Published in final edited form as:

Ann Surg. 2016 January ; 263(1): 153–161. doi:10.1097/SLA.0000000000001156.

Reliable Detection of Somatic Mutations in Fine Needle Aspirates of Pancreatic Cancer with Next-Generation Sequencing: Implications for Surgical Management

Vicente Valero III, MD¹, Tyler J. Saunders, BA², Jin He, MD¹, Matthew J. Weiss, MD^{1,3}, John L. Cameron, MD¹, Avani Dholakia, MD⁵, Aaron T. Wild, MD⁵, Eun Ji Shin, MD⁴, Mouen A. Khashab, MD⁴, Anne Marie O'Broin-Lennon, MD^{1,4}, Syed Z. Ali, MD², Daniel Laheru, MD³, Ralph H. Hruban, MD^{2,3}, Christine A. Iacobuzio-Donahue, MD, PhD^{1,2,3,6,*}, Joseph M. Herman, MD^{5,*}, and Christopher L. Wolfgang, MD, PhD^{1,2,3,†,*}

¹Department of Surgery, The Johns Hopkins University School of Medicine, Baltimore, MD, 21287

²Department of Pathology, The Johns Hopkins University School of Medicine, Baltimore, MD, 21287

³Department of Oncology, The Johns Hopkins University School of Medicine, Baltimore, MD, 21287

⁴Department of Gastroenterology and Hepatology, The Johns Hopkins University School of Medicine, Baltimore, MD, 21287

⁵Department of Radiation Oncology and Molecular Radiation Sciences, The Sol Goldman Pancreatic Cancer Research Center, The Johns Hopkins University School of Medicine, Baltimore, MD, 21287

Abstract

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

Background—FNA is a common method of diagnosis for pancreatic cancer, yet 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.

[†]Corresponding author: Christopher L. Wolfgang, MD, PhD, Department of Surgery, Johns Hopkins University School of Medicine, 600 N. Wolfe St, 685 Blalock, Baltimore, Maryland 21287, 410-502-4194, cwolfga2@jhmi.edu.

⁶Present address: Department of Pathology and the David Rubenstein Pancreatic Cancer Research Center, Memorial Sloan Kettering Cancer Center, New York, NY, 10065.

*All contributed equally as co-senior authors

Conflict of interests: The authors have no competing interests to disclose.

Results—Limiting dilution studies indicated that mutations present 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 one 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

Pancreatic Cancer; Pancreatic Surgery; Fine Needle Aspirates; Next-generation sequencing; Cancer Genetics; Genomics

INTRODUCTION

Pancreatic cancer (pancreatic ductal adenocarcinoma, PDAC) is a highly lethal disease with an overall 5-year survival of only 6.7%.¹ The only chance for long-term survival is through a potentially curative operation.^{1,2} However, even among patients with clinically localized disease who undergo resection, survival remains poor with the majority experiencing both local and systemic failure.^{3–7} Adjuvant and neoadjuvant treatment paradigms have been employed to reduce treatment failures - but these therapies provide only a modest benefit to survival.^{3,8–12} 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 following 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 prior to operation and acquisition of a surgical specimen. Experimentation with methods to accomplish this task have only recently been explored prior to this study.¹³

Recent developments in the understanding of the genetics of PDAC have lead to the potential for a personalized approach in operative patients. For example, the timing of surgery in relation to systemic therapy (i.e. 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.¹⁴ In addition, with the advent of targetable therapies for cancer, knowledge of a tumor's genetic signature will allow for therapy based on chemosensitivity.¹⁵ 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 PDAC obtained by endoscopic ultrasound has been useful in making a tissue diagnoses and has the potential to provide cancer DNA for this purpose. However, the ability to accurately characterize the global genetic mutational status from FNA specimens has not been definitively proven. It has been assumed that the extremely low quantity of cells obtained from FNA specimens, precludes accurate determination of a tumors genetic status. However, the development of next-generation sequencing offers the potential to overcome this limitation.¹⁶

