Variant Profiling of Candidate Genes in Pancreatic Ductal Adenocarcinoma

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BACKGROUND: Pancreatic ductal adenocarcinoma (PDAC) has a poor prognosis. Variant profiling is crucial for developing personalized treatment and elucidating the etiology of this disease.

METHODS: Patients with PDAC undergoing surgery from 2007 to 2012 (n = 73) were followed from diagnosis until death or the end of the study. We applied an anchored multiplex PCR (AMP)-based next-generation sequencing (NGS) method to a panel of 65 selected genes and assessed analytical performance by sequencing a quantitative multiplex DNA reference standard. In clinical PDAC samples, detection of low-level KRAS (Kirsten rat sarcoma viral oncogene homolog) mutations was validated by allele-specific PCR and digital PCR. We compared overall survival of patients according to KRAS mutation status by log-rank test and applied logistic regression to evaluate the association between smoking and tumor variant types.

RESULTS: The AMP-based NGS method could detect variants with allele frequencies as low as 1% given sufficient sequencing depth (>1500×). Low-frequency KRAS G12 mutations (allele frequency 1%–5%) were all confirmed by allele-specific PCR and digital PCR. The most prevalent genetic alterations were in KRAS (78% of patients), TP53 (tumor protein p53) (25%), and SMAD4 (SMAD family member 4) (8%). Overall survival in T3-stage PDAC patients differed among KRAS mutation subtypes (P = 0.019). Transversion variants were more common in ever-smokers than in never-smokers (odds ratio 5.7; 95% CI 1.2–27.8).

CONCLUSIONS: The AMP-based NGS method is applicable for profiling tumor variants. Using this approach, we demonstrated that in PDAC patients, KRAS mutant subtype G12V is associated with poorer survival, and that transversion variants are more common among smokers.

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Pancreatic ductal adenocarcinoma (PDAC)9 is the fourth most common cause of cancer-related death for both men and women in Europe (1). Accumulated genetic alterations play important roles in the tumorigenesis of PDAC, for example in the progression from a normal cell to precursor lesions and eventually to invasive and metastasizing carcinoma.

Since the 1970s, conventional sequencing platforms (e.g., Sanger sequencing) have revealed several somatic mutations in PDAC, including alterations in KRAS (Kirsten rat sarcoma viral oncogene homolog)10 and the tumor-suppressor genes TP53 (tumor protein p53), CDKN2A (cyclin-dependent kinase inhibitor 2A), and SMAD4 (SMAD family member 4). Mutations of these genes are considered to be drivers of genomic instability and tumor development (2–5). Recent advances in next-generation sequencing (NGS) have enabled detailed genetic characterization of cancers. In 2008, the Global Pancreatic Genomic study (6) described the genetic landscape of PDAC, including a range of molecular aberrations and mutations in 63 genes involved in 12 core signaling pathways. Four years later, Biankin et al. (7) reported 16 genes, including both known and novel mutations, on the basis of exome sequencing of 99 early-stage PDACs.
Materials and Methods

PATIENT POPULATION AND DESIGN
This study used materials collected in a population-based case-control study of pancreatic cancer performed in Stockholm, Sweden. Patients undergoing surgical resection from July 2007 to April 2012 were recruited. Fresh-frozen pancreatic cancer tissues from surgical resection samples, as well as matched peripheral blood samples, were collected at Karolinska University Hospital. Tumor cellularity was assessed by hematoxylin & eosin staining at the Department of Pathology, Karolinska University Hospital.

The study was approved by the Regional Ethical Review Board in Stockholm, Sweden. All participants provided written informed consent before sample collection.

Using the personal identity number allocated to all residents of Sweden, we collected data on tumor characteristics and survival of all participating patients through the electronic patient record system at Karolinska University Hospital, which contains information on tumor stage, chemotherapy, date of surgery, and date of death. In addition, in-person interviews with 57 of 73 PDAC patients were conducted by professional interviewers. The questionnaire was designed to collect detailed information about lifestyle and environmental factors.

