CANCER GENOMICS & PROTEOMICS *22*: 34-40 (2025)
doi:10.21873/cgp.20484

Whole-genome Sequencing Analysis of Bile Tract Cancer Reveals Mutation Characteristics and Potential Biomarkers

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Abstract. *Background/Aim: Bile tract cancer (BTC) is a malignant tumor with a poor prognosis. Recent studies have reported the heterogeneity of the genomic background and gene alterations in BTC, but its genetic heterogeneity and molecular profiles remain poorly understood. Whole-genome sequencing may enable the identification of novel actionable gene mutations involved in BTC carcinogenesis, malignant progression, and treatment resistance. Patients and Methods: We performed whole-genome sequencing of six BTC samples to elucidate its genetic heterogeneity and identify novel actionable gene mutations. Somatic mutations, structural variations, copy number alterations, and their associations with clinical factors were analyzed. Results: The average number of somatic mutations detected in each case was 53,705, with SNVs accounting for most of these mutations (85.02%). None of the 331 mutations related to BTC in The Cancer Genome Atlas (TCGA) database were found in the mutations identified in our study. A higher prevalence of gene mutations was observed in samples without vascular invasion than in those with vascular invasion. Several genes with differences in mutation accumulation between groups were identified, including ADAMTS7, AHNAK2, and CAPN10. Conclusion: Our study provides novel insights into the genomic landscape of BTC and highlights the potential of whole-genome sequencing analysis to identify actionable gene mutations and understand the molecular mechanisms underlying this malignancy. The high mutational burden, structural variations, and copy number alterations observed in BTC samples in this study underscore the genetic complexity and heterogeneity of this disease.*

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Key Words: Whole-genome sequencing analysis, bile tract cancer, heterogeneity, somatic mutations, structural variations, copy number alterations, biomarkers, vascular invasion.

Bile tract cancer (BTC) is a malignant tumor arising from the epithelial cells of the bile ducts, including the intrahepatic, perihilar, and distal bile ducts, as well as the gallbladder and ampulla of Vater (1). Despite advances in surgical techniques and chemotherapy, the prognosis of BTC remains poor, with a 5-year survival rate of only 39.7-40.9% (2, 3). To date, no effective biomarkers have been established to accurately predict the therapeutic effects of various BTC treatments.

Recent studies have reported heterogeneity in the genomic background and genetic alterations in a variety of BTC types (4, 5). However, the genetic heterogeneity of BTC remains poorly understood. The molecular profiles of BTC are as heterogeneous as its pathology and biology; therefore, a comprehensive analysis with clinical associations is necessary to understand its molecular carcinogenesis and heterogeneity.

In this study, we aimed to perform a whole-genome sequencing analysis of BTC samples. Whole-genome sequencing analysis of BTC may identify novel actionable gene mutations involved in carcinogenesis, malignant progression, and treatment resistance, and lead to the development of new biomarkers and therapeutic targets.

Patients and Methods

Clinical samples. Six patients with BTC who underwent surgical resection at Nagoya University Hospital (Nagoya, Japan) were enrolled in this study. Blood samples and resected BTC tissue samples were collected from each patient. Data were obtained between 2020 and 2021 with a median follow-up period of 24 months. The clinicopathological features of the patients are presented in Table I.

DNA was extracted from freshly frozen cancer tissue and blood samples for whole-genome sequencing. DNA quality was assessed

Table I. *Patient metadata and clinicopathological characteristics, including tumor subtype, histological features, presence of intraductal papillary neoplasm of the bile duct (IPNB), tumor size, and various pathological parameters.*

Case	Sex/Age	Subtype	Histology	IPNB	Tumor size (mm)	CSR	INF	ly	v	ne	T	N	LN	HM	EM	PV	A	Stage
A	M/64	DCC (BdC)	por	–	19	int	b	1	1	3	3	1	+	0	0	1	0	3B
B	F/80	PHC (Bp)	mod	–	13	int	b	0	1	0	2a	0	–	0	0	0	0	2
C	F/60	PHC (Bp)	mod	–	14	int	b	1	1	1	2b	0	–	0	0	0	0	2
D	M/54	PHC (BpC)	mod	–	24	int	b	1	0	2	4	1	+	0	0	1	1	4a
E	M/80	DCC (Bd)	pap	+	30	–	–	0	0	0	2	0	–	0	0	0	0	1b
F	M/71	PHC (Bp)	pap	+	15	–	–	0	0	0	1b	0	–	0	0	0	0	1

