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Clinical Cancer Research

Imaging, Diagnosis, Prognosis

Clinical Significance of the Genetic Landscape of Pancreatic Cancer and Implications for Identification of Potential Long-term Survivors

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Abstract

Purpose: Genetic alterations of *KRAS*, *CDKN2A*, *TP53*, and *SMAD4* are the most frequent events in pancreatic cancer. We determined the extent to which these 4 alterations are coexistent in the same carcinoma, and their impact on patient outcome.

Experimental Design: Pancreatic cancer patients who underwent an autopsy were studied ($n = 79$). Matched primary and metastasis tissues were evaluated for intragenic mutations in *KRAS*, *CDKN2A*, and *TP53* and immunolabeled for CDKN2A, TP53, and SMAD4 protein products. The number of altered driver genes in each carcinoma was correlated to clinicopathologic features. Kaplan–Meier estimates were used to determine median disease free and overall survival, and a Cox proportional hazards model used to compare risk factors.

Results: The number of genetically altered driver genes in a carcinoma was variable, with only 29 patients (37%) having an alteration in all 4 genes analyzed. The number of altered driver genes was significantly correlated with disease free survival ($P = 0.008$), overall survival ($P = 0.041$), and metastatic burden at autopsy ($P = 0.002$). On multivariate analysis, the number of driver gene alterations in a pancreatic carcinoma remained independently associated with overall survival ($P = 0.046$). Carcinomas with only 1 to 2 driver alterations were enriched for those patients with the longest survival (median 23 months, range 1 to 53).

Conclusions: Determinations of the status of the 4 major driver genes in pancreatic cancer, and specifically the extent to which they are coexistent in an individual patients cancer, provides distinct information regarding disease progression and survival that is independent of clinical stage and treatment status. *Clin Cancer Res; 18(22); 6339–47. ©2012 AACR.*

Introduction

Pancreatic ductal adenocarcinoma (PDAC) is one of the most lethal solid malignancies and a major cause of cancer-related deaths in developed countries (1), with a >95% mortality rate. Most patients present with locally advanced or metastatic disease at initial diagnosis leaving relatively few as candidates for a potentially curative resection. Unfortunately, even in patients who undergo pancreatic resection, both local and systemic recurrences are common with a median postresection survival of less than 18 months (2).

The recent completion of the pancreatic cancer exome marked a notable milestone (3). The coding regions of 20,661 genes were sequenced in 24 PDACs indicating that these neoplasms contain an average of 63 genomic alterations, the majority of which are point mutations. Moreover, the genetic landscape of the PDAC genomes is notable for 4 frequently mutated genes, designated "mountains," including *KRAS*, *CDKN2A (p16)*, *TP53*, and *SMAD4 (DPC4)*. Numerous candidate cancer genes altered at low frequency, designated "hills," were also identified such as *MLL3* and *ARID1A* (3, 4). These 4 mountain genes are well recognized as contributing to pancreatic carcinogenesis (5), and are thus classifiable as "driver" genes for this tumor type. Furthermore, based on comparative lesion sequencing these 4 genes are also classifiable as "founder" mutations

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Translational Relevance

Irrespective of clinical stage at diagnosis, most patients with pancreatic cancer will die of their disease. Although genomic efforts have now clarified the genetic basis for pancreatic cancer, the relationship of the genetic landscape to an individual patients' outcome is unknown. This study shows that there are distinct patterns and prevalence of the number of genetically altered driver genes in pancreatic cancer, a concept of significance for screening efforts based on identification of mutated alleles in body fluids. We also show that the number of altered driver genes is independently correlated with patient outcome, and that specific subsets of coexistent genes correspond to a greater incidence of metastatic failure. Finally, we show that carcinomas with 1 or 2 driver gene alterations identify a subset of patients with relatively more indolent disease, a finding of significance for early identification of long-term survivors.

in that they are present in the original parental clone that gave rise to the infiltrating carcinoma (6). Although additional genetic alterations accumulate during the ongoing clonal evolution of the carcinoma ("progressor" mutations), the constellation of founder mutations contained within the parental clone presumably constitutes the major characteristics for that carcinoma (6, 7).

The relationship between the genetic status of these 4 genes and clinicopathologic features, including survival have been previously studied. However, until now this work has focused on individual genes and has yielded conflicting results (8–14). Furthermore, although genetically engineered mouse models indicate that the concomitant expression of these mutated genes is crucial to progress to invasion and metastasis in PDACs (15–19), the extent to which the coexistence of 3 or more of these altered genes in the same PDAC influence the biologic behavior and survival outcome is unknown.

