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The potential role of comprehensive genomic profiling to guide targeted therapy for patients with biliary cancer

Hwajeong Lee and Jeffrey S. Ross

Abstract: Remarkable advancements in techniques of genomic profiling and bioinformatics have led to the accumulation of vast amounts of knowledge on the genomic profiles of biliary tract cancer (BTC). Recent largescale molecular profiling studies have not only highlighted genomic differences characterizing tumors of the intrahepatic and extrahepatic bile ducts and gallbladder, but have also revealed differences in genomic profiles pertaining to associated risk factors. Novel genomic alterations such as *FGFR2* fusions and *IDH1/2* mutations in intrahepatic cholangiocarcinoma (ICC) and *ERBB2* alterations in gallbladder cancer (GBCA) are emerging as targeted therapy options capable of advancing precision medicine for the care of these patients. Moreover, variable genomic alterations also appear to impact prognosis and overall disease outcome independent from their therapy selection value. High mutational burden and increased expression of immune checkpoint-related proteins observed in a subset of BTC also show a potential for guidance of immunotherapy. Thus, comprehensive genomic profiling (CGP) is rapidly achieving status as an integral component of precision medicine and is starting to become invaluable in guiding the management of patients with BTC, a rare disease with dismal outcome.

Keywords: biliary tract cancer, cholangiocarcinoma, comprehensive genomic profiling, gallbladder cancer, genomic alteration, next-generation sequencing, precision medicine, targeted therapy

Introduction

Biliary tract cancer (BTC), also known as cholangiocarcinoma, can be defined as an adenocarcinoma arising from epithelium of the intrahepatic and extrahepatic biliary tree, and gallbladder [Nakanuma *et al.* 2010; Malhi and Gores, 2006]. While the International Classification of Diseases for Oncology by the World Health Organization classifies hilar cholangiocarcinoma (Klatskin tumor) as extrahepatic [Welzel *et al.* 2006], the European Network for the Study of Cholangiocarcinoma classifies BTC into intrahepatic, perihilar and distal [Banales *et al.* 2016]. About 50–60% of BTC is located in the liver hilum with or without direct extension to the hepatic parenchyma [Malhi and Gores, 2006]. The incidence of extrahepatic BTC may be decreasing [Global Burden of Disease Cancer Collaboration, 2015], while the incidence of intrahepatic BTC appears to be increasing worldwide [Welzel *et al.* 2006].

BTC comprises about 3% of all gastrointestinal tract neoplasms [Augustine and Fong, 2014; Lee *et al.* 2016]. The rarity of BTC and overall poor prognosis make it challenging to design robust clinical trials designed to optimize treatment. Therefore, it would be of great benefit for patients with BTC to be able to identify targetable genomic alterations and offer individualized treatments. Recent advancement in comprehensive genomic profiling (CGP) technologies and bioinformatics have allowed us to have a better understanding of the pathobiology of BTC and have recently led to the discovery of numerous genomic alterations and mechanistic pathways that may be targetable. Herein, we review the recent developments and impact of CGP of BTC

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and how these discoveries are shaping modern treatment regimens employing targeted therapies and immunotherapies for this disease.

The literature was searched *via* PubMed using the combination of keywords including 'cholangiocarcinoma', 'bile duct cancer', 'biliary cancer', 'targeted therapy', 'next-generation sequencing' and 'genomic profiling', and reviewed by reading the articles and related articles. Foundation Medicine Inc. [(FMI) Cambridge, Massachusetts, USA] cases are the cornerstone of the manuscript and the updated data, including previously published and new cases, are integrated throughout the manuscript.

Epidemiology and risk factors

BTC is the second most common primary malignancy of the liver [Global Burden of Disease Cancer Collaboration, 2015]. Notably, there is a marked geographic variation for this cancer due to certain risk factors prevalent in some geographic areas and genetic predisposition of the population. For example, liver fluke including *Clonorchis sinensis* and *Opisthorchis viverrini*, and hepatolithiasis is endemic in Asia, contributing to increased incidence of BTC in this region [Shin *et al.* 2010]. In Northeast Thailand, BTC constitutes approximately 85% of primary liver malignancies [Poomphakwaen *et al.* 2009]. Additional well established risk factors of BTC include primary sclerosing cholangitis (PSC), bile duct cysts and exposure to thorotrast [Augustine and Fong, 2014]. Gallbladder cancer (GBCA) is frequent in the Andean region, in Native Americans, in Alaskan Natives, and in Mexican Americans, possibly in association with genetic predisposition and living conditions [Andia *et al.* 2008; Augustine and Fong, 2014; Jain *et al.* 2016]. Relatively well established risk factors of GBCA include cholelithiasis, infection, anomalous pancreaticobiliary duct junction and gallbladder polyps [Augustine and Fong, 2014].

Other postulated risk factors for BTC include viral hepatitis, human immunodeficiency viral infection, idiopathic inflammatory bowel disease independent of PSC, cirrhosis, alcohol intake, smoking, fatty liver disease, obesity and choledocholithiasis. Obesity, diabetes and genetic predisposition have been postulated to be additional risk factors of GBCA [Khan *et al.* 2008; Kongpetch *et al.* 2015; Zhou *et al.* 2012; Augustine and Fong, 2014; Jain *et al.* 2016].

