isolated from plasma, tissue samples and excretions, while maintaining sample integrity due to their stability [18–20]. Moreover, miRNAs are preserved after formalin fixation and can be isolated from paraffin embedded tissue, yielding similar results to fresh material [21].

Several microRNAs across numerous publications have been proposed as predictive factors for disease progression, chemotherapy outcome and patient survival. Of these markers, miR-21 has received the most attention, elevated expression levels being regularly suggested as a predictor of poor patient prognosis [22]. Jamieson et al. have also discovered the relationship of miR-21, alongside miR-146a, and miR-628 with tumor grade, stage and lymph node status [23]. Other miRNAs have shown promise as prognostic markers, as well; but were analyzed to a more limited degree, sometimes yielding inconsistent results among studies. Kong et al. have proposed elevated miR-196a serum levels as a predictor of poor survival, besides being able to differentiate resectable and unresectable patients [24]. MiR-155 has also been linked to advanced tumor stage and poor survival [25, 26]. Greither et al. have proposed a prognostic panel consisting of miR-155, -203, -210 and – 222, where their elevated expression is a predictor of poor outcome [27]. On the other hand, increased plasma levels of miR-210 were linked to better patient survival [28]

In this study, the expression of 7 miRNAs (miR-21, miR-96 miR-148a, miR-155, miR-196a and miR-210 and miR-217), described to be dysregulated in PDAC, was analyzed. Three of the selected miRNAs, miR-21, miR-155 and miR-217 were described to be differentially expressed in relationship with tumor progression [23, 25, 29]. MiR-21, miR-155, miR-196a and miR-210 were selected, due to them having been proposed as prognostic markers [22, 27, 30]. The role of miR-96 as well as miR-148a and miR-217 expression in patient prognosis has not been analyzed extensively yet. The aim of this work was evaluation of the relationship of miRNA expression levels with tumor morphology, progression and patients´ survival.

Materials And Methods

Patients and tissue specimens

For this study, tissue samples were collected from patients, who had undergone pancreatoduodenectomy (Whipple and Traverso-Longmire), distal pancreatectomy or total pancreatectomy for PDAC between 2007 and 2015. Formalin fixed paraffin embedded (FFPE) blocks with tumor and with normal pancreatic tissue used as negative control, were retrieved from the archive of the Department of Pathology of the University Hospital Kralovske Vinohrady in Prague. Tumor tissue represented at least two thirds of the volume of the histologic slide. Negative controls of non-neoplastic pancreatic tissue were procured at least 15 mm away from the tumor. The diagnosis of PDAC was confirmed by two pathologists (A. Sz. and V. M.) according to the WHO Classification of Tumors of the Gastrointestinal Tract, 4^{th} edition. Patient data, including age, gender, tumor grade and TNM status was collected for analysis. Patients were followed up until January of 2018, with a median follow-up time of 19 months.

Morphological analysis of tumors

Microscopic patterns of PDAC were classified into tubular, cribriform, solid-trabecular, mucinous, clear cell, dissociative and signet ring; quantified in increments of 5%, taking into account all the available slides with tumor. Tumor growth patterns are illustrated in Fig. 1. Nuclear atypia was semiquantitatively graded as low, medium or high. Mitotic count for each tumor was also performed in 10 HPF (Olympus microscope BX43 and objective Olympus Plan 40x/0.65).

MicroRNAs isolation and reverse transcription.

Three 6μm thick unstained sections from selected FFPE blocks where the tumor occupied at least two thirds, were procured for RNA extraction, using the miRNeasy FFPE kit (Qiagen), following the manufacturer's instructions. Reverse transcription was carried out as described in our previous work [31]. The stem-loop primer sequences for the examined pancreatic miRNAs and the internal control, alien spike miRNA (miR-39 from C. elegans) are listed in Table 1.

Real-time qPCR.

cDNA samples were amplified in duplicates**,** using the Applied Biosystems 7500 Fast real-time PCR system and Hot FirePol EvaGreen qPCR Mix Plus (Solis BioDyne). The reaction mix included 10 pmol of each primer (miRNA specific and the universal (Table 2) and 2μl of cDNA. Amplification of the cDNAs was performed at the following thermal conditions: denaturation at 94°C for 15 min, followed by 40 cycles consisting of denaturation at 94°C for 15 sec, annealing at 48°C for 60 sec and DNA synthesis at 72°C for 40 sec. Reaction product specificity was controlled with their respective melting curves. The ΔΔCt method was applied to measure the values of miRNA expression of interest [32].

Statistical analysis.

All statistical analyses were performed using GenEx 6 and S.A.S. software release 9.4 (SAS Inc., Cary, NC, USA). The expression of miRNAs in neoplastic and normal tissues was compared by a Wilcoxon's paired test. Spearman rank correlation was used to evaluate correlation between expression levels of different miRNAs. Cox proportional-hazards model was used for analyses of overall and progression-free survival. All tested hypotheses were two-sided. The level of significance was selected as alpha = 0.05, therefore p-values below 0.05 were considered as statistically significant.