Therefore, in order to facilitate the development of personalized surgical therapy for PDAC, we sought to determine if next-generation sequencing technology is capable of identifying somatically altered genes in FNA specimens obtained from patients with PDAC. We demonstrate here that this methodology has the potential to accurately identify relevant genetic alterations despite the limited DNA quantity of FNA specimens and the multi-clonal nature typical of PDAC. To our knowledge, this is among the first studies to demonstrate that deep-sequencing is possible in DNA isolated from FNAs of PDAC.¹³ 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 CAF-35 was established from surgically-resected pancreas tissue from a patient with sporadic PDAC as previously described.¹⁷ 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) and 2% penicillin-streptomycin (Invitrogen). For creation of Panc1-CAF35 cell line mixtures, cultured cells were first counted using the Countess® Automated Cell Counter (Life Technologies) 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 three 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 prior to surgery for sampling of resected tumor tissues in accordance with protocols approved by the Johns Hopkins University (JHU) Internal Review Board (IRB).

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 JHU IRB. 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 fiducial 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 15–30 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 lines and human tissues was extracted using phenol-chloroform, or QIAmp DNA Micro Kits (Qiagen) and quantified by calculating long interspersed nuclear elements (LINE) by real-time Polymerase Chain Reaction (PCR) as previously described.¹⁸ To minimize sequencing bias from using low-copy starting templates, only samples with DNA concentrations of ≥ 3.3 nanograms per microliter (1,000 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). Briefly, 10 nanograms (ng) of genomic DNA were 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 and J1003 clinical trial FNA samples, the Ion Ampliseq™ Comprehensive Cancer Panel® (Life Technologies) consisting of 15,992 amplicons covering all exons of 409 cancer related genes was used. The PCR amplification conditions were 99 °C for 2 min; 14 cycles of 99 °C for 15 s, 60 °C for 8 min with a 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). Libraries were quantified using Agilent High Sensitivity DNA Kit on the 2100 Bioanalyzer (Agilent) or using real-time PCR via the Taq-man based Ion Library Quantitation Kit (Life Technologies). 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). The Ion PGM™ OT 2 200 Kit (Life Technologies) was used for template preparation. Semiconductor amplicon sequencing was performed on the PGM™ using the Ion 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) and the

simulated FNA and clinical FNA samples were sequenced on Ion 318™ v.1 Chip Kits (Life Technologies).

Sequencing 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 (IGV) Version 2.3 (Broad Institute). The raw sequence was aligned and filtered on a tumor-normal pipeline (Ion Reporter (IR) 1.6, Life Technologies) that filtered out intronic and germline variants and provided a list of candidate variants. For the simulated FNA samples, somatic variants lists were independently generated for both the tumor-normal and FNA-normal pairs for each patient. True variants were required to have at least one mutant read in both the sense and anti-sense strand, a total read coverage of 100X, adequate mapping quality scores (>70), PHRED scores >20 and read lengths greater than 50 bp. All candidate variants were visually confirmed by reviewing raw sequencing data of the tumor/FNA and matched normal samples using IGV.

Immunohistochemistry

Immunolabeling for *TP53*, *SMAD4* and *CDKN2A* was performed as described in detail in our prior publications.¹⁹ Negative controls for each of the antibodies were obtained using nonimmune serum instead of the primary antibody.

Statistical Analysis

Statistical analyses were performed using SPSS v20 with p-values of <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 mutation²⁰, 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 >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). 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.²¹ 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). By contrast, absolute mutant allele frequencies for

the *KRAS* and *TP53* variants appeared interrelated to decreasing Panc1:CAF35 ratios, ranging from ~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 correspond to a neoplastic cellularity of 0.5% (Fig. 1B and Table S1). 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 (Fig. 1A, C and Table S2). 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 three pancreatic and one ampullary carcinoma in the surgical pathology suite immediately after surgical resection (Fig. 2A and Table S3). 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 292x. Sequencing metrics did not differ between the simulated FNAs and the tumor specimens of each patient (Table S4).

