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ORIGINAL ARTICLE

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Comprehensive analyses of the clinicopathological features and genomic mutations of combined hepatocellular-cholangiocarcinoma

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Abstract

Aim: Combined hepatocellular-cholangiocarcinoma (cHCC-CCA) is a rare primary liver cancer that has two different tumor phenotypes in a single tumor nodule. The relationship between genetic mutations and clinicopathological features of cHCC-CCA remains to be elucidated.

Methods: Whole-exome sequencing analyses were carried out in 13 primary and 2 recurrent cHCC-CCAs. The whole-exome analyses and clinicopathological information were integrated.

Results: *TP53* was the most frequently mutated gene in this cohort, followed by *BAP1*, *IDH1/2*, and *NFE2L2* mutations in multiple cases. All tumors with diameters <3 cm had *TP53* mutations. In contrast, six of seven tumors with diameters ≥3 cm did not have *TP53* mutations, but all seven tumors had mutations in genes associated with various pathways, including Wnt, RAS/PI3K, and epigenetic modulators. In the signature analysis, the pattern of mutations shown in the *TP53* mutation group tended to be more similar to HCC than the *TP53* nonmutation group. Mutations in recurrent cHCC-CCA tumors were frequently identical to those in the primary tumor, suggesting that those tumors originated from identical clones of the primary cHCC-CCA tumors. Recurrent and co-occurrent HCC tumors in the same patients with cHCC-CCA had either common or different mutation patterns from the primary cHCC-CCA tumors in each case.

Conclusions: The study suggested that there were two subtypes of cHCC-CCA, one involving *TP53* mutations in the early stage of the carcinogenic process and the other not involving such mutations. The comparison of the variants between primary and recurrent tumors suggested that cHCC-CCA was derived from an identical clone.

KEYWORDS

combined hepatocellular-cholangiocarcinoma, liver cancer, next-generation sequencing, *TP53*

Abbreviations: CCA, cholangiocarcinoma; cHCC-CCA, combined hepatocellular-cholangiocarcinoma; CNV, copy number variation; COSMIC, Catalog of Somatic Mutations in Cancer; EBCall, empirical Bayesian mutation calling; FFPE, formalin-fixed paraffin-embedded; HCC, hepatocellular carcinoma; iCCA, intrahepatic cholangiocarcinoma; LT, liver transplantation; TIL, tumor infiltrating lymphocyte; VAF, variant allele frequency; WES, whole-exome sequencing.

INTRODUCTION

Primary liver cancer, including HCC and iCCA, is the third leading cause of cancer-related deaths in the world.[1] Combined hepatocellular-cholangiocarcinoma is a rare primary liver cancer that has two different tumor components in a single tumor nodule: an HCC component and a CCA component.[2–4] The clinicopathological features of cHCC-CCA remain unclear because of its rarity. In addition, therapeutic strategies for cHCC-CCA, including chemotherapy and surgical procedures, remain to be elucidated.[5–12]

Comprehensive genomic analyses using next-generation genome sequencing have been developed recently. Several large-scale analyses were undertaken on HCC and iCCA, and some of them identified characteristic mutations of HCC, including *TERT* promoter, *CTNNB1*, *ARID1A*, and *TP53*, and of iCCA, including *KRAS*, *IDH1/2*, and *TP53*.[13-21] Although there have been a few comprehensive genomic analyses on cHCC-CCA,[22-25] their impact has been limited.

Genomic analyses of cHCC-CCA have been mainly focused on its clonality, namely the genomic difference between HCC and iCCA components.[22,24,25] Therefore, the relationship between genetic mutations and clinicopathological features of cHCC-CCA has been less thoroughly discussed.

In addition, because previous studies focused on only primary cHCC-CCA tumors, they did not compare genomic difference between primary and recurrent cHCC-CCA tumors. Although cHCC-CCA tumors have a higher recurrence rate than HCC, and postoperative recurrence is frequently observed, the histopathological types of recurrent tumors are diverse, and the patterns of recurrence have not been sufficiently established.[5] The discussion of recurrent tumors after surgery has been extremely limited, and genomic analyses of recurrent tumors are also critical.

In the present study, we undertook whole-exome analyses of pathologically diagnosed cHCC-CCAs and analyzed the relationship between genomic and clinical data. In addition, we compared genetic mutations of primary cHCC-CCA tumors with those of recurrent cHCC-CCA and HCC tumors in the same patients.

METHODS

Patients

Between January 2005 and December 2018, 21 patients were diagnosed with cHCC-CCA after hepatic resection or LT in our institution (previously reported as resection and transplantation cases).[5,26] The pathologic diagnosis was based on hematoxylin–eosin staining according to the WHO 2019 criteria,[4] and immunohistochemical examinations for hepatocytic (HepPar1) and cholangiocytic markers (CK7, CK19) were added as needed to confirm the diagnosis.

The study protocol was approved by the Ethics Committee of the Graduate School of Medicine, Kyoto University (R1737-2), and carried out in accordance with the 1964 Declaration of Helsinki and its later amendments.

Genomic DNA extraction from cHCC-CCA tumors

Frozen specimens preserved in our department as tumor/nontumor sections or FFPE tissues preserved in the pathology department were used as samples. Formalin-fixed paraffin-embedded specimens were dissected into tumor and nontumor sections from 10 μm-thick slides. Genomic DNA was extracted using bulk sampling throughout the cHCC-CCA tumors in order to reflect the genomic information of the entire tumor. Using fresh-frozen tissue specimens, genomic DNA was isolated using a NucleoSpin Tissue Kit (Macherey-Nagel), and for FFPE specimens, DNA was isolated using a GeneRead DNA FFPE Kit (Qiagen). A quality check of each DNA sample was carried out using a Qubit dsDNA HS Assay Kit, NanoDrop 2000 (Thermo Fisher Scientific) and 1% agarose gel electrophoresis.

