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MicroRNA-21 in Pancreatic Cancer: Correlation with Clinical Outcome and Pharmacologic Aspects Underlying Its Role in the Modulation of Gemcitabine Activity

Elisa Giovannetti[1,2], Niccola Funel[3], Godefridus J. Peters[1], Marco Del Chiaro[3], Leyla A. Erozenci[1], Enrico Vasile[4], Leticia G. Leon[1], Luca E. Pollina[3], Annemieke Groen[1], Alfredo Falcone[4], Romano Danesi[2], Daniela Campani[3], Henk M. Verheul[1], and Ugo Boggi[3]

Abstract

MicroRNA-21 (miR-21) was reported to be overexpressed and contributes to invasion and gemcitabine resistance in pancreatic ductal adenocarcinoma (PDAC). The aim of this study was to evaluate whether miR-21 expression was associated with the overall survival (OS) of PDAC patients treated with gemcitabine and to provide mechanistic insights for new therapeutic targets. miR-21 expression was evaluated in cells (including 7 PDAC cell lines, 7 primary cultures, fibroblasts, and a normal pancreatic ductal cell line) and tissues (neoplastic specimens from 81 PDAC patients and normal ductal samples) isolated by laser microdissection. The role of miR-21 on the pharmacologic effects of gemcitabine was studied with a specific miR-21 precursor (pre-miR-21). Patients with high miR-21 expression had a significantly shorter OS both in the metastatic and in the adjuvant setting. Multivariate analysis confirmed the prognostic significance of miR-21. miR-21 expression in primary cultures correlated with expression in their respective tissues and with gemcitabine resistance. Pre-miR-21 transfection significantly decreased antiproliferative effects and apoptosis induction by gemcitabine, whereas matrix metalloproteinase (MMP)-2/MMP-9 and vascular endothelial growth factor expression were upregulated. Addition of inhibitors of phosphoinositide 3-kinase and mammalian target of rapamycin resulted in decrease of phospho-Akt and prevented pre-miR-21–induced resistance to the proapoptotic effects of gemcitabine. miR-21 expression correlated with outcome in PDAC patients treated with gemcitabine. Modulation of apoptosis, Akt phosphorylation, and expression of genes involved in invasive behavior may contribute to the role of miR-21 in gemcitabine chemoresistance and to the rational development of new targeted combinations. *Cancer Res; 70(11); 4528–38. ©2010 AACR.*

Introduction

Pancreatic ductal adenocarcinoma (PDAC) is the fourth leading cause of cancer-related death, with only 3% of patients alive 5 years after diagnosis (1). The main reasons for this grim prognosis include early metastatic spread, high local recurrence rate, and multifactorial resistance to treatments (2).

In 85% of patients, PDAC is detected at advanced stages, characterized by infiltration of proximal lymph nodes and vascular structures, as well as metastasis to liver/peritoneum. The first-line agent gemcitabine produced some clinical benefit in the advanced setting but yields a limited disease control, with <15% of patients progression-free at 6 months from diagnosis (2, 3). Although several attempts have been made to increase the survival using combinations of chemotherapy and targeted therapy, only a marginal success was achieved with gemcitabine combined with capecitabine or erlotinib, and with a four-drug regimen (4–6). According to the results of CONKO-001 and ESPAC-3 trials (7, 8), gemcitabine also increased the disease-free survival (DFS) and overall survival (OS) in the adjuvant setting. However, the most effective adjuvant chemotherapy remains unclear, and the 5-year survival in patients undergoing resection still hovers between 10% and 20% (1).

Therefore, the identification of predictive factors for gemcitabine activity seems to be critical for maximizing therapeutic efficacy and minimizing useless treatment in PDAC. Pharmacogenetic studies showed correlations of polymorphisms or expression of DNA repair enzymes and nucleoside transporters with outcome in gemcitabine-treated patients (9, 10). However, prognosis of patients harboring favorable genotypes or expression levels for these candidate biomarkers

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is still poor and novel prognostic and therapeutic approaches are warranted.

Global genomic analysis showed that PDAC resulted from aberrations of genes that function through a relatively small number of core signaling pathways (11). In addition to yielding insights into pathogenesis, such studies suggest that the best hope for the development of agents targeting nodal points in the altered pathways lies in the study of mechanisms involved in gene expression regulation.

Recently, microRNAs (miRNA) have emerged as a critical class of negative regulators of gene expression through modulation of posttranscriptional activity of multiple target mRNAs by repression of translation or direct cleavage (12). The role of miRNAs in control of proliferation, differentiation, and apoptosis; the location of several miRNA genes at sites of translocation breakpoints or deletions; and their aberrant expression in many tumors indicated that they can function as tumor suppressors and oncogenes (13). Furthermore, selected miRNAs may influence response to chemotherapy (14–16).

Expression profiling identified several miRNAs aberrantly expressed in PDAC, including four miRNAs differentially expressed in other tumors: miR-155, miR-21, miR-221 and miR-222 (17). These results were corroborated by other studies, showing that miR-21 was among the top miRNAs with increased expression in PDAC (18–20).

miR-21 has been associated with ovarian cancer carcinogenesis (21), and a pivotal role in cancer is suggested by its widespread deregulation in various solid tumors, such as glioblastoma, cholangiocarcinoma, papillary thyroid, breast, esophageal, gastric, hepatocellular, colon, prostate, lung, head and neck, and cervical cancer, as well as in hematologic malignancies (22). The oncogenic properties of miR-21 are further supported by functional studies showing that inhibition of miR-21 expression reduced proliferation of several cancer cells, including breast, hepatocellular, and PDAC cells (23–25), and generated a proapoptotic response in different cell lines, including glioblastoma, cholangiocarcinoma, and PDAC cells (22, 26, 27). In contrast, transfection with miR-21 precursors stimulated invasion, extravasation, and metastasis in *in vivo* models of glioma, colorectal, and breast cancer (28–30), as well as in cellular models of PDAC (25).

These data suggested that elevated levels of miR-21 might be associated with tumor progression, and because miR-21 is one of the most abundant and easily detectable miRNAs (31), several studies evaluated its role as a prognostic biomarker. The expression of miR-21 has been correlated with clinical stage, lymph node, and distant metastasis as well as with poor prognosis in glioma, colon, breast, and tongue squamous cell cancers (24, 28, 32–34). High miR-21 expression was associated with more aggressive pancreatic endocrine tumors, characterized by increased Ki67 proliferation index and liver metastasis (35). Furthermore, miR-21 expression was significantly lower in the eight PDAC that clustered with the benign pancreas specimens in the study performed by Bloomston and colleagues (18). *In situ* hybridization showed that miR-21 overexpression was strictly localized to PDAC cells and predictive of shorter survival in node-negative patients, but this subset of patients was small and no data were available on treatment (36). In contrast, no correlation between miR-21 and clinicopathologic findings was observed using PCR on bulk tissues from 25 PDAC patients, without information on chemotherapy (25). Therefore, further studies on a larger number of better-characterized PDAC patients, using techniques to minimize contamination by surrounding stroma, such as laser microdissection (LMD), are warranted.

Recent studies also reported significant correlations between miR-21 expression and resistance to anticancer agents (15, 23, 37). In particular, inhibition of miR-21 increased sensitivity to gemcitabine in cholangiocarcinoma and PDAC cells (25–27). However, miR-21 did not affect gemcitabine-induced apoptosis in colon cancer cells (38), whereas other miRNAs, such as miR-200 and let-7, were involved in the reversal of epithelial-to-mesenchymal transition in gemcitabine-resistant PDAC cells (39).

The aim of the present study was to characterize miR-21 expression in a wide repository of PDAC tissues and cells, associated with clinical outcome and gemcitabine activity. We observed a significant correlation between outcome and miR-21 expression in laser-microdissected tumors from gemcitabine-treated patients, both in the metastatic and in the adjuvant setting, as well as a correlation with chemosensitivity in PDAC cells. Further, we characterized several factors, including modulation of apoptosis, Akt phosphorylation, and expression of PTEN and genes involved in invasive behavior, which may contribute to miR-21 role in gemcitabine chemoresistance and provide mechanistic insights for the rational development of new targeted combinations against PDAC.

Materials and Methods

Clinical study

Patients. From December 2001 to October 2004, a total of 81 patients affected by metastatic (n = 31) or nonmetastatic (n = 50) PDAC (median age, 63; range, 32–83) and treated with gemcitabine were enrolled in a retrospective study on determinants of gemcitabine activity (10). Treatment details are in Supplementary Materials and Methods.