Somatic alterations (n=15) were identified in the simulated FNAs of all four carcinomas (Table 1). All of the alterations were SBS. Missense mutations accounted for 13 of 15 variants and nonsense mutations for two of 15. Fourteen of 15 were also present in the matched carcinoma tissue for each patient and included *KRAS* mutations in four carcinomas, *TP53* mutations in two carcinomas and a *SMAD4* mutation in one 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 tumor²², or to differences in the depth of sequencing of the FNA versus that of the matched tissue (Table 1 and Table S5). Collectively, the concordance between the simulated FNAs and tissue was 100% for three patients and 80% for one patient, with all driver gene SBS correctly identified by deep sequencing.

While SBS are the most common somatic alteration in PDAC, some driver genes such as *CDKN2A*, *TP53* or *SMAD4* may also be inactivated by alternative mechanisms^{23–25} prompting us to immunolabel the matched paraffin embedded tissues of these four carcinomas for these gene products (Fig. 2B and Table 2). We noted TP53 protein accumulation in >75% of the neoplastic cells in the carcinoma of patient 1 (Fig. 2B), consistent with the Y220C missense variant identified (Table 1), whereas a complete loss of protein expression was observed in the carcinoma tissues of patients 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.^{23,24} Of interest, 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 stability²⁶, 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 pre-treatment FNA and peripheral blood samples from 19 patients with an unresectable, non-metastatic 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).²⁷ 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 168,164,747 bases sequenced and a mean depth of coverage of 298X per sample (Table S7). After filtering of sequencing data for germline variants 1448 candidate 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 comprised of 68 missense mutations, six nonsense mutations, two INDELs and one splice site mutation (Table S8). No mutations were identified in two 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 one mutation in a known driver gene (Table 3 and Table S8). The most commonly identified somatic alterations were in *KRAS* (n=14, 82%), followed by *TP53* (n=9, 53%), *SMAD4* (n=6, 35%) and *ARID1A* (n=3, 18%). Mutant allele frequencies ranged from as low as 0.84% to as high as 72.8% for these four genes (median 4.55%, interquartile range 2.4–22.6). The allele frequencies for different driver mutations present in the same FNA were highly concordant ($r=0.6689$, $p=0.017$) (Fig. 3) indicating that the allelic abundance of one mutant can be leveraged to assess the expected abundance of others in the same sample.

In addition to known and frequent driver genes, we noted recurrent SBS in other genes recently reported in PDAC.^{16,28} These include *GRM8* (n=2, 12%), *TRIM33* (n=2, 12%) and *SF3B1* (n=1, 6%). Mutations in a single carcinoma each were also noted in *CTNNB1*, *NOTCH1* and *NOTCH4*; as these three occurred outside the most commonly mutated domains or were conservative, we interpret these as passenger mutations occurring in a driver gene.²⁹ 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 two 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 two 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 kindreds³⁰ although it appears that *ATM* may be somatically inactivated in PDAC as well given these current data and that of others.¹⁶ Of interest, in both patients the abundance of the mutant *ATM* alleles was at least 3 standard deviations higher than that of the other driver mutations in the same samples (Table S8), 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 following ionizing-radiation^{31,32} suggesting that these patients would have enhanced benefit from radiotherapy. We therefore reviewed the clinical history of these two 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 (SBRT) 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 *MTOR*, *IDH2* and *TSC1*. This observation led us to hypothesize that the patient either had a non-ductal form of pancreatic neoplasia^{33,34}, or a metastasis to the pancreas from a different primary site. In this instance, the co-existence of both *MTOR* and *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.³⁵ The patients CA19-9 at diagnosis was 0 U/mL and thus noncontributory. While 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 following resection of PDAC may occur through adaptation of personalized treatment principles based on unique behavior of an individual's 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 tumors³⁶ partly due to the inability to obtain genetic data from patients prior to 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.¹⁴ As such, pretreatment determination of SMAD4 mutations could be used to stratify patients in clinical trials.¹⁴ Second, with the advent of targetable therapies for cancer, knowledge of a tumors genetic signature will allow for personalized therapy based on chemosensitivity.¹⁵ For example, patients with PALB2 mutations are exquisitely responsive to mitomycin C, a treatment that is not routinely employed against pancreatic tumors.^{15,37} Lastly, 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.¹⁹ 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 three had actionable mutations that could directly impact therapeutic management such as in *ATM*, *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 Phase I trials in rapid fashion. Finally, given the increasing role of neoadjuvant therapy in managing pancreatic cancers, sequencing of FNAs prior to treatment may aid identification of the genetic features that underlie treatment responses, particularly in those patients for which the resected tissues are rendered inadequate for sequencing.³⁸ This concept is illustrated by one patient in our study who was downstaged to resectable status following 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 effects³⁸, 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, based on our limiting dilution experiments we believe most FNA samples obtained in the clinical setting contain ample cancer cells to identify genetic alterations of significance.^{16,28} This is further supported by the finding of PDAC driver gene alterations in FNAs at frequencies expected based on large scale sequencing efforts.^{16,28} An important caveat of sequencing FNAs is that it should be performed in association with immunolabeling for gene expression in the same specimen as homozygous 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.