Conventional treatment and prognosis

Complete surgical resection or liver transplantation is potentially curative for resectable tumor, and conventional classifications of BTC according to anatomical location are relevant to surgical planning when the patients present with a localized resectable disease [Nathan *et al.* 2007; Rosen *et al.* 2010]. Unfortunately, only 13–55% of BTC patients are surgical candidates and most BTC is detected at an advanced, inoperable stage due to lack of specific symptoms and effective screening. Even after the resection, local recurrence rate is high and overall 5-year survival is in the range of 11–44% [Banales *et al.* 2016; Chong and Zhu, 2016; Skipworth *et al.* 2011]. Therefore, many patients receive palliative systemic chemotherapy for the disease management [Chong and Zhu, 2016; Banales *et al.* 2016]. To date, there is no site-specific or widely adopted standardized chemotherapy regimen for BTC, as high-quality data derived from clinical trials are scarce. Gemcitabine-based regimens have been extracted from pancreatic cancer management protocols, and used with or without combination with platinum agents or 5-fluorouracil, regardless of the site [Valle *et al.* 2010; Yang *et al.* 2013]. The prognosis of inoperable BTC remains dismal with $<$12 months of overall survival [Chong and Zhu, 2016; Lee *et al.* 2016].

Advancement in genomic profiling of biliary tract cancer

Variable methodologies have been employed to identify common molecular alterations of BTC in the past decade, which have contributed to continuous growth in understanding of the pathogenesis of this disease [Lee *et al.* 2016]. In 2012, Ong and colleagues carried out whole-exome sequencing of eight liver-fluke-related BTC. Using a hotspot mutation panel, the authors selected 15 major genes with somatic mutations, and validated the mutations in further 46 cases. In addition to common mutations that had been previously known, novel somatic mutations in *MLL3, ROBO2, RNF43, PEG3* and *GNAS* were identified [Ong *et al.* 2012]. Subsequently, further technical evolution of next-generation sequencing (NGS) techniques and the increased utilization of NGS clinical tests have led to an accumulation of a vast amount of genomic profiling data of BTC in a relatively short period of time [Borger *et al.* 2012; Chan-On *et al.* 2013; Jiao *et al.* 2013; Simbolo *et al.* 2014; Zou *et al.*

2014; Ross *et al.* 2014; Li *et al.* 2014; Lee *et al.* 2016; Churi *et al.* 2014; Jain *et al.* 2016]. These more recent studies have revealed differences in genomic profiling per anatomical location of the BTC as well as shedding light on the underlying risk factors for the disease and leading to the potential for the development of personalized targeted therapies.

Borger and colleagues showed that isocitrate dehydrogenase (*IDH1/2)* mutation is almost exclusively identified in intrahepatic cholangiocarcinomas (ICCs) (9 of 40 cases, 23%), but not in 22 extrahepatic cholangiocarcinomas (ECCs) and 25 GBCA [Borger *et al.* 2012]. Ong and colleagues' study was expanded to compare 108 ICC caused by liver-fluke infection and 101 cases without the infection by exome sequencing. This study showed that certain mutations were more frequent in liver-fluke-associated tumors compared with the tumors with other risk factors [Chan-On *et al.* 2013]. Jiao and colleagues reported frequent inactivating mutations of chromatin-remodeling genes in 32 ICC and frequent *TP53* mutation in 9 GBCA [Jiao *et al.* 2013]. Simbolo and colleagues carried out a mutational survey of 56 cancer-related genes in 70 ICC, 57 ECC and 26 GBCA. The molecular profiles of the tumors differed based on anatomical site, and 68% of tumors harbored targetable pathway alterations [Simbolo *et al.* 2014]. Zou and colleagues performed an exome sequencing of 102 ICC from Chinese patients, and reported the associations between HBsAg serology and gene mutation [Zou *et al.* 2014]. Site-specific (intrahepatic, extrahepatic and gallbladder) CGP studies by a deeper sequencing with broader coverage followed. These studies confirmed previous findings and identified additional clinically relevant genomic alterations [Ross *et al.* 2014; Lee *et al.* 2016; Li *et al.* 2014].

Differential genomic profile based on anatomical location and risk factors

A small series of genomic alterations are significantly enriched, based on the anatomical location of the BTC. Genomic alterations in *IDH1/2* (19%; 5–36%) and fibroblast growth factor receptor (*FGFR*) 2 fusion (6%; 4–20%) are almost exclusively identified in ICC [Jain and Javle, 2016; Jain *et al.* 2016; Javle *et al.* 2016; Ross *et al.* 2014; Chong and Zhu, 2016]. *BAP1* alteration is also common in ICC (12%; 1–38%) compared with ECC and GBCA [Jain and Javle, 2016; Jain *et al.* 2016; Javle *et al.* 2016; Ross *et al.* 2014; Chong and Zhu, 2016]. In contrast, *ERBB2* mutations are rare in ICC, while regularly identified in ECC (10%; 9– 25%) and GBCA (11%; 0–17%) [Jain and Javle, 2016; Jain *et al.* 2016; Javle *et al.* 2016]. *PRKACA* or *PRKACB* fusion was identified only in ECC, while *EGFR, ERBB3* and *PTEN* mutations preferentially occurred in GBCA [Nakamura *et al.* 2015]. Inactivating *TP53* mutations are more common in ECC (38%; 14–45%) and GBCA (57%; 46–59%) than in ICC (11%; 0–17%) [Jain and Javle, 2016; Jain *et al.* 2016; Javle *et al.* 2016; Ross *et al.* 2014; Chong and Zhu, 2016] (Figures 1–4).

Certain genomic alterations in BTC are associated with the pathogenesis of the disease (see Table 1). Liver-fluke-associated ICC shows higher somatic mutation burden compared with nonparasite-associated BTC [Chan-On *et al.* 2013].