CSR, Circumferential resection margin; INF, infiltrative growth pattern; ly, lymphatic invasion; v, vascular invasion; ne, perineural invasion; LN, lymph node metastasis; HM, hepatic metastasis; EM, extrahepatic metastasis; PV, portal vein invasion; A, hepatic artery invasion; por: poor; mod: moderated; pap: papillary.

using a Qubit fluorometer (Thermo Fisher Scientific, Waltham, MA, USA) and an Agilent 2100 Bioanalyzer (Agilent Technologies, Santa Clara, CA, USA). All patients provided informed consent to participate in the study. The institutional review boards at Nagoya University Hospital approved this study (approval number 2016-0268).

Library preparation and DNA sequencing. After preparing double-stranded DNA libraries according to the protocol provided by Illumina, whole-genome sequencing was performed using a NovaSeq6000 platform (Illumina, San Diego, CA, USA) with paired reads of 100-125 bp. Library quality was evaluated using an Agilent 2100 Bioanalyzer. Following quality control, the reads were mapped to the human reference genome hg38 using BWA mem (version 0.7.17) (https://github.com/lh3/bwa), and filtering was performed using GATK (version 4.1.9.0) (https://gatk.broadinstitute.org/hc/en-us) to remove germlines, slippage, normal artifacts, position, and FAIL variants.

Somatic mutation and structural variation analysis. Somatic mutations were detected using GATK from the BAM files of blood and cancer tissues and the reference genome sequence. For subsequent analyses, mutations that did not meet the filter criteria for germline, slippage, normal artifacts, position, or FAIL were excluded. Gene mutations were annotated using SnpEff version 5.0 (https://pcingola.github.io/SnpEff/) and aggregation was performed according to the mutation type: short insertions or deletions (INDELs), multiple nucleotide variants (MNV), and single nucleotide variants (SNV).

Tumor-specific somatic structural variations were detected using the "call" function of DELLY (version 0.9.1) (https://github.com/dellytools/delly) from the BAM files of blood and cancer tissues and the reference genome sequence. Regions unsuitable for analysis, such as telomeres, centromeres, pseudoautosomal regions (PAR) of the Y chromosome, and mitochondria, were excluded. The detected somatic structural variations were counted by total number and variation type: deletion (DEL), inversion (INV), insertion (INS), tandem duplication (DUP), and break end, interchromosomal translocation (BND).

Copy number alternation analysis. Tumor-specific copy number alterations were detected using Control-FREEC (version 11.6) (https://github.com/BoevaLab/FREEC) from the BAM files of blood and cancer tissues and the reference genome sequence. The minimum length of the detected copy number variations was set to 10,000 bp, and detection was not performed in the telomere and centromeric regions (50,000 bp), where false positives were likely to be detected. To avoid overestimation by counting only the number of copy number variations, aggregation was performed not only by the number of variations, but also by the length of the regions with copy number variations.

Mutation annotation analysis. Mutation IDs and frequency information from multiple databases were annotated using SnpSift (version 5.0) (http://pcingola.github.io/SnpEff/). The databases used were dbSNP (version 2021-05-25) (https://www.ncbi.nlm.nih.gov/snp/), Human Genetic Variation Database (version 2.30) (https://www.hgvd.genome.med.kyoto-u.ac.jp), ClinVar (version 20210912) (https://www.ncbi.nlm.nih.gov/clinvar/), ESP6500 (versionV2, GRCh38 liftover) (https://genome.ucsc.edu/cgi-bin/hgTables?db=hg19&hgta_group=varRep&hgta_track=evsEsp6500&hgta_table=evsEsp6500&hgta_doSchema=describe+table+schema), gnomAD (version 3.1.1) (https://gnomad.broadinstitute.org), PROVEAN precomputed scores (version 1.1) (http://provean.jcvi.org/index.php), and SIFT precomputed scores (version 1.1) (https://sift.bii.a-star.edu.sg/www/SIFT_nssnvs_help.html). Additionally, annotation was performed using mutation and gene information of patients with BTC registered in The Cancer Genome Atlas (TCGA) database on the GDC Portal (https://www.cancer.gov/ccg/research/genome-sequencing/tcga).

