Pancreatic Cancer–Related Mutational Burden Is Not Increased in a Patient Cohort With Clinically Severe Chronic Pancreatitis

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INTRODUCTION: Chronic pancreatitis is associated with an increased risk of developing pancreatic cancer, and patients with inherited forms of pancreatitis are at greatest risk. We investigated whether clinical severity of pancreatitis could also be an indicator of cancer risk independent of etiology by performing targeted DNA sequencing to assess the mutational burden in 55 cancer-associated genes.

METHODS: Using picodroplet digital polymerase chain reaction and next-generation sequencing, we reported the genomic profiles of pancreases from severe clinical cases of chronic pancreatitis that necessitated palliative total pancreatectomy with islet autotransplantation.

RESULTS: We assessed 57 tissue samples from 39 patients with genetic and idiopathic etiologies and found that despite the clinical severity of disease, there was no corresponding increase in mutational burden. The average allele frequency of somatic variants was 1.19% (range 1.00%–5.97%), and distinct regions from the same patient displayed genomic heterogeneity, suggesting that these variants are subclonal. Few oncogenic KRAS mutations were discovered (7% of all samples), although we detected evidence of frequent cancer-related variants in other genes such as TP53, CDKN2A, and SMAD4. Of note, tissue samples with oncogenic KRAS mutations and samples from patients with PRSS1 mutations harbored an increased total number of somatic variants, suggesting that these patients may have increased genomic instability and could be at an increased risk of developing pancreatic cancer.

DISCUSSION: Overall, we showed that even in those patients with chronic pancreatitis severe enough to warrant total pancreatectomy with islet autotransplantation, pancreatic cancer–related mutational burden is not appreciably increased.

SUPPLEMENTARY MATERIAL accompanies this paper at http://links.lww.com/CTG/A722, http://links.lww.com/CTG/A723, http://links.lww.com/CTG/A724, http://links.lww.com/CTG/A725, http://links.lww.com/CTG/A726, http://links.lww.com/CTG/A727, and http://links.lww.com/CTG/A728

INTRODUCTION
Chronic pancreatitis is a progressive inflammatory disease that is characterized by abdominal pain and can result in severe exocrine and endocrine insufficiencies. Individuals with chronic pancreatitis have an increased risk of developing pancreatic ductal adenocarcinoma (PDA) (1–4), which is poised to become the second leading cause of cancer-related deaths in the United States within a decade (5). The risk of developing PDA is even greater for individuals with inherited forms of pancreatitis (3), which involve severe chronic inflammation due to functional mutations in genes such as PRSS1, CFTR, CTRC, and SPINK1 (6).

Given this association, many hypothesize that key mutations that drive the development of PDA accumulate during active inflammatory episodes in these patients. Indeed, this hypothesis is supported by a series of articles documenting the presence of oncogenic KRAS mutations in patients with chronic pancreatitis.
However, these reports displayed wide discrepancy in the prevalence of these mutations, ranging from 0% (7–11) to between 9% and 53% (12–23). Thus, mutations in KRAS, found in greater than 95% of all human PDAs, are found at higher frequency in patients with chronic pancreatitis and inherited forms of chronic pancreatitis, compared with non-diseased controls, but there is variability in the estimate of this frequency, likely dependent on the assays used. Furthermore, few studies have examined the presence of additional mutations common in PDA in this patient population (8,11,16,18,20–22,24–28).

Although overall PDA risk is elevated among patients with hereditary pancreatitis compared with that among patients with idiopathic causes, it is unclear what other factors may affect cancer risk. We hypothesized that clinical severity of pancreatitis would be a key prognostic indicator of PDA risk independent of cause of pancreatitis. We reasoned that if this were the case, then patients with more clinical disease (and assumedly, inflammation) in the pancreas would contain more PDA-related mutations. However, there are currently no universally accepted clinical criteria to denote severity of chronic pancreatitis (29,30). Systems of classifications can include an assessment of endocrine and exocrine functions, pain, histopathology (including the degree of fibrosis), and imaging findings to score clinical severity, although current classifications do not accurately forecast the course of disease (29–31). Pain is frequently the most debilitating symptom and is the primary indicator for surgical intervention (32), although it is poorly predicted by imaging analyses (33). Severe fibrosis is a feature of advanced disease and is correlated with progressive pancreatic dysfunction (34,35). Calcification, resulting from recurrent inflammation, is also considered a classical hallmark of severe chronic pancreatitis (36). Because disease activity and inflammation can vary markedly, we focused on a cohort of patients undergoing palliative total pancreatectomy with islet autotransplantation (TPIAT) (37). Although these patients have various etiologies for their pancreatitis, they share severe clinical disease activity necessitating total pancreatectomy. This cohort comprised patients with debilitating pain that were refractory to other medical or endoscopic treatment and included individuals with severe pancreatic fibrosis and calcifications. During TPIAT, the pancreas is removed and islets are separated from exocrine tissue through digestion with collagenase and mechanical dispersion. To prevent onset of diabetes, the isolated islets are subsequently infused into the portal vein for transplantation to the hepatic parenchyma. Afterward, the remaining exocrine pancreas is usually discarded; however, we obtained this tissue for targeted DNA sequencing to assess mutational burden in a panel of genes commonly mutated in PDA. If clinical severity in this context affects PDA risk independent of etiology (i.e., hereditary vs idiopathic pancreatitis), we predict that the number of alterations in commonly mutated PDA genes would be similar. To enhance sensitivity of detection of mutations, we used picodroplet digital polymerase chain reaction (PCR), which allows for single-molecule emulsification and PCR before next-generation sequencing. This approach enabled the detection of low-frequency variants that may otherwise be lost through amplification bias of the wild-type sequence during bulk amplification (38). Using this approach, we estimated the prevalence of somatic alterations frequently associated with PDA in the absence of invasive carcinoma in patients with various forms of chronic pancreatitis. In this study, for the first time, we present the genomic profiles of pancreases from patients with severe forms of clinical pancreatitis where we found that clinical severity leading to TPIAT has no impact on mutational load. However, we identify other clinical variables that are associated with mutational burden, providing the basis for further work in genomics-based risk stratification for PDA in this patient population.

