FGFR mRNA Expression in Cholangiocarcinoma and Its Correlation with *FGFR2* Fusion Status and Immune Signatures

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ABSTRACT

Purpose: Selective FGFR inhibitors are effective against cholangiocarcinomas that harbor gene alterations in *FGFR2*. Clinical trials suggest that expression of wild-type FGFR mRNA can predict sensitivity to FGFR inhibitors, but this biomarker has not been well characterized in cholangiocarcinoma. This study explores the prevalence of FGFR mRNA overexpression in cholangiocarcinoma, its role in predicting sensitivity to FGFR inhibitors, and its association with immune markers.

Experimental Design: Tissue microarrays of intrahepatic (ICC) and extrahepatic cholangiocarcinomas (ECC) resected between 2004 and 2015 were used to evaluate FGFR1–4 mRNA expression levels by RNA *in situ* hybridization (ISH). Expression levels of FGFR2 mRNA were correlated with *FGFR2* fusion status and with patient outcomes. Immune markers expression was assessed by IHC and CSF1 and CSF1 receptor expression were examined by RNA ISH.

Results: Among 94 patients with resected cholangiocarcinoma, the majority had ICC (77%). *FGFR2* fusions were identified in 23% of ICCs and 5% of ECCs. High levels of FGFR mRNA in *FGFR2* fusion–negative ICC/ECC were seen for: FGFR1 (ICC/ECC: 15%/0%), FGFR2 (ICC/ECC: 57%/0%), FGFR3 (ICC/ECC: 53%/18%), and FGFR4 (ICC/ECC: 32%/0%). Overall, 62% of fusion-negative cholangiocarcinomas showed high levels of FGFR mRNA. In patients with advanced *FGFR2* fusion–positive ICC, high levels of FGFR2 mRNA did not correlate with clinical benefit. *FGFR2* fusion–positive tumors showed a paucity of PD-L1 on tumor cells.

Conclusions: FGFR mRNA overexpression occurs frequently in cholangiocarcinoma in the absence of genetic alterations in *FGFR*. This study identifies a molecular subpopulation in cholangiocarcinoma for which further investigation of FGFR inhibitors is merited outside currently approved indications.

Introduction

Cholangiocarcinoma is an aggressive malignancy of the bile ducts that in its advanced stages carries a poor prognosis. For more than a decade, chemotherapy has been the only treatment for patients with unresectable or metastatic cholangiocarcinoma. This changed in April 2020, when pemigatinib, an oral, selective FGFR inhibitor that competes for binding of ATP with the FGFR kinase domain, gained FDA approval for the treatment of patients with refractory *FGFR2* fusion–positive or rearrangement-positive cholangiocarcinoma. The oral, selective FGFR inhibitor infigratinib followed suit in May 2021 for treatment of that same population. In March 2022, the covalently-binding FGFR inhibitor futibatinib gained priority review by the FDA. The clinical success of FGFR inhibitors has sparked interest in the identification of additional biomarkers that may predict sensitivity to FGFR inhibitors and to drugs that can be combined with FGFR inhibitors to exploit known vulnerabilities (1, 2).

Biomarkers that most consistently predict sensitivity to FGFR inhibitors in cholangiocarcinoma include *FGFR2* fusions and rearrangements. These occur with a frequency of 13%–14% of patients with intrahepatic cholangiocarcinoma (ICC) and in approximately 1% of patients with extrahepatic cholangiocarcinoma (ECC), and they can be detected by tumor biopsy analysis or cell-free DNA analysis (1, 3–5). In the global FIGHT202 study, pemigatinib showed a 36% overall response rate (ORR) in patients with *FGFR*2 fusion–positive or rearrangement-positive cholangiocarcinoma. Other ATP-competitive inhibitors such as infigratinib and derazantinib have shown an ORR of 20%–23%. The irreversible covalent inhibitor futibatinib has shown an ORR of 42% in a similar population (6–8). Additional genetic alterations that can confer sensitivity of cholangiocarcinoma to FGFR inhibitors include mutations in *FGFR2* and less frequently amplifications in *FGF* or *FGFR2*. Most patients with these alterations experience stable disease at best (6, 9, 10), but certain FGFR2 extracellular domain in-frame deletions can lead to significantly more sensitivity to these drugs (11).

The clinical benefit of these selective FGFR inhibitors is encouraging and has heralded the first successful personalized medicine approach in cholangiocarcinoma. However, only 13%–21% of patients with ICC harbor genetic alterations in *FGFR* (1–3, 12–14). In other tumor types, expression of FGFR mRNA in the absence of genetic alterations in *FGFR* is predictive of a response to inhibition of FGFR. This widens the target patient population for FGFR inhibitors in other cancers. In patients with advanced solid tumors with positive FGFR mRNA expression treated with the pan-FGFR inhibitor rogaratinib, 15 of

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Translational Relevance

FGFR-driven cholangiocarcinoma is an important molecular subset of cholangiocarcinoma responsive to FDA-approved drugs, but gaps remain in identifying patients that may respond to these drugs. Here, we correlate genomic signatures in cholangiocarcinoma with gene expression and immune signatures to show that a significant percentage (62%) of *FGFR2* fusion–negative tumors show high FGFR mRNA expression levels, thus potentially identifying a subpopulation of patients with a tumor with a nongenetic biomarker that may predict sensitivity to FGFR inhibitors. We further show that tumors with high FGFR mRNA expression or *FGFR2* fusions showed a paucity of PD-L1 expression. These findings have relevance for immunotherapy strategies for patients with FGFR+ cholangiocarcinoma.

