Aberrant MicroRNA-155 Expression Is an Early Event in the Multistep Progression of Pancreatic Adenocarcinoma

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Key Words

MicroRNA · miR-21 · miR-155 · Pancreatic intraepithelial neoplasia · PanIN

Abstract

Background/Aims: Pancreatic intraepithelial neoplasia (PanIN) is the most common noninvasive precursor to invasive pancreatic adenocarcinoma. Misexpression of microRNAs (miRNAs) is commonly encountered in invasive neoplasia; however, miRNA abnormalities in PanIN lesions have not been documented. **Methods:** Three candidate miRNAs (miR-21, miR-155, and miR-221) previously reported as overexpressed in pancreatic cancers were assessed in 31 microdissected PanINs (14 PanIN-1, 9 PanIN-2, 8 PanIN-3) using quantitative reverse transcription PCR (qRT-PCR). Subsequently, miR-155 was evaluated by locked nucleic acid in situ hybridization (LNA-ISH) in PanIN tissue microarrays. **Results:** Relative to microdissected non-neoplastic ductal epithelium, significant overexpression of miR-155 was observed in both PanIN-2 (2.6-fold, $p = 0.02$) and in PanIN-3 (7.4-fold, $p = 0.014$), while borderline significant overexpression of miR-21 (2.5-fold, $p = 0.049$) was observed in PanIN-3 only. In contrast, no significant differences in miR-221 levels were observed between ductal epithelium and PanIN lesions by

qRT-PCR. LNA-ISH confirmed the aberrant expression of miR-155 in PanIN-2 (9 of 20, 45%) and in PanIN-3 (8 of 13, 62%), respectively, when compared with normal ductal epithelium (0 of 10) ($p < 0.01$). **Conclusions:** Abnormalities of miRNA expression are observed in the multistep progression of pancreatic cancer, with miR-155 aberrations demonstrable at the stage of PanIN-2, and miR-21 abnormalities at the stage of PanIN-3 lesions. Copyright © 2010 S. Karger AG, Basel and IAP

Introduction

Pancreatic adenocarcinoma (i.e., pancreatic cancer) has one of the lowest 5-year survival rates (<4%) amongst solid cancers [1]. The near uniform mortality of this disease arises from the fact that most patients present at an advanced stage of malignancy, rendering their carcinomas surgically inoperable. In contrast, the minor subset of patients who have pancreatic cancer localized to the organ have a significantly better prognosis, approaching 25–30% 5-year survival rates following surgical resection [2, 3]. These figures reiterate the critical importance of diagnosing pancreatic neoplasia at an early, and potentially curable, stage [4].

It is now established that invasive pancreatic cancers do not arise *de novo*, but are preceded by histologically distinct noninvasive precursor lesions within the pancreatic ducts [5]. The most common precursors are known as pancreatic intraepithelial neoplasias (PanINs), microscopic lesions of <5 mm in diameter [6, 7]. PanINs demonstrate a defined histological progression from low-grade PanIN-1 through intermediate grade PanIN-2, and culminating in the high-grade PanIN-3 ('carcinoma-in-situ'), which is thought to precede the onset of invasion. Not unexpectedly, this histological progression is mirrored by the sequential accumulation of molecular abnormalities within PanINs [8]. Some genetic alterations can be observed even in low-grade PanIN-1 lesions (for example, telomere attrition or activating mutations of the *KRAS2* gene) [9, 10], while other events are essentially restricted to the highest-grade PanIN-3 lesions and invasive cancers (such as, *BRCA2* gene mutations or expression of the GPI-anchored protein mesothelin) [11, 12]. Elucidation of molecular abnormalities in PanINs has implications for not only understanding the pathogenesis of invasive cancer, but also because it can lead to the development of better early detection tools as well as rational chemoprevention targets, both of which are critical for ameliorating the rather dismal prognosis of this disease [13].

