Genomic Profiling Of Blood-derived Circulating Tumor DNA From Patients With Advanced Biliary Tract Cancer
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Abstract
Background: Biliary tract cancer (BTC) is a highly lethal malignancy with high chemoresistance and poor clinical outcome. Accumulating evidence indicates targeted therapeutics may provide new hope for improving treatment response in BTC, hence better understanding the genomic profile is particularly important. Since tumor tissue may not be available for some patients, a complementary method is urgently needed. Circulating tumor DNA (ctDNA) provides a noninvasive means for detecting genomic alterations, and has been regarded as a promising tool to guide clinical therapies.

Methods: Next-generation sequencing (NGS) of 150 cancer-related genes was used to detect gene alterations in blood-derived ctDNA from 154 Chinese patients with BTC. Genomic alterations (GA) were analyzed and compared with an internal tissue genomic database (545 Chinese patients with BTC) and TCGA database (n=227).

Results: Nearly all patients (94.8%) had at least one change detected in their ctDNA. The median maximum somatic allele frequency (MSAF) across all cases was 6.47% (ranging 0.1%-34.8%). Pathologic type (p<0.001) and sex (p<0.001) were significantly related with MSAF. TP53 and KRAS were the most often mutated genes. The frequencies of single nucleotide variation in commonly mutated genes in ctDNA were similar to those detected in tissue samples, TP53 (35.1% vs 40.4%) and KRAS (20.1% vs 22.6%). Pathway analysis revealed that mutated genes were mapped to several key pathways including PI3K-Akt, p53, ERBB and Ras signaling pathway. In addition, patients harboring LRP1B, TP53, and ERBB family mutations presented significantly higher tumor mutation burden.
**Conclusion:** These findings demonstrated that ctDNA testing by NGS was feasible in revealing genomic changes and could be a viable alternative to tissue biopsy in patients with metastatic BTC.

**Keywords:** Biliary tract cancer; Circulating tumor DNA; Sequencing; Genomic feature

**Abbreviation**

<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Definition</th>
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<tbody>
<tr>
<td>BP</td>
<td>Biological process</td>
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<tr>
<td>BTC</td>
<td>Biliary tract cancer</td>
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<td>CC</td>
<td>Cellular component</td>
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<tr>
<td>cfDNA</td>
<td>Cell-free DNA</td>
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<tr>
<td>CNV</td>
<td>Copy number variation</td>
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<td>ctDNA</td>
<td>Circulating tumor DNA</td>
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<td>GA</td>
<td>Genomic alteration</td>
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<td>gDNA</td>
<td>Genomic DNA</td>
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<td>GO</td>
<td>Gene oncology</td>
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<tr>
<td>ICI</td>
<td>Immune checkpoint inhibitor</td>
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<tr>
<td>LRP1B</td>
<td>Low-density lipoprotein receptor-related protein 1B</td>
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<tr>
<td>MF</td>
<td>Molecular function</td>
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<td>MSAF</td>
<td>Maximum somatic allele frequency</td>
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<tr>
<td>SNV</td>
<td>Single nucleotide variant</td>
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<tr>
<td>TCGA</td>
<td>The Cancer Genome Atlas</td>
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<td>TMB</td>
<td>Tumor mutation burden</td>
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</table>
**Introduction**

Biliary tract cancer (BTC), including cholangiocarcinoma (intrahepatic and extrahepatic bile duct), gallbladder cancer and ampullary cancer, is an aggressive disease with poor prognosis [1]. Complete surgical resection provides the only chance for cure, but only 10% of patients are diagnosed at early-stage disease and are suitable for resection [2]; in addition, the recurrence rates are high [3,4]. Thus, for the majority of BTC patients, systemic chemotherapy is the mainstay of their treatment. Gemcitabine plus cisplatin (GemCis) is the standard regimen for first-line treatment, but the objective response rate is about 20% and the survival gain is limited [5]. What’s more, to date there is no standard second-line therapy. These highlight the need for the development of more effective treatment strategies.

