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Mutational profiling of 103 unresectable pancreatic ductal adenocarcinomas using EUS-guided fine-needle biopsy

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ABSTRACT

Background and Objective: Pancreatic ductal adenocarcinoma (PDAC) is among the most lethal cancers, with a 5-year survival rate of around 9%. Only 20% are candidates for surgery. Most unresectable patients undergo EUS-guided fine-needle biopsy (EUS-FNB) for diagnosis. Identification of targetable mutations using next-generation sequencing (NGS) is increasingly requested. Data on feasibility of EUS-FNB for NGS and knowledge regarding mutational profile of unresectable PDAC are scarce. We evaluated the "technical yield" of EUS-FNB for NGS in unresectable PDAC: relative fraction of diagnostic EUS-FNBs meeting technical criteria. We also investigated the "molecular yield": relative fraction of EUS-FNBs included in NGS containing sufficient DNA for detection of at least one mutation. Furthermore, we determined the relative frequency of cancer-associated mutations in unresectable PDAC.

Patients and Methods: Formalin-fixed and paraffin-embedded EUS-FNBs diagnostic of unresectable PDAC and fulfilling these criteria were included (n = 105): minimum 3-mm^2 tissue, minimum of 2-mm^2 tumor area, and minimum 20% relative tumor area. NGS was performed using Ion GeneStudio S5 Prime System and Oncomine™ Comprehensive Assay v.3 including 161 cancer-related genes.

Results: Technical yield was 48% (105/219) and molecular yield was 98% (103/105). Most frequently mutated genes were *KRAS* (89.3%) and *TP53* (69.9%), followed by *CDKN2A* (24.3%), *ARID1A* (9.7%), *SMAD4* (7.8%), *TSC2* (7.8%), and *CCND3* (6.8%).

Conclusion: EUS-FNB for NGS of unresectable PDAC is feasible. Our technical criteria for NGS, using leftovers in formalin-fixed and paraffin-embedded blocks after routine pathology diagnosis, were met by around half of EUS-FNBs. Almost all EUS-FNBs fulfilling the technical criteria yielded a successful NGS analysis.

Keywords: Pancreas; EUS; Fine-needle biopsy; Mutational profiling; Next-generation sequencing

INTRODUCTION

Pancreatic cancer (PC) is a highly lethal disease and currently the fourth leading cause of cancer-related death in Europe.[1,2] Most frequent type is pancreatic ductal adenocarcinoma (PDAC) with a 5-year survival rate of only 9%.[3] Surgical resection is the only treatment leading to long-term survival but can only be offered around 20%, and median survival is still only 24 months.[3] The high mortality is mainly due to the late disease stage at time of diagnosis, with metastases in 50% of cases.[4] Another plausible issue is that no predictive markers and no individualized treatment can be offered to the majority of PDAC patients. Therefore, identification of useful predictive markers is of great interest.

From a clinical perspective, PDAC is often viewed as a homogeneous disease, and patients are often offered the same standard treatment. Some PDACs, however, do harbor druggable genetic changes, some of which may be detected by next-generation sequencing (NGS), enabling personalized treatment.[5–7] O'Reilly et al. reported that patients with *gBRCA/PALB2*$^+$ PDAC had successful treatment with platinum-based chemotherapy (cisplatin and gemcitabine). However, poly(adenosine diphosphate–ribose) polymerase inhibitors (such as veliparib) did not improve the response rate.[6,7] Moreover, patients with microsatellite instability–high or mismatch repair deficiency have benefited from immune checkpoint inhibitor treatment.[5,6] Oncologists are increasingly requesting mutational profiling of PDAC.[8] Research regarding mutational profile of unresectable PDAC is sparse.[9,10]

Fine-needle aspiration (FNA) has traditionally been used for diagnosis of PDAC.[11,12] However, EUS-FNA seems to be less efficient for NGS studies compared with EUS-guided fine-needle biopsies (EUS-FNBs).[13,14] EUS-FNB can obtain primary tumor tissue from locally advanced and/or metastatic PDAC for ancillary studies, which previously has been complicated or impossible.[15–17] EUS-FNB produces tissue cylinders enabling additional immunohistochemical staining, special stains, and molecular analyses.[16–18]

The present study used EUS-FNBs from patients with unresectable PDAC for mutational profiling. The aims were to evaluate the utility of EUS-FNB for NGS, in terms of technical and molecular yield.

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We also evaluated frequency of cancer-associated mutations in unresectable PDAC.

MATERIALS AND METHODS

Study cohort and tissue specimens

All patients who underwent EUS-FNB at the Department of Surgery, Odense University Hospital, Denmark, in the period September 1, 2019, to December 31, 2022, were included in a search in the Danish Pathology Registry. The search terms used were "T59*" (pancreas) and "P30992" (FNB).

Technical criteria for inclusion of a given EUS-FNB in the NGS cohort were as follows: use of a 22-gauge SharkCore needle for EUS-FNB procedure and specimen diagnostic of PDAC. The formalin-fixed and paraffin-embedded (FFPE) block had to contain ≥3-mm^2 tissue, ≥2-mm^2 total tumor area, and ≥20% relative tumor area. Exclusion criteria were as follows: (malignant) EUS-FNB diagnosis other than PDAC and prior therapy against PDAC. Area measurements were done using digital whole-slide images produced by Hamamatsu Nanozoomer AQ2 S360 slide scanners (Hamamatsu Photonics, Hamamatsu, Japan), from 2021[19] using Image Management System (Sectra, Odense, Denmark) and prior to May 2021 using NDP.view 2.7.25 software (Hamamatsu Photonics). CT scans and EUS reports were checked by a pancreatic expert surgeon (MBM) who found no indications of another periampullary origin than the pancreas.

This project was approved by Danish National Ethics Committee (project ID 2006353, document ID 2226594), Region of Southern Denmark's Ethics Committee (project ID S-20220015), registry of research projects (journal ID 22/25602), and Strategic Research Council (journal ID 21/13792 and 23/19192). We ensured that the patients had not advocated against the use of their tissue for research ("Vævsanvendelsesregisteret").

EUS-FNB procedure and specimen preparation

The EUS-FNB procedure was carried out as described previously.[20] Specimens were fixed in formalin (6–24 hours) and embedded in paraffin. Eleven to 13 serial 3-µm-thick sections were cut from the paraffin blocks. Sections 1 and 11 (or 13) were stained with hematoxylin-eosin, whereas the sections between were initially left unstained. It was at the discretion of the pathologist whether additional hematoxylin-eosin, immunohistochemical, or special staining was necessary for routine diagnostics. Prior to section 11 (or 13), one 10-µm-thick section was cut and frozen at –80°C.

