Biology of Human Tumors

Very Long-term Survival Following Resection for Pancreatic Cancer Is Not Explained by Commonly Mutated Genes: Results of Whole-Exome Sequencing Analysis

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Abstract

Purpose: The median survival following surgical resection of pancreatic ductal adenocarcinoma (PDAC) is currently <20 months. However, survival 4 10 years is achieved by a small subset of patients who are defined as very long-term survivors (VLTS). The goal of this study was to determine whether specific genetic alterations in resected PDACs determined very long-term survival.

Experimental Design: We sequenced the exomes of eight PDACs from patients who survived 4 10 years. On the basis of the results of the exomic analysis, targeted sequencing of selected genes was performed in a series of 27 additional PDACs from VLTSs. A total of 53 cancers were sequenced.

Results: KRAS mutations were identified in 33 of 35 cancers (94%) from VLTSs and represented the most prevalent alteration in our cohort. TP53, SMAD4, and CDKN2A mutations occurred in 69%, 26%, and 17%, respectively. Mutations in RNF43, which have been previously associated with intraductal papillary mucinous neoplasms, were identified in four of the 35 cancers (11%). Taken together, our data show no difference in somatic mutations in carcinomas from VLTSs compared with available data from PDACs unselected for survival. Comparison of clinicopathologic features between VLTSs and a matching control group demonstrated that younger age, earlier stage, well/moderate grade of differentiation, and negative resection margins were associated with VLTS. However, more advanced stage, poor grade, or nodal disease did not preclude long-term survival.

Conclusions: Our results suggest that in most patients, somatic mutations in commonly mutated genes are unlikely to be the primary determinant of very long-term survival following surgical resection of PDAC.

Introduction

Pancreatic ductal adenocarcinoma (PDAC) is one of the deadliest solid human malignancies. It is estimated that more than 46,000 new patients have been diagnosed with PDAC in 2014, and only approximately 6% of those patients will survive 5 years (1). Such a dismal prognosis is attributed to the late stage at which most patients are diagnosed, together with the lack of effective systemic therapies to control the disease (2).

Surgical resection of PDAC at an early stage offers the best hope for improving survival rates, but despite advances in pancreatic surgery, surgically resected patients have a median survival <20 months (2–4). Long-term survival after surgery, however, is achieved by a subset of patients: up to 20% of all resected patients survive 5 years after their operation and approximately 10% are still alive after 10 years (5–17). Thus, long-term survival is uncommon even among patients eligible for surgical resection.

The factors responsible for long-term survival of patients with PDAC are poorly understood. Previous clinical studies focusing on 5-year and 10-year survivors have reported that low stage of disease, negative surgical margins, and negative lymph nodes are predictors of a more favorable prognosis (11, 13–16). Of note, these same studies also showed that positive resection margins or tumor metastasis to lymph nodes did not preclude long-term survival, as 20% to 40% of patients who survived at least 5 years after surgery had nodal disease and/or margin positivity. These findings suggest that pathologic staging is not the sole determinant of long-term survival in patients with pancreatic cancer. Hence, the less aggressive phenotype observed in a subset of pancreatic cancers may be dependent upon distinct genetic, epigenetic, or other biologic factors such as changes in the tumor microenvironment or enhanced immune response to the cancer by the host.

To determine whether specific somatic genetic alterations in resected carcinomas are associated with very long-term survival, we performed whole-exome sequencing of a series of well-characterized, surgically resected PDACs obtained from a group of patients who survived at least 10 years after surgical resection.

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Note: Supplementary data for this article are available at Clinical Cancer Research Online (http://clincancerres.aacrjournals.org/).

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doi: 10.1158/1078-0432.CCR-14-2600

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1944 Clin Cancer Res; 21(8) April 15, 2015
Pancreatic cancer is a deadly disease in dire need of new clinical approaches. Although the vast majority of patients with pancreatic cancer have a dismal prognosis, a very rare subset has long-term survival after surgery. Knowledge of the factors that mediate long-term survival could aid in the prognostication of patients with pancreatic cancer and provide insights into the underlying biology of this deadly cancer. In this study, we analyzed the genomes of 35 patients with pancreatic cancer who survived more than 10 years after surgery. We discovered that the somatic mutation profiles in these patients were very similar to those of “garden variety” pancreatic cancer, suggesting that somatic mutations are not the primary determinant of long-term survival in this disease.

