Figure S1. Quantification of immunostaining for CK7 and CA19-9 in #3HCC and #3iCCA components. A thin section adjacent to the DNA sampling section was subjected to immunohistochemical analysis to confirm the regions of HCC and iCCA subjected to laser capture microdissection. The positive area was measured in five fields per component. The percentage of positive area was determined and expressed as the mean ± standard deviation. HCC, hepatocellular carcinoma; iCCA, intrahepatic cholangiocarcinoma; CK7, cytokeratin 7; CA19-9, carbohydrate/cancer antigen 19-9.

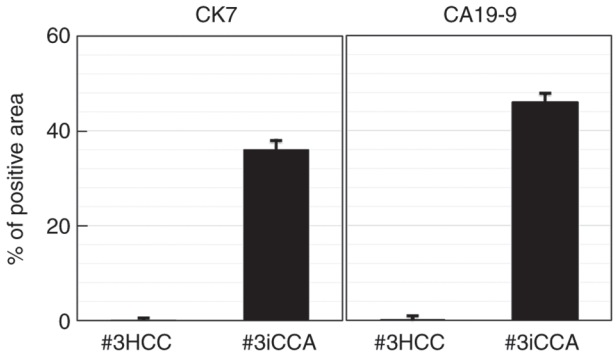


Figure S2. Amplification plot of TaqMan qPCR of HBV DNA in 8 samples. HBV DNA quantification was performed for DNA extracted from tumor (#1HCC, #2HCC and #3cHCC-CCA) and nontumorous (#1N, #2N and #3N) tissues from three cases. Nontumorous liver DNA from two HBV-positive HCC cases was used as a positive control. All assays were performed in duplicate and the results shown are comparable for the two repeats. HBV, hepatitis B virus; HCC, hepatocellular carcinoma; cHCC-CCA, combined hepatocellular-cholangiocarcinoma; N, nontumorous liver section; ΔRn, normalized reporter value of experimental reaction minus that of baseline signal.

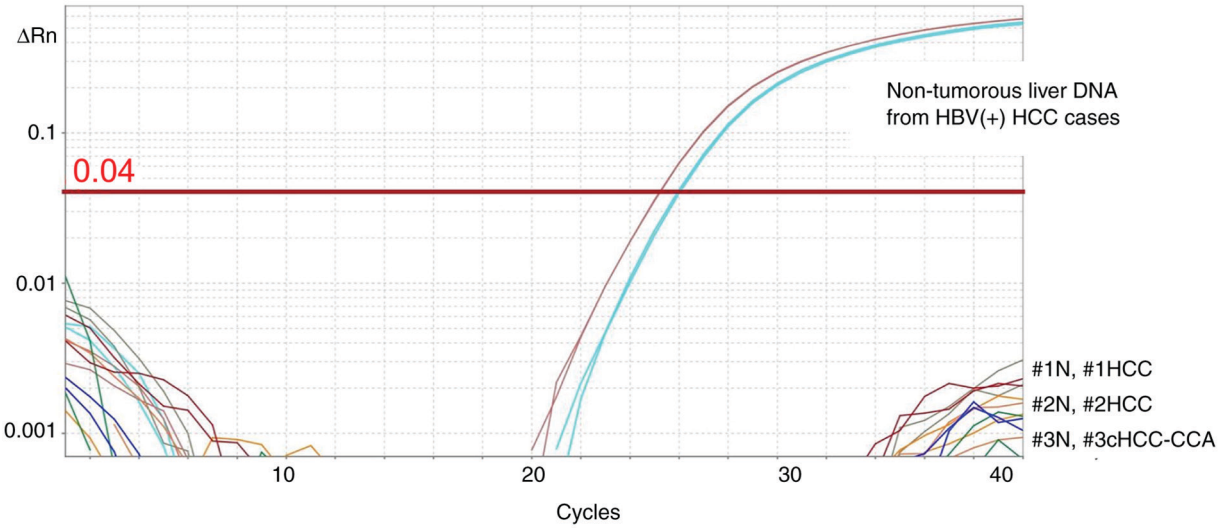


Figure S3. Scatterplot of mutant and wild-type alleles determined by digital PCR of the telomerase reverse transcriptase promoter mutation C228T in nontumorous (#1N, #2N, #3N) tissues and negative control liver and blood samples. FAM, mutant T allele; VIC, wild-type C allele.

