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Original Article

Differentially expressed miRNAs in the plasma may provide a molecular signature for aggressive pancreatic cancer

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Abstract: Pancreatic cancer (PC) has the poorest overall survival rate among all human cancers because of late diagnosis and absence of screening tools. We compared the expression profile of microRNAs (miRNAs) in the plasma of patients diagnosed with PC (n=50) with healthy volunteers (n=10). Data was further validated by quantitative real-time PCR and cell-based assays. Thirty-seven miRNAs were down-regulated and 54 were up-regulated in plasma from patients with PC. The expression of miR-21 was significantly higher, and the expression of let-7 family (especially let-7d) and miR-146a was significantly lower in PC. Most interestingly, the expression of miR-21 was correlated with worse survival, and the expression of let-7 was inversely correlated with survival in this pilot study with mixed patient population. Moreover, we found that miR-21 family was markedly over-expressed in chemo-resistant PC cell lines, which was consistent with the plasma data from PC patients. Our previous studies have shown increased expression of miR-21 with concomitant loss of PTEN expression in PC cells, which is consistent with our current findings showing the loss of three additional targets of miR-21 (PDCD4, Maspin and TPM1). These results suggest that identifying and validating the expression of miRNAs in newly diagnosed patients could serve as potential biomarker for tumor aggressiveness, and such miRNAs could be useful for the screening of high-risk patients, and may also serve as targets for future drug development.

Keywords: Pancreatic cancer, miR-21, miR-221, Drug Resistance, PTEN, let-7d

Introduction

The overall survival of patients diagnosed with pancreatic cancer (PC) is the worst among human malignancies because of lack of tools for its early detection and the aggressive nature of PC compared to other malignancies. PC remains the fourth leading cause of cancer-related deaths in the United States with an estimated 43,140 new cases and 36,800 deaths expected in 2010 [1]. The discovery of effective tools for diagnosing PC in its early stages may significantly improve the survival rates. One strategy would be the development of novel sensitive and non-invasive biomarkers by using plasma samples. Recent gene expression studies identified a set of small number of genes that are differentially expressed in PC [2] but no information is available for assessing their impact on disease prognosis. In recent years there has been a dramatic increase in the discovery of microRNAs (miRNAs) that are associated with cancer aggressiveness as summarized in our recent review articles [3,4,5]. The miRNAs are naturally occurring small non-coding molecules found in human that regulate gene expression and consequently have a potential functional role in a wide array of cellular processes, including differentiation, proliferation, and apoptosis [3,5]. The miRNAs are classified into different families based on location and function in various biological processes. Novel molecular technologies, like miRNAs profiling, have recognized more than 1000 miRNA sequences in the human genome as documented in the miRBase database (release 15; miRBase) [6]. Profiling of miRNAs from human tumors identified a number of miRNAs as either tumor suppressors or

oncogenes depending on whether they specifically target tumor suppressor genes or oncogenes, respectively.

Recent studies confirmed that miRNAs are present in remarkably stable form in blood and as biomarkers could distinguish cancer vs. non-cancer bearing subjects [7,8,9,10]. miRNAs that are dysregulated are referred to as *oncomiR*s and one such *oncomiR* is the miR-21 family that is over-expressed in nine non-hematological cancers including PC [11,12,13,14,15]. We and others have shown a significant correlation between miR-21 expression, tumor growth, and resistance to cytotoxic agents in a variety of malignancies [11,10,15]. Conversely, tumor suppressive miRNAs such as let-7, and miR-146a are usually under-expressed as documented in human lung and PC cell lines [16,17]. A recent study suggested that over-expression of miR-21 is linked to advanced tumor stage and poor survival of breast cancer patients [18]. There is also evidence that miR-21 may have anti-apoptotic properties [13].

Over-expression of miR-21 has been shown to enhance tumor growth in a xenograft model and was found to be regulated by activated androgen receptor in prostate cancer [19]. Moreover, it is believed that over-expression of miR-21 could lead to chemo-resistance in PC [20,21], and these findings are consistent with our published report showing that the down-regulation of miR-21 in PC cells may reverse drug resistance [11]. The findings suggest that the miRNAs in plasma may be useful prognostic or predictive biomarkers that could be useful especially in PC patients that may facilitate the development of novel strategies by which miRNAs could be down-regulated or up-regulated to improve the overall survival.

In this study, we initially determined the expression profiles of miRNAs in pooled plasma samples of 50 PC patients and compared to those from 10 normal healthy volunteers by miRNA microarray profiling technology and further validated by quantitative real-time PCR (RT-PCR) in individual plasma samples. We found 91 differentially expressed miRNAs in the plasma of PC patients compared to healthy controls. Further analysis by quantitative real-time PCR (qRT-PCR) focused on several miRNAs based on our previously published reports showing the biological significance of miR-21, let-7 family, miR-200 family and miR-146a with respect to tumor aggressiveness [11,16,3,5]. Consistent with published literature, we found higher expression of miR-21 and miR-221, and lower expression of let-7b, let-7d, miR-200b, miR-200c, and miR-146a in the plasma of PC patients compared to normal controls, which is consistent with the signature of tumor aggressiveness. The expression of miR-21 was correlated with worse survival, suggesting that over-expression of miR-21 could define biologic tumor behavior. We further tested this hypothesis experimentally by assessing differential expression of miRNAs in two parental and four chemo-resistant human PC cell lines by miRNA profiling and qRT-PCR. We found that miR-21 was significantly up-regulated in chemo-resistant cell lines compared to parental cell lines and that the over-expression of miR-21 was negatively correlated with the expression of its target genes such as PTEN, PDCD4, Maspin and TPM1. Based on these results, we conclude that miR-21 expression may predict tumor aggressiveness and may be exploited for the development of novel strategies by which miR-21 could be down-regulated for the successful treatment of patients diagnosed with PC.

Materials and methods

Collection of plasma

The study subjects consisted of newly diagnosed patients with pancreatic cancer (PC) and for whom we had the survival data. Patients who are newly diagnosed, undergoing surgery, or receiving treatment for PC at the Karmanos Cancer Center were considered eligible for this study. All blood samples were collected prior to any therapeutic procedures, including surgery. We enrolled 76 patients for the current study, and we collected eight milliliters of venous blood in CPT tubes (BD Vacutainer). The plasma was isolated within an hour by centrifugation at 1,500 x g at room temperature for 20 minutes and stored as multiple aliquots in fresh tubes at -80^{o}C. Blood from controls of ten normal healthy individuals with no evidence of any disease were also collected for comparison. Study was approved by the institutional human investigation review board and each subject provided signed informed consent.

Sample preparation and RNA isolation

Fifty PC patients were randomly selected from

the total 76 patients, and their plasma was pooled together along with 10 controls (normal subjects) into two separate groups and was subjected to miRNA microarray profiling to select miRNAs whose expression was differentially expressed in PC patients compared to the normal control subjects. Subsequently, we selected seven miRNAs for further validation in the individual plasma samples using TaqMan probe based qRT-PCR for only those PC patients (n=32) who were treated at Karmanos Cancer Center and for whom we had the survival data. Total RNA containing small RNA was isolated from plasma using Trizol LS reagent (Invitrogen Life Technologies) according to the manufacturer's protocol with the following modifications. The Trizol LS reagent was mixed with 3:1 ratio with plasma and incubated for 5 minutes. After the addition of chloroform, tubes were shaken well and centrifuged to separate the upper aqueous phase which was carefully transferred to a fresh tube. Isopropanol was then added to the aqueous phase for 30 minutes followed by centrifugation at 12,000 x g for 10 minutes. The RNA precipitate was then washed with 75% ethanol and centrifuged at 7,500 for 5 minutes. The RNA pellet was then purified using mirVana miRNA isolation kit (Ambion, Inc.) according to the manufacturer's protocol. Briefly, the pellet was lysed in lysis solution and incubated with 1/10 volume of miRNA homogenate additive for 10 minutes. Equal volume of Acid-Phenol:Chloroform was added to the mixture and vortexed for a minute before centrifugation to separate aqueous phase. About 1.25 volume of 100% ethanol was added to the aqueous phase before being applied directly to the filter cartridge. The RNA was washed with the buffers provided with the kit to remove impurities and eluted in a final volume of 100 µl.

Similarly total RNA was also isolated from human pancreatic cancer cell lines using Trizol as described above. The purified RNA samples from both plasma (normal and patient samples) and cell lines were analyzed by LC Sciences for miRNA microarray profiling (LC Sciences Houston, Tx).

TaqMan miRNA Real-Time Reverse Transcriptase-Polymerase Chain Reaction (RT-PCR)

Quantitative RT-PCR (qRT-PCR) is the standard method for validation of microarray profiling data which provides quantitative analysis of microRNA expression in real time for the miRNAs of interest from the samples analyzed by microarray profiling. To determine the expression of miRNA-21 family (miRNA-21, miR-210, and miR-214) and other oncogenes in six pancreatic cancer cell lines and plasma from normal individuals and PC patient, we used TaqMan MicroRNA Assay kit (Applied Biosystems) following manufacturer's protocol. About 10 ng of RNA from plasma or cells were reverse transcribed using 7 µl of master mix containing dNTPs, reverse transcriptase and RNase inhibitor and 3 µl of respective primer. The mixture was incubated at 16°C for 30 min, 42°C for 60 min, followed by 85°C for 5 minutes. Real-time PCR reactions were then carried out in a total volume of 25 µl reaction mixture containing 1.66 µl of RT product mixed with 1.25 µl Taqman primers, 12.5 µL of 2X Taqman universal PCR master mix, 9.58 µl of water and 1.25 µl of probe. All reactions, including controls were performed in triplicate using Smart Cycler II (Cepheid). Relative expression of miRNAs was analyzed using C_t method and was normalized by miRNA-16 expression for plasma samples and RNU6B expression for cell lines. RT-PCR is a sensitive and reproducible gene expression quantitative technique which is now being used to profile miRNA expression in cells and tissues. With rapid development of technology, detection of miRNA has become more easy, sensitive and credible.

