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Review

FGFR2 testing in cholangiocarcinoma: translating molecular studies into clinical practice

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Summary
Cholangiocarcinoma (CCA) is a heterogeneous group of neoplasms burdened by a dismal prognosis. Several studies have investigated the genomic profile of CCA and identified numerous druggable genetic alterations, including *FGFR2* fusions/rearrangements. Approximately 5-7% of CCAs and 10-20% of intrahepatic iCCAs harbor *FGFR2* fusions. With the recent advent of FGFR-targeting therapies into clinical practice, a standardization of molecular testing for *FGFR2* alterations in CCA will be necessary. In this review, we describe the technical aspects and challenges related to *FGFR2* testing in routine practice, focusing on the comparison between Next-Generation Sequencing (NGS) and FISH assays, the best timing to perform the test, and on the role of liquid biopsy.

Key words: cholangiocarcinoma, precision medicine, *FGFR2*, biomarkers

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Introduction

Cholangiocarcinoma (CCA) is a heterogeneous group of invasive adenocarcinomas arising from different locations within the biliary tree. CCA represents the second most common primary hepato-biliary malignancy and comprises 3% of all gastrointestinal cancers [1].
CCAs occurring within ductules or segmental ducts are classified as intrahepatic cholangiocarcinoma (iCCA), and those arising from the perihilar (pCCA) or distal portions of the biliary tract (dCCA) are classified as extrahepatic cholangiocarcinoma (eCCA). pCCA is the most common CCA, comprising 50-60% of cases, while iCCA is the least common, comprising 10-20% of CCA [2].
Risk factors for CCA include primary sclerosing cholangitis, Caroli's disease, hepatolithiasis, hepatobiliary fluke infections (*Opisthorchis viverrini* and *Clonorchis sinensis*), and comorbid hepatic disorders including chronic hepatitis B or C, non-alcoholic steatohepatitis (NASH), and non-alcoholic fatty liver disease (NAFLD), cirrhosis. However, most patients with cholangiocarcinoma have no identifiable risk factors [3].
Incidence and etiological factors vary between geographical regions. While CCA is a rare cancer in high-income countries, it is much more common in China and Thailand, due to the high prevalence of hepato-

biliary flukes and hepatolithiasis. Different from other cancer types, mortality has been increasing in patients with CCA in the last decades. Because CCA is often asymptomatic in its early stages, a significant proportion of patients present with locally advanced and unresectable disease. Patients presenting with resectable disease usually undergo potentially curative surgery, followed by adjuvant chemotherapy. However, due to early relapse rates after surgery, the median post-operative survival is 3 years [4,5].

Regarding iCCA, three different macroscopic growth patterns have been identified, namely mass forming (MF type), periductal infiltrating (PI type), and intraductal growing (IG type), with the MF type being the most common one. MF iCCA arises from peripheral small bile ducts, while PI and IG iCCA originate from large intrahepatic bile ducts [2,6].

Microscopically, iCCA shows several histological variants (conventional, cholangiolocarcinoma and rare variants) characterized by a different cells of origin and pathogenesis have been recognized. Conventional iCCA may be further classified into large duct iCCA and small duct iCCA. Large duct iCCA may arise from precancerous lesions, such as biliary intraepithelial neoplasia or intraductal neoplasms and are localized in the large intrahepatic bile ducts near the hepatic hilus; small duct iCCA mainly occurs in the peripheral hepatic parenchyma [2,6].

The molecular landscape of CCA

Numerous studies have investigated the genomic profile of CCA and have shown substantial molecular heterogeneity within this group of neoplasms (Fig. 1). In the largest genomic study reported in the literature, Javle et al. profiled 4371 CCA and found the most commonly altered genes to be *TP53*, *CDKN2A/B*, *KRAS*, *ARID1A*, *IDH1*, *BAP1*, *PBRM1*, and *FGFR2* (mostly fusions) [7].

Despite being limited by the relatively small number of eCCAs, many studies have revealed important differences between the molecular landscapes of iCCA and eCCA (Fig. 1). The genomic profile of iCCA and eCCA are different, with *FGFR* fusions, mutations, or amplifications and *IDH* mutations being much more common in iCCA than in eCCA, while *KRAS* mutations and *ERBB2* amplification and overexpression are more prevalent in eCCA [8-11].

The morphologic heterogeneity of iCCA reflects a substantial molecular heterogeneity. Small duct iCCA often harbors *IDH1* and *IDH2* mutations and *FGFR2* alterations. On the other hand, large duct iCCA, similar to eCCA, is frequently mutated in *KRAS* and/or *TP53* [12-14].