Results

Clinico-pathological characteristics of PDAC patients

Of 54 patients with PDAC, 27 (50%) were men and 27 (50%) were women The age of patients ranged from 34 to 83 years, median 63 years (Table 3). Four cancers were well differentiated, 27 cancers were moderately differentiated and 23 cancers were poorly differentiated. In one patient, the tumor originated from a mucinous cystic neoplasm (MCN); the analyzed sample was selected to contain only malignant

tumor. The tumor progression was classified in 4 patients pT1, in 7 patients pT2, in 41 patients pT3 and in 2 patients pT4. Lymph node metastases were discovered in 37 specimens. Perineural propagation was present in 47, and lymphovascular invasion in 29 cases. The resection margin was negative (R0) in 40 and positive (R1) in 14 patients (Table 3).

Correlation between tumor growth patterns

Analysis of the relations of particular histomorphological patterns of PDACs using Spearman's correlation showed negative correlation of tubular tumor pattern (being present in tumors with lower grade) with solid trabecular ($p<0.0001$) and dissociative ($p=0.0003$) growth patterns, high nuclear atypia ($p<0.0001$) and mitotic count ($p=0.0016$). Hallmarks of high tumor grade, which are solid trabecular and dissociative growth patterns, were, on the other hand, associated with higher degrees of nuclear atypia ($p=0.0002$, $p<0.001$). High mitotic count in PDAC was also related with nuclear atypia ($p=0.0004$).

Survival

Data on progression-free survival (PFS) were available in 42 patients, with a median of 13 months. Overall survival (OS) of the entire group ranged between 1-81 months, with a median of 19 months. Seven patients have shown no recurrence of the disease and have survived for 20-81 months, still being alive at the end of the follow-up period (Table 3).

Relationship of tumor parameters and patient survival.

Evaluation of prognostic significance of tumor stage was limited by the number of the patients. No difference in prognosis could be demonstrated between grade 2 and grade 3 PDACs ($p>0.05$). The small number of grade 1 tumors (n=4) in our group prevented us from further characterizing deviation in survival according to this parameter. Positive resection margin was associated with shorter PFS ($p=0.005$). Vascular invasion was significantly correlated with poor patient OS ($p=0.036$). No such relationship was apparent in perineural invasion and lymph node status. Microscopic tumor growth patterns, including tubular, cribriform, solid trabecular and dissociative, were not associated with patient prognosis. The strongest correlation of OS and PFS was with tumor mitotic count ($p=0.093$ and $p=0.063$). We identified a cut-off point of 3 mitoses on 10 HPF 40x to distinguish between patients with poor and good prognosis.

Abnormal miRNA expression in pancreatic cancers.

We observed significant overexpression of miR-21, miR-155 and miR-210 (up to 72.62-fold, 232.36 and 181.38-fold correspondently; $p<0.01$, Table 4) in PDACs in comparison with adjacent normal tissues. On the other hand, miR-96 was significantly downregulated in PDACs (-1.42-fold, $p<0.01$). Expression of the miR-217 often was inhibited, up to one hundred times and even was not detected in 15 PDAC samples (Table 4). However, we did not find any significant differences between cancer and normal tissues for the expression levels of miR-148a and miR-196a ($p>0.05$, Table 4).

We detected significant positive correlation between expression levels of different miRNAs. High levels of miR-21 correlated with high levels of the miR-155 (ρ=0.48, p<0.01) and miR-210 (ρ=0.36, p<0.01, Table 5). Downregulation of miR-96 correlated with miR-196a (ρ=0.42, p<0.01, Table 5). Correlation between miR-155 and miR-210 (ρ=0.30, p=0.029) as well as between miR-148a and miR-217 (ρ=0.27, p=0.048) was significant for the 95% and insignificant for 99% confidence interval (Table 5).

MiRNAs expression and clinico-pathological characteristics

Comparison of miRNA expression with clinico-pathological characteristics of patients disclosed positive correlation of miR-210 expression with patient age (ρ=0.35, p=0.0094; Table 6). Expression levels of all 7 miRNAs, failed to demonstrate significant correlation with other parameters, such as tumor stage, grade, nodal involvement, perineural and vascular invasion (Table 6).

MiRNA expression and microscopic tumor growth patterns.

Evaluation of the relationship between miRNA expression and microscopic tumor patterns, using Spearman's correlation discovered statistically significant association of miR-148a and miR-217 expression and tubular tumor growth pattern, characteristic for cancers of lower grade (ρ=0.39,p=0.0030;ρ=0.28,p=0.0396; Table 7). MiR-148a values have shown a negative correlation with nuclear atypia (ρ=-0.30,p=0.0274; Table 7) and dissociative growth pattern (ρ=-0.28,p=0.0338; Table 7). Additionally, miR-155 level had positive correlation with high tumor mitotic count (ρ=0.27,p=0.0468 Table VII).

MiRNA expression and patient's survival.

Analysis of a prognostic role of expression of tested miRNAs in PDAC did not discover any significant evidence for OS (p>0.05, Table 8). Correlation between miRNA levels and duration of PFS was also statistically insignificant for all seven selected miRNAs (p>0.05, Table 9).