Large molecular profiling studies have characterized the genomic landscape of BTC and indicated potentially targetable genomic alterations (e.g., FGFR fusion and IDH1/2 mutations) [6-8]. These findings potentiate the development of the use of personalized medicine in patients with BTC. In fact several targeted agents have showed meaningful activities in clinical trials. The most notable ones are Pemigatinib and Ivosidenib. Pemigatinib is an oral, selective inhibitor of FGFR1, 2 and 3. The multicentre phase II study FIGHT-202 enrolled patients with cholangiocarcinoma who had progressed after prior systemic therapy [9]. The objective response rate treated with pemigatinib for patients with FGFR2 fusions or rearrangements was 35.5% and the responses were durable [9]. Based on this results, FDA has granted accelerated approval to pemigatinib (Pemazyre), the first targeted treatment approved for advanced cholangiocarcinoma with an FGFR2 fusion or rearrangement who had received prior treatment [10]. Ivosidenib is a first-in-class small-molecule inhibitor of the mutant IDH1 protein [11]. In phase III ClarIDHy trial, Ivosidenib significantly improved PFS relative to placebo in previously treated cholangiocarcinoma patients with an IDH1 mutation [12]. These trials show us that some genetic subsets of patients can benefit from targeted therapies and we may select the right treatment for BTC patients based on their molecular features.

Hence, the precise molecular characterization of individual tumors is quite important. While tissue biopsy remains the gold-standard, tissue may not be available or limited for some patients. And the inter and intratumor heterogeneity is another pivotal challenge [13,14]. Liquid biopsy has emerged as a strategy to these challenges by detecting circulating tumor DNA (ctDNA) [15]. It is becoming a widely used diagnostic tool for identifying genomic alterations to guide therapy and prognosis. In BTC, several researches have been launched on assessing the sensitivity and positive predict value of ctDNA. Kinugasa et al. revealed that there was high concordance rate between bile ctDNA and tissue DNA samples and ctDNA might be used as a tool to diagnose gallbladder cancer [16]. In addition, changes in cell-free DNA correlated well with tumor marker dynamics in pancreatobiliary carcinoma, thus demonstrating the feasibility of cfDNA sequencing in identifying tumor-derived mutations [17].
Here we identified genomic alterations in blood-derived ctDNA from patients with BTC and assessed the concordance between alterations from ctDNA and their matched tumor tissue DNA. This work aids to prove that blood-derived ctDNA sequencing could be a potential complement to tissue testing, and might guide personalized cancer treatment.

Materials and Methods
Sample collection and clinicopathologic data
From January 2017 to December 2018, blood samples from 154 patients and the paired tissue and blood samples from 545 patients with metastatic BTC were collected in Hunan Provincial People's Hospital. All these samples were sent to a commercial company owning a CLIA-accredited/CAP-certified laboratory (3D Medicines Inc., Shanghai, China) for gene panel sequencing. The ethics committee of the Hunan Provincial People's Hospital approved this study, and all patients signed the waiver of informed consent form. In addition, the clinicopathologic characteristics, age and sex, were collected.

DNA isolation and sequencing
The methods of DNA extraction, sequencing and data analysis obeyed the published descriptions with some modifications [18]. Briefly, venous blood in STRECK tubes was centrifuged and kept the upper layer for the following genomic DNA (gDNA) extraction via using the QIAamp Circulating Nucleic Acid Kit (Qiagen, Germany). The cfDNA libraries were established by Accel-NGS 2S Plus DNA Library Kit (Swift BioSciences, USA), and then sequenced. The gDNA of tissue sample with quality control and white blood cells were extracted by the DNeasy Tissue or Blood Kit (Qiagen, Germany), respectively. After fragmenting gDNA, the sequencing libraries were prepared by KAPA Hyper Prep Kit (KAPA Biosystems, USA). After capturing, the libraries were loaded into NextSeq500 platform (Illumina, USA) and performed next-generation sequencing (targeted) 150 cancer-related genes. After eliminating duplicate or redundant information, the average coverage depth was 3000× for ctDNA and 500× for tissue sample.

