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Review Article

Genetic profiling of intrahepatic cholangiocarcinoma and its clinical implication in targeted therapy

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Abstract: Intrahepatic cholangiocarcinoma (iCCA) is a treatment-refractory primary liver cancer with an increasing incidence and mortality worldwide in recent years. Lack of a stereotyped genetic signature and limited understanding of genomic landscape make the development of effective targeted therapies challenging. Recent application of advanced technologies such as next-generation sequencing (NGS) has broadened our understanding of genetic heterogeneity in iCCA and many potentially actionable genetic alterations have been identified. This review explores the recent advances in defining genetic alterations in iCCAs, which may present potent therapeutic targets. Chromatin remodeling genes and genes encoding isocitrate dehydrogenase and tyrosine kinase receptors as well as their downstream effectors are among the most frequently altered genes. Clinical trials testing the effect of new targeted agents on iCCA patients, especially those with the above genetic markers are under way. However, the complex interplay of environmental and evolutionary factors contributing to the genetic variability in iCCA calls for a more cautionary use of NGS in tailoring targeted regimen to the patients. Next-generation functional testing may complement NGS to execute precision medicine in future.

Keywords: Cholangiocarcinoma, genetic heterogeneity, targeted therapy

Introduction

Intrahepatic cholangiocarcinoma (iCCA) is the second most common primary liver cancer, accounting for up to 15% of all primary liver cancers [1]. The incidence and mortality of iCCA have increased worldwide despite geographic variations during the past decades [2]. Surgical resection is the only potentially curative approach to iCCA. However, approximately 70% of patients are diagnosed with inoperable iCCA at advanced stage and are refractory to conventional chemotherapy [3]. The 5-year survival rate for these inoperable patients is lower than 10% [4]. Limited molecular understanding of iCCA has hindered the development of effective targeted therapies. Previously, genetic analyses of iCCA are very few and often restricted to selected oncogenes and tumor suppressors. The introduction of next-generation sequencing (NGS) to oncology practice has enabled better understanding of genetic landscapes and promoted identification of potential actionable genetic alterations in iCCA. iCCA is a distinct hepatobiliary malignancy that differs from hepatocellular carcinoma (HCC), hilar and distal bile duct cholangiocarcinoma in clinicopathologic and molecular features [4], and thus requires a distinct investigation. Herein, we briefly explored NGS-based genetic characterizations in iCCA as well as their impacts on patient prognosis and molecular targeted therapy.

Cancers often harbor multiple genetic alterations including somatic gene mutations, translocations and copy number variations (CNVs), but only one or few are responsible for induction and maintenance of tumorigenesis. These are referred to as “driver mutations”, while the remaining “passenger mutations” may have a more nuanced impact. Molecular pathogenesis of iCCA is a highly complex process involving multiple genetic alterations and signaling pathway changes. Although it is not fully understood, the genomic landscape of iCCA revealed by

Table 1. Recurrent mutations in iCCAs based on next generation sequencing

Pathway	Target	Prevalence of alterations (%)*	Reference (s)
P53	*TP53*	159/1015 (15.7)	[5-13, 15, 16, 18, 19]
Ras/Raf/MEK/ERK pathway	*EGFR*	11/492 (2.2)	[5-9]
	KRAS	122/1283(9.5)	[5-16, 18, 19]
	NRAS	21/576 (3.6)	[5-11, 18, 19]
	BRAF	24/723 (3.3)	[5-11, 16, 18, 19]
P38/MAPK pathway	*PTPN3*	51/124 (41.1)	[15]
	MAP3K4	10/124 (8.1)	[31]
PI3K/mTOR pathway	*PTEN*	26/597 (4.4)	[5-12, 18, 19]
	PIK3CA	26/597 (4.4)	[5-12, 18, 19]
Metabolic pathway	*IDH1*	121/1094 (11.0)	[5-8, 10-14, 17-19]
	IDH2	53/1094 (4.8)	[5-8, 10-14, 17-19]
Chromatin remodeling	*ARID1A*	72/501 (14)	[5-8, 11-13, 18, 19]
	PBRM1	34/501 (6.8)	[5-8, 11-13, 18, 19]
	BAP1	44/501 (8.8)	[5-8, 11-13, 18, 19]