The objective of this study was to clarify the clinical significance of the genetic landscape of pancreatic cancer, specifically the genetic status of the *KRAS*, *CDKN2A*, *TP53*, and *SMAD4* driver genes in a large series of nonfamilial advanced stage PDACs with known outcomes including patterns of failure and in a second set of xenografted PDACs. We now show that there are distinct patterns and prevalences to which these driver genes occur in the same carcinoma, and that these patterns are highly correlated with clinical features of patients.

Patients and Methods

Patients and tissue samples

Paraffin-embedded and snap-frozen tissue samples from 79 patients collected in association with the Gastrointestinal Cancer Rapid Medical Donation Program (GICRMDP) were used. This program was previously reported in detail (20). Among these 79 patients, 20 initially underwent surgical resection and the remaining 59 patients were initially diagnosed with Stage III/IV unresectable disease. On the basis of autopsy findings and clinical chart review, all patients died of causes directly related to their disease. The Johns Hopkins Institutional Review Board approved use of all patient samples for this study.

Sanger sequencing

Snap frozen tissue samples were embedded in OCT compound (Sakura Finetek), sectioned by a cryostat and stained by hematoxylin and eosin. Tumor tissues were dissected macroscopically if the neoplastic cellularity was at least 50%, or microscopically using a PALM MicroLaser System (Carl Zeiss MicroImaging) for cases with low neoplastic cellularity. Genomic DNA from dissected tissues was extracted using phenol-chloroform, or QIAmp DNA Micro Kits if microdissected (Qiagen). Genomic DNA from microdissected tissues was quantified by calculating long interspersed nuclear elements (LINE) by real-time PCR as described previously (6) and whole genome amplification (WGA) was carried out using 10 ng total template gDNA and an illustra GenomiPhi V2 DNA Amplification Kit (GE Healthcare). PCR amplification was carried out using 20 ng of gDNA for *KRAS* exons 1 and 2, *TP53* exons 5 to 9 and *CDKN2A* exons 1 and 2 using intronic primers flanking these exons (Supplementary Table 1). PCR products were sequenced by use of a M13F primer (5′-GTAAAACGACGGCCAGT-3′) or M13R primer (5′-CAGGAAACAGCTATGACC-3′) that was incorporated into the forward and reverse primer of each primer pair, respectively (Beckman Coulter Genomics). Sequencing data were analyzed with Sequencher 4.10 software (Gene Codes). Mutation analysis, confirmation and determination of somatic status were carried out using matched normal tissues from the same patient.

Immunohistochemistry

Paraffin-embedded samples of the primary carcinoma and matched metastases were immunolabeled for Cdkn2A, Tp53, and Smad4 as an adjunct to sequencing. At least 5 different distinct regions of the primary carcinoma were immunolabeled for each case to evaluate for potential heterogeneity. In the event of positive immunolabeling for Cdkn2A or Smad4 in the primary carcinoma, at least 5 different matched metastases, and local recurrences if available, were also labeled to assess for gene inactivation during disease progression. Immunohistochemical labeling was carried out using antibodies to Cdkn2A protein (ready-to-use, clone E6H4, MTM Laboratories), Tp53 protein (ready-to-use, Bp-53-11, Ventana) and Smad4 protein (clone B8, Santa Cruz Biotechnology) as reported (21). Nuclear labeling of Cdkn2A was scored as intact (positive, indicating the presence of an intact gene) or lost (negative, indicating a deletion, inactivating mutation, or promoter hypermethylation; refs. 22, 23). As previously described (21), Tp53 immunolabeling was considered abnormal when it showed robust nuclear accumulation of

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immunolabeled protein in ≥30% of the neoplastic cells compared with adjacent normal cells, or if the neoplastic cells showed a virtual absence of immunolabeling compared with immediately adjacent normal cells suggesting the presence of an intragenic deletion, nonsense or frameshift mutation (24–26). In all instances p53 labeling was evaluated within sections cut from at least 2 different paraffin blocks of the same carcinoma. Nuclear labeling of Smad4 was scored as intact (positive, indicating the presence of an intact gene) or lost (negative, indicating a deletion or inactivating mutation of the gene has occurred; ref. 27). Normal islets for Cdkn2A and normal acinar cells, islets, lymphocytes, and stromal cells for Tp53 and Smad4 were regarded as internal positive controls for each case. Negative controls for each of the antibodies were carried out using nonimmune serum instead of the primary antibody. Slides were scored by 2 of the authors (S.Y. and C.I.D.).