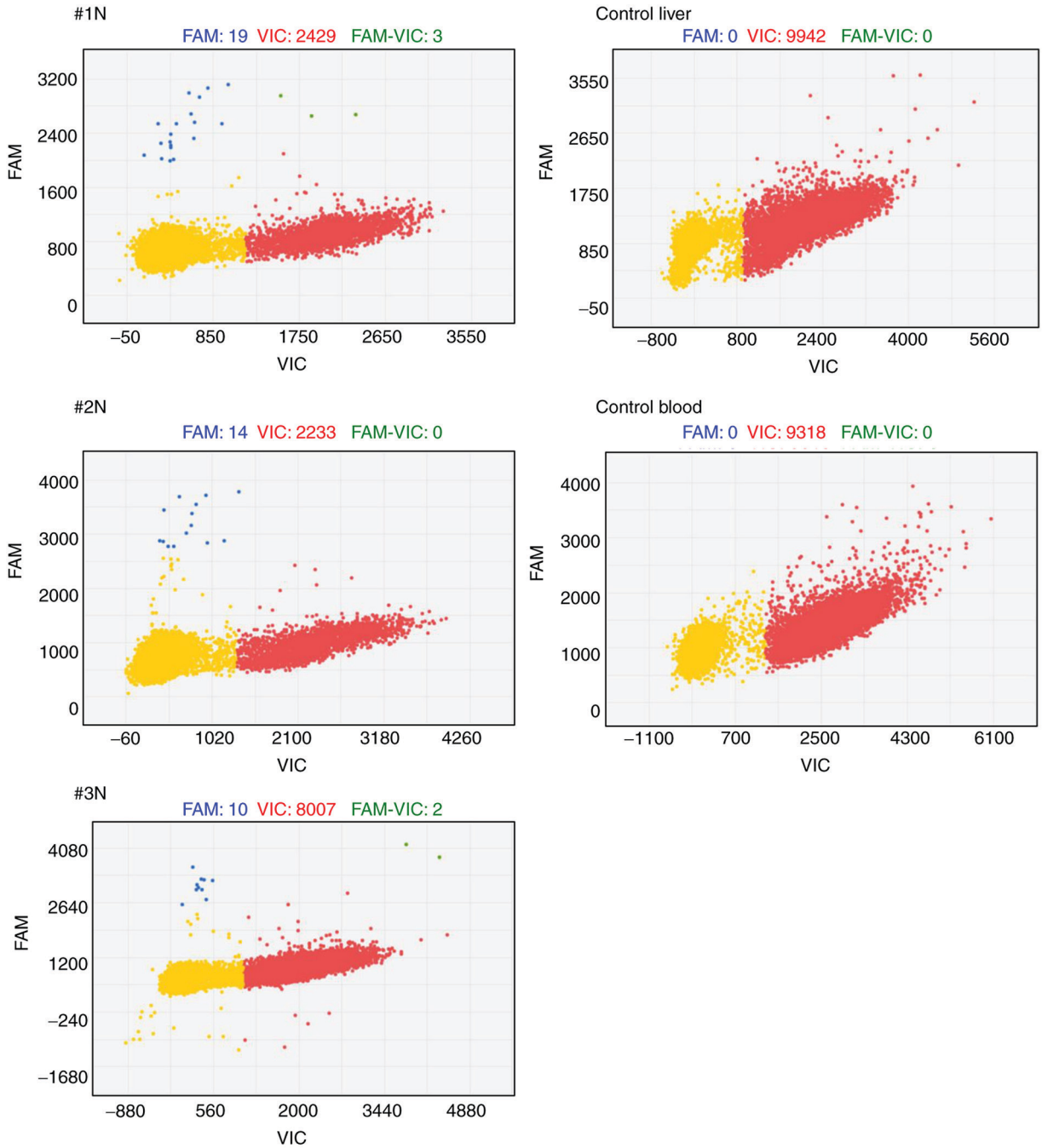
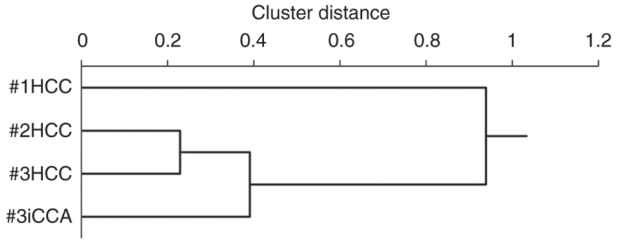


Figure S4. Cluster analysis of four cancers, #1HCC, #2HCC, #3HCC and #3iCCA, based on the allele frequency of six genes. Agglomerative clustering was performed using the allele frequency of five genes in Table I and the telomerase reverse transcriptase promoter mutation frequency in Fig. 2B. The Euclidean distance between two cancers each (lower table) was used for the clustering and the phylogenetic tree was constructed using the Ward method. HCC, hepatocellular carcinoma; iCCA, intrahepatic cholangiocarcinoma.