The current 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^{16,28}, for example those with unresectable, non-metastatic PDAC for which only an FNA may be available. Based on our initial data, locally-advanced non-metastatic carcinomas may harbor a different spectrum of mutations than resectable or metastatic carcinomas.¹⁶ For example, after exclusion of the suspected pancreatic metastasis, two of 16 patients had mutations in *GRM8*, an 8-fold higher rate than that reported in early stage pancreatic cancers¹⁶ (one 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.^{39,40} Analogous approaches have been successfully demonstrated by sequencing of DNA shed into pancreatic cysts for their accurate classification.³³

FNA is not required for diagnosis in patients with resectable disease, although they are more commonly being performed to confirm the diagnosis of cancer prior to administration of neoadjuvant therapy.⁴¹ The diagnostic accuracy of FNAs is high with a sensitivity and specificity reaching 87% and 99% respectively⁴², although accuracy is lost in the presence of inflammation as may be seen in chronic pancreatitis.⁴⁰ 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.^{33,39,43} The unique constellation of driver genes identified and the identification of actionable targets that confer chemosensitivity to available targeted therapies could personalize patient management.⁴⁴

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.³⁶ While some institutions perform routine molecular characterization of suspected PDAC, this is not the standard of care.⁴⁵ 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. While it is well known that the presence of a cytopathologist reduces the number of passes required to render an accurate diagnosis⁴¹ 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.

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, for example neoplasms of the thyroid, salivary gland, lung, cervical, breast or musculoskeletal system.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

Funding: Supported by NIH grants CA140599 (C.I.D.), CA179991 (C.I.D.), CA101955 (C.I.D.), CA062924 (R.H.H.), T32DK007713 (V.V.). Pilot funding from the Sol Goldman Pancreatic Cancer Research Center, The Claudio X. Gonzalez Family Foundation, The Natalie and David Lederman Foundation, and the Joseph C. Monastra Foundation. NIH T32 grant provided salary support for V.V. All other grants provided funding for the necessary supplies to perform the aforementioned experiments.

We thank Michael Goggins for provision of the CAF35 cell line.