Discussion

MicroRNAs are overexpressed or downregulated in pancreatic cancer.

For our analysis we have selected miRNAs, frequently described to be deregulated in various types of PDACs [20, 30, 33, 34]. Zhang et al. have demonstrated relative expression values of miRNAs spanning 6-logs (from 0.01–10,000) among individual cases [35]. In tumor samples we determined up to 232-fold increase variability in miR-21, miR-155 and miR-210 levels. On the other hand, miR-96 and miR-217 were inhibited up to one hundred times, or even undetected in PDACs (Table 4).

Expression levels of miR-21 vary greatly in different studies [30, 33, 36, 37]. For example, Bloomston et al. measured a median 2.2-fold increase in tumors [30], but Zhang et al. found upregulated expression of miR-21 up to 6888-fold in several tumors [35]. In our study, a mean 12.01-fold increase of miR-21 was observed and a maximum 72-fold elevation was present in tumors (Table 4). In addition, we detected

significant positive correlation between high expression levels of miR-21 and miR-155 (48%, $p < 0.01$) as well as miR-210 (36%, $p < 0.01$, Table 5).

The data about miR-96 expression in PDAC are controversial. Bloomston et al. measured an average 1.77-fold increase, when determining miR-96 levels in PDACs [30]. Kent et al. also demonstrated 2.7-fold upregulation of miR-96 in pancreatic cancer cell lines [38]. On the other hand, miR-96 has been shown to be frequently downregulated [19, 33, 39–41]. We found that miR-96 expression was significantly downregulated in cancers in comparison with normal tissues (up to -18-fold, mean was − 1.42-fold, $p < 0.01$, Table 4). Downregulation of miR-96 positively correlated with miR-196a expression levels (42%, $p < 0.01$, Table 5).

Decrease of miR-148a levels has been consistently identified in PDAC tissue samples, across several studies [19, 30, 42]. In contrast with the cited literature, we found slightly decreased miR-148a mean level in tumors (-1.63-fold), but insignificantly in comparison with normal tissues ($p > 0.05$, Table 4). However, this miRNA was inhibited up to -55-fold in several PDACs (Table 4). Positive correlation between miR-148a and miR-217 (27%, $p = 0.048$) activity was significant for the 95% and insignificant for 99% confidence interval (Table 5).

The miR-155 overexpression in PDACs and pancreatic cancer cell lines, measured by microarray, ranged from 1.8 to 2.9-fold in different studies [20, 30, 35, 43]. On the other hand, Zhang et al. reported up to 52-fold increase in individual cases [35]. In our group of samples a mean 22.9-fold increase was present ($p > 0.05$, Table 4). Positive correlation between miR-155 and miR-210 expression levels was found (30%, $p = 0.029$; Table 5).

Abnormalities in miR-196a expression have been described in pancreatic cancer as well as osteosarcoma, colonic, breast and esophageal carcinomas, including [34, 35, 44, 45].Wang et al. demonstrated 16.05-fold increase in plasma samples of patients with PDAC [34]. Xu et al. and Yu at al. detected up to 225-fold increase of miR-196a in plasma exosomes [28, 46]. In our group of PDAC patients we determined a great variation of miR-196a expression, from − 15-fold up to 25.9-fold in different tumors (Table 4). On the other hand, we did not find significant differences in miR-196a expression between cancer and normal tissues (mean was 0.913-fold, $p > 0.05$; Table 4).

Elevation in miR-210 levels have been consistently described across several studies [19, 27, 34]. Wang et al. reported about a 2-28-fold elevation in miR-210 plasma levels in PDAC patients [34]. Greither et al. detected up to 39.9-fold increase in tumors [27]. In our study we observed up to 181-fold increase of miR-210 expression in PDACs in comparison with normal tissues (mean 15.68-fold, $p < 0.01$; Table 4)

MiR-217 has potent tumor suppressor functions and is preferentially inhibited in PDACs. Greither et al. determined only a mean − 2-fold decrease of miR-217 expression [27] and Ma et al. demonstrated − 3.91-fold decrease [47], as well as Szafranszka et al. have shown downregulation up to -10-fold [19]. On the other hand, Hong et al. found downregulated expression of miR-217 to -62.5-fold in PDACs [33]. In our

samples, miR-217 expression was significantly downregulated, with up to-100-fold decrease or was even not detected in 15 PDACs ($p < 0.01$, Table 4).

Besides identifying abnormalities in the expression of single miRNAs, we have also discovered a positive correlation between high expression levels of the three onco-miRNAs: miR-21, miR-155a and miR-210 (Table 5). Acting together, these miRNAs may promote cancer development and progression [48–51]. Moreover, positive correlation was detected for downregulation of tumor suppressing miRNAs miR-148a and miR-217 (Table 5). Both of them inhibit cell proliferation [52, 53], therefore it may be necessary to deactivate them in tumors for successful cancerogenesis. Additionally, positive correlation was detected for downregulation of tumor suppressing miRNAs miR-96 and miR-196a (Table 5). Both of them inhibit cell proliferation [39, 40, 54], but miR-196a acts quite opposite, inhibiting apoptosis [55], promoting cell proliferation and migration [48]. So, downregulation of miR-96 and upregulation of miR-196a looks like a necessary condition for tumor survival.