Data and statistical analysis
Sequencing reads were mapped to the GRCh37/hg19 human reference genome, and analyzed for somatic genomic alterations (GAs) including single nucleotide variant (SNV), copy number variation (CNV) and fusion. The range of maximum somatic allele frequency (MSAF) was defined among 0.1% and 35% for all the somatic alterations per sample. Variants of unknown significance was included for calculating MASF, however nor was single nucleotide polymorphism. Clinically relevant GAs were defined as GAs that associated with response to currently available therapies or in target-driven clinical trials. TMB was defined as total number of somatic non-synonymous mutations in coding region. The raw data that support the findings of this study are available from the corresponding author upon reasonable request. In addition, data from the Cancer Genome Atlas (TCGA, https://www.cbioportal.org/) was extracted in December 2018 [19, 20]. Gene Oncology (GO) and pathway analysis on
gene alterations from ctDNA were performed using DAVID (https://david.ncifcrf.gov/) with the parameters P value cutoff = 0.05, and drawn in R by using the package “ggplot”. Demographic characteristics of patients were analyzed using the T test or Chi-Square ($\chi^2$) test. Two sided P-values were evaluated and P<0.05 was regarded as significance with statistical meaning. All the statistical analyses were performed by SPSS software, version 20.0 (SPSS Inc®, USA).

**Results**

**Patient characteristics and basic features of genomic alterations**

Hybrid capture-based genomic profiling were performed on ctDNA samples and tumor tissue DNA samples, respectively (Table 1). For patients who provided ctDNA samples, the median age was 61 years, ranging from 39 to 93 years old. Among them 66.2% were male. Cholangiocarcinoma was the most common pathologic subtype (72.1%), followed by gallbladder cancer (24.0%), and others (3.9%) such as ampullary carcinoma. ctDNA in the blood was detected in 94.8% of the cases as approximated using a maximum somatic allele frequency (MSAF) > 0. The median MSAF was 6.47%(range 0.1%-34.8%) and the average number of GAs was 4. As shown in Figure 1, highest median MSAF was observed in patients with gallbladder cancer, followed by patients with other pathological types and those with cholangiocarcinoma (P<0.05) (Figure 1A). Male patients showed significantly higher median MSAF compared to female patients (P=0.0001) (Figure 1B) while age had no significant effect(Figure 1C).

For patients who provided paired tumor tissue and blood samples, the median age was 59 years, ranging from 19 to 83 years old. The gender composition is relatively close to balance, with a distribution of 56.1% male and 43.9% female. Similar pathological types were observed, and cholangiocarcinoma (67.3%) was the dominant one. For patients providing paired tumor tissue and blood samples, ctDNA in the blood was detected in 520 (95.4%) of them and the median MSAF of tissue DNA was a little higher than that of ctDNA. The average GAs was 5. No significant correlation was observed between diverse baseline characteristics and TMB, including pathological subtype, sex and age (Figure 1D-F).

**Genomic alterations in blood-derived ctDNA**

Genomic alterations in ctDNA samples were identified using unique barcoding markers (Figure 2). TP53 (35.1%) and KRAS (20.1%) were found to be the most frequently altered genes. Using MutSigCV, other driver genes were also identified including EGFR (15.6%) and CDKN2A (9.7%). In patients with cholangiocarcinoma, TP53 was most frequently altered gene, followed by KRAS and EGFR (Supplementary Figure 1A). By contrast, gallbladder subtypes were significantly enriched for TP53, CDKN2A, and EGFR mutations (Supplementary Figure 1B). Patients harboring LRP1B, TP53, and ERBB family mutations showed significantly higher tumor mutation burden (TMB, Figure 1G-I).

