Reliable Detection of Somatic Mutations in Fine Needle Aspirates of Pancreatic Cancer With Next-generation Sequencing

Implications for Surgical Management

Vicente Valero III, MD,* Tyler J. Saunders, BA,† Jin He, MD,* Matthew J. Weiss, MD,‡ John L. Cameron, MD,* Avani Dholakia, MD,§ Aaron T. Wild, MD,§ Eun Ji Shin, MD,¶ Mouna A. Khshab, MD,¶ Anne Marie O’Broin-Lennon, MD,¶ Syed Z. Ali, MD,† Daniel Laheru, MD,¶ Ralph H. Hruban, MD,‡ Christine A. Iacobuzio-Donahue, MD, PhD,*† Joseph M. Herman, MD,§ and Christopher L. Wolfgang, MD, PhD‡†

Objective: To determine the feasibility of genotyping pancreatic tumors via fine needle aspirates (FNAs).

Background: FNA is a common method of diagnosis for pancreatic cancer, yet it has traditionally been considered inadequate for molecular studies due to the limited quantity of DNA derived from FNA specimens and tumor heterogeneity.

Methods: In vitro mixing studies were performed to deduce the minimum cellularity needed for genetic analysis. DNA from both simulated FNAs and clinical FNAs was sequenced. Mutational concordance was determined between simulated FNAs and that of the resected specimen.

Results: Limiting dilution studies indicated that mutations at allele frequencies as low as 0.12% are detectable. Comparison of simulated FNAs and matched tumor tissue exhibited a concordance frequency of 100% for all driver genes present. In FNAs obtained from 17 patients with unresectable disease, we identified at least 1 driver gene mutation in all patients including actionable somatic mutations in ATM and MTOR. The constellation of mutations identified in these patients was different than that reported for resectable pancreatic cancers, implying a biologic basis for presentation with locally advanced pancreatic cancer.

Conclusions: FNA sequencing is feasible and subsets of patients may harbor actionable mutations that could potentially impact therapy. Moreover, preoperative FNA sequencing has the potential to influence the timing of surgery relative to systemic therapy. FNA sequencing opens the door to clinical trials in which patients undergo neoadjuvant or a surgery-first approach based on their tumor genetics with the goal of utilizing cancer genomics in the clinical management of pancreatic cancer.

Keywords: cancer genetics, fine needle aspirates, genomics, next-generation sequencing, pancreatic cancer

(Pancreatic cancer (pancreatic ductal adenocarcinoma [PDAC]) is a highly lethal disease with an overall 5-year survival of only 6.7%. The only chance for long-term survival is through a potentially curative operation. However, even among patients with clinically localized disease who undergo resection, survival remains poor with the majority experiencing both local and systemic failure. Adjuvant and neoadjuvant treatment paradigms have been employed to reduce treatment failures—but these therapies provide only a modest benefit to survival. Currently, management of PDAC is not based on individual patient or tumor characteristics. Moreover, it is recognized that a spectrum of biological behavior exists for PDAC depending on the genetic alterations that drive tumor growth and dissemination. Improvements in outcome after resection of PDAC may occur through adaptation of personalized treatment principles based on unique behavior of an individual’s tumor. This approach will require reliable methodology to determine the genetic mutational status of a tumor before operation and acquisition of a surgical specimen. Experimentation with methods to accomplish this task have only recently been explored before this study.

Recent developments in the understanding of the genetics of PDAC have led to the potential for a personalized approach in operative patients. For example, the timing of surgery in relation to systemic therapy (ie, neoadjuvant versus adjuvant) may be impacted by the propensity for systemic spread. In this regard, it is known that an increased risk of systemic failure is correlated with the SMAD4 gene mutational status. In addition, with the advent of targetable therapies for cancer, knowledge of a tumor’s genetic signature will allow for therapy based on chemosensitivity. Although these advances are in the early stages of development, they have the potential to dramatically impact survival for future patients.

A major limitation in implementing treatment strategies based on the mutational status in the preoperative setting is the availability of tumor for detailed sequence analysis. A fine needle aspirate (FNA) of
This work will dramatically facilitate the development of personalized medicine in the surgical care of PDAC.

