Understanding and Overcoming Resistance to Selective FGFR Inhibitors across *FGFR2*-Driven Malignancies

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ABSTRACT

Purpose: Understanding resistance to selective FGFR inhibitors is crucial to improve the clinical outcomes of patients with *FGFR2*-driven malignancies.

Experimental Design: We analyzed sequential ctDNA, ± whole-exome sequencing, or targeted next-generation sequencing on tissue biopsies from patients with tumors harboring activating *FGFR2* alterations progressing on pan-FGFR–selective inhibitors, collected in the prospective UNLOCK program. FGFR2::BICC1 Ba/F3 and patient-derived xenograft models were used for functional studies.

Results: Thirty-six patients were included. In cholangiocarcinoma, at resistance to both reversible inhibitors (e.g., pemigatinib and erdafitinib) and the irreversible inhibitor futibatinib, polyclonal *FGFR2* kinase domain mutations were frequent (14/27 patients). Tumors other than cholangiocarcinoma shared the same mutated *FGFR2* residues, but polyclonality was rare (1/9 patients). At resistance to reversible inhibitors, 14 residues in the *FGFR2* kinase domain were mutated—after futibatinib, only the

molecular brake N550 and the gatekeeper V565. Off-target alterations in PI3K/mTOR and MAPK pathways were found in 11 patients, often together with on-target mutations. At progression to a first FGFR inhibitor, 12 patients received futibatinib or lirafugratinib (irreversible inhibitors), with variable clinical outcomes depending on previous resistance mechanisms. Two patients with *TSC1* or *PIK3CA* mutations benefited from everolimus. In cell viability assays on Ba/F3 and in pharmacologic studies on patient-derived xenografts, irreversible inhibitors retained better activity against *FGFR2* kinase domain mutations, with lirafugratinib active against the recalcitrant V565L/F/Y.

Conclusions: At progression to FGFR inhibitors, *FGFR2*-driven malignancies are characterized by high intra- and interpatient molecular heterogeneity, particularly in cholangiocarcinoma. Resistance to FGFR inhibitors can be overcome by sequential, molecularly oriented treatment strategies across *FGFR2*-driven tumors.

Introduction

Molecular alterations of FGFR family members (*FGFR1/2/3/4*) are frequent across cancers (1, 2). *FGFR* amplifications are the most frequent alterations observed, yet their inconsistent oncogenic potential raises questions about their suitability as targets for selective inhibition (3). *FGFR2* gene fusions occur in 10% to 15% of intrahepatic cholangiocarcinoma cases, whereas activating mutations in the extracellular domain account for only a minor fraction of this malignancy (4–6). Recent research has highlighted the importance of deletions in the extracellular domain and truncations in the intracellular C-terminal domain of *FGFR2* as key drivers and therapeutic targets in intrahepatic cholangiocarcinoma (6, 7).

Of note, the same molecular alterations can be found, with a lower incidence, across a variety of solid tumors, of almost any histology (7).

The development and availability of selective FGFR inhibitors for treating *FGFR2*-driven intrahepatic cholangiocarcinoma are transforming the therapeutic landscape for patients with this molecular subtype (8–10). Selective FGFR inhibitors can be categorized into reversible (e.g., infigratinib, pemigatinib, erdafitinib, derazantinib, zoligratinib, and fexagratinib) and irreversible (e.g., futibatinib and lirafugratinib), based on their binding to the tyrosine kinase domain. Erdafitinib has demonstrated effectiveness in inhibiting FGFR3 in urothelial cancer (11, 12) and shows activity across various *FGFR2*-driven tumors, reflecting the concept of molecularly driven, tumor-

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Translational Relevance

The clinical benefit generated by selective FGFR inhibitors in patients with *FGFR2*-driven cancer is hampered by the inevitable occurrence of resistance. We analyzed postprogression ctDNA and tissue biopsies from 36 patients suffering from *FGFR2*-driven cholangiocarcinoma or other tumor types, at resistance to FGFR inhibitors. We were therefore able to recognize molecular traits and characteristics of resistance to reversible inhibitors versus the irreversible agent futibatinib, especially in terms of variety of *FGFR2* kinase domain mutations involved in resistance. Compared with *FGFR2*-driven cholangiocarcinomas, polyclonal *FGFR2* kinase domain mutations were less frequent in other tumor types. Twelve patients were treated with an additional FGFR irreversible inhibitor (futibatinib or lirafugratinib), and two derived benefit from everolimus. We integrated longitudinal molecular data with the clinical outcomes on these sequential targeted treatments, with functional evidence using Ba/F3 cellular models and patient-derived xenografts, aiming to propose molecular treatment strategies to overcome resistance.

agnostic targeted therapy (13). Similarly, pemigatinib and the two irreversible FGFR inhibitors, initially developed for *FGFR2*-driven intrahepatic cholangiocarcinoma, have shown efficacy across multiple tumor types in dose-expansion cohorts of phase I/II clinical trials (14–17).

Studies have identified frequent on-target, polyclonal mutations in the tyrosine kinase domain of *FGFR2* as common mechanisms of resistance to selective FGFR inhibitors in *FGFR2*-driven cholangiocarcinoma (18, 19). These findings are supported by functional validation of specific *FGFR2* kinase domain mutations that confer resistance, with irreversible FGFR inhibitors designed to be effective against these mutations (20–26). Recently, off-target resistance mechanisms have been explored; pathogenic variants in the MAPK and PI3K/mTOR pathways have been detected at progression on selective FGFR inhibitors in patients with cholangiocarcinoma (21, 23, 27).

Importantly, resistance to FGFR inhibitors in the setting of *FGFR2*-driven disease has been predominantly reported in cholangiocarcinoma, except in four cases of *FGFR2*-driven tumors that progressed on pemigatinib (15, 28). The broader application of FGFR inhibitors across different histologies underscores the need to identify and address resistance mechanisms in non-cholangiocarcinoma tumors, potentially offering universal strategies to counteract resistance.

In this study, we report on- and off-target resistance mechanisms to reversible and irreversible FGFR inhibitors across *FGFR2*-driven tumor types, validated through functional studies. Furthermore, the outcomes from sequential molecular treatments, applied to one-third of the patients with longitudinal monitoring of molecular alterations, provide insights into strategies to overcome resistance across a spectrum of *FGFR2*-driven solid tumors.

Materials and Methods

Patients and treatments

UNLOCK is an institutional program which aims to decipher mechanisms of action and resistance to innovative drugs

To be included in this cohort of the UNLOCK program, patients had to satisfy the following criteria: (i) diagnosis of an advanced solid tumor requiring systemic treatment; (ii) molecular detection of an activating alteration (i.e., fusions/rearrangements and mutations) in the *FGFR2* gene; (iii) having received a selective FGFR inhibitor, either reversible (pemigatinib, erdafitinib, infigratinib, derazantinib, zoligratinib, rogaratinib, and fexagratinib) or irreversible (futibatinib or lirafugratinib); and (iv) having postprogression molecular analyses performed on ctDNA and/or tissue biopsies. In two cases showing primary resistance to futibatinib (**ST4455** and **MR719**), in the lack of the availability of postprogression samples, pretreatment tissue biopsy and ctDNA were analyzed.

The molecular analyses were performed within four institutional studies at Gustave Roussy, whose aim is the molecular characterization of tumors: MATCH-R (NCT02517892; ref. 29), MOSCATO (NCT01566019; ref. 30), STING (NCT04932525), and CTC (NCT02666612).

Patients were treated in the setting of clinical trials or compassionate use programs allowing treatment with FGFR inhibitors on the basis of molecular selection. Disease response was measured according to RECIST 1.1, and progression-free survival (PFS) was calculated from the date of targeted inhibitor start to the day of radiologic evidence of progression.

All patients participating in the mentioned studies were fully informed and signed a written informed consent. The studies have been approved by ethics committees in France (French National Agency for Medicines and Health Products Safety) and are being conducted in accordance with the Declaration of Helsinki.

Molecular analyses

Postprogression tissue biopsies, when possible, underwent whole-exome sequencing (WES), with or without concomitant RNA sequencing (RNA-seq). The lower limit for WES performance was a proportion of tumor cells ≥30% in the tissue sample. In cases with a proportion of tumor cells between 10% and 30%, molecular analyses with targeted next-generation sequencing panels (Mosc-4, Oncomine v3) were performed. For WES, the mean coverage was $140\times$.

With regard to ctDNA analyses, they were performed with Guardant Health, Illumina, Foundation Medicine, or IntegraGen liquid biopsy panels. For each patient with longitudinal ctDNA assessment, only analyses performed with the same platform were reported.

Among the findings of the molecular reports, only molecular events potentially implicated in resistance were reported in the present study. *FGFR2* kinase domain mutations were reported according to reference transcript NM_001144913.1, as previously reported by our group and others (20, 21, 31, 32).

Site-directed mutagenesis

Lentiviral vectors expressing *FGFR2:BICC1* fusions were created using the pLenti6/V5 directional TOPO Cloning Kit (#K495510, Thermo Fisher Scientific) according to the manufacturer's instructions. Point mutations in the *FGFR2* kinase domain of the *FGFR2::BICC1* fusion were introduced using the QuickChange XL Site-Directed Mutagenesis Kit (#200516, Agilent) according to the manufacturer's protocol.

Cell lines

Ba/F3 cells were infected with lentiviral constructs, as reported previously (33), to express the *FGFR2::BICC1* fusion, this latter with or without *FGFR2* kinase domain mutations. Ba/F3 cells harboring the fusion were selected in the presence of blasticidin (14 µg/mL) and IL3 (0.5 ng/mL) until recovery, and a second selection by culturing the

cells in the absence of IL3. *FGFR2* fusion and *FGFR2* kinase domain mutations were confirmed on the established cell lines by Sanger sequencing. The cells were not tested for *Mycoplasma* contamination, but cells were not maintained in culture for more than 2 months after establishment or thawing.