Thus, we observed that selected miRNAs were abnormally up- or downregulated in pancreatic cancers. Five of seven selected miRNAs demonstrated significant differences of expression levels in tumors in comparison with adjacent normal tissues (Table 4). Therefore, differential miRNAs expression may be a very sensitive tool for the pancreatic cancer diagnostics.

Correlation of clinicopathological features of tumors with microRNA expression

In surgical resection specimens, the relationship of abnormal miRNA expression with tumor morphology and progression has been investigated less frequently, compared with miRNA diagnostic and prognostic utility. Additionally, conclusions are inconsistent in different publications. Jamieson et al have demonstrated expression of miR-21, miR-146a, and miR-628 to be linked to tumor grade, stage and lymph node status [23]. Frampton et al. [56] and Giovanetti et al. [57], on the other hand, found statistically significant association between elevated miR-21 levels and poorly differentiated tumors, but not with other clinicopathological parameters. Dillhoff et al identified no correlation of miR-21 expression with tumor size, differentiation, nodal status, or tumor stage [58]. Dang et al. demonstrated in a group of 54 PDAC patients positive correlation of down-regulated miR-217 with late tumor stage, lymphatic invasion, vascular infiltration and distant metastasis [59]. Schultz et al, in microdissected samples in a group of 170 PDAC patients, could not identify a reliable miRNA profile to separate cancer samples according to tumor stage and lymph node status [60].

In our cases, we haven’t detected significant correlation of miRNA expression with tumor progression, grade, perineural, vascular invasion and lymph node status. Positive association was, however, discovered between patient age and miR-210 levels (Table 6). This finding could be in part related to the proposed role of miR-210 in cellular senescence [61]. Further investigation is warranted to analyze the potential of miRNA signatures in predicting the extent of tumor progression. Additionally, correlation of miRNA expression PDAC resection specimens and plasma samples in prospective studies could be beneficial for further understanding of the promise of miRNAs as predictors of tumor progression, therapy and prognosis.

Prognostic role of miRNAs expression profiles for PDAC patient's overall and progression-free survival.

In spite of significant progress in anticancer therapy in the recent decades, no breakthroughs in PDAC treatment have materialized. The only clinically available biomarker capable of assessing the prognosis of PDAC patients is CA19-9, yet its utility is limited by non-specific positivity and false negativity in multiple neoplastic and non-neoplastic diseases [62]. There is growing evidence that miRNA expression profiles have a potential to provide tumor-specific prognostic information. Several recent works have reported associations between microRNA expression and overall survival in PDAC patients [22, 57, 63, 64]. In our study we observed no correlation between expression of all selected miRNAs and OS or PFS (Tables 8 and 9).

Among the dysregulated miRNAs in PDAC, miR-21 has been the most widely studied potential prognostic factor. Abnormal expression of miR-21 as a marker of poor patient outcome has also been demonstrated in many other malignancies, including melanoma, glioma, carcinomas of breast, colorectal, lung, stomach, prostate, hepatocellular carcinoma and renal cell carcinoma [65]. In patients with PDAC, several meta-analyses, assessing more than 20 articles, published from 2007 to 2016, including more than 2000 PDAC cases [22, 63–66] have postulated that high miR-21 expression is consistently linked to poor OS and PFS. Similarly, Karasek et al. described association of poor patient OS and elevated plasma levels of miR-21 [67]. On the other hand, the literature is not in total agreement regarding the prognostic role of miR-21. Calatayud et al. and Schultz et al. did not find any significant associations between miR-21 expression and OS in their groups of 165 and 277 patients [60, 68]. Significant worse PFS survival was observed by Khan et al. in patients with inoperable PDAC, but no association of miR-21 levels with OS was detected [69]. Donahue et al. described worse OS in patients with elevated miR-21 expression receiving 5-fluoruracyl chemotherapy, but not in patients treated with gemcitabine [70]. Moreover, inconsistencies are also present in literature, whether miR-21 enriched in the cancer cells or in tumor-associated fibroblasts. Kadera et al. described elevated miR-21 expression in tumor-associated fibroblasts to be linked with poor prognosis and lymph node metastases. On the other hand, miR-21 expression in tumor cells was not significantly elevated [71]. Giovanetti et al. detected, however, significantly higher mir-21 levels in in microdissected tumor cells, correlating with significantly shorter OS [57]

The role of miR-96 expression levels in patient prognosis has not been analyzed extensively. Li et al. showed that downregulated expression of miR-96-5p was associated with a decreased overall survival in patients with PDAC [72]. MiR-96 was also associated with poor overall survival in liver and colorectal cancer [73, 74]. On the other hand, Cai et al. did not demonstrate any significant correlation between the expression level of miR-96 and OS in the lung cancer [75].