100 (15%) patients had a partial response. Ten of these 15 (67%) did not have a detectable genetic alteration in *FGFR* 1, 2, or 3 (15). The best response was seen in urothelial cancer where the ORR in FGFR3 RNA-expressing tumors was 24%. The majority of these responses occurred in patients with tumors without a genetic alteration in *FGFR*. Thus, patients with tumors that express FGFR3 mRNA constitute an additional population within urothelial cancer that is sensitive to FGFR inhibitors. This subgroup is beyond the current target population for erdaftinib, a selective FGFR inhibitor which has gained FDA approval for urothelial cancers harboring genetic alterations in *FGFR2* or *FGFR3* (16). Here we asked whether there may be a similar additional subset of patients with cholangiocarcinoma that may benefit from a strategy of FGFR inhibition. We therefore evaluated the frequency of mRNA overexpression of FGFR1–4 in fusion-negative cholangiocarcinoma.

Less than half of patients with *FGFR2* fusion–positive cholangiocarcinoma achieve objective responses when treated with FGFR inhibitors (7, 9, 17, 18). This stands in contrast to the response rates of 67%–80% seen in other oncogene-addicted tumors, such as *EGFR*-mutant or *ALK* fusion–positive non–small cell lung cancer (19, 20). The presence of the *FGFR2* fusion alone is therefore insufficient to predict which patients might derive benefit from FGFR inhibitors. Coalterations in tumor suppressor genes like p53 potentially serve as negative predictors of response as they have been associated with low ORRs with FGFR inhibitors (4). Additional positive predictors of response could help enrich target populations for those most likely to benefit. Here we study whether expression of FGFR mRNA could serve as a viable predictive biomarker for the response to treatment with an FGFR inhibitor in patients with *FGFR2* fusions or rearrangements.

Response rates to single-agent targeted therapy may be boosted by combination with an immunomodulatory agent (20). Overexpression of inhibitory immune checkpoint molecules (i.e., PD-L1) is correlated with increased tumor invasiveness and worse outcomes in patients with ICC (21, 22). A trial of single-agent PD-1 inhibitors has shown a modest benefit in advanced biliary tract cancers, with response rates of 6%–13%. An enrichment of responses in patients with PD-L1–expressing tumors was seen in a modest number of patients (23, 24). These studies did not stratify tumors according to FGFR status. Whether *FGFR*-positive tumors may be particularly suitable for the combination of FGFR inhibitors with checkpoint blockade is not known. Recently, it has been found that CSF1/CSF1 receptor (CSF1R) axis blockage by CSF1R inhibitor also enhances the efficacy of anti–PD-L1 therapy (24). Here we evaluate CD8, CD163, PD-L1, CSF1, and CFS1R in *FGFR2* fusion–positive and *FGFR2* fusion–negative tumors to assess a possible rationale for the combination of immunomodulatory agents with FGFR inhibitors to treat *FGFR2* fusion–positive cholangiocarcinoma.

In sum, *FGFR*-driven cholangiocarcinoma is an important molecular subset of cholangiocarcinoma responsive to FDA-approved drugs. We address knowledge gaps that could help predict a therapeutic response to FGFR inhibitors by correlating genomic signatures in cholangiocarcinoma with gene expression and immune signatures.

Materials and Methods

Study design

The study included patients who underwent surgical resection for cholangiocarcinoma at Massachusetts General Hospital (MGH) between 2004 and 2015 (primary cohort; Supplementary Fig. S1 illustrates the study design). Patients who had neoadjuvant therapy for cholangiocarcinoma were excluded. Available hematoxylin and eosin–stained slides from partial hepatectomy specimens were reviewed, and a representative slide and corresponding paraffin block was selected for tissue microarray (TMA) construction. One to four punches measuring 3 mm in diameter, each containing a representative tumor, were taken from donor blocks and arrayed into the recipient block. Hematoxylin and eosin staining of sections from TMA blocks was performed to confirm the presence of a tumor.

A second cohort of patients with advanced *FGFR2* fusion–positive or rearrangement-positive cholangiocarcinoma treated with at least one selective FGFR inhibitor was included to correlate baseline FGFR mRNA expression with response to FGFR inhibitor (Supplementary Fig. S1). Eligible patients were diagnosed with cholangiocarcinoma between May 2015 and May 2019, were treated on a clinical trial of a selective FGFR inhibitor at the Mass General Cancer Center, and had obtainable and sufficient tissue for FGFR mRNA expression analysis. Demographics, tumor staging, histologic and molecular pathology data, laboratory data, imaging findings, treatment histories, and survival outcomes were retrieved from the electronic medical record.

FISH for FGFR2 fusions

FISH was performed on 5 μmol/L sections of cholangiocarcinoma TMA blocks at Mayo Clinic, Rochester, MN, following the protocol described earlier (2). Separation of orange and green signals by more than two signal diameters apart in >20% tumor cells was considered as positive. The reading was done in all the evaluable cores and any tissue core showing positivity was taken as evidence of an *FGFR2* fusion.

RNA *in situ* hybridization (ISH)

RNA ISH was performed using RNAscope 2.5 LS assay [Advanced Cell Diagnostics (ACD)] as per the manufacturer's protocols. The staining was done on the fully automated Leica Biosystems BOND RX platform. In brief, 5 μmol/L sections from TMAs were baked at 60°C for 1 hour followed by deparaffinization in the automated instrument. Target retrieval was done for 15 minutes using Leica epitope retrieval buffer 2 at 95°C followed by protease treatment for 15 minutes at 40°C. Hybridization was done with target probes directed against FGFR1 (ACD, catalog no. 310078), FGFR2 (ACD, catalog no. 311178), FGFR3 (ACD, catalog no. 310798), and FGFR4 (ACD, catalog no. 412308), and also with a cocktail of probes recognizing FGFR 1–3 (FGFR 1–3 pool; ACD, catalog no. 454518), at 40°C for 3 hours. Diaminobenzidine-based chromogenic detection was used to visualize the signal.

