Targeted genomic profiling revealed a unique clinical phenotype in intrahepatic cholangiocarcinoma with fibroblast growth factor receptor rearrangement

Zhongzheng Zhu,1, Hui Dong,1, Jianguo Wu,1, Wei Dong,1, Xianling Guo,1, Hua Yu,2, Juemin Fang,2, Song Gao,2, Xuejun Chen,2, Huangbin Lu,3, Wenming Cong,1, Qing Xu,1,2

1 Department of Oncology, Shanghai Tenth People’s Hospital, Tongji University School of Medicine, 301 Middle Yanchang Road, Shanghai 200072, P.R. China
2 Department of Pathology, Eastern Hepatobiliary Surgery Hospital, Second Military Medical University, 225 Changhai Road, Shanghai 200438, P.R. China
3 Amoy Diagnostics Co., Ltd. 39 Dingshan Road, Xiamen 361027, P.R. China

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ABSTRACT
Genomic aberrations (GAs) in fibroblast growth factor receptors (FGFRs) are involved in the pathogenesis of intrahepatic cholangiocarcinoma (ICC), and clinical trials have shown efficacy of FGFR inhibitors in treating ICC patients with FGFR GAs such as FGFR2 rearrangement. To clarify the FGFRs GA profile and corresponding clinicopathological features in Chinese patients with ICC, a total of 257 cases were identified. Fourteen cases (5.45%) were positive for FGFR2 rearrangement. Further analysis on the 110 FGFR2 rearrangement negative cases showed that 13 patients present additional FGFRs GAs, including FGFR3 rearrangement (2.73%), and FGFRs mutations. When compared with patients without FGFRs GAs, those with FGFR2 or FGFR3 rearrangement presented more under the age of 58 years, female sex, HBsAb positivity, CD10 expression, and PD-L1 expression. The clinical characteristics between patients with FGFRs mutation and those without FGFRs GAs were similar, with the exception that cases with FGFRs mutation have more hepatolithiasis. We concluded that FGFR rearrangement is associated with unique clinical phenotypes in ICC.

INTRODUCTION
Intrahepatic cholangiocarcinoma (ICC) is the second most diagnosed hepatobiliary tumor, which is characterized by late diagnosis, extraordinary heterogeneity, limited treatment option and dismal prognosis [1]. Epidemiological studies have shown that the incidence of the deadly tumor has significantly increased in recent years [1,2]. Risk factors attributed to the tumorigenesis of ICC include parasitic infection, viral hepatitis, hepatolithiasis, choledochal cysts, primary sclerosing cholangitis, diabetes, obesity, smoking, alcohol-related disorders, and genetic susceptibility [3,4]. Surgical resection remains the gold standard treatment, however a surgical approach with curative intention may not be feasible in majority of ICC cases as the disease is typically diagnosed at advanced stage. For locally advanced or metastatic disease, the chemotherapy combination of gemcitabine and cisplatin remains the only preferred systemic treatment, with a median survival of less than one year [5,6]. Therefore, there is an urgent need for more treatment modalities for this severe tumor.

Recently, advances in integrated sequencing technology have provided a compendium of ICC genomic aberrations, which creates unprecedented opportunities for precision targeted therapy to the tumor [7]. Genomic aberrations in fibroblast growth factor receptors (FGFRs) are among the most frequent events during ICC development [8]. The FGFRs are part of the larger receptor tyrosine kinases family and contain...
four members: FGFR1, FGFR2, FGFR3, and FGFR4. Upon binding of fibroblast growth factors, the FGFRs undergo receptor dimerization and initiate downstream signaling, which is essential for diverse physiologic processes [9]. The altered FGFRs because of different genetic aberrations (GAs), including chromosomal translocation and activating mutation, have been proved to play a key role in tumor onset and progression in several human malignancies [10]. In ICC, chromosomal translocations involving FGFR2 have been frequently identified, resulting in the creation of oncogenic fusion proteins. The chimeric FGFR2 fusion proteins are assumed to undergo ligand-independent receptor dimerization resulting in a fully activated kinase, leading to activation of various oncogenic downstream pathways such as RAS/MAPK, PI3K/ AKT, and JAK/STAT [11]. As the FGFR2 rearrangement is particularly common in ICC as compared with other cancer types, it has been rapidly translated into a promising therapeutic target in this type of cancer [12, 13]. Several pre-clinical and clinical trials have shown the efficacy of FGFR inhibitors in treating ICC patients with FGFR2 rearrangement, as well as with FGFR3 rearrangement [14–22]. However, FGFRs GA profiling in ICC and corresponding clinico-pathological features remain unclear, which would hinder the optimal therapeutic application of the FGFR inhibitors.

