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Original Articles

Next generation sequencing of pancreatic cyst fluid microRNAs from low grade-benign and high grade-invasive lesions

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ABSTRACT

Intraductal papillary mucinous neoplasm (IPMN) is a precursor cystic lesion to pancreatic cancer. With the goal of classifying IPMN cases by risk of progression to pancreatic cancer, we undertook an exploratory next generation sequencing (NGS) based profiling study of miRNAs (miRNome) in the cyst fluids from low grade-benign and high grade-invasive pancreatic cystic lesions. Thirteen miRNAs (miR-138, miR-195, miR-204, miR-216a, miR-217, miR-218, miR-802, miR-155, miR-214, miR-26a, miR-30b, miR-31, and miR-125) were enriched and two miRNAs (miR-451a and miR-4284) were depleted in the cyst fluids derived from invasive carcinomas. Quantitative real-time polymerase chain reaction analysis confirmed that the relative abundance of tumor suppressor miR-216a and miR-217 varied significantly in these cyst fluid samples. Ingenuity Pathway Analysis (IPA) analysis indicated that the genes targeted by the differentially enriched cyst fluid miRNAs are involved in five canonical signaling pathways, including molecular mechanisms of cancer and signaling pathways implicated in colorectal, ovarian and prostate cancers. Our findings make a compelling case for undertaking in-depth analyses of cyst fluid miRNomes for developing informative early detection biomarkers of pancreatic cancer developing from pancreatic cystic lesions.

Introduction

Pancreatic cancer is associated with high rates of morbidity and mortality; the 5-year survival rate is <6% [1]. Early detection of pancreatic tumors amenable to surgical resection and adjuvant chemo/radiotherapy increases the 5-year survival rate by 15–40% [2]. Unfortunately, however, early detection of this malignancy remains a challenge in the absence of sensitive and specific early detection biomarker assays at this time. As a precursor lesion to pancreatic cancer, intraductal papillary mucinous neoplasm (IPMN), manifesting as cysts within the pancreas, offers an opportunity for development of such assays, since a significant number of these lesions either harbor or progress to invasive pancreatic cancer. IPMNs involving the main duct are most aggressive; ~62% of main duct IPMNs have foci of invasive carcinoma at the time of surgical resection, while ~24% of branch duct IPMNs progress to invasive cancer [3,4]. Current diagnostic technologies are unable to accurately distinguish high-risk from low-risk IPMNs. MicroRNA (miRNA) array profiling has recently been reported to augment diagnosis and management of pancreatic cysts [5].

Genetic studies have revealed that many of the seminal alterations observed in invasive pancreatic cancer, such as mutations in KRAS, DPC4/SMAD4, and TP53, are also present in a variable proportion of non-invasive IPMNs, thus validating their status as bona

Abbreviations: BC, breast cancer; BC#, bladder cancer; DLBCL, diffuse large B cell lymphoma; ESCC, esophageal squamous cell carcinoma; GC, gastric cancer; HCC, hepatocellular carcinoma; IPMN, intraductal papillary mucinous neoplasm; miRNA, microRNAs; NGS, next generation sequencing; Novel, novel miRNAs associated with cancer; NSCLC, non-small cell lung carcinoma; OC, ovarian cancer; Onc, oncogenic miR (oncomiR); PA, pancreatic adenocarcinoma; PC, prostate cancer; PDAC, pancreatic ductal adenocarcinoma; qRT-PCR, quantitative real-time polymerase chain reaction; RCC, renal cell carcinoma; RMS, rhabdomyosarcoma; TS, tumor suppressor miR; UBC, urinary bladder cancer.

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fide precursor lesions [6]. A recent publication identified recurrent mutations in the *GNAS* gene in invasive adenocarcinomas associated with IPMN, possibly defining a new pathway for pancreatic tumorigenesis [7]. Such gene mutation studies indicate that complex genetic pathways underlie the transformation of IPMN to invasive carcinoma. Until now, clinical diagnosis and evaluation of IPMNs have relied primarily on imaging technologies [8]. However, accurate diagnosis of these lesions is difficult due to substantial similarities in their morphology and limitation in current imaging technologies [9]. Although serum CA19-9 is a validated tumor marker in clinical use for pancreatic cancer, its use is limited by poor sensitivity in asymptomatic patients, false negativity in the Lewis-negative phenotype (5–10%), and false positivity in cases of obstructive jaundice [10]. Histological examination of cells aspirated from pancreatic cysts has high specificity but low sensitivity for the detection of invasive cancer [9]. The need for more sensitive and specific biomarker assays that can be applied routinely remains the biggest challenge in effective clinical management of this lethal disease.