*The percentage has been calculated by considering the number of samples presenting the somatic mutation over the total number of samples analyzed in all cohorts (discovery and prevalence set of samples). Abbreviations: TP53, tumor protein P53; MEK, mitogen-activated protein kinase/extracellular signal-regulated kinase kinase; ERK, extracellular regulated protein kinase; EGFR, epidermal growth factor receptor; MPAK, mitogen-activated protein kinase; PTPN3, tyrosine phosphatase non-receptor type 3; MAP3K4, mitogen-activated protein kinase kinase kinase 4; PI3K, phosphatidylinositol 3-kinase; mTOR, mammalian target of rapamycin; AKT, protein kinase B; PTEN, phosphatase and tensin homolog deleted on chromosome ten; PIK3CA, phosphatidylinositol-4,5-bisphosphate 3-kinase catalytic subunit alpha; IDH1, isocitrate dehydrogenase 1; IDH2, isocitrate dehydrogenase 2; ARID1A, AT-rich interactive domain-containing protein 1A; PBRM1, Protein polybromo-1; BAP1, BRCA1 associated protein-1.

NGS has facilitated the identification of novel driver mutations and certification of previously-recognized ones.

Potential driver somatic mutations in iCCA

The potential driver mutations were listed in **Table 1** after analyzing the results of NGS on approximately 1,000 iCCA cases [5-19]. Mutations in common oncogenes and oncosuppressors previously reported in various tumors including *KRAS, BRAF, EGFR, PI3CA, PTEN, TP53* have also been identified in iCCA. *KRAS* and *TP53* are among the most frequent mutated genes in iCCA. Michael et al. have successfully developed a genetically engineered mouse model of iCCA by incorporating tissue-specific activation of $Kras^{G12D}$ and deletion of *p53* [20]. The prevalence of *KRAS* mutations in patients with iCCA varied considerably ranging from 4 to 24% [5-16]. Activating mutations of *KRAS* were associated with a worse outcome in iCCA [6, 7]. EGFR is one of the major receptors that mediate activation of RAS/RAF/MEK/ERK signaling pathway. *EGFR* mutations occurred relatively rarely in iCCA with a frequency of 0-7.4% [5-9]. An addition of an orally active tyrosine-kinase inhibitor of EGFR erlotinib to traditional chemotherapy has proved to significantly prolong median progression-free survival in patients with cholangiocarcinoma including iCCA [21]. Rapid and robust anti-tumor activity of erlotinib has also been reported in an iCCA patient with somatic nonsense mutation in ERBB receptor feedback inhibitor 1 (*ERRFI1*), a direct negative regulator of EGFR activation [18]. However, *KRAS* mutations have been identified as a predictor for poor response to anti-EGFR therapy in colorectal cancer [22, 23]. Fortunately, various *KRAS*-driven cancers have been reported to respond to MEK inhibitors [24-27], and similar effect may also present in iCCA. Elumetinib, a small-molecule kinase inhibitor against MEK1/2, is currently under investigation about its therapeutic effect on patients with iCCA (NCT00553332). Mutations in genes coding for other components of RAS/RAF/MEK/ERK pathway including *NRAS, BRAF, ARAF, MAP3K1, MAP2K1* have also been described [5-11, 16, 18, 19]. Ras-induced oncogenesis could be promoted by interaction between a unique MAPK p38γ (also known as MAPK12) and its specific phosphatase PTPN3 (also known as PTPH1) [28, 29]. We have previously identified new *PTPN3* mutations in 51 iCCA patients after sequencing a cohort of 124 Chinese iCCA patients [15]. Gain-of-function mutations in *PTPN3* promoted proliferation and migration of iCCA cells in vitro, and correlated with increased postoperative recurrence in iCCA patients. More recently, the reciprocal allosteric regulation of p38γ and PTPN3 involving a

PDZ domain-modulated complex has been successfully defined, which will promote the development of structure-based anticancer drug targeting the PTPN3-p38γ interaction [30]. We have also reported that 8.06% of iCCA patients from the same cohort harbored mutations in *MAP3K4*, mostly inactivating mutations [31]. Interestingly, MAP3K4 served as a putative tumor suppressor and its deficiency promoted invasiveness of iCCA via induction of epithelial-mesenchymal transition (EMT) mediated by p38/NF-κB/snail signaling pathway. Accordingly, MAPK could serve as a novel marker for risk stratification of iCCA. PI3K/AKT/mTOR signaling pathway is another pathway frequently activated by EGFR and mutations in genes coding for its components have also been reported [5-12, 18, 19]. The therapeutic effect of mTOR inhibitor everolimus, which has been approved for use in renal carcinoma, on advanced cholangiocarcinoma is being studied in a Phase II clinical trial (NCT00973713). In addition, AKT inhibitor MK2206 applied to treat patients with advanced refractory biliary cancer including iCCA is also currently under investigation (NCT01425879).

