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Differential Expression of MicroRNAs in Tissues and Plasma Co-exists as a Biomarker for Pancreatic Cancer

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Abstract

Objective: Pancreatic cancer (PC) is a lethal disease with disappointing results from current treatment modalities, suggesting that novel therapeutic strategies are urgently needed. Since microRNAs (miRNAs) are important player in biology, the clinical utility of miRNAs for designing novel therapeutics is an active area of research. The objective of the present study was to examine differentially expressed miRNAs between normal and tumor tissues, and in plasma samples obtained from PC patients, chronic pancreatitis (CP) patients and healthy subjects (HC).

Material and methods: The miRNA expression profiling using formalin-fixed paraffin embedded (FFPE) tissues from normal and tumor specimens was accomplished using miRBase version 19 (LC Sciences, Houston, TX, USA). Quantitative real-time PCR (qRT-PCR) was subsequently performed in individual samples for 7 selected miRNAs. In addition, qRT-PCR was also performed for assessing the expression of 8 selected miRNAs in plasma samples.

Results: A significant difference in the expressions of *miR-21, miR-205, miR-155, miR-31, miR-203, miR-214* and *miR-129-2* were found in tumor tissue samples. Lower expression of *miR-214* was found to be associated with better overall survival. We also observed differential expression of 8 miRNAs in plasma samples of CP and PC patients compared to HC. Interestingly, over expression of *miR-21*, and *miR-31* was noted in both tumor tissues and in the plasma.

Conclusion: We found deregulated expression of miRNAs that could distinguish normal from PC in two different types of samples (tissues and plasma). Interestingly, lower expression of *miR-214* was found to be associated with better overall survival. Although not statistically significant, we also observed higher expression of *let-7a* and lower expression of *miR-508* to be associated with overall better survival. We conclude that our study nicely lays the foundation for detailed future investigations for assessing the role of these miRNAs in the pathology of pancreatic cancer.

Keywords: miRNAs; Chronic Pancreatitis; Pancreatic Cancer; Plasma; FFPE; qRT-PCR

Introduction

Despite considerable progress made in understanding the biology of pancreatic cancer (PC) during the past decade, PC still remains the most lethal cancer with a five-year overall survival of 7% [1]. There has been no measurable improvement in early detection and diagnosis, suggesting that identification of sensitive and precise non-invasive biomarkers at an early stage would be beneficial in distinguishing PC patients from healthy individual. To that end, differential expression of microRNAs (miRNAs) could become a promising strategy especially because miRNAs are more stable than mRNAs, known to regulate multiple target genes post-transcriptionally, and reported to play important roles in oncogenesis and tumor metastasis [2]. The miRNAs are valuable biomarker for investigating their levels in blood serum, plasma and archived material such as formalin-fixed paraffin embedded (FFPE) tissues. In addition, Shimizu et al has reported that carcinoma of the pancreas when resected earlier at stage I before the tumor size reaches 2 cm in diameter and remains confined to the pancreas has a better prognosis [3], which clearly suggest that early diagnosis and surgical management of this deadly disease is crucial because the treatment outcome with conventional chemotherapeutic agents are disappointing for patients diagnosed with PC at late stages.

One of the risk factors for PC is chronic pancreatitis (CP), a benign inflammatory disease which is also difficult to differentiate from early stage PC. An interesting observation was made by Bloomston et al., who observed a distinct pattern of miRNA expression from FFPE samples that may differentiate PC from CP and normal pancreas [4]. In line with this and other studies, Schultz et al reported the identification of two investigative panels based on the expression levels of miRNA from blood samples in a large study with the probability of distinguishing PC patients from CP and healthy individuals [5]. In addition, detection of a three-protein biomarker panel in urine samples of early stage PC patients compared to healthy individuals has been suggested to become a valuable non-invasive and inexpensive screening test for PC [6]. Moreover, the same study suggested that the three proteins REG1A, TFF1 and LYVE1 when combined with CA19-9 (the only PC biomarker in clinical use) may increase the accuracy of the screening test, confirming the biological importance of three-panel protein and CA19-9 as an early diagnostic biomarker for PC [6].

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The expressions of miRNAs such as *miR-21, miR-31*, and *miR-155* have been extensively studied by many investigators showing that the expression of these miRNAs in PC could be important [7-12]. The expression of the above three miRNAs was quantified in normal and tumor tissues of PC patients as well as in plasma samples of HC, CP and PC in the current study with an aim to differentiate their expression levels between different groups. In contrast, the expression of *miR-129-2* has been less extensively studied in PC [13]. The same group identified *miR-129-2* down-regulation occurs in most pancreatic carcinoma samples compared to tumor-uninvolved tissue samples from the same patient which was further correlated with increased expression of SOX4 mRNA [13]. These limited studies suggest that further investigations for assessing the role of differential expression of miRNAs between benign and tumor specimens and more importantly in serum or plasma samples is warranted. If confirmed, miRNAs could become a useful tool for early diagnosis and prognosis, and for designing and developing novel miRNA-based targeted therapeutics.

The aim of the present study was to assess differential expression of miRNAs among PC patients and healthy individuals in formalin-fixed paraffin-embedded (FFPE) tissues and plasma samples. The selection of seven miRNAs as discussed below was based on our miRNA microarray results for their differential expression, and also based on some limited literature. The relationship between overall survival and the differential expression of seven miRNAs in PC was examined quantitatively using FFPE samples that included the expressions of *miR-21, miR-205, miR-155, miR-31 miR-203, miR-214* and *miR-129-2-3p*. We also found that 5 miRNAs were up-regulated and 1 miRNA was down-regulated in PC compared to CP and normal plasma samples. Up-regulated miRNAs included *miR-21, miR-221, miR-181a, miR-935*, and *miR-508*, and down-regulated miRNA was *let-7a*. Interestingly we also observed two miRNAs (*miR-155, miR-31*) that were significantly up-regulated in CP compared to PC plasma samples. Although our study had limitations on sample size, nevertheless our results using tumor and normal tissues, and plasma led to the identification of three miRNAs (*miR-21, miR-155* and *miR-31*) that were commonly expressed in PC compared to normal. The panel of these three miRNAs may facilitate in differentiating HC from PC and that the expression of *miR-214, let-7a* and *miR-508* may also predict overall survival of PC patients. The knowledge gained from this study may lead to the development of miRNA-based tailored therapeutic strategy for improving the treatment outcome of patients diagnosed with PC.

Materials and Methods

Tissue Collection

Histopathology slides (H&E stained slides) from pancreatic adenocarcinoma (PC) patients who underwent surgical resection was evaluated by a pathologist, and the representative slides having predominantly tumors were selected (n=37) from the database of the Wayne State University. In addition, slides with only normal pancreatic tissue (n=24) were also selected. The representative formalin-fixed paraffin embedded (FFPE) tissue blocks corresponding to the pathology slide were pulled from the storage. Benign adjacent normal pancreatic tissue was not available from all 37 PC patients. For paired comparison of selected miRNAs, 24 cases of paired pancreatic non-tumor and tumor tissues were obtained. The institutional human investigation review board approved the study protocol. For normal and tumor tissue sections, four sections, each of 10 microns in thickness, were cut from the selected blocks and were used for the study.

Plasma Collection

The plasma samples from 20 healthy controls (HC), 20 chronic pancreatitis (CP) and 20 adenocarcinoma (PC) (Stage IIB) were processed and stored in the laboratory of Dr. Randall Brand at the University of Pittsburgh, Pennsylvania. Samples included about half male and half female. The institutional human investigation review board approved the study.

RNA Isolation from FFPE Tissue Blocks

The RNA was isolated from FFPE tissues using the RNeasy Kit (Qiagen, Valencia, CA, USA) in accordance with the manufacturer's protocol. Briefly, four tissue sections were placed in micro tubes, and 1 ml xylene was added and RNA was isolated as described previously [14]. RNA was eluted in a final volume of 25 µl, quantified and its purity was evaluated by the absorption ratio at 260/280 nm using NanoDrop 2000 (Thermo Scientific, Pittsburgh, PA, USA).