METHODS

Chronic pancreatitis samples

Exocrine pancreas tissue was obtained from 39 patients undergoing TPIAT (37) for palliation of pain from severe chronic pancreatitis at the University of Minnesota according to an Institutional Review Board–approved protocol. Informed consent or parental consent and patient assent were obtained for all cases. Tissue samples were immediately submerged in DNAgard (Sigma-Aldrich, St. Louis, MO), and DNA was isolated within 1–2 days after TPIAT. We also isolated DNA in a similar manner from a cadaveric donor to serve as a baseline assessment of the bioinformatics pipeline and to identify any recurring variant calls that are probable false positives. Multiple distinct representative tissue regions from each patient sample were selected for isolation of genomic DNA using the DNeasy Blood & Tissue Kit (Qiagen, Hilden, Germany) according to the manufacturer’s instructions. DNA concentrations and integrities were assessed using an Agilent TapeStation system (Agilent, Santa Clara, CA), which revealed consistently highly intact DNA, and samples were stored at −80°C until library preparation. Samples were labeled such that identical numbers denote samples from the same patient, and distinct tissue regions were denoted by letters. Severity of pancreatic fibrosis was assessed using a standardized approach by the islet laboratory facility on a scale of 0–10, as previously described (35,39). In brief, resected pancreases were assessed for firmness and texture and assigned an initial score of 1 (minimal change), 3 (mildly fibrotic), 5 (moderately fibrotic), 7 (very fibrotic), or 9 (severely fibrotic) to estimate the extent of fibrosis. Owing to the heterogeneity of pancreas presentation, the score was on occasion further refined ± 1 relative to other similarly scored pancreases based on parenchymal color and the presence of cysts, lipomas, and calcification such that the final scores estimate the extent of fibrosis to be less than half (scores 0–4), mostly fibrotic (scores 5–8), or more than 90% fibrotic (scores 9–10). Adjacent tissue was also collected in formalin at the time of surgery for histologic analysis, which confirmed these scores as a reasonable approximation of the extent of fibrosis. Paraffin–embedded tissue sections were stained with hematoxylin and eosin according to standard protocols and evaluated for the highest grade of pancreatic intraepithelial neoplasia (PanIN) identified. The presence of PanINs was used as an overall assessment of each patient but may not be representative of the separate tissue sample that was sequenced. In subgroup analyses, patients were classed as pediatric or adult, based on whether onset of illness occurred before age 19 years (40), and genetic or idiopathic, based on whether mutations were identified clinically in the known hereditary pancreatitis susceptibility genes CFTR, CTRC, PRSS1, or SPINK1.

Targeted sequencing

We adapted the ThunderBolts Cancer Panel (RainDance Technologies, Lexington, MA) to include coverage of 5 additional genes (CACNA1I, KMT2C, RNF43, STN1, and TTN) that were previously shown to be mutated in pancreatic disease (41–45) such that the amplicon panel used in this study comprises 267 target regions in 55 cancer-associated genes (see Supplemental Table, Supplemental Digital Content 1, http://links.lww.com/CTG/A722). Libraries were prepared from patient sample
genomic DNA according to the manufacturer’s instructions. In brief, 25–75 ng of DNA together with PCR master mix including cancer panel primers (see Table, Supplemental Digital Content 2, http://links.lww.com/CTG/A723) were emulsified into 5 pL droplets using the RainDance System Source instrument such that single-molecule separation was achieved. Droplets underwent PCR amplification, followed by de-emulsification and SPRSelect bead purification (Beckman Coulter, Brea, CA). Addition of adaptor and index sequences was achieved in a subsequent PCR, and DNA was again purified using SPRSelect beads. Completed libraries were sequenced using an Illumina MiSeq System (250 cycle paired-end sequencing; 10 samples per flow cell).

