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Feasibility of Comprehensive Genomic Profiling for Biliary Tract Cancer Using Transpapillary Biopsy Samples: A Prospective Study

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ABSTRACT

Background: Patients with biliary tract cancer (BTC) often have actionable mutations, and comprehensive genomic profiling (CGP) plays an important role. However, the feasibility of CGP using transpapillary biopsy (TPB) samples remains unclear.

Methods: Thirty patients with suspected BTC based on radiographic imaging were enrolled. Pre-analytical criteria for CGP suitability were based on the OncoGuide NCC Oncopanel System (NCCOP) and FoundationOne CDx (F1CDx). Each patient underwent six biopsies using an endoscopic introducer: five biopsy samples were preserved as formalin-fixed paraffin-embedded (FFPE) samples and one as a fresh frozen (FF) sample. DNA quality indicators were compared between the two groups.

Results: Malignancy was confirmed in 29 patients, and one had a benign biliary stricture. Suitability rate was 31% (9/29) for NCCOP and 3.4% (1/29) for F1CDx. Compared to FFPE samples, FF samples demonstrated significantly higher DNA concentration [ng/μL, interquartile range (IQR)], [0.34 (0.16–0.95) vs. 37.8 (11.6–67.6), $p < 0.001$] and DNA integrity number (IQR) [7.1 (6.8–7.3) vs. 8.9 (8.3–9), $p = 0.021$].

Conclusions: Introducer-assisted multipass TPB may increase the rate of obtaining adequate CGP specimens, but its suitability remains limited and strongly panel dependent. Since FF samples have better DNA quality, establishing a system detailing their use is desirable.

Trial Registration: ClinicalTrials.gov identifier: UMIN 000049826

1 | Introduction

Surgical resection is considered the only curative treatment for biliary tract cancer (BTC). As resection is not possible in many cases, chemotherapy plays an important role. However, the prognosis for patients who have undergone chemotherapy for advanced or recurrent BTC is limited to approximately 1 year, and treatment options are limited [1, 2]. Therefore, the development of new treatments is required. In recent years, mutation-specific therapy based on comprehensive genomic profiling (CGP) for

Abbreviations: BTC, biliary tract cancer; CE-CT, contrast-enhanced computed tomography; CGP, comprehensive genomic profiling; dCCA, distal cholangiocarcinoma; DIN, DNA integrity number; ERCP, endoscopic retrograde cholangiopancreatography; EST, endoscopic sphincterotomy; EUS, endoscopic ultrasonography; EUS-TA, endoscopic ultrasound-guided tissue acquisition; F1CDx, FoundationOne CDx; FF, fresh frozen; FFPE, formalin-fixed paraffin-embedded; H&E, hematoxylin and eosin; iCCA, intrahepatic cholangiocarcinoma; NCCOP, OncoGuide NCC Oncopanel System; POCS, peroral cholangioscopy; ROSE, rapid on-site cytological evaluation; TPB, transpapillary biopsy; WSIs, whole-slide images.

unresectable and recurrent cancers has become possible and is being actively performed for BTC [3, 4]. Approximately 40%–58% of patients with BTC have genetic mutations that could serve as therapeutic targets [5, 6].

CGP can be performed using tumor tissues or blood samples. In patients with unresectable BTC, tumor tissues can be obtained using ultrasound-guided liver biopsy or endoscopic ultrasound-guided tissue acquisition (EUS-TA) for intrahepatic cholangiocarcinoma (iCCA). In contrast, these methods are often difficult for extrahepatic cholangiocarcinoma, and transpapillary biopsy (TPB) during endoscopic retrograde cholangiopancreatography (ERCP) is therefore employed. When sufficient tumor tissue cannot be obtained, liquid biopsy may be considered; however, liquid biopsy may yield false-negative results due to factors such as sampling time and tumor volume [7]. In particular, detection of fusion genes has been reported to be poor, and the use of tumor tissue samples is recommended, whenever possible [8, 9].

Formalin-fixed paraffin-embedded (FFPE) tissues are widely used for CGP owing to their long-term storage stability, which preserves the structure of the tumor tissue. However, nucleic acid degradation during the fixation process can be disruptive to analysis when sample volumes are small [10]. DNA fragmentation results from chemical modifications of nucleic acids during formalin fixation. Prolonged fixation, in particular, exacerbates formalin-induced cross-linking, thereby causing greater fragmentation during nucleic acid extraction. Some studies have reported the usefulness of using non-FFPE samples such as stained smears, touch preparations, or liquid-based cytology for CGP [11, 12]. Fresh frozen (FF) samples are a type of non-FFPE samples, and it has been demonstrated that the data of FFPE and FF samples are highly correlated [13–16]. However, whether FF samples are feasible for CGP specifically in BTC, particularly when obtained endoscopically, has not been established.

