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Stromal MicroRNA-21 Levels Predict Response to 5-Fluorouracil in Patients with Pancreatic Cancer

Timothy R. Donahue^{1,2,*}, Andrew H. Nguyen^{1,*}, Jennifer Moughan³, Luyi Li¹, Sergei Tatishchev⁴, Paul Toste¹, and James J. Farrell⁵

¹Department of Surgery, David Geffen School of Medicine at UCLA

²Department of Molecular and Medical Pharmacology, David Geffen School of Medicine at UCLA

³RTOG Statistics Department, Philadelphia, PA

⁴Department of Pathology, David Geffen School of Medicine at UCLA

⁵Yale Center for Pancreatic Disease, Yale University School of Medicine

Abstract

Background & Aims—MicroRNA-21 (miR-21) is upregulated and inversely associated with survival in many cancer types, including pancreatic ductal adenocarcinoma (PDAC). We studied the predictive value of miR-21 levels for gemcitabine or 5-fluorouracil (5-FU) response in tumor cells (TCs) or cancer associated fibroblasts (CAFs) in a cohort of PDAC patients from the RTOG 9704 trial.

Methods—MiR21 expression in CAFs and TCs, determined by in situ hybridization, of the 229 PDAC subset from RTOG 9704 was correlated with (i) histopathology characteristics using a chi-square test and (ii) patient overall survival (OS) using the Cox proportional hazards model.

Results—MiR-21 was strongly expressed in TCs and CAFs in 137/182 (75%) and 152/181 (84%) PDACs, respectively. MiR-21 expression in CAFs for the group given 5-FU for OS: (i) approached significance in a univariate analysis (hazard ratio [HR], 1.57; 95% confidence interval [CI], 0.95 – 2.57; P = 0.07) and (ii) was significant in multivariate model (HR, 1.70; 95% CI, 1.03 – 2.82; P = 0.038).

Conclusions—MiR-21 expression in CAFs was associated with decreased OS in PDAC patients who received 5-FU but not gemcitabine. These findings begin to identify stromal miR-21 as a marker to guide chemotherapy choice in PDAC patients.

Keywords

Biomarkers; pancreatic cancer; microRNA-21; 5-fluorouracil

Address Correspondence to: Timothy Donahue, MD, 72-215 Center for the Health Sciences, 10833 Le Conte Avenue, David Geffen School of Medicine, Los Angeles, CA 90095-6904, Tel: (310) 206-7440, Fax: (310) 206-2472, tdonahue@mednet.ucla.edu.

*These authors contributed equally to this work.

Introduction

Despite the best current medical and surgical management, pancreatic ductal adenocarcinoma (PDAC) is associated with poor prognosis[1]. The median overall survival is one year for patients with advanced stage disease[2], and two years for those with early stage tumors who undergo surgical resection and receive current adjuvant chemotherapy[3]. A primary determinant of these disturbing statistics is PDAC's prominent resistance to chemotherapy. The chemoresistance in PDAC is due to intrinsic processes within the tumor cells (TC) and the dense stromal compartment through multiple mechanisms[4–6]. [7–9]. In the stroma, cancer associated fibroblasts (CAF) interact directly with TCs to improve their fitness and also synthesize the extracellular matrix that yields low vascular perfusion which is prohibitive of effective intratumoral drug levels[9, 10]. Together, the diverse TC and stromal chemoresistance mechanisms make PDAC particularly difficult to treat effectively. Therefore, there is an ongoing need to identify markers and mechanisms of chemoresistance to better personalize treatment regimens using currently approved chemotherapies (gemcitabine or 5-FU), direct future development of drugs against these targets, and begin to “crack” this notoriously untreatable cancer.

MicroRNAs (miR) are potent regulators of chemoresistance[11]. MiRs are short 22 nucleotide RNA molecules that inhibit translation of complementary messenger RNAs. MiRs have been found to regulate genes involved in cancer biology, including TC proliferation, invasion, and metastasis[12–14]. A variety of miRs are directly associated with tumor chemoresistance through TC intrinsic mechanisms by altering cell death processes[15] and promoting epithelial-to-mesenchymal transition[16], and also through the stroma by modulating angiogenesis[17]. As miRs are stable and resistant to degradation, they have emerged as measurable prognostic and predictive biomarkers in many solid organ cancers[18]. In addition, they are also being explored as direct anti-cancer targets for therapy[19, 20].

