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Available online 9 September 2022

Background: Targeted therapies have transformed clinical management of advanced biliary tract cancer (BTC). Cell-free DNA (cfDNA) analysis is an attractive approach for cancer genomic profiling that overcomes many limitations of traditional tissue-based analysis. We examined cfDNA as a tool to inform clinical management of patients with advanced BTC and generate novel insights into BTC tumor biology.

Patients and methods: We analyzed next-generation sequencing data of 2068 cfDNA samples from 1671 patients with advanced BTC generated with Guardant360. We carried out clinical annotation on a multi-institutional subset (n = 225) to assess intra-patient cfDNA–tumor concordance and the association of cfDNA variant allele fraction (VAF) with clinical outcomes.

Results: Genetic alterations were detected in cfDNA in 84% of patients, with targetable alterations detected in 44% of patients. Fibroblast growth factor receptor 2 (FGFR2) fusions, isocitrate dehydrogenase 1 (IDH1) mutations, and BRAF V600E were clonal in the majority of cases, affirming these targetable alterations as early driver events in BTC. Concordance between cfDNA and tissue for mutation detection was high for IDH1 mutations (87%) and BRAF V600E (100%), and low for FGFR2 fusions (18%). cfDNA analysis uncovered novel putative mechanisms of resistance to targeted therapies, including mutation of the cysteine residue (FGFR2 C492F) to which covalent FGFR inhibitors bind. High pre-treatment cfDNA VAF was associated with poor prognosis and shorter response to chemotherapy and targeted therapy. Finally, we report the frequency of promising targets in advanced BTC currently under investigation in other advanced solid tumors, including KRAS G12C (1.0%), KRAS G12D (5.1%), PIK3CA mutations (6.8%), and ERBB2 amplifications (4.9%).

Conclusions: These findings from the largest and most comprehensive study to date of cfDNA from patients with advanced BTC highlight the utility of cfDNA analysis in current management of this disease. Characterization of oncogenic drivers and mechanisms of therapeutic resistance in this study will inform drug development efforts to reduce mortality for patients with BTC.

Key words: cell-free DNA, liquid biopsy, biliary tract cancer, cholangiocarcinoma

INTRODUCTION

Biliary tract cancer (BTC) comprises intrahepatic cholangiocarcinoma, extradhepatic cholangiocarcinoma, and gallbladder carcinoma. Approximately 12,000 people are diagnosed with BTC each year in the United States, with the incidence and mortality for intrahepatic cholangiocarcinoma increasing both in the United States and...
globally.1-3 Although surgical resection of localized disease can be curative, most patients relapse.5 For patients with unresectable or metastatic BTC, gemcitabine plus cisplatin with or without durvalumab is the standard first-line treatment, and these regimens offer a median overall survival (OS) of ~1 year.5-7 Alternative therapeutic modalities are urgently needed.

Large-scale genomic profiling efforts have transformed the therapeutic opportunities for patients with BTC by identifying clinically actionable alterations in several genes, including FGFR2, IDH1, BRAF, NTRK, RET, and ERBB2 (HER2).9-10 The frequency of these alterations varies by anatomic subtype of BTC. Fibroblast growth factor receptor 2 (FGFR2) fusions and isocitrate dehydrogenase 1 (IDH1) mutations occur at a frequency of 13%-14% in intrahepatic cholangiocarcinoma and rarely in the other subtypes.9-14 Human epidermal growth factor receptor 2 (HER2) amplification is seen in ~14%-16% of gallbladder cancer and less frequently in the other subtypes.9-14 BRAF V600E mutations (3%-4%), NTRK fusions (<1%), and RET fusions (<1%) are rare across all subtypes.8-10,16-19

Precision medicine strategies have emerged to identify patients with these targets. The first approvals by the Food and Drug Administration (FDA) in cholangiocarcinoma were the selective FGFR inhibitors pemigatinib and iniflitratinib for advanced refractory FGFR2 fusion or rearrangement-positive cholangiocarcinoma. The IDH1 inhibitor ivosidenib was subsequently approved for IDH1-mutant cholangiocarcinoma. The National Comprehensive Cancer Network (NCCN) additionally recommends targeted therapies in the setting of a BRAF V600E mutation, NTRK fusion, RET fusion, or HER2 positivity based on either disease-agnostic FDA approvals or promising clinical trial data in BTC.19-25 Immune checkpoint inhibitors have gained FDA approval for treatment of tumors with high microsatellite instability (MSI), deficient mismatch repair, or high tumor mutational burden.29,30 Ongoing clinical trials are investigating targeted therapies in additional molecularly defined cohorts. Supplementary Table S1, available at https://doi.org/10.1016/j.annonc.2022.09.150, outlines the current NCCN guideline-recommended therapies for molecularly defined subsets of BTC and their approval status by the FDA and European Medicines Agency.

Given the importance of genomic information in guiding clinical management, it is critical to define the performance characteristics and added value of different genomic profiling approaches. Next-generation sequencing (NGS) of tumor tissue samples has advanced our understanding of the genomic alterations in many cancer types, including BTC. However, this approach has its limitations. Metastatic biopsies often have insufficient tumor cellularity for sequencing, leading to high failure rates of tissue profiling.31 Further, the capacity to examine tumor heterogeneity is limited by the spatial distribution of clonotypes, both between and within lesions.12 Lastly, while serial monitoring can identify tumor evolution and the emergence of treatment resistance, repeated invasive tissue biopsies carry risk of complications for patients.

Cell-free DNA (cfDNA) analysis is an attractive approach for cancer genomic profiling that can overcome many of the limitations of tissue-based analysis: (i) cfDNA can provide genomic information when tissue is unobtainable or insufficient in quantity or quality31; (ii) tumor DNA is shed into the blood from multiple lesions and thus may better capture intra-patient tumoral heterogeneity 23,34; (iii) serial cfDNA analysis can facilitate the study of tumor evolution and resistance35-37; (iv) cfDNA can also provide predictive and prognostic information.34-37,39 These characteristics are highly relevant in BTC. As a desmoplastic and often necrotic tumor, BTC is notoriously difficult to profile, with >25% of metastatic tumor biopsies yielding insufficient tumor content or DNA quality for analysis by NGS.31 Cholangiocarcinoma displays a significant degree of intra-tumoral heterogeneity, especially at the periphery of tumors, which may be missed with a single tumor biopsy from the center of a lesion.35,40-42 Capturing tumor heterogeneity may be particularly important for assessing resistance to FGFR inhibitors as the resistance is in part mediated by subclonal populations of FGFR2 kinase domain (KD) mutations.33,41,43,44 Finally, given the paucity of prognostic clinical biomarkers, non-invasive and rapidly available genetic biomarkers may improve management of these patients.