Based on the current evidence, the European Society for Medical Oncology (ESMO) and United States National Comprehensive Cancer Network (NCCN) guidelines recommend routine use of Next-Generation Sequencing (NGS) multigene panels on advanced CCAs to identify druggable genetic alterations [15,16]. ESMO recommends NGS testing for level I (i.e., improved

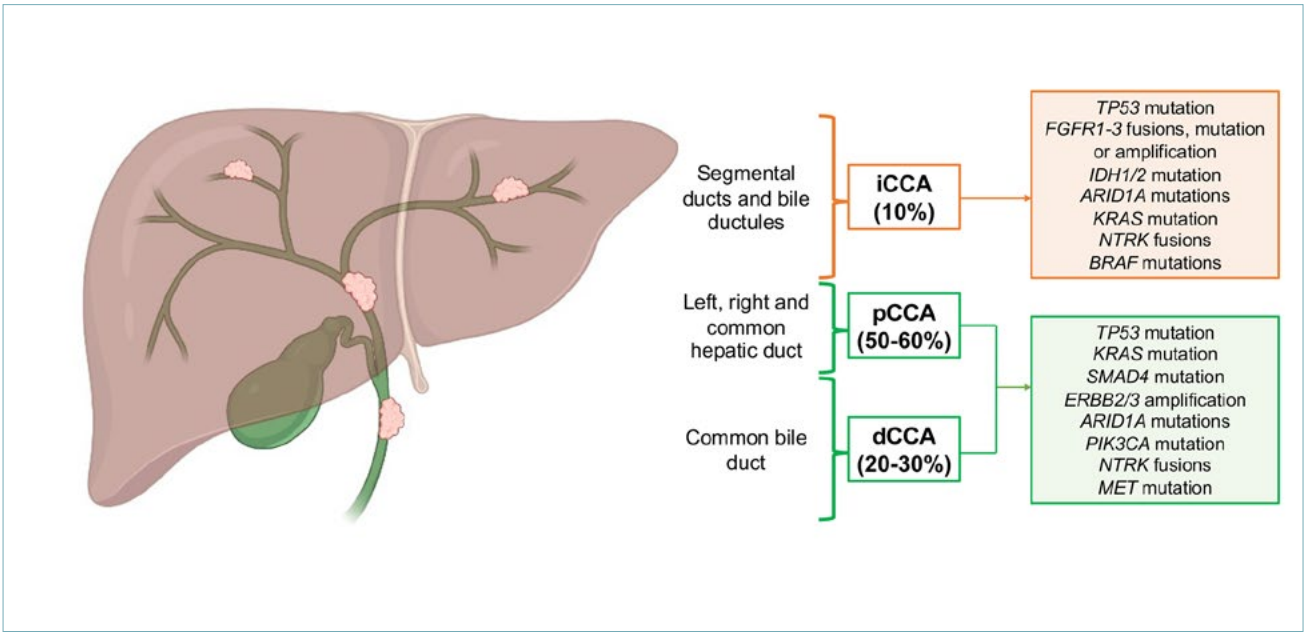

Figure 1. Common genetic alterations in intrahepatic and extrahepatic cholangiocarcinoma.

outcomes in clinical trials) genetic alterations according to the ESMO Scale for Clinical Actionability of molecular Targets (ESCAT), including *IDH1* mutations, *FGFR2* fusions, and *NTRK* fusions. The following level II and III actionable genetic alterations have available targeted therapies that do not have any indication for CCAs: microsatellite instability (MSI)/mismatch repair deficiency (dMMR), *BRAF* mutations, *ERBB2* amplifications or mutations, *PIK3CA* mutations, *BRCA1* and *BRCA2* mutations, *MET* amplifications.

Mutations in *IDH1/2* have been reported in 10-30% of iCCAs and 7% of eCCAs, with a higher prevalence of *IDH2* mutations in comparison with *IDH1* mutations (7-20% *vs* 3%) [15].

Ivosidenib is an anti-IDH1 targeted therapy that was approved by the United States Food and Drug Administration (FDA) in August 2021 for patients with previously treated, locally advanced, or metastatic CCA harboring an *IDH1* mutation [17]. According to the results of phase III ClarIDHy trial, ivosidenib significantly prolonged progression-free survival (PFS) (which was the primary endpoint of the study) in previously treated advanced or metastatic CCA [18]. At final overall survival (OS) analysis, the improvement in OS resulted not significant when comparing ivosidenib *versus* placebo group: however, when adjusted for crossover (as 70.5% of the patients assigned to placebo received ivosidenib after progression), the analysis revealed a significant OS benefit for ivosidenib [19].

At present, therapies (*i.e.,* larotrectinib, entrectinib) are available for patients with advanced or metastatic solid tumors, including CCA, harboring *NTRK* fusions and progressing after standard therapy [20,21]. However, *NTRK* fusion genes are a rare finding in CCA (approximately 4%) and limited data are available [22].

