MicroRNA-21 Is Induced Early in Pancreatic Ductal Adenocarcinoma Precursor Lesions

Mael C. du Rieu,1 Jérôme Torrisani,1 Janick Selves,2 Talal Al Saati,2 Anny Souque,1 Marlène Dufresne,3 Gregory J. Tsongalis,4 Arief A. Suriawinata,4 Nicolas Carrère,1,5 Louis Buscail,1,6 and Pierre Cordelier1*

BACKGROUND: Pancreatic ductal adenocarcinoma (PDAC) has the poorest overall prognosis among gastrointestinal cancers; however, curative resection in early-stage PDAC greatly improves survival rates, indicating the importance of early detection. Because abnormal microRNA production is commonly detected in cancer, we investigated noninvasive precursor pancreatic intraepithelial neoplasia (PanIN) lesions for microRNA production as a potential early biomarker of PDAC.

METHODS: Pathologists identified and classified ductal lesions. We extracted total RNA from laser-capture microdissected PanIN tissue samples from a conditional Kras(G12D) mouse model (n = 29) or of human origin (n = 38). MicroRNA production was quantified by quantitative real-time PCR. Internal controls included 5S and U6 RNAs.

RESULTS: Production of microRNAs miR-21, miR-205, and miR-200 paralleled PanIN progression in the Kras(G12D) mouse model, compared with microRNA production in samples of nonpathologic ducts. miR-21 demonstrated the highest relative concentrations in the precursor lesions. Interestingly, miR-205 and miR-21 up-regulation preceded phenotypic changes in the ducts. The production of microRNAs miR-21, miR-221, miR-222, and let-7a increased with human PanIN grade, with peak production occurring in hyperplastic PanIN-2/3 lesions. In situ hybridization analysis indicated miR-21 production to be concentrated in pathologic ductal cells. miR-21 production was regulated by Kras(G12D) and epidermal growth factor receptor in PDAC-derived cell lines.

CONCLUSIONS: Aberrant microRNA production is an early event in the development of PanIN. Our findings indicate that miR-21 warrants further investigation as a marker for early detection of PDAC.

Pancreatic ductal adenocarcinoma (PDAC) is the fourth-leading cause of cancer mortality, with a 5-year survival rate of <5% (1). The poor prognosis has been attributed to the inability to make a diagnosis while the tumor is still resectable and to a propensity toward early vascular dissemination and spread to regional lymph nodes. Up to 60% of patients have advanced PDAC at the time of diagnosis; the median survival time is only 3–6 months (1). PDAC is highly resistant to cytotoxic chemotherapy and ionizing radiation, but the fact that the 5-year survival rate can reach 40% with curative resection in early-stage PDAC emphasizes the importance of early detection (2). Improving early detection and treatment urgently requires the development of new PDAC markers. Substantial progress has been made in understanding the precursors that ultimately give rise to invasive PDAC. PDAC precursors comprise intraductal papillary mucinous neoplasm, mucinous cystic neoplasm, and pancreatic intraepithelial neoplasia (PanIN) (3). PanINs are noninvasive microscopic epithelial neoplasms, which are located in the smaller pancreatic ducts, and are characterized by cyto logical and architectural atypia. PanINs are divided into 3 grades according to the degree of epithelial atypia (3): flat and papillary lesions without dysplasia (PanIN-1A and PanIN-1B, respectively), papillary lesions with moderate dysplasia (PanIN-2), and lesions with marked atypia similar to in situ carcinoma (PanIN-3). Gene alterations of PanINs include activating point mutations.
in the KRAS8 (v-Ki-ras2 Kirsten rat sarcoma viral onco-
gene homolog) gene, and inactivation of the CDKN2A
(cyclin-dependent kinase inhibitor 2A (melanoma,
p16, inhibits CDK4)), TP53 (tumor protein p53), and
SMAD4 (SMAD family member 4) genes (3). Interest-
ingly, recently developed genetically engineered mouse
models expressing mutated KRAS in the pancreas have
been shown to develop PanIN lesions that are histologi-
cally identical to those in humans (4, 5). MicroRNAs
are endogenous small noncoding RNAs that negatively
regulate gene expression at the posttranscriptional
level by base-pairing to the 3′ untranslated region of
target mRNAs (6–8). These tiny but potent molecules
regulate various physiological and pathologic path-
ways, such as cell differentiation and cell proliferation.
Recently, microRNA alterations have been linked to
the initiation and progression of human cancer, in-
cluding PDAC (6–9). Consequently, profiling of
microRNA production in human tumors has identi-
fied signatures associated with diagnosis, staging, pro-
gression, prognosis, and response to treatment (6–8).
In addition, the utility of microRNAs surpasses that of
mRNAs for molecular characterization of archived
clinical samples, regardless of the fixation protocol
used (10–12); however, the profiles of microRNA pro-
duction in precursor lesions remain largely unknown.
Therefore, we evaluated the production of a panel of
candidate microRNAs in microdissected samples of
nonpathologic pancreatic ducts and PanIN lesions
from a conditional KRAS(G12D) mouse model and
from patients with PDAC.