CUSTOM TARGET DESIGN
A panel of 65 clinically relevant cancer genes (92,246 target bases in total), including a 39-gene subpanel of genes recurrently mutated in solid tumors obtained from the Catalogue of Somatic Mutations in Cancer (COSMIC) database (55,488 target bases) and an exploratory subpanel of 26 genes reported by the Global Pancreatic Genomic study (36,758 target bases), was selected for targeted sequencing of the full coding regions of tumor-suppressor genes and selected exons of oncogenes (see Supplemental Table 1, which accompanies the online version of this article at http://www.clinchem.org/content/vol61/issue11).

DNA EXTRACTION AND CONSTRUCTION OF SEQUENCING LIBRARIES
We extracted genomic DNA with the QIAamp DNA Mini Kit (Qiagen) and prepared DNA libraries with the AMP-based method (9). Briefly, 200 ng genomic DNA was sheared to 500-bp mean size on a Covaris S220 instrument with the following settings: 5% duty cycle, intensity 3, 200 cycles per burst, and 2 cycles of 100 s each in frequency-sweeping mode. Sheared fragments were followed by end repair (End-Repair Mix, Enzymatics), adenylation (Klenow Exo-, Enzymatics; Taq Polymerase, Life Technologies), and ligation (T4 DNA Ligase, Enzymatics) of a modified Illumina MiSeq adapter that incorporated a molecular index for tracking of template DNA fragments. Solid-phase reversible immobilization–cleaved ligated products underwent 2 rounds of nested PCR for target enrichment (Platinum Taq Polymerase, Life Technologies), yielding sequencing-ready libraries. These libraries were quantitated by quantitative PCR (Kapa Biosystems), pooled at equimolar amounts, and sequenced on a MiSeq (Illumina) with MiSeq Reagent Kit v2 (300 cycles). For each run, 32 samples were pooled and sequenced.

BIOINFORMATICS ANALYSIS
We initially processed sequence data by trimming adapter sequences from the 3’ end for both paired-end reads with a custom script. Processed reads were aligned to the human hg19 genome assembly with the Burrows-Wheeler Aligner MEM software (10). Single nucleotide variations (SNVs) and insertions and deletions (indels) were called with a custom script for SAMtools-based biconsortium variant calling. We used ANNOVAR for variant annotation (11). We identified somatic SNVs in tumor samples after filtering out control-sample SNVs and calculated copy number variation (target gain/loss) with a read-depth approach. Briefly, raw target read depth was extracted with Bedtools (12) and then normalized by individual sample sequencing yield and target/primer median read depth across all samples. Statistical significance of target gain and loss was tested on the basis of log-normal distribution of target read depth.

VALIDATION OF AMP WITH A QUANTITATIVE MULTIPLEX DNA REFERENCE STANDARD
To evaluate the analytical performance of the AMP-based NGS method, we sequenced a quantitative multiplex DNA reference standard (Horizon Diagnostics, DNA HD701) diluted with peripheral blood leukocyte DNA at 3 different ratios, each with 3 replicates. The reference DNA sample contained 6 genes and 11 mutations [BRAF (B-Raf proto-oncogene, serine/threonine kinase),
**SNV VALIDATION BY SANGER SEQUENCING**

We used Sanger sequencing to validate mutations in KRAS codons 12 and 13 that were initially detected with the AMP-based assay (primer sequences are listed in online Supplemental Table 2). Purified PCR products were subsequently sequenced on an ABI 3730XL (PE Applied Biosystems). Data were analyzed with Chromas Version 2.4.

**SNV VALIDATION BY ALLELE-SPECIFIC PCR**

We performed allele-specific PCR to validate mutations in KRAS codon 12 with allelic fractions too low (<5%) to be detected by the Sanger platform. In addition, as positive controls, a few samples with higher mutant allelic fractions were randomly selected for allele-specific PCR. The mutant-specific primers were designed as previously described (14) (see online Supplemental Table 3). In brief, the 3' terminal base of the allele-specific primer (G12D, G12R, or G12V) was identical to its corresponding mutation. To remove unincorporated primers, Exonuclease I/SAP (New England BioLabs) was applied to the PCR products and subsequently incubated at 37 °C for 30 min and then at 80 °C for 15 min. Finally, 1% agarose gel electrophoresis was performed to determine the presence or absence of the target amplicons.