References

1. SEER Research Data 1973–2011. 2014. Available at: www.seer.cancer.gov
2. Jemal A, Siegel R, Xu J, Ward E. Cancer statistics, 2010. *CA Cancer J Clin.* 2010; 60:277–300. [PubMed: 20610543]
3. Neoptolemos JP, Stocken DD, Friess H, et al. A randomized trial of chemoradiotherapy and chemotherapy after resection of pancreatic cancer. *N Engl J Med.* 2004; 350:1200–1210. [PubMed: 15028824]
4. Davila JA, Chiao EY, Hasche JC, Petersen NJ, McGlynn KA, Shaib YH. Utilization and determinants of adjuvant therapy among older patients who receive curative surgery for pancreatic cancer. *Pancreas.* 2009; 38:18–25.
5. Mayo SC, Austin DF, Sheppard BC, Mori M, Shipley DK, Billingsley KG. Adjuvant therapy and survival after resection of pancreatic adenocarcinoma: a population-based analysis. *Cancer.* 2010; 116:2932–2940. [PubMed: 20336787]
6. Slidell MB, Chang DC, Cameron JL, et al. Impact of total lymph node count and lymph node ratio on staging and survival after pancreatectomy for pancreatic adenocarcinoma: a large, population-based analysis. *Ann Surg Oncol.* 2008; 15:165–174. [PubMed: 17896141]
7. Mayo SC, Gilson MM, Herman JM, et al. Management of patients with pancreatic adenocarcinoma: national trends in patient selection, operative management, and use of adjuvant therapy. *Journal of the American College of Surgeons.* 2012; 214:33–45. [PubMed: 22055585]
8. Oettle H, Post S, Neuhaus P, et al. Adjuvant chemotherapy with gemcitabine vs observation in patients undergoing curative-intent resection of pancreatic cancer: a randomized controlled trial. *JAMA.* 2007; 297:267–277. [PubMed: 17227978]
9. Oettle H, Neuhaus P, Hochhaus A, et al. Adjuvant chemotherapy with gemcitabine and long-term outcomes among patients with resected pancreatic cancer: the CONKO-001 randomized trial. *JAMA.* 2013; 310:1473–1481. [PubMed: 24104372]
10. Regine WF, Winter KA, Abrams RA, et al. Fluorouracil vs gemcitabine chemotherapy before and after fluorouracil-based chemoradiation following resection of pancreatic adenocarcinoma: a randomized controlled trial. *JAMA.* 2008; 299:1019–1026. [PubMed: 18319412]
11. Conroy T, Desseigne F, Ychou M, et al. FOLFIRINOX versus gemcitabine for metastatic pancreatic cancer. *N Engl J Med.* 2011; 364:1817–1825. [PubMed: 21561347]
12. Hoff Von DD, Ervin T, Arena FP, et al. Increased survival in pancreatic cancer with nab-paclitaxel plus gemcitabine. *N Engl J Med.* 2013; 369:1691–1703. [PubMed: 24131140]

