Novel Biomarkers in Cholangiocarcinoma

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INTRODUCTION

Cholangiocarcinoma (CCA) is the second most common type of primary liver cancer, after hepatocellular carcinoma, and accounts for 10-25% of primary liver cancers (1, 2). CCAs are classified as intrahepatic CCA (iCCA) and extrahepatic CCA (eCCA), according to their anatomical location (3). CCA is more frequent in South Asia compared to Western countries, because of the increased prevalence of established risk factors, including fluke infections (2). In the USA, the incidence of CCA has increased over the past three decades (4). Surgical resection and transplantation are the only curative treatments for patients with CCA, however the majority of cases are not surgical candidates when diagnosed (5). Even after potentially curative surgery, the prognosis of patients with CCA remains poor with 5 year-survival rates of 20% (6). Gemcitabine in combination with cisplatin is widely accepted as a frontline standard therapy for advanced or metastatic CCA (7). However, beyond these treatments, there are limited effective systemic therapy options.

Recently, an array of actionable genetic alterations has been identified in CCA (8). Studies evaluating these biomarkers could provide insights into the mechanisms of CCA tumorigenesis and identify potential targets for therapy (9). Genome-wide characterization have highlighted differing molecular aberrations of CCA and they could help to stratify CCA patients for molecularly targeted therapies (10). Previous studies have investigated roles of genetic, epigenetic and transcriptomic alterations along with well characterized paradigms such as oncogene and tumor-suppressor alterations. Additionally, the comparison between CCA and benign disease has been useful in identifying differences in carcinogenic mechanisms (11). These studies identified genetic alterations in CCA that could potentially lead to early diagnosis and precision treatment. This review article discusses the potential therapeutic biomarkers in CCA and the steps for their clinical translation.

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INTEGRATED WHOLE-GENETIC PROFILING ANALYSIS FOR CCA

We have performed profiling studies of the genomic and transcriptomic landscapes of each CCA subtype, in an attempt to clarify their distinct biological behavior and use the information of patients' stratification for molecularly targeted therapies (10). In Jusakul et al., we proposed four main subtypes of CCA based on integrative clustering of mutation, copy number, gene expression, and epigenetic data from nearly 500 CCA samples (9). Fluke-positive CCAs, which were classified as cluster 1 and 2, are enriched in *ERBB2* amplifications and *TP53* mutations. Conversely, fluke-negative CCAs exhibit high copy-number alterations and *PD-1/PD-L2* expression (cluster 3), or epigenetic mutations (*IDH1/2*, *BAP1*) and *FGFR/PRKA*-related gene rearrangements (cluster 4). The cluster 1 or 2 of CCAs are characterized as elevated mutation rates and increased point-mutation sub-clonality, compared with other clusters, especially cluster 4 CCAs. Cluster 1 or 2 CCAs are driven by external carcinogenic agents and early epigenetic deregulation so that the processes of carcinogen-induced methylation, deamination, and mutation are inherently stochastic from cell to cell and lead to increased levels of intratumor heterogeneity. On the other hand, cluster 4 CCAs are likely driven by genetic events such as *IDH1/2* or *BAP1* mutations, with epigenetic aberrations arising as a downstream consequence. Therefore, dominant and critical somatic mutations (*IDH1/2* and *BAP1*) may occur as a primary event and drive both rapid clonal outgrowth, which directly induces DNA hypermethylation. Specifically, *IDH1/2* mutations have been shown to increase 2-hydroxyglutarate oncometabolite production, leading to DNA hypermethylation (12).

These signature genomic alterations in each subtype highlights potential therapeutic opportunities. For example, it is possible that CCAs in clusters 1 or 2 with *ERBB2* amplification may be appropriate for therapies targeting *ERBB2* (HER2) signaling (13). In cluster 4, *FGFR* fusion rearrangements and *IDH1/2* mutations are the most promising targeted treatments (14). The elevated expression of immune related genes in cluster 3 CCAs suggests a therapeutic opportunity for immunotherapy.