Statistics

Dichotomous variables were compared using Fisher's exact test or the χ^2 test, and continuous variables were compared using the Student *t* test or the Mann–Whitney *U* test, where appropriate. Multiple groups were compared by the Kruskal–Wallis test or the chi-square test, where appropriate. Survival analyses were carried out by the Kaplan–Meier method or Cox regression and survival curves were compared with the logrank test. A *P* value of ≤0.05 was considered statistically significant. Statistical analyses were carried out using SPSS 20.0 software (SPSS).

Results

Clinicopathologic features of autopsied patients

The clinicopathologic features of all 79 patients with lethal pancreatic ductal adenocarcinoma whose tissues were collected in association with the GICRMDP are summarized in Table 1. Detailed findings at autopsy of 60 of these patients were previously described (28). Among all 79 patients, 56% were male and 81% of the primary carcinomas developed in the head or body of the pancreas. Most patients (75%) had advanced stage disease at diagnosis (Stage III or IV), and this corresponded to a median overall survival of 10 months for all 79 patients. Nonetheless, when stratified by stage at diagnosis the median overall survival was 24 months for Stage I/II, 11.5 months for Stage III, and 6.5 months for Stage IV patients. At autopsy, 17 (85%) of Stage I/II patients had a local recurrence although for 3 of these it was the only site of disease found. The liver was the most common site of metastatic disease among all patients and was found in 76% of patients. However, the extent of metastatic disease burden among all patients varied greatly (less than 10 to >100), a reflection of the inherent "metastatic efficiency" of each patient's pancreatic cancer (28).

Table 1. Clinicopathologic features of patients

Characteristic	Autopsy patients (*n* = 79)
Age at diagnosis, years (mean ± SD)	62.2 ± 11.4
Gender (%)	
Male	44 (56%)
Female	35 (44%)
Tumor location (%)	
Head/body	64 (81%)
Tail	14 (18%)
NA	1 (1%)
Stage at diagnosis (%)	
I/II	20 (26%)
III	19 (24%)
IV	40 (50%)
Tumor differentiation (%)	
Well/moderate	27 (34%)
Poor	52 (66%)
Treatment (%)	
Chemoradiation	32 (41%)
Chemotherapy	34 (43%)
None	13 (16%)
Median overall survival, months (range)	10 (0.75–58)
Major sites involved by metastatic disease at autopsy[a] (%)	
Liver (*n* = 79)	60 (76%)
Lung (*n* = 65)[b]	31 (48%)
Peritoneum (*n* = 69)[c]	41 (59%)
Number of sites involved by metastatic disease (%)[b,c]	
0	8 (10%)
1	26 (33%)
2	29 (37%)
≥3	16 (20%)
Metastatic burden (%)	
0–10 (oligometastatic)	22 (28%)
11–100 (moderate)	27 (34%)
>100 (widely metastatic)	29 (37%)

Abbreviation: NA, not available.

[a]Refers to frequency at each site independently.

[b]Data regarding presence of lung metastasis not available for 14 patients.

[c]Data regarding presence of peritoneal metastasis not available for 10 patients.

Genetic features of pancreatic cancers obtained from autopsy

DNA was extracted from snap frozen samples of normal tissue, primary infiltrating ductal adenocarcinomas and multiple matched metastases for all patients and sequenced for *KRAS*, *CDKN2A*, and *TP53*. Multiple samples taken from distinct regions of each primary carcinoma were analyzed (mean 5.9 samples per carcinoma), as well as multiple different metastases (mean 6.3 matched metastases per patient) corresponding to a total of 884 individual samples and greater than 2.5 million bases of sequencing data analyzed.

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Activating mutations in *KRAS* were identified in 73 (92%) of 79 carcinomas analyzed (Supplementary Table 2). Mutations at codon 12 were most common (66 of 73 mutations, 90%), with G12D accounting for 38 (52%) of 73 carcinomas. For 6 carcinomas without a detectable *KRAS* mutation of codons 12, 13, or 61 we also analyzed for mutations of codon 146 (29), but no mutations were found.

High quality sequencing data were obtained for *CDKN2A* in 76 of 79 patient's carcinomas (Supplementary Table 2). Intragenic mutations were identified in 21 (28%) of 76 carcinomas analyzed, corresponding to 8 (38%) missense mutations, 7 (33%) nonsense mutations, and 6 (29%) frameshift mutations. All but 1 carcinoma with an intragenic mutation had loss of Cdkn2A protein expression. Because *CDKN2A* may undergo homozygous deletion or hypermethylation-induced silencing that would not be detected by sequencing (30), we also immunolabeled all 55 carcinomas in which no intragenic mutations were found. Of these, 48 (87%) had loss of Cdkn2A labeling. In total, loss of Cdkn2A secondary to any potential mechanism was detected in 72 of 79 (91%) carcinomas analyzed.

