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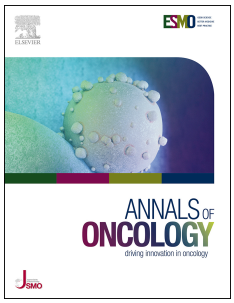

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Review

Practical considerations in screening for genetic alterations in cholangiocarcinoma

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Abstract

Cholangiocarcinoma (CCA) encompasses diverse epithelial tumors historically associated with poor outcomes due to an aggressive disease course, late diagnosis, and limited benefit of standard chemotherapy for advanced disease. Comprehensive molecular profiling has revealed a diverse landscape of genomic alterations as oncogenic drivers in CCA. *TP53* mutations, *CDKN2A/B* loss, and *KRAS* mutations are the most common genetic alterations in CCA. However, intrahepatic CCA (iCCA) and extrahepatic CCA (eCCA) differ substantially in the frequency of many alterations. This includes actionable alterations, such as *IDH1* mutations and a large variety of *FGFR2* rearrangements, which are found in up to 29% and approximately 10% of patients with iCCA, respectively, but are rare in eCCA. *FGFR2* rearrangements are currently the only genetic alteration in CCA for which a targeted therapy, the FGFR1-3 inhibitor pemigatinib, has been approved. However, favorable phase 3 results for *IDH1*-targeted therapy with ivosidenib in iCCA have been published, and numerous other alterations are actionable by targeted therapies approved in other indications. Recent advances in next-generation sequencing (NGS) have led to the development of assays that allow comprehensive genomic profiling of large gene panels within 2-3 weeks, including in vitro diagnostic tests approved in the US. These assays vary regarding acceptable source material (tumor tissue or peripheral whole blood), genetic source for library construction (DNA or RNA), target selection technology, gene panel size, and type of detectable genomic alterations. While some large commercial laboratories offer rapid and comprehensive genomic profiling services based on proprietary assay platforms, clinical centers may use commercial genomic profiling kits designed for clinical research to develop their own customized laboratory-developed tests. Large-scale genomic profiling based on NGS allows for a detailed and precise molecular diagnosis of CCA and provides an important opportunity for improved targeted treatment plans tailored to the individual patient's genetic signature.

Keywords

Actionable genetic alterations, cholangiocarcinoma, genomic profiling, next-generation sequencing, targeted therapy

Highlights

- Cholangiocarcinoma is a relatively rare, aggressive, heterogeneous malignancy associated with poor outcomes.
- Comprehensive molecular profiling has revealed a diverse landscape of oncogenic genomic alterations in cholangiocarcinoma.
- Advances in next-generation sequencing have allowed large gene panels to be assayed with high sensitivity, specificity, and at reduced cost.
- Large-scale genomic profiling has found actionable genomic alterations targeted by therapies approved in other indications.
- Large-scale genomic profiling may allow precise molecular diagnostics to guide treatment decisions in cholangiocarcinoma.

Introduction

Cholangiocarcinoma (CCA) represents diverse tumors originating from cholangiocytes in the bile ducts. Depending on their anatomical location, CCA is classified as intrahepatic (iCCA) or extrahepatic (eCCA), and eCCA is further classified as perihilar or distal eCCA.[1,2]

CCA is a relatively rare cancer with an incidence rate in the US of 1.20 per 100,000 person-years from 2000 and 2015, based on data from the National Cancer Institute (NCI) Surveillance, Epidemiology, and End Results (SEER) Program.[3] Estimated incidence rates in the US for iCCA and eCCA during this period were 0.77 and 0.43 per 100,000 person-years, respectively.[3] Recent retrospective data analyses suggest that the incidence of CCA has increased in past decades in both the US[3-5] and most European countries,[6] particularly of iCCA. In the US, the annual percentage increases from 2003 to 2015 in iCCA and eCCA were 7.0 and 2.1, respectively.[3] In Western and Central Europe, age-adjusted incidence rates (per 100,000 person-years) from 2008 to 2012 for iCCA were highest in the UK (1.15), France (1.13), and Germany (1.05), and those for eCCA were highest in Germany (0.74), the Netherlands (0.69) and Ireland (0.68).[6]

Although CCA is essentially a sporadic disease, diverse factors have been associated with increased risk of CCA, including bile duct cyst, Caroli's disease, primary sclerosing cholangitis, cholelithiasis or choledocholithiasis, parasitic liver infections, liver cirrhosis, hepatitis B or C virus infection, and hepatolithiasis (iCCA only).[7] The high incidence of CCA in some East Asian countries, such as South Korea and Thailand,[6] is due to the endemic presence of *Opisthorchis viverrini* and *Clonorchis sinensis* liver flukes[7] and vertical hepatitis B virus transmission.[8]

CCA is an aggressive cancer associated with a poor prognosis. SEER data from patients diagnosed with CCA between 1973 and 2008 suggested an estimated 5-year mortality rate of 70% to 91%, depending on age.[4] Major factors that contribute to poor outcomes in CCA are late-stage diagnosis and limited treatment options. Although complete surgical resection is

potentially curative, only about a third of patients diagnosed with CCA qualify for surgery.[1] Approved treatments for patients with unresectable, advanced CCA are largely limited to chemotherapy regimens tested in heterogeneous study populations of patients with biliary tract cancer (BTC). The standard first-line therapy, which consists of combination therapy with cisplatin and gemcitabine, was associated with a median overall survival (OS) of 11.7 months in a pivotal clinical trial in patients with locally advanced or metastatic BTC, 60% of whom had CCA.[9] For patients who experience disease progression after first-line therapy, available second-line combination chemotherapies provide only modestly improved survival benefit. In the phase 3 ABC-06 study in patients with advanced BTC (including 72% with CCA) who progressed after treatment with cisplatin plus gemcitabine, addition of modified FOLFOX to active symptom control was associated with a marginal improvement in the median OS compared with active symptom control alone (6.2 vs 5.3 months).[10] Results of a retrospective database analysis estimated the median OS from the time of second-line therapy initiation to be 13.4 months for patients with iCCA and 6.8 months for those with eCCA.[11]

Based on histologic criteria, CCAs can be classified as well, moderately, or poorly differentiated adenocarcinomas, or rare variants.[12] However, histopathologic criteria have proven to be insufficient tools for guiding treatment decisions to improve outcomes. Recent advances in comprehensive and integrative molecular profiling have revealed substantial molecular heterogeneity of CCA, even within anatomically or histologically defined subtypes, with important implications for diagnosis and disease classification,[13-15] prognosis,[13,16] and treatment.[16,17] The International Cancer Genome Consortium (ICGC) project is coordinating large-scale cancer genome studies in ~50 different cancers, including two projects focusing on BTC, to systematically characterize genomic alterations and to provide the cancer research community with access to the data.[18-20] The Pan Cancer Analysis of Whole Genome project (PCAWG) is a major initiative of the ICGC and the US National Cancer Institute's The Cancer Genome Atlas (TCGA),[21] and involves the collaboration of more than 1300 scientists and

clinicians from 37 countries. As of February 2020, more than 2600 genomes of 38 different tumor types have been analyzed and 16 working groups have been created to study multiple aspects of cancer development, progression, and classification.[20]

In light of the limited effectiveness of standard chemotherapy in patients with BTC and the complexity of the molecular landscape of CCA, recent technological advances in conducting rapid and comprehensive profiling of genomic alterations in individual patients offer the opportunity to include genomic profiling in the standard work-up during diagnosis and staging of patients suspected to have CCA. “Molecular diagnosis” at the individual patient level may provide useful information for treatment planning and selection of appropriate therapies, especially targeted therapy. In this review, we provide an overview of the molecular heterogeneity of CCA, the molecular profiling platforms available for molecular diagnosis, and the implication of molecular diagnosis for the management of CCA.

