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

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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.

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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 SPRIselect bead purification (Beckman Coulter, Brea, CA). Addition of adaptor and index sequences was achieved in a subsequent PCR, and DNA was again purified using SPRIselect 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 were 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 removed. 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 *KRAS*^{G12R} 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: ATTATAAGGCCTGCTGAAAATGACT; R: TCTGAATTAGCTGTATCGTCAAGG), allele-specific TaqMan probes (WT: VIC-TTGGAGCTGGTGGCGT-MGBNFQ; G12R: 6FAM-TGGAGCTCGTGGCGT-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: ATTATAAGGCCTGCTGAAAATGACT; R: GGTCCTGCACCAGTAATATGC) and oligonucleotide probes (sense: AGTTGGAGCTGGTGGCG; antisense: TCTTGCCTACG CCACCAG) 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

Table 1. Patient information of TPIAT samples that were sequenced using the amplicon panel[a]

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	9	Low grade	Yes	No	Yes	1	Alcoholism	
MN06	2	Male	22.9	25.1	2.2	Adult	9	Low grade	No	No	No		Genetic/pancreas divisum	CFTR (Y1014C)
MN07	2	Male	47.0	51.6	4.7	Adult	4	Low grade	No	No	No		Idiopathic	
MN08	2	Female	39.4	44.1	4.7	Adult	7	High grade	No	No	No		Idiopathic	
MN10	1	Female	52.4	59.6	7.1	Adult	7	Low grade	No	No	Yes	30	Idiopathic (SOD)	
MN11	2	Female	13.3	27.6	14.4	Pediatric	6	High grade	No	No	No		Idiopathic	
MN12	1	Female	13.7	29.1	15.4	Pediatric	9	N/A	Yes	No	No		Genetic	PRSS1 (N29I)
MN13	2	Female	54.2	56.0	1.8	Adult	4	Low grade	No	No	No		Genetic	PRSS1 (R122H)
MN14	1	Female	47.8	49.0	1.2	Adult	6	Low grade	No	No	No		Pancreas divisum	
MN16	2	Male	8.9	17.6	8.7	Pediatric	9	Low grade	Yes	No	No		Genetic	CFTR (R75Q), SPINK1 (N34S)
MN17	1	Female	16.2	16.9	0.7	Pediatric	9	Low grade	Yes	No	No		Genetic	PRSS1 (R122H)
MN18	2	Male	28.4	34.9	6.6	Adult	9	N/A	Yes	No	No		Pancreas divisum	
MN19	2	Female	30.2	31.8	1.6	Adult	9	Low grade	Yes	No	No		Iatrogenic duct obstruction	
MN20	2	Male	53.3	57.3	4.0	Adult	10	Low grade	Yes	No	Yes		Alcoholism	
MN21	1	Female	34.4	38.3	3.9	Adult	6	Low grade	No	No	Yes	2.5	Pancreas divisum	
MN22	1	Female	4.7	19.3	14.6	Pediatric	9	Low grade	Yes	No	No		Genetic	PRSS1 (R122H)
MN24	1	Female	34.3	36.3	2.1	Adult	5	High grade	No	No	Yes	15	Idiopathic	
MN25	1	Female	36.6	40.4	3.8	Adult	5	High grade	No	No	No		Idiopathic	
MN26	2	Male	14.5	22.2	7.6	Pediatric	8	Low grade	No	No	Yes	1	Idiopathic	
MN27	1	Female	21.3	25.6	4.3	Adult	9	N/A	No	No	No		Genetic	CFTR (R117H, ΔF508), CTRC (R254W), SPINK1 (N34S)
MN29	2	Female	21.0	52.7	31.7	Adult	8	Low grade	No	No	No		Idiopathic	
MN30	1	Male	2.2	8.9	6.7	Pediatric	9	Low grade	No	No	No		Genetic	PRSS1 (R122H)
MN31	2	Male	17.7	25.4	7.8	Pediatric	8	High grade	No	No	No		Idiopathic/gallstone	
MN32	3	Female	36.7	41.5	4.8	Adult	4	High grade	No	No	Yes	15	Idiopathic (SOD)	
MN35	2	Female	24.7	26.9	2.2	Adult	6	Low grade	No	No	No		Genetic	CFTR (R117H)
MN36	2	Female	5.5	27.4	21.9	Pediatric	8	High grade	Yes	No	Yes	1	Genetic	CTRC (R254W)
MN37	2	Male	15.1	37.0	21.9	Pediatric	9	High grade	Yes	Yes	Yes	10	Idiopathic	

Table 1. (*continued*)

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	Yes	32	Idiopathic	
MN39	1	Female	48.1	49.9	1.8	Adult	6	High grade	No	No	Yes	20	Pancreas divisum	
MN40	1	Female	19.0	22.0	2.9	Adult	8	Low grade	No	No	No		Idiopathic	
MN42	1	Male	3.2	10.2	7.0	Pediatric	8	Low grade	No	No	No		Genetic	PRSS1 (R122H)
MN43	1	Female	3.5	4.9	1.4	Pediatric	9	N/A	No	No	No		Genetic	PRSS1 (R122H)
MN44	1	Male	4.6	5.4	0.8	Pediatric	9	N/A	No	No	No		Genetic	PRSS1 (R122H)
MN45	1	Male	2.4	3.1	0.6	Pediatric	9	N/A	No	No	No		Genetic	PRSS1 (R122H)
MN46	1	Male	33.3	35.5	2.1	Adult	4	Low grade	No	No	No		Idiopathic	
MN47	1	Female	6.0	9.8	3.8	Pediatric	8	High grade	No	No	No		Idiopathic	
MN49	1	Female	32.0	34.0	2.0	Adult	2	Low grade	No	No	No		Pancreas divisum	
MN50	1	Female	43.4	47.0	3.6	Adult	9	Low grade	No	No	Yes		Genetic/pancreas divisum	CFTR (F1052V), SPINK1 (c.-147A > G)
MN51	2	Male	30.4	43.1	12.7	Adult	8	High grade	No	No	Yes	25	Idiopathic	

PanIN, pancreatic intraepithelial neoplasia; SOD, sphincter of Oddi dysfunction; TPIAT, total pancreatectomy with islet autotransplantation.
[a]Genes clinically confirmed to carry a mutation are listed for patients with genetic pancreatitis.