Genomic profiling and prognostic relevance

Wang and colleagues reported that *IDH1/2* mutation showed a better prognosis with a longer time-to-tumor-recurrence in ICC [Wang *et al.* 2013]. On the contrary, Jiao and colleagues reported reduced 3-year survival in ICC with these mutations, though they had only six cases with the mutations [Jiao *et al.* 2013]. Churi and colleagues performed NGS-based genomic profiling of 50 ICC and 25 ECC and correlated clinical outcome with genomic alterations. In ICC, *KRAS, TP53* or mitogen-activated protein kinase/mammalian target of rapamycin (MAPK/mTOR), alterations were associated with worse prognosis, whereas *FGFR* alterations (including amplification and fusion) were associated with a relatively indolent disease course. *IDH1* mutation did not confer a prognostic relevance in this study. In ECC, *BAP1* and *PBRM1* alterations were associated with worse prognosis with aggressive clinical course [Churi *et al.* 2014]. Subsequent case series study of 22 BTC (20 ICC and 2 ECC) with *BAP1* alteration reported that 59% of patients showed aggressive clinical course [Al-Shamsi *et al.* 2016]. Nakamura and colleagues showed that *TP53, KRAS* and *ARID2* mutation were associated with poor prognosis in BTC by univariate analysis [Nakamura *et al.* 2015]. A recent multi-institutional study of BTC confirmed the correlation of genomic profiles with clinical outcome. Javle and colleagues

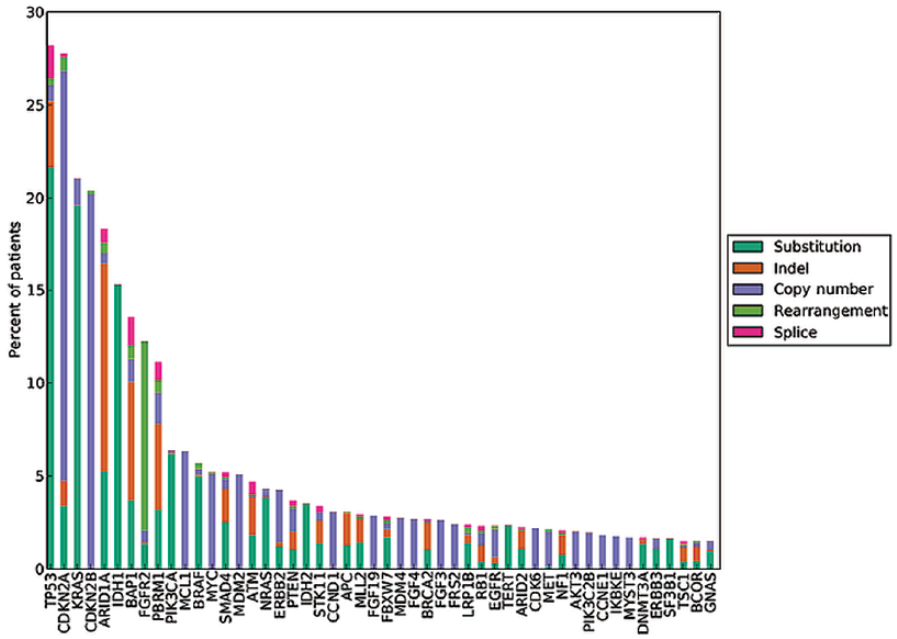


Figure 1. Long tail plot of the distribution of genomic alterations in 1682 cases of ICC (Provided by Foundation Medicine, Inc., Cambridge, MA, USA).

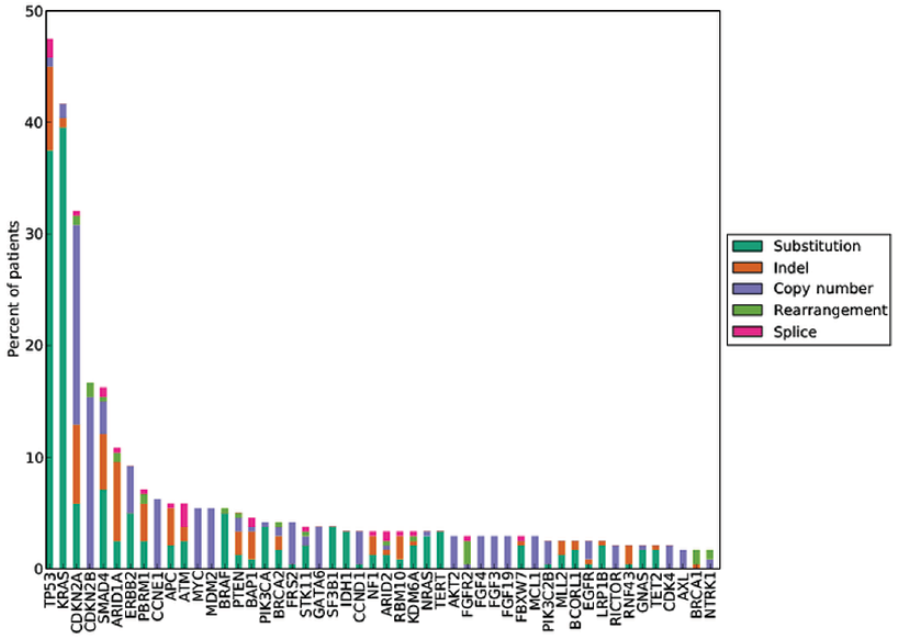


Figure 2. Long tail plot of the distribution of genomic alterations in 251 cases of ECC (Provided by Foundation Medicine, Inc., Cambridge, MA, USA).

performed genomic profiling for 554 cases of BTC (412 ICC, 57 ECC and 85 GBCA) and correlated the mutational profiles with clinical outcome for 321 patients. In keeping with previous studies, alterations in *TP53*, *KRAS*, *CDKN2A/B* and the MAPK/extracellular signal-regulated kinase (MAPK/ERK) pathway correlated with poor overall survival, whereas *FGFR2*