Materials and Methods

CLINICAL SAMPLES

The policies of the ethics committees at the Centre
Hospitale-Universitaires of Toulouse and Bordeaux
and at the Cancéropole Grand Sud-Ouest (France)
were followed throughout the study. We obtained
pancreatic tissue samples from patients undergoing
pancreatic surgery after they had provided informed
consent. Histopathology faculty sampled cancerous
pancreatic tissue along with matched adjacent non-
pathologic tissue. Total RNA was extracted with
the Qiagen RNeasy Micro Kit according to the manufac-
turer’s recommendations. RNA quality and concen-
tration were evaluated as described above. The miScript
PCR System (Qiagen) was used according to the manufac-
turer’s recommendations. RNA quality and concen-
tration were evaluated with the Experion automated elec-
trophoresis system (Bio-Rad Laboratories) and the
NanoDrop ND-1000 spectrophotometer (Thermo Sci-
entific), respectively.

MOUSE STRAINS

Conditional LSL-KRASG12D/+ and Pdx1-Cre+/−
mouse strains were bred to generate LSL-KRASG12D/+/
Pdx1-Cre+/− (KRAS8) mice, as previously described
(5). The LSL-KRASG12D/+ mouse strain was obtained
from the Mouse Models of Human Cancers Consor-
tium Repository at the National Cancer Institute. The
Pdx1-Cre+/− mouse strain was obtained from the lab-
ory of Douglas A. Melton (Department of Mole-
cular and Cellular Biology, Harvard University, Cam-
bridge, MA) (4). The IFR150 animal care committee
approved all procedures.

PanIN SAMPLES AND MICRODISSECTION

Two of the authors (M.C.R., J.S.) independently
examined pancreatic tissue by light microscopy for
the presence of PanINs; the 2 assessments agreed in all
cases. Ductal lesions were classified according to
the WHO classification (13). We used an Arcturus ARC
2000 microscope to microdissect 500 cells from frozen,
cryostat-embedded PanIN lesions generated in a con-
ditional KRAS(G12D) mouse model, (PanIN-1A, n =
6; PanIN-1B, n = 7; PanIN-2, n = 3; and PanIN-3, n =
3). Total RNA was extracted from 2 to 3 microdissec-
tion capsules with the RNAqueous-Micro Kit (Am-
bron/Applied Biosystems) according to the manufac-
turer’s recommendations. The study of human samples
was approved by the Dartmouth Committee for the
Protection of Human Subjects and The Norris Cotton
Cancer Center Research Committee. All patients
signed an informed-consent form. For human sam-
ple, we used the RecoverAll™ Total Nucleic Acid Isola-
tion Kit for FFPE (Applied Biosystems) to prepare
total RNA from formalin-fixed, paraffin-embedded
macrodissected PanIN lesions (nonpathologic pan-
creas, n = 4; PanIN-1A, n = 8; PanIN-1B, n = 13;
PanIN-2, n = 6; PanIN-3, n = 7). RNA quality and
concentration were evaluated as described above.