Materials and Methods

Patients

The study was approved by the Institution Review Board of The Johns Hopkins Hospital (Baltimore, MD). For the purpose of this study, very long-term survivors (VLTS) were defined as individuals who underwent surgical resection of an invasive ductal adenocarcinoma of the pancreas and lived 10 or more years following their surgery. Patients included in this study were selected from all consecutive patients who underwent a pancreatic resection for invasive ductal adenocarcinoma of the pancreas at The Johns Hopkins Hospital between 1989 and 2000.

An expert pancreatic pathologist carefully reviewed all of the available histologic slides to confirm the diagnosis. Variants of ductal adenocarcinoma, such as colloid carcinoma or adenosquamous carcinoma, were excluded (18). Ductal adenocarcinomas arising from intraductal papillary mucinous neoplasms (IPMN) or mucinous cystic neoplasms (MCN) were also excluded from the analysis, as it has been shown that pancreatic cancer originating from cystic precursor lesions may have a more favorable prognosis than conventional ductal adenocarcinoma (19, 20). All cases for which microscope slides or tissue blocks were not available were excluded from the analysis. Patients who had received chemotherapy and/or radiotherapy before surgical resection were also excluded from the analysis, to avoid potential confounding effects of treatment-induced DNA damage. The exclusion criteria were applied to all VLTSs included in our study (both discovery and validation sets).

Date of death or date of last follow-up (for patients who were still alive at the time the study was initiated) was confirmed by querying the Johns Hopkins Electronic Patient Record and the Social Security Death Index. After accurate selection, 37 patients that met our inclusion criteria were identified. Among these, 10 patients were selected on the basis of the availability of fresh-frozen neoplastic tissue with adequate cellularity for sequencing. Two patients were subsequently excluded due to low tumor neoplastic cellularity and insufficient DNA quality. Ultimately, PDACs from 8 patients were available for whole-exome sequencing (discovery set). Twenty-seven additional pancreatic cancers from VLTSs were included in the validation set. Non-neoplastic tissue was available for each of the cases analyzed.

Clinicopathologic data from our cohort of VLTSs were retrieved from the Surgical Pathology database. A separate group of 226 patients who underwent surgery for pancreatic cancer during the years 1989–2000 was chosen as control to explore clinical and pathologic correlations with long-term survival. None of the controls had experienced very long-term survival. Furthermore, patients in the control group with survival <30 days after the operation were excluded to rule out mortality related to surgical complications. Demographic and clinicopathologic data were retrieved from a prospectively maintained surgical database; in both the VLTSs and control groups, the staging of disease was reviewed and updated to comply with the 7th edition of the American Joint Committee on Cancer (AJCC) classification (21).

Sample acquisition/preparation

After pathology confirmation, each of the eight fresh-frozen surgically resected carcinomas was macrodissected to remove residual normal tissue and achieve a neoplastic cellularity of >50%. Normal tissue was analyzed by frozen section to confirm that no neoplastic tissue was present.

For each of the 27 cases included in the validation set, 20 slides were recut from formalin-fixed paraffin-embedded (FFPE) blocks of representative tumor tissue. After deparaffinization with xylene and hematoxylin and eosin staining, slides were manually micro-dissected to enrich tumor cellularity and avoid areas of non-neoplastic tissue. A cellularity ≥30% was achieved in each of these 27 samples.

DNA was purified from the macromacrossected frozen tumors using the AllPrep kit (Qiagen Inc., cat. #80204) and from micro-dissected FFPE tumors with a Qiagen FFPE kit (Qiagen Inc.; cat. #56494).

Whole-exome sequencing

We sequenced approximately 21,000 protein-coding genes (>37,000,000 base pairs of coding sequence) in matched tumor and normal DNA. Genomic DNA libraries were prepared and captured following Illumina’s (Illumina) suggested protocol. The Agilent SureSelect paired end version 2.0 human exome kit was used to capture the coding sequences from individual libraries for each sample. The captured libraries were then sequenced using the Illumina HiSeq Genome Analyzer (22, 23).