The data utilizing miR-148a as a prognostic marker for PDAC are inconsistent, as well. Schultz et al. identified low miR-148a expression as a predictor of short OS [60]. On the other hand, in a group of 78 patients, miR-148 expression levels where not statistically significant with regards to overall survival [47]. Also, miR-148a overexpression did not have any effect on cell proliferation and cell chemosensitivity in

four PDAC cell lines [76]. On the other hand, patients showing elevated expression of 6 genes targeted by miR-148a-5p had a significantly poorer overall survival as well as a shorter DFS [77].

Overexpression of miRNAs miR-155, miR-196a and miR-210 have been observed in pancreatic cancer patients who had a poor overall survival rate [27, 30]. These findings were confirmed by Ma et al. and Papaconstantinou et al. for miR-155 [25, 47] as well as by Greither et al. for miR-155 and miR-210 [27]. Mikamori et al. showed that both OS and DFS were significantly shorter in the high miR-155 expression group [26]. Bloomston et al. have linked high miR-196a with shorter OS [30]. Kong et al. also reported correlation of elevated miR-196a in blood sera of PDAC patients with poor survival and advanced disease stage [24].Yu et al. analyzed plasma levels of miR-196a and miR-210 in a cohort of 31 PDAC patients. High miR-196a expression was associated with poor OS, whereas high miR-210 expression was significantly associated with improved survival [28].

The effect of miR-217 expression levels on patient prognosis has been evaluated by Ma et al. and Vychytilova et al., showing no association with OS and PFS [47, 67].

In our study, we could not confirm significant prognostic value for OS and PFS for our panel of miRNAs (Tables 8 and 9). Therefore, the data about prognostic role of miRNAs expression in PDACs are insufficient and sometimes controversial. These discrepancies may be ascribed to several factors. Most of the studies have been performed on a wide variety of PDAC samples types, including frozen and FFPE tumor samples, plasma or blood serum. Tissue samples were analyzed with or without microdissection, which could affect the results by excluding miRNAs from tumor-associated fibroblasts. Detection of variations in miRNA expression was performed using several methods, including RT-PCR, FISH and microarrays, each requiring different protocols for sample preparation and varying greatly in their sensitivity and selectivity. The number of performed studies is low; as such the size and ethnicity of the PDAC patient cohort may also be regarded as a significant source of variability. Thus, further studies are needed to confirm whether miRNAs could serve as prognostic and predictive biomarkers for pancreatic cancer.

Conclusions

In conclusion, this study detected statistically significant differences in miRNA expression in PDAC, compared to normal pancreatic tissue. Thus, even though it has not been clinically implemented yet, detection of differential miRNAs expression may be a sensitive tool for the pancreatic cancer diagnostics. On the other hand, changes in miRNA expression in our group of patient could not be correlated with key tumor parameters, such as grade, stage, and lymph node status. We have, however, found significant correlation with histologic tumor growth patterns and miRNA expression, making this the first study to analyze this aspect of PDAC. Finally, our work could not confirm statistically significant relationship between miRNA expression and patient prognosis. Therefore, further investigations analyzing the potential of miRNAs to predict the extent of PDAC tumor progression and the possible prognostic role of miRNAs are needed.

Declarations

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Availability of Data

Data files, including raw CT values or fold change tables are available on request, please contact the corresponding author.

Authors' contributions

AS performed histologic revision of all cases and provided technical and material support, helped in collection and analysis of data and drafted the manuscript. AP designed and performed the molecular genetic studies, carried out a significant part of the statistical analysis and drafted the manuscript. RG provided the resection specimens and clinical data. ML, RS and ZV provided follow-up data.VM conceived of the study and participated in its design and coordination, performed histologic revision of all cases and helped to draft the manuscript. All authors read and approved the final manuscript.

Ethics approval and consent to participate

All patients provided informed written consent for their tissues to be used for scientific research and to publish their case details. The study was performed according to the Declaration of Helsinki and approved by the Ethics Committee of the Third Faculty of Medicine (Charles University in Prague, Czech Republic). The resolution 1006/2012 was signed by Dr. Marek Vacha, Ph.D., Head of the Ethics Committee.

Competing interests

The authors declare that they have no competing interests.

Abbreviations

PDAC: pancreatic ductal adenocarcinoma

RT-qPCR: reverse transcription quantitative polymerase chain reaction

FFPE: formalin-fixed, paraffin-embedded (tissues)

OS: overall survival

PFS: Progression-free survival

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Tables

Table 1. Stem-loop primers for the miRNAs.

miRNA name:	Stem-loop Primer sequence:
miR-39 C. elegans	GTCGTATCCAGTGCAGGGTCCGAGGTATTCGCACTGGATACGACTATTAC
mir-21	GTCGTATCCAGTGCAGGGTCCGAGGTATTCGCACTGGATACGACTCAACA
miR-96	GTCGTATCCAGTGCAGGGTCCGAGGTATTCGCACTGGATACGACAGCAAAAATGTG
miR-148a	GTCGTATCCAGTGCAGGGTCCGAGGTATTCGCACTGGATACGACAGTCGGAG
miR-155	GTCGTATCCAGTGCAGGGTCCGAGGTATTCGCACTGGATACGACACCCCTATCACG
miR-196a	GTCGTATCCAGTGCAGGGTCCGAGGTATTCGCACTGGATACGACCCCAACAACATG
miR-210	GTCGTATCCAGTGCAGGGTCCGAGGTATTCGCACTGGATACGACTCAGCCGCTGTC
miR-217	GTCGTATCCAGTGCAGGGTCCGAGGTATTCGCACTGGATACGACTCCAATCAGTTC

Table 2. Real-time qPCR primers.