Cell viability assays were performed in 96-well plates using the CellTiter Glo Luminescent Cell Viability Assay (G7570, Promega). We seeded 4,000 cells/well, and we treated cells for 48 hours. IC_{50} values were derived using GraphPad Prism software.

Reagents

Lirafugratinib was provided by Relay Therapeutics. Erdafitinib, infigratinib, fexagratinib, zoligratinib, derazantinib, and futibatinib were purchased from Selleck Chemicals. Pemigatinib and rogaratinib were purchased from MedChemExpress.

Development of patient-derived xenografts and *in vivo* pharmacologic studies

All animal procedures and studies have been approved by the French Ministry of "*Enseignement supérieur, de la Recherche et de l'Innovation*" (APAFIS#2790-2015112015055793 and APAFIS#2328-2015101914074846). Fresh tumor fragments were implanted in the subrenal capsule of 6-week-old female NOD/SCID gamma mice obtained from Charles River Laboratories. Patient-derived xenograft (PDX)–bearing NOD/SCID gamma mice were treated with the indicated doses of pemigatinib, erdafitinib, futibatinib, and lirafugratinib. Eight mice per group were treated for up to 50 days, and tumor volume and mouse weight were measured twice weekly.

Data availability

WES/RNA-seq raw data files from this study are deposited at the European Genome–phenome Archive (EGA) using the accession code EGAD50000000439. Access to this shared dataset is controlled by the institutional Data Access Committee, and requests for access can be sent to the corresponding author. Further information about EGA can be found at https://ega-archive.org/. Any additional information required to reanalyze the data reported in this article is available upon request from the corresponding author.

Results

Patient population and molecular treatments

We studied 36 patients with advanced solid tumors driven by *FGFR2*, all of whom were progressing on selective FGFR inhibitors (Supplementary Table S1). This cohort included 27 patients with intrahepatic cholangiocarcinoma and nine patients with various other tumor types: two with high-grade serous ovarian cancer, and one each with lung adenocarcinoma, urothelial cancer, triple-negative breast cancer, duodenal cancer, pancreatic ductal adenocarcinoma, adrenocortical carcinoma, and cancer of unknown primary. The majority, 31 patients, had tumors harboring *FGFR2* fusions, whereas five had tumors driven by *FGFR2* mutations located in the extracellular domain (specifically, three with FGFR2 C383R, one with FGFR2 S267P, and one with FGFR2 Y376C). *FGFR2* fusion partners included *BICC1* in five cases (all in intrahepatic cholangiocarcinomas), *TACC2* in three cases (n = 1 intrahepatic cholangiocarcinoma, n = 2 other tumor types), and *STRN4* and *CCSSER2* in two cases, one from each cohort. Other unique

fusion partners were found in the remaining 19 tumors, detailed in Supplementary Tables S2 and S3.

Twenty-three patients received a reversible FGFR inhibitor (n = 13 pemigatinib, n = 8 erdafitinib, n = 1 derazantinib, n = 1 zoligratinib), and 13 the irreversible inhibitor futibatinib (Supplementary Table S1). In the cholangiocarcinoma group, patients treated with reversible inhibitors and futibatinib showed 61% and 67% objective response rate, with median PFS of 8.7 and 11.1 months, respectively (Supplementary Table S4). Given the diversity in tumor origin, detailed clinical data of the non-cholangiocarcinoma patients are provided in Supplementary Table S3.

All patients underwent postprogression ctDNA analysis. Twenty-one of them had further molecular analyses performed on postprogression tissue biopsies, 16 via WES with or without RNA-seq, and five via targeted next-generation sequencing.

After disease progression on the first FGFR inhibitor, 12 patients received sequential targeted treatments. Eight patients with cholangiocarcinoma received futibatinib following a reversible inhibitor, with three receiving the mTOR inhibitor everolimus based on molecular findings. Two patients with cholangiocarcinoma and two with other tumor types, progressing respectively on pemigatinib and futibatinib, were switched to the FGFR2-selective inhibitor lirafugratinib (Supplementary Table S1).

Molecular alterations observed at resistance to selective FGFR inhibitors

In order to better approach the specificities of resistance mechanisms to reversible inhibitors versus the irreversible inhibitor futibatinib, and between intrahepatic cholangiocarcinoma and other tumor types, we separated below the different groups of patients analyzed at progression after a first FGFR inhibitor.

Intrahepatic cholangiocarcinoma on reversible inhibitors

The molecular alterations detected in 17 patients with intrahepatic cholangiocarcinoma progressing on reversible inhibitors are reported in **Fig. 1A**. Polyclonal kinase domain mutations (≥2 *FGFR2* mutations in the same blood sample) were detected in 10 of these patients (59%).

In three additional patients, a single *FGFR2* mutation was detected either in the tissue biopsy (**MR408** and **MR313**) or in ctDNA (**MR822**; FGFR2 N550T). Specifically, FGFR2 D651H was detected in both pre- and posttreatment biopsies of one patient (**MR313**). Another unique case (**MR488**) had two concurrent *FGFR2* kinase domain mutations in a single tissue biopsy (E566A and K642R). Concurrent pathogenic alterations in the MAPK and PI3K/mTOR pathways, suggesting off-target resistance mechanisms, were observed in five patients.

Other tumor types on reversible inhibitors

Five patients with non-cholangiocarcinoma *FGFR2*-driven tumors exhibited diverse resistance patterns after initial tumor shrinkage (**Fig. 1B**).

FGFR2 kinase domain mutations were detected in three of these patients, with one exhibiting polyclonal mutation. The latter (patient **ST1056**) was a *FGFR2::TACC2* rearranged lung adenocarcinoma that progressed on erdafitinib with FGFR2 N550K, V565L/F, C632Y, D651Y mutations, as well as KRAS G12A (**Fig. 1C**; Supplementary Fig. S1).

In two patients, no *FGFR2* kinase domain mutations aroused at progression to erdafitinib (**MR1035**, cancer of unknown primary; **ST238**, triple-negative breast cancer, **Fig. 1D**), but *KRAS/PIK3CA* and *HRAS/KRAS* mutations were detected in ctDNA at progression, respectively (**Fig. 1B**).
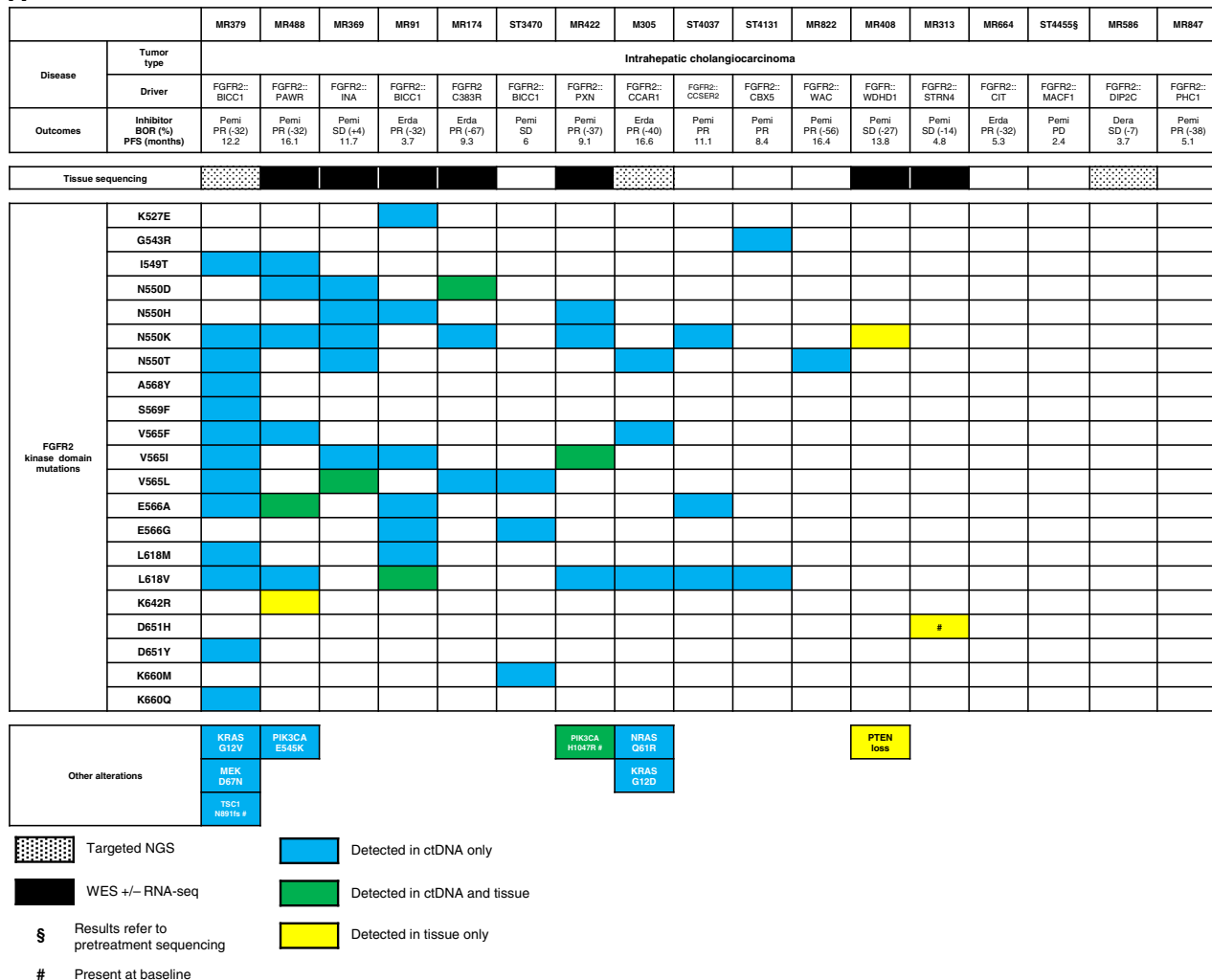
Figure 1.

