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Predictive Biomarkers and Personalized Medicine

Clinical Cancer Research

# Tumor Engraftment in Nude Mice and Enrichment in Stroma-Related Gene Pathways Predict Poor Survival and Resistance to Gemcitabine in Patients with Pancreatic Cancer

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## Abstract

**Purpose:** The goal of this study was to evaluate prospectively the engraftment rate, factors influencing engraftment, and predictability of clinical outcome of low-passage xenografts from patients with resectable pancreatic ductal adenocarcinoma (PDA) and to establish a bank of PDA xenografts.

**Experimental Design:** Patients with resectable PDA scheduled for resection at the Johns Hopkins Hospital were eligible. Representative pieces of tumor were implanted in nude mice. The status of the *SMAD4* gene and content of tumor-generating cells were determined by immunohistochemistry. Gene expression was carried out by using a U133 Plus 2.0 array. Patients were followed for progression and survival.

**Results:** A total of 94 patients with PDA were resected, 69 tumors implanted in nude mice, and 42 (61%) engrafted. Engrafted carcinomas were more often *SMAD4* mutant, and had a metastatic gene expression signature and worse prognosis. Tumors from patients resistant to gemcitabine were enriched in stroma-related gene pathways. Tumors sensitive to gemcitabine were enriched in cell cycle and pyrimidine gene pathways. The time to progression for patients who received treatment with gemcitabine for metastatic disease ($n = 7$) was double in patients with xenografts sensitive to gemcitabine.

**Conclusion:** A successful xenograft was generated in 61% of patients attempted, generating a pool of 42 PDA xenografts with significant biological information and annotated clinical data. Patients with PDA and *SMAD4* inactivation have a better engraftment rate. Engraftment is a poor prognosis factor, and engrafted tumors have a metastatic gene expression signature. Tumors from gemcitabine-resistant patients were enriched in stromal pathways. *Clin Cancer Res; 17(17); 5793–800. ©2011 AACR.*

## Introduction

Advanced pancreatic ductal adenocarcinoma (PDA) is a lethal disease (1). Most drugs tested in PDA clinical trials were selected on the basis of their activity in preclinical models, which is not generally predictive of clinical outcome (2). Traditional preclinical models are cell lines cultivated in monolayers or xenografts derived from them. Cells cultured in plastic may undergo substantial changes that deviate from their originator tumor, as recently shown with lung cancer cell lines (3). In addition, the stroma is underrepresented in these models. Several lines of evidence show that stroma has a role in cancer cell survival and metastases, particularly in pancreatic cancer (4, 5). Not surprisingly, targeting the stroma has recently been proposed as a novel strategy to improve drug delivery and chemotherapy efficacy in PDA (6, 7), and holds particular promise for this disease characterized by an intense desmoplastic reaction (6, 8).

Other preclinical models have been tested in PDA, including genetically engineered mouse models (GEMM). These models maintain an intact immune system and many are histologically akin to human pancreatic cancer, including a dense desmoplastic stroma (9). However, the predictive value of GEMM for assessing efficacy of novel anticancer agents in the clinic has not been extensively tested. Their dependence on a few critical genetic lesions, such as *KRAS*, *P53*, and *CDKN2A/P16*, might not reflect the

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## Translational Relevance

Current preclinical models of pancreatic cancer based on cell lines or xenografts derived from them are not predictive of drug activity in the clinic and do not represent the heterogeneity and complexity of this disease. We have established a platform of freshly generated pancreatic cancer xenografts annotated with clinical information and show that patients whose tumors engrafted had a significantly worse prognosis. The data show that these models predict response to gemcitabine in the metastatic setting and that the stromal compartment has a critical role in gemcitabine resistance. This collection of freshly generated pancreatic ductal adenocarcinoma models are a useful platform for preclinical drug screening, biomarker discovery, and delineating the biology of pancreatic cancer.

genetic diversity that exemplifies human PDA (10) and they failed to predict response to Ras inhibitors in one clinical trial (11). Early passages of freshly generated xenografts are probably better candidates for mimicking the heterogeneity of the disease and might be better predictors of response. We have previously established a collection of 23, extensively characterized PDA xenografts under an anonymized exempt tissue protocol that is being used as a platform for drug screening and biomarker development (12, 13). The initial tumor bank composed of these PDA xenografts did not include annotated clinical data such as clinical drug response.