Another whole-exome and transcriptome sequencing in a large cohort of 260 CCA patients proposed that nearly 40% of CCA patients obtains genetic alterations for potential therapeutic targets (15). In this study, recurrent mutations in *IDH1*, *IDH2*, *FGFR1*, *FGFR2*, *FGFR3*, *EPHA2*, and *BAP1* were identified predominantly in iCCA, whereas *ARID1B*, *ELF3*, *PBRM1*, *PRKACA*, and *PRKACB* mutations/alterations occurred preferentially in eCCA.

Notably, other reports have identified that *FGFR2* fusions result in ligand-independent activation of this receptor-tyrosine kinase exclusively in patients with iCCA (16-20). Novel gene fusions involving *PRKACA* or *PRKACB*, were discovered only in eCCA and these aberrations induced by gene fusions are considered potentially druggable. This study also reported that the subgroup with the poorest prognosis had significant enrichment of hypermutated tumors and a characteristic elevation in the expression of immune checkpoint molecules. Since the total number of somatic mutations (tumor mutation burden) are associated with clinical response to immune checkpoint blockade therapies in melanoma and lung cancer (21, 22), immune-modulating therapies might also be potentially promising options for this tumor subtype.

Sia et al. presented two subtypes of CCA, based on integrative genomic analysis using formalin-fixed CCA samples from 149 patients (23). One was represented by the proliferation type, which is characterized by activation of molecular characteristics such as stem-cell like iCCA, oncogenic pathways (*IGF1R*, *MET*, *EGFR*), and oncogenic mutations (*KRAS*, *BRAF* and *EGFR*). This type is associated with a poor prognosis, consistent with prior reports (24). The other was the inflammation-related type, which is associated with activation of inflammatory signaling, overexpression of chemokine and cytokines such as IL-6, IL-10 and IL-17, and *STAT3* constitutive activation. The overexpression mediators of Th2 immune responses, such as IL-4 and IL-10, downregulation of Th1 cytokines, and *STAT3* activation, a key transducer of cytokine signaling (25), suggest a potential role for immunotherapy targeting immunosuppressive factor expression by cancer cells.

Zhong et al. identified candidate biomarkers with high potential for clinical application in CCA using a meta-analysis-based approach (26). They collected the integrated analysis of 7 eligible datasets (9, 11, 23, 24, 27-29), which included a total of 428 cases and 46 controls, and identified a set of 1,080 differentially expressed genes, including 710 upregulated and 370 downregulated genes. The most upregulated genes associated with CCA were *SPP1*, *MMP11*, *COL1A1*, *TMSB10*, *AGRN*, and *COL4A1* (30-34). By evaluating the functional mechanism using gene ontology (GO) and Kyoto Encyclopedia of Gene and Genomes (KEGG) pathway enrichment analysis, the most significantly enriched terms are associated with activated cell cycle process and extracellular components. CCA has abundant of stroma and the genes of *SPP1*, *MMP11*, *COL1A1* may regulate the production and remodeling of stromal elements (35). This characteristic CCA

microenvironment may be a crucial component governing cancer progression and treatment response, and might represent an important target for new therapy approaches in CCA (36).

EMERGING MOLECULAR TARGETED THERAPIES

IDH1/2 as potential therapeutic targets in iCCA

Mutation in *IDH* genes in iCCA leads to abnormal enzymatic activity allowing them to convert α-ketoglutarate (αKG) to 2-hydroxyglutarate (2HG). This oncometabolite can inhibit the activity of multiple αKG-dependent dioxygenases, which results in alterations in cell metabolism, differentiation, survival, and extracellular matrix maturation (37-41). The oncogenic effects of *IDH* mutations appear to be primarily mediated through epigenetic alterations. The reduction of αKG production and reduced activity of αKG-dependent enzymes in *IDH* mutant cancers alters epigenetic states, which means histone and DNA hypermethylation. *IDH1* and *IDH2* mutations are detected predominantly in iCCAs, ranging from 18% to 36% of cases (18, 42-44). The vast majority of *IDH1* and *IDH2* mutations are novel substitution mutations recently targeted for molecule inhibition.