microRNA QUANTIFICATION BY QUANTITATIVE REAL-TIME PCR
(qRT-PCR)

The miScript PCR System (Qiagen) was used according
to the manufacturer’s instructions to quantify mature
microRNAs from 100 ng total RNA. U6 and 5S
RNAs were used as controls. cDNA samples were di-
luted to 1 part in 100 for microRNA detection or to 1
part in 10 000 for U6 and 5S RNA detection. Duplicate
qRT-PCR assays were then carried out in a StepOne-
Plus™ Real-Time PCR System (Applied Biosystems)
according to the manufacturer’s instructions with
SYBR Green PCR Master Mix (Qiagen). Relative
amounts of microRNA were calculated by the compar-

* Genes: KRAS, v-Ki-ras2 Kirsten rat sarcoma viral oncogene homolog; CDKN2A, cyclin-dependent kinase inhibitor 2A (melanoma, p16, inhibits CDK4); TP53, tumor protein p53; SMAD4, SMAD family member 4; MIR21, microRNA 21; Mus musculus gene Kras, v-Ki-ras2 Kirsten rat sarcoma viral oncogene homolog; BRCA2, breast cancer 2, early onset.
miR-21 in Pancreatic Intraepithelial Neoplasia

IN SITU HYBRIDIZATION FOR microRNA DETECTION
Locked nucleic acid in situ hybridization (LNA-ISH) analysis was performed on PanIN lesions with LNA™ probes for the microRNA miR-21 (Exiqon). Paraffin-embedded PanIN lesions were deparaffinized in xylene and rehydrated in descending grades of ethanol. The slides were incubated in proteinase K solution (10 mg/L) for 5 min at 37 °C and subsequently in 2 g/L glycine. The slides were then fixed in 40 g/L paraformaldehyde for 10 min at room temperature. After rinsing in PBS, the slides were prehybridized in hybridization buffer [500 mL/L formamide, 5× standard saline citrate (SSC) (0.75 mol/L NaCl and 0.075 mol/L sodium citrate), 1 mL/L Tween 20, 9.2 mmol/L citric acid, pH 6, containing 500 mg/L yeast RNA] for 2 h at room temperature in a humidified chamber and then incubated overnight in hybridization buffer containing 20 nmol/L digoxigenin-labeled LNA probe in an oven at 50 °C. Hybridization with a scrambled microRNA probe (Exiqon) was used as a negative control. Slides were rinsed in 2× SSC (0.30 mol/L NaCl and 0.030 mol/L sodium citrate) at 50 °C, washed 3 times under stringent conditions (500 mL/L formamide and 2× SSC at 50 °C for 30 min), and, finally, washed 3 times at room temperature in PBS containing 0.5 mL/L Tween 20 (PBST). Slides were incubated in blocking buffer (20 mL/L sheep serum and 2 g/L BSA in PBST) for 1 h and then incubated overnight with antidigoxigenin Fab fragment (1 part in 2000; Roche Diagnostics) in a humidified chamber at 4 °C. Slides were washed 7 times in PBST for 5 min each and then washed 3 times in alkaline phosphatase buffer (100 mmol/L Tris-HCl, pH 9.5, 50 mmol/L MgCl2, 100 mmol/L NaCl, and 1 mL/L Tween 20) for 5 min each. The colorimetric detection reaction was performed in the dark for 48 h with NBT/ BNI Ready-to-Use tablets (Roche Diagnostics). After 3 washes in PBST, coverslips were mounted on the slides with Glycergel Mounting Medium (Dako). Slides were examined with the aid of a Nikon E400 optical microscope coupled to an image analyzer (VisioLab 2000; Biocom). We analyzed 15 fields at 100× magnification for each sample.