13. Young G, Wang K, He J, et al. Clinical next-generation sequencing successfully applied to fine-needle aspirations of pulmonary and pancreatic neoplasms. *Cancer Cytopathol.* 2013; 121:688–694. [PubMed: 23893923]
14. Iacobuzio-Donahue CA, Fu B, Yachida S, et al. DPC4 gene status of the primary carcinoma correlates with patterns of failure in patients with pancreatic cancer. *Journal of Clinical Oncology.* 2009; 27:1806–1813. [PubMed: 19273710]
15. Villarroel MC, Rajeshkumar NV, Garrido-Laguna I, et al. Personalizing cancer treatment in the age of global genomic analyses: PALB2 gene mutations and the response to DNA damaging agents in pancreatic cancer. *Mol Cancer Ther.* 2011; 10:3–8. [PubMed: 21135251]
16. Biankin AV, Waddell N, Kassahn KS, et al. Pancreatic cancer genomes reveal aberrations in axon guidance pathway genes. *Nature.* 2012; 491:399–405. [PubMed: 23103869]
17. Walter K, Omura N, Hong SM, et al. Overexpression of Smoothed Activates the Sonic Hedgehog Signaling Pathway in Pancreatic Cancer-Associated Fibroblasts. *Clinical Cancer Research.* 2010; 16:1781–1789. [PubMed: 20215540]
18. Rago C, Huso DL, Diehl F, et al. Serial Assessment of Human Tumor Burdens in Mice by the Analysis of Circulating DNA. *Cancer Research.* 2007; 67:9364–9370. [PubMed: 17909045]
19. Yachida S, White CM, Naito Y, et al. Clinical Significance of the Genetic Landscape of Pancreatic Cancer and Implications for Identification of Potential Long-term Survivors. *Clinical Cancer Research.* 2012; 18:6339–6347. [PubMed: 22991414]
20. Deer EL, González-Hernández J, Coursen JD, et al. Phenotype and Genotype of Pancreatic Cancer Cell Lines. *Pancreas.* 2010; 39:425–435. [PubMed: 20418756]
21. Calhoun ES. Identifying Allelic Loss and Homozygous Deletions in Pancreatic Cancer without Matched Normals Using High-Density Single-Nucleotide Polymorphism Arrays. *Cancer Research.* 2006; 66:7920–7928. [PubMed: 16912165]
22. Yachida S, Jones S, Bozic I, et al. Distant metastasis occurs late during the genetic evolution of pancreatic cancer. *Nature.* 2010; 467:1114–1117. [PubMed: 20981102]
23. Hahn SA, Schutte M, Hoque ATMS, et al. DPC4, A Candidate Tumor Suppressor Gene at Human Chromosome 18q21.1. *Science.* 1996; 271:350–353. [PubMed: 8553070]
24. Schutte M, Hruban RH, Geradts J, et al. Abrogation of the Rb/p16 tumor-suppressive pathway in virtually all pancreatic carcinomas. *Cancer Research.* 1997; 57:3126–3130. [PubMed: 9242437]
25. Caldas C, Hahn SA, da Costa LT, et al. Frequent somatic mutations and homozygous deletions of the p16 (MTS1) gene in pancreatic adenocarcinoma. *Nat Genet.* 1994; 8:27–32. [PubMed: 7726912]
26. Iacobuzio-Donahue CA. Missense Mutations of MADH4: Characterization of the Mutational Hot Spot and Functional Consequences in Human Tumors. *Clinical Cancer Research.* 2004; 10:1597–1604. [PubMed: 15014009]
27. Herman JM, Chang DT, Goodman KA, et al. Phase 2 multi-institutional trial evaluating gemcitabine and stereotactic body radiotherapy for patients with locally advanced unresectable pancreatic adenocarcinoma. *Cancer.* 2014
28. Jones S, Zhang X, Parsons DW, et al. Core Signaling Pathways in Human Pancreatic Cancers Revealed by Global Genomic Analyses. *Science.* 2008; 321:1801–1806. [PubMed: 18772397]
29. Vogelstein B, Papadopoulos N, Velculescu VE, Zhou S, Diaz LA, Kinzler KW. Cancer Genome Landscapes. *Science.* 2013; 339:1546–1558. [PubMed: 23539594]
30. Roberts NJ, Jiao Y, Yu J, et al. ATM Mutations in Patients with Hereditary Pancreatic Cancer. *Cancer Discovery.* 2012; 2:41–46. [PubMed: 22585167]
31. Williams JR, Zhang Y, Zhou H, et al. Overview of Radiosensitivity of Human Tumor Cells to Low-Dose-Rate Irradiation. *International Journal of Radiation Oncology*Biology*Physics.* 2008; 72:909–917.
32. Sener SF, Fremgen A, Menck HR, Winchester DP. Pancreatic cancer: a report of treatment and survival trends for 100,313 patients diagnosed from 1985–1995, using the National Cancer Database11No competing interests declared. *Journal of the American College of Surgeons.* 1999; 189:1–7. [PubMed: 10401733]

