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Original Article

Molecular Detection of *FGFR2* Rearrangements in Resected Intrahepatic Cholangiocarcinomas: FISH Could Be An Ideal Method in Patients with Histological Small Duct Subtype

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Abstract

Background and Aims: Intrahepatic cholangiocarcinoma (ICC) is a subtype of primary liver cancer for which effective therapeutic agents are lacking. Fibroblast growth factor receptor 2 (*FGFR2*) has become a promising therapeutic target in ICC; however, its incidence and optimum testing method have not been fully assessed. This study investigated the rearrangement of *FGFR2* in intrahepatic cholangiocarcinoma using multiple molecular detection methods. ***Methods:*** The samples and clinical data of 167 patients who underwent surgical resection of intrahepatic cholangiocarcinoma in Zhongshan hospital, Fudan university were collected. The presence of *FGFR2* gene rearrangement was confirmed using fluorescence in situ hybridization (FISH) and targeted next-generation sequencing (NGS). *FGFR2* protein expression was determined using immunohistochemistry (IHC). The concordance between the methods was statistically compared. PD-L1 expression was also assessed in this cohort. The clinicopathological characteristics and genomic profile related to *FGFR2* rearrangements were also analyzed to assist candidate-screening for targeted therapies. ***Results:*** *FGFR2* rearrangement was detected in 21 of the 167 ICC cases (12.5%) using FISH. NGS analysis revealed that *FGFR2* rearrangement was present in 16 of the 20 FISH-positive cases, which was consistent with the FISH results (kappa value=0.696, $p<0.01$). IHC showed that 80 of the 167 cases (48%) were positive for *FGFR2* expression, which was discordant with both FISH and NGS results. By comparison, *FGFR2*-positivity tended to correlate with unique clinicopathological subgroups, featuring early clinical stage, histologically small duct subtype, and reduced mucus production (P<0.05), with improved overall survival ($p<0.05$). *FGFR2*-positivity was not associated with PD-L1 expression in ICCs. In genome research, we identified eight partner genes fused with *FGFR2*, among which *FGFR2-BICC1* was the most common fusion type. *BAP1, CDKN2A,* and *CDKN2B* were the most common concomitant genetic alterations of *FGFR2*, whereas *KRAS* and *IDH1* mutations were mutually exclusive to *FGFR2* rearrangements. ***Conclusions:*** FISH achieved satisfactory concordance with NGS, has potential value for *FGFR2* screening for targeted therapies. *FGFR2* detection should be prioritized for unique clinical subgroups in ICC, which features a histological small duct subtype, early clinical stage, and reduced mucus production.

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Introduction

Intrahepatic cholangiocarcinoma (ICC) is an aggressive malignancy with low incidence and poor prognosis. The 5-year survival for ICC is only 14–40%, whereas the rate of recurrence and metastasis is as high as 60–70%.[1,2] Radical surgical resection remains the sole curative method, which is seldom performed because of the lack of early diagnostic markers. The therapeutic effectiveness of traditional radiotherapy and chemotherapy are unsatisfactory for the treatment of ICCs. The median survival after first-line chemotherapy with gemcitabine plus platinum is less than 12 months in advanced ICC,[3] which presents considerable challenges.

The fibroblast growth factor (*FGF*) family plays an important role in tumorigenesis and comprises 18 secreted signaling proteins. In the human body, extracellular *FGF*s activate four receptor tyrosine kinases (*FGFR 1-4*) and participate in the early stages of embryonic development, in-

Keywords: Intrahepatic cholangiocarcinoma; Fibroblast growth factor receptor 2 (FGFR2); Fluorescence in situ hybridization (FISH); Clinicopathological subgroups; Prognosis.

Abbreviations: *FGFR2*, fibroblast growth factor receptor 2; FISH, fluorescence in situ hybridization; ICC, intrahepatic cholangiocarcinoma; IHC, immunohistochemistry; NGS, next-generation sequencing; PD-L1, anti-programmed cell death-ligand 1; TMAs, tissue microarrays.

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cluding organogenesis, glucose and lipid metabolism, tissue repair, and regeneration.[4] Given its essential role, any abnormality in *FGF-FGFR* signal transduction may cause tumor formation by affecting cell survival, apoptosis, and migration. In ICC, the *FGFR2* fusion protein promotes tumor generation, proliferation and angiogenesis by up-regulating the *RAS, JAK* and *PI3K/mTOR* pathways.[5,6]

Recently, study of molecular targets, such as *FGFR2* rearrangement, *NTRK* fusion, and *IDH1/2* mutations, has emerged as a focus in ICC. Inhibitors of *FGFR2* in particular have facilitated a recent breakthrough in ICC-targeted therapy.[7,8] Based on recent phase II clinical trials on patients with *FGFR2* rearrangement, *FGFR* targeted therapy has an objective remission rate of 14–35% and a disease control rate of 75–83% for advanced ICCs, along with a prolonged progression-free survival of 5.8–6.9 months.[9–11] Therefore, the USA's Food and Drug Administration (FDA) has recently approved pemigatinib, a targeted *FGFR* inhibitor, to treat ICCs with *FGFR2* rearrangement that have failed preceding chemotherapy.[12] Targeted therapy for *FGFR2* rearranged ICCs has shown promise to date, and several phase III clinical trials that are expected to bring radical changes to ICC treatment have been conducted.

In clinical diagnosis, molecular testing methods for *FGFR2* rearrangement are diverse, and include fluorescence in situ hybridization (FISH), next-generation sequencing (NGS), and immunohistochemistry (IHC). Currently, most large-scale clinical trials that target *FGFR2* rearrangements have established DNA-based NGS, which is costly in clinical practice, as one of the inclusion criteria. To date, there is a lack of well-established guidelines for *FGFR2* detection in targeted therapy of ICC. In addition, the incidence and clinicopathological features of ICCs with *FGFR2* rearrangement have not been fully assessed. *FGFR2* molecular detection is expected to provide evidence-based recommendations for better screening of potential candidates. This study investigated the rearrangement of *FGFR2* in ICCs using multiple molecular detection methods. We sought to determine 1) the ideal method for *FGFR2* screening in ICCs in clinical applications and 2) the clinicopathological and molecular features of ICCs with *FGFR2* rearrangements.

