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Accumulating Genetic Mutations from Primary to Secondary Biliary Tract Cancers: Analysis of Four Patients With Metachronous Biliary Tract Cancer Using Comprehensive Genomic Profiling

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Abstract

Background/Aim: Metachronous biliary tract cancer (BTC) is a rare occurrence after curative resection of primary BTC. The genetic alterations and pathogenesis associated with metachronous BTC remain poorly understood.

Patients and Methods: We analyzed four patients with metachronous BTC who underwent resection at the Nagoya University Hospital between 2010 and 2024. Gene panel examination was performed on both primary and secondary tumors using next-generation sequencing.

Results: The median interval between resection of the primary tumor and diagnosis of metachronous BTC was 24 months. Genetic alterations were observed in all paired primary and metachronous carcinomas. The number of genetic mutations was higher in metachronous lesions than in primary lesions. *CDKN2A* and *SMAD4* were the most frequently mutated genes in all metachronous lesions. Common genetic mutations between primary and metachronous lesions were confirmed in all four cases, suggesting a common clonal origin.

Conclusion: This study demonstrated that characteristic genetic alterations and their accumulation play important roles in metachronous BTC. This suggests that the increasing burden of gene mutations may play a crucial role in the carcinogenesis of metachronous BTC. Further investigation is required to validate these findings and elucidate the underlying molecular mechanisms.

Keywords: Metachronous biliary tract cancer, genetic mutation accumulation, comprehensive genomic profiling, next-generation sequencing, field cancerization, clonal origin, genetic predisposition, CDKN2A, SMAD4, carcinogenesis.

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Introduction

Biliary tract cancer (BTC) is a malignant neoplasm arising from the epithelial cells at any site in the biliary tree, including the intrahepatic and extrahepatic bile ducts, and gallbladder (1). Clinically, most patients with BTC have advanced disease at initial presentation, highly associated with poor prognosis (2). Surgical resection is the only potentially curative treatment; however, recurrence is common even after curative resection, with an incidence ranging from 57% to 67% (3, 4), thereby resulting in unsatisfactory survival rates of less than 30% (5, 6). Although tumor relapse after surgery generally includes distant and locoregional metastases, a rare mode of tumor relapse has been reported and referred to as metachronous BTC, which is defined as a new BTC development after the R0 resection of the initial BTC (7). With a prevalence approximately 7%, this rare event masks the pathologic nature of the secondary BTC: genuine new secondary lesions *versus* metastatic foci from the primary disease (8). However, its tumorigenesis is clinically important for deciding on the therapeutic approach; up-front definitive surgery should be considered for new secondary lesions whereas systemic chemotherapy is the first-line of treatment for metastases.

The researchers previously analyzed the relationship between primary and secondary tumors in six patients who underwent surgical resection, in which the definitive conclusion was challenging, even with immunochemical and morphologic approaches (9). Recently, the next-generation sequencing technologies have revealed genetic heterogeneity and multiple signaling pathways in BTC (10, 11). However, the specific genetic alterations and pathogenesis associated with metachronous BTC remains poorly understood (12).

In this study, we performed a gene panel examination of both primary and secondary tumors in four patients with metachronous BTC. The aim was to investigate the characteristics in genetic mutations and their correlations with clinicopathological findings.

Patients and Methods

Patient characteristics. Four patients with metachronous BTC who underwent resection at the Nagoya University Hospital between 2010 and 2024 were included in the study. All the patients agreed to participate in the study and provided informed consent. This study was approved by the Institutional Review Board of Nagoya University Hospital (2016-0268). There were two women and two men with a median age of 71 years (range=64–81 years), at the time of diagnosis of the primary tumor. No patient had underlying hepatobiliary diseases including pancreaticobiliary maljunction, primary sclerosing cholangitis, liver fluke parasitism, or hepatolithiasis. All patients underwent endoscopic retrograde cholangiography before primary and secondary surgeries. The histological types of primary and metachronous tumors were compared. The anatomical distribution of the paired carcinomas was assessed, and the distance between the primary and secondary tumors was evaluated.