EUS-FNB diagnosis

EUS-FNB pathology reports were reviewed, and the following data were extracted: date of biopsy, gender, age, histological diagnosis, macroscopic features, and microscopic findings. Every specimen was categorized using Papanicolaou Society of Cytopathology Terminology System, as described previously[15,21]: malignant, suspicious of malignancy, neoplastic: other, neoplastic: benign, atypical, benign, or nondiagnostic.

Purification of DNA

From each FFPE block, 6 × 10-µm sections were cut with a microtome. Macrodissection was not performed due to often diffuse distribution of tumor cells in the EUS-FNB material. A total of 12/105 specimens were rerun: Eight samples for technical reasons related to the sequencing procedure (even though sufficient amounts of DNA were extracted) and four samples because no mutations were detected in the first NGS analysis. From these specimens, an additional 6 × 10-µm FFPE sections were cut for DNA extraction. Sequencing of the eight samples rerun due to technical reasons was all successful. From two of the four samples rerun because no mutations were detected initially, not enough tumor tissue was left precluding a new analysis, whereas from the remaining two specimens, sufficient DNA was extracted and NGS was successfully performed.

Purification of DNA from paraffin-embedded tissue blocks was carried out using GeneRead™ DNA FFPE kit (Qiagen, Hilden, Germany) cat. no. 180134, n = 15) or QiAamp DNA FFPE Advanced Kit (cat. no. 56604, n = 43), which are identical. Due to temporary supply shortage, the QiAamp DNA FFPE Tissue Kit (cat. no. 56404, n = 47) also was used. Two DNA concentration measurements were made, the first after tissue purification (using qualitative polymerase chain reaction and obtained as ng/µL) and the second prior to Ion Chef setup (using qualitative polymerase chain reaction, obtained as pM). We succeeded in purifying DNA for all samples, with great variation between DNA concentrations (Table 1).

GeneRead DNA FFPE procedure was used to remove paraffin and reverse formalin cross-links from the DNA before it was bound to the QIAamp MinElute column (Qiagen). Subsequently, deaminated cytosine residues were removed. Residual contaminants such as salts were removed by washing with two different wash buffers followed by ethanol. Any residues from the ethanol were removed by a supplemental centrifugation step. At last, DNA was eluted and extracted in a total volume of 100 µL.

Table 1

Overview of total tissue area, total tumor area, relative tumor area, and DNA concentrations achieved in 103 EUS-FNBs with unresectable PDAC.

		Total tissue area, mm^2	Total tumor area, mm^2	Relative tumor area, %	DNA concentration, RNaseP ng/µL	DNA concentration, pM
Median		9.5	5	63	0.2	675.5
Range	Minimum	3	2	20	0.1	0.1
	Maximum	48	43	100	1.9	5930.5
Interquartile range	Q1	6	3	47	0.1	36.0
	Q2	9.5	5	63	0.2	675.5
	Q3	12	9	80	0.3	1840.6

EUS-FNB: EUS-guided fine-needle biopsy; PDAC: pancreatic ductal adenocarcinoma.

Targeted NGS

For NGS, sequencing platform Ion GeneStudio S5 Prime and Oncomine™ Comprehensive Assay v. 3 including 161 cancer-related genes were used. Sequencing depth was set to 2.000x to 6.000x. The following materials were included: Ion AmpliSeq Library Kit 2.0, Ion Express Barcode Adapter Kit, Ion Library Quantitation Kit, and Ion 540 Kit Chef (Life Technologies (Carlsbad, California, USA)). All raw data were stored in the IonReporter server, and variants identified were visualized by Golden Helix GenomeBrowser V.3.0.0 software (GoldenHelix, Montana, USA). After processing raw data in GenomeBrowser software, ClinVar, OncoKB, and dbSNP were applied to examine all variants, which were classified using the American College of Medical Genetics and Genomics guidelines as pathogenic, likely pathogenic, variants of uncertain significance (VUS), likely benign, or benign.[22] Pathogenic variants are well established as disease-causing, whereas VUS hold uncertain significance, meaning that evidence is insufficient or conflicting. A benign variant is defined as not responsible for causing disease.[22] All hotspot mutations in *KRAS*, *TP53*, *SMAD4*, and *CDKN2A* were included, and for other variants, a 10% cutoff was set in relation to allele ratio, as well as a minimum coverage of 500. Identified mutations were classified as missense, frameshift, or nonsense: missense mutations result in the substitution of one amino acid with another due to a single nucleotide being replaced. Frameshift mutations are insertions or deletions of nucleotides in numbers not multiples of three. Nonsense mutations are insertions or deletions resulting in a stop codon.

RESULTS

Patient characteristics, demographics, and EUS-FNB diagnoses

The original search included 631 EUS-FNBs from 546 patients [Figure 1]. Sixty-two patients underwent two, 10 patients underwent three, and one patient underwent four EUS-FNB procedures. Five hundred twenty-six specimens did not fulfill the inclusion criteria. Hence, 105 EUS-FNBs were included in the NGS analysis [Figure 1]. Demographics and absolute and relative frequencies of diagnoses are shown in Table 2. Of the 12 patients with an initial EUS-FNB diagnosis of "suspicious of malignancy," 10 patients were rebiopsied at a later date. Of these, seven were diagnosed with PDAC, one with adenosquamous carcinoma, one with atypical changes, and one was nondiagnostic.