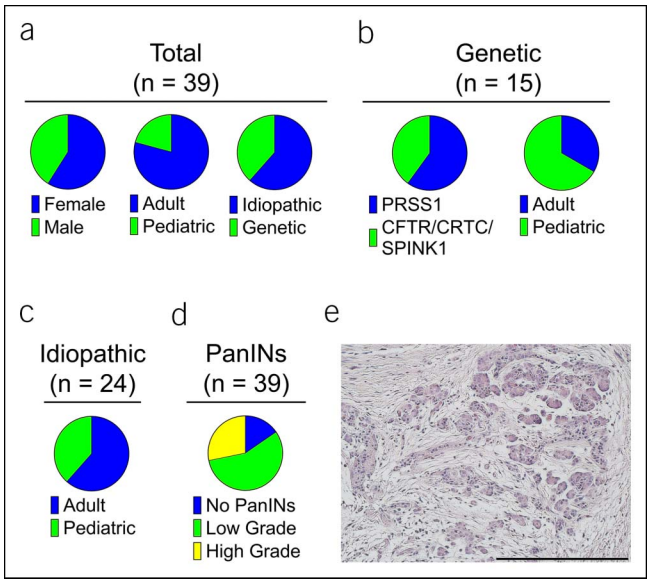

Figure 1. Characteristics of TPIAT patient cohort. (**a**) Proportion of all patient cases according to sex, age at onset (pediatric younger than 19 years), and etiology. (**b**) Proportion of genetic cases only, classified by contributing gene mutations and age at onset. (**c**) Age at onset of idiopathic cases only. (**d**) Total patients according to the highest PanIN grade identified in tissue sections. (**e**) Representative hematoxylin and eosin–stained tissue section from a patient undergoing TPIAT (scale bar = 200 μm). PanIN, pancreatic intraepithelial neoplasia; TPIAT, total pancreatectomy with islet autotransplantation.

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 5,293× depth of target coverage and an overall average 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 mutational 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 oncogenic *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 nonsynonymous 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 *SMAD4* (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 nonsynonymous COSMIC variants were reported in 9 of the 55 amplicon panel genes, and a further 20 genes included only 1 or 2 nonsynonymous COSMIC variants. A higher average number of total variants was detected in samples containing oncogenic *KRAS* variants (Figure 4a), although a corresponding increase in the number of COSMIC variants in those cases was not significant ($P = 0.1662$) (Figure 4b). There were no differences in clinical parameters such as fibrosis score and disease duration (Figure 4c,d), suggesting that activating *KRAS* mutations alone may correlate with increased mutational burden.

We next confirmed the existence of low-prevalent oncogenic *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