Genomic Alterations in CCA

Frequently Altered Genes

A number of recent studies have used comprehensive genomic profiling to determine the frequency of different genomic alterations in patients with CCA, including those with prior histopathologic diagnosis of iCCA vs eCCA.[17,22-28] Although these studies varied with respect to the numbers of genes and patients analyzed, the overall findings suggest substantial heterogeneity among the molecular profiles of individual patients, and important differences between the molecular landscapes of iCCA and eCCA (Figure 1). In the largest study reported to date, Javle et al. profiled tumor samples from 4371 patients with CCA to identify alterations in exons and select introns of up to 404 genes and to determine tumor mutational burden (TMB), microsatellite instability (MSI), and genomic loss of heterozygosity.[27] Although 75% of the tumor samples originated from liver biopsies, the primary tumor location (iCCA vs eCCA) was not

disclosed. The most commonly altered genes (in at least 10% of patients) were *TP53* (mostly truncations and mutations), *CDKN2A/B* (mostly copy number loss), *KRAS* (mostly mutations), *ARID1A* (mostly truncations), *IDH1* (mutations), *BAP1* (mostly truncations), *PBRM1* (mostly truncations), and *FGFR2* (85% fusions) (**Figure 1A**).[27]

Comprehensive genomic profiling of altered genes in more than 1000 patients with CCA was performed during screening for enrollment in FIGHT-202 (NCT02924376), a phase 2 study of the selective oral FGFR 1-3 inhibitor pemigatinib in patients with previously treated advanced CCA.[29] Most of the patients (>80%) had iCCA, although the precise percentage could not be determined as primary tumor location at diagnosis was not available for all patients [Silverman I, personal communication]. Similar to the study by Javle et al.,[27] the most commonly altered genes (in at least 10% of patients) were *TP53*, *CDKN2A/B*, *KRAS*, *CDKN2B*, *ARID1A*, *IDH1*, *SMAD4*, *IDH1*, *BAP1*, and *PBRM1*; *FGFR2* alterations were found in 7% of patients (Figure 1A).[25] An overall similar distribution of altered genes was also observed in a comprehensive molecular profiling study of 410 cancer-associated genes in 195 patients with CCA, including 158 (81%) with iCCA (Figure 1A).[24]

Several molecular profiling studies determined the frequency of genomic alterations separately in patients with iCCA and eCCA.[17,24,28] Although the studies were limited by relatively small numbers of patients with eCCA, they revealed important differences between the molecular landscapes of iCCA and eCCA. In the study by Lowery et al.[24] genes preferentially altered in iCCA (*n* = 158; Figure 1B) vs eCCA (*n* = 37) were *IDH1* (29% vs 5%), *BAP1* (19% vs 0%), and *FGFR2* (13% vs 0%), whereas genes preferentially altered in eCCAs vs iCCAs were *TP53* (49% vs 18%), *KRAS* (38% vs 7%), *SMAD4* (30% vs 5%), and *STK11* (11% vs <1%). Genomic profiling of 412 patients with iCCA and 57 patients with eCCA identified *IDH1* mutations and *FGFR2* alterations in 16% and 9%, respectively, of patients with iCCA (Figure 1B), but failed to detect such alterations in eCCA.[28] Genomic profiling of 73 genes in 150 patients with eCCA found that the most common alterations (in at least 10% of patients) were

KRAS, *TP53*, *ARID1A*, and *SMAD4* mutations (Figure 1C).[15] In addition, eCCA was associated with recurrent chromosomal amplifications in *YEATS4* (6.0%), *MDM2* (4.7%), *CCNE1* (2.7%), *CDK4* (1.3%) and *ERBB2* (1.3%).[15] Similar findings were previously reported by Lee et al. for 99 patients with eCCA (Figure 1C).[23] Of note, a particularly high rate of *TP53* alterations (68%) was found in 80 Chinese patients with eCCA (Figure 1C).[26]

Along with the apparent differences in molecular landscapes between CCA subtypes, the molecular profile of CCA is also geographically heterogeneous, which may reflect divergent extrinsic risk factors and etiologies.[6] In this regard, a recent NGS study compared genomic profiles of patients with iCCA located in the US (n = 283) with those in China (n = 164).[30] Divergent genomic signatures were found: *BRCA1/2*, *DDR*, *KMT2C*, *NF1*, *RB1*, *RBM10*, *SPTA1*, *TERT*, *TGFBR2*, and *TP53* were significantly more prevalent in Chinese patients, and *BAP1*, *CDKN2A/B*, and *IDH1/2* were significantly more common in US patients.[30] Also consistent with this, a particularly high rate of *TP53* alterations was observed in 80 Chinese patients with eCCA (Figure 1C).[26] Notably, an integrative clustering analysis of copy number, gene expression, mutation, and epigenetic data from 489 CCA samples spanning 10 countries, yielded 4 clusters characterized by divergent clinicopathologic and molecular profiles.[13] Clusters 1 and 2 primarily encompassed liver fluke-positive CCAs and were enriched in *ERBB2* amplification and *TP53* mutation; clusters 3 and 4 primarily encompassed liver fluke-negative CCAs, with cluster 3 displaying immune checkpoint gene upregulation (*PD1*, *PD-L1*, and *BTLA*), and cluster 4 exhibiting *IDH1/2* and *BAP1* mutations, as well as *FGFR* alterations.[13] Another study also reported significant differences in genomic profiles between liver fluke-positive and -negative CCA, including *BAP1* mutations which were more prevalent in liver fluke-negative iCCA.[31] Elsewhere a whole-exome sequencing (WES) study of iCCA samples from 103 Chinese patients provided evidence for an association between somatic *TP53* mutations and HBsAG-seropositivity,[32] suggesting a role of p53-mediated signaling in iCCA resulting from HBV infection.[8]

The question whether the genomic signature of CCA changes longitudinally and whether the sample assessed is derived from primary tumor or metastases biopsies remains in debate. Evidence supporting this was provided by a larger comparative comprehensive genomic profiling study, which demonstrated that the frequency of *KRAS* mutations was significantly (~2-fold) greater in metastasis versus primary tumor biopsies.[33] However, contrary to these findings, a recent NGS study did not find any significant differences in the frequencies of genetic alterations in primary tumor biopsies (n = 141) versus metastasis biopsies (n = 54).[24] Of note, *FGFR2* fusions have been reported to occur in surgical resections from patients with early-stage cholangiocarcinoma, indicating that *FGFR2* fusions may occur early in oncogenesis and may drive subsequent disease progression.[34] Clonal mutations, such as *FGFR* fusions, would be expected to be present in most re-biopsies performed longitudinally, including those performed at metastases. Moreover, for similar reasons, it is unlikely that *FGFR* fusions would emerge from chemotherapy.

Clinically Actionable Genetic Alterations

Although *FGFR2* rearrangements, which are found in approximately 10% to 13% of patients with iCCA,[17,22,24,35] are currently the only genetic alterations with an approved targeted therapy for CCA, an estimated 40% to 50% of patients with CCA have at least one genetic alteration that may be clinically actionable.[24,25] "Actionable genes" are often differentiated based on information provided by the oncoKB database, which categorizes them by level of evidence supporting treatment, including treatment approved by the US Food and Drug Administration (FDA) (level 1), standard of care (level 2), clinical evidence (level 3), and biological evidence (level 4).[36,37] A ranking system developed by the European Society of Medical Oncology (ESMO), the ESMO Scale for Clinical Actionability of molecular Targets (ESCAT), distinguishes five levels of treatment evidence, including improved outcomes in clinical trials (level I), antitumor activity associated with unknown magnitude of benefit (level II), improved benefit in

other tumor types (level III), preclinical evidence (level IV), and objective response without improved outcomes in clinical trials (level V).[38] Based on the current evidence, ESMO further recommended routine use of NGS multigene panels covering level I alterations on tumor samples in a number of epithelial cancers including cholangiocarcinoma.[39] For the purpose of this review, we considered genetic alterations in CCA to be actionable if a targeted therapy is approved for any indication or under investigation in a pivotal phase 2 or 3 trial in patients with BTC or CCA (Table 1).