Positive staining was identified as brown, punctuate dots representing individual RNA signals. TMA glass slides were digitally scanned using Aperio ScanScope scanner at 40×.

Currently, there is no universal scoring system for RNA ISH signals. We therefore scored ISH results semiquantitatively as 0 = negative (no appreciable staining or <5 dots/cell in <50% tumor cells), 1+ = low expression (1–5 dots/cell in >50% tumor cells), 2+ = moderate expression (6–10 dots/cell in >50% tumor cells), and 3+ = high expression (>10 dots/cell in >50% tumor cells).

We also performed RNA ISH for CSF1 (ACD, catalog no. 313008) and CSF1R (ACD, catalog no. 310818) in a subset of cases ($n = 58$) on automated BondRx platform (Leica Biosystems) using same protocol as described above. RNA ISH expression of CSF1 and CSF1R was evaluated in the tumor cells and immune cells and expression was semiquantitatively graded into two broad categories of low (consistent signals at low level in most of the cells) and high (consistent signals at high level in most of the cells).

Genetic studies via SNaPSHOT and solid fusion assays

The mutational landscape of a subset of cholangiocarcinoma cases was done on the DNA isolated from formalin-fixed paraffin-embedded tissues. SNaPSHOT was performed for targeted mutational analysis as described previously (25). The Solid Fusion Assay, a clinically validated laboratory-developed internal assay at MGH was used to detect gene fusions. The assay is based on Anchored Multiplex PCR and detects fusion transcripts involving 29 genes as one of the fusion partners (26). ArcherDx FusionPlex Solid Tumor Kit primers were used in two heminested PCR reactions and the library so prepared was sequenced on an Illumina NextSeq (2×150 bp paired-end sequencing). Fusion transcript detection and annotation was done using a laboratory-developed algorithm (version 2.1.0).

IHC

IHC for PD-L1, (catalog no. 13684; clone E1L3N, rabbit mcl., dilution 1:400, Cell Signaling Technology), CD8 (NCL-L-CD8-4B11; mouse mcl., dilution 1:400, Leica), and CD163 (NCL-L-CD163; mouse mcl., dilution 1:500, Leica) was performed on automated BondRx platform (Leica Biosystems) as per standard IHC protocol. PD-L1 staining was called positive if it is expressed on >1% tumor cells or immune cells. CD8- and CD163-positive immune cells were counted on Halo image analysis platform (Halo 2.3; Indica Laboratories) per tissue area (μm^2).

Statistical analysis

The quantitative data were expressed as median and the qualitative data were enumerated as frequencies. The comparisons of different clinicopathologic features with respect to FGFR mRNA expression status were done using Kruskal–Wallis test. A P value of <0.05 was considered to be significant. All data were recorded and analyzed using the SPSS v20.0 software package (SPSS Inc.).

Data availability

The data generated in this study are available upon request from the corresponding author.

Results

Baseline patient and tumor characteristics

A total of 94 patients with resected cholangiocarcinoma were available for analysis (primary cohort, **Table 1**; Supplementary Fig. S1). The majority of patients had ICC (77%), with the remainder having ECC. Tumors across all four stages were included: 23% were stage I, 43% stage II, 33% stage III, and 1% stage IV. The median age of the cohort was 65 years (range, 37–88 years), and the majority were female (60%). Most tumors were moderately differentiated (64%), and the median CA19-9 at diagnosis was 26.5 U/mL (range, 0–100,410 U/mL). Most patients had no known risk factors, with only 5% having cirrhosis ($n = 3/43$), 4% ($n = 1/25$) chronic hepatitis B, 5% ($n = 3/65$) chronic hepatitis C, and 3% ($n = 2/65$) primary sclerosing cholangitis.

Table 1. Patient characteristics.

Parameters	ICC (*N*, %)	ECC (*N*, %)	Combined ICC+ECC cohort
Total cases	73	21	94
Median age at diagnosis (years, range)	64 (37–80)	73 (44–88)	65 (37–88)
Female gender	46 (63%)	10 (48%)	56
FGFR fusion positive	17 (23%)	1 (5%)	18
Tumor differentiation[a]			
Poorly	17 (23%)	6 (29%)	23 (24%)
Moderately	48 (66%)	12 (57%)	60 (64%)
Well	6 (8%)	1 (5%)	7 (7%)
Presence of cirrhosis	4 (5%)	1 (5%)	5 (5%)
Median tumor size (range, cm)	5.5 (1.5–16)	2.5 (0.7–5)	
Lymph node positive	13 (18%)	8 (38%)	21 (22%)
Presence of lymphovascular invasion	42 (58%)	12 (57%)	54 (57%)
Presence of perineural invasion	21 (29%)	17 (81%)	38 (40%)
Stage (AJCC 8th):			
I	12 (16%)	1 (5%)	13 (14%)
II	29 (40%)	13 (62%)	42 (45%)
III	10 (14%)	4 (19%)	14 (15%)
IV	22 (30%)	2 (10%)	24 (25%)
Recurrence-free survival, (median, months)	12.4	15.83	13.9
Overall survival, (from diagnosis, months)	44.2 (0.2–173)	18.2 (0.06–91)	35.2

Abbreviations: ECC, extrahepatic cholangiocarcinoma; ICC, intrahepatic cholangiocarcinoma.
[a]May not sum to 100% due to missing or unavailable values.

The median size of ICC tumors was larger at 5.5 cm (range, 1.5–16) compared with ECCs at 2.5 cm (range, 0.7–5 cm) but ECCs did have a higher rate of nodal involvement (38% vs. 18%, $P < 0.01$) and perineural invasion (PNI; 81 vs. 29%, $P < 0.01$) compared with ICC. Both groups showed extensive lymphovascular invasion (LVI; ICC vs. ECC: 58 vs. 57%, $P > 0.05$).

