Diagnosis of pancreatic lesions collected by endoscopic ultrasound-guided fine-needle aspiration using next-generation sequencing

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Abstract. Endoscopic ultrasound-guided fine-needle aspiration (EUF-FNA) has improved the diagnosis of pancreatic lesions. Next-generation sequencing (NGS) facilitates the production of millions of sequences concurrently. Therefore, in the current study, to improve the detectability of oncogenic mutations in pancreatic lesions, an NGS system was used to diagnose EUS-FNA samples. A total of 38 patients with clinically diagnosed EUS-FNA specimens were analyzed; 27 patients had pancreatic ductal adenocarcinoma (PDAC) and 11 had non-PDAC lesions. DNA samples were isolated and sequenced by NGS using an Ion Personal Genome Machine system. The Cancer Hotspot Panel v2, which includes 50 cancer-related genes and 2,790 COSMIC mutations, was used. A >2% mutation frequency was defined as positive. *KRAS* mutations were detected in 26 of 27 PDAC aspirates (96%) and 0 of 11 non-PDAC lesions (0%). The G12, G13, and Q61 *KRAS* mutations were found in 25, 0, and 1 of the 27 PDAC samples, respectively. Mutations were confirmed by TaqMan® polymerase chain reaction analysis. *TP53* mutations were detected in 12 of 27 PDAC aspirates (44%). *SMAD4* was observed in 3 PDAC lesions and cyclin-dependent kinase inhibitor 2A in 4 PDAC lesions. Therefore, the current study was successfully able to develop an NGS assay with high clinical sensitivity for EUS-FNA samples.

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Abbreviations: AIP, autoimmune pancreatitis; CP, chronic pancreatitis; EUS-FNA, endoscopic ultrasound-guided fine-needle aspiration; IPMN, intraductal papillary mucinous neoplasm; NET, neuroendocrine tumors; NGS, next generation sequencing; PDAC, pancreatic ductal adenocarcinoma; SCN, serous cystic neoplasm; TP53, tumor protein 53

Key words: next-generation sequencing, pancreatic ductal adenocarcinoma, endoscopic ultrasound-guided fine-needle aspiration, *KRAS, TP53*

Introduction

Pancreatic ductal adenocarcinoma (PDAC) is a leading cause of cancer-associated mortality in the United States and Japan, and its incidence continues to increase (1,2). PDAC has one of the lowest survival rates, with a 5-year survival rate of <10% (1,2). Therefore, improved methods of diagnosing PDAC are required.

A number of diagnostic modalities for PDAC have been developed. Among them, endoscopic ultrasound-guided fine-needle aspiration (EUS-FNA) has become an important method of diagnosing pancreatic tumors due to its high safety, cost-effectiveness and accuracy (3,4). EUS-FNA demonstrates high sensitivity for histological diagnosis (75-94%) and a specificity approaching 100% in the majority of studies (5,6). However, false-negative, atypical and suspicious cytopathological diagnoses remain relatively frequent (5,7,8).

Activating mutations in the *KRAS* gene are observed in >95% of patients with PDAC and may be one of the earliest steps in the formation of pancreatic intraepithelial neoplasia (PanIN) (9-11). Molecular profiling studies have demonstrated that PanIN-to-PDAC progression occurs by inactivation of the tumor suppressor genes tumor protein 53 (*TP53*), cyclin-dependent kinase inhibitor 2A (*CDKN2A*) and/or *SMAD4* (12-15). With a few rare exceptions, the genetic mutations in patients with cancer that activate *KRAS* proteins predominantly result from one of three single point mutations at residues G12 (98%), G13 (<1%) or Q61 (<1%) (9).

Mutation profiling has been attempted using traditional single-gene analysis, which is commonly performed with Sanger sequencing. However this is costly, relatively low in sensitivity, and time- and labor-intensive. Furthermore,

substantial amounts of DNA are required; thus the simultaneous evaluation of several genes within a small specimen is not possible. Next-generation sequencing (NGS) solves this problem due to its ability to perform multiplex, high-throughput sequencing of many samples for multiple genes. NGS allows druggable mutations to be identified and a more complete genotype of a given type of cancer to be generated. The use of NGS and the combined analysis of separate sets of data thus enable a more detailed picture of a specific disease to be established (7,16).