**METHODS**

**Cell Lines**

The PDAC cell line Panc1 was purchased from the American Type Culture Collection (Manassas, VA). The stromal fibroblast cell line cancer associated fibroblasts 35 (CAF35) was established from surgically resected pancreas tissue from a patient with sporadic PDAC as previously described. Cells were grown at 37 °C in a humidified atmosphere containing 5% CO₂ and cultured in Dulbecco’s modified Eagle medium supplemented with 10% fetal bovine serum (Gibco, Thermo Fisher Scientific, Waltham, MA) and 2% penicillin-streptomycin (Invitrogen, Thermo Fisher Scientific). For creation of Panc1-CAF35 cell line mixtures, cells were first counted using the Countess Automated Cell Counter (Life Technologies, Thermo Fisher Scientific) according to the manufacturer instructions.

**Human Tissues**

Tumor specimens were obtained from resection specimens of newly diagnosed and untreated patients, who underwent pancreaticoduodenectomy for PDAC at The Johns Hopkins Hospital. After a frozen section was performed to confirm the presence of adenocarcinoma, a pancreatic FNA was simulated by inserting a 19-gauge needle attached to a 10-mL syringe into the tumor specimen in the histologically confirmed region of cancer. Up to 3 passes were obtained per carcinoma, and phosphate-buffered saline was used to flush cells from the FNA needle into 2-mL cryogenic tubes followed by immediate storage at −80 °C until use. Matched samples of the cancer and of normal duodenal tissue were also obtained for each resection specimen. All patients had consented before surgery for sampling of resected tumor tissues in accordance with protocols approved by the Johns Hopkins University institutional review board.

**FNA Samples**

Pancreatic FNAs were obtained from treatment naïve patients with newly diagnosed unresectable PDAC. The samples were part of clinical trial J1003 previously approved by the Johns Hopkins University internal review board. The trial included gemcitabine, followed by stereotactic body radiation therapy, and followed by additional gemcitabine until progression or toxicity. FNA samples for sequencing were obtained by endoscopic ultrasound and standard techniques by a certified gastroenterologist at the time of a second-look placement for stereotactic body radiation therapy. In selected cases, a cytotechnologist performed on-site specimen adequacy. The FNA samples were blown through the FNA needle into 1.5-mL cryogenic vials using air. The aspirates were then immediately flash-frozen in the endoscopy suite using liquid nitrogen. Blood was also obtained and processed for each patient within 15 to 30 minutes of being drawn. Processing entailed spinning for 10 minutes at 3000 RPM followed by extracting the plasma and buffy coat cells in 1.25-mL aliquots into 1.5-mL cryogenic vials. All FNA-blood pairs were stored at −80 °C until sequencing.

**DNA Extraction and Quantification**

Genomic DNA from cell line and human tissues was extracted using phenol-chloroform, or QIAmp DNA Micro Kits (Qiagen) and quantified by calculating long interspersed nuclear elements by real-time polymerase chain reaction (PCR) as previously described. To minimize sequencing bias from using low-copy starting templates, only samples with DNA concentrations of more than or equal to 3.3 ng/μL (1000 genome equivalents) were used as a starting template for library construction.

**DNA Library Construction, Quantification, and Sequencing**

Libraries were prepared using the protocols for the Personal Genome Machine (PGM) provided by Ion Torrent (Life Technologies, Thermo Fisher Scientific). Briefly, 10 ng of genomic DNA was used as the starting template for library construction. For limiting dilution studies, the Ion AmpliSeq Cancer Panel Primer Pool (Life Technologies) designed to amplify 190 amplicons covering 739 COSMIC mutations in 46 genes was used. For the simulated FNA and J1003 clinical trial FNA samples, the Ion AmpliSeq Comprehensive Cancer Panel (Life Technologies, Thermo Fisher Scientific) consisting of 15,992 amplicons covering all exons of 409 cancer-related genes was used. The PCR amplification conditions were 99 °C for 2 minutes; 14 cycles of 99 °C for 15 seconds, 60 °C for 8 minutes; and 10 °C hold. Amplicons generated were then ligated with barcodes and P1 adaptors using Ion Xpress Barcode Adaptors (Life Technologies). Target amplicons were purified from the genomic DNA using Agencourt AMPure XP Reagent (Beckman-Coulter, Brea, CA). Libraries were quantified using Agilent High Sensitivity DNA Kit on the 2100 Bioanalyzer (Agilent, Santa Clara, CA) or using real-time PCR via the Taqman based Ion Library Quantitation Kit (Life Technologies, Thermo Fisher Scientific). Library concentrations of 14–20 pM were then used for emulsion PCR. DNA was bound to Ion Sphere Particles for clonal expansion of template DNA via emulsion PCR using the Ion PGM One Touch 2 system (Life Technologies, Thermo Fisher Scientific). The Ion PGM OT 2 200 Kit (Life Technologies) was used for template preparation. Semiconductor amplicon sequencing was performed on the PGM instrument. For PGM Sequencing 200 Kit (Life Technologies) following the protocols provided by Ion Torrent. Cell line mixtures were sequenced on Ion 314 v.1 Chip Kits (Life Technologies, Thermo Fisher Scientific) and the simulated FNA and clinical FNA samples were sequenced on Ion 318 v.1 Chip Kits (Life Technologies, Thermo Fisher Scientific).