RNA Isolation from Plasma Samples

About 200 µl of plasma from each sample was mixed with 750 µl of QIAzol (Life Technology) and 1µg of carrier RNA (MS2 RNA, Roche Diagnostic) and was incubated for 5 minutes at room temperature. The addition of carrier RNA preceding to RNA extraction improves recovery of miRNA from plasma samples and allows use of very small amount of clinical samples. To this 200 µl of chloroform was added, mixed well, and centrifuged for 15 minutes at 12,000 x g which was described previously [15]. About 1.5 volume of ethanol was added to the upper aqueous phase and the solution was then transferred onto the RNeasy Mini Spin Column and centrifuged at 13,000 x g for 30 seconds. The flow-through was discarded, and the steps were repeated until the entire sample was used. The RNeasy Mini Column was then washed with buffers provided with the kit, centrifuged for 13,000 x g for 1 minute and the RNA (containing miRNAs) was then eluted with about 25 µl of water. The RNA obtained from 200 µl of plasma samples cannot be quantified due to low yield compared to tissue samples. Hence, the miRNAs of interest was reverse transcribed using standard curve from the template mature miRNAs available commercially (Applied BioSystems).

MicroRNA Profiling

Initial screening of miRNAs in normal and tumor samples from FFPE using miRNA microarray profiling provides a comprehensive way of determining a large number of miRNAs from very small amounts of difficult samples such as FFPE. Equal quantity of extracted RNA from all samples was pooled into two tubes for example pancreas normal and pancreas tumor. LC Sciences then qualitatively and quantitatively analyzed the RNA for miRNA microarray profiling, using miRBase version 19 (LC Sciences, Houston, TX, USA). The data was normalized using selected housekeeping genes. Furthermore, the web-based Ingenuity pathway analysis software was used to perform network analysis (Ingenuity Systems, Redwood City, CA, USA).

Quantitative Real-time Polymerase Chain Reaction (qRT-PCR) from FFPE tissue samples:

Quantitative-RT-PCR was performed on the individual samples in order to validate the miRNA profiling results of 7 selected miRNAs using TaqMan Universal PCR Master Mix, no AmpErase UNG. Selected miRNAs were *miR-21, miR-205, miR-155, miR-31, miR-203, miR-214* and *miR-129-2-3p*. The High Capacity cDNA Reverse Transcription Kit (Applied BioSystems, Foster City, CA, USA) was

used per manufacturer's protocol. Approximately 10 ng of RNA from the respective tissue specimens was reverse transcribed using 7 µl of master mix and 3 µl of respective RT primers as described earlier [16]. PCR reactions were then carried out using resulting cDNA, miRNA specific probes and the TaqMan Universal PCR Master Mix in triplicate using Step One Plus Real-Time PCR (Applied BioSystems, Foster City, CA, USA) as described previously [16]. Relative expression of miRNAs was analyzed using the Ct method and was normalized by RNU48 expression.

Quantitative Real-time Polymerase Chain Reaction (qRT-PCR) using plasma samples

Quantitative-RT-PCR was performed on the individual plasma samples (60) from three different groups (HC, CP and PC) with 8 selected miRNAs using Exiqon-Universal cDNA Synthesis kit (Exiqon, Woburn, MA). Three of the selected miRNAs were common from FFPE tissue samples *miR-21, miR-155* and *miR-31*, and the other five miRNAs were chosen based on other investigators findings, *let-7a, miR-221, miR-181a, miR-935* and *miR-508*. The quantification of all the above recognized 8 miRNAs that are known to express at different levels was investigated in all 60 plasma samples from three different groups. The cDNA for standard curve was synthesized by reverse transcriptase using the template mature miRNAs from Applied BioSystems. The RT reaction included 20 µl of sample containing 4 µl of 5X RT buffer, 2 µl of enzyme mixture, 10 µl of water and 4 µl of either the plasma miRNA or 4 µl (250 nM) of standard miRNA. The mixture was incubated for 60 minutes at 42°C and 5 minutes at 95°C. PCR reactions were performed in equivalent with standard miRNAs to evade batch effects. The standard cDNA prepared earlier was diluted with water and the standard curve was set up in triplicate with five points starting at 10,000, 5000, 2500, 1250 and 625 copy numbers. The plasma cDNA was diluted to 20 folds. The PCR reaction was set up with a total volume of 10 µl containing 5 µl of SYBR Green (Applied BioSystems), 1 µl of PCR primer mix, 4 µl of either the diluted standard cDNA or the plasma cDNA using standard curve model. The plasma miRNA concentration was calculated in 10^{-2} pM units using the quantity value *3.125/6.02/1000.

Statistical Analyses

Differences in the expression levels of miRNAs between normal and tumor paired samples were statistically evaluated by non-parametric Wilcoxon matched pairs test using GraphPad StatMate software (GraphPad Software Inc.). The *p* values that represent differences between normal and tumor samples are displayed in the graph. In addition, differences in the expression levels of miRNAs between HC *vs* CP, and HC *vs* PC were statistically evaluated by GraphPad StatMate software using unpaired t-test. The *p* values between those two comparisons are shown above the bar.

Survival Analyses

The correlation between survival outcome and the miRNAs expression levels was determined by the Kaplan-Meier survival analysis of PC patients from both study sites. The survival was defined as time from diagnosis to any cause of death. The Log-rank test was used to evaluate the survival difference among the low *vs* high groups defined by median value of miRNA expression.

Results

Description of study patients

Archival FFPE tissue blocks of 37 patients were both clinically and pathologically confirmed for pancreatic cancer (PC). We also collected FFPE tissue blocks of normal pancreas from 24 patients out of 37 from the same study group. Two patients were removed from the survival curve analysis due to lack of the availability of the survival data. The median age was 63 and gender count was 40.5% male and 59.5 % female. For the miRNA study from the blood samples, 20 healthy controls (HC), 20 chronic pancreatitis (CP) and 20 pancreatic adenocarcinoma (PC) (Stage IIB) were selected, and the plasma were isolated in Dr. Randall Brand's laboratory at the University of Pittsburgh, Pennsylvania. Samples included about half male and half female.

The results of miRNA expression profiling

Purified RNA samples were pooled (equal amount of RNA from each patients material) separately from FFPE tissue samples of tumor and normal, and were analyzed by LC Sciences for miRNA microarray profiling using miRBase version 19 (LC Sciences, Houston, TX). Expression profiling revealed 291 miRNAs that were differentially expressed between normal and tumor of PC patients. Based on the above results, we selected best seven miRNAs that were found to be significantly deregulated for further validation using qRT-PCR in each samples. These miRNAs included *miR-21, miR-205, miR-155, miR-31, miR-203, miR-214* and *miR-129-2-3p*. The analyses of the profiling results on these miRNAs are illustrated in the following sections.

Ingenuity Pathway Analysis for deregulated miRNAs

Network analysis was performed with the web-based bioinformatics tool Ingenuity pathway Analysis (IPA) software (Ingenuity Systems) to understand the target genes and pathways involved in PC. The analysis revealed the influence of many frequently studied pathways such as NF-κB, MAP kinase, Pro-inflammatory Cytokines, insulin and EIF2C2, as illustrated in supplementary Figure 1A & 1B.

Seven potential miRNAs to differentiate Normal from Tumor of the FFPE samples as assessed by qRT-PCR

Seven miRNAs were selected for further validation individually in 37 PC patient samples compared to pooled normal tissue (with no signs of cancer present in the specimens) obtained from FFPE tissue block that were further away anatomically from the pancreatic tumor and the results are shown in Figure 1-3 (A & D) and Figure 4A using qRT-PCR. The expression analyses of seven miRNAs were also performed using 24 paired samples of normal and tumor tissues from the same PC patient as presented in Figure 1-3 (B & E) and Figure 4B. The relative differences in the expression level of the selected miRNAs in PC were established by setting the expression level of normal samples at unit value (1.0).