MiR-21 is an oncomiR that is upregulated in many solid cancers, including PDAC. In PDAC, increased miR-21 expression is an early change that occurs during tumor initiation in premalignant, pancreatic intraepithelial neoplasia-1 (PanIN-1) lesions[21]. In addition, miR-21 is also involved in the biology of fully transformed PDAC [22–25]. Staining of primary human PDAC samples revealed that this impact may be from both the TC and stromal compartments; higher miR-21 expression in the TCs[22, 24, 25] or CAFs[23] is associated with shorter survival. While cell culture studies suggest that higher miR21 expression is associated with gemcitabine or 5-FU chemoresistance in PDAC or non-PDAC immortalized TC cell lines, the precise mechanisms in both TCs and CAFs remain to be well-defined[24, 26, 27].

In this study, we extend the current literature which has defined the prognostic significance of higher miR21 expression in PDAC and investigate whether it is specific to TCs or CAFs in patients specifically treated with adjuvant gemcitabine or 5-fluorouracil (5-FU) in the prospective Radiation Therapy Oncology Group 9704 (RTOG 9704) clinical trial[28]. RTOG 9704 was a phase III, randomized, adjuvant PDAC therapeutic trial comparing 5-FU to gemcitabine before and after 5-FU-based chemoradiation therapy (RT). We find that

higher levels of miR-21 expression in CAFs correlate with worse OS and DFS specifically for the group of patients receiving 5-FU in the RTOG 9704 trial.

Methods

Patient Selection and Consent

Patients entering the RTOG 9704 gave consent for use of formalin-fixed tissue for future planned translational research as part of the informed consent process. The RTOG tissue bank received tumor blocks from a total of 217 of the 538 patients who had undergone surgical resection and were entered into the RTOG 9704 prospective adjuvant treatment trial. Tissue microarrays (TMAs) were constructed from these blocks. Clinicopathologic factors were obtained as part of the patients' enrollment in the study. Treatment schedules and follow-up clinical information including outcome (overall survival, disease-free survival) and toxicity were recorded as end points in the study. Permission to perform this study was obtained by the study sites' and investigators' institutional review boards.

TMA Construction

A TMA was constructed using tissue core samples from the patients enrolled in the RTOG 9704 study. Each patient's tumor was represented by 3 cores (each core is 6 mm in size) from different regions within the tumor block and placed on 3 separate arrays to exclude effects of heterogeneous antigen expression. H&E staining as performed on each of the 3 TMAs to confirm tumor presence.

In situ hybridization and scoring

Three separate formalin-fixed paraffin-embedded pancreatic TMA sections (4- to 6- μ m thick, each containing the 217 patients' pancreatic tumor cores) were incubated at 60°C for 1 hour, deparaffinized in xylene, and rehydrated with graded alcohol washes. Slides were then washed 3 times with diethyl pyrocarbonate-treated PBS, digested with 5 μ g/mL proteinase K at 37°C for 30 minutes, washed then dehydrated in graded alcohol. Slides were hybridized at 55°C for 2 hours with 50 nmol/L locked-nucleic acid (LNA)-modified DIG-labeled probes for miR-21 (Exiqon, Vedbæk, Denmark). After stringency washes (5 $\times$, 1 $\times$, 0.2 $\times$ SSC), slides were placed in blocking solution for 1 hour at room temperature followed by overnight incubation at 4°C in alkaline phosphatase conjugated anti-DIG Fab fragment solution. Antibody signal was amplified with NBT and BCIP substrate (Roche, Mannheim, Germany) and then tissue was counterstained with Nuclear Fast Red (Vector Laboratories, Burlingame, CA).

Two M.D. readers (C.W. and S.T.) experienced with ISH staining interpretation assessed and scored miR-21 staining intensities. All miR-21 staining was scored, including cytoplasmic and nuclear. Tumor cell and peritumoral spindle shaped cells (likely fibroblasts) were scored separately. Both readers were blinded to the clinical characteristics of the outcome data. An intensity histoscore with the following categories was used: 0 negative, 1 weakly positive, 2 moderately positive, and 3 strongly positive. Because each patient's tumor was represented on each of the 3 TMAs, the maximum score for TC or CAFs in all 3 of the TMAs was used as the final miR-21 scores for that patient. Any discrepancies in

scoring were reviewed by both scorers and a consensus score was agreed upon. Interobserver variability was determined by unweighted κ scores. Good interobserver concordance was seen; $\kappa = 0.55$ (95% CI: 0.43 – 0.66).