Cells culture, drugs and reagents

Human PC cell lines MIAPaCa-2, and AsPc-1, were chosen for this study. Both the cell lines were exposed to gemcitabine, oxaliplatin or tarceva every other week for six months to create the resistant cell lines. We named these cell lines as MIAPaCa-GR (gemcitabine resistant), AsPc-1OR (oxaliplatin resistant), MIAPaCa-GTR, AsPc-1GTR (gemcitabine and tarceva resistant) based on their exposure to chemo-drugs. The parental cell lines MIAPaCa-2 and AsPc-1 have been tested and authenticated through our core facility; Applied Genomics Technology Center at Wayne State University on March 13, 2009. The method used for testing was short tandem repeat (STR) profiling using the PowerPlex® 16 System from Promega (Madison, WI). The cells were frozen in liquid nitrogen in multiple aliquots and the cells in cultures were used for six months after which new aliquot of frozen cells was used to initiate new culture.

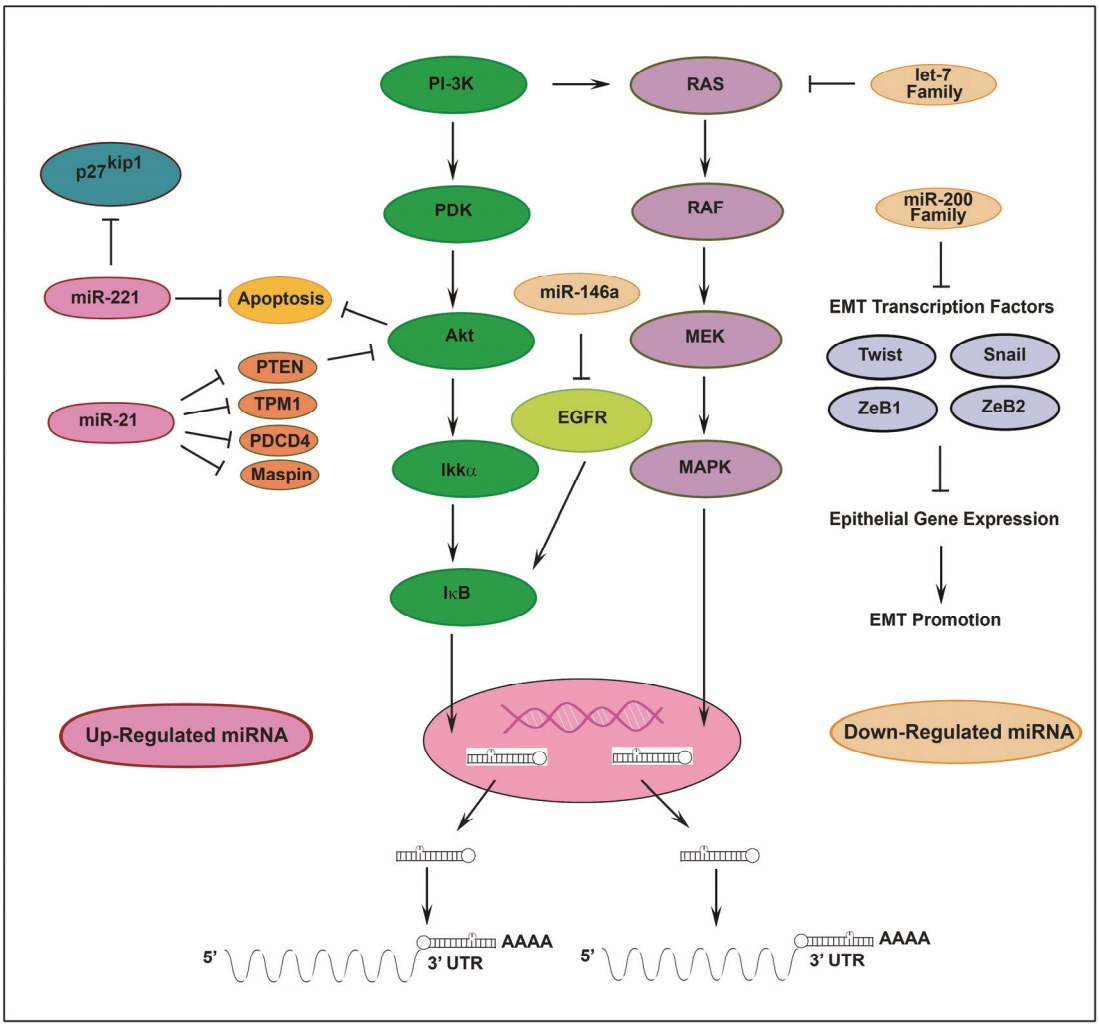

Figure 1. A schematic presentation of microRNAs in pancreatic cancer (PC). Various families of microRNAs are shown as either up-regulated or down-regulated miRNAs with their corresponding targeted genes.

Protein extraction and Western blot analysis

BxPC-3, MIAPaCa-2, MIAPaCa-GR, MIAPaCa-GTR, AsPc-1, AsPc-1OR, AsPc-1GTR cells were used to evaluate the basal level of E-cadherin, vimentin, FEN-1, PTEN, PDCD4, Tropomyosin 1, Maspin, and β-actin expression. Western blot analysis was performed as described previously [22] and the signal intensity was measured using chemiluminescent detection system (Pierce Rockford, IL).

Statistical methods

Expression levels of the seven miRNAs in PC patients and normal subjects were stratified by the median value, and the correlation between survival outcome and miRNA levels was determined by the Kaplan-Meier survival analysis. The correlations among the seven biomarkers were calculated using the Pearson's correlation coefficient. The survival was defined as time from diagnosis to any cause of death. The Regression Tree method was used to find the best threshold for each biomarker's expression with respect to its prognostic value. The Log-rank test was used to evaluate the survival difference among the subgroups defined by marker expression (low vs high) and tumor stage. The Cox model was fit to estimate the prognostic

value of each marker stratified on tumor stage with different baseline hazards. We assumed that all patients received best care. The performance status (ps) was 0 or 1 for all the patients. The statistical significance level was adjusted to 0.01 for multiple testing.

Results

Description of patient study

All 76 patients enrolled in the study were both clinically and pathologically diagnosed for pancreatic cancer (PC). Among the 32 patients who had survival data, one patient (patient ID 62) with ps=3 was removed from the survival analysis. The median age was 57 and gender count was 51.6% male and 48.4% female. Five (16%) patients had ps=0 and 25(84%) had ps=1. Nine had (29%) locally advanced tumor and 22 (71%) had metastatic disease. Only one out of 31 patients was censored. The remaining patients were all followed up to the event of death. The median survival for overall group of 31 patients was 7 months (CI 5.65, 12.3).

Expression profiling of plasma miRNAs characterized 91 miRNAs that were differentially expressed in PC patients

In this study, plasma samples (about 100 µl each) from 50 PC patients and 10 normal (about 500 µl each) healthy individuals were pooled separately. Total RNA was then extracted from both the pooled samples. Plasma miRNA expression profiling revealed 91 miRNAs that were differentially expressed of which 54 were up-regulated and 37 were down-regulated compared to control subjects (**Table 1**). The molecular mechanisms identified with the seven selected miRNAs for subsequent analysis and their corresponding target genes are shown in **Figure 1**. Analysis of miRNA microarray profiles showed that the miR-21 family was significantly up-regulated (**Figure 2A**) whereas let-7 family and miR-146a were significantly down-regulated (**Figure 2B** and **2C**). Real-time qRT-PCR was conducted on 32 patient plasma samples and compared to 10 healthy controls individually to validate the miRNA profiling results by TaqMan miRNA based assay as shown below.

Real-time RT-PCR of seven miRNAs of 32 PC patients and 10 normal controls

Of the seven miRNAs chosen for further analysis, two were oncogenes (miR-21 and miR-221) and five functioned as tumor suppressors (miR-200b, miR-200c, miR-146a, let-7b, and let-7d). The RT-PCR analysis was blinded to the source of the samples. The samples from control and PC patients were tested in parallel to avoid batch effects. The reproducibility of the RT-PCR assay showed that miRNAs can be efficiently extracted from plasma and could be compared across multiple samples. Compared to miRNA levels from controls, the expression levels of miR-21 and miR-221 were increased to more than 10 folds in some patients (**Figure 3A** and 3B) whereas the expression levels of tumor suppressor miRNA such as miR-146a were significantly reduced in most PC patients (**Figure 3C**). Moreover, analysis clearly indicated that the expression levels of miR-200b, miR-200c, let-7b and let-7d were significantly down-regulated in more than 80% of the PC patients compared to normal controls whose expression was set at 1.0 (**Figure 4**). We subsequently assessed the value of miR-21 and miR-221 in defining tumor aggressiveness using drug-resistant PC cell lines compared to parental cells as shown below.