Molecular findings at resistance to reversible FGFR inhibitors. **A,** Patients suffering from intrahepatic cholangiocarcinoma. *(Continued on the following page.)*

Intrahepatic cholangiocarcinoma on futibatinib

Among nine patients with intrahepatic cholangiocarcinoma progressing on futibatinib, fewer *FGFR2* kinase domain mutations were observed than in those on reversible inhibitors, involving key regions like the molecular brake (N550) and gatekeeper (V565; **Fig. 2A**). Only three patients had polyclonal mutations, which were limited to N550K and V565F/L/Y.

One patient (**MR553**) showed pretreatment *FGFR2* kinase domain mutations that disappeared during response and re-emerged at progression (**Fig. 2B**; Supplementary Fig. S2).

Interestingly, the patient **MR332** first experienced an isolated bone progression, whose biopsy revealed a FGFR2 V565L mutation (not detectable in blood), followed by a hepatic progression harboring a FGFR2 V565F mutation (**Fig. 2C**).

Other tumor types on futibatinib

Four patients with various tumor types showed resistance mechanisms to futibatinib (**Fig. 2D**), including a patient with duodenal cancer (**MR1271**) who exhibited a monoclonal FGFR2 V565L mutation concurrent with progression in the lung and liver (see **Fig. 5C**).

Global analysis of candidate resistance mechanisms

Comparing the spectrum of putative resistance mechanisms occurring in *FGFR2*-driven cholangiocarcinoma or other tumor types, we hypothesized that the two entities converged toward overlapping ways to escape targeted FGFR2 inhibition. We therefore pooled the molecular data of the two populations to allow a global view on resistance to a first FGFR inhibitor among *FGFR2*-driven tumor types (**Fig. 3**).

Across the 36 patients, 14 residues in the FGFR2 kinase domain (K527, G543, I549, N550, L551, A568, S569, V565, E566, L618, C623, K642, D651, and K660) were found mutated at progression. For six of them (N550, V565, E566, L618, D651, and K660), at least two possible substitutions were observed, thus representing 24 possible mutations (**Fig. 3A**). FGFR2 C623Y and L551F were the
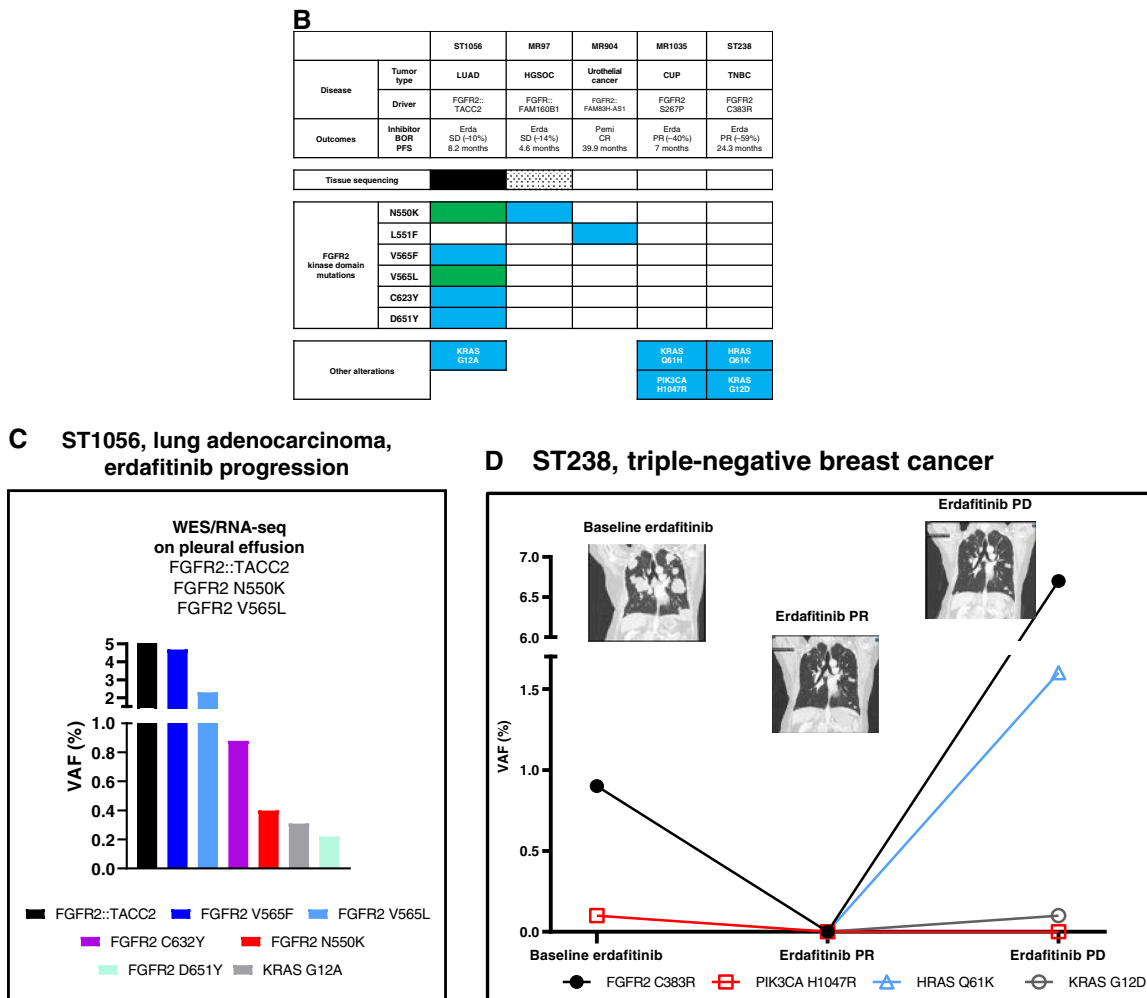
B

		ST1056	MR97	MR904	MR1035	ST238
Disease	Tumor type	LUAD	HGSOC	Urothelial cancer	CUP	TNBC
	Driver	FGFR2::TACC2	FGFR::FAM160B1	FGFR2::FAM83H-AS1	FGFR2 S267P	FGFR2 C383R
Outcomes	Inhibitor BOR PFS	Erda SD (–10%) 8.2 months	Erda SD (–14%) 4.6 months	Pemi CR 39.9 months	Erda PR (–40%) 7 months	Erda PR (–59%) 24.3 months

Tissue sequencing					

FGFR2 kinase domain mutations	N550K					
	L551F					
	V565F					
	V565L					
	C623Y					
	D651Y					

Other alterations		KRAS G12A			KRAS Q61H / PIK3CA H1047R	HRAS Q61K / KRAS G12D

C ST1056, lung adenocarcinoma, erdafitinib progression

D ST238, triple-negative breast cancer

Figure 1.

(*Continued.*) **B,** Patients suffering from other tumor types. **C,** Molecular findings of patient ***ST1056***, suffering from a lung adenocarcinoma harboring a *FGFR2::TACC2* fusion, at acquired progression to erdafitinib. **D,** Clinicoradiologic and molecular evolution of patient ***ST238***, suffering from a FGFR2 C383R–driven triple-negative breast cancer. The ctDNA findings are reported, and ctDNA findings are reported as VAF (%). BOR, best objective response; CR, complete response; CUP, cancer of unknown primary; Dera, derazantinib; Erda, erdafitinib; HGSOC, high-grade serous ovarian cancer; LUAD, lung adenocarcinoma; PD, progressive disease; Pemi, pemigatinib; PR, partial response; SD, stable disease; TBNC, triple-negative breast cancer.

only mutations found exclusively in non-cholangiocarcinoma cases (**Fig. 1B**). FGFR2 C623Y has not previously been reported, whereas L551F has been described in the setting of cholangiocarcinoma progressing on infigratinib (19).

Polyclonal kinase domain mutations were detected in half (11/22) of the patients progressing on reversible inhibitors, almost exclusively with cholangiocarcinoma, whereas only three patients (23%) revealed polyclonal *FGFR2* mutations after futibatinib (**Fig. 3B** and **C**). When assessable due to amplicon sizes, the polyclonal *FGFR2* kinase domain mutations were always detected *in trans* (i.e., on different alleles).

Overall, 61 *FGFR2* kinase domain mutations were detected after reversible inhibitors, whereas only nine mutations were observed after futibatinib (**Fig. 3A**). The most frequently mutated residues were the molecular brake N550 and the gatekeeper V565.

In 13/22 (59%) of the patients progressing on reversible inhibitors, at least one mutation affecting either of these two residues was

found, whereas the two residues were comutated in eight cases (36%). N550 and V565 were also the residues with the highest number of different substitutions, as N550D/H/K/T and V565F/I/L. L618V/M occurred in nine cases, followed by E566 (E566A/G) that was mutated in six patients.

In contrast, the N550 and V565 residues were the unique sites of *FGFR2* kinase domain mutations found at progression to futibatinib, namely N550K (n = 2), V565F (n = 2), V565L (n = 4), and V565Y (n = 1; **Figs. 2A** and **3A**). Of note, the mentioned molecular brake and gatekeeper mutations have been previously reported, with the exception of FGFR2 V565Y (34). This mutation is a novel entity, emerging from a double-base substitution in the corresponding valine codon GTT, for which we hypothesize the sequential occurrence of single-nucleotide substitutions, from V565F (F being coded by the codon TTT) to V565Y (Y being coded by TAT).