In this report, we analyze results from 2068 cfDNA samples obtained as a routine part of clinical care of 1671 patients with advanced BTC. We assess the utility of cfDNA to detect clinically actionable alterations and also provide insights into BTC tumor biology. This represents the largest and most comprehensive evaluation to date of the clinical utility of cfDNA analysis in patients with BTC.

PATIENTS AND METHODS

**cfDNA cohort**

The primary cfDNA cohort consisted of 1671 consecutive de-identified patients with advanced BTC who had blood samples collected for clinical testing using the Guardant360 cfDNA NGS assay (Guardant Health, Inc., Redwood City, CA) between 1 October 2015 and 28 January 2019. All patients underwent testing with the 70-gene and 73-gene assays (Supplementary Table S2, available at https://doi.org/10.1016/j.annonc.2022.09.150). A total of 2068 samples were analyzed from this cohort. Analytical cohorts are described in Figure 1A and B and Supplementary Figures S1 and S2, available at https://doi.org/10.1016/j.annonc.2022.09.150. The assay is a Clinical Laboratory Improvement Amendments (CLIA)-certified targeted sequencing panel designed to detect single-nucleotide variants (SNVs), insertions and deletions (indels), amplifications, and fusions. The Guardant360 CDx test is now approved by the FDA, though the study cohort received laboratory-developed testing before this approval. We carried out detailed clinical annotation on a subset of 225 patients from 6 institutions: Mass General Cancer Center, Mayo Clinic in Arizona, Mayo Clinic in Florida, Washington University...
Siteman Cancer Center, University of California San Francisco Helen Diller Family Comprehensive Cancer Center, and Gustave Roussy Cancer Campus. This research was conducted in accordance with the Declaration of Helsinki and was carried out with institutional review board approval.

Defining clinically actionable alterations

To determine the prevalence of clinically actionable alterations in cfDNA, we classified each alteration based on the OncoKB Therapeutic Levels of Evidence V2. Each cfDNA alteration was assigned to therapeutic level 1, 2, 3A, 3B, or 4. Only alterations specifically characterized as ‘oncogenic’ or ‘likely oncogenic’ in OncoKB were included in levels 1-4. Alterations not listed in OncoKB or characterized as ‘likely neutral’ or ‘inconclusive’ were considered ‘unclassified’. Classifications were made as of 15 June 2021.

Comparison of cfDNA and tissue alterations

Alterations are reported for the top 20 most frequently altered genes in BTC categorized by alteration type, including non-synonymous SNVs (missense and nonsense mutations), indels, amplifications, and fusions. To compare the frequency of clinically actionable alterations in cfDNA with tissue genomic profiling, we downloaded sequencing results from the AACR Project GENIE v8.0, a publicly accessible international cancer registry of real-world data assembled through data sharing between 19 cancer centers worldwide. To allow for the most comparable prevalence calculations between cfDNA and tissue cohorts, we included only metastatic samples from tissue datasets that analyzed all exons of genes sequenced by the cfDNA assay (n = 349) (Supplementary Table S3, available at https://doi.org/10.1016/j.annonc.2022.09.150).
**Statistical analysis**

Proportions and 95% confidence intervals (CIs) for binomial proportions were calculated for genes in the cfDNA and tissue cohorts based on the frequency and number of patients studied using the modified Wald method. Linear regression was used to evaluate the relationship between gene mutation frequencies in the cfDNA versus tissue cohorts. For time-to-event analyses, OS was defined as the time from advanced BTC diagnosis to death or censor and progression-free survival (PFS) was defined as the time from treatment initiation to investigator-assessed radiographic or clinical progression. OS and PFS were calculated using the Kaplan–Meier method with log-rank test. Unadjusted hazard ratios (HRs) were estimated using Cox regression models. Student’s t-test was used to compare the mean number of mutations in first versus last cfDNA samples. Fisher’s exact test was used to compare differences between two categorical variables. All tests were two-sided and considered statistically significant at P < 0.05. Statistical analysis was carried out using JMP Pro 14 (JMP, Cary, NC).

**RESULTS**

**cfDNA landscape and prevalence of clinically actionable alterations**

To molecularly characterize cfDNA in patients with advanced BTC, we analyzed results from patients who underwent cfDNA NGS with the Guardant360 assay between October 2015 and February 2019 as part of routine clinical care for a diagnosis of BTC. Only the first sample from each of the 1671 patients was used for all analyses of the primary cfDNA cohort unless otherwise specified (Figure 1A; Supplementary Figures S1 and S2, available at https://doi.org/10.1016/j.annonc.2022.09.150). The Guardant360 panel included 70 or 73 genes (Supplementary Table S2, available at https://doi.org/10.1016/j.annonc.2022.09.150). SNVs, small indels, fusions, and copy number amplifications were assessed with an average sequencing depth of 15 000x. The median age in the cohort was 65 years, 52% of patients were female, and 91% had cholangiocarcinoma versus 9% with gallbladder carcinoma (Figure 1A and B).

We first determined the prevalence of alterations that represent non-synonymous SNVs, indels, amplifications, and fusions for the 20 most frequently altered genes. In cases where multiple alterations were detected in an individual gene, we report the data as a single alteration, with SNVs taking precedence over other genetic alterations (Figure 1C). Consistent with prior analyses of BTC by NGS of tissue samples, TP53 and KRAS were the most frequently altered genes. Two therapeutic targets, FGFR2 and IDH1, were each altered in 9.3% of patients, but the frequency of these alterations by anatomic subtype could not be determined as the data form accompanying the clinical test did not capture this information. Other frequently altered genes included oncogenes and tumor suppressor genes previously identified in BTC tissue-based analyses such as PIK3CA mutations and ERBB2 amplification.