Whole-exome sequencing

Exome capture was carried out using SureSelect Human All Exon V6 (Agilent Technologies) according to the manufacturer's instructions. Whole-exome sequencing was carried out using a NovaSeq 6000 platform (Illumina) with a standard 150 bp paired-end read protocol, as previously reported.[27,28] Sequencing reads were first aligned to NCBI Human Reference Genome Build 38 (hg38), and the sequencing data were analyzed for somatic mutation calling using the Genomon2 pipeline (https://genomon.readthedocs.io/ja/latest/), as previously described.[29] A paired analysis of both tumor tissue and nontumoral liver tissue was carried out. Candidate mutations were adopted using the EBCall algorithm[30] with the following filtering process: (i) *p*-value by EBCall <0.01, (ii) variant number of tumor ≥4, (iii) variant number of nontumor ≤2, (iv) variants only present in single-direction reads were excluded, (v) *p* value by local realignment <0.05, and (vi) VAF ≥ 0.05. In cases 6 and 12, which were both recurrent cases, variants identified in primary or recurrent tumor were recalled in the other tumor even if they were excluded due to incompatibility regarding the VAF or local realignment *p* value. Cancer-related alterations were searched from the candidate mutations according to the COSMIC database (https://cancer.sanger.ac.uk/signatures). A pathway analysis was undertaken mainly based on the Kyoto Encyclopedia of Genes and Genomes pathway database and the oncogenic signaling pathway database reported by Sanchez-Vega et al.[31,32]

Copy number analysis

A copy number variation analysis was carried out using the CNVkit software program, version 0.9.9, at the default setting, with paired tumor/nontumor bam data.[33] We set the *q* value cut-off at 0.25 using

GISTIC2.0 on the Genepattern pipeline and detected significantly amplified or deleted regions/genes.[34,35]

Mutational signature analysis

For the mutational signature analysis, the number of mutations assigned to each of 96 possible substitution classifications, defined by the substitution class and sequence context immediately 5′ and 3′ to each mutated base in coding regions, was counted for each sample. The frequency of each mutation was calculated by dividing each count by the total number of mutations. That analysis was undertaken using the MutationalPatterns package in the R software program at the default setting[36,37] and based on Mutational Signatures version 3 in the COSMIC database.

Sanger sequencing

To detect hotspot mutations of the *TERT* promoter region and identical alterations in synchronous/metachronous recurrent tumors, we adopted a direct sequencing technique.

Polymerase chain reaction was carried out on extracted genomic DNA, and direct sequencing by an Applied Biosystems 3730xl DNA analyzer (Thermo Fisher Scientific) was undertaken on those products. The primer list is provided in Table S1.

Statistical analysis and visualization

Statistical analyses were undertaken using the R software program, version 4.1.2. Continuous data are presented as the median with the range and were analyzed using Wilcoxon's signed-rank test. Categorical data were analyzed using Fisher's exact test. All analyses were two-tailed, and differences with a p value of <0.05 were defined as statistically significant. The summary of the data analyses was visualized using the maftools package in the R software program.[38]

RESULTS

Clinical characteristics of cHCC-CCA patients

Thirteen of the total 21 cHCC-CCA patients were enrolled in the study. Regarding the cases that were not included, genomic DNA specimens of sufficient quality for WES were not available. Fifteen cHCC-CCA tumors from these 13 patients, including 13 primary tumors and 2 recurrent tumors, were examined. The clinicopathological features are summarized in Table 1. The histopathological findings of each tumor are summarized in Table S2, including the percentages of the HCC and CCA components in each tumor, the degree of differentiation in each component, and the appearance of TILs.

TABLE 1 Clinicopathological characteristics of patients with combined hepatocellular-cholangiocarcinoma (cHCC-CCA).

	Total (n = 13)
Sex, *n* (%) (male/female)	7(53.8)/6(46.2)
Age, years	63 (45–81)
Background liver disease, *n* (%)	
HCV	4 (30.8)
HBV	3 (23.1)
NASH	2 (15.4)
Alcoholic	1 (7.7)
Normal liver	3 (23.1)
Child–Pugh, *n* (%)	
A	8 (61.5)
B	3 (23.1)
C	2 (15.4)
AFP, ng/mL	14.2 (1.7, 2075.0)
DCP, mAU/mL	28.0 (15.0, 22480.0)
CEA, ng/mL	2.6 (0.6, 13.1)
CA19-9, U/mL	36.0 (0.6, 121.1)
Tumor number, *n*	1 (1–10)
Tumor number of cHCC-CCA, *n*	1
Tumor size of cHCC-CCA, cm	3.6 (1.4–11.7)
Tumor subtype, *n* (%) (combined/mixed)	9(69.2)/4(30.8)
Ratio of CCA component, %	30 (0–90)
TIL appearance, *n* (%)	5 (38.5)
Operation, *n* (%)	
Hepatic resection	10 (76.9)
Living-donor liver transplantation	3 (23.1)

Note: Numbers are described as median (range) or *n* (%) unless otherwise indicated.

Abbreviations: AFP, alpha-fetoprotein; CA19-9, carbohydrate antigen 19-9; CEA, carcinoembryonic antigen; DCP, des-gamma-carboxy prothrombin; HBV, hepatitis B virus; HCV, hepatitis C virus; NASH, nonalcoholic steatohepatitis; TIL, tumor infiltrating lymphocyte.

Ten patients had background liver disease. Alpha-fetoprotein and des-gamma-carboxy prothrombin levels were extremely high in some cases, similar to the characteristics of HCC patients. No patients had multiple cHCC-CCA nodules, although there were synchronous HCC nodules with a cHCC-CCA nodule in some cases. Three LT cases were included in this analysis.

Somatic mutational analysis of cHCC-CCA

The averaged coverage was 111.5-fold in all samples, and those in the tumor sample and the control sample were 139.3- and 77.2-fold, respectively, which was sufficient for a mutational analysis by WES

(including recurrence) (Table S3). We identified 976 mutations, including 699 nonsilent mutations (1152 mutations, including 828 nonsilent mutations if recurrence samples were included) (Table S4). The median number of nonsilent mutations was 48 per case (maximum 116, minimum 4), and single-nucleotide variations accounted for most of the mutations (640 mutations), followed by insertions and deletions (59 mutations). These included 585 missense, 36 nonsense, 43 frameshift, and 12 in-frame mutations (Figures S1, S2).