Aberrant expression of several miRNAs has been detected in pancreatic cancers [11–14]. Deregulated tumor-associated miRNAs have been implicated in tumor initiation and progression. Profiling and functional characterization studies have identified miRNAs that have either oncogenic functions (oncomiRs) or tumor suppressor functions (tumor suppressor miRs) mediated by targeting genes and genetic networks deregulated in cancers [15]. We have reported that profiling of a miRNA signature circulating in plasma can identify pancreatic cancer patients [16] and that oncomiRs in pancreatic cancer are associated with early steps of immortalization of human pancreatic ductal epithelial cells, which are involved in genetic pathways deregulated in this malignancy [17]. Profiling of miRNomes in blood and body fluids has yielded encouraging results supporting the feasibility of developing informative diagnostic and prognostic biomarkers in cyst fluids with this approach [15,18]. Absolute quantification of differentially enriched miRNAs using next generation sequencing (NGS) offers a promising approach for risk stratification of IPMN cases. We therefore undertook an NGS study of miRNAs in cyst fluid derived from IPMN, with the goal of identifying differences between cases suggestive of benign or low grade dysplasia as opposed to those suggestive of high grade dysplasia or invasive pancreatic cancer.

Materials and methods

Study cohort and sample collection

The study cohort consisted of patients with imaging-confirmed IPMN who had been selected for either active surveillance or surgery at the University of California, San Francisco. Surgical patients had pathologically confirmed disease. The patients were generally in their sixth to eighth decade of life. We collected cyst fluid samples from patients with IPMN, mucinous cystic neoplasm, and pancreatic cancer. Given the risk of cyst infection, only a single cyst component was sampled within each IPMN. The cyst component with the highest risk features for advanced dysplasia was sampled, as is the conventional approach pursued by clinicians at these centers. Risk features included size (larger size, higher risk), mural nodularity, debris, or direct involvement of the main pancreatic duct. The collection and banking of cyst fluids were performed with signed patient consent in accordance with the CHR, Institutional Review Board, and HIPAA. Cyst fluid collection was guided by diagnostic endoscopic ultrasound and the specimens were immediately stored at –80 °C. All samples were stripped of patient-identifying information prior to delivery for analysis. The clinic and the research laboratories coordinated to allow processing of cyst fluids immediately after collection of the samples. The volume of cyst fluid obtained for each sample was variable and often very small. When the volume was limited, testing was done selectively and focused on the most clinically informative parameters, as determined by the endoscopist. Thus, we have CEA levels and cytology reports for some but not all of the samples.

miRNA extraction and purification

Cyst fluid was cleared through centrifugation at 1300 × *g* at 4 °C for 10 minutes and stored at –80 °C. Total RNA was extracted from each cyst fluid sample and purified by the following method: cleared cyst fluid was mixed with Trizol LS (1:3 ratio; Life Technologies, Grand Island, NY), and after phase separation by centrifugation, the aqueous phase was extracted once with phenol/chloroform and added to ethanol before being applied directly to a mirVana miRNA column (Ambion, Austin, TX) according to the manufacturer's instructions. The concentration of RNA samples was quantified by using NanoDrop 2000 (NanoDrop, Wilmington, DE).

Next generation sequencing

The NGS analysis of cyst fluids was performed at the non-coding RNA sequencing core facility at The University of Texas MD Anderson Cancer Center on a SOLID™ platform according to the SOLiD small RNA Sequencing protocol, recommended by Life Technologies. In brief, small RNAs were enriched by PAGE fractionation and collected for library construction and barcoded individually. The barcoded libraries were pooled in equal for multiplexing. The sequencing temples were generated by EZ beads system. The sequencing was performed in 35 nts on SOLiD 5500XL genome analyzer (Applied Biosystems, Foster City, CA). Each sample provided approx. 10–15 million reads in 35 nts. The sequence data generated were analyzed for small non-coding RNA and miRNA bioinformatics was performed. For all the sequenced libraries, filtering with a stringent cutoff of 50 read counts/million was applied to identify the differentially enriched circulating miRNAs following match alignment with the most recent miRbase release.

Quantitative real-time polymerase chain reaction analysis

Taqman miRNA assays (Applied Biosystems, Foster City, CA) were used to quantify the expression levels of mature miR-216a (Assay ID 002220, Applied Biosystems) and miR-217 (Assay ID 002337, Applied Biosystems). Twenty ng of RNA from each sample of cyst fluid was reverse-transcribed by the TaqMan microRNA reverse transcription kit (Applied Biosystems, Foster City, CA) in a reaction mixture containing a miR-specific stem-loop reverse transcription (RT) primer. The quantification of mature miRNAs was performed using the TaqMan miRNA assay kit (Applied Biosystems) containing TaqMan primers in a universal PCR master mix [19]. Expression levels of miRNAs were quantified by using the ViiA 7 Real-Time PCR System (Life Technologies). Relative differences in mature miRNA expression (expressed as fold change) between the high risk samples and the low risk samples were calculated by using the comparative CT ($2^{-\Delta\Delta CT}$) method, using RNU6B (Assay ID 001093, Applied Biosystems) as the endogenous control to normalize the data. Primers for detection of RNU6B RNA expression were RNU6B-Fw, GTGCTCGCTTCGGCAGCACATAT and RNU6B-Rev, AAAAATATGGAACGCTTCACGAA.