**Variant calling**

Variants in sequenced DNA were identified using a custom informatics pipeline. In brief, Illumina adapter sequences were removed from the 3′ end of reads using Cutadapt 1.8.1 (46). Quality of raw reads was assessed and combined into integrated reports using MultiQC v0.4 (47) and aligned to the GRCh37 (hg19) human genome assembly using the default parameters of BWA-MEM (48). Read coverage on target regions was calculated using BEDTools v2.22.1 (49). Primers on aligned reads were soft-clipped using Katana, and variants were called by FreeBayes 1.0.2 (50) and VarScan 2.4.0 (51) using parameters listed in Table, Supplemental Digital Content 3. http://links.lww.com/CTG/A724. Variants across all samples were assembled and aggregated using VarSeq v1.3.5 (Golden Helix, Bozeman, MT). A strand bias filter (SAF/[SAF + SAR], where SAF and SAR = the number of alternate observations in the forward and reverse strands, respectively) was applied to minimize allele bias, and variants were accepted within the 0.2–0.8 filter range. Ambiguous variants, 1bp insertions or deletions adjacent to homopolymer regions consisting of 5 or more identical bases, and variants identified within 5bp of primer locations, where base quality is expected to decline (52,53), were discarded. Individual variants at genomic coordinates below the target sequencing depth (2,000×) were also discarded. Finally, somatic variants detected in the cadaver sample and also detected in chronic pancreatitis samples were removed because of their recurrent nature. Variants called by FreeBayes, unless otherwise indicated, are reported.

**Sequencing quality and validation of targeted sequencing panel**

We sought to detect variants above 1% allelic frequency because many somatic mutations below 5% allelic frequency are known to have clinical relevance (54), and variants <1% require additional experimental considerations to ensure accuracy such as molecular barcoding (55). Therefore, using a minimum sequencing depth of 2,000× and 5 alternate calls per base pair, we calculated the binomial probability of a false negative to be <0.0001% (56). This sequencing depth, using the reported error rate for Illumina MiSeq of 0.1% (53,57,58), will also result in a 0.1% probability of a false positive from sequencing alone (59) but does not account for additional errors that may result from PCR amplification and library preparation (52,60). The nature of microdroplet-based amplification eliminates the likelihood of PCR errors introduced by multiplexing PCR reactions through discrete encapsulation of each DNA molecule before thermal cycling (61). The resulting PCR errors are equivalent to traditional singleplex PCRs (61,62). To assess the accuracy of the amplicon panel and our bioinformatics pipeline, we sequenced a genomic DNA reference standard (HD701; Horizon Discovery, Cambridge, UK) that includes verified single-nucleotide variants and deletions at allelic frequencies as low as 1%. We confirmed the presence of all 11 expected variants (Table, Supplemental Digital Content 4, http://links.lww.com/CTG/A725), and linear regression analysis of the observed allelic frequencies yielded a coefficient of determination of 0.9881, indicating a high goodness of fit.

**Confirmation of low-prevalence variants**

Mutant KRASG12R was quantified using a RainDrop Plus Digital PCR System (RainDance Technologies) using an optimized protocol. In brief, DNA (5 ng) was partitioned into picoliter-sized droplets containing KRAS-specific primers (F: ATTA- TAAAGCCTGCTGAAAATGACT; R: TCTGAATTAGCTG- TATCGTCAGG), allele-specific TaqMan probes (WT: VIC-TTGGAGCTGGTGGGT-MGBNFQ; G12R: 6FAM- TGGAGCTGTGGC-T-MGBNFQ), and TaqMan Genotyping Master Mix (ThermoFisher, Waltham, MA). DNA was subsequently amplified according to the following PCR protocol: 95°C for 10 minutes; 45 cycles of 95°C for 15 seconds, 60°C for 1 minute; and 98°C for 10 minutes. Finally, absolute quantification of wild-type or mutant KRAS-positive droplets was performed by detecting fluorescence within individual droplets and analyzed by the RainDrop Analyst II software. DNA isolated from cell lines PSN-1 (CRM-CRL-3211) and BxPC-3 (CRL-1687) from the ATCC served as controls.

Nuclease-assisted minor allele enrichment with probe overlap was performed according to a previously published protocol (63). KRAS primers (F: ATTATAAGGCCCTGCTGAAAATGACT; R: GGTTCTCGACCAGTAATATGC) and oligonucleotide probes (sense: AGTGGAGGCTGTTGGCG; antisense: TCTCGCCTACGCCACAG) were used to specifically amplify low-prevalent KRAS mutations. Replicate assessments were pooled together, and 25 ng of DNA was evaluated by Sanger sequencing.

**Statistical analyses**

Unpaired 2-sample t tests were used to compare differences in the number of somatic variants between subgroups. Simple linear regression analysis was performed to assess the significance of the number of somatic variants with variables, including age, disease duration, smoking duration, and fibrosis score. Statistical analyses were performed in GraphPad Prism v 8.3.0.