Comprehensive analysis of mutation accumulation and clinical information. The patients were divided into groups according to their clinical information [presence or absence of lymph node metastasis (ly), presence or absence of vascular invasion (v), and intraductal papillary neoplasm of the bile duct (IPNB)]. To explore genes with differences in mutation accumulation between groups,

Table II. *Distribution of somatic mutations, including insertions and deletions (INDEL), multiple nucleotide variants (MNV), and single nucleotide variants (SNV) in bile tract cancer samples.*

Case	A	B	C	D	E	F
INDEL	6,472	5,399	5,565	5,986	7,918	6,051
MNV	2,009	1,652	1,773	1,655	1,999	1,582
SNV	50,097	40,370	36,474	49,003	54,717	43,505
Total	58,578	47,421	43,812	56,644	64,634	51,138

Table III. *Average number of somatic structural variations, including deletions (DEL), inversions (INV), insertions (INS), tandem duplications (DUP), and interchromosomal translocations (BND) in bile tract cancer samples.*

Case	Average	Range
DEL	9,413	(8,952-9,880)
INV	2,322	(2,207-2,395)
INS	838	(785-900)
DUP	3,717	(3,515-3,942)
BND	6,543	(2,765-10,583)
Total	22,832	(18,692-27,577)

Table IV. *Average number of somatic structural mutations, including deletions (DEL), inversions (INV), insertions (INS), tandem duplications (DUP), and interchromosomal translocations (BND) with breakpoints in coding regions.*

Case	Average	Range
DEL	2,664	(2,557-2,781)
INV	153	(136-170)
INS	381	(364-402)
DUP	227	(192-253)
BND	58	(42-71)
Total	3,482	(3,326-3,655)

Table V. *Distribution of copy number variations across six bile tract cancer samples. Each row represents a different copy number (from 0 to 7 or more), and each column represents an individual sample (A to F).*

Copy number	A	B	C	D	E	F
0	1	0	0	0	3	0
1	1	2	2	5	47	15
3	92	227	35	47	59	32
4	3	34	0	1	9	4
5	0	2	0	2	5	4
6	3	0	0	1	2	5
>7	7	0	0	0	1	4
Total	107	265	37	56	126	64

oncoplots of the detected somatic mutations were created using the maftools package (version 2.10.05) (https://bioconductor.org/packages/release/bioc/html/maftools.html) and Bernard's test was performed.

Results

Somatic mutations. The number of somatic mutations ranged from 43,812 to 64,634, with an average of 53,705 mutations detected in each case (Table II). These somatic mutations included an average of 6,232 INDELs (range=5,399-7,918), 1,778 MNVs (range=1,582-2,009), and 45,694 SNVs (range=36,474-54,717). In most cases, SNVs accounted for a high proportion of somatic mutations, ranging from 83.25% to 85.52%, with an average of 85.02%.

Somatic structural variations. The number of structural variations ranged from 18,692 to 27,577, with an average of 22,832 structural variations confirmed (Table III). The average number of structural variations was 9,413 for DEL, 2,322 for INV, 838 for INS, 3,717 for DUP, and 6,543 for BND.

Somatic structural variations with breakpoints in the coding regions were analyzed to explore the candidate fusion genes. The number of somatic structural variations with breakpoints ranged from 3,326 to 3,655, with an average of 3,482 (Table IV). The average number of structural variations was 2,664 for DEL, 153 for INV, 381 for INS, 227 for DUP, and 58 for BND. DEL, the most common somatic structural variation, accounted for 75.15%-77.73% of the total cases, with an average of 76.50%.

Copy number variations. Control-FREEC analysis revealed copy number changes in regions 37-265, and an average of 109 regions were confirmed to have copy number changes (Table V). In the analysis of the regions with copy number variation, the copy number increase in each case ranged from 22,404,167 to 790,561,141 bp, whereas the copy number decreased from 30,000 to 670,287,019 bp (Table VI). Visualization of regions with large copy number changes revealed amplifications spanning several hundred thousand bp in multiple cases.