Inactivating mutations in *TP53* were identified in 58 of 79 (73%) carcinomas, of which 28 (48%) were missense mutations, 11 (19%) were frameshift mutations, 9 (16%) were nonsense mutations, 6 (10%) were intragenic deletions, and 4 (7%) were splice-site mutations (Supplementary Table 2). Carcinomas found to be *TP53* wild type by sequencing were also immunolabeled for Tp53 protein to assess for potential large homozygous deletions or mutations outside of the analyzed region. Of these, 3 had robust nuclear accumulation of Tp53 and 5 of 21 had complete absence of Tp53 protein. Overall, *TP53* was altered in 66 of 79 (84%) carcinomas.

Finally, we also determined Smad4 immunolabeling patterns, which is a strong marker of *SMAD4* genetic status (27, 31). Of 79 carcinomas analyzed, 39 (49%) showed loss of Smad4 immunolabeling consistent with inactivation of the *SMAD4* gene (Supplementary Table 2).

In 73 patients analyzed (92%), there was complete concordance for genetic status and/or immunolabeling patterns of all genes in the primary carcinoma and the matched metastases. Of the remaining 6 patients, 1 showed intact Smad4 labeling in the primary carcinoma and peritoneal metastases, whereas the matched liver metastases in this patient showed loss of labeling, indicating genetic inactivation of *SMAD4* occurred during subclonal evolution and metastatic progression (Fig. 1A and B). In an additional 5 patients intratumoral heterogeneity for Cdkn2A labeling was observed in the primary carcinoma in that regions of both strong positive and complete loss of labeling were seen (Fig. 1C–E). True heterogeneity versus a labeling artifact was confirmed by use of a second antibody to Cdkn2A raised against a different epitope of the protein that showed the identical pattern of labeling in these 5 carcinomas. One of these carcinomas contained a 6 bp in-frame deletion of the *CDKN2A* gene, and the matched liver metastases showed complete loss of Cdkn2A labeling. In the remaining 4 carcinomas no mutations were found, and the matched liver metastases also had loss of Cdkn2A labeling.

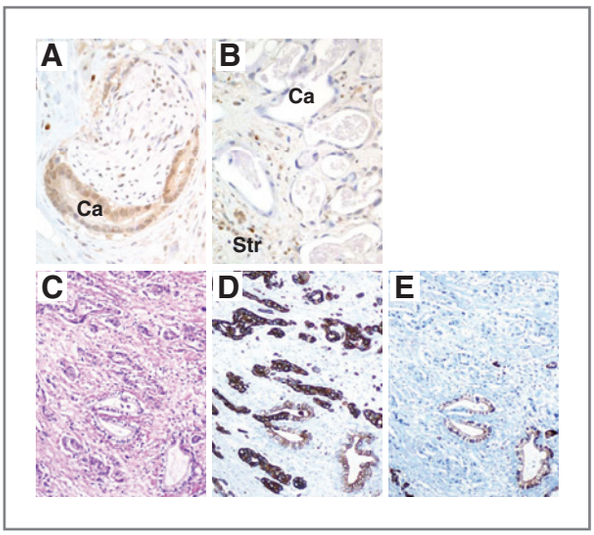

Figure 1. Deviant Smad4 and Cdkn2A immunohistochemical labeling patterns in pancreatic cancer tissues. A, intact Smad4 immunolabeling in a primary carcinoma. Both nuclear and cytoplasmic labeling for Smad4 is present within the neoplastic glands (**Ca**) in an area of perineural invasion. B, loss of Smad4 immunolabeling in a liver metastasis derived from the carcinoma shown in A. In this example, no labeling of Smad4 is seen within the neoplastic glands (**Ca**). By contrast, positive labeling of surrounding stromal cells (**Str**) is present. C, hematoxylin and eosin stained section of infiltrating pancreatic carcinoma. D, CK19 labeling of the carcinoma shown in C indicating strong positive labeling throughout the neoplastic epithelium. E, example of focal loss of Cdkn2A immunolabeling in the carcinoma shown in C. No labeling of Cdkn2A is seen in the neoplastic epithelium within the upper half of the shown section, whereas strong positive labeling is seen within scattered neoplastic glands in the lower half.