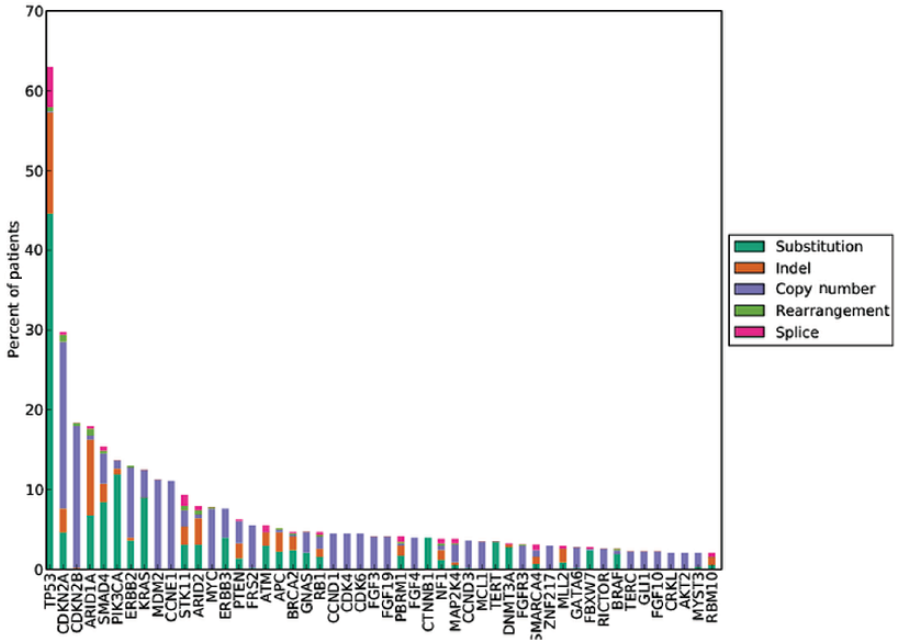


Figure 3. Long tail plot of the distribution of genomic alterations in 593 cases of GBCA (Provided by Foundation Medicine, Inc., Cambridge, MA, USA).

mutations were associated with improved overall survival. Twenty ICC patients with *FGFR* mutations received *FGFR*-specific targeted therapy, and showed superior overall survival compared with patients treated with conventional chemotherapy. *IDH1* again did not show prognostic relevance [Javle *et al.* 2016].

Clinically relevant genomic alterations

When 'clinically relevant' genomic alterations are defined as alterations for which targetable treatment or registered clinical trials are available, up to 83% of BTC feature clinically relevant and potentially actionable alterations [Simbolo *et al.* 2014; Lee *et al.* 2016; Ross *et al.* 2014].

(1) *FGFR2* fusions

Fibroblast growth factor receptor (*FGFR* 1–4) is a transmembrane receptor tyrosine kinase family that regulates cell proliferation, migration, differentiation and angiogenesis *via* binding to the ligands (fibroblast growth factors, FGFs) and affecting subsequent signaling responses through multiple pathways [Theelen *et al.* 2016]. Variable *FGFR2* fusion gene products paired with *-BICC1, -KIAA1598, -TACC3, -PARK2, -AHCYL1, -MGEA5, -KCTD1, -PPHLN1, -CCDC6* and *-TXLNA* have been identified in ICC [Banales *et al.* 2016; Jain *et al.* 2016].

The *FGFR2* fusion is diagnostically useful with its exclusive association with ICC [Arai *et al.* 2014]. In addition, ICCs with *FGFR2* fusion were associated with female predilection, younger age at onset, improved survival and relatively indolent disease course [Javle *et al.* 2016; Graham *et al.* 2014]. This fusion is also of therapeutic significance given that nonselective *FGFR* inhibitors such as brivanib, nintedanib, lenvatinib, pazopanib, regorafenib, dovitinib, lucitanib and ponatinib are on the market as well as under consideration in mechanism-driven clinical trials for expansion of original approvals [Ang, 2015; Abou-Alfa *et al.* 2016]. Also, selective novel *FGFR*-targeted tyrosine kinase inhibitors such as BGJ398, AZD4547, and JNJ42756493 are under investigation. Especially, BGJ398 is being evaluated in a phase II clinical trial, with promising response in patients with *FGFR2*-altered advanced or metastatic ICC [Jain *et al.* 2016]. A study using mouse xenograft model with *FGFR2-CCDC6* fusion protein showed that BGJ398 may outperform nonselective *FGFR* inhibitors ponatinib and dovitinib [Wang *et al.* 2016].