Results from larger genomic profiling studies suggest that *IDH1* and *FGFR2* are the most common genes with actionable alterations in iCCA (Figure 2). In FIGHT 202, an estimated 45% of the 1206 patients screened had clinically actionable genetic alterations.[25] The most common were *IDH1* missense mutations (10%), *ERBB2* alterations (8%: 48% amplifications, 24% missense mutations, 28% multiple alterations), *FGFR2* alterations (7%: 59% fusions, 17% other rearrangements, 12% missense mutations, 12% multiple alterations), *PIK3CA* alterations (7%: 91% missense mutations, 9% multiple alterations), and *BRAF* alterations (5%: 92% missense mutations, 5% rearrangements, 3% multiple alterations) (Figure 2A).[25] Similar findings were reported by Javle et al., who profiled more than 4000 CCA samples obtained primarily through liver biopsies, and by Lowery et al. who profiled 195 patients of whom 81% had been diagnosed with iCCA (Figure 2A).[24,27] Results from these two studies further suggest that *IDH1* and *FGFR2* alterations are largely mutually exclusive,[24,27] although co-occurring *IDH1* mutations were found in 5.1% of patients with *FGFR2*-rearranged CCA in the FIGHT-202 prescreening analysis by Silverman et al.[25]

The larger profiling studies in CCA by Silverman et al.[25] and Javle et al.[27] revealed that many genes with actionable alterations are found in less than 2% of patients (e.g., *EGFR*, *FGFR3, FGFR1*, *RET*, *ALK*, and *ROS1*). Furthermore, in both studies, MSI-high was observed in approximately 1% of patients, and TMB >20 mutations per megabase was observed in slightly more than 1% of patients;[25,27] 3% of patients profiled by Javle et al. had TMB >10

mutations per megabase.[27] MSH-high and high TMB were highly correlated,[25,27] but did not commonly coincide with other actionable alterations.[25] In the FIGHT-202 analysis, no patient with an *FGFR2* rearrangement had MSI-high status or TMB >20 mutations per megabase.[25]

Quantitative data for actionable genetic alterations in patients with eCCA are limited, due to the small number of studies specifically conducted in this patient population, relative small sample sizes, and variable criteria used to identify actionable genetic alterations.[15,23,26] Important differences between the molecular profiles of eCCA and iCCA are the low frequency of *IDH1* mutations and apparent lack of *FGFR2* fusions/rearrangements in eCCA.[15,23,24,26] Results of a recent study in 189 patients with eCCA suggest that approximately 25% of the patients harbor actionable mutations.[15] *ERBB2* and *PIK3CA* each were altered in 5% of patients, *BRCA1/2* and *IDH2* each in 3% of patients, *BRAF* and *IDH1* each in 2% of patients, and *EGFR* in 1% of patients (Figure 2B).

Because the genomic profile of CCA is governed by the underlying etiology, the presence (or absence) of actionable genetic alterations may also vary across geographic regions of the world. For example, the integrative clustering analysis of 489 CCA samples spanning 10 countries described above found that *IDH1/2* and *FGFR2* rearrangements were exclusive to a cluster described by liver fluke-negative CCA etiologies.[13]

Of note, the comparative comprehensive genomic profiling study described above demonstrated that among actionable genetic alterations in metastasis versus primary tumor biopsies, respectively, the KRAS G12C mutation was significantly more frequent, whereas *IDH1* mutations and *FGFR2* alterations were significantly less frequent.[33] Because *IDH1* and *FGFR2* alterations are highly characteristic of iCCA, the authors concluded that the metastasis biopsies assessed may have included metastatic lesions derived from primary tumors that had been misclassified as iCCA.[33]

Targeted Therapies

Increasing understanding of the unique and complex mutational landscape of CCA has fueled efforts to develop therapies targeting specific genetic alterations in CCA. The complexity and heterogeneity of the mutational landscape in CCA is best illustrated by the large and growing number of different novel *FGFR2* fusions that have been detected in patients with iCCA.[25,27] While Silverman et al. found 63 unique *FGFR2* fusion partners in 1206 patients with iCCA,[25] profiling of 4371 patients with CCA identified 144 different *FGFR2* fusion partners, 131 of which were each found in less than five patients.[27] Pemigatinib was recently approved in the US, Europe, and Japan for the treatment of patients with previously treated unresectable, locally advanced CCA with *FGFR2* fusions and other rearrangements[40] based on results of FIGHT-202 showing an objective response rate (ORR) of 35.5% (95% confidence interval [CI], 26.5-45.4), a median progression-free survival (PFS) of 6.9 months (95% CI, 6.2-9.6), and a median OS of 21.1 months (95% CI, 14.8-not estimable).[29] A randomized phase 3 study investigating the efficacy and safety of pemigatinib vs chemotherapy as first-line therapy in unresectable or metastatic cholangiocarcinoma with *FGFR2* rearrangement (FIGHT-302, NCT03656536)[41] is currently recruiting patients. In addition to pemigatinib, other *FGFR* inhibitors, including derazantinib,[42-44] infigratinib,[45,46] Debio 1347,[47,48] and futibatinib[49-51] have provided encouraging results in early-phase clinical studies for patients with CCA with *FGFR2* alterations, and most of these agents are now in phase 2 or 3 clinical development (Table 1). In a phase 2 study (NCT02150967) of infigratinib in patients with previously treated CCA with *FGFR2* alterations, those with *FGFR2* fusions (*n* = 71) had an ORR of 31.0% (95% CI, 20.5-43.1%), a median PFS of 6.8 months (95% CI, 5.3-7.6), and a median OS of 12.5 months (95% CI, 9.9-16.6).[46] An ongoing randomized controlled phase 3 study (PROOF 301, NCT03773302) is comparing first-line therapy with infigratinib vs gemcitabine plus cisplatin in patients with advanced cholangiocarcinoma with *FGFR2* translocations.[52]

IDH1 missense mutations currently are the most common actionable genetic alterations in iCCA,[24] and thus of particular importance as therapeutic targets in CCA. *IDH1* missense

mutations are known oncogenic drivers in acute myeloid leukemia, which can be treated with the FDA-approved IDH1 inhibitors ivosidenib and enasidenib.[53] The efficacy and safety of the selective IDH1 inhibitor ivosidenib in patients with CCA harbouring *IDH1* mutations was assessed in the global phase 3 ClarIDHy study (NCT02989857).[54] The primary analysis in 185 patients (92% with metastatic CCA) demonstrated significantly longer PFS with ivosidenib vs placebo (hazard ratio [HR] = 0.37, 95% CI: 0.25-0.54; $P < 0.001$; median, 2.7 vs 1.4 months).[54] However, the ORR with ivosidenib was low (2.4% vs 0% with placebo).[54] The difference in OS between ivosidenib and placebo arms in this analysis was not statistically significant (HR 0.69 [95% CI, 0.44-1.10]; $P = 0.060$; median 10.8 vs 9.7 months);[54] this difference remained non-significant in a later final analysis in 187 patients when OS maturity had been achieved (HR = 0.79 [95% CI, 0.56–1.12; $P = 0.093$; median 10.3 vs 7.5 months).[55] This apparent lack of a significant difference in OS between arms likely reflects the high crossover rate from placebo to ivosidenib in the trial (70%).[55]Many genes altered in CCA are potentially actionable with targeted therapies that have approved indications in other cancers, such as non-small-cell lung cancer (NSCLC), breast cancer, and melanoma (Table 1). Some of the targeted therapies established in other cancers are currently being investigated in CCA or BTC with DNA repair gene mutations including *BRCA1* and *2, ERBB2, and PIK3CA* (Table 1). Furthermore, a phase 2 basket trial of dabrafenib plus trametinib provided encouraging results in patients with previously treated advanced *BRAF*V600E-positive BTC, including a median OS of 11.3 months (95% CI, 7.3-17.6).[56] Of note, the immune checkpoint inhibitor pembrolizumab was recently approved in the US for the treatment of patients with solid tumors with MSI-high or TMB >10 mutations per megabase who have no alternative treatment options,[57] and thus is also available for patients with CCA who have these alterations. In addition, FDA-approved therapies are available for the treatment of patients with solid tumors harboring *NTRK* fusions (Table 1). Although the US clinical studies leading to the approval of these therapies included a few

patients with CCA with *NTRK* fusions,[58,59] *NTRK* fusions appear to be rare among patients with CCA treated in the US.[17,25,27]