Primer name:	Primer sequence:
Universal primer	ATCCAGTGCAGGGTCCGAGG
mir-39 C. elegans	GCGGCGGAGCTGATTTCGTCTTG
mir-21	GCGGCGGTAGCTTATCAGACTG
miR-96	GCGGCGGTTTGGCACTAGCAC
miR-148a	GCGGCGGAAAGTTCTGAGACACTCC
miR-155	GCGGCGGTTAATGCTAATCGTG
miR-196a	GCGGCGGTAGGTAGTTTCATGTTG
miR-210	GCGGCGGCTGTGCGTGTGACAG
miR-217	GCGGCGGTACTGCATCAGGAAC

Table 3. Clinico-pathological characteristics of 54 PDAC patients.

Age (Median)	63
Gender (male/female)	27/27
Tumor grade (1/2/3)	4/27/23
Tumor stage (pT1/T2/T3/T4)	4/7/41/2
Lymph node metastasis (N1/N0)	37/16
Perineural invasion (yes/no)	47/7
Lymph vessel invasion (yes/no)	29/25
Resection margin (R1/R0)	14/40
PFS length (in months) median	0-81 13
OS length (in months) median	5-81 19

Table 4. Average miRNAs fold change values in pancreatic cancers in comparison with normal tissues.
MicroRNA expression was measured relative to the alien spike (C. elegans miR-39) as the internal control. Negative fold change values indicate downregulation of the miRNAs in cancer samples. Data are presented as means±standard deviation (SD). P-value $p<0.05$ is considered as statistically significant. P-values of the Wilcoxon's test for the significant differences are shown in bold. *Expression of miR-217 was not detected in 15 PDACs.

miRNAs	miRNAs expression fold change		
	Min	Max	Mean±SD
miR-21	-16.12	72.62	12.01±14.242 **p=0.0000035**
miR-96	-18.52	6.22	-1.42±3.981 **p=0.00006**
miR-148a	-55.56	42.3	-1.63±10.762 p=0.08348
miR-155	-13.16	232.36	22.91±38.526 **p=0.00015**
miR-196a	-15.38	25.9	0.913±5.574 p=0.91119
miR-210	-4.0	181.38	15.68±28.869 **p=0.00059**
miR-217	-100 (0*)	15.87	-7.45±16.537 **p=0.00055**
Total number of patients 54			

Table 5. Correlation of expression levels between different miRNAs. Values of the Spearman rank correlation for the significant differences are shown in bold (A). P-value $p<0.05$ is considered as statistically significant. They are shown in bold, too (B).

A. Values of the Spearman rank correlation.

	miR-21	miR-96	miR-148a	miR-155	miR-196a	miR-210	miR-217
miR-21	1	0.1197	0.1167	**0.4827**	0.1786	**0.3601**	-0.0415
miR-96	0.1197	1	0.1142	0.1193	**0.4281**	-0.0047	0.0150
miR-148a	0.1167	0.1142	1	0.0871	-0.1575	0.0843	**0.2747**
miR-155	**0.4827**	0.1193	0.0871	1	-0,0630	**0.3023**	-0.1114
miR-196a	0.1786	**0.4281**	-0.1575	-0.0630	1	0.0478	0.0409
miR-210	**0.3601**	-0.0047	0.08435	**0.3023**	0.0478	1	-0.0557
miR-217	-0.0415	0.0150	**0.27475**	-0.1114	0.0409	-0.0557	1

B. P-values for the Spearman rank correlation.

	miR-21	miR-96	miR-148a	miR-155	miR-196a	miR-210	miR-217
miR-21	0	0.39775	0.40961	**0.00028**	0.20512	**0.00871**	0.76992
miR-96	0.39775	0	0.42014	0.39928	**0.00154**	0.97316	0.91539
miR-148a	0.40961	0.42014	0	0.53890	0.26478	0.55215	**0.04869**
miR-155	**0.0002**	0.39928	0.53890	0	0.65716	**0.02933**	0.43166
miR-196a	0.20512	**0.00154**	0.26478	0.65716	0	0.73616	0.77322
miR-210	**0.00871**	0.97316	0.55215	**0.02933**	0.73616	0	0.69476
miR-217	0.76992	0.91539	**0.04869**	0.43166	0.77322	0.69476	0

Table 6. Correlation of clinico-pathological characteristics with miRNAs expression levels. Values of the Spearman rank correlation (ρ) were used to assess the relationship of morphological tumor parameters with miRNAs expression. P-value $p<0.05$ is considered as statistically significant. This value is shown in bold.

