

Genetic alterations in Japanese extrahepatic biliary tract cancer

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Abstract. Biliary tract cancer (BTC) is one of the most devastating types of malignant neoplasms worldwide. However, the mechanisms underlying the development and progression of BTC remain unresolved. BTC includes extrahepatic bile duct carcinoma (EBDC), gallbladder carcinoma (GBC) and ampulla of Vater carcinoma (AVC), named according to the location of the tumor. Although genetic alterations of intrahepatic cholangiocarcinoma have been investigated, those of EBDC, GBC and AVC have not yet been fully understood. The present study analyzed somatic mutations of 50 cancer-associated genes in 27 Japanese BTC cells, including: 11 EBDC, 14 GBC and 2 AVC. Next-generation sequencing using an Ion AmpliSeq Cancer Panel identified a total of 44 somatic mutations across 14 cancer-associated genes. Among the 44 mutations, 42 were judged as pathological mutations. Frequent mutations were identified in tumor protein 53 (*TP53*) (14/27), SMAD family member 4 (*SMAD4*) (6/27), phosphatidylinositol-4,5-bisphosphate 3-kinase, catalytic subunit α (*PIK3CA*) (6/27), and Kirsten rat sarcoma (*KRAS*) (6/27); no significant differences were identified between EBDC and GBC tissues. Notably, the frequency of the *PIK3CA* mutation was higher when compared with previous reports. This result may suggest that the activation of the *PIK3CA*-protein kinase B signaling pathway, in addition to the abrogation of p53, *SMAD4* and RAS mitogen-activated protein kinase may have a crucial

role in the carcinogenesis of Japanese BTC. These findings may be useful for the development of personalized therapies for BTC.

Introduction

Biliary tract cancer (BTC) is the sixth most common cause of cancer-associated mortalities in Japan and >18,000 patients succumbed to this disease in 2013 (1). BTC is anatomically classified into three groups: Extrahepatic bile duct carcinoma (EBDC), gallbladder carcinoma (GBC) and ampulla of Vater carcinoma (AVC) (2). Previous epidemiological studies have revealed that the incidence of EBDC is high in Japan and Korea, and that of GBC is high in Chile, Argentina, India, Peru, Ecuador and Eastern Europe, including the Czech Republic and Slovakia (3). The etiology of intrahepatic and extrahepatic cholangiocarcinoma in Thailand is associated with infection by a liver fluke, *Opisthorchis viverrini* (4,5), whereas BTCs that develop in Japanese patients do not typically involve infection with parasites. Although complete surgical resection of tumors allows for improved prognosis, there are limited cases of its use due to the vast majority of patients who are diagnosed at an advanced cancer stage, with the exception of cases diagnosed incidentally at the time of elective cholecystectomy for gallbladder stones (4). Furthermore, effective molecular-targeted drugs have not yet been developed for BTC (6). Combined chemotherapies using gemcitabine, platinum agents and docetaxel regimens are used for patients with tumors that cannot be removed by resection surgery; however, their efficacies are far from satisfactory (7). The prognosis of patients with advanced BTC remains poor, and the five-year survival rates remain low at 5-15% for advanced stage BTC (8,9). Therefore, the development of effective molecular targeted drugs is a matter of pressing concern.

A number of previous studies have identified that chronic and continuous stimulation of the biliary tract serves a role in the process of carcinogenesis (10). A case-control study of intrahepatic cholangiocarcinoma in the USA demonstrated that choledocholithiasis and cholangitis were risk factors for BTC, with odds ratios of 4.0 and 8.8, respectively (11). In addition, it was reported that choledocholithiasis increased the risk

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of extrahepatic and intrahepatic cholangiocarcinoma 34-fold and 22.5-fold, respectively, and that cholangitis also increased the risk of extrahepatic and intrahepatic cholangiocarcinoma 45.7-fold and 64.2-fold, respectively (12). Notably, the risk of BTC increases ~400-fold in patients with PSC, in comparison with the general population (13). Additionally, in patients with pancreaticobiliary maljunction (PBM), the incidence of GBC and BTC are ~14.8 and 4.9%, respectively (14). Patients with PBM suffer from frequent refluxes of pancreatic juice into the biliary tract, which causes damage to the epithelium of the biliary tract (14). Chronic inflammation exposes epithelial cells to cytokines and interferons, which causes genotoxic stress through the production of reactive oxygen species (14). Therefore, with the rapid renewal of damaged cells, epithelial cells with genotoxic stresses may happen to acquire unrepaired genetic changes, leading to the accumulation of mutations in oncogenes and tumor suppressor genes, including Kirsten rat sarcoma (*KRAS*) and tumor protein 53 (*TP53*) (14).