**RESULTS**

**Patient population**

Fifty-seven exocrine pancreas samples from 39 patients with chronic pancreatitis of varying etiologies were sequenced (Table 1). All patients had clinically severe disease. Most of the patients had severe fibrosis, and a subset of them also presented with pancreatic calcifications. Among the patient population, there was a slight female predominance (23 of the 39; 59.0%), and more patients (24 of the 39; 61.5%) were classified as adults based on onset of illness (older than 19 years; Figure 1a) (40). The patients had a range of clinically defined etiologies, including 15 (38.5%) with an origin attributable to genetic causes (Figure 1a), most of which harbored a PRSS1 mutation (9 of the 15; 60.0%) (Figure 1b). Of the remaining idiopathic patient cases, approximately half (11 of the 24; 45.8%) had a defined contributing factor at diagnosis. Of note, most of the cases with defined genetic etiology were also classified with pediatric onset of illness (10 of the 15; 66.7%) (Figure 1b), whereas most of the idiopathic cases (19 of the 24; 79.2%) were classified as adults based on age at onset (Figure 1c). The overall average ages at onset and at TPIAT treatment, including both genetic and idiopathic
| Patient identifier | Number of samples sequenced | Sex | Age at onset (years) | Age at TPIAT (years) | Duration (years) | Classification (by age at onset) | Severity of fibrosis (0–10) | PanIN score | Calcification | History of diabetes | History of smoking | Smoking duration (years) | Pancreatitis classification | Affected gene(s) |
|--------------------|-----------------------------|-----|---------------------|---------------------|-----------------|-------------------------------|-----------------------------|-------------|--------------|---------------------|---------------------|--------------------------|-----------------------------|----------------|
| MN05               | 1                           | Male| 28.2                | 36.6                | 8.5             | Adult                         | Low grade                   | 9           | Yes          | No                  | Yes                 | 1                        | Alcoholism                  | CFTR (Y1014C)   |
| MN06               | 2                           | Male| 22.9                | 25.1                | 2.2             | Adult                         | Low grade                   | 9           | No           | No                  | No                  | No                       | Genetic/pancreas divisum | CFTR (Y1014C)   |
| MN07               | 2                           | Male| 47.0                | 51.6                | 4.7             | Adult                         | Low grade                   | 4           | No           | No                  | No                  | No                       | Idiopathic                  | CFTR (Y1014C)   |
| MN08               | 2                           | Female| 39.4               | 44.1                | 4.7             | Adult                         | Low grade                   | 7           | No           | No                  | No                  | No                       | Idiopathic                  | CFTR (Y1014C)   |
| MN10               | 1                           | Female| 52.4               | 59.6                | 7.1             | Adult                         | Low grade                   | 7           | No           | No                  | Yes                 | 30                       | Idiopathic (SOD)            | CFTR (Y1014C)   |
| MN11               | 2                           | Female| 13.3               | 27.6                | 14.4            | Pediatric                      | High grade                  | 6           | No           | No                  | No                  | No                       | Idiopathic                  | CFTR (Y1014C)   |
| MN12               | 1                           | Female| 13.7               | 29.1                | 15.4            | Pediatric                      | Low grade                   | 9           | Yes          | No                  | No                  | No                       | Genetic                     | CFTR (Y1014C)   |
| MN13               | 2                           | Female| 54.2               | 56.0                | 1.8             | Adult                         | Low grade                   | 4           | No           | No                  | No                  | No                       | Genetic                     | CFTR (Y1014C)   |
| MN14               | 1                           | Female| 47.8               | 49.0                | 1.2             | Adult                         | Low grade                   | 6           | No           | No                  | No                  | No                       | Idiopathic                  | CFTR (Y1014C)   |
| MN16               | 2                           | Male| 8.9                 | 17.6                | 8.7             | Pediatric                      | Low grade                   | 9           | Yes          | No                  | No                  | No                       | Genetic                     | CFTR (Y1014C)   |
| MN17               | 1                           | Female| 16.2               | 16.9                | 0.7             | Pediatric                      | Low grade                   | 9           | Yes          | No                  | No                  | No                       | Genetic                     | CFTR (Y1014C)   |
| MN18               | 2                           | Male| 28.4                | 34.9                | 6.6             | Adult                         | Low grade                   | 9           | Yes          | No                  | No                  | No                       | Pancreas divisum            | CFTR (Y1014C)   |
| MN19               | 2                           | Female| 30.2               | 31.8                | 1.6             | Adult                         | Low grade                   | 9           | Yes          | No                  | No                  | No                       | Idiopathic (SOD)            | CFTR (Y1014C)   |
| MN20               | 2                           | Male| 53.3                | 57.3                | 4.0             | Adult                         | Low grade                   | 10          | No           | Yes                 | Yes                 | 2.5                      | Alcoholism                  | CFTR (Y1014C)   |
| MN21               | 1                           | Male| 34.4                | 38.3                | 3.9             | Adult                         | Low grade                   | 6           | No           | No                  | Yes                 | 2.5                      | Pancreas divisum            | CFTR (Y1014C)   |
| MN22               | 1                           | Female| 4.7                | 19.3                | 14.6            | Pediatric                      | Low grade                   | 9           | Yes          | No                  | No                  | No                       | Genetic                     | CFTR (Y1014C)   |
| MN24               | 1                           | Female| 34.3               | 36.3                | 2.1             | Adult                         | High grade                  | 5           | No           | Yes                 | 15                  | 15                       | Idiopathic                  | CFTR (Y1014C)   |