Expression of miR-21 and miR-221 in resistant cell lines was altered by chronic exposure to conventional anti-cancer drugs

MIAPaCa-2 and AsPc-1 cells were continuously exposed to gemcitabine, oxaliplatin or tarceva for a period of six months to create MIAPaCa-GR, MIAPaCa-GTR, AsPc-1OR, AsPc-1GTR cell lines. Profiling of these cell lines revealed miRNAs that were significantly altered and are presented in **Table**s **2** and **3**. We chose miR-21 and miR-221 and compared their expression with their drug treated cells versus the parental cells. The levels of miR-21 and miR-221 were significantly increased in the drug treated cells (**Figure 5A**). We further confirmed this finding with real-time qRT-PCR and included the BxPC-3 cell line that is sensitive to conventional drugs. We found higher expression of miR-21 in MIAPaCa-2, and AsPc-1 cells compared to BxPC-3 cells. Interestingly, when these cells were exposed to chemo-therapeutic drugs described above, highly significant increase in the expression levels of miR-21 was observed in MIAPaCa-GR (2 fold), MIAPaCa-GTR (4 fold), AsPc-1OR (2 fold) and AsPc-1GTR (4 fold). Similarly, the expression of miR-221 in all drug-resistant cell lines was higher compared to drug-sensitive and the parental cell lines; however, the effect was

Table 1. Statistical and clustering analysis of miRNA data between the two groups

			Group 1	Group 2	
			Normal	Patient	Log2 (G2/G1)
No.	Reporter Name	p-value	Mean	Mean	
706	hsa-miR-574-5p	1.93E-07	215	10,953	5.67
431	hsa-miR-32*	2.36E-07	25	961	5.24
67	hsa-miR-1228*	7.71E-07	113	3,535	4.97
4	hsa-let-7b	8.84E-07	827	104	-2.99
254	hsa-miR-188-5p	9.05E-07	348	1,249	1.84
60	hsa-miR-1224-5p	1.26E-06	301	882	1.55
814	hsa-miR-671-5p	1.78E-06	282	1,565	2.47
280	hsa-miR-193b*	1.94E-06	742	37	-4.32
295	hsa-miR-1977	2.35E-06	5,647	1,831	-1.62
833	hsa-miR-765	1.04E-05	425	1,282	1.59
520	hsa-miR-423-5p	1.05E-05	3,260	336	-3.28
6	hsa-let-7c	1.07E-05	599	60	-3.31
110	hsa-miR-1268	1.23E-05	867	3,409	1.97
435	hsa-miR-320d	1.27E-05	2,070	258	-3.01
55	hsa-miR-1207-5p	2.33E-05	137	778	2.51
242	hsa-miR-1826	3.13E-05	2,406	793	-1.60
434	hsa-miR-320c	3.16E-05	3,324	749	-2.15
153	hsa-miR-1305	3.26E-05	8,440	2,878	-1.55
433	hsa-miR-320b	3.45E-05	3,353	746	-2.17
169	hsa-miR-134	4.76E-05	494	1,028	1.06
209	hsa-miR-149*	5.12E-05	854	1,848	1.11
80	hsa-miR-1246	5.42E-05	383	658	0.78
812	hsa-miR-670	1.44E-04	24	454	4.23
211	hsa-miR-150*	2.19E-04	309	609	0.98
731	hsa-miR-595	2.73E-04	23	629	4.75
432	hsa-miR-320a	3.09E-04	2,610	576	-2.18
780	hsa-miR-638	3.51E-04	3,979	8,649	1.12
821	hsa-miR-711	6.67E-04	1,803	162	-3.48
679	hsa-miR-550	9.78E-04	587	58	-3.33
549	hsa-miR-483-5p	1.15E-03	6,038	10,071	0.74
43	hsa-miR-1183	1.18E-03	834	403	-1.05
486	hsa-miR-371-5p	1.41E-03	282	477	0.76
297	hsa-miR-1979	1.50E-03	15,069	10,529	-0.52
831	hsa-miR-762	1.66E-03	480	814	0.76
273	hsa-miR-1915	4.54E-03	996	1,472	0.56
195	hsa-miR-1469	7.94E-03	306	452	0.56
Following transcripts are statistically significant but have low signals (signal < 500)					
746	hsa-miR-610	2.43E-07	14	194	3.75
1	hsa-let-7a	5.71E-07	343	37	-3.20
648	hsa-miR-539	1.30E-06	12	161	3.71
772	hsa-miR-630	2.51E-06	99	370	1.91
761	hsa-miR-623	4.11E-06	22	108	2.29
154	hsa-miR-1306	4.53E-06	21	289	3.77
767	hsa-miR-627	6.60E-06	199	40	-2.33
322	hsa-miR-206	1.10E-05	24	270	3.48
372	hsa-miR-23a*	1.11E-05	57	148	1.36
755	hsa-miR-617	4.28E-05	27	158	2.57
868	hsa-miR-92a	5.32E-05	436	163	-1.42
737	hsa-miR-601	6.43E-05	229	346	0.59
699	hsa-miR-568	1.01E-04	73	21	-1.76
393	hsa-miR-297	3.82E-04	13	90	2.75
174	hsa-miR-136	4.13E-04	6	47	2.90
40	hsa-miR-1180	4.97E-04	53	185	1.81
592	hsa-miR-510	5.08E-04	43	26	-0.75
371	hsa-miR-23a	5.11E-04	164	60	-1.44
17	hsa-let-7i	5.51E-04	102	49	-1.06
318	hsa-miR-205*	5.64E-04	9	63	2.82
744	hsa-miR-608	5.70E-04	22	68	1.64
64	hsa-miR-1226*	6.37E-04	24	123	2.34
270	hsa-miR-1913	6.48E-04	178	123	-0.53
247	hsa-miR-185	1.15E-03	382	33	-3.55
73	hsa-miR-1237	1.18E-03	167	93	-0.84

718	hsa-miR-584	1.80E-03	51	120	1.23
380	hsa-miR-26a	1.98E-03	56	30	-0.87
707	hsa-miR-575	2.12E-03	154	351	1.19
696	hsa-miR-564	2.17E-03	29	69	1.26
95	hsa-miR-125a-3p	2.17E-03	37	64	0.81
290	hsa-miR-1972	2.32E-03	29	113	1.98
329	hsa-miR-21	2.36E-03	60	127	1.08
404	hsa-miR-300	2.76E-03	41	90	1.12
745	hsa-miR-609	2.77E-03	32	73	1.18
298	hsa-miR-198	3.77E-03	236	169	-0.48
196	hsa-miR-146a	3.77E-03	98	24	-2.04
871	hsa-miR-92b	3.96E-03	97	59	-0.71
893	hsa-miR-99b	4.62E-03	39	22	-0.80
518	hsa-miR-422a	4.66E-03	20	44	1.11
597	hsa-miR-513a-5p	5.08E-03	15	59	1.98
42	hsa-miR-1182	6.56E-03	175	382	1.13
540	hsa-miR-451	7.21E-03	61	39	-0.65
8	hsa-let-7d	7.25E-03	129	29	-2.17
90	hsa-miR-1255b	7.35E-03	11	27	1.32
705	hsa-miR-574-3p	7.40E-03	150	75	-1.01
462	hsa-miR-342-3p	7.63E-03	38	19	-0.95
364	hsa-miR-223	8.08E-03	70	21	-1.72
163	hsa-miR-1321	8.32E-03	11	32	1.48
343	hsa-miR-214	8.40E-03	34	169	2.30
392	hsa-miR-296-5p	8.53E-03	143	91	-0.65
260	hsa-miR-1908	8.68E-03	116	135	0.21
554	hsa-miR-486-5p	8.76E-03	229	160	-0.52
178	hsa-miR-138-1*	8.82E-03	36	68	0.94
127	hsa-miR-1281	9.17E-03	186	240	0.37
131	hsa-miR-1285	9.64E-03	26	62	1.29

Table 2. Statistical and clustering analysis of miRNA data between the three groups

			Group 1	Group 2	Group 3
			MIAPaCa-2	MIAPaCa-GR	MIAPaCa-GTR
No.	Reporter Name	p-value	Mean	Mean	Mean
156	hsa-miR-1308	4.32E-10	173	745	569
852	hsa-miR-886-5p	2.82E-09	587	132	90
293	hsa-miR-1975	1.69E-08	1,715	5,918	4,848
247	hsa-miR-185	6.34E-08	291	673	674
273	hsa-miR-1915	8.97E-08	173	651	527
868	hsa-miR-92a	9.62E-08	3,823	1,596	1,754
100	hsa-miR-126	1.10E-07	534	971	902
157	hsa-miR-130a	1.12E-07	351	802	634
432	hsa-miR-320a	1.12E-07	3,847	2,072	2,084
119	hsa-miR-1275	1.16E-07	666	188	291
817	hsa-miR-7	1.38E-07	1,610	565	423
824	hsa-miR-720	1.73E-07	1,134	364	370
433	hsa-miR-320b	1.95E-07	4,053	2,110	2,051
434	hsa-miR-320c	2.27E-07	4,185	2,193	2,141
435	hsa-miR-320d	4.31E-07	3,040	1,599	1,624
126	hsa-miR-1280	5.48E-07	1,175	478	668
355	hsa-miR-22	5.80E-07	236	495	482
223	hsa-miR-15a	6.85E-07	759	1,399	1,806
417	hsa-miR-30a	7.29E-07	912	2,288	2,541
780	hsa-miR-638	9.17E-07	489	853	881
473	hsa-miR-361-5p	1.01E-06	807	1,524	1,608
426	hsa-miR-30e	1.42E-06	420	896	1,169
424	hsa-miR-30d	1.76E-06	552	1,210	1,306
80	hsa-miR-1246	1.86E-06	3,605	8,071	7,683
871	hsa-miR-92b	2.02E-06	1,397	637	720
327	hsa-miR-20b	2.88E-06	2,375	1,239	1,247
521	hsa-miR-424	3.83E-06	250	540	667
385	hsa-miR-27a	4.38E-06	1,538	2,819	3,093
292	hsa-miR-1974	6.60E-06	15,945	9,498	8,684