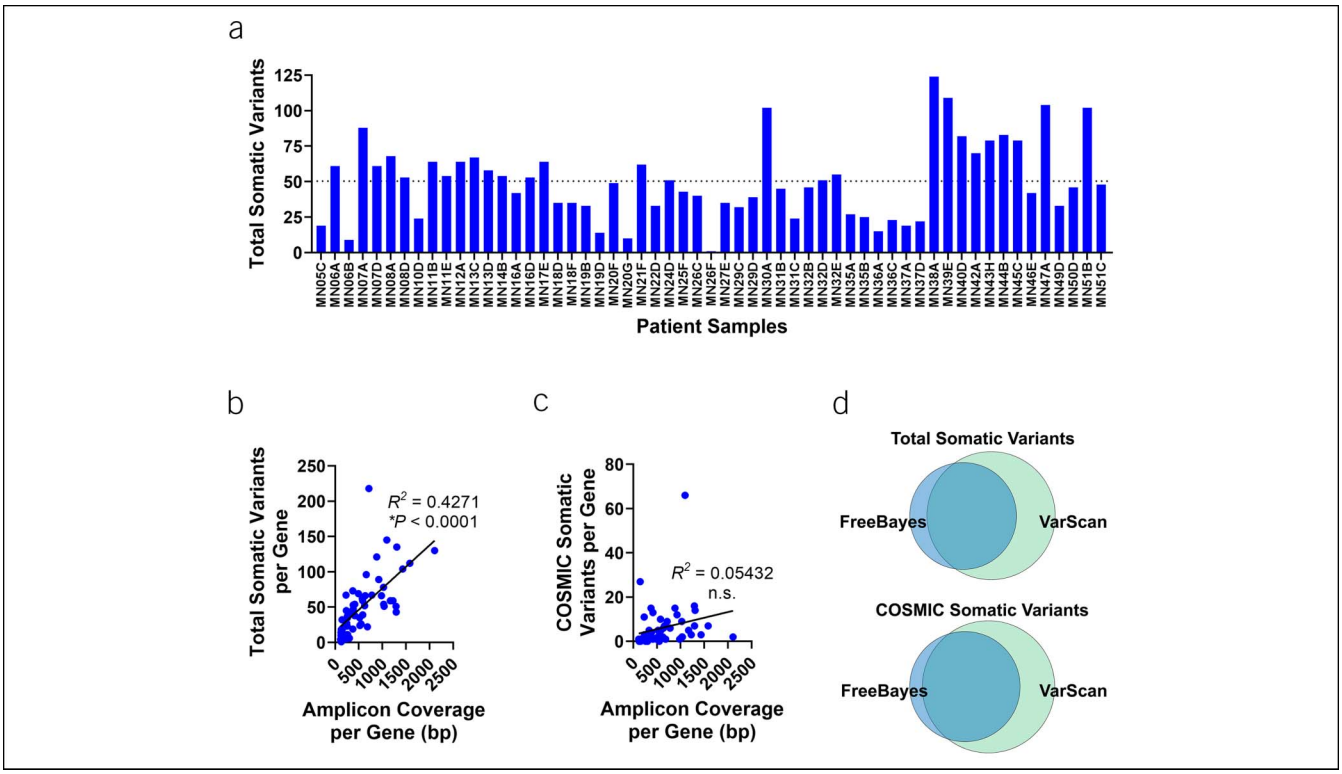

Figure 2. Identification of somatic variants. (**a**) Total somatic variants per patient sample. (**b**) Total and (**c**) COSMIC somatic variants from all patient samples in relation to amplicon coverage for each gene. (**d**) Venn diagrams demonstrating overlap between FreeBayes and VarScan variant callers. COSMIC, Catalogue of Somatic Mutations in Cancer.

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 cancer-related genes in 57 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.

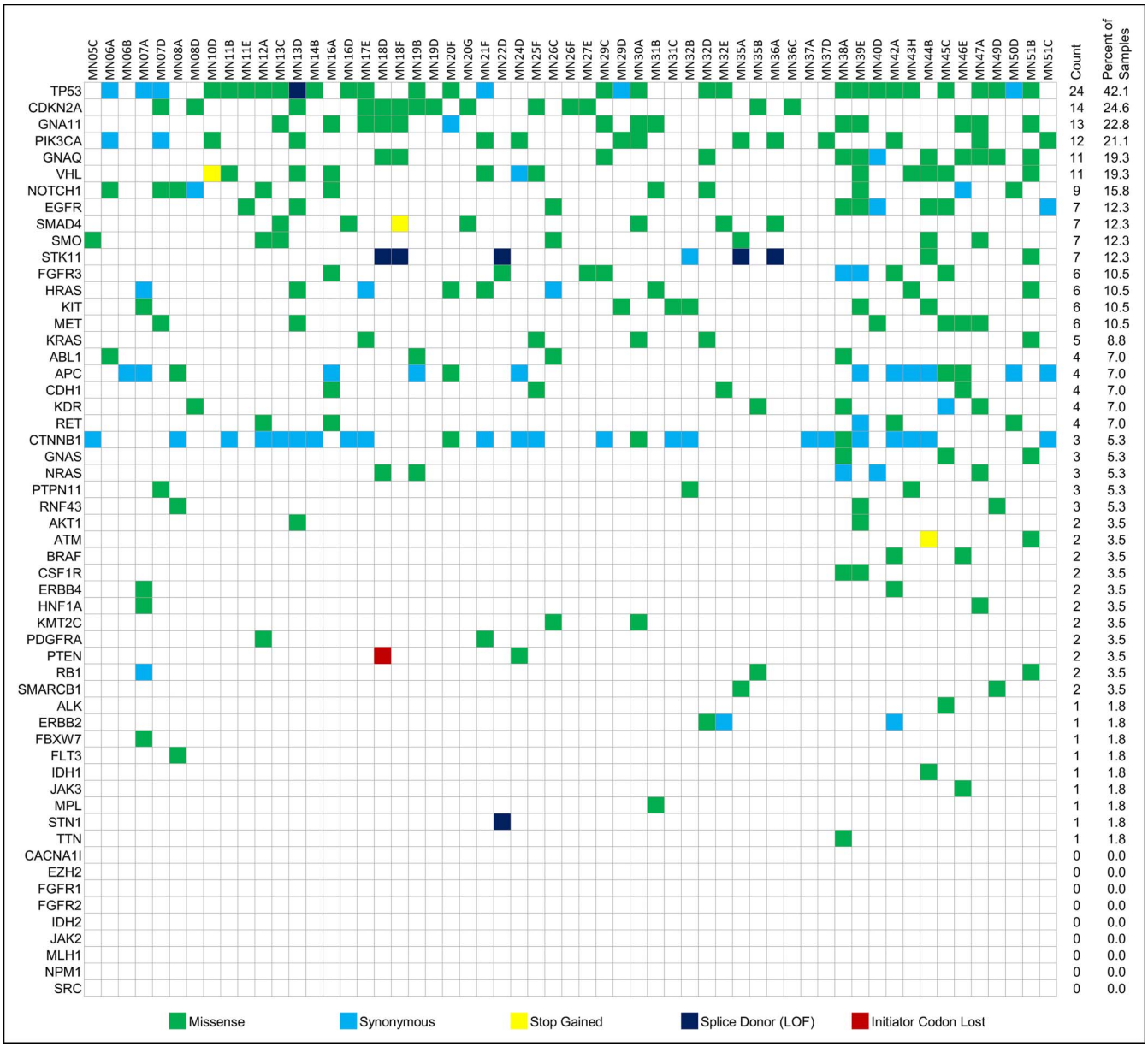

Figure 3. COSMIC somatic variants in chronic pancreatitis tissue samples. Genes harboring 1 or more COSMIC somatic variants, as determined by our amplicon panel, are highlighted. Genes are sorted by prevalence of nonsynonymous variants. COSMIC, Catalogue of Somatic Mutations in Cancer; LOF, loss of function.