Numerous previous studies have performed genetic analyses of BTC (15,16), and identified *TP53* mutations in 8.2-35.7% of GBC cases (17,18), and *KRAS* mutations in 2-20% of GBC cases (18). *KRAS* mutations were identified in 20-67% of EBDC cases (19,20), and in 28.6-37.0% of AVC cases (21-23). Although previous studies have revealed that genetic alterations in *TP53* and *KRAS* are involved in tumorigenesis (24-27), the number of reports of global mutation profiles in BTC is limited.

The present study analyzed genetic alterations in 11 EBDC, 14 GBC and 2 AVC tissues, using an Ion AmpliSeq Cancer Panel and covering 50 cancer-associated genes. The results may be useful for the establishment of personalized therapies, and the development of novel anticancer drugs for this devastating disease.

Materials and methods

This study was approved by the institutional review board of the Institute of Medical Science, University of Tokyo (Tokyo, Japan; approval no. IMSUT-IRB #24-56). Written informed consent was obtained from all patients prior to the study.

Patients and clinical tissues. Tumor tissues and corresponding non-cancerous tissues were obtained from 27 patients with BTC in the Chiba Cancer Center Hospital (Chiba, Japan) and Kanagawa Cancer Center (Yokohama, Japan). The tissues were resected during surgeries that occurred between June 1997 and August 2013, snap frozen in liquid nitrogen, and stored at -80°C until required for DNA extraction. These tissues included 11 with EBDC, 14 with GBC and 2 with AVC, and kept frozen until analysis. The 27 patients included 12 females (44.4%) and 15 males (55.6%), with a median age of 69 years (range, 44-82 years). All tumors were histologically diagnosed as BTC according to the WHO criteria (28). Disease stages were determined according to the UICC Tumor-Node-Metastasis (TNM) system (29). Clinicopathological information is summarized in Table I.

Extraction and quantification of DNA. Frozen tissue sections (10 µm in thickness) of tumorous and non-tumorous tissues were fixed in ice cold 4% formalin for 10 min, washed using

water for 5 min and subsequently stained with hematoxylin. Tumorous and non-tumorous cells were collected from the hematoxylin-stained tissue sections. Genomic DNA was extracted from the cells using a QIAamp DNA formalin-fixed, paraffin-embedded tissue kit (Qiagen GmbH, Hilden, Germany), according to the protocol of the manufacturer. The concentration of DNA was measured by e-SPECT (Malcom, Tokyo, Japan) and a Qubit2 fluorometer (Invitrogen; Thermo Fisher Scientific Inc., Waltham, MA, USA). All genomic DNA was stored at -20°C until use.

