Direct molecular evidence for both multicentric and monoclonal carcinogenesis followed by transdifferentiation from hepatocellular carcinoma to cholangiocarcinoma in a case of metachronous liver cancer

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Abstract. Frequent recurrence is a major issue in liver cancer and histological heterogeneity frequently occurs in this cancer type. However, it has remained elusive whether such cancers are multicentric or monoclonal. To elucidate the clonal evolution of hepatocellular carcinoma (HCC) recurrence and combined hepatocellular-cholangiocarcinoma (cHCC-CCA) development, the somatic mutation frequency and signatures in a patient with triple occurrence of liver cancer every three years were examined, with samples designated as #1HCC, #2HCC and #3cHCC-CCA, respectively. A total of four tumor regions, including HCC (#3HCC) and intrahepatic CCA (#3iCCA) components of #3cHCC-CCA, and three nontumor regions (#1N, #2N and #3N) were precisely dissected from formalin-fixed paraffin-embedded tissues of each surgical specimen. DNA was extracted and subjected to tumor-specific somatic mutation determination. Of note, five nonsynonymous single-nucleotide variants (SNVs), namely those of KMT2D, TP53, DNMT3A, PKHD1 and TLR4, were identified in #3cHCC-CCA. All five SNVs were detected in both #3HCC and #3iCCA and #2HCC but not in #1HCC. The telomerase reverse transcriptase (TERT) promoter mutation C228T, but not C250T, was observed in all tumors. Digital PCR of C228T also indicated the presence of the TERT promoter mutation C228T in nontumorous liver tissues (#1N, #2N and #3N) at a frequency of 0.11-0.83% compared with normal liver and blood samples. These results suggest the following phylogenetic evolution of three metachronous liver cancers: #1HCC was not related to #2HCC, #3HCC and #3iCCA; both #3HCC and #3iCCA arose from #2HCC. From the above, three novel findings were deduced: i) Both multicentric occurrence and intrahepatic metastasis may be involved in liver cancer in a three-year interval; ii) transdifferentiation from HCC to iCCA is a possible pathogenic mechanism of cHCC-CCA; and iii) a nontumorous, noncirrhotic liver may contain a preneoplastic region with a cancer driver mutation in the TERT promoter.

Introduction

Hepatocellular carcinoma (HCC) is one of the most common types of primary malignant tumor. HCC usually develops in the background of chronic liver diseases such as chronic hepatitis or liver cirrhosis caused by hepatitis B virus (HBV) or hepatitis C virus (HCV) infection, alcohol intake or metabolic syndrome (1). The challenging problem of HCC is its easy recurrence after curative resection; indeed, the recurrence rate within 5 years is 70% (2). There are two different mechanisms of HCC recurrence: Intrahepatic metastasis (IM) of primary HCC and multicentric carcinogenesis (MC) of HCC independent of primary HCC. Several discriminating
factors of IM and MC have been identified and reviewed (3,4). IM is characterized by early recurrence of HCC (e.g., within 2 years), pathological similarity and a more advanced grade of HCC. MC is characterized by late recurring HCC, different pathological features and relatively early-stage HCC. However, despite the clinical importance of discrimination in treatment and prognosis, it is difficult to precisely discriminate these two types of recurrence. Recently, molecular omics approaches, such as genomics, transcriptomics, proteomics and metabolomics, have been used to discriminate between MC and IM (3,5).

A similar challenging issue regarding carcinogenesis exists in combined hepatocellular-cholangiocarcinoma (cHCC-CCA). cHCC-CCA is a primary liver carcinoma with unequivocal features of both hepatic and cholangiocytic differentiation within the same tumor based on morphology revealed by hematoxylin and eosin staining (6). There are three possible models for the origin and evolution of HCC and intrahepatic CCA (iCCA) components in cHCC-CCA: i) Hepatocytes and cholangiocytes synchronously transform to HCC and iCCA, respectively, even in the same nodule; ii) HCC develops first and then partial HCC transdifferentiates into iCCA in the same nodule (6-8); iii) hepatic stem or progenitor cells (HPCs) expand to progenitor-like tumors and then synchronously differentiate into HCC and iCCA in the same nodule. HPC directly develops into cHCC-CCA via a single-cell origin (6,8,9). The first model involves a different origin of carcinoma, whereas the second and third models involve the same origin. Molecular analysis, such as genomics and transcriptomics, is useful for clarifying the evolution of cHCC-CCA (10,11).