miRNA	Patient parameters						
	Age	Grade	Tumor	Lymph node metastasis	Perineural invasion	Vascular invasion	Resection margin
miR-21	0.21643 p=0.11598	0.13555 p=0.32841	0.08242 p=0.55349	-0.06524 p=0.63931	0.02653 p=0.84897	0.01311 p=0.92506	-0.03796 p=0.78523
miR-96	0.03486 p=0.80239	0.00322 p=0.98155	0.14026 p=0.31172	0.22004 p=0.10987	0.06899 p=0.62012	-0.05362 p=0.70017	0.05152 p=0.71139
miR-148a	-0.09795 p=0.48104	-0.14682 p=0.28941	-0.00647 p=0.96297	0.12408 p=0.37138	0.05483 p=0.69375	-0.08936 p=0.5205	0.15726 p=0.25611
miR-155	0.18552 p=0.17925	0.02586 p=0.85272	0.13994 p=0.31286	0.0243 p=0.86152	0.16095 p=0.24497	-0.00596 p=0.9659	-0.0949 p=0.49489
miR-196a	0.1392 p=0.31543	0.04928 p=0.72342	0.14689 p=0.28917	0.15736 p=0.25579	-0.08668 p=0.53313	0.01907 p=0.89116	0.19932 p=0.14848
miR-210	0.35047 **p=0.00937**	0.03133 p=0.82208	0.03515 p=0.80078	0.03198 p=0.81844	0.08666 p=0.53321	-0.01787 p=0.89794	0.02982 p=0.83048
miR-217	-0.24358 p=0.07591	-0.06054 p=0.66365	0.15461 p=0.26429	0.01408 p=0.91954	0.046 p=0.74117	-0.20857 p=0.13014	0.09764 p=0.48243

Table 7. Correlation of microscopic tumor growth patterns, nuclear atypia and mitotic activity with miRNAs expression levels. Values of the Spearman rank correlation (ρ) were used to assess the relationship of morphological tumor parameters with miRNAs expression. P-value $p<0.05$ is considered as statistically significant. They are shown in bold.

Tumor parameters	miRNAs						
	miR-21	miR-96	miR-148a	miR-155	miR-196a	miR-210	miR-217
Tubular pattern	-0.06396 p=0.6459	0.20166 p=0.1437	0.39621 **p=0.0030**	-0.18439 p=0.1820	0.07923 p=0.5690	-0.17178 p=0.2142	0.28100 p=0.0396
Cribriform pattern	-0.00316 p=0.9819	0.04965 p=0.7215	0.01063 p=0.9392	0.06643 p=0.6332	-0.07216 p=0.6041	0.16552 p=0.2317	-0.07146 p=0.6076
Solid trabecular pattern	0.02782 p=0.8417	-0.09591 p=0.4903	-0.21358 p=0.1210	0.15416 p=0.2657	-0.06176 p=0.6573	-0.01352 p=0.9227	-0.10583 p=0.4463
Dissociative pattern	-0.04564 p=0.7431	-0.08164 p=0.5573	-0.28938 **p=0.0338**	0.03988 p=0.7747	-0.05832 p=0.6753	0.01061 p=0.9393	-0.11410 p=0.4113
Clear cell pattern	0.15876 p=0.2515	0.05180 p=0.7099	-0.00503 p=0.9712	0.17032 p=0.2182	0.09870 p=0.4777	0.19978 p=0.1475	-0.15013 p=0.2786
Nuclear atypia	0.09231 p=0.5068	-0.03809 p=0.7845	-0.30015 **p=0.0274**	0.11659 p=0.4011	-0.05003 p=0.7194	0.04269 p=0.7592	-0.01895 p=0.8918
Mitotic activity	0.18985 p=0.1691	-0.16938 p=0.2208	-0.10428 p=0.4530	0.27182 **p=0.0468**	-0.01885 p=0.8924	0.08383 p=0.5467	-0.24747 p=0.0712

Table 8. Overall survival and miRNAs expression levels in PDAC patients.

Cox proportional-hazards model was utilized to estimate prognostic functions of miRNAs. P-value of $p<0.05$ is considered as statistically significant.

miRNA	Parameter Estimate	Standard Error	Chi-Square	P-value	Hazard Ratio	95% Hazard Ratio Confidence Limits	
miR-21	0.00419	0.01060	0.1562	0.692; **p>0.05**	1.004	0.984	1.025
miR-96	0.02320	0.14363	0.0261	0.871; **p>0.05**	1.023	0.772	1.356
miR-148a	0.00340	0.02204	0.0238	0.877; **p>0.05**	1.003	0.961	1.048
miR-155	0.00299	0.00401	0.5562	0.455; **p>0.05**	1.003	0.995	1.011
miR-196a	-0.01748	0.04550	0.1475	0.70; **p>0.05**	0.983	0.899	1.074
miR-210	0.00427	0.00510	0.6998	0.402; **p>0.05**	1.004	0.994	1.014
miR-217	0.07048	0.08000	0.7761	0.378; **p>0.05**	1.073	0.917	1.255

Table 9. Progression-free survival and miRNAs expression levels in PDAC patients.