The miRNA expression analysis exhibited 6 miRNAs that were up-regulated in most of the tumor samples compared to the normal samples which included *miR-21, miR-205* (Figure 1A & 1D), *miR-155, miR-31* (Figure 2A & 2D) and *miR-203, miR-214* (Figure 3A & 3D). Only one miRNA (miR-129-2-3p) out of seven showed significant down-regulation (Figure 4A) in tumor tissue of most of the PC patient samples tested compared to normal samples. The expression levels in 24 paired tumor and normal tissue samples of *miR-21, miR-205* (Figure 1B & 1E), *miR-155, miR-31* (Figure 2B & 2E), *miR-203, miR-214* (Figure 3B & 3E) showed significant up-regulation of all 6 miRNAs when compared to their paired normal tissue samples, suggesting the oncogenic role of all six miRNAs. The overall trend of up-regulation of all six miRNAs between the paired samples is clear from the figures even though the sample size was small. The expression level of *miR-*

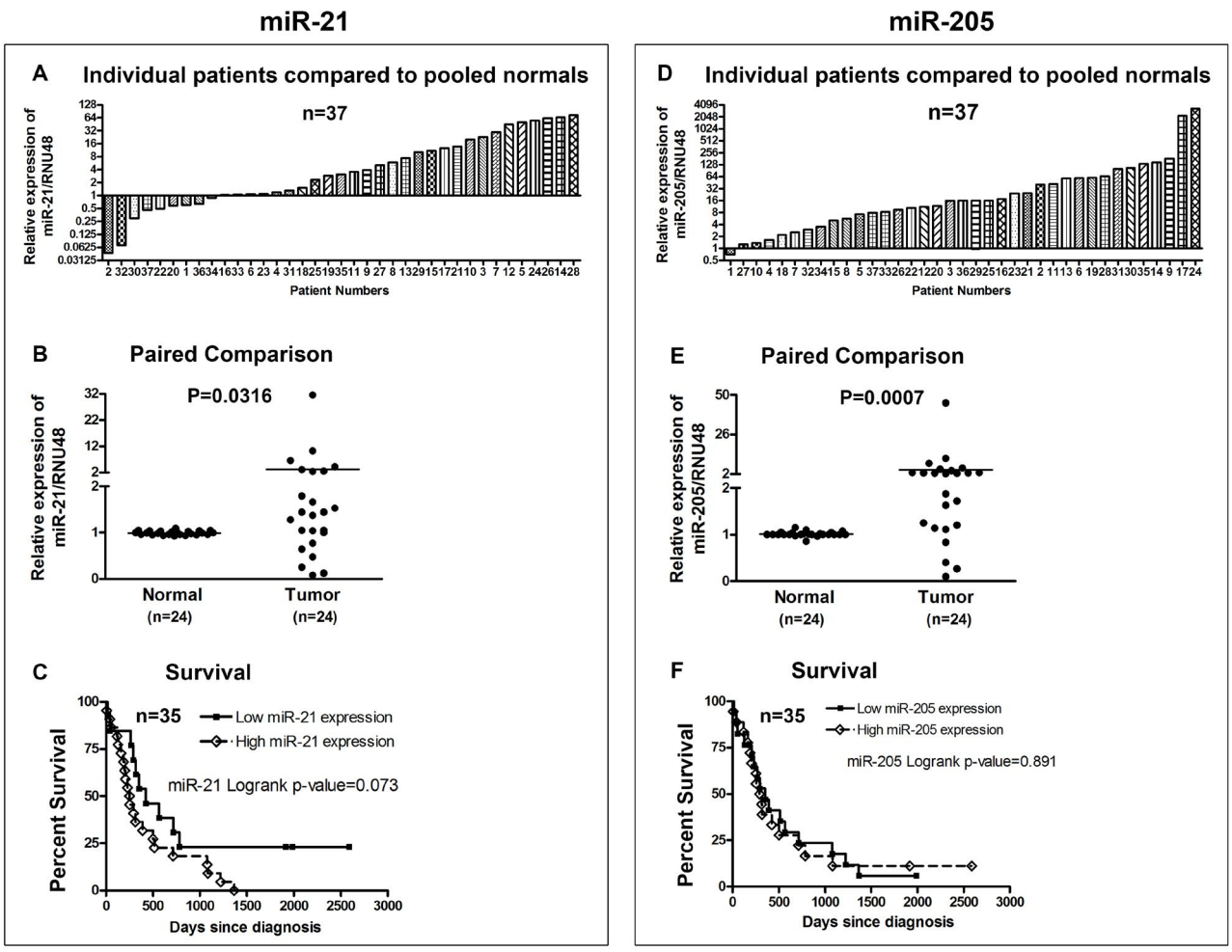

Figure 1: Comparative expression analysis of *miR-21* and *miR-205* in 37 pancreatic cancer patient's tumor specimens compared to 24 pooled normal samples of FFPE tissue blocks (1A&1D), comparative expression analysis in 24 paired samples of FFPE tissue blocks of tumor and normal tissue samples from the same patient (1B&1E) quantitated individually using qRT-PCR. The Kaplan-Meier curve and Log-ranks tests for *miR-21* (1C) and *miR-205* (1F) expression and survival of patients are also presented. There was a significant up-regulation of *miRNA-205* in almost all tumor samples when compared to Normal (1D&1E). Overall, a larger percentage of *miR-21* appears to be up-regulated, in tumor samples than normal samples (1A&1B). The miRNAs expression was normalized using RNU48 miRNA. P values represent comparison between normal and tumor paired samples (1B,1E) using Wilcoxon matched pairs t-test.

205, miR-155 and *miR-203* was significantly enhanced in all except few PC patients as depicted in Figure 1D, 2A, 3A.

Conversely, *miR-129-2-3p* showed down-regulation in its expression in most of the PC patient samples when compared to normal pooled samples (Figure 4A). This general trend of lower miR-129-2-3p expression was also observed in paired tumor samples when compared to their paired normal tissue specimens (Figure 4B), indicating that *miR-129-2-3p* may be a tumor suppressor miRNA. RNU48 was used as control miRNA to normalize the miRNA expression of all samples.

Correlation between miRNAs expression from FFPE samples and the survival of 35 PC patients

The correlation between survival outcome and the seven miRNAs expression levels was determined by the Kaplan-Meier survival analysis for 35 PC patients by using median miRNA expression value which has been represented in Figures 1-3 (C & F) and Figure 4C. The survival was defined as time from diagnosis to any cause of death. The Log-rank test was used to evaluate the survival difference among the low vs high expressing miRNA relative to the mean expression of each miRNA by qRT-PCR. Interestingly, one miRNA *(miR-214)* showed the Log-rank *p* value as statistically significant when compared between low miRNA vs high miRNA (Figure 3F), suggesting that m*iR-214* may serve as an important prognostic marker for PC patients. Although *miR-21* (Figure 1C) Log-rank p value was not significant, it had a wider curve and the patients with low miRNA expression tend to survive longer than the PC patients with high *miR-21* expression. This data is consistent with our previous finding on plasma miRNA study [17]. No such observation was observed for *miR-205* (Figure 1F), *miR-155* (Figure 2C), miR-31 (Figure 2F), *miR-203* (Figure 3C) and *miR-129-2-3p* (Figure 4C).

Eight potential miRNAs to differentiate PC from CP and HC in plasma samples as assessed by qRT-PCR

Eight miRNAs were selected to measure their expression levels, and quantitated the amount of miRNAs present in each of the 20 HC, 20 CP and 20 PC plasma samples. Three of the selected miRNAs (*miR-21, miR-155, and miR-31*) were based on findings showing that their expressions were found to be common in FFPE tissue samples, and the