A number of studies have assessed the genetic changes in resected PDAC specimens by NGS (15,17). However, the majority of PDACs are inoperable due to disease progression. To overcome this issue, the use of specimens obtained by EUS-FNA is preferable and promising for clinical application.

The aim of the current study therefore, was to establish an NGS assay for genetic alterations in pancreatic specimens obtained via EUS-FNA.

Materials and methods

Patients. A total of 38 patients with pancreatic disease were eligible for the current study, and underwent EUS-FNA at the Gastroenterological Center, Yokohama City University Medical Center (Yokohama, Japan) between September 2013 and March 2015. Eligible patients were pathologically or clinically diagnosed as having pancreatic disease and provided written informed consent to participate in the current study. None of the patients had previously undergone chemotherapy or radiotherapy. Out of all the patients, 27 were diagnosed with PDAC and 11 with non-PDAC lesions. Non-PDAC lesions included autoimmune pancreatitis (AIP), intraductal papillary mucinous neoplasms (IPMN), serious cystic neoplasms (SCN), pancreatic neuroendocrine tumors (NETs) and tumor-forming chronic pancreatitis (CP) (Table I). There were 26 male and 12 female patients with an average age of 66.5 years (range, 39-86 years). EUS-FNA was performed using a linear echo-endoscope and the aspirated material was smeared onto microscope slides for on-site examination. Hematoxylin and eosin staining was performed and a pathologist reviewed the slides. A portion of the tissues was stored at -80˚C until DNA extraction. The study protocol was approved by the Ethics Committee at Yokohama City University.

DNA extraction and library preparation. DNA from direct EUS-FNA material was extracted using a ReliaPrep™ gDNA Tissue Miniprep system (Promega Corporation, Madison, WI, USA), according to the manufacturer's instructions. The highly intact and nondegraded RNA-free genomic DNA was subjected to library preparation prior to sequencing.

Multiplex polymerase chain reaction (PCR) was performed by amplifying 10 ng of DNA using the Ion AmpliSeq™ Cancer Hotspot Panel v2.0 and the Ion AmpliSeq™ Library kit v2.0 (Thermo Fisher Scientific, Inc., Waltham, MA, USA). Somatic mutations (substitutions, insertions, or deletions) were designed to amplify 207 amplicons covering ~2790 Catalogue of Somatic Mutations in Cancer (COSMIC) mutations from the 50 most commonly reported oncogenes and tumor suppressor genes (Ion Torrent™; Thermo Fisher Scientific, Inc.). Sequencing library preparation was performed according to the manufacturer's instructions (18,19).

Emulsion PCR and Ion Torrent personal genome machine (PGM)™ sequencing. Pooled, barcoded libraries were clonally amplified using the Ion OneTouch™ 2 instrument from the Ion PGM™ Template OT2 200 kit (Thermo Fisher Scientific, Inc.) according to the manufacturer's instructions (20). Ion sphere particles were enriched using the Ion One Touch™ enrichment system (Thermo Fisher Scientific, Inc). The subsequently enriched template-positive ion sphere particles were loaded onto an Ion 316™ chip and sequenced on the PGM using the Ion PGM sequencing 200 kit v2 (Thermo Fisher Scientific, Inc.). Data from sequencing runs on the Ion Torrent PGM™ were automatically transferred to the Torrent Server hosting the Torrent Suite™ Software v4.4.3 (Thermo Fisher Scientific, Inc.). The Torrent Suite Software uses the Torrent Browser, which includes the Torrent Mapping Alignment Program and Torrent Variant Caller for alignment and variant detection. A human genome reference sequence (hg19) was used as a reference. Ion Reporter™ Software (Thermo Fisher Scientific, Inc.) was used to perform variant calling and mapping. A >2% mutation frequency was defined as positive.