33. 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 US A*. 2011; 108:21188–21193.
34. Jiao Y, Shi C, Edil BH, et al. DAXX/ATRX, MEN1, and mTOR pathway genes are frequently altered in pancreatic neuroendocrine tumors. 2011; 331:1199–1203.
35. Ross JS, Wang K, Al-Rohil RN, et al. Advanced urothelial carcinoma: next-generation sequencing reveals diverse genomic alterations and targets of therapy. *Mod Pathol*. 2013; 27:271–280. [PubMed: 23887298]
36. Crane CH, Iacobuzio-Donahue CA. Keys to Personalized Care in Pancreatic Oncology. *Journal of Clinical Oncology*. 2012; 30:4049–4950. [PubMed: 23045599]
37. Jones S, Hruban RH, Kamiyama M, et al. Exomic sequencing identifies PALB2 as a pancreatic cancer susceptibility gene. *Science*. 2009; 324:217–217. [PubMed: 19264984]
38. Hartman DJ, Krasinskas AM. Assessing Treatment Effect in Pancreatic Cancer. *Archives of Pathology & Laboratory Medicine*. 2012; 136:100–109. [PubMed: 22208494]
39. Ogura T, Yamao K, Hara K, et al. Prognostic value of K-ras mutation status and subtypes in endoscopic ultrasound-guided fine-needle aspiration specimens from patients with unresectable pancreatic cancer. *J Gastroenterol*. 2012; 48:640–646. [PubMed: 22983505]
40. Siddiqui AA, Kowalski TE, Shahid H, et al. False-positive EUS-guided FNA cytology for solid pancreatic lesions. *Gastrointestinal Endoscopy*. 2011; 74:535–540. [PubMed: 21737075]
41. Varadarajulu S, Hasan MK, Bang JY, Hebert-Magee S, Hawes RH. Endoscopic ultrasound-guided tissue acquisition. *Digestive Endoscopy*. 2013; 26:62–69. [PubMed: 24033879]
42. Hébert-Magee S, Bae S, Varadarajulu S, et al. The presence of a cytopathologist increases the diagnostic accuracy of endoscopic ultrasound-guided fine needle aspiration cytology for pancreatic adenocarcinoma: a meta-analysis. *Cytopathology*. 2013; 24:159–171. [PubMed: 23711182]
43. Wu J, Matthaei H, Maitra A, et al. Recurrent GNAS Mutations Define an Unexpected Pathway for Pancreatic Cyst Development. *Science Translational Medicine*. 2011; 3:92.
44. Wagle N, Berger MF, Davis MJ, et al. High-Throughput Detection of Actionable Genomic Alterations in Clinical Tumor Samples by Targeted, Massively Parallel Sequencing. *Cancer Discovery*. 2012; 2:82–93. [PubMed: 22585170]
45. Raut CP, Grau AM, Staerckel GA, et al. Diagnostic accuracy of endoscopic ultrasound-guided fine-needle aspiration in patients with presumed pancreatic cancer. *J Gastrointest Surg*. 2003; 7:118–26. discussion 127–8. [PubMed: 12559193]

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 1×10^4 cells.

Figure 2. Histology and immunolabeling features of resected pancreatic cancer specimens used for simulated fine needle aspirates

(A) Hematoxylin and eosin staining of representative sections for each of the four 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.

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 one driver gene mutation was detected. For all patients a *KRAS* mutation was found, with the second gene either *TP53* (n=7, red), *SMAD4* (n=4, blue), or *TGFB2* (n=1, green). In the event that *TP53* and *SMAD4* mutations or *TP53* and *CDKN2A* mutations were both present, *TP53* values were used. The two-sided Spearman correlation coefficient (r) between the *KRAS* allele frequency and the second gene allele frequency was 0.45 ($p=0.017$).

Figure 4. Histology of a resected pancreatic cancer treated with stereotactic body radiation therapy

Abundant stromal fibrosis is present with only rare carcinoma cells seen (arrows). The extreme low cellularity of this specimen precluded targeted exome sequencing.

TABLE 1

Somatic variants identified in simulated fine needle aspirates and matched tissues. Three out of four simulated fine needle aspirates (FNA) 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 (^ahuman genome build 19), coding change and amino acid change is listed. Driver genes are underlined.

