Evaluation and Diagnostic Value of Next-Generation Sequencing Analysis of Residual Liquid-Based Cytology Specimens of Pancreatic Masses

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BACKGROUND: Liquid-based cytology (LBC) is a widely used method for processing specimens obtained by endoscopic biopsy. This study evaluated next-generation sequencing (NGS) analysis of LBC specimens to improve the diagnostic accuracy of pancreatic lesions. **METHODS:** Upon the diagnosis of a suspected pancreatic mass, LBC residues were used retrospectively. The quantity and quality of DNA extracted from residual LBC samples were evaluated, and an NGS analysis targeting 6 genes (*KRAS*, *GNAS*, *TP53*, *CDKN2A*, *SMAD4*, and *PIK3CA*) was performed. **RESULTS:** The library was prepared from LBC specimens taken from 52 cases: 44 were successful, and 8 preparations failed. An analysis of DNA quantity and quality suggested that the success or failure of NGS implementation depended on both properties. The final diagnosis was achieved by a combination of the pathological analysis of the surgical excision or biopsy material with clinical information. Among the 33 cases of pancreatic ductal adenocarcinoma (PDAC), *KRAS*, *TP53*, *CDKN2A*, and *SMAD4* mutations were identified in 31 (94%), 16 (48%), 3 (9%), and 2 (6%), respectively. Among the 11 benign cases, only a *KRAS* mutation was identified in 1 case. On the basis of NGS results, 18 of 33 PDACs (55%) were classified as highly dysplastic or more, and 10 of 11 benign lesions were evaluated as nonmalignant, which was consistent with the final diagnosis. **CONCLUSIONS:** NGS analysis using LBC specimens from which DNA of appropriate quantity and quality has been extracted could contribute to improving the assessment of pancreatic tumor malignancies and the application of molecular-targeted drugs. ***Cancer Cytopathol* 2022;130:202-214**.

KEY WORDS: liquid-based cytology; mutation; next-generation sequencing; pancreatic ductal carcinoma; pancreatic lesions; proto-oncogene proteins p21(ras).

INTRODUCTION

Pancreatic cancer is the fourth leading cause of cancer-related mortality in Japan with a 5-year survival rate below 8%.[1] Although surgical intervention for most pancreatic ductal adenocarcinoma (PDAC) is difficult in its advanced stages, endoscopic ultrasound–guided fine-needle aspiration (EUS-FNA) is an important technique used to histologically distinguish PDAC from inflammatory diseases and rare primary pancreatic tumors and

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thus prevent unnecessary surgery. The advantages of EUS-FNA include safety, cost-effectiveness, and accuracy, with a reported diagnostic accuracy of more than 70% for solid pancreatic masses.[2-5] Other definitive diagnostic methods include bile duct brushing during endoscopic retrograde cholangiopancreatography and forceps biopsy. Depending on the report, the sensitivity of this technique for detecting malignancy varies widely from a few percent to several tens of percent.[6,7] These methods cannot easily distinguish between neoplasia and inflammation or reactivity, and they may result in atypical cytopathological diagnoses.

Liquid-based cytology (LBC) is a thin-layer slide preparation that is widely used to process gynecological specimens, but it also has high diagnostic sensitivity, negative predictive values, and accuracy rates for pancreatic specimens.[2,4,5] LBC was developed to overcome the shortcomings of conventional smears, such as cell clouding and blood contamination.[8]

Gene variants identified in PDAC mainly include *KRAS*, *TP53*, *SMAD4*, and *CDKN2A* (70%, 53%, 19%, and 9%, respectively),[9] but some infrequent variants also occur.[10-13] Several studies have reported on the application of targeted next-generation sequencing (NGS) of pancreatic cancer using surgical materials and frozen/fixed specimens derived from EUS-FNA.[14-18] The advantages of targeted NGS include its use in the analysis of hotspot mutations in cancer-related genes; this can be achieved even with poor-quality DNA derived from formalin-fixed, paraffin-embedded samples. Several studies have described the application of NGS analysis of DNA obtained from fine-needle aspiration (FNA) materials and pathological specimens for searching for mutations as therapeutic targets and for evaluating the benign or malignant status of pancreatic masses.[6,19,20]

LBC specimens are an ideal tool for obtaining genetic information to guide diagnosis, prognosis, and treatment for the following reasons: tumor cells can be identified by morphological diagnosis; DNA can be extracted; handling, storage, and transportation are easy; and patients are not burdened beyond normal routine medical practice. On the other hand, NGS can detect mutated DNA even in the presence of large amounts of normal DNA, and this makes it suitable for the analysis of samples containing noncancerous cells. In fact, several reports have described NGS analysis using LBC-derived DNA.[21-24] For example, it has been reported that BiliSeq can be performed prospectively from alcohol-based preservative solutions used for biliary strictures and combined with pathological diagnosis to obtain high specificity.[6] Furthermore, DNA extracted from scrape samples of specimens preserved with ThinPrep LBC allowed the analysis of 39 genes by NGS, and it was successful in 65 of 73 specimens.[25] We previously reported that *KRAS* gene mutation analysis could be conducted with the fluorescence resonance energy transfer–based preferential homoduplex formation assay (F-PHFA) in LBC specimens of pancreatic FNA.[26] Although *KRAS* mutations are also found in chronic inflammation and pancreatic intraepithelial neoplasms,[27,28] NGS is expected to increase the specificity of diagnosis for malignancy by analyzing multiple gene mutations simultaneously. In addition, NGS is expected to provide information enabling the prediction of clinical prognosis and informing molecular targeted therapy. In this study, we examined the utility of NGS of pancreatic LBC specimens and the contribution that mutations in 6 genes could make toward the diagnosis of pancreatic masses.

MATERIALS AND METHODS

Patients

Seventy-nine patients with suspicious pancreatic or periampullary lesions were subjected to EUS-FNA, brushing, or bile juice collection to obtain LBC specimens from January 2017 to July 2020 at Nara Medical University Hospital (Kashihara, Japan) and Minami-Nara General Medical Center (Ooyodo, Japan). All patients provided informed consent for inclusion before participation in the study. Clinical information was obtained from medical records, and the study protocol was approved by the ethics committees of Nara Medical University (IRB2657) and Minami-Nara General Medical Center (IRB31).