Off-target mutations that are potentially implicated in resistance, such as those affecting the MAPK (i.e., *HRAS*, *KRAS*,
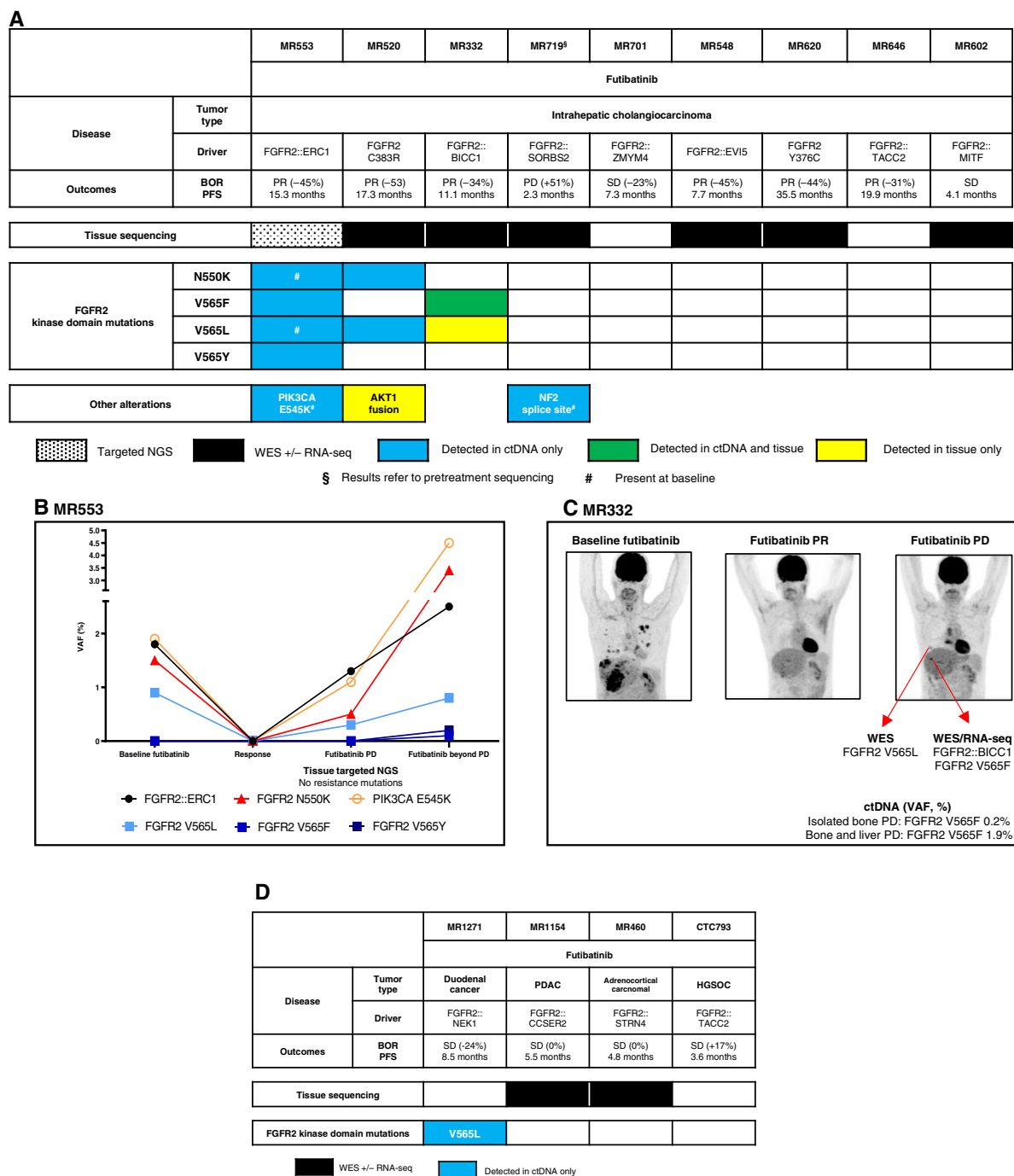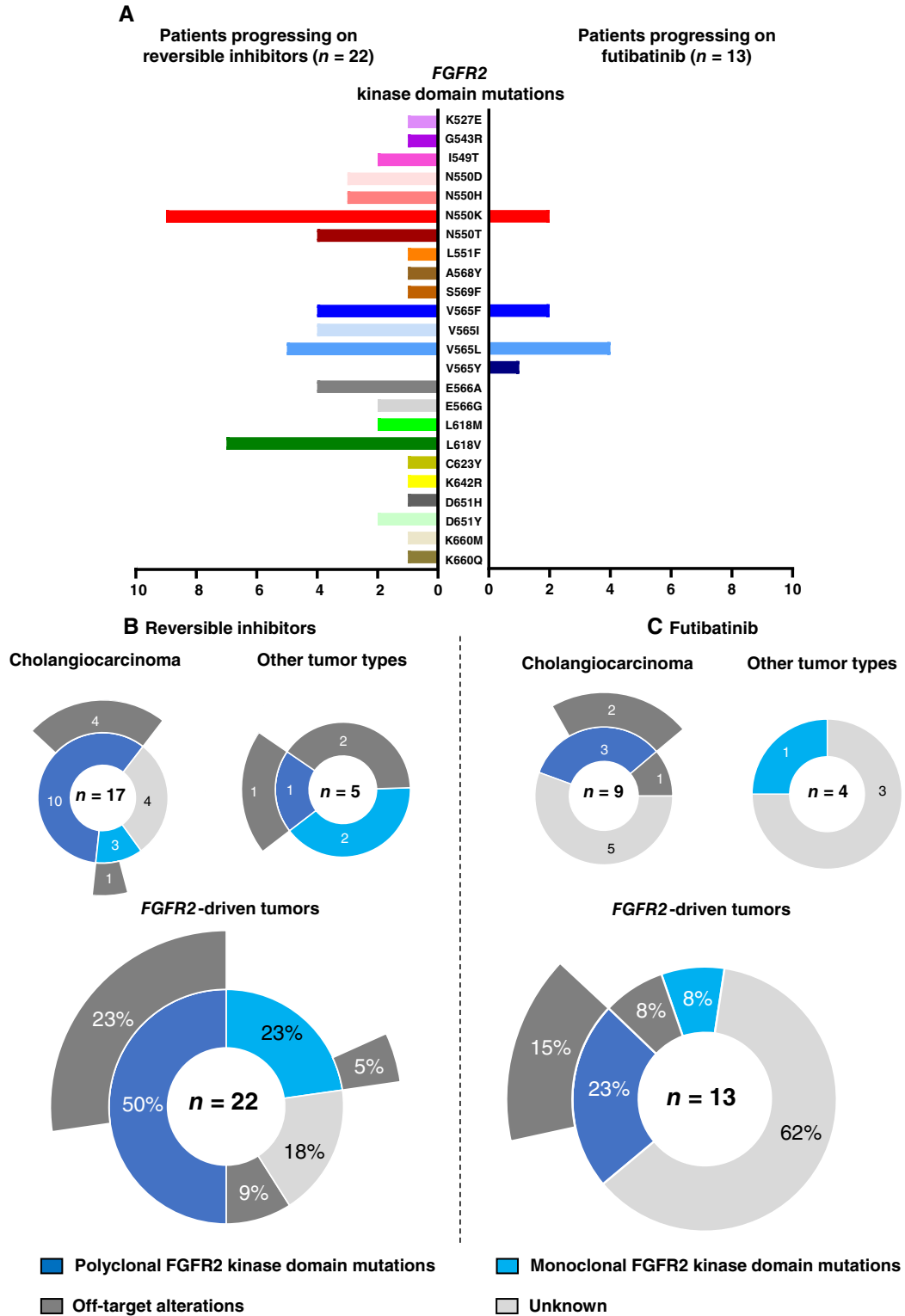
Figure 2.
Molecular findings at resistance to the irreversible FGFR inhibitor futibatinib. **A,** Patients suffering from intrahepatic cholangiocarcinoma. **B,** Molecular evolution of patient **MR553**, suffering from an intrahepatic cholangiocarcinoma harboring a *FGFR2::ERC1* fusion. **C,** Clinicoradiologic and molecular evolution of patient **MR332**, suffering from an intrahepatic cholangiocarcinoma driven by a *FGFR2::BICC1* fusion. **D,** Patients suffering from other tumor types. The ctDNA findings are reported as VAF (%). BOR, best objective response; HGSOC, high-grade serous ovarian cancer; PD, progressive disease; PDAC, pancreatic ductal adenocarcinoma; PR, partial response; SD, stable disease.

NRAS, and *MEK*) and PI3K/mTOR pathways (i.e., *PIK3CA*, *PTEN*, and *TSC1*), were found in 8/22 (36%) and 3/13 (23%) cases progressing on reversible inhibitors and futibatinib, respectively (**Figs. 1A**, **B**, **2A**, **3B**, and **C**). In eight patients (23%), these mutations co-occurred with *FGFR2* kinase domain mutations. In two patients with other tumor types progressing on erdafitinib, they emerged without concomitant on-target alterations (**Fig. 1B**).

Figure 3.
Global view on candidate resistance mechanisms to FGFR inhibitors across *FGFR2*-driven malignancies. **A,** Spectrum of FGFR2 kinase domain mutations detected across patients progressing to reversible inhibitors and futibatinib. **B,** Overview of the molecular alterations found at progression to reversible inhibitors in cholangiocarcinoma and other tumor types (top), and pooled across all cases. **C,** Overview of the molecular alterations found at progression to the irreversible inhibitor futibatinib in cholangiocarcinoma and other tumor types (top), and pooled across all cases.

Pooling the molecular data obtained at progression, we observed molecular candidates for resistance in 77% and 38% of the patients progressing on reversible inhibitors and futibatinib, respectively (**Fig. 3B** and **C**).

Sequential treatment strategies

Within our study cohort, 33% of the patients ($n = 12$, including 10 with intrahepatic cholangiocarcinoma and one each from pancreatic and duodenal cancers) underwent sequential targeted therapy regimens (with longitudinal sampling) that included irreversible FGFR inhibitors and the mTOR inhibitor everolimus.

Sequential treatments including futibatinib and everolimus

After progressing on reversible inhibitors, three patients with intrahepatic cholangiocarcinoma received a sequential treatment with futibatinib and everolimus based on specific molecular findings (**Fig. 4A**).