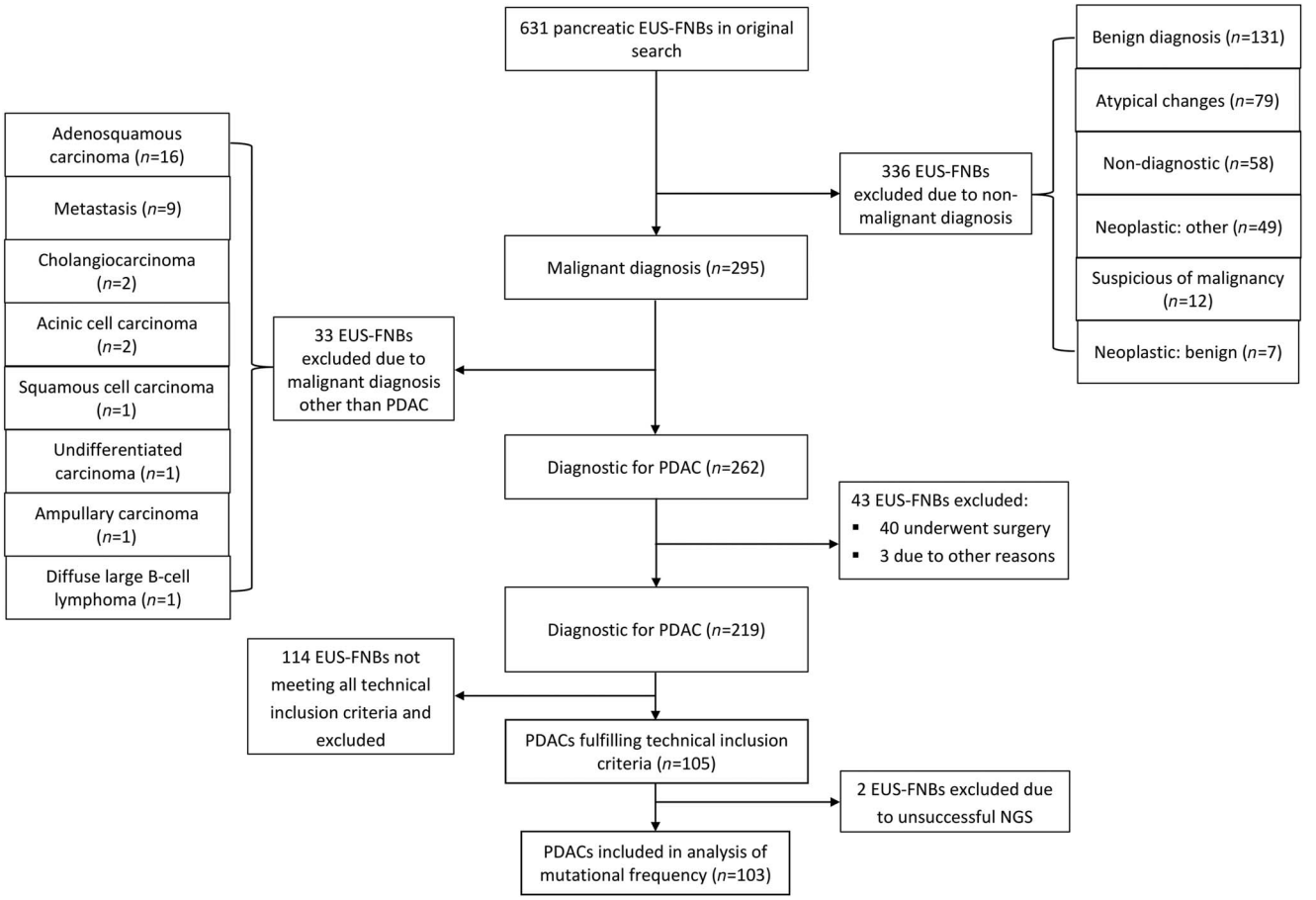

Figure 1. Flowchart illustrating our series of consecutive pancreatic EUS-guided fine-needle biopsies (FNBs). The original search included 631 EUS-FNBs. Due to a nonmalignant diagnosis, 336 specimens were excluded. Thirty-three specimens were excluded due to malignant diagnosis other than pancreatic ductal adenocarcinoma (PDAC). In total, 262 EUS-FNBs were diagnostic of PDAC. Of these, 40 patients underwent pancreatic surgery, three were excluded for other reasons, and 114 did not fulfill the technical specimen inclusion criteria. Hence, in total, 105 pancreatic SharkCore EUS-FNBs with a diagnosis of PDAC were included in the next-generation sequencing (NGS) analysis, whereas two EUS-FNBs were excluded due to unsuccessful sequencing.

Table 2

Demographics and absolute and relative frequencies of diagnoses established on 631 pancreatic EUS-FNBs from 546 patients.

Patients/diagnosis	*n* (%)
Age, median (range), y	70 (12–87)
Sex, male	288 (53)
Sex, female	258 (47)
EUS-FNB diagnosis, *n* (%)	631 (100)
Malignant	**295 (46.7)**
PDAC	262 (89)
Adenosquamous carcinoma	16 (5.4)
Metastasis	9 (3.0)
Cholangiocarcinoma	2 (0.7)
Acinic cell carcinoma	2 (0.7)
Squamous cell carcinoma	1 (0.3)
Undifferentiated carcinoma	1 (0.3)
Ampullary carcinoma	1 (0.3)
Diffuse large B-cell lymphoma	1 (0.3)
Suspicious of malignancy, *n* (%)	**12 (1.9)**
Neoplastic: benign, *n* (%)	**7 (1.1)**
SCA	7 (100)
Neoplastic: other, *n* (%)	**49 (7.8)**
NET	26 (53.0)
IPMN	14 (28.6)
PanIN*	4 (8.2)
MCN	4 (8.2)
SPN	1 (2.0)
Atypical, *n* (%)	**79 (12.5)**
Atypical	34 (43.0)
Dysplasia	34 (43.0)
Unspecific reactive changes	11 (14.0)
Benign, *n* (%)	**131 (20.8)**
Chronic pancreatitis	60 (45.8)
Unspecific fibrosis	28 (21.4)
AIP†	16 (12.2)
Pseudocyst	13 (9.9)
Normal pancreas	9 (6.9)
Fat necrosis	4 (3.0)
GPA	1 (0.8)
Nondiagnostic, *n* (%)	**58 (9.2)**

*PanIN, two PanIN 1 and two PanIN 2.

†Eleven patients had type 1 AIP (68.8%, seven level 1 International Consensus Diagnostic Criteria (ICDC), four level 2 ICDC), and five patients had type 2 AIP (31.2%, two level 1 ICDC and three level 2 ICDC).

AIP: autoimmune pancreatitis; EUS-FNB: EUS-guided fine-needle biopsy; GPA: granulomatosis with polyangiitis; IPMN: intraductal papillary mucinous neoplasm; MCN: mucinous cystic neoplasm; NET: neuroendocrine tumor; PanIN: pancreatic intraepithelial neoplasia; PDAC: pancreatic ductal adenocarcinoma; SCA: serous cystadenoma; SPN: solid-pseudopapillary neoplasm.

Technical and molecular yield

Of EUS-FNBs fulfilling clinical and diagnostic criteria, 48% (105/219) fulfilled the technical inclusion criteria ("technical yield") and were included in NGS analysis. The "molecular yield," defined as the relative number of EUS-FNBs from which NGS detected at least one variant, was 98% (103/105). Patient characteristics and demographics for these 103 patients are shown in Table 3.

Mutational profiling using targeted NGS

Targeted NGS detected 314 mutations in 49 different genes, of which 64.3% (202/314) were classified as pathogenic variants and 35.7% (112/314) as VUS [Figure 2]. The most frequently mutated gene *KRAS* was altered in 89.3% (92/103), followed by *TP53* in 69.9% (72/103), *CDKN2A* in 24.3% (25/103), *ARID1A* in 9.7% (10/103), *SMAD4* and *TSC2* each in 7.8% (8/103), and *CCND3* in 6.8% (7/103) [Figure 3A]. In Figure 3B, the relative frequency of identified pathogenic mutations and of VUS are shown separately.

Figure 4A illustrates the number of variants found within each of the tumors. Figure 4B represents the number of driver gene mutations (*KRAS*, *TP53*, *CDKN2A*, and *SMAD4*) per tumor.