MicroRNAs (miRNAs) are a class of 18–24 nucleotide non-coding RNAs, whose principal function is to regulate the translation of coding mRNA transcripts [14]. Physiologic regulation of the cellular transcriptome by miRNAs plays a critical role during development and in mature tissue homeostasis. Aberrant expression of miRNA is common in human cancers, and miRNAs can be over- or underexpressed in neoplastic cells compared to their normal counterparts [15, 16]. The underlying basis for aberrant miRNA expression in cancer can be manifold, including genomic alterations (amplifications and deletions) [17–19], epigenetic mechanisms [20–22], or altered transcription factor regulation [23, 24]. In many instances, the coding mRNA targets of aberrant miRNAs have been elucidated, and include transcripts whose protein products regulate critical cell growth, cell death, and metastatic machineries in cancer cells [25–29]. Not surprisingly, recent studies have also identified multiple aberrantly expressed miRNAs in pancreatic cancers compared to normal pancreatic tissues [30–35]. The patterns of miRNA abnormalities in the noninvasive precursor lesions of pancreatic cancer, however, remain largely unknown. The objective of our study was to profile three candidate miRNAs reported to be overexpressed in inva-

sive adenocarcinoma of the pancreas (miR-21, miR-155, and miR-221) in microdissected PanIN lesions of various histological grades, and to compare their relative expression to that of microdissected non-neoplastic pancreatic ductal epithelium. We demonstrate that significant overexpression of miRNAs, and in particular miR-155, can be observed in PanINs, occurring as early as at the stage of PanIN-2 lesions. Thus, comparable to genetic alterations, miRNA expression abnormalities are another integral feature of the multistep progression model of pancreatic ductal adenocarcinoma.

Materials and Methods

PanIN Samples and Laser Microdissection

Cryostat embedded frozen sections of 31 PanIN specimens were obtained from surgical resection specimens at the Johns Hopkins Hospital. In each case, the PanIN lesion had been observed at the margin of resection during intraoperative consultation by an expert pancreatic pathologist (R.H.H.), and was assigned a histological grade according to previously described consensus criteria [6, 7]. Briefly, the margin specimens were snap-frozen in liquid nitrogen, and embedded in Tissue-Tek OCT compound medium (Sakura Finetek USA, Torrance, Calif., USA). Upon confirmation of a PanIN lesion at the margin, additional unstained sections were sectioned onto UV-treated PALM® membrane slides (Carl Zeiss MicroImaging, Inc., Thornwood, N.Y., USA) for the purpose of laser microdissection using the PALM® MicroBeam platform (fig. 1). The slides were kept frozen at –70°C until they were microdissected. Of the 31 PanIN samples, there were 14 PanIN-1, 9 PanIN-2, and 8 PanIN-3 lesions. Non-neoplastic ductal epithelium adjacent to PanIN lesions was microdissected from 8 independent specimens as control. The microdissected tissue samples were then subjected to small RNA extraction using the mirVana™ miRNA Isolation kit (Ambion/Applied Biosystems, Austin, Tex., USA), according to the manufacturer's protocol [36]. The minimum RNA concentration required for quantitative reverse transcription PCR (qRT-PCR) is in the range of ~10 ng/μl. Therefore, we microdissected a minimum of 1,000 cells within a given duct profile by laser capture, using 4–5 serial 5-μm sections from cryostat embedded sections of PanIN lesions.

Quantitative Reverse Transcription PCR for miRNA

qRT-PCR of miRNAs were performed using pre-designed TaqMan® miRNA assays (Applied Biosystems, Foster City, Calif., USA) with the 7300 real-time RT-PCR system (Applied Biosystems), as previously described [36]. The assays are a two-step protocol, including reverse transcription with human mature miRNA-specific RT primers, followed by real-time PCR with miRNA-specific primers. We selected 3 candidate miRNAs – miR-21, miR-155, and miR-221 for qRT-PCR. The non-coding RNU6B (U6 control) was used as the normalization control, as previously described [36, 37]. Each sample was assessed in triplicate for any given miRNA, and the mean relative fold expression for each histological grade of PanIN compared to ductal epithelium was calculated using the $2^{-\Delta\Delta C_t}$ method, as previously described [38].