Tissues. RNA was extracted from biopsies and primary tumors, resected before chemotherapy, using the LMD7000 instrument (Leica Microsystems), as described previously (40). LMD was also used to obtain cells of epithelium ducts from 5 normal pancreatic tissues, obtained from the "Organ Donor Program," whereas in 10 cases RNA was extracted from the whole tumor without microdissection. All specimens were obtained according to a protocol approved by the Local Ethics Committee.

Reverse transcription and quantitative PCR analysis of miR-21. RNA (10–100 ng) was reverse transcribed and the resulting cDNA was amplified using the specific Taqman MicroRNA assays (Applied Biosystems) for miR-21 and RNU43 (assay ID, 000397 and 001095, respectively). The PCRs were performed in the 7500HT sequence detection system (Applied Biosystems), in accordance with the manufacturer's instructions. Specimens were amplified in triplicate with appropriate nontemplate controls. Amplification data were

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normalized to RNU43 expression. Quantification of relative expression [reported as arbitrary units (a.u.)] was performed using the ΔC_t method. Quantitative PCR data showed a variability coefficient of C_t always lower than 2% of mean values.

In vitro studies

Cells and cytotoxicity studies. Seven PDAC cell lines, the human pancreatic duct epithelial-like cell line hTERT-HPNE, and skin fibroblasts Hs27 were obtained from the American Type Culture Collection, whereas seven primary PDAC cultures (LPc006, LPc028, LPc033, LPc067, LPc111, LPc167, and PP437) were isolated from patients at Pisa Hospital (40). The cell growth–inhibitory effect of 72-hour gemcitabine exposure was studied as described previously (10).

Quantitative PCR analysis of miR-21. RNA was extracted according to the Trizol-chloroform protocol, and the miR-21 basal expression as well as its modulation after gemcitabine treatment using IC_{50} concentrations were assessed by quantitative PCR, as described above. Data were normalized to RNU43, and quantification of miR-21 expression compared with untreated controls was assessed using the $\Delta\Delta C_t$ method (10).

miR-21 transfection. The effect of miR-21 on chemosensitivity and apoptosis was evaluated by transfecting the PDAC cells with pre-miR-21 precursors (pre-miR-21) or antisense oligonucleotides (anti-miR-21) purchased from Ambion (assay ID, PM10206 and AM10206, respectively) at 30 nmol/L final concentration. Cells were plated at 200,000 per well in 3 mL RPMI 1640 with 10% fetal bovine serum (FBS) and 1% antibiotics. After 24 hours, cells were exposed to 9 μL Oligofectamine (Invitrogen) in serum-free medium and mixed for 10 minutes, followed by addition of 3 μL miR-21 precursor/inhibitor. Cells were also incubated with miRNA-negative controls and FAM-labeled pre-miR/anti-miR (Ambion). After 24 hours, the medium was replaced with RPMI 1640 with 10% FBS, without antibiotics. To evaluate the effects on cell growth, cells were allowed to grow for additional 48 or 72 hours in drug-free medium or treated with gemcitabine, as described previously (10, 40). To evaluate apoptosis induction

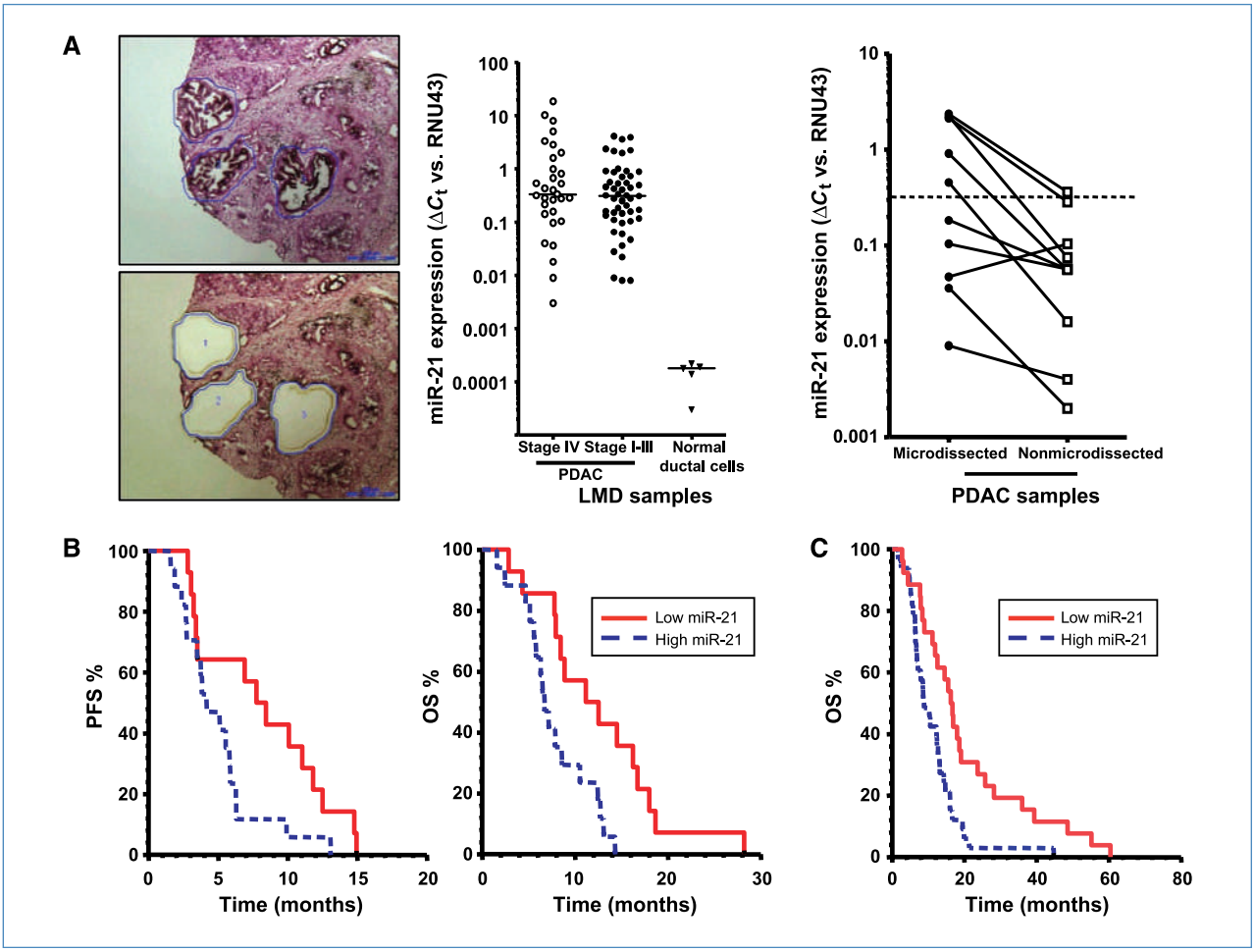

Figure 1. A, left, example of extracted tumor epithelium and stroma before and after LMD. H&E staining of 5-μm frozen sections. Original magnification, ×10. Middle, miR-21 expression in the cohort of 81 patients (31 in metastatic and 50 in stage I–III) and 5 normal pancreatic ductal tissues. Right, comparison between miR-21 expression in microdissected and nonmicrodissected samples from 10 PDAC. B, Kaplan-Meier curves of PFS (left) and OS (right) according to miR-21 in the PDAC patients in the metastatic setting. C, OS curve including both treatment settings.

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and modulation of cell signaling and invasion, cells were allowed to grow for additional 48 hours in drug-free medium or treated with 50 μmol/L gemcitabine, 10 μmol/L LY294002, and 200 nmol/L rapamycin, alone and in combinations (26), as described in Supplementary Materials and Methods. Additional control wells were used for RNA extraction, as described above, whereas the transfection efficiency with FAM-labeled pre-miR/anti-miR controls was evaluated with fluorescence microscopy.

Statistics. All experiments were performed in triplicate and repeated thrice. Data were expressed as mean ± SE and analyzed by Student's *t* test or ANOVA followed by the Tukey's multiple comparison. Comparison of clinical information and miR-21 expression was made using Pearson χ^2 test and Wilcoxon test. The relationship between miR-21 expression and outcome was evaluated by stratifying the patients with respect to the median expression value.

OS was calculated from the date of pathologic diagnosis (i.e., the date of surgery/biopsy) to the date of death, DFS was defined as the time from the date of diagnosis to the date of first relapse or death in radically resected patients, and progression-free survival (PFS) was defined as the time from the date of diagnosis to the date of progression or death in metastatic patients. OS, PFS, and DFS curves were constructed using Kaplan-Meier method, and differences were analyzed using log-rank test. The significant prognostic variables of OS in univariate analysis were included in multivariate analyses using Cox's proportional hazards model. Data were analyzed using SPSS v.17 statistical software (SPSS, Inc.). Statistical significance was set at $P < 0.05$.