Since small-molecule inhibitors of mutant *IDH1* or *IDH2* have shown favorable efficacy in leukemia (45, 46), orally bioavailable inhibitors (AG120) have also moved to clinical trials for patients with *IDH1* mutations-harboring CCA. Preliminary results from a phase I trial of AG-120 have shown an acceptable safety profile in dose-escalation and dose-expansion cohorts. Moreover, among 20 patients with CCA treated with AG-120, one had a partial response and 11 showed disease stabilization. A multicenter, double-blind, placebo-controlled phase III trial in 186 patients with *IDH1*-mutant CCA is currently ongoing (NCT02989857). On the other hand, enasidenib, an oral selective inhibitor of mutant *IDH2*, has shown activity against acute myeloid leukemia (47-49). Enasidenib is also currently being evaluated in a multicenter phase I/II trial in patients with *IDH2*-mutant advanced cancers, including iCCA (NCT02273739) (table 1).

FGFR2 as a potential therapeutic target in CCA

FGFR2 is another attractive target in iCCAs. *FGFR2* alterations are frequently found (in 13-20% of iCCAs)

(17, 42). *FGFR2* fusions represent a majority of *FGFR2* arrangement in CCA and are associated with longer survival compared with CCA without *FGFR2* translocation (17). As discussed above, *FGFR2* translocations have been identified as a potential target for tyrosine kinase inhibitor therapies.

The pan-FGFR inhibitor NVP-BGJ398 has shown potential anti-tumor efficacy in preclinical models of CCA (50), and a phase II study in patients with advanced CCA with *FGFR* alterations is ongoing (NCT02150967) (table 1). Preliminary data from this study showed that NVP-BGJ398 may indeed have promising anti-tumor activity, with a disease-control rate of 82% and a manageable safety profile (51). Erdafitinib, which is another orally pan-FGFR inhibitor (52), is currently being tested in a phase II trial (NCT02699606). In a previous phase I dose-escalation study (NCT01703481) (table 1), erdafitinib showed a manageable safety profile at the doses tested and favorable clinical responses. Among the 23 patients with solid tumors harboring FGFR alterations enrolled in this trial, four patients had a confirmed response to erdafitinib treatment, one had an unconfirmed partial response and 16 had stable disease (53). Other FGFR-selective inhibitors are currently evaluated in patients with advanced solid-organ malignancies, for example ARQ 087 (NCT03230318), TAS-120 (NCT02052778) and INCB054828 (NCT02924376, NCT02393248) (table 1). Finally, ponatinib, a multikinase inhibitor with anti-FGFR activity, has also shown promising efficacy in patients with advanced iCCA with *FGFR2* fusions (16), and is currently being evaluated in a phase II trial (NCT02265341) (table 1).

KRAS and BRAF mutations as a target in CCA

Activating mutations in the *KRAS* gene are oncogenic driver mutations that frequently occur in CCA (11-25% of cases) (15, 18, 42). *KRAS* activation upregulates downstream pathways including the RAF/MEK/ERK (MAPK) pathway and is associated with poor survival in CCA patients (18, 44, 54). Phase Ib and II studies of selumetinib, a selective MEK inhibitor, demonstrated median PFS rates of 6.4 and 3.7 months, respectively, in advanced CCA patients (55, 56). *BRAF* mutations also occur in some CCAs (3-5% of cases) (44, 54, 57) (table 1). The most common activating mutations are V600E *BRAF* mutations. In melanoma patients with V600E *BRAF* mutations, *BRAF* inhibition leads to robust responses in a majority of patients. Among eight CCA patients with *BRAF* mutation, treatment with the oral