Methods

Case selection and sample preparation

In this study, 167 consecutive ICC cases archived at Zhongshan Hospital, Fudan University between January 2014 and December 2017 were retrospectively analyzed. All ICCs were surgically resected with formalin-fixed paraffin-embedded (FFPE) tissues and hematoxylin and eosin (H&E)-stained slides. The inclusion and exclusion criteria were pathologically confirmed ICC after surgical resection, and no evidence of preoperative distant metastasis, and having received no anti-cancer treatment. Tissue microarrays (TMAs) were constructed from all FFPE specimens using a TMA instrument (Bonan Co. Ltd., Shanghai, China). According to the H&E slides marked by a pathologist, two 0.2 mm cores from tumor areas and one from the normal liver were punched out of each FFPE tissue and arrayed into recipient TMA blocks. FISH and IHC analyses were performed for all 167 cases using TMA blocks. Four unstained FFPE tumor sections (4 μm thick) and paired normal liver sections were prepared for targeted DNA sequencing. All study procedures were approved by the Zhongshan Hospital Research Ethics Committee (B2020-194) and performed in accordance with the Declaration of Helsinki. Written informed consent was obtained from each patient.

Pathology and clinical data

Clinical data, including age, sex, symptoms of jaundice, serum hepatitis B surface antibody positivity, liver cirrhosis, primary biliary cholangitis, primary sclerosing cholangitis, hepatolithiasis, liver fluke infection, and presurgery serum CA199, AFP, and CEA levels, were retrieved from the hospital medical records. Pathological data, including gross features, tumor grade, histological subtype, microvascular invasion, perineural invasion, perihilar invasion, portal tract invasion, liver capsule invasion, mucus production, and major necrosis, were interpreted carefully by two pathologists (YJ and YZ) according to the literature.[13] The histological subtype was determined according to the 2019 World Health Organization diagnostic criteria for ICC classification.[14] Pathological tumor-node-metastasis stage (pTNM) was determined according to the eighth edition of the American Joint Committee on Cancer/Union for ICC.[15]

FISH analysis

FISH for *FGFR2* rearrangement was performed on the 4 μm thick TMA sections using a commercially available probe kit in accordance with the manufacturer's recommendations (10q26 gene break-apart probe set, Linked Biotech Pathology Co. LTD, Guangzhou, China). In brief, xylene was used to deparaffinize the sections. Sections were rehydrated in a 100%, 85%, 70% alcohol gradient and then boiled for 20 m and air dried, followed by digestion in a proteinase K solution (0.05 mg/mL, pH 7.0). After that, the *FGFR2* testing probe was added, and hybridization was carried out at 80°C for 5 m and 37°C overnight. Before hydration and air-drying, sections were immersed in 2× saline sodium citrate (2 × SSC) buffer for 5 m and washed in NP40/2×SSC for 7 m. Sections were counterstained with 4′,6-diamidino-2-phenylindole, DAPI. The dual-labeled probe hybridized with the neighboring 5′-telomere (STSG-72444, labeled with spectrum red) and 3′-centromere (SHGC-85446, labeled with spectrum green) *FGFR2c* sequences.

FISH slides were interpreted by two pathologists using a fluorescence microscope (Olympus BX43, Olympus Optical Co. LTD, Tokyo, Japan). Owing to the colocalization of the green and red spectra, the unsplit 5′/3′ spectrum was shown in yellow. We defined the 5′ and 3′ probe signals located at a distance greater than 1× the single signal size as split positive signals. Thus, the rearrangement-positive cell contained any split signals above (YGR type) and an isolated red signal (YR type). Cells with an isolated green signal (YG type), were considered negative, as that denoted deletion of the 5′ sequence of the *FGFR2* gene.[16] Using those criteria, at least 100 non-overlapping nuclei of tumor cells were examined for each case, and the percentage of rearrangement-positive cells was calculated. Based on the results of a previous study,[5] a 15% positive cell ratio was adopted as the cutoff value for FISH analysis.

IHC analysis

IHC staining was performed on 4 μm thick TMA sections using an Ascend Aliya autostainer (Ascend Microsystems, Guangzhou, China). A commercially available rabbit monoclonal FGFR2 antibody (#23328, clone D4L2V, 1:500; Cell Signaling Technology, Danvers, MA, USA) was used for the FGFR2 IHC staining. FGFR2 protein expression was evaluated by two pathologists blindly and separately. According to the manufacturer's protocol, cytoplasmic and/or membrane staining was considered positive and nuclear staining was considered negative. In accordance with the IHC grading in a previous study,[17] IHC-FGFR2 staining was classified as 0

(negative staining), 1+ (poor to moderate staining), and 2+ (strong staining). Cases with 1-2+ staining were defined as IHC-FGFR2 positive, and as negative otherwise.

An anti-programmed cell death-ligand 1 (PD-L1) 22C3 antibody (M3653; Dako, Glostrup, Denmark) was used for the PD-L1 IHC test. Two standardized counting methods, the tumor proportion score (TPS) and combined positive score (CPS),[18] were adopted for PD-L1 interpretation. The optimal cutoff values of PD-L1 positivity, estimated using X-tile software,[19] were 5 for CPS and 5% for TPS interpretation.

Targeted DNA sequencing

Genomic DNA was extracted from three or four 4 μm FFPE tumor tissue slides and paired peritumoral normal liver tissue. FFPE samples containing at least 20% tumor cells were used for NGS detection. At least 50 ng of cancer tissue DNA was extracted from each 40 mm^3 FFPE tumor sample. Targeted DNA sequencing was performed at OrigiMed Co. Ltd. (Shanghai, China). SU-450 panel and Illumina platforms were used for sequencing. Hybridization capture was used in the NGS platform. The detection targets included all known and unknown *FGFR2* rearrangements, and 450 cancer-related genes. Gene variants, such as single nucleotide variation (SNV), long indels, and substitutions, were detected with an effective sequencing depth of 800–1,000×. Genomic DNA was isolated by using QIAamp DNA FFPE Tissue Kit and QIAamp DNA Blood Midi Kit (Qiagen, Hilden, Germany) following the manufacturer's instructions. The concentration of DNA was measured by Qubit and normalized to 20–50 ng/μL. All the sequencing data were obtained by using Illumina NextSeq 500 (Illumina, Inc., San Diego, CA, USA) in the laboratory certified by College of American Pathologists (CAP) and Clinical Laboratory Improvement Amendments (CLIA).

Statistical analysis

The chi-square and Fisher's exact tests were used to analyze the association between *FGFR2* rearrangement and clinicopathological features. Univariate survival analysis for ICC with rearranged *FGFR2* was performed using the Kaplan-Meier curve and log-rank test. The McNemar paired chi-square and kappa consistency tests were used to compare the concordance of results detected using IHC, FISH, and NGS. Statistical significance was set at $p<0.05$.