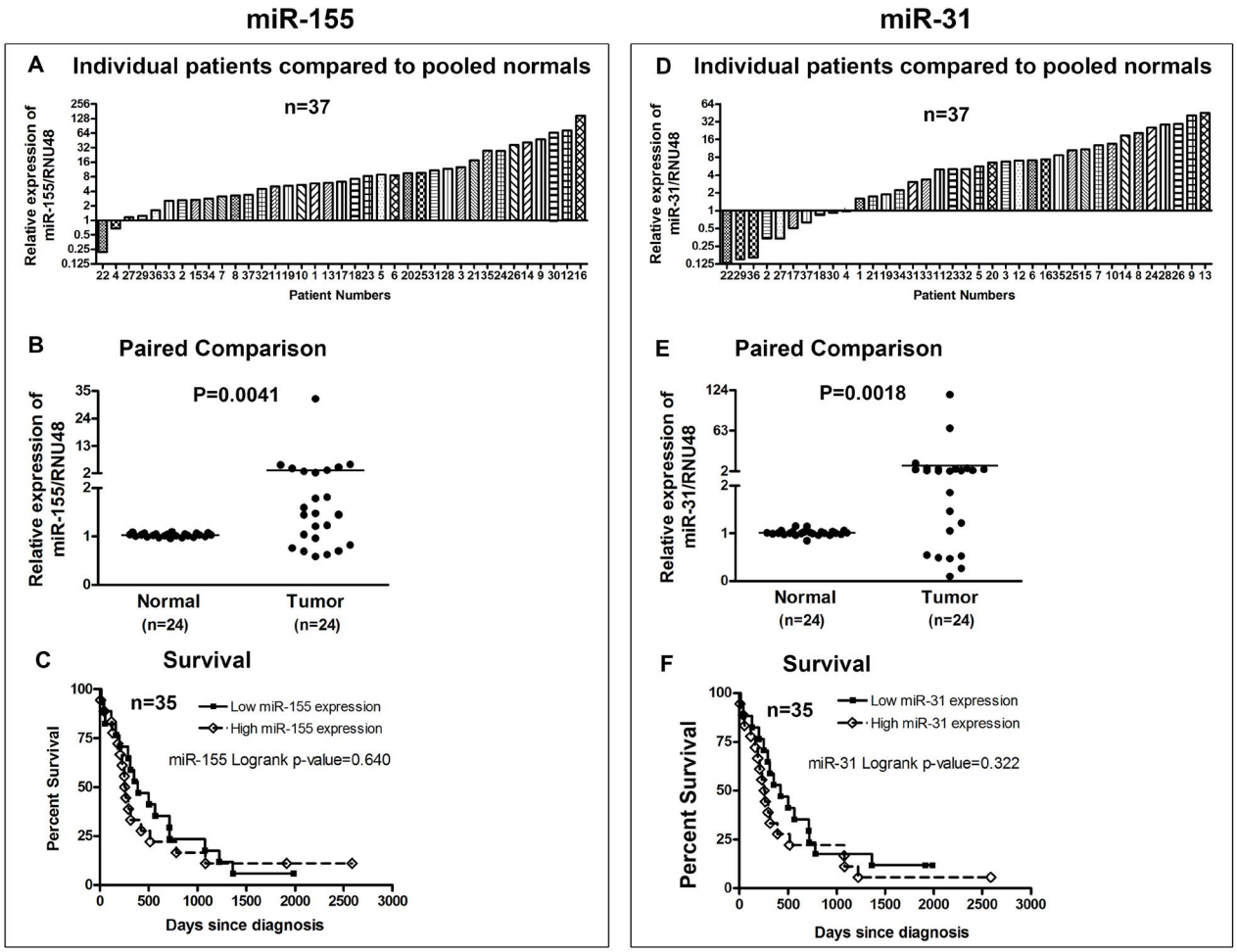

Figure 2: Comparative expression analysis of *miR-155* and *miR-31* in 37 pancreatic cancer patient's tumor specimens compared to 24 pooled normal samples of FFPE tissue blocks (2A&2D), comparative expression analysis in 24 paired samples of FFPE tissue blocks of tumor and normal tissue samples from the same patient (2B&2E) quantitated individually using qRT-PCR. The Kaplan-Meier curve and Log-ranks tests for *miR-155* (2C) and *miR-31* (2F) expression and survival of patients are also presented. There was a significant up-regulation of *miRNA-155* in all except two tumor samples when compared to normal (2A). Similarly, *miR-31* appears to be up-regulated in most of the tumor samples compared to normal samples (2D&2E). The miRNAs expression was normalized using RNU48 miRNA. P values represent comparison between normal and tumor paired samples (2B, 2E) using Wilcoxon matched pairs t-test.

other five miRNAs were chosen (*let-7a, miR-221, miR-181a, miR-935* and *miR-508*) based on our previous research and other investigators findings for further quantification individually in 20 samples in each of the group.

As can be seen in Figure 5A, the plasma samples from CP and PC showed significant up-regulation of *miR-221* when compared to HC. This miRNA is known to be an oncogenic miRNA in PC and other cancers as reported by our group and also by other investigators [7,18-21]. In contrast, *let-7a* was significantly down-regulated in CP and PC plasma samples when compared to HC (Figure 5B) which is consistent with published results, suggesting the global tumor suppressive role of *let-7a* [22-25]. Expression levels were also compared for *miR-155* and *miR-31* between all HC, CP and PC plasma samples, as shown in Figure 5C & 5D. The expression levels of these two miRNAs surprisingly showed overwhelming differences in CP samples compared to HC which were then decreased in PC samples. This interesting finding deserves further in-depth research. When *miR-31* was examined in tumor samples isolated from FFPE tissue samples (Figure 2) and also PC plasma samples (Figure 5D) there appeared to be an overall increase in their expression when compared to controls, suggesting that *miR-31* may serve as oncogenic miRNA and can be measured and detected in a variety of patient samples especially in plasma.

As depicted in Figure 6 (A,B,C and D) the expression levels of *miR-21, miR-181a, miR-935,* and *miR-508* gradually increased from HC to CP to PC, suggesting their roles in tumor progression. It's noteworthy that the differential expression from HC to CP was not significant; however, as we move from CP to PC, the enhancement in their expression became very significant. Further assessment of the expression levels of *miR-21* from FFPE tissue samples (Figure 1 A & 1B) appeared to show overall up-regulation in FFPE tumor samples and also in PC plasma samples (Figure 6A), suggesting that *miR-21* is a global oncogenic miRNA that can be discovered in patient samples, and these results are consistent with our previous findings on plasma samples [17].

Correlation between miRNAs expression in plasma samples and the survival of 20 PC patients

The correlation between survival outcome and the expression of

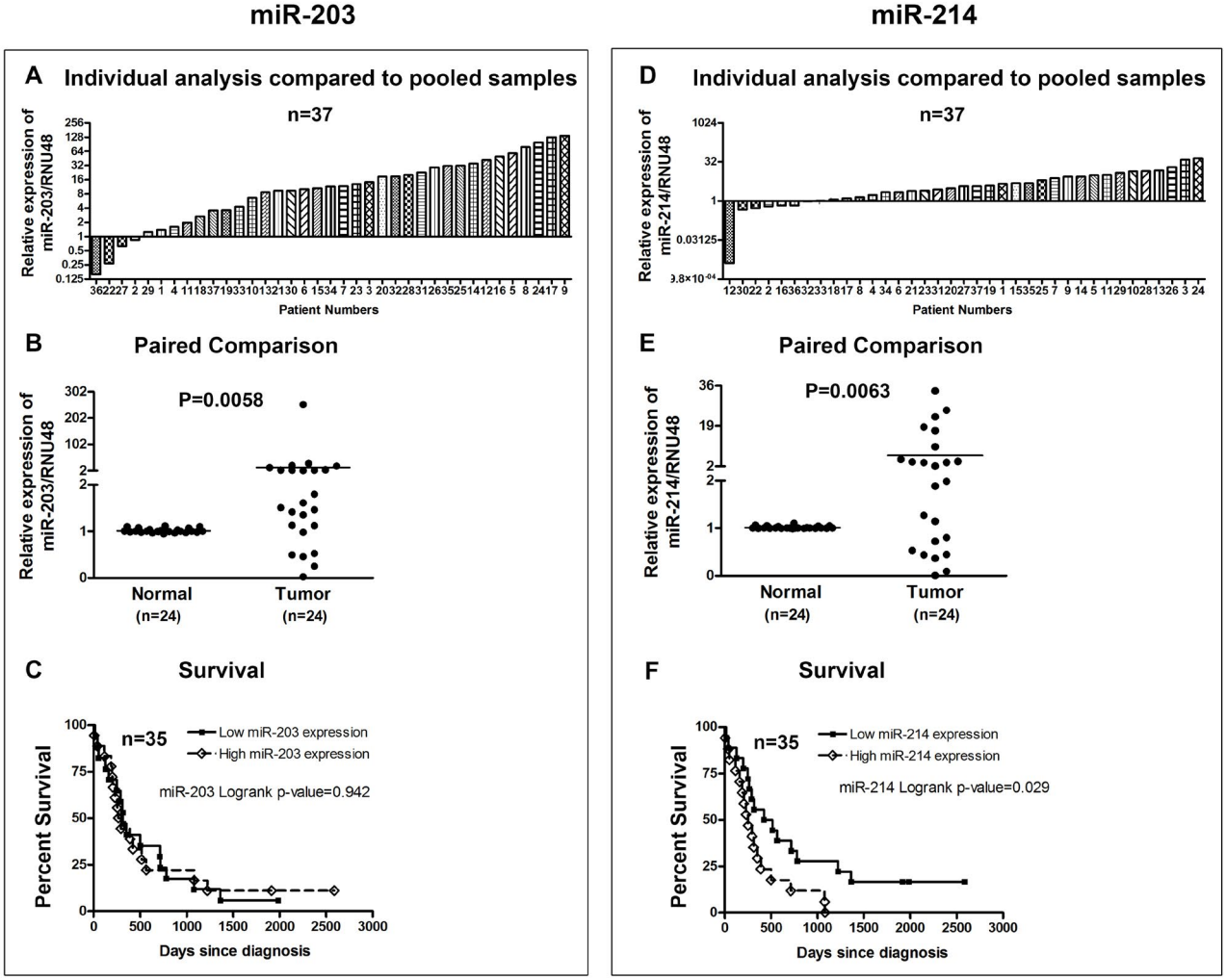

Figure 3: Comparative expression analysis of *miR-203* and *miR-214* in 37 pancreatic cancer patient's tumor specimens compared to 24 pooled normal samples of FFPE tissue blocks (3A&3D), comparative expression analysis in 24 paired samples of FFPE tissue blocks of tumor and normal tissue samples from the same patient (3B&3E) quantitated individually using qRT-PCR. The Kaplan-Meier curve and Log-ranks tests for *miR-203* (3C) and *miR-214* (3F) expression and survival of patients are also presented. There was a significant up-regulation of *miRNA-203* and *miR-214* in all except few tumor samples when compared to normal (A,B,D and E). Furthermore, Kaplan-Meier curve for *miR-214* (3F) demonstrated a significant longer survival of patients with low *miR-214* expression. The miRNAs expression was normalized using RNU48 miRNA. P values represent comparison between normal and tumor paired samples (3B,3E) using Wilcoxon matched pairs t-test.