297	hsa-miR-1979	6.90E-06	6,301	5,431	3,752
419	hsa-miR-30b	8.50E-06	453	854	903
213	hsa-miR-151-5p	1.00E-05	883	1,432	1,313
230	hsa-miR-17	1.04E-05	3,486	2,408	1,865
29	hsa-miR-106a	1.11E-05	3,465	2,424	1,880
360	hsa-miR-221	1.59E-05	5,421	7,191	8,200
325	hsa-miR-20a	2.04E-05	3,911	2,789	2,245
34	hsa-miR-10a	2.32E-05	480	279	200
227	hsa-miR-16	2.43E-05	8,590	13,443	11,781
380	hsa-miR-26a	3.02E-05	2,496	3,740	3,922
10	hsa-let-7e	4.98E-05	2,287	1,338	2,377
329	hsa-miR-21	6.57E-05	16,363	18,437	24,832
387	hsa-miR-27b	8.97E-05	565	950	1,069
523	hsa-miR-425	1.06E-04	335	674	613
890	hsa-miR-98	1.21E-04	492	436	635
283	hsa-miR-195	1.85E-04	221	416	408
399	hsa-miR-29b	2.39E-04	373	569	662
478	hsa-miR-365	2.91E-04	466	164	147
31	hsa-miR-106b	7.19E-04	1,724	2,563	2,211
6	hsa-let-7c	8.52E-04	4,155	3,708	4,967
893	hsa-miR-99b	1.14E-03	730	677	550
33	hsa-miR-107	1.23E-03	1,931	2,816	2,544
492	hsa-miR-374b	1.27E-03	586	645	841
4	hsa-let-7b	1.83E-03	2,002	2,375	2,684
24	hsa-miR-103	1.90E-03	2,130	3,091	2,599
397	hsa-miR-29a	2.11E-03	7,701	11,078	9,797
718	hsa-miR-584	2.21E-03	895	655	617
242	hsa-miR-1826	2.28E-03	19,150	20,015	15,659
17	hsa-let-7i	2.72E-03	9,341	12,161	11,956
418	hsa-miR-30a*	2.89E-03	578	739	853
244	hsa-miR-183	4.36E-03	573	574	456
225	hsa-miR-15b	4.40E-03	4,731	3,494	3,966
125	hsa-miR-128	4.99E-03	558	671	748
239	hsa-miR-182	5.19E-03	1,049	1,367	1,142
264	hsa-miR-191	7.20E-03	1,812	2,120	2,091
421	hsa-miR-30c	7.23E-03	1,699	2,165	2,428
873	hsa-miR-93	7.92E-03	2,239	2,187	1,772
402	hsa-miR-29c	7.98E-03	544	661	776
362	hsa-miR-222	8.28E-03	5,701	5,760	6,566
249	hsa-miR-186	8.85E-03	429	493	382
Following transcripts are statistically significant but have low signals (signal < 500)					
232	hsa-miR-181a	6.03E-09	66	289	177
204	hsa-miR-148a	1.92E-08	33	215	211
741	hsa-miR-605	5.62E-08	8	219	34
255	hsa-miR-18a	2.63E-07	441	281	189
161	hsa-miR-132	1.27E-06	49	90	100
464	hsa-miR-345	1.78E-06	51	137	150
36	hsa-miR-10b	2.03E-06	264	106	81
127	hsa-miR-1281	2.14E-06	20	86	77
405	hsa-miR-301a	2.50E-06	100	209	95
475	hsa-miR-362-5p	2.92E-06	103	185	172
770	hsa-miR-629	3.58E-06	58	109	107
199	hsa-miR-146b-5p	4.32E-06	28	76	73
285	hsa-miR-196a	4.57E-06	42	20	9
275	hsa-miR-192	4.94E-06	122	319	232
214	hsa-miR-152	5.89E-06	177	327	284
647	hsa-miR-532-5p	6.80E-06	186	389	381
803	hsa-miR-660	9.13E-06	153	310	330
22	hsa-miR-101	1.02E-05	125	272	272
110	hsa-miR-1268	1.12E-05	191	89	86
159	hsa-miR-130b	1.29E-05	203	368	283
195	hsa-miR-1469	1.80E-05	88	197	183
707	hsa-miR-575	1.82E-05	26	64	51
137	hsa-miR-1290	1.85E-05	30	103	95
737	hsa-miR-601	1.92E-05	47	29	21
851	hsa-miR-886-3p	2.01E-05	231	99	77

257	hsa-miR-18b	2.08E-05	171	126	87
281	hsa-miR-194	2.32E-05	105	225	216
176	hsa-miR-137	2.54E-05	93	43	43
814	hsa-miR-671-5p	5.15E-05	186	104	88
439	hsa-miR-324-5p	5.50E-05	72	139	135
356	hsa-miR-22*	5.93E-05	90	183	213
117	hsa-miR-1274a	6.22E-05	91	62	61
291	hsa-miR-1973	6.79E-05	33	102	78
196	hsa-miR-146a	8.06E-05	11	35	11
9	hsa-let-7d*	8.26E-05	312	167	180
576	hsa-miR-500*	8.41E-05	87	126	130
518	hsa-miR-422a	9.15E-05	106	48	71
474	hsa-miR-362-3p	9.80E-05	32	62	68
764	hsa-miR-625	1.19E-04	14	24	35
546	hsa-miR-455-3p	1.45E-04	90	189	140
472	hsa-miR-361-3p	1.52E-04	26	47	47
829	hsa-miR-760	1.53E-04	107	67	41
26	hsa-miR-103-as	1.61E-04	44	73	76
847	hsa-miR-877	1.86E-04	109	51	50
554	hsa-miR-486-5p	1.89E-04	235	114	177
771	hsa-miR-629*	3.80E-04	24	52	47
553	hsa-miR-486-3p	4.53E-04	97	57	109
831	hsa-miR-762	4.92E-04	180	271	339
149	hsa-miR-1301	5.00E-04	60	114	81
778	hsa-miR-636	5.15E-04	20	17	35
537	hsa-miR-450a	5.94E-04	44	63	99
236	hsa-miR-181c	6.10E-04	24	55	37
882	hsa-miR-940	6.14E-04	105	112	181
879	hsa-miR-937	6.24E-04	19	30	34
289	hsa-miR-197	6.38E-04	149	92	100
254	hsa-miR-188-5p	7.43E-04	69	34	35
182	hsa-miR-140-3p	7.62E-04	124	197	171
834	hsa-miR-766	7.77E-04	20	45	51
83	hsa-miR-1249	7.99E-04	117	44	131
878	hsa-miR-936	8.40E-04	36	13	20
724	hsa-miR-589*	8.64E-04	9	27	23
511	hsa-miR-409-3p	1.03E-03	13	37	25
470	hsa-miR-34c-3p	1.03E-03	16	42	35
646	hsa-miR-532-3p	1.12E-03	139	183	256
583	hsa-miR-505	1.17E-03	53	71	88
706	hsa-miR-574-5p	1.28E-03	354	236	211
575	hsa-miR-500	1.30E-03	75	94	108
806	hsa-miR-663	1.40E-03	98	139	209
183	hsa-miR-140-5p	1.57E-03	38	52	60
160	hsa-miR-130b*	1.76E-03	85	58	69
677	hsa-miR-548q	2.00E-03	27	43	34
73	hsa-miR-1237	2.53E-03	61	37	90
888	hsa-miR-96	2.82E-03	66	90	97
209	hsa-miR-149*	2.91E-03	99	139	166
794	hsa-miR-652	3.29E-03	46	64	52
490	hsa-miR-374a	3.40E-03	449	291	349
88	hsa-miR-1254	3.52E-03	66	14	15
877	hsa-miR-935	3.76E-03	31	17	27
172	hsa-miR-135b	4.12E-03	87	47	54
881	hsa-miR-939	4.15E-03	43	24	26
567	hsa-miR-494	4.67E-03	20	38	51
579	hsa-miR-502-3p	4.91E-03	79	98	119
48	hsa-miR-1201	4.92E-03	32	30	17
74	hsa-miR-1238	5.15E-03	40	28	68
446	hsa-miR-331-3p	5.30E-03	81	136	135
822	hsa-miR-718	5.52E-03	29	36	59
449	hsa-miR-335*	5.55E-03	9	32	14
238	hsa-miR-181d	5.97E-03	78	90	60
651	hsa-miR-542-3p	6.70E-03	31	50	71
208	hsa-miR-149	6.86E-03	44	55	89
206	hsa-miR-148b	7.01E-03	294	377	380
270	hsa-miR-1913	7.25E-03	65	44	130

524	hsa-miR-425*	7.49E-03	19	31	35
496	hsa-miR-376a*	7.57E-03	4	22	0
887	hsa-miR-95	7.61E-03	12	17	30
245	hsa-miR-183*	7.69E-03	55	47	34
699	hsa-miR-568	7.90E-03	19	21	10
16	hsa-let-7g*	7.92E-03	26	13	20
372	hsa-miR-23a*	9.40E-03	38	22	23
377	hsa-miR-24-2*	9.41E-03	51	71	75

Table 3. Statistical and clustering analysis of miRNA data between the three groups