In many cases of BTC, TPB is commonly performed during ERCP, which is performed for diagnosis and drainage. However, the amount of tissue obtained via TPB is limited compared with that obtained surgically, and the clinical application of CGP has not progressed [17]. Increasing the number of biopsy passes may improve yield, yet repeated cannulation can raise concerns about pancreatitis or ductal injury. Recently, the introduction of endoscopic introducers has enabled safe, rapid multipass TPB [18]. Accordingly, we conducted a prospective study to evaluate the feasibility of CGP using TPB specimens obtained with an endoscopic introducer in patients with suspected BTC and to directly compare DNA quality indicators between matched FFPE and FF samples.

2 | Methods

2.1 | Patients

This prospective study was conducted from December 2022 to February 2025 at Okayama University Hospital in Japan. Patients were enrolled if they required examination or treatment with ERCP for suspected BTC. The inclusion criteria were as follows: (1) ability to provide written informed consent and (2) age 20 years or older. The exclusion criteria were as follows: (1) Eastern Cooperative Oncology Group performance status 3 or 4; (2) severe complications in other organs (such as cardiac failure, respiratory failure, or impaired consciousness); (3) severe coagulation dysfunction; and (4) judged inappropriate by the research leader. This study was conducted in compliance with the Declaration of Helsinki and was approved by the Ethics Committee Review Board for Human Research (approval number: 2301-018; approval date: November 19, 2022). This study was registered in the UMIN Clinical Trials Registry (identifier: UMIN 000049826; registration date: November 19, 2022; start date: November 19, 2022).

2.2 | Endoscopic Procedures

ERCP was performed using a side-viewing duodenoscope (TJF-260 V or TJF-Q290V; Olympus Medical Systems, Tokyo, Japan), following standard procedures. After biliary cannulation, biliary strictures were observed using cholangiography. As part of other research, this study included many cases in which endoscopic sphincterotomy (EST) was performed in the previous session and peroral cholangioscopy (POCS) was performed in the same session. In cases with naïve papillae, EST was performed using a sphincterotome (CleverCut3V; Olympus Medical System). Bile duct biopsy was then performed using an endoscopic introducer (EndoSheather; Piolax, Kanagawa, Japan) [18]. Biopsy forceps that are 2.0 mm in diameter (Radial Jaw 4 Pediatric Biopsy Forceps; Boston Scientific Japan, Tokyo, Japan) were used in this study (Figure 1). Six biopsies were performed using an endoscopic introducer; the first through fifth specimens were pooled and processed as FFPE samples, whereas the sixth specimen was processed as an FF sample. All five FFPE samples were placed in formalin bottles and used as tissue specimens and were evaluated by a pathologist. The FF samples were frozen in liquid nitrogen and evaluated by an experienced laboratory technician (HI) (Figure 2). Regarding stent selection, self-expandable metallic stents were preferentially used in patients receiving best supportive care, whereas plastic stents were used in the remaining patients. For plastic stents, either inside or transpapillary stents were selected according to the location and extent of the biliary stricture. In patients scheduled for surgical resection, biliary drainage was performed in the future liver remnants. In unresectable cases, drainage was attempted to cover at least

FIGURE 1 | Image of the endoscopic introducer system. Image of the tip of the system after the insertion of the biopsy forceps into the outer sheath.