**Sequencing and Statistical Analysis**

Torrent Suite Server Software 3.4.2 and 3.6.2 were employed for processing the signal output from the PGM. Variant analysis was executed using the Variant Caller plugin version 3.4 provided by the Torrent Server and visualized using the Integrative Genomics Viewer (Broad Institute, Cambridge, MA). The raw sequence was aligned and filtered on a tumor-normal pipeline (Ion Reporter 1.6, Life Technologies, Thermo Fisher Scientific) that filtered out somatic and germline variants that were provided a list of candidate variants. For the simulated FNA samples, somatic variant lists were independently generated for both the tumor-normal and FNA-normal pairs for each patient. True variants were required to have at least 1
mutant read in both the sense and antisense strand, a total read coverage of 100×, adequate mapping quality scores (>70), Phred scores greater than 20, and read lengths greater than 50bp. All candidate variants were visually confirmed by reviewing raw sequencing data of the tumor/FNA and matched normal samples using Integrative Genomics Viewer.

**Immunohistochemistry**

Immunolabeling for TP53, SMAD4, and CDKN2A was performed as described in detail in our prior publications. Negative controls for each of the antibodies were obtained using nonimmune serum instead of the primary antibody. Statistical analyses were performed using SPSS v20 with \( P < 0.05 \) considered significant.

**RESULTS**

**Determination of the Limits of Detection for FNA Sequencing**

A typical FNA specimen from pancreatic cancer contains neoplastic epithelial cells admixed with stromal cells and blood. To simulate a low neoplastic cellularity FNA sample and to determine the minimal neoplastic cellularity required to detect somatic variants, the human PDAC cell line Panc1 that contains a KRAS G12D mutation and a TP53 R273H mutation was mixed with the cancer-associated fibroblast cell line CAF35 in decreasing ratios while maintaining the total number of cells at 200,000 (Fig. 1A). DNA extracted from each mixture was then subjected to targeted exome sequencing to assess 739 mutations in 46 known cancer genes, achieving an average depth of coverage of more than 2000× per sample. Sequencing of the undiluted Panc1 DNA alone identified the KRAS variant at 70% and the TP53 variant at 100% mutant allele frequencies, respectively (Fig. 1B and Table S1, available at http://links.lww.com/SLA/A742). Such patterns are consistent with the presence of a heterozygous activating mutation in KRAS and mutation accompanied by loss of the wild-type allele of the TP53 tumor suppressor gene. No alterations using this panel were identified in CAF35 cells.

Decreasing cell ratios were not correlated with differences in total coverage per sample, total bases sequenced, or accuracy (Table S1, available at http://links.lww.com/SLA/A742). By contrast, absolute mutant allele frequencies for the KRAS and TP53 variants appeared interrelated to decreasing Panc1:CAF35 ratios, ranging from approximately 50% of mutant calls for both KRAS and TP53 in 1:1 mixtures to as few as 0.12% calls in 1:200 mixtures that

![Figure 1](https://example.com/figure1.png)

**FIGURE 1.** Limit of detection of mutant KRAS and TP53 alleles in Panc1:CAF35 admixtures. (A) Schematic of limiting dilution experiment. The pancreatic cancer cell line Panc1 that contains a KRAS G12D mutation and a TP53 R273H mutation was mixed with the normal fibroblast cell line CAF35 in decreasing ratios while maintaining the total number of cells at 200,000, or by maintaining the ratio at 1:25 while decreasing the total cell number. All cell admixtures were prepared in duplicate and each independently sequenced (B). The progressive decrease in Panc1 cellularity corresponded to a stepwise reduction in mutant allele frequency (MAF) with as little as 0.12 MAF for the TP53 allele detected in the 1:200 mixtures. (C) Panc1 and CAF35 cells were mixed at 1:25 ratios but at decreasing numbers of cells to evaluate the effect of total cell number on MAF detection. Each mutant allele was confidently detected until cell numbers reached a minimum of \( 1 \times 10^4 \) cells.
correspond to a neoplastic cellularity of 0.5% (Fig. 1B and Table S1, available at http://links.lww.com/SLA/A742). Given the ability to detect rare mutant alleles, we next determined the effects of decreasing the total cell number of Panc1:CAF35 mixtures while maintaining the cell ratio at 1:25 (Figs. 1A, C and Table S2, available at http://links.lww.com/SLA/A742). A decrease in total cell number did not affect total coverage for KRAS and TP53 to any great extent until the cell numbers fell below 25,000 total cells. Thus, we conclude that we can identify single base substitutions of significance regardless of the limited quantity of DNA derived from FNA specimens.