Statistical analysis

The consensus miR-21 TC and CAF ISH scores were submitted to the RTOG Statistical Core for analysis without knowledge of patient demographics, treatment arm randomization, or outcome. A statistical comparison to assess whether missing miR-21 data were associated with baseline characteristics was performed using the chi-square test. MiR-21 expression in TCs or CAFs was separately dichotomized as low vs. high miR-21 expression (maximum histoscore of 1 or 2 vs. 3, respectively). The following baseline characteristics were dichotomized: pathological t-stage (T1, T2 vs. T3, T4) and American Joint Committee on Cancer stage (I, II vs. III, IV), and primary tumor location (head vs. body / tail). Race was categorized as white vs. other. Patient performance status was measured via the Karnofsky score and categorized as healthy (90 or 100) vs. less healthy (60, 70, or 80). The failure event for overall survival was defined as death from any cause. Survival time was measured from the date of randomization to the date of death or last follow-up evaluation. The failure event for disease-free survival was defined as disease relapse (local or regional), distant disease (including abdominal ascites, peritoneal seeding, and other abdominal sites), second primary or death from any cause. Disease-free time was measured from the date of randomization to the date of first disease-free failure event occurrence.

Associations between maximum TC or CAF miR-21 ISH scores, either ungrouped or dichotomized (low vs. high miR-21) with tumor demographic details and treatment outcome (OS and DFS) were sought by unconditional logistic regression analysis using the chi-square test and the Cox proportional hazards model. Patients with TC or CAF miR-21 ISH scores of 0 were excluded from analysis due to poor tissue preservation and staining. Both treatment arms of the study were analyzed. Univariate and multivariate analysis for correlation between OS and DFS and patient clinical features were calculated according to the Kaplan-Meier method and compared by the log-rank test based on the pattern of TC or CAF miR-21 ISH. The following variables were included in the multivariate analysis: maximum TC or CAF miR-21 ISH score (low vs. high), treatment (gemcitabine/cisplatin vs. 5-FU), age, gender, race, tumor location, nodal involvement (no vs. yes), tumor diameter (<3 vs. ≥3 cm), and surgical margin status (negative vs. positive vs. unknown). Because there were 3 possible responses for surgical margin status (negative, positive, or unknown), this variable was broken into 2 dummy variables with a value if negative as the reference level. Only maximum miR-21 ISH score was forced into the models and a stepwise selection procedure was used to choose the other variables using $\alpha=0.05$ level as the entry and exit criteria for the model building. Results were expressed as a hazard ratio (HR) (HR <1 denoting survival benefit) and were considered significant at a P value of 0.05 or less.

Results

Patient Population and Study Design

The study opened on July 20, 1998 and closed on July 26, 2002, with a total of 538 patients, and the final results have been published[28]. The flow of trial patients in our study is outlined in Figure 1. In brief, there were 270 patients randomized to the RT + 5-FU group and 268 to the RT + gemcitabine group. Subsets of patients in each of these groups, 40 in 5-FU and 47 in gemcitabine, were either determined to be ineligible or withdrew consent yielding a total of 230 5-FU and 221 gemcitabine enrolled patients who entered the trial, were included in the primary demographic and survival analysis, and eligible for miR-21 expression analysis. However, an additional 260 patients, 130 in 5-FU and 130 in gemcitabine, were later determined to be ineligible or did not have cores included on the TMA, resulting in 100 patients evaluated for miR-21 expression in TC or CAFs in the 5-FU arm, and 91 in the gemcitabine arm. In addition, after staining for miR-21, there were additional patients omitted from the miR-21 analysis due to poor tissue preservation or miR-21 staining in TCs or CAFs. Ultimately, the number of patients with analyzable miR-21 expression for the stroma or tumor cell compartments in the 5-FU or gemcitabine arms included: (i) 5-FU, CAF: 94 or TC: 98; and (ii) gemcitabine, CAF: 85 or TC: 84. Possible associations between baseline characteristics, OS, or DFS and determination of miR-21 CAF or TC levels in 5-FU or gemcitabine groups were investigated to determine if missing data may have influenced the analysis. No positive statistical associations were identified between patients with missing and determined miR-21 expression in each of the groups assessed (data not shown).