Sample	Gene	Genomic Coordinate ^a	Variant	AA change	Mutational Concordance
Patient 1	<i>HIF1A</i>	chr14:62199164	c.802G>T	p.Glu268*	80%
	<i>KRAS</i>	chr12:25398284	c.35C>T	p.Gly12Asp	
	<i>MTOR</i>	chr1:11187858	c.6039G>A	p.Ser2013Ser	
	<i>SMAD4</i>	chr18:48604750	c.1572G>T	p.Trp524Cys	
	<i>TP53</i>	chr17:7578190	c.659T>C	p.Tyr220Cys	
Patient 2	<i>BRD3</i>	chr9:136916706	c.477C>T	p.Prol159Pro	100%
	<i>KEAP1</i>	chr19:10602913	c.665T>C	p.Asn222Ser	
	<i>KRAS</i>	chr12:25398284	c.35C>A	p.Gly12Val	
	<i>TP53</i>	chr17:7577022	c.916G>A	p.Arg306*	
	<i>ZNF521</i>	chr18:22805083	c.2799G>A	p.Asn933Asn	
Patient 3	<i>EPHA7</i>	chr6:94120364	c.687G>C	p.Glu229Asp	100%
	<i>KRAS</i>	chr12:25398284	c.35G>A	p.Gly12Asp	
	<i>MLL3</i>	chr7:151896487	c.4150G>A	p.Asp1384Asn	
Patient 4	<i>KRAS</i>	chr12:25398284	c.35G>A	p.Gly12Asp	100%
	<i>XPC</i>	chr3:14199543	c.1840T>C	p.Phe614Leu	

TABLE 2
Immunohistochemical features of resected carcinomas

CDKN2A, TP53 and SMAD4 were all immunolabeled to observe the protein expression in the four simulated fine needle aspirates (FNA). When combined with sequencing data these provide ample molecular characterization of pancreatic tumors.

Patient ID	CDKN2A	TP53	SMAD4
1	Intact	Nuclear Accumulation	Intact
2	Lost	Lost	Lost
3	Lost	Lost	Lost
4	Lost	Normal	Lost

TABLE 3

Somatic alterations identified in clinical trial J1003 patients

Common variants identified in more than one 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.

Patient	KRAS	CDKN2A	TP53	SMAD4	ATM	TRIM33	ARID1A	GRM8	Other Variants
J1003-JH01	G12V	-	-	p.X553Arg	p.Glu73Gly	-	p.Ser402Pro	-	SMO
J1003-JH16	G12V	-	-	p.Ser242Pro	-	-	-	-	-
J1003-JH38	No mutations	-	-	-	-	-	-	-	-
J1003-JH10	G12V	-	-	p.Ile478Val	p.Trp488X	-	-	-	MLL3, FBXW7, SF3B1
J1003-JH12	G12V	p.Trp110X	p.Arg273His	-	-	-	-	-	KAT6B, KDM6A, PDE4DIP
J1003-JH04	-	-	p.Arg175His	-	-	-	-	-	NOTCH1, LTF, LPHN3
J1003-JH19	G12V	-	p.Ser215Ile	-	-	p.Ser422Gly	-	p.Ser219Leu	FOXO2, PAX3, UBR5
J1003-JH22	G12R	-	p.Arg100Gly	-	-	-	-	-	IKZF1, ZNF521
J1003-JH24	G12V	-	p.Arg342*	-	-	-	-	-	ADAMTS20, EP400, LTK, PDGFRB
J1003-JH35	G12L	-	-	-	-	-	-	-	CTNNB1, FLT4, IRS2, TET2, WT1
J1003-JH29	G12V	-	p.Ala69Val	p.Asp404Gly	-	-	p.Ser1488Pro	-	ROS1
J1003-JH30	-	-	-	-	-	-	p.Thr1198Ala	-	NF1
J1003-JH42	G12D	-	-	-	-	-	-	p.Arg607Cys	ERBB3, FLCN
J1003-JH45	G12D	-	-	-	-	-	-	-	TGFBR2
J1003-JH09	G12V	-	p.Leu194Arg	p.Arg361His	-	p.Gln521Arg	-	-	NOTCH4, MAGI1
J1003-JH34	G12D	-	p.Ile162Asn	-	-	-	-	-	SOX11
J1003-JH36	-	-	-	-	-	-	-	-	MTOR, IDH2, TSC1, AFF3, RPS6KA2
J1003-JH33	No mutations	-	-	-	-	-	-	-	-
J1003-JH41	G12D	-	p.Arg175His	p.Cys115Ser	-	-	-	-	EPHB4