**SNV CONFIRMATION BY DIGITAL PCR**

Digital PCR was performed on samples with low-level KRAS mutations at codon 12 (<5%). KRAS G12D, G12V, and G12R mutations were assayed separately with TaqMan SNP Genotyping Assays optimized for digital PCR on a QuantStudio 3D Digital PCR System (Life Technologies).

**STATISTICAL ANALYSES**

Survival analysis was performed on PDAC patients having stage T3 tumors without evidence of distant metastasis. Patients were followed from the date of surgery until their deaths or the end of the study (January 1, 2014), whichever occurred first. The Kaplan–Meier method was used to create overall survival curves stratified by KRAS mutation subtype or mutation status (yes/no) of TP53 and genes involved in the transforming growth factor (TGF)-β signaling pathway [SMAD4 or TGFBR2 (transforming growth factor, β receptor II, 70/80 kDa)]. We used log-rank test to compare survival curves and applied logistic regression models to investigate the associations between smoking and overall genetic transversion or transition rates, adjusted for sex and age at surgery. All statistical analyses were performed with SAS software (version 9.3, SAS Institute).

**Results**

Table 1 lists the clinical characteristics of the PDAC patients recruited for the study; 73 samples and 55 matched peripheral blood samples were included. Seventy patients were untreated before pancreatectomy, whereas 3 patients had been treated with neoadjuvant chemotherapy with the intent of tumor downstaging.

The mean age at surgery was 68.2 years, and 53.4% of the participants were men. The predominant tumor stage was T3 (80.8%). Of the 57 patients with available smoking data, 75.4% had ever smoked.

**VALIDATION OF THE AMP METHOD BY SEQUENCING A QUANTITATIVE MULTIPLEX DNA REFERENCE STANDARD**

To examine the analytical performance of the AMP-based assay, with the 39-gene subpanel, we sequenced 9 spike-in samples (3 dilution ratios, performed in triplicate) of the reference DNA (see online Supplemental Table 4) in 1 MiSeq run. With 1 exception, >90% of target regions in all samples had a minimum of 250× depth of coverage (see online Supplemental Fig. 1). In the 1 sample with worse performance, only 75% of target regions had a minimum of 250× depth of coverage. This reduced depth of coverage in multiple target regions was likely due to pipetting errors during processing of this sample. The early drop in coverage curves was mainly due to the presence of difficult targets such as the TERT (telomerase reverse transcriptase) promoter and FOXL2 (forkhead box L2), which have high GC content (data not shown).

All 31 variants with expected allele frequencies, ranging from 6.30% to 18.38%, were successfully detected by the AMP-based assay. For the 49 variants with allele frequencies ranging from 1% to 5%, the AMP-based assay detected 44 and rejected 5 (1 variant with 2.63% allele frequency and 336× depth; 4 variants with ≤158× depth). About half (9 of 19) of the very-low-allele-frequency (0.16% to 0.96%) variants were detected. Notably, the AMP-based assay detected a 15-bp deletion of EGFR exon 19 in all 9 samples, with low allele frequencies of 0.3% to 0.5%. Allele frequencies derived from the AMP-based assay were highly correlated with the expected frequencies across the range (see online...
Supplemental Fig. 2). Sequencing depth greater than approximately 1000× was needed to detect variants at allele frequency of 2.5%, and approximately 1500× depth was needed to detect variants with a frequency of 1% (see online Supplemental Fig. 3).