The demonstrated efficacy of FGFR inhibitors exerts selection pressure favoring clonal evolution and acquired resistance; a knowledge of the associated resistance mechanisms is vital for guiding salvage treatment decisions. To allow this, it is recommended to obtain biopsies of primary tumors as well as any metastases after progression on treatment. Several acquired *FGFR2* mutations have been identified in patients with CCA who have progressed on FGFR inhibitor treatment.[25,51,60] Among 8 patients progressing on pemigatinib in FIGHT-202, all acquired resistance mutations spanning 5 amino acid residues (residues numbered according to FGFR2-IIIb splice isoform: *FGFR2* p.N550H/K, p.E566A, p.K660M, p.L617V, p.K641R), with 3 patients acquiring polyclonal mutations.[25] The *FGFR2* p.N550H mutation was also identified in a separate case study of a patient with iCCA harboring an *FGFR2-CLIP1* fusion who had progressed on pemigatinib.[61] A case series of 3 patients with iCCA harboring *FGFR2* fusions who had progressed on infigratinib detected the same 5 point mutations as well as an additional *FGFR2* p.V564F variant.[60] Point mutations were also detected in another case series of 4 patients with iCCA progressing on infigratinib (*FGFR2* p.K660M, p.K715R, p.N550H/K/T, p.V565F, and p.E566A), or Debio1347 (*FGFR2* p.H683L and p.M538I). Importantly, in vitro assays demonstrated that *FGFR2* point mutations including p.N549H, p.E565A, and p.L617M reduce the potency of FGFR inhibitors including AZD4547, erdafitinib, Debio1347, pemigatinib, and infigratinib.[51,61,62] In keeping with this, N549, E565 (and V564) residues participate in the ATP-binding site and may perturb the ATP-competitive binding of FGFR inhibitors.[25,51] The effects of acquired resistance mutations on FGFR inhibitor potency remain to be fully determined, and there is an unmet need for inhibitor molecules rationally designed to overcome such acquired resistance.

Genomic Profiling to Guide Treatment Decisions

Information on whether and when NGS-based genomic testing should be performed in patients with CCA is currently lacking in the literature. However, this question has been examined more generally in patients with cancer,[63-67] and reference to these findings shed light on when NGS might be useful in patients with CCA. In general, it has been suggested that NGS may not be clinically warranted in patients with early-stage cancer, because molecular profiling in these patients is unlikely to yield actionable genetic alterations other than those that could be identified from conventional approaches (see below).[66] Because of the high costs associated with administering expensive anticancer agents in the off-label setting, it has also been posited that, in general, NGS use might be more suited to clinical trials and investigational/research studies rather than community practice.[63,64,68] Nevertheless, given the genetic heterogeneity of CCA and large number of potentially actionable genetic alterations,[17,22-28] patients with early stage CCA might benefit from multiplex NGS after diagnosis. The consensus in the literature for patients with locally advanced or metastatic cancer, including CCA, is that many could benefit from early NGS-testing to guide treatment planning, given that few standard-of-care treatment options are available to them.[65-67] In keeping with this, treatment guidelines recommend considering molecular profiling for CCA at advanced stage, when the tumor is unresectable or metastatic[69,70] with the motivation of matching these patients to basket trials recruiting for corresponding genetic alterations. On a practical note, if resection is planned for a patient with CCA, a preoperative biopsy for molecular profiling may not be routinely recommended to avoid treatment delays.[71] In this situation, given the high rate of relapse after surgery, patients with advanced CCA should be encouraged to undergo a biopsy for molecular profiling as soon as possible after diagnosis to proactively establish a treatment plan.[65-67] The authors also recommend requesting the surgical team to reserve a tissue sample during surgery, which may then be used for molecular profiling.

Two main considerations for selecting molecular screening tests for a specific type of cancer are the need for comprehensive coverage of altered genes that are potentially actionable and the ability to identify commonly occurring types of genomic alterations, including single-nucleotide variants (SNVs), insertions and deletions (indels), gene amplifications, and chromosomal rearrangements. CCA has a diverse landscape of oncogenic drivers, representing all types of these genomic alterations (Figure 3). Although some genomic alterations found in CCA have been well characterized in other cancer types and may be identified with established conventional tests, others, such as the large and growing number of *FGFR2* rearrangements, are not easily detected by conventional testing approaches. In addition, the molecular profile in individual patients varies substantially and is a priori unknown. Thus, a useful molecular diagnosis to aid therapeutic decision making would require an unbiased and comprehensive testing strategy.

In the US, the FDA has recognized the importance of NGS-based genomic profiling in cancer diagnostics, as indicated by the clearance or approval of a number of such assays for in vitro diagnostics (IVD).[72,73] Of note, FoundationOne CDx, a large-scale genomic profiling assay targeting up to 324 genes, was recently approved as a companion diagnostic test for pemigatinib therapy in patients with CCA with *FGFR2* fusions or other rearrangements.[74] Furthermore, the ESMO Precision Medicine Working Group recently issued recommendations for the use of NGS in patients with specific types of metastatic cancer, including advanced CCA.[39] For CCA, it was recommended to use targeted multigene NGS-based genomic profiling for the detection of ESCAT level I actionable alterations, such as *IDH1* mutations, *FGFR2* and *NTRK* fusions, and MSI-high.[39]

Conventional Genetic Tests

A large number of established conventional tests, including FDA-cleared or approved companion diagnostic tests for approved therapies,[72] are available for alterations that have been

well characterized in other cancer types, including but not limited to *BRAF*V600 mutations, *ERBB2* amplifications, *EGFR* and *KRAS* mutations, and *ALK, ROS1,* and *EGFR* rearrangements.[75-82] Common conventional tests are based on immunohistochemistry (IHC), fluorescence in situ hybridization (FISH), or various strategies involving DNA or RNA sequence amplification via polymerase chain reaction (PCR). Although most conventional tests are inexpensive and rapid to perform (within a day), they are not suitable for high-throughput profiling of multiple genes and generally require knowledge of the targeted alterations.

IHC is widely used to detect overexpression of specific oncogenic proteins, where the underlying genetic cause for phenotypical changes in protein expression are a priori known. However, IHC test results may be difficult to quantify,[83] and test quality depends on the sensitivity and specificity of available antibody probes. In practice, IHC is used to confirm well-characterized amplification events of specific genes, such as *HER2* (*ERBB2*) amplifications in breast cancer,[83,84] and validated IHC tests are available to determine the presence of established rearrangements associated with overexpression of the resulting fusion protein in tumor tissue compared with the native protein in control tissue (e.g., established *ALK* and *ROS1* fusions).[85,86] IHC may be used as a screening method to detect *NTRK* fusions in CCA.[87] However, it must be emphasized that no IHC techniques have been validated up to now for the detection of *FGFR2* fusions. In addition, some mutations, including *EGFR* mutations associated with non-small-cell lung cancer[88] and the *BRAF*V600E mutation[75,77] can be detected by IHC using mutation-specific antibodies.

FISH, which relies on direct hybridization of a fluorescence-labeled DNA-probe with genomic DNA, is used to quantify gene amplifications and test for known rearrangements, including gene fusions, in specific genes.[82] Break-apart FISH is a common approach to detect gene fusions. It requires the use of two differently labeled DNA probes (red and green fluorescence) encompassing the fusion breakpoint to create a signal of overlapping red and green fluorescence that is specific to the unaltered gene. Rearrangements at the fusion breakpoint

increase the distance between the 5' and 3' probes, resulting in the separation of the red and green fluorescent signals.[82] Break-apart FISH does not require knowledge of the precise fusion breakpoint or the identity of the possible fusion partners. However, the reliability of break-apart FISH is sensitive to the distance between the 5' and 3' probes before and after rearrangement, requiring careful probe design and validation for each assay to avoid false negative results. Especially, intrachromosomal rearrangements, which account for approximately 50% of all *FGFR2* rearrangements in CCA,[89] may not be detected if the distance between the 5' and 3' probes after rearrangement remains too short.