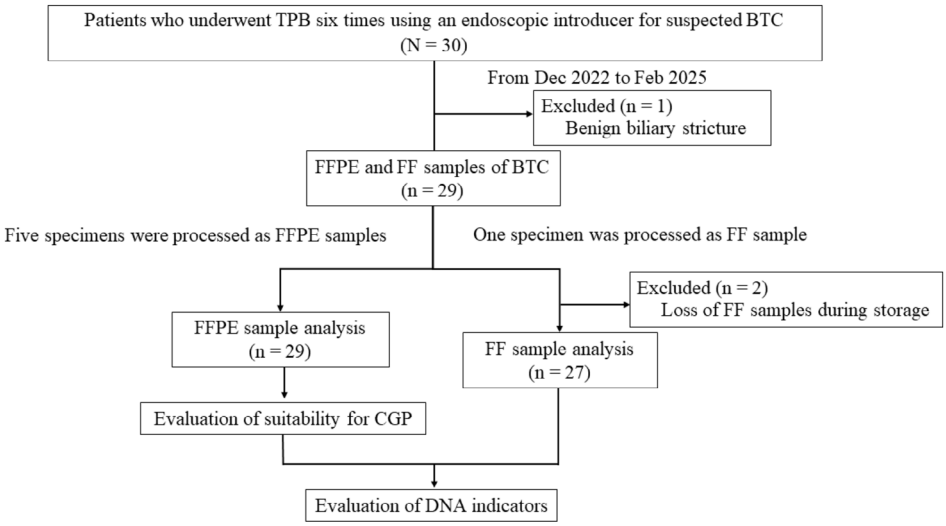

FIGURE 2 | Study flow diagram. BTC, biliary tract cancer; CGP, comprehensive genomic profiling; DNA, deoxyribonucleic acid; FF, fresh frozen; FFPE, formalin-fixed, paraffin-embedded; TPB, transpapillary biopsy; WSI, whole-slide image.

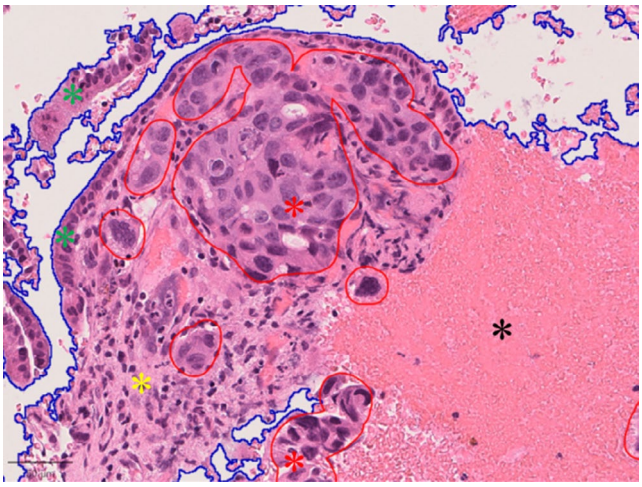

FIGURE 3 | A schema of a whole slide image. Screenshot of a whole slide image. The sample area is delineated in blue, and the tumor cells are outlined in red. The sample area consists of tumor cells (red asterisks), non-tumor epithelial cells (green asterisks), stroma (yellow asterisk), and blood clots (black asterisk). Scale bar indicates 50 μm.

50% of the functional liver volume. In this study, obstructive jaundice was observed in all patients with gallbladder cancer ($n = 1$) and iCCA ($n = 3$), and TPB targeting bile duct strictures was performed in these patients.

2.3 | Processing and Quality Assessment of FFPE Samples

In accordance with the guidelines, formalin fixation was performed for 6–48 h [19]. For each case, a representative hematoxylin and eosin (H&E)-stained section from the FFPE block used for genomic profiling was evaluated. H&E slides were converted to whole-slide images (WSIs) using a NanoZoomer S60 (Hamamatsu Photonics, Shizuoka, Japan) (Figure 3), and the WSIs were analyzed using the open-source software QuPath [20]. The sample areas were automatically detected using the "Pixel classifier" command with parameters optimized for this study: resolution, high; channel, average channels; prefilter, Gaussian; smoothing sigma, 1; threshold, 220; minimum object size, 200; and minimum hole size, 200. Blood clots and acellular mucin were included in the detected sample areas.

Tumor cells were manually annotated by a pathologist (MF) with reference to an optical microscope. Nuclear detection was performed automatically using QuPath's built-in "Cell detection" algorithm, which identifies individual nuclei based on hematoxylin staining (background radius: 10 μm; median filter radius: 1 μm; sigma: 1 μm; minimum area: 5 μm^2; maximum area: 200 μm^2; others: default settings). The number of nuclei located within the tumor cell annotation and the total number of nuclei within the pixel-classified sample area were automatically obtained. Tumor cellularity was defined as the proportion of nuclei located within the tumor cell annotation relative to the total number of nuclei detected within the sample area (the number of nuclei in the tumor cell area divided by the number of nuclei in the sample area).