Distribution of miR-21 expression

ISH was performed to separately assess miR-21 expression in TCs or CAFs across tumors. Expression was quantified via an intensity histoscore ranging from 0 (no staining) to 3 (strongly positive staining). TC or CAF with a maximum histoscore of 0 across three tumor sections on the TMA were omitted for reasons previously discussed. Representative examples of TCs and CAFs at each histoscore and the distribution of overall staining are illustrated in Figure 2. MiR-21 staining in the stroma localized to stellate shaped fibroblasts with long projections surrounding malignant epithelial cells. Using a histoscore of 2 as a cutoff, miR-21 was increased in the TCs or CAFs of 137/182 (75%) or 152/181 (84%) patients, respectively. Dichotomizing miR-21 expression into high (histoscore 3) vs. low (histoscore 1 or 2), there was an overall greater percentage of patients with high miR-21 expression in CAFs than TCs (59% vs. 41%, respectively). This was predominantly due to significantly more patients with high CAF miR-21 in the 5-FU than gemcitabine arm (68% vs. 48%, respectively).

Despite multiple grouping strategies, TC miR-21 expression did not correlate with DFS or OS for patients treated with gemcitabine or 5-FU (data not shown). Therefore, the remainder of the study focuses on the relationship between CAF miR-21 expression and treatment outcome in the RTOG 9704 trial.

Analysis of baseline demographics

Basic demographic characteristics were similar between dichotomized high vs. low CAF miR-21 expression with the exception of (i) gender which approached significance in the 5-FU arm ($p=0.063$) and (ii) surgical margins which was significant in patients who received gemcitabine ($p=0.027$) (Table 1).

Analysis of Outcome

Univariate analysis—Univariate Cox proportional hazards models were performed to determine the hazard ratios (HR) for the association between CAF miR-21 expression and OS or DFS in patients treated with 5-FU or gemcitabine (Table 2). While high CAF miR-21 expression approached significantly shorter survival in 5-FU treated patients, there was no correlation in gemcitabine treated patients. The HR for 5-FU patients of high vs. low maximum CAF miR-21 ISH scores on OS was 1.57 (95% CI: 0.95–2.57; $p=0.076$), while for gemcitabine patients, it was 0.94 (0.59, 1.49; $p=0.78$). As illustrated in Figure 3, the median OS for 5-FU patients with high CAF miR-21 was 1.28 years as compared with 1.70 years for patients with low CAF miR-21. Estimated 4 year OS followed these trends with 33% survival for maximum CAF miR-21 ISH scores of 1/2 vs. 13% for scores of 3 (Table 3).

Similarly, for DFS, the HR of high vs. low maximal CAF miR-21 ISH scores for 5-FU treated patients was 1.55 (95% CI: 0.96–2.50; $p=0.071$), while for gemcitabine, it was 1.08 (0.69, 1.68, $p=0.75$). As illustrated in Figure 3, the median DFS for 5-FU patients with high CAF miR-21 was 0.77 years as compared with 1.03 years for patients with low CAF miR-21. Estimated 4 year DFS were similar; high CAF miR-21 scores had poorer survival (Table 3).

As maximum CAF miR-21 expression did not correlate with prognosis in gemcitabine treated patients in the RTOG 9704 study (data not shown), the remainder of the study focused on the 5-FU treated group.

Multivariate analysis with adjustments for race and nodal involvement—Cox proportional hazard models were then used to perform a multivariate analysis of maximum CAF miR-21 ISH scores on OS and DFS for 5-FU treated patients (Table 5). For OS, significant predictor variables included high vs. low maximum miR-21 (HR=1.70, 95% CI: 1.03–2.82, $p=0.038$), race (HR=1.91, 95% CI: 1.02–3.57, $p=0.043$), and nodal involvement (HR=1.66, 95% CI: 1.03–2.67, $p=0.038$). For DFS, significant predictor variables included high vs. low maximum miR-21 (HR=1.63, 95% CI: 1.01–2.65, $p=0.047$) and race (HR=1.88, 95% CI: 1.01–3.49, $p=0.048$).