eight miRNAs from plasma samples was determined by the Kaplan-Meier survival analysis for 20 PC patients by using median miRNA expression value as depicted in supplementary Figure 2. The survival was defined as time from diagnosis to any cause of death. The Log-rank test was used to evaluate the survival difference among the low vs high expressing miRNA relative to the mean expression of each miRNA by qRT-PCR. Based on the sample size limitation in the plasma study, the survival analysis does not claim to provide definitive conclusion on any of the miRNAs that were tested. However, it does aim to draw scientific attention to the possibility of revealing differences in two miRNAs of which one is oncogenic (*miR-508*) and other one is tumor suppressor (*let-7a*), and their correlation with overall survival is presented (Supplementary Figure 2). Although the Log-rank p value was not statistically significant when compared between low and high expression of *miR-508*, a wider curve was observed and the patients with low *miR-508* expression tend to survive longer than the PC patients with high *miR-508* expression. In contrast, *let-7a* higher expression had a wider curve and the patients with high *let-7a* expression tend to survive longer than patients with low *let-7a* expression. No such observation was observed for *miR-221, miR-155, miR-31, miR-21, miR-181a* and *miR-935* (Supplementary Figure 2). Even though our sample size was small our results may facilitate further studies in the determination of which miRNAs may serve as biomarkers and are essential for future development of miRNA-targeted therapies to optimize personalized treatments of PC patients.

Discussion

Despite intensive efforts and innovative discoveries in the development of early stage biomarkers, none has yet been accurately translated in the clinical setting. Due to the lack of early clinical symptoms of the disease, the majority of PC patients are diagnosed very late when the disease diagnosed very late when the disease is either locally advanced or metastatic. Hence, development of early stage biomarkers are crucial for PC patients. Differential expression of

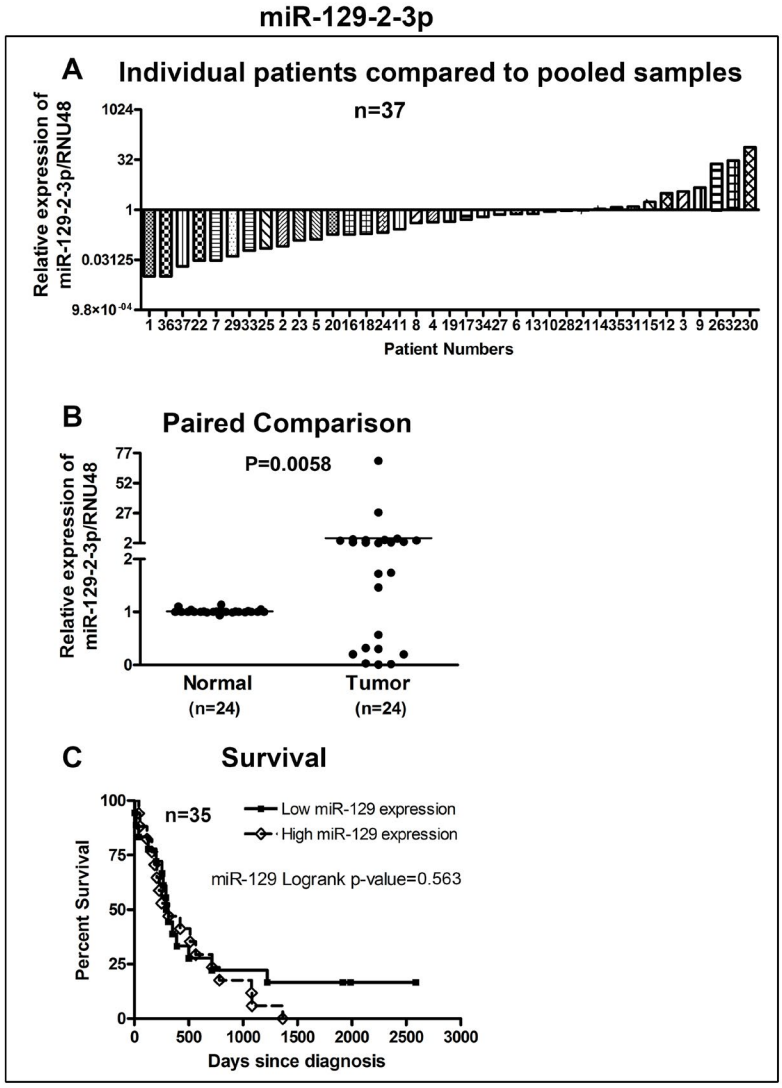

Figure 4: Comparative expression analysis of *miR-129-2-3p* in 37 pancreatic cancer patient's tumor specimens compared to 24 pooled normal samples of FFPE tissue blocks (4A), comparative expression analysis in 24 paired samples of FFPE tissue blocks of tumor and normal tissue samples from the same patient (4B) quantitated individually using qRT-PCR. The Kaplan-Meier curve and Log-ranks tests for *miR-129-2-3p* expression and survival of patients are also presented in 4C. There was a significant down-regulation of *miRNA-129-2-3p* in most of the tumor samples when compared to normal (4A). The miRNAs expression was normalized using RNU48 miRNA. P values represent comparison between normal and tumor paired samples (4B) using Wilcoxon matched pairs *t* test.

specific miRNAs between normal and PC have been studied in recent years showing that it could serve as biomarkers to identify PC patients at an early stage. The miRNAs are known to regulate cell proliferation, differentiation and carcinogenesis for a number of malignancies including PC [2,15].

In this study, we assessed and found comprehensive expression profile of several miRNAs that are altered during tumor progression. We have previously reported the activation of many frequently studied signaling pathways such as NF-κB, and MAP kinase in other malignant diseases [16]. Similarly, we observed the activation of NF-κB, MAP kinase, Pro-inflammatory Cytokines, insulin and EIF2C2 in PC as demonstrated in supplementary Figure 2A and 2B. This result supports the notion that NF-κB activation may be involved in the progression of several malignancies including PC.

Of the several miRNAs that were found to be altered from our profiling results between normal and tumor samples, we chose seven miRNAs for qRT-PCR validation individually in each of the samples. Six miRNAs appear to be up-regulated and one down-regulated in tumor tissue in most of the samples in our cross validation study by qRT-PCR. We also observed a significantly lower expression of *miR-129-2* in many tumor samples which is consistent with another published study [13]. The same published study demonstrated decreased expression of *miR-129-2* in PC tumor samples compared to that of non-tumor samples, which was associated with over expression of SOX4 transcription factor with shorter survival patients diagnosed with PC (13). These authors further demonstrated that re-expression of *miR-129-2* in PC cells by transient transfection had minimal effect on SOX4 expression, suggesting the involvement of other miRNAs along with *miR-129-2* in the regulation of SOX4 [13].

