Table 2 | Candidate driver genes in hepatocellular carcinoma with recurrent genetic alterations

Gene	Frequency (%)	Total number of cases analysed	Number of mutation-positive cases	Genetic alteration	Pathway
TP53	31	2,720	844	Mutation, LOH	TP53
ARID1A	28.2	85	24	Mutation, LOH	Chromatin modifying
CTNNB1	18.8	3,238	609	Mutation	WNT
MTDH	14.7	286*	42	Amplification	Cell adhesion
AXIN1	14.2	466	66	Mutation, LOH	WNT
CDKN2A	11.7	686	80	Mutation, LOH	Cell cycle
ARID2	10.9	202	22	Mutation, LOH	Chromatin modifying
CHD1L	10.7	286*	31	Amplification	Chromatin modifying
BCL9	8.7	286*	25	Amplification	Chromatin modifying
NFE2L2	7.4	162	12	Mutation	Oxidative stress
ATM	6.9	72	5	Mutation, LOH	TP53
PIK3CA	6.3	631	40	Mutation	Growth factor signalling
SMARCA4	6.2	129	8	Mutation, LOH	Chromatin modifying
TSC2	5.2	77	4	Mutation, LOH	Growth factor signalling
CCND1	4.7	286*	14	Amplification	Cell cycle
APC	4.7	107	5	Mutation, LOH	WNT
JAK2	4.7	85	4	Mutation	Growth factor signalling
PTEN	4.4	451	20	Mutation, LOH	Growth factor signalling
BRAF	4.4	360	16	Mutation	Growth factor signalling
FGF19	4.3	286*	13	Amplification	Growth factor signalling
RB1	4.3	94	4	Mutation, LOH	Cell cycle
COL1A1	4.2	71	3	Mutation	Cell adhesion
HNF1A	3.9	233	9	Mutation	Chromatin modifying
KRAS	2.7	672	18	Mutation	Growth factor signalling
NRAS	1.6	426	7	Mutation	Growth factor signalling

*Copy number change. Abbreviation: LOH, loss of heterozygosity.

in HCC and hepatoblastoma.[93–95] Frequent epigenetic inactivation of SFRPs and SOX1, both of which are negative regulators of WNT signalling, has also been detected.[96,97] Alterations in the *CTNNB1, APC* and *AXIN1* genes occur in a mutually exclusive way and activate downstream signals, including transcriptional activation of the *MYC* and *CCND1* genes, which are also amplified in HCC.[98–100] *CTNNB1* mutation is reported to be associated with HCV-related HCC.[28]

Chromatin modifying factors

DNA is tightly associated with proteins, mainly various types of histones, and compactly packed in the nucleus. This DNA-protein complex is called chromatin, and its structure (open or closed) or position is dynamically regulated by histone modifications or ATP-dependent mobilization, which affect gene expression and convey epigenetic information beyond DNA replication. The SWI/SNF (switch/sucrose non-fermentable) protein complex regulates chromatin structure by altering the position of the nucleosome, the basic unit of the DNA-histone complex, and participates in a wide range of biological phenomena, such as differentiation, growth, DNA repair, and reprogramming.[101,102] *ARID1A, ARID1B* and *ARID2* encode core proteins of SWI/SNF complexes and are frequently altered in HCC.[26,31] Alterations of these ARID family members have been reported in other tumour types, including ovarian cancer, renal cell cancer and gastric cancer.[103] In addition, the presence of frame-shift mutations, copy number loss and homozygous deletions observed in *in vitro* studies demonstrated that members of the ARID family function as tumour suppressor genes.

Alterations of other epigenetic regulators have also been reported in HCC. As an epigenetic writer (functioning in histone modification), mutations in the gene encoding histone-lysine N-methyltransferase 2A (*KMT2A*; also known as *MLL*)[104,105] and its family members (*MLL3* and *MLL4*) are frequent.[28] A group of genes encoding epigenetic readers (specifically recognizing histone modification) including *BPTF*[106] and other histone binding proteins (*RNF20* [also known as *BRE1A*] and BRDT) are also altered in certain HCCs.[31] Alterations in these epigenetic regulators account for >50% of HCC cases.[31]

Growth factor signalling pathway

Copy number analyses of HCC identified focal gene amplification of the genes encoding the receptor tyrosine