Multiplex polymerase chain reaction (PCR) and DNA sequencing. A total of 10 ng DNA was used for multiplex PCR amplification of a panel covering 207 areas in 50 cancer-associated genes, including: *ABL1*, *AKT1*, *ALK*, *APC*, *ATM*, *BRAF*, *CDH1*, *CDKN2A*, *CBF1R*, *CTNNB1*, *EGFR*, *ERBB2*, *ERBB4*, *EZH2*, *FBXW7*, *FGFR1*, *FGFR2*, *FGFR3*, *FLT3*, *GNAI1*, *GNAQ*, *GNAS*, *HNFI1A*, *HRAS*, *IDH1*, *IDH2*, *JAK2*, *JAK3*, *KDR*, *KIT*, *KRAS*, *MET*, *MLH1*, *MPL*, *NOTCH1*, *NPM1*, *NRAS*, *PDGFRA*, phosphatidylinositol-4,5-bisphosphate 3-kinase, catalytic subunit α (*PIK3CA*), *PTEN*, *PTPN11*, *RBI*, *RET*, SMAD family member 4 (*SMAD4*), *SMARCB1*, *SMO*, *SRC*, *STK11*, *TP53* and *VHL* (Ion AmpliSeq Cancer Panel v2; Thermo Fisher Scientific, Inc.). The library construction and subsequent enrichment of the paired DNA samples was performed using an Ion OneTouch system (Thermo Fisher Scientific, Inc.), according to the manufacturer's protocol. Sequencing was performed on a 316 chip with a capacity of 300-500 megabases, using the Ion PGM™ System (Thermo Fisher Scientific, Inc.). Sequencing reads were mapped to the University of California, Santa Cruz (UCSC) human genome (GRCh37/hg19) using Torrent Suite™ software (version 4.0.2; Thermo Fisher Scientific, Inc.).

Variant calling and classification of somatic mutations. Sequence data were analyzed using Variant Caller™ (version 4.0-r76860) and Ion Reporter™ (version 4.0-r77897; both Thermo Fisher Scientific, Inc.). Calls of single nucleotide variants (SNV) <2%, and calls of insertions and deletions (indels) <5% in tumor tissues were excluded from further analysis. Calls at positions with sequence coverage >50 reads in tumorous and non-tumorous cells were analyzed, and those present in the tumor sample but not in the matched normal sample, were regarded as somatic mutations. Fisher's exact test was carried out for the variants present in the tumor and corresponding normal tissue, and those detected with a significantly higher frequency ($P < 0.01$) in tumor tissues than the matched normal controls were classified as somatic mutations. All somatic mutations were reviewed by the Integrative Genomic Viewer (IGV; version 2.3; Broad Institute, Cambridge, MA, USA).

According to their locations, somatic mutations were classified into three groups as follows: Intronic, splice site and exonic mutations. The exonic mutations were further divided into four types, namely exonic indels, nonsense, synonymous and non-synonymous mutations. The pathological significance of the mutations was evaluated using several databases: ClinVar, COSMIC, ONCOMINE, Human Gene Mutation Database (HGMD), Leiden Open Variation Database (LOVD) in International Society for Gastrointestinal

Table I. Clinicopathological features of the 27 patients with BTC.

Patient ID	Age	Gender	Location ^a	Maximum tumor size (cm)	TNM ^b	Stage ^b
1	82	M	EBDC	6.0	T3N1M0	IIB
2	68	M	EBDC	9.0	T1N0M0	IA
3	65	M	EBDC	10.0	T1N0M0	IA
4	73	M	EBDC	15.0	T1N0M0	IA
5	67	M	EBDC	2.7	T1N0M0	IA
6	69	M	EBDC	2.0	T2N1M0	IIB
7	61	M	EBDC	1.8	T1N1M0	IIB
8	70	F	EBDC	Unknown	T1N1M0	IIB
9	69	M	EBDC	4.0	T1N0M0	IA
10	71	M	EBDC	1.8	T1N0M0	IA
11	65	M	EBDC	5.0	T1N0M0	IA
12	69	M	GBC	6.5	T3N0M0	IIA
13	74	M	GBC	3.6	T1N1M0	IIB
14	62	F	GBC	5.5	T1N1M0	IIB
15	80	F	GBC	3.0	T1N0M0	IA
16	73	F	GBC	3.5	T2N0M0	IB
17	71	F	GBC	8.5	T1N0M0	IA
18	70	M	GBC	Unknown	T1N0M0	IA
19	71	F	GBC	3.0	T1N0M0	IA
21	65	M	GBC	2.3	T4N0M0	III
20	58	F	GBC	5.5	T2N0M0	IB
22	76	F	GBC	6.0	T4N1M0	III
23	48	M	GBC	5.0	T1N0M0	IA
24	49	F	GBC	3.0	T2N0M0	IB
25	44	F	GBC	3.5	T2N1M0	IIB
26	66	F	AVC	5.0	T3N1M0	IIB
27	70	F	AVC	4.0	T4N0M0	III

^aEBDC, extrahepatic bile duct carcinoma; GBC, gallbladder carcinoma; AVC, Ampulla of Vater carcinoma; BTC, biliary tract cancer. ^bTNM and stage were determined based on the international union against cancer staging system.