To clarify the cancer origin and evolution of the two issues described above (i.e., the mechanism of HCC recurrence and the origin of cHCC-CCA), it is necessary to precisely separate cancer samples to be used for mutational profiling, including mutation frequency determination. In the present study, a case of triple occurrence of liver cancer with a three-year interval was examined; the first and second liver cancers were diagnosed as HCC; the third was diagnosed as cHCC-CCA. To precisely separate cancer samples, laser capture microdissection (LCM) of sample tissues was performed and they were histopathologically diagnosed. To obtain precise mutational profiles, next-generation sequencing (NGS) of 409 cancer-associated genes was performed and the mutation frequency was then quantified by allele-specific quantitative PCR (qPCR) and digital PCR (dPCR). The two aspects of the phylogenetic tree of liver cancer components were analyzed as follows: i) Whether the three metachronous liver cancers were of the same origin; and ii) whether the two synchronous components of cHCC-CCA, i.e., HCC and iCCA, were of the same origin.

Materials and methods

Liver tissue samples and DNA extraction. The patient was a 71-year-old male with a primary HCC (#1HCC) in 2007 who subsequently developed two recurrent liver cancers every three years; the second was HCC (#2HCC) and the third was cHCC-CCA (#3cHCC-CCA). Noncancerous regions of #1N (2 cm away from the tumor), #2N (1 cm from the tumor) and #3N (2 cm from the tumor) exhibited chronic hepatitis with a fibrosis score of F2. The patient was consistently positive for anti-HCV and hepatitis B surface (HBs) antibodies and negative for serum HBV DNA (Table SI) and received curative resection as a standard clinical treatment after each diagnosis. The patient had no history of alcohol abuse and did not receive any antiviral therapy for HCV infection. Formalin-fixed, paraffin-embedded (FFPE) liver tissues collected during surgery for pathological diagnosis were sliced into thin sections of 10 µm in thickness. LCM was performed with a PALM-MBIII-N (Carl Zeiss AG) to obtain #3HCC and #3iCCA lesions (Fig. 1). Macrodissection was performed to obtain #1HCC, #2HCC and #3cHCC-CCA lesions (Fig. 1). Nontumorous tissue sections (#1N, #2N and #3N) were also obtained from the terminal FFPE tissues of resected specimens. DNA was extracted from the FFPE samples using the RecoverAll Total Nucleic Acid Isolation Kit for FFPE (Thermo Fisher Scientific, Inc.) with certain modifications as described previously (12,13). DNA was also extracted from fresh-frozen liver specimens, nontumorous liver of a patient with liver metastasis of colon cancer, to use as controls for TaqMan quantitative PCR (qPCR) (12,13). The present study was approved by the Ethics Committee of Nihon University School of Medicine (approval no. 237-1). Informed consent was obtained from the patients prior to the start of the study.

TaqMan qPCR. Quality assessment of FFPE DNA was performed by TaqMan qPCR of glyceraldehyde-3-phosphate dehydrogenase (GAPDH) and the ratio of FFPE DNA quality to frozen tissue DNA was calculated by the Δ quantification cycle (ΔCq) method, as described previously (12,13); PCR was performed in a 10-µl reaction mixture containing Premix Ex Taq (Probe qPCR; Takara Bio, Inc.) with an initial denaturation step at 95°C for 20 sec, followed by 45 cycles at 95°C for 1 sec and 60°C for 20 sec using StepOnePlus (Thermo Fisher Scientific, Inc.). HBV-DNA was also quantified using a TaqMan Gene Expression Assay (Thermo Fisher Scientific, Inc.) and QuantStudio 3 System (Thermo Fisher Scientific, Inc.). Assay IDs for GAPDH and HBV DNA are presented in Table SII. All assays were performed in duplicate.

Comprehensive Cancer Panel (CCP) amplicon sequencing. DNA samples from #3cHCC-CCA and #3N were subjected to amplicon sequencing using Ion AmpliSeq Comprehensive Cancer Panel (Thermo Fisher Scientific, Inc.), as described previously (13). Candidates for cancer (#3cHCC-CCA)-specific single-nucleotide variants (SNVs) were identified by Tumor-Normal Pair Analysis version 5.2 of Ion Reporter (https://ionreporter.thermofisher.com/ir/).