Statistical analysis

We used the nonparametric Mann–Whitney U test to compare the miRNA expression levels between two groups and the Kruskal–Wallis test for comparisons involving more than two groups. The Cuzick's test was performed to identify trends. All tests of statistical significance were two sided. P values of <0.05 were considered statistically significant. All statistical analyses were done using the Stata 10.1 software (Stata Corporation, College Station, TX).

Pathway analysis

To determine the potential specific pathways involving differentially abundant miRNAs and their target gene transcripts, we used the Ingenuity Pathway Analysis (IPA) software program as described elsewhere [17].

Results

Patient risk categories

For this study, we collected 17 cyst fluid samples from patients with IPMN, mucinous cystic neoplasm, and pancreatic cancer. We categorized the samples by cancer risk into three groups: low-risk, high-risk, and pancreatic adenocarcinoma, as follows: IPMN and mucinous cystic neoplasm with low grade or moderate grade dysplasia was categorized as low risk, while IPMN and mucinous cystic neoplasm with high grade dysplasia was categorized as high risk and frank invasive carcinoma was categorized as such. The numbers of samples in the low-risk, high-risk, and pancreatic adenocarcinoma groups were 6, 8, and 3, respectively.

Next generation sequencing analyses of miRNome

As a first step in developing a cyst fluid miRNA biomarker signature predictive of aggressive pancreatic carcinoma, we performed

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Table 1
Cyst fluid miRNA profiles identified from high grade-invasive (HG-I) and low grade-benign (LG-B) groups by NGS analysis.

miRNA	Base mean (LG-B)	Base mean (HG-I)	Fold change (HG-I/LG-B)	P value	miR function	Pancreatic cancer	Circulating miRNA in cancer	Target gene transcripts
mir-216a	5.88	846.92	143.95	0.007	TS	√	–	PTEN, SMAD7, TSLC1, Ybx1
mir-217	164.22	6360.85	38.73	0.010	TS	√	–	PTEN, SMAD7, KRAS
mir-802	3.10	407.22	131.21	0.023	Novel	–	–	Caveolin-1, HNF1B
mir-204	11.77	980.79	83.35	0.011	TS	√	–	ANKRD13A, SMAD4, BCL2
mir-218-1	30.26	957.82	31.65	0.048	TS	√	GC	EZH2, LEF1
mir-218-2	32.27	955.58	29.61	0.051	TS	√	GC	EZH2, LEF1
mir-155	8.73	215.09	24.64	0.194	Onc	√	BC, NSCLC, DLBCL, ESCC, PDAC	SOCS1, c-Jun, CK1α, MMP16, TP53INP1, CKAP5,
mir-214	157.13	3821.22	24.32	0.030	Onc/TS	√	BC, RMS	ING4, TFAP2, LTF
mir-195	623.71	10,851.43	17.40	0.051	TS	–	BC, HCC, PC, GC	Raf-1, IKKα, TAB3, cyclin D1, cyclin E1
mir-138-2	98.66	956.70	9.70	0.126	TS	–	–	NGAL, Mst1, FAK
mir-138-1	146.45	1100.66	7.52	0.159	TS	–	–	NGAL, Mst1, FAK
mir-26a-2	2089.43	18,247.40	8.73	0.122	Onc/TS	–	HCC, OC, BC[#]	E2F7, EZH2 GSK-3β, ABCA1, ARL7,
mir-26a-1	2098.22	18,242.92	8.69	0.123	Onc/TS	–	HCC, OC, BC[#]	E2F7, EZH2, ABCA1, ARL7, GSK-3β
mir-30b	2275.55	18,371.75	8.07	0.139	TS	–	PDAC	CaMKIIδ, Snail1, GALNT7
mir-31	2292.17	15,098.90	6.59	0.161	Onc/TS	√	ESCC, PDAC	hMLH1, CREG, RASA1
mir-125a	11172.41	86,177.34	7.71	0.102	TS	√	Oral cancer	FYN, MMP11, VEGF
mir-125b-1	13470.42	67,539.52	5.01	0.210	TS	√	PC	FYN, MMP11, VEGF
mir-451a	367293.41	30,117.14	0.08	0.084	Onc/TS	√	BC, RCC, GC, NSCLC	LKB1/AMPK, RAB14
mir-4284	343.39	6.16	0.02	0.183	Novel	–	–	–