FREQUENCY AND DISTRIBUTION OF GENETIC ABERRATIONS
Two subpanels of genes, the 39-gene solid-tumor subpanel (see online Supplemental Fig. 4A) and the 26-gene PDAC-specific exploratory subpanel (see online Supplemental Fig. 4B), were sequenced with the AMP-based assay in 73 PDAC samples and 55 matched blood samples. All samples except 1 were sequenced, with ≥80% of the target bases in the solid-tumor subpanel >100× depth (72 samples had mean sequencing coverages ranging from 341× to 1314×) (see online Supplemental Fig. 4A). In the same 72 samples, >80% of the target bases in the PDAC exploratory subpanel had >30× depth (mean coverages of 274× to 1011×) (see online Supplemental Fig. 4B).

We identified 95 nonsynonymous variants, 7 synonymous variants, and 5 indels in the 73 PDACs (Table 1). Variants in 13 genes were identified in ≥1 patient (Fig. 1). In 84% of PDACs (61 of 73), ≥1 variant was detected in the targeted regions.

The prevalence of KRAS mutations in PDACs was 78% (57 of 73); 33% (24 of 73) of the samples had low tumor cellularity (<15%); and 8% (6 of 73) had very low tumor cellularity (≤5%). A list of KRAS mutations is shown in online Supplemental Table 1. The most prevalent KRAS mutations were GGT>GAT (G12D), GGT>CGT (G12R), and GGT>GGT (G12V). Four samples without KRAS mutations harbored mutations in TP53, SMAD4, TGFB2, EGFR, or CDH1 (cadherin 1, type 1) (Fig. 1). Mutations in KRAS codons 12 and 13, which were detected by the AMP-based assay and were either present at high allelic frequency (>5%) or negative in 59 of 73 samples, were validated and confirmed by Sanger sequencing (Fig. 2, online Supplemental Table 5). The remaining 14 cases had low-frequency KRAS mutations (<5%) as determined by the AMP-based assay, which is below the detection limit for conventional Sanger sequencing. These cases were confirmed by allele-specific PCR (see online Supplemental Fig. 5) and digital PCR (see online Supplemental Table 5 and Supplemental Figs. 6–8). The validation results showed that the AMP-based assay was completely consistent with Sanger sequencing for high-allelic-frequency variants and was consistent with allele-specific PCR and digital PCR assays for low-frequency (1%–5%) variants.

TP53 mutations, the second most common alterations in PDACs, were observed in 25% (18 of 73) of samples; 6 of 18 coexisted with KRAS G12D, and 7 of 18 coexisted with G12R. SMAD4 mutations were observed in 8.2% (6 of 73) of PDACs, and 4 of the 6 coexisted with KRAS G12R. GNAS (GNAS complex locus) mutations were observed in 4.1% of PDACs (3 of 73), and all 3 were concurrent with KRAS G12D.

Less frequently affected genes and the clinicopathologic features of the corresponding patients are shown in Fig. 1 (see online Supplemental Table 6 for comparisons). Seven samples had variant allele frequencies of <2% in CDH1, EGF, GNAS, PTEN (phosphatase and tensin homolog), and TP53, and 1 sample contained 2 low-frequency variants (CDH1 and TP53); the allele frequencies of these variants ranged from 0.21% to 1.71%.
These variants met the following criteria: (a) they affected both strands of the original DNA template and (b) the total number of mutant reads (forward plus reverse strands) was ≤4. Genes without detectable variants are listed in online Supplemental Table 1.

Among the 3 patients treated with neoadjuvant chemotherapy, 1 harbored an *EGFR* mutation; 1 had mutations in multiple genes (*KRAS*, *TP53*, *DDR2* (discoidin domain receptor tyrosine kinase 2), and *EGFR*), and 1 had no detectable mutations.

**SURVIVAL ANALYSIS ACCORDING TO MUTATION STATUS**

Overall, the median survival time of patients with wild-type *KRAS* was 26.2 months, longer than the 15.7 months for patients with any *KRAS* G12 mutation (*P* = 0.067) (Fig. 3A). The corresponding values for each *KRAS* mutant subtype were 11.9 months for G12V, 16.5 months for G12R, and 19.6 months for G12D (*P* = 0.0197) (Fig. 3B).