Given the challenges associated with tissue acquisition, turnaround time, and patient preference, non-invasive genomic profiling through analysis of cfDNA could play an important role in identifying patients who may benefit from targeted therapy. To determine the prevalence of clinically actionable alterations in cfDNA, we first classified each cfDNA alteration observed in the primary cfDNA cohort using the OncoKB Therapeutic Levels of Evidence V2 (Figure 1D). Based on compelling clinical evidence that ERBB2 amplifications (originally classified as level 3B) predict response to pertuzumab plus trastuzumab, and that select FGFR2 mutations (originally classified as level 4) predict a response to FGFR inhibitors, these alterations were both re-classified as 3A (Supplementary Table S4, available at https://doi.org/10.1016/j.annonc.2022.09.150). MSI, an OncoKB level 1 biomarker for all solid tumors, was not assessed in this study, as MSI testing was not introduced to the Guardant360 panel until September 2018. cfDNA samples from 250 patients were processed after this date, and of these, only 4 (1.6%) were classified as MSI-high.

To determine the proportion of patients with advanced BTC with clinically actionable alterations detected in cfDNA, we assessed the frequency of level 1-3B alterations in the primary cfDNA cohort. Overall, 84% of patients had at least one genomic alteration detected in their first cfDNA sample collected, and 44% of the total population had at least one level 1-3B alteration (Figure 1D). Of these, 13% had more than one level 1-3B alteration. We next assessed the prevalence of specific alterations with therapeutic relevance in BTC. The five level 1 alterations—IDH1 mutations, FGFR2 fusions, BRAF V600E mutations, NTRK fusions, and RET fusions—were present in 9.1%, 1.4%, 1.3%, 0.1%, and 0.1% of patients, respectively (Figure 1E). The frequency of other notable gene alterations included ERBB2 amplification (4.9%), PIK3CA mutations (6.8%), KRAS G12D (5.1%), and KRRAS G12C (1.0%). Supplementary Table S4, available at https://doi.org/10.1016/j.annonc.2022.09.150, displays a comprehensive list of all alterations detected in the primary cfDNA cohort and their frequencies.

**Concordance between cfDNA and tissue genomic profiling**

While cfDNA detects clinically actionable alterations in a high percentage of patients with advanced BTC, the correlation between cfDNA and tissue genomic profiling has not been characterized in a large study. We therefore compared the prevalence of alterations in the primary cfDNA cohort with data from the AACR Project GENIE database, a publicly accessible international cancer registry from 19 cancer centers. As the cfDNA cohort comprised patients with advanced disease, we only included metastatic tumor biopsy samples from the GENIE cohort, which included 349 of 1152 BTC tumor samples (Supplementary Table S3, available at https://doi.org/10.1016/j.annonc.2022.09.150). For the most comparable analysis, we analyzed the 13 genes with full exon coverage in both cohorts and focused on non-synonymous SNV mutations (Supplementary Tables S2 and
cfDNA BTC samples; while most were likely somatic, mutations can also be due to clonal hematopoiesis.53

Notable similarities and differences were seen between cfDNA and tissue in the frequency of actionable alterations (Figure 2B). Similar frequencies were observed for IDH1 mutations (9.3% versus 9.5%, in cfDNA and tissue, respectively; \( P = 0.92 \)), ERBB2 amplifications (4.9% versus 5.6%; \( P = 0.66 \)), and BRAF V600E mutations (1.3% versus 2.3%; \( P = 0.14 \)). FGFR2 fusions, however, were detected at a lower frequency in cfDNA samples (1.4% versus 4.3%; \( P = 0.0018 \)).

For IDH2 mutations, which are clinically actionable in acute myeloid leukemia and are a target of drug development efforts in cholangiocarcinoma, were identified in 1.2% of cfDNA BTC samples; while most were likely somatic, IDH2 mutations can also be due to clonal hematopoiesis.53

Having established the correlation of mutational frequencies between cfDNA and tissue in large populations, we next sought to characterize intra-patient cfDNA–tissue concordance for individual patients. The cfDNA–tissue concordance cohort comprised a subset of 194 patients with advanced BTC (Figure 2D).34,50-52 IDH2 mutations, which are clinically actionable in acute myeloid leukemia and are a target of drug development efforts, were detected in 1.2% of cfDNA BTC samples; while most were likely somatic, IDH2 mutations can also be due to clonal hematopoiesis.53

We first focused on IDH1 and BRAF V600E mutations. Of the patients with IDH1 mutations detected in tissue, these mutations were also detected in cfDNA in 87% (41/47) of patients (Figure 3A). Two additional patients whose tumor genomic profiling failed due to insufficient tissue had IDH1 mutations detected in cfDNA. Detection rates of IDH1 mutations differed by disease status at the time of cfDNA collection (Figure 3B), with the highest concordance (100%) observed when cfDNA was collected before initiation of systemic therapy and the lowest concordance (56%) observed when cfDNA was collected on systemic therapy or during a period of stable disease. The majority of IDH1 mutations in the primary cfDNA cohort were R132C (Figure 3C). There was complete intra-patient concordance (100%) for BRAF V600E mutations between cfDNA and tissue, albeit numbers were small (n = 4). The high intra-patient concordance for IDH1 mutations and BRAF V600E mutations is consistent with similar concordance for these mutations seen in the populations analyzed above in the primary cfDNA cohort and GENIE tissue database (Figure 2B).

In contrast, of the patients with FGFR2 fusions detected in tissue, only 18% (12/67) had fusions identified in cfDNA (Figure 3D). This discordance is consistent with the larger cohorts, in which a significantly lower proportion of cfDNA samples than tissue samples harbored FGFR2 fusions (Figure 2B). The low detection rate for FGFR2 fusions is not explained by low circulating tumor DNA levels, as 88% (59/67) of patients with FGFR2 fusions had at least one mutation detected in cfDNA, similar to the 84% fraction seen in the primary cfDNA cohort. Rather, detection rates correlated with the identity of the FGFR2 fusion partner. BICC1 was the most common fusion partner among 42 unique partners observed in tissue, present in 28% (19/67) of