Tumor cells were carefully delineated from stromal components as precisely as possible, although complete separation from intratumoral inflammatory cells or capillaries was frequently unattainable. All tumor annotations were performed by a single pathologist using a predefined workflow with fixed QuPath parameters, and all image analysis procedures were performed using identical parameters across all cases to maintain consistency.

2.4 | DNA Extraction and Quality Assessment of FFPE and FF Samples

Fifty microliters of DNA was extracted from each 10 μm section of FFPE tissues and a whole piece of FF tissues. In FFPE tissues, five biopsy samples were prepared as a single FFPE block, and the most appropriate 10 μm section was selected based on the tumor cellularity and tissue area. DNA was quantified using a Qubit 4.0 Fluorometer (Thermo Fisher Scientific, Tokyo, Japan)

and a Qubit 1X dsDNA HS Assay Kit (Thermo Fisher Scientific). DNA quality was determined using a TapeStation 4150 (Agilent Technologies Japan, Tokyo, Japan) with a Genomic DNA (Agilent Technologies Japan). The DNA integrity number (DIN) was used as a quality control indicator for DNA samples. The DIN reflects the degree of DNA degradation. It ranges from 1.0 to 10.0, with lower values indicating more advanced degradation [19].

2.5 | Diagnostic Criteria

Malignancy was defined as histopathological evidence of malignancy or suspicious for malignancy and/or cytological findings classified as Class 4 or 5 according to the Papanicolaou classification [21]. For cases in which surgery was performed, the postoperative pathology results were used as the final diagnosis. If a diagnosis could not be made using these methods, the lesion was classified as benign or malignant based on the patient's 6-month clinical follow-up. The primary site of BTC was classified according to the Union for International Cancer Control TNM classification (8th edition), using contrast-enhanced computed tomography (CE-CT). The macroscopic type of the primary tumor was classified as nodular, flat, or other based on imaging findings such as those of CE-CT, endoscopic ultrasonography (EUS), and cholangiography. Technical success was defined as the successful completion of all six TPBs. Procedural adverse events were evaluated using the American Society for Gastrointestinal Endoscopy Lexicon [22]. For CGP, we used the OncoGuide NCC Oncopanel System (NCCOP; Sysmex, Hyogo, Japan) and FoundationOne CDx (F1CDx; Foundation Medicine, Cambridge, MA, USA), which are generally used in Japan. Based on previous studies, the eligibility criteria for NCCOP were tumor cellularity $\geq 20\%$ and tissue area $\geq 4\,mm^2$ [23, 24]. The eligibility criteria for F1CDx were cellularity $\geq 20\%$ and tissue area $\geq 25\,mm^2$ [25]. The other quality control indicators for DNA assessment included DNA concentration $> 0.5\,ng/\mu L$ or DIN ≥ 2 [26]. The criterion of DNA concentration was set as the condition that would achieve a minimum nucleic acid yield of 200 ng for 10 slides when extracted with 50 μL of solvent.

2.6 | Outcomes

The proportion of patients who met the CGP eligibility criteria based on TPB samples among patients with BTC was evaluated. The technical success rate, rate of adequate sampling for malignant diagnosis, tumor cellularity, tissue size, rate of procedure-related adverse events, and proportion of successful CGP analyses performed in practice were also evaluated. Furthermore, DNA quality indicators, such as DNA concentration and DIN between the FFPE and FF samples, were compared.

2.7 | Statistical Analysis

Given the exploratory nature of this study and the difficulty of reliably estimating the effect size in advance, we did not perform a power analysis and instead consecutively enrolled the maximum feasible number of patients. Cases with missing data for the variables of interest were excluded from the analysis. All statistical analyses were performed using the JMP Pro software version 18 (SAS Institute, Cary, NC, USA). Continuous variables are expressed as medians (interquartile ranges). Categorical variables are expressed as numbers (percentages). Pearson's $\chi 2$ test was used for categorical variables, whereas the Mann–Whitney *U*-test was used for continuous data. Univariate analysis was performed using logistic regression to identify significant risk factors for adverse events. p values < 0.05 were considered statistically significant.

3 | Results

3.1 | Patient Characteristics

A total of 30 consecutive patients were prospectively enrolled in this study. A flow diagram of the study is shown in Figure 2. One patient was excluded because of a benign biliary stricture, and FFPE samples from 29 patients were analyzed. Of these, two patients were excluded due to loss of FF samples during storage, and FF samples from 27 patients were analyzed.