| MN25               | 1                           | Female| 36.6               | 40.4                | 3.8             | Adult                         | High grade                  | 5           | No           | No                  | No                  | No                       | Idiopathic                  | CFTR (Y1014C)   |
| MN26               | 2                           | Male| 14.5                | 22.2                | 7.6             | Pediatric                      | Low grade                   | 8           | No           | Yes                 | 1                   | 1                        | Idiopathic                  | CFTR (Y1014C)   |
| MN27               | 1                           | Female| 21.3               | 25.6                | 4.3             | Adult                         | N/A                         | 9           | No           | No                  | No                  | No                       | Genetic                     | CFTR (Y1014C)   |
| MN29               | 2                           | Female| 21.0               | 52.7                | 31.7            | Adult                         | Low grade                   | 8           | No           | No                  | No                  | No                       | Idiopathic                  | CFTR (Y1014C)   |
| MN30               | 1                           | Male| 2.2                 | 8.9                 | 6.7             | Pediatric                      | Low grade                   | 9           | No           | No                  | No                  | No                       | Genetic                     | CFTR (Y1014C)   |
| MN31               | 2                           | Male| 17.7                | 25.4                | 7.8             | Pediatric                      | High grade                  | 8           | No           | No                  | No                  | No                       | Idiopathic/gallstone       | CFTR (Y1014C)   |
| MN32               | 3                           | Female| 36.7               | 41.5                | 4.8             | Adult                         | High grade                  | 4           | Yes          | No                  | No                  | Yes                      | Idiopathic (SOD)            | CFTR (Y1014C)   |
| MN35               | 2                           | Female| 24.7               | 26.9                | 2.2             | Adult                         | Low grade                   | 6           | No           | No                  | No                  | No                       | Genetic                     | CFTR (Y1014C)   |
| MN36               | 2                           | Female| 5.5                | 27.4                | 21.9            | Pediatric                      | High grade                  | 8           | Yes          | No                  | Yes                 | 1                        | Genetic                     | CFTR (Y1014C)   |
| MN37               | 2                           | Male| 15.1                | 37.0                | 21.9            | Pediatric                      | High grade                  | 9           | Yes          | Yes                 | Yes                 | 10                       | Idiopathic                  | CFTR (Y1014C)   |
| Patient identifier | Number of samples sequenced | Sex | Age at onset (years) | Age at TPIAT (years) | Duration (years) | Classification (by age at onset) | Severity of fibrosis (0–10) | PanIN score | Calcification | History of diabetes | History of smoking | Smoking duration (years) | Pancreatitis classification | Affected gene(s) |
|-------------------|-----------------------------|-----|---------------------|---------------------|-----------------|-------------------------------|----------------------------|-------------|---------------|-------------------|--------------------|-------------------|--------------------------|------------------|
| MN38              | 1                           | Male| 44.9                | 50.8                | 5.9             | Adult                        | 8                          | Low grade   | No            | No                | No                 | Yes               | 32                       | Idiopathic       |
| MN39              | 1                           | Female| 48.1              | 49.9                | 1.8             | Adult                        | 6                          | High grade  | No            | No                | No                 | Yes               | 20                       | Pancreas divisum  |
| MN40              | 1                           | Female| 19.0              | 22.0                | 2.9             | Adult                        | 8                          | Low grade   | No            | No                | No                 | No                | Idiopathic               |
| MN42              | 1                           | Male| 3.2                 | 10.2                | 7.0             | Pediatric                    | 8                          | Low grade   | No            | No                | No                 | No                | Genetic                  | PRSS1 (R122H)   |
| MN43              | 1                           | Female| 3.5               | 4.9                 | 1.4             | Pediatric                    | 9                          | N/A         | No            | No                | No                 | No                | Genetic                  | PRSS1 (R122H)   |
| MN44              | 1                           | Male| 4.6                 | 5.4                 | 0.8             | Pediatric                    | 9                          | N/A         | No            | No                | No                 | No                | Genetic                  | PRSS1 (R122H)   |
| MN45              | 1                           | Male| 2.4                 | 3.1                 | 0.6             | Pediatric                    | 9                          | N/A         | No            | No                | No                 | No                | Genetic                  | PRSS1 (R122H)   |
| MN46              | 1                           | Male| 33.3                | 35.5                | 2.1             | Adult                        | 4                          | Low grade   | No            | No                | No                 | No                | Idiopathic               |
| MN47              | 1                           | Female| 6.0               | 9.8                 | 3.8             | Pediatric                    | 8                          | High grade  | No            | No                | No                 | No                | Idiopathic               |
| MN49              | 1                           | Female| 32.0              | 34.0                | 2.0             | Adult                        | 2                          | Low grade   | No            | No                | No                 | No                | Pancreas divisum        |
| MN50              | 1                           | Female| 43.4              | 47.0                | 3.6             | Adult                        | 9                          | Low grade   | No            | No                | Yes                | No                | Genetic/pancreas divisum | CFTR (F1052V), SPINK1 (c.-147A > G) |
| MN51              | 2                           | Male| 30.4                | 43.1                | 12.7            | Adult                        | 8                          | High grade  | No            | No                | No                 | Yes               | 25                       | Idiopathic       |