MSI is the molecular fingerprint of MMRd. Microsatellites are repetitive sequences distributed throughout the human genome, which are prone to the accumulation of mutations. MSI/MMRd can be assessed by immunohistochemistry (IHC) or molecular assays. CCA can be MSI/MMRd but to a lower extent in comparison with other gastrointestinal neoplasms (i.e., colorectal or gastroesophageal cancer). In fact, previous works have reported rates of MSI/MMRd ranging from 1% to 3%. Pembrolizumab is currently indicated for the treatment of MSI/MMRd unresectable or metastatic pre-treated biliary cancer [7,23,24].

Tumor mutational burden (TMB) is defined as the number of mutations per megabase of coding DNA [25]. High TMB (TMB-h), defined as TMB ≥ 10 mutations per megabase, is associated with MSI in some cancer types. In CCA the association between MSI and TMB-h is still debated [26]. Javle et al. [7] found that 1% of the CCAs profiled had a TMB > 20 mutations per megabase and 3% had a TMB > 10 mutations per megabase.

Following the promising results of the KEYNOTE-158[27], the anti-PD-1 pembrolizumab has been approved for the treatment of patients with solid tumors with MSI or TMB ≥ 10 mutations per megabase [28].

BRAF mutations and rearrangements occur in between 1% and 7% of CCAs, with a higher prevalence in iCCAs [9,11,24]. The phase II Rare Oncology Agnostic Research study reported encouraging results in CCA patients with $BRAF^{V600E}$ mutations treated with dabrafenib in combination with trametinib. However, at present, there is no indication for targeted anti-BRAF therapy in CCA [29].

Furthermore, some of the targeted therapies currently in use in other cancer types are being investigated for use in CCA with *BRCA1/2* mutations (olaparib, NCT04042831), *ERBB2* amplifications and mutations (trastuzumab, NCT00478140; trastuzumab emtansine, NCT02999672) and *PIK3CA* hotspot mutations (copanlisib, NCT02631590) [30].

Different testing modalities can be used to identify the large spectrum of genetic alterations in CCAs in tissue samples. Some alterations (i.e., $BRAF^{V600}$ mutations, *ERBB2* amplifications, *EGFR* and *KRAS* mutations, and ALK, ROS1, and EGFR rearrangements) have been well characterized in other cancer types and may be identified with established conventional tests. Conventional tests are based on IHC, fluorescent in situ hybridization (FISH) and PCR-based RNA or DNA sequencing. Despite being rapid and less expensive than NGS, these methods are not suitable to identify multiple genetic alterations in samples with limited amount of tissue [31].

Targeted NGS approaches allow molecular profiling of selected gene panels with improved coverage of relevant tumor-specific genes. Due to the lower costs, shorter turnaround time, and simplified data analysis, targeted NGS is more suitable in routine molecular diagnostics than whole genome, whole exome, or transcriptome sequencing [32].

The role of FGFR2 in CCA

The fibroblast growth factor receptor (FGFR) family is a family of tyrosine kinase receptors that include FGFR1, FGFR2, FGFR3, and FGFR4 [33]. Following the binding of growth factors, FGFRs dimerize and activate intracellular signaling pathways responsible for cellular proliferation, survival, and angiogenesis [34]. The *FGFR2* gene contains at least 24 exonic sequences, however, only subgroups of these are used for different isoforms through alternative splicing.

To the present day, more than 25 isoforms of FGFR2 have been described [34].

The binding of an FGF and heparin/heparan sulfates as co-factors to FGFR2 results in dimerization and subsequently trans autophosphorylation of the receptor at its cytoplasmic component. The activated intracellular kinase domain phosphorylates downstream targets, leading to the activation of numerous signaling pathways, including JAK-STAT, MAPK, and PI3K-AKT [35].

FGFR2 gene is located on chromosome 10, and approximately half of *FGFR2* fusions evolve through intrachromosomal rearrangements [34]. Over 150 different *FGFR2* fusion partners have been observed in CCA, with the most common partner being *BICC1* [36,37]. All FGFR2 fusions in CCA are "type 2" fusions with transmembrane-type FGFRs with C-terminal substitution to the region of fusion partners [38]. *FGFR2* fusions are mutually exclusive with FGFR2 mutations and commonly co-occur with *BAP1* alterations [39]. Of note, no association between *FGFR2* rearrangement and high TMB or MSI/dMMR has been reported [24,40].

In a study by Helsten et al. [41], 4,853 solid tumors were profiled by NGS and *FGFR* aberrations were found in 7.1% of all cancers, with the majority (66%) being gene amplifications, followed by single-nucleotide variants (26%), and fusions (8%). Among the 115 CCAs included in the study, 7% harbored *FGFR* aberrations, mostly in the *FGFR2* gene. Alterations in the *FGFR1* and *FGFR3* genes have also been described in CCA [40]. Various studies aimed at profiling CCAs estimated the frequency of *FGFR2* fusions to be approximately 5-7% in patients with any CCA and in 10-20% of patients with iCCA [42]. The prevalence of CCA harboring *FGFR2* fusions may vary in different geographical areas and is influenced by etiological factors (i.e., FGFR2 fusions are rare in liver fluke-associated CCAs) [43].