For the second cohort (Supplementary Fig. S1), among 27 patients with unresectable or metastatic *FGFR2* fusion–positive ICC who were treated with an FGFR inhibitor, 13 had sufficient tissue for analysis of FGFR mRNA expression. In this subset, all patients were treated with futibatinib, a covalent irreversible FGFR inhibitor, which gained FDA breakthrough designation for refractory *FGFR2* fusion–positive or rearrangement-positive ICC. All were participants in a clinical trial (NCT02052778). The median ORR of this group was 30%, and the median PFS was 6.9 months (range, 2.5–17 months). The specimen evaluated for FGFR mRNA expression prior to treatment with FGFR inhibitor was a resection specimen in 30% of cases and a metastatic disease biopsy in 70% of cases. Samples were obtained prior to any systemic therapy in 69% of cases and post-chemotherapy but immediately prior to treatment with FGFR inhibitor in 31% of cases.

Frequency of high FGFR mRNA expression in FGFR2 fusion–positive and FGFR2 fusion–negative tumors

In the TMAs of the 94 patient samples, we first assessed the *FGFR2* fusion status by break apart FISH. We then assessed FGFR mRNA expression by RNA ISH. High expressors were defined as those with 2+ or 3+ staining by RNA ISH.

FGFR2 fusions were present in 23% (17/73) of ICCs and in 5% (1/21) of ECCs. *FGFR2* fusion–positive tumors showed a higher expression of FGFR2 ($P = 0.03$), confirming previous reports (27), but did not associate with higher expression of FGFR1 ($P = 0.98$), FGFR3 ($P = 0.26$), or FGFR4 ($P = 0.59$). In the *FGFR2* fusion–negative cohort of 76 patients, we detected high mRNA expression for FGFR1 (15%), FGFR2 (57%), FGFR3 (53%), and FGFR4 (32%; **Fig. 1**). Overall, 62% of fusion-negative cholangiocarcinomas showed high levels of FGFR mRNA expression.

The results were similar in the subset of patients with *FGFR2* fusion–negative ICCs: FGFR1 (15%), FGFR2 (54%), FGFR3 (50%), and FGFR4 (30%). Among these ICCs, 61% had high expression of at least one isoform of FGFR, and 48% had high expression of more than one isoform of FGFR. The most commonly co-highly-expressed isoforms were FGFR2 and FGFR3. All ECC *FGFR2* fusion–negative tumors showed only low expression (0 or 1+) of FGFR1, FGFR2, and FGFR4, and 18% showed high expression of FGFR3 mRNA.

When using a pooled FGFR probe, which measures FGFR1–3 mRNA expression simultaneously, 81% of ICC *FGFR2* fusion–negative tumors showed high levels of FGFR mRNA expression, including all cases identified as high expressors by individual probes. Only 25% of ECC *FGFR2* fusion–negative tumors showed high levels of FGFR mRNA expression when examined with the FGFR pool probe.

Correlation of FGFR expression and fusion status with clinical features

We assessed associations between clinical features and (i) FGFR1, 2, 3, or 4 expression status and (ii) *FGFR2* fusion status. We specifically assessed associations with age, gender, tumor size, presence of LVI or PNI, presence or absence of cirrhosis, and tumor differentiation. With regard to FGFR expression status, high FGFR1 and high FGFR2