**GO enrichment and signaling pathway analysis of genomic alterations**

To better understand the biological function of these frequent alterations, gene ontology enrichment and signaling pathway analysis were performed. Figure 3 showed the significant enriched GO terms on three aspects, namely biological process (BP), cellular
component (CC), and molecular function (MF). The top one enriched GO terms of BP was related to cell cycle and regulation, including regulation of transcription, regulation of cell proliferation, and cell cycle arrest. Most of the genes located in the nucleus, and the MF of calcium ion binding and chromatin binding enriched the most number of genes. As shown in Figure 4, a number of pathways that may be implicated in BTC were commonly mapped, including PI3K-Akt signaling pathway, p53 signaling pathway, ERBB signaling pathway, and Ras signaling pathway.

**Comparison of alterations in ctDNA versus tissue and TCGA database**

The frequencies of SNVs in commonly mutated genes in ctDNA samples were compared with the frequencies detected in tissue samples and TCGA database (Figure 5). TP53 was the most commonly mutated gene in all the three data source (35.1% vs 40.4% vs 24.2%), followed by KRAS (20.1% vs 22.6% vs 10.1%). And for most genes the mutation frequencies in ctDNA were similar with those detected in tissue samples and were relative higher than in TCGA database, with the exception of ARID1A and IDH1 which were most highly mutated in TCGA database.

**Discussion**

The present study identified the genomic alterations of ctDNA from patients with advanced BTC, analyzed the possible biological processes regulated by altered genes, and assessed the concordance between blood sample and tumor tissue sample. These results demonstrated the feasibility of NGS-based ctDNA mutation profiling to guide treatment decisions in cancer.

Somatic mutations were analyzed in blood samples of patients with advanced BTC, and ctDNA somatic mutations could be detected in 94.8% of all the cases. This result is consistent with other publications. Oliver et al reported the fraction is 84.6% (22/26) in patients with pancreatobiliary carcinomas[21]. In our study, TP53 and KRAS were the most frequently mutated genes, followed by EGFR. A whole-exome and targeted gene sequencing result identified that genes with a significantly frequency of mutations included TP53 (47.1%) and KRAS (7.8%); the ERBB signaling was the most extensively mutated pathway affecting 36.8% of the GBC patients[20]. TP53 and KRAS were also identified as the significantly mutated genes in a cohort of ICC patients and Ras/PI3K signaling was one of the most affected pathways, followed by cell cycle signaling pathway[22]. These are basically consistent with our results on GO and pathway analysis.

We identified that there was no significant difference on TMB among diverse pathological subtypes, which is consistent with the previous publication [23]. We also found that patients with LRP1B, TP53 or ERBB family member mutations had a significantly higher TMB than patients with wild-type genes respectively. LRP1B (low-density lipoprotein receptor-related protein 1B) gene mutations were frequently seen in multiple types of human cancer [24] and had been recognized as driver mutations in liver cancer and pancreatic cancer [25, 26]. Higher TMB was found in LRP1B mutated patients with melanoma and non-small cell lung cancer [27]. TP53 is a key tumor
suppressor gene. The encoded protein plays a key role in the regulation of cell cycle arrest, apoptosis, senescence, DNA repair and changes in metabolism. Mutations in this gene are associated with a variety of human cancers [28]. An integrated analysis on the genomic, transcriptomic, proteomic, and clinical data from cohorts of lung adenocarcinoma patients revealed that TP53-mutated tumors showed prominently increased mutation burden[29]. Although several researches have discussed the relationship between LRP1B/TP53 gene mutation and TMB, no definite conclusions have been reached in BTC. The ERBB family of receptor tyrosine kinases comprises four members, EGFR/ERBB1, ERBB2, ERBB3, ERBB4. Mutation of these members occurred in nearly 15% of BTC patients and was associated with higher TMB[30]. This was consistent with our discovery.