Mutation annotation analysis. The TCGA database contained information on 331 somatic mutations and 321 genes related to BTC. While none of these 331 mutations were found in the somatic mutations (INDELs, MNVs, SNVs) identified in our study, we observed a total of 180-256 somatic structural variations in these 321 genes. These structural variations had

Table VI. *Length of regions with increased or decreased copy numbers for each sample (A to F). 'Gain' represents gene amplification, while 'loss' represents gene deletion.*

Copy number	A	B	C	D	E	F
Gain	126,506,007	790,561,141	22,404,167	91,842,803	188,567,422	192,338,636
Loss	30,000	10,260,000	30,000	90,000	670,287,019	110,803,285

an average of 221 occurrences, including an average of 101 for DEL, 67 for INV, 11 for INS, 36 for DUP, and 5 for BND (Table VII).

Table VII. *Somatic structural variations, including deletions (DEL), inversions (INV), insertions (INS), tandem duplications (DUP), and interchromosomal translocations (BND) on 321 genes encoding in BTC.*

Case	Average	Range
DEL	101	(88-108)
INV	67	(49-88)
INS	11	(5-15)
DUP	36	(27-48)
BND	6	(5-7)
Total	221	(180-256)

Mutation accumulation and clinical factors. To elucidate the gene and mutation trends associated with clinical factors, such as vascular invasion, lymph node metastasis, perineural invasion, and portal vein invasion, we generated a comprehensive visual analysis integrating these multiple factors. Accumulated gene mutations were identified extensively in samples without vascular invasion compared to samples with vascular invasion (Figure 1). Bernard's test revealed a statistically significant association between vascular invasion and gene mutations that showed a difference in mutation accumulation of 0.60 or higher between groups, such as *ADAMTS7*, *AHNAK2*, *ANKRD36C*, *CAPN10*, *HSPG2*, *MEGF8*, *MST1*, *OBSCN*, and *PRSS3* (p<0.05). However, no specific genes with accumulated mutations were identified based on the presence or absence of lymph node metastasis, perineural invasion, or portal vein invasion. In addition, no specific genes with accumulated mutations were identified in IPNB.

Discussion

In this study, we performed whole-genome sequencing analysis of six BTC samples to elucidate the genetic heterogeneity of BTC and identify novel actionable gene mutations associated with its carcinogenesis, malignant progression, and treatment resistance. Our results revealed a high number of somatic mutations, structural variations, and copy number alterations in the BTC samples, highlighting the complex genomic landscape of this malignancy. Nakamura *et al*. revealed the genetic diversity and complex genomic structure of BTC, which supports our findings regarding genetic complexity and heterogeneity in BTC (4).

Approximately 30-35% of patients with advanced biliary tract cancer receive second-line treatment, but the optimal therapeutic approach has not been established (6). The development of molecular targeted therapies focusing on FGFR2, IDH1, HER2, and other targets is rapidly progressing, and the importance of genomic profiling is recognized for personalized treatment. However, the diagnosis of BTC remains challenging. Liquid biopsy, especially circulating tumor DNA (ctDNA) analysis, is expected as a new diagnostic method that is minimally invasive and can be repeatedly performed (7). For its application to BTC, sensitivity and specificity have to improve against the genetic complexity and heterogeneity.

Previous studies have reported a high frequency of gene mutations and structural variations in BTC (4). Several studies have also reported that genetic abnormalities, such as inactivating mutations in multiple genes, may contribute to the development and progression of BTC (5). Additionally, multiple studies have shown that SNVs account for most somatic mutations in BTC (8, 9). In our study, the average number of somatic mutations detected in each case was 53,705; SNVs accounted for most of these mutations (85.02%). This high mutational burden suggests the involvement of multiple genetic alterations in the development and progression of BTC. Furthermore, the identification of an average of 22,832 structural variations per case, with DEL being the most common type, indicated the potential role of gene fusion and rearrangement in BTC pathogenesis.

Copy number analysis revealed significant amplifications and deletions spanning several hundred thousand bp in multiple cases. These findings suggest the presence of potential oncogenes or tumor suppressor genes within these amplified and deleted regions, which may contribute to the development and progression of BTC (10). In addition, a higher frequency of copy number loss was observed in the IPNB-positive groups (cases E and F). The copy number