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kinase MET, FGF19 (which is a ligand for FGFR4), and downstream signalling components (MYC and RPS6KB1). Furthermore, HCC genome sequencing studies have revealed recurrent somatic mutations in genes encoding other kinases (*RPS6KA3* and *JAK1*). Epigenetic silencing of SOCS-1, a negative regulator of the JAK/STAT pathway, occurs frequently in HCC.[107] Compared to other epithelial cancers, such as lung or colorectal cancer, activating mutations in the RAS (*KRAS, NRAS* and *HRAS*) and *PIK3CA* genes are rarely reported in HCC, but occur more frequently in IHCC.[108–110] Activation of other growth factors including TGF-β,[111] IGF[112] and VEGF[113] are also involved in hepatocarcinogenesis. These genomic alterations, especially *JAK1/PIK3CA* mutations,[32] are potential therapeutic targets in liver cancer.

KEAP1–NFE2L2 pathway

The *NFE2L2* gene encodes a sequence-specific transcriptional factor that upregulates genes associated with oxidative stress and other metabolic pathways.[114] The level of the NFE2L2 protein is regulated by the ubiquitin-proteasome pathway, and KEAP1 functions as an E3 ubiquitin ligase. Activating missense mutations in the *NFE2L2* gene,[115] which disrupt direct NFE2L2–KEAP1 interaction, or inactivating mutations of the *KEAP1* gene are recurrently reported in HCC.[26,28] These alterations result in the accumulation of the NFE2L2 protein and promote aberrant activation of downstream genes that confer resistance to oxidative stress and induce metabolic transformation in cancer cells.[114,116]

NOTCH pathway

The role of the NOTCH cascade in solid tumours is controversial. Comparative functional genomics integrating transcriptome data from mice and human HCC samples indicate that NOTCH is activated in this cancer,[117,118] whereas other reports identified activation of NOTCH signalling as a suppressor feedback mechanism during HCC progression.[119,120] These contradictions suggest that biological activities of NOTCH signalling during hepatocarcinogenesis largely depend on the cellular contexts, as reported in other tumour types.[121]

Genomic changes during tumour progression

Midorikawa *et al.*[72] analysed copy number changes during multistep hepatocarcinogenesis and found that 1q21.3–44 gain and loss of heterozygosity on 1p36.21–36.32 and 17p13.1–13.3 were frequently observed in the early stage of HCC, whereas the combination of chromosomal gains on 5q11.1–35.3 and 8q11.1–24.3 and loss of heterozygosity on 4q11–34.3 and 8p11.21–23.3 are late molecular events in advanced HCC.

Roessler *et al.*[20] combined array comparative genomic hybridization and gene expression data in 76 HBV-positive HCCs and attempted to elucidate genomic signatures associated with tumour progression and the prognosis of patients. These authors found a substantial correlation between copy number aberration and gene expression. In particular, a cluster of six genes located on chromosome 8p were deleted in tumours from patients with a poor prognosis; these genes included *PROSC, SH2D4A* and *SORBS3*, which showed tumour suppressive activities, along with *DLEC1* (also known as *DLC1*), a known tumour suppressor gene.

Classification and prognosis prediction

In clinical settings, prognosis assessment and decisions regarding treatment are made on the basis of various tumour staging systems. The Edmondson–Steiner grading system has been applied to assess tumour aggressiveness in HCC, but data supporting its independent prognostic impact are quite weak.[122] Therefore, new approaches and methodologies are needed to develop independent prognostic and predictive tools that might finally assist the clinical decision-making process to further improve curative strategies in HCC.

Genomic profiling, such as gene expression profiling, has been applied to classify HCCs.[123,124] Copy number alterations have also been integrated for classification and therapeutic target identification.[125] In prognosis prediction, the expression pattern from the adjacent non-tumour tissue, which reflects "carcinogenic field effect",[126] was previously reported to correlate with patient survival.[127] A large collection of human HCC samples from patients undergoing curative resection was analysed by microarray profiling. A panel of five genes, including *TAF9, RAMP3, HN1, KRT19* and *RAN*, showed the strongest prognostic relevance and was selected for further analysis.[128] The five-genes score was further validated in an independent, large cohort and also increased its prognostic accuracy when combined with the expression pattern in non-tumour tissues as described above.[127]

Integrative genomic analysis with gene mutation profiles will enable us to elucidate the genetic and epigenetic mechanism of HCC for better classification and to construct a better scoring system for prognosis prediction and treatment selection.