Concordance of Somatic Variants Between FNAs and Pancreatic Cancer Tissues

To assess the genetic concordance of an FNA to its matched primary tumor, we performed simulated (bench-top) FNAs on 3 pancreatic and 1 ampullary carcinoma in the surgical pathology suite immediately after surgical resection (Fig. 2A and Table S3, available at http://links.lww.com/SLA/A742). Libraries of each sample were then prepared from 10 ng of genomic DNA for sequencing of 15,992 amplicons covering all exons of 409 cancer-related genes. Tumor tissue from each cancer was also sequenced as well as each patient’s normal duodenal mucosa to facilitate the distinction between germline variants and somatic mutations. Using this panel, a total of 57,081,679 reads were generated with an average of 4,875,449 reads per sample and an average depth of coverage of 292×. Sequencing metrics did not differ between the simulated FNAs and the tumor specimens of each patient (Table S4, available at http://links.lww.com/SLA/A742).

Somatic alterations (n = 15) were identified in the simulated FNAs of all 4 carcinomas (Table 1). All of the alterations were single base substitution (SBS). Missense mutations accounted for 13 of 15 variants and nonsense mutations for 2 of 15. Fourteen of 15 were also present in the matched carcinoma tissue for each patient and included KRAS mutations in 4 carcinomas, TP53 mutations in 2 carcinomas, and a SMAD4 mutation in 1 carcinoma. In patient 1, a HIF1A c.802G>T variant was identified in the carcinoma tissue that was not identified in the matched FNA. Such occurrences may be secondary to clonal variation in the primary tumor,24 or to differences in the depth of sequencing of the FNA versus that of the matched tissue (Table 1 and Table S5, available at http://links.lww.com/SLA/A742). Collectively, the concordance between the simulated FNAs and tissue was 100% for 3 patients and 80% for 1 patient, with all driver gene SBS correctly identified by deep sequencing.

Although SBS are the most common somatic alteration in PDAC, some driver genes such as KRAS, TP53, or SMAD4 may be re-inactivated by alternative mechanisms23–25 prompting us to immunolabel the matched paraffin embedded tissues of these 4 carcinomas for these gene products (Fig. 2B and Table 2). We noted TP53 protein accumulation in more than 75% of the neoplastic cells in the carcinoma of patient 1 (Fig. 2B), consistent with the Y220C missense mutation identified, whereas SMAD4 showed nuclear protein expression despite the findings of a W524C missense mutation.

In patient 2, no expression was seen for any gene consistent with a homozygous deletion or methylation of CDKN2A, the R306X mutation in TP53, and a homozygous deletion of SMAD4. Arrowheads indicate positive labeling of adjacent normal stromal cells or lymphocytes.

FIGURE 2. Histology and immunolabeling features of resected pancreatic cancer specimens used for simulated FNAs. (A) Hematoxylin and eosin staining of representative sections for each of the 4 resected PDAC specimens used to perform simulated FNAs. In each example, infiltrating carcinoma glands (arrows) are present in association with a robust desmoplastic stromal response (Str). (B) Immunolabeling of resected PDAC specimens for CDKN2A, TP53, and SMAD4 in patients 1 and 2. The results of targeted exome sequencing for each gene are indicated under each image. Patient 1 showed expression of CDKN2A in the absence of any mutations consistent with wild type gene status. TP53 showed nuclear accumulation consistent with the Y220C missense mutation identified, whereas SMAD4 showed nuclear protein expression despite the findings of a W524C missense mutation. In patient 2, no expression was seen for any gene consistent with a homozygous deletion or methylation of CDKN2A, the R306X mutation in TP53, and a homozygous deletion of SMAD4. Arrowheads indicate positive labeling of adjacent normal stromal cells or lymphocytes.
Table 1: Somatic Variants Identified in Simulated Fine Needle Aspirates and Matched Tissues