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Fig. 1. Microdissection of pancreatic intraepithelial neoplasia lesions. **a** HE-stained PALM[®] frozen slide shows the PanIN-1 lesions. **b** After laser microdissection, the PanIN lesions were selectively removed without surrounding cell contamination.

Locked Nucleic Acid in situ Hybridization

Locked nucleic acid in situ hybridization (LNA-ISH) was performed on archival PanIN lesions using LNA[™] probes for miR-155 (Exiqon, Woburn, Mass., USA), in order to validate the results of qRT-PCR. The PanIN tissue microarrays (TMAs) used in this analysis were created by 1 of the authors (A.M.), and have been previously described [12, 39–41]. We and others have described the use of LNA-ISH on archival tissues for semiquantitative assessment of miRNA expression [36, 42]. Briefly, after deparaffinization, the slides were incubated in proteinase K solution, and subsequently, in 0.2% glycine. Thereafter, the slides were fixed in 4% paraformaldehyde and then washed in phosphate-buffered saline (PBS). After rinsing in PBS, the slides were pre-hybridized with hybridization buffer for 2 h at room temperature, and then incubated with hybridization buffer containing the digoxigenin labeled LNA[™] probe in an oven at 53°C overnight. A parallel set of PanIN TMAs was hybridized with a scrambled miRNA probe (negative control; Exiqon), as previously described [36]. After stringent washes with 50% formamide and 2× SSC at 53°C, the slides were blocked with blocking buffer for 1 h and incubated with anti-digoxigenin Fab fragment (1:2,000) overnight in a humid chamber at 4°C. The colorimetric detection reaction was performed using NBT/BNI ReadyMix (Thermo-Scientific, Rockford, Ill., USA) in the dark for 48 h. After stringent washes, the slides were stained with Mayer hematoxylin, and then mounted with cover-slips using CytoSeal 60 (Thermo Scientific). The TMAs were scored on a multi-headed microscope by 2 of the authors (J.K.R and S-M.H.). The LNA-ISH results were scored based on the intensity of hybridization as 0 (negative), 1 (weak), or 2 (strong), and based on the percentage of positive epithelial cells as 0 (<1%), 1 (focal, 1–50%) or 2 (diffuse, >50%), respectively. The relative intensity of the scrambled probe was used as background control. An arbitrary 'ISH-score' for each lesion was generated as the product of intensity multiplied by area, as we have recently described [36]. The 'ISH-score' was

then binned into a 2-tier classification of 'negative' (score 0), and 'positive' (score ≥1).

Statistical Analysis

Statistical significance was assessed by the Mann-Whitney U test and Fisher's exact tests to compare the data of qRT-PCR and ISH, respectively, using Prism version 4.00 (GraphPad Software Inc., San Diego, Calif., USA), and $p < 0.05$ was considered as statistically significant.

Results

We performed qRT-PCR to assess the mean relative levels of 3 candidate miRNAs (miR-21, miR-155, and miR-221) in 31 microdissected PanIN lesions stratified by histological grade, compared to 8 microdissected samples of non-neoplastic ductal epithelium. We observed that miR-155 was significantly overexpressed in both PanIN-2 (2.6-fold, $p = 0.02$) and in PanIN-3 (7.4-fold, $p = 0.014$) lesions, while no significant differences in expression were seen in the PanIN-1 lesions versus non-neoplastic ductal epithelium (fig. 2). We also observed borderline significant overexpression of miR-21 (2.5-fold, $p = 0.049$) in PanIN-3 lesions compared to non-neoplastic ductal epithelium, while these differences were not significant for PanIN-1 and 2 lesions. In contrast, no significant differences were observed for miR-221 levels between ductal epithelium and any of the 3 histological grades of PanIN lesions. Overall, our qRT-PCR data suggested that miR-155 dem-