Conclusions

As summarized in this Review, advances in sequencing technologies have enabled the examination of liver cancer genomes at high resolution. In addition to copy number changes and mutations, analyses have identified additional genome alterations, including structural alterations, HBV integration, and retrotransposon changes. Integrated analyses of trans-omics data (genome, transcriptome and methylome data) have identified multiple critical genes and pathways implicated in hepatocarcinogenesis.

These comprehensive genomic analyses have already identified many potential therapeutic targets in liver cancer, including growth factor signalling/kinases (MET, FGF9/FGFR, PIK3CA/AKT/mTOR and JAK/STAT), the NFE2L2-mediated oxidative pathway and chromatin modifying factors. Functional analysis of these targets and the identification of novel potential driver mutations, and the construction of *in vitro* and *in vivo* therapeutic models to evaluate new molecular-targeting compounds are necessary for effective translational research connecting basic molecular science to the clinic.

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The aetiological factors associated with liver cancer (for example hepatitis infection, alcohol and obesity) are well known, and ethnic differences in the prevalence of this disease are prominent. However, the effect of these factors on the accumulation of somatic changes in the liver and the influence of ethnic variation in risk factors on the susceptibility to this tumour remain unknown. In this sense, the international collaboration of cancer genome sequencing projects, such as the International Cancer Genome Consortium (ICGC), will contribute to an improved understanding of this tumour.

Review criteria

We initially selected the articles by searching PubMed, COSMIC and OMIM with the following keywords: "HCC", "sequencing", "exome", "mutation", "CGH", "copy number", "methylation", and "HBV integration". We then searched the reference lists of the identified papers or the above databases by using specific keywords, including "TP53", "CTNNB1" and "RB" among others. Selected papers included mainly full-text papers, and abstracts if we could not access the full text. Only papers published in English were selected, with no publication date restrictions.

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Fibroblast Growth Factor Receptor 2 Tyrosine Kinase Fusions Define a Unique Molecular Subtype of Cholangiocarcinoma

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Cholangiocarcinoma is an intractable cancer, with limited therapeutic options, in which the molecular mechanisms underlying tumor development remain poorly understood. Identification of a novel driver oncogene and applying it to targeted therapies for molecularly defined cancers might lead to improvements in the outcome of patients. We performed massively parallel whole transcriptome sequencing in eight specimens from cholangiocarcinoma patients without *KRAS/BRAF/ROS1* alterations and identified two fusion kinase genes, *FGFR2-AHCYL1* and *FGFR2-BICC1*. In reverse-transcriptase polymerase chain reaction (RT-PCR) screening, the *FGFR2* fusion was detected in nine patients with cholangiocarcinoma (9/102), exclusively in the intrahepatic subtype (9/66, 13.6%), rarely in colorectal (1/149) and hepatocellular carcinoma (1/96), and none in gastric cancer (0/212). The rearrangements were mutually exclusive with *KRAS/BRAF* mutations. Expression of the fusion kinases in NIH3T3 cells activated MAPK and conferred anchorage-independent growth and *in vivo* tumorigenesis of subcutaneous transplanted cells in immune-compromised mice. This transforming ability was attributable to its kinase activity. Treatment with the fibroblast growth factor receptor (FGFR) kinase inhibitors BGJ398 and PD173074 effectively suppressed transformation. *Conclusion: FGFR2* fusions occur in 13.6% of intrahepatic cholangiocarcinoma. The expression pattern of these fusions in association with sensitivity to FGFR inhibitors warrant a new molecular classification of cholangiocarcinoma and suggest a new therapeutic approach to the disease. (HEPATOLOGY 2014;59:1427-1434)

Cholangiocarcinoma (CC) is a highly malignant invasive carcinoma that arises through malignant transformation of cholangiocytes.[1] It is an intractable tumor with poor prognosis, whose incidence and mortality rates are high in East Asia and have been rapidly increasing worldwide.[1,2] CC can be subdivided into intrahepatic (ICC) and extrahepatic (ECC) types, which show distinct etiological and clinical features.[2] ICC is the second most common primary hepatic malignancy after hepatocellular carcinoma, and is associated with hepatitis virus infection. Somatic mutations of *KRAS* and *BRAF* are the most common genetic alterations in CC.[3,4] Surgical resection is the only curative treatment for CC, and no standard chemotherapy regimens have been established for inoperative cases or those showing recurrence after surgical resection.[5,6]

Abbreviations: CC, cholangiocarcinoma; ECC, extrahepatic cholangiocarcinoma; FGFR, fibroblast growth factor receptor; FISH, fluorescent in situ hybridization; ICC, intrahepatic cholangiocarcinoma; TKI, tyrosine kinase inhibitor.