The mutational landscape is presented in Figure 1. *TP53* was the most frequently mutated gene in this study, which was compatible with previous findings.[22,24,25] Copy number losses of *TP53* were also detected in many cases. Each base substitution on *IDH1* was identical in three cases (Figure 2a), and this *IDH1* R132C mutation was a hotspot mutation that has been reported frequently in iCCA and acute myeloid leukemia.[39] *NFE2L2*, an HCC oncogene, was also mutated in several cases, and *CTNNB1* and *ARID1A* mutations were observed as well. *ARID1A* has been reported to be present in not only HCC but also iCCA, and *EPHA2*, which has been reported in iCCA, was mutated in one case; however, there were no cases with *KRAS* mutations. A hotspot mutation of the *TERT* promoter, identified by Sanger sequencing, was found in six cases (Figure S3). The mutated genes were similar between the primary and recurrent nodules in cases 6 and 12.

Copy number variations and mutational signature analysis

The heatmap of CNVs for each case suggested the presence of several arm-level gains (1q, 2p, 6p, 7p-q, 8q, 19q, and 20q) and losses (1p, 4q, 6q, 8p, 9p-q, 10q, 13q, 14q, 16q, 17p, and 21q) in the samples (Figure 3a). This tendency was similar to that reported in HCC and iCCA. The CNVs were also similar between the initial and recurrent nodules in cases 6 and 12. Gistic2 analysis revealed that the CNVs in certain regions were gained in 8q24.13 (*MYC*) and lost in 17p13.2 (*TP53*, *BRCA1*, *NCOR1*, and *MAP2K3*), 13q14.2 (*RB1*), and 1p36.13 (*ARID1A*) (Figures 1,3b). Those regions include cancer-related genes, and a subset of them, such as *TP53*, *ARID1A*, and *RB1*, is known to be more closely related to oncogenesis in HCC than iCCA.

In the mutational signature analysis, the overall signature in this cohort was considered similar to that of iCCA based on the proportion of C > T at the CpG island and T > C base mutations (Figure 4a).

TP53 mutations and clinical characteristics

All tumors with diameters <3 cm had *TP53* alterations (Figure 1). In contrast, seven tumors with diameters ≥3 cm, only one of which carried a *TP53* mutation, had mutations in genes associated with various pathways, including Wnt, RAS/PI3K, and epigenetic modulators. A similar tendency was observed in the pathway analysis using maftools, based on the oncogenic signaling pathway reported by Sanchez-Vega et al.[31] Namely, *TP53* mutations were found in all tumors <3 cm in diameter. In contrast, tumors ≥3 cm in diameter rarely had *TP53* mutations and instead tended to have mutations in other carcinogenic pathway-related genes. Hippo pathway- and Notch pathway-related gene mutations were found in both smaller and larger tumors (Figure 2b).

Therefore, cHCC-CCA patients were categorized into two subgroups based on the presence of *TP53* mutations, and their clinical data were comparatively analyzed. The background liver disease and laboratory data showed no significant differences between the subgroups (Table 2). The tumor size was significantly larger in the *TP53* wild group than in the *TP53* mutation group. The tumors of the *TP53* wild patients had a significantly higher grade of histopathologic biliary tract invasion than those of the *TP53* mutation patients, suggesting that those tumors had iCCA-like properties. There was no significant difference in the overall or recurrence-free survival between these subgroups (Figure 4b), despite the significant difference in their tumor sizes. The sites of recurrence in the *TP53* mutated and *TP53* nonmutated groups did not differ to a statistically significant extent. In the histopathological features, TILs appeared more frequently in tumors with TP53 mutations than in tumors without TP53 mutations. The former accounted for four of seven cases, while the latter comprised one of six cases. No other significant findings were found in the percentage of HCC or CCA components of the tumors or the degree of differentiation. In the mutational signature analysis, the overall signature in this cohort was similar to that of iCCA, as mentioned above, but the *TP53* mutation group showed a decreased proportion of C > T at the CpG island and increased proportion of T > C, a similar tendency to that seen in HCC (Figures 4c, S4).

Comparison of genetic mutations between primary and recurrent cHCC-CCA tumors

To examine whether or not recurrent and co-occurrent tumors originated from primary cHCC-CCA tumors genetically, genomic sequence data were compared among primary, recurrent, and co-occurrent tumors (Table S5). The recurrent tumors in cases 5, 6, and 12 were cHCC-CCA pathologically (Figure 5). The WES data on the primary and recurrent tumors in cases 6 and 12 were compared, respectively, and the sequence data obtained by the Sanger method for the primary and recurrent tumors in case 5 were compared. In contrast, the recurrent tumors in cases 4 and 16 were pathologically diagnosed to be HCC, and the co-occurrent tumors in case 16 were pathologically diagnosed to be HCC (Figure 5b). These recurrent and co-occurrent tumors were examined by the Sanger method.

The recurrent cHCC-CCA tumors in cases 6 and 12 had many alterations from their primary tumors in common, including in some