SYBR green allele-specific qPCR. Mutation frequency was determined by allele-specific qPCR, as described previously (13); allele-specific qPCR was quantitatively performed using THUNDERBIRD SYBR qPCR Mix (Toyobo Life Science) with the QuantStudio 3 System (Thermo Fisher Scientific, Inc.). PCR was performed in a 10-µl reaction in duplicate by preheating at 95°C for 10 min, followed by 45 cycles of 95°C for 15 sec and 60 to 64°C for 20 sec. Allele-specific primers for wild-type (Wt) and mutant (Mu) sequences and a single opposite-directed primer were designed
as previously described (13) and as presented in Table S11, together with each annealing temperature. The ratio of the Mu allele to the Wt allele was calculated as $2^{-\Delta Cq}$, where $\Delta Cq$ is the result of subtracting the Cq value of the PCR for the Wt from that of the Mu. The Mu allele frequency was calculated as $2^{-\Delta Cq}/(1+2^{-\Delta Cq})$, as described previously (13). The Mu allele frequency was used for cluster analysis of cancers with the agglomerative clustering method and the phylogenetic tree was constructed using the Ward method (14). To determine the Mu cell population, the two-hit theory of the mutation was hypothesized, whereby all Mu cells are heterozygous with the first-hit SNV when the Mu allele frequency is >0.5 and all cells consist of Mu heterozygotes and hemizygotes, which undergo Wt allele loss as the second hit when the Mu allele frequency is <0.5. In the former case, the Mu heterozygote population was calculated by the equation of heterozygote population=$2x$ allele frequency. In the latter case, the populations of Mu heterozygotes and hemizygotes were calculated by the equations of Mu heterozygotes population=1/allele frequency-1 and hemizygote population=2-1/allele frequency, respectively.

**Results**

**Histological findings for three metachronous liver cancers.** In the present case, three metachronous liver cancers developed with intervals of 28 and 41 months. The first and second liver cancers were diagnosed as HCC (#1HCC, #2HCC) and the third as cHCC-CCA (#3cHCC-CCA) (Fig. 1). All of the metachronous liver cancers were of the nodular type (Fig. 1). The

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Histological grade was well-differentiated [Edmondson and Steiner grade I (15)] for #1HCC and moderately differentiated (Edmondson and Steiner grade II) for #2HCC. #3cHCC-CCA mainly consisted of a mixed HCC/iCCA area and partial HCC and iCCA components were separately located in the same tumor nodule (Fig. 1). Immunohistochemistry for CK7 and CA19-9, known as iCCA markers (16), clearly indicated #3HCC and #3iCCA to be regionally separated (Figs. 1 and S1).

To perform a precise molecular analysis of cancer evolution, multiple cancerous and noncancerous regions were obtained by histological examination of FFPE tissues used in the pathological diagnostic division of the hospital, followed by dissection or LCM techniques. DNA was extracted from the dissected tissue specimens of three metachronous liver cancers, including noncancerous tissues: #1N, #1HCC, #2N, #2HCC, #3N and #3cHCC-CCA (whole cancerous region of Table I. Allele frequency of five SNVs in five samples from three metachronous liver cancers.