Sequencing reads were analyzed and aligned to human genome 18 (hg 18) using the Eland algorithm in CASAVA 1.6 software (Illumina). The data were filtered for quality, and alterations in the matched tumor and normal tissues were then compared to identify tumor-specific somatic mutations as has been described (22, 23). A mismatched base was identified as a somatic mutation only if the following conditions were met: (i) it was identified by five or more distinct pairs; (ii) it was identified in reads in both the forward and reverse directions; (iii) the number of distinct tags containing a particular mismatched base was at least 15% of the total distinct tags; (iv) it was not present in any tags in the matched normal samples; and (v) the matched normal sample had sufficient coverage to identify the mutation. In addition, for this study, we only considered nonsynonymous mutations, which altered the protein sequence of the encoded product. A subset of mutations was verified by visual inspection of the sequencing data. In addition, 44 mutations were validated by conventional Sanger sequencing.

Targeted sequencing of the 27 additional PDACs was performed using SafeSeqS, an approach in which template molecules are individually assessed via massively parallel sequencing (24, 25). The mutational status of the following nine genes (listed in

Translational Relevance

Pancreatic cancer is a deadly disease in dire need of new clinical approaches. Although the vast majority of patients with pancreatic cancer have a dismal prognosis, a very rare subset has long-term survival after surgery. Knowledge of the factors that mediate long-term survival could aid in the prognostication of patients with pancreatic cancer and provide insights into the underlying biology of this deadly cancer. In this study, we analyzed the genomes of 35 patients with pancreatic cancer who survived more than 10 years after surgery. We discovered that the somatic mutation profiles in these patients were very similar to those of “garden variety” pancreatic cancer, suggesting that somatic mutations are not the primary determinant of long-term survival in this disease.
The validation panel included genes that were mutated in more than one sample in the exomic analysis and genes known to be commonly mutated in cystic neoplasms of the pancreas (to test whether some of our PDACs could have originated from a cystic precursor). The entire coding sequence of *CDKN2A*, *PIK3CA*, *RNF43*, *SMAD4*, *VHL*, *PIK3CA*, and *TP53* was investigated. Analysis of *KRAS*, *GNAS*, and *BRAF* was limited to the hotspot locations (*KRAS* exons 2 and 3; *GNAS* exon 8 and *BRAF* exon 15).

A more detailed description of library preparation, exome capture, and the SafeSeqS approach is provided in the Supplementary Methods.

### Statistical analyses
Continuous variables were presented as mean and SD and compared using the unpaired *t* test. Categorical variables were compared using the Fisher exact test. A P value <0.05 was considered as statistically significant. Median survival was calculated using the Kaplan–Meier method. All statistical analyses were performed using GraphPad Prism version 5.04 (GraphPad Software) and R version 3.1.1.

### Results
The set of 35 PDACs from the VLTSs included tumors from 21 female (60%) and 14 male patients (40%; Table 1). The average age at the time of surgical resection was 59.1 years. Twenty-nine patients (83%) had undergone a Whipple procedure, 4 (11%) a distal pancreatectomy with splenectomy, and 1 (6%) a total pancreatectomy. Twenty-two patients (63%) were stage IB disease (R0 disease), whereas 5 patients (17%) had positive margins (R1 disease). Twenty-one patients had a stage IIB disease (R0 disease), four patients were stage IIA (11%), 4 patients were stage IB (11%), and 6 patients were stage IA (17%). Data on adjuvant therapy were available on 17 of the 35 patients (48.5%).

### Sequencing analysis
Whole-exome sequencing was performed on eight PDACs surgically resected from VLTSs. A total of 50 MB of captured DNA was sequenced with an average depth of coverage of 122-fold in the targeted region, and >93.6% of targeted bases were present at least 10 reads (Supplementary Table S1). These carcinomas had a mean of 37.6 nonsynonymous somatic mutations and were all enriched for C:G-to-T:A transitions (78.7% of mutations).