Patient **MR379** suffered from a tumor harboring both *FGFR2::BICC1* fusion and a *TSC1* frameshift mutation in the baseline tissue sample. At progression to pemigatinib, the two alterations were found in ctDNA together with 13 different *FGFR2* kinase domain mutations, *KRAS* and *MEK1* mutations (**Fig. 4B**). Futibatinib did not induce any clinical benefit (stable disease; PFS 2.7 months), whereas everolimus, administered due to the *TCS1* loss-of-function alteration, led to stable disease with a PFS of 7.6 months. The clinical benefit was accompanied by the reduction of the allele frequencies of all the alterations in ctDNA (**Fig. 4B**).

Patient **MR422** experienced oligo-progression while on pemigatinib. A liver biopsy revealed a new FGFR2 V565I mutation alongside the preexisting *FGFR2::PNX* fusion and PIK3CA H1047R mutation (**Fig. 4A** and **C**). Despite continued progression, pemigatinib treatment was extended, resulting in increased variant allele frequency (VAF) of PIK3CA H1047R, FGFR2 V565I, N550H, and N550K mutations. Everolimus, initiated due to the *PIK3CA* mutation, provided stable disease for 11 months, corresponding to a decrease in the VAF of the documented mutations. Their VAF increased again at everolimus progression, and subsequent futibatinib administration allowed the achievement of disease stabilization with a PFS of 7.8 months.

Patient **MR408** progressed to pemigatinib with an isolated lung nodule showing a FGFR2 N550K mutation and *PTEN* loss. Although everolimus showed no clinical activity, subsequent futibatinib treatment led to tumor shrinkage and a PFS of 7.2 months, despite the baseline documentation of a FGFR2 L618V mutation (Supplementary Fig. S3).

Sequential treatments with reversible FGFR inhibitors followed by futibatinib

Five additional patients with *FGFR2*-driven cholangiocarcinoma were treated with futibatinib after experiencing resistance to reversible FGFR inhibitors (**Fig. 4D**).

Two patients showed clinical benefit from futibatinib (**MR586** and **ST1748**). Upon progression on futibatinib, FGFR2 V565F/L mutations were identified in patient **ST1748**, consistent with mutations typically seen in FGFR inhibitor–naïve patients. Three other patients experienced primary resistance to futibatinib after acquired resistance to reversible inhibitors. Of notice, FGFR2 N550D/K and V565L were present at futibatinib baseline in *MR174,* and could explain its lack of benefit (**Fig. 4E**).

Sequential treatments with lirafugratinib

Four patients received lirafugratinib after progressing on a previous inhibitor (pemigatinib or futibatinib), without other intervening therapies (**Fig. 5A**).

Lirafugratinib outcomes were divergent among the two patients with *FGFR2*-rearranged cholangiocarcinoma progressing on pemigatinib

Patient **MR822**, with a prolonged initial response to pemigatinib, developed an FGFR2 N550T mutation along with a persistent driver fusion *FGFR2::WAC*. Lirafugratinib treatment resulted in another prolonged response, highlighting its effectiveness against this specific mutation (Supplementary Fig. S4A). Patient **ST3470** encountered primary progression on lirafugratinib despite no detectable *FGFR2* kinase domain mutations in ctDNA, suggesting an alternative resistance mechanism (**Fig. 5B**). Importantly, three FGFR2 mutations V565L, E566G, and K660M present before lirafugratinib were lost at progression, suggesting their sensitivity to lirafugratinib.

Two other patients suffering from tumors other than cholangiocarcinoma also benefited from lirafugratinib after futibatinib progression (**Fig. 5A**).

Patient **MR1271** suffered from a FGFR2::NEK1–driven duodenal carcinoma, who progressed on futibatinib with the acquisition of FGFR2 V565L in ctDNA, and the major disease response was observed with lirafugratinib (**Fig. 5C**).

Patient **MR1154** suffered from a pancreatic carcinoma harboring *FGFR2::CCSER2* fusion. No molecular events potentially implicated in resistance to futibatinib were detected, but *FGFR2::CCSER2* VAF was no longer detectable 3 weeks after lirafugratinib initiation (Supplementary Fig. S4B).

FGFR2 kinase domain mutations exert a differential spectrum of resistance according to selective FGFR inhibitors

To explore how specific *FGFR2* kinase domain mutations affect resistance to FGFR inhibitors, we used 18 Ba/F3 cell lines, engineered to express the *FGFR2::BICC1* fusion with various secondary mutations. *FGFR2::BICC1* was chosen, being the most frequent fusion observed in our cohort and in other series (35). We exposed each Ba/F3 cell line to increasing concentrations of seven selective, reversible FGFR inhibitors and to the irreversible agents futibatinib and lirafugratinib, in order to establish their IC_{50} values (**Fig. 6A** and **B**; Supplementary Fig. S5).

In our experiments, erdafitinib emerged as the most potent inhibitor across all mutants, followed closely by infigratinib and futibatinib, achieving sub-nanomolar IC_{50} values against the wild-type FGFR2::BICC1 Ba/F3 cell line (**Fig. 6A** and **B**).

The profiles of sensitivity and resistance conferred by individual *FGFR2* kinase domain mutations matched with the spectrum of mutations emerging in patients treated with either reversible agents or futibatinib, respectively (**Figs. 3A, 6A,** and **B**). FGFR2 D651H did not confer resistance to any of the inhibitors, suggesting its role as a passenger event in patient **MR313**.

A significant finding from our study was the variable resistance patterns conferred by mutations at key residues within FGFR2, notably the molecular brake N550 and the gatekeeper V565. Mutations at these sites—N550K and V565F/L/Y—broadly conferred resistance across several reversible inhibitors, casting doubt on the effectiveness of using these drugs sequentially in patients with these mutations. However, other mutations like N550T and V565I seemed to result in a lesser degree of

A

Patient	Baseline tissue	Reversible inhbitor BOR PFS	Postprogression tissue analysis	Postprogression ctDNA	Second molecular therapy	Outcomes BOR PFS	Postprogression ctDNA	Third molecular therapy	Outcomes BOR PFS	Postprogression ctDNA
MR379	FGFR2::BICC1 TSC1 N891fs	Pemigatinib PR (−32%) 12.2 months	Tissue NGS No resistance mutations	FGFR2::BICC1 5.4% FGFR2 I549T 0.3% FGFR2 N550K 0.1% FGFR2 N550T 0.1% FGFR2 V565F 1% FGFR2 V565I 1.8% FGFR2 V565L 0.3% FGFR2 E566A 2.7% FGFR2 A568Y 0.1% FGFR2 S569F 0.6% FGFR2 L618M 0.6% FGFR2 L618V 0.4% FGFR2 D651Y 0.2% FGFR2 K660Q 0.6% KRAS G12V 0.1% MEK1 D67N 0.7% TSC1 N891fs 14.7%	Futibatinib	PD 2.7 months	FGFR2::BICC1 5.1% FGFR2 I549T 0..2 FGFR2 N550K 0.1% FGFR2 N550T 0.2% FGFR2 V565I 1.3% FGFR2 V565F 0.8% FGFR2 E566A 2.5% FGFR2 A568Y 0.1% FGFR2 S569F 0.1% FGFR2 L618M 1.8% FGFR2 L618V 0.5% FGFR2 K642R 0.1 FGFR2 K660Q 0.7% MEK1 D67N 0.5% TSC1 N891fs 16.3%	Everolimus	SD 7.6 months	Not performed
MR422	FGFR2::PXN FGFR2 H1047R	Pemigatinib PR (−37%) 9.1 months	WES/RNA-seq FGFR2::PNX FGFR2 V565I PIK3CA H1047R	FGFR2 N550H 0.1% FGFR2 N550K 1.2% FGFR2 V565I 0.4% FGFR2 L618V 0.1% PIK3CA H1047R 3%	Everolimus	SD 11 months	FGFR2 N550H 0.5% FGFR2 N550K 0.6% FGFR2 V565I 0.2% PIK3CA H1047R 4.4%	Futibatinib	SD (0%) 7.8 months	Not performed
MR408	FGFR2::WDHD1	Pemigatinib SD (−27%) 13.8 months	WES FGFR2 N550K PTEN loss	Nothing detected	Everolimus	PD 3 months	#FGFR2::WDHD1 0.7% FGFR2 L618V 0.2%	Futibatinib#	SD (−10%) 7.2 months	FGFR2::WDHD1 3.9% FGFR2 L618V 0.3%

In patient **MR408**, the interval time between everolimus and futibatinib was one year and three months, during which two chemotherapy lines were administered. The ctDNA data correspond to futibatinib baseline.

B MR379

C MR422
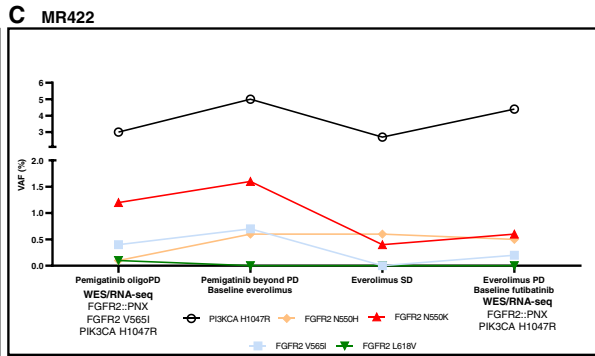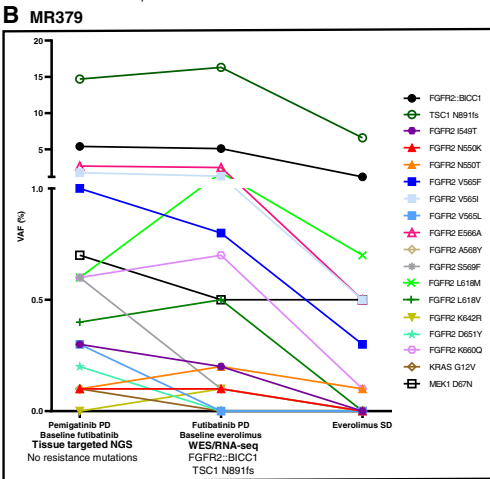
D

