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The Canary in the Coal Mine: The Growth of Patient-Derived Tumorgrafts in Mice Predicts Clinical Recurrence after Surgical Resection of Pancreatic Ductal Adenocarcinoma

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Abstract

Background—Recurrence after resection of pancreatic ductal adenocarcinoma (PDAC) is common, thus postoperative surveillance is critical for detection and treatment of recurrent disease. The development of biologically based techniques for early recurrence detection may enable more timely and effective treatment of such recurrences.

Methods—Tumor fragments derived from patients who underwent potentially curative resection of PDAC were heterotopically implanted into NOD/SCID mice. Engraftment success rates and growth parameters were matched to clinicopathologic data, preoperative treatment status, and oncologic outcomes to correlate disease-free survival (DFS) and overall survival.

Results—Seventy patients consented to participate with 56 (80 %) developing a mouse PDAC tumorgraft. Patients with successful engraftment had a shorter median DFS compared with patients whose tumorgrafts failed to engraft (9.8 vs. 40.9 mo, respectively; $p \leq 0.01$). Fifty patients received preoperative therapy with 36 (72 %) successful tumorgrafts from this cohort. On multivariate analysis, lymph node metastasis (hazard ratio [HR] 3, 95 % CI 1.4–6.7, $p \leq 0.01$) and successful engraftment (HR 5.8, 95 % CI 2–16.9, $p \leq 0.01$) were predictive of a shorter DFS in the preoperative therapy cohort. In patients who recurred, tumorgraft formation was identified at a median of 134.5 days before standard methods of radiographic recurrence detection ($p \leq 0.01$).

Conclusions—Patient-derived tumorgrafts from resected PDAC may potentially predict recurrence months before currently available surveillance modalities. This lead-time advantage may allow for earlier implementation or changes in therapy as successful engraftment, particularly in those having undergone preoperative therapy, may indicate a more biologically aggressive disease.

Postoperative surveillance is critical after resection of pancreatic ductal adenocarcinoma (PDAC), because the majority of patients develop recurrence after resection.^{1–4} Current National Comprehensive Cancer Network (NCCN) guidelines recommend a history and physical exam for new symptom assessment, serum carbohydrate antigen 19-9 (CA19-9) measurement, and abdominal/pelvic CT every 3–6 months for 2 years postoperatively and annually thereafter, but presents significant challenges.⁵ As a biomarker, CA19-9 is neither sensitive nor specific for PDAC, is not secreted by every tumor, and is most often elevated with advanced disease.^{6–8} Additionally, when recurrence is diagnosed radiographically, patients often have a reduced performance status or significant tumor burden that prevents the initiation of systemic chemotherapy.^{7–9} Developing a surveillance program that detects disease earlier is therefore critical to offer the practitioner an opportunity for timely therapeutic interventions.

We evaluated the potential role of a patient-derived tumorgraft program in predicting recurrence and survival in patients who underwent potentially curative resection for PDAC and compared it to preexisting surveillance methods of serum CA19-9 and imaging. Because the majority of patients treated for PDAC at our institution receive preoperative therapy, we evaluated the impact of this treatment on tumor engraftment rates in mice and recurrence prediction compared to treatment-naïve tumors. We hypothesized that successful patient-derived tumorgraft growth, irrespective of preoperative treatment status, correlates with clinical recurrence. Additionally, if preoperative treatment induces cellular death in the primary tumor, we hypothesized that the engraftment rates would be reduced in this patient cohort and that successful engraftment despite preoperative therapy would equate to more aggressive biology with earlier recurrence and reduced patient survival.

METHODS

The Institutional Review Board and the Institutional Animal Care and Use Committee at The University of Texas MD Anderson Cancer Center approved all aspects of this study. After informed consent, patients with pathologically confirmed PDAC underwent potentially curative pancreatic resection using standardized techniques and patient demographics and clinicopathologic factors were recorded.¹⁰ A standardized pathological evaluation was performed as previously described.^{11,12} Preoperative therapy involved the administration of systemic chemotherapy and radiation based on a multidisciplinary consensus.