DISCUSSION

Despite best current medical and surgical treatment, the overall prognosis of patients with PDAC remains poor[1]. Patients who present with early stage disease survive a median of two years while those with more advanced tumors live approximately half that duration. These disappointing statistics can be partially explained by PDAC's resistance to molecular or cytotoxic therapies. To explain PDAC's chemoresistance, two leading theories have been

proposed. The first theory is centered on the TCs, which develop innate or acquired mechanisms to resist drug-induced killing. The second theory focuses on the tumor-associated stroma (TAS) which is comprised of a supportive matrix and is populated with protumorigenic accessory cells including CAFs, immune suppressive cells, and few blood vessels. CAFs are the predominant cell type; they interact directly with TCs and also synthesize the dense matrix that precludes drug delivery[10, 29]. The identification of molecules and proteins responsible for chemoresistance in each of these tumor compartments is a promising approach to begin to improve responses of PDAC to treatment.

MiRs, in general, regulate genes involved in many pathways and processes associated with tumor progression. Numerous well-conducted studies point to their role in metastasis development[14, 30]. MiR-21 is an oncomiR whose expression is increased in many solid malignancies[31–34]. In the context of PDAC, TC or CAF miR-21 expression is associated with poor prognosis[22–25] and renders TCs resistant to gemcitabine in cell culture[35]. The RTOG 9704 clinical trial was a prospective trial that randomized PDAC patients who underwent surgical resection plus 5-FU-based radiation to gemcitabine or 5-FU adjuvant treatment arms[36]. Therefore, we utilized ISH of the RTOG 9704 TMA to investigate whether TC or CAF miR-21 expression in early stage PDAC patients was associated with survival in the context of gemcitabine or 5-FU therapies, the two most frequently used chemotherapies for patients with this disease.

We find that elevated miR-21 expression was prevalent in the TCs and CAFs of patients in the RTOG 9704 study. In the stroma, it was localized to fibroblast-shaped cells which have previously been confirmed as α -SMA and nestin positive[23]. Using a histoscore of 2 as a cutoff, miR-21 was increased in 75% and 84% of patients in TCs and CAFs, respectively. Dichotomizing miR-21 into high (histoscore 3) vs. low (histoscore 1/2), we further find that strong miR-21 expression in CAFs of 5-FU treated patients was associated with significantly shorter DFS and OS. Interestingly, strong TC miR-21 expression was not correlated with survival in gemcitabine or 5-FU treated patients.

A significantly greater percentage of patients in the gemcitabine group with high miR21 expression had positive surgical margins than those with low miR21 expression. While this could potentially confound the negative results, we do not think this is the case. The prognostic implications of positive surgical margins has not been consistent in the literature. Most notably, in the overall RTOG 9704 survival analysis, positive margin status was not correlated with worse survival [28]. Moreover, a recent large analysis from the Massachusetts General Hospital revealed that a margin clearance of < 1 mm is equivalent to a microscopic positive (i.e. R1) margin resection[37]. While the RTOG required gross negative margins as an inclusion criteria, analyses of this trial do not distinguish the distance of margin clearance.

There are numerous studies that have identified the prognostic significance of strong miR-21 expression in PDAC TCs. Giovanetti et al. determined miR-21 expression via laser capture microdissection and quantitative reverse transcription polymerase chain reaction (qRT-PCR) of TCs from 81 patients with early and late stage PDACs who were treated with gemcitabine[25]. Higher miR-21 expression in TCs was associated with significantly shorter

OS in the metastatic and adjuvant settings. Furthermore, in cell culture, overexpression of miR-21 increased gemcitabine resistance, which was abrogated by dual PI3K/mTOR inhibition. Hwang et al. studied two independent cohorts of patients[24]. Higher TC miR-21 expression, determined via qRT-PCR of whole tissue blocks, was the strongest predictor of death or recurrence on multivariate analysis, which controlled for other prognostically significant clinicopathologic variables. The CAFs and TCs were not separated in this study. Dillhoff et al. determined miR-21 status of 80 resected PDACs via ISH of tumor samples and found that it was expressed (>0 histoscore) in 79% of patients[22]. Furthermore, strong miR-21 expression was associated with shorter survival in patients with node negative disease. We speculated that these results are in contradistinction to our prior analysis[24] which did not identify prognostic significance of miR21 expression in TCs due to poor preservation of the TCs on the UCLA TMA and a lower percentage of highly expressing tumors. In the current study, an analysis of the subset of patients with node negative disease was not performed, as the significance of higher miR21 expression in CAFs was stronger and applied to the whole group of patients treated with 5-FU.