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

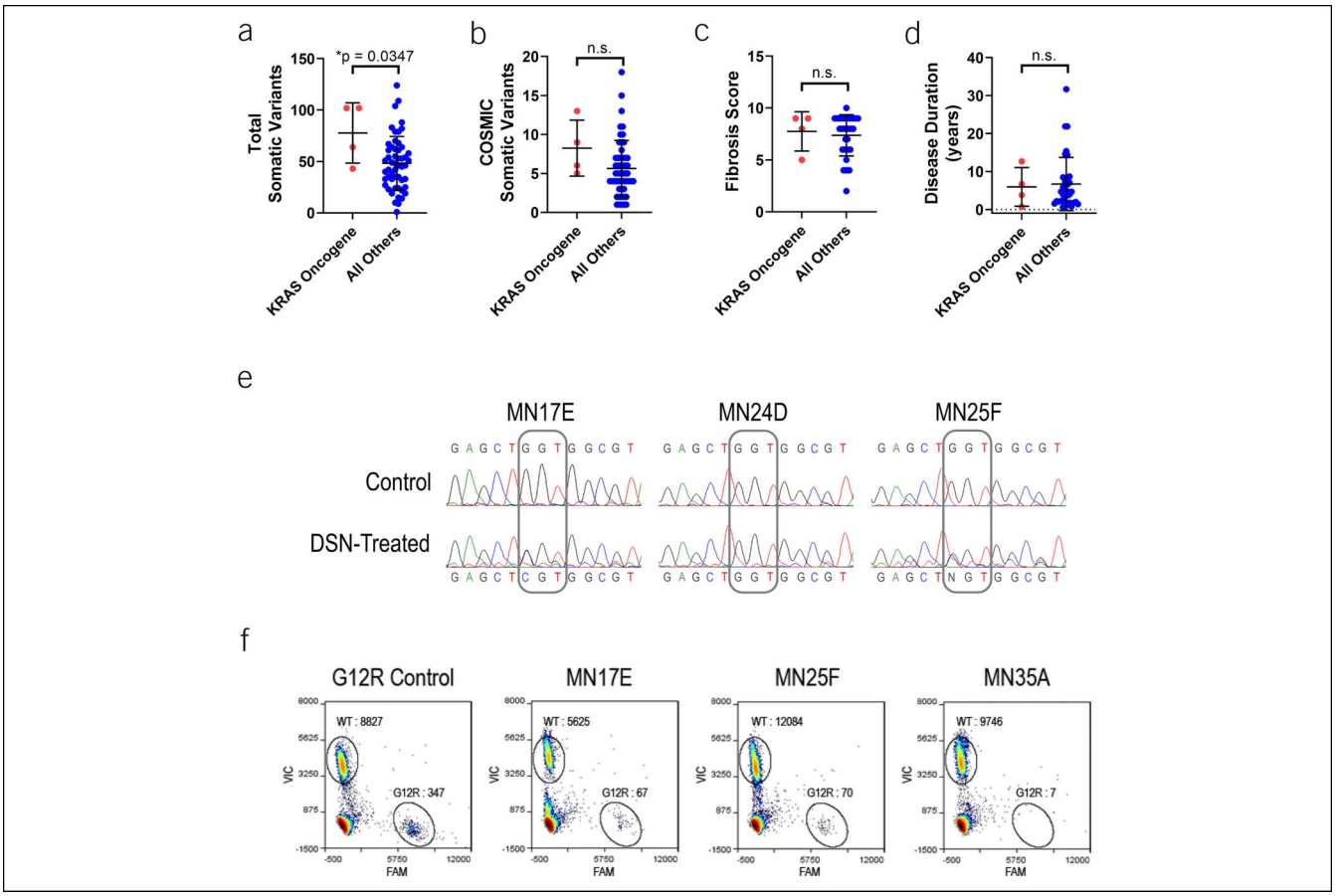

Figure 4. *KRAS* mutations in TPIAT patient cohort. Graphs show mean ± SD. (**a**) Total somatic variants from *KRAS* mutation-bearing patients (p.G12R or p.Q61R) compared with other samples and (**b**) COSMIC somatic variants between those groups. (**c**) Fibrosis score and (**d**) disease duration 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.