Sample preparation. The tissue samples were acquired directly after surgical removal of the specimens, placed immediately in 10% neutral-buffered formalin, and fixed for 48 h at room temperature. The formalin-fixed tissues were embedded in paraffin. The tissues were cut into 10 μm-thick sections containing areas ≥25 mm2 in size, which had ≥50% cancer components. From each tumor specimen (primary and metachronous), six sections were prepared and were stained with hematoxylin and eosin (HE). The extent of cancer invasion was then highlighted under microscopic visual control. All samples were anonymized, and individual information was masked.

DNA extraction and quality control. Using HE-stained slides as a guide, we identified the cancer component areas in the unstained tissue sections from the surgical samples. These areas were carefully scraped using a sterilized razor blade. The scraped tissue fragments transferred into a safe-lock tube (Eppendorf, Hamburg, Germany). For DNA

Table I. *Clinical characteristics, surgical interventions, and long-term outcomes of 4 patients with primary and metachronous biliary tract cancer.*

	Case 1	Case 2	Case 3	Case 4
Age (y)/Sex*	72/F	81/M	64/F	69/M
Primary cancer				
Primary site	Distal bile duct	Cystic duct (Invasion to CHD)	Hilar bile duct	Hilar bile duct
Stage (pTN)	IA (T2N0)	IIIC (T3aN1)	IA (T1aN0)	II (T2aN0)
SR status	0	0	0	1
Operation**	SSPPD	S1,5,6,7,8	S1,2,3,4	S1,5,6,7,8
Adjuvant therapy	-	-	-	-
Interval (months)***	63	19	20	6
Secondary cancer				
Secondary site	Intrahepatic bile duct (B6)	Intrapancreatic bile duct	Intrapancreatic bile duct	Intrapancreatic bile duct
Stage (pTN)	II (T2aN0)	IIB (T3aN0)	I (T1bN0)	IIB (T3aN0)
R status	0	0	0	0
Operation**	S5,6,7,8	SSPPD	SSPPD	SSPPD
Follow up (months)****	57, alive (without recurrence)	6, dead (local recurrence)	29, dead (liver+peritoneum recurrence)	31, dead (paraaortic LN recurrence)

*At operation for primary lesion. **Expressed as Couinaud's hepatic segments resected. ***Time to secondary cancer. ****After resection for secondary cancer. CHD: Common hepatic duct; SSPPD: subtotal stomach preserving pancretoduodenectomy.

extraction, we used the QIAamp DNA FFPE Tissue Kit (QIAGEN, Hilden, Germany). The quality of the extracted DNA was subsequently assessed by determining its DNA integrity number.

Next-generation sequencing. Fragmented DNA libraries were constructed using 50 to 150 ng of DNA and enriched with the clinically validated 435-gene panel, CANCER PLEX-JP (Denka Kew Genomics, Tokyo, Japan). This panel focuses on coding regions and selected introns of genes known to be associated with cancer. Sequencing was conducted on the Illumina MiSeq and NextSeq platforms (Illumina, San Diego, CA, USA) with an average sequencing depth of 500×. The methodology for processing artifact and mutation data has been outlined in a previous publication (13). For somatic mutations (including single-nucleotide substitutions, indels, or both), we used 5% mutant allele frequency threshold for the artifact determination and 10% mutant allele frequency threshold for comparison between the paired samples.

Statistical analysis. Owing to the small sample size, only descriptive statistics were used to summarize the clinical, histopathological, and genetic characteristics of tumors. No statistical analyses were performed.