Among conventional tests, PCR-based assays provide the greatest versatility in detecting mutations. DNA-based quantitative PCR can be used to screen for specific deletion mutations and SNVs in exon sequences.[75,90,91] In addition, RNA-based real-time reverse transcription PCR is a fast and sensitive method to detect transcribed gene fusions, where both fusion partners and the location of the breakpoint are known.[92] The use of multiplex PCR may allow simultaneous detection of different known alterations at the same time.[93] An example is the *therascreen* FGFR RGQ RT-PCR Kit, which is a companion diagnostic test for erdafitinib use in urothelial cancer that allows the simultaneous detection of disease-specific *FGFR3* mutations and fusions.[94] Furthermore, combining real-time PCR with subsequent amplicon sequencing can be used to test for unknown mutations in specific target regions of genomic DNA.[75] Because of the large number of potential *FGFR2* fusion partners in CCA (>150), real-time PCR is not feasible for *FGFR2* fusion testing in this disease.

NGS-Based Molecular Profiling

The development of next-generation sequencing (NGS) platforms that allow massive parallel sequencing of large numbers of genes with unprecedented sensitivity, specificity, accuracy, and speed[95] provides oncologists with the opportunity for comprehensive unbiased molecular

screening of individual patients to detect genetic alterations that may inform treatment decisions.

NGS-Based Assay Technologies

Although WES and whole transcriptome sequencing are available options for genomic profiling (e.g., Caris Molecular Intelligence[96]), technologies for targeted NGS (e.g., MSK-IMPACT[97] and FoundationOne CDx[98]) have been developed that allow comprehensive genomic profiling of select gene panels to improve coverage of relevant tumor-specific genes, reduce the amount of input material needed, and shorten turnaround time.[99]

Targeted NGS-based tests may differ in multiple respects, including the type of acceptable source material (e.g., formalin-fixed paraffin-embedded tumor tissue or peripheral whole blood), the genetic source material used for library construction (genomic DNA, RNA, or circulating cell-free DNA [cfDNA]), the technology used for target selection (hybrid selection or amplicon-based technologies), the size of available gene panels, and the type of genetic alterations that can be detected.

Genomic profiling assays for solid tumors generally require formalin-fixed paraffin-embedded (FFPE) tissue samples obtained by tumor biopsy. However, biliary tract tumors may not always be easily accessible, as is often the case for primary cholangiocarcinoma, rendering them operationally difficult to biopsy.[100] In this regard, a recent retrospective analysis of 149 tumor samples from 104 patients with advanced biliary tract cancers demonstrated a high sample failure rate of 26.8% in tissue biopsies, which was mainly due to inadequate tumor content in the sample (91.2%).[101] Although repeat biopsy might remedy sample failure due to inadequate tumor content, this is also accompanied by correspondingly increased morbidity risk.[102] In addition, FFPE processing and storage of tumor tissue for RNA-based assays may pose practical challenges due to the instability of RNA, which is easily degraded by omnipresent

RNases. Thus, ensuring the integrity of biopsy materials (e.g., FFPE tissue samples) is critical for the performance of RNA-based tests.

To overcome these practical challenges with sample integrity and failure, NGS-based genomic profiling assays using blood samples (liquid biopsy) to extract cfDNA are being developed and validated for clinical applications in patients with advanced solid tumors.[103,104] However, although liquid biopsies are minimally invasive, circulating tumor DNA (ctDNA) constitutes only a fraction of cfDNA, and limited quantities of ctDNA retrieved from blood samples pose challenges to the sensitivity of liquid biopsy-based assays for solid tumors. Recent studies of NGS-based genomic profiling in patients with advanced tumors have shown variable concordance (<60% to 100%) in the detection of clinically relevant genetic alterations in plasma-derived ctDNA versus genomic DNA derived from tumor biopsies.[101,105-110] Low concordance has been attributed, in part, to intra-tumoral genomic heterogeneity (e.g., between primary and metastatic sites).[105,109-111] However, a high degree of concordance between plasma- and tissue-based NGS has been reported for established validated biomarkers in gastrointestinal cancer,[111] and results from a large observational screening study in patients with advanced gastrointestinal cancer showed that plasma-based vs tumor tissue-based testing within the same centers significantly shortened screening time without compromising patient selection for targeted therapies.[112] The increasing clinical relevance of ctDNA testing is further illustrated by the recent approval of two cfDNA-based genomic profiling assays, FoundationOne Liquid CDx[113] and Guardant360 CDx,[114] as companion diagnostics for the treatment of various solid tumors.[73] Taken together, plasma derived cfDNA testing could facilitate identification of additional therapeutically targetable genetic alterations not identified due to biopsy and/or sample failure.

The types of genetic alterations that are preferentially detected by NGS-based molecular profiling tests depend to a large extent on whether genomic DNA or RNA is used as the genetic source material (Table 2). DNA-based tests are capable of determining essentially any type of

genomic alteration, including SNVs, indels, rearrangement, amplifications, TMB, and MSI-high. However, the specific capabilities of a given test also depend on the size of the available gene panel and the type of sequences targeted, such as promoter regions and other intronic sequences in addition to exons. The main drawback of tests targeting genomic DNA is that effects on gene expression and the sequences of transcripts resulting from rearrangements have to be predicted for novel alterations. In contrast, RNA-based assays detect genomic alterations manifest in the transcriptome, including those more difficult to detect with DNA-based assays, such as alternative splicing events and complex gene fusions.[115-118] Furthermore, they allow quantification of gene expression to provide direct phenotypic evidence of activating genetic alterations. However, because mutations in genes expressed at low level can be missed, and heterozygous loss-of-function mutations cannot be detected, DNA-based NGS methods are generally preferred for the detection of exonic mutations.

Targeted NGS-based genomic profiling platforms using DNA or RNA can roughly be distinguished by two main strategies used for target enrichment (Table 2), namely hybrid capture (e.g., FoundationOne[95,98] and MSK-IMPACT[97,119]) and amplicon-based approaches (e.g., Oncomine Dx Target Test[120] and Archer FusionPlex Solid Tumor panel[121,122]). Target enrichment via hybrid capture is achieved by using gene-specific hybridization probes to select the desired target sequences from shotgun genomic DNA libraries (DNA-based method)[95] or from libraries of expressed transcripts (RNA-based method).[116] Hybrid capture-based NGS has been used for comprehensive genomic profiling of large gene panels (>400 genes) in large patient populations, including patients with CCA,[25,27] to screen for genetic alterations that included base substitutions, indels, rearrangements, gene amplifications, MSI, and TMB.[95,119,123]

Amplicon-based approaches targeting genome or transcriptome sequences use target-specific primers for PCR amplification of target sequences during library construction. Amplicon-based approaches are rapid and require relatively little DNA;[120] however, hybrid capture-based NGS has been reported to provide more uniform coverage and yield higher sensitivity for variant

calling.[124] For anchored multiplex PCR, a universal adapter sequence is added to the target sequence to allow PCR amplification with a gene-specific primer at one end and a universal primer at the other end.[121] RNA-based anchored multiplex PCR is particularly useful for the profiling of gene fusions,[118] especially of genes that have a large number of known and unknown fusion partners, as in the case of *FGFR2* fusions in patients with iCCA.[25] RNA-based NGS technologies combining the use of universal and gene-specific primers in multiplexed assays, such as the Archer FusionPlex NGS assay, allow the simultaneously detection of any known or unknown 5' or 3' prime fusion partner of multiple targets.[125] Comparison of RNA-based NGS technologies for the detection of fusions demonstrated that the performance in detecting unknown fusions and known fusions with unknown breakpoints was affected by library preparation technology and exon coverage.[126]

Despite the sensitivity and accuracy of NGS-based platforms for detecting gene fusions, including *FGFR2* fusions,[25] the question arises whether a second platform, based on different architecture should be used to confirm the presence or absence of a gene fusion, if the presence of that fusion is suspected. If the initial platform that generated a negative result was suboptimal for detecting fusions (ie RT-PCR-based), then use of a different platform would be warranted. However, this should be weighed against current data suggesting a high degree of concordance between platforms in the results generated.[127,128]

NGS-Based In Vitro Diagnostic Tests

NGS-based assays exist in a variety of formats, including FDA-cleared or approved IVDs or internally validated laboratory-developed tests (LDTs). As of November 2020, a number of "tumor profiling" assays have been cleared or approved by the FDA for use as an IVD, with panels of up to several hundred genes (Table 3).[72] Most of these tests are or will become available for routine diagnostic testing through commercial clinical testing laboratories.