| SNV/sample | Allele frequency | Mutant cell population |
|------------|------------------|------------------------|
|            | CCP | qPCR | Hetero | Wt-loss |
| KMT2D G>C p.Arg5432Gly | 0.097 (193/1991) | | |
| #1HCC | 0.000±0.000 | 0.00±0.00 | |
| #2HCC | 0.063±0.027 | 0.13±0.05 | |
| #3cHCC-CCA | 0.082±0.006 | 0.16±0.01 | |
| #3HCC | 0.088±0.011 | 0.18±0.02 | |
| #3iCCA | 0.085±0.010 | 0.17±0.02 | |
| TP53 C>G p.Glu204Ter | 0.096 (192/1994) | | |
| #1HCC | -0.009±0.009 | 0.00±0.02 | |
| #2HCC | 0.262±0.043 | 0.52±0.09 | |
| #3cHCC-CCA | 0.191±0.015 | 0.38±0.03 | |
| #3HCC | 0.310±0.017 | 0.62±0.03 | |
| #3iCCA | 0.208±0.010 | 0.42±0.02 | |
| DNMT3A C>G p.Glu426Gln | 0.094 (187/1994) | | |
| #1HCC | 0.000±0.000 | 0.00±0.00 | |
| #2HCC | 0.248±0.034 | 0.50±0.07 | |
| #3cHCC-CCA | 0.206±0.025 | 0.41±0.05 | |
| #3HCC | 0.288±0.012 | 0.58±0.02 | |
| #3iCCA | 0.236±0.032 | 0.47±0.06 | |
| PKHD1 G>C p.Leu2479Val | 0.093 (185/1998) | | |
| #1HCC | 0.000±0.000 | 0.00±0.00 | |
| #2HCC | 0.758±0.133 | 0.36±0.24 | 0.64±0.24 |
| #3cHCC-CCA | 0.335±0.039 | 0.67±0.08 | 0.00 |
| #3HCC | 0.688±0.029 | 0.46±0.06 | 0.54±0.06 |
| #3iCCA | 0.460±0.013 | 0.92±0.03 | 0.00 |
| TLR4 C>T p.His456Tyr | 0.054 (79/1456) | | |
| #1HCC | 0.000±0.000 | 0.00±0.00 | |
| #2HCC | 0.172±0.114 | 0.34±0.23 | |
| #3cHCC-CCA | 0.179±0.037 | 0.36±0.07 | |
| #3HCC | 0.280±0.019 | 0.56±0.04 | |
| #3iCCA | 0.116±0.005 | 0.23±0.01 | |

* Gene symbols, nucleotide changes and corresponding amino acid changes and positions are presented. * DNA samples are from cancer lesions from Fig. 1. * Allele frequency (mutant coverage/total coverage) was determined by amplicon sequencing of the CCP using #3cHCC-CCA DNA. * Allele frequency was determined by SYBR green allele-specific qPCR and is expressed as the mean ± standard deviation. The mutant allele frequency was used for cluster analysis of cancers with the agglomerative clustering and Ward method (Fig. S4). * The mutant cell population was determined assuming that all mutant cells were heterozygotes and is expressed as the mean ± standard deviation. * In cases with an allele frequency >0.5 (only PKHD1 SNV), assuming that the second hit of Wt allele loss occurred in partially mutant cells (Wt-loss), populations of heterozygotes (Mu/Wt) and hemizygotes (Mu/-) were individually determined and presented as the mean ± standard deviation. KMT2D, histone-lysine N-methyltransferase 2D; TP53, tumor protein p53; DNMT3A, DNA (cytosine-5')-methyltransferase 3α; PKHD1, polycystic kidney and hepatic disease 1; TLR4, toll like receptor 4; CCP, comprehensive cancer panel; Wt, wild-type; Mu, mutant; HCC, hepatocellular carcinoma; eHCC-CCA, combined hepatocellular-cholangiocarcinoma; iCCA, intrahepatic cholangiocarcinoma; SNV, single-nucleotide variant.
#3HCC and #3iCCA. The mutant cell populations of variant similar among the three dissected cancer samples, #2HCC, for PKHD1, but the frequency pattern of the five SNVs was SNVs varied from 0.063-0.088 for KMT2D to 0.335-0.758 in #2HCC but not in #1HCC. The allele frequency of the five SNVs were then subjected to Sanger sequencing. An arrow indicates the C228T single nucleotide variant. (B) dPCR of the C228T mutation. The numbers of Mu/WT/both amplifications in each nontumorous DNA sample (#1N, #2N and #3N) were as follows: 19/2429/3, 14/2223/0 and 10/8007/2, respectively. The levels in two negative control DNA samples, liver (nontumorous liver of a patient with metastasis of gallbladder cancer) and blood (healthy donor blood) were 0/9942/0 and 0/9318/0, respectively. TERT, telomerase reverse transcriptase; Wt, wild-type; Mu, mutant/mutation; N, nontumorous liver section; HCC, hepatocellular carcinoma; iCCA, intrahepatic cholangiocarcinoma; dPCR, digital PCR.