Results

Baseline characteristics of 167 ICC patients are shown in Table 1.

FGFR2 gene rearrangement detected by FISH

Of the 167 cases, 21 were deemed *FGFR2* rearrangement-positive (FISH-*FGFR2* positive). The vast majority of FISH patterns showed typical positive signals, as previously defined. More specifically, 18/21 exhibited the YGR pattern (Fig. 1A, B), whereas the other two had the YR pattern (Fig. 1C, D). The remaining one case, was atypical, with separate red and green signals as described, accompanied by clusters of green signals (Fig. 1E, F). The percentage of positive cells in FISH-*FGFR2* positive cases ranged from 17–80%, with an average of 53%. FISH-*FGFR2* negative cases had background cell signal ratios that ranged from 0–14%, with an average of 7.8%. Among the 21 FISH-*FGFR2* positive cases, men had a slight advantage over women (12 vs. 9). Histologically, the small duct subtype accounted for the majority of cases (18/21, 86%; Table 2).

Table 1. Patient and tumor characteristics of the cohort

Characteristics	Frequencies, *n*=167
Sex	
Male	104 (62)
Female	63 (38)
Age in years	
>65	56 (34)
≤65	111 (66)
pTNM stage	
I	49 (29)
II	52 (31)
III	47 (28)
IV	19 (11)
Cirrhosis	
Yes	16 (10)
No	151 (90)
PBC	
Yes	4 (2)
No	163 (98)
PSC	
Yes	3 (2)
No	164 (98)
Hepatolithiasis	
Yes	8 (5)
No	159 (95)
Serum HBsAb positivity	
Positive	63 (38)
Negative	104 (62)
Tumor grade	
Poorly differentiated	56 (34)
Moderate/well differentiated	111 (66)
Histological subtype	
Large duct	55 (33)
Small duct	93 (56)
Unclassified	19 (11)
Median overall survival in months (range)	34 (0–67)

Data are presented as *n* (%) unless indicated otherwise. HBsAb, hepatitis B surface antibody; PBC, primary biliary cholangitis; PSC, primary sclerosing cholangitis; pTNM stage, pathological tumor-node-metastasis stage.

FGFR2 gene rearrangement detected by targeted NGS

Targeted NGS was successfully performed on all 20 FISH-*FGFR2* positive cases and eight matching FISH-*FGFR2*-negative cases. One FISH-positive sample (case 21) failed the NGS test because of DNA extraction. DNA sequencing was used to target 450 cancer-related genes, and identified 191 somatic mutations, including 102 single nucleotide variants, 59 copy number variations, 26 fusions/rearrangements, and

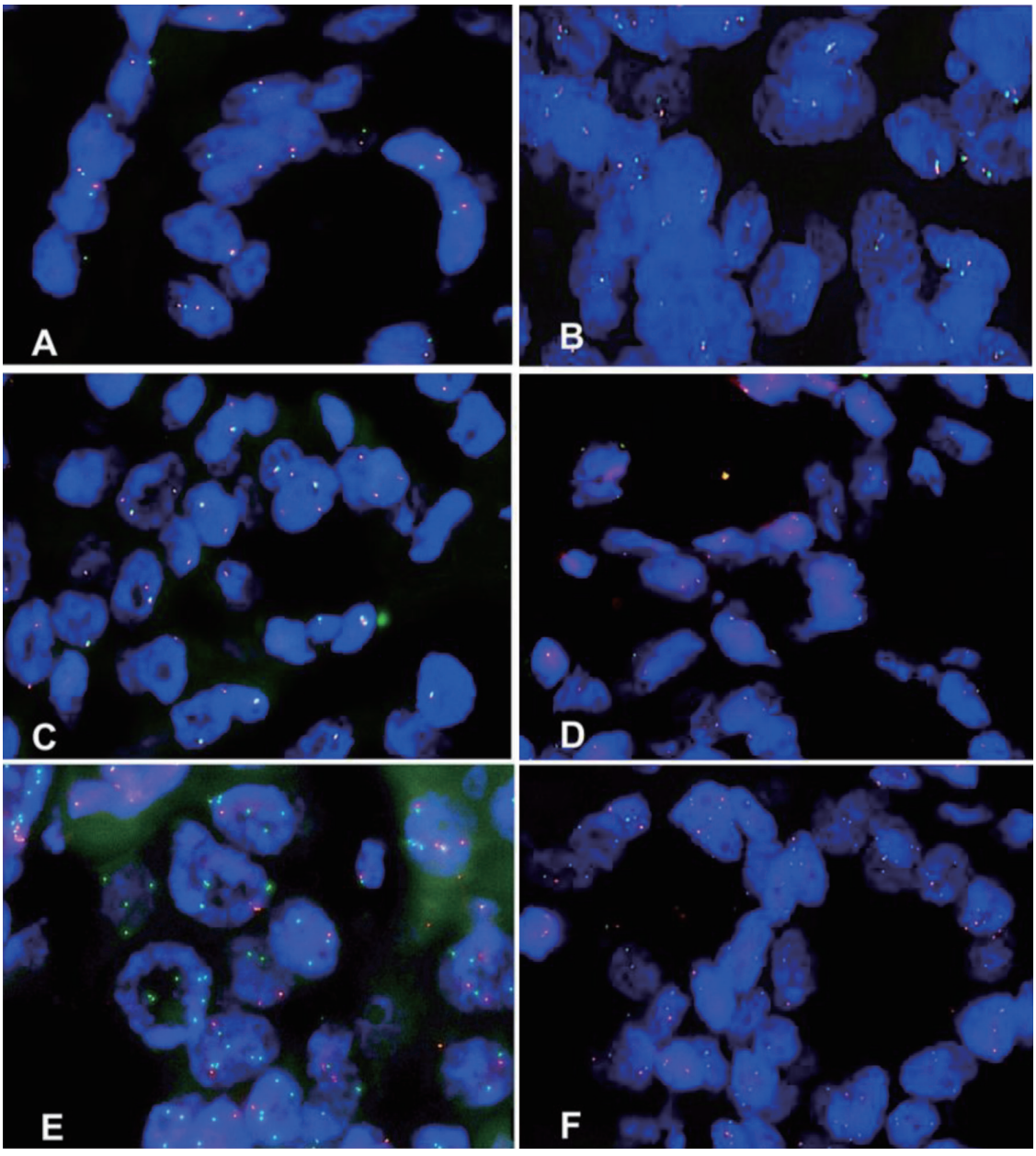

Fig. 1. Schematic representation of *FGFR2* rearrangement-positive in ICCs, by *FGFR2* dual–colored break-apart FISH probe. (A, B) Typical split signals with any 5′-red and 3′-green probe located at a distance greater than 1 time the single signal size (YGR pattern); (C, D) Isolated red signal besides unsplit yellow signal (YR pattern); (E, F) Atypical FISH pattern with a pair of separated red and green signals, accompanied by clusters of 3′-green signals. *FGFR2*, fibroblast growth factor receptor 2; FISH, fluorescence in situ hybridization; ICC, intrahepatic cholangiocarcinoma.