The primary goal of this prospective clinical trial was to use freshly generated xenografts from patients with resectable PDA to determine the engraftment rate and dynamics of the process, the biological characteristics and prognostic implications of tumor engrafting as well as the correlation between patient response to gemcitabine and their corresponding xenografts. A secondary objective was to generate a clinically annotated and biologically characterized collection of PDA xenografts for future studies.

## Materials and Methods

### Patient eligibility

Patients with pancreatic masses suspicious of a diagnosis of PDA were enrolled, prior to surgery, in J0507, a Johns Hopkins University clinical trial (NCT00276744). Tissue samples of PDA not needed for diagnosis were xenografted into nude mice.

Eligibility criteria also included ECOG performance status 0–1, age more than 18 years, expected survival more than 12 weeks, no prior treatment for PDA, and adequate liver, renal, and bone marrow function (absolute neutrophil count $\geq$ 1,500/μL; platelets $\geq$ 100,000/μL; hemoglobin $\geq$ 9 g/dL; serum creatinin $\leq$ 2 mg/dL; bilirubin $\leq$ 2 mg/dL; alanine aminotransferase, aspartate aminotransferase, and alkaline phosphatase $\leq$5 times the upper limit of normal). The study was approved by the Johns Hopkins University Institutional Review Board.

### Xenografts

PDA tumor specimens from resected patients were implanted subcutaneously on each flank of 5- to 6-week-old athymic nude mice and expanded as previously described (12).

### Treatment

When tumors reached approximately 200 $mm^3$ in volume, mice were randomized to treatment and control arms. Gemcitabine was administered at 100 mg/kg intraperitoneally to third-generation passages (F3) 3 times per week for 4 weeks. Tumor size was evaluated 2 times a week by caliper measurements by using the following formula: tumor volume = (length $\times$ width$^2$)/2. Relative tumor growth inhibition/regression was calculated as $T/C = (T_i - T_0/C_i - C_0)$; $T_i$ and $C_i$ represent tumor size of treatment and control group at the end of experiments, respectively; $T_0$ and $C_0$ represent tumor size at initiation of experiments, respectively. $T/C > 0$ represents growth inhibition, $T/C < 0$ represents tumor regression. The research protocol was approved by the Johns Hopkins University Animal Care and Use Committee, and animals were maintained in accordance to guidelines of the American Association of Laboratory Animal Care.

### SMAD4 and aldehyde dehydrogenase staining

Immunolabeling was carried out on Bond-Leica autostainer (Leica Microsystems) by using a standard immunohistochemistry (IHC) protocol (Supplementary Methods).

### Microarray gene expression

Primary tumors were profiled when possible by using Affymetrix U133 Plus 2.0 GeneChip arrays (Affymetrix) in duplicates. In 4 patients, primary tumors (F0) and paired samples from F5 (passage 5) and F10 (passage 10) were profiled. Sample preparation and processing were carried out as described in the Affymetrix GeneChip Expression Analysis Manual (Affymetrix, Inc.). The gene expression data will be deposited at the National Center for Biotechnology Information Gene Expression Omnibus.

### Gene set enrichment analysis

Gene set enrichment analysis was carried out by using gene set enrichment analysis (GSEA) software, version 2.0.1, obtained from the Broad Institute (14). Gene set permutations were done 1,000 times for each analysis. The nominal *P* value and normalized enrichment score were used to rank the pathways enriched in each phenotype. We used 199 pathways defined by the Kyoto Encyclopedia of Genes and Genomes (KEGG) database as the gene set in this study. One hundred fifty-seven gene sets passed the gene set size filter criteria (min = 10, max = 500).