Results

Clinical study

miR-21 expression in pancreatic specimens. LMD was performed on 81 samples from PDAC patients, including 7 tumor specimens from which primary cultures were derived. LMD was also performed on five specimens from normal ducts. For each sample, the precision of the focus of the laser beam allowed to pick up 5,000 cells, with high degree of accuracy and extremely low risk of contamination (Fig. 1A, video). miR-21 was detectable in all samples, and Fig. 1A shows its large variability across the tissues, with median value of 0.315 a.u. (range, 0.003–18.336). Remarkably, miR-21 expression profile differed significantly between grade 1/2 ($n = 33$) and grade 3 ($n = 35$) tumors ($P = 0.01$, Wilcoxon rank sum test). In contrast, no difference was detected in miR-21 expression levels according to stage or other clinicopathologic parameters (Table 1).

The mean miR-21 expression of normal pancreatic duct samples was ~1,000-fold lower than the levels in microdissected tumors (Fig. 1A). Furthermore, the nonmicrodissected tumor tissues had a significantly lower expression of miR-21 than their respective microdissected samples ($P = 0.014$; Fig. 1A).

miR-21 overexpression correlated with worse outcome in PDAC patients treated with gemcitabine. Clinical data were available from 31 patients in the metastatic and 28 in the adjuvant setting, followed-up until December 31, 2009, with follow-up ranging from 1.6 to 60.5 months (median, 17.3 mo). Response data are in Supplementary Table S1.

Table 1. Association of miR-21 expression with clinicopathologic covariates

Characteristic	Low miR-21 (%)	High miR-21 (%)	*P* (Wilcoxon)
No. patients			
Age (median years)			
≤63	18 (46.2)	21 (53.8)	0.64
>63	22 (52.4)	20 (47.6)	
Sex			
Male	20 (46.2)	23 (53.5)	0.96
Female	20 (52.6)	18 (47.4)	
Clinical stage			
I–II	16 (48.5)	17 (51.5)	0.39
III–IV	23 (50.0)	23 (50.)	
Lymph node			
Negative	4 (57.1)	3 (42.9)	0.47
Positive	18 (48.6)	19 (51.4)	
Vascular infiltration			
No	14 (66.7)	17 (70.8)	0.68
Yes	7 (33.3)	7 (29.2)	
Neural infiltration			
No	17 (81.0)	15 (62.5)	0.91
Yes	4 (19.0)	9 (37.5)	
PanIN			
No	7 (33.3)	10 (43.5)	0.17
Yes	14 (66.7)	13 (56.5)	
Grading			
1–2	21 (63.6)	12 (36.4)	0.01
3	9 (25.7)	26 (74.3)	
Setting			
Metastatic	14 (45.2)	17 (54.8)	0.82
Adjuvant	12 (42.9)	16 (57.1)	

NOTE: Data on age and sex were available from 81 patients, on stage from 79 patients, on grading from 68 patients, on vascular and neural infiltration from 45 patients, and on lymph node infiltration and pancreatic intraepithelial neoplasia from 44 patients.
Abbreviation: PanIN, pancreatic intraepithelial neoplasia.

Table 2 summarizes the clinicopathologic characteristics and their relation with outcome in metastatic patients. The median PFS and OS were 5.5 and 8.4 months, respectively. The high miR-21 expression group had a poorer prognosis than the low expression group. Patients with miR-21 expression above median had a significantly shorter median OS [6.7; 95% confidence interval (95% CI), 5.5–7.9 mo] compared with patients with miR-21 expression lower than median [11.2; 95% CI, 4.5–17.8 mo; hazard ratio (HR), 3.1; 95% CI, 1.4–7.1; $P = 0.01$]. Similar results were obtained with the PFS curves of patients with miR-21 expression above median, with a median of 4.2, compared with 7.8 months in patients with the lowest miR-21 expression (HR, 2.4; 95% CI, 1.1–5.3;

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$P = 0.03$). The OS and PFS Kaplan-Meier curves are shown in Fig. 1B.

Table 3 summarizes the clinicopathologic characteristics and their accordance with OS in radically resected patients. The median OS was 16.0 months (95% CI, 14.3–17.7). No association was observed between OS and the studied characteristics, except miR-21. Patients with miR-21 expression above median had a median OS of 13.2 months (95% CI, 8.3–18.0), whereas the remaining patients had a median OS of 23.7 months (95% CI, 12.3–35.0; HR, 3.1; 95% CI, 1.4–7.3, $P = 0.008$). Similar data were reported for DFS [8.8 mo (95% CI, 4.7–18.0) versus 23.6 mo (95% CI, 12.3–35.0)], for miR-21 above and below the median, with HR = 4.4 (95% CI, 1.8–10.7; $P = 0.001$).

Univariate analysis of patients in the adjuvant and in the metastatic setting ($n = 59$) showed that the treatment setting was a significant prognostic factor of OS, whereas stage showed a trend toward significant association, and age, gender, and infiltration were not correlated with outcome (Table 3). However, a significant difference in survival curves was still found according to miR-21 expression levels, with OS of 8.8 (95% CI, 5.8–11.8) versus 16.2 (95% CI, 13.3–19.2) months (HR, 2.3; 95% CI, 1.3–4.1; $P = 0.007$; Fig. 1C). Multivariate analysis indicated that the adjuvant setting of therapy and the high miR-21 expression were independent predictors of PDAC prognosis (HR, 0.3; 95% CI, 0.1–0.6, with $P < 0.001$ for adjuvant setting, and HR, 3.1; 95% CI, 1.2–5.3, with $P = 0.003$ for miR-21 expression above median, respectively).

In vitro studies

Gemcitabine cytotoxicity. A dose-dependent inhibition of cell growth was observed after gemcitabine treatment in all PDAC cells (Fig. 2A), with IC_{50}s ranging from 5.5 ± 0.7 nmol/L (LPc028) to 38.1 ± 3.0 nmol/L (PL45).

miR-21 is expressed in all PDAC cells and significantly increased after gemcitabine treatment. The expression of miR-21 was detectable in all PDAC cell lines/cultures as well as in hTERT-HPNE cells and Hs27 fibroblasts. However, this expression differed among cells, ranging from 4.5 a.u. in PL45 to 0.1 a.u. in Hs27 cells (Fig. 2A). miR-21 expression levels in primary cultures were correlated to the expression detected in their respective tissues (Supplementary Fig. S1).

Although the small sample size of cells used in this study precluded the assessment of the predictive value of miR-21 expression data as validated determinants of chemosensitivity, the Spearman test showed a trend toward significant correlation ($P = 0.08$) between the miR-21 expression and gemcitabine IC_{50}s (Fig. 2B). Furthermore, cells with miR-21 expression below the median had significantly lower IC_{50}s than cells with miR-21 expression above the median (Fig. 2C).

To evaluate whether gemcitabine affects miR-21 expression *in vitro*, we measured the levels of miR-21 in 13 PDAC cells/cultures after 72-hour exposure to gemcitabine at IC_{50}. This treatment resulted in a significant increase of miR-21 expression, ranging from 2.1- to 19.1-fold, in comparison with basal expression (Fig. 2D).

miR-21 inhibits gemcitabine antiproliferative effects and apoptosis induction in PDAC cells. To explore the role of miR-21 on antiproliferative effects and apoptosis induction after gemcitabine, relatively sensitive (LPc028 and LPc067) and resistant (LPc111 and LPc006) cells were transfected with miR-21–specific inhibitor and precursor. Transfection efficiency was evaluated by analysis of fluorescent microscope images of cells transfected with specific FAM-labeled pre-miR/anti-miR. These tests showed at least 60% efficiency for both transfection conditions in each cell type, with >70% cell viability. Furthermore, we studied miR-21 expression by quantitative PCR in three of the four transfected cultures,

Table 2. Clinical outcome according to clinical characteristics and miR-21 expression in the patients in the metastatic setting

Characteristic	Patients, *n* (%)*	Response/evaluable patients, *n* (%)*	*P*	PFS, mo (95% CI)	*P*	OS, mo (95% CI)	*P*
No. patients	31	3/31 (9.7)		5.5 (3.4-7.7)		8.4 (7.1-9.7)	
Baseline characteristics							
Age (y)							
≤63	18 (58.1)	2/18 (11.1)	0.99	4.2 (2.9–8.4)	0.67	7.9 (7.5–8.3)	0.22
>63	13 (41.9)	1/13 (7.7)		5.8 (2.9–8.7)		11.2 (3.2–19.1)	
Sex							
Male	22 (71.0)	1/22 (4.5)	0.19	5.1 (2.9–7.4)	0.53	7.9 (5.8–9.9)	0.96
Female	9 (29.0)	2/9 (22.2)		9.9 (4.3–10.7)		12.4 (1.1–23.8)	
miR-21 expression							
≤Median	14 (45.2)	2/14 (14.3)	0.58	7.8 (5.0–10.5)	0.03	11.2 (4.5–17.8)	0.01
>Median	17 (54.8)	1/17 (5.9)		4.2 (2.3–6.0)		6.7 (5.5–7.9)	

NOTE: Response, OS, and PFS data were available from all the 31 patients. No individuals were alive at last contact (event rate, 100%).