four long indels (Fig. 2). The average number of somatic mutations in each case was 3.8 (range, 0–45), and the average mutation Mb was 2.23 (range: 0–7.3). G>A, G>T, A>G, and C>T transitions were primarily detected in abnormal *FGFR2* gene samples. The mutation spectrum of the 20 most common genes in ICCs is shown in Figure 3. Mutations of the *BAP1* gene were the most common, second only to *FGFR2*. *TP53* is the most commonly mutated gene in ICCs with normal *FGFR2* genes.

NGS-*FGFR2* alteration was detected in 18 cases, of which 15 were rearrangements, two were substitutions, and one had both *FGFR2* rearrangements and mutations. Of the 16 *FGFR2* rearranged cases, 11 fused *FGFR2* partner genes were identified, including *GPHN, NRBF2, BICC1, MAPK1IP1L, NOL4, SYT1, AHCYL1, SHTN1, ETV6, INA,* and *SORBS1*. Among those, *FGFR2-BICC1* was the most common fusion type (6/16, 36%; Table 2). *KRAS* and *IDH1* mutations and *FGFR2* rearrangements were mutually exclusive; *BAP1* (28%), *CDKN2A* (28%), and *CDKN2B* (17%) were common concomitant genetic alterations of *FGFR2* (Fig. 3).

FGFR2 protein expression detected by IHC

Cases were divided by IHC into FGFR2-negative (Fig. 4A) and positive groups (+/++; Fig. 4C, D). Of the 167 patients,

Table 2. Cases with *FGFR2* rearrangements by FISH and NGS

Case	Sex	Age	Stage	HBV/ HCV	Cir- rhosis	Histological Subtype	FISH+ cell rate, %	FISH sig- nal type	IHC	NGS		
										Type	Partner	
1	M	61	IV	(+)	N	Large	68	YGR	Weak	Fusion	*FGFR2*	*GPHN*
2	F	67	IIIB	(−)	N	Small	76	YGR	Strong	Fusion	*FGFR2*	*NRBF2*
3	M	30	IB	(+)	N	Small	32	YGR	Strong	Fusion	*FGFR2*	*BICC1*
4	F	66	IA	(+)	Y	Small	60	YGR	Negative	Rearrangement	*MAPK1IP1L*	*FGFR2*
5	F	73	IA	(+)	N	Small	72	YGR	Negative	Fusion+Substitution	*FGFR2*	*BICC1*
6	M	53	IA	(−)	N	Small	47	YGR	Negative	Fusion	*FGFR2*	*NOL4*
7	F	42	IIIB	(−)	N	Small	74	YGR	Nuclear	Rearrangement	*SYT1*	*FGFR2*
8	M	50	II	(−)	N	Unclassified	49	YGR	Weak	Fusion	*FGFR2*	*AHCYL1*
9	F	41	IA	(+)	N	Small	61	YGR	Negative	Fusion	*FGFR2*	*AHCYL1*
10	M	69	IA	(−)	N	Small	71	YGR	Negative	Rearrangement	*FGFR2*	*intergenic*
11	F	52	II	(−)	N	Unclassified	80	YR	Negative	Fusion	*FGFR2*	*SHTN1*
12	M	41	IA	(+)	N	Small	40	YGR	Negative	Fusion	*FGFR2*	*ETV6*
13	F	53	II	(−)	N	Small	60	YGR	Weak	Fusion	*FGFR2*	*INA*
14	M	60	IA	(+)	Y	Small	36	YR	Weak	Rearrangement	*FGFR2*	*SORBS1*
15	F	67	IIIB	(−)	N	Small	56	YGR	Weak	Fusion	*FGFR2*	*BICC1*
16	M	74	II	(−)	N	Small	56	YGR	Weak	Fusion	*FGFR2*	*BICC1*
17	M	57	II	(−)	N	Small	28	YGR	Weak	Substitution	–	–
18	M	53	II	(−)	N	Small	17	YGR	Weak	Not detected	–	–
19	M	62	II	(+)	N	Small	22	YGR	Negative	Not detected	–	–
20	M	60	II	(+)	Y	Small	56	Atypical	Negative	Not detected	–	–
21	F	71	IA	(−)	N	Small	62	YGR	Weak	Not tested	–	–

FISH, fluorescence in situ hybridization; F, female; IHC, immunohistochemistry; M, male; N, no; NGS, next-generation sequencing; Y, yes; YGR, yellow green red pattern; YR, yellow red pattern.

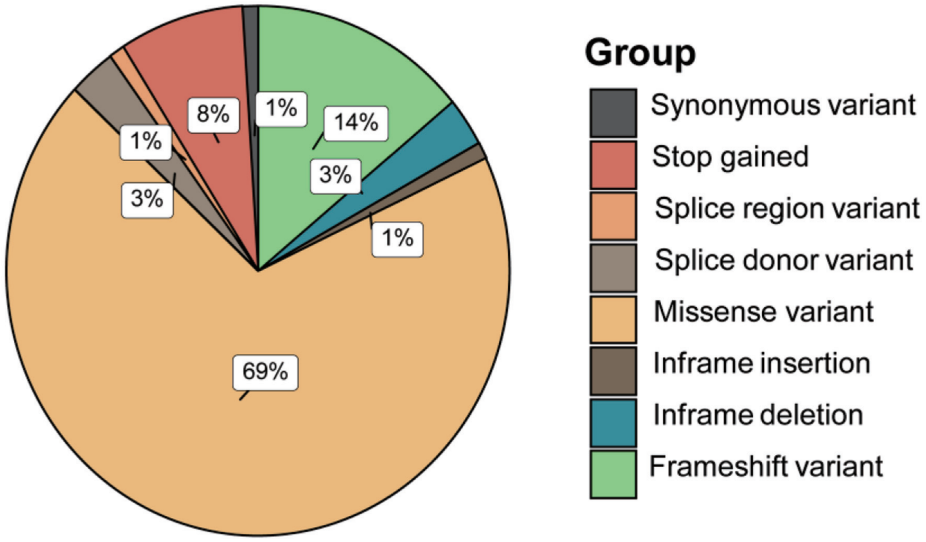

Fig. 2. SNV type proportion of the 28 ICC cases detected by targeted DNA sequencing. DNA sequencing was used to target 450 cancer-related genes and identified 191 somatic mutations, including 102 SNVs, and 4 long indels. ICC, intrahepatic cholangiocarcinoma; SNV, single nucleotide variation.