Table 1 - The selected biomarker and clinical trials in cholangiocarcinoma

Biomarker	Pathway	Incidence (%)	Agents	Clinical trial	Phase
FGFR2 fusion	FGFR	13-20 (iCCA)	ARQ 087	NCT03230318	II
			BGJ398	NCT02150967	II
			Ponatinib	NCT02265341	II
			INCB054828	NCT02924376	II
			Dovitinib, gemcitabine and capecitabine	NCT01497392	I
			H3B-6527	NCT02834780	I
IDH1/2 mutation	Krebs cycle	18-36 (iCCA)	Erdafitinib	NCT02699606	II
			TAS-120	NCT02052778	I/II
			AG-120	NCT02989857	III
			AG-221	NCT02273739	I/II
			IDH305	NCT02381886	I
BRAF mutation	RAF/MEK/ERK	3-5 (iCCA)	Vemurafenib	NCT02304809	II
			Dabrafenib and trametinib	NCT02034110	II
			Gemcitabine, cisplatin and MEK162	NCT01828034	I/II
MET overexpression	MET	12 (iCCA), 16 (eCCA)	Cabozantinib	NCT01954745	II
MET amplification			Merestinib	NCT03027284	I
EGFR overexpression	EGFR	100 (iCCA), 52.6 (eCCA)	Erlotinib	NCT00033462	II
EGFR mutation			Panitumumab + gemcitabine/irinotecan	NCT00948935	II
DKK-1	Wnt/b catenin	38.5 (iCCA)	DKN-01 + gemcitabine/cisplatin	NCT02375880	I
BAP1 mutation	Deubiquitinase	9-26 (iCCA), 5-10 (eCCA)	Tazemetostat	None	Pre-clinical
PI3CA amplification/mutation	PI3K/AKT/mTOR	25.5 (iCCA), 40 (eCCA)	Everolimus	NCT00973713	II
Notch	Notch	17-65 (iCCA) 6-56 (eCCA)	Copanlisib+gemcitabine/cisplatin	NCT02631590	II
PARP	PARP		LY3039478+other anticancer agents	NCT02784795	I
HER2 amplification/overexpression	ERBB2	1 (iCCA), 5-9 (eCCA)	Niraparib	NCT03207347	II
			Trastuzumab	NCT00478140	II
			Lapatinib	None	Case series
			Pertuzumab	None	Case series
			ASLAN001	NCT02609958	II
VEGFR2		60 (iCCA)	Ramucirumab	NCT02520141	II
			Apatinib	NCT03521219	II
VEGFR/PDGFR/RAF			Pazopanib+GSK1120212 (MEK inhibitor)	NCT01438554	I
			Pazopanib+gemcitabine	NCT01855724	II
EGFR/VEGFR/PDGFR/RAS/RAF			Regorafenib	NCT02053376	II
TrkA/B/C/ALK/ROS1			Entrectinib	NCT02568267	II

iCCA, intrahepatic CCA; eCCA, extrahepatic CCA

BRAF inhibitor vemurafenib led to a partial response in one patient (58).

MET as a target in CCA

Signaling of hepatocyte growth factor (HGF) through its tyrosine kinase receptor MET is important in cellular processes mediating survival and invasion and is thought to be a key mediator of cancer metastasis (23). Previous studies have indicated that MET is commonly expressed in both iCCA (20–58% of cases) and eCCA (0–68% of cases) (59–61), and that iCCA cell proliferation is characterized by activation of MET, EGFR, and MAPK signaling (23). However, early phase clinical trials

of MET inhibitors in patients with CCA have failed to show significant activity. A phase I study (62) of the MET inhibitor tivantinib in combination with gemcitabine in patients with CCA exhibited partial responses and stable disease in 20% and 46% of patients, respectively (*table 1*). Cabozantinib, a multikinase inhibitor against MET and VEGFR2, had shown a median PFS of 1.8 months with limited activity and substantial toxicity in unselected CCA patients (63). Moreover, plasma soluble MET, not tissue MET expression, correlated with patient outcome in this study. It remains to be unclear whether tissue or plasma soluble MET could be useful biomarkers for the selection of patients for treatment using MET inhibitors.

EGFR as a target in CCA

Increased EGFR expression has been associated with poor prognosis, and there is a subpopulation with significant enrichment related to EGFR signaling based on genomic analyses in CCA patients (24). EGFR activation promotes cell proliferation, migration and tumor angiogenesis. EGFR expression was identified in 38–100% of CCA patients (64). These analyses suggested that anti-EGFR treatment might benefit certain CCA subpopulations. However the results of early phase clinical trial of EGFR inhibitors have been disappointing. A phase II trial of EGFR inhibitor erlotinib in CCA patients showed an overall response rate (ORR) of 8% and PFS of 17% at 6 months and was terminated early (65) (*table 1*). Thus, EGFR expression by IHC is not necessarily associated with treatment response to EGFR inhibitors and has not been a reliable biomarker thus far.