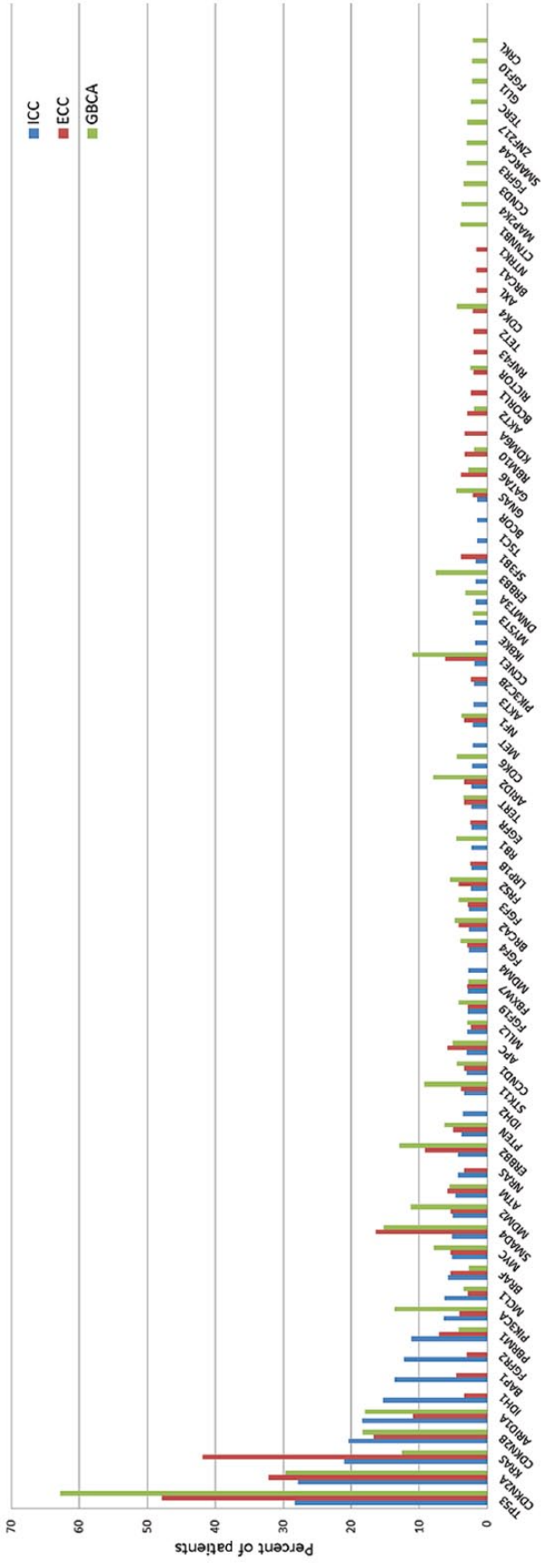
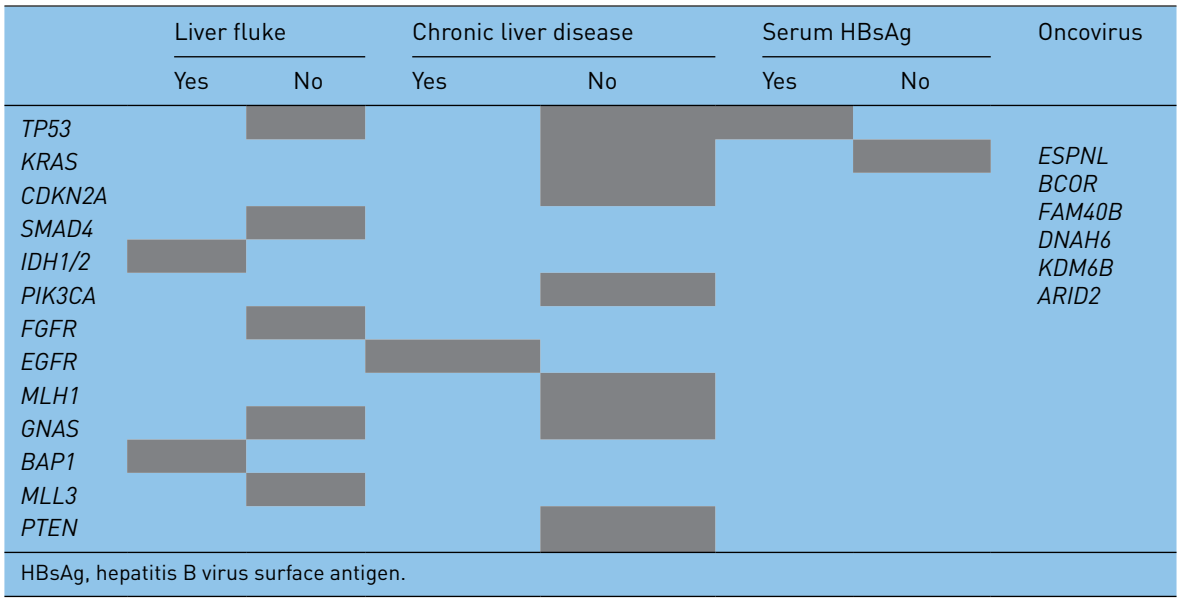


Figure 4. Combined long tail plot (combination of Figures 1–3) of ICC (intrahepatic cholangiocarcinoma), ECC (extrahepatic cholangiocarcinoma) and GBCA (gallbladder cancer).

Table 1. Genomic alterations pertaining to associated risk factors (grey). Oncovirus-hepatitis B and C, human papilloma virus and human T-lymphotrophic virus 1. Ong *et al.* [2012]; Chan-On *et al.* [2013]; Jang *et al.* [2014]; Zou *et al.* [2014]; Nakamura *et al.* [2015]; Jain and Javle [2016].



HBsAg, hepatitis B virus surface antigen.

(2) *IDH1/2* mutations

IDH1 and *IDH2* encode enzymes involved in conversion of isocitrate to alpha-ketoglutarate (α-KG) while reducing NADP to NADPH. *IDH1/2* mutations lead to production of oncometabolite D-2-hydroxyglutarate (D-2HG) instead of α-KG. This metabolite inhibits α-KG-dependent dioxygenases and contributes to epigenetic alterations such as hypermethylation of histone and DNA, and inhibits cell differentiation. Moreover, D-2HG may be used as surrogate biomarker to predict *IDH* mutations in cancer [Mondesir *et al.* 2016].

Mutations in *IDH1/2* have been identified in gliomas, hematologic malignancies, chondrosarcoma, and 30–40% of ICC [Jain and Javle, 2016; Jain *et al.* 2016; Javle *et al.* 2016; Ross *et al.* 2014; Chong and Zhu, 2016]. Currently, selective *IDH1/2* inhibitors, AG-120 and AG-221 are in phase I clinical trials using *IDH1/2* genomic alterations as a trial entry requirement for ICC patients. For example, a clinical trial is recruiting patients with *IDH1*-mutated ICC to evaluate firstly, the safety and tolerability, and secondly, the efficacy of AG-120, which is showing encouraging preliminary results [ClinicalTrials.gov identifier: NCT02073994]. One of twenty patients with ICC showed partial response and eleven of them showed stable disease [Mondesir *et al.* 2016; Chong and Zhu, 2016].