**Genetic evolution of liver cancers.** DNA quality from the FFPE samples was determined by GAPDH qPCR to be 0.006 to 0.118 compared with frozen tissue DNA (set as 1). The DNA quality of #1HCC and #2HCC was 0.006 and 0.008, respectively, and was <1/10 of that of #3cHCC‑CCA and #3N (0.118 and 0.059, respectively), which was due to the higher concentration of formalin used for tissue fixation (13). The DNA quality of #1HCC and #2HCC was 0.006 and 0.008, respectively, and was <1/10 of that of #3cHCC‑CCA and #3N (0.118 and 0.059, respectively), which was due to the higher concentration of formalin used for tissue fixation (13). Therefore, #3cHCC‑CCA and #3N were subjected to amplicon sequencing to determine tumor-specific nonsynonymous SNVs of all exons of 409 cancer-associated genes (Table SIII). A total of 173 SNVs were detected by Tumor-Normal Pair Analysis version 5.2 of Ion Reporter. A total of 5 nonsynonymous SNVs [histone-lysine N-methyltransferase 2D (KMT2D), tumor protein p53 (TP53), DNA (cytosine-5')-methyltransferase 3α (DNMT3A), polycystic kidney and hepatic disease 1 (PKHD1) and toll like receptor 4 (TLR4)] were detected in #3HCC-CCA after filtering by the following three factors: i) Allele coverage in the nontumorous sample of 0; ii) total coverage in the tumor sample of >100; and iii) allele frequency in the tumor sample of >0.05 (Table SIV). The allele-specific qPCR results for these SNVs validated the presence of somatic mutations in not only #3cHCC-CCA but also in both #3HCC and #3iCCA (Table I). Furthermore, all five SNVs were detected in #2HCC but not in #1HCC. The allele frequency of the five SNVs varied from 0.063-0.088 for KMT2D to 0.335-0.758 for PKHD1, but the frequency pattern of the five SNVs was similar among the three dissected cancer samples, #2HCC, #3HCC and #3iCCA. The mutant cell populations of variant heterozygotes and hemizygotes were also calculated when the allele frequency was >0.5; the PKHD1 variant frequencies of #2HCC and #3HCC were 0.758 and 0.688, respectively. For HCC cells containing the second hit of wild-type allele loss of the PKHD1 gene, variant frequencies were predicted to be 0.64 and 0.54 in the population, respectively. #3iCCA contained no or a very low number of hemizygous cancer cells. The TERT promoter mutation C228T but not C250T was detected in all of these cancers (#1HCC, #2HCC, #3HCC and #3iCCA). By contrast, no mutations were detected in nontumorous samples (#1N, #2N and #3N) by Sanger sequencing (Fig. 2A). dPCR of the C228T mutation indicated a variable mutation frequency from 0.217 to 0.467 in the individual samples (#1N, #2N and #3N) all contained TERT promoter mutations C228T but not C250T. A total of 173 SNVs were detected by Tumor-Normal Pair Analysis version 5.2 of Ion Reporter. A total of 5 nonsynonymous SNVs [histone-lysine N-methyltransferase 2D (KMT2D), tumor protein p53 (TP53), DNA (cytosine-5')-methyltransferase 3α (DNMT3A), polycystic kidney and hepatic disease 1 (PKHD1) and toll like receptor 4 (TLR4)] were detected in #3HCC-CCA after filtering by the following three factors: i) Allele coverage in the nontumorous sample of 0; ii) total coverage in the tumor sample of >100; and iii) allele frequency in the tumor sample of >0.05 (Table SIV). The allele-specific qPCR results for these SNVs validated the presence of somatic mutations in not only #3cHCC-CCA but also in both #3HCC and #3iCCA (Table I). Furthermore, all five SNVs were detected in #2HCC but not in #1HCC. The allele frequency of the five SNVs varied from 0.063-0.088 for KMT2D to 0.335-0.758 for PKHD1, but the frequency pattern of the five SNVs was similar among the three dissected cancer samples, #2HCC, #3HCC and #3iCCA. The mutant cell populations of variant heterozygotes and hemizygotes were also calculated when the allele frequency was >0.5; the PKHD1 variant frequencies of #2HCC and #3HCC were 0.758 and 0.688, respectively. For HCC cells containing the second hit of wild-type allele loss of the PKHD1 gene, variant frequencies were predicted to be 0.64 and 0.54 in the population, respectively. #3iCCA contained no or a very low number of hemizygous cancer cells.