In the present study from a Chinese ICC population, we sought to determine: 1) the FGFR2 rearrangement status in a total of 257 cases; 2) the FGFRs GA profile in 110 FGFR2 rearrangement-absent cases; and 3) the clinical and pathological features in cases with FGFRs GAs.

Materials and methods

Patients and specimens

A total of 257 ICC cases were enrolled in this study. We retrieved archived formalin-fixed, paraffin-embedded (FFPE) diagnostic material from surgical cases of ICC diagnosed at Eastern Hepatobiliary Surgery Hospital, Second Military Medical University, Shanghai, China, between September 2015 and August 2018. None of the enrolled patients received radiation therapy, chemotherapy, or other anticancer therapy before surgery. Glass slides were reviewed by pathologists (Drs. H. Dong and W. Cong) to confirm the pathological diagnosis of adenocarcinoma, determine the tumor grade, and select an appropriate paraffin block for ancillary studies. Cases with insufficient tumor for testing were excluded. Information including age, gender, cigarette smoking, alcohol drinking, hepatolithiasis, serum CA19-9, and radiological and pathological reports, was obtained when available. An ever-smoker was defined as a smoker of at least 1 cigarette/day for 6 months or longer. An ever-drinker was defined as a person who reported drinking alcoholic beverages at least once a week for 6 months or longer. The serologic tests for hepatitis B virus (HBV) and hepatitis C virus (HCV) infection were performed using commercially available products (ELISA Processor III, Behring, Germany). Anti-programmed cell death-ligand 1 (PD-L1) expression was measured by using commercially available products (ELISA Processor III, Behring, Germany). Anti-programmed cell death-ligand 1 (PD-L1) expression was measured by using commercially available products (ELISA Processor III, Behring, Germany). Anti-programmed cell death-ligand 1 (PD-L1) expression was measured by using commercially available products (ELISA Processor III, Behring, Germany).

Library preparation and Next-Generation Sequencing (NGS) assay

DNA and total RNA extraction

DNA and total RNA from FFPE tissue sections were extracted and purified using the MagMAX™ FFPE DNA/RNA Ultra Kit (ThermoFisher, A31881) and processed following the manufacturer’s instructions. The quality of isolated DNA and total RNA was verified using QuantiFluor dsDNA System (Promega, E2760) and QuantiFluor RNA system (Promega, E3310) in Quantus™ Fluorometer E6150. RNA integrity was checked by running on the Agilent Bioanalyzer (RNA Nano Chip).

Fluorescence in Situ Hybridization (FISH)

FGFR2 rearrangement was identified using a break-apart FISH probe kit (5’ flank and 3’ flank of FGFR2 were labeled in green and orange, respectively) from AmoyDx (Amoy Diagnostics, Xiamen, China), and the performance was done according to the manufacturer’s instruction. In briefly, the 4 μm-thick sections cut from FFPE tissue block were deparaffinized in xylene, rehydrated in gradient ethanol (100%, 85%, 70%) to deionized water. Sections were then boiled in the pretreatment solution (pH 7.0) for 20 min, and air-dried. Sections were digested in proteinase K working solution (final concentration was 0.05 mg/mL, pH 7.0) for 6 min, dehydrated in gradient ethanol (70%, 85%, 100%), and air-dried. 10 μL FGFR2 break-apart probe described above was added on each slide. After sealing, slides were put on the hybridizer (Abbott Molecular, Des Plaines, IL), and codenaturation and hybridization were carried out at 85 °C for 5 min and 37 °C for overnight, respectively. After hybridization, slides were immersed in 2 × saline sodium citrate buffer (2 × SSC, pH 7.0) for 5 min, following washed in 0.1% NP40/2 × SSC at 46 °C for 7 min, then dehydrated and air-dried. Finally, DAPI solution was used as a counterstain, and slides were cover slipped.