Alternatively, therapeutic options targeting hypermethylation induced by downstream product D-2HG are also of interest. In an *IDH1*-mutant glioma-xenograft mouse model study, tumor regression was observed only in the xenograft group that was treated with hypomethylating agent, 5-azacytidine. There was no tumor regression in the group without the treatment [Borodovsky *et al.* 2013]. Furthermore, *IDH1* mutation may be a potential target for immunotherapy given its ubiquitous expression and potent antigenicity [Schumacher *et al.* 2014]. The prognostic significance of *IDH1/2* mutations in ICC has not been established [Wang *et al.* 2013; Javle *et al.* 2016].

(3) *ERBB2*(*HER2*) mutation

Anti-HER2-targeted therapies have been the mainstay of treatment for breast cancer since 1998. Routine HER2 overexpression and amplification testing achieved standard of care for management of both early- and late-stage breast cancer. In addition, anti-HER2-targeted therapy has successfully been adopted for gastric and gastroesophageal cancer management [Bang *et al.* 2010]. *ERBB2* alterations, either gene amplifications or sequence mutations, are seen in approximately 10–11% of ECC and GBCA, while they are rare in ICC [Jain and Javle, 2016; Jain *et al.* 2016; Javle *et al.* 2016; Chong and Zhu, 2016]. In a study of 187 GBCA, 13% of the GBCAs demonstrated

HER2 overexpression by immunohistochemistry when commonly accepted scoring criteria were used, and these patients showed overall worse survival [Roa *et al.* 2014].

Although slightly less frequent than seen in breast and upper gastrointestinal cancers, *ERBB2* gene amplifications may emerge as an attractive target in BTC, especially GBCA. However, the efficacy of anti-HER2 targeted therapies in BTC currently remains unclear [Lee *et al.* 2016; Jain *et al.* 2016]. Javle and colleagues retrospectively reviewed the clinical responses of anti-HER2 antibody (trastuzumab)-directed therapy in 9 GBCA (8 with *ERBB2* amplification or overexpression) and 5 ICC (3 with *ERBB2* amplification) patients. Although tumors with *ERBB2* gene amplification showed promising responses (Figure 5), those with *ERBB2* sequence mutations showed mixed responses or no radiological responses [Javle *et al.* 2015]. Therefore, while anti-HER2-directed therapy is promising in BTC with *ERBB2* amplification, further investigation of small molecule anti-ERBB2 tyrosine kinase inhibitors such as lapatinib, neratinib and canertinib may be worthwhile for BTC with *ERBB2* mutations in the kinase domain [Lee *et al.* 2016; Bose *et al.* 2013; Jain *et al.* 2016].

(4) *EGFR* (*HER1*) and its signaling pathway

Alterations in *EGFR* and its downstream signaling pathways including *KRAS* and *PIK3CA*, have been a subject of extensive research. EGFR overexpression and amplification is seen in 20–30% of BTC, and its overexpression may portend a poor prognosis in ICC [Yoshikawa *et al.* 2008; Yang *et al.* 2014].

In a multicenter randomized phase III study of BTC, no significant difference in progression-free survival (PFS) was noted between the group with and without the addition of the anti-EGFR tyrosine kinase inhibitor erlotinib to gemcitabine and oxaliplatin. However, the addition of erlotinib showed an increased rate of objective tumor response in this group [Lee *et al.* 2012]. Subsequent molecular subgroup analysis of the same cohort revealed that tumors with wild-type *KRAS* showed improved response rate toward the addition of erlotinib compared with tumors harboring a *KRAS* mutation. Tumors with wild-type *PIK3CA* also showed a favorable trend for overall response [Kim *et al.* 2015]. These studies indicate that similar to other types of cancer, response to anti-EGFR therapy is associated with a complex interplay between various genomic alterations that await further investigation.

(5) *VEGF*

Overexpression of VEGF was reported in 55–60% of BTC [Chong and Zhu, 2016]. Several clinical trials are underway to evaluate the efficacy of variable VEGF inhibitors such as bevacizumab, sorafenib, sunitinib and cediranib in combination with conventional gemcitabine-based regimens on BTC but have not, as yet, demonstrated benefit in overall survival [Chong and Zhu, 2016; Lee *et al.* 2013]. Notably, Valle and colleagues showed that the addition of cediranib in combination with conventional regimen improved overall survival when the baseline PDGFbb concentration level was high [Valle *et al.* 2015]. PDGFbb has been shown to induce VEGF secretion in ovarian cancer [Matei *et al.* 2007].

(6) *MET*

Alteration of the proto-oncogene *MET* promotes survival of neoplastic cells by increasing cell motility and angiogenesis in variable solid tumors. MET overexpression is known to confer a poor prognosis, and may contribute to resistance to anti-EGFR treatment [Chong and Zhu, 2016]. However, when MET expression detected by immunohistochemistry was used as a requirement for clinical trial entry in non-small cell lung cancer, anti-*MET* targeted therapies did not achieve their endpoints and regulatory approval for these agents was not granted [Scagliotti *et al.* 2015; Spigel *et al.* 2013]. *MET* amplification was detected in 2–7% of ICC using CGP (Figure 6) [Ross *et al.* 2014; Churi *et al.* 2014; Javle *et al.* 2016]. Also, a patient with *MET* amplification confirmed by NGS experienced a metabolic response with a MET inhibitor [Churi *et al.* 2014]. In a xenograft mouse model study, LY2801653, a small-molecule inhibitor with potent activity against MET kinase, led to suppression of the proliferation of cholangiocarcinoma cell line [Barat *et al.* 2016].