As further support of TC miR-21 as a mediator of tumor progression, Moriyama et al. showed that overexpression of miR-21 in PDAC TCs resulted in increased TC proliferation, invasion, and gemcitabine resistance in cell culture. Despite having a similar frequency of strong miR-21 expression in PDAC TCs as previous reports[22], our results from the RTOG 9704 trial do not identify the prognostic significance of TC miR-21. These negative findings are consistent with our previous results on an independent TMA which also measured miR-21 using ISH. Therefore, we conclude that the prognostic significance of miR-21 in TCs is not consistent across studies. We speculate that this variability may be due to the technique used to determine miR-21 status (PCR vs. ISH), the scoring system to assess ISH staining, and the availability of prospectively collected, well characterized patient tissue cohorts. We believe that ISH is a better technique to characterize miR-21 expression both quantitatively via the histoscore and in each specific compartment – stroma vs. tumor cells; PCR of bulk homogenized tissue is not able to do the latter.

The prognostic significance of miR-21 in the stromal compartment of solid gastrointestinal tumors has also been examined. Kjaer-Frifeldt et al. evaluated 764 resected colorectal cancers via ISH[38]. MiR-21 was predominantly localized to the stroma where strong expression was associated with shorter recurrence free cancer specific survival. Nelson et al. further examined 130 stage II colon and 67 rectal cancers[39]. MiR-21 was localized to the stroma where it predicted shorter DFS in the colon cancer patients only. As discussed earlier, our previous findings also reveal a PDAC relevant prognostic stromal story for miR-21. In this study, we identify that it may be specific for early stage patients receiving 5-FU therapy.

A specific mechanism to explain how miR-21 induces resistance to 5-FU and not gemcitabine remains to be determined. To our knowledge, there are only two published reports examining miR-21 and 5-FU[26]REF. Valeri et al. identified that miR-21 induces chemoresistance specifically to 5-FU by downregulating the human DNA mismatch repair recognition protein, DNA Mut S homolog 2. Tominaru et al. found that expression level of miR21 in hepatocellular carcinoma (HCC) cells directly correlated with IFN α /5-FU

resistance in vitro (REF). While these mechanisms may immediately seem more pertinent to TCs, it can also be speculated from our study that 5-FU resistance of CAFs can lead to their survival and continued direct support of TCs and synthesis of the supportive extracellular matrix[40]. Based on our correlative findings, further experiments with miR21 over- or under-expressed in TAFs co-cultured or –implanted (in vivo) with TCs exposed to 5-FU chemotherapy which are designed to identify this precise mechanism are warranted.

Taken together with previous findings on stromal miR-21 expression in colon cancer and PDAC, these results support the prognostic significance of miR-21 expression in the CAFs of the PDAC stromal compartment. Furthermore, they suggest that this significance may be specific to early stage patients treated with adjuvant 5-FU, who should be considered for alternative therapies. This study further confirms the importance of the stroma in PDAC in determining outcome and response to treatment.

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Figure 1.
Study design.

Figure 2.

MiR-21 Maximum Histoscore TC/CAF Distributions. (A) ISH of miR-21 was performed on the RTOG 9704 TMA and miR-21 was quantified with an intensity histoscore ranging from 0 (no staining) to 3 (strongly positive staining) in TCs and CAFs. Arrows indicate tumor cells or CAFs. (B) Distributions of miR-21 expression in TCs and CAFs were determined for gemcitabine treated, 5-FU treated, and the total patients.

Figure 3.

OS and DFS Survival Curves for 5-FU treated patients. Kaplan-Meier survival estimates for 5-FU treated patients demonstrating the relationship between dichotomized high vs. low CAF miR-21 expression and (A) Overall Survival and (B) Disease Free Survival.

Table 1
Characteristics of Patients Entering Into Gemcitabine and 5-FU Treatment Arms Based on Maximum CAF miR-21 ISH Score.