*Percentage was calculated with respect to *n* of the correspondent characteristic.

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Table 3. Survival according to clinical characteristics and miR-21 expression

Characteristic	Adjuvant setting			Adjuvant + palliative setting		
	n	OS, mo (95% CI)	*P*	*n*	OS, mo (95% CI)	*P*
No. patients	28	16.0 (14.3–17.7)		59	12.4 (9.7–15.2)	
Age (median years)						
≤63	12	16.7 (9.8–23.5)	0.66	34	12.3 (8.6–15.9)	0.97
>63	16	13.2 (3.5–22.9)		25	12.5 (8.5–16.5)	
Sex						
Male	13	16.0 (13.5–18.5)	0.59	35	10.5 (6.0–15.0)	0.19
Female	15	16.7 (9.3–24.0)		24	13.1 (10.6–15.5)	
Clinical stage						
I–II	19	15.5 (10.3–20.8)	0.77	19	15.5 (10.3–20.8)	0.05
III–IV	8	19.0 (14.5–23.5)		39	10.5 (5.8–15.3)	
Lymph node						
Negative	2	6.4	0.71	2	6.4	0.71
Positive	25	16.7 (14.5–18.8)		25	16.7 (14.5–18.8)	
Vascular infiltration						
No	19	16.0 (12.0–20.0)	0.93	19	16.0 (12.0–20.0)	0.93
Yes	9	19.0 (12.2–25.8)		9	19.0 (12.2–25.8)	
Neural infiltration						
No	22	16.7 (12.7–20.6)	0.70	22	16.7 (12.7–20.6)	0.70
Yes	6	13.2 (2.2–24.2)		6	13.2 (2.2–24.2)	
PanIN						
No	9	16.7 (14.7–18.6)	0.71	9	16.7 (14.7–18.6)	0.71
Yes	19	16.0 (13.0–19.0)		19	16.0 (13.0–19.0)	
Grading						
1–2	12	19.5 (11.9–27.1)	0.13	18	16.7 (14.0–19.4)	0.12
3	15	14.7 (10.1–19.4)		18	12.3 (9.7–14.9)	
Setting						
Metastatic	—	—	—	31	8.4 (7.2–9.7)	<0.001
Adjuvant	—	—		28	16.0 (14.3–17.7)	
miR-21 expression						
≤Median	12	23.7 (12.3–35.0)	0.008	26	16.2 (13.3–19.2)	0.003
>Median	16	13.2 (8.3–18.0)		33	8.8 (5.8–11.8)	

NOTE: Data on age, sex, treatment, and miR-21 were available from all; on stage from 27 of 28 patients in the adjuvant and from all in the metastatic setting; on grading from 27 of 28 patients in the adjuvant and from 9 of 31 patients in the metastatic setting; on lymph node infiltration from 27 of 28 patients in the adjuvant setting; and on vascular/neural infiltration and pancreatic intraepithelial neoplasia from the 28 patients in the adjuvant setting.

showing a 3-, 2-, and 1.8-fold increase of miR-21 expression in LPc067, LPc111, and LPc028 cells, respectively (Supplementary Fig. S2). The increased expression of miR-21 was associated with ~15% increased proliferation and reduced apoptosis in cells transfected with pre-miR-21, suggesting that aberrant expression of this miRNA enhanced cell growth.

Transfection with pre-miR-21 resulted in significant reduction of gemcitabine antiproliferative effects, with increase of 72-hour exposure to gemcitabine at IC_{50}s from 1.3 ± 0.3 nmol/L (LPc028), 7.2 ± 0.6 nmol/L (LPc006), 5.5 ± 0.9 nmol/L (LPc067), and 16.6 ± 2.0 nmol/L (LPc111) to 15.6 ± 2.2, 44.5 ± 3.1, 37.6 ± 4.2, and 85.1 ± 11.4 nmol/L, respectively (Fig. 3A).

The effects on apoptosis induction by gemcitabine were studied on LPc028 and LPc067 cells, which were relatively sensitive to gemcitabine antiproliferative effects but characterized by miR-21 expression above and below the median, respectively. The different assays allowed the evaluation of early apoptosis, late apoptosis, and global cell death, showing similar results (Supplementary Fig. S3). Both cultures showed decreased gemcitabine-induced apoptosis when transfected with pre-miR-21. In LPc067 cells, early apoptosis was reduced from 38% to 23%, whereas late apoptosis was reduced from 8% to 5%, as detected by Annexin V assay. In contrast, LPc067 cells transfected with anti-miR-21 had increased cell death, and analysis of typical apoptotic morphology showed ~10% increased apoptotic index in gemcitabine-treated cells. Similarly, only 12% of LPc028 underwent apoptosis after gemcitabine treatment in cells transfected with pre-miR-21, whereas a higher percentage (28%) was found after gemcitabine

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exposure in cells treated with miRNA control (Fig. 3B). Gemcitabine exposure in cells transfected with anti-miR-21 significantly increased apoptotic index up to 39%.

To further investigate the effects of miR-21 on pathways involved in inhibition of apoptosis, cells were transfected with pre-miR-21 and treated with gemcitabine and agents targeting Akt/protein kinase B/mammalian target of rapamycin (mTOR) pathway, such as LY294002 and rapamycin. The apoptotic index after LY294002 or rapamycin treatment was <8% in both cell cultures. These values were reduced around 3% to 5% in cells transfected with pre-miR-21. The combination of gemcitabine with rapamycin slightly increased the apoptotic index in both cell cultures, whereas the combination of gemcitabine with LY294002 resulted in an additive effect on apoptosis induction (+6%) only in LPc067 cells. These combinations were not able to reverse the antiapoptotic effect of pre-miR-21 transfection. However, the combination of all the three drugs (i.e., gemcitabine + LY294002 + rapamycin)