The *miR-21* and *miR-221* has been extensively studied in PC and other cancers [7,19,21,26]. In our current study, we found a significant

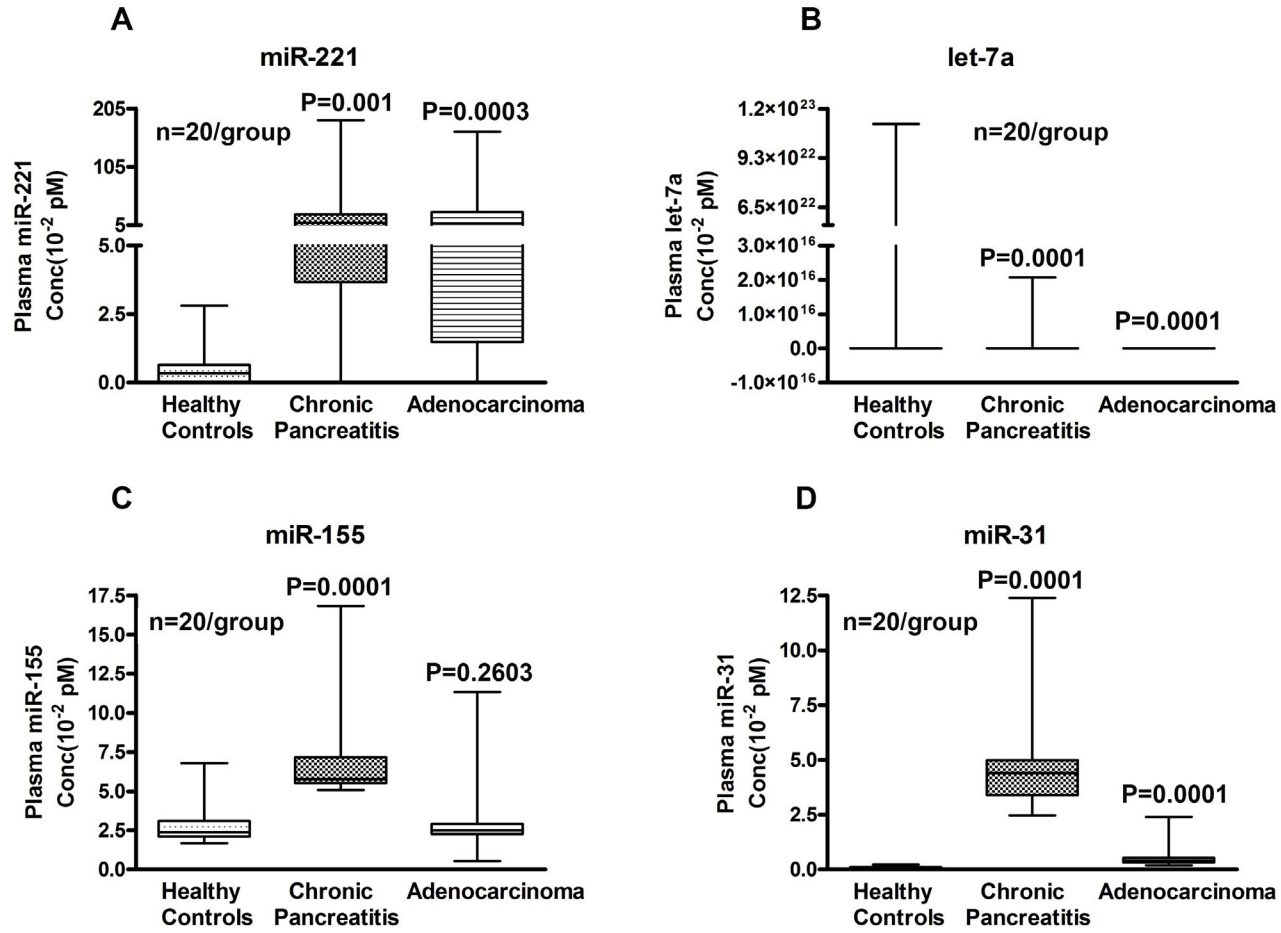

Figure 5: Comparative expression analysis of *miR-221* (5A), *let-7a* (5B), *miR-155* (5C) and *miR-31* (5D) from plasma samples of HC, CP and PC quantitated individually using qRT-PCR (n=20/group). In *miR-221*, there appears to be an increase in the expression in CP and PC compared to HC, suggesting an oncogenic role of *miR-221* (5A). In contrast, the expression of *let-7a* was significantly decreased in PC compared to both HC and CP patient's plasma samples indicating a tumor suppressor role of *let-7a* (5B). Both *miR-155* and *miR-31* expression was mostly found to be up-regulated in CP patient samples followed by PC and primarily lower expression was found in HC plasma (5C&5D). The plasma miRNAs concentration was calculated using the standard miRNA concentration in 10^{-2} pM units using the Quantity value *3.125/6.02/1000. P values represent comparison between HC vs CP and HC vs PC using t-test.

increase in the expression of both *miR-21* and *miR-221* in PC samples. We and others have demonstrated over expression of the above two miRNAs in different cells derived from PC patients such as like stellate cells, cancer-associated fibroblast (CAF) and cancer stem-like cells [7,26]. The inhibition of these two miRNAs by antagomir transfection significantly reduced cell migration, invasion, [7] cell differentiation and downstream gene regulation (26), suggesting that the assessment of these two miRNAs in the plasma could be useful marker of early disease and tumor progression.

One less studied miRNA in PC is *miR-205* [27,28]. We observed a significant increase in the expression of *miR-205* in most of the tumor samples. One study examined *miR-205* expression level in exocrine pancreatic secretions from PC patients and observed a marked increase in the expression of *miR-205* along with *miR-210* compared to non-pancreatic, non-healthy as controls, which was further correlated with decreased overall survival, and differentiated tumors and lymph node metastasis [28].

Other miRNAs, for example *miR-31* and *miR-155* were found to be up-regulated in PC and CP regardless of sample type. The observed up-regulation of *miR-31* and *miR-155* reported by Wang et al in next generation sequencing studies of pancreatic cyst fluid from low grade-benign and high grade-invasive lesions suggested that these miRNAs may serve as early detection biomarkers of PC developing from pancreatic cystic lesions [29]. A meta-review of published studies comparing miRNA expression between PC tissues and neighboring non-cancerous tissues identified 10 miRNAs to be used as diagnostic markers and therapeutic targets [30]. Amongst the 10 miRNAs identified, 7 miRNAs are up-regulated and 3 are down-regulation. Some of the up-regulated miRNAs such as *miR-21, miR-31, miR-221* and *miR-155* are consistent with the findings reported in this study. Not only in PC patient samples but in a comprehensive study involving 16 PC cell lines, *miR-31* was identified as a unique miRNA whose expression level was extremely high in 10 of the 16 PC cell lines. Interestingly, both the inhibition and re-expression of *miR-31* in AsPC-1 PC cell line reduced cell proliferation, migration and invasion associating *miR-31* as an exceptional miRNA whose level is critical for these phenotypes [31]. The expression of *miR-155* when inhibited with antagomir transfection increased the expression of Suppressor of cytokine signaling 1(SOCS1) and simultaneously decreased the invasion and migration of PC cells as