The characteristics of the 30 patients are summarized in Table 1. The biliary stricture was approximately half the hilar site and half the distal end. The macroscopic type was nodular (18/30, 60%), followed by flat (8/30, 26.7%). The most common bismuth classification was type I (12/30, 40%), followed by II (8/30, 26.7%) and IV (7/30, 23.3%) [27]. In the final diagnosis, perihilar cholangiocarcinoma and distal cholangiocarcinoma (dCCA) together accounted for just over 80% of the cases (25/30, 83.3%). Only two (6.7%) patients were undergoing chemotherapy.

3.2 | Procedure Details and Procedural Adverse Events

The procedural details of the biopsies are presented in Table 2. Technical success was achieved in all the 30 (100%) patients. The method of malignant diagnosis was TPB in 24/29 (82.8%) of patients, and the remaining five patients were diagnosed using the following methods: surgery, 2; clinical course, 2; and imprint cytology, 1. Sixteen of the 30 (53.3%) patients had undergone EST in the previous session, and 19 of the 30 (63.3%) patients underwent POCS before TPB in the same session. The rate of procedure-related adverse events was 10% (3/30): mild cholangitis, 6.7% (2/30) and moderate liver abscess, 3.3% (1/30). All patients were managed conservatively. The analysis of the risk factors for adverse events is shown in Table S1. No involvement of POCS was found (OR, 1.18; 95% CI, 0.10–27.2; $p = 0.899$), while the placement of three stents was identified as a significant risk factor (OR, 16; 95% CI, 1.20–417; $p = 0.036$).

3.3 | Eligibility Criteria for CGP

Figure 4 presents the proportion of patients who met the eligibility criteria for CGP. The proportion of patients meeting each

TABLE 1 | Characteristics of study patients ($N = 30$).

Age (IQR), years	74.5	(71.5–80.5)
Sex, *n* (%)		
Male	19	(63.3)
Female	11	(36.7)
Biliary stricture site, *n* (%)		
Hilar	16	(53.3)
Distal	14	(46.7)
Macroscopic type, *n* (%)		
Nodular type	18	(60)
Flat type	8	(26.7)
Others	4	(13.3)
Bismuth classification, *n* (%)		
1	12	(40)
2	8	(26.7)
3a	1	(3.3)
3b	2	(6.7)
4	7	(23.3)
Final diagnosis, *n* (%)		
pCCA	13	(43.3)
dCCA	12	(40)
iCCA	3	(10)
GBC	1	(3.3)
Benign biliary stricture	1	(3.3)
Cholangitis at the time of ERCP, *n* (%)	0	(0)
Undergoing chemotherapy, *n* (%)	2	(6.7)

Abbreviations: dCCA, distal cholangiocarcinoma; GBC, gallbladder cancer; iCCA, intrahepatic cholangiocarcinoma; IQR, interquartile range; pCCA, perihilar cholangiocarcinoma.

TABLE 2 | Procedure details and procedural adverse events ($N = 30$).

Technical success, n/N (%)	30/30	(100)
Adequate sampling for malignant diagnosis, n/N (%)	24/29	(82.8)
Ampullary intervention, n/N (%)		
Post EST	16/30	(53.3)
EST in the same session	11/30	(36.7)
EPBD	3/30	(10)
Stent placement, n/N (%)	27/30	(90)
Stent type, n/N (%)		
PS	23/27	(85.2)
SEMS	4/27	(14.8)
Number of stents, n/N (%)		
1	17/27	(63)
2	5/27	(18.5)
3	5/27	(18.5)
POCS in the same session, n/N (%)	19/30	(63.3)
Procedure time (IQR), min	60	(43–78.8)
Procedure-related adverse event, n/N (%)	3/30	(10)
Cholangitis	2/30	(6.7)
Liver abscess	1/30	(3.3)

Abbreviations: EPBD, endoscopic papillary balloon dilatation; EST, endoscopic sphincterotomy; IQR, interquartile range; POCS, peroral cholangioscopy; PS, plastic stent; SEMS, self-expandable metallic stent.

eligibility criterion was as follows: 48.3% (14/29), tumor cellularity ≥ 20%; 65.5% (19/29), tissue area ≥ 4 mm^2; and 3.4% (1/29), tissue area ≥ 25 mm^2. For NCCOP and F1CDx analyses, 31% (9/29) and 3.4% (1/29), respectively, were eligible. The characteristics and outcomes of patients who met the NCCOP analysis eligibility criteria are shown in Table S1.