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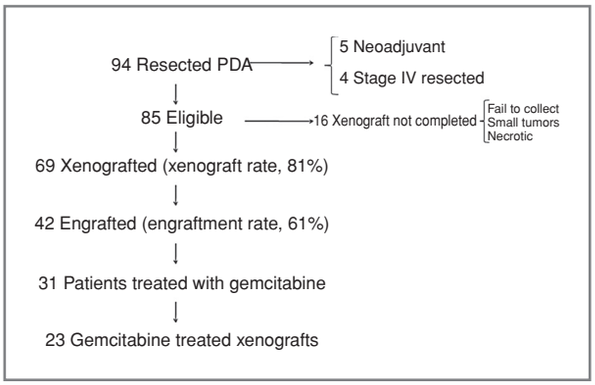


Figure 1. Patient flow chart. A total of 94 patients with resected PDA were included in this study; 85 patients were eligible for xenografting. Patients who received neoadjuvant therapy or had stage IV resected PDA were excluded. Of the 69 xenografted tumors, 42 were able to engraft and grew in nude mice.

### Statistical analysis

Overall survival (OS) was calculated by using the Kaplan–Meier method (15). OS was computed from the date of surgery to the date of death or last follow-up. Median OS was reported in days with 95% CIs. Survival analyses were conducted in patients who survived more than 30 days to exclude perioperative mortality. Univariate and multivariate Cox proportional hazards models were fit to assess the associations between patient characteristics and clinical outcomes. The following variables were used for univariate analysis: tumor size, grade, margin status, perineural invasion, *SMAD4* status, engraftment rate, and adjuvant therapy. Variables that were marginally significant in the univariate analysis were included in the Cox model multivariate analysis. The results of the Cox model are reported with HRs and 95% CIs. $P < 0.05$ was considered significant for all statistical analysis. Statistical analyses were carried out by using the statistical analysis package SPSS version 17 (SPSS).

## Results

### Overall patient and xenograft characteristics

Figure 1 depicts the flow of patients. A total of 94 patients with PDA were operated on and 85 were eligible to have their tumors xenografted into nude mice. These were patients with resected PDA who had not received neoadjuvant treatment. Of these 85, 69 were xenografted. The flow chart describes the reasons why patients could not be xenografted. Of the 69 implanted cancers, 42 engrafted for an engraftment rate of 61%. Table 1 summarizes the principal clinical characteristics of patients and Supplementary Table S1 lists detailed information about tumor stage, treatment, the xenograft generated from these patients, and the principal biological information available from these tumors. This collection of well-annotated PDA xenografts can form the basis of drug screening and biomarker development.

**Table 1.** Characteristics of 69 xenografted patients

| | |
|---|---|
| Age, y | |
| Mean (SD) | 66 (9.96) |
| Range | 30–84 |
| Gender | |
| Male | 29 (42%) |
| Female | 40 (58%) |
| Tumor size, cm, mean (SD) | 3.3 (1.17) |
| Lymph node status | |
| Positive | 53 (77%) |
| Negative | 16 (23%) |
| Margins | |
| Positive | 29 (42%) |
| Negative | 40 (58%) |
| Adjuvant | |
| No | 18 (26%) |
| Yes | 51 (74%) |
| Type of adjuvant | |
| CR | 43 (84%) |
| CT | 7 (14%) |
| RT | 1 (2%) |

### Biological characteristics of engrafted tumors

To determine biological features associated with a higher rate of engraftment, we first estimated whether the proportion of tumor initiating cells (TIC), determined by the expression of aldehyde dehydrogenase (ALDH), was related to a higher engraftment rate. Our group recently showed a correlation between the tumor initiating compartment in PDA and the expression of this intracellular enzyme (16). However, we found no differences in the expression of ALDH in carcinomas that engrafted in mice compared with those that did not (data not shown).