Figure 2. Correlation between genomic profiling of cell-free DNA (cfDNA) and tissue in advanced biliary tract cancer (BTC) cohorts. (A) Correlation between non-synonymous single-nucleotide variant (SNV) mutation frequencies for 13 genes with full exon coverage in all samples from both the Guardant360 BTC primary cfDNA cohort (\( n = 1671 \)) and the AACR Project GENIE BTC tissue metastatic cohort (\( n = 349 \)); the fitted linear regression line is shown in red. (B) Comparison of frequencies for OncoKB level 1-3A alterations in BTC in these cfDNA and tissue cohorts; error bars represent 95% confidence intervals; asterisks denote genes whose mutation frequency significantly differed (\( P < 0.05 \)) between cfDNA and tissue. (C) Comparison of frequencies for TP53 and KRAS mutations in BTC in these cfDNA and tissue cohorts. (D) Comparison of frequency of JAK2 V617F mutations in these cfDNA and tissue cohorts.
patients (Figure 3E). FGFR2–BICC1 fusions present in tissue were detected in the first cfDNA sample in 58% (11/19) of cases, whereas only 2.1% (1/48) of non-BICC1 fusions were identified in cfDNA (P = 6.1 × 10−7) (Figure 3F). The lone non-BICC1 fusion detected in cfDNA was FGFR2–TACC2. Unlike IDH1 mutations, FGFR2–BICC1 fusion detection in cfDNA was not clearly dependent on the timing of sample collection, albeit numbers were small (Figure 3G). To assess if repeated sampling improved detection of FGFR2 fusions, we analyzed 259 samples from 35 patients with known FGFR2 fusions who underwent serial cfDNA analysis (median 7 samples per patient, range 2-19; BICC1 = 6, non-BICC1 = 29). Overall, 97% (34/35) of patients were concordant for the serial cfDNA samples, with a fusion either always present or always absent. Of the six patients with an FGFR2–BICC1 fusion, consistent detection in all samples (range 2-8) was seen in three patients, a consistent absence of FGFR2–BICC1 fusions in all samples (range 3-10) was seen in two patients, and one patient harbored a fusion in the first sample but none of the subsequent three. Of the 29 patients with a non-BICC1 partner and availability of serial samples, no FGFR2 fusions were detected in any of the 230 cfDNA samples (range 2-19 samples per patient).

Intra-patient concordance analysis of FGFR2 mutations provides insights into the biology of FGFR2-altered BTC. We evaluated 22 paired tissue–cfDNA samples from 20 patients with either an FGFR2 extracellular domain mutation (n = 10) or FGFR2 KD mutation(s) (n = 12) who had tissue and cfDNA collected within 60 days and no therapy in between. Of the 48 FGFR2 mutations detected across 22 paired samples, 69% were present in cfDNA only, 25% in cfDNA and tissue, and 6% in tissue only (Figure 4A). We next assessed intra-patient concordance separately for extracellular domain and KD mutations. FGFR2 extracellular domain mutations were detected in both cfDNA and tissue in 70% (7/10) of patients and in cfDNA only in 30% (3/10) of patients (Figure 4B). Of the seven extracellular domain mutations detected in both cfDNA and tissue, six were F276C and one was S372C, both of which are reported to be pathogenic.54-56 Among the three FGFR2 extracellular domain mutations detected in cfDNA only, one was F276C while the other two, P47T and L58Q, were variants of unknown significance.56 No FGFR2 extracellular domain mutations detected in tissue were missed by cfDNA analysis. For FGFR2 KD mutations, however, detection was significantly better in cfDNA than in tissue. These mutations can emerge in distinct tumor foci as a secondary polyclonal resistance mechanism to FGFR inhibitors and may therefore be more readily represented in cfDNA than in biopsies of single lesions (Figure 4C).33,41 Lack of full coverage of all exons in the FGFR2 KD in targeted NGS tissue profiling panels may also contribute to under-recovery of mutations. A total of 38 FGFR2 KD mutations were detected in 12 patients, all of whom had been previously treated with a selective FGFR inhibitor for FGFR2 fusion-positive or FGFR2-mutant cholangiocarcinoma. The majority (79%) of the FGFR2 KD mutations (30/38) were detected in cfDNA only.

Given the success of cfDNA in detection of FGFR2 mutations, we evaluated cfDNA as a discovery platform to characterize the spectrum of FGFR2 mutations in the extracellular domain, transmembrane domain, and KD. To maximize discovery, we included all 2068 cfDNA samples

Figure 3. Intra-patient concordance of clinically actionable alterations between cell-free DNA (cfDNA) and tissue in a clinically annotated cohort of 194 patients with advanced biliary tract cancer (BTC). Concordance in detection of IDH1 mutations (n = 49 patients) (A) overall and (B) by clinical scenario at cfDNA collection. (C) Spectrum of IDH1 mutations present in 163 of 1671 patients with advanced BTC. (D) Concordance in detection of fibroblast growth factor receptor 2 (FGFR2) fusions among 67 patients. (E) Frequency of non-BICC1–FGFR2 fusion partners detected on tissue profiling. (F) Concordance in detection of FGFR2 fusions for BICC1 fusions versus non-BICC1 fusions. (G) Concordance in detection of FGFR2–BICC1 fusions by clinical scenario at cfDNA collection.
from the 1671 patients (Supplementary Figure S2, available at https://doi.org/10.1016/j.annonc.2022.09.150). In total, 148 samples from 107 patients harbored FGFR2 nonsynonymous SNV mutations. We identified 70 unique mutations involving 62 amino acids, including residues in the KD, extracellular domain, transmembrane domain, and outside of these defined regions (Figure 4D). These comprised several oncogenic or likely oncogenic FGFR2 extracellular domain and transmembrane domain mutations that may be sensitive to FGFR inhibitors in select cases.

Among the 31 unique FGFR2 KD mutations, the most common was V565L/F/I, followed by N550K/H/D/T, present in 43% (18/42) and 40% (17/42) of patients, respectively (Figure 4E). FGFR2 V565 is the gatekeeper residue and N550 is a molecular brake residue. Mutations at these sites are known to confer clinically acquired resistance to FGFR inhibitors. These mutations require functional characterization to see whether they contribute to oncogenicity and to explore their role in conferring resistance to FGFR inhibitors. Futibatinib (TAS120) covalently binds cysteine 492 in the P loop of the FGFR2 kinase domain. The fusions contain the FGFR2-Tllb splice isofrom (NM_001144913.1), and the amino acids are numbered accordingly. If an amino acid was mutated in more than one patient, the number of patients with a mutation at a given residue is noted in parentheses. FGFR2 mutations that confer resistance to the ATP-competitive FGFR inhibitors pemigatinib and infigratinib and the irreversible FGFR inhibitor futibatinib are noted. FGFR2 mutations not previously reported are also noted.8-10,16,31,41,43-46,58-60 These mutations require functional characterization to see whether they contribute to oncogenicity and to explore their role in conferring resistance to FGFR inhibitors.