The ZytoLight® SPEC FGFR2 Dual Color Break Apart Probe from ZytoVision (ZytoVision GmbH, Bremerhaven, Germany) was selected to confirm the result of the AmoyDx’s probe. The probe was performed in a similar way described above according to the manufacturer’s instruction. In particular, the denaturation and hybridization condition were 75 °C for 10 min and 37 °C for overnight, respectively.

Analysis was performed using 100 × 1.4 NA oil objective under Olympus BX53 (Olympus, Tokyo, Japan) microscopy equipped with the appropriate filter sets including DAPI single bandpass, Green single bandpass and Orange single bandpass. For each specimen, 50–100 non-overlapping tumor cells were analyzed, positive was considered if separate green and orange signals or single green signal besides undivided signals had to be present in at least 15% of nuclei throughout the tumor. On the contrary, specimens with qualified FISH signals but not meeting the criteria were considered as negative. The representative images of each specimen were acquired with ProgRes cooled CCD camera (Analytik Jena AG, Jena, Germany) in monochromatic layers that were subsequently merged by the FISH 3.0 software (ImStar Thapeutics, Paris, France).

The NGS assay was performed using a laboratory developed test kit from AmoyDx (Amoy Diagnostics, Xiamen, China), which was designed to sequence the whole coding sequences of FGFR1, FGFR2, FGFR3, and FGFR4. Sequencing data was processed using a customized bioinformatics pipeline designed to detect several classes of genomic alterations, including nucleotide substitution, indel, and genomic rearrangement. The DNA (100 ng) was sheared using a Covaris M220 instrument. The input amount of RNA used for library preparation was 100 ng. The RNA fragmentation time was accounted for the degree of fragmentation determined by the RNA integrity check. After first and second strand synthesis, the dsDNA were mixed with fragmented DNA and purified using AMPure XP Beads (Beckman, A63880). The dsDNA and RNA mix were then repaired to make them blunt and phosphorylated, followed by dA-tailing and adaptor ligation. Sample indexes were added during the PCR...
enrichment step. FGFR capture was conducted by hybridizing the pre-PCR libraries with biotinylated DNA baits at 65 °C for 16–24 h followed by extraction using Dynabeads MyOne™ Streptavidin T1 beads (Thermo Fisher, 65,601). The capture libraries were pooled and sequenced on Illumina Novaseq6000 with PE150 cycles. FASTQ files obtained from different samples were first processed by FormatFastq to complete basic QC and generate high-quality clean data. Valid sequencing data were then mapped to the human genome (UCSC hg19) by Burrows-Wheeler Aligner (BWA) to generate original alignment in BAM format. Then, a custom pipeline was used to do the variant calling and identify single nucleotide variant (SNV), insertion and deletion. For rearrangement detecting analysis, valid sequencing data were then mapped to the human genome (UCSC hg19) by STAR (Spliced Transcripts Alignment to A Reference) to generate original mapping results in BAM format, and Chimeric reads. Then, STAR-Fusion was used to call and filter candidate rearrangements with Chimeric reads. The most arguments in STAR and STAR-Fusion are default, but some arguments are optimized according our pre-experiments. The SNV or indel mutation was filtered out using 1000genome database supporting reads. The fusion was defined positive as ≥5 unique fusion reads. Germline mutation was filtered out using 1000genome database (allele frequency ≥1%) and ExAC and GnomAD database (allele count ≥2), and the remaining mutations except synonymous mutations were exported.