Hereditary Tumors (InSiGHT), *TP53* Database in International Agency for Research on Cancer (IARC), dbSNP and Human Genetic Variation Database (HGVD) in Kyoto University. Mutations reported to play a role in tumorigenesis, and those regarded as deleterious in the ClinVar, COSMIC, ONCOMINE, HGMD, LOVD and *TP53* databases, were judged as pathological mutations. In addition, nonsense mutations, splice site mutations and exonic indels were included as pathological mutations. Among the remaining mutations, missense mutations were evaluated using three prediction tools: SIFT, PolyPhen and PANTHER. In the present study, those predicted to be damaged, deleterious or pathological by all three methods were considered as pathological mutations, and other missense mutations as variants of uncertain significance (VUS). The remaining synonymous mutations were regarded as non-pathological alterations.

Statistical analysis. Statistical differences were analyzed using the Fisher's exact test. For the detection of somatic mutations, $P < 0.01$ was considered to indicate a statistically

significant difference for the comparison between normal and cancerous tissues. For the comparison of mutation profiles between EBDCs and GBCs, $P < 0.05$ was considered to indicate a significant difference.

Results

Genetic analysis. Genetic analysis of the 27 tumors and matched normal tissues was performed by amplicon sequencing. The average sequence throughput per sample was ~26 Mb, and the average number of reads per amplicon was 1,099. Among the 207 regions analyzed, all regions (207/207) were covered by ≥ 100 reads on average, and 86.9% of all the amplicons were covered by ~500 reads. Subsequent mutation analysis using Valiant Caller™ and Ion Reporter™ detected a total of 92 variant calls that were candidates for somatic mutations. However, 37 of the 92 calls were filtered out for further analysis as no significant differences were identified ($P > 0.01$) in matched normal tissues, and the frequency was not statistically different between the tumors and noncancerous tissues.

Among the remaining 55 alterations, 11 variants were located within homopolymers, which were identified as miscalls by reviewing with IGV. Finally, 44 variants were judged to be somatic mutations. Among the 44 mutations, 42 were located in exons and the two in splice sites. The exonic mutations were comprised of 38 non-synonymous, 2 synonymous, 1 deletion and 1 insertion. The two synonymous mutations were identified as non-pathological, as they did not induce amino acid changes or were not predicted to cause aberrant splicing.

Among the 42 mutations, the most frequently mutated gene was *TP53* (14/27) and the second was *PIK3CA* (6/27), followed by: *KRAS* (6/27), *SMAD4* (6/27), *RBI* (2/27), *APC* (1/27), *ATM* (1/27), *CDKN2A* (1/27), *CTNBN1* (1/27), *KIT* (1/27), *NRAS* (1/27), *SMO* (1/27) and *VHL* (1/27; Table II). All 14 *TP53* mutations are included in the IARC *TP53* database (R17). The six *PIK3CA* mutations, including one p.E542 K, four p.E545Ks and one p.Q546K, were reported to be oncogenic mutations (30), and are repeatedly observed in the COSMIC database (v74). Five of the six *KRAS* mutations are activating mutations (p.G12D, p.G12R and p.G13D), and are located at codon 12 or codon 13, the two major hot spots (24,25). Although the single remaining missense mutation (p.L19F) is located outside of the hot spots, this mutation was reported to exhibit oncogenic activities (31). Therefore, it was considered to be a pathological mutation. The six *SMAD4* mutations included: One nonsense mutation, one 24-bp deletion and four missense mutations (p.D351H, p.G352E, p.R361H and p.A532D). Two of the four mutations (p.D351H and p.R361H) are well-known pathological mutations, and the other two missense mutations (p.G352E and p.A532D) are relatively infrequent mutations. The presence of p.G352E was reported in colorectal cancer (32), squamous cell lung cancer (33) and pancreatic cancer in the COSMIC (v74) database (34); p.A532D was identified in colorectal and pancreatic cancer in the COSMIC (v74) database (35). These two mutations were identified as pathological mutations following *in silico* analysis, including SIFT, PolyPhen and PANTHER. Finally, it was judged that all *SMAD4* mutations may result in the loss of *SMAD4* function, and that they may have a vital role in tumorigenesis.