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A better understanding of the molecular basis of cancer would help develop targeted therapeutic agents against druggable genetic aberrations identified in cancer genomes.[7,8] Tyrosine kinase inhibitors (TKIs) that target anaplastic lymphoma kinase (ALK) are particularly effective in the treatment of a distinct subset of lung adenocarcinoma carrying *ALK* fusions.[9] *FIG-ROS1*, the first identified targetable fusion kinase in CC, has so far been reported in two patients.[10] Very recently, a novel kinase fusion, *FGFR2-BICC1*, was detected in two CC cases.[11] Thus, only a few cases harboring targetable fusion kinase genes have been reported, and the clinical characteristics of fusion-positive CC cases have not yet been described.

In the present study, we identified fibroblast growth factor receptor 2 (*FGFR2*) rearrangements including a novel *FGFR2-AHCYL1* fusion using whole transcriptome high-throughput sequencing of tumor specimens, and determined the prevalence of *FGFR2* rearrangements in CC. Our data indicate that *FGFR2*-fusions arise exclusively in ICC. *In vitro* studies suggest that *FGFR2* fusion kinase is a promising candidate for targeted therapy in CC.

Materials and Methods

Clinical Samples. Clinical specimens of cholangiocarcinoma, gastric cancer, hepatocellular carcinoma, and colorectal cancer were provided by the National Cancer Center Biobank, Japan. Total RNA was extracted from grossly dissected, snap-frozen tissue using RNAspin (GE Healthcare, Little Chalfont, UK) according to the manufacturer's instructions, and RNA quality was examined using a Bioanalyzer 2100 (Agilent Technologies, Santa Clara, CA). The study protocol was approved by the Ethics Committee of the National Cancer Center, Tokyo, Japan.

Analysis of Whole Transcriptome Sequence Data. Complementary DNA (cDNA) libraries composed of 150-200 bp inserts were prepared from 2 μg of total RNA using the TruSeq RNA Sample Preparation Kit (Illumina, San Diego, CA). The libraries were subjected to paired-end sequencing of 50-100 bp fragments on the HiSeq2000 instrument (Illumina) according to the manufacturer's instructions. Paired-end reads were mapped to known RNA sequences in the RefSeq, Ensembl, and LincRNA databases using the Bowtie program (v. 0.12.5) as basically described previously.[12] The detailed algorithm for fusion transcript detection is described in the Supporting Methods.

RT-PCR and Quantitative Real-Time PCR. Total RNA was reverse-transcribed to cDNA using SuperScript III (Life Technologies, Carlsbad, CA). The cDNA was subjected to PCR amplification using Ex-Taq (Takara Bio, Tokyo, Japan) with the following primers: FR2AHC-CF (GGACTCGCCAGAGATATCAACAATATAGAC) and FR2AHC-CR (GGACTG TGAGATCGAGCGAGAC) for *FGFR2-AHCYL1* fusion, FR2BIC-CF2 (GTGTTAATGTGGGAGATCT TCACTTTAGG) and FR2BIC-CR2 (CATCCATCTT CAGTGTGACTCGATTG) for *FGFR2-BICC1* fusion, FIG-e2CF1 (ACTGGTCAAAGTGCTGACTCTGGT) and ROS-e36CR2 (CAGCAAGAGACGCAGAGTCAGTTT) for *FIG-ROS1* fusion, ACTB-S (CAAGAGATGGCCACGGCTGCT) and ACTB-A (TCCTTC TGCATCCTGTCGGCA) for *β-actin*. The PCR products were directly sequenced by Sanger sequencing using the BigDye terminator kit (Life Technologies). The expression of the *FGFR2* transcript was assayed by quantitative real-time PCR (qPCR) using the LC480 thermal cycler (Roche, Penzberg, Germany). *FGFR2* expression was normalized to β-actin expression. Primers used for qPCR are as follows: FGFR2 (Fwd-GGACCCAAAATGGGAGTTTC, Rev-ACCACTTG CCCAAAGCAA), β-actin (Fwd-CCAACCGCGAGA AGATGA, Rev-CCAGAGGCGTACAGGGATAG).

Fluorescent* In Situ *Hybridization. To identify *FGFR2* rearrangements, break-apart fluorescent *in situ* hybridization (FISH) was performed on formalin-fixed, paraffin-embedded tumors using BAC clones corresponding to the 5′ (RP11-78A18) and 3′ (RP11-7P17) sequences flanking the *FGFR2* gene and labeled by nick translation in green and red, respectively.