			Group 1	Group 2	Group 3
			AsPC-1	AsPC-1-OR	AsPC-1-GTR
No.	Reporter Name	p-value	Mean	Mean	Mean
196	hsa-miR-146a	5.42E-14	50	803	1,163
156	hsa-miR-1308	3.23E-09	541	166	996
232	hsa-miR-181a	7.36E-09	581	260	853
293	hsa-miR-1975	1.30E-08	1,209	1,148	2,965
311	hsa-miR-200c	6.91E-08	488	778	1,486
17	hsa-let-7i	1.69E-07	6,313	2,572	4,862
295	hsa-miR-1977	2.43E-07	494	513	1,125
273	hsa-miR-1915	2.75E-07	396	360	880
385	hsa-miR-27a	4.55E-07	2,159	2,484	3,886
399	hsa-miR-29b	5.84E-07	395	319	695
285	hsa-miR-196a	8.73E-07	465	533	323
29	hsa-miR-106a	9.65E-07	5,619	3,240	2,793
891	hsa-miR-99a	1.05E-06	472	800	417
435	hsa-miR-320d	1.21E-06	1,891	1,185	1,105
817	hsa-miR-7	1.27E-06	1,792	1,619	703
297	hsa-miR-1979	1.63E-06	2,708	1,505	2,623
868	hsa-miR-92a	1.71E-06	4,903	3,497	2,902
80	hsa-miR-1246	1.77E-06	493	1,264	1,504
824	hsa-miR-720	1.79E-06	625	1,023	424
230	hsa-miR-17	1.86E-06	5,860	3,254	2,916
718	hsa-miR-584	2.02E-06	550	446	227
871	hsa-miR-92b	2.32E-06	2,070	1,450	1,197
433	hsa-miR-320b	3.09E-06	2,516	1,632	1,408
10	hsa-let-7e	3.94E-06	3,747	4,542	2,438
434	hsa-miR-320c	4.03E-06	2,578	1,697	1,445
387	hsa-miR-27b	4.85E-06	1,553	1,521	2,065
20	hsa-miR-100	5.33E-06	1,269	2,118	1,270
893	hsa-miR-99b	5.83E-06	715	732	410
235	hsa-miR-181b	6.36E-06	906	516	663
292	hsa-miR-1974	7.10E-06	3,357	5,102	5,715
426	hsa-miR-30e	7.56E-06	389	318	778
325	hsa-miR-20a	8.05E-06	6,502	4,076	3,463
242	hsa-miR-1826	8.17E-06	11,001	9,759	15,410
780	hsa-miR-638	1.17E-05	1,219	1,192	2,307
304	hsa-miR-19b	1.60E-05	2,040	1,031	1,023
397	hsa-miR-29a	1.91E-05	8,567	9,222	14,033
383	hsa-miR-26b	1.96E-05	2,074	2,817	1,573
97	hsa-miR-125b	2.13E-05	1,382	2,274	1,816
360	hsa-miR-221	2.25E-05	2,869	2,202	3,833
706	hsa-miR-574-5p	2.33E-05	190	454	159
432	hsa-miR-320a	2.73E-05	2,292	1,598	1,350
366	hsa-miR-224	2.93E-05	1,379	1,578	997
327	hsa-miR-20b	3.15E-05	4,047	2,600	1,967
225	hsa-miR-15b	3.27E-05	1,666	2,408	1,390
873	hsa-miR-93	3.71E-05	1,096	784	889
307	hsa-miR-200a	3.75E-05	502	334	694
302	hsa-miR-19a	6.86E-05	573	321	328
424	hsa-miR-30d	7.13E-05	763	692	1,263
1	hsa-let-7a	1.23E-04	12,377	16,225	11,303
4	hsa-let-7b	2.08E-04	5,034	6,342	3,822
281	hsa-miR-194	2.58E-04	4,993	6,724	8,032
264	hsa-miR-191	3.31E-04	1,079	1,537	1,425

473	hsa-miR-361-5p	5.53E-04	420	482	363
419	hsa-miR-30b	5.61E-04	1,304	2,092	2,015
8	hsa-let-7d	5.88E-04	7,252	9,036	6,765
421	hsa-miR-30c	8.23E-04	1,100	1,416	1,613
96	hsa-miR-125a-5p	1.83E-03	2,796	2,369	2,049
6	hsa-let-7c	2.14E-03	9,427	11,381	8,282
126	hsa-miR-1280	2.61E-03	1,413	1,554	1,039
239	hsa-miR-182	2.86E-03	987	1,303	1,076
223	hsa-miR-15a	4.42E-03	847	691	857
31	hsa-miR-106b	4.74E-03	867	726	936
212	hsa-miR-151-3p	6.59E-03	776	653	595
Following transcripts are statistically significant but have low signals (signal < 500)					
199	hsa-miR-146b-5p	2.61E-10	34	118	146
221	hsa-miR-155	4.46E-09	15	63	54
184	hsa-miR-141	1.16E-08	39	45	276
119	hsa-miR-1275	2.63E-08	206	385	115
195	hsa-miR-1469	3.37E-08	189	404	228
478	hsa-miR-365	6.99E-08	346	91	117
761	hsa-miR-623	7.19E-08	19	59	16
888	hsa-miR-96	1.10E-07	70	74	143
22	hsa-miR-101	1.63E-07	89	57	139
296	hsa-miR-1978	1.69E-07	235	178	387
255	hsa-miR-18a	2.22E-07	265	172	94
546	hsa-miR-455-3p	2.22E-07	146	263	91
490	hsa-miR-374a	2.66E-07	338	427	167
544	hsa-miR-454	4.63E-07	397	447	223
472	hsa-miR-361-3p	6.88E-07	35	20	53
278	hsa-miR-193a-5p	7.21E-07	250	129	83
460	hsa-miR-340	1.06E-06	68	33	43
677	hsa-miR-548q	1.11E-06	52	91	94
214	hsa-miR-152	1.45E-06	93	35	53
741	hsa-miR-605	1.65E-06	104	10	22
209	hsa-miR-149*	1.68E-06	155	162	351
567	hsa-miR-494	1.75E-06	33	18	106
102	hsa-miR-1260	1.89E-06	107	105	46
254	hsa-miR-188-5p	1.96E-06	43	87	27
521	hsa-miR-424	2.08E-06	189	189	357
832	hsa-miR-764	2.35E-06	47	12	17
707	hsa-miR-575	2.39E-06	57	55	172
814	hsa-miR-671-5p	4.28E-06	113	237	88
890	hsa-miR-98	4.67E-06	270	334	161
492	hsa-miR-374b	5.03E-06	302	404	235
349	hsa-miR-218	5.09E-06	5	43	69
260	hsa-miR-1908	6.42E-06	47	32	111
160	hsa-miR-130b*	6.67E-06	59	38	23
110	hsa-miR-1268	6.72E-06	108	180	79
257	hsa-miR-18b	7.32E-06	105	72	54
852	hsa-miR-886-5p	7.70E-06	91	98	185
696	hsa-miR-564	9.49E-06	16	49	22
572	hsa-miR-498	1.01E-05	31	76	27
236	hsa-miR-181c	1.18E-05	172	47	282
211	hsa-miR-150*	1.25E-05	42	81	43
249	hsa-miR-186	1.30E-05	195	103	127
392	hsa-miR-296-5p	1.44E-05	41	81	37
100	hsa-miR-126	2.04E-05	325	203	187
764	hsa-miR-625	2.35E-05	49	54	93
784	hsa-miR-642	2.90E-05	98	34	53
523	hsa-miR-425	3.77E-05	162	191	295
287	hsa-miR-196b	4.20E-05	93	46	68
486	hsa-miR-371-5p	4.64E-05	28	53	28
244	hsa-miR-183	4.94E-05	211	313	195
56	hsa-miR-1208	5.11E-05	27	62	39
716	hsa-miR-582-5p	6.62E-05	147	106	81
118	hsa-miR-1274b	7.89E-05	57	86	82
517	hsa-miR-421	8.12E-05	37	58	23
501	hsa-miR-378	9.22E-05	203	114	208