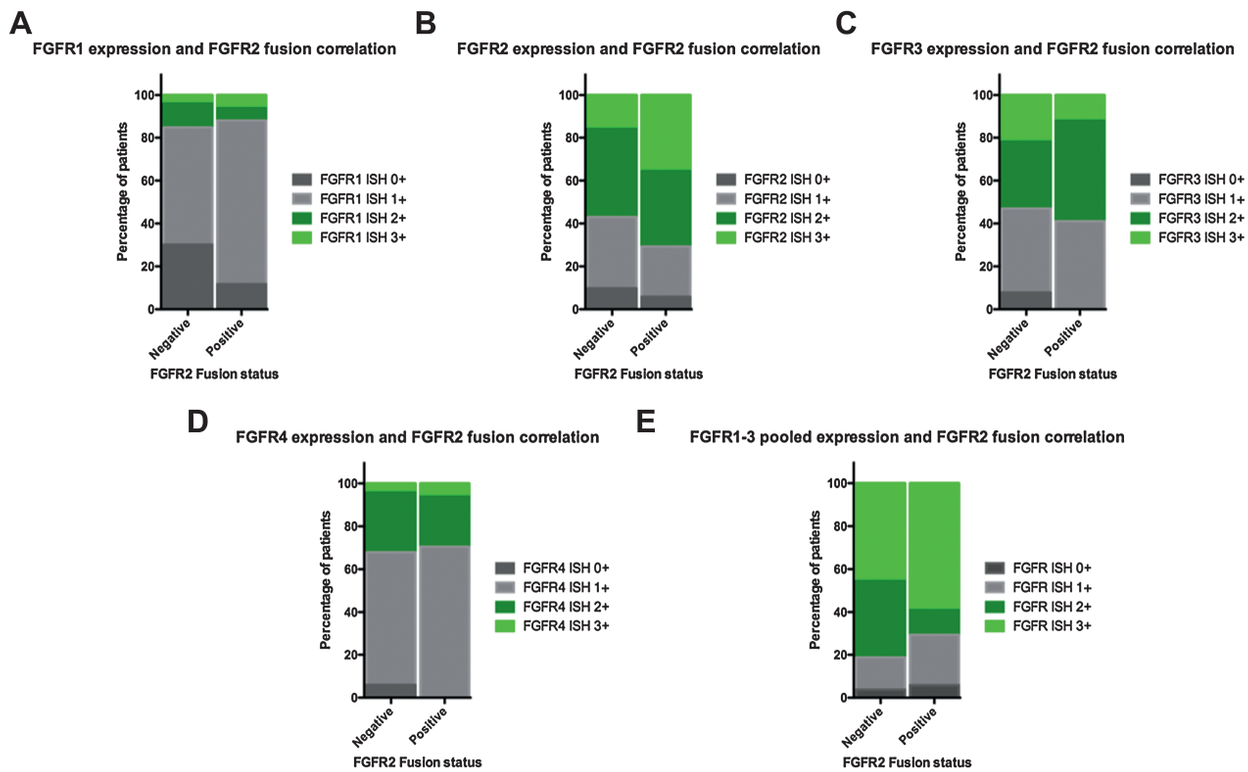

Figure 1.

A–E, Correlation between FGFR expression and FGFR2 fusion status in cholangiocarcinoma.

expression were associated with a lower PNI ($P = 0.02$, $P = 0.01$), but these associations did not hold for FGFR3 or FGFR4 ($P = 0.07$, $P = 0.11$). There were no other associations between FGFR expression levels and age, gender, LVI, presence or absence of cirrhosis, and tumor differentiation. There was no correlation between any of the clinical parameters and *FGFR2* fusion status: age ($P = 0.38$), gender ($P = 0.70$), size ($P = 0.64$), presence of PNI ($P = 0.75$), presence of LVI ($P = 0.37$), presence of cirrhosis ($P = 0.46$), and differentiation status ($P = 0.18$).

Correlation between FGFR2 mRNA expression levels and outcomes on FGFR inhibitors in FGFR2 fusion–positive cholangiocarcinoma

Given the modest response rate of *FGFR2* fusion–positive cholangiocarcinoma when treated with selective FGFR inhibitors, we examined FGFR2 mRNA expression levels as a possible predictor of the response to FGFR inhibitors in this population. We looked for a possible correlation of FGFR mRNA expression levels with the best overall response and progression-free survival (PFS). First, to obtain a baseline frequency for high FGFR2 mRNA expression levels in *FGFR2* fusion–positive cholangiocarcinoma, we assessed this correlation in the fusion-positive cases ($n = 18/94$) in the TMAs. In the 17 patients with *FGFR2* fusion–positive ICC, 70% had high levels of FGFR2 mRNA expression (2+ or 3+ as estimated by FISH), and 30% had low mRNA levels (0 and 1+). In the single patient with *FGFR2* fusion–positive ECC, FGFR2 mRNA expression was low.

Next, we evaluated FGFR2 mRNA expression levels in samples of 13 patients with *FGFR2* fusion–positive cholangiocarcinoma, prior to treatment with inhibitor, but who were subsequently treated with futibatinib (second cohort, **Table 2**). Among 13 patients, 38% had high levels of FGFR2 mRNA expression, 54% had low levels, and 7% had no detectable FGFR2 mRNA expression. The ORR in the high versus low/nonexpressors was 22% versus 33% ($P = 0.61$), and the PFS was 11.0 versus 7.0 months ($P = 0.88$). No significant correlation was seen between FGFR mRNA levels and clinical benefit for this small cohort of patients. We also assessed associations between ORRs and various clinical characteristics. There was no correlation between ORR and gender ($P = 0.70$), number of prior lines of therapy ($P = 0.36$), or presence of metastatic disease in the lungs ($P = 0.69$), bone ($P = 0.51$), or peritoneum ($P = 0.94$).

Correlations between immune-related biomarkers and FGFR2 fusion status

To characterize the immune landscape of *FGFR*-positive cholangiocarcinoma, we assessed the correlation of expression of immune markers and *FGFR2* fusion status in a subset of patients of the primary cohort ($n = 58/94$; **Fig. 2**). Expression of PD-L1 was examined on tumor cells and immune cells. Expression of CSF1 and CSF1R were also measured for tumor cells and immune cells, respectively.

None of the *FGFR2* fusion–positive ($n = 17$) cases were positive for PD-L1 on tumor cells (defined at ≥ 1%) while 20% (8/41) of *FGFR2* fusion–negative samples were positive ($P = 0.05$). No difference in expression of PD-L1 was seen for immune cells in *FGFR2* fusion–positive and fusion–negative tumors (12% vs. 29%, $P = 0.15$, Fisher). We also compared CD8 expression on immune cells in *FGFR2* fusion–positive and *FGFR2* fusion–negative cases ($P = 0.121$) and CD163 expression on immune cells in *FGFR2* fusion–positive and *FGFR2* fusion–negative cases ($P = 0.267$), and no statistically significant differences were noted.

Eighteen percent of *FGFR2* fusion–positive tumors showed high levels of CSF1R expression, compared with 5% of *FGFR2* fusion–negative tumors on immune cells ($P = 0.11$). Similarly, no significant difference was seen between *FGFR2* fusion–positive and *FGFR2* fusion–negative tumors (35% vs. 51%, respectively, $P = 0.27$) for expression of CSF1 in tumor cells.

When stratified for high versus low levels of FGFR2 mRNA expression, no difference was detected in the expression of PD-L1 in the tumor ($P = 0.14$), PD-L1 in the immune cells ($P = 0.05$), CD8 in the immune cells ($P = 0.277$), CD163 in the immune cells ($P = 0.112$), CSF1 in tumors ($P = 0.15$), or CSF1R in immune cells ($P = 0.69$).