Fig. 2. Relative fold expression of miRNAs in microdissected PanIN lesions compared to non-neoplastic ductal cells. **a** Micro-RNA-21 showed borderline significant overexpression in only PanIN-3 lesion compared to non-neoplastic ductal epithelium (2.5-fold, ^a $p = 0.049$). **b** Significant overexpression of miR-155 was noticed in both PanIN-2 (2.6-fold, ^a $p = 0.02$) and in PanIN-3 (7.4-fold, ^b $p = 0.014$), and significant differences in expression were also seen in the PanIN-3 versus PanIN-2 lesions (^c $p = 0.003$). **c** No significant differences were observed for relative miR-221 levels between ductal epithelium and any of the three histological grades of PanIN lesions.

Table 1. Frequency of miR-155 expression in PanIN lesions by LNA-ISH

miRNA	Ductal epithelium	PanIN-1	PanIN-2	PanIN-3	Total (PanIN)
miR-155	0/10 (0%)	0/4 (0%)	9/20 (45%)*	8/13 (62%)**	17/37 (46%)**

* $p < 0.05$; ** $p < 0.01$ compared with ductal epithelium.

onstrated the earliest aberration amongst the 3 candidate miRNAs, being significantly upregulated even at the level of PanIN-2, with a progressive elevation in PanIN-3 (the differences in mean relative expression levels between the PanIN-2 and PanIN-3 grades were also statistically significant, $p = 0.027$). Of the 8 PanIN-3 lesions, 6 demonstrated >5-fold relative expression compared to non-neoplastic ductal epithelium.

Based on the qRT-PCR results, we wanted to confirm the overexpression of miR-155 in a larger panel of

PanINs, which we accomplished using LNA-ISH on archival TMAs. Overall, 37 evaluable PanIN lesions (with matched scrambled miRNA control) were available for analysis, including 4 PanIN-1, 20 PanIN-2, and 13 PanIN-3 lesions. None of the 10 non-neoplastic ductal epithelial cores on the TMA labeled for miR-155; similarly, the 4 PanIN-1 lesions were also negative (table 1). In contrast, 9 of 20 PanIN-2 (45%) and 8 of 13 PanIN-3 (62%) labeled with the miR-155 probe (fig. 3), validating the trend observed in the qRT-PCR results.

Discussion

A series of recent studies in pancreatic adenocarcinoma tissues and cell lines has established that most pancreatic cancers aberrantly express miRNAs [30–35]. Although these individual studies have used different strategies including qRT-PCR and array-based approaches to elucidate differentially expressed miRNAs, certain candidates have emerged as a common thread across multiple laboratories. For example, miR-21, miR-155 and miR-221, the 3 candidates selected here for analysis in PanIN lesions, were each found to be differentially upregulated in pancreatic cancer compared to benign pancreatic tissues in 4 or more independent reports [30–35]. To the best of our knowledge, this is the first study to explore miRNA expression in PanINs, which are the most common noninvasive precursor lesions of invasive pancreatic cancer [5].

We demonstrate that a subset of the miRNAs differentially overexpressed in invasive pancreatic cancers are also overexpressed in noninvasive PanIN lesions [8, 12]. Specifically, within our panel of miRNAs, miR-155 appears to be an ‘early’ event in the multistep progression model, as significant overexpression can be observed even at the stage of PanIN-2 lesions. In contrast, miR-21 is a ‘late’ event, mostly overexpressed in PanIN-3 and beyond, while the overexpression of miR-221 appears to be restricted to invasive adenocarcinoma. Of note, none of these 3 miRNAs were overexpressed in PanIN-1, which could either be reflection of the fact that these low-grade lesions lack miRNA abnormalities or that such alterations are present in non-coding RNAs outside our selected panel. Given that PanIN-1 lesions harbor discernible genetic abnormalities including telomere attrition and *KRAS2* gene mutations [9, 10], the latter is a distinct possibility.