Coexistent genetic alterations in pancreatic cancer

We next determined the specific genes altered in pancreatic cancers with 1, 2 and 3 total genetic alterations (Table 2), as well as the type of alterations for these genes in each category. Of interest, for 1 aggressive carcinoma (patient A68) only a *KRAS* mutation was found, despite analysis of 8 different microdissected samples of the primary carcinoma and 24 different matched metastases. Among carcinomas with 2 genetic alterations, all 14 had a *KRAS* or *CDKN2A* alteration and 9 of 14 (64%) harbored an alteration in both *KRAS* and *CDKN2A*. The remaining 5 carcinomas had either a *KRAS* or *CDKN2A* alteration in combination with a *TP53* alteration. Among the 35 carcinomas with 3 genetic alterations, all 35 had a *KRAS* or *CDKN2A* alteration and for 28 of 35 (80%) carcinomas *KRAS* and *CDKN2A* were coexistent. Moreover, 25 of these 28 carcinomas (89%) contained *TP53* as the third genetic alteration, and the remaining 3 carcinomas contained loss of *SMAD4* as the third genetic alteration. The remaining 7 of 35 (20%) carcinomas had a *KRAS* or *CDKN2A* alteration in association with both *TP53* and *SMAD4* alterations.

Given the observations made in autopsied patients, we further explored the extent to which these driver gene

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Table 2. Coexistence of *KRAS*, *CDKN2A*, *TP53*, and *SMAD4* alterations in pancreatic cancer

Category	Autopsy patients ($n = 79$)	Xenografts ($n = 84$)
One gene		
KRAS	1 (100%)	—
Two genes		
KRAS/CDKN2A	9 (64%)	9 (75%)
KRAS/TP53	2 (14%)	2 (17%)
CDKN2A/TP53	3 (21%)	1 (8%)
Three genes		
KRAS/CDKN2A/TP53	25 (71%)	33 (85%)
KRAS/CDKN2A/SMAD4	3 (9%)	5 (13%)
KRAS/TP53/SMAD4	4 (11%)	1 (2%)
CDKN2A/TP53/SMAD4	3 (9%)	0
Four genes		
KRAS/CDKN2A/TP53/SMAD4	29 (100%)	33 (100%)

alterations are coexistent in a second and more uniform set of xenografts derived from 84 pancreatic cancer patients with Stage I/II disease seen at our institution. The specific genetic features of *KRAS*, *CDKN2A*, *TP53* and *SMAD4* in these xenografts have previously been reported in association with whole exome sequencing of a large series of pancreatic cancers (3). These xenografts were also previously analyzed as part of a larger series of xenografted carcinomas evaluating the relationship of each of these genes to overall survival (8). However as the frequency and prevalence of coexistent mutations in xenografts from these patients were not addressed, we focused specifically on that aspect.

The genetic features of *KRAS*, *CDKN2A*, *TP53*, and *SMAD4* in these xenografts were similar to that found for the autopsy cohort. All but 1 carcinoma (99%) had a mutation in *KRAS* with G12D the most common mutation identified in 40 of 84 (48%) carcinomas analyzed. Inactivating mutations or homozygous deletions of *CDKN2A* were found in 81 of 84 carcinomas (96%), and of *TP53* in 71 of 84 (83%) of these same cases. Inactivation of *SMAD4* by mutation or homozygous deletion was identified in 39 of 84 (46%) carcinomas and was most often seen in association with *TP53* mutation (34 of 39, 87%). The frequency at which these driver gene alterations were coexistent in a single pancreatic cancer was also similar to the autopsy cohort, with the majority of carcinomas also having 3 (46%) or 4 (39%) coexistent alterations. Thus, our findings of the frequency and coexistence of driver genes in autopsied patients is likely correct and not an underestimate due to our sample type analyzed.