Clinical centers also may design their own LDTs to aid clinical decision making in the absence of appropriate commercially available tests. NGS-based LDTs derived from

commercially available test kits (designated “for research only”), such as Oncomine Comprehensive Assay Plus[129] and TruSight Oncology 500,[130] may require extensive customization and validation to be able to serve as suitable and reliable molecular diagnostic tests for specific cancers. Memorial Sloan Kettering Cancer Center (MSKCC) was one of the first academic centers to develop its own large-scale genomic profiling platform, MSK-IMPACT, in order to provide molecular diagnostic services for its patients, and MSK-IMPACT was cleared in 2017 by the FDA for use as an IVD (Table 3).[97] For oncologists associated with clinical institutions that do not have the resources to provide suitable validated NGS-based LDTs, large commercial laboratories, such as Foundation Medicine (Cambridge, MA), Caris Life Sciences (Irving, TX) , Tempus (Chicago, IL), NeoGenomics (Fort Myers, FL), and Kew (Cambridge, MA), offer comprehensive genomic profiling services with turnaround times of approximately 2 to 3 weeks. Most of these services involve proprietary targeted NGS platforms currently designed for clinical research only (e.g., Tempus xT,[131] Neotype Discovery Profile,[132] and CANCERPLEX[133] (Table 3).

In the UK, the National Health Service (NHS) England is establishing a National Genomic Medicine Service through a network of genomic laboratory hubs tasked to coordinate services for different parts of the country.[134] NHS England recently activated its genomic testing services for patients with cancer. The 2019/2020 National Genomic Test Directory for cancer specifies the genomic tests commissioned by the NHS England for each cancer type, including conventional and NGS-based tests, patients’ eligibility criteria for each test, and the scope of each test.[135]

Cost of NGS-Based Tests

Since the sequencing of the Human genome in 2003, which was the culmination of an approximately decade-long international collaboration costing several hundreds of millions of dollars, the cost of genomic sequencing has decreased exponentially with the advent of NGS and improved sample handling and analysis efficiency.[136] The current cost of the NGS-based

molecular profiling tests varies widely and the turnaround times range from a few days to ~2 weeks (Table 3). Current research focused on the overall cost and cost-effectiveness of NGS-based molecular profiling has been reviewed extensively elsewhere.[100,101,137-143] Overall, there is a paucity of real world evidence for the cost-effectiveness of the use of NGS in routine clinical practice, upon which payers rely for making coverage decisions.[144,145] In keeping with this, a qualitative study published in 2015 which canvassed U.S. payers for their opinions regarding challenges associated with coverage of NGS-based tests cited a lack of evidence-based support leading to a belief that NGS should be considered an investigational/experimental modality rather than a medical necessity.[146] Nevertheless, the coverage policies of private and governmental payers are starting to include NGS-based testing, in part in recognition of recent FDA approvals of NGS-based companion diagnostic tests including FoundationOne CDx, FoundationOne Liquid CDx[98], and Oncomine DX Target Test[120] for patients with CCA.[73,147] Studies of the overall cost and cost-effectiveness of NGS have drawn primarily on data from the US; the important question of how these data translate to financial burden of NGS-based testing on patients located in other countries, particularly in those of middle- and low-income, remains to be resolved. Finally, any cost-effectiveness evaluation should take into consideration the clinical scenario. Most patients with iCCA have only limited biopsy sample available for molecular profiling; NGS allows the detection of multiple biomarkers in a single analysis, thus reducing the amount of tissue needed for testing. When using standard techniques analyzing a single biomarker per test, a significant fraction of patients will need an additional biopsy to obtain sufficient tissue to test for all the approved biomarkers, as has been already shown in lung cancer.[148]

Conclusion

CCA is characterized by significant molecular heterogeneity, with implications for prognosis and treatment. The molecular landscape of CCA, especially iCCA, is unique, as it includes alterations not commonly found in other solid tumors, such as *IDH1* mutations and a large variety of *FGFR2* rearrangements. Although *FGFR2* rearrangements are the only genetic alteration in CCA for which a targeted therapy has been approved, favorable phase 3 results for an *IDH1*-targeted therapy have recently been published, and large-scale genomic profiling has identified numerous actionable alterations for which targeted therapies are approved in other indications. Given the paucity of current treatment options and the limited effectiveness of standard chemotherapy in CCA, “molecular diagnosis” provides an important opportunity for improved personalized treatment plans in CCA.

Large-scale genomic profiling based on NGS allows a comprehensive and precise molecular diagnosis of CCA in individual patients that cannot be achieved with conventional molecular diagnostic tools. Due to the progress in sequencing technologies in the last decade, NGS-based tests can now produce results for individual patients within 1 to 2 weeks.

Currently available tests vary with regard to the technologies used and the number of genes that can be covered. DNA-based tests using hybrid selection or amplicon-based technology for target enrichment can cover essentially any genomic alteration and are offered for the profiling of standard or custom gene panels that may include more than 400 genes. RNA-based tests using anchored multiplex PCR to generate amplicons are ideal to screen for *FGFR2* fusion transcripts with large numbers of different or unknown fusion partners.

Recent approvals of NGS-based genomic profiling assays as IVDs illustrate their increasing relevance in cancer diagnosis to enable personalized medicine. The recent successes and continuing efforts in the development of targeted therapies for patients with CCA suggest that genomic profiling will become an integral diagnostic tool to guide treatment decisions in CCA. However, future development of precision medicine in CCA will likely require the integration of information from multiple omics approaches. For example, response rates to FGFR and IDH

inhibitors in iCCA are lower than response rate to targeted therapy in other oncogene-addicted cancer, such as EGFR-mutated lung cancer. In addition to genomic profiling, transcriptomics, proteomics, and metabolomics may provide valuable information to improve treatment stratification of patients with CCA.

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Table 1. Actionable genetic alterations and associated targeted therapies in CCA

Gene	Genetic alteration	Targeted therapy	Approved indication[a]	Clinical trial in BTC or CCA
IDH1	Mutation	Ivosidenib	AML	Phase 3 – CCA (NCT02989857); with results[54]
IDH2	Mutation	Enasidenib	AML	
FGFR2	Rearrangement, mutation, amplification	Pemigatinib	CCA	Phase 2 – CCA (previously treated, NCT02924376)[29] Phase 3 – CCA (first line, NCT03656536)
		Infigratinib		Phase 2 – CCA (previously treated, NCT02150967) Phase 3 – CCA (first line, NCT03773302)
		Futibatinib		Phase 3 – CCA (first line, NCT04093362)
		Derazantinib		Phase 2 – CCA (previously treated, NCT03230318)
		E7090		Phase 2 – CCA (previously treated, NCT04238715
		Erdafitinib	Urothelial carcinoma	
FGFR3	Mutation, rearrangement	Erdafitinib	Urothelial carcinoma	
BRAF	Mutation, rearrangement	Encorafinib	Colorectal cancer, melanoma	
		Dabrafenib	Melanoma, NSCLC, anaplastic thyroid cancer	Phase 2 – iCCA (NCT02465060)
		Vemurafenib	Melanoma	
BRCA1/2	Mutation	Olaparib	Breast cancer	Phase 2 – BTC (NCT04042831)
ERBB2	Amplification,	Trastuzumab	Breast cancer	Phase 2 – CCA:

	mutation			Trastuzumab (NCT00478140) Trastuzumab emtansine (NCT02999672)
		Tucatinib	Breast cancer	
		Lapatinib	Breast cancer	Phase 2 – BTC/Liver cancer (NCT00101036, NCT00107536)
		Neratinib	Breast cancer	
		Afatinib, dacomitinib	NSCLC	
PIK3CA	Mutation	Alpelisib	Breast cancer	
		Copanlisip	Follicular lymphoma	Phase 2 – CCA (NCT02631590)
CDK4/ CDK6	Amplification	Palbociclib, ribociclib, abemaciclib,	Breast cancer	
MET	AMP	capmatinib	NSCLC	
RET	Rearrangement, mutation	Selpercatinib	NSCLC, thyroid cancer	
EGFR	Mutation	Erlotinib, gefitinib	NSCLC	
ALK	Rearrangement	Alectinib, lorlatinib, ceritinib, brigatinib, crizotinib	NSCLC	
ROS1	Rearrangement	Entrectinib, crizotinib	NSCLC	
NTRK	Fusion	Larotrectinib, entrectinib	Solid tumors	