PanIN, pancreatic intraepithelial neoplasia; SOD, sphincter of Oddi dysfunction; TPIAT, total pancreatectomy with islet autotransplantation.

*Genes clinically confirmed to carry a mutation are listed for patients with genetic pancreatitis.
causes, were 25.5 and 32.2 years, respectively. However, the average age at onset attributable to genetic causes, 15.4 years, is similar to previously reported analyses of hereditary pancreatitis cohorts (64,65) and that of idiopathic cases, 31.8 years, is consistent with the literature where age at onset is in the range of 30–40 years (66–68). Histologic analysis of hematoxylin and eosin–stained adjacent tissue sections revealed that most of the patients (28 of the 39; 71.8%) harbored only low-grade PanINs or lacked preneoplastic lesions entirely (Figure 1d,e).

Identification of single-nucleotide variants in exocrine pancreas tissue
All patient samples were successfully sequenced with a minimum of 5,293 × depth of target coverage and an overall average of 10,388 × depth of target coverage. We detected an average of 73.0 variants per chronic pancreatitis sample, including likely single-nucleotide polymorphisms owing to their approximate allelic frequencies of 50% or 100%, their detection in separate samples from the same individual, and their inclusion in the Single Nucleotide Polymorphism Database (69). We, therefore, focused on variants with allelic frequencies less than 30%, which we considered to be somatic variants.

Somatic variants were predominantly low-prevalent single-nucleotide transitions with an average allele frequency of 1.19% (range 1.00%–5.97%). To assess potential cancer risk, we examined total somatic variants and the subset of variants annotated in the Catalogue of Somatic Mutations in Cancer (COSMIC) database (version 90) (70), which are thoroughly curated mutations derived from published reports of sequencing data from a range of human cancers. On average, we detected 50.4 somatic variants and 5.8 COSMIC somatic variants per chronic pancreatitis sample (Figure 2a and Table, Supplemental Digital Content 5, http://links.lww.com/CTG/A726). Different tissue regions from the same patient showed distinct mutualional profiles, suggesting genomic heterogeneity. The total number of somatic variants identified per gene across all samples was correlated with increasing amplicon coverage (Figure 2b), although amplicon coverage did not affect the number of somatic COSMIC variants per gene (Figure 2c). We also assessed whether an independent variant caller, VarScan (51), can identify the variants established by FreeBayes. VarScan, although not as sensitive as FreeBayes, is more precise (71) and replicated 83.0% of the somatic variants, including 88.6% of somatic COSMIC variants identified by FreeBayes (Table, Supplemental Digital Content 6, http://links.lww.com/CTG/A727), indicating a high degree of concordance between the 2 variant callers (Figure 2d).

Low prevalence of oncogetic KRAS mutations in patients undergoing TPIAT
The COSMIC variants were of particular interest due to their previously reported association with cancer genomes. We noted the COSMIC variants in all 57 chronic pancreatitis samples and determined the frequency of each gene harboring at least 1 non-synonymous somatic variant in our sample set (Figure 3). Surprisingly, activating KRAS mutations, affecting greater than 95% of patients with pancreatic cancer, were not nearly as prevalent in our data set and were only detected in 4 patients (2 p.G12R and 2 p.Q61R variants). By contrast, other PDA-associated genes such as TP53, CDKN2A, and SMAD (72) more frequently contained somatic variants in these samples. Indeed, the prevalence of somatic COSMIC variants is seemingly enriched in PDA-related genes because no non-synonymous COSMIC variants were reported in 9 of the 55 amplicon panel genes, and a further 20 genes included none of the 55 amplicon panel genes, and a further 20 genes included.

We next confirmed the existence of low-prevalent oncogetic KRAS mutations in samples from patients with chronic pancreatitis using picodroplet digital PCR with TaqMan probes (73) and also using nuclease-assisted minor allele enrichment with probe overlap analysis (63). This technique uses wild-type sequence DNA probes that bind to an area of interest, which, if it contains a mutation, will result in a mismatch that prevents subsequent digestion from a double-stranded DNA–specific nuclease. The undigested mutation-containing DNA is then available for PCR amplification and Sanger sequencing. Using both independent methods, we confirmed the presence of p.G12R mutations in the 2 samples annotated by our variant caller and were unable to detect KRAS codon 12 mutations in other samples (Figure 4e,f).

Few correlations with clinical factors
Based on clinical characteristics including sex, overall disease duration, and age at disease onset or at TPIAT (Figure 5a–d), the only significant differences in either the total number of somatic variants or the number of COSMIC somatic variants was a negative association between disease duration and the number of
somatic variants, indicating that these attributes mostly had no impact on mutational load. In addition, there were no increases in the number of somatic variants with markers of disease severity, such as the degree of fibrosis or presence of PanINs (Figure 5e,f and Supplemental Figure, Supplemental Digital Content 7, http://links.lww.com/CTG/A728). Indeed, the presence of calcification, a hallmark of severe chronic pancreatitis (36), was actually associated with a reduced number of both total and COSMIC somatic variants (Figure 5g). However, among smokers, we did note a weak positive correlation between smoking duration and the number of somatic variants (Figure 5h). Therefore, overall, clinical presentation and indicators of disease severity do not correlate with increased mutational load. However, when we examine patient subgroups that may be at increased risk of developing PDA (such as the aforementioned KRAS mutant-positive cases), we find significant differences in the number of somatic variants.

**Increased mutational burden in patients with PRSS1 mutations**

We, therefore, assessed the mutational burden in other patient subgroups and noted an increased number of average total somatic variants and total COSMIC somatic variants in PRSS1 mutation–containing cases of hereditary pancreatitis compared with all other cases (Figure 6a). In addition, patients with PRSS1 mutations also harbored an increased number of total somatic and COSMIC variants than other patients with mutations in CFTR, CTRC, or SPINK1 (Figure 6b). There was also a significant increase in the total number of somatic variants in patients with PRSS1 mutations compared with idiopathic cases alone, although the number of COSMIC somatic variants did not reach significance ($P = 0.0730$). Interestingly, there were no differences between the number of somatic variants detected in CFTR, CTRC, and SPINK1 mutation–containing patients compared with all other non-PRSS1 cases ($P = 0.0968$). An analysis of other clinical parameters from these patients, such as disease duration and degree of fibrosis, revealed no differences (Figure 6c,d), suggesting the PRSS1 mutations alone may correlate with increased mutational load.