We examined *SMAD4* alterations to see whether they were associated with a higher take rate in the mouse. The primary cancers from 58 of the 69 xenografted patients were analyzed and *SMAD4* status was determined by Smad4 immunolabeling patterns, a strong marker of *SMAD4* genetic status (17, 18). The incidence of Smad4 protein loss was statistically higher in engrafted patients than in nonengrafted patients (67% vs. 36%, $P = 0.024$; Fig. 2A). We also show that Smad4 loss was not a marker of tumor grade given the fact that Smad4 might be deleted in low-grade tumors while preserved in poorly differentiated ones (Fig. 2B).

To explore further previous work from our group showing that *SMAD4*-mutant PDAs have a higher metastatic potential (18), we examined the presence of a metastasis-associated gene signature developed by Ramaswamy and colleagues. (19) This gene signature contains 17 genes that were identified by comparing adenocarcinoma metastases from multiple tumor types to unmatched primary adenocarcinomas. In this analysis, we used the gene expression profiles from 4 primary tumors and 2 different passages of their matching xenografts. We found that 5 of 8 genes from

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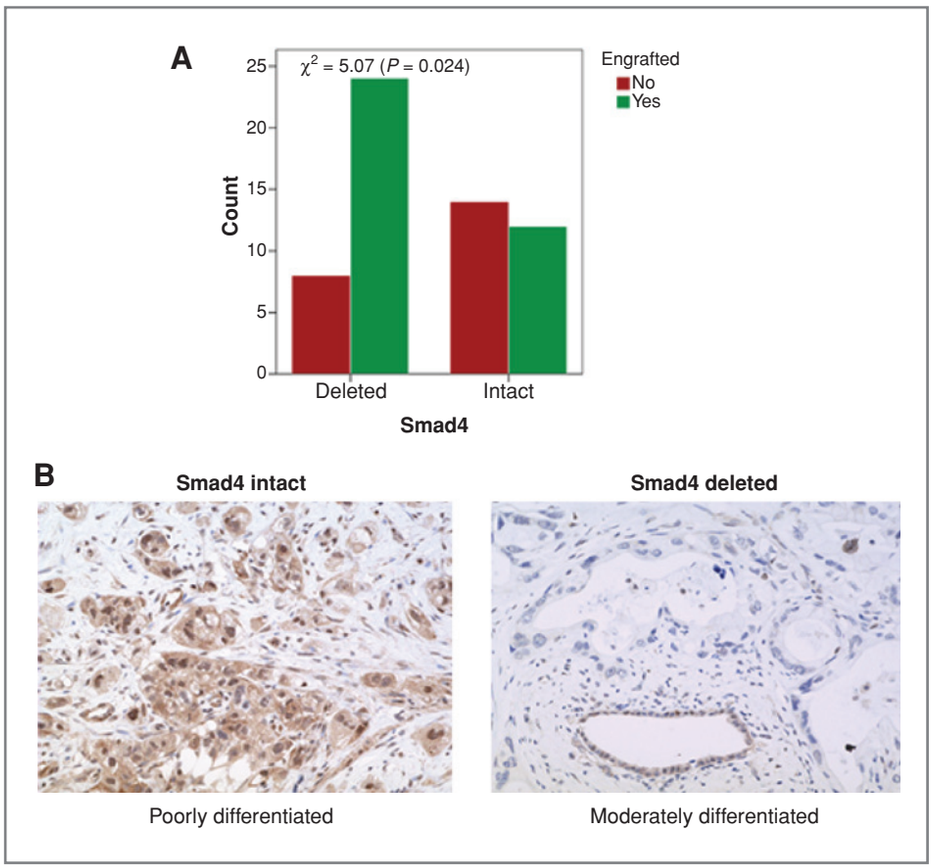


Figure 2. A, engraftment rate was higher in patients with *SMAD4* deletions. B, *SMAD4* deletions were unrelated to the differentiation grade of the tumor.

the gene signature of metastatic adenocarcinoma (*SNRPF*, *EIF4E2*, *HNRPAB*, *DHPS*, and *PTTG1*) were also upregulated in the xenografts compared with their counterpart primary tumors (Fig. 3). Additionally, 4 of the 9 genes that were downregulated in this gene signature were also downregulated in xenografts versus in primary tumors (F0).