**Statistical analysis**

The associations between the occurrence of FGFRs GAs and clinicopathologic features were assessed utilizing χ² or Fisher exact tests, as appropriate. All reported P values were 2-sided, and P < 0.05 was considered significant. All statistical analyses were performed with Stata 16.0 (Stata, College Station, USA).

**Results**

In total, 257 ICC cases were subjected to FISH screening for FGFR2 rearrangements based on the AmoyDx platform. Fourteen cases were observed positive for the presence of the rearrangement, which accounts for 5.45% of the cohort here analyzed. The FGFR2 rearrangement status of 122 cases (114 negative and 8 positive patients) from our screening cohort was also explored by a second FISH platform (ZytoVision), and 100% concordance result between the two FISH platforms were obtained (Fig. 1).

The demographic and clinical characteristics of the cases harboring FGFR2 rearrangement are summarized in Table 1. The median age of patients with FGFR2 rearrangement was 49 years (range, 39 to 69 years). The number of women with FGFR2 rearrangement (n = 7, 50.0%) was equal to the number of men (n = 7, 50.0%). Most of patients with FGFR2 rearrangement were non-smoker (n = 8, 66.7%) and non-drinker (n = 9, 81.8%). All the FGFR2 rearrangement positive patients had hepatolithiasis. Eleven patients (78.6%) with FGFR2 rearrangement presented a normal serum level of CA19-9 (< 39 U/mL). Patients with FGFR2 rearrangement predominantly had positive HBsAb (n = 9, 69.2%), and smaller numbers of patients had positive HBsAg (n = 3, 23.1%) or negative HBsAb/HBsAg (n = 1, 7.7%). Thirteen patients (92.9%) presented with earlier disease (stage I or II) at the time of diagnosis. The tumor grade of the analyzed specimens was predominantly moderately differentiated (n = 12, 85.7%). By immunohistochemistry, 6 cases (46.1%) with FGFR2-rearrangement showed positive for CD10. Eight cases (57.1%) were positive for PD-L1 expression. Examples of CD10 and PD-L1 immunoactivity are shown in Fig. 2.

Overall, there were no significant differences between patients with and without FGFR2 rearrangement regarding gender, cigarette smoking, alcohol drinking, hepatolithiasis, serum CA19-9, tumor grade, and tumor stage. However, some significant differences were noted. When compared with patients without FGFR2 rearrangement, more FGFR2 rearrangement patients presented before the age of 58 years (P = 0.028), HBsAb positivity (P = 0.008), CD10 expression (P = 0.013), and PD-L1 expression (P = 0.024) (Table 2).

Next, we performed NGS assay on 12 FGFR2 rearrangement positive and 110 negative cases from the cohort, achieving the same result on FGFR2 rearrangement status between NGS and FISH platform. For FGFR2 rearrangement positive cases, no further FGFR1–4 GA was observed. Multiple FGFR2 rearrangement partners were discovered including CUX1 (n = 2), SORB1, SHROOM3, WAC, TNL4A, CBX5, COL16A1, ALAD, POF1B, FILIP1, and POC1B (Fig. 3). For FGFR2-POC1B and FGFR2-SORB1, respectively, 2 rearrangement transcripts were identified in a single case, because of the diversity of genomic breakpoints. To our knowledge, most of the FGFR2 rearrangement partners have not previously been reported in ICC, except for SHROOM3, SORB1, and WAC [22-24]. Among the 110 FGFR2 rearrangement negative cases, 13 (11.8%) patients present FGFRs GAs, including FGFR3 rearrangement (n = 3) (Fig. 3), and mutations at FGFR1 (n = 1), FGFR2 (n = 3), FGFR3 (n = 2), and FGFR4 (n = 4) (Table 3). The FGFR3 rearrangements were MYT1L-FGFR3, FGFR3-TACC3, and FGFR3-MSRB2 (Fig. 3), and only FGFR3-TACC3 has been reported previously in ICC [24] and other cancers [25]. One 72-year-old male had coexisting 2 FGFR2 mutations (Table 3). Rearrangement at FGFR2–3 (FGFRs probe; the dominant positive signal pattern displays as single orange signal besides undivided signals (5′ flank of FGFR2 was labeled in green). F Enlargement of boxed area in panel E. G Tumor positive for FGFR2 rearrangement with ZytoVision FGFR2 Break-apart probe; the dominant positive signal pattern displays as single orange signal besides undivided signals (5′ flank of FGFR2 was labeled in orange). H, Enlargement of boxed area in panel G.