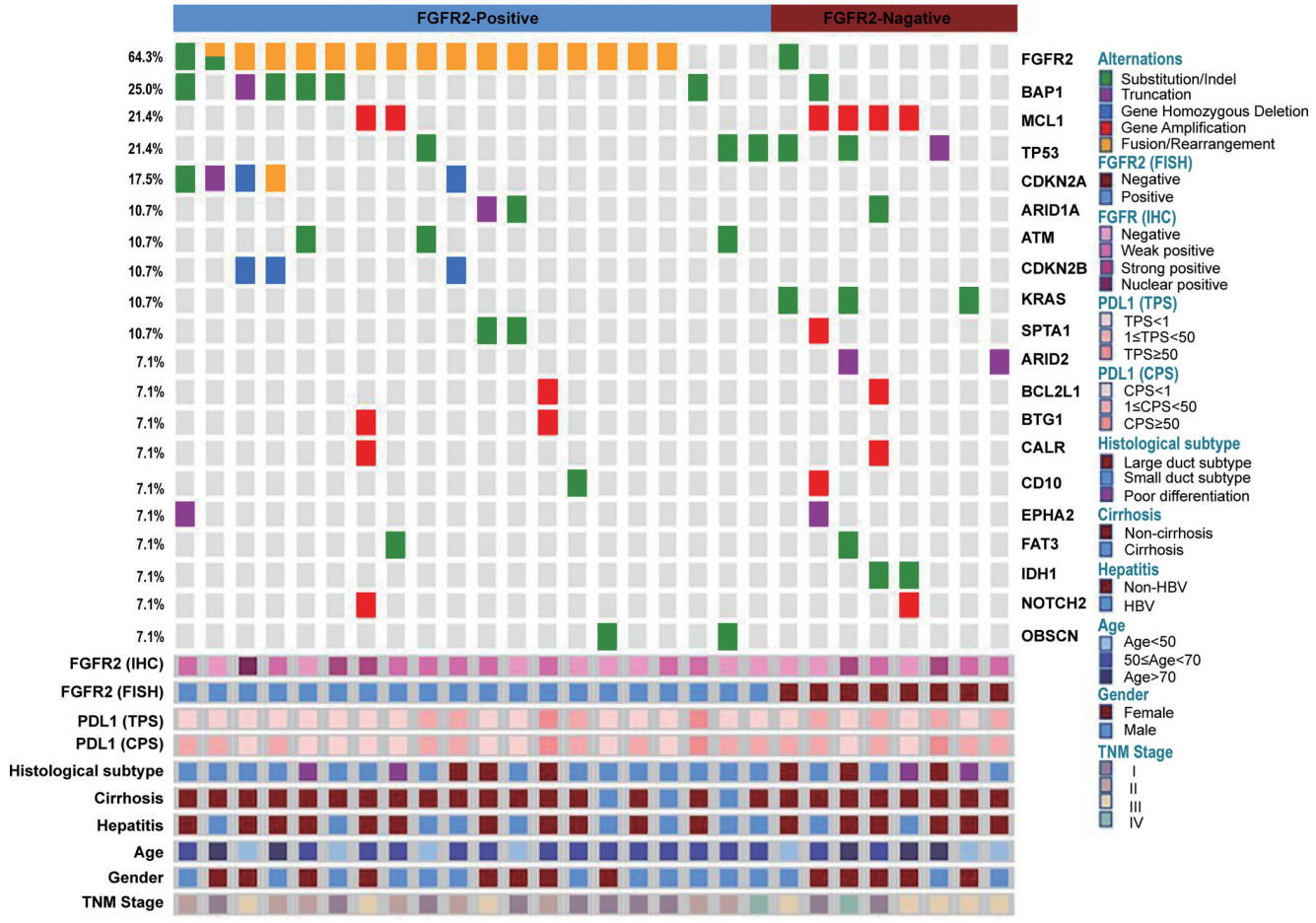

Fig. 3. Genomic profiling of the 28 ICCs detected by NGS and their clinicopathological characteristics. *FGFR2-BICC1* was the most common fusion type. *BAP1, CDKN2A* and *CDKN2B* were the most frequent concomitant genetic alterations of *FGFR2* in ICCs. *KRAS, IDH1* mutations and *FGFR2* rearrangements were mutually exclusive. *BAP1* is the most common mutated gene except *FGFR2*. *TP53* was the most commonly mutated gene among NGS-*FGFR2* negative ICCs. ICC, intrahepatic cholangiocarcinoma; NGS, next-generation sequencing.