A portion of the resected primary tumor was removed for heterotopic implantation into the flank of five separate NOD/SCID mice (Charles River Laboratories International, Wilmington, MA), aged 4–8 weeks.¹³ Mice were monitored for the development of tumors and harvested when it reached 1 cm in diameter, which was the primary experimental

endpoint and used for comparison to matched clinicopathologic data and cancer-related endpoints.

A tissue microarray (TMA) was generated from formalin-fixed, paraffin-embedded tissue from each original patient tumor and its subsequent matched tumorgraft. Immunohistochemistry (IHC) of each TMA slide was performed to confirm uniformity and preservation of expression patterns for the following markers between the primary tumor and paired tumorgrafts: Beta-catenin (BD Biosciences), Caspase-3 (Cell Signaling), E-cadherin (Invitrogen), p16 (Ventana Medical), and SMAD4 (Santa Cruz Biotechnology).¹⁴ IHC scoring was performed in a blinded fashion based on staining intensity (0 = absent/weak, 1 = moderate, 2 = strong) and the percentage of cells staining positively (0 = 0, 1 = \30 and 2 = C30 %). The product of the scores was tabulated, and it was from this numerical value that inter-rater agreement was calculated. Positivity by IHC was defined a priori as staining intensity C1 and C30 % of cells staining positively. The inter-rater agreement was thus calculated between each primary tumor and its paired tumorgraft with staining correlation receiving a score of 1 and absence of correlation receiving a score of 0.

Statistical analyses were performed using MedCalc (v12.3) statistical software (MedCalc Software, Mariakerke, Belgium). Summary statistics were used to describe the clinical and demographic characteristics of the cohort that underwent tumorgraft implantation. Differences between continuous and categorical variables were assessed utilizing the Wilcoxon and Pearson's Chi squared tests, respectively. The inter-rater agreement (K) was calculated to compare the correlation of IHC staining between primary tumor and paired tumorgraft.¹⁵ Time to formation (TTF) was defined as the time from implantation to palpable tumor formation with growth confirmation in subsequent biweekly evaluations. The engraftment ratio was the ratio of mice with successful engraftment to the total number of mice implanted for any individual patient. Disease-free survival (DFS) was defined as the time from surgery to the date of clinically documented local, regional, or distant disease recurrence, death attributed to PDAC, or last follow-up, at which point the data were censored. Overall survival (OS) was defined as the time from surgery to the date of death or last follow-up, at which point the data were censored. The Kaplan–Meier method was used to estimate OS and DFS based on clinicopathologic and tumorgraft factors. Cox proportional hazards regression was used to model the association between tumorgraft parameters and DFS or OS. A two-sided p value of 0.05 and a 95 % confidence interval (CI) were used for all statistical analyses.

RESULTS

Patient Demographics and Tumorgraft Metrics

A total of 228 patients underwent potentially curative pancreatic resection for PDAC between February 2008 and July 2012, of which 70 (30.7 %) patients consented for the study with a median follow-up of 15.5 (range 1.6–54.4) months. Figure 1 summarizes the cohorts based on surgery first versus preoperative therapy followed by surgery (preoperative therapy cohort). There were no differences in patient demographics or clinicopathologic parameters between those who had successful engraftment and those who did not except preoperative therapy (Table 1). Of the 70 tumors that were implanted, 56 (80 %) had

successful engraftment and growth. Successful engraftment was achieved in 36 patients (76 %) in the preoperative therapy cohort compared with all 20 patients (100 %) who underwent surgery first ($p = 0.02$). The median TTF for all successful tumorgrafts was 29.5 (range 8–312) days, and there was no TTF difference between the preoperative therapy cohort (33 days, range 12–312) and surgery first cohort (22.5 days, range 8–164; $p = 0.2$). The median engraftment ratio for the entire cohort was 0.7 (mean 0.59) and was lower for the preoperative therapy cohort versus surgery first (0.55 vs. 0.8, respectively; $p \leq 0.01$).

Stability of PDAC Pathways in Patient-derived Tumorgrafts

Multiple genetic alterations have been documented in PDAC affecting numerous cellular pathways.¹⁶ Patient-derived tumorgrafts have been previously demonstrated to recapitulate the altered pathways of the primary tumor from which they were derived.^{17,18} In order to demonstrate similar pathway stability in our cohort and to ensure that any expression differences were not responsible for differences in engraftment, IHC was performed and the correlation between samples was determined. There was excellent inter-rater agreement ($K = 0.81$ – 1.00) for IHC scoring between each primary tumor and paired tumorgraft for every marker tested, thus demonstrating expression stability (Table 2).