<table>
<thead>
<tr>
<th>Sample</th>
<th>Gene</th>
<th>Genomic Coordinate</th>
<th>Variant</th>
<th>AA Change</th>
<th>Mutational Concordance</th>
</tr>
</thead>
<tbody>
<tr>
<td>Patient 1</td>
<td>HIF1A</td>
<td>chr14:62199164</td>
<td>c.802G&gt;T</td>
<td>p.Glu268+</td>
<td>80%</td>
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<tr>
<td></td>
<td>KRAS</td>
<td>chr12:25398284</td>
<td>c.35C&gt;T</td>
<td>p.Gly12Asp</td>
<td></td>
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<tr>
<td></td>
<td>MTO1</td>
<td>chr11:1117858</td>
<td>c.6039G&gt;A</td>
<td>p.Ser2013Ser</td>
<td></td>
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<tr>
<td></td>
<td>SMAD4</td>
<td>chr18:48604750</td>
<td>c.1572G&gt;T</td>
<td>p.Try524Cys</td>
<td></td>
</tr>
<tr>
<td></td>
<td>TP53</td>
<td>chr17:7578190</td>
<td>c.659T&gt;C</td>
<td>p.Try220Cys</td>
<td></td>
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<tr>
<td>Patient 2</td>
<td>RBRD1</td>
<td>chr9:136916706</td>
<td>c.477C&gt;T</td>
<td>p.Pro159Pro</td>
<td>100%</td>
</tr>
<tr>
<td></td>
<td>KEAP1</td>
<td>chr19:10602913</td>
<td>c.66ST&gt;C</td>
<td>p.Asn222Ser</td>
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<tr>
<td></td>
<td>TP53</td>
<td>chr17:757022</td>
<td>c.916G&gt;A</td>
<td>p.Arg306+</td>
<td></td>
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<tr>
<td></td>
<td>ZNF512</td>
<td>chr18:22805083</td>
<td>c.279G&gt;A</td>
<td>p.Asp933Asn</td>
<td></td>
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<tr>
<td>Patient 3</td>
<td>EPHA7</td>
<td>chr9:94120364</td>
<td>c.687G&gt;C</td>
<td>p.Glu229Asp</td>
<td>100%</td>
</tr>
<tr>
<td></td>
<td>KRAS</td>
<td>chr12:25398284</td>
<td>c.35G&gt;A</td>
<td>p.Gly12Asp</td>
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<td>MLL3</td>
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<td>p.Asp1384Asn</td>
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<td>TP53</td>
<td>chr17:757022</td>
<td>c.35G&gt;A</td>
<td>p.Gly12Asp</td>
<td></td>
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<tr>
<td></td>
<td>XPC</td>
<td>chr13:1499545</td>
<td>c.1840T&gt;C</td>
<td>p.Phe614Leu</td>
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</tbody>
</table>

Three out of 4 FNAs displayed a 100% mutational concordance (patients 2, 3, and 4) with the resected cancer specimen while the mutational concordance in patient 1 was 80%. For each variant identified, the appropriate gene, genomic coordinate (+human genome build 19), coding change, and amino acid change is listed. Driver genes are underlined.

Table 2: Immunohistochemical Features of Resected Carcinomas

<table>
<thead>
<tr>
<th>Patient ID</th>
<th>CDKN2A</th>
<th>TP53</th>
<th>SMAD4</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>Intact</td>
<td>Nuclear accumulation</td>
<td>Intact</td>
</tr>
<tr>
<td>2</td>
<td>Lost</td>
<td>Lost</td>
<td>Lost</td>
</tr>
<tr>
<td>3</td>
<td>Lost</td>
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<td>Lost</td>
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<tr>
<td>4</td>
<td>Normal</td>
<td>Lost</td>
<td>Lost</td>
</tr>
</tbody>
</table>

CDKN2A, TP53, and SMAD4 were all immunolabeled to observe the protein expression in the 4 simulated FNAs. When combined with sequencing data, these provide ample molecular characterization of pancreatic tumors.

2 and 3 (Table 2). The loss of expression in patient 2 is consistent with the p.Arg306X mutation found, whereas in patient 3 the loss of expression suggests the homozygous deletion of TP53 with only wild type sequences contributed by the normal cells in the sample being sequenced. SMAD4 and CDKN2A expression was also lost in 3 of the 4 samples, none of which exhibited a sequencing variant, also suggesting homozygous deletions (SMAD4, CDKN2A) or methylation-induced silencing (CDKN2A) in these samples. Although the carcinoma of patient 1 had intact SMAD4 protein expression despite the finding of a W524C mutation. In this instance, the variant may perturb protein function but not protein stability, although the possibility of a passenger mutation occurring in SMAD4 must also be considered in light of the extreme 3′ location of this SBS.