The TERT promoter mutation C228T but not C250T was detected in all of these cancers (#1HCC, #2HCC, #3HCC and #3iCCA). By contrast, no mutations were detected in nontumorous samples (#1N, #2N and #3N) by Sanger sequencing (Fig. 2A). dPCR of the C228T mutation indicated a variable mutation frequency from 0.217 to 0.467 in the individual cancers: #3iCCA<#3HCC<#1HCC<#2HCC (Fig. 2B). Compared with normal liver and blood, nontumorous regions (#1N, #2N and #3N) all contained TERT promoter mutations in a small number of cells (Figs. 2B and S3).

To summarize, the molecular evolution of three metachronous liver cancers was illustrated, as presented in Figs. 3 and S4, based on the phylogenetic principle that the truncal mutations present in the common ancestor are present in all descendants at higher frequencies. The different allele frequencies of six SNVs, including the TERT promoter mutation, are presented in doughnut charts and used for the cluster analysis (Fig. S4). Mutant cancer cell populations were predicted as indicated in Table I and cancer cell evolution by accumulation of somatic mutations is presented from right to left in ellipses. Bar charts indicate the population of two types of PKD1-mutant cells: Heterozygous with SNV (colored) and hemizygous with wild-type loss as the second hit (blank); the % population of the mutant hemizygote is also presented. There are two possibilities for #3iCCA generation: #3iCCA was directly generated from #2HCC or indirectly generated from #2HCC through #3HCC, as indicated by a narrow arrow in parentheses. SNV, single-nucleotide variant; HCC, hepatocellular carcinoma; iCCA, intrahepatic cholangiocarcinoma; cHCC-CCA, combined hepatocellular-cholangiocarcinoma; iCCA, intrahepatic cholangiocarcinoma; TERT, telomerase reverse transcriptase; KMT2D, histone-lysine N-methyltransferase 2D; TP53, tumor protein p53; DNMT3A, DNA (cytosine-5')-methyltransferase 3α; PKHD1, poly cystic kidney and hepatic disease 1; TLR4, toll like receptor 4.
the following evolutionary cancer history: i) #1HCC was not related to #2HCC and #3cHCC-CCA; ii) #2HCC was generated independently of #1HCC in MC mode; iii) #3cHCC-CCA originated from #2HCC; #2HCC already intrahepatically metastasized prior to curative resection and developed into #3cHCC-CCA in IM mode; iv) #3iCCA was generated directly from #2HCC or indirectly from #3HCC.

Discussion

In the present study, three major findings were obtained from the phylogenetic analysis of three metachronous liver cancers with three-year intervals: i) There were two modes of cancer occurrence, MC and IM, in a single case; ii) it is possible that iCCA is generated from HCC in cHCC-CCA; iii) the TERT promoter mutation C228T was not only a common event in the three cancers but also an early event in the noncancerous chronic hepatitis liver without liver cirrhosis. The mutant cell population was 0.22-1.66% in chronic hepatitis liver cells, which probably existed prior to carcinogenesis. Of note, these results were obtained through the combination of two methods: i) Precise dissection of target tissue samples based on histopathology; and ii) the mutation frequency of multiple gene mutations determined by previously established allele-specific qPCR (13) and dPCR protocols.