Results

Clinical course. The clinical characteristics of patients are summarized in Table I. The primary tumor sites included the distal bile duct (n=1), cystic duct with invasion of the common hepatic duct (n=1), and hilar bile duct (n=2). Case 1 underwent subtotal stomach preserving pancreaticoduodenectomy (SSPPD), and the other three patients underwent major hepatobiliary resection. Case 4 was the only patient who had positive margins at the distal bile duct stump during primary surgery; the remaining three patients underwent R0 resection. No patient received adjuvant chemotherapy.

The median interval between resection of the primary tumor and diagnosis of metachronous BTC was 24 months (range=3–62 months). Metachronous BTC was detected

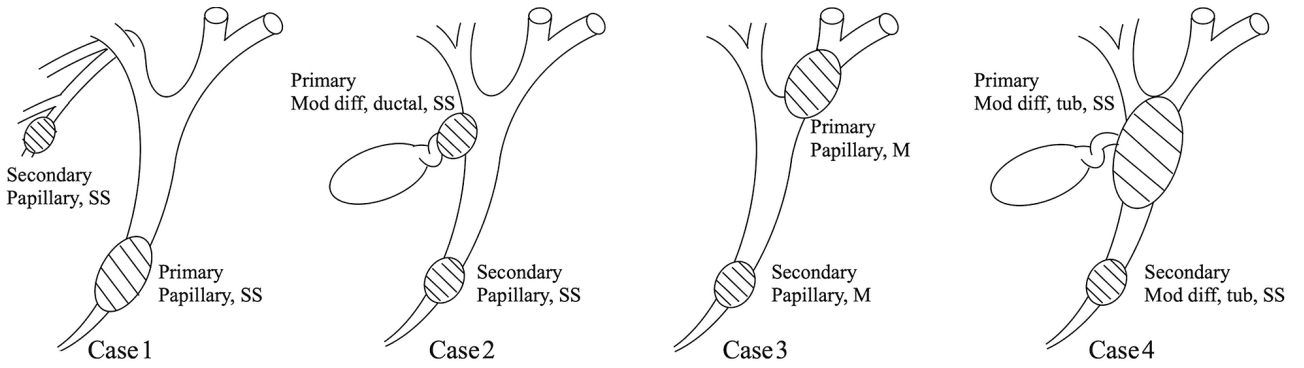

Figure 1. *Anatomical distributions and histological types of paired primary and metachronous lesions of metachronous BTC. Paired lesions are depicted by their anatomical location, histological type (papillary or moderately differentiated tubular or ductal), and pathological stage SS or M. BTC: Biliary tract cancer; SS: superficial spreading; M: muscle-invasive.*

using follow-up computed tomography and found in the intrahepatic (n=1) and intrapancreatic (n=3) bile ducts. Case 1 had a secondary tumor at the intrahepatic bile duct (B6) and underwent right posterior segmentectomy. The other three patients had secondary tumors in the remnant intrapancreatic bile ducts, thus received SSPPD. All patients underwent R0 resection at the second surgery, and two received adjuvant therapy. Case 4 received radiotherapy at the edge of the intrahepatic bile duct. Despite postoperative complications arising, such as pancreatic fistula, bile leakage, intra-abdominal bleeding, or intestinal perforation, all patients were discharged from the hospital in good health.

Only Case 1 showed no recurrence 57 months after the second surgery, whereas the remaining three patients died of the disease at 6, 29, and 31 months after the second operation.

Anatomical distribution of paired carcinomas. The anatomical distributions of paired primary and metachronous carcinomas and their histological types and depths are depicted in Figure 1.

Histological types of paired carcinomas. Except in Case 2, the histological type was the same between the primary and secondary cancers. In Cases 1 and 3, the histological type of both cancers was papillary adenocarcinoma. In Case 4, both cancers were moderately differentiated tubular adenocarcinomas.