(7) RAS/RAF/MEK/ERK pathway

Aberrant signaling of RAS/RAF/MEK/ERK pathway is frequent in BTC [O'Neill and Kolch, 2004], and alteration of this pathway including KRAS mutation may confer a poor prognosis [Javle *et al.* 2016; Chong and Zhu, 2016]. A phase II study of selumetinib, an MEK inhibitor, in

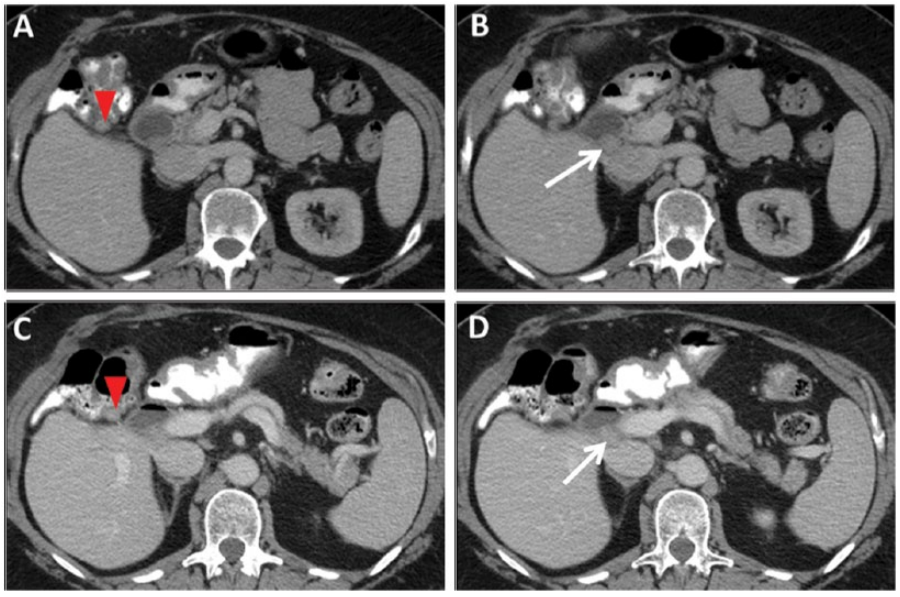


Figure 5. *ERBB2* amplified gallbladder carcinoma responds to combination therapy of trastuzumab with chemotherapy. A 64-year-old female with recurrent gallbladder carcinoma. Axial contrast-enhanced computed tomography images demonstrate (A) a 1.2 cm nodule in the gallbladder fossa adjacent to the hepatic flexure, and (B) a 1.6 cm nodule in the portocaval region. Both nodules were new from the postoperative scan (following resection of recurrent tumor in the gallbladder fossa), in keeping with recurrence. In (C) and (D), 8 months later, both nodules are stable. (Case provided by Dr Milind Javle, The University of Texas MD Anderson Cancer Center, Houston, TX, USA.)

metastatic BTC showed that 3 (12%) of 28 patients demonstrated objective response and 17 (68%) had stable disease [Bekaii-Saab *et al.* 2011]. A recent phase Ib study of selumetinib in combination with gemcitabine and cisplatin in advanced or metastatic BTC showed manageable toxicities [Bridgewater *et al.* 2016]. Also, patients with *BRAF* V600E-mutated BTC showed partial response to treatment with BRAF-inhibitor [Churi *et al.* 2014]. Moreover, a patient with *BRAF*-mutated ICC showed a dramatic response to dual inhibition of BRAF (dabrafenib) and MEK (trametinib) [Loaiza-Bonilla *et al.* 2014]. Although widely anticipated, the development of an effective direct KRAS inhibitor for diseases such as BTC is yet to be achieved.

(8) PI3K/AKT/mTOR pathway

A study with 39 patients with advanced, metastatic or recurrent BTC that progressed despite chemotherapy (mostly combination of gemcitabine and oxaliplatin) were enrolled in a phase II trial of mTOR inhibitor everolimus. One patient showed partial response and one showed complete response that sustained up to 8 months, with a favorable toxicity profile [Buzzoni *et al.* 2014].

A recent experiment using human cholangiocarcinoma cell line demonstrated that targeting AKT by MK2206, an AKT inhibitor, resulted in suppression of cellular growth [Wilson *et al.* 2015]. In another cell-line study, combined targeting of AKT and mTOR by MK2206 and RAD001 enhanced the efficacy of BTC treatment [Ewald *et al.* 2013].

The oral PI3K inhibitor BKM120 in combination with mFOLFOX6 (5-fluorouracil/leucovorin + oxaliplatin) was administered to 17 patients with advanced solid tumors, including four patients with BTC. Although one patient with ICC showed stable disease and remained on treatment for 26 weeks, further study was not recommended due to intolerable toxicity [McRee *et al.* 2015].

Mutational burden and immunotherapy

Some BTCs are enriched with significantly high mutational load. Transcriptome sequencing and hierarchical clustering of gene expression levels classified BTC into four subgroups with prognostic implication. Notably, the tumors in the worst prognosis group (39%; 74 of 188 tumors) showed high mutational burden, with increased expression of immune-checkpoint molecules and enrichment

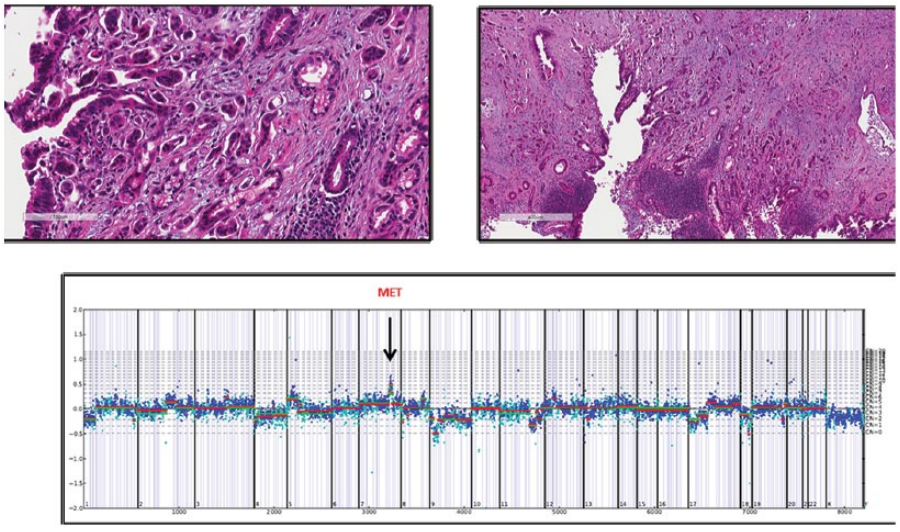


Figure 6. *MET* amplification of grade 3 Stage III gallbladder carcinoma in a female patient. *MET* amplification was detected using the comparative genomic hybridization plot (shown) generated by the Illumina Hi-Seq system and the FMI copy number algorithm (arrow: *MET* amplification spike at chromosome 7) (Case provided by Foundation Medicine, Inc. Cambridge, MA, USA).