Conventional EUS-FNA Analysis and Brush and Bile Juice Collection

An experienced gastroenterologist performed FNA during endoscopic ultrasound, whereas bile juice or biliary brush samples were obtained during standard endoscopic retrograde cholangiopancreatography. The EUS-FNA procedure has been reported previously (see the supporting information).[2,26] The EUS-FNA sample was pressed out into the preservation solution, and the visible solids were removed with a dropper and fixed in 10% neutral buffered formalin to prepare paraffin-embedded tissue specimens (Fig. 1). Methods of specimen preparation

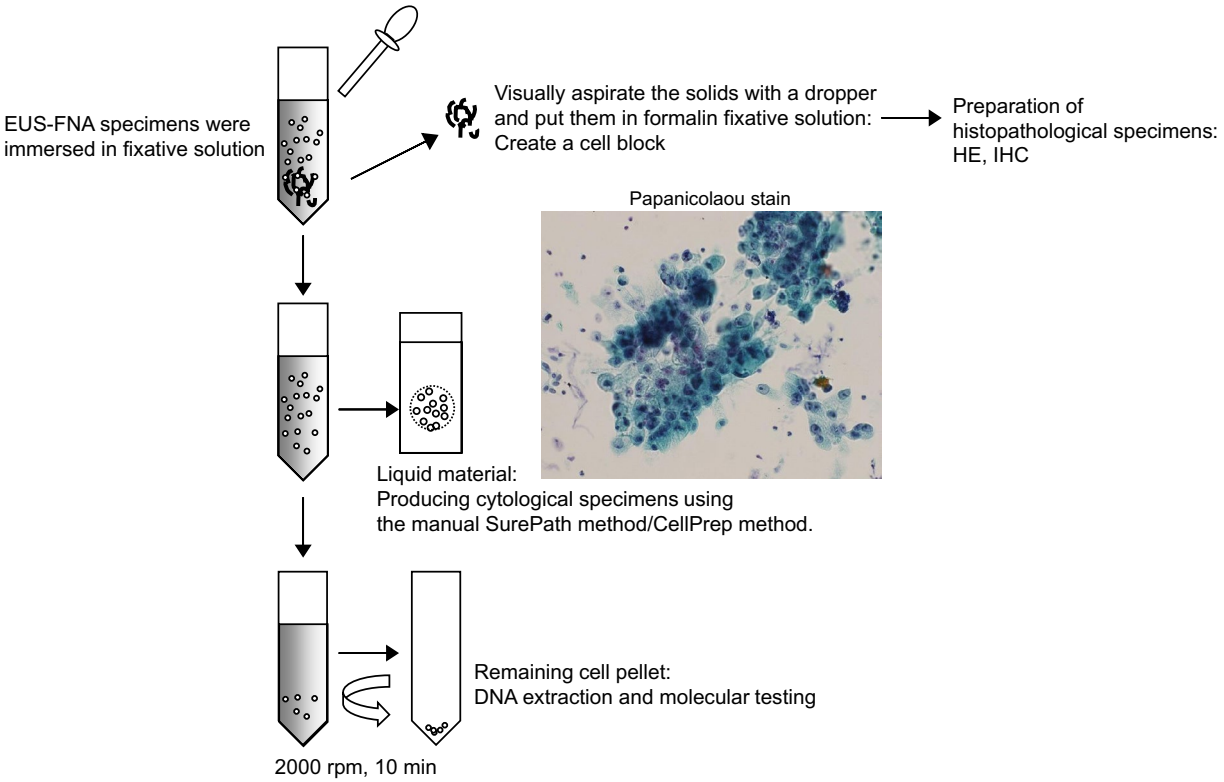

Figure 1. Preparation of LBC specimens for EUS-FNA and subsequent genetic testing. After the preparation of histological and cytological specimens, a gene analysis was performed with LBC samples stored at 4°C. EUS-FNA indicates endoscopic ultrasound–guided fine-needle aspiration; HE, hematoxylin and eosin; IHC, immunohistochemistry; LBC, liquid-based cytology.

from brushes and bile juice are described in the supporting information (Supporting Fig. 1).

Preparation of Cytology Specimens and Residual LBC Specimens

Cytology specimens were prepared from EUS-FNA by manual SurePath and from brushes and bile juice by CellPrep methods, and in this way, residual samples were obtained (see the supporting information; Supporting Fig. 2). The residual specimens were stored at 4°C until DNA extraction. The median storage time after pathological specimen preparation was 66.5 days (Table 1).

Pathological Analysis

As a part of routine practice, tissue specimens were evaluated by 3 experienced pathologists, and cytological specimens were diagnosed by 2 experienced cytologists and 1 pathologist. According to the findings of the histopathological specimens, the results were classified as follows: 1) no evidence of malignancy, 2) inflammation, 3) atypia, and 4) adenocarcinoma (AC). As for definitions, *inflammation* was defined as the absence of malignant cells but the presence of inflammatory findings, whereas *no evidence of malignancy* was defined as the absence of malignant cells and inflammatory findings. The evaluation of cytology specimens is described in the supporting information.

TABLE 1. Characteristics of Residual LBC-Derived DNA

Specimens	No.	52
Storage period	Median (range), d	66.5 (14-437)
Amount of extracted DNA	Median (IQR), ng	166.4 (121.4-305.3)
DIN value	Median (IQR)	5.5 (3.1-6.5)
Amount of DNA below the calculation limit	No.	7

Abbreviations: DIN, DNA integrity number; IQR, interquartile range; LBC, liquid-based cytology.
LBC specimens were stored in the refrigerator after routine diagnosis. The storage period ranged from the period of pathology sample preparation up to processing for DNA extraction.

Final Diagnosis

The final diagnosis was made through the combination of the pathological diagnosis from the resected and LBC processed specimens (EUS-FNA, biliary brush, and bile juices) with clinical information (imaging, prognosis, and metastasis),