Characterization of KRAS variants

As expected, *KRAS* was the most frequently mutated gene (89.3% [92/103]). Most frequent *KRAS* variants are shown in Figure 5A.

Characterization of TP53 mutations

Targeted NGS detected 80 *TP53* mutations in 72 different PDACs. Of these, 79% (62/80) were missense mutations, with the remaining being frameshift (11% [9/80]) or nonsense (10% [8/80]) mutations [Figure 5B]. Localization of mutations in the coding sequence of exons 2–10 is shown in Figure 5B. Frameshift mutations were most frequently observed in exon 4 (45% [4/9]) and exon 7 (22% [2/9]). One *TP53* frameshift mutation was detected in exons 5, 6, and 9 (11% [1/9]). Nonsense mutations were most prevalent in exons 6 (50% [4/8]) and 10 (37.5% [3/8]). One nonsense mutation was found in exon 5 (12.5% [1/8]). In 93% (66/71) of tumors that harbored a *TP53* mutation, also a *KRAS* mutation was identified [Figure 2].

Characterization of CDKN2A and SMAD4 mutations

We found a *CDKN2A* mutation in 24.3% (25/103) and a *SMAD4* mutation in 7.8% (8/103) [Figure 2A]. All PDACs with *SMAD4* mutations had a concurrent *KRAS* mutation. Among tumors with a *CDKN2A* mutation, a concomitant *KRAS* mutation was found in 95.8% (23/24) [Figure 1]. Because *SMAD4* had a relatively low mutational frequency, we reviewed the pathology records for each of the 103 EUS-FNBs and found that SMAD4 protein expression by immunohistochemistry was examined in 58.1% (60/103) of PDACs. Among these cases, SMAD4 expression was abnormal (absent) in 73.3% (44/60).

Homologous recombination DNA damage repair gene mutations in PDAC

Several genes serve in the homologous recombination repair, such as *ARID1A*, *BRCA1*, *BRCA2*, *PALB2*, *ATM*, *ATR*, *CHEK2*,

Table 3

Characteristics and demographics of 103 patients who underwent EUS-FNB with unresectable PDAC and who were included in our mutational profiling.

Patients number, total		103 (100)
Age, median (range), y		72 (44–86)
Sex, *n* (%)	Male	52 (51)
	Female	51 (49)
Location, *n* (%)	Head	64 (62)
	Neck	5 (5)
	Body	26 (25)
	Tail	8 (8)
Needle type		SharkCore
Needle size		22-gauge

EUS-FNB: EUS-guided fine-needle biopsy; PDAC: pancreatic ductal adenocarcinoma.

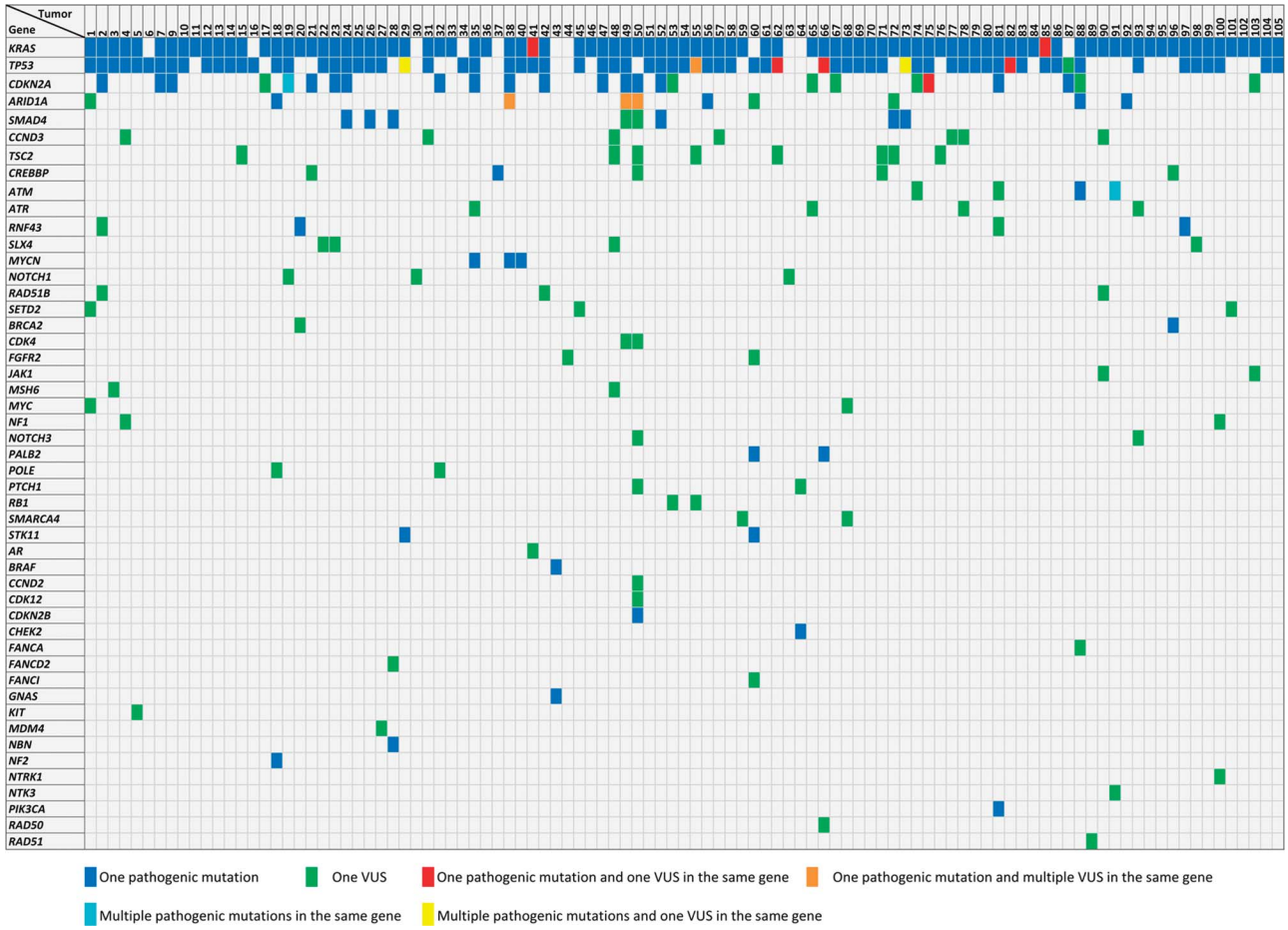

Figure 2. Results from mutational profiling of 103 pancreatic EUS-guided fine-needle biopsies (EUS-FNBs) with a diagnosis of unresectable pancreatic ductal adenocarcinoma (PDAC). Oncoplot representing gene mutations found in each tumor. VUS: variant of uncertain significance.