NGS of small non-coding RNAs from four blinded samples, two from the low grade-benign group and two from the high grade-invasive group. We successfully generated the sequencing libraries from these small non-coding RNAs. The library quality was assessed to ensure the quality control according to the manufacturer's instructions. The short read was aligned to the miRBase database (V19) (ftp://mirbase.org/pub/mirbase/CURRENT/miRNA.dat.gz) using CLC Genomics WorkBench. The alignment related statistics were reported in Appendix: Supplementary Fig. S1 and Table S1. NGS analyses of miRNomes isolated from the four cyst fluid samples identified multiple miRNAs that were differentially enriched in high grade-invasive lesions compared with low grade-benign lesions. As shown in Table 1, we found 13 miRNAs (miR-138, miR-195, miR-204, miR-216a, miR-217, miR-218, miR-802, miR-155, miR-214, miR-26a, miR-30b, miR-31, and miR-125) that were differentially elevated in cyst fluid samples from pancreatic tumors and two miRNAs (miR-451a and miR-4284) that were depleted in these samples. It should be mentioned, however, that because of limited sample size in this preliminary study, differences in miRNA expression did not appear significant for all of the listed miRNAs after adjusting for false discovery rate.

Quantitative real-time polymerase chain reaction analysis of candidate miRNAs

To validate the differential enrichment of cyst fluid miRNAs identified by NGS, we used quantitative real-time polymerase chain reaction (qRT-PCR) assay to quantify two miRNAs (miR-216a and miR-217) in all the 17 cyst fluid samples. The relative abundance of these two miRNAs, normalized to the level of RNU6B, varied significantly between patients in the low-risk group, the high-risk group, or the pancreatic adenocarcinoma group. There was a significant difference in relative levels of miR-216 among the cyst fluids of different risk groups (Kruskal–Wallis test, P = 0.014) (Table 2). The Cuzick's trend test revealed a trend for increased miR-216 expression with higher grade of the lesions (P = 0.006). The miR-216 level was significantly elevated in the high-risk group compared with the low-risk group (Mann–Whitney test, P = 0.011). It also was higher in the pancreatic adenocarcinoma group than in the low-risk group (Mann–Whitney test, P = 0.020). There was no difference between the high-risk group and the pancreatic adenocarcinoma group (Mann–Whitney test, P = 0.540). The miR-217 level did not differ significantly among the three groups (Kruskal–Wallis test, P = 0.100). However, it showed a tendency to be more elevated in the lesions of higher histological grade (trend test, P = 0.035). The miR-217 level did not differ significantly in any pairwise comparison of the three groups (Table 2), although the P values for the variations between the low-risk group vs high-risk group and the low-risk group vs the pancreatic adenocarcinoma group were close to significant (Mann–Whitney test, P = 0.093 and P = 0.070, respectively). These differences might become significant with a larger sample size. Further study with a larger sample cohort is, therefore, warranted.

Functional networks and pathways of cyst fluid identified miRNA target genes

The functional networks and cellular pathways of the genes targeted by the miRNAs, identified in pancreatic cancer cyst fluids, were analyzed with IPA. The 15 differentially abundant miRNAs in cyst fluids and their targets were analyzed for their involvement in the

Table 2
Relative expression level of miRNAs in cyst fluid samples from low-risk, high-risk, and pancreatic adenocarcinoma (PA) patient groups.

miRNA	Risk group (N)	Expression, median	P value			
			Low/high	Low/PA	High/PA	Trend test
miR-216	Low (6)	0.175	0.011	0.020	0.540	0.006
	High (8)	3.89				
	PA (3)	8.08				
miR-217	Low (6)	0.94	0.093	0.070	0.414	0.035
	High (8)	3.12				
	PA (3)	26.9				

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Table 3
Five canonical pathways involving the greatest number of genes targeted by miRNAs differentially expressed in cyst fluid.

Signaling pathway	P value	Ratio	Molecules
Molecular mechanisms of cancer	1.10 E-06	7/388 (0.018)	FYN,SMAD7,SMAD4,LEF1,KRAS,RASA1,BCL2
Colorectal cancer metastasis signaling	2.38 E-06	6/268 (0.022)	VEGFA,MMP16,SMAD4,LEF1,KRAS,MMP11
Ovarian cancer signaling	2.39 E-06	5/152 (0.038)	VEGFA,LEF1,KRAS,PTEN,BCL2
Ephrin receptor signaling	9.15 E-06	5/210 (0.024)	VEGFA,FYN,ADAM10,KRAS,RASA1
Prostate cancer signaling	1.01 E-05	4/103 (0.039)	LEF1,KRAS,PTEN,BCL2

genetic networks and cellular pathways (Table 3). A total of 46 genes have been found in multiple independent studies to be targeted by these 15 miRNAs. Interestingly, comprehensive network and pathway analysis of the deregulated genes revealed that they are associated with five canonical pathways involved in cancer development signaling (Table 3) and three network functions relevant to the development of pancreatic cancer (Table 4). The target genes of the differentially enriched miRNAs constituted about half of the molecules involved in network-associated cellular functions of these three networks and included genes related to (1) tissue morphology, cellular growth and proliferation, and cellular development; (2) cellular movement, cancer, and cellular development; and (3) cell morphology, cancer and carbohydrate metabolism (Table 4). These genes also belong to five canonical signaling pathways, which are involved in regulation of molecular mechanisms of cancer, colorectal cancer metastasis signaling, ovarian cancer signaling, prostate cancer signaling and ephrin receptor signaling (Table 3).