Given that *SMAD4* loss was commonly seen in association with *TP53* inactivation, we further explored this relationship. *SMAD4* inactivation always occurred in association with 2 or 3 coexistent driver gene alterations, and the vast majority of *SMAD4* inactive carcinomas had coexistent *TP53* mutations (36 of 39, 92%). By contrast, *TP53* alterations were equally likely to be found independent of *SMAD4* inactivation with 35 of 66 (53%) in *SMAD4* wild type carcinomas versus 31 of 66 (47%) in association with *SMAD4* loss. *SMAD4* status alone was significantly correlated with high metastatic burden ($P = 0.008$), as was *TP53* status ($P = 0.039$). However, as these 2 gene alterations are commonly coexistent we compared the features among pancreatic cancers with *TP53* alterations only, with *SMAD4* alterations only, with alterations in both genes and in neither gene. To our surprise, *TP53* alterations were similarly correlated with high metastatic burden disease when they occurred with or without coexistent *SMAD4* alterations ($P = 0.170$), and differed from carcinomas without *TP53* and *SMAD4* alterations in which metastatic burden was more commonly oligometastatic ($P = 0.008$). To determine if the types of *TP53* alterations differ among these groups to explain this observation, we assessed the frequency of *TP53* missense versus null mutations (nonsense, deletion or frameshift) in the 58 carcinomas with complete sequencing data available. Of interest, null mutations were significantly more common in *SMAD4* intact carcinomas (18/28, 64%) than in carcinomas with *SMAD4* loss (7/22, 38%, $P = 0.046$). Collectively, this suggests that pancreatic cancers with high metastatic efficiency may be represented by at least 2 genetic subtypes, i.e., *TP53* null mutant and *TP53* missense mutant in association with *SMAD4* loss.

Relationships of genetic features to clinical features in pancreatic cancer patients

Among all 79 carcinomas analyzed, 1 (1%) had a single detectable gene alteration, 14 (18%) had 2 gene alterations, 35 (44%) had 3 gene alterations and 29 (37%) had an alteration in all 4 genes analyzed (Table 2). Carcinomas with 1 or 2 alterations only were combined into a single group, as were carcinomas with 3 or 4 alterations, and the relationships of the number of genetic alterations to clinical features of each patient's carcinoma was analyzed (Table 3). There were no differences in mean age or gender distribution among patients in relation to number of gene alterations, nor were there differences in tumor size, location or differentiation at initial diagnosis. No relationship was found either with clinical stage at diagnosis, although 1 to 2 gene mutant carcinomas were twice more commonly observed in association with Stage I/II disease (30% of patients, vs. 15% of Stage III and 15% of Stage IV). By univariate analysis the number of altered genes was significantly correlated with both median disease free survival ($P = 0.008$) in patients with Stage I/II disease, and median overall survival ($P = 0.041$; Fig. 2) among all patients although this was not maintained when separated out by stage. However, a greater number of altered genes was also significantly correlated with high metastatic burden at autopsy with 10 of 15 (66%) patients with 1 to 2 altered genes having oligometastatic failure compared with 2 of 29 (14%) of patients with widespread metastatic failure ($P =$

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Table 3. Relationship of number of genetic alterations to clinical features in 79 autopsied pancreatic cancer patients

	Number of altered genes		
Feature	**1–2 ($n = 15$)**	**3–4 ($n = 64$)**	***P* value**
Age (yrs)	66.1 ± 9.0	61.3 ± 11.7	0.147
Gender			
Male	9 (20%)	35 (80%)	0.469
Female	6 (17%)	29 (83%)	
Clinical stage at diagnosis			
I/II	6 (30%)	14 (70%)	0.347
III	3 (15%)	16 (85%)	
IV	6 (15%)	34 (85%)	
Tumor size at diagnosis (cm)			
I/II	2.7 ± 0.8	3.2 ± 1.5	0.468
III	4.7 ± 2.8	3.6 ± 1.0	0.195
IV	4.9 ± 2.0	4.3 ± 1.5	0.429
Tumor location[a]			
Head/body	12 (80%)	52 (81%)	0.865
Tail	3 (20%)	11 (19%)	
Tumor differentiation			
Well/moderate	6 (40%)	43 (67%)	0.404
Poor	9 (60%)	21 (33%)	
Median disease free survival, stage I/II (mo)	20	7	0.008
Median overall survival (mo)			
All stages	23	9	0.041
I/II only	24	24	0.448
III only	18	10	0.134
IV only	2	6	0.428

[a]Info on 1 patient not available.

0.002; Table 4). This relationship was also maintained when patients were stratified by tumor stage. Of interest, when controlling for clinical stage at diagnosis the number of altered genes remained significantly correlated to patient survival ($P = 0.046$; Table 5).

Discussion

The pancreatic cancer progression model illustrates the approximate timing of accumulation of genetic alterations during PanIN progression (32). *KRAS* mutations are an early event and are followed by inactivating mutations in *CDKN2A*, whereas *TP53* and *SMAD4* alterations occur relatively later during PanIN-3. Although our data are in agreement with this model, they also suggests that this mode of genetic progression likely occurs for only a subset of patients in that only 37% to 39% of carcinomas contain alterations in all genes. Thus, a more complete understanding of the extent to which alterations of these genes are coexistent in pancreatic cancer should not only provide insight into the dynamics by which they occur during pancreatic carcinogenesis, but also the biologic features of the infiltrating carcinomas that developed from those precursors.