RAD51, *NBN*, *RAD50*, *RAD51(B)*, and *FANC*.[23,24] Mutations in these genes can result in HR deficiency (HRD). Our NGS analysis revealed mutations in several of these genes: *ARID1A* in 9.7% (10/103), *ATR* in 3.9% (4/103), *ATM* in 3.9% (4/103), *RAD51B* in 2.9% (3/103), *BRCA2* in 1.9% (2/103), *PALB2* in 1.9% (2/103), and *CHEK2*, *NBN*, *RAD50*, *RAD51*, *FANCA*, *FANCD2*, and *FANCI* each in 1% (1/103) [Figure 2]. In total, 24.3% (25/103) of the PDACs showed at least one mutation in these genes.

DISCUSSION

Based on our targeted NGS study of unresectable PDAC using EUS-FNBs, the relative fraction of PDAC EUS-FNBs that met our technical inclusion criteria was 48% (105/219). Sufficient DNA for mutational profiling enabling detection of at least one mutation could be achieved from 98% (103/105). Most frequently mutated genes were *KRAS* (89.3%), *TP53* (69.9%), *CDKN2A* (24.3%), *ARID1A* (9.7%), *SMAD4* and *TSC2* (each in 7.8%), and *CCND3* (6.8%). *KRAS* mutations most often occurred at codon 12 (91%), and the most frequent variants were p.Gly12Asp (45%), p.Gly12Val (28%), and p.Gly12Arg (15%). Mutations of *TP53* were mainly found at exons 4–8.[25,26]

Most genomic testing of PDAC, to date, has been conducted using surgical resection specimens. However, of all patients with this malignancy, only 20% have resectable disease, whereas most patients present locally advanced or metastatic disease.[27,28] Hence, our knowledge regarding mutational profile in primary tumors of unresectable PDACs is limited. However, targeted NGS based on EUS-FNBs has been used in a few recent studies.[13,14,29,30] This is also relevant to identify targetable genes, such as *KRASwt*, (opening for targeting of for example EGF receptor), *KRASG12C*, HRD genes (ie, *BRCA1/2*, *PALB2*, *CHECK2*, *ATM*).[7,31–33] The technical yield in the present study was rather low (48%), but biopsies were performed with the best EUS-FNB needle available at the moment. This is primarily due to needle design (cutting end of needle) rather than size. We know that EUS-FNB increases the risk of complications (eg, bleeding and acute pancreatitis) when compared with EUS-FNA.[15] This means that the needle size probably cannot be increased without sacrificing the safety profile of EUS biopsy. Number of punctures could be increased, but this may also lead to more complications. Research that may result in improved technical yield must include not only needle design but also biopsy technique (fanning *vs.* 1-step), specimen handling from needle to transport medium, and the subsequent analysis. Target location, transduodenal/gastric biopsy, and EUS experience are other important factors that may influence technical yield.

Park et al. reported a relative inclusion rate of 69.9%, which is a higher rate than in the present study, but precise technical inclusion

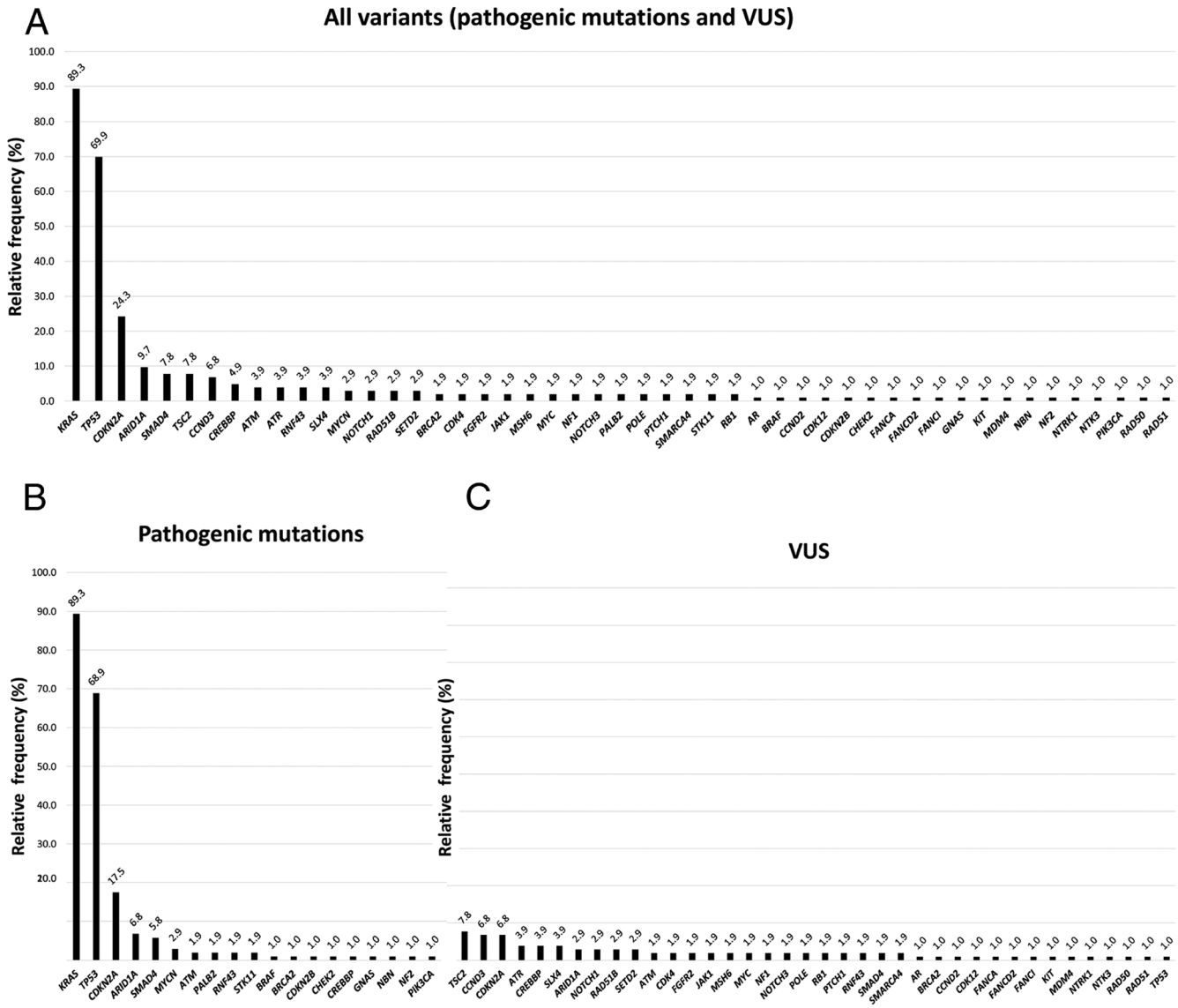

Figure 3. Relative frequency of 103 unresectable pancreatic ductal adenocarcinomas (PDACs) harboring alterations in cancer-associated genes, identified by next-generation sequencing (NGS). (A) Relative frequency of tumors harboring at least one pathogenic mutation and/or at least one variant of unknown significance (VUS). (B) Relative frequency of tumors harboring at least one pathogenic mutation. (C) Relative frequency of tumors harboring one or several VUS but no concomitant pathogenic mutations.