### Table 1. Clinicopathologic data of 35 patients with pancreatic cancer who survived more than 10 years after surgery

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<th>Type of surgery</th>
<th>Tumor size (cm)</th>
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Abbreviations: Disc, discovery; Val, validation; G1, well differentiated; G2, moderately differentiated; G3, poorly differentiated; R0, negative resection margin; R1, microscopic positive margin.
Three hundred and one somatic mutations were identified in 274 genes in the eight carcinomas (Supplementary Table S2). Forty-four somatic mutations in 27 genes were confirmed with conventional Sanger sequencing.

Six of eight carcinomas harbored KRAS mutations (75%) and six of eight had TP53 mutations (75%). Only one of the eight carcinomas harbored a mutation in the SMAD4 gene (12.5%). Two mutations were identified in the CDKN2A gene (25%) and three carcinomas had mutations in the RNF43 gene (37.5%; Table 2).

The BRAF, CDKN2A, GNAS, KRAS, PIK3CA, RNF43, SMAD4, TP53, and VHL genes were sequenced using Safe-SeqS in a panel of 27 additional surgically resected ductal adenocarcinomas of the pancreas obtained from VLTSs. KRAS was the most commonly mutated gene, as alterations were found in 27 of 27 (100%) of these validation cancers. Four of the 27 validation cancers harbored CDKN2A mutations (11%), eight harbored SMAD4 mutations (29%), and 18 had TP53 mutations (68%). GNAS, RNF43, and BRAF were each found mutated in one sample (4%). No mutations were found in the PIK3CA and VHL genes (Supplementary Table S3).

When the results from the whole-exome and targeted sequencing were combined, KRAS proved to be the most commonly altered gene, with activating mutations identified in 33 (94%) of the 35 carcinomas. TP53 mutations were found in 24 (69%) of 35 cases, SMAD4 mutations in nine cases (26%), and CDKN2A mutations in six cases (17%). RNF43 mutations were identified in four (11%) of the carcinomas (Table 2).

**Clinicopathologic correlations**

Clinical and pathologic characteristics of the cohort of 35 VLTSs were compared with a control group of 226 surgically resected patients matched by years of surgery (1990–2000; Table 3). The VLTS group was significantly younger at the time of surgery (mean age 59.1 vs. 65.7, \( P = 0.001 \)). The mean tumor size was significantly smaller in the group of VLTSs than in the control group (2.8 cm vs. 3.1 cm). Compared with the control group, VLTSs were more likely to have stage IA-IB disease (\( P < 0.001 \)), well or moderately differentiated tumor grade (\( P = 0.002 \)), and negative resection margins (\( P = 0.011 \); Table 3). The VLTSs also had a higher rate of negative nodal status than the controls (\( P = 0.036 \)). The median survival for VLTSs and controls was 196 months and 14 months, respectively. Of note, none of the VLTSs was known to experience a tumor recurrence in the 10-year follow-up period, although we did not have these data on all patients. None of the VLTSs had a family history of pancreatic cancer, although several had personal histories of other tumor types, including breast, prostate, and lung cancers. However, none of these patients had mutations suggestive of an inherited cancer predisposition syndrome (e.g., BRCA2 mutation in patient with breast and pancreatic cancer).

**Discussion**

The characterization of the coding sequences of pancreatic cancer has greatly advanced our understanding of the genetic alterations that underpin this devastating disease (26). The genetic landscape of PDAC is defined by four mutational "mountains" (KRAS, TP53, CDKN2A, SMAD4), which are thought to be the main drivers of carcinogenesis. In addition, numerous other genes harbor mutations at much lower rates, most of which are considered of little functional significance (passenger genes; ref. 26).

More recent studies have improved the identification of driver events by integrating sequencing data with data obtained from functional screens and animal models, or targeting selected groups of patients, such as those with genetic predisposition to PDAC (27–29). These studies have identified additional candidate driver genes that are potentially relevant in sporadic (MLL3, USP9X, MAP2K4) and familial (BRCA2, PALB2, ATM) pancreatic cancer (27–31), but which are each mutated in only a small fraction of the cancers.