In addition to identifying previously described FGFR2 mutations, this analysis identified 13 new FGFR2 KD mutations at 12 amino acids that have not previously been reported in OncoKB, The Cancer Genome Atlas (TCGA), AACR Project GENIE, COSMIC, or published BTC tissue and cfDNA genomic profiling papers: C492F, A499V, A512G, A512V, D531E, M538I/M539L, M541I, L552F, Y609H, L634V, P701L, V703I, K715R, R760G, S908L, and L955F.8-10,16,31,41,43-46,58-60

Figure 4. Intra-patient concordance of fibroblast growth factor receptor 2 (FGFR2) mutations between cell-free DNA (cfDNA) and tissue in 22 patients and a summary of FGFR2 mutations in 2068 cfDNA samples of advanced biliary tract cancer (BTC). Concordance in detection of FGFR2 mutations (A) overall and divided into (B) extracellular domain (ECD) mutations versus (C) kinase domain (KD) mutations. (D) Lollipop plot showing all non-synonymous single-nucleotide variant mutations in FGFR2 in 2068 cfDNA samples. Mutations are annotated with OncoKB oncogenic classifications. (E) Ribbon diagram of the FGFR2 kinase domain. The fusions contain the FGFR2-Tllb splice isofrom (NM_001144913.1), and the amino acids are numbered accordingly. If an amino acid was mutated in more than one patient, the number of patients with a mutation at a given residue is noted in parentheses. FGFR2 mutations that confer resistance to the ATP-competitive FGFR inhibitors pemigatinib and infigratinib and the irreversible FGFR inhibitor futibatinib are noted. FGFR2 mutations not previously reported are also noted.8-10,16,31,41,43-46,58-60
Of the 194 patients who underwent tissue and cfDNA analysis, we identified 9 patients (4.6%) in whom tumor genomic profiling failed. Of those, two (22%) had an OncoKB level 1 alteration (both IDH1 R132C mutations), three (33%) had a level 3B alteration (FGFR2 N550H, ERBB2 L726F, and MET amplification), three (33%) had unclassified alterations, and one (11%) had no alteration. One of the patients with an IDH1 mutation was subsequently treated with an IDH1 inhibitor and achieved a PFS of 7.5 months. These data highlight the value of cfDNA analysis for the identification of clinically actionable alterations when tissue genomic profiling fails or is not possible.

Assessment of cfDNA clonality

Clonality of cfDNA alterations provides insight into tumor biology and can have clinical significance. Clonal alterations often represent early, truncal events, enriched for oncogenic drivers, while subsequent alterations tend to be either acquired mechanisms of resistance or non-significant passenger alterations with a high proportion of variants of unknown significance. To further characterize cfDNA results for clinically actionable alterations in BTC, we evaluated the proportion of alterations in the first sample of the patients in the primary cfDNA cohort were clonal versus subclonal for the OncoKB level 1-3B alterations. Alterations were defined as clonal if the variant allele fraction (VAF) for that alteration was $\geq 25\%$ of the highest VAF in the sample, whereas VAF $<25\%$ was considered subclonal, as previously defined.

We hypothesized that known oncogenic driver alterations would show a higher proportion of clonal alterations. The three alterations that predict benefit from targeted therapy in BTC—BRAF V600E mutations, IDH1 mutations, and FGFR2 fusions—were clonal in 100%, 88%, and 88% of patients, respectively (Figure 5A). Of all 73 genes included on the Guardant cfDNA panel, which includes mutations in the canonical oncogene KRAS (76% of which were clonal), these three targetable alterations were most likely to be clonal. In contrast to these highly clonal oncogenic drivers, deleterious alterations in BRCA1/2 were clonal in only 57% of patients. This is consistent with results from the intra-patient concordance analysis, wherein 70% of BRCA1/2 mutations were present in cfDNA only, suggesting that most, if not all, were subclonal (Supplementary Figure S3A, available at https://doi.org/10.1016/j.annonc.2022.09.150).

A further example of clonality that reflects the underlying biology of the tumor is seen for FGFR2 mutations. FGFR2 extracellular domain mutations were significantly more likely to be clonal than KD mutations (73% versus 47%; $P = 0.020$). This is consistent with the findings that demonstrate the emergence of subclonal FGFR2 KD mutations as a secondary mechanism of acquired resistance to FGFR inhibitors. A characterization of the clonality of cfDNA alterations can thus provide insight into their clinical and biological significance.

To complement the clonality analysis, we assessed the clinical significance of mutations detected in cfDNA. Mutations that predict a possible response to a drug in patients with BTC—OncoKB level 1 and level 3A—were considered...
clinically significant. Nearly all IDH1 mutations (98%) were clinically significant, as well as 39% of FGFR2 mutations and 31% of BRAF mutations. The remaining 69% of BRAF mutations were non-V600E and not clinically significant (Figure 5B). In contrast, no BRCA1/2 mutations were clinically significant, and the vast majority (90%) were unclassified. Of the 142 BRCA1/2 alterations, including mutations and indels, only 12% were both clonal and pathogenic (Supplementary Figure S3B, available at https://doi.org/10.1016/j.annonc.2022.09.150). These findings have clinical and biological implications. That nearly all mutations in IDH1 are clinically significant reinforces its role as an oncogenic driver. Conversely, the high proportion of BRCA1/2 mutations that are unclassified and/or subclonal raises the question of the biological and clinical significance of these alterations. Finally, clinicians must be aware that not all alterations in clinically actionable genes (e.g. FGFR2 and BRAF) are clinically significant.