Some studies reported a positive association between the presence of *FGFR2* fusions and a better prognosis. Graham et al. [14] evaluated 152 CCAs and 4 intraductal papillary biliary neoplasms of the bile duct for the presence of *FGFR2* fusions by FISH. According to the results of the study, the median cancer-specific survival for the 30 patients whose tumors harbored *FGFR2* translocations was 123 months compared to 37 months for negative cases without FGFR2 translocations ($p = 0.039$). In an additional study by Jain et al. [39], out of 377 CCAs, 95 harbored *FGFR* genetic alterations, including 63 fusions. The presence of FGFR2 aberrations was associated with longer OS compared with patients without *FGFR* aberrations (37 *vs* 20 months, respectively; $p < 0.001$). Furthermore, the rate of *FGFR2* genetic alterations was higher among younger patients (≤ 40 years; 20%). Rizzato et al. [24] detected *FGFR2* fusions and *FGFR3* aberrations in 15/286 (5.2%) and 5/286 (1.5%), respectively, locally advanced or metastatic biliary tract cancers included in the study. At multivariate analysis, *FGFR2/3* altered patients had a median OS of 29.2 months compared to 14.4 months for wild-type patients. In the same study, PFS following the start of second-line therapy, was relatively longer in patients with FGFR2 rearrangements (5.0 *vs* 3.0 months). Another work by Abou-Alfa et al. reported a longer OS and PFS among patients with iCCAs harboring *FGFR2* fusions on second-line, but not first-line systemic therapy [44].

Targeting FGFR2 in CCA

Several candidate drugs are currently under development and investigations in randomized clinical trials in patients with CCA harboring FGFR pathway alterations, including non-selective and selective FGFR tyrosine kinase inhibitors (TKIs), anti-FGF/FGFR monoclonal antibodies, and FGF traps [45]. However, the use of non-selective FGFR TKIs comes with various complications in clinical practice, including off-target side effects [45]. For this reason, a plethora of selective FGFR inhibitors have been evaluated in early-phase clinical trials in patients with refractory iCCA harboring *FGFR2* gene fusions, either in randomized clinical trials for iCCA patients or in basket trials [46].

Pemigatinib is a small molecule inhibitor of FGFR1, FGFR2, and FGFR3 and represents the first targeted treatment to be approved in the CCA setting. Pemigatinib has received accelerated approval as second-line treatment in April 2020 by the FDA in CCA patients harboring *FGFR2* gene fusions or other rearrangements, following the results of the seminal clinical trial FIGHT-202 [47]. The drug has been subsequently approved by EMA [48] and is currently reimbursed by the Italian National Health Service (SSN) for locally advanced or metastatic CCA patients harboring *FGFR2* fusions or rearrangements after at least one line of systemic therapy.

The open-label phase II study FIGHT-202 study evaluated the efficacy and safety of pemigatinib in 146 patients with locally advanced or metastatic previously treated iCCA, including 107 with *FGFR2* fusions or rearrangements, and found a marked difference in overall response rate (ORR) between patients with *FGFR2* fusions or rearrangements (35.5%), and those with other *FGF* or *FGFR* alterations or no *FGF*/*FGFR* alterations (0% in both groups). Moreover, 82% of patients harboring *FGFR2* fusions or rearrangements achieved disease control (i.e., objective response or

disease stabilization as best response). Patients with *FGFR2* fusions or rearrangements had a PFS of 6.9 months; PFS was 2.1 months for patients with other *FGF*/*FGFR* alterations, and 1.7 months for patients without *FGFR* alterations [49]. Data from FIGHT-202 compare favorably with those achieved with cytotoxic chemotherapy among unselected patients [50], even when a potential prognostic impact of *FGFR* alterations is considered [44]. Final data from FIGHT-202 (Vogel A, et al – data presented at ESMO GI 2022) pinpointed an ORR of 37% (95% CI: 28-47) a disease control rate (DCR) of 82% (95% CI: 74-89) and a median duration of response (DOR) of 9.1 months (95% CI: 6-14.5) for tumors with *FGFR2* fusions or rearrangements, followed for a median follow-up of 42.9 months. Taken together, these results confirm that therapeutic targeting of *FGFR* fusions and rearrangements is an opportunity that should not be missed in CCA patients.