Relationship of Survival to Tumorgraft Growth

At last follow-up, the median DFS and OS of all 228 resected patients was 15.9 months and 27.9 months, respectively. There was no difference in DFS or OS (Figs. 2a, b) between the patients who did not undergo tumor engraftment versus those who did [(16.6 vs. 14.1 months, $p = 0.2$) and (28 vs. 27.9 months, $p = 0.9$), respectively]. Patients whose tumors successfully engrafted experienced a shorter median DFS versus those whose did not (9.8 vs. 40.9 months, respectively; $p \leq 0.01$). The median OS for patients with successful engraftment was shorter compared with those whose tumor did not engraft but was not statistically significant, likely because the median was not yet reached (20.7 months vs. Median not reached, respectively; $p = 0.1$; data not shown). Of the 70 attempted tumor implantations, no differences in DFS or OS were found between patients who received preoperative therapy versus those who did not (data not shown). However, when patients who underwent preoperative therapy were stratified based on tumorgraft growth, the median DFS and OS were shorter for patients who had successful tumor engraftment versus those who did not [(DFS 6.4 vs. 40.9 months, respectively; $p \leq 0.01$, HR 3.4, 95 % CI 1.7–6.7; Fig. 2c) and (OS 20.7 months vs. median not reached, respectively; $p = 0.03$, HR 2.8, 95 % CI 1.1–6.8; Fig. 2d)]. Finally, of the patients who had successful engraftment, the median DFS was significantly shorter for the preoperative therapy cohort versus patients who underwent surgery first (6.4 vs. 18.3 months, respectively; $p = 0.04$), but there was no difference in median OS between these groups (20.7 months vs. median not reached, respectively; $p = 0.2$).

Predictors of DFS and OS

Previous studies have demonstrated that CA19-9 normalization is a predictor of survival in patients undergoing pancreatectomy for PDAC.^{6,19–22} The effect of CA19-9 normalization after preoperative therapy in regards to tumor engraftment and thus survival was evaluated to help establish a link between tumor engraftment and CA19-9 normalization. In patients who

underwent preoperative therapy, there was a greater proportion of successful tumorgrafts in patients who failed to normalize CA19-9 (40) versus those who had normalization but this was not statistically significant [$n = 17$ (89.5 %) vs. $n = 18$ (66.7 %), respectively; $p = 0.15$]. Patients who underwent preoperative therapy and failed to have CA19-9 normalization had a significantly decreased DFS than patients who did (6.6 vs. 26.8 months, respectively; $p = 0.02$). In addition, failure to normalize CA19-9 resulted in a shorter OS compared with patients who had CA19-9 normalization after preoperative treatment (18.8 vs. 43.8 months, respectively; $p < 0.01$).

Univariate analysis revealed that failure of serum CA19-9 normalization, lymph node metastasis, engraftment ratio [75 %, and successful tumor engraftment negatively predict DFS in patients who received preoperative therapy. Factors predictive of a shorter OS in the preoperative therapy cohort included failure of serum CA19-9 normalization, lymph node metastasis, primary tumor size [2.5 cm, and successful tumor engraftment (Table 3). After adjusting for factors identified on univariate analysis, lymph node metastasis (HR 3, 95 % CI 1.4–6.7, $p < 0.01$) and successful engraftment (HR 5.8, 95 % CI 2–16.9, $p < 0.01$) were predictive of a shorter DFS in the preoperative therapy cohort on multivariate analysis. Only the presence of lymph node metastasis (HR 3.3, 95 % CI 1.04–10.7, $p = 0.04$) and CA19-9 normalization post-treatment (HR 0.36, 95 % CI 0.13–1.01) were predictors of OS on multivariate analysis.