criteria were not specified.[10] Park et al. found at least one mutation in 114 of 116 samples (98.3%), consistent with our findings. Redegalli et al. reported that mutational profiling was successful in 88% (67/76) of cytological smears from EUS-FNAs, corresponding to their molecular yield, whereas the technical yield was not reported.[9]

Several studies have examined the mutational profile of PDAC in resectable and, to a much lesser extent, unresectable PDAC. Park et al. examined a total of 166 patients with PDAC, of both resectable (n = 13) and unresectable (n = 103) stage, using the CancerScan panel v1 consisting of 83 genes.[10] They found mutations in *KRAS* (90%), *TP53* (77%), *CDKN2A* (31%), *SMAD4* (29%), *ARID1A* (14%), *ATM* (11%), and *STK11* (7%). Their higher frequency of *SMAD4* mutations, compared with the present study, could in part be explained by that they were able to include copy number variation analysis of several genes, including loss of *SMAD4*.[10]

Redegalli et al. examined the mutational profile of two independent PDAC cohorts, using the Oncomine Comprehensive Assay v.3 DNA panel. Cohort 1 consisted of 77 patients with resectable PDAC, from 56 of whom cytological EUS-FNAs obtained at the time of diagnosis were analyzed. Cohort 2 consisted of 20 EUS-FNAs with unresectable PDAC.[9] For cohort 1, mutations in cytological smears were reported in *KRAS* (90%), *TP53* (52%), *CDKN2A* (19%), *SMAD4* (7%), *ATM* (5%), *BRCA2* (5%), and *RB1* (5%). For cohort 2, mutations were found in *KRAS* (80%), *TP53* (45%), *CDKN2A* (15%), *RNF43* (10%), *SMAD4* (5%), *SF3B1* (5%), *ERBB2* (5%), *BRCA2* (5%), and *PIK3CA* (5%). We found low-frequent variants in several additional genes.[9] Although data show conflicting results, we believe that EUS-FNB is superior compared with EUS-FNA, as data show that histological specimens have a higher diagnostic yield.[9,13,30,34]

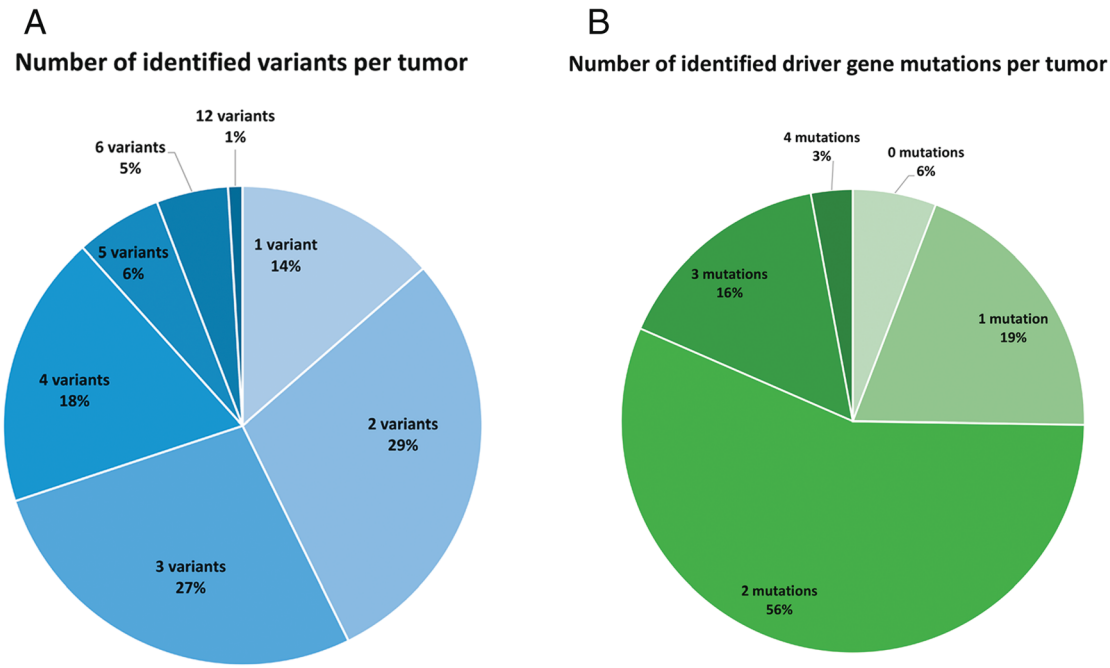

Figure 4. Number of genetic alterations per tumor, identified by targeted next-generation sequencing (NGS) in 103 unresectable pancreatic ductal adenocarcinomas (PDACs). (A) Total number of identified genetic alterations per tumor. Between 0 and 12 variants were identified. The figure includes both pathogenic mutations and variants of unknown significance (VUS). (B) Total number of identified pathogenic driver gene mutations, *KRAS*, *TP53*, *CDKN2A*, and *SMAD4*, per tumor. Between 0 and 4 pathogenic driver gene mutations were identified.

We observed *KRAS* mutations to most frequently occur at codon 12 or 61, which is consistent with prior studies, reporting codons 12, 13, and 61 as hotspots in PDAC.[35] *KRAS* mutations are mostly observed in G12D (>40%), G12V (>30%), and G12R (>15%),[36–40] consistent with our results.

In our cohort, *TP53* was the second most frequently mutated gene, in accordance with previous studies.[9,10,41] Rivlin et al. established that mutations in *TP53* are distributed in all coding exons and observed a strong predominance in exons 4–9, the DNA-binding domain of the protein.[42] In many types of cancer including PDAC, around 30% of *TP53* mutations can be designated to 6 "hotspot" residues (Arg175, Gly245, Arg248, Arg249, Arg273, and Arg282).[42,43] We detected five of these six residues, in 30% (24/80) of *TP53* gene mutations, which all were located in exons 4–9.