miR-21 PROMOTER ANALYSIS
We PCR-amplified the full length of the MIR21 (microRNA 21) gene promoter (~410 to +38) from genomic DNA prepared from PDAC-derived MIA PaCa-2 cells, as described previously (14). The miR-21 promoter was cloned into a pGL3 luciferase reporter vector (Promega) to yield pGL3-pmiR21. We then transfected 2.5 × 10^5 PDAC-derived MIA PaCa-2 cells with 4 μg of the pGL3-pmiR21 construct. We also transfected HPNE cells either stably expressing or not expressing a retroviral copy of Kras(G12D) (15) (kind gift of Dr. M. Ouellette, University of Nebraska Medical Center, Omaha, NE). Control cells were transfected with pGL3 plasmid. Cells were cotransfected with 1 μg control vector encoding the Renilla luciferase (Promega) with the aid of Fugene® 6 (Roche Diagnostics; 1 part Fugene and 3 parts cells), according to the manufacturer’s recommendation. Transforming growth factor β (TGF-β) (10 μg/L; Euromedex) or cetuximab (5 mg/L Erbitux®; Merck Liphra Santé) were added to the culture 24 h after transfection. Firefly and Renilla luciferase activities were measured consecutively with the Dual-Glo® Luciferase Assay System (Promega) 24 h after TGF-β, Erbitux, or vehicle treatment.

STATISTICAL ANALYSIS
Results are expressed as the mean and SE. Statistical analyses were performed on ΔCT data. GraphPad InStat software (GraphPad Software) was used for statistical analysis of data with the unpaired 2-tailed Student t-test. P values <0.05 were considered statistically significant.

Results
We first assessed the production of a panel of candidate microRNAs for which the concentrations are altered in cancer and used a training set of 7 PDAC tumor samples matched with adjacent nonpathologic tissue samples. 5S and U6 small RNAs were used as internal controls. We selected the following microRNAs: miR-21, which promotes cell survival and cell proliferation (16); miR-221 and miR-222, which are involved in tumor angiogenesis (17); let-7a, a well-characterized inhibitor of cell proliferation (8); miR-29c, which regulates the epigenome (18); and miR-200 and miR-205, which regulate the epithelial-to-mesenchymal transition (19). With qRT-PCR analysis, we demonstrated the up-regulation of miR-21 [20.6-fold (0.6-fold), P < 0.005], miR-221 [8.75-fold (0.8-fold), P < 0.005], miR-222 [13.5-fold (0.8-fold), P < 0.005], miR-200 [4.5-fold (0.6-fold), P < 0.01], and miR-205 [65.1-fold (3-fold), P < 0.005] in PDAC samples, compared with the matching adjacent nonpathologic tissue samples. miR-29c was minimally up-regulated, and let-7a production was down-regulated [5-fold (0.6-fold), P < 0.005] (Fig. 1). Several different genetically engineered mouse models of pancreatic exocrine neoplasia have been developed (20). Hingorani et al. generated a mouse model of PanIN with conditional KRAS(G12D) mice in which endogenous production of mutant KRAS in the pancreas is activated by a Pdx1-Cre transgene (5). We confirmed that this model develops the entire panel of PanIN lesions (Fig. 2). We next ex-
tracted total RNA from frozen microdissected PanIN lesions. Nonpathologic ducts from LSL-KRAS\(^{G12D/+}\)/ Pdx1-Cre\(^{-/-}\) littermates were used as a control. Included in this study were nonpathologic ducts surrounding the PanIN lesions from KRAS\(^*\) mice. qRT-PCR analysis revealed that miR-21, miR-205, and miR-200 production paralleled PDAC progression in this mouse model (Fig. 3). On the other hand, miR-222 production decreased in PDAC precursor lesions, and miR-29c, miR-221, and let-7a production remained unchanged. Interestingly, miR-205 and miR-21 up-regulation preceded phenotypic changes in the ducts. Next, we measured the production of the candidate microRNAs in dissected PanINs of human origin. Ducts from nonpathologic pancreas were used as a control. Fig. 4 shows that miR-21, miR-221, miR-222, miR-200, and miR-205 production increased with human PanIN grade, with peak production occurring in PanIN-2/3 lesions. Interestingly, the production of the antiproliferative microRNA let-7a was also increased in high-risk PanIN lesions. With these results in mind and because this microRNA is a key oncogenic factor in many cancers (16), we focused our attention on miR-21, which demonstrated the highest relative concentration in both experimental models. We used in situ hybridization on archived PDAC tumors to determine the cellular localization of miR-21. Overall, 20 PanIN lesions were available for analysis. Scramble microRNA was used as a control. The LNA-ISH results (Fig. 5) indicated that miR-21 production also occurs in human PanIN lesions, but the scramble probes failed to display specific staining. miR-21 was readily detectable in 2 (40%) of 5 PanIN-1 lesions, in 8 (67%) of 12 PanIN-2 lesions, and in 3 (100%) of 3 PanIN-3/in situ