A comparison of DNA quality indicators between the FFPE and FF samples is shown in Figure 5. The median DNA concentration was 0.34 (0.16–0.95) ng/μL and 37.8 (11.6–67.6) ng/μL in the FFPE and FF groups, respectively, and was significantly higher in the FF group than in the FFPE group ($p < 0.001$). The proportion of patients with DNA concentration > 0.5 ng/μL was also significantly higher in the FF group than in the FFPE group [FFPE group: 27.6% (8/29) versus FF group: 100% (27/27); $p < 0.001$]. The median DIN was significantly higher in the FF group than in the FFPE group [FFPE group: 7.1 (6.8–7.3) versus FF group: 8.9 (8.3–9), $p = 0.021$]. The proportion of patients with DIN > 2 was also significantly higher in the FF group than in the FFPE group [FFPE group: 6.9% (2/29) versus FF group: 81.5% (22/27); $p < 0.001$].

3.4 | Actual CGP

Actual CGP was attempted using TPB samples from four patients. The success rate of CGP using the TPB specimens was 50% (2/4). Details of the pre-analytical criteria for the two successful cases are presented in Table S2 (Cases 2 and 7). In Case 2, although the tissue area measured 8.1 mm^2, falling short of the 25 mm^2 threshold, F1CDx was selected owing to a favorable tumor cellularity of 50%. Case 7 satisfied all eligibility criteria; therefore, F1CDx was performed. Both successful cases were dCCA. Mutations in *BRAF*, *RNF43*, *SMAD4*, and *TP53* were detected in Case 2, and mutations in *CDKN2A*, *CDKN2B*, *ERBB3*, *STK11*, *KEL*, *DIS3*, and *SMAD2* were detected in Case 7. Both failed cases were classified as pre-analytical rejections due to insufficient tumor cellularity; therefore, a liquid biopsy was selected. In failure case 1, the tumor cellularity was 10%, tissue area was 16.6 mm^2, DNA concentration was 0.272 ng/μL, and DIN was unmeasurable. In failure case 2, the tumor cellularity was 5.1%, tissue area was 5.7 mm^2, DNA concentration was 0.126 ng/μL, and DIN was unmeasurable.

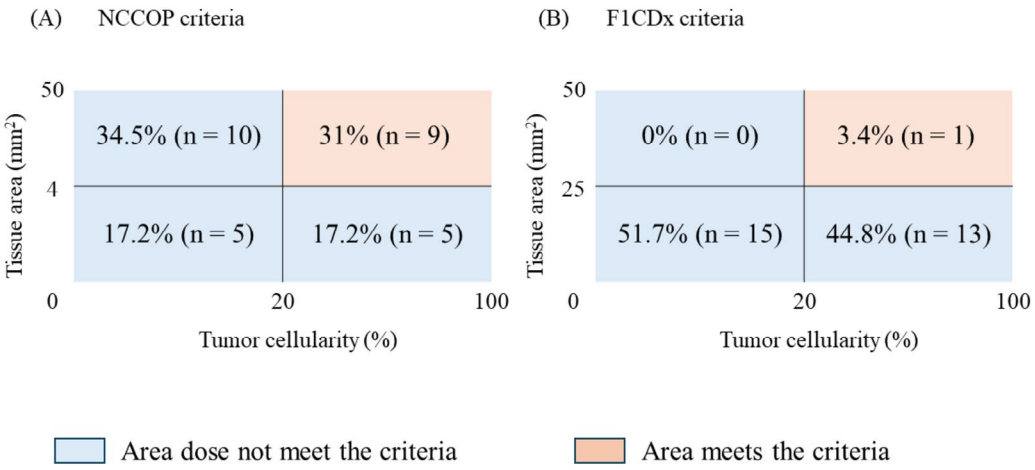

FIGURE 4 | Adequacy of sample for each CGP analysis according to each eligibility criterion ($N = 29$). (A) NCCOP criteria. (B) F1CDx criteria. CGP, comprehensive genomic profiling; F1CDx, FoundationOne CDx; NCCOP, OncoGuide NCC Oncopanel System.