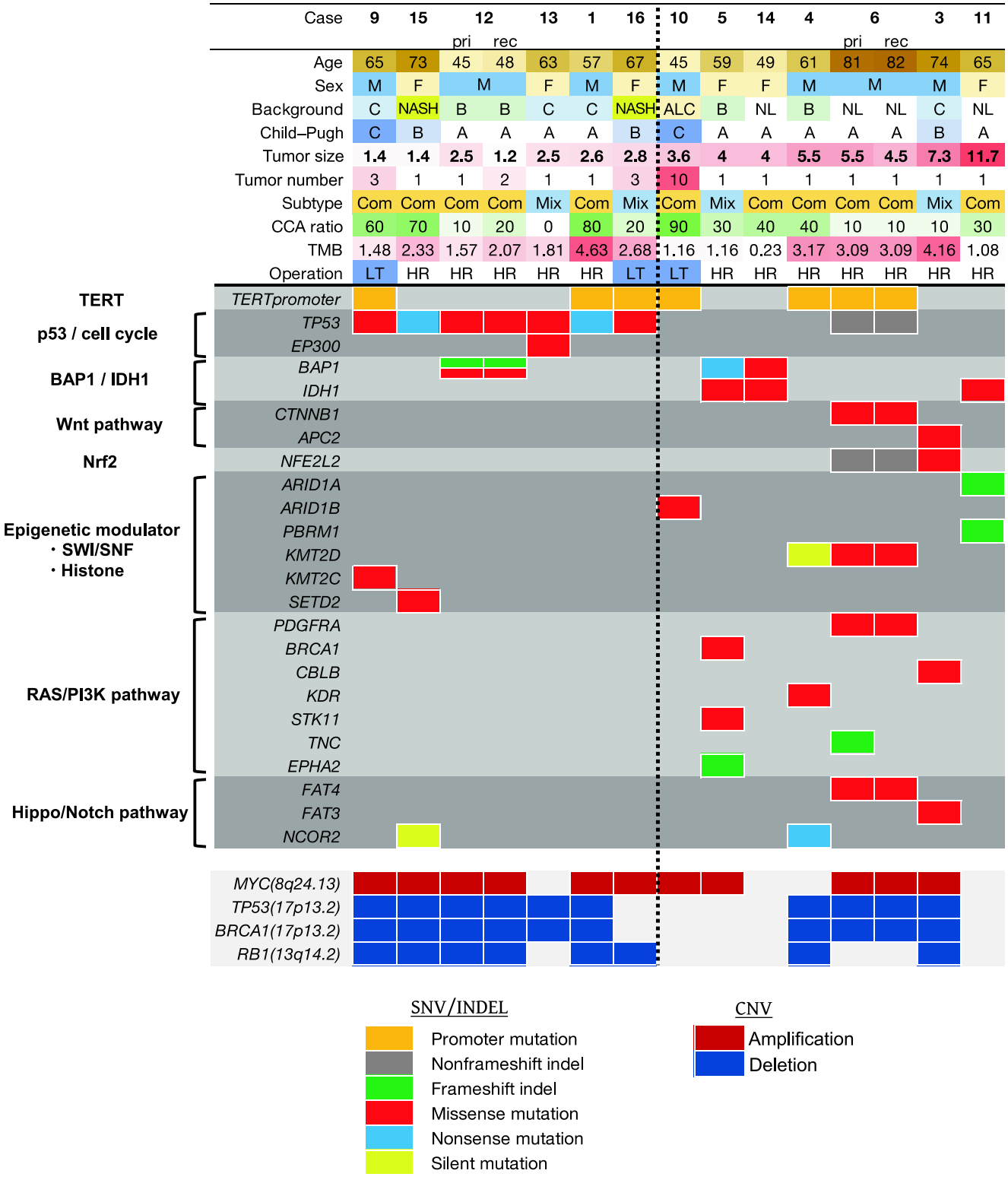


FIGURE 1 Clinicopathological mutational landscape of combined hepatocellular-cholangiocarcinomas. Top panel shows the case number and clinical features. Cases 6 and 12 have both primary (pri) and recurrent (rec) data available. Categories in descending order: Age, years. Sex: F, female; M, male. Background: ALC, alcoholic; B, hepatitis B virus; C, hepatitis C virus; NASH, nonalcoholic steatohepatitis; NL, normal liver. Tumor size, cm. Subtype: Com, combined type; Mix, mixed type. CCA ratio, %. TMB, tumor mutation burden. Operation: HR, hepatic resection; LT, liver transplantation. Middle panel shows the mutated genes, and the bottom panel shows copy number variations, with mutation types indicated at the bottom. On the left side of the panel, mutated genes are categorized by function, pathway, and intrahepatic CCA-specific mutation group. Cases are listed in order of tumor size. Dotted line represents the cut-off of the tumor size (3 cm). CNV, copy number variation; INDEL, insertion/deletion; SNV, single nucleotide variation.