Fig. 3. In situ hybridization for miR-155 in PanIN lesions. **a, b** No staining is seen with both miR-155 (**a**) and scramble probe (**b**) in PanIN-1 lesion. $\times 400$. **c, d** In situ hybridization shows strong miR-155 expression (**c**; $\times 400$) in PanIN-2 lesion. Note both cytoplasmic and nuclear staining in ductal epithelial cells contrast to negative staining of scramble probe (**d**; $\times 400$). **e, f** In situ hybridization shows strong miR-155 expression (**e**; $\times 200$) in PanIN-3. Note both cytoplasmic and nuclear staining in ductal epithelial cells contrast to negative staining of scramble probe (**f**; $\times 200$).

Although initially identified as an overexpressed miRNA in hematological malignancies [43, 44], miR-155 has now been reported as upregulated in multiple solid cancers in addition to pancreatic cancer [45–49]. While the precise mechanism(s) by which miR-155 might contribute to tumorigenesis remains under investigation, a recent study has shown that overexpressed miR-155 in pancreatic cancer appears to repress the function of tumor protein 53-induced nuclear protein 1 (TP53INP1); the latter is a pro-apoptotic, p53-induced protein, and the miR-155-mediated downregulation of TP53INP1 in pancreatic cancer enhances tumorigenicity in vivo [50]. Although miR-155 levels were not directly examined in PanIN lesions in the aforementioned study, an immunohistochemical analysis demonstrated loss of TP53INP1 in PanINs, which potentially may have been secondary to miR-155 overexpression [50]. This miRNA has also recently shown to be a target of the transforming growth factor β (TGF β) pathway [51]. These accumulating lines of evidence, in conjunction with our own findings, suggest that miR-155 is likely to play an important role in both tumor initiation and tumor progression vis-à-vis pancreatic cancer.

In addition to its biological significance, miR-155 also harbors the potential to emerge as a potential biomarker for early detection of pancreatic neoplasia. For example, we have previously shown that it is also overexpressed in another precursor lesion to invasive pancreatic cancer, namely intraductal papillary mucinous neoplasm (IPMN) [36]. In this previous study, we had demonstrated that miR-155 levels are elevated in cyst fluid samples obtained from patients with noninvasive IPMNs, but not in other benign pancreatic cysts [36]. Elevated miR-155 levels may also be detectable in other clinically relevant samples (e.g., pancreatic juice or serum) obtained from patients at risk for pancreatic cancer. miRNAs are shorter than coding mRNAs, and therefore more resistant to ribonuclease degradation, thus remaining amenable to quantitative measurement not only in archival tissues, but also in serum [52]. One example of such an at risk cohort would be asymptomatic individuals from high-risk familial pancreatic cancer kindreds that are undergoing endoscopic screening for early disease [53, 54]; many of these patients harbor multi-focal PanINs (including high-grade lesions) or IPMNs in their pancreata [55], and miR-155 expression in the pancreatic juice might provide a diagnostic adjunct in cases borderline for malignancy. In this context, miR-21, whose expression appears to be restricted to PanIN-3 lesions, might prove an even more specific biomarker for lesions at highest risk of progres-

sion to invasive cancer. We caution, however, that we cannot predict on the utility of either miR-155 or miR-21 as a biomarker for pancreatic cancer based on this pilot analysis restricted to archival PanIN samples. We anticipate that future studies in a larger patient cohort, including individuals being sampled for chronic pancreatitis (the most common clinical mimic of adenocarcinoma), would be warranted to better address the issue of miRNAs as an early detection biomarker.

In conclusion, we have profiled 3 miRNAs known to be overexpressed in invasive pancreatic cancer in a panel of PanIN lesions of varying histological grades. Our findings identify miR-155 deregulation as an early event in the multistep progression of pancreatic cancer, while

miR-21 and miR-221 abnormalities occur later, either at the stage of carcinoma-in-situ or in invasive adenocarcinomas. In addition to expanding the repertoire of molecular alterations described in PanINs, this study lays the foundation for pursuing miRNA expression in clinical samples as a strategy for the early diagnosis of pancreatic cancer.

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