The major clinical implication of this work is that knowledge of the gene status of the 4 major driver genes in pancreatic cancer, and specifically the extent to which they are coexistent in an individual patients cancer, provides distinct information regarding patterns of disease progression, metastatic failure and survival outcome. It is important to emphasize that other genes also play an important role in the biology of pancreatic cancer, for example inactivating *BRCA2*, *PALB2*, or *FANC* gene mutations that may confer susceptibility to cisplatin or PARP inhibitors (33, 34). However, because mutations in those genes are relatively uncommon our rationale was to identify genetic factors that influence outcomes for a greater number of patients. For example, among Stage I/II patients' carcinomas with 2 driver gene alterations were associated with relatively longer median disease-free survival, and carcinomas with 2 driver gene alterations were significantly more likely to develop oligometastatic failure. Ultimately, although the demographics of these patients are entirely in keeping with the epidemiology and clinical features of larger cohorts of patients in well-controlled studies, additional validations in a controlled setting will be necessary.

The most common initiating genetic events in pancreatic cancer are oncogenic mutations in *KRAS* and inactivating

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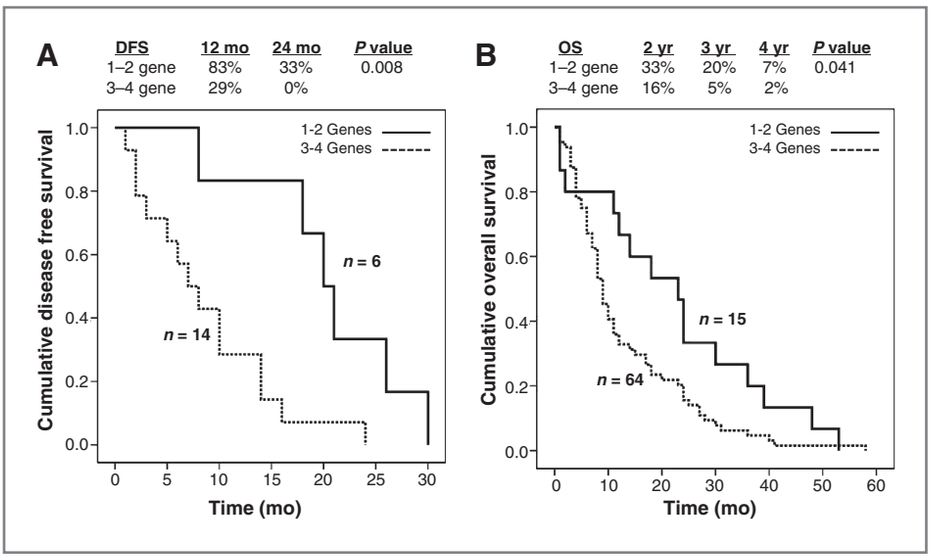

Figure 2. Kaplan–Meier survival curves showing the relationship of number of driver gene alterations (1 to 2 vs. 3 to 4) to disease free survival in 20 Stage I/II patients specifically (A) and overall survival among all 79 patients (B). Survival curves were compared by a log rank test. The percentage of patients alive at interval time points are also indicated for each arm.

mutations, deletions or methylation of *CDKN2A* (30), and the sole identification of these 2 driver genes accounted for many of these cases. However, in other carcinomas the 2 driver gene alterations corresponded to alternative combinations, for example *KRAS* and *TP53*, but importantly never included *SMAD4*. Overall, these carcinomas with "two" driver genes had significantly longer disease free and median overall survival, suggesting the subset of patients whose carcinomas have these genetic features may be enriched for long-term survivors. Of note, it is highly likely that additional genes may be mutated in the *TP53* (apoptotic) and TGFβ pathways in these carcinomas that were not evaluated by our approach. For example, Jones and colleagues proposed that the significance of genetic alterations in pancreatic cancer were largely for their indication of the core signaling pathways they occurred in, and that although more than 1 gene may be targeted in a pathway only 1 gene of the pathway is targeted per carcinoma (3). Moreover, it is conceivable that these alternative genetic alterations may not have the same effects on survival or progression as for *TP53* and *SMAD4* that are the most frequent genetic targets in their respective pathways. Consistent with this notion, Blackford and colleagues found that among all members of the TGFβ signaling pathway that may be genetically inactivated in pancreatic cancer, only *SMAD4* loss is associated with worse overall survival (8). By contrast, in 1 patient in our study only a *KRAS* mutation was found despite careful methodology, and this patient had