### Engrafted patients had poorer overall survival

Because cancers that successfully engrafted putatively have a higher metastatic potential, we investigated the prognostic role of engraftment in addition to other well-known prognostic factors. We conducted a univariate analysis to determine impact on survival (Supplementary Table S2) and found that *SMAD4* status, tumor size $\geq$3.5 cm, positive margins, no adjuvant treatment, and ability to engraft were associated with shorter survival. In contrast, only tumor engraftment ($P < 0.006$) and adjuvant therapy ($P < 0.008$) were significant predictors of shorter survival in the multivariate analysis. The median survival for engrafted patients was 299 days (95% CI, 230–369), whereas the median survival for patients whose cancers failed to engraft has not been reached (>800 days). Patients whose cancers failed to engraft had an 81% reduction in the risk of death (HR = 0.19; 95% CI, 0.060–0.632; Supplementary Table S3; Fig. 4). As PDA patients typically die of metastatic disease, this finding is consistent with the *SMAD4* status and metastatic signature of engrafted cancers.

### Gene set enrichment analysis identified upregulation of stromal and Notch pathways in patients resistant to gemcitabine

GSEA was used to investigate gene pathways that might be involved in resistance to gemcitabine by using the gene expression profiles from primary tumors from patients treated with gemcitabine (Supplementary Table S1). GSEA provides statistical measurements for the gene pathways that are enriched when gene expression profiles from 2 different phenotypes are compared (i.e., sensitive versus resistance to gemcitabine). At baseline, 79 and 76 pathways were upregulated in patients sensitive and resistant to gemcitabine, respectively. Gemcitabine-resistant tumors showed enrichment in gene pathways related to stroma (ECM receptor interaction, focal adhesion, cell communication, Gap junction, cell adhesion molecules) and stem cells (Notch signaling pathways; Supplementary Table S4). Gemcitabine-sensitive tumors showed upregulation of cell cycle and pyrimidine pathways (Supplementary Table S5). Overall, these results suggest that coordinated overexpression of genes in stromal and Notch signaling pathways may confer resistance to gemcitabine.

### Prediction of clinical outcome with gemcitabine

A xenograft was successfully developed in 23 patients who were clinically treated with gemcitabine. The activity of gemcitabine in the matching xenografts is shown in

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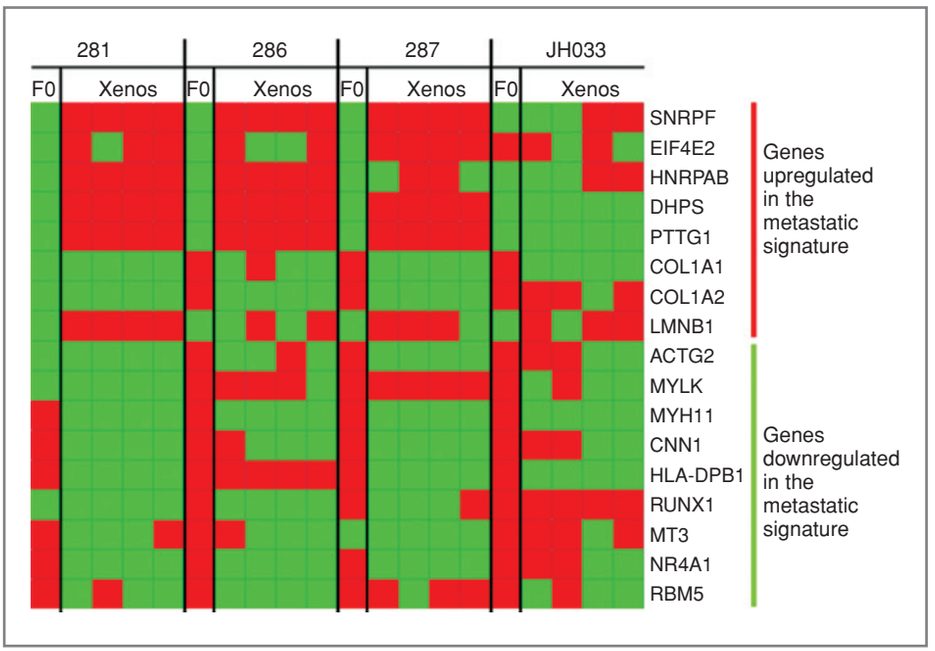