The present study identified a *KIT* mutation (c.2411G>A, p.R804Q) that had not been reported in previous studies or public databases. *In silico* analysis predicted that this was a pathological mutation; possibly damaged (score: 1) by PolyPhen, deleterious (score: 0.01) by SIFT and deleterious (subPSEC: -3.301; P_{deterious}: 0.575) by PANTHER. As a result, 42/44 mutations were identified to be pathological.

Mutation profiles of EBDCs and GBCs. Mutation profiles between the 11 EBDC and the 14 GBC samples were compared further. It is of note that *TP53*, *KRAS*, *PIK3CA* and *SMAD4* mutations were observed in the two types of tumors (Table III). No significant differences ($P>0.05$) were identified between the frequencies of mutations of the four genes in the various types of cancer tumors.

Discussion

The current study performed a genetic analysis of 27 BTC tissue samples consisting of: 11 EBDC, 14 GBC and 2 AVC.

Using NGS, it was discovered that the tumors frequently carry mutations in *TP53*, *KRAS*, *PIK3CA* and *SMAD4*.

TP53 mutations in 45.6% of EBDC and 64.3% of GBC tissue samples were identified. These frequencies are consistent with previous studies for Caucasian EBDC (17.5-75%) (16,36,37) and GBC (46.2-63%) (15,36,38). Additionally, the frequencies of *KRAS* mutations in Japanese EBDC (27.2%) and GBC (14.3%) are in agreement with previous studies (16,19,20,36,37). Notably, an *NRAS* mutation was identified in a tumor without *KRAS* mutation, corroborating mutual exclusiveness between the mutations in *KRAS* and *NRAS*. In total, *TP53* and *KRAS* mutations in 14 and 7 of the 27 tumors, respectively, were identified, suggesting that the inactivation of *TP53* and activation of the RAS-MAPK pathway serve a crucial role in biliary tract carcinogenesis.

Notably, the present study revealed relatively high frequency (22.2%) of *PIK3CA* mutations in Japanese BTCs, compared with previous reports (15,16,18,23,36,37,39-41). In Caucasian BTC, the frequencies of *PIK3CA* mutation are limited (16,23,36,37). Simbolo *et al* (36) identified a *PIK3CA* mutation in 5/57 (8.8%) of EBDC samples. By contrast, a number of previous studies did not identify any *PIK3CA* mutations in EBDC (16,37). Regarding GBC, numerous previous studies revealed *PIK3CA* mutations at frequencies of 7.7% and 5.9% (36). These results may suggest that mechanisms underlying the pathogenesis of BTC vary between Japanese and Caucasian populations. In addition, molecular agents targeted to the activated PI3K/AKT signaling pathway may be more applicable for Japanese patients with BTC, as compared with Caucasian patients.

The present study identified six *SMAD4* mutations, including: One nonsense mutation, one in-frame deletion and four missense mutations (p.D351H, p.G352E, p.R361H and p.A532D). *SMAD4* is a tumor suppressor gene containing two evolutionarily conserved regions as follows: Mad homology 1 and 2 domains (MH1 and MH2, respectively) (42,43). The four mutations, p.D351H, p.G352E, p.R361H and p.A532D, are located in the MH2 domain (codon 319-552), which serves an important role in heteromerization and transactivation functions (43). The mutations at codons 351 and 361 are frequently reported (43). The two residues, Asp351 and Arg361, are located in the loop-helix region of *SMAD4*, and are involved in the interaction with Asp450 in *SMAD2* (43). Therefore, these mutations may change the loop-helix structure, disrupting the heterodimerization between *SMAD2* and *SMAD4*. Mutations at codons 352 and 532 are relatively infrequent (43); a total of 10 cases of a mutation at codon 352 and three cases at codon 532 are present in the COSMIC database. Although p.G352E is one of the ten cases, it was identified as a pathological mutation as the germ line p.G352E mutation has been reported in patients with juvenile polyposis and hereditary hemorrhagic telangiectasia (44). Although Ala532 is located outside of mutation cluster region (MCR) between codons 330 and 370, it is within the three-helix bundle (codon 445-540), which is reported to be functionally important (45). Missense mutations at Ala532 are assumed to disrupt the packing between the three-helix bundle and the β -sandwich (45). Additionally, *in silico* analysis of the mutation using the three algorithms identified it to be a pathological mutation. Therefore, p.A532D was considered to be a pathological mutation.