Discussion

Current clinical parameters, imaging, and laboratory analytic criteria are insufficient to distinguish benign pancreatic cystic lesions from those predisposed to malignant progression. IPMNs present both a strong need and a unique opportunity for development of biomarkers that would allow early detection of lesions that are predisposed to becoming pancreatic cancer from those that are benign or low grade and less likely to progress.

Since genetic evolution of pancreatic cancer from the precursor lesion with initiating mutation to metastasis requires more than a decade [20], development of appropriate biomarkers capable of identifying IPMN lesions before progression to pancreatic cancer is going to be critical within the window of opportunity for effective clinical intervention [3]. Thus, characterizing the diagnostic and prognostic value of pancreatic cystic fluid biomarkers is an area of active investigation with great potential for improving outcomes in pancreatic cancer.

Our NGS analysis revealed 15 miRNAs that were differentially enriched in cyst fluid samples from patients with pancreatic adenocarcinoma. The miRNAs were identified from 18 to 40 nt long small RNA derived bar coded libraries yielding 10–15 million reads from each sample. This amount of read data is expected to detect even low expressing transcripts, thus allowing robust identification of differentially abundant miRNAs, irrespective of their expression levels, among the samples analyzed. It is relevant to point out here that if the samples in a group were to be combined, we would lose the power to estimate the variations within the group and statistical P-values for differentially expressed genes. A recent study shows that sequencing less reads and performing more biological replication are an effective strategy to increase power and accuracy in differential expression RNA-seq studies [21]. Results revealed differential abundance of multiple miRNAs between high grade-invasive (HG-I) and low grade-benign (LG-B) samples and five of these (miR-125, miR-195, miR-26, miR-30 and miR-217) were consistent with those reported in a RT-PCR array based miRNA profiling study of IPMN, published earlier [5]. We also observed that a number miRNAs (miR-216, miR-217, miR-155, miR-195, miR-204, and miR-214) depleted in the cyst fluid of patients with mucinous cystic neoplasm (data not shown). Expression of some tumor suppressor miRNAs in circulation has earlier been reported to show an inverse correlation with their abundance in tumor tissue [15]. Similar trend of elevated putative tumor suppressor miRNAs (miR-138, miR-195, miR-204, miR-216a, miR-217, and miR-218) in cyst fluid was also observed in this study. For example, miR-204 deregulated in various tumors [22–24] that suppresses cancer cell migration and invasion, as well as epithelial–mesenchymal transition [22,23] was found elevated in the cyst fluid samples from patients with high grade-invasive lesions, similar to higher levels detected in the serum of endometrial cancer patients compared with healthy controls [25]. Furthermore, qRT-PCR validation of NGS data in our study confirmed that the relative expression levels of miR-216a and miR-217 were elevated significantly in the patients with high grade-invasive lesions. Down-regulation of miR-216a and miR-217 in mouse pancreatic tumors has validated their role as tumor suppressors [26]. miR-217, which functions as a potential tumor suppressor in pancreatic cancer by targeting KRAS, was found repressed in about 76% of pancreatic adenocarcinoma tissues and cell lines [27]. miR-217 can inhibit invasion of hepatocellular carcinoma cells by targeting E2F3 [28] and correlates with resistance of Philadelphia chromosome-positive leukemia cells to ABL tyrosine kinase inhibitors [29]. Unlike the inverse correlation observed

Table 4
Genetic networks associated with genes targeted by miRNAs differentially expressed in cyst fluid.