Relationship of Tumorgraft Growth Metrics to Detection of Clinical Recurrence

Ninety-one of the 158 patients (57.6 %) who did not undergo tumor engraftment developed a clinical recurrence a median of 253 (range 37–1207) days postoperatively. Of the 70 patients who had attempted tumor engraftment, 41 %) developed a clinical recurrence a median of 192 (range 29–1330) days after surgery. There was no statistical difference in the recurrence rates or time to clinical recurrence between these groups. The clinically documented time to recurrence (TTR) was statistically shorter for patients who developed a clinical recurrence and had successful tumorgraft growth ($n = 33$, 58.9 %) versus those who developed a recurrence but their tumorgraft did not grow ($n = 8$, 57.1 %) [177 (range 29–1092) days vs. 593 (range 41–1330) days, respectively; $p = 0.02$]. Matched analysis of the 33 patients who recurred and had successful tumor engraftment, comprised of 24 preoperative therapy patients and 9 surgery-first patients, determined that the median TTF was 38 (range 14–293) days. Pairing the TTR of each clinical case to the TTF of each corresponding tumorgraft revealed a median lead-time advantage of 134.5 days (mean 193.1, range –185 to 871; $p < 0.01$) for the tumorgrafts to predict recurrent disease (Fig. 3).

DISCUSSION

Despite a possible curative resection, the majority of patients with PDAC will develop recurrence.^{2,4,6} This report describes a patient-specific and potentially clinically relevant system that appears associated with recurrence after resection for PDAC. This is most likely relevant in patients whose tumors successfully engrafted despite preoperative exposure to cytotoxic therapy. This association was demonstrated in that tumorgraft growth was a stronger predictor of clinical recurrence than lymph node metastasis on multivariate analysis

(HR 5.8 vs. 3, respectively). Thus, successful tumor engraftment after preoperative therapy is a particularly ominous prognostic indicator.

Various surveillance strategies after resection for PDAC reflect a lack of consensus with current NCCN guidelines recommending serum CA19-9, imaging, and symptom assessment every 3–6 months postoperatively.⁵ This is based on studies demonstrating that CA19-9 levels after preoperative therapy or surgical resection for PDAC can serve as a surrogate for tumor burden with increases indicative of recurrence.^{6,19–22} CA19-9 is not PDAC specific and some tumors do not produce the antigen but is the best serum marker available.⁸ We have demonstrated a correlation between CA19-9 normalization after preoperative therapy, tumorgraft growth, and survival which suggests that like failure of CA19-9 to normalize, successful engraftment of patient-derived PDAC may also be a harbinger of recurrence in resected patients after preoperative therapy. Similar to CA19-9, utilizing imaging as a means of surveillance does not allow preemptive institution or alteration of treatment regimens as once symptoms or radiographically evident disease develops during the surveillance period, there is often substantial tumor burden or a decline in performance status rendering any potential therapy as palliative.⁹ To our knowledge, this is the first report that links engraftment of patient-derived PDAC tumorgrafts to the time of clinical recurrence, thereby providing a potential lead-time advantage to identify patients at high risk for recurrence before clinical detection.

While this study sought to evaluate the utility of patient-derived tumorgraft as a surveillance strategy, further research is needed. In order to confirm the utility of such a program, a larger cohort of patients, including both individuals who received preoperative therapy and others who proceeded directly to surgery will be needed, as our cohort was skewed towards patients who received preoperative therapy. This may be accomplished through multi-institutional recruitment or regionalization of tumorgraft programs. Regionalization, however, would delay implantation, increase false-negative engraftment rates, and likely lose the correlation demonstrated with timing of recurrence. Improvements in tissue preservation to make regionalization feasible would be invaluable. Additional studies also should be centered on the use of patient-derived tumorgraft programs to test novel therapeutics and patient (i.e., tumor) specific treatment during the lead-time prior to any clinical recurrence.^{23–27}

Clinicians would be able to use this information to guide treatment at the time of recurrence, either by using established regimens or to better select patients for clinical trials. Generating tumor-grafts from initial diagnostic core biopsies of pancreatic tumors to determine real-time chemosensitivity for adjuvant use also can expand such a program. Finally, patients may be enrolled prospectively to undergo additional systemic therapy should they have successful tumor engraftment. This may be particularly useful for patients who underwent preoperative therapy and had a good pathologic response (negative nodes and margins) yet had successful tumor engraftment, as they normally would not receive adjuvant therapy. In this way, the true utility of such a surveillance program may be established.