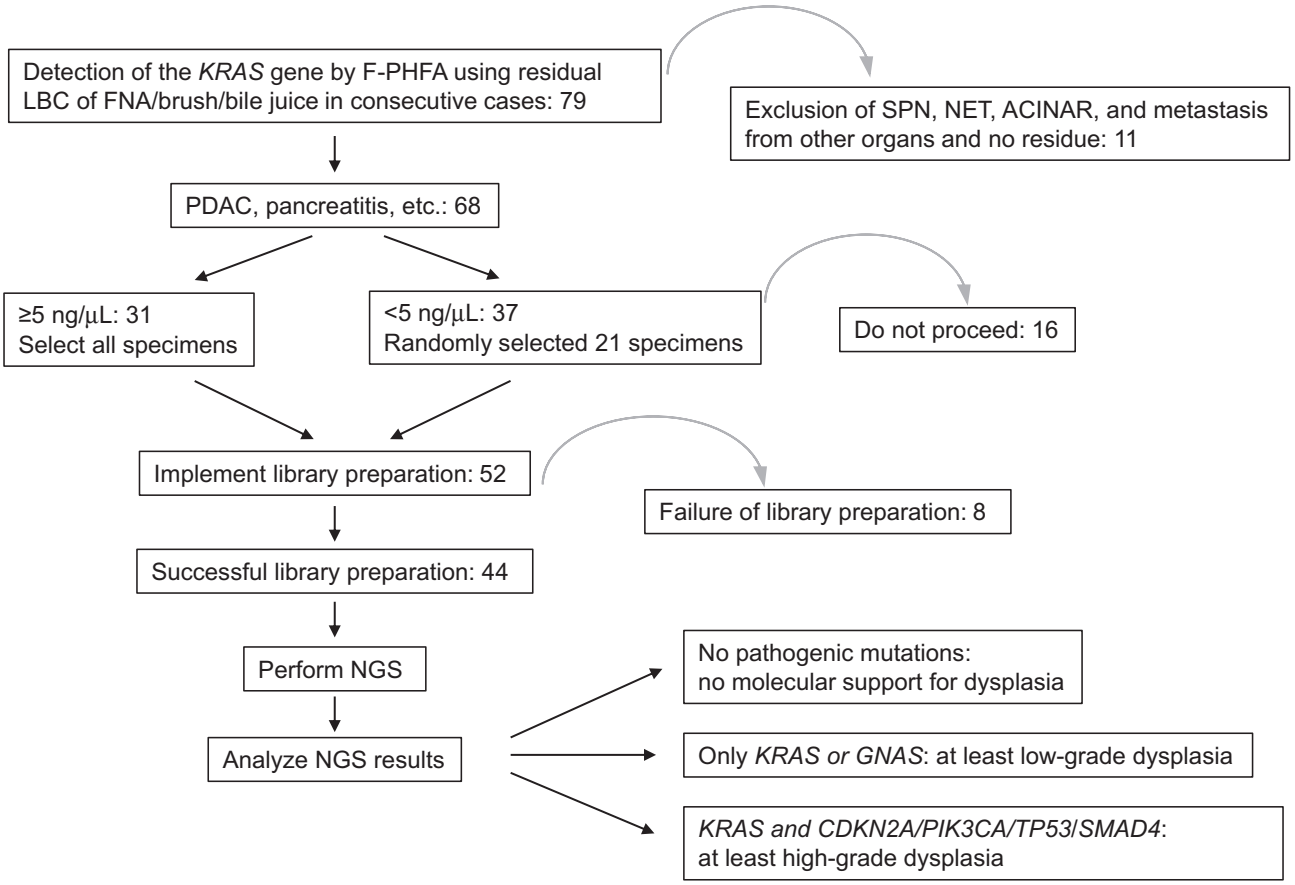

Figure 2. Schematic diagram of NGS sample selection, result implementation, and interpretation. ACINAR indicates acinar cell carcinoma; FNA, fine-needle aspiration; F-PHFA, fluorescence resonance energy transfer–based preferential homoduplex formation assay; LBC, liquid-based cytology; NET, neuroendocrine tumor; NGS, next-generation sequencing; PDAC, pancreatic ductal adenocarcinoma; SPN, solid pseudopapillary tumor.

and it was interpreted as follows: 1) if the biopsy result and/or surgical reports indicated AC, the masses were considered PDAC; 2) if the biopsy results indicated atypia or benign cells, the masses were considered malignant if the clinical and imaging follow-up data were consistent with malignancy, such as clinical progression or metastasis; and 3) if the biopsy results indicated atypia and benign cells, the masses were considered benign on the basis of the clinical manifestation (suspected inflammation or a lack of progression).

DNA Extraction

Residual LBC specimens stored at 4°C were centrifuged at 2000 rpm for 10 minutes (Fig. 1). The details of the DNA extraction are described in the supporting information.

Selection of Specimens for NGS

Figure 2 presents the selection of samples from the residual LBC specimens and the NGS protocol. Genetic mutational analysis for the malignancy assessment was performed with the exception of 9 of the 79 original samples, which were excluded because of a diagnosis of solid pseudopapillary tumor, neuroendocrine tumor, acinar cell carcinoma, or metastasis from other organs. Samples with no residues were also excluded. Preliminary studies predicted that small amounts of DNA samples could lead to failure in the NGS library preparation; thus, the remaining 68 specimens were divided into 2 groups based on the DNA content: >5 ng/μL (high) and <5 ng/μL (low). The subsequent series of operations was performed in a blinded fashion. Library preparation was performed for all 31 cases with high concentrations of DNA, and 21 random cases were selected from the 37 cases with lower DNA concentrations. Library preparation succeeded for 44 of the 52 specimens and failed for 8. NGS sequencing was performed for the 44 successfully prepared libraries.

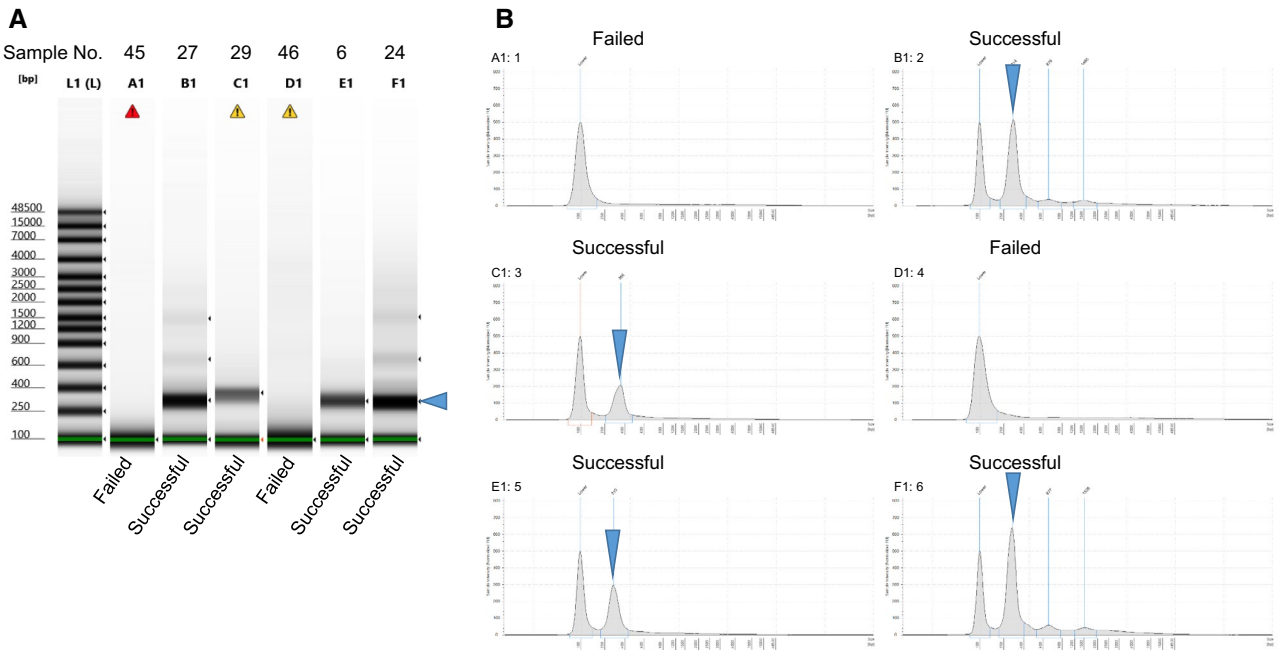

Figure 3. Confirmation of the library after preparation. (A) Representative electrophoretic images of successful and failed library preparations. (B) Representative electropherogram of successful and failed library preparations. (A,B) The large blue arrow shows the band peak of the synthesized library amplicon of the target area. (A) The small black arrow indicates the band peak detected by the TapeStation device. (A) The red and yellow triangles indicate samples whose DNA concentration was too low for the TapeStation device to calculate the amount of DNA or the degree of degradation. (A) The green line indicates the control DNA contained in the loading dye reagent. bp indicates base pair.