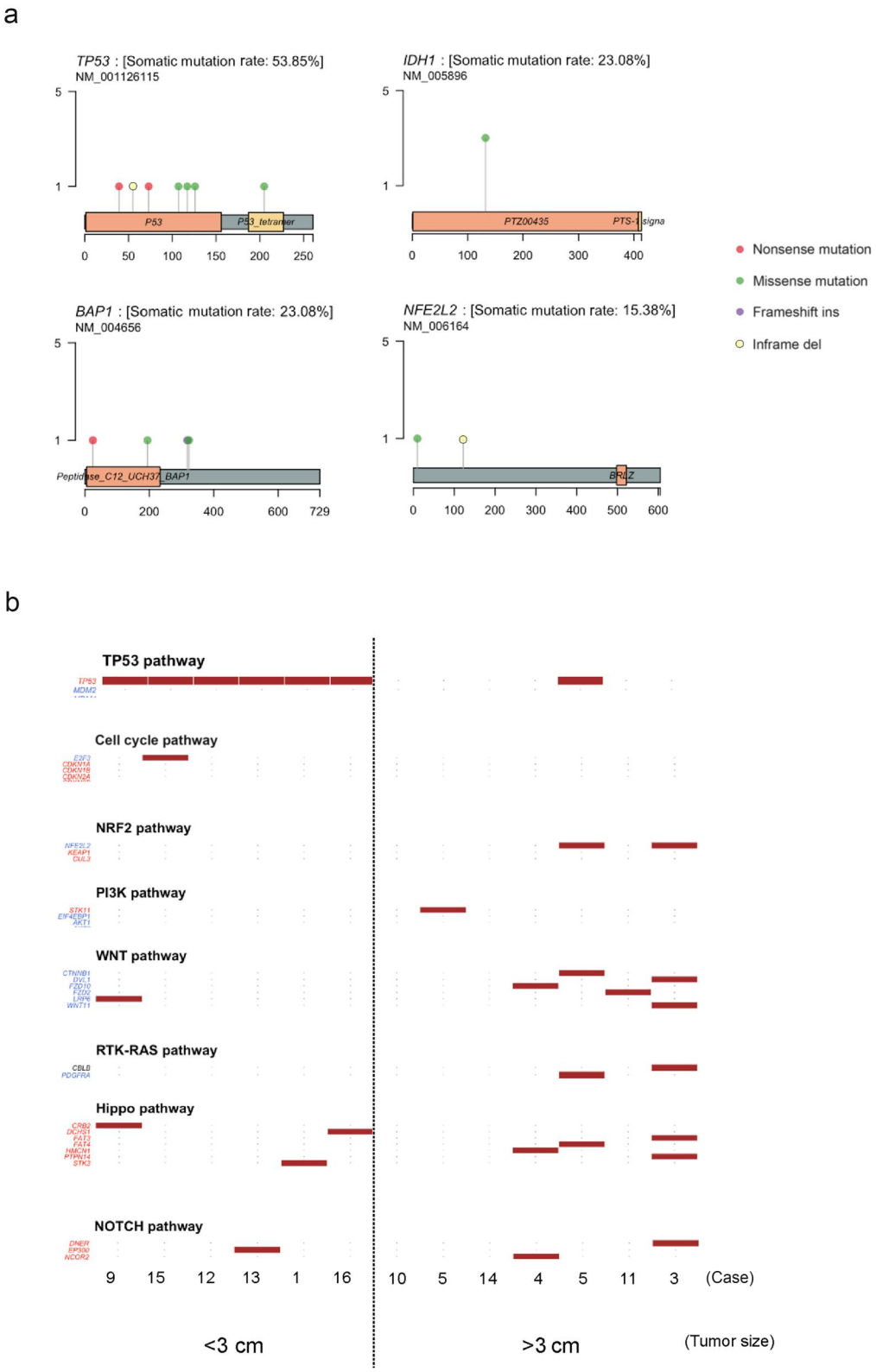


FIGURE 2 Maftools analyses of genomic mutations in patients with combined hepatocellular-cholangiocarcinoma. (a) Lollipop plots displaying the mutation distribution and protein domains of the four genes, *TP53*, *IDH1*, *BAP1*, and *NFE2L2*, that were mutated in multiple cases. Base-substitutions on *IDH1* were consistent in all three cases with *IDH1* mutations. (b) A pathway analysis by maftools among the primary cases. Only mutated genes are presented, excluding nonmutated genes in the pathway. As with the pathway analysis by Kyoto Encyclopedia of Genes and Genomes, tumors <3 cm in size had mutations in the TP53/cell cycle pathway, while tumors ≥3 cm had more mutations in other pathways. del, deletion; ins, insertion.

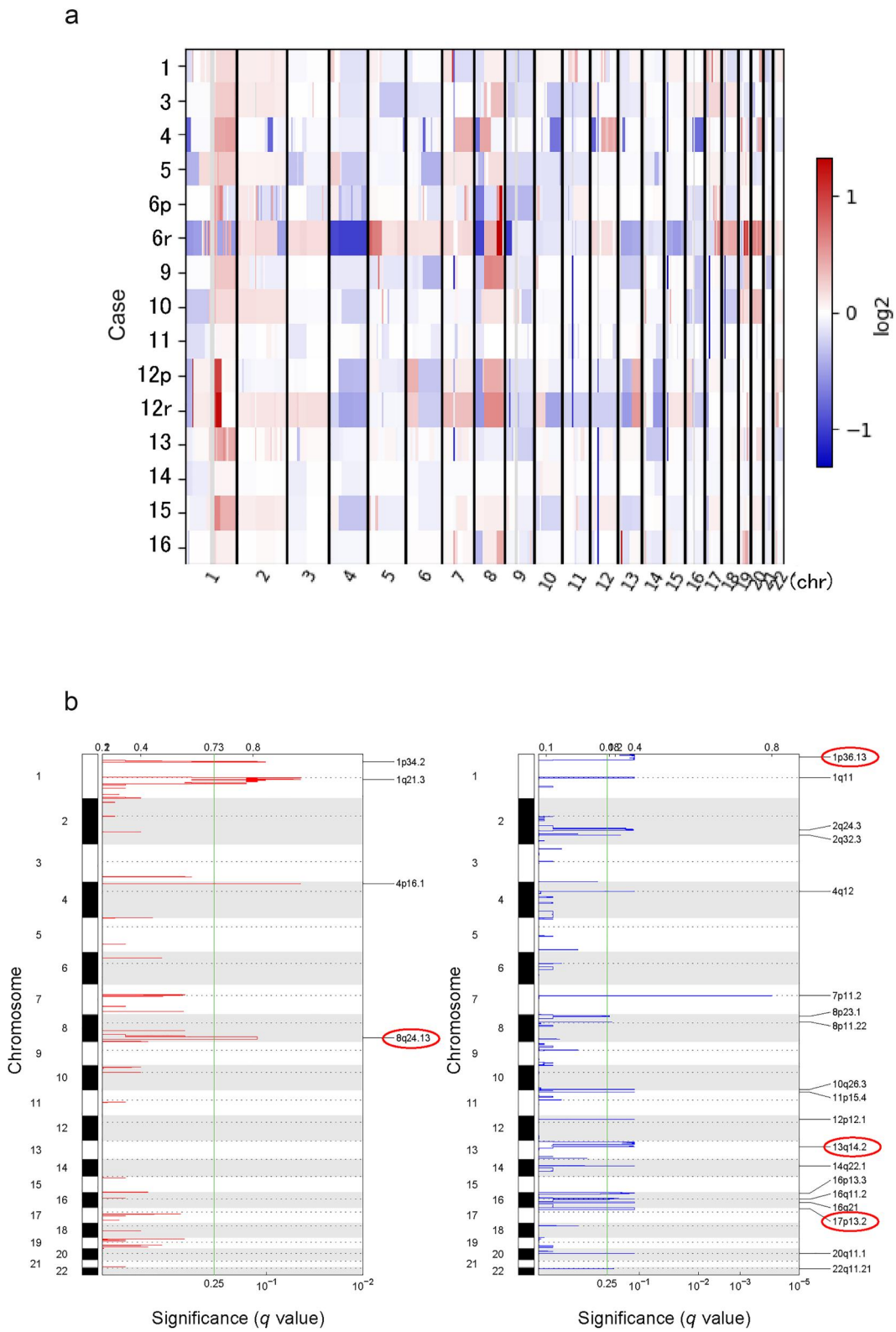


FIGURE 3 Copy number analyses of patients with combined hepatocellular-cholangiocarcinoma. (a) Heatmap of copy number variations (CNVs). Primary and recurrent tumors in case 12 have similar CNV characteristics, as do those in case 6. (b) GISTIC plot of CNVs. Positions indicating CNVs associated with *MYC*, *TP53*, *RB1*, and *ARID1A* are circled in red.