Patient	Baseline tissue	Baseline ctDNA	Reversible inhbitor BOR PFS	Postprogression tissue analysis	Postprogression ctDNA	Intervevening therapy, timeline	Futibatinib outcomes BOR PFS	Postprogression ctDNA
ST1748	FGFR2 fusion	Not performed	Zoligratinib SD (−22%) 2.9 months	Not performed	Not performed	One chemo line 6 months	SD (+1%) 11.6 monts	FGFR2::BICC1 41.8% FGFR2 V565F 0.2% FGFR2 V565L 1.1 %
MR586	FGFR2::DIP2C	FGFR2::DIP2C 0.3%	Derazantinib SD (−7%) 3.7 months	WES/RNA-seq FGFR2::DIP2C	FGFR2-DIP2C 4.2%	Two chemo lines 7 months	SD (0%) 8.6 months	
MR313	FGFR2::STRN4 FGFR2 D651H	FGFR2::CTNNA3 0.1%	Pemigatinib SD (−14%) 4.8 months	WES/RNA-seq FGFR2::STRN4 FGFR2 D651H	Nothing detected (D651 not covered)	No	PD 2.7 months	
MR174	FGFR2 C383R	FGFR2 C383R 3.2%	Erdafitinib PR (−67%) 9.3 months	WES FGFR2 C383R FGFR2 N550D	FGFR2 C383R 12.2% FGFR2 N550D 2.8% FGFR2 N550K 0.3% FGFR2 V565L 1.8%	No	PD 1.6 months	
MR664	FGFR2::CIT	FGFR2::CIT 2.3%	Erdafitinib PR (−32%) 5.3 months	Not performed	FGFR2::CIT 3.6% (increasing to 8%)	No	PD 1 month	

E MR174
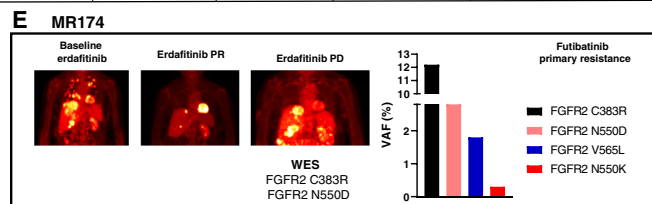
Figure 4.

Clinical and molecular evolution of patients with *FGFR2*-driven intrahepatic cholangiocarcinoma receiving sequential targeted treatments including futibatinib and everolimus. **A,** Three patients received a sequential treatment of futibatinib and everolimus, with the latter administered given the molecular finding of alterations in the PI3K/mTOR pathway. **B,** Molecular evolution of patient **MR379**, suffering from an *FGFR2::BICC1*–driven disease, with a concomitant pathogenic *TSC1* frameshift mutation. **C,** Molecular evolution of patient **MR422**, suffering from a *FGFR2*-rearranged disease, with a concomitant PIK3CA H1047R mutation. **D,** Additional five patients received a sequence of reversible FGFR inhibitor followed by futibatinib. **E,** Clinicoradiologic and molecular evolution of patient **MR174**, suffering from a FGFR2 C383R–driven disease. The ctDNA findings are reported as VAF (%). BOR, best objective response; Chemo, chemotherapy; PD, progressive disease; PR, partial response; SD, stable disease.

resistance. Interestingly, zoligratinib demonstrated relatively lower IC_{50} values against V565F/Y mutations than those against V565I/L, indicating specific interactions between the inhibitor and variant amino acids at this site.

The irreversible inhibitors generally showed superior activity compared with reversible agents in our Ba/F3 models. Both futibatinib and lirafugratinib exhibited efficacy within the 2 to 20 nmol/L range against various mutations, although they displayed slight differences in activity against certain mutations. Notably, lirafugratinib was particularly effective against FGFR2 V565F/Y mutations, in which futibatinib showed reduced activity due to steric hindrances from bulky amino acids like phenylalanine and tyrosine (IC_{50} > 200 nmol/L; refs. 25, 36). In contrast, the two irreversible inhibitors showed an opposite profile of activity against FGFR2 V565I and V565L mutants, with futibatinib being more active on V565I and lirafugratinib on V565L. The slight difference in the tridimensional structure between leucine (V565L) and isoleucine (V565I) likely explains the activity of the two inhibitors. In addition, FGFR2 V565I is known to increase basal activity of the kinase domain (37), and the higher potency of futibatinib could prevail in this setting.

The two irreversible inhibitors maintained an IC_{50} in the 10 to 20 nmol/L range for FGFR2 N550K, the most common mutation arising after a reversible inhibitor. Because FGFR2 N550K occurred in two patients progressing on futibatinib, and in five patients the gatekeeper FGFR2 V565L emerged (**Figs. 2A** and **4D**), we suppose that these two mutations cannot be overcome with clinically achievable concentrations of the agent.

In order to further validate our *in vitro* preclinical analyses on more clinically relevant models *in vivo*, we established PDXs from biopsies of patients with cholangiocarcinoma, collected at the time of acquired resistance to FGFR inhibitors. We treated three PDX models with pemigatinib, erdafitinib, futibatinib, and lirafugratinib (**Fig. 6C**). The FGFR2 N550D mutation in ***MR174*** PDX, established at progression to erdafitinib, could be overcome by futibatinib and lirafugratinib. In addition, a dose effect was noticed for lirafugratinib, with tumor growth abrogated only at the dose of 60 mg/kg (and not 20 mg/kg), which is in line with the IC_{50} observed in the Ba/F3 models. ***MR369*** PDX, established at progression to pemigatinib, harbored a FGFR2 V565L mutation, whereas ***MR332*** PDX, established at futibatinib resistance, harbored FGFR2 V565F (see **Fig. 2C**). In both cases, only lirafugratinib (even at low doses) was able to prevent tumor growth in this *in vivo* model, confirming our suggestion that gatekeeper mutations FGFR2 V565L/F can be difficult to overcome with futibatinib in the clinical setting, while retaining sensitivity to lirafugratinib.

Of note, two of the PDX models (***MR174*** and ***MR369***) were established from patients progressing with polyclonal *FGFR2* kinase domain mutations detected in ctDNA, but only one mutation was found in the corresponding tissue biopsy and PDX (**Fig. 6C**). These observations underscore the limitation of tissue biopsies to fully recapitulate the molecular spectrum of heterogeneity observed in patients at resistance.

Discussion

FGFR inhibition in *FGFR2*-driven malignancies marks a significant advance in precision oncology, emphasizing the need to understand molecular mechanisms behind drug resistance to develop new treatment strategies. Our study integrates extensive clinical and molecular data, along with *in vitro* and *in vivo*

validation assays, to explore resistance mechanisms in patients with *FGFR2*-driven cancers, including cholangiocarcinoma and other tumor types.

Our findings confirm that polyclonality of *FGFR2* kinase domain mutations is commonly observed in ctDNA from patients at progression on reversible inhibitors, particularly in cholangiocarcinoma (18–20). Indeed, *FGFR2* kinase domain mutations were undetectable or found as isolated entities in tissue analyses, compared with multiple alterations in ctDNA, highlighting the "polyclonal" nature of tumor progression and the fundamental role of liquid over tissue biopsy. Interestingly, such mutations were less common after treatment with the irreversible inhibitor futibatinib, which primarily affected the molecular brake N550 and gatekeeper V565 residues.

Recently, Wu and colleagues (21) gathered data on resistance mechanisms in patients with *FGFR2*-driven cholangiocarcinoma, pooling evidence from published articles and meeting abstracts. In our study, we were able to differentiate between resistance to reversible inhibitors and futibatinib, providing clinical proof to their functional observations. As predicted by their evaluation of clinically achievable doses of futibatinib, in our cohort, FGFR2 N550K frequently emerged at progression to the irreversible agent. Interestingly, we did not detect any mutation in the binding site for irreversible inhibitors (FGFR2 C492), in line with the reduced cellular fitness caused by these mutations, which somehow suggests their limited frequency of occurrence, such as the FGFR2 C492F found in the patient reported by Berchuck and colleagues (32). On the other hand, FGFR2 V565L, labeled by Wu and colleagues as sensitive to futibatinib, was the mutation most frequently observed at progression to the irreversible inhibitor in our cohort.

Similarly, resistance mechanisms in tumors other than cholangiocarcinoma mirrored those observed in cholangiocarcinoma, involving known *FGFR2* residues and off-target resistance mechanisms. If considering the nine patients with other tumor types in our cohort, together with the report from Nicolò and colleagues (ref. 28; FGFR2 V565L detected at pemigatinib progression in a patient with breast cancer), the emergence of polyclonal *FGFR2* mutations was limited to only one patient with a lung adenocarcinoma progressing on erdafitinib (**Fig. 1C**). More recently nevertheless, Rodón and colleagues (15) detected polyclonal *FGFR2* mutations in two patients with non-cholangiocarcinoma tumors progressing on pemigatinib. Nevertheless, the overall small number of patients with *FGFR2*-driven other tumor types evaluated at resistance challenges the conclusion that the propensity of developing polyclonal *FGFR2* mutations is a feature more common in *FGFR2*-driven cholangiocarcinoma.

Furthermore, in concomitance with *FGFR2* kinase domain mutations or not, alterations in genes of the PI3K/mTOR pathway were frequently present at progression to reversible inhibitors and futibatinib. Interestingly, in three patients, clinical benefit was obtained from reversible FGFR inhibitors or futibatinib, despite the presence of PI3K/mTOR alterations at baseline (***MR379***, ***MR422***, and ***MR553***), which were maintained at progression (**Figs. 1A** and **2A**). In line with our clinical observations, Wu and colleagues (21) recently reported that the PI3KCA E545K mutation does not impact futibatinib sensitivity in the context of *FGFR2*-driven cholangiocarcinoma cell lines.