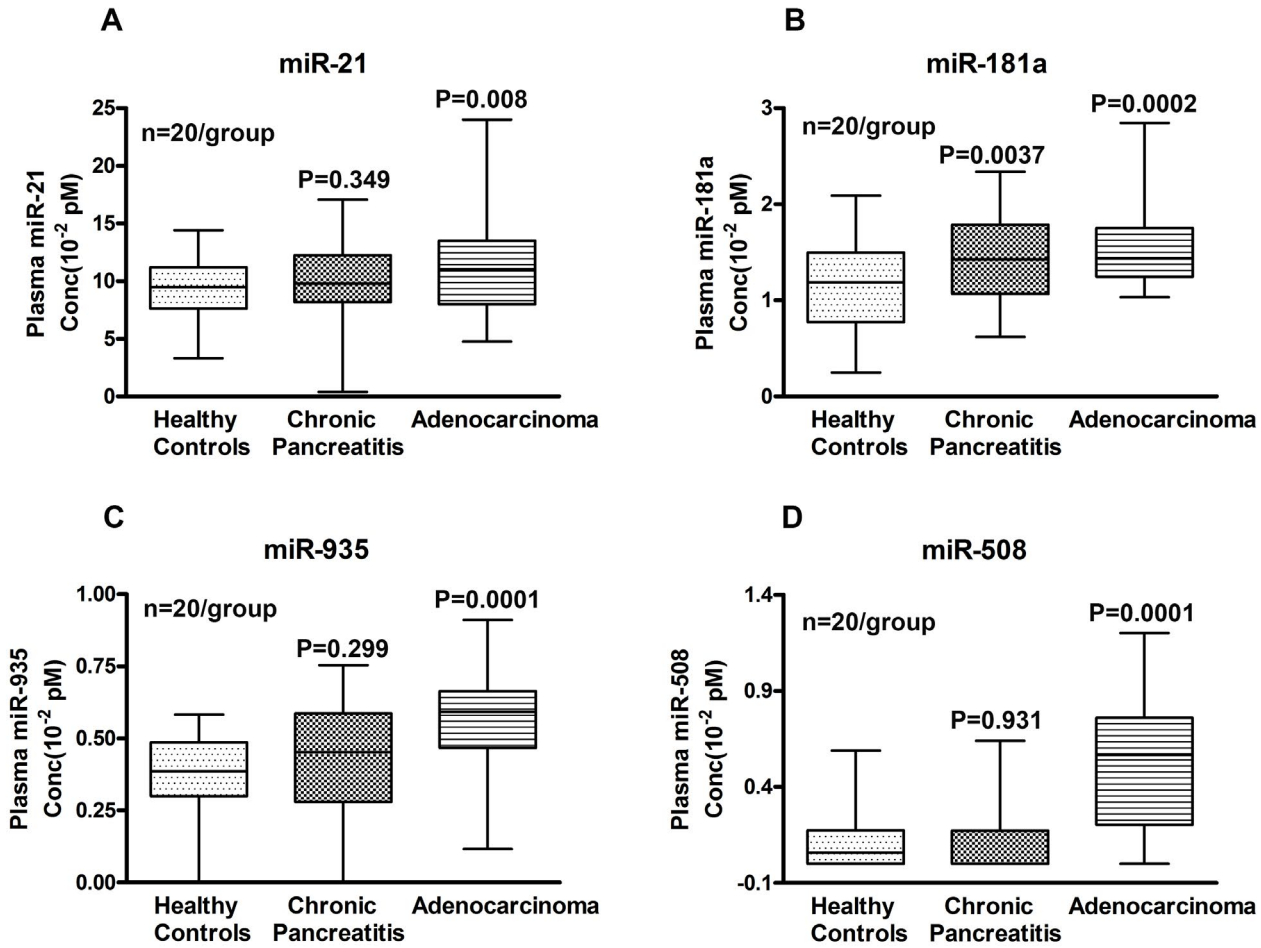

Figure 6: Comparative expression analysis of *miR-21* (6A), *miR-181a* (6B), *miR-935* (6C) and *miR-508* (6D) in plasma samples of HC, CP and PC subjects quantitated individually using qRT-PCR (n=20/group). There appears to be a gradual increase in the expression in CP and PC compared to HC in all four miRNAs as presented in Figure 6. The plasma miRNAs concentration was calculated using the standard miRNA concentration in 10^{-2} pM units using the Quantity value *3.125/6.02/1000. P values represent comparison between HC *vs* CP and HC *vs* PC using t-test.

demonstrated by Huang et al [32]. Previous research has demonstrated *miR-155* as a negative regulator of Mut homologue 1 (MLH1) protein expression, and the over expression of this protein was found to be associated with less lymph node metastasis [33]. The observed up regulation of *miR-155* in tumor samples by the above investigators is consistent with our findings in both the samples types tested.

Another over expressed miRNA was *miR-203*, which showed increased expression in most of the tumor samples. Up regulation of *miR-203* was also detected by Ikenaga et al in FFPE tissue samples of PC compared to CP and normal pancreas using qRT-PCR, which was associated with shorter survival time [34]. Although we also observed over expression of *miR-203* in PC samples there was no change in the survival difference which may be due to limited sample size.

Interestingly, the increased expression of *miR-214* was observed in human heart failure [35]. PTEN, TP53, TWIST1 are some of the representative targets of *miR-214* as highlighted in the same review article. The *miR-214* was distinguished as a bi-functional cardio-miR that plays good and bad roles [35]. Over expression of *miR-214* was also demonstrated in gastric cancer and PTEN expression was negatively regulated through a *miR-214* binding site within the 3'-UTR at the posttranscriptional level [36]. Our current pilot study showed increased expression of *miR-214* in tumor samples which was associated with overall survival, and thus deserves further future study in a larger patient population.

We also have identified over expression of *miR-181a*, *miR-508* and *miR-935* in plasma samples of PC patients. Most notably, *miR-181* was uniquely increased in CP and PC compared to HC. The other two miRNAs (*miR-508* and *miR-935*) were over expressed in PC only compared to CP and HP, suggesting that these two miRNAs are specific for PC. Our findings of increased expression of *miR-181a* in tumor samples were consistent with another study relative to normal samples [4]. In hepatocellular carcinoma (HCC), *miR-181a* was also identified as circulating miRNA that would differentiate HCC patients from chronic liver disease and from normal controls [37]. Interestingly, *miR-181a* was negatively correlated with tumor suppressors PTEN and MAP2K4 expression as proposed by another study in PC [38]. Both previous study and the results of our current study suggesting that *miR-935* appear to be up regulated in PC compared to HC [5]. The expression of *miR-508* was also found to be up regulated in PC plasma samples. When compared to other miRNAs, *miR-508* has been briefly

studied in cancer. Lin et al. observed direct suppression of multiple phosphatases such as PTEN with *miR-508* expression in esophageal squamous cell carcinoma consistent with activated PI3K/Akt signaling pathway [39].

Finally, we found that *let-7a* was significantly down regulated in PC plasma samples comparted to HC and also in CP compared to HC. Although not significant but over expression of *let-7a* in PC plasma samples showed association with overall better survival. Alterations of this miRNA in our study were similar to those described in the literature earlier by our group as well as by other investigators [22,23,40-42]. The aberrant expression of *let-7a* in many types of human cancers including PC indicates its tumor suppressor role [22,24,43,44]. For example, over-expression of *let-7a* with diflourinated-curcumin (CDF) mediated tumor regression with reduced EZH2, Notch-1, and EpCAM expression in PC in our previous study [22]. Moreover, the expression of doublecortin-like kinase 1 (DCLK1) in PC was inhibited by XMD8-92, a kinase inhibitor via up regulation of tumor suppressor miRNA *let-7a* [45].

Although our study had limitations on sample size, the present report contributes to the rising understanding of the role that miRNAs may play in differentiating PC patients from healthy individuals or patients with CP. Our results from both sample resources identified three miRNAs (*miR-21, miR-155, and miR-31*) that were common and presented at similar expression levels in PC compared to normal. The panel of these three miRNAs may facilitate in differentiating PC from HC, and that *miR-214* expression may predict overall survival. In conclusion, we anticipate that plasma miRNA research being non-invasive will have a prevailing clinical consequence for the development of miRNA-based targeted therapies that may lead to a much improved therapeutic outcome in patients diagnosed with PC. Finally, we conclude that our study nicely lays the foundation for detailed future investigations for assessing the role of these microRNAs in the pathology of pancreatic cancer.