Correlations between FGFR2 fusion status, mRNA expression levels, and survival

We evaluated the prognostic value of *FGFR2* fusions and FGFR2 mRNA expression levels in patients with resected cholangiocarcinoma. The recurrence rates in the entire cohort of 94 patients were 50%, similarly distributed over ICC (47%) and ECC (57%). When evaluated by *FGFR2* fusion status in patients with ICC, recurrence rates were similar in *FGFR2* fusion–positive versus *FGFR2* fusion–negative ICC (41% vs. 50%, respectively, $P = 0.26$). In the entire cohort, the

Table 2. FGFR RNA ISH expression in pretreatment tumor tissue of patients with FGFR2 fusion–positive ICC treated on futibatinib as the first FGFR inhibitor.

Patient	Fusion type	Gender	Age at diagnosis (years)	Sites of metastasis prior to treatment	Number of prior lines of therapy	FGFR2, Pretreatment RNA ISH+	Progression-free survival on first FGFR inhibitor (months)	Best overall response (%)
1	FGFR2-SORBS1 Fusion	F	69.7	Lung, peritoneal	3	2	15.8	−77%
2	FGFR2-POC1B Fusion	F	61.2	Lung, bone	1	1	7.0	−42%
3	FGFR2-WAC Fusion	F	55.5	Lung, bone, peritoneal	0	0	4.8	−7%
4	FGFR2-AHCYL1 Fusion	F	55.4	Lung, bone	1	1	4.7	−25%
5	FGFR2-POC1B Fusion	F	31.0	Lung	2	1	7.2	−50%
6	FGFR2-NRAP Fusion	M	44.2	Lung, bone, peritoneal	4	1	17.2	−48%
7	FGFR2-SORBS1 Fusion	M	63.5	Lung	1	2	13.4	−29%
8	FGFR2-POC1B Fusion	F	40.6	Lung, bone	1	2	11.0	−19%
9	FGFR2-ZMYM4 Fusion	F	59.8	Lung, bone, peritoneal	2	1	6.7	8%
10	FGFR2-BICC1 Fusion	F	52.9	Lung, bone	2	1	7.2	−46%
11	FGFR2-INA Fusion	M	26.5	Lung, bone, peritoneal	3	2	2.9	−22%
12	FGFR2-DBP Fusion	F	46.6	None	1	1	5.2	−22%
13	FGFR2-PHGDH Fusion	M	74.1	Lung	1	2	2.6	−3%

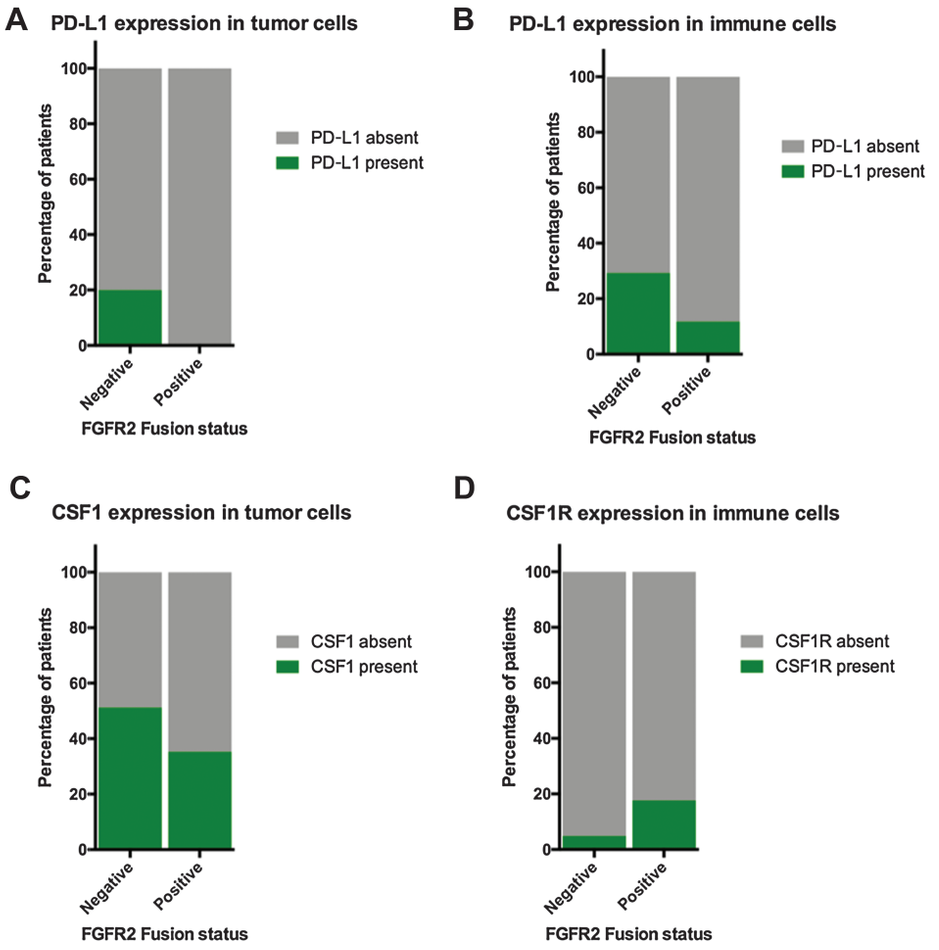

Figure 2.
A–D, Correlation of PD-L1, CSF1, and CSF1R expression with FGFR2 fusion status in patients with ICC.

median relapse-free survival (RFS) was 14.0 months (range, 1.5–64.2 months); in ICC, it was 12.4 months [95% confidence interval (CI) = 11.0–20.0], and in ECC, it was 15.8 months (95% CI = 10.0–34.0). *FGFR2* fusion status in patients with ICC did not significantly impact their median RFS (11 in fusion-positive vs. 14 months in fusion-negative, $P = 0.52$). Similarly, the differences in median RFS in patients with ICC with high versus low FGFR2 mRNA levels were not significant (12 vs. 13 months, $P = 0.36$). The number of ECC patients who had *FGFR2* fusion–positive tumors or with high levels of FGFR mRNA expression was too small for a meaningful comparative survival analysis. Overall, *FGFR2* fusion status and FGFR2 mRNA expression levels did not significantly impact recurrence rates or RFS, albeit the numbers were small.