![Fig. 1. Schematic representation of FGFR2 rearrangement in intrahepatic cholangiocarcinoma, by FGFR2 break-apart FISH probe.](image-url)
Table 1
Clinicopathological features of patients with intrahepatic cholangiocarcinoma with FGFR2 rearrangement.

| Sample no. | Gender | Age (years) | HBV status | CA19–9 (U/mL) | Tumor grade | TNM stage | CD10 expression | PD-L1 expression |
|------------|--------|-------------|------------|---------------|-------------|-----------|-----------------|-----------------|
| 1          | Male   | 65          | HBsAg+, HBeAg+, HBcAb+ | 33.6          | Moderately differentiated | I         | Negative         | Negative         |
| 2          | Female | 56          | HBsAg+, HBeAg+, HBcAb+ | 106.3         | Poorly differentiated   | I         | Negative         | Positive         |
| 3          | Female | 54          | HBsAg+, HBeAg+, HBcAb+ | 0.8           | Moderately differentiated | I         | n/a             | Positive         |
| 4          | Female | 46          | HBsAg+, HBeAg+, HBcAb+ | 66.8          | Moderately differentiated | I         | Negative         | Positive         |
| 5          | Male   | 56          | HBsAg+, HBeAg+, HBcAb+ | 24.2          | Moderately differentiated | II        | Positive         | Negative         |
| 6          | Male   | 65          | HBsAg+, HBeAg+, HBcAb+ | 21.0          | Moderately differentiated | I         | Positive         | Positive         |
| 7          | Female | 45          | HBsAg+, HBeAg+, HBcAb+ | 422.2         | Moderately differentiated | I         | Positive         | Positive         |
| 8          | Male   | 39          | HBsAg+, HBeAg+, HBcAb+ | 7.6           | Moderately differentiated | II        | Positive         | Negative         |
| 9          | Female | 47          | HBsAg+, HBeAg+, HBcAb+ | 0.6           | Moderately differentiated | III       | Negative         | Negative         |
| 10         | Male   | 48          | n/a         | 25.2          | Moderately differentiated | I         | Negative         | Positive         |
| 11         | Female | 45          | HBsAg+, HBeAg+, HBcAb+ | 11.7          | Moderately differentiated | I         | Negative         | Positive         |
| 12         | Male   | 69          | HBsAg+, HBeAg+, HBcAb+ | 8.6           | Moderately differentiated | I         | Negative         | Negative         |
| 13         | Female | 49          | HBsAg+      | 16.1          | Moderately differentiated | II        | Positive         | Positive         |
| 14         | Male   | 58          | HBsAg+      | 8.3           | Poorly differentiated   | I         | Positive         | Positive         |

n/a, not available; HBV, hepatitis B virus; HBsAg, hepatitis B surface antigen; HBsAb, anti-hepatitis B surface antibody; HBeAb, anti-hepatitis B virus e antibody; HBcAb, anti-hepatitis B virus core antibody; TNM, tumor-node-metastasis; CD10, cluster of differentiation 10; PD-L1, programmed cell death-ligand 1.