Genetic alterations. Genetic alterations were observed in paired primary and metachronous carcinomas (Table II). The number of genetic mutations was higher in metachronous lesions than in the primary lesions, and the genetic mutations identified in the primary lesions were confirmed in the metachronous lesions. *CDKN2A* and *SMAD4* were the most frequently mutated genes in all metachronous lesions. *APC* and *CDKN2B* mutations were detected in three metachronous lesions. In addition, gene mutations in both primary and secondary cancers were detected: *AXIN1* in Cases 1 and 2; *CDKN2A* in Cases 1 and 3; and *APC* in Cases 3 and 4.

Common genetic mutations between the primary and metachronous lesions were confirmed in all four cases. In Case 1, *AXIN1* G508fs, *ERBB* gain, *ERBB2* L755S, *MSH2* E260fs, *RNF43* G659fs, and *TGFBR2* K128fs mutations were shared. In Case 2, *AXIN1* H516fs and *PIK3CA* E545K were common. In Case 3, *APC* Q999X, *CD274* G-14-1A Splice variant, *CDKN2A* loss, *CDKN2B* loss, and *MDM2* gain were shared. In Case 4, *AMER1* loss, *APC* K2052fs, *JAK1* S294L, *RB1* P23L, *SMAD4* D351V, and *TP53* G266M mutations were common. Six gene mutations were

Table II. *Genetic alternations in primary and secondary lesion of metachronous BTC.*

	Case 1		Case 2		Case 3		Case 4	
	Primary	Secondary	Primary	Secondary	Primary	Secondary	Primary	Secondary
ACVR2A	-	p.K437fs	-	-	-	-	-	-
AMER1	-	-	-	-	-	-	Loss	Loss
APC	-	-	-	Loss	p.Q999X	p.Q999X	p.K2052fs	p.K2052fs
ARID1A	-	-	-	Loss	-	Loss	-	-
ATR	-	p.I774fs	-	-	-	-	-	-
AXIN1	p.G508fs	p.G508fs	p.H516fs	p.H516fs	-	-	-	-
CCND1	-	-	-	Gain	-	-	-	-
CD274	-	-	-	-	c.G14-1A, splice variant	c.G14-1A, splice variant	-	-
CDKN1A	-	-	-	Loss	-	-	-	-
CDKN1B	-	-	-	-	Loss	Loss	-	-
CDKN2A	Loss	p.G136fs	-	Loss	Loss	Loss	-	Loss
CDKN2B	Loss	-	-	Loss	Loss	Loss	-	Loss
CDK12	-	Gain	-	-	-	-	-	-
ERBB2	Gain	Gain	-	-	-	-	-	-
ERBB2	p.L755S	p.L755S	-	-	-	-	-	-
FBXW7	-	-	-	Loss	-	Loss	-	-
FLCN	-	-	-	Loss	-	-	-	-
JAK1	-	-	-	-	-	-	p.S294L	p.S294L
KEAP1	-	-	-	-	-	Loss	-	-
KRAS	-	-	-	-	-	-	-	Gain
MDM2	-	-	-	-	Gain	Gain	-	-
MSH2	p.E260fs	p.E260fs	-	-	-	-	-	-
NF1	-	p.N78fs	-	-	-	-	-	-
PALB2	-	-	-	Loss	-	-	-	-
PIK3CA	-	-	p.E545K	p.E545K	-	-	-	-
PIK3R1	-	-	-	Loss	-	Loss	-	-
PTEN	-	-	-	Loss	-	-	-	-
PTPRD	-	-	-	Loss	-	Loss	-	-
RB1	-	-	-	-	-	-	p.P23-L	p.P23L
RNF43	p.G659fs	p.G659fs	-	-	-	-	-	-
SMAD4	-	p.P203fs	-	Loss	-	Loss	p.D351V	p.D351V
STK11	-	-	-	-	cGT290+1-290+2AA, splice variant	Loss	-	-
TP53	p.R306X	-	-	-	-	-	p.G266E	p.G266E
TGFBR2	p.K128fs	p.K128fs	-	-	-	-	-	-
UBR5	p.E2121fs	-	-	-	-	-	-	-
XPO1	-	-	-	Gain	-	-	-	-

'Gain' represents gene amplification, while 'Loss' represents gene deletion. BTC: Biliary tract cancer.

matched in Case 1, two in Case 2, five in Case 3, and six in Case 4. Notably, all cases exhibited a high degree of similarity in their mutation profiles, suggesting a common clonal origin for their metachronous lesions. These findings suggest that accumulation of genetic mutations plays a crucial role in the pathogenesis of metachronous BTC.