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

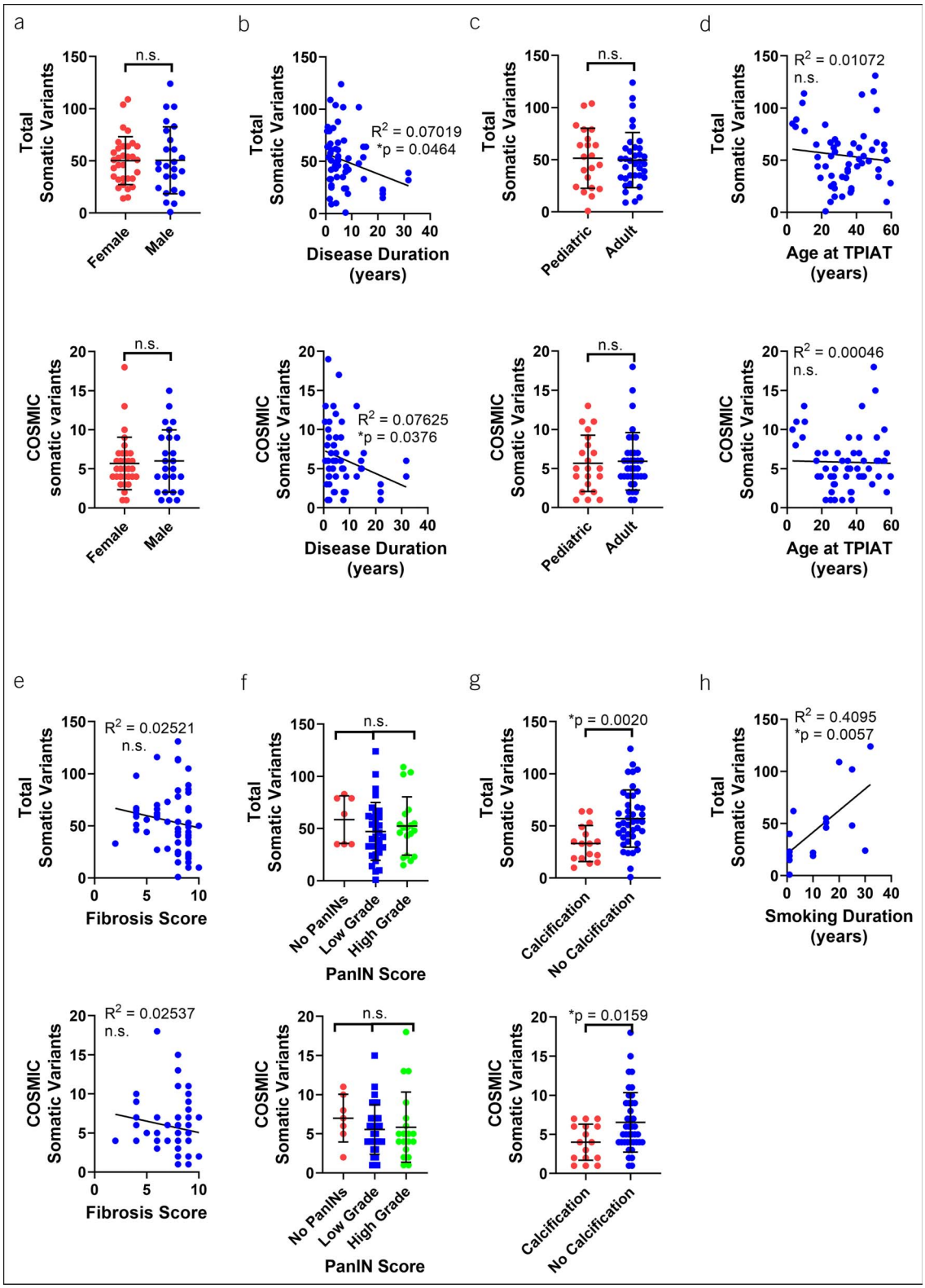

Figure 5. Single-nucleotide variant correlation with clinical characteristics. Total somatic variants and COSMIC somatic variants are compared (**a**) between female and male patients (mean ± SD); (**b**) with disease duration; (**c**) according to age at onset of disease (pediatric younger than 19 years; 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.

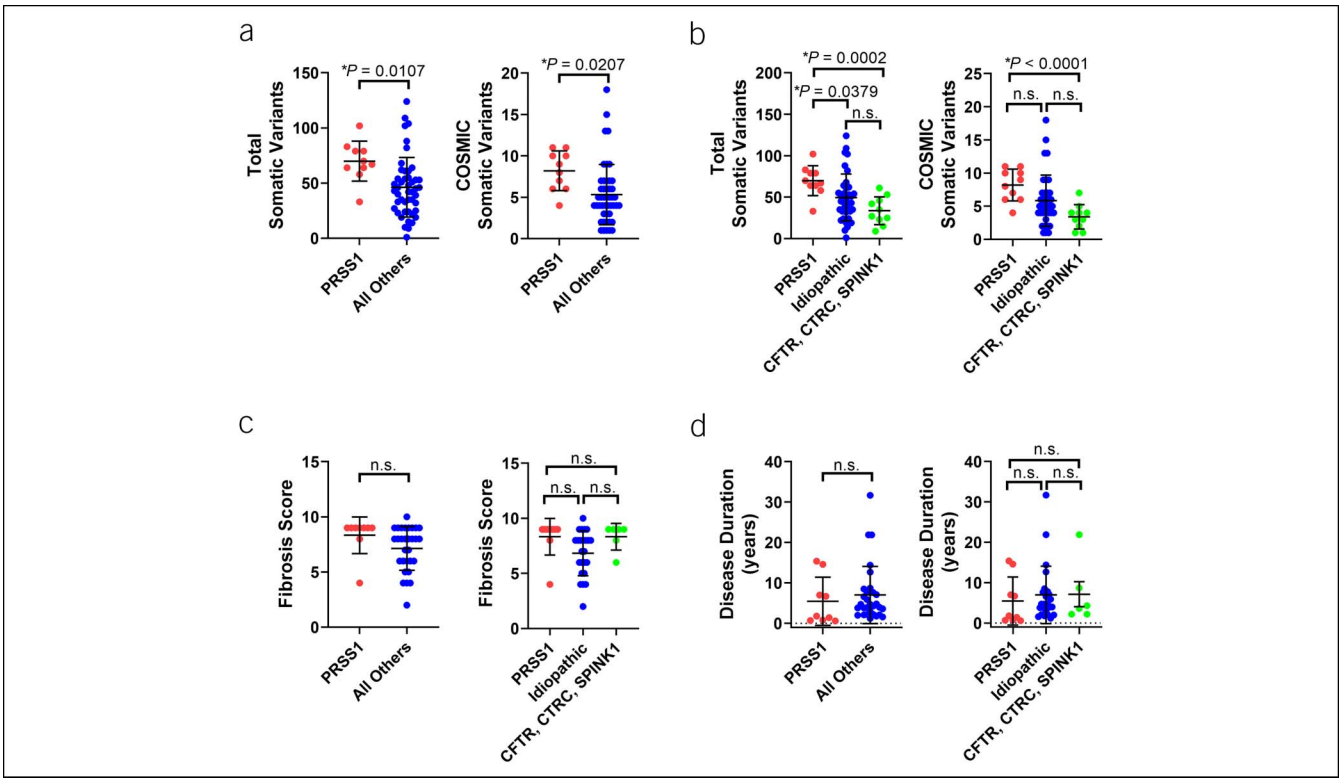

Figure 6. Increased mutational burden in patients with *PRSS1* mutations. (**a**) Samples from *PRSS1* mutation-bearing patients have a higher average number of somatic variants and COSMIC somatic variants than all other samples together (mean ± SD). (**b**) Samples from *PRSS1* mutation-bearing patients also have increased total and COSMIC somatic variants compared with other hereditary pancreatitis samples harboring mutations in *CFTR*, *CTRC*, or *SPINK1* and a higher average number of somatic variants than idiopathic cases. (**c**) Fibrosis scores and (**d**) disease duration show no differences among *PRSS1* mutation-bearing patients, idiopathic cases, or other hereditary pancreatitis cases (mean ± SD). COSMIC, Catalogue of Somatic Mutations in Cancer.