**Assessment of clonal evolution**

The ease with which cfDNA enables serial sampling allowed an assessment of clonal evolution. It provided insight into the development of tumor heterogeneity, a known driver of resistance to commonly used treatments in BTC.4,33 We had access to 192 patients for whom multiple cfDNA samples were available. We analyzed the first and last sample for these 192 patients, termed the serial cfDNA cohort (Figure 1A). The median number of days between the first and last sample was 226 (range 32-1128). The lack of clinical annotation in the primary cfDNA cohort precluded an evaluation of possible mechanisms of resistance to specific systemic therapies. Nonetheless, we could establish an increase in tumor heterogeneity over time. The average number of alterations in the final sample was significantly greater than in the first sample (3.0 versus 2.2; \(P = 0.0041\)) (Figure 5C). Eight or more alterations were observed in 2% of the first samples compared to 9% of the final samples (\(P = 0.0020\)). The greater number of alterations as disease progressed reflects the emergence of subclonal resistance alterations and indicates an increase in tumor complexity. Indeed, a significantly higher proportion of mutations in the final samples compared to the first samples were subclonal (47% versus 38%; \(P = 0.015\)) (Figure 5D). The most frequently altered genes in the final samples were TP53, KRA5, and FGFR2 (Figure 5E). Numerically higher frequencies of TP53 (47% versus 39%) and KRA5 (16% versus 12%) alterations in final compared to first samples are consistent with their known association with cancer aggressiveness and resistance to anticancer therapy.57,68 FGFR2 also shows an increase in the number of mutations from first (11%) to final (17%) sample, likely reflecting the emergence of polyclonal resistance to FGFR inhibitor therapy.

**Association of cfDNA characteristics with clinical outcomes**

Specific cfDNA characteristics are predictive of clinical outcomes in cancer patients treated with systemic therapy. In one of the largest studies of cfDNA in a clinically annotated cohort of patients with solid tumors, high VAF, specifically the top quartile of maximum VAF (>8.6%), was the only cfDNA characteristic independently associated with worse OS.69 As only 36 patients in that cohort had BTC, the prognostic significance of cfDNA VAF in patients with BTC is unclear. We therefore evaluated the association of the top quartile of maximum VAF (defined as the highest VAF for any alteration in a given sample) with OS in our cfDNA clinical outcomes cohort of 105 patients with systemic therapy-naïve advanced BTC (Figure 1A). The median OS for this cohort was 16.0 months (95% CI 9.8-22.1 months) (Figure 6A). Patients were divided into quartiles by maximum VAF: quartile 1 (0.0%-0.7%), quartile 2 (0.8%-3.2%), quartile 3 (3.3%-9.0%), quartile 4 (9.1%-92%). OS significantly differed by quartile with the poorest OS for patients in quartile 4 (\(P = 0.0498\)) (Figure 6B). We then compared quartile 4 to quartiles 1-3 and found that quartile 4 was associated with significantly worse OS (8.2 versus 20.1 months, respectively; \(HR = 1.7, 95\% CI 1.03-3.0; P = 0.0441\)) (Figure 6C).

In addition to its established prognostic value, there is a correlation of pre-treatment cfDNA levels with PFS in patients who receive systemic therapy.36,37 Given the demonstrated prognostic value of high VAF (maximum VAF >9.0%) in advanced BTC, we investigated whether the >9.0% cut-off in a pre-treatment sample was predictive of PFS in patients who receive chemotheraphy and in patients who receive targeted therapy, when compared to a low VAF (maximum VAF ≤9.0%). Among 86 patients with systemic therapy-naïve advanced BTC, treated with first-line gemcitabine/platinum chemotherapy, patients with a high VAF had a significantly shorter median PFS of 2.6 months compared to 7.6 months among those with a low VAF (HR 1.9, 95% CI 1.1-3.2; \(P = 0.035\)) (Figure 6D and E). Median OS from the start of first-line chemotherapy was also shorter for patients with a high VAF (7.7 months) compared to a low VAF (19.9 months) (HR 2.1, 95% CI 1.1-4.0; \(P = 0.030\)) (Figure 6F).

Similarly, among 29 patients with FGFR2 fusion or rearrangement positive cholangiocarcinoma (Figure 6G), patients with a high pre-treatment VAF had significantly shorter median PFS of 3.2 months on FGFR inhibitor therapy compared to 7.9 months among those with low VAF (HR 6.1, 95% CI 2.1-18.9; \(P = 0.001\)) (Figure 6H). Median OS from the start of FGFR inhibitor therapy was also shorter for patients with a high VAF compared to a low VAF (10.5 versus 15.3 months, respectively; HR 4.4, 95% CI 1.3-14.9; \(P = 0.019\)) (Figure 6I). The association of a high VAF with inferior outcomes suggests a predictive and prognostic role for cfDNA to inform clinical care for patients with advanced BTC.

**DISCUSSION**

Efficient genomic profiling to identify actionable alterations is critical to maximizing survival for patients with advanced BTC. We report the largest and most comprehensive study...
to date on the clinical utility of cfDNA analysis in BTC. We analyzed genomic results from 2068 plasma samples from 1671 patients with advanced BTC, as well as from a subset of 225 patients for whom detailed clinical annotation was available. Several findings have clinical relevance for management of patients with advanced BTC. Profiling of cfDNA (i) identifies similar frequencies of clinically actionable alterations compared to tissue profiling; (ii) detects most IDH1 mutations, BRAF V600E mutations, and FGFR2 extracellular domain mutations, but is suboptimal for detecting FGFR2 fusions; (iii) reveals a high degree of clonality for the most clinically actionable alterations in BTC—IDH1 mutations, BRAF V600E mutations, and FGFR2 fusions—suggesting that these are early driver events in BTC; (iv) uncovers novel mechanisms of resistance to targeted therapy not detected on tumor biopsy; and (v) provides prognostic and predictive data to integrate into clinical decision making.

Patients with advanced BTC are prime candidates for molecular profiling by cfDNA analysis. Rates of tumor shed are high with 84% of patients in this study having detectable somatic variants in cfDNA, comparable to 85% of 21,000 patients with various solid tumors. The frequency of clinically actionable variants is high, with 44% of patients in this study having such variants, compared to 25%-47% in BTC tissue profiling studies and 43%-55% in smaller BTC cfDNA studies.9,66,71,72 The variability in frequency across studies may be explained by differences in the definition of therapeutic relevance and differences in proportion of intrahepatic cholangiocarcinoma tumors included. BTC also harbors many other genetic alterations of unclear significance, and as clinical and functional characterization of these variants improves, the utility of genomic profiling can only increase. With >50 active clinical trials investigating targeted therapies in biomarker-selected patients with BTC (Supplementary Table S7, available at https://doi.org/10.1016/j.annonc.2022.09.150).
1016/j.annonc.2022.09.150), there is cause for hope that the number of patients with BTC who can benefit from molecularly guided treatment will grow substantially in the coming years.