Table II. List of the somatic mutations.

Gene	Nucleotide	Amino acid	Type of mutation	Pathogenicity	Evidence of pathogenicity ^a	Number of cases
<i>TP53</i>	c.293_294insA	p.P98Pfs50X	Insertion	Pathological	IARC	1
	c.329G>T	p.R110L	Missense	Pathological	IARC, CSMC	1
	c.451C>G	p.P151A	Missense	Pathological	ONC, IARC, CSMC	1
	c.473G>C	p.R158P	Missense	Pathological	ONC, IARC, CSMC	1
	c.493C>T	p.Q165X	Nonsense	Pathological	IARC, CSMC	1
	c.574C>T	p.Q192X	Nonsense	Pathological	ONC, IARC, CSMC	1
	c.613T>G	p.Y205D	Missense	Pathological	ONC, IARC, CSMC	1
	c.614A>G	p.Y205C	Missense	Pathological	ONC, IARC, CSMC	1
	c.853G>A	p.E285K	Missense	Pathological	IARC, CSMC	1
	c.994-1G>A	-	Splice site	Pathological	SPL, IARC, CSMC	1
	c.1024C>T	p.R342X	Nonsense	Pathological	NS, IARC, CSMC	1
	c.839G>A	p.R280K	Missense	Pathological	ONC, IARC, CSMC	1
	c.824G>A	p.C275Y	Missense	Pathological	ONC, IARC, CSMC	1
	c.721T>C	p.S241P	Missense	Pathological	ONC, IARC, CSMC	1
<i>PIK3CA</i>	c.1623T>C	p.S541=	Synonymous	Non-pathological	-	1
	c.1624G>A	p.E542K	Missense	Pathological	dbSNP, ONC, CSMC	1
	c.1633G>A	p.E545K	Missense	Pathological	dbSNP, ONC, CSMC	4
	c.1636C>A	p.Q546K	Missense	Pathological	dbSNP, CSMC	1
<i>SMAD4</i>	c.346C>T	p.Q116X	Nonsense	Pathological	NS, CSMC	1
	c.535_558delATTCA AACCATCCAGCATC CACCA	p.I179_P186del	Deletion	Pathological	INDEL	1
<i>KRAS</i>	c.1051G>C	p.D351H	Missense	Pathological	ONC, IS, CSMC	1
	c.1055G>A	p.G352E	Missense	Pathological	dbSNP, CSMC, Rep	1
	c.1082G>A	p.R361H	Missense	Pathological	dbSNP, ONC, CSMC	1
	c.1595C>A	p.A532D	Missense	Pathological	CSMC, Rep, IS	1
	c.34G>C	p.G12R	Missense	Pathological	dbSNP, ONC, CSMC	1
<i>NRAS</i>	c.35G>A	p.G12D	Missense	Pathological	dbSNP, ONC, CSMC	3
	c.38G>A	p.G13D	Missense	Pathological	dbSNP, ONC, CSMC	1
	c.57G>T	p.L19F	Missense	Pathological	Rep, CSMC	1
	c.181C>A	p.Q61K	Missense	Pathological	dbSNP, ONC, CSMC	1
<i>CDKN2A</i>	c.358G>T	p.E120X	Nonsense	Pathological	NS, CSMC	1
<i>RBI</i>	c.596T>A	p.L199X	Nonsense	Pathological	NS, CSMC	1
	c.607+2T>G	-	Splice site	Pathological	SPL	1
<i>APC</i>	c.4339C>T	p.Q1447X	Nonsense	Pathological	NS, CSMC	1
<i>CTNNB1</i>	c.134C>T	p.S45F	Missense	Pathological	dbSNP, CSMC	1
<i>KIT</i>	c.2411G>A	p.R804Q	Missense	Pathological	IS	1
<i>SMO</i>	c.961G>A	p.V321M	Missense	Pathological	CSMC, IS	1
<i>ATM</i>	c.1044G>C	p.L348F	Missense	Pathological	IS	1
<i>FLT3</i>	c.1746C>T	p.T582=	Synonymous	Non-pathological	-	1
<i>VHL</i>	c.286C>T	p.Q96X	Nonsense	Pathological	NS, CSMC	1