Examination of DNA Quality Using Real-Time Polymerase Chain Reaction

Apart from the analysis of DNA properties using TapeStation, DNA was evaluated with the QIAseq DNA QuantiMIZE Assay Kit (Qiagen).[22,29] This kit uses 2 types of quantitative polymerase chain reaction (PCR) assays, the details of which are described in the supporting information. Two values were calculated from the cycle threshold (Ct) values of 100- and 200-bp amplicons, and the DNA quality of the specimen was evaluated as follows: 1) the amplifiable DNA concentration (ng/μL; $1/2^{(\Delta Ct100 + \Delta Ct200)/2 \times 5}$), which denoted the absolute amount of amplifiable DNA, and 2) the quality check (QC) score ($\Delta Ct200 - \Delta Ct100/200 - 100$), which was derived from the slope of Ct200 and Ct100.

Confirmation of KRAS Gene Mutations and Percentages of Tumor Cells in Specimens

At our laboratory, we routinely checked for *KRAS* gene mutations by using the F-PHFA method (Riken Genesis Co, Ltd, Tokyo, Japan). F-PHFA can detect common *KRAS* mutations, such as G12D/V/R/C, Q61H, and G13D substitutions. The details of the method are described in the supporting information.

To estimate the proportions of tumor cells in the LBC specimens, we compared the numbers of cancerous and benign cells in the LBC-derived pathological specimens. The numbers of benign and tumor cells in 5 randomly selected fields at ×200 magnification were counted, and the proportions of tumor cells were calculated.

Library Preparation, QC, and NGS Analysis

The library preparation for NGS was performed with 10 ng of DNA, and the details are provided in the supporting information.

Amplicon peaks were evaluated with the Agilent 4200 TapeStation system to confirm the success or failure of NGS library preparation. The AmpliSeq for Illumina Cancer Hotspot Panel is designed to generate synthetic amplicons in the target region around 300 bp. Amplicon peak sizes ranged from 307 to 396 bp (large blue arrowheads in Fig. 3A,B). Library samples for which synthetic amplicons could not be identified were rejected for further analysis. The manipulations after

library confirmation are described in the supporting information.

NGS Data Analysis

A postsequencing data analysis, including alignment to the GRCh38 human reference genome and variant calling, was conducted to validate mutations with VariantStudio 3.0 (Illumina, Inc). A filter was used in VariantStudio 3.0 to select mutations that met the following criteria: minimum sequencing coverage depth > 100 and variant quality score > 30. Additionally, putative germline mutations, synonymous mutations, and variants common to >1% of the East Asian population were excluded. Each variant was prioritized according to the 2017 collaborative consensus guidelines, a hierarchy-based system for interpreting sequence variants in cancer, from the Association for Molecular Pathology, the American Society of Clinical Oncology, and the College of American Pathologists.[30] Only variations registered in the Catalogue of Somatic Mutations in Cancer for the purpose of extracting tier I and II scores were reported and analyzed. Grading from the NGS analysis was compared with histopathologic reports with the final diagnosis used as a reference. The selection and classification of mutations to evaluate the malignancy of the mass were based on the report by Sibinga Mulder et al[20] with some modifications. We focused on the pathological mutations identified in the 6 genes and classified variants as follows: no mutation was classified as "no molecular support for dysplasia" (NMSD); a *KRAS* or *GNAS* mutant alone was classified as "at least low-grade dysplasia" (LGD); and the combination of *KRAS* with a *CDKN2A*, *PIK3CA, TP53,* or *SMAD4* variant was classified as "at least high-grade dysplasia" (HGD; Fig. 2).[6,19,20]

Statistical Analysis

The first objective was to evaluate the factors determining the success or failure of library preparation, and the evaluation items were the total amount of DNA, the DNA integrity number (DIN) values, the concentration of amplifiable DNA, and the QC score. Continuous variables were described as medians and interquartile ranges. We compared 2 variables with a 2-tailed Mann-Whitney *U* test. The same test was performed to evaluate the difference in DNA by the type of preservation solution. The Fisher exact test was performed to evaluate the difference in the number below the DIN calculation limit between the samples that succeeded and failed in the library preparation. Group comparisons were performed with the Kruskal-Wallis test with the Dunn multiple comparison test to evaluate the differences among the 3 specimen types. A P value < .05 was considered statistically significant. Data analyses were performed with GraphPad Prism (version 8; GraphPad Software, La Jolla, California). We referred to the final diagnosis to verify the sensitivity and specificity of each morphological diagnosis and NGS-based classification.

RESULTS

Assessment of the Quantity and Quality of DNA Extracted From Residual LBC Specimens

The DNA extracted from the LBC specimens of 52 patients was evaluated with TapeStation. The median amount of DNA was 166.4 ng, and the median DIN value was 5.5 (Table 1). As shown in an electrophoretic image and graph, the amount of DNA was low in many LBC specimens (Fig. 4A,B). DIN values, indicative of the degree of DNA degradation, varied widely (Fig. 4C). In 7 samples, the DIN values could not be calculated because of insufficient DNA. The total amount of DNA obtained did not change with the type of preservative used or the sample type, but the DIN values tended to be superior for bile (Supporting Fig. 3A,B and Supporting Table 1).

Influence of the Quantity and Quality of DNA for the Success of the Library Preparation

Among the 52 cases, library preparation was successful in 44 (33 PDAC cases and 11 benign cases), whereas it failed in 8 (6 PDAC cases and 2 benign cases; Table 2). The percentage of tumor cells per specimen did not differ between the successful and failed preparations (Supporting Table 2).