Accurate and effective genomic profiling is key to realizing this goal. More than 25% of metastatic tumor biopsies fail to provide sufficient material for genomic profiling in BTC. Our work establishes cfDNA profiling as a viable alternative. The correlation between cfDNA and tissue genomic profiling for non-synonymous SNVs was excellent ($R^2 = 0.96$), consistent with a similar finding in colorectal cancer ($R^2 = 0.95$). For IDH1 mutations and BRAF V600E mutations, which are the two SNVs with standard-of-care therapies available in BTC, it was gratifying to see high concordance of cfDNA results with tissue profiling in this study. However, sensitivity of cfDNA profiling notably dropped when carried out on samples collected on treatment or during a period of stable disease. Overall, these data strongly support cfDNA as a reliable source for detecting clinically actionable IDH1 mutations and BRAF V600E mutations in the appropriate clinical setting for patients with advanced BTC.

FGFR2 fusions, in contrast, eluded detection by cfDNA in the majority of cases. The sensitivity in patients with a known fusion on tissue profiling was only 18%. With the recent FDA approval of pemigatinib and infigratinib and other FGFR inhibitors on the way, understanding the analytical performance and limitations of different tumor profiling approaches is critical. Prior studies that evaluated intra-patient concordance between cfDNA and tissue samples in BTC were small and contained few patients with FGFR2 fusions. The intra-patient concordance analysis in our study included 194 patients, 67 of whom had been proven to harbor FGFR2 fusions. We identified the diversity of FGFR2 fusion partners as a key factor that hampered detection. The sensitivity of cfDNA profiling for FGFR2—BICC1 fusions, the most common FGFR2 fusions in cholangiocarcinoma among >50 identified, was 58%, but only 2% for non-BICC1 fusions. Other fusions with promiscuous anchor genes, as seen with ROS1, also have modest detection rates by cfDNA. Interestingly, ALK, which partners with EML4 in >80% of ALK fusions, has detection rates of 86%-94%. These notions suggest that the frequency of specific rearrangement events or breakpoints may correlate with sensitivity in cfDNA.

The discrepancy in FGFR2 fusion detection in this study highlights how assay design can impact the ability to detect clinically significant tumor alterations. Guardant360 utilizes a DNA-based hybrid capture platform. This approach has limitations for fusion detection inherent to both the hybrid capture method and analyzing DNA. Fusion detection using hybrid capture requires sequence-specific probes targeting near the breakpoint in one of the involved genes. Probes also targeting the fusion partner are required for optimal sensitivity; however, panel size may constrain the number of probes allocated to fusion partners and breakpoints, particularly for promiscuous genes, such as FGFR2. Design elements that may enhance the performance of cfDNA-based fusion detection include incorporating probes that target common fusion breakpoints and/or a broad range of fusion partner genes, as well as using bioinformatics tools to detect non-targeted fusion partners. The Illumina (Illumina, Inc., San Diego, CA) TruSight Oncology 500 cfDNA assay detected FGFR2 fusions in 87% of a recently described cohort, likely due to its specific inclusion of sequence-specific probes targeting two introns in the FGFR2 gene where the majority of FGFR2 fusions occur. Encouragingly, reprocessing past Guardant360 samples with updated fusion detection methods identified FGFR2 fusions not previously detected in cfDNA samples, highlighting the power of bioinformatics tools to detect non-targeted fusion partners. Alternative target enrichment methods may also improve fusion detection by cfDNA. Anchored multiplex PCR, for example, permits enrichment based on one known partner (e.g. FGFR2), thus eliminating the need to target all fusion partners and facilitating the identification of novel fusion events. Assay analyte—DNA or RNA—is another important design consideration. Currently, most clinical liquid biopsy tests analyze DNA due to its stability. However, RNA analysis offers an advantage for fusion detection as fusions are overrepresented in the transcriptome relative to the genome. The poor stability of RNA in blood currently limits its clinical utility, but cell-free RNA analysis holds promise for profiling in the future. Assay manufacturers and clinicians must consider how these design elements impact detection of clinically actionable tumor alterations. Specifically, clinicians caring for patients with BTC must be mindful that cfDNA analysis may not identify all FGFR2 fusions, and tissue genomic profiling should be considered to detect these clinically actionable alterations.

Our study shows the value of cfDNA for the characterization of tumor heterogeneity and acquired mechanisms of resistance. We previously reported FGFR2 KD mutations as an acquired mechanism of resistance to FGFR inhibitors in patients with fusion-positive cholangiocarcinoma. Analysis of cfDNA identified several FGFR2 KD mutations not present in tumor samples obtained from these patients. Exploiting the size of the cfDNA BTC cohort to annotate a more expansive list of FGFR2 KD mutations, we identified 31 unique FGFR2 KD mutations, including 13 previously unrecognized variants. The most commonly mutated codons—V565 and N550—confer resistance to ATP-competitive inhibitors. The clinical relevance of most variants remains to be established. A previously uncharacterized variant present in a single patient was FGFR2 C492F, an alteration of the cysteine residue to which covalent FGFR2 inhibitors irreversibly bind. While cysteine residue mutations are an established mechanism of acquired resistance to covalent kinase inhibitors, such as the EGFR C797S mutation that confers resistance to osimertinib in lung cancer, or BTK C481S for resistance to ibrutinib in chronic lymphocytic leukemia, the C492F mutation is not a common driver of resistance to FGFR inhibitors in FGFR2 fusion-positive cholangiocarcinoma. Our compendium of FGFR2 KD mutations provides an important resource for future functional characterization.
and highlights the value of translational research wherein patient samples collected at the bedside facilitate insights into tumor biology. With multiple FGFR inhibitors approved and others in development, understanding the clinical relevance of the spectrum of FGFR KD mutations will be critical to develop the next generation of FGFR inhibitors.