Fig. 1. Production of candidate microRNAs in PDAC tumors.
Results are expressed as the -fold change in the microRNA produced in PDAC tissue, compared with matching adjacent tissue. Data are presented as the mean and SE. **P < 0.01; ***P < 0.005.

Fig. 2. Histologic features of early-stage (top panels) and advanced-stage (bottom panels) PanIN in mice with endogenous KRAS(G12D) expression.
Fig. 3. Production of candidate microRNAs in laser-capture material of PanIN lesions developed in the pancreas of mice bearing the KRAS(G12D) mutation.

Normal duct, results from a duct from a LSL-KRAS<sup>G12D</sup>/Pdx1-Cre<sup>−/−</sup> mouse; KRAS<sup>*</sup> duct, results from a nonpathologic duct from a KRAS<sup>*</sup> mouse. Data are presented as the mean and SE. *P < 0.05; **P < 0.01; ***P < 0.005.
Fig. 4. Production of candidate microRNAs in human PanIN lesions.
Normal duct, results from a duct from a LSL-KRAS<sup>G12D</sup>+/Pdx1-Cre<sup>−/−</sup> mouse. Data are presented as the mean and SE. *P < 0.05; **P < 0.01; ***P < 0.005.
PDAC lesions, but it was not detectable in nonpathologic ducts (data not shown). These data confirm the qRT-PCR results. miR-21 production was detected mainly in pathologic ductal cells, as well as a few positive stromal cells. We next investigated the mechanisms by which miR-21 was induced in pancreatic carcinogenesis. We constructed a reporter plasmid in which the miR-21 promoter was fused to the 5’ end of the firefly luciferase gene. This plasmid was transfected into PDAC-derived MIA PaCa-2 cells and from human nestin-positive cells purified from ducts, expressing or not expressing a retroviral copy of Kras(G12D) (15).

We found that the miR-21 promoter was active in pancreas-derived cell lines. Fig. 6A shows that the miR-21 promoter displays clear Kras(G12D) induction. Erbitux, a well-characterized inhibitor of the epidermal growth factor receptor (EGFR), significantly reduced miR-21 promoter activity. Erbitux also reduced miR-21 concentrations in MIA PaCa-2 cells, confirming the positive effect of the EGFR transduction pathway on miR-21 production (Fig. 6C). On the other hand, TGF-β significantly reduced the activity of the miR-21 promoter but failed to alter miR-21 production in MIA PaCa-2 cells (Fig. 6, B and C).