Immunohistochemistry. Four-micrometer-thick sections from formalin-fixed paraffin-embedded block were used for immunohistochemistry. Epitope retrieval was performed with trypsin (T7168, Sigma, St. Louis,

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Additional Supporting Information may be found in the online version of this article.

MO) for 20 minutes at pH 7.7. The slides were then washed with phosphate-buffered saline (PBS) and incubated overnight with FGFR2 antibody at 4°C (1:500, ab10648, Abcam, Cambridge, UK). Immunoreactions were detected using the EnVision-FLEX system (DAKO, Glostrup, Denmark).

cDNA Cloning and Generation of Kinase-Deficient Mutants. The full-length *FGFR2-AHCYL1* and *FGFR2-BICC1* cDNAs were isolated from the corresponding tumor specimens by RT-PCR using PrimeSTAR GXL polymerase (Takara Bio) and primers FGFR2-H5F1 (ATGGTCAGCTGGGGTCGTTTCATCTGCCTGGTCG), AHCYL-H6R1 (GTATCTGTAATAATTAGGTTTGAATGGCCC), and BICC1-H6R1 (CCAGCGGCCACTGACACTAGCAATGTCTGA). *EZR-ROS1* cDNA was reported previously.[13] Each cDNA was subcloned into a pMXs vector (Cell Biolabs, San Diego, CA) to generate recombinant retrovirus expressing the fusion protein with a FLAG epitope tag. The kinase activity-deficient mutants were constructed by replacing tyrosine with phenylalanine at codons 568 and 569 in the *FGFR2-AHCYL1* and *FGFR2-BICC1* genes using a PrimeSTAR site-directed mutagenesis kit (Takara Bio).

Transforming Activity of FGFR2 Fusions. Mouse NIH3T3 fibroblast cells were infected with EZR-ROS1, FGFR2-AHCYL1, FGFR2-AHCYL1-KD, FGFR2-BICC1, or FGFR2-BICC1-KD-expressing retroviruses. Quantification of anchorage-independent growth was performed on day 12 in soft agar with the CytoSelect-96 kit (Cell Biolabs) in the presence or absence of FGFR inhibitors BGJ398 (#S2183, Selleck, Houston, TX) and PD173074 (#S1264, Selleck). The compound solution was added to the top layer of soft agar every 3 days.

Subcutaneous Transplantation in Immune-Compromised Mice. A total of 1×10^6 transduced NIH3T3 cells were injected subcutaneously into nude mice (BALB/c-nu/nu, CLEA Japan, Tokyo, Japan). Tumor formation was measured after 18 days. All animal procedures were performed with the approval of the Animal Ethics Committee of the National Cancer Center, Tokyo, Japan.

Immunoblot Analysis. To analyze signaling, retrovirally transduced NIH3T3 cells were serum-starved for 2 hours, after which vehicle (DMSO), BGJ398, or PD173074 was added for a further 2 hours. The culture medium was then changed to standard medium containing 10% fetal bovine serum (FBS) for 10 minutes. Whole cell lysates were subjected to sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) followed by transfer to a PVDF membrane. Western blot detection was performed with the WesternBreeze Chemiluminescent Immunodetection kit (Life Technologies) using primary antibodies against FLAG tag (#1E6, Wako Chemicals, Tokyo, Japan), phospho-FGFR1-4 (Tyr653, 654) (#AF3285, R&D Systems, Minneapolis, MN), STAT3 (#610189, BD, Becton Drive, NJ), phospho-STAT3 (Tyr705) (#9138, Cell Signaling Technology, Danvers, MA), p44/42 MAPK (#4695, Cell Signaling Technology), and phospho-p44/42 MAPK (Thr202/Tyr204) (#9106, Cell Signaling Technology), AKT1 (#2967, Cell Signaling Technology), and phospho-AKT (Ser473) (#4051, Cell Signaling Technology).

Statistical Analysis. All data analyses were performed using JMP v. 8.02 (SAS Institute, Cary, NC). Fisher's exact test was used for categorical data, and the Student t-test was used for continuous data. Overall survival, measured from the date of surgery, was determined using the Kaplan-Meier method, and survival difference was compared using the log-rank test. Two-sided significance level was set at $P < 0.05$.