JAK2	Mutation	Ruxolitinib, fedratinib	Myelofibrosis
MSI-high		Pembrolizumab	Tumor-agnostic
		Nivolumab	Colorectal cancer
TMB >10 mutations/megabase		Pembrolizumab	Tumor-agnostic

AML, acute myeloid leukemia; BTC, biliary tract cancer; CCA, cholangiocarcinoma; MSH, microsatellite instability; NSCLC, non-small-cell lung cancer; TMB, tumor mutational burden.

[a]See prescribing information for details.

Table 2. Comparison of DNA- and RNA-based NGS assays for targeted molecular profiling in CCA

	DNA-based	RNA-based
Sequences targeted	Genome: exons, introns, promoter regions	Transcriptome: exons, transcribed rearrangements
Size of gene panels	<20 to >600	<100
Alterations detected	SNVs, indels, CNVs, rearrangements, MSI, TMB	SNV, indels, alternative splicing events, gene fusions
Limitations	Effects of genetic alterations on gene expression have to be predicted	Quality of biopsy material is critical due to RNA instability
	Only hybrid-capture based assays may identify unknown fusion partners	Alterations in genes expressed at low level may be missed
Main applications in CCA	Comprehensive profiling of genetic alterations	Identification of fusion transcripts, including novel fusions[b]

CNV, copy number variation; indel, insertion/deletion; MSI, microsatellite instability; NGS, next-generation sequencing; TMB, tumor mutation burden; SNV, single-nucleotide variation.

[a]Using multiplexed amplicon-based approaches combining universal and gene-specific primers.

Table 3. Targeted NGS-based molecular profiling assays

Company	Test	Specimen	Template	Enrichment technology	Gene panel size	Genetic alterations	FDA Approved?[a]	Turnaround	Cost[c]
Memorial Sloan Kettering Cancer Center	MSK-IMPACT[97]	FFPE	DNA	Hybrid capture	468	SNVs, indels, MSI	Yes	NR	NR
Foundation Medicine	FoundationOne CDx[98]	FFPE	DNA	Hybrid capture	324	SNVs, substitutions, indels, CNAs, rearrangements, MSI, TMB, LOH	Yes[b]	~2 weeks	3500 USD
	FoundationOne Liquid CDx[113]	Whole blood plasma	cfDNA	Hybrid capture	311	SNVs, substitutions, indels, CNAs, rearrangements; blood TMB, MSI	Yes	~2 weeks	3500 USD
	FoundationOne Heme[149]	Whole blood, bone marrow aspirate	DNA + RNA	Hybrid capture	406 (DNA), 265 (RNA)	SNVs, substitutions, indels, CNAs, rearrangements, MSI, TMB	No	~2 weeks	3500 USD
Guardant Health	Guardant360 CDx[114]	Whole blood plasma	cfDNA	Hybrid capture	55	SNVs, indels, CNAs, fusions	Yes	1 week	3500 EURO
ArcherDX	FusionPlex Solid Tumor[122]	FFPE	RNA	Anchored multiplex PCR	53	Fusions	No	3-5 days	NR
Tempus	Tempus xT[131]	FFPE	DNA + RNA	NR	596	SNVs, indels, CNAs,	No	~2 weeks	NR

			(for transcriptome sequencing)			rearrangements, MSI, TMB			
ThermoFisher Scientific	Oncomine Comprehensive Assay Plus[129]	FFPE	DNA, RNA	AmpliSeq	>500	SNVs, indels, CNA, fusions, MSI, TMB	No	5 days	14,570.00 USD
	Oncomine Dx Target Test[120]	FFPE	DNA + RNA (for fusions only)	AmpliSeq	23	SNVs, deletions, *ROS1* fusions	Yes	4 days	NR
Illumina	TruSight Oncology 500[130]	FFPE	DNA + RNA (for transcriptome sequencing)	Hybrid capture	523	SNVs, indels, CNA, rearrangements, MSI, TMB	No	4-5 days	17,999 USD
NeoGenomics Laboratories	NeoType Discovery Profile for Solid Tumors[132]	FFPE	DNA, RNA	NR	323	SNVs, indels, rearrangements, MSI, TMB	No	~2 weeks	NR
KEW	CANCERPLEX[133]	FFPE, biopsy, aspirate	DNA	NR	435	SNVs, indels, CNA, rearrangements, MSI, TMB	No	7-10 days	NR
Caris Life Sciences	CARIS Molecular Intelligence[150]	FFPE, biopsy aspirate	DNA, RNA	Microdissection	~22,000	SNVs, indels, CNA, fusions, MSI, TMB	No	10-14 days	NR
Paradigm	PCDx[151]	FFPE	DNA, RNA	NR	234	SNVs, indels, CNA,	No	4-5 days	NR

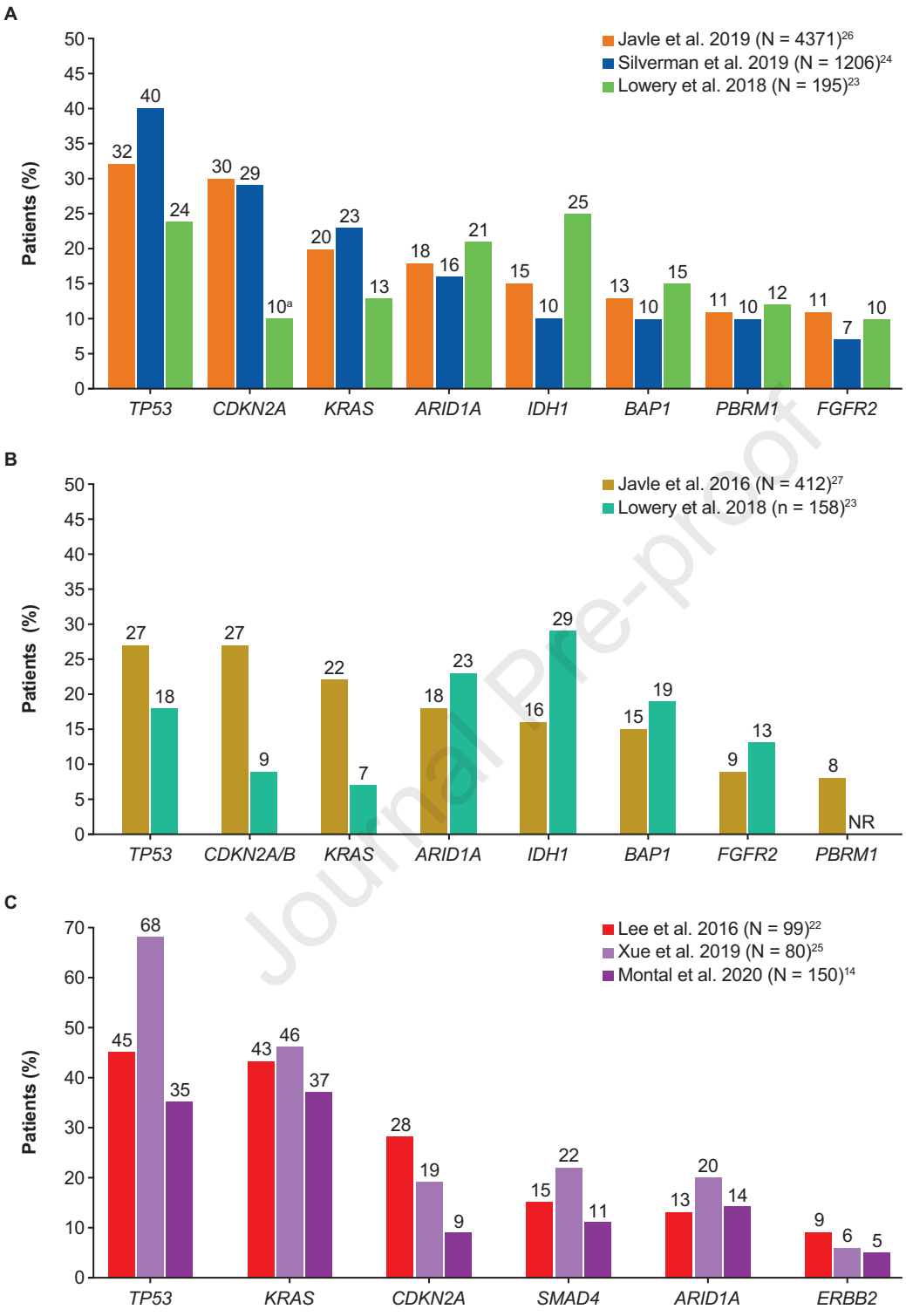
						rearrangements, TMB			
OmniSeq Corporation	OmniSeq Comprehensive[152]	FFPE	DNA, RNA		144	SNVs, indels, CNA, fusions, MSI, TMB	No	10-15 days	NR
PathGroup	SmartGenomics[153]	FFPE	DNA	NR	160	SNVs, indels, fusions	No	NR	NR