Analysis of genetic mutations and clinicopathological features. The correlation between genetic mutations and clinicopathological features were also investigated. No specific gene mutations were found to be associated with histologic type, depth, disease stage, or prognosis of primary and metachronous BTC. In addition, site-specific genetic alterations were not identified in either tumor type.

Discussion

This study investigated the genetic features of primary and metachronous lesions in four patients, observing two findings: accumulating genetic mutations in the secondary tumor, compared to the primary tumor, and considerably overlapping mutations. Thus, the increasing burden of gene mutations may play a crucial role in the carcinogenesis of metachronous BTC.

Several mechanisms explain carcinogenesis, such as field cancerization, clonal spread, and genetic predisposition theories. Field cancerization involves the exposure of the whole biliary epithelium to carcinogenic factors, leading to the development of multiple independent tumors over time (14), chronic inflammation, and bile acid exposure (15). Meanwhile, clonal spread occurs when residual cancer cells from the primary tumor spread through the biliary tract, leading to new tumors at the remote site in the biliary system (16). This may occur through intraluminal dissemination or *via* the lymphatic or vascular system. Finally, genetic predisposition occurs when genetic mutations or polymorphisms increase an individual's susceptibility to BTC (17). The anatomical distribution of the paired carcinomas revealed that the primary and secondary cancers were sufficiently separated in most cases, indicating that the metachronous lesions were not derived from local recurrence.

Shinohara *et al*. (9) reported that primary and metachronous lesions showed similarities in histological morphology and immunohistochemical profile. They suggested that the two tumors may arise from the same epithelial precursor lesion, *i.e.*, multicentric origin. In our results, histological features were shared in the three patients, suggesting that the metachronous lesions may have originated from similar cellular origins. This suggests that genetic factors and environmental exposure may interact to promote the carcinogenesis of metachronous BTC. In addition, several researchers reported that the accumulation of gene mutations is commonly found in the carcinogenesis of various cancers, such as colorectal cancer, pancreatic cancer, and hepatic cellular carcinoma (18–21). Murali *et al*. (22) reported that the progression from low-grade to high-grade epithelial lesions involves additional gene mutations and copy number alterations. Our results consistently followed this finding. A high mutation burden in patients with long intervals between primary and metachronous tumors suggests that the accumulation of genetic mutations increases over time. Furthermore, because metachronous lesions developed relatively shortly after resection of the primary lesions, the genetic mutations necessary for the development of metachronous BTC may have already accumulated, additionally to the primary lesions. These findings support the concept that accumulation of genetic alterations over time contributes to the development of metachronous BTC.

Common genetic mutations between the primary and metachronous lesions included *CDKN2A* and *SMAD4*, suggesting that these genes may play a crucial role in the pathogenesis of metachronous BTC. The considerable similarity in the mutational profiles of all the cases further supports the concept of a common clonal origin for metachronous lesions, although a limited number of samples was examined. This common clonal origin enables increased precise prediction of the risk of metachronous lesion occurrence and overall survival rate based on the characteristics of the primary tumor.

Conclusion

This study demonstrates that characteristic genetic alterations and their accumulation play important roles in metachronous BTC. Further investigation is required to validate the findings of the present study and elucidate the molecular mechanisms underlying the development of metachronous BTC.

Conflicts of Interest

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

Authors' Contributions

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

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