Table 2
Clinicopathological characteristics of patients with intrahepatic cholangiocarcinoma with and without FGFR2 rearrangement [n (%)].

| Characteristics | No FGFR2 rearrangement (n = 243) | FGFR2 rearrangement (n = 14) | P-value* |
|-----------------|----------------------------------|------------------------------|----------|
| Gender          | Male 85 (34.0) 7 (50.0)          | 158 (65.0) 7 (50.0)          | 0.265    |
| Age (years)     | ≤58 115 (47.3) 11 (78.6)          | 128 (52.7) 3 (21.4)          | 0.028    |
|                | >58 120 (50.4) 11 (78.6)          | 118 (49.6) 3 (21.4)          | 0.003    |
| Hepatolithiasis | Positive 66 (27.9) 9 (69.2)      | 171 (72.1) 4 (30.8)          | 0.053    |
| Serum CA19–9 (U/mL) <39 120 (50.4) 11 (78.6) | 118 (49.6) 3 (21.4) | 0.079 |
|                | ≥39 118 (49.6) 3 (21.4)          | 120 (50.4) 11 (78.6)         | 0.003    |
| Smoker          | No-smoker 148 (63.3) 8 (66.7)     | 63 (26.9) 2 (16.7)           | 0.540    |
|                | Current smoker 23 (9.8)           | 23 (9.8) 2 (16.7)            | 1.000    |
|                | Former smoker 164 (73.2) 9 (61.8)| 46 (20.5) 2 (18.2)           | 0.492    |
|                |-former smoker 4 (1.7)             | 0 (0.0)                      | 0.492    |
| Tumor grade     | Well differentiated 167 (68.7) 12 (85.7)| 4 (1.7) 0 (0.0) | 1.000    |
|                | Moderately differentiated 72 (29.6)| 2 (14.3)                      | 0.013    |
|                | Poorly differentiated 191 (78.6) 13 (92.9)| 52 (21.4) 1 (7.1) | 0.013    |
|                | CD10 expression 189 (84.4) 7 (53.9) | 35 (15.6) 6 (46.1) | 0.024    |
|                | PD-L1 expression 127 (71.8) 6 (42.9) | 50 (28.2) 8 (57.1) | 0.024    |

TNM, tumor-node-metastasis; HBsAb, anti-hepatitis B surface antibody; CD10, cluster of differentiation 10; PD-L1, programmed cell death-ligand 1.

* P-value from Fisher exact test, as appropriate.

Discussion

Our results demonstrated that patients with ICC with FGFRs rearrangement have distinct clinical phenotype compared with the general population of patients with ICC. Specifically, we observed significant enrichment for FGFR2–3 rearrangement in patients age ≤ 58 years, of female, with positive serum HBsAb, and whose tumor expressed CD10 and PD-L1. Since FGFRs rearrangement-positive tumors can be sensitive to FGFR inhibitors [14–22], these observations suggest that molecular testing to detect FGFRs rearrangement in ICC should be prioritized for patients with these clinical and pathological features. In addition, our
Table 3

| Sample no. | Gene       | Transcript | Coding sequence change | Clinical significance |
|------------|------------|------------|-------------------------|-----------------------|
| 1          | FGFR1      | NM_023110  | Exon6, c.742G > A: p. V248M | Likely benign         |
| 2          | FGFR2      | NM_000141  | Exon 7, c.870G > T: p. W290C | Pathogenic            |
| 3          | FGFR2      | NM_000141  | Exon 3, c.185G > A: p. G62Y | Uncertain significance |
| 4          | FGFR2      | NM_000141  | Exon 14, c.1976A > T: p. K659M | Pathogenic            |
| 5          | FGFR2      | NM_000141  | Exon 14, c.1880T > A: p. L627 | Likely significance   |
| 6          | FGFR3      | NM_000142  | Exon 6, c.713G > A: p. R238Q | Uncertain significance |
| 7          | FGFR3      | NM_000142  | Exon 7, c.796G > A: p. V266M | Uncertain significance |
| 8          | FGFR3      | NM_000142  | Exon 9, c.1183C > T: p. L395F | Uncertain significance |
| 9          | FGFR4      | NM_213,647 | Exon 10, c.1310G > A: p. V437H | Uncertain significance |
| 10         | FGFR4      | NM_213,647 | Exon 3, c.187G > T: p. G66C | Uncertain significance |

* Nonsense mutation.