The sequencing of cancers has helped facilitate the recognition of histologically indistinguishable molecular subgroups that might determine specificity to a specific therapy (32) or have prognostic significance. For example, previous genetic analyses of surgically resected PDACs have demonstrated that SMAD4 protein loss correlates with patterns of metastatic spread and worse prognosis (33–35). The patients whose pancreatic cancer had SMAD4 loss were more likely to die with widespread (in some cases
thousands of metastases, whereas the patients whose pancreatic cancers had intact SMAD4 were more likely to die with localized disease (33, 34).

We hypothesized that genetic analysis of a group of pancreatic cancers characterized by unconventional clinical behavior, such as those from patients who survived 10 years or longer after surgery, could identify genetic determinants of long-term survival. Over 1,700 surgical resections for pancreatic cancer have been performed at The Johns Hopkins Hospital (5), providing us with a unique patient population that includes a number of patients who survived more than 10 years after surgery. In an effort to define the genetic changes that characterize long-term survival, we applied whole-exome and targeted sequencing to a series of well-characterized PDACs resected from VLTS.

To our surprise, we found no significant differences in the mutational profile of this unique cohort of pancreatic cancers, compared with the mutational profile that has been previously published by our group and others in “garden variety” PDAC (26, 27, 36). After merging the discovery and validation sets, KRAS was confirmed as the most commonly mutated gene (94%) in the PDACs from VLTSs, at a rate that is comparable with rates reported in literature. Similarly, TP53, SMAD4, and CDKN2A were also commonly mutated at rates comparable with those published in the literature for nonselected PDACs (Table 2). The overall prevalence of RNF43 mutations in our cohort was 11% (4 out of 35 cases). A similar prevalence (10%) was also reported by the International Cancer Genome Consortium, for a large cohort of pancreatic cancers not selected based on long-term survival (37).

The RNF43 gene, which encodes a protein with intrinsic U3 ubiquitin ligase activity, is relatively understudied in pancreatic cancer (38). However, inactivating mutations in the RNF43 gene have been reported in IPMNs of the pancreas (38, 39). It has been suggested that IPMN-associated invasive carcinomas are less aggressive than carcinomas that do not arise in association with an IPMN (19, 40). Origin in an IPMN, as evidenced by the presence of RNF43 mutations, could therefore explain some of the VLTS in our cohort. Although careful pathologic reevaluation of all cases included in our analysis showed no evidence of IPMN, it is possible that in some instances, the invasive carcinoma overgrew a preexisting noninvasive component, resulting in loss of the IPMN.

Recent studies have shown that IPMNs commonly harbor GNAS mutation, which are very specific for this tumor type (38, 39, 41, 42). GNAS was included in our validation panel to verify whether some of the cancers had indeed originated from IPMNs. No GNAS mutations were identified in the eight carcinomas subjected to exome sequencing, and only one of the 27 samples analyzed at targeted sequencing harbored a GNAS mutation (Supplementary Table S2). Interestingly, that one sample did not harbor an RNF43 mutation. It should be noted that the absence of GNAS mutations in the carcinomas from VLTSs might be the result of the histologic inclusion criteria used in this study. GNAS mutations are associated with intestinal differentiation in IPMNs and intestinal-type IPMNs often give rise to colloid-type invasive carcinomas, which were excluded from this study (39, 41, 42). Therefore, we may have selected for IPMN-associated cancers that harbor RNF43 mutations but not GNAS mutations.

Theoretically, a specific genetic alteration may confer prolonged survival to patients with PDAC by either rendering the cancer less aggressive or determining increased sensitivity to therapies that target specific genetic abnormalities. The latter instance is exemplified by alterations in the Fanconi anemia/BRCA2 pathways, which render cancer cells hypersensitive to interstrand cross-linking agents (43). A dramatic response to therapy with mitomycin C and other DNA-damaging agents has been occasionally reported in patients with metastatic pancreatic cancer resistant to gemcitabine that harbored mutations in the BRCA2 or PALB2 genes (44, 45). Our analysis did not reveal biallelic inactivation of the BRCA2 or PALB2 genes in any of the eight samples subjected to whole-exome sequencing, excluding the possibility that this could have been a mechanism of very long-term survival in this portion of our VLTS cohort.