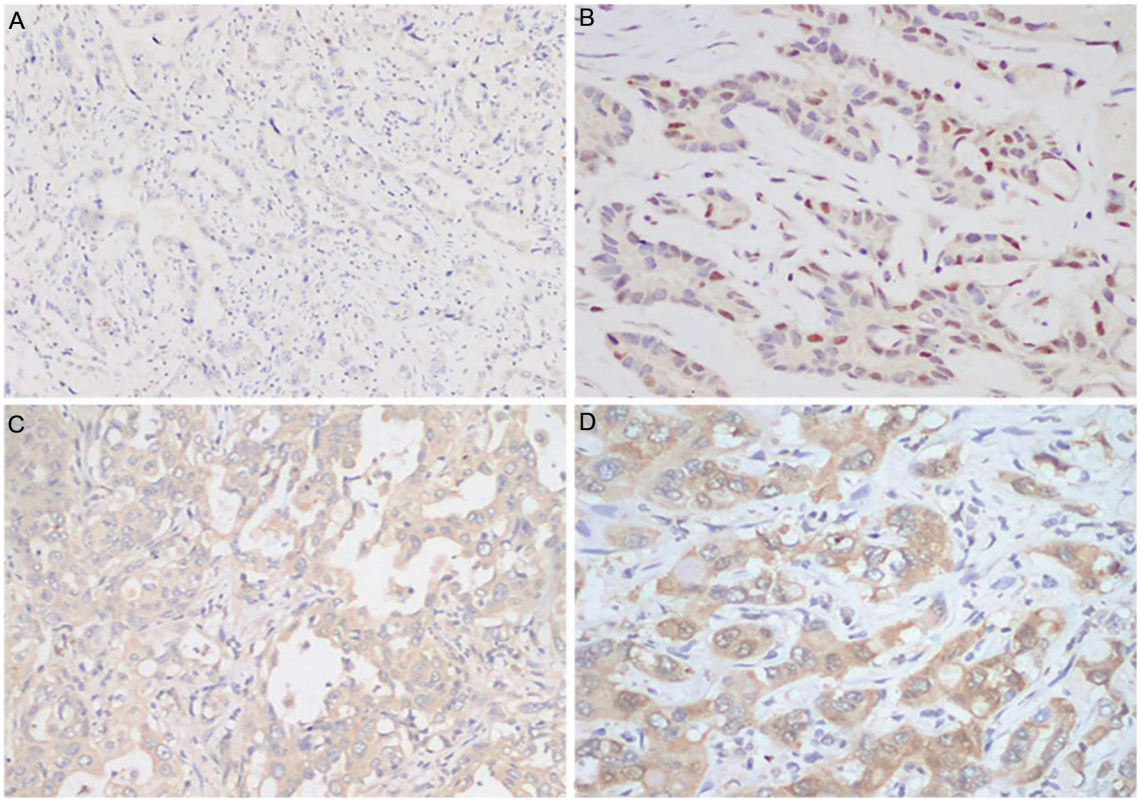

Fig. 4. Representative images of *FGFR2* IHC (200×) positive cases. (A) Negative immunostaining (0) of case 6 with *FGFR2*:*NOL4* fusion; (B) Nuclear immunostaining of case 7 with *SYT*:*FGFR2* rearrangement; (C) Poor to moderate cytoplasmic positive immunostaining(1+) of case 13 with *FGFR2*:*INA* fusion; (D) Strong cytoplasmic positive immunostaining(2+) of case 3 with *FGFR2*:*BICC1* fusion. *FGFR2*, fibroblast growth factor receptor 2; IHC, immunohistochemistry.

80 (48%) were positive for IHC-FGFR2,and 87 (52%) were negative. One case had nuclear staining (Fig. 4B). As stated above, nuclear staining was classified as negative. However, this case with nuclear staining was detected *SYT1*:*FGFR2* rearrangement by NGS, which is worthy of further study.

Concordance of FGFR2 status by FISH, NGS, and IHC

Based on our data, the results of FISH consistence with those obtained by NGS using the consistency test (Kappa value=0.696, $p<0.001$; Table 3). The four cases with discordance in FISH and NGS are shown in Table 2, including case 17 (FISH+/NGS− with substitution), cases 18 and 19 (FISH+/NGS−), and case 20 (FISH+ with atypical pattern/NGS−). However, the IHC results failed the consistency test for both FISH (Kappa value=0.048, $p=0.365$; Table 3) and NGS (Kappa value=0.125, $p=0.508$; Table 3). IHC results were discordant with those of FISH and NGS.

Clinicopathological characteristics and prognosis related to FGFR2 positivity in ICCs

The clinicopathological characteristics of cases with *FGFR2* positivity were evaluated. Histologically, the small duct subtype was significantly related to *FGFR2* positivity ($p=0.003$; Table 4) with markedly reduced mucus secretion ($p=0.025$; Table 4). In addition, *FGFR2* rearrangements were more common in cases of early-stage disease (stage I–II, $p=0.041$; Table 4). Macroscopic mass-forming type, no history of hepatolithiasis or liver fluke, and low preoperative serum AFP level (<20 ng/mL) were found in all 21 cases of *FGFR2* rearrangement, although statistical significance was not achieved when compared with cases of normal *FGFR2*. Possible factors evaluated in other studies, including younger age and serum HBsAb positivity, were unrelated to the *FGFR2* status in our cohort ($p>0.05$; Table 4). Kaplan-Meier survival analysis showed that *FGFR2*-positive cases had better overall survival ($p=0.013$; Fig. 5).

According to the PD-L1 interpretation criteria, 84 cases (50%) were estimated as PD-L1-CPS positive in this study, and 47 cases (28%) were estimated as PD-L1-TPS positive. PD-L1 positivity, interpreted using both TPS and CPS, was not correlated with *FGFR2* rearrangement in our cohort ($p=0.131$ and $p=0.336$, respectively; Table 4).

Discussion

ICC is a rare malignancy of the biliary tract associated with poor prognosis. As mentioned, long-term survival of ICC can be improved by surgical resection or first-line chemotherapy with gemcitabine and cisplatin in rare cases. With the recent emergence of sequencing technology, molecular therapies targeting biliary malignancies have developed significantly.[20] *FGFR2* rearrangement is considered an important oncogenic change in ICC. Therapies targeting the *FGFR2* gene have achieved satisfactory results in several clinical trials and are expected to be applied in clinical practice soon.[21–23]

At present, molecular testing of tumor-related fusions, such as *FGFR2* and *ALK*, are diverse and include targeted DNA sequencing, RNA sequencing, multiplex RT-PCR, FISH, and IHC. Compared to the well-established guidelines for *ALK* detection in targeted therapy of non-small cell lung cancer,[24] *FGFR2* targeted therapy lacks clear evidence-based recom-

Table 3. Consistency test for FISH, NGS, and IHC

Test	FISH	Positive	Negative
	positive	12	68
IHC			
	negative	9	78
	McNemar's chi-squared=43.688, df=1, *p*-value=3.851e-11		
	Kappa value	0.048	*p*=0.365
	FISH	positive	negative
	positive	16	0
NGS			
	negative	4	8
	McNemar's chi-squared=2.25, df=1, *p*-value=0.1336		
	Kappa value	0.696	*p*<0.0001
	IHC	positive	negative
	positive	10	6
NGS			
	negative	6	6
	McNemar's chi-squared=0, df=1, *p*-value=1		
	Kappa value	0.125	*p*=0.508

FISH, fluorescence in situ hybridization; IHC, immunohistochemistry; NGS, next-generation sequencing.