**Figure 3.** Xenografts (Xenos) show a metastatic signature of adenocarcinoma. We collected the gene expression profiles of primary tumors (F0) and corresponding xenografts (F5 and F10). For each xenograft the first column corresponds to primary tumor (F0). Second and third column are duplicates of passage 5 (F5), whereas fourth and fifth column are duplicates of passage 10 (F10). We tested a gene expression signature from metastatic adenocarcinoma and found that our xenografts were enriched in this metastatic gene signature.

Fig. 5. In 7 of the 23 patients for whom gemcitabine was used to treat metastatic disease, the response of the xenografted cancers to gemcitabine correctly predicted longer time to progression. In patients whose xenografts were sensitive to gemcitabine, the median time to progression was 80 versus 46 days (log-rank $P = 0.037$). Gemcitabine was administered in the adjuvant setting to 16 patients as part of varied complex multimodality regimens, precluding correlation between activity in the preclinical model with clinical outcome. Anecdotally, however, one patient with a T3N1M0 cancer had a rather long disease-free survival after surgery and 6 cycles of conventional gemcitabine (1,203 days) but that patient's xenograft was resistant to gemcitabine. To gain further insight into this finding we conducted an orthotopic implantation of the patient's tumor and observed that, in contrast to the subcutaneously implanted tumor, the orthotopic model was exquisitely sensitive to gemcitabine. In fact, the primary patient tumor and orthotopically implanted tumor had a similar gemcitabine response signature but diverse from the subcutaneously implanted tumor (Supplementary Fig. S1). Moreover, whereas a desmoplastic reaction was present in the orthotopic model, it was significantly decreased in the subcutaneous xenograft (Supplementary Fig. S2). Although this discrepancy was only observed in one patient, the evidence above suggest that orthotopic models may be better predictors of response in this disease.

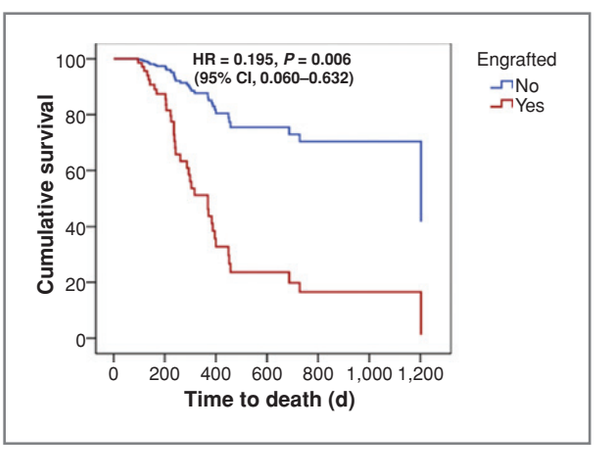


**Figure 4.** Kaplan–Meier survival curves as a function of engraftment. Nonengrafted patients had a decrease in the risk of death of 81%.

## Discussion

The goal of this clinical trial was to prospectively generate fresh PDA xenografts with annotated clinical information and significant biological data. The main results of the study indicate that patients whose tumors engrafted have a poor outcome, which is likely related to the loss of Smad4 and increased metastatic potential. Because of the inherent nature of complex adjuvant protocols, we were not able to correlate response to gemcitabine in the xenograft model and time to progression-free survival given that few patients received gemcitabine alone adjuvantly. Nonetheless, the data confirm that response to gemcitabine in the xenograft model did correlate with response to gemcitabine in the advanced metastatic setting. However, an important limitation of this study was that only 7 patients received single-agent gemcitabine, abrogating definitive statistical correlations.