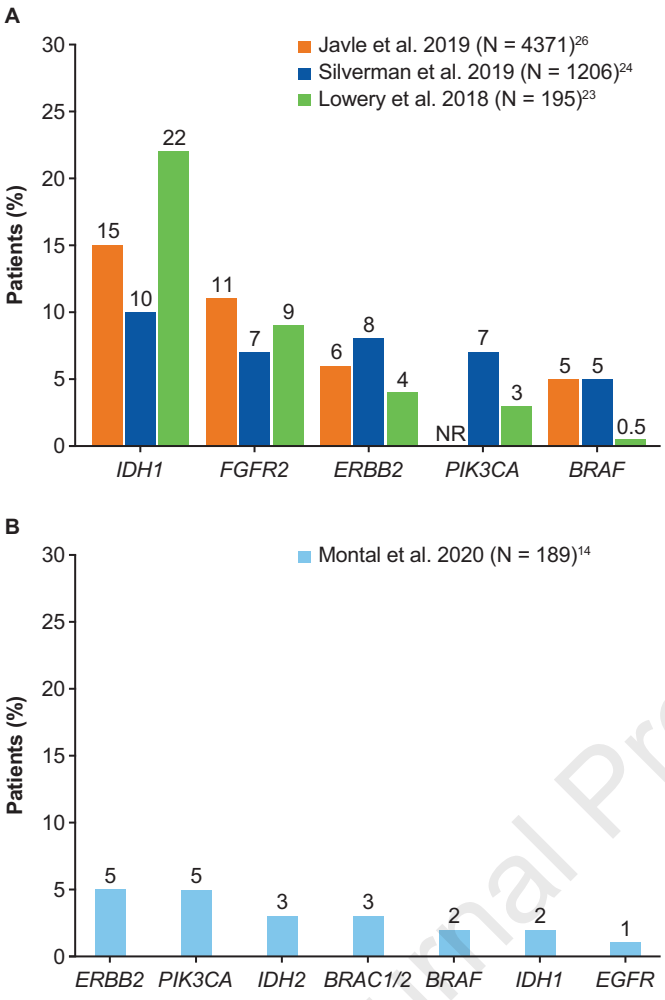
cfDNA, cell-free DNA; CNA, copy number alteration; FFPE, formalin-fixed paraffin-embedded; GIS, genomic instability score; indel, insertion or deletion of a DNA sequence into a genome; IVD, in vitro diagnostic; LOH, loss of heterozygosity; MSI, microsatellite instability; NR, not reported; PCR, polymerase chain reaction; SNV, single-nucleotide variant; TMB, tumor mutational burden; USD, United States dollars.

[a]FDA-cleared/approved IVD test.[72]

[b]FDA-approved as companion diagnostic for pemigatinib-eligibility in patients with cholangiocarcinoma.[74]

[c]Costs are those publicly disclosed in the company's website or literature.





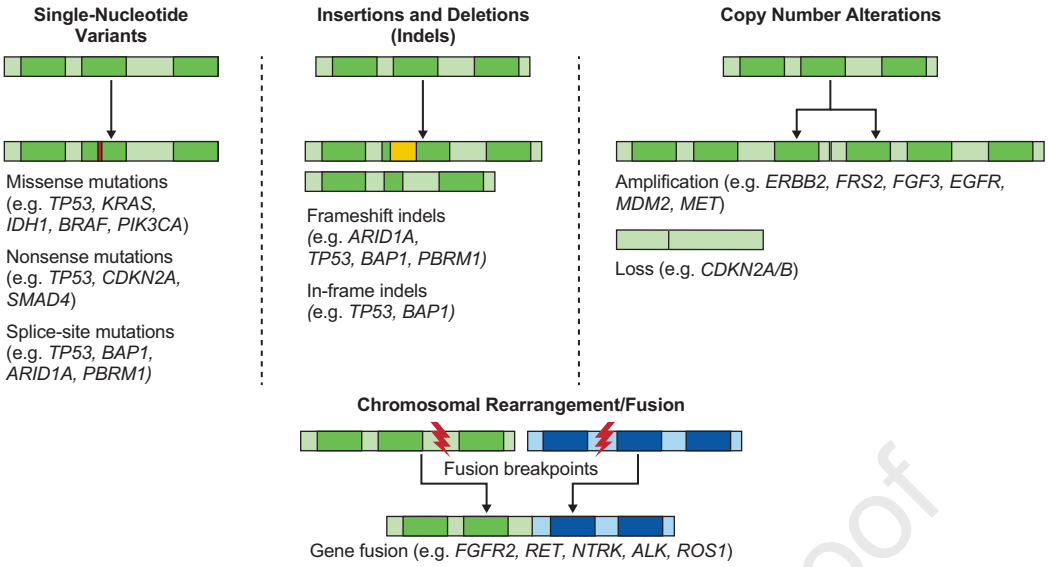


Figure Captions

Figure 1. Frequently altered genes in CCA (A), iCCA (B), and eCCA (C). In two of the studies listed in panel A, Silverman et al 2019[25] and Lowery et al. 2018,[24] >80% of patients had iCCA. The percentage of patients with iCCA in the study by Javle et al. 2019,[27] was not disclosed. Some patient percentages were estimated based on graphic rather than numerical data presented in the original studies. NR, not reported. [a]Also includes patients with *CDKNB* alterations.

Figure 2. Commonly altered genes with actionable alterations in CCA/iCCA (A) and eCCA (B). In two of the studies listed in panel A, Silverman et al 2019[25] and Lowery et al. 2018,[24] >80% of patients had iCCA. The percentage of patients with iCCA in the study by Javle et al. 2019,[27] was not disclosed. Some patient percentages were estimated based on graphic rather than numerical data presented in the original studies. The most common actionable alterations (in each gene) were *IDHI* missense mutations, *FGFR2* fusions/rearrangements, *ERBB2* amplifications and mutations; *PIK3CA* missense mutations; *BRAF* missense mutations, *IDH2* mutations, *BRCA1/2* mutations, and *EGFR* amplifications and mutations. NR, not reported.

Figure 3. Genetic alterations in CCA.