TaqMan® PCR. TaqMan® Mutation Detection assays (Thermo Fisher Scientific, Inc.) were used to confirm the *KRAS* codon-12 mutations (c.34 G>Cp.pG12R, c.35 G>A p.12D, c.35 G>T p.G12 V) revealed by NGS analysis. Briefly, detection experiments were performed using reaction mixtures comprising a DNA template, one Genotyping Master Mix and one TaqMan® Detection assay containing an allele-specific forward primer, locus-specific reverse primer, allele-specific blocker and locus-specific TaqMan® probe. Quantitative PCR (qPCR) was performed on an Applied Biosystems® 7,500 fast real-time PCR system (Applied Biosystems; Thermo Fisher Scientific, Inc.). A >0.1% mutation frequency was defined as positive.

Results

Clinicopathological characteristics. Final diagnoses of solid pancreatic lesions based on EUS-FNA following cytopathology and clinical passage are presented in Table I. Of the 38 patients, 27 had PDAC (19 males, 8 females) and mean patient age was 66.5 years (range, 39-86 years). Histological analysis of EUS-FNA samples indicated that 19 of the 27 PDAC samples were diagnosed with adenocarcinoma and 1 sample was diagnosed as suspicious for adenocarcinoma. Of the remaining samples, 6 were diagnosed as atypical epithelium and 1 sample was diagnosed as exhibiting no malignancy (Table II).

The mean size of the PDAC tumors at the greatest diameter was 36.8 mm (range, 16-64 mm). Of all PDAC lesions, 12 were located in the pancreatic head, 7 were in the pancreatic body to tail, 6 were in the pancreatic body and 2 were in the pancreatic tail. (Table II). According to the UICC (Union International Cancer Control) clinical staging system, 16 PDACs were stage IV, 7 were stage III, 3 were stage IIA, and 1 was stage IA (Table II). The diagnoses of the 11 patients who did not have PDAC were as follows: 2 cases of metastatic cancer, 2 cases

of NET, 1 case of IPMN, 1 case of SCN, 3 cases of AIP and 2 cases of CP (Table III).

Prevalence of gene mutations in EUS-FNA samples by NGS. Using the Ion Ampliseq™ Cancer Hotspot Panel v2 (Thermo Fisher Scientific, Inc.), which includes the 50 most common cancer-related genes and 2,790 COSMIC mutations, 10 ng of DNA was analyzed according to the manufacturer's protocol. The average sample loading obtained was 67% (range, 46-91%) and the average number of total reads was 481,049 with an average read length of 105 bp.

Following the analysis, putative germline mutations and nonsignificant mutations, including synonymous ones, were excluded. Among the 27 PDACs, 26 tumors (96%) had *KRAS* mutations; of these, 15 had G12D (c.35 G>A), 6 had G12 V (c.35 G>T), 3 had G12R (c.34 G>C), 1 had G12C (c.34 G>T) and 1 had Q61H (c.183 A>T) mutations. *TP53* mutations were detected in 12 of the 27 samples (44%). The details of the *TP53* mutations are presented in Table IV. These mutations were evaluated by TransFIC (TRANSformed Functional Impact for Cancer) (21) and their level of impact was assessed; those determined to have a high impact are presented (Table IV). *SMAD4* and *CDKN2A* mutations were identified in 3 of 27 samples (11%; #2: c.1487G>A, #10: c.1558G>T, #19: c.984C>G) and 4 of 27 samples (15%; #7: c.152T>A, #12: c.242C>T, #16:c.197A>G, #26:c.238C>T), respectively (Table II). MET (#26:c1124A>G) and KIT (#26:c.1621A>C) mutations were each identified in 1 sample (3.7%; Table II). In the remaining 9 samples, 1 sample from metastatic carcinoma exhibited *VHL*, *BRAF* and *GNAS* mutations, suggesting that the tumor was not derived from the pancreas but from another organ, such as the colon or kidney (Table III). One sample from a metastatic pancreatic tumor harbored the *TP53* mutation, however the primary locus could not be determined. The remaining 9 samples exhibited no putative driver mutation (Table III).

Validation analysis of KRAS mutation by TaqMan® assay. A TaqMan® gene expression PCR assay was used to validate the *KRAS* mutations detected by NGS (17,22,23). PCR primers were only used for the G12 V, G12D and G12R, and all mutations detected by NGS were also detected by the TaqMan® assay. However, neither NGS nor the TaqMan assay was able to detect a KRAS mutation in 1 PDAC sample (sample ID 15; Table II). Mutations of Q61H or G12C detected by NGS (sample ID 18 or 27; Table II) had not been determined. The G12D mutation was detected in a sample from a patient with AIP. However, the TaqMan assay is able to detect mutations with a frequency of <0.1%, whereas the mutation frequency must be >2% to be defined as positive by NGS.