The comparison of clinicopathologic characteristics between the VLTSs and an independent group of well-characterized, surgically resected pancreatic adenocarcinomas confirmed the results of previous clinical studies: VLTSs had more favorable features, such as smaller and better differentiated tumors, lower stage of disease, and higher rate of negative surgical margins. However, the majority of VLTSs in our cohort had cancer spread to lymph nodes (66%); furthermore, a poorly differentiated cancer or positive resection margin were not uncommon in the VLTS group (Table 3), suggesting that biologic rather than clinical or pathologic factors are likely the main determinants of prognosis.

Although this study suggests that very long-term survival in patients with pancreatic cancer is not dependent upon specific genetic alterations, it should be noted that our analyses were limited to the exomes of these cancers. Other types of genetic changes, such as chromosomal rearrangements, translocations, large deletions and insertions, inversions, chromothripsis, and intronic alterations, would have been missed by our approach, as would epigenetic changes as well as changes in gene and miRNA expression. We, therefore, cannot exclude the possibility that one of these alterations is driving very long-term survival. In addition, we cannot exclude the possibility that coding mutations were missed in our approach (so called false negatives) due to variability in coverage or mutation calling. However, the identification of somatic alterations in frequently mutated genes in PDAC at rates similar to those previously described argues against significant false negatives in our analysis. Finally, we did not examine the contribution of tumor microenvironment and host immune response, which could also have been responsible for the improved survival.

Although only 35 VLTSs were analyzed in our study, this is a relatively large number, given the exceptionally low number of patients with PDAC who survive 10 or more years. We would have expected that, if a significant fraction of the VLTSs were dependent upon specific alterations in coding DNA sequences, our analysis would have been able to appreciate them. Still, since we only performed whole-exome sequencing on eight VLTSs, it is possible that our analysis missed an uncommon mutation that contributes to long-term survival. Considering the diversity of possible genomic alterations, additional studies incorporating multiple mutation detection approaches with additional samples as part of multi-institutional efforts are required to validate our findings. Analyses that consider mutations on a pathway rather than individual gene level may also identify determinants of long-term survival not evident from our initial gene-based analysis.

In summary, our results suggest that nonsynonymous somatic mutations in commonly mutated genes are unlikely to be the primary determinant of very long-term survival following the surgical resection of pancreatic cancer.
Disclosure of Potential Conflicts of Interest

N. Papadopoulos, B. Vogelstein, and K.W. Kinzler are co-founders of, have ownership interest (subject to certain restrictions under Johns Hopkins University policy) in, and are consultant/advisory board members for Sysmex Inostics and Personal Genome Diagnostics. L.D. Wood is a consultant/advisory board member for Personal Genome Diagnostics. R.H. Hruban receives royalty payments from Myriad Genetics for the PALB2 invention. Under agreements between the Johns Hopkins University, Genzyme, Exact Sciences, Sysmex Inostics, Qiagen, Invitrogen and Personal Genome Diagnostics, N. Papadopoulos, B. Vogelstein, and K.W. Kinzler are entitled to a share of the royalties received by the Johns Hopkins University on sales related to products and the technologies described in this manuscript. No potential conflicts of interest were disclosed by the other authors.

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Grant Support

This work was supported by Blum-Kovler Foundation, NIH grant CA69294, Lustgarden Foundation for Pancreatic Cancer Research, Sol Goldman Pancreatic Cancer Research Center, and The Virginia and D. K. Ludwig Fund for Cancer Research.

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Received October 10, 2014; revised December 23, 2014; accepted December 28, 2014; published OnlineFirst January 26, 2015.

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Analysis and interpretation of data (e.g., statistical analysis, biostatistics, computational analysis): J.M. Dal Molin, M. Zhang, C.L. Wolfgang, A. Blackford, K.W. Kinzler, N. Papadopoulos, R.H. Hruban, A. Maitra, L.D. Wood

Very Long-term Survival in Pancreatic Cancer


Clinical Cancer Research

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