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References

1. Siegel R, Ma J, Zou Z, Jemal A (2014) Cancer statistics, 2014. CA Cancer J Clin 64: 9-29.
2. Dillhoff M, Wojcik SE, Bloomston M (2009) MicroRNAs in solid tumors. J Surg Res 154: 349-354.
3. Shimizu Y, Yasui K, Matsueda K, Yanagisawa A, Yamao K (2005) Small carcinoma of the pancreas is curable: new computed tomography finding, pathological study and postoperative results from a single institute. J Gastroenterol Hepatol 20: 1591-1594.
4. Bloomston M, Frankel WL, Petrocca F, Volinia S, Alder H, et al. (2007) MicroRNA expression patterns to differentiate pancreatic adenocarcinoma from normal pancreas and chronic pancreatitis. JAMA 297: 1901-1908.
5. Schultz NA, Dehlendorff C, Jensen BV, Bjerregaard JK, Nielsen KR, et al. (2014) MicroRNA biomarkers in whole blood for detection of pancreatic cancer. JAMA 311: 392-404.
6. Radon TP, Massat NJ, Jones R, Alrawashdeh W, Dumartin L, et al. (2015) Identification of a Three-Biomarker Panel in Urine for Early Detection of Pancreatic Adenocarcinoma. Clin Cancer Res 21: 3512-3521.
7. Ali S, Suresh R, Banerjee S, Bao B, Xu Z, et al. (2015) Contribution of microRNAs in understanding the pancreatic tumor microenvironment involving cancer associated stellate and fibroblast cells. Am J Cancer Res 5: 1251-1264.
8. Frampton AE, Giovannetti E, Jamieson NB, Krell J, Gall TM, et al. (2014) A microRNA meta-signature for pancreatic ductal adenocarcinoma. Expert Rev Mol Diagn 14: 267-271.
9. Hong TH, Park IY2 (2014) MicroRNA expression profiling of diagnostic needle aspirates from surgical pancreatic cancer specimens. Ann Surg Treat Res 87: 290-297.
10. Pang W, Su J,, Wang Y, Feng H, et al. (2015) Pancreatic cancer-secreted miR-155 implicates in the conversion from normal fibroblasts to cancer-associated fibroblasts. Cancer Sci 106: 1362-1369.
11. Vychytilova-Faltejskova P, Kiss I, Klusova S, Hlavsa J, Prochazka V, et al. (2015) MiR-21, miR-34a, miR-198 and miR-217 as diagnostic and prognostic biomarkers for chronic pancreatitis and pancreatic ductal adenocarcinoma. Diagn Pathol 10: 38.
12. Wang P,, Zhu CF, Ma MZ, Chen G, et al. (2015) Micro-RNA-155 is induced by K-Ras oncogenic signal and promotes ROS stress in pancreatic cancer. Oncotarget 6: 21148-21158.
13. Huang HY, Cheng YY, Liao WC, Tien YW, Yang CH, et al. (2012) SOX4 transcriptionally regulates multiple SEMA3/plexin family members and promotes tumor growth in pancreatic cancer. PLoS One 7: e48637.
14. Ali S, Saleh H, Sethi S, Sarkar FH, Philip PA (2012) MicroRNA profiling of diagnostic needle aspirates from patients with pancreatic cancer. Br J Cancer 107: 1354-1360.
15. Sethi S, Ali S, Kong D, Philip PA, Sarkar FH (2013) Clinical implication of microRNAs in molecular pathology. Clin Lab Med 33: 773-786.
16. Suresh R, Sethi S, Ali S, Giorgadze T, Sarkar FH (2015) Differential Expression of MicroRNAs in Papillary Thyroid Carcinoma and Their Role in Racial Disparity. Journal of Cancer Science & Therapy 7: 145-154.
17. Ali S, Almhanna K, Chen W, Philip PA, Sarkar FH (2010) Differentially expressed miRNAs in the plasma may provide a molecular signature for aggressive pancreatic cancer. Am J Transl Res 3: 28-47.
18. Eissa S, Matboli M, Sharawy A, El-Sharkawi F3 (2015) Prognostic and biological significance of microRNA-22 in breast cancer. Gene 574: 163-167.
19. Matsuzaki J, Suzuki H2 (2015) Role of MicroRNAs-221/222 in Digestive Systems. J Clin Med 4: 1566-1577.
20. Sarkar S, Dubaybo H, Ali S, Goncalves P, Kollepara SL, et al. (2013) Down-regulation of miR-22 inhibits proliferation of pancreatic cancer cells through up-regulation of PTEN, p27(kip1), p57(kip2), and PUMA. Am J Cancer Res 3: 465-477.
21. Zhao JJ, Chu ZB, Hu Y, Lin J, Wang Z, et al. (2015) Targeting the miR-221-222/PUMA/BAK/BAX Pathway Abrogates Dexamethasone Resistance in Multiple Myeloma. Cancer Res 75: 4384-4397.
22. Bao B, Ali S, Banerjee S, Wang Z, Logna F, et al. (2012) Curcumin analogue CDF inhibits pancreatic tumor growth by switching on suppressor microRNAs and attenuating EZH2 expression. Cancer Res 72: 335-345.
23. Bhutia YD, Hung SW, Krentz M, Patel D, Lovin D, et al. (2013) Differential processing of let-7a precursors influences RRM2 expression and chemosensitivity in pancreatic cancer: role of LIN-28 and SET oncoprotein. PLoS One 8: e53436.
24. Kelly BD,, Miller N, Sweeney KJ, Durkan GC, et al. (2015) A Circulating MicroRNA Signature as a Biomarker for Prostate Cancer in a High Risk Group. J Clin Med 4: 1369-1379.
25. Wang G, Wang J, Zhao H, Wang J, Tony To SS3 (2015) The role of Myc and let-7a in glioblastoma, glucose metabolism and response to therapy. Arch Biochem Biophys 580: 84-92.
26. Zhao Y, Zhao L, Ischenko I, Bao Q, Schwarz B, et al. (2015) Antisense inhibition of microRNA-2 and microRNA-22 in tumor-initiating stem-like cells modulates tumorigenesis, metastasis, and chemotherapy resistance in pancreatic cancer. Target Oncol.
27. Bera A, VenkataSubbaRao K, Manoharan MS, Hill P, Freeman JW (2014) A miRNA signature of chemoresistant mesenchymal phenotype identifies novel molecular targets associated with advanced pancreatic cancer. PLoS One 9: e106343.
28. Wang J, Raimondo M, Guha S, Chen J, Diao L, et al. (2014) Circulating microRNAs in Pancreatic Juice as Candidate Biomarkers of Pancreatic Cancer. J Cancer 5: 696-705.

29. Wang J, Paris PL, Chen J, Ngo V, Yao H, et al. (2015) Next generation sequencing of pancreatic cyst fluid microRNAs from low grade-benign and high grade-invasive lesions. Cancer Lett 356: 404-409.

30. Ma MZ, Kong X, Weng MZ, Cheng K, Gong W, et al. (2013) Candidate microRNA biomarkers of pancreatic ductal adenocarcinoma: meta-analysis, experimental validation and clinical significance. J Exp Clin Cancer Res 32: 71.

31. Laurila EM, Sandström S, Rantanen LM, Autio R, Kallioniemi A (2012) Both inhibition and enhanced expression of miR-3 lead to reduced migration and invasion of pancreatic cancer cells. Genes Chromosomes Cancer 51: 557-568.

32. Huang C, Li H, Wu W, Jiang T, Qiu Z (2013) Regulation of miR-155 affects pancreatic cancer cell invasiveness and migration by modulating the STAT3 signaling pathway through SOCS1. Oncol Rep 30: 1223-1230.

33. Liu WJ, Zhao YP, Zhang TP, Zhou L, Cui QC, et al. (2013) MLH as a direct target of MiR-155 and a potential predictor of favorable prognosis in pancreatic cancer. J Gastrointest Surg 17: 1399-1405.

34. Ikenaga N, Ohuchida K, Mizumoto K, Yu J, Kayashima T, et al. (2010) MicroRNA-203 expression as a new prognostic marker of pancreatic adenocarcinoma. Ann Surg Oncol 17: 3120-3128.

35. Katoh M (2014) Cardio-miRNAs and onco-miRNAs: circulating miRNA-based diagnostics for non-cancerous and cancerous diseases. Front Cell Dev Biol 2: 61.

36. Yang TS, Yang XH, Wang XD, Wang YL, Zhou B, et al. (2013) MiR-214 regulate gastric cancer cell proliferation, migration and invasion by targeting PTEN. Cancer Cell Int 13: 68.

37. Jiang L, Cheng Q, Zhang BH, Zhang MZ (2015) Circulating microRNAs as biomarkers in hepatocellular carcinoma screening: a validation set from China. Medicine (Baltimore) 94: e603.

38. Liu J, Xu D, Wang Q, Zheng D, Jiang X, et al. (2014) LPS induced miR-181a promotes pancreatic cancer cell migration via targeting PTEN and MAP2K4. Dig Dis Sci 59: 1452-1460.

39. Lin C, Liu A, Zhu J, Zhang X, Wu G, et al. (2014) miR-508 sustains phosphoinositide signalling and promotes aggressive phenotype of oesophageal squamous cell carcinoma. Nat Commun 5: 4620.

40. Bao B, Wang Z, Ali S, Ahmad A, Azmi AS, et al. (2012) Metformin inhibits cell proliferation, migration and invasion by attenuating CSC function mediated by deregulating miRNAs in pancreatic cancer cells. Cancer Prev Res 5: 355-364.

41. Ma J, Fang B, Ma C, Pang H, Zeng F, et al. (2015) [Proanthocyanidins inhibit pancreatic cancer AsPC- cell growth and migration through up-regulation of let-7a]. Nan Fang Yi Ke Da Xue Xue Bao 35: 1110-1115.

42. Oh JS, Kim JJ, Byun JY, Kim IA (2010) Lin28-let7 modulates radiosensitivity of human cancer cells with activation of K-Ras. Int J Radiat Oncol Biol Phys 76: 5-8.

43. Wu J, Li S, Jia W, Deng H, Chen K, et al. (2015) Reduced Let-7a Is Associated with Chemoresistance in Primary Breast Cancer. PLoS One 10: e0133643.

44. Yu L,, Xiong J,, Guo L, et al. (2015) The effects of lanthanum chloride on proliferation and apoptosis of cervical cancer cells: involvement of let-7a and miR-34a microRNAs. Biometals 28: 879-890.

45. Sureban SM, May R, Weygant N, Qu D, Chandrakesan P, et al. (2014) XMD8-92 inhibits pancreatic tumor xenograft growth via a DCLK1-dependent mechanism. Cancer Lett 351: 151-161.