	5-FU		Gemcitabine	
	1/2 (n=30)	3 (n=64)	1/2 (n=45)	3 (n=42)
CAF miR-21 ISH score				
Age (years)				N/A
Median (Min-Max)	58 (37-76)	62 (36-80)	65 (35-77)	58 (40-74)
Gender				0.57
Male	22 (73.3%)	34 (53.1%)	23 (51.1%)	24 (57.1%)
Female	8 (26.7%)	30 (46.9%)	22 (48.9%)	18 (42.9%)
Race				0.12
White	24 (80.0%)	57 (89.1%)	41 (91.1%)	42 (100.0%)
Other	6 (20.0%)	7 (10.9%)	4 (8.9%)	0 (0.0%)
Pancreas Tumor Location				0.34
Head	25 (83.3%)	56 (87.5%)	35 (77.8%)	36 (85.7%)
Elsewhere	5 (16.7%)	8 (12.5%)	10 (22.2%)	6 (14.3%)
Karnofsky Score (KPS)				0.81
80, 70, 60	12 (40.0%)	22 (34.4%)	16 (35.6%)	16 (38.1%)
100, 90	18 (60.0%)	42 (65.6%)	29 (64.4%)	26 (61.9%)
T-Stage				0.54
T1, T2	9 (30.0%)	18 (28.1%)	11 (24.4%)	8 (19.0%)
T3, T4	21 (70.0%)	46 (71.9%)	34 (75.6%)	34 (81.0%)
N-stage				0.82
N0	12 (40.0%)	22 (34.4%)	15 (33.3%)	15 (35.7%)
N1	18 (60.0%)	42 (65.6%)	30 (66.7%)	27 (64.3%)
AJCC Stage				0.82
I, II	10 (33.3%)	22 (34.4%)	15 (33.3%)	15 (35.7%)
III, IV	20 (66.7%)	42 (65.6%)	30 (66.7%)	27 (64.3%)
Largest tumor dimension				0.25
<3 cm	10 (33.3%)	25 (39.1%)	16 (35.6%)	20 (47.6%)

	5-FU			Gemcitabine		
	1/2 (n=30)	3 (n=64)	p-value	1/2 (n=45)	3 (n=42)	p-value
CAF miR-21 ISH score						
3 cm	20 (66.7%)	39 (60.9%)		29 (64.4%)	22 (52.4%)	
Primary Tumor Status			0.90			0.027
Negative margins	13 (43.3%)	31 (48.4%)		19 (42.2%)	15 (35.7%)	
Positive margins	10 (33.3%)	19 (29.7%)		10 (22.2%)	20 (47.6%)	
Unknown margins	7 (23.3%)	14 (21.9%)		16 (35.6%)	7 (16.7%)	

Table 2

Maximum CAF miR-21 ISH Score and Survival in Patients Treated with 5-FU or Gemcitabine Expressed as Hazard Ratio (H.R.) (95% CI)

	5-FU	Gemcitabine
	CAF miR-21 ISH score 1/2 vs.3	CAF miR-21 ISH score 1/2 vs.3
Overall Survival	1.57 (0.95, 2.57) (p=0.076)	0.94 (0.59, 1.49) (p=0.78)
Disease Free Survival	1.55 (0.96, 2.50) (p=0.071)	1.08 (0.69, 1.68) (p=0.75)

Table 3

Estimated 4-Year Overall Survival and Disease Free Survival in 5-FU Treated Patients.

		Maximum CAF miR-21 score of	
		1/2	3
Overall Survival	4-Yr(95% CI)	33% (18%, 50%)	13% (6%, 22%)
	MOS (95% CI)	1.70 yrs (1.07, 5.76)	1.28 yrs (1.08, 1.67)
	Log-rank p-value	0.076	
Disease Free Survival	4-Yr (95% CI)	23% (10%, 39%)	6% (2%, 14%)
	MDFS (95% CI)	1.03 yrs (0.48, 1.82)	0.77 yrs (0.49, 1.02)
	Log-rank p-value	0.071	

MOS: median overall survival time; MDFS: median disease free survival

Table 4

Multivariate Analysis of 5-FU Treated Patients.

Endpoint	Adjustment Variables	Comparison	Adjusted HR ^a	95% C.I. LL	95% C.I. UL	p-value ^b
Overall Survival	CAF miR-21 score	1/2 vs. 3	1.70	1.03	2.82	0.038
	Race	White vs. Other	1.91	1.02	3.57	0.043
	Nodal Involvement	No vs. Yes	1.66	1.03	2.67	0.038
Disease Free Survival	CAF miR-21 score	1/2 vs. 3	1.63	1.01	2.65	0.047
	Race	White vs. Other	1.88	1.01	3.49	0.048