mendations for molecular testing to screen potential candidates. Drawing on experiences using FISH in *ALK* detection, FISH analysis enables visual observation of the cell morphology and direct detection of the *FGFR2* gene break status at the DNA level. This offers high sensitivity and specificity, regardless of the limitation caused by specimen preservation. Although FISH cannot identify specific fusion partners and breakpoints when compared with NGS, FISH is more feasible in large groups owing to short turnaround time and lower cost. Therefore, this study adopted dual-color break-apart probes for FISH-*FGFR2* detection throughout the cohort. DNA-based targeted second-generation sequencing was used to verify the FISH results. Consequently, 12.6% of our ICC cohort was deemed FISH-*FGFR2* positive, which was in accordance with the 9–14% reported previously.[8] Compared with the NGS results, four FISH false-positive cases emerged, which made us focus on FISH interpretation, including both the cutoff percentage of tumor nuclei with *FGFR2* positivity and the discrimination of atypical FISH patterns. Three of the FISH false-positive cases had reduced positive cell ratios of <30%, but the ratios in positive cases confirmed by NGS were >30%. In fact, most were >50%. A similar situation was observed in a recent study by Maruki *et al*.[16] Based on the results of a small preliminary test, Maruki *et al*.[16] defined a cutoff value of 7%, but RNA sequencing indicated that two FISH false-positive cases had reduced FISH ratios that were close to the cutoff value, with other positive cases all having distinct values of >15%. This study found that false positivity in FISH may be derived from setting the cutoff value. Based on our results, the indicated cutoff value was 30%. The remaining false positive FISH case had an atypical FISH pattern that was characterized by a pair of separated red and green signals and accompanied by clusters of 3′-green signals. In contrast to typical break-apart signals (i.e. YGR or YR patterns), the interpretation of atypical FISH signals requires extra clinical attention and practice.[25,26] Eventually, experience with targeted therapy may set the standard for interpreting these atypical signals. Overall, FISH is an ideal method to achieve preferable consistency with NGS when detecting *FGFR2* rearrangements. In the future, we expect more evidence-based clinical trials to establish FISH as a recommendation for *FGFR2* screening. Meanwhile, the results of our study suggest that when the positive cell ratio is <30% or an atypical FISH pattern appears, the ultimate discrimination of the results should be comprehensively considered with multiple methods.

Most large-scale clinical trials that targeted *FGFR2* rearrangements established DNA-based NGS rather than FISH as a standardized screening method.[12] NGS accurately identifies genomic breakpoints and precise fusion partners. *FGFR2* alteration can be classified as a known fusion partner or in-frame with *FGFR2*, and the second with no identifiable partner or a partner out-of-frame with *FGFR2* in the intergenic region.[10] In this study, 14 of 16 NGS-*FGFR2* positive cases had the first type of fusion/rearrangement. One case had the second type mentioned above, and one case had both *FGFR2-BICC1* fusion and a long indel mutation. Current studies indicate that different types of rearrangement partners are expected to benefit comparably from *FGFR2* targeted therapy. Whether there is a difference in therapeutic effect between the two rearrangement types remains to be studied.[10] Cases of simultaneous *FGFR2* fusion and mutation are rarely reported. A case study we performed illustrates the case of a 73-year-old woman with stage IA ICC and a history of hepatitis B but no liver cirrhosis, whose tumor was a histologically small duct subtype. No chemotherapy or targeted therapy was administered after surgery, and no recurrence was found during the 41-month follow-up. The clinical value of this case awaits further study through a long-term follow-up. Approximately 40 fusion partners have been identified.[27] In our current study, we identified 11 fusion partners. *FGFR2-BICC1*, was the most common fusion type in our study,

Table 4. Univariate analysis of *FGFR2*-positive patients determined by FISH (*n*=167)

Parameter	Positive, *n*=21	Negative, *n*=146	*p*-value
Sex			
Female	9	54	0.604
Male	12	92	
Age			
>65	7	49	0.984
≤65	14	97	
Jaundice			
Yes	1	3	0.419
No	20	143	
Serum HBsAb			
Positive	9	54	0.604
Negative	12	92	
Hepatolithiasis			
Yes	0	8	0.598
No	21	138	
Liver fluke infection			
Yes	0	13	0.375
No	21	133	
Cirrhosis			
Yes	2	14	0.992
No	19	132	
PBC			
Yes	1	3	0.419
No	20	143	
PSC			
Yes	0	3	0.507
No	21	143	
pTNM stage			
I–II	17	84	0.041*
III–IV	4	62	
T Stage			
T1–2	20	125	0.315
T3–4	1	21	
N Stage			
N0	18	110	0.411
N1	3	36	
M Stage			
M0	20	128	0.473
M1	1	18	
Serum CA199 in U/mL			
<37	11	70	0.703

(continued)

Table 4. *(continued)*

Parameter	Positive, *n*=21	Negative, *n*=146	*p*-value
≥37	10	76	
Serum AFP in ng/mL			
<20	21	141	0.861
≥20	0	5	
Serum CEA in ng/mL			
<5	17	109	0.531
≥5	4	37	
Gross feature			
Mass forming	21	125	0.349
Periductal infiltrating	0	6	
Mixed	0	15	
Tumor grade			
Poorly differentiated	4	52	0.133
Moderate/well differentiated	17	94	
Histological subtype			
Large duct	1	54	0.003*
Small duct	18	75	
Unclassified	2	17	
Margin positivity			
Positive	0	5	0.901
Negative	21	141	
Major necrosis			
Yes	10	59	0.531
No	11	87	
Microvascular invasion			
Positive	10	47	0.163
Negative	11	99	
Perineural invasion			
Positive	5	49	0.372
Negative	16	97	
Portal tract invasion			
Positive	3	24	0.802
Negative	18	122	
Perihilar invasion			
Positive	1	19	0.474
Negative	20	127	
Liver capsule invasion			
Positive	13	99	0.591
Negative	8	47	
Mucin production			
Yes	2	49	0.025*

(continued)

Table 4. *(continued)*

Parameter	Positive, *n*=21	Negative, *n*=146	*p*-value
No	19	97	
PD-L1TPS			
Positive	3	44	0.131
Negative	18	102	
PD-L1CPS			
Positive	8	76	0.336
Negative	13	70	

*$p<0.05$. AFP, alpha fetoprotein; CA199, Carbohydrate antigen199; CEA, carcinoembryonic antigen; CPS, combined positive score; FISH, fluorescence in situ hybridization; HBsAb, hepatitis B surface antibody; PBC, primary biliary cholangitis; PD-L1, Programmed cell death 1 ligand 1; PSC, primary sclerosing cholangitis; pTNM stage, pathological tumor-node-metastasis stage; TPS, tumor proportional score.

in concordance with the literature. A phase II clinical trial[12] showed that *FGFR2-BICC1* fusion cases exhibited no significant difference in response to *FGFR2* inhibitors compared to other *FGFR2* fusion types to date. *FGFR2* mutations, however, are not considered genomic aberration (GA) candidates for targeted therapy,[12] which were also found in two cases in our cohort. Considering that the diversity of aberration types may lead to different therapeutic effects of molecular inhibitors, we believe that in-depth research on specific GA classifications will be of great value.