Promising results have also been reported with other anti-FGFR agents among CCA patients with *FGFR2* fusions or rearrangements. In a multicenter, open-label, phase II study (NCT02150967), the FGFR inhibitor infigratinib demonstrated an ORR of 23.1% in a series of 108 previously treated, locally advanced or metastatic patients [51,52]. Derazantinib, a multikinase pan-FGFR inhibitor, was associated with an ORR of 20.7% and a DCR of 82.8% in a phase I/II study (NCT01752920) among 29 patients with unresectable iCCA with *FGFR2* fusion, who experienced disease progression or were intolerant or not eligible to first-line chemotherapy [53]. More recently, futibatinib (TAS-120, a highly selective irreversible FGFR1-4 inhibitor) confirmed the value of FGFR-targeting in CCA in the phase II FOENIX-CCA2 study (NCT02052778). The trial design was similar to that of FIGHT-202 and enrolled 103 patients with pretreated unresectable or metastatic iCCA with FGFR2 fusions or rearrangements. Futibatinib demonstrated an ORR of 42% and a DCR of 83%, reporting a median PFS of 9.0 months [54]. On the basis of these data, futibatinib received FDA approval as salvage treatment of molecularly selected iCCA patients. Notably, futibatinib also demonstrated potent activity against some *FGFR2* kinase domain mutations associated with resistance to ATP-competitive FGFR-inhibitors, and preliminary reports suggest potential activity after progression to previous FGFR-inhibitors [55]. If confirmed, these data open the way towards a continuum-of-care with anti-FGFR agents in this small molecularly-defined subset of CCA patients.

Moving from the activity observed among pretreated patients, FGFR inhibitors are currently being tested as first-line therapy in CCA patients with *FGFR2* fusions or rearrangements. The ongoing phase III study FIGHT-302 (NCT03656536) is assessing the efficacy and safety of pemigatinib *versus* standard-of-care gemcitabine plus cisplatin in the first-line treatment of patients with metastatic CCA harboring *FGFR2* rearrangements [56]. Similarly designed trials are ongoing in order to compare infigratinib (NCT03773302) and futibatinib (NCT04093362) with first-line chemotherapy.

FGFR2 fusion testing in CCA: a practical approach

The development of targeted therapies is significantly impacting the diagnostic and therapeutic decision-making process of CCA patients. With the advent of personalized medicine, modern pathology has gone way beyond traditional morphological evaluations of tissue specimens. The pathologist has become a central figure who is responsible for the delivery of a morpho-molecular report [57,58]. In this new era, the delivery of personalized medicine and oncology strongly relies upon personalized diagnostics. The selection of the most appropriate sample, diagnostic technology and test are crucial factors when detecting patient-to-patient variations in genes or protein expression levels, which act as prognostic or predictive biomarkers [57,58].

What is the best sample?

CCA is a relatively rare neoplasm with a dismal prognosis and is highly heterogeneous from a molecular standpoint. A substantial proportion of CCAs, especially iCCAs, harbor somatic alterations with therapeutic implications. For this reason, treatment guidelines recommend using molecular profiling for metastatic and unresectable advanced CCA, allowing patients to receive biomarker-directed therapy or clinical trial enrollment [30]. In the diagnostic scenario, a non-neglible percentage of routine practice samples are classified as “scant samples” (including small biopsy or cytological specimens) due to low quality and quantity of nucleic acids available for molecular testing. In this regard, an optimized workflow based on harmonized pre-analytical and analytical procedures play a pivotal role in improving successful rate of molecular techniques [59,60]. Bekaii-Saab et al. suggested that preoperative biopsy for molecular profiling should be encouraged without delay even in patients with advanced resectable CCA, due to the high rates of relapse after surgery [31].

Collecting adequate tissue samples for molecular profiling in advanced or metastatic CCA patients is often challenging. According to Lamarca et al. [61], one in four archived tissue samples may have insufficient neo-

plastic cell content for molecular profiling, resulting in the failure of molecular analysis regardless of the platform employed. This may be attributed to the unique location of the tumors and the desmoplastic nature of CCA. The involvement of a pathologist can minimize the failure rate of molecular analysis, but the main obstacle remains the low amount of material collected during endoscopic retrograde cholangiopancreatography (ECRP) or biliary brush citology [61].

Additionally, tumor location and pattern of growth may influence the amount of material to be dedicated to molecular studies. For example, in periductal infiltrating iCCA and pCCA, surgery is rarely performed, and molecular profiling relies more frequently on biopsy or brushing samples [2].

Since the majority of the tumor samples of CCA are small biopsy or cytological specimens, an important aspect that the pathologist must take into account is the suitability of the sample to analyze. The number of cells required for successful DNA/RNA extraction for molecular mutation screening is not defined in CCA, but for other neoplasms (i.e., lung cancer) a range of at least 200-400 cells is desirable [62]. Moreover, the percentage of tumor cells in a given specimen is a crucial parameter to consider and should be correlated with the sensitivity of the downstream molecular test performed [63]. Nevertheless, the quality of DNA/RNA extraction should be assessed before molecular testing to determine the DNA fragmentation index and RNA integrity.