Table 4. Relationship of number of genetic alterations to metastatic burden in autopsied pancreatic cancer patients

	Number of altered genes		
Feature	**1–2 ($n = 15$)**	**3–4 ($n = 64$)**	***P* value**
All Patients ($n = 79$)			
Metastatic burden (all patients)			
Oligometastatic (≤10)	10 (43%)	13 (52%)	
Moderate (11–100)	3 (11%)	24 (89%)	0.002
Widely metastatic (>100)	2 (7%)	27 (93%)	
Stage I/II patients only ($n = 20$)			
Metastatic burden			
Oligometastatic (≤10)	4 (80%)	1 (20%)	
Moderate (11–100)	1 (13%)	7 (87%)	0.019
Widely metastatic (>100)	1 (14%)	6 (86%)	
Stage III/IV patients only ($n = 59$)			
Metastatic burden			
Oligometastatic (≤10)	6 (33%)	12 (66%)	
Moderate (11–100)	2 (11%)	17 (89%)	0.033
Widely metastatic (>100)	1 (5%)	21 (95%)	

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Table 5. Cox regression analysis of driver genes versus clinical stage

	Hazard ratio	95.0% CI	*P* value
Clinical stage at diagnosis (I/II vs. III vs. IV)	0.211	0.114–0.390	0.000
Number of driver genes (1/2 vs. 3 vs. 4)	1.392	1.006–1.927	0.046

widespread metastatic disease at autopsy following a mere 5 month overall survival, suggesting relatively rare genetic events occurred during carcinogenesis leading to a particularly aggressive phenotype (35).

We have previously shown that *SMAD4* status of the primary carcinoma correlates with patterns of failure in pancreatic cancer (28), and now extend this observation by illustrating that *SMAD4* loss is most often seen in the setting of coexistent mutations in *TP53*. In this regard, *SMAD4* loss is a marker of genetically complex pancreatic cancers (i.e., those with all 4 driver gene mutations). These data also clarify prior observations that not all patients with widespread metastatic disease at autopsy have *SMAD4* loss, and provide evidence that mutations that specifically abolish *TP53* gene expression may also promote widespread metastatic failure independently of *SMAD4* loss in some patients. Thus, determinations of both *SMAD4* and *TP53* status may have value in identifying patients at risk for widespread metastatic failure. Furthermore, as additional genes are functionally validated as drivers in this tumor type (3, 4), it is conceivable that they will provide added information regarding prognosis and risk of metastatic failure for pancreatic cancer patients.

KRAS mutations in normal cells leads to replicative senescence (36), and it has been suggested that *CDKN2A* inactivation provides a selective advantage to *KRAS* mutant cells by allowing cell division to proceed unhampered through the G1 checkpoint (37). That the vast majority of pancreatic cancers in this study have coexistent *KRAS* and *CDKN2A* mutations (89%) provides support to this concept. Beyond *KRAS* and *CDKN2A*, the frequencies by which alterations in *TP53* or *SMAD4* occur are relatively lower. *SMAD4* loss most often occurred in a background of *TP53* mutations yet *TP53* mutations occurred at similar frequency in the presence or absence of *SMAD4* loss, suggesting *SMAD4* inactivation follows *TP53* during the genetic progression of PanINs. In this context *SMAD4* loss may provide a selective advantage to cells with coexistent *KRAS*, *CDKN2A*, and *TP53* mutations. In support of this hypothesis, we noted that *TP53* null mutations were less commonly found in association with *SMAD4* inactivation suggesting that *TP53* null mutations select against *SMAD4* loss. Alternatively, *TP53* null mutations may have similar "potency" in progressing to an infiltrating carcinoma as coexistent *TP53* missense mutations and *SMAD4* loss. Consistent with this concept the metastatic burden of patients whose carcinomas corresponded to these 2 genetic categories (*KRAS/CDKN2A/TP53*-null vs. *KRAS/CDKN2A/TP53*-missense/*SMAD4*) were similar to each other and significantly different from carcinomas that did not have *TP53* or *SMAD4* mutations.

The significance of exomic sequencing can only be realized by translational studies that include well-annotated patient data. We now show the clinical significance of such data for patients with pancreatic cancer. In time, these data may also have value for personalized approaches to management of pancreatic cancer patients.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

Authors' Contributions

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