We show that tumor engraftment is an independent predictor of survival in resected PDA. Patients whose tumors failed to engraft had an 81% reduced risk of death. To further investigate this issue we tested 2 complementary

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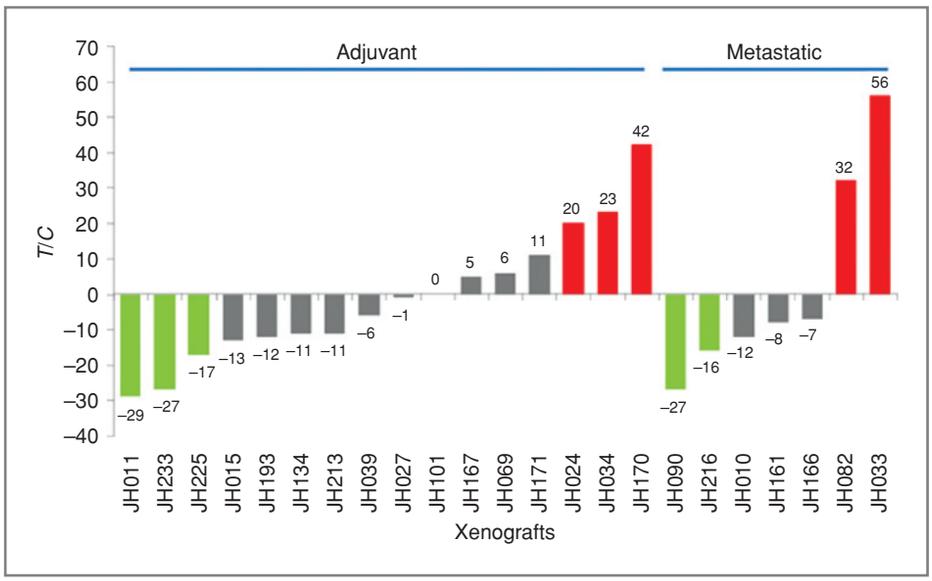


**Figure 5.** Waterfall plot with gemcitabine activity in xenografts. We applied RECIST criteria to *T/C* to define sensitivity/resistance. We reduced the threshold of sensitivity to −15% to have sensitive xenografts, because none of them had a $T/C \leq 30\%$. Green bars represent sensitive cases ($T/C \leq 15\%$), gray bars represent stable disease ($T/C = -15\%$ to 20%) and red bars represent progressive disease ($T/C > 20\%$).

hypotheses. One is that engrafting tumor contains higher proportion of TICs as these cells have been linked to treatment resistance and development of metastasis (20). No differences were observed in the number of TICs as assessed by IHC for ALDH (16). However, other markers that have been associated with TICs in pancreatic cancer were not tested in this study (21). The second hypothesis was that engrafting tumors lack Smad4, based on previous work from our group, which showed that Smad4-defective tumors have a worse prognosis and a higher metastatic potential (18, 22, 23). Indeed, engrafted tumors more frequently had Smad4 loss (67% vs. 36%, $P = 0.024$). Development of metastatic disease is the ultimate determinant of survival in cancer patients. This is further supported by the finding that the gene expression of xenografted tumors is similar to metastatic adenocarcinomas, suggesting that passage of the tumor in mice selects a clonal population of cells with a predilection to colonize new microenvironments. Altogether, the higher prevalence of Smad4 loss and the presence of a metastatic signature of adenocarcinoma in engrafted patients suggest that patients whose tumors engraft are more likely to have metastatic disease. This is consistent with evidence from different disease types showing that engraftment is a marker of poor prognosis (24, 25).