DNA/RNA could be heavily affected by degradation and fragmentation during pre-analytical phases of tissue handling. For example, studies conducted on the preservation status of nucleic acids in FFPE tissues generally agree on the relatively good (though not optimal) preservation of DNA [64]. On the other, RNA has been found to be heavily degraded and fragmented so that only short sequences (approximately 100-200 nucleotides) can be recognized [5]. The main effect of formaldehyde in tissues is linked to the formation of methylol groups on amino groups first, followed by the establishment of cross-linking methylene groups that lead to proper fixation [66]. Bases of nucleic acids are involved in this process, resulting in cross-linking with side-chain amino groups of proteins.

Bussolati et al. demonstrated that RNA degradation can be inhibited by maintaining a low temperature through the entire fixation process (so-called "cold fixation" [CF]) [67]. The CF procedure is linked to a lower significantly lower degree of nucleic acid fragmentation, especially of mRNA, while keeping the basic advantages that make formalin the fixative of choice in diagnostic histopathology.

When is the best timing?

At present, there is no consensus about whether and when NGS-based genomic testing should be carried out in patients with CCA.

In general, NGS profiling is not recommended in early-stage cancer patients undergoing potentially curative treatment, because it is unlikely to yield actionable alterations beyond those that can be detected using conventional approaches (i.e., IHC, FISH and PCR-based molecular assays) [68-70]. However, because only a small percentage of CCA patients are candidates for definitive treatment, there is a high rate of relapse after receiving surgical treatment and CCA are molecularly heterogeneous with a large number of potentially actionable genetic alterations, patients with early-stage CCA might benefit from NGS profiling after diagnosis [7,71].

Additionally, due to the few standard-of-care treatment options available, early molecular profiling for locally advanced or metastatic CCA is recommended for matching patients to basket trials recruiting for a specific genetic alteration [15,72]. Thus, the authors recommend performing NGS profiling as reflex testing on all CCAs, regardless of stage at presentation.

NGS testing is becoming a crucial step in the diagnostic and therapeutic decision-making process of oncologic patients. The routine use of NGS in CCA and other solid tumors requires efforts from governmental institutions to allocate resources toward the delivery of NGS testing and the creation of a functional laboratory network [73]. On 22nd December 2022, the Italian Government allocated 600,000 euros (2023-2025) to guarantee access to early NGS testing to all CCA patients [74].

Which is the best method?

FISH uses fluorescence-labeled DNA probes to target specific chromosomal locations within the nucleus to detect and quantify gene amplifications and known rearrangements, including gene fusions [75].

At present, two FISH approaches broadly in use are the break-apart probes and dual fusion-specific probes. Break-apart FISH requires the use of two differently labeled DNA probes (red and green fluorescence) near the FGFR2 locus. The absence of alterations within the *FGFR2* locus creates a combined fluorescence signal (yellow), while the presence of a DNA break caused by rearrangements results in separate fluorescence signals for each probe (green and red) [75].

In the dual fusion probe approach, two gene-specific probes are used. The wild-type situation results in separate fluorescence signals (red and green), conversely, if that specific gene fusion is present the fluo-

rescence signals overlap and result in a yellow fusion signal [75].

Break-apart FISH is able to identify rearrangements in a partner-agnostic manner, however, it lacks the detailedness of sequencing-based methods and provides no data on fusion gene partners nor on the expression of the fusion protein. Of note, the break-apart assay may lead to false negative results. In fact, in order to order to ensure the visibility of the red and green fluorescence signals, the two probes have to be separated far enough from each other, resulting in an inadequate detection of intrachromosomal rearrangements, which represent 50% of all *FGFR2* rearrangements. Conversely, dual-FISH approaches can only detect fusion involving a specific fusion gene partner and cannot assess the expression of the fusion protein [34].

In the era of precision oncology, highly selective single-gene testing has been outdated by NGS and other multiplexed platforms. At present, targeted NGS panels find application in routine diagnostics because they target genes of clinical significance, and have greater sensitivity, faster turnaround time and lower cost [76].

Different types of genetic sources of material (i.e., DNA or RNA) can lead to the detection of different types of genetic alterations through NGS analysis. DNA-based NGS can detect any type of genomic alteration, including single-nucleotide variants (SNVs), indels, rearrangements, amplifications, TMB, and MSI. However, the performance of a specific test is influenced by the size of the gene panel and the type of sequences targeted. RNA-based NGS can determine exons and transcribed rearrangements, including alternative splicing events and complex gene fusions, which often go undetected by DNA-based NGS, and can also quantify gene expression levels. On the contrary, when using DNA-based NGS, the transcripts resulting from rearrangements and gene expression levels have to be predicted using computational levels. With RNA-based NGS, mutations with low variant allele frequency and heterozygous loss-of-function mutations can be missed [77-79].