Median survival times were 14.1 and 20.8 months for patients with and without mutations in *SMAD4* or *TGFBR2*, respectively, but the difference was not statistically significant (*P* = 0.138) (Fig. 3C). Moreover, survival did not differ significantly between patients with and without *TP53* mutations (*P* = 0.391) (Fig. 3D).

**ASSOCIATION BETWEEN SMOKING AND GENETIC ALTERATIONS**

Smoking was associated with a higher frequency of transversion variants (odds ratio 5.7, 95% CI 1.2–27.8), but not with transition variants (odds ratio 0.7, 95% CI 0.2–2.9) (Table 2).

**Discussion**

Our results show that, given sufficient sequencing depth, the AMP-based NGS method can achieve analytically sensitive variant profiling in PDAC. Using this method, we found that *KRAS* mutant subtype G12V is associated with a poorer prognosis in PDAC patients. Our results confirm the predominance of *KRAS* mutations in PDAC, in agreement with previous studies. In addition, our data suggest that smoking is related to transversion variants in PDAC.
A number of target enrichment and deep sequencing methods and variant-calling algorithms have been developed (15). However, background noise generally limits the analytical sensitivities of these methods. In this study, we used the AMP method for targeted NGS sequencing and sequenced selected genes to a high depth of coverage. A performance test with a DNA reference standard demonstrated that the assay could detect variants with allele frequencies as low as 1%, given sufficient sequencing depth. A more comprehensive study including more samples, various sample types, and more variant types is needed to fully evaluate the clinical and analytical performance of the AMP-based assay. In the clinical PDAC samples, the detected KRAS mutations were completely confirmed by Sanger sequencing for high allele frequencies and by allele-specific PCR and digital PCR for low allele frequencies down to 1%. This validation demonstrates the reliability of the AMP-based assay for detection of a broad range of variants. A previous study of KRAS mutations that used fine needle aspiration material from various pancreatic lesions showed that the NGS-based method had higher sensitivity than Sanger sequencing and allele-specific locked nucleic acid PCR (16). The ability of NGS-based assays to use small amounts of input material, e.g., from fine needle aspiration and cyst fluid, while simultaneously testing a panel of important genes, makes this method potentially useful for preoperative clinical management of pancreatic lesions.

Some targeted regions had poor depth of coverage; these included the TERT promoter and FOXL2, which have very high GC content (data not shown). Because the TERT promoter is recurrently mutated in cancers of the central nervous system, bladder, thyroid, and skin, but not the pancreas (17), and FOXL2 genetic variants are not common in PDAC, we did not attempt to optimize these regions in this study. Possible optimizations aimed at improving coverage of GC-rich regions in future studies may involve addition of PCR additives such as DMSO and betaine.
In previous reports from European studies, \textit{KRAS} mutations in PDAC samples ranged from 72% to 83\% (18–22). Consistent with those results, we observed a \textit{KRAS} mutation frequency of 78\%, which could be an underestimate due to inadequate tissue sampling. Of the 73 PDACs diagnosed by original histopathologic examination, 3 frozen biopsy samples used for DNA extraction were found upon histopathologic reexamination to contain no tumor cells (see online Supplementary Table 7). In 12 PDAC samples (16\%), we did not detect a tumor-specific variant in any of the 65 analyzed genes.

![Fig. 3. Overall survival analyses by the Kaplan-Meier method for PDAC patients restricted to T3 stage. (A), Overall survival by \textit{KRAS} mutation status. (B), Overall survival by \textit{KRAS} mutation subtype. (C), Overall survival by mutation status in TGF-\(\beta\) signaling pathway genes (\textit{SMAD4} or \textit{TGFBR2}). (D), Overall survival by \textit{TP53} mutation status.](image)

<table>
<thead>
<tr>
<th>Smoking status</th>
<th>G:C&gt;T:A</th>
<th>G:C&gt;C:G</th>
<th>All transversions</th>
<th>All transitions$^b$</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>No</td>
<td>Yes</td>
<td>OR (95% CI)</td>
<td>No</td>
</tr>
<tr>
<td>Never</td>
<td>10</td>
<td>3</td>
<td>1.0</td>
<td>12</td>
</tr>
<tr>
<td>Ever</td>
<td>32</td>
<td>11</td>
<td>2.3 (0.4–11.6)</td>
<td>32</td>
</tr>
</tbody>
</table>

$^a$ Logistic regression model adjusted for age and sex. One case was excluded for lack of tumor stage information; 16 cases were excluded for lack of smoking information.