418	hsa-miR-30a*	1.34E-04	210	348	189
238	hsa-miR-181d	1.64E-04	368	228	254
276	hsa-miR-192*	1.77E-04	53	57	93
881	hsa-miR-939	1.90E-04	50	78	41
60	hsa-miR-1224-5p	1.97E-04	40	77	45
446	hsa-miR-331-3p	1.99E-04	97	64	120
806	hsa-miR-663	3.08E-04	186	135	260
518	hsa-miR-422a	3.20E-04	134	72	147
554	hsa-miR-486-5p	3.49E-04	184	138	105
289	hsa-miR-197	4.43E-04	72	43	42
125	hsa-miR-128	4.75E-04	206	174	129
820	hsa-miR-7-1*	6.23E-04	117	118	74
388	hsa-miR-27b*	6.32E-04	48	41	29
169	hsa-miR-134	6.43E-04	19	42	14
14	hsa-let-7f-2*	6.53E-04	35	19	14
331	hsa-miR-210	6.65E-04	33	43	56
151	hsa-miR-1303	6.83E-04	35	37	19
502	hsa-miR-378*	7.01E-04	22	16	28
869	hsa-miR-92a-1*	8.47E-04	48	32	23
475	hsa-miR-362-5p	8.99E-04	36	24	39
520	hsa-miR-423-5p	9.19E-04	249	319	185
182	hsa-miR-140-3p	9.31E-04	151	200	238
400	hsa-miR-29b-1*	9.53E-04	114	141	89
149	hsa-miR-1301	9.99E-04	39	25	23
688	hsa-miR-556-3p	1.19E-03	40	45	28
35	hsa-miR-10a*	1.32E-03	138	97	144
579	hsa-miR-502-3p	1.38E-03	28	14	35
280	hsa-miR-193b*	1.54E-03	71	48	25
703	hsa-miR-572	1.54E-03	31	44	54
66	hsa-miR-1228	1.62E-03	63	32	36
2	hsa-let-7a*	1.64E-03	42	28	23
9	hsa-let-7d*	1.72E-03	82	94	59
429	hsa-miR-31*	1.79E-03	37	52	47
83	hsa-miR-1249	1.84E-03	70	122	70
771	hsa-miR-629*	1.92E-03	51	29	31
803	hsa-miR-660	1.93E-03	54	34	54
831	hsa-miR-762	2.01E-03	207	273	297
851	hsa-miR-886-3p	2.22E-03	37	39	59
427	hsa-miR-30e*	2.27E-03	233	288	179
206	hsa-miR-148b	2.55E-03	213	134	175
157	hsa-miR-130a	2.76E-03	38	27	22
69	hsa-miR-1231	2.92E-03	37	37	70
511	hsa-miR-409-3p	3.35E-03	32	15	18
277	hsa-miR-193a-3p	3.40E-03	27	11	22
797	hsa-miR-654-5p	3.72E-03	43	18	53
322	hsa-miR-206	4.31E-03	10	34	9
291	hsa-miR-1973	4.57E-03	12	8	28
270	hsa-miR-1913	4.68E-03	61	95	53
5	hsa-let-7b*	4.96E-03	67	53	46
576	hsa-miR-500*	5.37E-03	29	21	36
172	hsa-miR-135b	5.43E-03	208	194	275
553	hsa-miR-486-3p	5.58E-03	77	84	56
117	hsa-miR-1274a	6.19E-03	29	29	48
882	hsa-miR-940	6.27E-03	95	151	93
448	hsa-miR-335	6.28E-03	97	99	133
229	hsa-miR-16-2*	6.46E-03	118	105	137
449	hsa-miR-335*	6.54E-03	72	51	45
183	hsa-miR-140-5p	6.61E-03	53	39	68
161	hsa-miR-132	7.00E-03	32	44	49
847	hsa-miR-877	7.23E-03	35	47	34
541	hsa-miR-452	7.56E-03	182	173	134
389	hsa-miR-28-3p	7.84E-03	101	85	85
241	hsa-miR-1825	8.22E-03	44	22	27
550	hsa-miR-484	8.70E-03	91	58	64
368	hsa-miR-2276	9.29E-03	14	29	15
40	hsa-miR-1180	9.34E-03	41	37	24

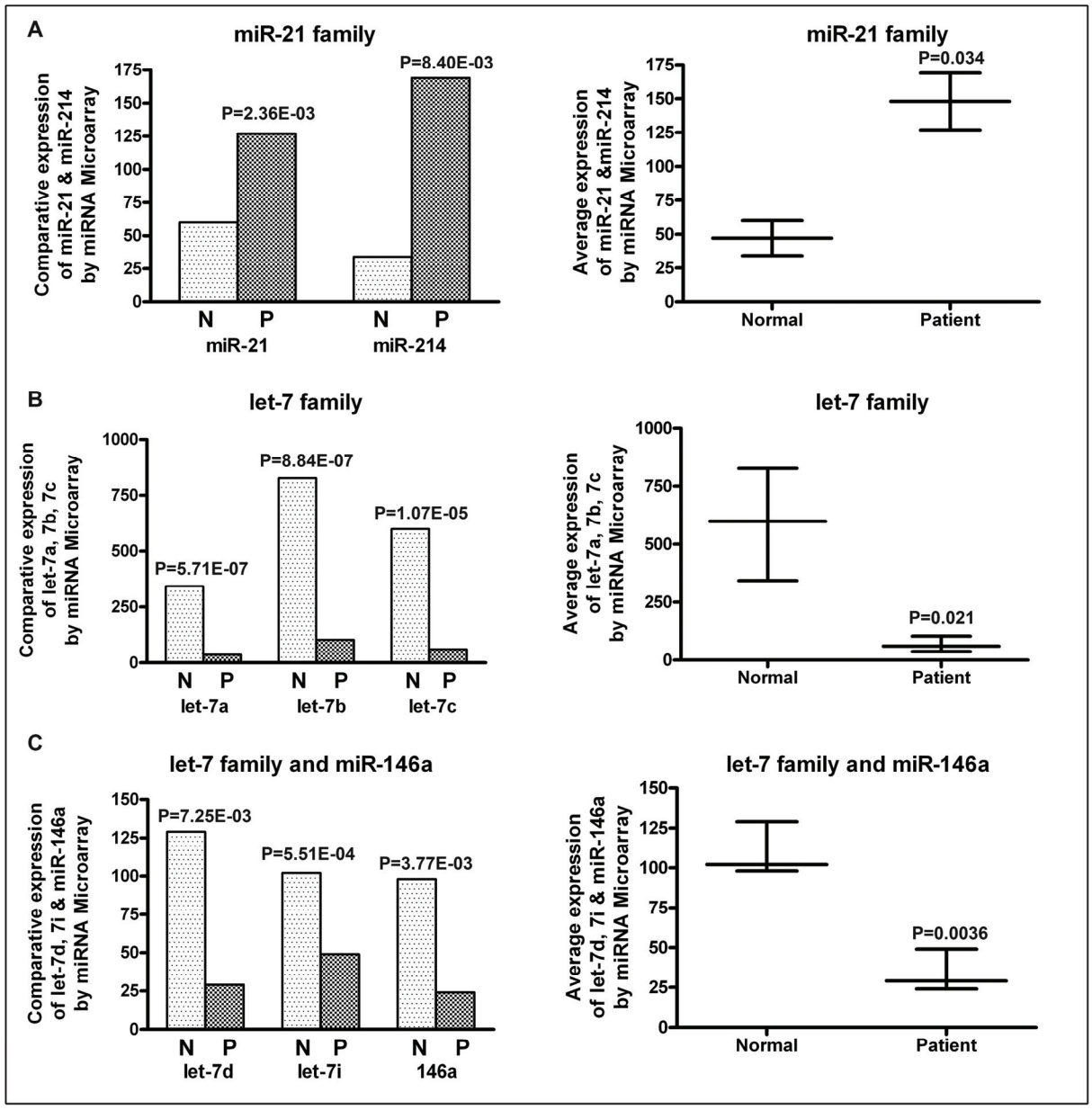

Figure 2. Comparative expression analysis of differentially expressed miRNAs by microarray profiling in plasma from PC patients and healthy controls. (A) The expression of miR-21 and miR-214 (left panel) and the miR-21 family (right panel), (B) The expression of let-7a, let-7b and let-7c (left panel) and the let-7 family (right panel), (C) The expression of let-7d, let-7i, miR-146a (left panel) and combined let-7 family and miR-146a (right panel). There was a significant up-regulation of miR-21 family in PC patients compared to normal subjects. Conversely, let-7 family and miR-146a expression showed a significant down-regulation compared to normal subjects.

more pronounced in MIAPaCa-GTR and AsPc-1GTR cell lines compared to MIAPaCa-GR and AsPc-1OR (**Figure 5B**). These results confirmed that drug resistance could be mechanistically linked with increased expression of miR-21 and may be explained on the basis of loss of PTEN, PDCD4, Maspin, and TPM1 gene expression that are known targets of miR-21. To further validate this notion, we extracted total proteins from all the seven PC cell lines and measured the basal level of expression of E-cadherin, vimentin, FEN-1, PTEN, PDCD4, Maspin, and