Here, the mTOR inhibitor everolimus provided clinical benefit in two patients with alterations in *TSC1* or *PIK3CA* at progression to FGFR inhibition. The reduction in VAF of concomitant *FGFR2*

A

Patient	Tumor type	Baseline tissue	Baseline ctDNA	First FGFR inhibitor BOR PFS	Post-progression tissue analysis	Post-progression ctDNA	Outcomes on lirafugratinib BOR PFS
MR822	Intrahepatic cholangiocarcinoma	FGFR2::WAC	FGFR2::WAC 0.6% PIK3CA G106V 0.1%	Pemigatinib PR (−56%) 16.4 months	Not performed	FGFR2::WAC 0.3% FGFR2 N550T 0.5%	PR (−53%) 16.8 months
ST3470		FGFR2::BICC1	Not performed	Pemigatinib SD 6 months	Not performed	FGFR2::BICC1 38.7% FGFR2 V565L 0.5% FGFR2 E566G 0.2% FGFR2 K660M 1.8%	PD 1.9 months
MR1271	Duodenal cancer	FGFR2::NEK1	FGFR2::NEK1 0.4%	Futibatinib SD (−24%) 8.5 months	Not performed	FGFR2::NEK1 2.4% FGFR2 V565L 8.5%	PR (−40%) 3.9 months
MR1154	Pancreatic ductal adenocarcinoma	FGFR2:CCSER2	FGFR2::CCSER2 0.2%	Futibatinib SD (0%) 5.5 months	WES/RNA-seq FGFR2::CCSER2	FGFR2::CCSER2 1%	PR (−32%) 5.5 months

B ST3470, intrahepatic cholangiocarcinoma
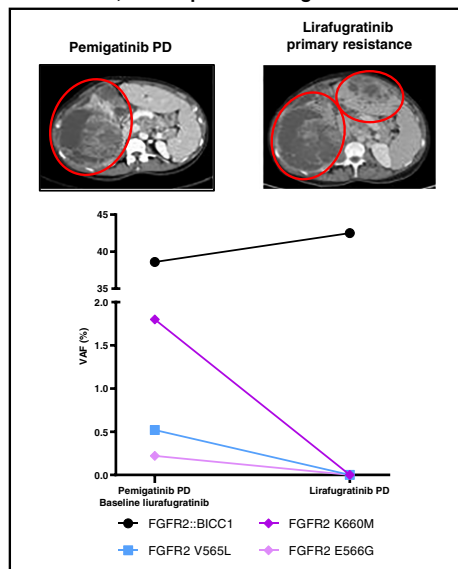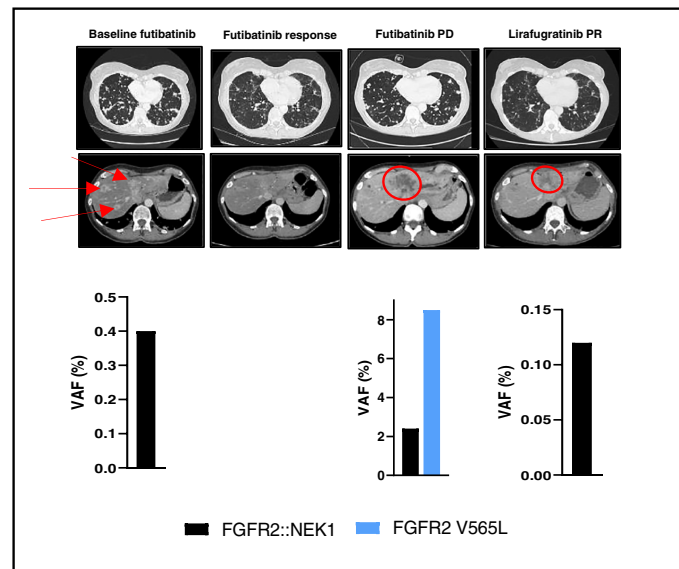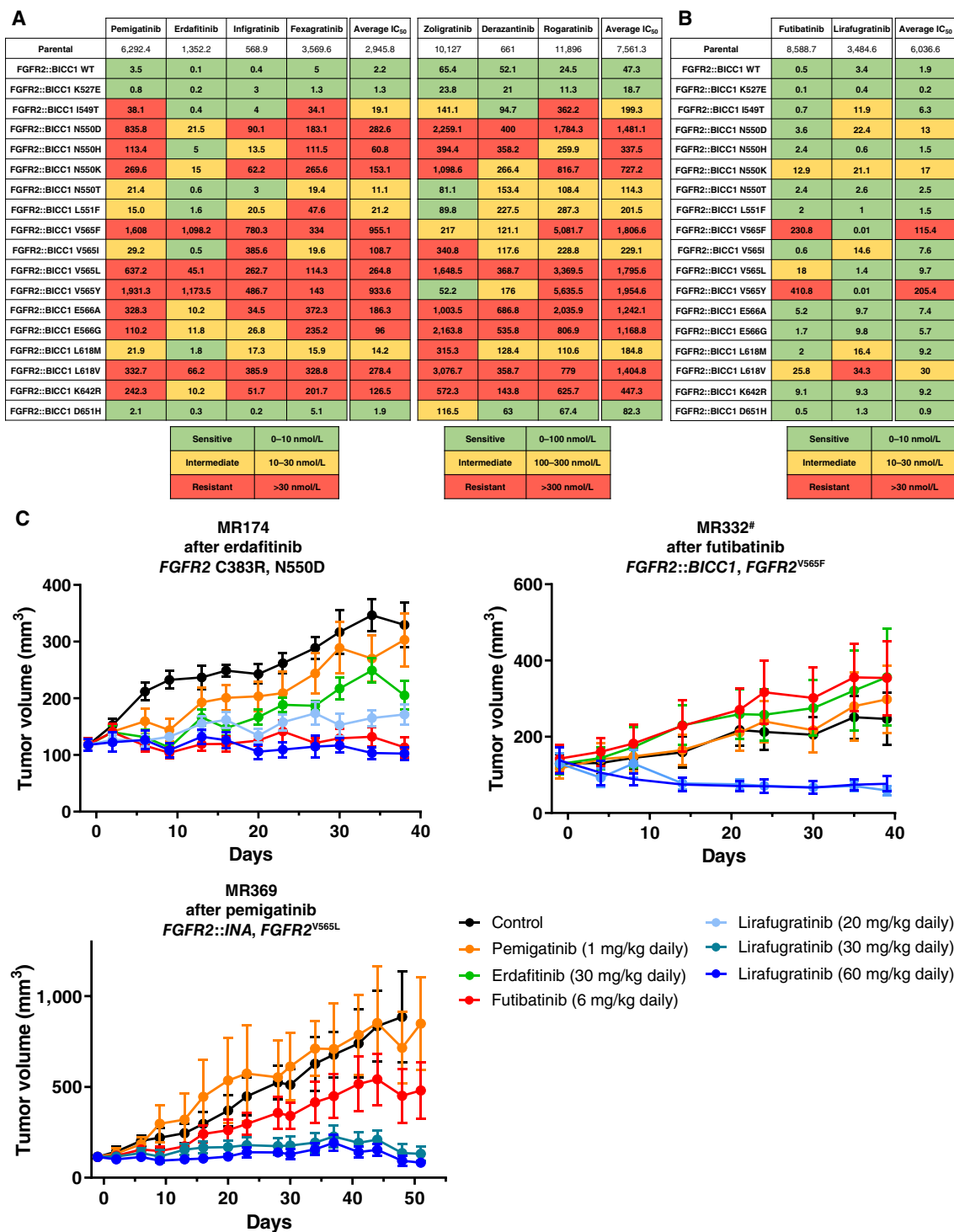
C MR1271, duodenal cancer

Figure 5.
Clinical and molecular evolution of patients receiving a first FGFR inhibitor followed by the irreversible, highly selective FGFR2 inhibitor lirafugratinib. **A,** Four patients harboring a *FGFR2* fusion received lirafugratinib as a second FGFR inhibitor. None received intercurrent treatment between the FGFR inhibitors. **B,** Clinicoradiologic and molecular evolution of patient **ST3470**, with a cholangiorcarcinoma driven by *FGFR2::BICC1* progressing on pemigatinib. **C,** Patient **MR1271** had duodenal cancer progressing on futibatinib with emergence of FGFR2 V565L mutation that was cleared by lirafugratinib. The ctDNA findings are reported as VAF (%). BOR, best objective response; PD, progressive disease; PR, partial response; SD, stable disease.

kinase domain mutations during everolimus treatment suggests that the on-target alterations probably emerged as an early event in a tumor clone already harboring the corresponding *TSC1* or *PI3KCA* mutations (**Fig. 4B** and **C**). It therefore seems that the loss of function of *TSC1* and the activation of *PIK3CA* do not represent the *bona fide* molecular mechanisms responsible for resistance to FGFR inhibitors, still explaining the clinical benefit from everolimus.

The enrichment in MAPK pathway alterations in a setting similar to ours has been recently reported by DiPeri and colleagues (27) in cholangiocarcinoma. Whether these mutations can be overcome by combination treatments in the clinical setting is still to be proven, as according to their data, only an *in vitro* synergistic effect of FGFR/MEK inhibition was achieved, with no meaningful effect in the *in vivo* model.