The genomic landscape and molecular features of BTC have been reported in several papers[20, 22, 31]. However, almost all these researches use tumor tissues as the sequencing samples, and limited data on ctDNA profiling of BTC has been reported. On 2019 ASCO meeting, a research revealed the basic rudiment of the ctDNA genomic alteration landscape of BTC, and indicated that 55% of the patients harbored targetable genetic alterations[32]. Another blood-based genomic profiling also showed a subgroup of patients with BTC may benefit from targeted therapy and TP53 and KRAS were the most frequently altered genes [33]. In these papers the sequencing panel was relative small, thus may limit our understanding of genomic features. Researchers from Germany analyzed the correlation between ctDNA alterations and disease progression in BTC using a 710 cancer-related-genes panel [34]. However, only 8 patients were detected by this panel, making the admissibility of the result quite weak.

To our knowledge, this work firstly revealed the genomic landscape of ctDNA in BTC with a large sample size, and directly compared it with the genomic landscape of tumor tissue DNA. These results indicated that ctDNA could be used as a potential complementary tool for gene sequencing, aiding to screen patients who may benefit from targeted therapies.

One limitation of this paper is the lack of baseline clinical characteristics and therapeutic regimens. As the clinical features may affect the detection of ctDNA[35, 36], further researches are needed to study the association between clinical information and molecular information.

**Author contribution**

All authors contributes to all the following: (1) the conception and design of the study, or acquisition of data, or analysis and interpretation of data, (2) drafting the article or revising it critically for important intellectual content, (3) final approval of the version to be submitted.

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**Conflict of Interest**

The authors declare no potential conflicts of interests in this work.
Reference


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Dong ZY, Zhong WZ, Zhang XC, et al. Potential Predictive Value of TP53 and


Legend

Figure 1. Association between baseline characteristics and ctDNA alteration in clinical samples. The impact of pathological subtype (A), sex (B), and age (C) on MSAF; the impact of pathological subtype (D), sex (E), and age (F) on TMB; comparison of TMB between patients with LRP1B (G), TP53 (H), ERBB family (I) mutation and wild-type, respectively.

Figure 2. Genomic alteration landscape of ctDNA in clinical samples.

Figure 3. GO Enrichment Analysis of frequently mutated genes categorized by biological process (A), cellular component (B) and molecular function (C).

Figure 4. Signaling pathways mapped by frequently mutated genes.

Figure 5. Genomic alterations in ctDNA versus tumor tissue from clinical samples and the TCGA database.

Table 1. Characteristics of BTC patients who provided ctDNA or tissue samples

<table>
<thead>
<tr>
<th>Characteristic</th>
<th>ctDNA samples</th>
<th>Tissue samples</th>
</tr>
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<tbody>
<tr>
<td>Cases</td>
<td>154</td>
<td>545</td>
</tr>
<tr>
<td>Median age, year (range)</td>
<td>61 (39-93)</td>
<td>59 (17-81)</td>
</tr>
<tr>
<td>Sex (male vs female)</td>
<td>102 vs 52</td>
<td>306 vs 239</td>
</tr>
<tr>
<td>Subtype (cholangiocarcinoma, vs gallbladder carcinoma vs other)</td>
<td>105 vs 37 vs 4</td>
<td>367 vs 161 vs 17</td>
</tr>
<tr>
<td>MSAF&gt;0, n (%)</td>
<td>146 (94.8%)</td>
<td>520 (95.4%)</td>
</tr>
<tr>
<td>Median MSAF</td>
<td>6.47% (0.1%-34.8%)</td>
<td>19.9% (0.8%-35.0%)</td>
</tr>
<tr>
<td>Average GA/case</td>
<td>4</td>
<td>5</td>
</tr>
</tbody>
</table>

Note:
MSAF: maximum somatic allele frequency;
GA: genomic alteration.