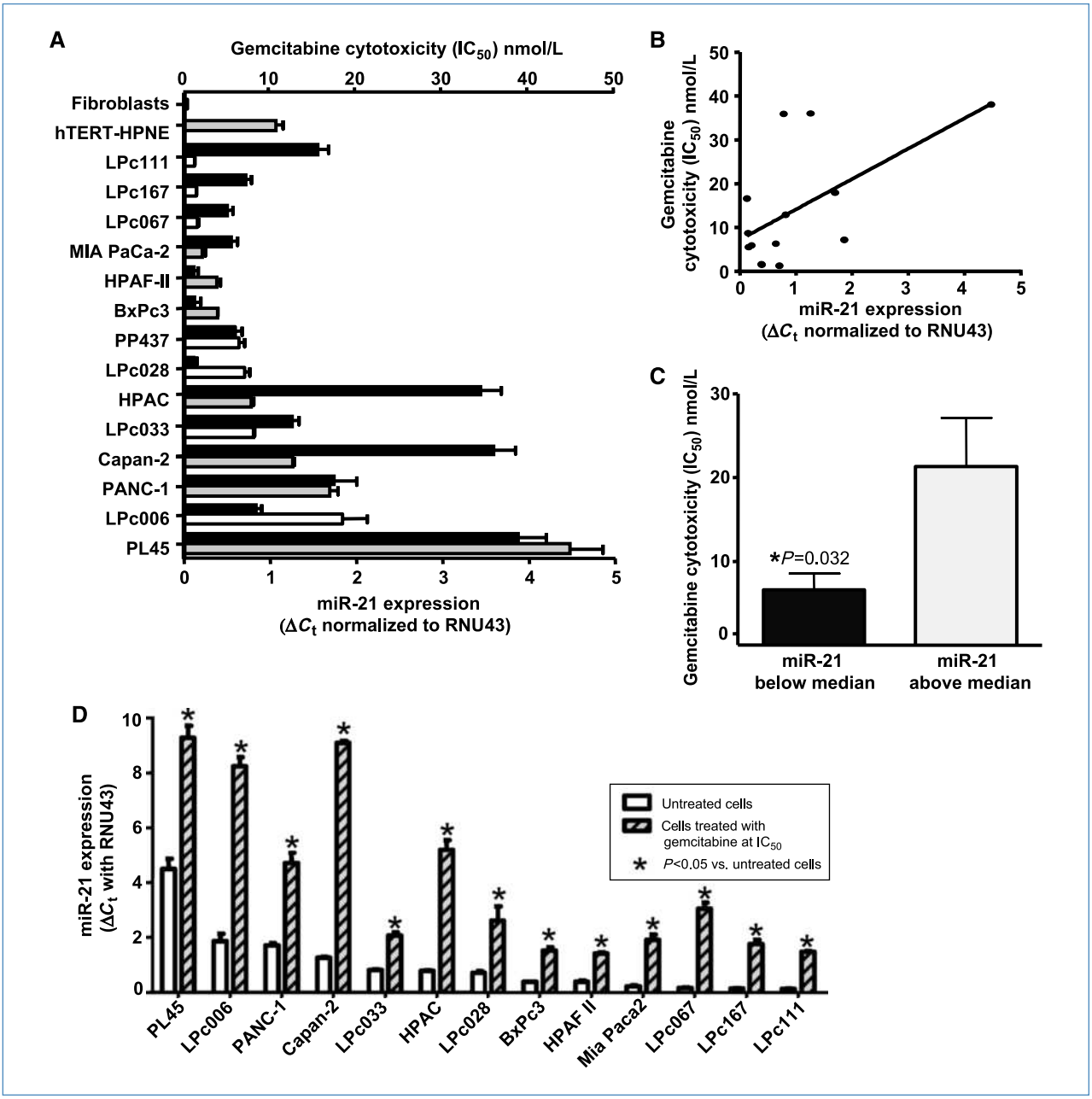

Figure 2. A, gemcitabine IC_{50}s (black columns) and expression values of miR-21 in PDAC cell lines (gray columns), primary cultures (white columns), hTERT-HPNE cells, and Hs27 fibroblasts. B, correlation between miR-21 expression and gemcitabine cytotoxic activity. C, analysis of median IC_{50} in cells with miR-21 expression above and below the median. D, significant modulation of miR-21 expression in PDAC cells treated with gemcitabine at IC_{50}. Columns, mean from three independent experiments; bars, SE.

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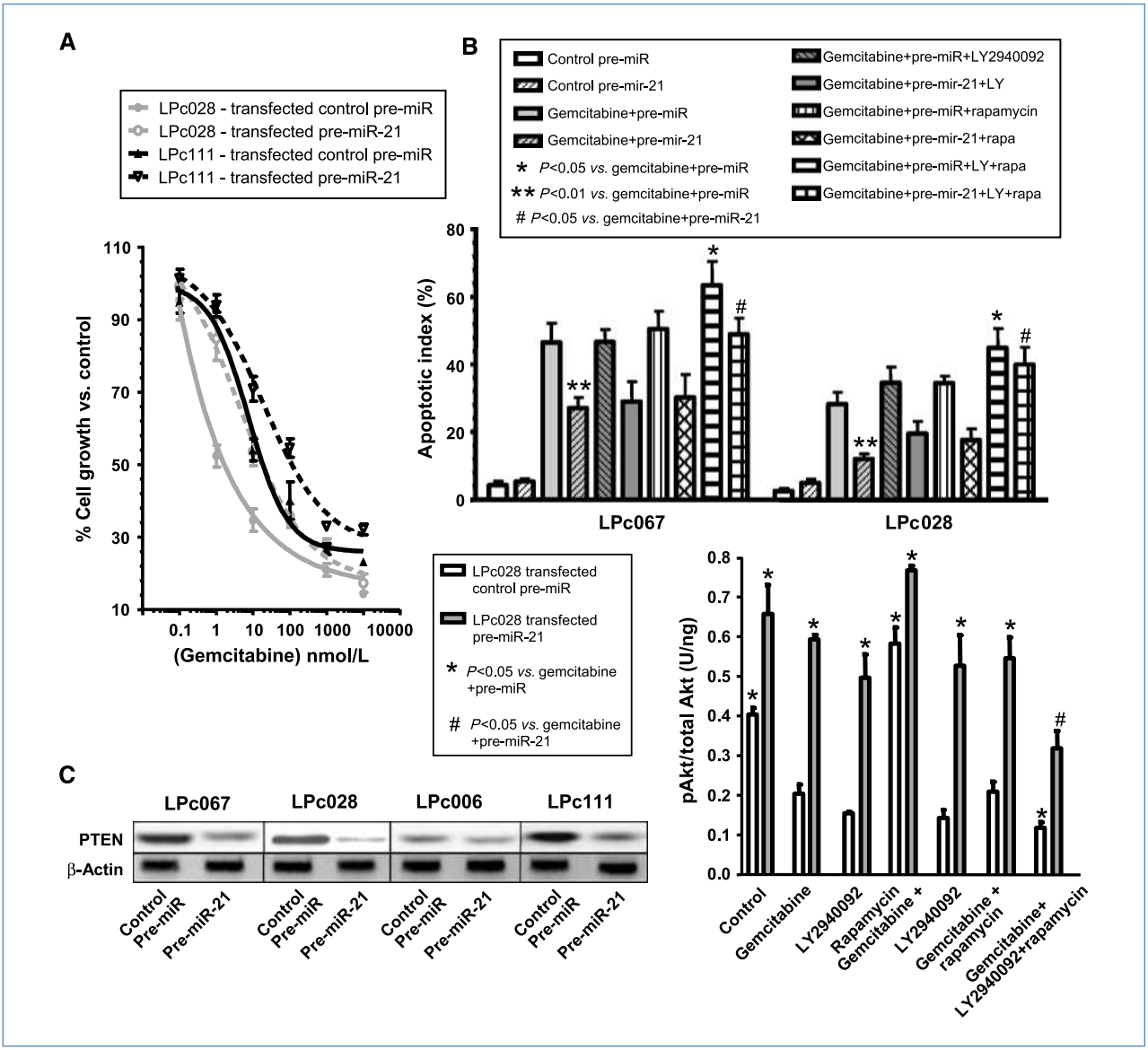

Figure 3. A, representative curves of growth-inhibitory effects of 72-h gemcitabine exposure in cells transfected with pre-miR-21. B, apoptosis after pre-miR-21 transfection and exposure to gemcitabine, LY294002, rapamycin, and their combinations. C, representative blots of Western blotting analyses of modulation of PTEN expression (left) and modulation of Akt phosphorylation by pre-miR-21 transfection, gemcitabine, LY294002, rapamycin, and their combinations (right). Columns, mean; bars, SE.

significantly increased the apoptotic index with respect to gemcitabine alone and reversed the antiapoptotic effect observed in the cells transfected with pre-miR-21. The apoptotic index in LPc067 cells transfected with pre-miR-21 and treated with gemcitabine + LY294002 + rapamycin was 49% ($P < 0.05$ versus 28% of LPc067 cells transfected with pre-miR-21 treated with gemcitabine). Similar results were observed in the LPc028 cells (Fig. 3B).

miR-21 affects PTEN and Akt expression. Previous studies showed that (*a*) miR-21 regulates expression of PTEN and phosphorylation of its downstream kinase Akt (24, 26, 33) and (*b*) the reduction of phospho-Akt (pAkt) correlated with the enhancement of gemcitabine-induced apoptosis and antitumor activity *in vitro* and *in vivo*, suggesting that Akt pathway plays a significant role in mediating drug resistance in PDAC cells (41). Therefore, we investigated the PTEN expression and Akt phosphorylation status before and after pre-miR-21 transfection and drug treatment.

Pre-miR-21 transfection reduced PTEN expression in all the cell cultures (Fig. 3C), whereas tumors with high miR-21 expression had lower PTEN expression (Supplementary Fig. S4).

Akt phosphorylation was evaluated in two cell cultures, including LPc028, described previously, and LPc006 (relatively resistant to gemcitabine and with high miR-21 expression). Transfection with pre-miR-21 resulted in an increase of pAkt/Akt ratio, ranging from +24% to +63% in LPc006 and

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LPc028 cells, respectively. In contrast, transfection with anti-miR-21 significantly reduced pAkt/Akt ratio, from 0.198 to 0.092 U/ng in LPc006 and from 0.403 to 0.142 U/ng in LPc028 cells.

Gemcitabine exposure slightly reduced pAkt/Akt ratio in LPc006 but significantly affected this ratio in LPc028 cells. Similarly, LY294002 significantly impaired the activation status of Akt in both cell cultures. In contrast, in LPc006 cells, rapamycin hardly affected Akt phosphorylation at serine residue pS473 nor total Akt levels but increased pAkt/Akt ratio in LPc028 cells. In the LPc006 cells, the lowest levels of pAkt/Akt compared with control were observed after exposure to the combination of gemcitabine and LY294002. However, pAkt/Akt ratio was potently (>50%) downregulated by the combination of rapamycin with gemcitabine and LY294002 in both LPc006 and LPc028 cells (Fig. 3C).