Different NGS approaches can be used on both DNA and RNA level. The imbalance assay is fusion partner-agnostic. RNA molecules are quantified by looking for an imbalance between the 5' and 3' region of the FGFR2 mRNA (i.e., the two ends of the transcript). Amplicon-based approaches are regarded as "closed" because they can only find gene fusions for which a

Figure 2. Comparison of different methods for the detection of *FGFR2* spectrum of translocations/fusions.

specific primer pair is included in the panel. The single-primer extension approach is another partner-agnostic approach and can identify gene fusion transcripts through the ligation of an adaptor. Hybrid capture-based assays can be used both on RNA and DNA level and rely on target enrichment before sequencing by using sequence-specific hybridization probes [34].
Overall, NGS may represent a more impacting diagnostic method than FISH in the detection of *FGFR2* fusions and allows the detection of multiple genetic alterations in tissue samples with scarce material (Fig. 2) [59,80].

Are all NGS panels the same?

DNA-based NGS is preferred for the detection of exonic mutations, while RNA-based NGS can interrogate directly the fusion transcript and is preferred for the detection of fusion genes, including *FGFR2* fusions [31].
Additionally, RNA-based assays are more sensitive because transcription leads to a signal augmentation due to larger numbers of RNA molecules compared to DNA molecules [34].
However, RNA extracted from FFPE is more unstable and prone to degradation, resulting in higher rates of failure of NGS analyses in comparison with DNA-based assays [81].
The theoretical performances of the previously mentioned NGS assays for specific fusion events are different. Thus, false negative and discordant results can be encountered when applying all the different assays. The single-primer extension and hybrid capture-based assays (DNA and RNA) have the broadest spectrum of detection of *FGFR2* fusion events [34].
When choosing an RNA-based NGS panel, only fusion partner-agnostic assays should be considered. In fact, amplicon-based panels should not be used for the detection of *FGFR2* fusions because they rely on gene-specific primer pairs and can only detect a predefined set of fusions [34].
Another significant aspect to consider when choosing the most suitable *FGFR2* fusion detection method is the amount of sufficient tumor DNA/RNA (i.e., detection limits) for the different assays [34].
For *FGFR2* rearrangements, the location of the breakpoint has clinical implications. All *FGFR2* fusions in iCCA are "type 2" fusions with C-terminal fusion partners. Because the C-terminal end of the kinase domain is encoded by exon 17, only rearrangements involving exon 17, intron 17, or the protein-coding region of exon 18 will maintain the kinase domain and thus act as oncogenic [34].
According to ESMO guidelines, gene fusions involving the *FGFR2* gene should preferably be interrogated at the transcriptomic level using a panel that can detect fusion transcripts of known and unknown fusion partners. Ideally, small biopsies of cholangiocarcinoma are well suited for combinatorial DNA and RNA profiling by single-primer extension and hybrid capture-based assays to identify breakpoints involving mainly exons 17 and 18 of *FGFR2*. If the tumor content is not sufficient for NGS-based analysis, a break-apart FISH can be performed, since it requires a minimum of 50-100 cells [82]. Considering these aspects, harmonized bioinformatic pipelines are required to successfully interpret clinically meaningful variants. Bioinformatic tools should consider the heterogeneous landscape of fusion partners that promote clinically relevant aberrant transcripts. In particular, several strategies are available to detect unknown fusion partners taking into account variant calling, unbalanced normalized ratio and quality score. Accordingly, false positive results derived from low-quality samples may occur when low stringency analytic pipeline is approached. In these challenging cases, secondary analysis levels may improve success rate for data interpretation and clinical administration of iCCA patients [83].

What is the role of liquid biopsy?

Liquid biopsy is emerging as a promising minimally invasive tool for biomarker testing in solid tumors. Liquid biopsy approaches may overcome challenges associated with molecular profiling of tissue samples, including insufficient tumor cellularity, molecular intratumor heterogeneity and the impossibility to perform serial biopsy sampling to monitor the onset of resistance to targeted therapy [84]. However, the low fraction of ctDNA retrieved from blood samples may challenge the sensitivity of liquid biopsy-based assays for solid tumors, including CCA. Interestingly, Lamarca et al. reported a significantly lower failure rate of molecular profiling of ctDNA in comparison with tissue samples, highlighting that ctDNA may be a valid way of accessing molecular analysis for patients with insufficient tissue [61].
According to ESMO guidelines [85], *IDH1* mutation and *FGFR2* fusion testing in circulating tumor (ctDNA) is recommended when tissue testing is not feasible or when urgent decision-making for fast therapeutic intervention is required. Although repeat biopsy is the gold standard in case of failure of molecular analysis due to inadequate tumor content, this may be not feasible in locally advanced and metastatic patients [86].
Guardant360® CDx (Guardant Health, Redwood city, CA, USA) and FoundationOne® Liquid CDx (Foundation Medicine, Cambridge, MA, USA) are commercial FDA-approved blood-based companion diagnostics. While the Guardant360® CDx panel detects fusions exclusively in *FGFR2* and *FGFR3*, the Foundation-

One® Liquid CDx panel detects fusions in *FGFR1, FGFR2, FGFR3, FGFR4* [87,88].