Discussion

Recent studies of populations at high risk for PDAC [family history of PDAC or a genetic defect that increases PDAC risk, such as BRCA2 (breast cancer 2, early onset) mutation] demonstrated that individuals with a diagnosed precursor lesion could be treated before their curable pancreatic neoplasm progresses to incurable invasive disease (21–23). Thus, early detection is a paramount challenge to ameliorate PDAC prognosis. Imaging technologies currently are sensitive enough to identify intraductal papillary mucinous neoplasms but are insufficient for detecting PanIN lesions. Canto et al. recently suggested that endoscopic ultrasound–based screening of asymptomatic high-risk individuals can detect prevalent resectable pancreatic neoplasia; however, false-positive diagnoses also frequently occur (24). In addition, the differential diagnosis of PDAC and pseudotumoral forms of chronic
pancreatitis is frequently difficult because of the similar imaging and clinical presentations (25). Several protein and nucleic acid markers have been shown to have diagnostic potential, but these markers have failed to demonstrate diagnostic specificity and sensitivity in clinical applications (26). The broad changes in microRNA profiles at late stages of PDAC have recently been reported (6–8). Szafranska et al. found that the ratio of the production of miR-196 to that of miR-217 provides a reliable index for identifying diseased pancreatic tissues (27, 28). Moreover, Bloomston et al. demonstrated that few microRNAs could differentiate PDAC from chronic pancreatitis or predict poor survival (29). We found that let-7 production was lost in advanced PDAC (9); however, microRNA production patterns in PDAC precursor lesions have scarcely been investigated. Habbe et al. recently proposed miR-155 as a potential biomarker for intraductal papillary mucinous neoplasms (30), but no studies have elucidated microRNA production across the progression of PanIN stages to PDAC.

We confirmed that 6 of the 7 candidate microRNAs that we selected for analysis in PanINs are differentially regulated in PDAC, compared with matching adjacent nonpathologic tissue. Although downregulated in lung cancer (18), miR-29c production is unaffected in PDAC. We next asked whether the production of our candidate microRNAs was altered within the different histologic grades of PanIN lesions. We first examined the feasibility of our approach in the KRAS* murine model, which corresponded perfectly with pancreatic carcinogenesis (4, 5). Because the pancreas consists primarily of acinar cells that can mask discrete preneoplastic lesions, we microdissected fresh frozen PanIN lesions and analyzed them by qRT-PCR.
for their microRNAs. We found that miR-221 and let-7 concentrations were not altered in PanIN progression relative to the results for nonpathologic duct cells. These results strongly suggest that altered production of these 2 microRNAs is restricted to invasive PDAC. miR-21 and miR-200 production increased with PanIN grade, whereas miR-205 was up-regulated regardless of the PanIN grade. Interestingly, miR-21 and miR-205 overproduction appears to be a very early event in the multistep progression model, because substantial up-regulation of these microRNAs preceded phenotypic changes in duct cells in KRAS* mice. Remarkably, we identified similar increases in the production of miR-21, miR-200, and miR-205 in KRAS(G12D)/Pdx1-Cre mice that developed the entire spectrum of PanIN lesions (data not shown). The latter finding excludes the possibility of interexperimental errors in our approach. What remains intriguing is the decrease in miR-222 production with PanIN grade in this model. This finding may be evidence for a late “angiogenic switch” in PDAC. We completed our experimental approach by analyzing formalin-fixed, paraffin-embedded human PanINs and demonstrated that microRNAs were not significantly affected by formalin fixation, a finding that shows the clear advantage of this class of nucleic acids for biomarker discovery over the use of mRNAs (10–12). We found that miR-21, miR-221, miR-222, let-7a, miR-205, and miR-200 production increased with human PanIN grade, with peak production occurring in high-risk PanIN-2/3 lesions. With the exception of miR-221 and miR-222, these results are consistent with the data obtained with the mouse model. The discrepancy regarding the differential production of miR-221 and miR-222 may be due to the multiple genetic changes in the human lesions compared with the Kras(G12D) mouse model. Interestingly, let-7a was increased in advanced PanIN lesions. Such overproduction may reflect an attempt at negative feedback by the ductal epithelium in the face of activation of other mitogenic pathways (e.g., Kras-mediated signaling). Although we have described, for the first time, the production patterns for 7 microRNAs in microdissected pancreatic cancer precursor lesions, we have provided only a snapshot of the overall microRNA signature of these lesions. We performed microRNA microarray hybridization, but the paucity of the collected material prevented more than only a small fraction of the tested lesions from being informative. In these assays, we did not use the step of preamplifying small RNAs, because the linearity of microRNA measurements could not be guaranteed. Future studies based on the use of highly sensitive, validated microRNA qRT-PCR arrays are needed to extend our work.