for the genes involved in cytokine activity and antiapoptosis [Nakamura *et al.* 2015]. Recent studies have confirmed that patients with hypermutated tumors have greater chance of benefitting from immune-checkpoint inhibitors (ICPI) in non-small cell lung cancer, melanoma, and bladder cancer [Reck *et al.* 2016]. Therefore, the BTC patients whose tumors demonstrate a high mutational burden may be candidates for ICPI immunotherapy.

While mismatch repair protein (MMR)-deficient (microsatellite instability [MSI] high) tumors with their uniformly high mutational burden are also considered to be good targets for ICPI treatment, <10% of BTC was reported to be MMR deficient by immunohistochemistry [Goyal *et al.* 2014]. A study of PD-1 blockade in tumors with MMR deficiency enrolled one patient with MMR-deficient BTC. This patient showed partial response with 93% of biochemical response [Le *et al.* 2015]. Currently ongoing phase II study of pembrolizumab (ML-3495; anti PD-1 antibody) in noncolorectal MMR-deficient tumors includes three patients with BTC. Pembrolizumab appears to be well tolerated in patients with advanced BTC [Chong and Zhu, 2016].

In addition, recent studies in colorectal cancer suggest that tumor mutation burden (TMB), the algorithm-based calculation of nongermline mutations per megabase of sequenced DNA, will outperform MMR status as a biomarker for predicting response to checkpoint-inhibitor-based therapies, and tumors with high TMB may be a potential cohort for immunotherapy [George *et al.* 2016]. When subjected to a hybrid capture-based CGP assay, the prevalence of high TMB, defined by a >20 mutations per megabase TMB score in BTC, was quite low at 2% (unpublished data, Table 2).

Genomic alteration and resistance to chemotherapy

Genomic alterations in BTC may serve as biomarkers in predicting response to chemotherapy. Current standard regimen for advanced or recurrent BTC constitutes a combination of gemcitabine and platinum, with a variable and generally overall poor response. The mechanism of chemoresistance in BTC and relevant genes can be divided into five categories; (1) by reduction of the amount of intracellular drug: *SLCO2A1, SLC22A3, SLC29A1, SLC28 A1, SLC31 A1, ABCB1, ABCC1, ABCC3, ABCC4, ABCC5*, (2) by decreased activation of prodrug or inactivation of active agents: *TYMP, UPP1, UMPS, GSTP1*, (3) by changing molecular targets: *TYMS, ESR1, ESR2, EGFR, IGF1R*, (4) by interfering drug-induced DNA lesions: *ERCC1, RAD51, MSH2, MSH3, MSH6, MLH1, PMS2, RRM2B, TLK1*, and (5) by downregulation of apoptosis: *NK4, MET, TNFSF10, FAS, TP53, BCL2, XIAP, BIRC5, AKT1* [Banales *et al.* 2016].

Table 2. Tumor mutation burden of biliary tract cancer. (Data provided by Foundation Medicine, Inc., Cambridge, MA).

Tumor type	Case number	% > 10 mut/Mb	% > 20 mut/Mb
ICC	1018	3.4%	1.7%
ECC	176	5.7%	1.7%
Gallbladder carcinoma	344	5.5%	1.2%

mut, mutations; Mb, megabase; ICC, intrahepatic cholangiocarcinoma; ECC, extrahepatic cholangiocarcinoma.

Ahn and colleagues profiled 183 BTC using NGS. Nine common somatic mutations were selected, and their association with overall survival was studied. While mutations in *CDKN2A*, *TP53* and *ARID1A* were mostly mutually exclusive, dual loss of function mutations of these were associated with PFS and overall survival in patients treated with gemcitabine and platinum-based therapy. For example, patients with dual mutations in *CDKN2A* and *TP53* with wild-type *ARID1A* showed shorter PFS compared with those who were wild type for all three. On the other hand, *ARID1A* mutation slightly improved PFS in patients with *TP53* and/or *CDKN2A* mutation. A single patient with all three mutations demonstrated greatly improved PFS. *KRAS* mutation did not show prognostic relevance [Ahn *et al.* 2016].

Summary

BTC is a relatively rare but aggressive form of cancer which typically presents at an advanced clinical stage and is refractory to standard chemotherapy regimens. However, recent advancement of CGP revealed that BTC is enriched with multiple targetable genomic alterations and that the three types of BTC, ICC, ECC and GBCA, differ greatly in their molecular signatures. While its rarity makes it challenging to design robust clinical trials, BTC may be an ideal type of tumor to apply and test targeted therapy and precision medicine given its diverse genomic landscape.

Limitations of CGP and its application are firstly, many genomic alterations are not targetable; secondly, identified clinical trials may not be locally available and thirdly, CGP has not, to date, been proven to predict responses to chemo- or radiation therapy and has not been used to predict combination therapy benefits. Lastly, long-term clinical-outcome data are required to show true impact of targeted therapy.

Incorporation of genomic profiling in clinical practice and multidisciplinary approach to this intriguing tumor will enhance our knowledge about it and lead to the accumulation of long-term outcome data.

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