Furthermore, the combination of gemcitabine and LY294002 after pre-miR-21 transfection resulted in 18% decrease of pAkt/Akt ratio with respect to untreated pre-miR-21–transfected cells, but a more pronounced inhibition was detected after the combination of all three drugs (−32%) in LPc006-transfected cells. Similar results were found after the combination of gemcitabine, rapamycin, and LY294002 in LPc028 cells, with pAkt/Akt ratio reduced to 0.318 U/ng.

miR-21 enhanced metalloproteinase expression and vascular endothelial growth factor expression/secretion. Because miR-21 has been reported to have proinvasion and proangiogenic effects, we evaluated the expression of possible markers of these activities.

As marker for invasion, we investigated mRNA expression of matrix metalloproteinase-2 (*MMP-2*) and *MMP-9* in LPc067 cells transfected with miR-21, showing a 5.6- to 5.9-fold increase ($P < 0.05$). As marker for angiogenesis, we evaluated both vascular endothelial growth factor (*VEGF*) mRNA expression levels and VEGF secretion into the medium, showing an increase after miR-21 transfection of +20% and +104% (LPc028) and +36% and +58% (LPc067), respectively (Supplementary Fig. S5).

Discussion

This study evaluated the effect of miR-21 on the outcome of PDAC and, to our knowledge, is the first to show its association with PFS and OS in advanced PDAC patients treated with gemcitabine. Furthermore, we observed a significant association between miR-21 expression and DFS/OS in patients who underwent radical resection and were treated with gemcitabine in the adjuvant setting.

Recent trials supported the use of chemotherapy in radically resected patients, but the most effective regimen (gemcitabine or 5-fluorouracil/leucovorin) remains unclear (7, 8). Similarly a phase 3 trial showed that a cisplatin–epirubicin–5-fluorouracil–gemcitabine regimen obtained a 1-year survival rate of 38.5%, which was significantly better than single-agent gemcitabine (6). However, there are still no guidelines for selecting treatment for PDAC both in the adjuvant and in the metastatic setting.

Several molecular predictors of response and toxicity to chemotherapy in PDAC are being investigated, including germ-line markers such as polymorphisms (9), tumor-related molecular markers such as mutations, and aberrations in mRNA/protein expression (11, 42). Over the last few years, miRNA emerged as a prominent class of gene regulators, and their aberrant expression was linked to different tumors, including PDAC (13, 17 18). Several studies suggested their use for diagnostic purpose, showing that expression pattern of 217 miRNAs classified poorly differentiated tumors better than data from 16,000 mRNA (43). However, a miRNA can regulate multiple coding genes related to tumor growth and is also likely to more effectively reflect the status and outcome of a disease.

More than 500 miRNAs are expressed in human cells, but high-throughput screenings identified a limited number of key miRNAs. Since its identification as the miRNA most strongly upregulated in glioblastoma, miR-21 has attracted the attention of researchers in various fields (22). *In situ* hybridization showed strong miR-21 expression only in PDAC cells but not in the surrounding stroma (36). Interestingly, in the present study, we observed a significant lower expression of miR-21 in 10 tumor samples, which were not obtained by LMD, than in their corresponding laser-microdissected specimens. This suggests that LMD succeeded in eliminating the stroma, which can mask the true expression of miR-21. The study of Dillhoff and colleagues (36) also reported that miR-21 overexpression was predictive of shorter survival in node-negative but not in all the patients. However, this subset of patients was small ($n = 17$), and they had a significantly longer OS than patients with positive lymph nodes. Previous studies reported controversial data about the prognostic role of lymph node and staging, and most PDAC patients have American Joint Committee on Cancer stage >2A at diagnosis (44), as in our population.

Several studies showed that more advanced/malignant tumors expressed higher levels of miR-21 (32–35). However, in gastric carcinomas, in which miR-21 can serve as a diagnostic marker, its levels did not seem to have prognostic value (45), whereas reports in patients with diffuse large B-cell lymphoma suggested that high levels of miR-21 in tumor and serum were associated with better prognosis (46).

These controversial data suggested that the prognostic role of miR-21 is possibly tumor specific as well as treatment related. Indeed, miR-21 expression was correlated with resistance to several anticancer agents in different models (15, 23, 26, 37). In particular, inhibition of miR-21 increased sensitivity and apoptosis induction by gemcitabine in PDAC but not in colon cancer cells (25, 38).

The present study revealed that PDAC cells with miR-21 expression below the median had significantly lower gemcitabine IC_{50}s than cells with miR-21 expression above the median. miR-21 expression was similar in PDAC cultures and their respective tissues, as reported in glioblastoma cultures and tumors (22), suggesting the suitability of these *ex vivo* models for further molecular analysis. However, miR-21 expression was detectable at similar levels in PDAC and hTERT-HPNE cells. These results can be explained by the fact that although hTERT-HPNE cells (47) have a normal phenotype

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(diploid, with wild-type $p16^{INK4a}$, K-Ras, and p53), miR-21 expression may be related to the immortalization of these cells by the ectopic expression of hTERT, as reported previously in the immortalized pancreatic ductal cells HPDE (25).

Of note, miR-21 expression was increased after exposure to gemcitabine in all PDAC cells, suggesting that this miRNA can also contribute to acquired chemoresistance and explain the short time of response/stabilization in most PDAC patients. However, no tissues from previously treated patients were available, and high levels of miR-21 may not only characterize cancers but also represent a common feature of pathologic growth or stress, as observed in models of mouse hypertrophic heart (48).

Further, we studied the activity of miR-21 on pharmacologic effects of gemcitabine and molecular pathways involved in its activity. Increased expression of miR-21 following transfection with a specific precursor led to a significant reduction of antiproliferative effects and apoptotic index in cells treated with gemcitabine, as reported previously (25, 27). Computational algorithms predict hundreds of mRNA as possible targets for miR-21, but only a few of them have been experimentally validated and are involved in apoptosis regulation. Previous studies showed that both miR-21 and anti-miR-21 modulated a luciferase construct containing PTEN 3′ untranslated region and the expression of PTEN *in vitro*, whereas the staining of PTEN was reversely correlated with miR-21 in tongue squamous cell carcinoma (24, 26, 33). Other reports did not find changes in PTEN expression after transfection with miR-21 precursors in PDAC cells (25), but in the present study, transfection with pre-miR-21 resulted in reduction of PTEN expression, which was also negatively correlated with miR-21 expression in 14 PDAC samples. Furthermore, in agreement with the hypothesis that overexpression of miR-21 leads to downregulation of PTEN and a more active signaling through the phosphoinositide 3-kinase (PI3K)–Akt pathway, rendering the cancer cells less susceptible to apoptosis, we found that increased miR-21 expression was associated with activation of PI3K/Akt/mTOR pathway. Then, we showed that drugs targeting PI3K/Akt/mTOR pathway reduced pAkt levels and enhanced apoptosis when used in combination with gemcitabine. These findings are consistent with studies showing that addition of PI3K inhibitors to gemcitabine-treated cells decreased pAkt and increased apoptosis (41). In contrast, rapamycin treatment alone resulted in highly increased pAkt levels compared with untreated cells and cells treated with gemcitabine. These results can be explained by the ability of rapamycin to inhibit the mTORC1-mediated pathway through dephosphorylation of several downstream effectors, including S6K1, which acted as antagonist of PI3K/Akt/mTOR pathway (49). Therefore, rapamycin-mediated inhibition of mTORC1 resulted in induction of Akt activity (50). However, the pAkt/Akt ratio was strongly downregulated by the combination of rapamycin with gemcitabine and LY294002, and the combination of the three drugs overcame the resistance to apoptosis caused by pre-miR-21 transfection, yielding useful information on critical targets to reduce chemoresistance.

Another recently identified miR-21 target is RECK, which might mediate miR-21 invasiveness and angiogenesis by inhibiting MMPs (22). Therefore, we studied mRNA expression of *MMP-2* and *MMP-9*, as well as mRNA and protein expression of VEGF, which were positively correlated with miR-21 expression, as reported in other PDAC cells (25).

The consistency and strength of the accumulating preclinical data, together with our clinical data on correlation with outcome, strongly suggest that PDAC cells are more aggressive and resistant to gemcitabine if they have high expression of miR-21, which therefore represents a promising target for prognostic and therapeutic approaches.