Identification of Somatic Variants in Pretreatment FNA Samples of Unresectable Pancreatic Tumors

To determine the feasibility of next-generation sequencing of diagnostic FNAs in real-time clinical practice, we prospectively obtained pretreatment FNA and peripheral blood samples from 19 patients with an unresectable, nonmetastatic pancreatic mass. These patients were all enrolled in a Phase II clinical trial at our institution evaluating gemcitabine and stereotactic body radiation therapy for locally advanced unresectable pancreatic adenocarcinoma (Table S6, available at http://links.lww.com/SLA/A742). All patients successfully met the inclusion criteria for this trial based on imaging studies. Analysis of all samples by targeted exon sequencing resulted in a total of 168,144,747 bases sequenced and a mean depth of coverage of 298× per sample (Table S7, available at http://links.lww.com/SLA/A742). After filtering of sequencing data for germline variants, 1448 candidates of somatic variants were identified of which 77 (5%) remained valid after visualization of the raw sequencing data for all variants. These 77 somatic alterations were composed of 68 missense mutations, 6 nonsense mutations, 2 INDELs, and 1 splice site mutation (Table S8, available at http://links.lww.com/SLA/A742). No mutations were identified in 2 of the 19 (11%) FNA samples, likely a result of sampling error.

We first determined the feasibility of identifying driver mutations in each FNA. All 17 FNA samples with a mutation had at least 1 mutation in a known driver gene (Table 3 and Table S8, available at http://links.lww.com/SLA/A742). These 17 samples were then considered in light of the extreme 3′ location of this SBS.

In addition to known and frequent driver genes, we noted recurrent SBS in other genes recently reported in PDAC. 27-28 These include GEMM (n = 2, 12%), TRIM33 (n = 2, 12%), and SF3B1 (n = 1, 6%). Mutations in a single carcinoma were also noted in CTNNB1, NOTCH1, and NOTCH4; as these 3 occurred outside the most commonly mutated domains or were conservative, we interpret these as passenger mutations occurring in a driver gene. One exception is the p.C147R variant we identified in NF1 in a carcinoma with wild-type KRAS, BRAF, NRAS, and HRAS genes.

We noted 2 examples by which sequencing of FNA samples could rapidly inform clinical management. First, FNA sequencing can identify actionable targets. For example, we identified somatic mutations in ATM (ataxia-telangiectasia mutated) in 2 of 17 (12%) unresectable carcinomas (JH01 and JH10), and both mutations are predicted to be deleterious in nature. Germline inactivating mutations in ATM have recently been reported to cause the familial aggregation of PDAC in some kindreds, although it seems that ATM mutations may be somatically inactivated in PDAC as well given these current data and that of others. Of interest, in both patients the abundance of the mutant ATM allele was at least 3 standard deviations higher than that of the other driver mutations in the same samples (Table S8, available at http://links.lww.com/SLA/A742), evidence they arose early during carcinogenesis. Importantly, ATM mutations have been shown to confer increased radiosensitivity owing to the central role of ATM in...
DNA damage repair after ionizing-radiation,

suggesting that these patients would have enhanced benefit from radiotherapy. We therefore reviewed the clinical history of these 2 patients. One patient (JH10) developed pneumonia shortly after enrollment that precluded treatment on trial and was subsequently lost to follow-up. The second patient whose cancer harbored a somatic ATM\(^*\) mutation (JH01) had a favorable response to stereotactic body radiation therapy with a drop of serum CA19-9 levels from 1119 to 100 U/mL and no evidence of local or distant progression over 19 months of follow-up.

As opposed to relying on imaging alone to diagnose and stage patients with localized PDAC, FNA sequencing may also aid diagnosis by classifying neoplasms based on their genetic features. We found that the FNA of patient JH36 did not have mutations in any of the common PDAC driver genes but did have somatic mutations in \(\text{MTOR}\), \(\text{IDH2}\), and \(\text{TSC1}\). This observation led us to hypothesize that the patient either had a nonductal form of pancreatic neoplasia, or a metastasis to the pancreas from a different primary site. In this instance, the co-existence of both \(\text{MTOR}\) and \(\text{TSC1}\) mutations suggested a metastatic urothelial primary as the source of the pancreatic mass as mutations in these genes are recurrently altered in this tumor type.