Results

Identification of a Novel FGFR2 Fusion Gene. Whole transcriptome high-throughput sequencing of tumor specimens is one of the most effective methods for the identification of fusion oncogenes. Eight primary cholangiocarcinomas without *KRAS/BRAF* mutations or *FIG-ROS1* fusion (Supporting Table 1) were analyzed to identify novel molecular alterations by massively parallel paired-end transcriptome sequencing. Aberrant paired reads that mapped to different transcription units were identified, and 17 potential fusion transcripts were predicted by our algorithm[12] (Supporting Table 2). Sequence reads spanning the junctions of eight fusion candidate transcripts indicated in-frame gene fusion (Fig. 1A-C; Supporting Table 3) and were verified by direct sequencing of RT-PCR products spanning the breakpoints. Among these, fusion transcripts of the receptor kinase gene were detected as *FGFR2-AHCYL1*, *FGFR2-BICC1*, *AHCYL1-FGFR2*, and *BICC1-FGFR2*. However, two transcripts of *AHCYL1-FGFR2* and *BICC1-FGFR2* did not encode a functional protein of relevance to cancer, and conversely *FGFR2-AHCYL1* and *FGFR2-BICC1* were predicted to form chimeric proteins carrying the kinase domain of FGFR2 (Fig. 1D). Transcriptome sequencing showed a specific increase in the expression of the fused 3′ portion of *AHCYL1* and *BICC1* (Supporting Fig. 1A,B). Therefore, the formation of *FGFR2-AHCYL1* or *FGFR2-BICC1* might play important roles in cancer transformation.

From the tumor specimens, CC64 and CC73, we obtained cDNAs corresponding to *FGFR2-AHCYL1*

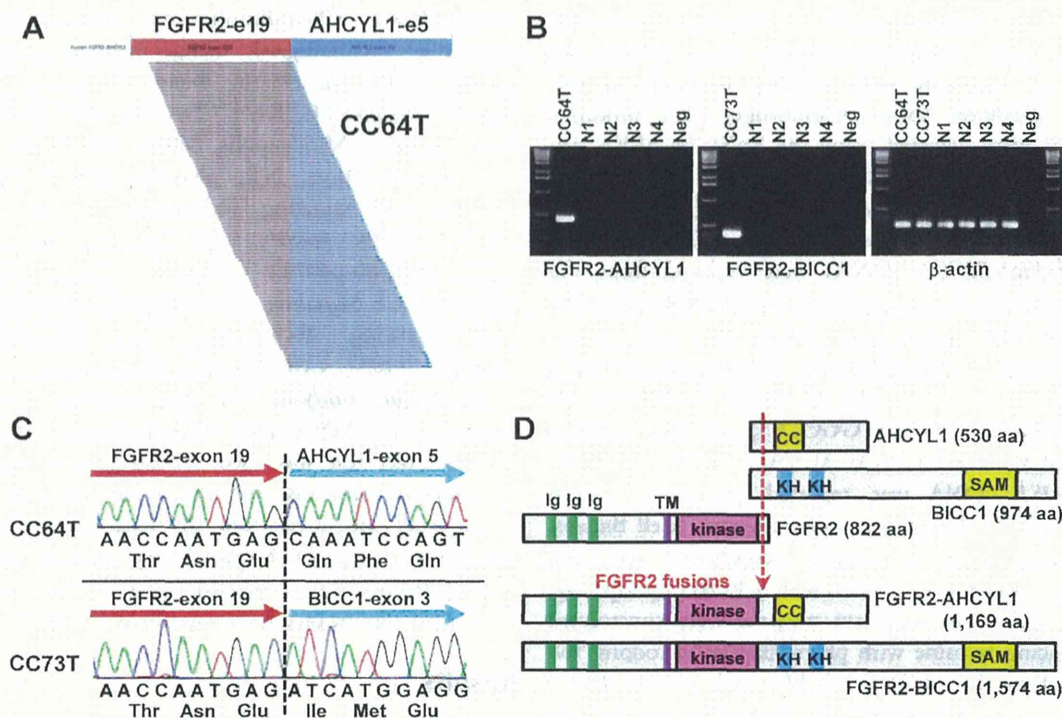

Fig. 1. *FGFR2* fusion genes in cholangiocarcinoma. (A) Junction reads representing *FGFR2-AHCYL1* fusion transcripts in CC64T samples. (B) Confirmation of tumor specific fusion transcripts by RT-PCR. Fusion transcripts were detected only in tumor tissues (CC64T and CC73T), but not in normal liver tissues (N1-N4). Neg: no template. β-Actin expression was used as a control. (C) Sanger sequencing of the RT-PCR product validated in-frame fusion transcripts. (D) Schematic representation of FGFR2-AHCYL1 and FGFR2-BICC1 fusion proteins. Ig: immunoglobulin-like domain, TM: transmembrane domain, kinase: protein tyrosine kinase domain, CC: coiled-coil domain, KH: K homology RNA binding domain, SAM: sterile alpha motif. The dotted vertical line indicates break points.