The frequency of FGFR2 rearrangement were lower in Asian ICC patients than that in Caucasian patients, possibly reflecting the differences in ethnicity, causative etiology, and compositions of various clinical characteristics. Importantly, in ICC lacking FGFR2 rearrangement, we revealed FGFR3 rearrangement and FGFR1–4 mutations, with frequencies of 2.7% and 9.1%, respectively. Several pre-clinical and clinical trials have shown the efficacy of FGFR inhibitors in treating ICC patients with FGFR3 rearrangement or certain FGFRs mutations, as well as with FGFR2 rearrangement [14–17]. Thus, the findings of biomarker profile in our data may expand the proportion of potential FGFR-targetable cases in ICC. Several studies have already identified secondary resistance mutations to FGFR-targeted therapies, most of which occurred in the Tyrosine Kinase Domain (TKD) of the FGFR genes [35–37]. However, in our dataset, all the FGFRs mutations were detected in non-TKD and none of the ICC patients received FGFR inhibitors, representing primary mutations. The associations of these primary mutations with the sensitivity and resistance to FGFR inhibitors merit further studies.

FGFR pathway GAs have been examined in relation to clinical and pathological characteristics in biliary tract cancers, with several studies on Caucasian patients reporting an association. Jain et al. [21] have reported that FGFR2 rearrangements or mutations in ICC were associated with younger age at onset and female sex. Graham et al. [27] have reported that FGFR2 rearrangement in mixed intrahepatic, perihilar and extrahepatic cancer was associated with a female predominance. Jain et al. [22] have reported in mixed intrahepatic, extrabiliary and gallbladder cancer, FGFR and FGFI9 GAs occurred more frequently in younger patients and presented at an earlier tumor stage. In the present study, we provided further valuable information that FGFR2-3 rearrangement in ICC was associated with younger age (< 58 years), female sex, serum HBsAb positivity, and tumoral CD10 and PD-L1 expression, while FGFRs GAs were associated with an earlier tumor stage. HBV infection has been proved to be associated with an increased risk of ICC incidence [38]. It has been reported that FGFR2 rearrangement positive cases had a propensity for hepatitis virus infection (HCV or HBV) in
mixed intrahepatic and extrahepatic cancer from a Japanese population [29]. However, we observed significant enrichment for FGFR rearrangement in patients with positive HBsAb, rather than those with positive HBsAg (P = 0.381), consistent with the findings from another study in China reporting no significant association between FGFR2 rearrangement in ICC and HBV infection [34]. Although PD-L1 expression, as a potential predictor for anti-PD-1/PD-L1 immune checkpoint inhibitors (ICIs) treatment, was enriched in FGFR rearrangement-positive ICCs, it is still unknown whether implications for combining FGFR inhibitor and anti-PD-1/PD-L1 agent could enhance treatment response for these patients. It has been reported that, in patients with advanced urothelial cancer with FGFR alterations, sequential application of FGFR inhibitor and PD-1/PD-L1 inhibitor enhanced the ICI response rate to approximately 30% in contrast to only 3.6% for patients receiving initial PD-1/PD-L1 inhibitors [39]. CD10, a cell surface ectoenzyme, is widely expressed on different types of cancers, and has been associated with tumor progression, therapeutic resistance, and molecular dysregulation in the tumor microenvironment [40]. Further studies are needed to explore whether the CD10 and/or PD-L1 expression impact the response of targeted therapy and immunotherapy in ICCs.

Several limitations should be noted in the present study. Firstly, the number of samples remains relatively small, especially for the FGFRs GA subsets, which reflects the relatively rare molecular subsets of ICC. Despite these limitations, this study is important, as it is, to our knowledge, the first profiling of FGFR GAs in a cohort of patients with ICC. Secondly, the cohort investigated is made up of surgery patients who are mostly in earlier tumor stage (stage I/II: 79.4%). Since the incidence of FGFRs GAs in ICC may be higher in surgically resectable disease stages [31], future studies should address the rate of these GAs in patients with later tumor stages, as well as in different tumor locations (primary tumor vs. metastases). Thirdly, the FGFRs GAs detected by NGS array was not validated by other methods such as PCR-based first-generation sequencing. Finally, all the patients in the present study did not received the FGFR inhibitors, which hindered our further analysis on the association between FGFRs GAs and clinical response to FGFR inhibitors. We recognize that the detection of candidate FGFRs GAs does not necessarily indicate its relevance as a potential therapeutic target. Thus, the functional consequences of these FGFRs GAs, especially mutations, await further investigation.