Table I. Final clinical diagnosis of pancreatic lesions reference for EUS guided FNA.

Final diagnosis	No. of lesions
Pancreatic adenocarcinoma	27
Metastatic pancreatic tumor	2
Neuroendocrine tumor	2
Intraductal papillary mucinous neoplasm	1
Serous cystic neoplasm	1
Autoimmune pancreatitis	3
Chronic pancreatitis	2
Total	38

Discussion

In the current study, an NGS mutational analysis system was established for EUS-FNA tissue samples. The diagnostic accuracy of EUS-FNA by histological analysis is sometimes insufficient. Associated factors include the size of the tissue sample, size or location of the tumor, and operator's technique (7,16,24). The current study revealed that 7 of 27 PDAC samples were not diagnosed as adenocarcinoma by histological analysis; furthermore, PNAC diagnosis may differ according to the pathologist involved. For example, in the current study, 2 PDAC patients (sample IDs 21 and 22; Table II) were not diagnosed as adenocarcinoma by pathological analysis in EUS-FNA samples and therefore underwent surgical biopsy. A method for objective and accurate diagnosis of EUS-FNA samples should be established, which would result in more widespread use of the EUS-FNA technique. The present study has demonstrated that NGS and TaqMan analysis are able to detect *KRAS* mutations in EUS-FNA samples. If detection of *KRAS* mutations is established as a method of PDAC diagnosis, the rate of successful PDAC diagnosis may improve and patients may receive effective treatment more promptly.

In general, pancreatic tumor tissue contains fibroblasts or hematopoietic cells and in some cases, may even contain more fibroblasts than tumor cells (25). Therefore, the use of Sanger sequencing for PDAC is difficult due to possible contamination by fibroblasts. It has been demonstrated that detecting *KRAS* mutations, the most common and important mutations for the development of PDAC, by a qPCR-based method is accurate and highly sensitive (26). However, mutational analysis of other PDAC driver genes including *TP53,* is relatively difficult. Additionally, these genetic mutations may provide information to aid the diagnosis of PDAC, furthermore, certain mutations including *TP53* and *SMAD4*, are predictive of a poorer prognosis of patients with PDAC (27,28). However, *TP53* or *SMAD4* mutations have been found in several exons; thus, Sanger sequencing of many loci or analysis at the protein level is necessary. NGS may overcome these problems; indeed, the present study demonstrated that the *TP53* mutation locus differed among the patients assessed. In addition, it is difficult to perform a protein analysis using EUS-FNA samples due to the low quantity of protein they contain.

There has been a recent focus on personalizing PDAC treatment by analyzing genetic alterations or molecular biomarkers. Despite intensive research, personalized therapy for patients with PDAC has not yet been established. Erlotinib is an important drug approved for the treatment of PDAC and is very effective. However, no biomarker for its use has been established. A previous *in vivo* study has suggested that epidermal growth factor signaling, the target of erlotinib, is more active in the presence of the *TP53* mutation (29); therefore, erlotinib may not be effective in patients with PDAC

Table II. Clinicopathological features and details of gene mutations in PDAC.