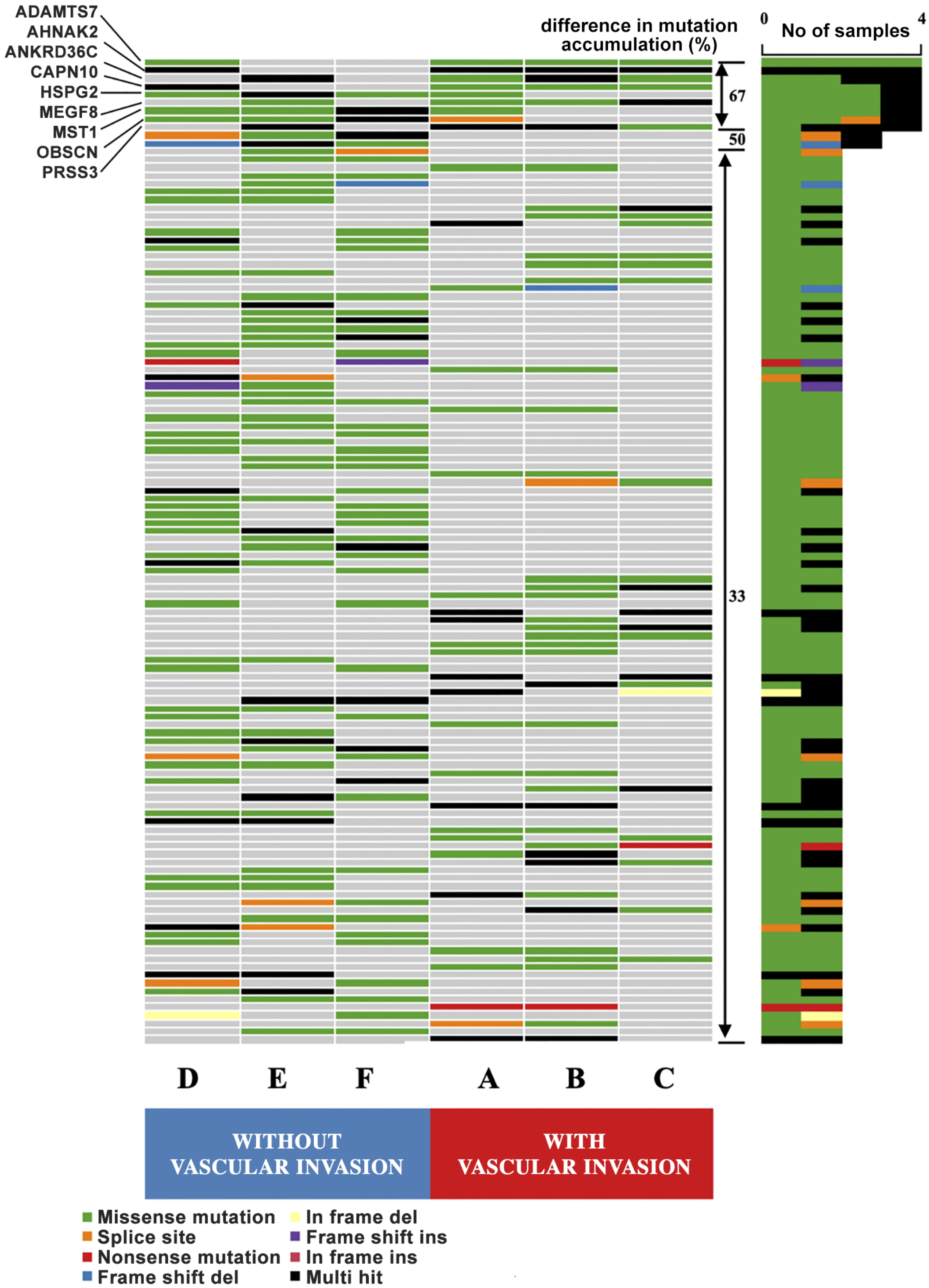

Figure 1. *Accumulated gene mutations with or without vascular invasion. Accumulated gene mutations were observed in three cases with (N=3) and without vascular invasion (N=3).*

losses observed in the IPNB-positive groups suggest that the deletion of tumor suppressor gene regions may be involved in the development and pathogenesis of IPNB.

Notably, none of the 331 mutations related to BTC in the TCGA database were identified in our study. This discrepancy may be attributed to the genetic heterogeneity of BTC and the limited sample size of our study. The incidence of BTC varies significantly across regions, with a very high incidence in East Asia and a relatively low incidence in Western countries. Furthermore, the genetic characteristics of bile tract cancers differ between Western and East Asian populations. For instance, mutations in *CDKN2A/B*, *IDH1/2*, and *BAP1* are more common in Western populations, whereas mutations in *TP53*, *KRAS*, and *SMAD4* are more prevalent in East Asian populations (11). Ethnic differences may also contribute to this discrepancy.