The amounts of DNA and the DIN values were significantly higher for the specimens that were suitable for library preparation than those of the failed preparations (Fig. 5A and Table 2). DIN values could not be calculated for 5 successful cases and 2 failed cases because of the extremely low amount of residual DNA. A second test to determine DNA quantity and quality was conducted in 36 DNA specimens with the QIAseq DNA QuantiMIZE Assay Kit. Sixteen samples with no residual DNA (all successful cases) were omitted from the analysis. For all cases investigated, the genomic DNA was rated as high quality with a QC score of <0.04, and there was no significant association in terms of the success or failure of the library preparation (Fig. 5B and Supporting Table 3). The amplifiable

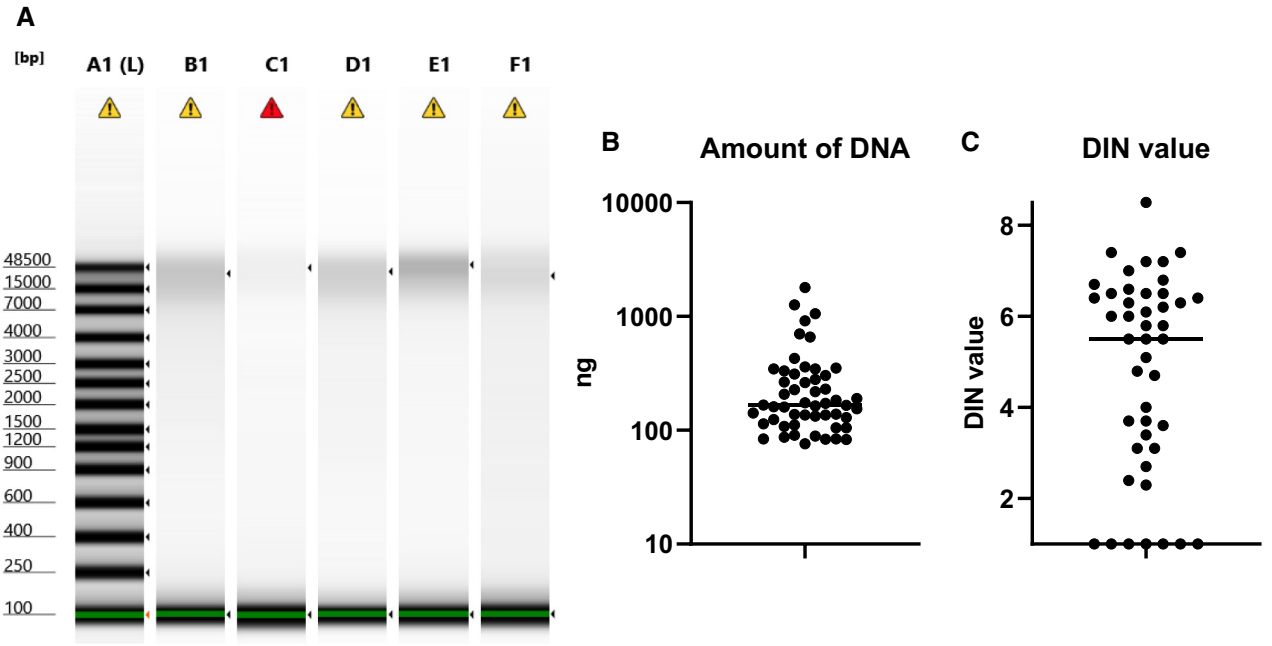

Figure 4. Characteristics of DNA extracted from LBC specimens. (A) Electrophoretic image of DNA from TapeStation. The small black arrow indicates the band peak detected by the TapeStation device. The red and yellow triangles at the top show samples whose DNA concentration was too low for the TapeStation device to calculate the amount of DNA and/or the degree of degradation. The green line indicates the control DNA present in the loading dye reagent. (B) Total amount of DNA in all specimens. (C) DIN values for evaluable samples obtained from the electrophoretic pattern from TapeStation. Bars indicate median values. bp indicates base pair; DIN, DNA integrity number; LBC, liquid-based cytology.

TABLE 2. Comparison of Properties, Including the Quantity and Quality of DNA, Between Successful and Failed Library Preparations

Specimens	No.	S	44	
		F	8	
Evaluation by TapeStation				*P*
Amount of extracted DNA	Median (IQR), ng	S	179.0 (136.4-336.8)	.0076[a]
		F	106.5 (91.5-125.3)	
DIN value	Median (IQR)	S	6 (3.6-6.5)	.0090[a]
		F	1 (1.0-5.6)	
Amount of DNA below DIN calculation limit	No. (%)	S	5 (11.4)	.2915
		F	2 (25)	
QIAseq DNA QuantiMIZE assay				
Amplifiable DNA	Median (IQR), ng/μL	S	3.91 (2.39-8.78)	.0001[b]
		F	0.53 (0.1-0.78)	
QC score	Median (IQR)	S	0.006 (0.003-0.008)	.3312
		F	0.004 (0.003-0.007)	

Abbreviations: DIN, DNA integrity number; F, failed; IQR, interquartile range; QC, quality check; S, successful.
[a]$P < .01$.
[b]$P < .0001$.

DNA concentration was higher in the successful samples and lower in the failed samples (Fig. 5B and Table 2).

Clinicopathological Characteristics of the Patients

Table 3 presents the final diagnoses of solid pancreatic lesions in the 44 cases for which successful library preparation was achieved from LBC residues of EUS-FNA, brushing, and bile juice. Patients with PDAC and 11 others with benign lesions were examined (mean age for patients with PDAC, 75 years; mean age for others, 67 years). Table 4 presents the clinicopathological characteristics of the 33 patients with a final diagnosis of PDAC classified according to the Union for International Cancer Control.

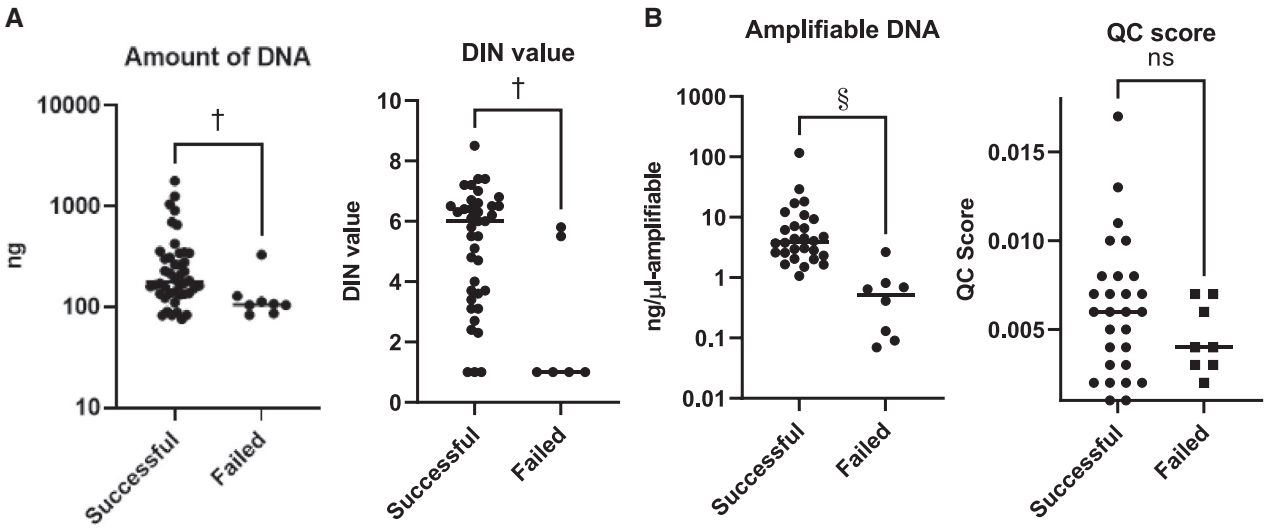

Figure 5. Comparison of the quantity and quality of DNA in successful and failed library preparations. (A) (*Left*) Amount of DNA in LBC specimens and (*Right*) DIN values. (B) Evaluation of DNA quality via the QuantiMIZE polymerase chain reaction method. (*Left*) Amplifiable DNA of LBC specimens and (*Right*) QC scores are shown. Bars indicate mean values. $^{\dagger}P < .01$; $^{\S}P < .0001$. DIN indicates DNA integrity number; LBC, liquid-based cytology; ns, not significant; QC, quality check.