In a study by Berchuck et al., cell-free DNA (cfDNA) samples of 1671 patients with advanced biliary tract cancers were analyzed using Guardant360® CDx. cfDNA analysis detected *IDH1* mutations and *BRAF*^{V600E} at similar rates to tissue biopsies, but the concordance rate for *FGFR2* fusions detection was only 18%. The sensitivity of cfDNA profiling for *FGFR2* fusions was affected by the diversity of *FGFR2* fusion partners. In fact, the sensitivity for *FGFR2-BICC1* fusions was 58%, but only 2% for non-*BICC1* fusions [89]. According to the authors, the performance of cfDNA-based fusion detection could be improved by incorporating probes that target common fusion breakpoints and/or a broad range of fusion partner genes and using bioinformatics tools to detect non-targeted fusion partners [89]. To increase the capability of detecting *FGFR2* fusions and rearrangements, a novel fusion partner agnostic algorithm was applied to the Guardant360® CDx test on 73 plasma samples from CCA patients. The novel algorithm reached an agreement of 84% between tissue NGS and cfDNA profiling, with 24 additional *FGFR2* fusions detected in comparison with the standard algorithm [90].

Conclusion

CCA still remains an aggressive and deadly neoplasm, due to the lack effective of conventional treatments. Several studies have provided a greater understanding of the complex and heterogenous molecular landscape, identifying several druggable genetic alterations, including a large variety of FGFR2 rearrangements. With the recent approval of pemigatinib targeting of *FGFR2* fusions in CCA, a standardization of molecular profiling of these tumors will be necessary.

Due to the advancement of sequencing technologies, NGS-based tests have now lower costs, shorter turnaround time, and simplified data analysis. Overall, NGS outperforms alternative conventional methods like FISH in the identification of *FGFR2* rearrangements and allows the detection of multiple genetic alterations in CCA biopsies with low tumor content. Ideally, the best test is combinatorial DNA and RNA profiling by hybrid capture-based assays and single-primer extension panels, in order to cover the broadest spectrum of *FGFR2* fusion events. If the tumor content is not sufficient for NGS-based analysis, a break-apart FISH can be perforMed In the setting of CCA, liquid biopsy is emerging as a promising minimally invasive tool for biomarker testing as a way of accessing molecular analysis for patients with insufficient tissue. However, *FGFR2* rearrangement detection by ctDNA analysis is still suboptimal but this versatile, dynamic and easily-managing source of nucleic acids may reveal other clinical applications for the management of patients [31]. Personalized diagnostics (i.e., the selection of the most appropriate sample, diagnostic technology and test when detecting patient-to-patient variations) has become the cornerstone of personalized oncology. Thus, pathologists and oncologists must be equipped to navigate the complexity of the evolving diagnostic scenario of predictive biomarkers, including *FGFR2*. Moreover, ongoing clinical trials are evaluating the clinical performance of emerging FGFR inhibitors involving FGFR1-2-3 isoforms. Further investigations about this topic will provide new insights for the comprehensive evaluation of molecular hallmarks in iCCA patients [91,92].

Conflicts of interest

MF reports research funding (to Institution) from QED, Macrophage pharma, Astellas, Diaceutics; personal honoraria as invited speaker from Roche, Astellas, AstraZeneca, Incyte, Bristol Myers Squibb, Merck Serono, Pierre Fabre, GlaxoSmithKline, Novartis, Amgen; participation in advisory board for Amgen, Astellas, Roche, Merck Serono, GlaxoSmithKline, Novartis, Janssen. UM has received personal fees (as consultant and/or speaker bureau) from Boehringer Ingelheim, Roche, MSD, Amgen, Thermo Fisher Scientifics, Eli Lilly, Diaceutics, GSK, Merck and AstraZeneca, Janssen, Diatech, Novartis and Hedera unrelated to the current work. GP reports research funding (to Institution) from Exact Sciences, Astrazeneca, Novartis, Merck Serono, Boehringer Ingelheim; personal honoraria as invited speaker from Roche, Lilly, AstraZeneca, Incyte, Diatech Pharmacogenetics, Veracyte, GlaxoSmithKline, Novartis, Amgen, Exact Sciences, Bio-Optica, Janssen; participation in advisory board for Amgen, Astrazeneca, Novartis, Exact Sciences. LF reports personal honoraria as invited speaker from Incyte, Bristol Myers Squibb, EliLilly; research funding (to Institution) from MSD, Bristol Myers Squibb, AstraZeneca, Incyte, BeiGene, Astellas, Daiichi Sankyo, Roche; participation in advisory board for MSD, AstraZeneca, Incyte, Taiho, Servier, Daiichi Sankyo, EliLilly.

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Authors' contributions

Conceptualization, MF, GP and UM; methodology LF, FP and GP; data curation, AV; writing-original draft preparation, AV, LF, SMR, and FP; writing-review and editing, MF, GP and UM. All authors have read and agreed to the published version of the manuscript.

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