cancer-related genes (Figure 5a). Evolutionary analyses indicated that the primary and recurrent tumors in each case had a common origin. Sanger sequencing was carried out in recurrent and co-occurrent tumors to compare the gene mutations from their primary tumors in cases 4, 5, and 16 (Figures S5,S6). The results revealed that the primary and recurrent cHCC-CCA tumors in case 5

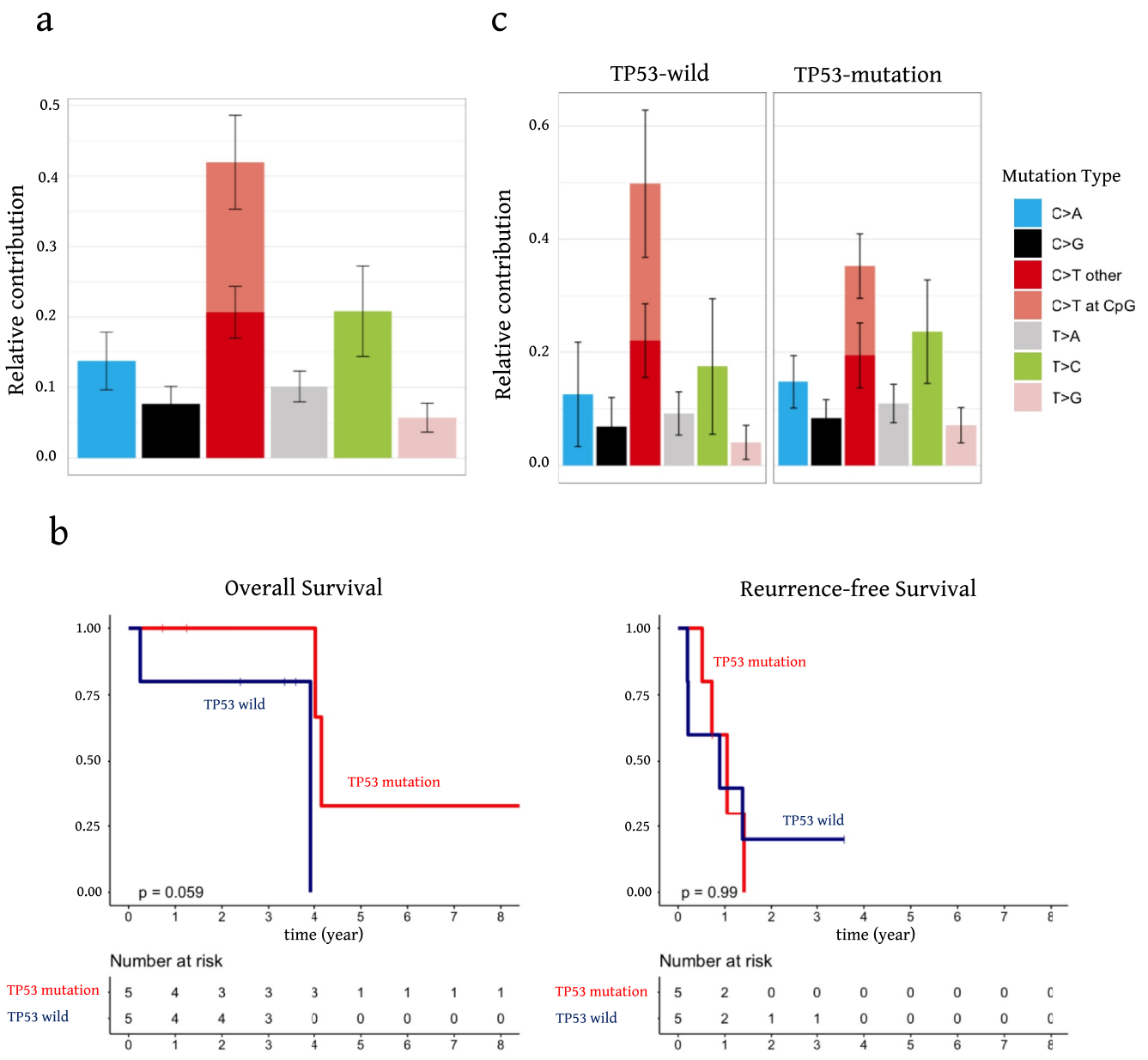


FIGURE 4 Assessment based on *TP53* mutations in patients with combined hepatocellular-cholangiocarcinoma (cHCC-CCA). (a) Mutational signatures of the whole primary cHCC-CCAs in this cohort. This signature of C > T at the CpG island being dominant and followed by T > C was similar to that found in intrahepatic cholangiocarcinoma. (b) Comparison of prognosis in cHCC-CCA patients with and without *TP53* mutations. There was no significant difference in the overall survival or recurrence-free survival between the groups with and without *TP53* mutations. (c) Comparison of mutational signatures in cHCC-CCA tumors with and without *TP53* mutations. The signature of the tumor group with *TP53* mutations showed a decreased proportion of C > T at the CpG island and increased T > C, which resembled that of HCC.

had common base-substitutions in two genes. The primary cHCC-CCA tumor and recurrent HCC tumor in case 4 had common base-substitutions in two genes, suggesting that the recurrent HCC tumor in case 4 shared a common origin with the primary cHCC-CCA tumor. These findings led us to speculate that the tumor was most likely metastatic recurrence from cHCC-CCA, rather than de novo HCC. In case 16, by contrast, the co-occurrent and recurrent HCC tumors did not have common base-substitutions with the primary cHCC-CCA tumor, suggesting that the co-occurrent and recurrent HCC tumors might have originated from different cell origins from the primary cHCC-CCA tumor.

DISCUSSION

Combined HCC-CCA is a rare tumor with a unique phenotype, and there have been few reports integrating clinical and genomic data on this tumor. In this study, we analyzed the clinical characteristics and

TABLE 2 Comparison of clinicopathological characteristics in combined hepatocellular-cholangiocarcinoma (cHCC-CCA) with and without *TP53* mutation.