WNT/DKK1 as a target in CCA

Dickkopf-1 (Dkk1) is an antagonist of Wnt signaling that is overexpressed in many cancers, including CCA, and has been demonstrated to promote cell migration, tumor growth and angiogenesis. The positive expression of Dkk1 was identified in 38.5% of iCCA and considered an unfavorable predictor for overall survival (66). The oncogenic mechanism of action of Dkk1 occurs in part through the activation of non-canonical Wnt signaling and PI3K/Akt signaling in cancer cells. In addition, recent studies have demonstrated that Dkk1 has immune modulatory activity that promotes tumorigenesis (67). Dkk1 contributes to an immunosuppressive tumor microenvironment in syngeneic lung and melanoma models by inhibiting canonical Wnt signaling in myeloid-derived suppressor cells (MDSCs) (68). Neutralization of Dkk1 blocked MDSC activity and results in tumor regression. Furthermore, Dkk1 also promotes immune evasion of metastatic cancer cells by downregulating NK activating ligands (69). Inhibiting Dkk1 expression results in NK recognition and eradication of these cells. Moreover, in CCA patients, anti-Dkk1 antibodies (DKN-01) showed greater than expected efficacy in recent phase I trials (NCT02375880) (*table 1*).

IMMUNOTHERAPY IN CCA

Immunotherapy using immune checkpoint blockade (ICB) has shown great promise in oncology, but its impact in GI cancers has been more limited so far, including in CCA. Among many candidate biomarkers of a response

ICB, the most investigated candidate has been expression of the PD-1 ligand, PD-L1. The expression of PD-L1 on tumor cells has shown inconsistent correlations with response to ICB monotherapy in melanoma and non-small-cell lung cancer (NSCLC). In CCA, recent reports have shown widely variable PD-L1 expression (9–72% of specimens) and immune cell infiltration within the CCAs (46–63%) (70, 71). However, similar to pancreatic cancer, a substantial proportion of CCAs have a highly immunosuppressive tumor-associated stroma, consisting of activated cancer-associated fibroblasts, endothelial cells, MDSCs and tumor-associated macrophages. This characteristic CCA microenvironment may modulate anticancer immune responses and impair the efficacy of ICB treatment. Further investigation of PD-L1 expression and the immunosuppressive cues in CCA microenvironment are warranted to clarify their roles as biomarkers of response or resistance to ICB therapies.

The presence of tumor DNA mismatch repair (MMR) deficiency and/or microsatellite instability (MSI) is associated with high rates and durability of responses to ICB therapy across multiple tumor types since the accumulation of genetic aberrations might relate to the expression of neoantigens capable of eliciting an anti-tumor T-cell response (72–74). Indeed, MMR deficiency was detected in 5–10% of CCA (75). Moreover, the cumulative mutational burden in tumor has been proposed as a biomarker for responsiveness to ICB in melanoma and NSCLC (21, 22). According to a whole-exome-sequencing study of 231 CCA tumor samples (15), a median of 39 and 35 somatic nonsynonymous mutations were identified in iCCA and eCCA, respectively. Nearly 6% of CCAs show hypermutated status and 36% of these hypermutated tumors has concurrent MMR deficiency and/or MSI. On the other hand, the median number of non-synonymous mutations was 302 in patients with NSCLC who are considered to obtain durable clinical benefit from pembrolizumab (21). These data suggest that ICB and immunomodulating therapies are possibly promising, especially for the subgroup of patients with CCA with high mutational loads.