The median overall survival (OS) for the entire primary cohort was 35.2 months (range, 0.06–173.0 months), which was significantly higher in the ICC group at 44.2 months than in the ECC group (18.2 months; $P = 0.02$). As expected, patients with higher T-stage ($P = 0.001$), presence of nodal disease ($P = 0.01$), and/or cirrhosis ($P = 0.001$) showed worse OS. No statistically significant median difference in OS was seen between FGFR2 fusion–positive and FGFR2 fusion–negative patients in the ICC cohort (44.0 vs. 28.0 months, respectively, $P = 0.24$). No difference in median OS was seen when comparing patients with ICC with tumors that have high versus low FGFR2 mRNA expression levels (45.0 vs. 43.0 months, respectively, $P = 0.17$; **Fig. 3**). Overall, *FGFR2* fusion status and FGFR2 mRNA expression levels did not significantly impact OS.

RFS and OS were assessed for correlation with expression of PD-L1, CSF1R, and CSF1 in the tumor and tumor microenvironment in patients with ICC. Patients with PD-L1–positive versus PD-L1–negative tumors had a median RFS of 5 versus 15 months, respectively (log-rank $P = 0.26$) and a median OS of 21 versus 45 months, respectively (log-rank $P = 0.12$). CSF1R-positive versus -negative tumors showed a median RFS of 12 versus 15 months (log-rank, $P = 0.45$) and median OS of 32 versus 44 months (log-rank, $P = 0.97$). CSF1 status in tumors also did not correlate with OS ($P = 0.53$ and $P = 0.78$, respectively).

Discussion

The recent approval of FGFR inhibitors for *FGFR2* fusion–positive cholangiocarcinoma inspires the characterization of additional biomarkers that may predict response to these inhibitors. Broadening the indication of these drugs could provide access to treatment for more patients with this rare and aggressive malignancy. Here we examine FGFR mRNA expression as a biomarker in ICC and ECC, and we integrate these data with genomic data on *FGFR* and mRNA and protein expression data on immune markers. Our data demonstrate that a sizable fraction (62%) of *FGFR2* fusion–negative tumors show

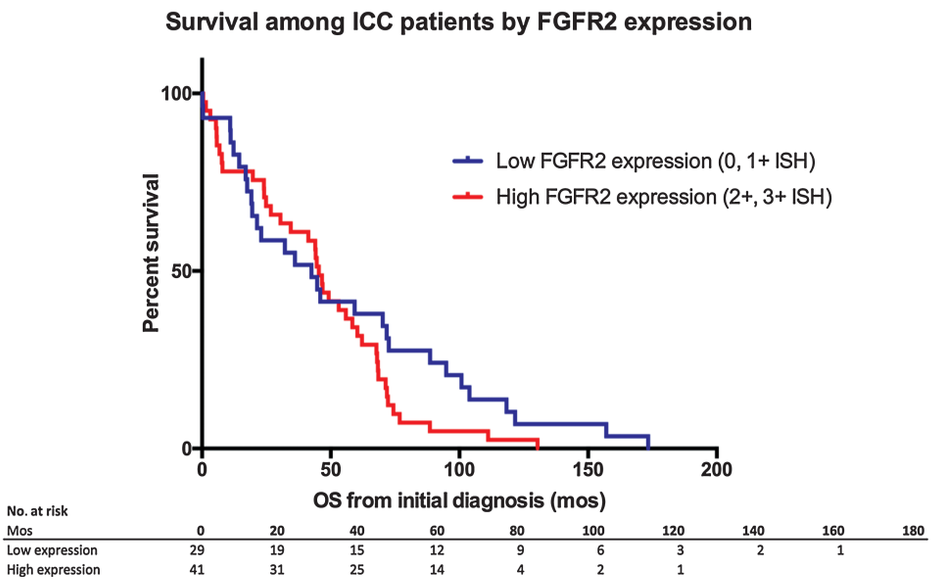

Figure 3.
Survival difference among patients with ICC with high versus low FGFR2 expression (45.0 vs. 43.0 months, respectively, $P = 0.17$).

high levels of FGFR mRNA expression. This trait may identify a subpopulation of patients with a biomarker that may predict sensitivity to FGFR inhibitors in the absence of any obvious genetic alterations. We further examined the relationship between the tumor microenvironment and *FGFR* status to explore the rationale of combining FGFR inhibitors with immunomodulatory agents. Among the immune markers we examined, we saw no significant correlation between *FGFR2* fusion status and expression of these markers in ICC.