IARC, international agency for research on cancer; CSMC, COSMIC; *TP53*, tumor protein 53; ONC, oncology; *PIK3CA*, phosphatidylinositol-4,5-bisphosphate 3-kinase, catalytic subunit α ; dbSNP, single nucleotide polymorphism database; *SMAD4*, SMAD4 family member 4; SPL, splice site mutations; NS, nonsense mutations; INDEL, exonic insertions/deletions; Rep, reports; IS, *in silico* analyses; *KRAS*, Kirsten rat sarcoma; *NRAS*, neuroblastoma RAS viral oncogene homolog; *CDKN2A*, cyclin-dependent kinase inhibitor 2A; *RBI*, retinoblastoma 1; *APC*, adenomatous polyposis coli; *CTNNB1*, catenin β -1; *KIT*, KIT proto-oncogene receptor tyrosine kinase; *SMO*, Smoothed frizzled class receptor; *ATM*, ataxia-telangiectasia mutated; *FLT3*, FMS-related tyrosine kinase 3; *VHL*, von Hippel-Lindau tumor suppressor. ^aEvidence of pathogenicity is judged from i) the type of mutation including nonsense mutations (NS); splice site mutations (SPL) and exonic indels (INDEL); ii) reports (Rep); iii) databases including ONCOMINE (ONC), dbSNP (dbSNP), TP53 database in international agency for research on cancer (IARC) and COSMIC (CSMC); and iv) *in silico* analyses (IS).

Table III. Mutation profile of 27 BTCs.

Gene name	Total n=27 (%)	EBDC n=11 (%)	GBC n=14 (%)	AVC n=2 (%)
<i>TP53</i>	14 (51.9)	5 (45.5)	9 (64.3)	0 (0)
<i>SMAD4</i>	6 (22.2)	3 (27.3)	2 (14.3)	1 (50)
<i>PIK3CA</i>	6 (22.2)	3 (27.3)	3 (21.4)	0 (0)
<i>KRAS</i>	6 (22.2)	3 (27.3)	2 (14.3)	1 (50)
<i>NRAS</i>	1 (3.7)	1 (9.1)	0 (0)	0 (0)
<i>CDKN2A</i>	1 (3.7)	0 (0)	1 (7.1)	0 (0)
<i>RBI</i>	2 (7.4)	1 (9.1)	1 (7.1)	0 (0)
<i>APC</i>	1 (3.7)	1 (9.1)	0 (0)	0 (0)
<i>CTNNB1</i>	1 (3.7)	1 (9.1)	0 (0)	0 (0)
<i>ATM</i>	1 (3.7)	0 (0)	1 (7.1)	0 (0)
<i>KIT</i>	1 (3.7)	1 (9.1)	0 (0)	0 (0)
<i>SMO</i>	1 (3.7)	1 (9.1)	0 (0)	0 (0)
<i>VHL</i>	1 (3.7)	0 (0)	1 (7.1)	0 (0)

TP53, tumor protein 53; *SMAD4*, SMAD family member 4; *PIK3CA*, phosphatidylinositol 3-kinase catalytic alpha; *KRAS*, Kirsten rat sarcoma; *NRAS*, neuroblastoma RAS viral oncogene homolog; *CDKN2A*, cyclin-dependent kinase inhibitor 2A; *RBI*, retinoblastoma 1; *APC*, adenomatous polyposis coli; *CTNNB1*, catenin β -1; *ATM*, ataxia-telangiectasia mutated gene; *KIT*, KIT proto-oncogene receptor tyrosine kinase; *SMO*, smoothened, frizzled class receptor; *VHL*, von Hippel-Lindau tumor suppressor.