We next focused our attention on miR-21, which demonstrated the highest relative concentration in both PanIN models, because this microRNA is a key oncogenic factor in many cancers (16). Still, miR-21 is an early event in the multistep progression of PDAC precursor lesions. Finally, we used LNA-ISH analysis to confirm the increased production of miR-21 in human PDAC. We conclude that miR-21 may emerge as a potential molecular marker for detecting PDAC; however, its value for detecting cancers in general, and PDAC in particular, remains to be established. The measurement of tumor-derived microRNAs in serum or plasma has recently emerged as an important approach for blood-based detection of human cancers (31), including PDAC (32). Future studies aimed at detecting miR-21 in the blood of mice diagnosed with PanINs will help to better define the potential of this microRNA as an early molecular marker of PDAC. The reasons for the increase in miR-21 production with PDAC progression remain unknown. miR-21 has recently been linked to the activation of the transcription factor AP-1 in response to RAS (33). Thus, it is not surprising that PanINs originating in the KRAS(G12D) model display high concentrations of this microRNA; however, activating point mutations in codon 12 of the Kras gene occur in about half of the nonpapillary duct lesions but occur in the vast majority of more-advanced papillary lesions (34, 35). Accordingly, we demonstrated that activated Kras(G12D) stimulates the miR-21 promoter in human pancreatic cells. On the other hand, EGFR production is intensified in PDAC, and EGFR activation appears to play an important role in the growth and differentiation of this type of neoplasia. In this study, we demonstrated that EGFR promotes miR-21 production in PDAC-derived cells, as has previously been described in lung carcinogenesis (36). Interestingly, alterations at the EGFR level have major importance in the response to EGFR tyrosine kinase inhibitors, whereas modifications of downstream effectors could lead to treatment resistance. Future studies need to identify whether the analysis of miR-21 production can increase the power of patient selection for anti-EGFR therapy.

In summary, our study demonstrates that miR-21 deregulation is a very early event in the multistep progression of PDAC. We conclude that miR-21 warrants further investigation as a molecular marker for PanIN, particularly for the most advanced PanIN-3 lesions, before they become invasive.

Author Contributions: All authors confirmed they have contributed to the intellectual content of this paper and have met the following 3 requirements: (a) significant contributions to the conception and design, acquisition of data, or analysis and interpretation of data; (b) drafting or revising the article for intellectual content; and (c) final approval of the published article.
References


Authors’ Disclosures of Potential Conflicts of Interest: Upon manuscript submission, all authors completed the Disclosures of Potential Conflict of Interest form. Potential conflicts of interest:

Employment or Leadership: None declared.
Consultant or Advisory Role: None declared.
Stock Ownership: None declared.
Honoraria: None declared.

Research Funding: This work was supported by grants from INSERM, Region Midi-Pyrenees, Association pour la Recherche contre le Cancer, Agence Nationale de la Recherche (ANR), and Cancérople Grand Sud-Ouest (GSO). J. Torrisani was funded by the Institut National du Cancer and the Ligue Nationale Contre le Cancer.

Expert Testimony: None declared.

Role of Sponsor: The funding organizations played no role in the design of study, choice of enrolled patients, review and interpretation of data, or preparation or approval of manuscript.

Acknowledgments: We thank Jean-Jose Maoret (molecular biology facility, IFR150, Toulouse) for his expertise in RNA detection and quantification. We thank Hubert Lulka, Pascal Clerc, and Véronique Gigoux for their management of the mouse models. Tumor samples were obtained through the Department of Pathology and the Tumor Banks of CHU de Bordeaux, CHU de Toulouse, and Cancérople Grand Sud Ouest. We thank Laurence Lamant and Cécile Desjoubert for their technical expertise for the microRNA ISH analyses.