TABLE 3. Final Diagnosis of Pancreatic Lesions in a Successfully Prepared Library

Final Diagnosis	No. of Cases
PDAC	33
AP	6
Pancreatitis	3
Cholangitis	2
Total	44

Abbreviations: AP, autoimmune pancreatitis; PDAC, pancreatic ductal adenocarcinoma.

TABLE 4. Clinicopathological Characteristics of Pancreatic Ductal Adenocarcinomas (n = 33)

Characteristic	Value
Age, mean (range), y	75 (57-91)
Sex, No. (%)	
Male	19 (58)
Female	14 (42)
Clinical stage, No. (%)[a]	
IA	2 (6)
IB	1 (3)
IIA	7 (21)
IIB	3 (9)
III	4 (12)
IV	16 (48)
Tumor location, No. (%)	
Head	10 (30)
Body/tail	23 (70)
Tumor size, mean (range), mm	30.6 (12-66)

[a]*TNM Classification of Malignant Tumors*, 7th edition, from the Union for International Cancer Control.

Evaluation of NGS of LBC Specimens and Gene Mutation Status

The average total number of leads for each sample was 987,439 (Supporting Table 4). The percentage of aligned reads exceeded the vendor-recommended level of 80% in all cases. The mean coverage of the amplicon in most specimens reached the vendor-recommended level (2500-3000 times); however, in 2 cases, the coverage was low (sample 23, 2469.9; sample 36, 1941.5). The uniformity of the coverage percentage exceeded the vendor-recommended level of 95% in all but 3 samples (samples 23, 40, and 44).

The NGS analysis was confirmed with the *KRAS* experiments using F-PHFA. The concordance rates of *KRAS* mutations in PDAC for the 2 methods using the residual LBC samples were 85% (28 of 33) for PDAC and 100% (11 of 11) for the others (Fig. 6). Among these discrepancies, 2 *KRAS* mutations were detected only in F-PHFA (not in NGS), and 3 mutations were detected only in NGS (not in F-PHFA). The *KRAS* Q61R mutant was detected only in NGS because it was not covered by the F-PHFA method. For the other 4 cases, the reason may be that the quantity and quality of LBC-derived DNA varied greatly among the samples, as shown in Figure 4B,C. First, it is possible that the amount of DNA was too small to be detected by the F-PHFA method. The recommended amount of DNA per PCR reaction

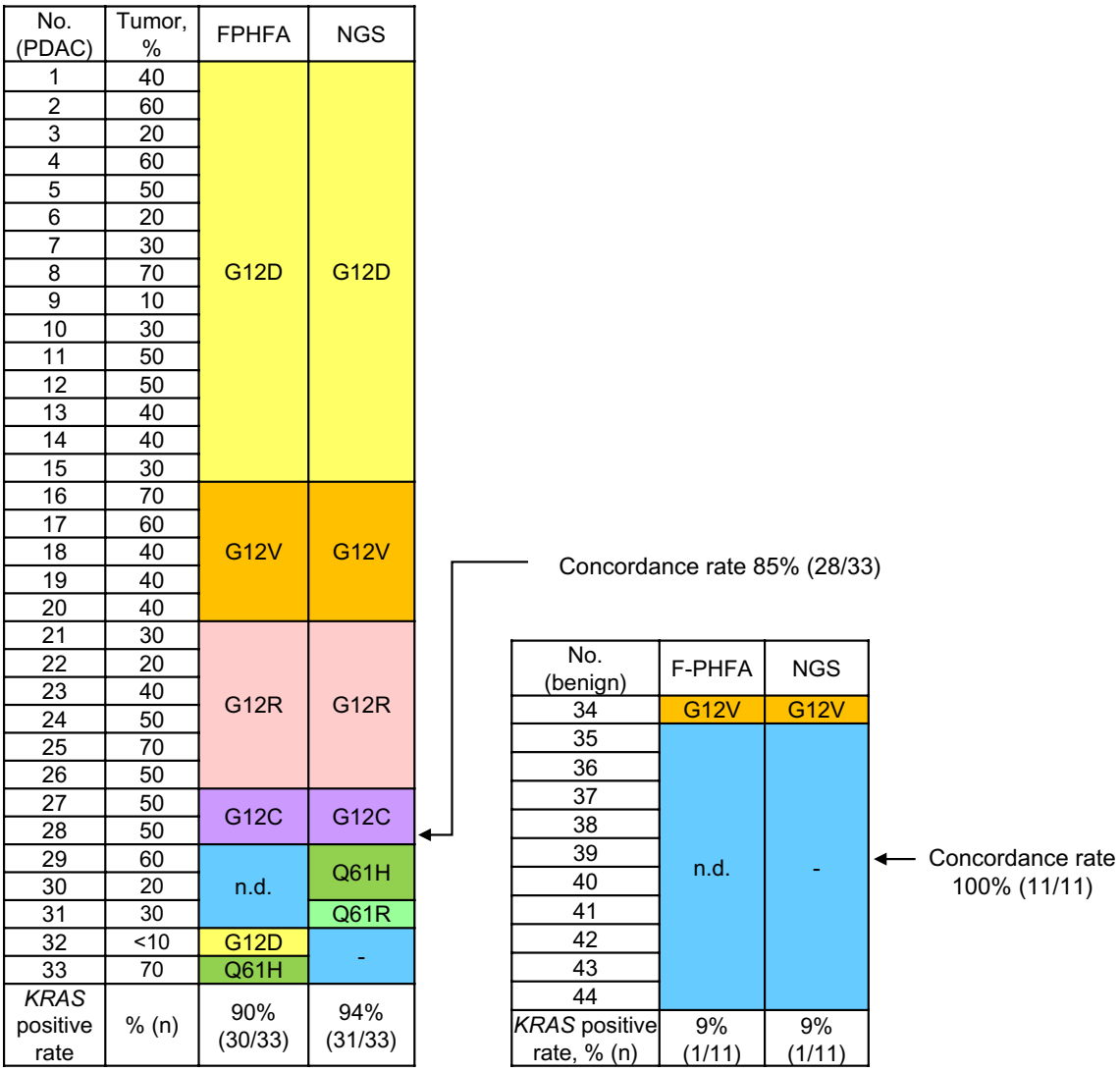

Figure 6. Concordance rate of *KRAS* mutations. Two methods were used to detect *KRAS* mutations: F-PHFA and NGS. The first column reports the proportion of tumor cells detected by light microscopy. *KRAS* positivity and the mutation type are indicated; 1 to 33 are PDACs, and 34 to 44 are benign lesions. F-PHFA indicates fluorescence resonance energy transfer–based preferential homoduplex formation assay; n.d., not determined; NGS, next-generation sequencing; PDAC, pancreatic ductal adenocarcinoma.