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 *PRSS1* and *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, histology 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 *PRSS1* or oncogenic *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

Guarantor of the article: Robert W. Cowan, PhD
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.

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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.

REFERENCES

1. Kirkegard J, Mortensen FV, Cronin-Fenton D. Chronic pancreatitis and pancreatic cancer risk: A systematic review and meta-analysis. Am J Gastroenterol 2017;112:1366–72.
2. Lowenfels AB, Maisonneuve P, Cavallini G, et al. Pancreatitis and the risk of pancreatic cancer. International Pancreatitis Study Group. N Engl J Med 1993;328:1433–7.
3. Raimondi S, Lowenfels AB, Morselli-Labate AM, et al. Pancreatic cancer in chronic pancreatitis; aetiology, incidence, and early detection. Best Pract Res Clin Gastroenterol 2010;24:349–58.
4. Tong GX, Geng QQ, Chai J, et al. Association between pancreatitis and subsequent risk of pancreatic cancer: A systematic review of epidemiological studies. Asian Pac J Cancer Prev 2014;15:5029–34.
5. Rahib L, Smith BD, Aizenberg R, et al. Projecting cancer incidence and deaths to 2030: The unexpected burden of thyroid, liver, and pancreas cancers in the United States. Cancer Res 2014;74:2913–21.
6. Raphael KL, Willingham FF. Hereditary pancreatitis: Current perspectives. Clin Exp Gastroenterol 2016;9:197–207.
7. Bournet B, Souque A, Senesse P, et al. Endoscopic ultrasound-guided fine-needle aspiration biopsy coupled with KRAS mutation assay to distinguish pancreatic cancer from pseudotumoral chronic pancreatitis. Endoscopy 2009;41:552–7.
8. Gansauge S, Schmid RM, Muller J, et al. Genetic alterations in chronic pancreatitis: Evidence for early occurrence of p53 but not K-ras mutations. Br J Surg 1998;85:337–40.
9. Hsiang D, Friess H, Buchler MW, et al. Absence of K-ras mutations in the pancreatic parenchyma of patients with chronic pancreatitis. Am J Surg 1997;174:242–6.
10. Orth M, Gansauge F, Gansauge S, et al. K-ras mutations at codon 12 are rare events in chronic pancreatitis. Digestion 1998;59:120–4.
11. Salek C, Benesova L, Zavoral M, et al. Evaluation of clinical relevance of examining K-ras, p16 and p53 mutations along with allelic losses at 9p and 18q in EUS-guided fine needle aspiration samples of patients with chronic pancreatitis and pancreatic cancer. World J Gastroenterol 2007;13:3714–20.
12. Kamisawa T, Takuma K, Tabata T, et al. Long-term follow-up of chronic pancreatitis patients with K-ras mutation in the pancreatic juice. Hepatogastroenterology 2011;58:174–6.
13. Luttges J, Diederichs A, Menke MA, et al. Ductal lesions in patients with chronic pancreatitis show K-ras mutations in a frequency similar to that in the normal pancreas and lack nuclear immunoreactivity for p53. Cancer 2000;88:2495–504.
14. Maluf-Filho F, Kumar A, Gerhardt R, et al. Kras mutation analysis of fine needle aspirate under EUS guidance facilitates risk stratification of patients with pancreatic mass. J Clin Gastroenterol 2007;41:906–10.
15. Queneau PE, Adessi GL, Thibault P, et al. Early detection of pancreatic cancer in patients with chronic pancreatitis: Diagnostic utility of a K-ras point mutation in the pancreatic juice. Am J Gastroenterol 2001;96:700–4.
16. Costentin L, Pages P, Bouisson M, et al. Frequent deletions of tumor suppressor genes in pure pancreatic juice from patients with tumoral or nontumoral pancreatic diseases. Pancreatology 2002;2:17–25.
17. Furuya N, Kawa S, Akamatsu T, et al. Long-term follow-up of patients with chronic pancreatitis and K-ras gene mutation detected in pancreatic juice. Gastroenterology 1997;113:593–8.
18. Lohr M, Muller P, Mora J, et al. p53 and K-ras mutations in pancreatic juice samples from patients with chronic pancreatitis. Gastrointest Endosc 2001;53:734–43.