Sample ID	A	G	FNA histology	CEA, ng/ml	CA19-9, IU/ml	Location in pancreas	Tumor size, mm	Stage (UICC)	KRAS mutation (NGS)	Amino acid substitution (NGS)	Amino acid substitution (TaqMan)	TP53 mutation (NGS)	Other mutations
1	78	F	AC	3.8	1	Ph	25	IV	+	G12V	G12V	+	
2	67	M	AC	12.6	1,130	Pbt	64	IV	+	G12V	G12V	+	SMAD4
3	64	M	AC	7.8	504	Pb	53	IV	+	G12V	G12V	+	
4	69	M	AC	10.1	70,890	Ph	34	IV	+	G12D	G12D	+	
5	47	M	AC	2.4	105	Ph	25	IV	+	G12D	G12D	+	
6	68	M	AC	20.2	150	Pbt	29	IV	+	G12D	G12D	+	
7	77	F	AC	37.3	24,421	Pb	41	IV	+	G12V	G12V	+	CDKN2A
8	64	M	AC	151	1,039	Ph	51	IV	+	G12D	G12D	+	
9	53	F	AT	37.4	1,064	Pbt	60	IV	+	G12D	G12D	-	
10	73	M	AC	196.8	37,107	Ph	49	IV	+	G12D	G12D	-	SMAD4
11	67	M	AC	7.3	67	Ph	38	IV	+	G12R	G12R	-	
12	41	M	AC	0.9	110	Pbt	29	IV	+	G12D	G12D	-	CDKN2A
13	73	M	AC	4	403	Pb	39	IV	+	G12D	G12D	-	
14	74	M	AT	17.1	566	Ph	20	IV	+	G12D	G12D	-	
15	74	M	AC	3.6	2,770	Ph	22	IV	-	-	-	-	
16	49	M	AT	2.6	403	Pb	63	IV	+	G12D	G12D	-	CDKN2A
17	65	M	AT	3.7	630	Ph	26	III	+	G12D	G12D	+	
18	75	F	AC	1.7	331	Pb	40	III	+	Q61H	-	-	
19	66	M	AT	11.5	174	Pbt	64	III	+	G12R	G12R	-	SMAD4
20	79	M	AC	12.7	80	Pt	32	III	+	G12D	G12D	-	
21	77	F	AT	2.1	24	Pbt	30	III	+	G12V	G12V	-	
22	74	M	NM	62.5	1	Ph	35	III	+	G12D	G12D	-	
23	84	F	SFAC	24	3	Pt	22	III	+	G12V	G12V	-	
24	73	M	AC	2.6	38	Ph	20	IIA	+	G12D	G12D	+	
25	86	F	AC	6.1	147	Pbt	32	IIA	+	G12R	G12R	+	
26	84	M	AC	3.9	563	Ph	37	IIA	+	G12D	G12D	-	CDKN2A, MET, KIT
27	39	F	AC	1	46	Pb	16	IA	+	G12V	-	+	

All patients had the same final diagnosis of pancreatic ductal adenocarcinoma. G, gender; A, age; M, male; F, female; FNA, fine-needle aspiration; CEA, Carcinoembryonic antigen; CA19-9, carbohydrate antigen 19-9; UICC, Union for international cancer control; NGS, next generation sequencing; TP53, tumor protein 53; AC, adenocarcinoma; AT, atypical; SFAC, suspicious for adenocarcinoma; NM, no malignancy. Ph, pancreatic head; Pb, pancreatic body; Pt, pancreatic tail; Pbt, pancreatic body to tail.

Table III. Clinicopathological features and details of genetic mutations in non-PDAC patients.

Sample ID	Final diagnosis	Age	Gender	Histology	KRAS mutation (NGS)	Amino acid substitution (TaqMan)	TP53 mutation (NGS)	Other mutations
28	Metastatic pancreatic cancer	45	F	No malignancy	-	-	+	
29	Metastatic pancreatic cancer	86	F	Adenocarcinoma	-	-	-	BRAF, VHL, GNAS
30	NET	55	M	NET	-	-	-	
31	NET	46	M	No malignancy	-	-	-	
32	IPMN	64	F	No malignancy	-	-	-	
33	SCN	68	M	No malignancy	-	-	-	
34	AIP	77	M	No malignancy	-	-	-	
35	AIP	71	F	No malignancy	-	G12D	-	
36	AIP	67	M	No malignancy	-	-	-	
37	CP	52	M	No malignancy	-	-	-	
38	CP	47	M	No malignancy	-	-	-	

M, male; F, female; AIP, autoimmune pancreatitis; CP, chronic pancreatitis; EUS-FNA, endoscopic ultrasound-guided fine-needle aspiration; IPMN, intraductal papillary mucinous neoplasm; NET, neuroendocrine tumors; NGS, next generation sequencing; PDAC, pancreatic ductal adenocarcinoma; SCN, serous cystic neoplasm; TP53, tumor protein 53; BRAF, proto-oncogene B-RAF; VHL, von Hippel-Lindau tumor suppressor; GNAS, GNAS complex locus.