**DISCUSSION**

Patients with chronic pancreatitis are at increased risk of developing PDA (1–4). We hypothesized that severe clinical cases of chronic pancreatitis, from patients requiring total pancreatectomy, may have a higher incidence of cancer-related mutations. We examined the presence of somatic variants in 55 freshly dissected samples of exocrine pancreas from 39 patients with chronic pancreatitis and found that clinical severity does not seem to be relevant in assessing PDA risk. In our study, we identified somatic variants with an average allele frequency of only 1.19% (range 1.00%–5.97%). Moreover, distinct regions from the same patient displayed genomic heterogeneity, suggesting these variants are subclonal and have not yet undergone selection. There were no increases in the number of somatic variants that correlated with clinical markers of disease severity, and a reduction of the number of somatic and COSMIC variants was actually noted in patients presenting with pancreatic calcifications. Together, these results indicate that the process by which exocrine cells sustain mutations is somewhat independent of the processes that lead to clinically severe symptoms.
Furthermore, we identified a surprisingly low prevalence of oncogenic KRAS variants than might be expected if clinical severity correlated with PDA risk. Only 2 KRAS p.G12R and 2 p.Q61R variants were detected (7.0% of all samples), with an average allelic frequency of 1.21%. An additional KRAS p.C51R variant, catalogued in squamous cell carcinoma (74) and colorectal carcinomas (75,76), but having unknown significance, was also detected. Two previous studies have quantified the allelic frequencies of KRAS mutations from pancreatic juice (77) and peripheral blood (78) from patients with chronic pancreatitis and reported results as most often in the 0.2%–1% range. In this study, we have quantified KRAS variants from freshly dissected pancreas tissue and show a similarly low allelic frequency. The sensitivity of the assay, with a calculated false negative of <0.0001%, suggests that additional somatic variants in KRAS are unlikely to be found above the 1% threshold in this sample cohort. Previous studies have reported higher KRAS mutation incidences (12–23) among their patient populations with chronic pancreatitis. This discrepancy may be attributable to differences in patient populations because we focused on a TPIAT cohort, whereas others studied a broader general population with chronic pancreatitis. In fact, some studies demonstrating KRAS mutations in chronic pancreatitis reveal coincidence of PDA or development of PDA shortly after mutational analyses (12,14,15), suggesting undiagnosed PDA at the time of analyses that may influence results. Some patients with severe chronic pancreatitis may additionally be ineligible for TPIAT, particularly those with advanced disease resulting in insulin-deficient diabetes, and may represent a separate cohort that was not evaluated in this study. Moreover, experimental procedures may also account for differences because

**Figure 3.** COSMIC somatic variants in chronic pancreatitis tissue samples. Genes harboring 1 or more COSMIC somatic variants, as determined by our amplicon panel, are highlighted. Genes are sorted by prevalence of nonsynonymous variants. COSMIC, Catalogue of Somatic Mutations in Cancer; LOF, loss of function.
microdissection or enrichment for PanINs specifically might increase the positivity rate, although previous studies that used these methods also show low KRAS mutation rates (13,22,23). Of note, other analyses have not found KRAS mutations in their patient populations with chronic pancreatitis (7–11). In this study, we have demonstrated through sensitive picodroplet digital PCR analysis that even in severe clinical cases of pancreatitis, incidence of oncogenic KRAS mutations is low. Moreover, although we detected evidence of frequent COSMIC variants in other genes known to be mutated in the PanIN-to-PDA progression model (72), such as TP53, CDKN2A, and SMAD4, these variants also reinforce the notion that at this stage of disease, before any histologic evidence of PDA, the somatic alterations we detect are of low prevalence. Therefore, clinical severity does not appreciably increase the PDA-related mutational burden in patients with chronic pancreatitis.

Of interest, we also noted a higher average number of total somatic variants and COSMIC-listed variants in PRSS1 mutation-bearing patients. Patients with detectable oncogenic KRAS variants similarly harbored an elevated average number of total somatic variants. Given that oncogenic KRAS mutations are central to most pancreatic cancers (79) and that the risk of developing cancer is greater for hereditary pancreatitis than chronic pancreatitis in general (3), this suggests that these patient populations have greater genomic instability and may be more likely to develop PDA. Curiously, we did not detect a similar difference in higher average somatic variants compared with other idiopathic cases in samples from patients with CFTR, CTRC, and SPINK1 mutations, suggesting that PRSS1 mutations specifically may affect genomic instability in this TPIAT cohort. However, larger patient cohorts are required to discern whether the differences we noted in average number of somatic variants among patient subgroups is maintained on a broader scale, particularly because the association of increased somatic variants with oncogenic KRAS is based on a small sample size due to the aforementioned lack of KRAS mutations in this cohort. Moreover, the low frequency of somatic mutations and the potential for false positives using picodroplet digital PCR, which are estimated to be comparable with traditional singleplex PCRs (61,62), may require additional experimental considerations such as molecular barcoding for ultralow variant calling (55) to further distinguish these subgroups. Nevertheless, we have confirmed the presence of oncogenic KRAS mutations in our