in the F-PHFA method is 20 to 100 ng, but there were samples that contained <10 ng as the maximum amount. Conversely, the amplified DNA size used in F-PHFA was ~60 bp, and this suggested that it may be possible to analyze even degraded DNA. In addition, Nishikawa et al[21] used the same DNA derived from cultured cells to compare the detection of *EGFR* mutations by NGS and F-PHFA. The sensitivity of the F-PHFA method was superior to that of NGS for both exon 19 deletions and T790M/L858R mutations. This may explain, in part, the discrepancy between the F-PHFA and NGS *KRAS* mutations in this study.

Table 5 presents the protein changes and the number of cases in which mutations were detected by NGS for *CDKN2A*, *PIK3CA*, *KRAS*, *SMAD4*, and *TP53*.[9,31] The *GNAS* mutations were not detected in all specimens in this study. The details of each case are described in Supporting Table 5. Figure 7 shows the final diagnoses, pathological examination of the LBC-treated specimens, types of genes with mutations in NGS, and grade classification by gene mutation for individual cases of PDAC and other lesions. Among the 33 PDACs, gene mutations were identified in 31 (94%) for *KRAS*, in 16 (48%) for *TP53*, in 3 (9%) for *CDKN2A*, in 2 (6%) for *SMAD4*,

TABLE 5. Details of the Detected Target Gene Mutations in the 6 Genes

Gene	Protein Change (No. of Cases)	COSMIC Genomic Mutation ID (No. of Registered Cases)	ClinVar Significance
CDKN2A	R80* (1)	COSV58682746 (307)	Pathogenic
	H83Y (2)	COSV58682852 (127)	Uncertain significance
KRAS	G12C (2)	COSV55497469 (5340)	Pathogenic
	G12D (15)	COSV55497369 (15848)	Pathogenic
	G12R (6)	COSV55497582 (1548)	Pathogenic
	G12V (6)	COSV55497419 (10797)	Pathogenic
	Q61H (2)	COSV55499223 (143)	Pathogenic
	Q61R (1)	COSV55498739 (166)	Pathogenic
PIK3CA	N345K (1)	COSV55873276 (279)	Pathogenic
	T1025A (1)	COSV55873252 (53)	Pathogenic
SMAD4	A118V (1)	COSV61684095 (40)	—
	W524G (1)	COSV100747680 (1)	—
TP53	E11Q (1)	COSV52746799 (15)	Uncertain significance
	G245V (1)	COSV52666323 (135)	Pathogenic
	R175H (1)	COSV52661038 (1911)	Pathogenic
	P177S (1)	COSV52688422 (15)	Uncertain significance
	H179R (1)	COSV52661712 (278)	Conflicting interpretations of pathogenicity
	I195T (1)	COSV52664264 (187)	Pathogenic
	I195S (1)	COSV52661172 (17)	Pathogenic
	E204* (1)	COSV52679869 (76)	Pathogenic
	T230Hfs (1)	COSV52738764 (3)	—
	T236C (1)	COSV52662150 (141)	Uncertain significance
	N239S (1)	6COSV5261127 (48)	Likely pathogenic
	R248Q (1)	COSV52661580 (1280)	Likely pathogenic: uncertain significance: pathogenic
	I251Sfs (1)	COSV52688126 (11)	Pathogenic
	R282W (2)	COSV52662048 (906)	Pathogenic/likely pathogenic
	E343* (1)	COSV52683572 (28)	Pathogenic
	E346* (1)	COSV52688134 (8)	Pathogenic

Abbreviation: COSMIC, Catalogue of Somatic Mutations in Cancer.
The number in parentheses in the protein change column indicates the number of cases with mutations in this study. The number in parentheses for the COSMIC genomic mutation ID indicates the number of cases registered in the database. Data were extracted from the COSMIC and ClinVar databases[9,31] (accessed from November to December 2020).

and in 2 (6%) for *PIK3CA*. Only 1 benign case harbored a *KRAS* mutation.

Comparison Between Histological Diagnosis and Grading Based on Gene Mutations Using NGS Analysis

Thirty-two of the 33 cases of PDAC (97%) were histopathologically diagnosed as AC, and 1 was diagnosed as atypia (Fig. 7 and Supporting Fig. 4A). Among the 11 benign cases, 5 were diagnosed as inflammation, 4 were diagnosed as no evidence of malignancy, and 2 were diagnosed as atypia. Among the 33 cases with a final diagnosis of PDAC, there were 18 cases of HGD (55%), 13 cases of LGD (39%), and 2 cases of NMSD according to the mutation-based grading by NGS analysis (Supporting Fig. 4B). Only 1 of the 11 benign cases was defined as having a mutation by NGS defined as LGD, and all others were classified as NMSD.

In comparison with the final diagnosis, the sensitivity of the morphological diagnosis was 97%, and the sensitivity of the NGS mutation-based classification of HGD was 55% (Supporting Fig. 4A,B). The specificity was 100% for both morphological diagnosis and classification by NGS. The sensitivity of LGD or HGD by NGS classification was 94%, which approached the sensitivity of morphological diagnosis, but the specificity was 91%.

DISCUSSION

The distinction between benign and malignant pancreatic masses remains a diagnostic challenge because of the importance of early detection of cancer and avoidance of unnecessary surgery. The usefulness of NGS analysis of LBC samples stored at 4°C for several months was investigated. The NGS library was successfully prepared in 44 of 52 cases, and suitable NGS analysis results were obtained. The results of the NGS analysis using LBC specimens were reliable and could support a morphological diagnosis.