The patient’s CA19-9 at diagnosis was 0 U/mL and thus noncontributory. Although these features were only noted after the patient expired, they nonetheless suggest that FNA sequencing can aid the clinician in guiding appropriate management based on the tumor’s unique genetic signature beyond simple radiographic characteristics.

**DISCUSSION**

Currently, management of PDAC is generally not based on individual patient or tumor characteristics. Treatment is often delivered in a “one size fits all” approach with evidence that was based on an average effect on population of patients with heterogeneous tumor behaviors. It would follow that improvement in outcome after resection of PDAC may occur through adaptation of personalized treatment principles based on unique behavior of an individual’s genes.

**TABLE 3.** Somatic Alterations Identified in Clinical Trial J1003 Patients

<table>
<thead>
<tr>
<th>Patient</th>
<th>(\text{KRAS})</th>
<th>(\text{CDKN2A})</th>
<th>(\text{SMAD4})</th>
<th>(\text{TP53})</th>
<th>(\text{ATM})</th>
<th>(\text{ARID1A})</th>
<th>Other Variants</th>
<th>Common variants identified in more than 1 sample are displayed with the corresponding amino acid change observed in each individual patient. Gene symbols with unique variants for each sample are also displayed.</th>
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<td>J1003-JH01</td>
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Common variants identified in more than 1 sample are displayed with the corresponding amino acid change observed in each individual patient. Gene symbols with unique variants for each sample are also displayed.

**FIGURE 3.** Correlation of driver gene allele frequencies in a single sample. Shown is the comparison of allele frequencies for 12 samples in which more than 1 driver gene mutation was detected. For all patients, a \(\text{KRAS}\) mutation was found, with the second gene \(\text{TP53}\) (\(n=7\), red), \(\text{SMAD4}\) (\(n=4\), blue), or \(\text{TGFBR2}\) (\(n=1\), green). In the event that \(\text{TP53}\) and \(\text{SMAD4}\) mutations or \(\text{TP53}\) and CDKN2A mutations were both present, \(\text{TP53}\) values were used. The 2-sided Spearman correlation coefficient (\(r\)) between the \(\text{KRAS}\) allele frequency and the second gene allele frequency was 0.45 (\(P = 0.017\)).
tumor. As such, sequencing methods to characterize the genetic fingerprint of a tumor must be optimized. Personalized management of PDAC based on genetic or molecular parameters continues to lag behind other solid organ tumors partly due to the inability to obtain genetic data from patients before treatment initiation. We demonstrate here the ability to identify somatic mutations in FNA samples obtained from patients with PDAC. The results exhibit the ability of next-generation sequencing technology to overcome the potential technical limitations inherent to obtaining sequence data from FNA specimens of PDAC. These findings will dramatically facilitate the development of personalized medicine in the surgical care of PDAC.

In particular, this work has immediate application in the design of trials that evaluate the sequence of surgical resection, use of radiation, and systemic therapy. Several recent developments in the genetics of PDAC have the potential to improve the outcome of patients undergoing surgical resection. First, it is known that pancreatic cancers exist as distinct genetic subtypes with different patterns of disease failure as exemplified by the SMAD4 gene mutational status. As such, pretreatment determination of SMAD4 mutations could be used to stratify patients in clinical trials. Second, with the advent of targetable therapies for cancer, knowledge of a tumors’ genetic signature will allow for personalized therapy based on chemosensitivity. For example, patients with PALB2 mutations are exquisitely responsive to mitomycin C, a treatment that is not routinely employed against pancreatic tumors. Finally, beyond providing insight into the biology or therapeutic options for PDAC, determinations of the genetic landscape of PDAC may have prognostic value for an individual patient. Although these advances are in the early stages of development, they have the potential to dramatically impact survival for future patients.

The advent of targetable agents in cancer therapeutics has fueled scientists to identify tumor-specific biomarkers that will allow personalized therapy for patients. Among our patients, at least 3 had actionable mutations that could directly impact therapeutic management such as in AT, NF1, or MTOR. That these mutations were found in pancreatic FNAs that inherently contain limited quantity of cancer DNA implies this approach could be implemented into true clinical pipelines or pharmacokinetic ratelimiting fashion. Finally, given the increasing role of neoadjuvant therapy in managing pancreatic cancers, sequencing of FNAs before treatment may aid identification of the genetic features that underlie treatment responses, particularly in those patients for whom the resected tissues are rendered inadequate for sequencing. This concept is illustrated by a patient in our study who was downstaged to resectable status after stereotactic body radiation therapy. Sequencing of the resected tumor tissue in this patient did not reveal any of the mutations identified in the matched pretreatment FNA sample. Histologic sections were therefore reviewed of the resection specimen indicating it contained abundant fibrosis and rare single carcinoma cells consistent with treatment effects, indicating that the extreme low cellularity of the specimen precluded the confident identification of mutations (Fig. 4).