Network	Associated network functions	Score	Focus molecules	Molecules in network
1	Tissue morphology, cellular growth and proliferation, cellular development	23	11	**ADAM10**, Ap1, BCR (complex), **CADM1**, Collagen type IV, **CREG1**, Cyclin A, Cyclin E, E2f, Efna dimer, ERK1/2, **EZH2**, Hdac, **HNF1B, LEF1**, MAP2K1/2, **MMP11, MST1**, NFAT (complex), p70 S6k, Pdgf (complex), PDGF BB, phosphatase, **RASA1**, Rxr, Smad, **SMAD7**, Smad2/3, Smad2/3-Smad4, Sos, **TAB3**, TCF, Tgf beta, TGFBR, VAV
2	Cellular movement, cancer, cellular development	18	9	14-3-3, Akt, Alp, AMPK, **ANKRD13A, BCL2**, caspase, CD3, Cofilin, Creb, cytochrome C, estrogen receptor, Fcer1, **FYN**, Ige, Ikb, IKK (complex), IL1, **KRAS,** LDL, **LTF**, Mek, Nfat (family), P110, p85 (pik3r), **PTEN**, Ras, Shc, **SOCS1**, STAT5a/b, TCR, **TFAP2A**, Tnf (family), ubiquitin, **VEGFA**
3	Cell morphology, cancer, carbohydrate metabolism	17	8	26s Proteasome, **ABCA1**, Alpha catenin, Alpha tubulin, **ARL4C**, calmodulin, Cg, collagen type I, **E2F7**, ERK, focal adhesion kinase, FSH, Histone h3, IL12 (complex), **ING4**, insulin, integrin, Jnk, Lh, Mapk, Mmp, **MMP16**, NFkB (complex), P38 MAPK, Pdgfr, PI3K (complex), Pka, Pkc(s), pro-inflammatory cytokine, **RAB14**, Ras homolog, RNA polymerase II, **SMAD4**, Vegf, **YBX1**

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between the levels of tumor suppressor miRNAs in tumors and body fluids, the level of miR-155, a well characterized oncomiR, has always been found elevated in both tumors and serum/plasma [16,30–36], similar to their elevated levels detected in the cyst fluid from pancreatic adenocarcinoma patients in this study.

In contrast to the consistent functional roles of the above mentioned miRNAs as tumor suppressors or oncomiRs, a number other miRNAs such as miR-26, miR-31, and miR-214 display both tumor suppressor as well as oncomiR like properties in different organ specific cancers. For example, increased expression of miR-31 has been detected in colorectal, lung, and pancreatic cancers, head and neck squamous cell carcinoma, and osteosarcoma, but repression of miR-31 has been reported in bladder cancer, breast cancer, glioma, melanoma, ovarian cancer, and prostate cancer [37]. Elevated levels of miR-31 were reported in plasma samples of patients with oral carcinoma [38] or breast cancer [39], which suggested that miR-31 may be a potential circulating diagnostic marker in blood. The involvement of miR-26, miR-31, and miR-214 in various tumors depends on their target genes and cognate pathways.

These findings highlight the importance of characterizing the functional significance of miRNAs implicated in deregulated cancer-relevant pathways. Our results reveal the robust involvement of miRNAs, differentially enriched in cyst fluids, in deregulating cancer-associated genetic networks, and we anticipate that miRNA signatures in cyst fluids constituted oncomiRs (miR-155) and tumor suppressor miRNAs (miR-138, miR-195, miR-204, miR-216a, miR-217, and miR-218) underlying aberrantly expressed genetic networks and pathways would constitute sensitive and specific biomarkers for pancreatic cancers. The functional pathway analyses would help prioritize differentially abundant cyst fluid miRNAs for inclusion in a biomarker signature predictive of aggressive IPMN. Such signatures may serve in early detection of pancreatic cancer, especially for detection of aggressive IPMN and invasive pancreatic cancers with the help of a cyst fluid miRNA biomarker profiling assay.

Finally, we recognize that count data from NGS analyses as the readout for differential RNA expression or copy enrichment are not entirely reliable under all circumstances. It is important to undertake such studies in large sample cohorts and have stringent quality control standards for cyst fluid sample preparation and filtering of sequence data for significant read number cutoffs in order to ensure that sample size is adequate for selecting the candidate biomarker miRNAs capable of discriminating patients with benign vs malignant disease.

Conflict of interest

All authors have no conflict of interest.

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Appendix: Supplementary material

Supplementary data to this article can be found online at doi:10.1016/j.canlet.2014.09.029.