The impetus to study FGFR mRNA expression as a biomarker that could predict sensitivity to FGFR inhibitors in cholangiocarcinoma was based on the viability of this strategy for other tumors and activity of FGFR inhibition in patients with cholangiocarcinoma without detectable *FGFR* gene alterations in FGFR. In patients with advanced solid tumors, a phase I study showed the utility of measuring the expression of FGFR mRNA to predict the responsiveness to rogaratinib. This applied in particular to FGFR3-overexpressing urothelial cancer. A disease-specific trial confirmed the viability of this strategy (27). In the phase II FIGHT study, patients with unresectable or metastatic gastroesophageal cancer whose tumors showed expression of FGFR2b mRNA and/or amplification of *FGFR2* had a longer median OS with FOLFOX combined with bemarituzumab, an afucosylated anti-FGFR2b mAb, compared with FOLFOX alone (28). The majority of patients had tumors that showed expression of FGFR2b without concurrent genetic alterations, although the presence of *FGFR2* mutations and *FGFR2* fusion status were not reported or universally assessed. Tumors may have differential sensitivity to FGFR inhibitors depending on the expression levels of FGFR. The gradient of expression was evaluated in the FIGHT study, and patients with tumors in which >10% of cells showed high levels of FGFR2b mRNA had a higher median OS compared with the intent-to-treat population that received the bemarituzumab (28). Returning to cholangiocarcinoma, while tumor FGFR expression was not reported in the FIGHT202 study, 22% of patients in the cohort without FGF/FGFR alterations had tumor shrinkage on pemigatinib, including one unconfirmed response (9). There is thus rationale for a prospective assessment of FGFR mRNA expression as a biomarker to predict a response to inhibition of FGFR activity in cholangiocarcinoma.

We also explored possible connections between the tumor immune microenvironment and *FGFR* fusion and expression status. About 30% of ICC tumors may express PD-L1 (29). Defects in expression of class I HLA antigens in combination with expression of PD-L1 may allow immune escape, despite the presence of tumor-infiltrating lymphocytes (29). An analysis of 489 cholangiocarcinomas showed that the cluster enriched for *FGFR* alterations did not show elevated expression of PD-1 and PD-L2 expression (14). In our study, *FGFR2* fusion–positive ICCs were negative for PD-L1 expression. A minority of *FGFR2* fusion–negative ICCs expressed PD-L1. Data from prior studies on the interplay between PD-L1 and FGFR give a mixed message; *in vitro* studies in colorectal cancer ($n = 90$) have indicated that expression of PD-L1 was positively correlated with that of FGFR2. Expression of FGFR2 also promoted the expression of PD-L1 in a mouse xenograft model of colorectal cancer (30). However, analysis of urothelial carcinoma samples from patients ($n = 310$) found that *FGFR3*-mutated tumors were less likely to express PD-L1 compared with *FGFR3* wild-type tumors (31). The interplay between expression of FGFR and immune checkpoints may thus depend on histologic context.

When examining potential combinations of targeted therapy and immune therapy, the combination of FGFR inhibitors with PD-1 blockade produced a synergistic response in a FGFR2/p53-mutant lung cancer mouse model (32). Tumors treated with erdafitinib showed decreased numbers of terminally exhausted ($PD1^+$ $TIM3^+$ $LAG3^+$) T cells. The combination of erdafitinib with a PD-1 inhibitor led to more activated, more proliferative T and natural killer (NK) cells, relative to erdafitnib alone. In our cohort, when examining tumor cells, none of the samples tested positive for both PD-L1 expression and an *FGFR2* fusion. More patient samples may be needed to determine which subsets of patients with cholangiocarcinoma may benefit from dual-pathway targeting.

There are several limitations to this study. The *FGFR* mutation and amplification status of the resected tumors on the TMAs was not known, as next-generation sequencing was performed rarely in these early-stage tumors. Some tumors with high levels of FGFR mRNA expression could harbor genetic alterations in *FGFR*. However, mutations in *FGFR* are present in approximately 5% of cholangiocarcinomas, and instances of *FGFR* amplifications are even fewer. The majority of fusion-negative tumors with expression of FGFR mRNA were thus unlikely to harbor *FGFR* genetic alterations. Also, we had a

limited number of patients with *FGFR2* fusion–positive cholangiocarcinoma treated with FGFR inhibitors that had sufficient residual biopsy material for FGFR2 mRNA expression assessment, and no patients with *FGFR* wild-type cholangiocarcinoma at our institution were treated with a selective FGFR inhibitor to allow comparison in this analysis. This is a clear limitation of our analyses on the utility of FGFR expression as a complementary predictive biomarker in this *FGFR2* fusion–positive population. Finally, although our study does include patients with extrahepatic cholangiocarcinoma, the numbers were small, and the majority of our findings were based on the analysis of ICC. We addressed this by analyzing ICC and ECC separately.

Our study correlates DNA and mRNA level biomarker data for FGFR and expression data for immune signatures to inform therapeutic strategies for patients with FGFR-positive cholangiocarcinoma. A significant proportion of *FGFR2* fusion–negative ICC tumors shows high levels of FGFR mRNA expression, most commonly that of FGFR2 or FGFR3. This provides baseline molecular epidemiologic data to evaluate the usefulness of selective FGFR inhibitors in this population. Further studies are needed to determine whether FGFR mRNA expression levels at baseline can be used to select patients with *FGFR2* fusion–positive tumors and boost response rates to FGFR inhibitors. Finally, more studies are needed to understand the immune environment of FGFR-driven cholangiocarcinomas to bring immunotherapy, alone or in combination with FGFR inhibitors, to bear as a strategy to treat these patients.

Authors' Disclosures

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Authors' Contributions

V. Sridharan: Formal analysis, investigation, writing–original draft, writing–review and editing. **A. Neyaz:** Data curation, formal analysis, methodology, writing–review and editing. **A. Chougule:** Data curation, formal analysis, methodology. **I. Baiev:** Data curation, writing–original draft. **S. Reyes:** Methodology, project administration. **E.G. Barr Fritcher:** Data curation, methodology. **J.K. Lennerz:** Data curation, formal analysis, methodology. **W. Sukov:** Data curation, methodology. **B. Kipp:** Data curation, methodology. **D.T. Ting:** Conceptualization, supervision, writing–review and editing. **V. Deshpande:** Conceptualization, supervision, investigation, methodology, writing–original draft, writing–review and editing. **L. Goyal:** Conceptualization, supervision, writing–original draft, writing–review and editing.

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Note

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