In the present study, irreversible inhibitors were also administered after progression to reversible ones in one third of the patients. We integrated the case-by-case analysis of the clinical response in the presence of precise *FGFR2* kinase domain mutations, with the dynamics of resistance mutations in ctDNA during treatment sequencing, and exploring functional data in Ba/F3 cellular models and matched PDX models. Nevertheless, the unique complexity of *FGFR2*-driven tumors at progression to reversible inhibitors, in terms of high levels of molecular heterogeneity, hampers the definition of precise patterns of resistance suitable for the sequential treatment with an irreversible agent. This is in contrast with single on-target mutations in *EGFR*- and *ALK*-driven lung cancer, overcome by the respective third generation inhibitors in the clinical setting (38, 39). In our cohort and in line with other reports (6, 40), in case of progression to a first FGFR inhibitor (reversible or

A

	Pemigatinib	Erdafitinib	Infigratinib	Fexagratinib	Average IC$_{50}$	Zoligratinib	Derazantinib	Rogaratinib	Average IC$_{50}$
Parental	6,292.4	1,352.2	568.9	3,569.6	2,945.8	10,127	661	11,896	7,561.3
FGFR2::BICC1 WT	3.5	0.1	0.4	5	2.2	65.4	52.1	24.5	47.3
FGFR2::BICC1 K527E	0.8	0.2	3	1.3	1.3	23.8	21	11.3	18.7
FGFR2::BICC1 I549T	38.1	0.4	4	34.1	19.1	141.1	94.7	362.2	199.3
FGFR2::BICC1 N550D	835.8	21.5	90.1	183.1	282.6	2,259.1	400	1,784.3	1,481.1
FGFR2::BICC1 N550H	113.4	5	13.5	111.5	60.8	394.4	358.2	259.9	337.5
FGFR2::BICC1 N550K	269.6	15	62.2	265.6	153.1	1,098.6	266.4	816.7	727.2
FGFR2::BICC1 N550T	21.4	0.6	3	19.4	11.1	81.1	153.4	108.4	114.3
FGFR2::BICC1 L551F	15.0	1.6	20.5	47.6	21.2	89.8	227.5	287.3	201.5
FGFR2::BICC1 V565F	1,608	1,098.2	780.3	334	955.1	217	121.1	5,081.7	1,806.6
FGFR2::BICC1 V565I	29.2	0.5	385.6	19.6	108.7	340.8	117.6	228.8	229.1
FGFR2::BICC1 V565L	637.2	45.1	262.7	114.3	264.8	1,648.5	368.7	3,369.5	1,795.6
FGFR2::BICC1 V565Y	1,931.3	1,173.5	486.7	143	933.6	52.2	176	5,635.5	1,954.6
FGFR2::BICC1 E566A	328.3	10.2	34.5	372.3	186.3	1,003.5	686.8	2,035.9	1,242.1
FGFR2::BICC1 E566G	110.2	11.8	26.8	235.2	96	2,163.8	535.8	806.9	1,168.8
FGFR2::BICC1 L618M	21.9	1.8	17.3	15.9	14.2	315.3	128.4	110.6	184.8
FGFR2::BICC1 L618V	332.7	66.2	385.9	328.8	278.4	3,076.7	358.7	779	1,404.8
FGFR2::BICC1 K642R	242.3	10.2	51.7	201.7	126.5	572.3	143.8	625.7	447.3
FGFR2::BICC1 D651H	2.1	0.3	0.2	5.1	1.9	116.5	63	67.4	82.3

Sensitive	0–10 nmol/L
Intermediate	10–30 nmol/L
Resistant	>30 nmol/L

Sensitive	0–100 nmol/L
Intermediate	100–300 nmol/L
Resistant	>300 nmol/L

B

	Futibatinib	Lirafugratinib	Average IC$_{50}$
Parental	8,588.7	3,484.6	6,036.6
FGFR2::BICC1 WT	0.5	3.4	1.9
FGFR2::BICC1 K527E	0.1	0.4	0.2
FGFR2::BICC1 I549T	0.7	11.9	6.3
FGFR2::BICC1 N550D	3.6	22.4	13
FGFR2::BICC1 N550H	2.4	0.6	1.5
FGFR2::BICC1 N550K	12.9	21.1	17
FGFR2::BICC1 N550T	2.4	2.6	2.5
FGFR2::BICC1 L551F	2	1	1.5
FGFR2::BICC1 V565F	230.8	0.01	115.4
FGFR2::BICC1 V565I	0.6	14.6	7.6
FGFR2::BICC1 V565L	18	1.4	9.7
FGFR2::BICC1 V565Y	410.8	0.01	205.4
FGFR2::BICC1 E566A	5.2	9.7	7.4
FGFR2::BICC1 E566G	1.7	9.8	5.7
FGFR2::BICC1 L618M	2	16.4	9.2
FGFR2::BICC1 L618V	25.8	34.3	30
FGFR2::BICC1 K642R	9.1	9.3	9.2
FGFR2::BICC1 D651H	0.5	1.3	0.9

Sensitive	0–10 nmol/L
Intermediate	10–30 nmol/L
Resistant	>30 nmol/L

C

Figure 6.

In vitro and *in vivo* evaluation of the activity of selective FGFR inhibitors against *FGFR2* kinase domain mutations. **A,** IC$_{50}$ values of seven reversible FGFR inhibitors (and their average) against parental Ba/F3, FGFR2::BICC1 Ba/F3 (wild-type, WT), and 17 mutants. **B,** Graphical representation of the IC$_{50}$ values of the two irreversible FGFR inhibitors futibatinib and lirafugratinib (and their average) against parental Ba/F3, FGFR2::BICC1 Ba/F3 (WT), and 17 mutants. We created two different cut-off thresholds, given the lower potency of zoligratinib, derazantinib, and rogaratinib against WT FGFR2::BICC1 Ba/F3. In **A** and **B**, IC$_{50}$ values (nmol/L) are reported as means of ≥3 independent datasets. **C,** Tumor growth kinetics in PDX models established from patients with cholangiocarcinoma, exposed to four FGFR inhibitors. # *MR332* PDX was established from the liver tissue biopsy harboring FGFR2 V565F, whereas the bone lesion harbored FGFR2 V565L (see **Fig. 2C**). q.d.: *quaque die* (i.e., daily).

futibatinib) mediated by a unique *FGFR2* kinase domain mutation, the clinical activity profiles of futibatinib and lirafugratinib corresponded well to our functional assessment. We were indeed able to overcome resistance to reversible inhibitors and futibatinib due to mutations occurring in the *FGFR2* gatekeeper residue (FGFR2 V565F/L). In line with the initial proofs from Subbiah and colleagues (25), the FGFR2-selective inhibitor lirafugratinib was active in our Ba/F3 cellular models, PDX, and in patient *MR1271* (FGFR2 V565L).

On the other hand, objective responses to futibatinib and lirafugratinib were observed even in cases with polyclonal *FGFR2* kinase mutations (20, 25). The relative abundance of each individual resistance mutation at the baseline of the irreversible inhibitors is suspected to influence the clinical response on a systemic scale. Emblematic in this sense is the evolution of our patient *ST3470*, experiencing primary resistance to lirafugratinib despite clearance of three *FGFR2* kinase domain mutations (**Fig. 5B**). Considering their better on-target activity, it is possible that progression to irreversible FGFR inhibitors occurs without detectable *FGFR2* kinase domain mutations or off-target alterations, suggesting the implication of additional mechanisms, as indicated by the resistance study to futibatinib in our cohort (**Fig. 3C**).

Given the unpredictability of resistance mechanisms and the corresponding activity of irreversible inhibitors administered in a sequential way, their administration as first anti-FGFR agents seems appropriate, particularly considering the outcomes of clinical activity reported in clinical trials in this setting (9, 10). As shown here in two cases (**Fig. 5**), switching from an irreversible inhibitor to another can also be a suitable therapeutic option.

This study, however, is not without limitations. Primarily, it relies on genomic analyses, potentially overlooking nongenetic factors like epithelial–mesenchymal transition or activation of alternate resistance pathways, as recently reported for EGFR in *FGFR2*-driven cholangiocarcinoma (41, 42). Moreover, our focus is mainly on on-target resistance mechanisms, with less emphasis on proving the role of off-target events such as MAPK and PI3K/mTOR alterations. The lack of systematic tissue biopsy and ctDNA analysis at multiple timepoints for all patients may also have constrained the depth of our insights into resistance mechanisms. Finally, the lack of clinical data of resistance to lirafugratinib limits our observations of resistance to irreversible FGFR inhibitors.

In summary, the present work provides a global approach to apprehend resistance mechanisms to FGFR inhibitors across *FGFR2*-driven diseases, a clinical entity of major current interest given the development of active targeted agents. Our clinical and molecular findings are corroborated by functional analyses of *FGFR2* kinase domain mutations in conferring resistance to different FGFR inhibitors. The additional clinical experience with sequential treatment with FGFR inhibitors or everolimus, together with the concomitant longitudinal study on resistance mechanisms, provides further valuable information both on the potential clinical management of patients and on the molecular correlates of resistance in this setting.

Authors' Disclosures

Authors' Contributions

F. Facchinetti: Conceptualization, resources, data curation, formal analysis, investigation, visualization, methodology, writing–original draft. **Y. Loriot:** Resources, supervision, funding acquisition, investigation, methodology, writing–review and editing. **F. Brayé:** Resources, data curation, investigation. **D. Vasseur:** Resources, software, formal analysis, investigation, writing–review and editing. **R. Bahleda:** Resources. **L. Bigot:** Resources, investigation, writing–review and editing. **R. Barbé:** Resources, data curation, software, writing–review and editing. **C. Nobre:** Resources, investigation, writing–review and editing. **D. Combarel:** Data curation, validation, writing–review and editing. **S. Michiels:** Formal analysis, supervision, methodology, writing–review and editing. **A. Italiano:** Resources, writing–review and editing. **C. Smolenschi:** Resources, investigation, writing–review and editing. **L. Tselikas:** Resources, investigation, writing–review and editing. **J.-Y. Scoazec:** Resources, methodology, writing–review and editing. **S. Ponce-Aix:** Resources, investigation. **B. Besse:** Resources, funding acquisition, validation, methodology, writing–review and editing. **F. André:** Funding acquisition, validation. **K.A. Olaussen:** Supervision, validation, visualization, writing–review and editing. **A. Hollebecque:** Conceptualization, resources, funding acquisition, investigation. **L. Friboulet:** Conceptualization, resources, supervision, funding acquisition, validation, investigation, visualization, methodology, writing–review and editing.

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Note

Supplementary data for this article are available at Clinical Cancer Research Online (http://clincancerres.aacrjournals.org/).

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