References

[1] R. Siegel, J. Ma, Z. Zou, A. Jemal, Cancer statistics, CA Cancer J. Clin. 64 (2014) (2014) 9–29.
[2] C.J. Yeo, J.L. Cameron, K.D. Lillemoe, J.V. Sitzmann, R.H. Hruban, S.N. Goodman, et al., Pancreaticoduodenectomy for cancer of the head of the pancreas. 201 patients, Ann. Surg. 221 (1995) 721–731.
[3] R.M. Thomas, J.B. Fleming, MicroRNA dissects out dangerous pancreatic cysts from all the rest, Clin. Cancer Res. 18 (2012) 4482–4484.
[4] M. Tanaka, C. Fernandez-del Castillo, V. Adsay, S. Chari, M. Falconi, J.Y. Jang, et al., International consensus guidelines 2012 for the management of IPMN and MCN of the pancreas, Pancreatology 12 (2012) 183–197.
[5] H. Matthaei, D. Wylie, M.B. Lloyd, M. Dal Molin, J. Kemppainen, S.C. Mayo, et al., miRNA biomarkers in cyst fluid augment the diagnosis and management of pancreatic cysts, Clin. Cancer Res. 18 (2012) 4713–4724.
[6] A. Maitra, N. Fukushima, K. Takaori, R.H. Hruban, Precursors to invasive pancreatic cancer, Adv. Anat. Pathol. 12 (2005) 81–91.
[7] J. Wu, H. Matthaei, A. Maitra, M. Dal Molin, L.D. Wood, J.R. Eshleman, et al., Recurrent GNAS mutations define an unexpected pathway for pancreatic cyst development, Sci. Transl. Med. 3, 3 (2011) 92ra66.
[8] A. Mohammed, N.B. Janakiram, S. Lightfoot, H. Gali, A. Vibhudutta, C.V. Rao, Early detection and prevention of pancreatic cancer: use of genetically engineered mouse models and advanced imaging technologies, Curr. Med. Chem. 19 (2012) 3701–3713.
[9] B.G. Turner, W.R. Brugge, Diagnostic and therapeutic endoscopic approaches to intraductal papillary mucinous neoplasm, World J. Gastrointest. Surg. 2 (2010) 337–341.
[10] M.J. Duffy, C. Sturgeon, R. Lamerz, C. Haglund, V.L. Holubec, R. Klapdor, et al., Tumor markers in pancreatic cancer: a European Group on Tumor Markers (EGTM) status report, Ann. Oncol. 21 (2010) 441–447.
[11] M. Gironella, M. Seux, M.J. Xie, C. Cano, R. Tomasini, J. Gommeaux, et al., Tumor protein 53-induced nuclear protein 1 expression is repressed by miR-155, and its restoration inhibits pancreatic tumor development, Proc. Natl. Acad. Sci. U.S.A. 104 (2007) 16170–16175.
[12] A.E. Szafranska, M. Doleshal, H.S. Edmunds, S. Gordon, J. Luttges, J.B. Munding, et al., Analysis of microRNAs in pancreatic fine-needle aspirates can classify benign and malignant tissues, Clin. Chem. 54 (2008) 1716–1724.
[13] E. Giovannetti, N. Funel, G.J. Peters, M. Del Chiaro, L.A. Erozenci, E. Vasile, et al., MicroRNA-21 in pancreatic cancer: correlation with clinical outcome and pharmacologic aspects underlying its role in the modulation of gemcitabine activity, Cancer Res. 70 (2010) 4528–4538.
[14] N.B. Jamieson, D.C. Morran, J.P. Morton, A. Ali, E.J. Dickson, C.R. Carter, et al., MicroRNA molecular profiles associated with diagnosis, clinicopathologic criteria, and overall survival in patients with resectable pancreatic ductal adenocarcinoma, Clin. Cancer Res. 18 (2012) 534–545.
[15] J. Wang, K.Y. Zhang, S.M. Liu, S. Sen, Tumor-associated circulating MicroRNAs as biomarkers of cancer, Molecules 19 (2014) 1912–1938.
[16] J. Wang, J. Chen, P. Chang, A. LeBlanc, D. Li, J.L. Abbruzzesse, et al., MicroRNAs in plasma of pancreatic ductal adenocarcinoma patients as novel blood-based biomarkers of disease, Cancer Prev. Res. 2 (2009) 807–813.
[17] J. Wang, S. Sen, MicroRNA functional network in pancreatic cancer: from biology to biomarkers of disease, J. Biosci. 36 (2011) 481–491.
[18] P.S. Mitchell, R.K. Parkin, E.M. Kroh, B.R. Fritz, S.K. Wyman, E.L. Pogosova-Agadjanyan, et al., Circulating microRNAs as stable blood-based markers for cancer detection, Proc. Natl. Acad. Sci. U.S.A. 105 (2008) 10513–10518.
[19] C. Chen, D.A. Ridzon, A.J. Broomer, Z. Zhou, D.H. Lee, J.T. Nguyen, et al., Real-time quantification of microRNAs by stem-loop RT-PCR, Nucleic Acids Res. 33 (2005) e179.
[20] S. Yachida, S. Jones, I. Bozic, T. Antal, R. Leary, B.J. Fu, et al., Distant metastasis occurs late during the genetic evolution of pancreatic cancer, Nature 467 (2010) 1114–1127.
[21] Y. Liu, J. Zhou, K.P. White, RNA-seq differential expression studies: more sequence or more replication?, Bioinformatics 30 (2014) 301–304.
[22] L. Zhang, X. Wang, P. Chen, MiR-204 down regulates SIRT1 and reverts SIRT1-induced epithelial-mesenchymal transition, anoikis resistance and invasion in gastric cancer cells, BMC Cancer 13 (2013) 290.
[23] Y.H. Qiu, Y.P. Wei, N.J. Shen, Z.C. Wang, T. Kan, W.L. Yu, et al., miR-204 inhibits epithelial to mesenchymal transition by targeting slug in intrahepatic cholangiocarcinoma cells, Cell. Physiol. Biochem. 32 (2013) 1331–1341.
[24] L. Ma, X. Deng, M. Wu, G. Zhang, J. Huang, Down-regulation of miRNA-204 by LMP-1 enhances CDC42 activity and facilitates invasion of EBV-associated nasopharyngeal carcinoma cells, FEBS Lett. 588 (2014) 1562–1570.
[25] W. Jia, Y. Wu, Q. Zhang, G. Gao, C. Zhang, Y. Xiang, Identification of four serum microRNAs from a genome-wide serum microRNA expression profile as potential non-invasive biomarkers for endometrioid endometrial cancer, Oncol. Lett. 6 (2013) 261–267.
[26] S. Ali, S. Banerjee, F. Logna, B. Bao, P.A. Philip, M. Korc, et al., Inactivation of Ink4a/Arf leads to deregulated expression of miRNAs in K-Ras transgenic mouse model of pancreatic cancer, J. Cell. Physiol. 227 (2012) 3373–3380.
[27] W.G. Zhao, S.N. Yu, Z.H. Lu, Y.H. Ma, Y.M. Gu, J. Chen, The miR-217 microRNA functions as a potential tumor suppressor in pancreatic ductal adenocarcinoma by targeting KRAS, Carcinogenesis 31 (2010) 1726–1733.
[28] J. Su, Q. Wang, Y. Liu, M. Zhong, miR-217 inhibits invasion of hepatocellular carcinoma cells through direct suppression of E2F3, Mol. Cell. Biochem. (2014).
[29] C. Nishioka, T. Ikezoe, J. Yang, A. Nobumoto, M. Tsuda, A. Yokoyama, Downregulation of miR-217 correlates with resistance of Ph(+) leukemia cells to ABL tyrosine kinase inhibitors, Cancer Sci. 105 (2014) 297–307.
[30] B.C. Onyeagucha, M.E. Mercado-Pimentel, J. Hutchison, E.K. Flemington, M.A. Nelson, S100P/RAGE signaling regulates microRNA-155 expression