About the prognostic use of miR-21, further validation in prospective studies is warranted, and more accessible samples sources, such as miR-21–enriched tumor-derived exosomes from blood, should be investigated. Similarly, the modulation of miR-21 targets seems as a promising adjuvant to current therapies of PDAC in selected patients, thus providing a potential new tool for treatment optimization.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

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References

1. Jemal A, Siegel R, Ward E, Hao Y, Xu J, Thun MJ. Cancer statistics. CA Cancer J Clin 2009;59:225–49.
2. Li D, Xie K, Wolff R, et al. Pancreatic cancer. Lancet 2004;363:1049–57.
3. Burris HA III, Moore MJ, Andersen J, et al. Improvements in survival and clinical benefit with gemcitabine as first-line therapy for patients with advanced pancreas cancer: a randomized trial. J Clin Oncol 1997;15:2403–13.
4. Rivera F, Lopez-Tarruella S, Vega-Villegas ME, Salcedo M. Treatment of advanced pancreatic cancer: from gemcitabine single agent to combinations and targeted therapy. Cancer Treat Rev 2009;35:335–9.
5. Cunningham D, Chau I, Stocken DD, et al. Phase III randomized comparison of gemcitabine versus gemcitabine plus capecitabine in patients with advanced pancreatic cancer. J Clin Oncol 2009;27:5513–8.
6. Reni M, Cordio S, Milandri C, et al. Gemcitabine versus cisplatin, epirubicin, fluorouracil, and gemcitabine in advanced pancreatic cancer: a randomised controlled multicentre phase III trial. Lancet Oncol 2005;6:369–76.
7. Neuhaus P, Riess H, Post S, et al. CONKO-001: final results of the randomized, prospective, multicenter phase III trial of adjuvant chemotherapy with gemcitabine versus observation in patients with resected pancreatic cancer (PC) [abstract 4504]. J Clin Oncol 2008;26 Suppl 15:214s.

Published OnlineFirst May 11, 2010; DOI: 10.1158/0008-5472.CAN-09-4467

8. Neoptolemos J, Büchler M, Stocken DD, et al. ESPAC-3(v2): a multicenter, international, open-label, randomized, controlled phase III trial of adjuvant 5-fluorouracil/folinic acid (5-FU/FA) versus gemcitabine (GEM) in patients with resected pancreatic ductal adenocarcinoma [abstract LBA4505]. J Clin Oncol 2009;27 Suppl 18:203s.
9. Li D, Frazier M, Evans DB, et al. Single nucleotide polymorphisms of RecQ1, RAD54L, and ATM genes are associated with reduced survival of pancreatic cancer. J Clin Oncol 2006;24:1720–8.
10. Giovannetti E, Del Tacca M, Mey V, et al. Transcription analysis of human equilibrative nucleotide transporter-1 predicts survival in pancreas cancer patients treated with gemcitabine. Cancer Res 2006;66:3928–35.
11. Jones S, Zhang X, Parsons DW, et al. Core signaling pathways in human pancreatic cancers revealed by global genomic analyses. Science 2008;321:1801–6.
12. Carthew RW, Sontheimer EJ. Origins and mechanisms of miRNAs and siRNAs. Cell 2009;136:642–55.
13. Iorio MV, Croce CM. MicroRNAs in cancer: small molecules with a huge impact. J Clin Oncol 2009;27:5848–56.
14. Passetti F, Ferreira CG, Costa FF. The impact of microRNAs and alternative splicing in pharmacogenomics. Pharmacogenomics J 2009;9:1–13.
15. Blower PE, Chung JH, Verducci JS, et al. MicroRNAs modulate the chemosensitivity of tumor cells. Mol Cancer Ther 2008;7:1–9.
16. Boren T, Xiong Y, Hakam A, et al. MicroRNAs and their target messenger RNAs associated with ovarian cancer response to chemotherapy. Gynecol Oncol 2009;113:249–55.
17. Lee EJ, Gusev Y, Jiang J, et al. Expression profiling identifies microRNA signature in pancreatic cancer. Int J Cancer 2006;120:1046–54.
18. Bloomston M, Frankel WL, Petrocca F, et al. MicroRNA expression patterns to differentiate pancreatic adenocarcinoma from normal pancreas and chronic pancreatitis. JAMA 2007;297:1901–8.
19. Szafranska AE, Doleshal M, Edmunds HS, et al. Analysis of microRNAs in pancreatic fine-needle aspirates can classify benign and malignant tissues. Clin Chem 2008;54:1716–24.
20. Mardin WA, Mees ST. MicroRNAs: novel diagnostic and therapeutic tools for pancreatic ductal adenocarcinoma? Ann Surg Oncol 2009;16:3183–9.
21. Nam EJ, Yoon H, Kim SW, et al. MicroRNA expression profiles in serous ovarian carcinoma. Clin Cancer Res 2008;14:2690–5.
22. Krichevsky AM, Gabriely G. miR-21: a small multi-faceted RNA. J Cell Mol Med 2009;13:39–53.
23. Si ML, Zhu S, Wu H, et al. MiR-21-mediated tumor growth. Oncogene 2007;26:2799–803.
24. Meng F, Henson R, Wehbe-Janek H, Ghoshal K, Jacob ST, Patel T. MicroRNA-21 regulates expression of the PTEN tumor suppressor gene in human hepatocellular cancer. Gastroenterology 2007;133:647–58.
25. Moriyama T, Ohuchida K, Mizumoto K, et al. MicroRNA-21 modulates biological functions of pancreatic cancer cells including their proliferation, invasion, and chemoresistance. Mol Cancer Ther 2009;8:1067–74.
26. Meng F, Henson R, Lang M, et al. Involvement of human micro-RNA in growth and response to chemotherapy in human cholangiocarcinoma cell lines. Gastroenterology 2006;130:2113–29.
27. Park JK, Lee EJ, Esau C, Schmittgen TD. Antisense inhibition of microRNA-21 or -221 arrests cell cycle, induces apoptosis, and sensitizes the effects of gemcitabine in pancreatic adenocarcinoma. Pancreas 2009;38:e190–9.
28. Gabriely G, Wurdinger T, Kesari S, et al. MicroRNA 21 promotes glioma invasion by targeting matrix metalloproteinase regulators. Mol Cell Biol 2008;28:5369–80.
29. Lu Z, Liu M, Stribinskis V, et al. MicroRNA-21 promotes cell transformation by targeting the programmed cell death 4 gene. Oncogene 2008;27:4373–9.
30. Asangani IA, Rasheed SA, Nikolova DA, et al. MicroRNA-21 (miR-21) post-transcriptionally downregulates tumor suppressor Pdcd4 and stimulates invasion, intravasation and metastasis in colorectal cancer. Oncogene 2008;27:2128–36.
31. Landgraf P, Rusu M, Sheridan R, et al. A mammalian microRNA expression atlas based on small RNA library sequencing. Cell 2007;129:1401–14.
32. Yan LX, Huang XF, Shao Q, et al. MicroRNA miR-21 overexpression in human breast cancer is associated with advanced clinical stage, lymph node metastasis and patient poor prognosis. RNA 2008;14:2348–60.
33. Li J, Huang H, Sun L, et al. MiR-21 indicates poor prognosis in tongue squamous cell carcinomas as an apoptosis inhibitor. Clin Cancer Res 2009;15:3998–4008.
34. Schetter AJ, Nguyen GH, Bowman ED, et al. Association of inflammation-related and microRNA gene expression with cancer-specific mortality of colon adenocarcinoma. Clin Cancer Res 2009;15:5878–87.
35. Roldo C, Missiaglia E, Hagan JP, et al. MicroRNA expression abnormalities in pancreatic endocrine and acinar tumors are associated with distinctive pathologic features and clinical behavior. J Clin Oncol 2006;24:4677–84.
36. Dillhoff M, Liu J, Frankel W, Croce C, Bloomston M. MicroRNA-21 is overexpressed in pancreatic cancer and a potential predictor of survival. J Gastrointest Surg 2008;12:2171–6.
37. Li Y, Li W, Yang Y, et al. MicroRNA-21 targets LRRFIP1 and contributes to VM-26 resistance in glioblastoma multiforme. Brain Res 2009;1286:13–8.
38. Wang P, Zou F, Zhang X, et al. MicroRNA-21 negatively regulates Cdc25A and cell cycle progression in colon cancer cells. Cancer Res 2009;69:8157–65.
39. Li Y, Vanden Boom TG II, Kong D, et al. Up-regulation of miR-200 and let-7 by natural agents leads to the reversal of epithelial-to-mesenchymal transition in gemcitabine-resistant pancreatic cancer cells. Cancer Res 2009;69:6704–12.
40. Funel N, Giovannetti E, Del Chiaro M, et al. Laser microdissection and primary cell cultures improve pharmacogenetic analysis in pancreatic adenocarcinoma. Lab Invest 2008;88:773–84.
41. Ng SS, Tsao MS, Nicklee T, Hedley DW. Wortmannin inhibits PKB/Akt phosphorylation and promotes gemcitabine antitumor activity in orthotopic human pancreatic cancer xenografts in immunodeficient mice. Clin Cancer Res 2001;7:3269–75.
42. Streit S, Michalski CW, Erkan M, Friess H, Kleeff J. Confirmation of DNA microarray-derived differentially expressed genes in pancreatic cancer using quantitative RT-PCR. Pancreatology 2009;9:577–82.
43. Lu J, Getz G, Miska EA, et al. MicroRNA expression profiles classify human cancers. Nature 2005;435:834–8.
44. Pawlik TM, Gleisner AL, Cameron JL, et al. Prognostic relevance of lymph node ratio following pancreaticoduodenectomy for pancreatic cancer. Surgery 2007;141:610–8.
45. Chan SH, Wu CW, Li AF, Chi CW, Lin WC. miR-21 microRNA expression in human gastric carcinomas and its clinical association. Anticancer Res 2008;28:907–11.
46. Lawrie CH, Gal S, Dunlop HM, et al. Detection of elevated levels of tumour-associated microRNAs in serum of patients with diffuse large B-cell lymphoma. Br J Haematol 2008;141:672–5.
47. Lee KM, Nguyen C, Ulrich AB, Pour PM, Ouellette MM. Immortalization with telomerase of the Nestin-positive cells of the human pancreas. Biochem Biophys Res Commun 2003;301:1038–44.
48. Thum T, Gross C, Fiedler J, et al. MicroRNA-21 contributes to myocardial disease by stimulating MAP kinase signalling in fibroblasts. Nature 2008;456:980–4.
49. Haruta T, Uno T, Kawahara J, et al. A rapamycin sensitive pathway downregulates insulin signaling via phosphorylation and proteosomal degradation of insulin receptor substrate-1. Mol Endocrinol 2000;14:783–94.
50. O'Reilly KE, Rojo F, She Q, et al. mTOR inhibition induces upstream receptor tyrosine kinase signaling and activates Akt. Cancer Res 2006;66:1500–8.