We found *CDKN2A* mutations in 24.3%, in accordance with previous reports (14%–40%) of resectable[6,9,38,41,44,45] and unresectable PDACs.[9,41] Takano et al. examined 58 EUS-FNA or EUS-FNB specimens, predominately with unresectable PDAC, and found a *CDKN2A* mutation in 14%.[41] Redegalli et al. reported *CDKN2A* mutation in 15% of unresectable and 14%–19% of surgically resected PDACs.[9]

The fourth most frequently mutated gene identified in our study was *ARID1A* (9.7%). Analysis of 24 surgically resected PDACs by targeted NGS of 116 genes found *ARID1A* to be altered in 9%.[46] Likewise, Witkiewicz et al. examined 109 surgically resected PDACs with whole-exome sequencing and found *ARID1A* mutations in 6%.[38] Hence, our findings in unresectable PDAC are similar to those reported in surgically treated PDAC.

We identified *SMAD4* mutations in 7.8% (8/103), even though *SMAD4* is reported to be inactivated in 20%–50% of PDACs, due to homozygous deletion (up to 30%) or loss of one allele, coupled with intragenic alteration in the second allele (around 20%).[47,48] Redegalli et al. reported *SMAD4* mutations in 7%–17% of resectable PDAC and 5% of unresectable tumors[9]. Another study found *SMAD4* mutations in up to 26% of unresectable PDACs.[41] In this study, also copy number variation analyses, enabling inclusion of gains and losses of *SMAD4*, were included, which may explain the relatively high rate. We found loss of SMAD4 protein in 73.3% of the EUS-FNBs where immunohistochemistry for this protein was available, supporting that homozygous deletion of *SMAD4* may, in fact, account for a high number of the *SMAD4* alterations often reported in the literature.[48–50]

We also detected mutations in *TSC2* (7.8%) and *CCND3* (6.8%). Singhi et al. performed targeted NGS of primary and metastatic PDAC using EUS- or computed tomography (CT)–guided biopsy or surgical resection specimens. They reported *CCND3* mutations in 2%, which is at a slightly lower frequency than what we observed. Interestingly, Singhi reported that *CCND3* mutations were often related to liver metastasis, which could indicate that mutational events in the *CCND3* gene occur at late stages.[51] *TSC2* mutations have been detected in 1.5% of PDACs.[52] In PDAC, also mutations in HR-DDR (homologous recombination DNA damage repair) genes may occur. *BRCA1/2*, *PALB2*, and other HRD genes are found in 5%–10% of all patients with PDAC.[44,53,54] Park et al. reported HRD mutations in *ARID1A* (14%), *ATM* (11%), and *BRCA2* (4%).[10] We observed *BRCA2* mutations in 1.9%. *BRCA* mutations are commonly mutated in familial PC and increase the susceptibility to PDAC.[53] *BRCA1/2* mutations are

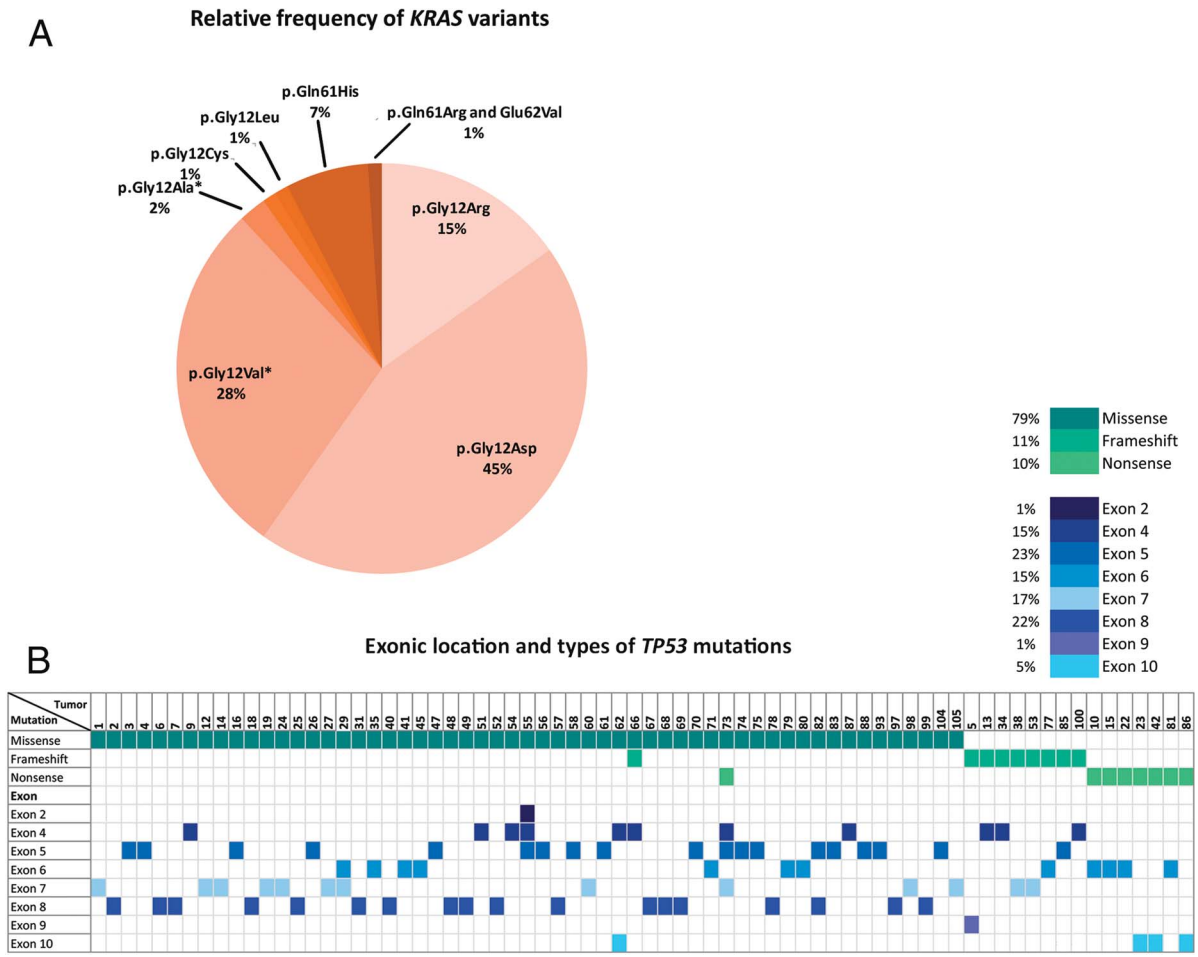

Figure 5. Detailed information regarding *KRAS* and *TP53* mutations in our series of 103 unresectable pancreatic ductal adenocarcinomas (PDACs). (A) Distribution of 92 *KRAS* variants observed. *Two PDAC had, in addition to a pathogenic mutation, also a VUS (p.Val9Ala and p.Val160Met). (B) Exonic location and mutation types of *TP53* mutations identified in 72 PDACs.