	TP53 wild ($n = 6$)	*TP53* mutation ($n = 7$)	*p* value
Age, years	60 (45, 74)	65 (45, 81)	0.431
Sex, *n* (%) (male/female)	3 (50.0)/3 (50.0)	3 (42.9)/4 (57.1)	1.000
Child–Pugh (%)			1.000
A	4 (66.7)	4 (57.1)	
B	1 (16.7)	2 (28.6)	
C	1 (16.7)	1 (14.3)	
Platelet number, $\times 10^4/\mu L$	14.4 (3.1, 49.0)	12.9 (5.6, 18.4)	0.886
Total bilirubin, mg/dL	0.95 (0.6, 4.9)	1.3 (0.7, 2.8)	0.829
Albumin, g/dL	3.2 (2.5, 4.3)	4.3 (2.7, 4.6)	0.116
Prothrombin time, %	73 (44, 120)	81 (65, 99)	0.720
AFP, ng/mL	5.90 (1.7, 1517.0)	78.6 (2.0, 2075.0)	0.731
DCP, mAU/mL	234.0 (17.0, 22480.0)	28.0 (15.0, 2610.0)	0.830
CEA, ng/mL	1.6 (1.2, 11.1)	3.25 (0.6, 13.1)	0.662
CA19-9, U/mL	23.8 (0.6, 39.3)	40.0 (22.7, 121.1)	0.056
Background liver disease, *n* (%)			0.517
HCV	1 (16.7)	3 (42.9)	
HBV	2 (33.3)	1 (14.3)	
NASH	0 (0.0)	2 (28.6)	
Alcoholic	1 (16.7)	0 (0.0)	
Normal liver	2 (33.3)	1 (14.3)	
Tumor size of cHCC-CCA, cm	4.75 (3.6, 11.7)	2.5 (1.4, 5.5)	0.015
Tumor number, *n*	1 (1, 10)	1 (1, 3)	0.846
Major biliary tract invasion, *n* (%)	4 (66.7)	0 (0)	0.021
Major portal vein invasion, *n* (%)	1 (16.7)	0 (0)	0.462
Tumor subtype, *n* (%) (combined/mixed)	5(71.4)/2(28.6)	4(66.7)/2(33.3)	1.000
Ratio of CCA component, %	20 (0, 80)	35 (10, 90)	0.601
TIL appearance, *n* (%)	4 (57.1)	1 (16.7)	0.266
Operation, *n* (%)			1.000
Hepatic resection	5 (83.3)	5 (71.4)	
Living-donor liver transplantation	1 (16.7)	2 (28.6)	

Note: Numbers are described as median (range) or *n* (%) unless otherwise indicated.

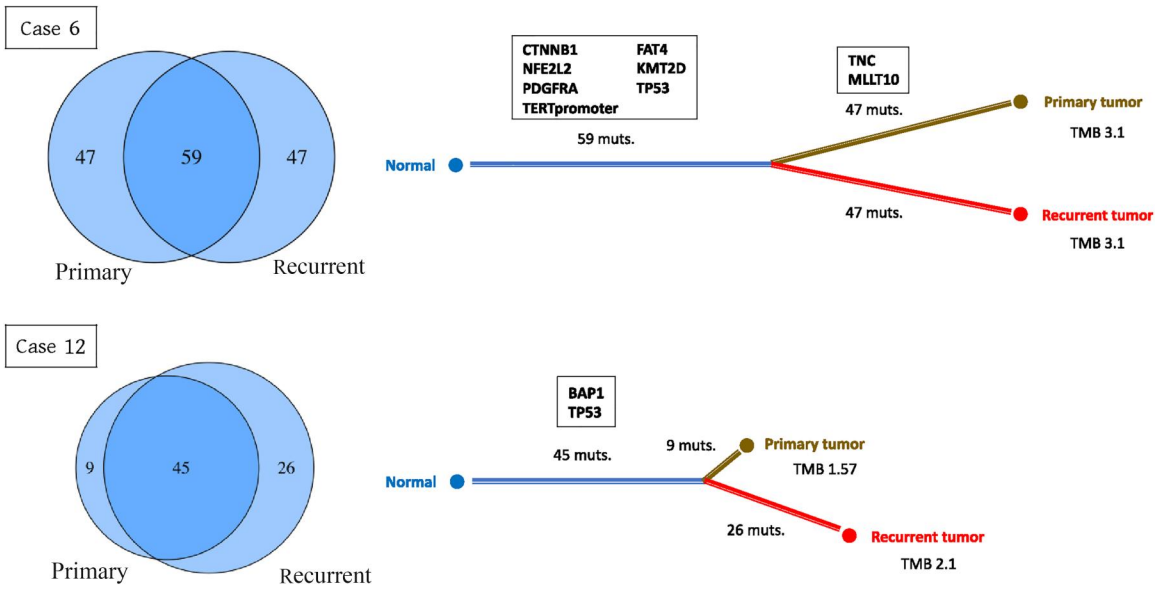
Abbreviations: AFP, alpha-fetoprotein; CA19-9, carbohydrate antigen 19-9; CEA, carcinoembryonic antigen; DCP, des-gamma-carboxy prothrombin; HBV, hepatitis B virus; HCV, hepatitis C virus; NASH, nonalcoholic steatohepatitis; TIL, tumor infiltrating lymphocyte.

recurrence pattern of this rare cancer with genetic information at the whole-exome level. Few genomic analyses of cHCC-CCA have so far been published. Some reports have suggested that cHCC-CCA has a similar mutation pattern to that of HCC, while others have suggested that cHCC-CCA has mutations specific to iCCA, making the position of cHCC-CCA in primary liver cancer unclear from a genetic perspective.[24,40] Furthermore, there have been few reports on genomic analyses associated in detail with the clinical background. In this study, we analyzed the mutations of 10 patients with cHCC-CCA who underwent HR, including 12 nodules (10 nodules from primary lesions and two from recurrent lesions), and three nodules in three patients with cHCC-CCA who underwent LT, in relation to their clinical backgrounds.