Several lines of evidence suggest that the stroma plays an important role in the pathophysiology of cancer. Enrichment of stroma-related genes was observed in the progression from preinvasive disease to invasive gastrointestinal cancers (26). This mechanism is not disease specific as mesenchymal stem cells within the stroma promote breast cancer growth and metastases (27). Moreover, shifting toward a stromal phenotype is an intrinsic property of chemotherapy-resistant tumors (28). An additional finding in our work was that stromal pathways were enriched in gemcitabine-resistant patients. We acknowledge that the number of samples included in the gene expression analysis was too low to draw definitive conclusions (Supplementary Table S1); however, this finding needs to be examined in the context of earlier work showing that stromal depletion may increase tumor permeability to gemcitabine and result in increased drug activity (6, 7). A phase III trial is testing this "stromal collapse" strategy based on promising activity in the phase I setting (29).

The work presented here also augments information from previous preclinical models by developing tumors from clinically well-annotated cases. For many of these tumors, there is significant information available from "omics" technologies, including global exonic sequencing (10). This is a unique platform for conducting additional research in PDA, although the development of these platforms has not been limited to PDA. Recently, several papers have reported similar efforts in other diseases such as lung, melanoma, and breast cancers (24, 30, 31). It is anticipated that these models will progressively become more utilized in early drug development discovery.

A fundamental question that must be answered to justify the use of freshly generated xenograft platforms for drug development is whether findings in the model correlate with clinical findings. Our first attempt to see whether such a correlation existed was to analyze the results of adjuvant treatment. However, most patients included in this series had been treated with complex multimodality treatments that were impossible to replicate in a murine model (Supplementary Table S1). In retrospect, it became apparent that the ability to appropriately assess correlations between the model and clinical responses in patients rested upon protocol-guided treatments. In the group of patients who received gemcitabine in a setting of advanced cancer, the time to treatment failure was longer in patients whose xenografts responded better to gemcitabine. A difficulty

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we encountered was the lack of efficacy of gemcitabine in these xenografts and, indeed, as borne out by applying RECIST criteria to the *T*/*C* data and finding that not a single case reached the 30% partial response mark (Fig. 5). This lack of response highlights the major issue in PDA treatment, which is the lack of minimally effective treatments. Although overall these data are encouraging, additional work is needed to properly determine the predictive capabilities of these models. This point is illustrated by the potentially better predictive power of orthotopic implantation versus subcutaneous implantation.

In summary, we have developed and characterized a set of 42 PDA-engrafted xenografts with annotated clinical information. Interestingly, successfully engrafted tumors are more likely to lack Smad4 and show a gene expression profile similar to cancer metastasis, which might explain the finding that these patients have a poorer prognosis. The stromal compartment has a putative role in resistance to gemcitabine. Despite the limitations in the number of patients and the overall lack of efficacy of gemcitabine treatment, the model seems to be predictive of response to gemcitabine in the metastatic setting. Given the complexities of generating these models and testing drugs in real time, it is unlikely that this approach will be broadly applicable to personalized cancer treatment, at least in PDA with its rapid clinical course and very few effective agents to address this refractory tumor. Moreover, our findings suggest that host microenvironments are better mimicked by orthotopic models. Although our data are limited to one patient, they suggest that orthotopic xenografts may be better predictors of response. Additional work is needed to continue developing and exploring the role of freshly generated xenografts in cancer research.

## Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

## Authors' Contributions

Conception and design: A. Maitra, B. Rubio-Viqueira, A. Jimeno, and M. Hidalgo. Financial support: M. Hidalgo and A. Maitra. Administrative support: M. Hidalgo and G. Taylor. Provision of study materials or patients: I. Garrido-Laguna, M. Uson, G. Taylor, D. Laheru, A. Maitra, R.H. Hruban, A. Jimeno, and M. Hidalgo. Experiments, collection, and assembly of data: I. Garrido-Laguna, M. Uson, N.V. Rajeshkumar, A.C. Tan, E. de Oliveira, C. Karikari, M.C. Villaroel, A. Salomon, and R. Sharma. Data analysis and interpretation: I. Garrido-Laguna, M. Uson, R.H. Hruban, A. Maitra, and M. Hidalgo. Manuscript writing: I. Garrido-Laguna, M. Uson, N.V. Rajeshkumar, R.H. Hruban, A. Maitra, and M. Hidalgo.

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