In conclusion, our data demonstrate that a patient-derived tumorgraft program may have utility as part of a pancreatic cancer surveillance program in patients who have undergone potentially curative resection, especially after undergoing preoperative therapy. We demonstrate that successful patient-derived tumorgraft growth portends a poor DFS and OS

in resected individuals. Such a program may detect recurrence approximately 4 months before the clinical detection, allowing a window of opportunity to assess tumor-sensitivity to experimental or known therapeutic regimens with in vivo testing. Finally, patients who have undergone preoperative therapy prior to resection, yet have successful engraftment of their tumors, demonstrate their unfavorable tumor biology and may benefit the most from stricter surveillance. Implementing a patient-derived tumorgraft program therefore may be one avenue by which disease recurrence can be detected earlier allowing for the timely initiation of therapies in the hope of altering outcomes.

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FIG. 1.

Flow diagram of the cohort based on the treatment strategy of surgery first (a) versus preoperative therapy followed by surgery (b). Each arm illustrates the number of primary tumor implantations attempted and the number of subsequent tumorgrafts established. Total number patients in each group (n), number of recurrence events with median DFS, and number of deaths with median OS are indicated for each patient group

FIG. 2.

Kaplan-Meier analysis comparing the entire cohort of patients who underwent potentially curative resection of PDAC from February 2008 to July 2012, based on attempted tumorgraft implantation, did not show a statistical difference in DFS (a) or OS (b) between the two groups. Patients who underwent preoperative therapy and had successful tumorgraft growth had a significantly shorter DFS (c) and OS (d) compared with patients who underwent preoperative therapy yet did not have successful growth of their tumorgraft

FIG. 3.

The timing of each patient's postoperative PDAC recurrence was matched to the timing of their corresponding individual tumorgraft growth. This paired analysis demonstrated that tumorgraft formation occurred a median of 134.5 days prior to the detection of a clinical recurrence ($p < 0.01$)

TABLE 1

Clinicopathologic parameters of the patient cohort

	Tumorgraft			p
	Entire cohort n = 70	No growth n = 14	Growth n = 56	
Gender, n (%)				NS (0.95)
Male	32 (45.7)	7 (50)	25 (44.6)	
Female	38 (54.3)	7 (50)	31 (55.4)	
Age (year)				NS (0.65)
Mean (range)	64.6 (39.4–81.7)	65.6 (45–81.7)	64.4 (39.4–81.4)	
Median	63.1	67.6	62.6	
SD	8.8	9.7	8.6	
Preoperative CA 19-9				NS (0.16)
Mean (range)	140.8 (0–2174)	37.8 (1–159)	167.1 (0–2174)	
Median	38.9	29.7	54.9	
SD	303.1	41.2	334.4	
Procedure, n (%)				NS (0.94)
Pancreaticoduodenectomy	53 (75.7)	10 (71.4)	43 (76.8)	
Distal/total pancreatectomy	17 (24.3)	4 (28.6)	13 (23.2)	
Operative time				NS (0.76)
Mean (range)	448.5 (161–702)	439.6 (206–586)	450.7 (161–702)	
Median	453	467.5	450	
SD	119.9	110.6	122.9	
Tumor size (cm)				NS (0.98)
Mean (range)	2.87 (1–6)	2.63 (1.5–4)	2.93 (1–6)	
Median	2.9	2.75	2.9	
SD	1.12	0.85	1.17	
Lymph node status, n (%)				NS (0.58)
Negative	28 (40)	7 (50)	21 (37.5)	
Positive	42 (60)	7 (50)	35 (62.5)	
Margin status, n (%)				NS (0.56)
Negative	65 (92.9)	14 (100)	51 (91.1)	
Positive	5 (7.1)	0 (0)	5 (8.9)	
Perineural invasion, n (%)				NS (0.69)
No	10 (14.5)	2 (14.3)	8 (14.5)	
Yes	59 (85.5)	12 (85.7)	47 (85.5)	
Lymphovascular invasion, n (%)				NS (0.23)
No	27 (39.7)	8 (57.1)	19 (35.2)	
Yes	41 (60.3)	6 (42.9)	35 (64.8)	
Tumor viability, n (%)				NS (0.63)
0–10 %	9 (17.6)	3 (21.4)	6 (16.2)	
11–50 %	22 (43.1)	7 (50)	15 (40.5)	
51–100 %	20 (39.2)	4 (28.6)	16 (43.2)	