via AP-1 activation in colon cancer, Exp. Cell Res. 319 (2013) 2081–2090.
[31] Y. Zhang, W. Wei, N. Cheng, K. Wang, B. Li, X. Jiang, et al., Hepatitis C virus-induced up-regulation of microRNA-155 promotes hepatocarcinogenesis by activating Wnt signaling, Hepatology 56 (2012) 1631–1640.
[32] M. Yang, H. Shen, C. Qiu, Y. Ni, L. Wang, W. Dong, et al., High expression of miR-21 and miR-155 predicts recurrence and unfavourable survival in non-small cell lung cancer, Eur. J. Cancer 49 (2013) 604–615.
[33] C. Huang, H. Li, W. Wu, T. Jiang, Z. Qiu, Regulation of miR-155 affects pancreatic cancer cell invasiveness and migration by modulating the STAT3 signaling pathway through SOCS1, Oncol. Rep. 30 (2013) 1223–1230.
[34] Q. Liu, J. Chen, J. Wang, C. Amos, A.M. Killary, S. Sen, et al., Putative tumor suppressor gene SEL1L was downregulated by aberrantly upregulated hsa-mir-155 in human pancreatic ductal adenocarcinoma, Mol. Carcinog. 53 (9) (2013) 711–721.
[35] C. Eichelser, D. Flesch-Janys, J. Chang-Claude, K. Pantel, H. Schwarzenbach, Deregulated serum concentrations of circulating cell-free microRNAs miR-17, miR-34a, miR-155, and miR-373 in human breast cancer development and progression, Clin. Chem. 59 (2013) 1489–1496.
[36] F. Gao, J. Chang, H. Wang, G. Zhang, Potential diagnostic value of miR-155 in serum from lung adenocarcinoma patients, Oncol. Rep. 31 (2014) 351–357.
[37] E.M. Laurila, A. Kallioniemi, The diverse role of miR-31 in regulating cancer associated phenotypes, Genes Chromosomes Cancer 52 (2013) 1103–1113.
[38] C.J. Liu, S.Y. Kao, H.F. Tu, M.M. Tsai, K.W. Chang, S.C. Lin, Increase of microRNA miR-31 level in plasma could be a potential marker of oral cancer, Oral Dis. 16 (2010) 360–364.
[39] Z.D. Lu, Y.P. Ye, D.C. Jiao, J.H. Qiao, S.D. Cui, Z.Z. Liu, miR-155 and miR-31 are differentially expressed in breast cancer patients and are correlated with the estrogen receptor and progesterone receptor status, Oncol. Lett. 4 (2012) 1027–1032.

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