found in approximately 2%–9% of all PDACs.[44,55–57] Patients with germline mutations have 3- to 10-fold increased risk of developing PDAC,[58–61] which is why patients (and first-degree relatives) who have been diagnosed with a *BRCA* germline mutation should be offered genetic counseling. In addition to EUS-FNB, peripheral blood is needed, to determine whether the mutation is hereditary or sporadic. In the two *BRCA2*-mutated PDACs in our series, the allele ratio was roughly 50%, which may support that these were germline mutations. Olaparib has been approved by the European Medicines Agency and the US Food and Drug Administration as monotherapy for the maintenance treatment of adult patients with germline *BRCA1/2* mutations who have metastatic PDAC and have not progressed after a minimum of 16 weeks of platinum-based treatment within a first-line chemotherapy regimen.[62]

We found *ATM* mutation in 3.9%, a gene indispensable in sensing and repair of DNA damage.[63] Our findings are comparable to published studies, reporting *ATM* variants in up to 6%.[64,65] Loss of *ATM* function is an independent prognostic factor associated with poor overall survival in resectable PC.[65–67] *ATM* exerts its functions together with *ATR*, which in our cohort also was mutated in 3.9%. In our series, *PALB2* was mutated in 1.9%. Others reported *PALB2* mutations in 0.2% to 1.1%.[64] Studies based on families with germline *PALB2* pathogenic variants and intraductal papillary mucinous neoplasms harboring a germline *PALB2* mutation reported increased risk of PDAC[68]. The prevalence of *PALB2* mutations in patients with familial PC is 1%–4%.[69,70] Other HR-DDR gene mutations observed in our cohort had relative frequencies in agreement with previously reported studies.[24,64,71] Mutations in such genes can be predictive of increased sensitivity to platinum and poly(adenosine diphosphate–ribose) polymerase inhibitors.[72] However, whether targeted treatment of such mutations may benefit patients with unresectable PDAC remains to be elucidated in future prospective trials. This also concerns *KRASwt* and *KRASG12C* PDACs.[7,31] Our data support that such trials may include targeted NGS of FFPE EUS-FNBs. It is also of principal interest to investigate whether certain combinations of mutations hold value as predictive markers, as the most frequent mutations are currently not targets for therapy of PDAC. However, it is still relevant to investigate for mutations in the driver genes of PDAC, as *KRAS*wt patients hold a better prognosis.[73,74] Furthermore, in *KRAS*wt patients, often alternative driver events are present, potentially representing targets for treatment.

Our 112 different VUS were observed in 39 different genes. VUS were included in our study as we at the time of interpretation did

not know whether these variants are pathogenic or benign, and based on the scant knowledge of these genes and the few studies available regarding the mutational profile of unresectable PDAC, we do not presently know their clinical significance. Because the molecular profile of unresectable PDAC has so far only been rarely studied, we chose to include VUS in our report. Many other studies in the field do not mention how VUS were handled.

Dorman et al. examined 165 PC specimens using either the Oncomine Focus Assay including 52 genes or the Oncomine Comprehensive Assay v3.[75,76] At initial diagnosis, 60% of patients presented metastatic and 37% resectable or locally advanced disease (3% missing). PDAC was the diagnosis in the large majority of patients (96.4%), whereas 3.6% had rare histological subtypes, such as adenosquamous carcinoma, sarcomatoid carcinoma, and acinic cell carcinoma. This study is an example of "clinical action taken" on identified targetable mutations in PDAC. Although 95 patients presented targetable mutations (such as *KRASwt*, *KRASG12C*, and *BRCA1/2)*, only three patients were recommended a corresponding treatment.[75] Reasons for this may be due to the short survival and rapid deterioration of the disease. Moreover, off-label treatments may be delayed due to the need to apply for cost coverage by insurance companies in advance. In addition, doctors' decisions are also influenced by low levels of evidence, which only emphasizes the need for more clinical trials regarding targeted treatments in PC.

Among possible limitations of this study, it should be mentioned that it was retrospective and that most tumors were located in the pancreatic head. As the tumors were unresectable, it cannot be totally excluded that a few of them may have origin in another periampullary organ, such as the ampulla of Vater or the intrapancreatic common bile duct, which may have affected the results.[77–79] However, we find this risk rather low,[77,78] as all our patients were thoroughly examined, with EUS in addition to contrast-enhanced CT, and as the patient records including CT scans and EUS reports were checked by a pancreatic expert surgeon who found no indications of another periampullary origin than the pancreas. Other possible limitations are the use of FFPE tissue, which has its challenges compared with fresh frozen tissue when performing NGS analysis. Besides, we had to use three DNA extraction kits in this study, and one of them did not contain Uracil-DNA glycosylase (UNG) and was applied to almost half of the samples, but it was our impression that this did not perform significantly different. Among the strengths of the present study, the relatively large study cohort and strict inclusion and exclusion criteria should be mentioned, resulting in a homogeneous cohort of EUS-FNBs from patients with unresectable PDAC. Furthermore, we consequently classified all identified variants according to the American College of Medical Genetics and Genomics. This study represents a real-life setting of leftovers in FFPE blocks with EUS-FNBs with PDAC after completion of diagnostic pathology. Only few other studies have used such diagnostic pancreatic biopsies with unresectable PDAC for mutational profiling, even though the vast majority of PDAC patients are not candidates for surgery.

CONCLUSION

Based on our data, it can be concluded that targeted NGS based on EUS-FNB is feasible in unresectable PDAC, using leftovers in the FFPE block, after initial histological diagnosis. We found that almost 50% of EUS-FNBs diagnostic of PDAC met our technical criteria to be included in NGS analysis. At least one mutation was detected in almost all EUS-FNBs in which NGS was performed (98%). The most frequently mutated genes in our cohort of unresectable PDAC were *KRAS* (89.3%), *TP53* (69.9%), *CDKN2A* (24.3%), *ARID1A* (9.7%), *SMAD4* (7.8%), *TSC2* (7.8%), and *CCND3* (6.8%). Future studies should evaluate whether the technical inclusion criteria for EUS-FNB specimens to be included in NGS analysis can be less strict, which would enable targeted mutational profiling in a higher number of patients with unresectable PDAC. It should also be investigated which mutation constellations could lead to new therapeutic approaches in PDAC patients.

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Conflicts of Interest

The authors declare that they have no financial conflict of interest with regard to the content of this report.

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Author Contributions

Julie Buchberg did the data curation, investigation, validation, methodology, visualization, writing-original draft, editing, and writing—approval of final version of manuscript. Karin de Stricker did the conceptualization, investigation, validation, methodology, and writing—approval of final version of manuscript. Per Pfeiffer did the conceptualization, methodology, and writing—approval of final version of manuscript. Michael Bau Mortensen did the conceptualization, methodology, writing—approval of final version of manuscript. Sönke Detlefsen did the conceptualization, data curation, investigation, methodology, visualization, writing—review, supervision (main supervisor), editing, primary responsibility for final content, writing-approval of final version of manuscript.

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