	Tumorgraft			p
	Entire cohort n = 70	No growth n = 14	Growth n = 56	
Any preoperative therapy, n (%)				0.021
No	20 (28.6)	0 (0)	20 (35.7)	
Yes	50 (71.4)	14 (100)	36 (64.3)	
Any adjuvant therapy, n (%)				NS (0.83)
No	15 (22.4)	3 (21.4)	13 (23.2)	
Yes	52 (77.6)	11 (78.6)	43 (76.8)	

NS not significant

IHC correlation of PDAC pathway expression between primary tumor and F1 tumorgraft

TABLE 2

Marker	Primary tumor negative	F1 tumorgraft negative	Primary tumor positive	F1 tumorgraft positive	Primary versus F1 agreement (K)	SE	95 % CI
Beta-catenin	0	0	13	13	1.00	0.00	1.00–1.00
Caspase-3	0	0	13	13	1.00	0.00	1.00–1.00
E-cadherin	0	0	12	12	1.00	0.00	1.00–1.00
p16	9	8	5	6	0.85	0.14	0.57–1.00
SMAD4	9	9	2	2	1.00	0.00	1.00–1.00
All markers	18	17	45	46	0.96	0.04	0.88–1.00

TABLE 3

Univariate analysis of patient survival in the cohort that underwent preoperative therapy

Preop Tx cohort n = 50		Univariate analysis			
		Disease-free survival		Overall survival	
		HR (95 % CI)	p	HR (95 % CI)	p
Gender, n (%)					
Male (ref)	21 (42)	1		1	
Female	29 (58)	1.05 (0.52–2.1)	NS	1.22 (0.49–3)	NS
Age [64 year, n (%)					
No (ref)	26 (52)	1		1	
Yes	24 (48)	0.82 (0.41–1.65)	NS	2.04 (0.83–5)	NS
CA19-9 normalization (40), n (%) *					
No (ref)	19 (40.4)	1	0.02	1	0.01
Yes	28 (59.6)	0.44 (0.2–0.96)		0.3 (0.11–0.77)	
Procedure, n (%)					
Pancreaticoduodenectomy (ref)	38 (76)	1		1	
Distal/total pancreatectomy	12 (24)	1.84 (0.74–4.6)	NS	1.65 (0.51–5.4)	NS
Tumor [2.5 cm, n (%)					
No (ref)	25 (50)	1	NS	1	
Yes	25 (50)	1.82 (0.91–3.7)	3.1 (1.26–7.6)	0.02	
Lymph node status, n (%)					
Negative (ref)	24 (48)	1		1	
Positive	26 (52)	2.1 (1.03–4.1)	0.04	4.1 (1.7–10.1)	0.01
Perineural invasion, n (%)					
No (ref)	10 (20)	1		1	
Yes	40 (80)	1.76 (0.74–4.2)	NS	4.5 (1.5–14)	NS
Lymphovascular invasion, n (%)					
No (ref)	24 (49)	1		1	
Yes	25 (51)	1.4 (0.71–2.9)	NS	1.86 (0.76–4.6)	NS
Tumor viability [50 %, n (%)					

Preop Tx cohort n = 50		Univariate analysis			
		Disease-free survival		Overall survival	
		HR (95 % CI)	p	HR (95 % CI)	p
No (ref)	33 (67.3)	1		1	NS
Yes	16 (32.7)	1.76 (0.8–3.8)	NS	2.3 (0.84–6.2)	
Any adjuvant therapy, n (%)					
No (ref)	13 (26)	1		1	
Yes	37 (74)	1.6 (0.76–3.4)	NS	2.9 (1–8.1)	NS
Tumorgraft growth, n (%)					
No (ref)	14 (28)	1	0.01	1	0.03
Yes	36 (72)	3.4 (1.7–6.7)		2.8 (1.1–6.8)	
Tumorgraft engraftment ratio [75 %, n (%)					
No (ref)	29 (58)	1	0.04	1	NS
Yes	21 (42)	2 (0.9–4.2)		1.6 (0.6–4.3)	

* Denominator does not equal 50 because 3 patients did not have detectable serum CA19-9 levels prior to or after preoperative therapy and were thus excluded from analysis