Cancer Research

Correction: Online Publication Dates for *Cancer Research* April 15, 2010 Articles

The following articles in the April 15, 2010 issue of *Cancer Research* were published with an online publication date of April 6, 2010 listed, but were actually published online on April 13, 2010:

Garmy-Susini B, Avraamides CJ, Schmid MC, Foubert P, Ellies LG, Barnes L, Feral C, Papayannopoulou T, Lowy A, Blair SL, Cheresh D, Ginsberg M, Varner JA. Integrin $\alpha4\beta1$ signaling is required for lymphangiogenesis and tumor metastasis. Cancer Res 2010;70:3042–51. Published OnlineFirst April 13, 2010. doi:10.1158/0008-5472.CAN-09-3761.

Vincent J, Mignot G, Chalmin F, Ladoire S, Bruchard M, Chevriaux A, Martin F, Apetoh L, Rébé C, Ghiringhelli F. 5-Fluorouracil selectively kills tumor-associated myeloid-derived suppressor cells resulting in enhanced T cell-dependent antitumor immunity. Cancer Res 2010;70:3052–61. Published OnlineFirst April 13, 2010. doi:10.1158/0008-5472.CAN-09-3690.

Nagasaka T, Rhees J, Kloor M, Gebert J, Naomoto Y, Boland CR, Goel A. Somatic hypermethylation of *MSH2* is a frequent event in Lynch syndrome colorectal cancers. Cancer Res 2010;70:3098–108. Published OnlineFirst April 13, 2010. doi:10.1158/0008-5472.CAN-09-3290.

He X, Ota T, Liu P, Su C, Chien J, Shridhar V. Downregulation of HtrA1 promotes resistance to anoikis and peritoneal dissemination of ovarian cancer cells. Cancer Res 2010;70:3109–18. Published OnlineFirst April 13, 2010. doi:10.1158/0008-5472.CAN-09-3557.

Fiorentino M, Judson G, Penney K, Flavin R, Stark J, Fiore C, Fall K, Martin N, Ma J, Sinnott J, Giovannucci E, Stampfer M, Sesso HD, Kantoff PW, Finn S, Loda M, Mucci L. Immunohistochemical expression of BRCA1 and lethal prostate cancer. Cancer Res 2010;70:3136–9. Published OnlineFirst April 13, 2010. doi:10.1158/0008-5472.CAN-09-4100.

Veronese A, Lupini L, Consiglio J, Visone R, Ferracin M, Fornari F, Zanesi N, Alder H, D'Elia G, Gramantieri L, Bolondi L, Lanza G, Querzoli P, Angioni A, Croce CM, Negrini M. Oncogenic role of *miR-483-3p* at the *IGF2/483* locus. Cancer Res 2010;70:3140–9. Published OnlineFirst April 13, 2010. doi:10.1158/0008-5472.CAN-09-4456.

Lu W, Zhang G, Zhang R, Flores LG II, Huang Q, Gelovani JG, Li C. Tumor site–specific silencing of *NF-κB p65* by targeted hollow gold nanosphere–mediated photothermal transfection. Cancer Res 2010;70:3177–88. Published OnlineFirst April 13, 2010. doi:10.1158/0008-5472.CAN-09-3379.

Geng H, Rademacher BL, Pittsenbarger J, Huang C-Y, Harvey CT, Lafortune MC, Myrthue A, Garzotto M, Nelson PS, Beer TM, Qian DZ. ID1 enhances docetaxel cytotoxicity in prostate cancer cells through inhibition of p21. Cancer Res 2010;70:3239–48. Published OnlineFirst April 13, 2010. doi:10.1158/0008-5472.CAN-09-3186.

Yoo BK, Chen D, Su Z-z, Gredler R, Yoo J, Shah K, Fisher PB, Sarkar D. Molecular mechanism of chemoresistance by astrocyte elevated gene-1. Cancer Res 2010;70:3249–58. Published OnlineFirst April 13, 2010. doi:10.1158/0008-5472.CAN-09-4009.

Lu ZH, Shvartsman MB, Lee AY, Shao JM, Murray MM, Kladney RD, Fan D, Krajewski S, Chiang GG, Mills GB, Arbeit JM. Mammalian target of rapamycin activator RHEB is frequently overexpressed in human carcinomas and is critical and sufficient for skin epithelial carcinogenesis. Cancer Res 2010;70:3287–98. Published OnlineFirst April 13, 2010. doi:10.1158/0008-5472.CAN-09-3467.

Hattermann K, Held-Feindt J, Lucius R, Müerköster SS, Penfold MET, Schall TJ, Mentlein R. The chemokine receptor CXCR7 is highly expressed in human glioma cells and mediates antiapoptotic effects. Cancer Res 2010;70:3299–308. Published OnlineFirst April 13, 2010. doi:10.1158/0008-5472.CAN-09-3642.

Nadiminty N, Lou W, Sun M, Chen J, Yue J, Kung H-J, Evans CP, Zhou Q, Gao AC. Aberrant activation of the androgen receptor by NF-κB2/p52 in prostate cancer cells. Cancer Res 2010;70:3309–19. Published OnlineFirst April 13, 2010. doi:10.1158/0008-5472.CAN-09-3703.

Acu ID, Liu T, Suino-Powell K, Mooney SM, D'Assoro AB, Rowland N, Muotri AR, Correa RG, Niu Y, Kumar R, Salisbury JL. Coordination of centrosome homeostasis and DNA repair is intact in MCF-7 and disrupted in MDA-MB 231 breast cancer cells. Cancer Res 2010;70:3320–8. Published OnlineFirst April 13, 2010. doi:10.1158/0008-5472.CAN-09-3800.

McFarlane C, Kelvin AA, de la Vega M, Govender U, Scott CJ, Burrows JF, Johnston JA. The deubiquitinating enzyme USP17 is highly expressed in tumor biopsies, is cell cycle regulated, and is required for G_1-S progression. Cancer Res 2010;70:3329–39. Published OnlineFirst April 13, 2010. doi:10.1158/0008-5472.CAN-09-4152.

Dudka AA, Sweet SMM, Heath JK. Signal transducers and activators of transcription-3 binding to the fibroblast growth factor receptor is activated by receptor amplification. Cancer Res 2010;70:3391–401. Published OnlineFirst April 13, 2010. doi:10.1158/0008-5472.CAN-09-3033.

Cho SY, Xu M, Roboz J, Lu M, Mascarenhas J, Hoffman R. The effect of CXCL12 processing on $CD34^+$ cell migration in myeloproliferative neoplasms. Cancer Res 2010;70:3402–10. Published OnlineFirst April 13, 2010. doi:10.1158/0008-5472.CAN-09-3977.

Published OnlineFirst 05/11/2010.

doi: 10.1158/0008-5472.CAN-10-1347