The whole-exome analyses revealed that all of the cHCC-CCAs had common gene mutations with HCC, including *TERT* promoter, *TP53*, and *CTNNB1*. However, they also showed mutations specific to

a

Case	Primary		Recurrent
6	**cHCC-CCA**	→	**cHCC-CCA**
12	**cHCC-CCA**	→	**cHCC-CCA** + HCC



b

Case 5	(ref)	cHCC-CCA	→	cHCC-CCA	→	cHCC-CCA(ly)
IDH1 chr 2:20824389	**G**	**G > A**		**G > A**		**G > A**
BAP1 chr3:52409603	**T**	**T > A**		**T > A**		**T > A**

Case 4	(ref)	cHCC-CCA	→	HCC
KDR chr4:551043	**C**	**C > T**		**C > T**
KMT2D chr12:49038530	**G**	**G > T**		**G > T**

Case 16	(ref)	cHCC-CCA	+	HCC1	+	HCC2	→	HCC
RUNX1T1 chr8:92014808	**G**	**G > A**		**G**		**G**		**G**
NUP98 chr11:375334	**T**	**T > C**		**T**		**T**		**T**

FIGURE 5 Genetic comparison among primary, recurrent, and co-occurrent combined hepatocellular-cholangiocarcinoma (cHCC-CCA) tumors. (a) In cases 6 and 12, both the primary and recurrent tumors were analyzed by whole-exome sequencing. Venn diagrams and evolutional trees show base-substitutions in cases 6 and 12. In both cases, the primary and recurrent nodules shared a number of base-substitutions in common. In the evolutional tree, cancer-related gene variants are shown. (b) Sanger sequencing results of the primary, recurrent, and co-occurrent tumors in cases 5, 4, and 16. ly, lymph node recurrence; muts, mutations; ref, reference; TMB, tumor mutation burden. [Correction added on 09 October 2023, after first online publication: Figure 5 has been corrected.]

iCCA, such as *IDH1* and *BAP1*, and their mutational signatures showed similar patterns to those of iCCA.

The presence of *TP53* mutations in small tumors <3 cm in size suggested that *TP53* mutations played an important role in the early stages of the carcinogenic process. In contrast, *TP53* mutations were rare in tumors ≥3 cm in diameter in this study. The mutations of the larger tumors were found in genes associated with diverse signaling pathways, including the WNT/β-catenin pathway and the Hippo pathway. These findings suggested that they had acquired various gene mutations during the carcinogenic process. In addition, TILs were more frequently found in tumors with *TP53* mutations. These findings might be associated with the small tumor size.[41,42] Although the correlation between tumor size and *TP53* mutation was clear, there was no clear correlation between the histopathological phenotypes that were reported by Xue et al (i.e., combined and mixed type) and the gene mutation pattern.[22] A further large-scale genome analysis is desirable.

In a mutational signature analysis, the pattern of the *TP53* nonmutant group more closely resembled that of iCCA than that of the *TP53* mutant group. These results indicated that there were two subtypes of cHCC-CCA: one that was affected by *TP53* mutations in the early stage of the oncogenic process, and another without *TP53* mutations that had various gene mutations accumulated during carcinogenetic progression. Although there have been several reports that cHCC-CCA has a higher frequency of *TP53* mutations than HCC or iCCA, this is the first report to describe *TP53* mutations in relation to tumor size. Hepatocellular carcinoma with *TP53* mutations has been reported to be associated with hepatitis virus infection, high tumor marker levels, high proliferative potential, and a poor prognosis.[43,44] In many reports, cHCC-CCA was found to have a poorer prognosis than HCC. Taken together, these findings suggest that *TP53* mutations might be related to a poor prognosis in both cHCC-CCA and HCC.

The HCC and CCA components of cHCC-CCA reportedly share a common mutation in many studies.[22,24,25] This implies that most cHCC-CCA tumors are derived from a single clone or a small number of clones. In the present study, we compared mutations between primary and recurrent cHCC-CCA nodules and found that they shared common mutations in many genetic loci. If cHCC-CCA were a tumor composed of multiple clones, it would be highly unlikely that the recurrent nodule would have the same mutation as the primary nodule. These results suggest that cHCC-CCA has clonality, which is consistent with the findings of previous reports.[22,24,25]

Notably, in cases of recurrence as HCCs, two recurrence patterns were noted: a multicentric recurrence pattern, as in case 16, and an intrahepatic metastatic pattern, as in case 4, which might have occurred as a result of metastasis of only the HCC component of cHCC-CCA. However, the condition of the preserved specimens in this study inhibited the extraction of genomic DNA from each component of a single tumor nodule. If cHCC-CCA is indeed derived from a single clone, more detailed studies will be needed to determine the mechanism underlying intratumor phenotypic variation.

Several limitations associated with the present study warrant mention. It was a single-center analysis, and a whole-genome analysis was not carried out. In addition, statistical analyses were insufficient due to the small sample size. We were also unable to identify any gene mutation that might be therapeutic target for cHCC-CCA.

CONCLUSIONS

We undertook a whole-exome analysis for cHCC-CCA. The findings suggested that there were two subtypes of cHCC-CCA: one involving *TP53* mutations on the early stage of the carcinogenic process, and the other with various gene mutations that accumulated during carcinogenic progression. A comparison of variants between primary and recurrent tumors suggested that cHCC-CCA was derived from a single clone.

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CONFLICT OF INTEREST STATEMENT

The authors declare that they have no competing interests.

DATA AVAILABILITY STATEMENT

The sequence data have been deposited in the Japanese Genotype–Phenotype Archive (https://trace.ddbj.nig.ac.jp/jga), which is hosted by the DDBJ, under accession number JGAS000599.

ETHICS STATEMENTS

Approval of the research protocol by an institutional review board: The study protocol was approved by the Ethics Committee of the Graduate School of Medicine, Kyoto University (R1737-2), and performed in accordance with the 1964 Declaration of Helsinki and its later amendments.

Informed consent: The approval for this retrospective study was obtained on an opt-out method.

Registry and registration no. of the study/trial: N/A.

Animal studies: N/A.

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