Euclidean distance

	#1HCC	#2HCC	#3HCC	#3iCCA
#1HCC	0.000	0.866	0.868	0.602
#2HCC		0.000	0.229	0.397
#3HCC			0.000	0.312
#3iCCA				0.000

Table SI. Serum hepatitis virus markers in the patient.

	HBV		HCV	
Liver cancer	HBs Ag, COI (1.0)[a]	HBs Ab, mIU/ml (10)[a]	HBV DNA, log IU/ml (2.1)[a]	HCV Ab, COI (1.0)[a]
#1HCC	0.4	556.1	<2.1	100.0
#2HCC	0.5	646.9	<2.1	84.0
#3cHCC-CCA	0.1	413.3	<2.1	72.3

[a]Cut-off values, assessed with Lumipulse Presto HBsAg-HQ (Fujirebio) for HBs Ag, Lumipulse Presto HBsAb-N (Fujirebio) for HBs Ab, COBAS6800/8800 system HBV (COBAS amplicor-COBAS TaqMan; Roche) for HBV DNA and Lumipulse Presto HCV (Fujirebio) for HCV Ab. HBs, hepatitis B surface antigen; HBV, hepatitis B virus; COI, cut-off index (signal to cut-off); Ab, antibody; HCC, hepatocellular carcinoma; cHCC-CCA, combined hepatocellular-cholangiocarcinoma.

Table SII. Primer sequences and TaqMan probes used in this study.

A, SYBR green allele-specific qPCR

Gene/primer type	Sequence (5'-3')[a]	Annealing temperature (˚C)
KMT2D		62
F-Wt	AGTACATTGGCACCATCATTC	
F-Mu	TACATTGGCACCATCATTG	
R	gactatgcaccacaatggc	
TP53		62
F	gcctctgattcctcactg	
R-Wt	GTGTTTCTGTCATCCAAATACTC	
R-Mu	AGTGTTTCTGTCATCCAAATACTA	
DNMT3A		60
R-Wt	ggtgccctcatttacCTTC	
R-Mu	ggtgccctcatttacCTTG	
F	AGAACAAGCCCATGATTGA	
PKHD1		64
F	GGAAGTCTGTGTATGTCATCTG	
R-Wt	GGCCACACAGTTGATGAG	
R-Mu	GGCCACACAGTTGATGAC	
TLR4		63
F	GGCTTAGAACAACTAGAACATCTG	
R-Wt	AAGCAACTCTGGTGTGAGTATG	
R-Mu	GAAAGCAACTCTGGTGTGAGTATA	

B, Sanger sequencing

Gene/primer type	Sequence (5'-3')[a]	Annealing temperature (˚C)
TERT		68
F1	cgtcctgccccttcacctt	
R1	CCACGTGCGCAGCAGGA	
F2	ccttccagctccgcctcct	
R2	CAGCGCTGCCTGAAACTCG	

C, TaqMan qPCR

Gene/primer type	Sequence (5'-3')[b]	Annealing temperature (˚C)
GAPDH	Hs02758891_g1	60
HBV	Pa03453406_s1	60

[a]Upper and lower cases indicate exon and intron sequences, respectively. [b]Assay ID (Thermo Fisher Scientific, Inc.) is provided for TaqMan qPCR. For SYBR green allele-specific qPCR, two allele-specific primers for Wt and Mu sequences and a single opposite-directed primer were designed as described previously (13), by adjusting their melting temperatures to a similar temperature between 60 and 66˚C, as based on the Nearest Neighbor's method for melting temperature calculation. Primer sequences for TERT promoter amplification were designed using Primer3 (https://bioinfo.ut.ee/primer3-0.4.0/). The specificity of all the primers was checked using Primer-BLAST (https://www.ncbi.nlm.nih.gov/tools/primer-blast/). 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; TERT, telomerase reverse transcriptase; HBV, hepatitis B virus; F, forward primer; R, reverse primer; Wt, wild type; Mu, mutant type.

Table SIII. Mapping of amplicon sequencing of the Comprehensive Cancer Panel.

Sample	Mapped reads	% Reads on target	Average base coverage depth
#3N	2330855	97.4	1384
#3cHCC-CCA	1799353	98.7	1103

cHCC-CCA, combined hepatocellular-cholangiocarcinoma; N, nontumorous liver section.