and *FGFR2-BICC1* encoding 1,169 and 1,574 amino acids, respectively. The chimeric genes consisted of the in-frame fusion of the *FGFR2* amino terminus (exons 1-19) and the *AHCYL1* carboxyl terminus (exons 5-21) or the *BICC1* carboxyl terminus (exons 3-21) (Fig. 1C,D; GenBank/DDBJ accession numbers AB821309 and AB821310). *FGFR2-AHCYL1* is a novel *FGFR2* fusion. *AHCYL1* encodes an S-adenosyl-L-homocysteine hydrolase and inositol 1,4,5-trisphosphate binding protein, and contains a coiled-coil motif in the central domain.[14] *BICC1* encodes an RNA binding protein with a sterile alpha motif (SAM) protein-interaction and dimerization module at the carboxyl terminus.[15] The FGFR2-AHCYL1 and FGFR2-BICC1 fusion proteins are likely to form homodimers through the coiled-coil motif of AHCYL1 and the SAM motif[16] of BICC1, respectively. *FGFR2*, *AHCYL1*, and *BICC1* mapped to chromosome 10q26.1, 1p13.2, and 10q21.1, respectively (Fig. 2A). *FGFR2* and *BICC1* are located on the long arm of chromosome 10 in opposite directions, suggesting that the *FGFR2-BICC1* fusion is generated by intrachromosomal inversion (Supporting Fig. 1B). Gross rearrangement of the *FGFR2* gene locus was verified by FISH with break-apart probes, which showed a split in the signals of the probes flanking the *FGFR2* breakpoint in CC64 and CC73 tumors (Fig. 2B).

Prevalence of FGFR2 Fusions. RT-PCR and Sanger sequencing analysis of 102 cholangiocarcinoma specimens (66 ICCs and 36 ECCs) from Japanese individuals, including eight who had been subjected to whole transcriptome sequencing, identified seven *FGFR2-AHCYL1*-positive and two *FGFR2-BICC1*-positive cases (Table 1; Supporting Table 4). The nine *FGFR2*-fusion-positive cases were ICC type tumors (9/66, 13.6%). *KRAS* mutations were detected in 19 cases (19/102, 17.8%) and *BRAF* mutations in one (1/102, 1%); these mutations were mutually exclusive with the *FGFR2* fusions (Fig. 3A; Supporting Table 4). Although two cases of *FIG-ROS1* fusion (2/23, 8.7%) have been reported by other researchers in cholangiocarcinoma,[10] we did not detect such fusion in this cohort. No significant differences in age, gender, tumor differentiation, clinical stage, and prognosis were detected between fusion-positive and -negative cases. (Table 2, Fig. 3B). Overall survival of ICC cases