There have been several reports regarding how to discriminate between the two different mechanisms of recurrence, IM and MC (3,4); early recurrence is indicative of IM and late recurrence is indicative of MC occurrence. The recurrence-free time is the most differentiating factor between IM and MC, and 18 months is the best cutoff time point (17). Recently, somatic SNV information has provided accurate diagnosis of MC and IM using whole-genome sequencing of multiple liver cancers, including synchronous and metachronous cancers (5). In particular, this comprehensive molecular diagnosis is powerful compared to previous techniques due to its high sensitivity and it is useful for cases with inconsistent clinicopathological diagnoses. It was also demonstrated here that mutational profiling is the most definitive approach for discriminating between IM and MC recurrence. Despite an approximate three-year interval from #1HCC (well-differentiated) to #2HCC (moderate-differentiated) (28-month interval) and from #2HCC to #3cHCC-CCA (41-month interval), the former recurrence was MC and the latter was IM. SNVs specifically detected in only #1HCC are convincing. Unfortunately, #1HCC and #2HCC were not subjected to NGS using a comprehensive cancer panel, but the following information supports a rational explanation for the conclusion in the absence of #1HCC-specific SNVs. Intratumor heterogeneity and branched evolution have been reported and the evolutionary history of cancer metastasis has been established by several articles, including those by Gerlinger et al (18) and Gundem et al (19). Cancer evolutionary genomics using clinical samples from metastatic cancers, including the cancer cell fraction (CCF) plot, indicates that ubiquitous mutations are present in all primary and metastatic (metachronous and synchronous) cancers, constructing a trunk phylogenetic tree. The CCF (the fraction of cancer cells within a sample containing a mutation) plot also indicates that the mutation frequency of truncal mutations is higher than that of branch and leaf mutations. Therefore, if #1HCC had been the origin of #2HCC and #3HCC-CCA, the top 5 SNVs detected in #2HCC and #3cHCC-CCA would have also been detected in #1HCC. The top 5 SNVs were at least ubiquitous mutations in #2HCC and #3HCC-CCA. Thus, truncal mutations were not detected in #1HCC; i.e., #1HCC has no relationship with #2HCC. Furuta et al (5) used whole-genome sequencing analyses to demonstrate similar results for three metachronous HCCs with intervals of 21 and 30 months; the second HCC was MC and the third was IM from the second HCC. Yamamoto et al (20) also performed whole-exome sequencing of 41 multiple HCCs: 18 genomic IMs and 23 genomic MCs. Among 10 clinical MCs with recurrence >2 years after initial resection, 3 were genomic IMs. Thus, the recurrence-free time is ambiguous when attempting to discriminate MC and IM. The present study indicated that patients with MC occurrence have a lower risk of death following surgery than those with IM (4,20). In the present case, the patient died at 6 months after the third operation due to multiple bone metastases and recurrent liver cancer in S4. Thus, the precise discrimination of IM and MC by cancer genomics is valuable for prognosis.