Indeed, many studies are now testing the use of ICBs in CCA patients (see *table 2*). The KEYNOTE-028 study is testing pembrolizumab to CCAs harboring $\geq 1\%$ PD-L1 expression in the tumor. The ORR was 17% and the disease control rate was 34%. There were also a number of durable responders (22%) that continued to benefit from pembrolizumab over 40 weeks. This promising safety and efficacy of pembrolizumab in the KEYNOTE-028 prompted the next enrollment for 100 CCAs patients in the ongoing KEYNOTE-158 trial

Table 2 - Clinical trials of immunotherapy for cholangiocarcinoma

Proposed therapy	Phase	Clinical trial
Single ICB		
Pembrolizumab (anti-PD-1 antibody)	II	NCT03110328
Pembrolizumab (anti-PD-1 antibody)	II	NCT02628067
Nivolumab (anti-PD-1 antibody)	II	NCT02829918
Durvalumab (anti-PD-L1 antibody)	I	NCT01938612
Dual ICB		
Nivolumab + ipilimumab (anti-CTLA-4 antibody)	II	NCT03101566
Nivolumab + ipilimumab (anti-CTLA-4 antibody)	II	NCT02923934
Nivolumab + ipilimumab (anti-CTLA-4 antibody)	II	NCT02834013
Durvalumab + tremelimumab (anti-CTLA-4 antibody)	I	NCT01938612
ICB + immunomodulatory agent		
Pembrolizumab + GC-CSF	II	NCT02703714
Pembrolizumab + Peg-IFN α -2b	II	NCT02982720
ICB + local treatment		
Tremelimumab + TACE, RFA, cryoablation or SBRT	I	NCT01853618
Durvalumab + tremelimumab + TACE, RFA or cryoablation	II	NCT02821754
ICB + chemotherapy		
Pembrolizumab + mFOLFOX6	I	NCT02268825
Pembrolizumab + capecitabine/oxaliplatin	II	NCT03111732
Nivolumab + gemcitabine/cisplatin	II	NCT03101566
Durvalumab + tremelimumab + gemcitabine/cisplatin	II	NCT03046862
SHR-1210 + gemcitabine/oxaliplatin	II	NCT03486678
Durvalumab + guadecitabine	I	NCT03257761
ICB + molecularly targeted therapy		
Pembrolizumab + INCB054828 (FGFR inhibitor)	I/II	NCT02393248
Pembrolizumab + XL888(HSP90 inhibitor)	I	NCT03095781
Pembrolizumab + Ramucirumab (anti-VEGFR2 antibody)	I	NCT02443324
Atezotizumab (anti-PD-L1 antibody) + Cobimetinib (MEK inhibitor)	II	NCT03201458
Nivolumab + ABBV-368	I	NCT03071757
ICB + adoptive cell		
Pembrolizumab + tumor infiltrating lymphocytes	II	NCT01174121

ICB, immune checkpoint blockade

(NCT02628067). On the other hand, in a study in 86 patients with MMR-deficient tumors, including 4 CCAs, the ORR for pembrolizumab treatment was 53% (74). One CCA patient (25%) had a complete response and the other three showed stable disease (74). A number of clinical trials evaluating immunotherapy approaches are currently ongoing and these promising preliminary data warrant larger studies and bring significant effect for combination therapies to this intractable disease.

SUMMARY AND FUTURE PERSPECTIVE

With substantial understanding of the genetic aberrations in subtypes of CCA from recent studies, a precision medicine approach should be established and implemented in this largely intractable disease. The recently described molecular pathogenesis based on whole-gene sequence analysis in CCA needs further investigation in oncological function and in correlative

studies in clinical trials. Therapies targeting FGFR2 and IDH1 have recently yielded promising initial results in iCCA, and ICB immunotherapy has also shown activity in stabilizing disease progression. Potential biomarkers for treatment selection have been identified and multiple clinical trials are ongoing to test this approach. Despite the relatively low prevalence of oncogenic drivers, such as *FGFR*, *IDH*, *BRAF* or *EGFR* mutations, when considered together, a meaningful subset can be considered actionable mutations to targeted therapy. A prior study estimated that about 60% patients may have actionable mutations for targeting therapy in CCA (18). However, the role of combination therapies, especially including those targeting specific targets in the tumor cells and the immune environment, remains to be established in future studies. These developments might bring renewed hope for a breakthrough in overcoming the dismal prognosis of CCA.

Conflicts of Interest

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