Cox proportional hazards model was used to estimate prognostic functions of miRNAs. P-value $p<0.05$ is considered as statistically significant.

miRNA	Parameter Estimate	Standard Error	Chi-Square	P-value	Hazard Ratio	95% Hazard Ratio Confidence Limits	
miR-21	0.00704	0.00993	0.5029	0.478; **p>0.05**	1.007	0.988	1.027
miR-96	0.01405	0.13169	0.0114	0.915; **p>0.05**	1.014	0.783	1.313
miR-148a	0.04633	0.02440	3.6051	0.057; **p>0.05**	1.047	0.999	1.099
miR-155	0.00357	0.00358	0.9915	0.319; **p>0.05**	1.004	0.997	1.011
miR-196a	-0.02901	0.04370	0.4407	0.506; **p>0.05**	0.971	0.892	1.058
miR-210	0.00565	0.00457	1.5276	0.216; **p>0.05**	1.006	0.997	1.015
miR-217	0.04751	0.07628	0.3879	0.533; **p>0.05**	1.049	0.903	1.218

Figures

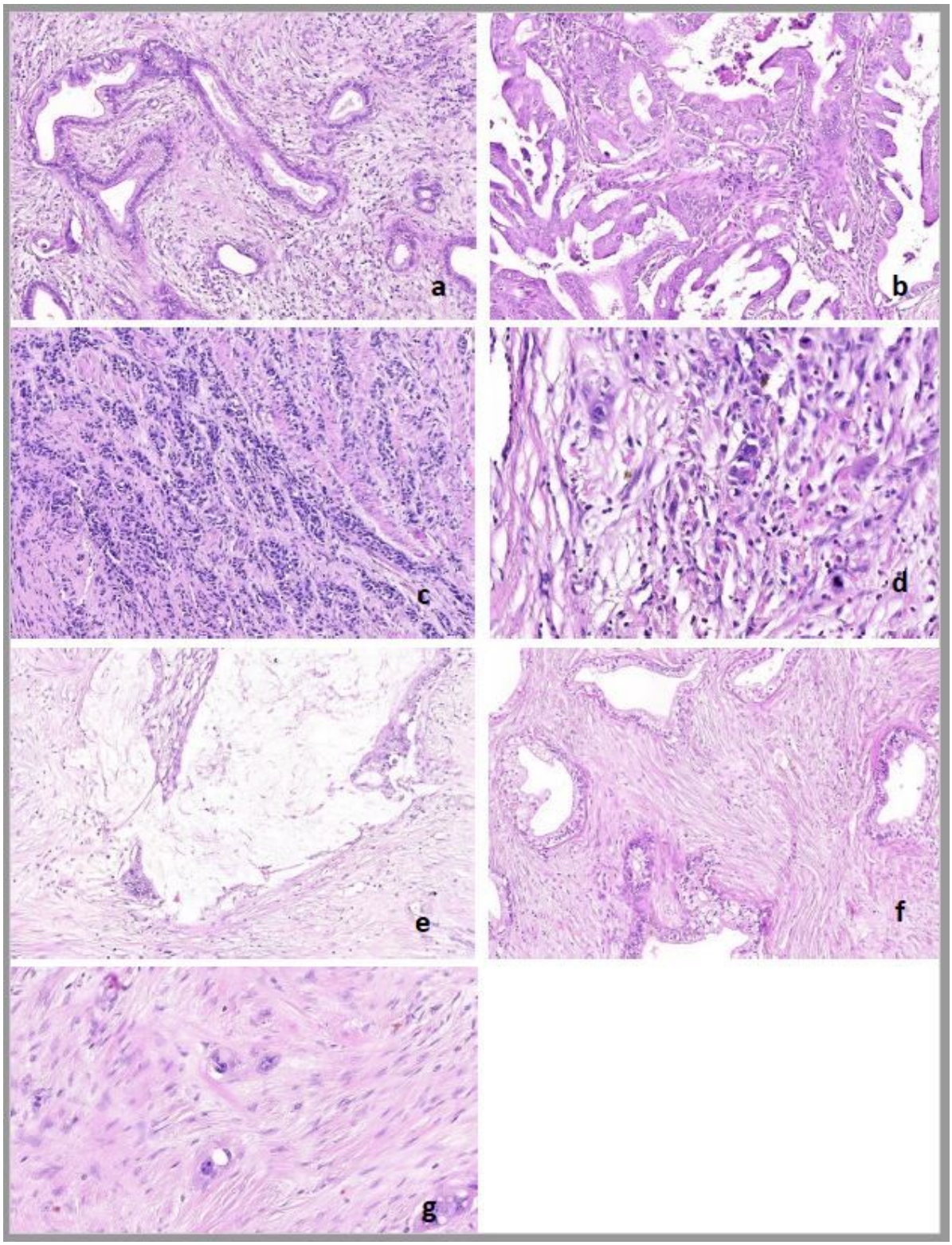

Figure 1

Growth patterns in PDAC. a – tubular (20x magnification). b – cribriform (20x). c- solid trabecular (20x). d – dissociative (40x). e – mucinous (20x). f – clear cell (20x). g – signet ring (40x.)