19. Maire F, Micard S, Hammel P, et al. Differential diagnosis between chronic pancreatitis and pancreatic cancer: Value of the detection of KRAS2 mutations in circulating DNA. Br J Cancer 2002;87:551–4.
20. Popovic Hadzija M, Korolija M, Jakic Razumovic J, et al. K-ras and Dpc4 mutations in chronic pancreatitis: Case series. Croat Med J 2007;48:218–24.
21. Rashid S, Singh N, Gupta S, et al. Progression of chronic pancreatitis to pancreatic cancer: Is there a role of gene mutations as a screening tool? Pancreas 2018;47:227–32.
22. Talar-Wojnarowska R, Gasiorowska A, Smolarz B, et al. Usefulness of p16 and K-ras mutation in pancreatic adenocarcinoma and chronic pancreatitis differential diagnosis. J Physiol Pharmacol 2004;55(Suppl 2): 129–38.
23. Talar-Wojnarowska R, Gasiorowska A, Smolarz B, et al. Clinical significance of K-ras and c-erbB-2 mutations in pancreatic adenocarcinoma and chronic pancreatitis. Int J Gastrointest Cancer 2005;35:33–41.
24. Casey G, Yamanaka Y, Friess H, et al. p53 mutations are common in pancreatic cancer and are absent in chronic pancreatitis. Cancer Lett 1993;69:151–60.
25. Rosty C, Geradts J, Sato N, et al. p16 Inactivation in pancreatic intraepithelial neoplasias (PanINs) arising in patients with chronic pancreatitis. Am J Surg Pathol 2003;27:1495–501.
26. Takano S, Fukasawa M, Maekawa S, et al. Deep sequencing of cancer-related genes revealed GNAS mutations to be associated with intraductal papillary mucinous neoplasms and its main pancreatic duct dilation. PLoS One 2014;9:e98718.
27. Talar-Wojnarowska R, Gasiorowska A, Smolarz B, et al. Comparative evaluation of p53 mutation in pancreatic adenocarcinoma and chronic pancreatitis. Hepatogastroenterology 2006;53:608–12.
28. Yu J, Sadakari Y, Shindo K, et al. Digital next-generation sequencing identifies low-abundance mutations in pancreatic juice samples collected from the duodenum of patients with pancreatic cancer and intraductal papillary mucinous neoplasms. Gut 2017;66:1677–87.
29. Esposito I, Hruban RH, Verbeke C, et al. Guidelines on the histopathology of chronic pancreatitis. Recommendations from the working group for the international consensus guidelines for chronic pancreatitis in collaboration with the International Association of Pancreatology, the American Pancreatic Association, the Japan Pancreas Society, and the European Pancreatic Club. Pancreatology 2020;20:586–93.
30. Rahman A, O'Connor DB, Gather F, et al. Clinical classification and severity scoring systems in chronic pancreatitis: A systematic review. Dig Surg 2020;37:181–91.
31. Beyer G, Habtezion A, Werner J, et al. Chronic pancreatitis. Lancet 2020; 396:499–512.
32. Kempeneers MA, Issa Y, Ali UA, et al. International consensus guidelines for surgery and the timing of intervention in chronic pancreatitis. Pancreatology 2020;20:149–57.
33. Wilcox CM, Yadav D, Ye T, et al. Chronic pancreatitis pain pattern and severity are independent of abdominal imaging findings. Clin Gastroenterol Hepatol 2015;13:552–60; quiz e28–9.
34. Ammann RW, Heitz PU, Kloppel G. Course of alcoholic chronic pancreatitis: A prospective clinicomorphological long-term study. Gastroenterology 1996;111:224–31.
35. Young MC, Theis JR, Hodges JS, et al. Preoperative computerized tomography and magnetic resonance imaging of the pancreas predicts pancreatic mass and functional outcomes after total pancreatectomy and islet autotransplant. Pancreas 2016;45:961–6.
36. Sarner M, Cotton PB. Classification of pancreatitis. Gut 1984;25:756–9.
37. Kuroki T, Adachi T, Ono S, et al. Pancreatic islet autotransplantation with total pancreatectomy for chronic pancreatitis. Surg Today 2013;43:715–9.
38. Barnard R, Futo V, Pecheniuk N, et al. PCR bias toward the wild-type k-ras and p53 sequences: Implications for PCR detection of mutations and cancer diagnosis. Biotechniques 1998;25:684–91.