The carcinogenesis of cHCC-CCA is also debated with regard to cancer clonality. Wang et al (11), Joseph et al (21) and Xue et al (22) investigated the sequence-based molecular pathogenesis of cHCC-CCA by separately sequencing two components, HCC and iCCA, of 6, 9, and 41 cases, respectively. All cases carried ubiquitous mutations shared by HCC and iCCA, as well as substantial individual mutations, suggesting the monoclonal origin of cHCC-CCA and intratumor heterogeneity. In particular, Xue et al (22) performed not only genomic but also transcriptomic profiling of multiple cHCC-CCA cases classified into three subtypes: Separate, combined (corresponding to the present case) and mixed. All cases were of monoclonal origin, except for 2 of 6 separate subtype cases. Both HCC and iCCA components of the combined subtype also exhibited a similar global gene expression pattern, and of note, high Nestin expression in both components was indicated to be a biomarker for cHCC-CCA. Thus, the combined type cHCC-CCA is certainly monoclonal, as in the present case. Next, the genesis of monoclonal but heterologous cHCC-CCA may be debated with respect to two mechanisms: Transdifferentiation of HCC or HPC origin (6,8). Regarding the two mechanisms of monoclonal origin, Wang et al (11) suggested the HPC origin theory, whereby two components of cHCC-CCA originate from a common cell with stem cell-like features. On the other hand, Joseph et al (21) demonstrated that the genetics of cHCC-CCA are similar to those of HCC and distinct from iCCA and described a possible mechanism of transdifferentiation of partial HCC to iCCA via interplay between genetic factors and the surrounding tumor microenvironment (23,24). The novel finding reported in the present study based on mutational profiles is a potential mechanism of transdifferentiation of partial HCC to iCCA via interplay between genetic factors and the surrounding tumor microenvironment (23,24). The novel finding reported in the present study based on mutational profiles is a potential mechanism of transdifferentiation of partial HCC to iCCA via interplay between genetic factors and the surrounding tumor microenvironment (23,24). The novel finding reported in the present study based on mutational profiles is a potential mechanism of transdifferentiation of partial HCC to iCCA via interplay between genetic factors and the surrounding tumor microenvironment (23,24). The novel finding reported in the present study based on mutational profiles is a potential mechanism of transdifferentiation of partial HCC to iCCA via interplay between genetic factors and the surrounding tumor microenvironment (23,24). The novel finding reported in the present study based on mutational profiles is a potential mechanism of transdifferentiation of partial HCC to iCCA via interplay between genetic factors and the surrounding tumor microenvironment (23,24). The novel finding reported in the present study based on mutational profiles is a potential mechanism of transdifferentiation of partial HCC to iCCA via interplay between genetic factors and the surrounding tumor microenvironment (23,24).
C228T is the most frequent TERT promoter mutation (25). The frequency of TERT promoter mutations in HCV-positive HCC is high, at 64-72%, compared to that of HBV, at 37-39% (26,27). By contrast, TERT promoter mutations are rare in iCCA (5%) (28). In addition, considering this point of view, the iCCA component of cHCC-CCA in the present study was not similar to iCCA but was similar to HCC. The mutant cell population was predicted from the mutation frequency. The C228T mutant cells comprised 77% in #1HCC and 93% in #2HCC; on the other hand, they were 48% in the #3HCC component and 43% in the #3iCCA component. Most cancer cells in #1HCC and #2HCC were positive for the C228T mutation, but the mutant cell population similarly decreased in both components of #3cHCC-CCA, resulting in similar mutant cell populations with TP53 and DNMT3A mutations. These results suggest that after cancer evolution, #2HCC contained an early HCC clone harboring the C228T mutation but not the other mutations. The early clone was probably not metastatic and was filtered out, resulting in the absence of the early clone in #3cHCC-CCA. Digital PCR of C228T clearly indicated no mutation in the negative control consisting of normal liver and normal blood samples but the presence of mutation signals in nontumorous tissues. This is a novel finding and suggests that small foci with TERT promoter mutations may be generated in nontumor lesions. Carcinogenesis may even be initiated in the chronic hepatitis state without cirrhosis. Recently, Kim et al (29) performed deep sequencing of various tumor-related genes, including the TERT promoter region, in regenerative nodules of liver cirrhosis, with an average sequence depth of 958; low-abundance mutations of TP53 and ARID1A were detected in independent nodules, but no TERT promoter mutation was observed in any of the 205 regenerative nodules from 10 cirrhotic livers examined. Although it is a novel finding that clonal expansion within a cirrhotic regenerative nodule occurs in the absence of TERT promoter mutation, it does not exclude the present results for the presence of TERT promoter mutation with lower frequency, 0.11 to 0.83%, which was determined by dPCR based on 2,247-8,021 amplifications. Thus, it is still possible that the TERT promoter mutation occurs followed by small subclonal expansion during chronic liver inflammation, even without cirrhosis.

Finally, the present study was a retrospective analysis of comprehensive cancer genomics by separate sequencing of each component of liver cancers, including cHCC-CCA, suggesting the usefulness not only for clonal evolution analysis but also for prognosis through the discrimination of IM or MC. In the future, targeted therapy based on cancer driver mutations may be useful.

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Availability of data and materials
All the data analyzed during the present study are included in this published article. The datasets collected using the Ion AmpliSeq Comprehensive Cancer Panel are not publicly available as the patient died and it is therefore impossible to obtain consent. The patient consent for publication obtained contains the genetic analysis results but not NGS data containing the personal information, as NGS technology had not yet been included in the genetic analysis at that time consent was given. However, the dataset used for analysis in the present study is available from the corresponding author upon reasonable request.

Authors' contributions
SO, HY, YH and YN performed the experimental work. SO and MS performed pathological diagnoses of the tissue samples. YM was involved in the surgical treatment and diagnosis. SO, HY, HN and TN performed the molecular genetic studies and analyzed the data. SO drafted the manuscript. ME designed the study and edited the manuscript. MM reviewed the study design, interpreted data and critically revised the manuscript for scientific content. ME and MM confirm the authenticity of all the raw data. All authors read and approved the final manuscript.

Ethics approval and consent to participate
The present study was approved by the Ethics Committee of Nihon University School of Medicine (Tokyo, Japan; approval no. 237-1). Written informed consent for use and analysis of samples was obtained from the patients and a healthy subject including those for control samples prior to the start of the study.

Patient consent for publication
Written informed consent for publication of data was obtained from the patient.

Competing interests
The authors declare that they have no competing interests.

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