Figure 4. KRAS mutations in TPIAT patient cohort. Graphs show mean ± SD. (a) Total somatic variants from KRAS mutation-bearing patients (p.G12R or p.Q61R) compared with other samples and (b) COSMIC somatic variants between those groups. (c) Fibrosis score and (d) disease duration revealed no discernable differences between KRAS mutation-bearing patients and other patients. (e) Representative Sanger sequencing results demonstrating enrichment of KRAS p.G12R variants in MN17E and MN25F DNA, but not MN24D DNA, on treatment with double-stranded nuclease (DSN) as part of nuclease-assisted minor allele enrichment with probe overlap analysis. (f) Representative digital droplet PCR confirmation of KRAS p.G12R variants in MN17E and MN25F samples and the absence of variants in MN35A using fluorescently labeled TaqMan probes that were optimized using PSN-1 and BxPC-3 cell lines that contain KRAS p.G12R or wild-type KRAS, respectively, and serve as a control. COSMIC, Catalogue of Somatic Mutations in Cancer; TPIAT, total pancreatectomy with islet autotransplantation.
Figure 5. Single-nucleotide variant correlation with clinical characteristics. Total somatic variants and COSMIC somatic variants are compared (a) between female and male patients (mean ± SD); (b) with disease duration; (c) according to age at onset of disease (pediatric younger than 19 years; mean ± SD); (d) with age at TPIAT; (e) with patient fibrosis scores; (f) according to PanIN score (mean ± SD); and (g) according to the presence of calcification (mean ± SD). (h) For smokers, smoking duration is compared with total somatic variants. COSMIC, Catalogue of Somatic Mutations in Cancer; PanIN, pancreatic intraepithelial neoplasia; TPIAT, total pancreatectomy with islet autotransplantation.
samples at frequencies as low as 1% using independent protocols. Increased smoking duration was also associated with an increased total number of somatic variants in our study, and smoking is an established risk factor of PDA (80). Therefore, our findings of increased mutational burden in patient subgroups suggests that clinical severity alone is not prognostic for PDA risk, and other assessments, including \textit{PRSS1} and \textit{KRAS} mutation status, may be required.

There are several advantages to using picodroplet digital PCR, followed by next-generation sequencing. Foremost is the ability to quantitatively assess the mutational status of multiple genes of interest simultaneously, including tumor suppressor genes that have a broader mutational profile than oncogenes. This protocol could also be adapted to other methods of pancreatic tissue collection, including endoscopic ultrasound-guided fine-needle biopsies, which would permit long-term longitudinal follow-up and enable an assessment of a wider patient cohort. A potential limitation of our study surrounds the use of patients undergoing TPIAT: the removal and processing of pancreases for islet autotransplantation may introduce unforeseen confounding factors. However, the pancreatectomy process, from a single institution, is standardized and any changes are likely to affect samples uniformly. Moreover, pathology of samples collected for analysis showed no detectable effects on pancreas morphology, and highly intact DNA, as assessed by Agilent TapeStation, was consistently isolated. The use of resected pancreas also afforded some advantages in that it allowed multiple distinct regions and larger tissue specimens to be assessed, thereby expanding the search for mutations in these patients.

In summary, we find that genomic characterization of exocrine pancreas from patients with chronic pancreatitis is feasible and informative. In our TPIAT patient cohort, we show that even in severe clinical cases of chronic pancreatitis, PDA-related mutational burden is not appreciably increased. However, we noted significant differences in the average number of total somatic mutations in patient subgroups such as \textit{PRSS1} or oncogenic \textit{KRAS}-bearing samples, which may suggest that these patients have greater genomic instability and are at increased risk of cancer development. Using an amplicon-based mutational panel may assist in stratifying PDA risk in patients with chronic pancreatitis. Further analysis of a broader patient cohort without total pancreatectomies and monitoring for cancer development may be beneficial for informing clinical decisions on patients with chronic pancreatitis.

**CONFLICTS OF INTEREST**

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Specific author contributions: R.W.C., E.D.P., J.M.K., E.M.Q., L.P.B.: designed and performed experiments. J.J.W., M.A., M.D.B., A.D.R.: designed experiments and provided critical resources. R.W.C., E.D.P., J.M.K., J.Z., P.J.U.: performed analyses. R.W.C., E.D.P., J.M.K., P.J.U., A.D.R.: interpreted data. M.D.B. and A.D.R.: conceived the study design. A.D.R.: conceived the project. R.W.C.: drafted the manuscript; all other authors revised the manuscript. All authors approved the final version of the manuscript.
Financial support: A.D.R. is supported by the Doris Duke Foundation (Clinical Scholar Grant), Andrew Sabin Family Foundation, National Institutes of Health, MD Anderson Cancer Center (Physician Scientist Program, Pancreatic Cancer Moonshot) and Cancer Prevention and Research Institute of Texas (Rising Stars Award).

Potential competing interests: None to report.

Study Highlights

WHAT IS KNOWN

- Patients with chronic pancreatitis are at increased risk of developing pancreatic cancer.
- Pancreatic cancer is driven by numerous gene mutations.

WHAT IS NEW

- Pancreatic cancer–related mutational burden is not appreciably increased in a cohort with clinically severe chronic pancreatitis.
- Samples with oncogenic KRAS mutations and from patients with PRSS1 mutations had increased mutational burden.

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