Jusakul *et al*. reported that gene mutations and cholangiocarcinoma (CCA) subtypes reflect different mutational pathways (12). Molecular differences between subtypes are thought to be related to the frequency of gene mutations rather than differences in the sets of mutated genes. This heterogeneity may contribute to the diverse mechanisms underlying the carcinogenesis, malignant progression, and treatment resistance of cholangiocarcinoma.

Lowery *et al*. reported that BTC has a wide range of actionable gene mutations (13). The diversity of these gene mutations suggests that they can serve as therapeutic targets. Therefore, therapies based on actionable gene mutations are challenging for the treatment of BTC. Hence, developing more effective personalized treatments and precision medicines targeting gene mutations based on clinical factors is crucial.

A higher prevalence of gene mutations was observed in samples without vascular invasion than in those with vascular invasion. This suggests that the absence of vascular invasion is associated with a more genetically unstable BTC phenotype. However, no specific genes with accumulated mutations were identified based on the presence or absence of lymph node metastasis, perineural invasion, portal vein invasion, or IPNB. The small sample size in our study may have limited the statistical power to detect significant associations between specific gene mutations and clinical factors.

Nonetheless, our study identified several genes with a difference in mutation accumulation of 0.60 or higher between groups with and without vascular invasion. Bernard's test revealed a statistically significant association between vascular invasion and gene mutations, such as *ADAMTS7*, *AHNAK2*, *ANKRD36C*, *CAPN10*, *HSPG2*, *MEGF8*, *MST1*, *OBSCN*, and *PRSS3* ($p<0.05$).

ADAMTS7 is one of the metalloproteases involved in the remodeling of the extracellular matrix. *ADAMTS7* is highly expressed in various cancers, including renal cell carcinoma, lung adenocarcinoma, and breast cancer (14). It is thought to be involved in cancer progression and metastasis. *AHNAK2* is a large scaffolding protein that has been implicated in various cellular processes, such as cell migration, invasion, and epithelial-mesenchymal transition (EMT). *AHNAK2* is also associated with poor prognosis and is believed to contribute to tumor progression and metastasis by regulating key signaling pathways involved in cancer development (15). The expression of *CAPN10*, a member of the calpain (CAPNs) family, is reported a positive correlation with overall survival (OS) and recurrence-free survival (RFS) in pancreatic cancer (16). These gene mutations are considered to be closely related to vascular invasion. However, these mutations and their impact on tumor biology and clinical outcomes have not yet been investigated. In addition, the lack of a validation cohort and functional experiments precludes confirmation of the identified genes as biomarkers or therapeutic targets. These genes may represent potential biomarkers or therapeutic targets for BTC and warrant further investigation in larger cohorts and functional studies.

Conclusion

In conclusion, our study provides novel insights into the genomic landscape of BTC and highlights the potential of whole-genome sequencing to identify actionable gene mutations and understand the molecular mechanisms underlying this malignancy. The high mutational burden, structural variations, and copy number alterations observed in the BTC samples in this study underscore the genetic complexity and heterogeneity of this disease. Further research is needed to validate our findings in larger cohorts and to explore the functional significance of the identified gene mutations in the context of BTC pathogenesis and treatment response. We believe that integrating genomic data with clinical information may ultimately lead to the development of personalized treatment strategies and improved outcomes for patients with BTC.

Funding

This study was supported by JSPS KAKENHI (grant number 21K08796).

Conflicts of Interest

The Authors declare no competing interests in relation to this study.

Authors' Contributions

Toshio Kokuryo, Masaki Sunagawa, Takashi Mizuno, and Tomoki Ebata conceived and designed the study. Junpei Yamaguchi and Taisuke Baba performed the experiments and acquired data. Nobuyuki Watanabe, Shunsuke Onoe, and Shoji Kawakatsu analyzed the data. Toshio Kokuryo and Takashi Mizuno wrote the manuscript. All the Authors have read and approved the final version of the manuscript.

Acknowledgements

We thank Amelieff (https://amelieff.jp) for technical assistance.

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Received September 11, 2024
Revised October 9, 2024
Accepted October 17, 2024