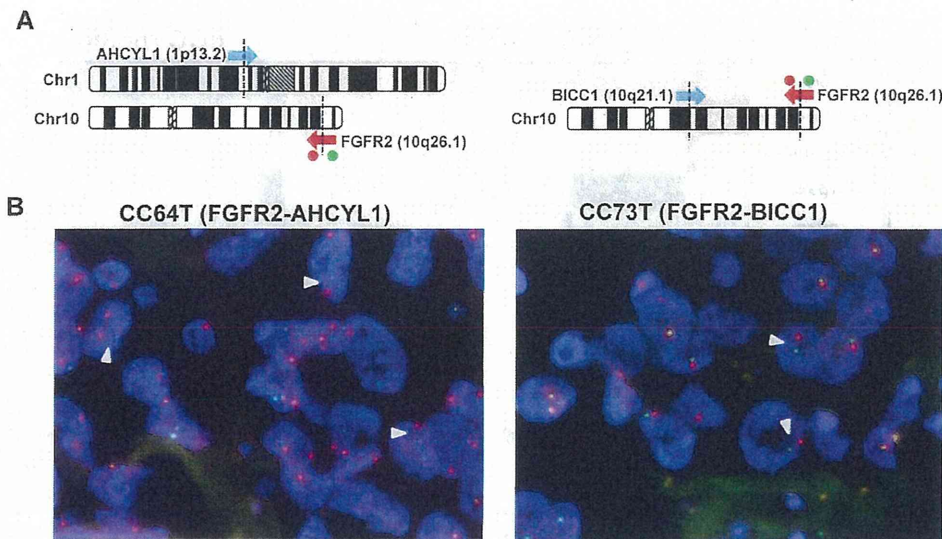

Fig. 2. Detection of *FGFR2* rearrangements. (A) Schematic representation of *FGFR2* gene rearrangements: *FGFR2-AHCYL1* (left) and *FGFR2-BICC1* (right). Arrows indicate the position and direction of the fused genes. Green and red spots indicate the genomic location of 5′ and 3′ FISH probes for the *FGFR2* gene. (B) Representative FISH pattern of *FGFR2* probes in *FGFR2-AHCYL1* and *FGFR2-BICC1*-positive cases. Arrows indicate a split of 5′ green and 3′ red signals.

also showed no great distinction between the two groups. However, fusion positive cases had a propensity for hepatitis virus infection (Table 2). Expression of *FGFR2* mRNA was significantly higher in fusion-positive cases than in fusion-negative ones (Supporting Fig. 2). Especially, *KRAS/BRAF* mutant cases showed reduced *FGFR2* expression. This might afford collateral evidence of mutually exclusive relationships between *FGFR2* fusion and *KRAS/BRAF* mutation. Immunohistological analysis revealed prominent FGFR2 protein expression at both cytoplasm and plasma membrane in fusion-positive cases (Supporting Fig. 3). We further screened 212 gastric cancers, 149 colorectal cancers, and 96 hepatocellular carcinomas by RT-PCR for the presence of these FGFR2 fusion transcripts. The *FGFR2-BICC1* fusion gene was detected in one colorectal cancer (0.7%) and one hepatocellular carcinoma (1.0%). These fusion-positive non-ICC cases were also hepatitis virus-positive (Table 1).

***FGFR2 Fusions Transform NIH3T3 Cells Both* In Vitro *and* In Vivo.** To assess the oncogenic activity of the FGFR2 fusion proteins, stable NIH3T3 clones expressing the retrovirally transfected wild-type fusion proteins or their kinase activity-deficient mutants (KD mutant) were established. As shown in Fig. 4A, wild-type FGFR2-AHCYL1 or FGFR2-BICC1-expressing cells showed anchorage-independent colony formation in soft agar, which was severely suppressed in KD mutant expressing cells. Subcutaneous transplantation of these clones into immunodeficient mice resulted in the formation of tumors from FGFR2-AHCYL1 and FGFR2-BICC1 expressing clones, whereas those expressing KD mutants did not form tumors (Fig. 4B).

To investigate the mechanisms by which the FGFR2 fusion drives oncogenesis, downstream FGFR signaling was analyzed *in vitro* (Fig. 5A; Supporting Fig. 4). The wild-type fusion expressing cells showed constitutive tyrosine phosphorylation in the activation loop of the FGFR kinase domain. FGFR2 signaling activates multiple downstream pathways, including RAS/MAPK and PI3K/AKT.[17] Immunoblot analysis revealed that activation of MAPK, but not AKT or STAT3, was induced in clones expressing FGFR2-AHCYL1 and FGFR2-BHCC1. These results indicate that FGFR2 fusion proteins activate

Table 1. Clinical Features of FGFR2 Fusion Positive Cases

FGFR2 fusion	Gender	Age	Virus status	Pathlogy	Differentiation
FGFR2-AHCYL	F	72	HCV	ICC	mod
FGFR2-AHCYL	F	59		ICC	well
FGFR2-AHCYL	M	62	HCV	ICC	mod
FGFR2-AHCYL	M	73		ICC	well
FGFR2-AHCYL	F	52		ICC	mod
FGFR2-AHCYL	M	59		ICC	well
FGFR2-AHCYL	F	49		ICC	mod
FGFR2-BICC1	M	65	HBV	ICC	mod
FGFR2-BICC1	F	68		ICC	well
FGFR2-BICC1	F	66	HCV	CRC	mod
FGFR2-BICC1	F	46	HBV	HCC	por

ICC: Intrahepatic cholangiocarcinoma
CRC: colorectal cancer
HCC: hepatocellular carcinoma