FGFR2 aberrations are currently believed to occur mainly in ICC rather than other segments of the biliary tree.[16,28] Several studies have shown that *FGFR2* gene abnormalities are mutually exclusive to *KRAS*, *IDH*, and *BRAF* mutations.[5,29,30] Our results found that one case of *FGFR2* substitution was concomitant with a *KRAS* mutation, whereas no *KRAS* gene abnormality was found in *FGFR2* rearrangement cases. *IDH1* mutation was found in 2 cases, none of which was accompanied by *FGFR2* aberration. In this study, we observed that *BAP1* (28%), CDKN2A (28%), and *CDKN2B* (17%) were common concomitant genetic alterations of *FGFR2*. Among these, *BAP1* is considered the most common concomitant alteration of *FGFR2*, which indicates a favorable prognosis and relatively indolent disease.[27,31] Our *FGFR2*-impaired cases concomitant with *BAP1* mutation had an average survival of 36 months, which was higher than that of patients with normal *BAP1* (32 months). Nevertheless, the concomitant presence of *FGFR2* in ICCs and its prognostic value need to be confirmed further using larger samples.

The IHC results in this present study were inconsistent that of both NGS and FISH. The low expression of FGFR2 fusion protein and deviation caused by tumor heterogeneity on TMA could be reasons for the blame. Therefore, this study suggests that IHC analysis using this antibody is not recommended as an alternative screening method before NGS or FISH.

Clinicopathologically, this study showed that the molecular subtype of *FGFR2* positivity was associated with early clinical stage and predominately histological small-duct subtype with diminished mucus secretion, and is associated with favorable overall survival. The findings are consistent with those in previous reports.[5,32] In addition, *FGFR2* positivity is reportedly correlated to age, serum HBsAb positivity, and ductal growth pattern,[5,13,32–34] were not confirmed in this study. However, the propensity for *FGFR2*-positive cholangiocarcinoma patients with HBV infection was found in a cohort with both

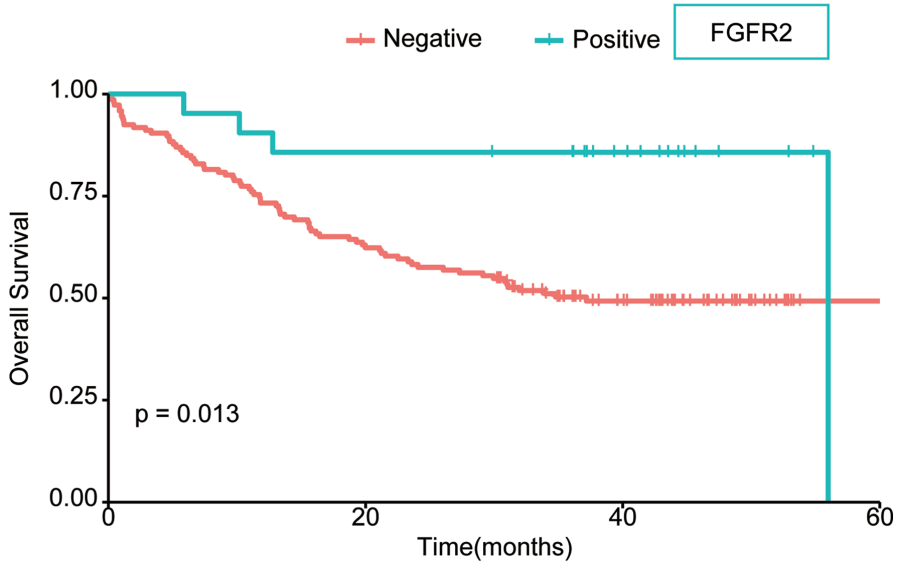

Fig. 5. Survival analysis of FISH-positive ICCs. Comparatively, ICCs with *FGFR2* translocation had improved overall survival (p=0.01). *FGFR2*, fibroblast growth factor receptor 2; FISH, fluorescence in situ hybridization; ICC, intrahepatic cholangiocarcinoma.

intra- and extrahepatic tumors.[5] Our cohort included only ICCs, which may have caused the difference in HBV infection propensity between the studies. Sequential application of *FGFR2* and PD-L1 inhibitors can reportedly enhance the response to immune checkpoint inhibitors in patients with advanced urothelial cancer.[35] However, PD-L1 positivity in ICCs with *FGFR2* rearrangements has rarely been studied. Zhu *et al*.[36] reported that PD-L1 positivity is enriched in ICCs with *FGFR2* rearrangements, in contrast to our findings. Considering that the percentage of *FGFR2* rearrangement in Zhu's study was lower than reported, and their interpretation of PD-L1 positivity differed from ours, the association between PD-L1 expression and *FGFR2* rearrangement in ICCs remains to be studied further. Overall, this study demonstrates that *FGFR2*-positivity is related to the unique clinicopathological features of ICCs. Patients with an early clinical stage, histologically small duct subtype, and diminished mucus secretion should be prioritized for *FGFR2* screening.

Our study has some limitations. First, the sample size was relatively small, and multicenter studies including larger ICC cohorts need to be conducted. Second, none of the ICC cases with *FGFR2* rearrangement in our study received *FGFR* inhibitor treatment. The corresponding response to *FGFR* inhibitors remains the decisive factor when choosing detection methods in targeted therapy, and data on ICCs treated with *FGFR* inhibitors should be collected further.

Conclusions

FISH achieved satisfactory concordance with NGS and has potential value for *FGFR2* detection in targeted therapies. *FGFR2* detection should be prioritized for unique clinical subgroups in ICC, which features a histological small duct subtype, early clinical stage, and reduced mucus production.

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Conflict of interest

The authors have no conflict of interests related to this publication.

Author contributions

Conceived the idea and designed the study (YJ), performed the study and drafted the article (YZ), revised the article (YJ), and conducted the data acquisition, analysis and interpretation (YZ, KZ, YP, JH, XZ, ZJ, YH, WG). All authors discussed the results and agreed to be accountable for all aspects of the work. All authors read and approved the final manuscript.

Ethical statement

The study procedures were approved by the Zhongshan Hospital Research Ethics Committee (B2020-194) and performed in accordance with the Declaration of Helsinki. Written informed consent was obtained from each patient.

Data sharing statement

The original contributions presented in the study are included in the article/Supplementary Material. Further data and calculation tools are available by request via ji.yuan@zs-hospital.sh.cn.

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