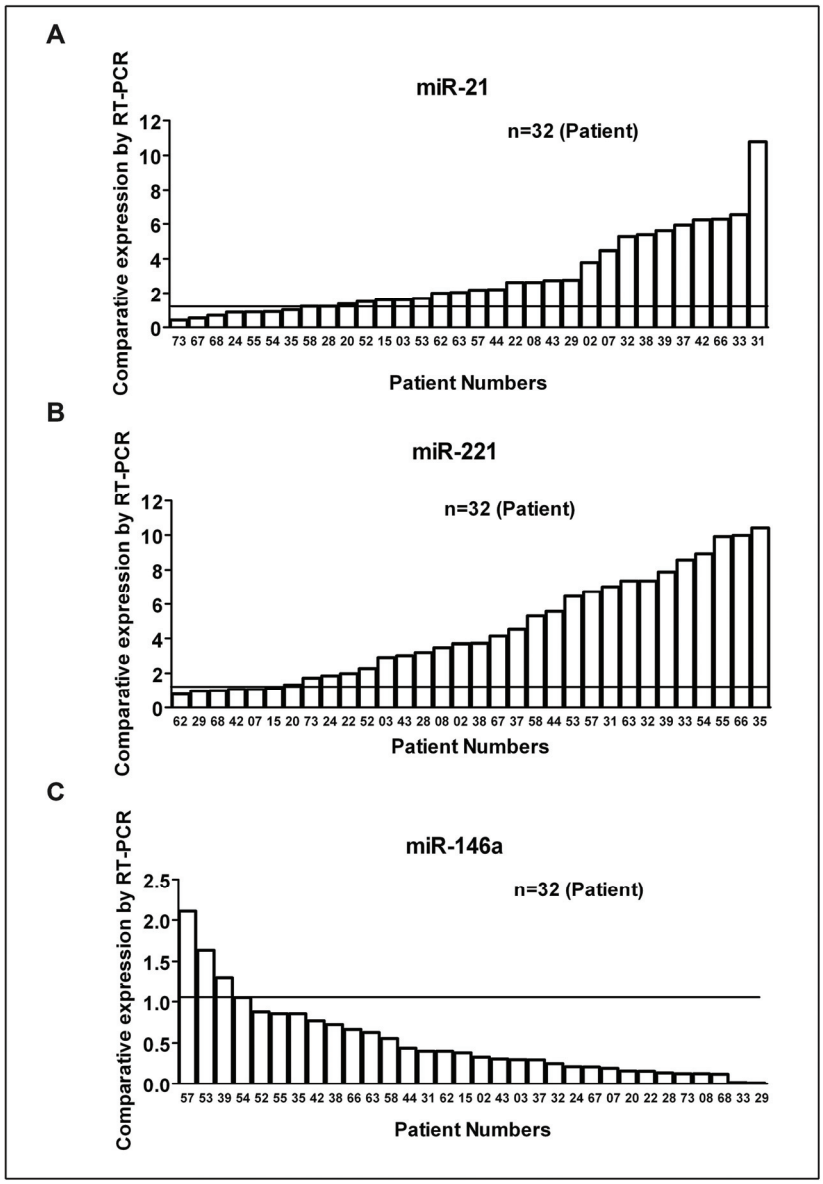

Figure 3. Comparative expression analysis of miRNAs (miR-21, miR-221 and miR-146a) in the plasma of 32 PC patients analyzed individually compared to plasma samples obtained from 10 normal subjects by using qRT-PCR. The line drawn at 1.0 represents average of normal subjects (n=10). The results showed a significant increase in the expression of miR-21 and miR-221 (oncogene) over the cutoff of 1.0 by almost all PC patients. In contrast, a significant down-regulation of miR-146a (tumor suppressor gene) was observed in PC patients compared to normal subjects.

TPM1 (**Figure 5C**). The level of expression of PTEN, PDCD4, Maspin, and TPM1 was found to be significantly reduced in drug-resistant cell lines. In contrast, the level of FEN-1 and vimentin was significantly up-regulated in drug-resistant cell lines.

Correlation between miR-21 and let-7d expression and the survival of PC patients

The expression levels of all seven miRNAs are presented as box plot in **Figure 6A**. The miR-21 and let-7d plasma expression levels in patients with PC were associated with overall survival (**Figure 6B** and **6C**). These results suggest that higher expression of miR-21 could serve as a biomarker for worse survival of PC patients, and thus could serves as an important prognostic marker. In contrast, the overall low level of let-7d expression could serve as an independent prognostic marker for PC patients. While the survival rate of PC patients with low expression levels of miR-221 trended to be longer than patients with higher levels, the difference was not statistically significant. Similarly, low expression levels of let-7b, miR-146a, miR-200b, and miR-200c were not statistically significant when compared between PC to healthy controls. This would strengthen the argument that miR-21 would be a useful plasma marker for predicting tumor aggressiveness and overall survival in patients with PC.

Discussion

Although, many miRNAs are expressed in tissues and tumor cells, their development as biomarkers requires tissue collection by invasive methods as opposed to the more convenient approach of studying peripheral blood. A high correlation of miRNA expression between tumor tissue and matching plasma was demonstrated in patients with breast cancer [23]; however, such studies

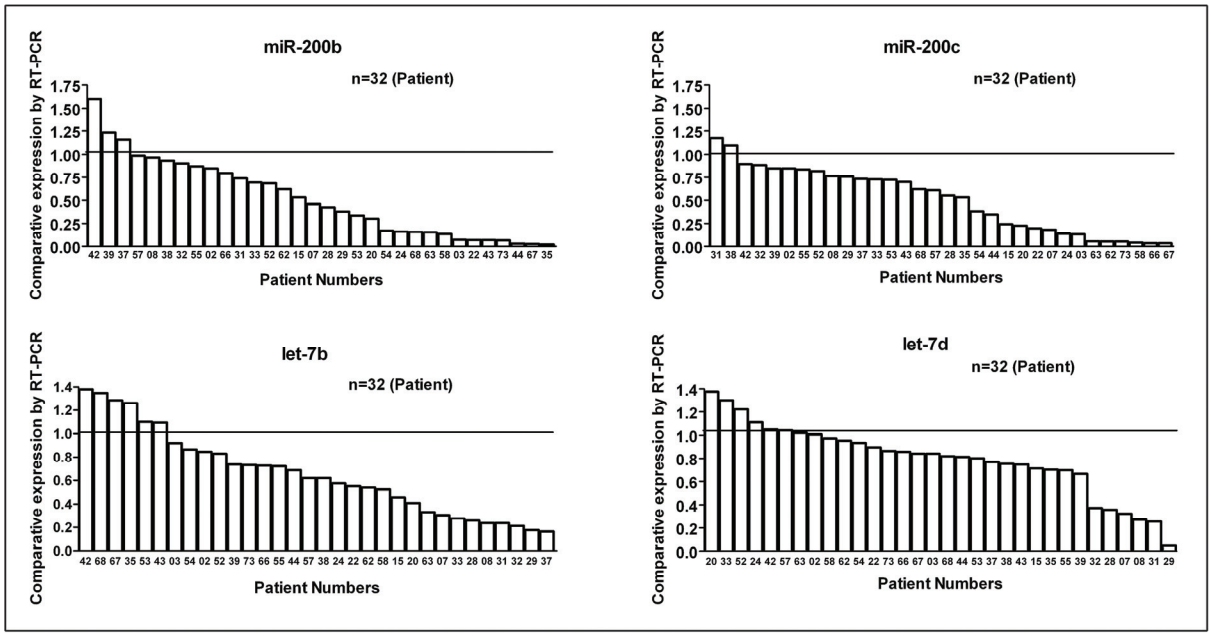

Figure 4. Comparative expression analysis of miRNAs (miR-200b, miR-200c, let-7b and let-7d) in the plasma of 32 PC patients analyzed individually compared to plasma obtained from 10 normal subjects by using qRT-PCR. The line drawn at 1.0 represents average of normal subjects (n=10). The results showed a significant decrease in the expression of miR-200b, miR-200c, let-7b, let-7d (tumor suppressor genes) in almost all PC patients compared to normal subjects.

are lacking in PC. Since early diagnosis may hold the key for our success in improving the outcome of patients with PC the identification and validation of miRNA as potential biomarkers for early detection and predicting tumor aggressiveness may have a major impact on design of future strategies in the diagnosis and treatment of PC. Emerging evidence clearly support the importance of circulating miRNAs in many tumor types including breast, prostate, colorectal, lung, ovarian, and pancreatic cancer [24,9,25,26,10]. Studies have shown that miR-21 is up-regulated in most of these cancers [13,27,28,29,30,31,32,33,34,35,15,36] support the concept that discovery of other miRNAs from the plasma of PC patients would be useful for earlier diagnosis and, predicting tumor behavior. In addition the discovery of agents that can favorably alter the expression of miRNAs would provide opportunities for novel strategies to improve the survival of patients with PC.

Identification of differentially expressed plasma miRNAs from pooled plasma samples by miRNA profiling followed by qRT-PCR validation of individual patient samples may serve as a novel approach for specific tumor types. In this report, we found 91 miRNAs that were differentially expressed in the plasma of PC patients compared to normal controls. This finding supports the utility of miRNA profiling helping differentiating PC patients from healthy individuals and a potential novel approach for non-invasive biomarker test for diagnosis and risk stratification. Similar to other studies, our results demonstrated that miR-21 was up-regulated in PC patients supporting its suggested oncogenic role. Our data are also consistent with four resistant cell lines data as presented in this report. We and other investigators have reported earlier that miR-21 targets PTEN and inversely regulates its expression, which in turn, inversely regulates the Akt pathway [11,32]. Here we show that there are several other genes that are regulated by miR-21, which not only includes the PTEN but also PDCD4, Maspin and TPM1 that were significantly decreased in all four drug -resistant PC cell lines (**Figure 5C**), resulted from the increased expression of miR-21 in all drug-resistant aggressive cell lines.

Our results on the high expression of miR-21 in the plasma and its correlation with worse sur-