Regarding the potential problem when working with limited quantity of DNA, our work shows that somatic variants can be successfully identified in FNAs by deep sequencing technology. In fact, on the basis of our limiting dilution experiments, we believe most FNA samples obtained in the clinical setting contain ample cancer cells to identify genetic alterations of significance. This is further supported by the finding of PDAC driver gene alterations in FNAs at frequencies expected based on large scale sequencing efforts. An important caveat of sequencing FNAs is that it should be performed in association with immunohistochemistry for the expression in the same specimen as homologous deletions may be missed on sequencing. Together, this approach can accurately identify and classify the genetic alterations of significance for each patient.

Tumor heterogeneity limits the study of PDAC for subclonal events; however, these data indicate that FNA may be sufficient to establish the clonal mutations for each individual’s tumor. Robust regional sampling of the mass by the endoscopist may provide a more complete picture of the tumors’ genetic landscape.

This study has further implications beyond the management of resectable patients. First, sequencing of FNAs will allow studies of patient populations not routinely included in genome-sequencing efforts. For example, those with unresectable, nonmetastatic PDAC for which only an FNA may be available. On the basis of our initial data, locally advanced nonmetastatic carcinomas may harbor a different spectrum of mutations than resectable or metastatic carcinomas. For example, after exclusion of the suspected pancreatic metastasis, 2 of 16 patients had mutations in GRM3, an eightfold higher rate than that reported in early-stage pancreatic cancers (1 of 99, P = 0.048, Fisher exact test). To our knowledge, this study is also the first to report an NF1 mutation in a patient with a high clinical suspicion of PDAC based on radiographic characteristics and CA19-9 biomarker levels. Second, unbiased sequencing of FNAs may increase the sensitivity and specificity of diagnosing cancer, particularly in the background of intense chronic inflammation such as pancreatitis. Analogous approaches have been successfully demonstrated by sequencing of DNA shed into pancreatic cysts for their accurate classification.

FNA is not required for diagnosis in patients with resectable disease, although they are more commonly being performed to confirm the diagnosis of cancer before administration of neoadjuvant therapy. The diagnostic accuracy of FNAs is high with a sensitivity and specificity reaching 97% and 99%, respectively, although accuracy is lost in the presence of inflammation as may be seen in chronic pancreatitis. Given the specificity of somatic alterations for neoplasia, this approach could also be used to supplement...
cytological diagnoses and increase sensitivity and specificity of FNAs. The unique constellation of driver genes was identified, and the identification of actionable targets that confer chemosensitivity to available targeted therapies could personalize patient management. The implementation of FNA sequencing into clinical trials and personalized therapies for pancreatic cancer patients will require a change in culture within the clinical oncology community. Although some institutions perform routine molecular characterization of suspected PDAC, this is not the standard of care. Some of our research FNAs were performed without a cytopathologist present in the endoscopy suite due to financial constraints involved with the study budget. However, on-site cytopathology support needs to be present in the endoscopy suite during sample acquisition as the standard of care. Although it is well known that the presence of a cytopathologist reduces the number of passes required to render an accurate diagnosis, it will also ensure the quality of material aspirated for molecular studies including FNA sequencing. It should be noted that the FNA specimens we sequenced were fresh frozen samples that were obtained from a clinical trial and sequenced at a later date. Given that current clinical practice employs routine alcohol fixation of FNAs, future studies should optimize sequencing directly from alcohol fixed samples.

CONCLUSIONS
In summary, we show that targeted deep sequencing of FNAs is feasible and can be utilized in the course of real-time clinical management. Moreover, routine sequencing of FNAs will afford new opportunities for study of previously uncharacterized pancreatic cancer patient populations by linking the tumor’s genetics to clinical outcome. In the future, FNA sequencing may help clinicians develop personalized therapy for patients with pancreatic cancer with potential to improve outcomes for this morbid disease. These findings also have direct relevance to any tumor type for which FNA samples are acquired in the course of diagnosis or treatment (eg, neoplasms of the thyroid, salivary gland, lung, cervical, breast, or musculoskeletal system).

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