In the present study, fewer frequencies of mutations were identified in tyrosine kinase receptor (TKR) genes than other studies (36). The mutations in TKRs, including *ALK*, *EGFR*, *ERBB2*, *ERBB4*, *FGFR3*, *MET*, *KIT*, *KDR* and *VEGFR2*, were reported with a higher prevalence in GBCs (6/26; 23.1%), compared with intrahepatic bile duct carcinoma IHBDCs (4/70; 5.7%) and EBDCs (4/57; 7.0%) (36). Although amplicon sequencing covering these genes was carried out, alterations were not detected in the BTC samples used in the present study. Therefore, the mechanisms underlying Japanese BTC may differ from Caucasian BTC, and therapeutic drugs for Japanese patients must be selected according to the specific mutations in their tumors.

The present study identified two *RBI* mutations in an EBDC and a GBC tissue sample. However, previous studies did not detect any *RBI* mutations in EBDC (16,36,37). Additionally, a *CDKN2A* mutation was revealed in one GBC. *CDKN2A* serves a role in cell cycle regulation through the inhibition of cyclin dependent kinases (CDK), including CDK4 and CDK6, and acts as a tumor suppressor gene in melanoma (46), esophageal cancer (47) and pancreatic cancer (48). In combination with cyclins, CDK4 and CDK6 phosphorylate the retinoblastoma protein (RB), which results in the transactivation of E2F transcription factors (49). The data suggest that disrupted cell cycle regulation at the G₁-S transition is involved in the tumorigenesis of Japanese BTC. Furthermore, immunohistochemical staining demonstrated loss of p16 (*CDKN2A*) expression in 74.1% (20/27) of GBC tissues, and loss of pRB expression in 3.7% (1/27) of GBC tissues (50). Recently, the FDA approved palbociclib, an inhibitor of CDK4/6, for the treatment of patients with HR-negative and HER2-negative metastatic breast cancer (51). Notably, a melanoma cell line with mutations in *CDKN2A* was predicted to be sensitive to the CDK4/6 inhibitor (52). Therefore, patients with BTC

carrying a *CDKN2A* mutation are expected to benefit from the use of palbociclib.

In addition, a nonsense mutation of *VHL*, p.Q96X, was identified in one GBC case. *VHL* is a tumor suppressor gene responsible for the hereditary disease, von Hippel Lindau syndrome. Inactivation of *VHL* by mutation, deletion or hypermethylation, is frequently observed in renal cell carcinomas (RCC) (53). In the COSMIC (v74) database, p.Q96X has been reported in 10 cases, including nine RCCs (54,55) and one hemangioblastoma. Although mutations of *VHL* have been less frequently reported in BTC than RCC, immunohistochemical staining revealed decreased *VHL* expression levels in (31/33; 93.9%) of GBC cases (56). Genetic alterations induce loss of *VHL*, which increases survival rate through the increased expression of *VEGF*, *PDGF*, *TGF- β* , *GLUT1* and *FGF* genes (57). Patients suffering from RCC with a *VHL* mutation are often treated by tyrosine kinase inhibitors, anti-vascular endothelial growth factor (VEGF) antibodies and mechanistic target of rapamycin inhibitors (58). Therefore, patients with BTC and a *VHL* mutation may also benefit from these three treatments.

In conclusion, it was revealed in the present study that *TP53*, *RAS* family genes, *PIK3CA* and/or *SMAD4* are frequently mutated in Japanese BTC. In addition, it was revealed that *PIK3CA* mutations are more frequently identified in Japanese BTC than Caucasian BTC, suggesting that the PI3K-AKT signaling pathway may have a crucial role in the tumorigenesis of Japanese BTC. The data may be useful not only for the comprehensive understanding of tumorigenesis, but also for the development of personalized therapies for this type of tumor.

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