39. Berger M, Bellin MD, Kirchner V, et al. Laparoscopic-assisted versus open total pancreatectomy and islet autotransplantation: A case-matched study of pediatric patients. J Pediatr Surg 2020;55:558–63.
40. Morinville VD, Lowe ME, Ahuja M, et al. Design and implementation of INSPPIRE. J Pediatr Gastroenterol Nutr 2014;59:360–4.
41. Furukawa T, Kuboki Y, Tanji E, et al. Whole-exome sequencing uncovers frequent GNAS mutations in intraductal papillary mucinous neoplasms of the pancreas. Sci Rep 2011;1:161.
42. Jones S, Zhang X, Parsons DW, et al. Core signaling pathways in human pancreatic cancers revealed by global genomic analyses. Science 2008;321: 1801–6.
43. Tan H, Bao J, Zhou X. Genome-wide mutational spectra analysis reveals significant cancer-specific heterogeneity. Sci Rep 2015;5:12566.
44. Wu J, Jiao Y, Dal Molin M, et al. Whole-exome sequencing of neoplastic cysts of the pancreas reveals recurrent mutations in components of ubiquitin-dependent pathways. Proc Natl Acad Sci USA 2011;108: 21188–93.
45. Yachida S, Jones S, Bozic I, et al. Distant metastasis occurs late during the genetic evolution of pancreatic cancer. Nature 2010;467:1114–7.
46. Martin M. Cutadapt removes adapter sequences from high-throughput sequencing reads. EMBnet.journal 2011;17:10–12.
47. Ewels P, Magnusson M, Lundin S, et al. MultiQC: Summarize analysis results for multiple tools and samples in a single report. Bioinformatics 2016;32:3047–8.
48. Li H. Aligning sequence reads, clone sequences and assembly contigs with BWA-MEM. ArXiv 2013. arXiv:1303.3997v2 [q-bio.GN].
49. Quinlan AR, Hall IM. BEDTools: A flexible suite of utilities for comparing genomic features. Bioinformatics 2010;26:841–2.
50. Garrison E, Marth G. Haplotype-based variant detection from short-read sequencing. ArXiv 2012. arXiv:1207.3907 [q-bio.GN].
51. Koboldt DC, Chen K, Wylie T, et al. VarScan: Variant detection in massively parallel sequencing of individual and pooled samples. Bioinformatics 2009;25:2283–5.
52. Ma X, Shao Y, Tian L, et al. Analysis of error profiles in deep next-generation sequencing data. Genome Biol 2019;20:50.
53. Schirmer M, Ijaz UZ, D'Amore R, et al. Insight into biases and sequencing errors for amplicon sequencing with the Illumina MiSeq platform. Nucleic Acids Res 2015;43:e37.
54. Shin HT, Choi YL, Yun JW, et al. Prevalence and detection of low-allele-fraction variants in clinical cancer samples. Nat Commun 2017;8:1377.
55. Salk JJ, Schmitt MW, Loeb LA. Enhancing the accuracy of next-generation sequencing for detecting rare and subclonal mutations. Nat Rev Genet 2018;19:269–85.
56. Jennings LJ, Arcila ME, Corless C, et al. Guidelines for validation of next-generation sequencing-based oncology panels: A joint consensus recommendation of the association for molecular pathology and college of American pathologists. J Mol Diagn 2017;19:341–65.
57. Fox EJ, Reid-Bayliss KS, Emond MJ, et al. Accuracy of next generation sequencing platforms. Next Gener Seq Appl 2014;1:1000106.
58. Glenn TC. Field guide to next-generation DNA sequencers. Mol Ecol Resour 2011;11:759–69.
59. Petrackova A, Vasinek M, Sedlarikova L, et al. Standardization of sequencing coverage depth in NGS: Recommendation for detection of clonal and subclonal mutations in cancer diagnostics. Front Oncol 2019;9:851.
60. Filges S, Yamada E, Stahlberg A, et al. Impact of polymerase fidelity on background error rates in next-generation sequencing with unique molecular identifiers/barcodes. Sci Rep 2019;9:3503.
61. Tewhey R, Warner JB, Nakano M, et al. Microdroplet-based PCR enrichment for large-scale targeted sequencing. Nat Biotechnol 2009;27: 1025–31.
62. Nikiforova MN, LaFramboise WA, Nikiforov YE. Amplification-based methods. In: Kulkarni S, Pfeifer J (eds). Clinical Genomics. Academic Press, 2015, pp 57–67.
63. Song C, Liu Y, Fontana R, et al. Elimination of unaltered DNA in mixed clinical samples via nuclease-assisted minor-allele enrichment. Nucleic Acids Res 2016;44:e146.
64. Howes N, Lerch MM, Greenhalf W, et al. Clinical and genetic characteristics of hereditary pancreatitis in Europe. Clin Gastroenterol Hepatol 2004;2:252–61.
65. Rebours V, Boutron-Ruault MC, Schnee M, et al. The natural history of hereditary pancreatitis: A national series. Gut 2009;58:97–103.
66. Agarwal S, Sharma S, Gunjan D, et al. Natural course of chronic pancreatitis and predictors of its progression. Pancreatology 2020;20: 347–55.
67. Bhasin DK, Singh G, Rana SS, et al. Clinical profile of idiopathic chronic pancreatitis in North India. Clin Gastroenterol Hepatol 2009;7:594–9.
68. Liu Y, Wang D, Cai YL, et al. Classification of early-onset and late-onset idiopathic chronic pancreatitis needs reconsideration. Sci Rep 2020;10:10448.
69. Smigielski EM, Sirotkin K, Ward M, et al. dbSNP: A database of single nucleotide polymorphisms. Nucleic Acids Res 2000;28:352–5.
70. Bamford S, Dawson E, Forbes S, et al. The COSMIC (Catalogue of Somatic Mutations in Cancer) database and website. Br J Cancer 2004;91:355–8.
71. Sandmann S, de Graaf AO, Karimi M, et al. Evaluating variant calling tools for non-matched next-generation sequencing data. Sci Rep 2017;7: 43169.
72. Hruban RH, Goggins M, Parsons J, et al. Progression model for pancreatic cancer. Clin Cancer Res 2000;6:2969–72.
73. Taly V, Pekin D, Benhaim L, et al. Multiplex picodroplet digital PCR to detect KRAS mutations in circulating DNA from the plasma of colorectal cancer patients. Clin Chem 2013;59:1722–31.
74. South AP, Purdie KJ, Watt SA, et al. NOTCH1 mutations occur early during cutaneous squamous cell carcinogenesis. J Invest Dermatol 2014; 134:2630–8.
75. Kovaleva V, Geissler AL, Lutz L, et al. Spatio-temporal mutation profiles of case-matched colorectal carcinomas and their metastases reveal unique de novo mutations in metachronous lung metastases by targeted next generation sequencing. Mol Cancer 2016;15:63.
76. Ling C, Wang L, Wang Z, et al. A pathway-centric survey of somatic mutations in Chinese patients with colorectal carcinomas. PLoS One 2015;10:e0116753.
77. Shi C, Fukushima N, Abe T, et al. Sensitive and quantitative detection of KRAS2 gene mutations in pancreatic duct juice differentiates patients with pancreatic cancer from chronic pancreatitis, potential for early detection. Cancer Biol Ther 2008;7:353–60.
78. Le Calvez-Kelm F, Foll M, Wozniak MB, et al. KRAS mutations in blood circulating cell-free DNA: A pancreatic cancer case-control. Oncotarget 2016;7:78827–40.
79. Almoguera C, Shibata D, Forrester K, et al. Most human carcinomas of the exocrine pancreas contain mutant c-K-ras genes. Cell 1988;53:549–54.
80. Lynch SM, Vrieling A, Lubin JH, et al. Cigarette smoking and pancreatic cancer: A pooled analysis from the pancreatic cancer cohort consortium. Am J Epidemiol 2009;170:403–13.