We compared the DNA quantity and quality and examined the reasons for the success or failure of the library preparation. Kim et al[32] reported that PCR of DNA from LBC specimens stored for ≥9 months failed

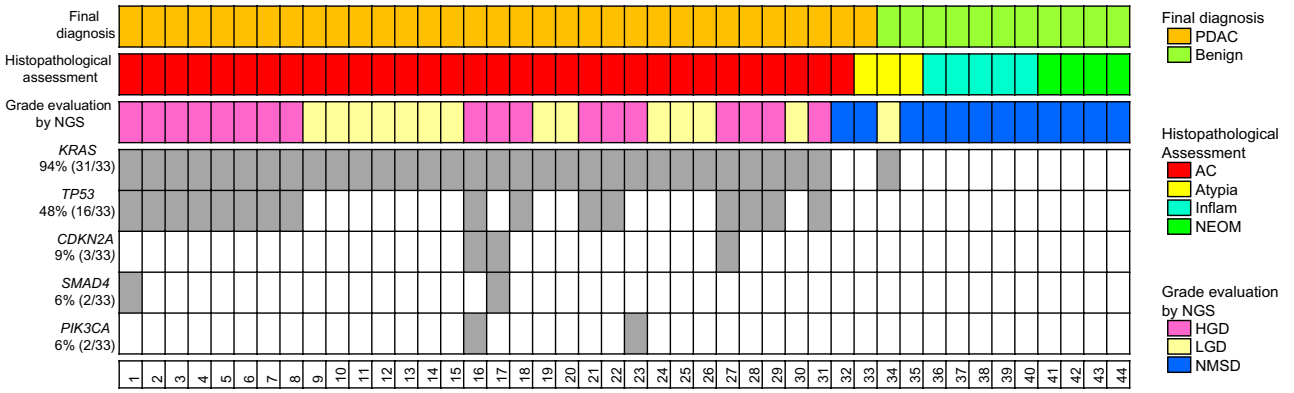

Figure 7. Correlative findings for the pathological examination of endoscopic ultrasound–guided fine-needle aspiration, brushes, and bile juice and grade evaluation by individual genomic alterations from NGS testing in PDAC and other lesions. Nonsynonymous and indel mutation rates are shown for *KRAS*, *TP53*, *CDKN2A*, *SMAD4*, and *PIK3CA* mutations. AC indicates adenocarcinoma; HGD, at least high-grade dysplasia; Inflam, inflammation; LGD, at least low-grade dysplasia; NEOM, no evidence of malignancy; NGS, next-generation sequencing; NMSD, no molecular support for dysplasia; PDAC, pancreatic ductal adenocarcinoma.

because of DNA degradation with an amplification product of 226 bp. We considered the possibility that DNA degradation due to long-term storage might prevent library preparation, but the average storage period of the residual LBC specimens used in this study was 103 days, and the storage period of most of the specimens did not exceed 9 months. In addition, the preservation period was not significantly longer for the failed specimens than the successful specimens (Supporting Fig. 5). DNA analysis using TapeStation and real-time PCR showed that there were significant differences in the amount of DNA and DNA degradation and in the concentration of amplifiable DNA in terms of the success or failure of library preparation and NGS. These showed that both the quantity and quality of DNA are crucial for the success of NGS. It is important to ensure the quality of the sequence reads to guarantee the reliability of the results, and generally, sequence variants derived from libraries with >100× coverage in the target region and a robust amplification profile may be considered reliable.

The 4 major driver gene mutations of PDAC are *KRAS*, *TP53*, *SMAD4*, and *CDKN2A*.[9-13] A simple and economical test is needed to identify different gene mutations to effectively contribute to the accurate diagnosis of tumors and the search for therapeutic agents. In this study, instead of a comprehensive evaluation of all genetic mutations occurring in pancreatic cancer, we selected and evaluated 6 genes (*KRAS*, *GNAS*, *TP53*, *CDKN2A*, *SMAD4*, and *PIK3CA*) frequently identified by previous studies as contributing to the distinction between benign and malignant disease. A single mutation may be detected in borderline lesions such as pancreatic intraepithelial neoplasms,[28,33-35] so combining multiple genetic mutations could reduce overdiagnosis and increase specificity. Mutation-based grading by NGS analysis revealed that 18 of the 33 PDAC cases were HGD, but 13 were LGD. In comparison with the final diagnosis, the sensitivity of HGD or LGD (94%) was higher than that of HGD alone (55%). The NGS classification did not overdiagnose nonmalignant lesions. Thus, we propose that it would be reasonable to use NGS as an adjunct to the morphological diagnosis of pancreatic LBC-derived specimens rather than NGS alone to determine malignancy.

In addition, it has been reported that a large number of mutations in the major driver genes of pancreatic cancer (*KRAS*, *TP53*, *SMAD4*, and *CDKN2A*) correlates with a poor prognosis[18] and that the *KRAS* G12D mutation is an independent prognostic factor for advanced pancreatic ductal carcinoma.[36] Fifteen of the 31 *KRAS* mutation–positive patients in this study were of the G12D subtype; additional clinical follow-up data would be needed to evaluate the prognostic value of NGS analysis.

At present, there are few effective molecular targeted therapies for pancreatic cancer. Although infrequent, sotorasib has been reported to be effective in various solid tumors with *KRAS* G12C mutations.[37] Two cases reviewed in this study had this variant of *KRAS* mutation, and this suggests that LBC specimens are useful material

not only for diagnosis but also for selecting molecular targeted drugs.

Despite its positive findings, our study had several limitations. It is difficult to draw definitive conclusions on the possibility of using LBC for diagnostic purposes in routine work because of the small number of biliary brush and bile specimens. However, we believe that the total number of the 3 types of specimens examined in this study meets the minimum number to evaluate the possibility of pancreatic LBC. If we assume that the quality of nucleic acids was low in specimens that were originally stored at 4°C in LBCs with a small number of cells, there were some regions that could not be evaluated because hotspots of 6 frequent genes were evaluated. Because the examination was performed after the *KRAS* mutation was confirmed by F-PHFA, it was difficult to fully evaluate the success or failure of the library preparation for the specimens with no remaining volume. Surgical specimens were not compared with the LBC-derived mutation analysis because the tumor cells had regressed on account of preoperative chemo/radiation therapy.

In conclusion, we examined the feasibility of an NGS assay on residual LBC specimens and found that they were likely to be successful if the quantity and quality of DNA were acceptable. Cancer-related mutations evaluated by NGS using LBC specimens suggested that this approach could contribute to the diagnosis of malignancy. The assay could be integrated into clinical laboratories as a routine test for diagnosis and prognosis and to identify potential molecular targeted therapies.

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AUTHOR CONTRIBUTIONS

Yoko Sekita-Hatakeyama: Conceptualization, survey, data analysis, and writing–original draft. **Tomomi Fujii:** Conceptualization, survey, data analysis, and writing–review and editing. **Takeshi Nishikawa:** Methodology. **Akira Mitoro:** Conceptualization, methodology, and survey. **Masayoshi Sawai:** Methodology and survey. **Hiroe Itami:** Methodology. **Kouhei Morita:** Methodology. **Tomoko Uchiyama:** Methodology. **Maiko Takeda:** Methodology. **Masayuki Sho:** Methodology and survey. **Hitoshi Yoshiji:** Methodology and survey. **Kinta Hatakeyama:** Conceptualization and writing–review and editing. **Chiho Ohbayashi:** Conceptualization and writing–review and editing.

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