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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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