In conclusion, our data showed that FGFR2 and FGFR3 rearrangement in ICC is associated with unique clinical phenotypes, with features of younger age at onset, female sex, serum HBsAb positivity, and tumoral CD10 and PD-L1 expression.

### Table 4

Clinicopathological characteristics of patients with FGFR2–3 rearrangement versus those with FGFR1–4 mutation or those without rearrangement and mutation.

|                          | No FGFRs rearrangement or mutation (n = 97) | FGFR2–3 rearrangement (n = 17) | FGFR1–4 mutation (n = 10) | p-value1 | p-value2 |
|--------------------------|-------------------------------------------|-------------------------------|--------------------------|----------|----------|
| Gender                   |                                           |                               |                          |          |          |
| Female                   | 26 (26.8)                                 | 9 (52.9)                      | 5 (50.0)                 | 0.034    | 0.598    |
| Male                     | 71 (73.2)                                 | 8 (47.1)                      | 5 (50.0)                 |          |          |
| Age (years)              |                                           |                               |                          |          |          |
| ≤58                      | 49 (50.5)                                 | 14 (82.4)                     | 3 (30.0)                 | 0.017    | 0.013    |
| >58                      | 48 (49.5)                                 | 3 (17.6)                      | 7 (70.0)                 |          |          |
| Hepatolithiasis           |                                           |                               |                          |          |          |
| Negative                 | 81 (83.5)                                 | 17 (100.0)                    | 4 (40.0)                 | 0.124    | 0.001    |
| Positive                 | 16 (16.5)                                 | 0 (0.0)                       | 6 (60.0)                 |          |          |
| HBsAb status             |                                           |                               |                          |          |          |
| Positive                 | 25 (26.6)                                 | 11 (68.8)                     | 2 (22.2)                 | 0.003    | 0.041    |
| Negative                 | 69 (73.4)                                 | 5 (31.2)                      | 7 (77.8)                 | 0.021    | 0.022    |
| CD10 expression          |                                           |                               |                          |          |          |
| Negative                 | 76 (80.9)                                 | 8 (50.0)                      | 9 (100.0)                | 0.009    | 0.069    |
| Positive                 | 18 (19.1)                                 | 8 (50.0)                      | 0 (0.0)                  |          |          |
| PD-L1 expression         |                                           |                               |                          |          |          |
| Negative                 | 58 (71.6)                                 | 6 (37.5)                      | 6 (85.7)                 |          |          |
| Positive                 | 23 (28.4)                                 | 10 (62.5)                     | 1 (14.3)                 |          |          |

1 FGFR2–3 rearrangement vs. FGFRs negative.

2 FGFR2–3 rearrangement vs. FGFR1–4 mutation.

### Prior presentation

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### Authors’ contribution

Conceptualization, Z.Z., H.D., J.W., W.C. and Q.X.; methodology, X. C. and H.L.; investigation, W.D., X.G., H.Y., J.F., S.G., X.C. and H.L.; resources, W.D. and H.Y.; writing—original draft preparation, Z.Z., H. D., X.C. and H.L.; writing—review and editing, W.D., W.C. and Q.X.; supervision, Z.Z.; funding acquisition, Z.Z.. All authors have read and agreed to the published version of the manuscript.

### Declaration of Competing Interest

Two authors, X.C. and H.L, work at the Amoy Diagnostics Co., Ltd. whose product, the break-apart FISH probe kit, was used to detect FGFR2 rearrangement in the manuscript. Other authors listed in the authorship declared they had no competing financial interests.

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