*FGFR2* fusions and *IDH1* mutations have been proposed as early driver events in cholangiocarcinoma. Compelling evidence to support this long-held assumption is lacking. cfDNA now provides an avenue to assess clonality. A high degree of clonality correlates with truncality.90 Of the 73 genes included on the Guardant cfDNA panel, three genetic alterations predictive of clinical benefit from targeted therapy in BTC—*IDH1* mutations, *BRAF* V600E mutations, and *FGFR2* fusions—showed the highest extent of clonality, and thus truncality, in cfDNA.21,23,24 This implicates these alterations as cancer-initiating events in BTC, akin to *KRAS* mutations in the development of colon cancer. *KRAS* mutations are the most frequent clonal alteration in cfDNA from patients with colon cancer.34,91

We found few *BRCA1/2* alterations that were clonal and pathogenic. This may be relevant for drug development as poly (ADP-ribose) polymerase (PARP) inhibitors are currently being trialed for *BRCA1/2*-deficient BTC (Supplementary Table S7, available at https://doi.org/10.1016/j.annonc.2022.09.150).92 As clinical trials increasingly allow cfDNA profiling for trial entry, consideration of clonality may be important for patient selection. *BRCA1/2* alterations in cfDNA may also arise from clonal hematopoiesis, as can other clinically actionable alterations, such as *IDH1* mutations.93 Approximately 10% of patients with advanced cancer may harbor clonal hematopoiesis mutations in DNA repair genes used to determine PARP inhibitor eligibility.94 In order to drive clonal selection in the white blood cell population, clonal hematopoiesis mutations are often pathogenic and indistinguishable from tumor-derived alterations, unless a whole blood control is included.52,94 Clinicians should therefore be cognizant that not all deleterious *BRCA1/2* alterations in cfDNA will confer sensitivity to PARP inhibitors. *BRCA1/2* are tumor suppressor genes that require ‘two hits’ for loss of function. The detection of copy number loss in cfDNA, a common event resulting in loss of the second *BRCA1/2* allele, is challenging in samples with low tumor content.95,96 Further exploration of the impact of clonality, the confounding effect of clonal hematopoiesis, and the ability to accurately assess loss of heterozygosity in the context of therapeutic efficacy is warranted as cfDNA analysis becomes more mainstream for profiling.

Analysis of cfDNA can inform therapeutic decision making. Gemcitabine—platinum regimens and FGFR inhibitors are effective for some patients, but yield no benefit with significant toxicity in others. As treatment options expand for BTC, biomarkers to guide therapeutic decision making will be important. Our study builds upon reports that high baseline tumor VAF in cfDNA is associated with poor PFS on systemic therapy,36,37,38 showing that a VAF >9.0% before gemcitabine—platinum chemotherapy or initiation of FGFR inhibitor therapy was associated with a short median PFS. With further validation, this parameter may be useful to select patients for early radiological assessment and/or guide discussions with patients who are borderline candidates for systemic therapy. Similarly, the association of a high baseline VAF with poor OS—an association also observed for other metastatic solid tumors—can serve as another datapoint to guide patient-physician decision making.69 However, some patients with a high VAF may still benefit from systemic therapy, so additional biomarkers are necessary to nuance these findings.

Our study has several limitations. The primary cfDNA cohort lacks full clinical annotation such as cholangiocarcinoma subtype (intrahepatic versus extrahepatic), treatment history, and disease status at the time of sample collection. We therefore could not report frequencies for clinically actionable alterations aggregated by BTC subtype. It was not possible to evaluate tumor evolution and mechanisms of resistance. To overcome these limitations, we collaborated with multiple institutions that see a high volume of patients with this rare cancer to create a subset of 225 patients with detailed clinical annotation, which enabled the intra-patient concordance analysis and allowed correlation of the results from cfDNA analysis with clinical outcomes. This cohort had sufficient power not only to validate prior discoveries, but also to identify new associations. The lack of assessment of MSI status and tumor mutation burden on earlier versions of the Guardant360 platform precluded detection of another 2%-3% of clinically actionable alterations that can now be detected on the current version.49 Finally, we analyzed cfDNA data only from Guardant360. Other commercial cfDNA tests may yield different results.

In summary, our findings strongly support the utility of genomic profiling of cfDNA to inform clinical care for patients with advanced BTC. As targeted therapies enter frontline and neoadjuvant clinical trials, cfDNA analysis will be an important tool to facilitate timely enrollment, especially as the technology improves for key drivers such as *FGFR2* fusions. In the meantime, clinicians should recognize the limitations of cfDNA for the detection of *FGFR2* fusions and consider tumor biopsies as a complementary approach. Collectively, the findings in this study support a role for cfDNA analysis in current clinical practice and highlight the importance of incorporating cfDNA into clinical trials to further define its utility as a non-invasive predictive and prognostic biomarker in patients with BTC.

**ACKNOWLEDGEMENTS**

This work was supported by the RARE Initiative, and the authors would like to acknowledge the support of Jacqueline Lewis and Joe and Katie Comeau.

**FUNDING**

JEB is supported by the Department of Defense [grant number W81XWH-20-1-0118]. FF is supported by the Philanthropia-Lombard Odier Foundation. JKL is supported...
by the NIH [grant number R37 CA225655]. LG and NB are supported by the NIH/NCI Gastrointestinal Cancer SPORE [grant number P50 CA127003] and V Foundation for Cancer Research Translational Grant. LG is supported by the Cholangiocarcinoma Foundation Andrea Marie Fuqay Research Fellowship, MGH Fund for Medical Discovery Award, the American Cancer Society Institutional Research Grant, and the American Cancer Society Clinical Scientist Development Grant [grant number 134013-0SGD-19-163-01-TBG]. Opinions, interpretations, conclusions, and recommendations are those of the authors and are not necessarily endorsed by the American Cancer Society.

DISCLOSURE

JEB: advisor/consultant: Genome Medical, VetOncoDx; equity: Genome Medical, VetOncoDx, Cityblock Health (spouse); patents: institutional patent filed on methods to detect neuroendocrine prostate cancer through tissue-informed cell-free DNA methylation analysis. DVTC: advisor/consultant: Genentech/Roche, Eli Lilly, Merck, Daiichi Sankyo, BMS, Ono, Five Prime, Seattle Genetics, Amgen, Taiho, Astellas, Novartis, Gritstone, Pieris, Zymeworks, Basilea, QED, Foundation Medicine, Pierian, Silverback Therapeutics, Servier, Blueprint Medicines, Arcus Biosciences, Catamaran Bio, Tempus, Guardant Health, Archer & Natera; speaker: Genentech/Roche, Eli Lilly, Merck, Daiichi, Astrazeneca, Tempus, Guardant Health. ARP: research funding (to institution): Puretech, PMV Pharma, Plexxicon, Takeda, BMS, Novartis, Mirati, Genentech, Daiichi Sankyo; equity: C2i Genomics; consulting/advisor: Checkmate Pharmaceuticals, Eli Lilly, Pfizer, Invivata, Biofidelity; Data and Safety Monitoring Committee: Roche. LAK: employee and interest.

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