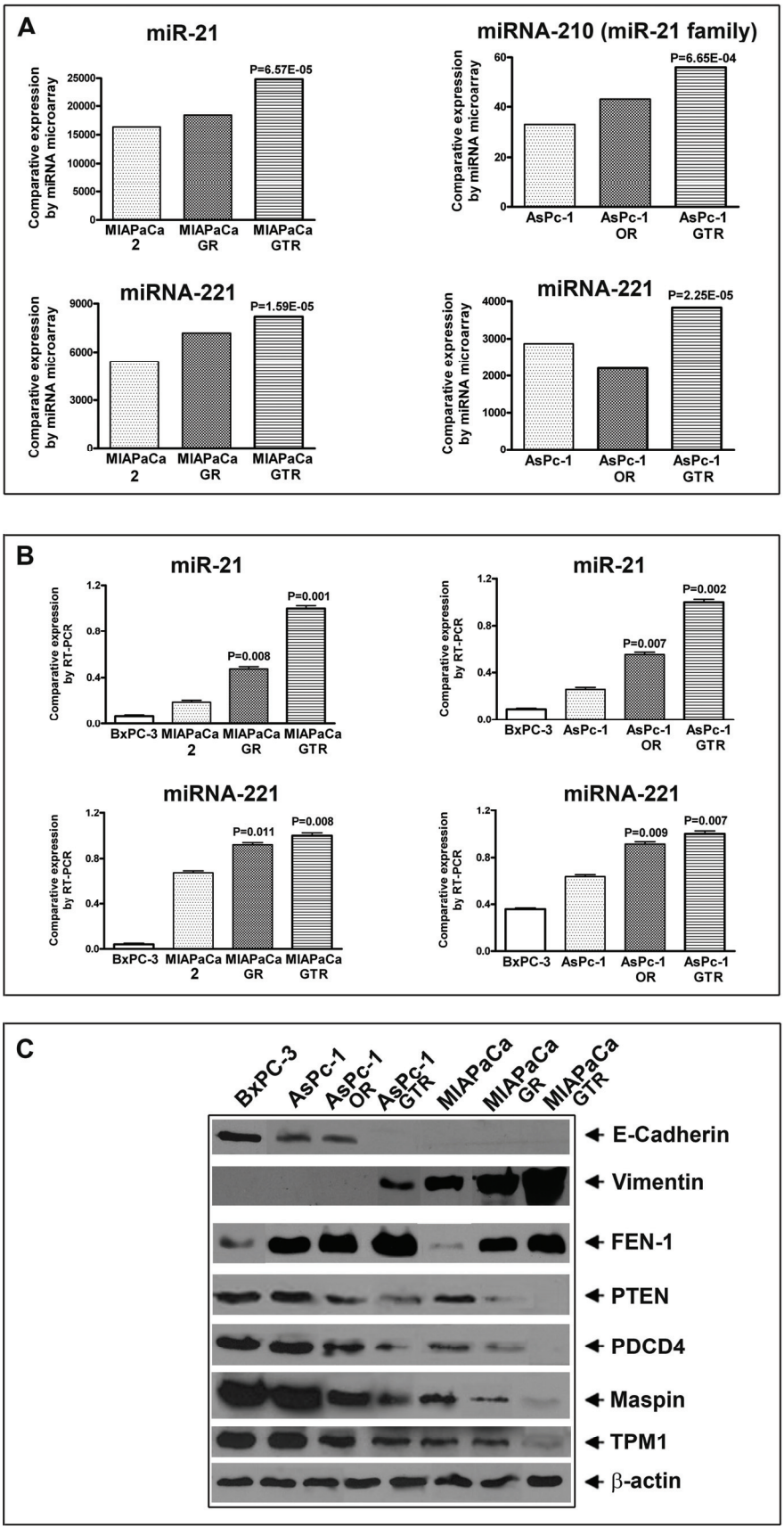

Figure 5. Comparative expression analysis of (A) The expression of miR-21 family and miR-221 in MIAPaCa-2, MIAPaCa-GR, MIAPaCa-GTR, AsPc-1, AsPc-1OR, AsPc-1GTR as determined by miRNA microarray profiling; (B) Comparative expression analysis of miR-21 and miR-221 in BxPC-3, MIAPaCa-2, MIAPaCa-GR, MIAPaCa-GTR, Aspc-1, AsPc-1OR, AsPc-1GTR cells by qRT-PCR. There was a significant up-regulation of both miR-21 and miR-221 by both the methods of microarray profiling and qRT-PCR methods. P values were calculated by the paired t-test (C) Western blots analysis showing the basal level expression of several proteins some of which are targets of miR-21 (PTEN, PDCD4, Maspin and TPM1).

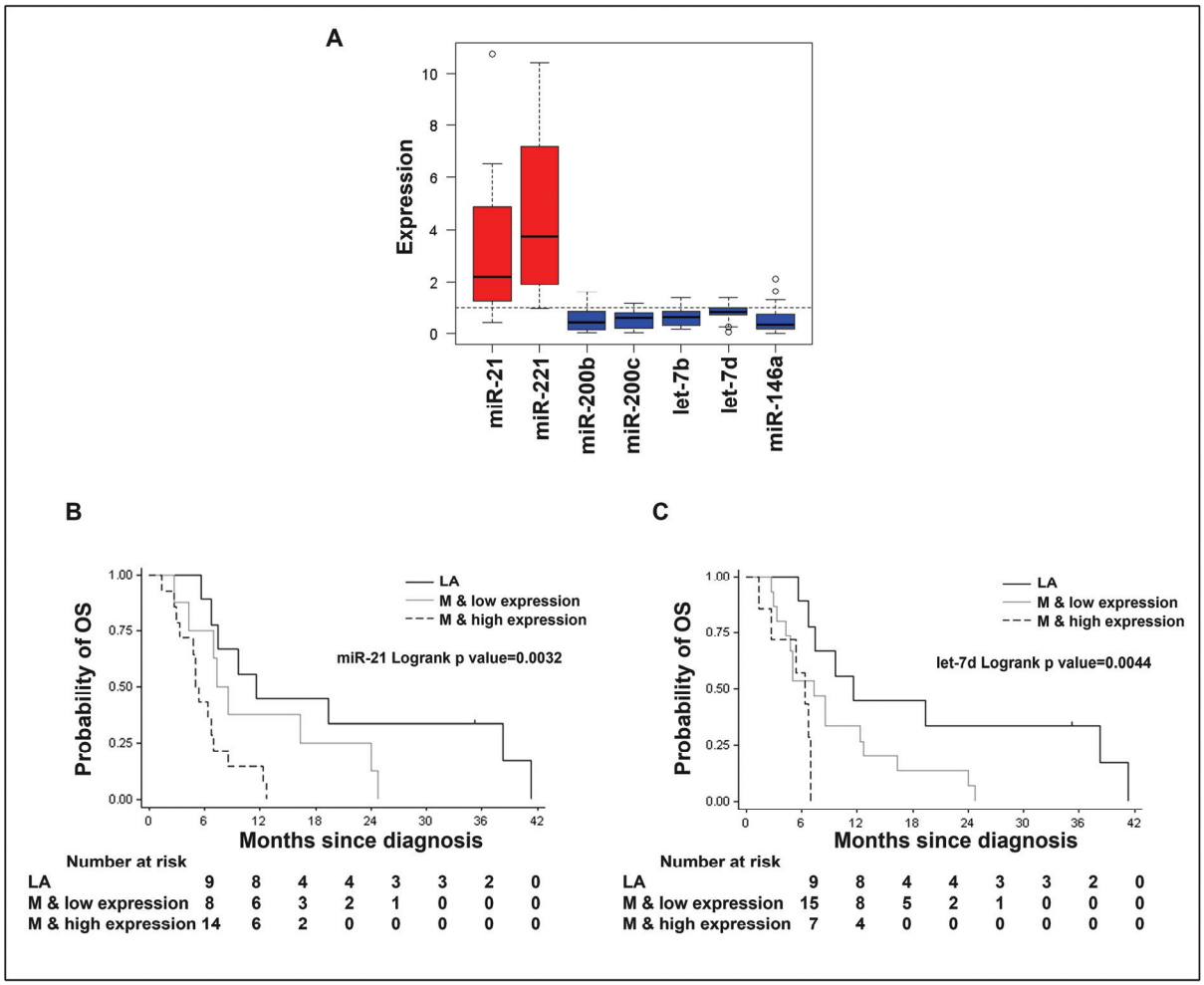

Figure 6. (A) Box plot representing the expression of seven miRNAs as assessed by qRT-PCR. (B). The Kaplan-Meier curves and log-rank tests for miR-21 expression and survival; (C) The Kaplan-Meier curves and log-rank tests for let-7d expression and survival.

vival are consistent with several other reports. A recent study showed that the loss of PTEN function may cause accelerated cancer progression, and decreased survival in the K-ras mouse model of pancreatic cancer [37]. Others have shown that miR-21 serves as a driver and negative regulator of the Ras/MEK/ERK pathway in transgenic mice of NSCLC model [38]. Zhu *et al* demonstrated that ionizing radiation up-regulates miR-21, which activates AP-1 and ErbB/Stat3 pathway and promotes liver carcinogenesis [39]. Up-regulation of miR-21 is not limited to pancreatic, lung, or liver cancers but also seen in colon carcinoma cells as downstream effectors of TGF-β by directly targeting Rac GTPase and facilitating invasion and metastasis [40]. Moreover, studies have shown shorter disease-free interval in patients with a higher expression of miR-21 in colorectal carcinoma tissue and colorectal cancer liver metastasis relative to adjacent normal colonic tissues [41]. Furthermore, growth of laryngeal squamous cell carcinoma xenograft tumors and Ras protein expression was significantly reduced by anti-sense miRNA (ASO-miR-21 lentivirus) [42]. Surprisingly, the functional role of miR-21 is not only limited to cancer because it has been found to be up-regulated in cardiovascular diseases by affecting fibrosis and heart failure [43]. Taken together, miR-21 appears to be an interesting molecular target for the development of therapeutic strategies against many forms of cancer as well as for cardiovascular diseases.

Drug-resistance is considered as one of the major reasons for treatment failure in patients with PC [4]. At this time no therapeutics are able to overcome drug-resistance. However, recent studies have shown that low expression of miR-21 was associated with benefit from adjuvant treatment of PC and consistent with increased anticancer drug activity *in vitro*, suggesting that miR-21 may allow stratification for adjuvant therapy [44]. We and others have reported earlier that PC cells are more aggressive and resistant to gemcitabine if they have higher expression of miR-21 [11,45], and thus strategies for down-regulation of miR-21 could improve the outcome of conventional therapeutics. In fact, Mei *et al* reported that combining miR-21 inhibitor gene therapy with paclitaxel may represent a promising novel therapeutic approach for the treatment of breast cancer [46].

Based on our data on the expression profiles of miRNAs in plasma from patients with PC as well as the relationship between miRNA expression and drug sensitivity in PC cell lines, we suggest that miR-21 is predictive marker of clinical drug-resistance and tumor aggressiveness through regulation of several genes, such as those regulating cell survival. Consequently, strategies to down-regulate miR-21 by natural or synthetic agents, gene-based therapies, or synthetic miRNA antagonists, could be useful for overcoming drug-resistance, in patients with PC, which will lead to improve the overall survival of patients diagnosed with this deadly disease.

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