Table IV. TP53 mutations identified in PDAC patients.

Sample ID	Mutation	Protein change	Type	transFIC prediction
1	g.chr17:7578553	p.T126S	SNP	Driver mutation
2	g.chr17:7577141	p.G266V	SNP	Driver mutation
3	g.chr17:7577120	p.R273C	SNP	Driver mutation
4	g.chr17:7578406	p.A175H	SNP	Driver mutation
5	g.chr17:7577547	p.G245N	SNP	Driver mutation
6	g.chr17:7579388	p.G1100Ter	SNP	Strongly affecting mutation
7	g.chr17:7577553	p.C242S	SNP	Strongly affecting mutation
7	g.chr17:7578434	p.S166A	SNP	Strongly affecting mutation
8	g.chr17:7577570	p.M237I	SNP	Driver mutation
10	g.chr17:7577103	p.R280fs	INDEL	Putative affecting
17	g.chr17:7577559	p.S241F	SNP	Driver mutation
24	g.chr17:7578448	p.A161N	SNP	Strongly affecting mutation
25	g.chr17:7578201	p.H214A	SNP	Strongly affecting mutation

PDAC, pancreatic ductal adenocarcinoma; SNP, single nucleotide polymorphisms; INDEL, small insertions and deletions; transFIC, TRANSformed functional impact for cancer.

exhibiting *TP53* mutations. Future studies should clarify whether the *TP53* mutation may be used as a biomarker for the administration of erlotinib.

The EUS-FNA technique is used worldwide and is considered to be the most powerful method for the histological diagnosis of pancreatic tumors. PDAC has a markedly unfavorable prognosis; the 5-year survival rate is <10% and the disease is usually diagnosed at an advanced, inoperable stage (1,2). Therefore, EUS-FNA is the sole method of obtaining tumor tissue in the majority of patients with advanced-stage disease. A method for the diagnosis and determination of the appropriate therapy using EUS-FNA samples is required.

Such a method is also important for the diagnosis of other pancreatic masses, including metastatic tumors, lymphoma, IPMC, NET and benign lesions. In the current study, 11 samples from patients with other pancreatic masses (non-PDAC) were analyzed. As described above, >95% of PDACs exhibit the *KRAS* mutation; therefore, its absence reduces the probability of PDAC diagnosis. This information may facilitate the differential diagnosis of a malignancy and a benign disorder.

Accurately diagnosing solid pancreatic tumors is critical to avoid chemotherapy or unnecessary resections in patients with benign lesions, including AIP and focal lesions of CP.

Differential diagnosis between PDAC and other malignancies is sometimes difficult. In the current study, samples from metastatic tumors of unknown origin were used. One sample harbored *BRAF* and *VHL* mutations but no *KRAS* mutations, suggesting that the tumor was unlikely to be PDAC and may have been metastatic pancreatic cancer. However, *KRAS* mutations have been detected in other types of cancer, including those of the lung, colon and stomach (30). Determinating the tumor origin by genetic analysis alone is difficult in such cases.

The present study had several limitations, the most prominent of which was the relatively small sample size. However, the study did establish genetic mutational analysis by NGS with EUS-FNA samples. In addition, the Ampliseq cancer panel was used to analyze 50 genes containing 1650 hotspots. This may not be sufficient to profile the landscape of PDAC. Following the establishment of genetic diagnosis-based treatment methods, even more accurate methods of genetic diagnosis should be established.

The current study eliminated the results of suspected germline mutations. However, determining whether a mutation is germ line or somatic is difficult, and both tumor samples and normal tissue control samples must therefore be used. Furthermore, the mutation should be identified as either driver or passenger to determine whether mutational analysis is clinically appropriate.

In conclusion, an NGS assay of EUS-FNA samples was successfully established in the present study. Analysis of oncogenic mutations using NGS enabled very high clinical sensitivity. The assay developed herein may be incorporated into clinical laboratories as a routine test for diagnosing and constructing personalized therapy for patients with PDAC.

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