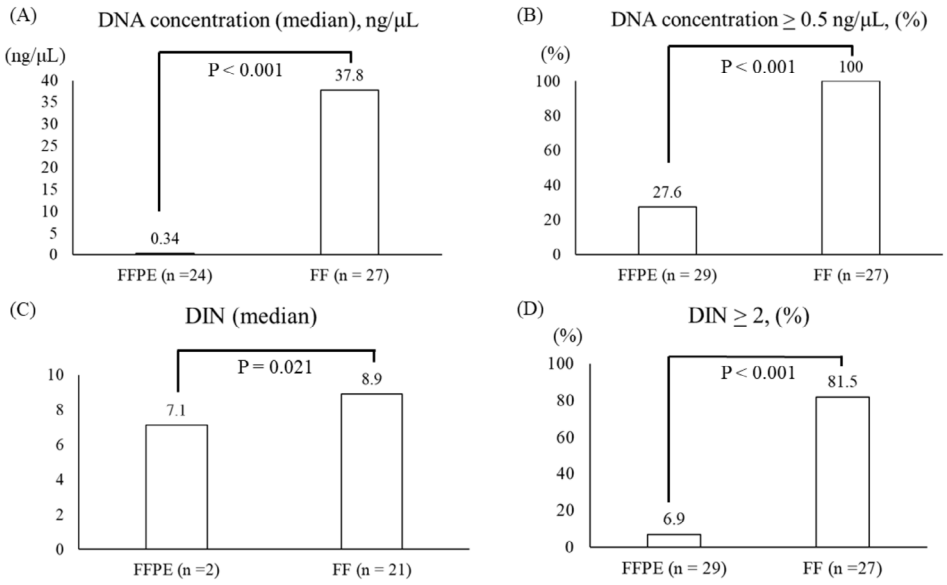

FIGURE 5 | Comparison of DNA quality indicators between FFPE and FF samples. (A) DNA concentration (median), ng/μL. (B) DNA concentration > 0.5 ng/μL, (%). (C) DIN (median). (D) DIN > 2, (%). DIN, deoxyribonucleic acid integrity number; DNA, deoxyribonucleic acid; FF, fresh frozen; FFPE, formalin-fixed paraffin-embedded.

4 | Discussion

In this prospective study, we show that introducer-assisted multipass TPB can yield tissue suitable for CGP in BTC, albeit in a panel-dependent manner. CGP suitability was achieved in 31% of cases for NCCOP and 3.4% for F1CDx, and FF processing produced markedly higher DNA quality than FFPE. Taken together, these findings indicate that combining multi-pass TPB with FF handling can improve the likelihood of successful CGP from endoscopically acquired specimens. To our knowledge, this is the first prospective evaluation of TPB samples for CGP in BTC.

A meta-analysis reported that the diagnostic sensitivity of TPB for malignant biliary strictures was 48.1% [28]; however, in this study, the sensitivity improved to 82.8% (24/29). A retrospective study reported that the suitability of TPB samples for NCCOP analysis in patients with BTC was 8.6% [17] whereas the suitability rate was 31% (9/29) in this study. This improvement may be attributed to performing TPB five times using an endoscopic introducer, which likely increased the total sample volume. The incidence of procedure-related adverse events associated with TPBs using an endoscopic introducer was 10% (3/30); however, all patients were managed successfully with conservative treatment. POCS was performed in the same session in 63.3% (19/30) of the patients. Because this raised concerns about the impact on sample quality (bleeding, inflammation, tissue fragility, and contamination), we compared the outcomes using FFPE samples between the POCS and non-POCS groups (Table S3). No significant differences were observed between the two groups in terms of tumor cellularity, tissue area, or DNA quality indicators. The sensitivity for diagnosing malignancies was 78% (14/18) in the POCS group and 91% (10/11) in the non-POCS group, indicating no apparent improvement with POCS guidance. In the risk factor analysis for adverse events, no influence of POCS was observed (OR, 1.18; 95% CI, 0.10–27.2; $p = 0.899$). Importantly, no

adverse events were directly attributed to the TPBs, suggesting that the procedure is safe when performed with an introducer. A previous study evaluated the feasibility of CGP using EUS-TA for primary BTC lesions and reported a suitability rate of 34.9% for NCCOP analysis [17]. However, this was comparable to the 31% suitability rate observed for the TPB in our study, indicating that both approaches still have room for improvement.