$^b$ At 50\% cutoff, <3 total transitions at low frequency; \(\geq\)3 total transitions at high frequency.
We found that the *KRAS* mutant G12V subtype was associated with worse prognosis, and this association was still significant after separate adjustments for smoking, chemotherapy treatment, or *TP53* mutation status (data not shown). The reported effects of *KRAS* mutations on overall survival of PDAC vary substantially between studies, possibly because of variations in study design, sample size, source population, and tumor stages. In a Norwegian study (23) of 36 patients with resectable PDAC, individuals with the G12V mutation had a longer median survival time (23.5 months) than those with G12D (9.5 months) or G12R (7.5 months). In another European study of 66 patients with resectable PDAC, median survival was longer among patients with the G12V mutation (12.5 months) than in patients with G12D (8.7 months) or G12R (6.7 months) (19). A recent Japanese study of 242 patients with resectable pancreatic cancer reported a poorer prognosis for carriers of the G12D or G12R mutation relative to those with the G12V mutation (24). In a German study of 135 patients with resectable PDAC, the G12D and G12V mutations were associated with poorer prognosis than the G12R subtype (18). However, our results are consistent with those of earlier studies on other cancer types, including colorectal and lung cancers, which reported that the *KRAS* mutant G12V subtype is associated with more aggressive biological behavior and shorter survival (25–27). In addition, in vitro studies showed that the basal GTPase activity of G12V is one-quarter that of G12D and one-tenth that of wild-type *KRAS*, keeping the mutant *KRAS* protein in the GTP-bound active state for a longer period of time (28, 29). Furthermore, cells with the *KRAS* G12V mutation were shown in vitro to be significantly more invasive than those with G12D or wild-type *KRAS* (30). TGF-β signaling is another critical pathway in PDAC development (31, 32). Our results showed that patients with mutations in *SMAD4* or *TGFBR2* have poorer prognosis than patients lacking these mutations, but the difference was not statistically significant.

Smoking is a well-established risk factor for pancreatic cancer and is associated with genetic alterations (21, 33, 34). A familial pancreatic cancer study showed that smokers develop pancreatic cancer a decade earlier than nonsmokers (35). Furthermore, smoking and genetic variants are more important risk factors for early-onset pancreatic cancer than for pancreatic cancer later in life (36). Pancreatic cancers from smokers harbor more *KRAS* and *TP53* mutations than those from nonsmokers (21, 37). However, some studies do not support these findings (34, 38). One study reported that transition variants were 24% more common in smokers than in nonsmokers, and transversion variants were 53% more frequent in smokers (33). In lung cancer, G-to-T transversion in *TP53* is a signature of DNA damage by tobacco smoke (39). In colon cancer, smoking was associated with transversion variants (40) and poorer prognosis, and this effect was most prominent in patients whose tumors contained wild-type *BRAF* or mutated *KRAS* (41). This study was underpowered for evaluation of the association between smoking and genetic changes, as well as the influence of genetic changes on PDAC prognosis. Investigation of the interactions between smoking and a comprehensive set of candidate genes on PDAC prognosis awaits larger cohort studies.

In conclusion, the AMP-based NGS method can be used for analytically sensitive PDAC variant profiling, even for low-tumor-cellularity samples. On the basis of this method, we demonstrated that the presence of *KRAS* mutant subtype G12V is an indicator of worse prognosis, and that smoking is associated with transversion variants in PDAC. Future studies on the basis of larger cohorts are warranted to confirm these findings, to examine the influence of genetic alterations on PDAC prognosis, and to evaluate the associations of genetic alterations with environment and lifestyle factors.

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