To date, most genotyping studies have been performed using FF samples and have provided great insights into the molecular biology of cancer [29]. However, FF samples are expensive to collect and maintain, so they have not been used for CGP. A meta-analysis evaluating the utility of EUS-tissue acquisition for CGP in pancreatic cancer showed that the success rate of CGP using FF and FFPE samples was similar, at 87% and 80%, respectively [30]. Moreover, the DNA quality indicators of FF samples were clearly superior to those of FFPE samples in this study. When the sample volumes are small, such as in BTC, nucleic acid degradation during the fixation process disrupts the analysis [10], and FF samples are more advantageous. We performed five multipass TPBs for FFPE samples; however, consistently meeting the eligibility criteria for CGP proved difficult. Because further increasing the number of passes is impractical, it would be desirable to develop a system that allows CGP using FF samples, depending on the case. However, there is no guarantee that the tumor tissue has been collected from frozen specimens; thus, it seems necessary to take measures such as performing rapid on-site cytological evaluation (ROSE) when collecting samples. In clinical practice, the CGP workflow using FF samples is envisioned as follows. First, to account for false negatives, at least two biopsies should be performed for histopathological diagnosis using FFPE samples. Next, ROSE should be performed using imprint cytology to confirm the presence of tumor cells. If tumor cells are identified, an additional sample should be obtained for FF processing. The samples are immediately frozen in liquid nitrogen and transported to the laboratory under frozen conditions. Upon arrival, DNA extraction is performed after thawing. Thus, it is conceivable to perform at least four multipass TPBs.

This study had several limitations. First, this was a single-center study with a small number of cases, which is a major limitation. Second, for DNA extraction and quality assessment, there was a difference in the sample volume of the FFPE and FF groups (FFPE: 10 μm section, FF: whole piece). Therefore, there are certain limitations to a strict comparison between the two groups. At our hospital, when performing CGP analysis using FFPE specimens, we prepare 10 μm sections (usually 5–10) for DNA extraction. In this study, DNA was extracted from a 10-μm section of FFPE specimens prepared from five biopsy samples, while assuming that at least five 10-μm sections could be prepared from one biopsy sample of FF specimen. In this case, the analysis was conducted under the assumption that the sample volumes of both groups were approximately equivalent. Third, the primary endpoint was not the actual success rate of CGP, and samples from only a few patients were used for CGP. Fourth, we did not verify whether tumor tissue was present in the FF samples; thus, there was a risk of false negatives. Because the biopsies were obtained from the same site under endoscopic introducer guidance, we assumed that if tumor tissue was identified in the FFPE samples on histopathological examination, it would also be present in the corresponding FF sample. Fifth, tumor cell annotation was performed by a single pathologist, and a formal assessment of intraobserver or interobserver variability was not conducted. Although all cases were evaluated using a standardized annotation workflow with fixed QuPath parameters to maintain consistency, the precise separation of tumor cells from stromal cells, inflammatory cells, or small capillaries can be challenging. Therefore, some degree of variability in tumor cell delineation may remain. In the future, multicenter studies are needed to verify whether actual CGP is possible using FF samples collected using TPB in patients with BTC.

Taken together, introducer-assisted multipass TPB provides a viable route to obtain tissue suitable for CGP in BTC, although suitability remains panel dependent. Across matched specimens, FF processing yielded consistently superior DNA quality compared with FFPE. These findings suggest that a workflow that prioritizes FF handling for endoscopically acquired small-volume specimens may help improve the feasibility of CGP.

Author Contributions

K. Miyamoto and K. Matsumoto conceived and designed the research and wrote the paper. T.O. and M.F. evaluated the pathological outcomes of the FFPE samples. H.I. extracted and evaluated DNA from FFPE and FF samples. R.S., A.M., Y.F., and D.U. collected the data. M.O. provided the final approval for this article. All the authors have read and approved the final version of this manuscript.

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Ethics Statement

This study was approved by the Okayama University Graduate School of Medicine, Dentistry, and Pharmaceutical Sciences, Okayama University Hospital and the Ethics Committee Review Board for Human Research (approval number: 2301-018; approval date: November 19, 2022).

Consent

Written informed consent was obtained from all participants.

Conflicts of Interest

The authors declare no conflicts of interest.

Data Availability Statement

The data that support the findings of this study are available on request from the corresponding author. The data are not publicly available due to privacy or ethical restrictions.

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Supporting Information

Additional supporting information can be found online in the Supporting Information section. **Table S1:** Risk factors for AEs. **Table S2:** Characteristics and outcomes of the patients who met the NCCOP analysis eligibility criteria. **Table S3:** Comparison of outcomes using FFPE samples between the POCS group and the non-POCS group.