IDH1 and IDH2 encoding the NADP+-dependent isocitrate dehydrogenases represent the most frequently mutated metabolic genes in human cancers [32-35]. Mutations in *IDH1/2* occurred to approximately 10-20% of iCCA cases, which had not been identified in HCC [5-8, 10-14, 17-19]. Recurrent mutations in *IDH1/2* resulted in the conversion of normal metabolite α-ketoglutarate (α-KG) to oncogenic counterpart R(-)-2-hydroxyglutarate (2HG), which inhibited the activity of multiple α-KG-dependent dioxygenases [36-38]. According to the study from Saha et al. [39], mutant *IDH* blocked liver progenitor cells from undergoing hepatocyte differentiation through accumulation of 2HG and suppression of HNF-4α, and promoted the development of iCCA with cooperative function of activated Kras. *IDH1/2* mutations were associated with longer overall survival and were independent factors for longer time to tumor recurrence after surgical resection of iCCAs, suggesting a molecular sub-class with a better prognosis [14]. Circulating 2HG, a potential surrogate biomarker in iCCA, has found to be directly correlated with the tumor burden of iCCA [40]. Serum level of 2HG ≥170 ng/ml could predict the presence of *IDH1/2* mutation in iCCA with high sensitivity and specificity [40]. Specific IDH1 inhibitor AG-120 has shown a promising therapeutic effect on acute myeloid leukemia (AML) harboring *IDH1* mutation in a phase I clinical trial. Whether patients with iCCA can gain parallel benefit from specific inhibitors to IDH1 (AG-120) and IDH2 (AG-221) are currently being investigated in a phase I (NCT02073994) and a phase I/II (NCT02273739) clinical trial respectively.

Chromatin remodeling genes regulate the organization of DNA into chromatin, and disruption of their expression can interfere with the control of gene expression. Approximately 25-50% of iCCAs had genetic alterations involving at least one of the chromatin modulating genes (i.e. *BAP1, ARID1A* and *PBRM1*) [5-8, 11-13]. *ARID1A* encodes the AT-rich interactive domain-containing protein 1A, which is a member of the SWI/SNF chromatin-remodeling complexes. *PBRM1* also encodes component of the SWI/SNF-B (PBAF) chromatin-remodeling complexes whereas *BAP1* encodes a nuclear deubiquitinase. *ARID1A, PBRM1* and *BAP1* functioned as tumor suppressors and were enriched in inactivating mutations in iCCA [12]. Subjects with mutations in any one of these genes tended to have shorter survival time than subjects in whom all three genes were wild type [12]. Besides, *BAP1* mutations were also associated with an increased risk of early postoperative tumor recurrence [6, 11]. These mutations may qualify additional therapies for iCCA, as drugs targeting chromatin remodeling like histone deacetylase (HDAC) inhibitors may be of therapeutic benefit [41]. Mutations in genes involved in DNA repair pathway like *MSH6, BRCA1, ATM, MLH1* and other novel genes like *GNAS, SMAD4* have also been identified [5, 13].

Actionable gene fusions in iCCA

Somatic gene fusions can drive the development of human cancers and function as druggable targets, though their translational relevance has been mostly limited to hematological malignancies. Recently, various studies have revealed that more than 10% of iCCAs featured fibroblast growth factor receptor 2 (*FGFR2*) fusions including *FGFR2-PPHLN, FGFR2-AHCYL1, FGFR2-BICC1, FGFR2-KIAA1598, FGFR2-TACC3, FGFR2-NOL4, FGFR2-PARK2, FGFR2-MGEA5* [6, 7, 18, 19]. *FGFR2* encodes a member of the fibroblast growth factor receptor family. Constitutive activation of FGFR2 and downstream MAP kinase ERK1/2 by *FGFR2-*

Table 2. Recurrent focal amplifications or deletions in iCCAs based on next generation sequencing

Pathway	Target	Prevalence of alteration (%)*	Reference (s)
High-level amplification			
HGF signaling	*c-MET*	2/28 (3.6)	[7]
FGF signaling	*FGF 19*	6/202 (3.0)	[6, 16]
Cell cycle control	*CKD6*	3/102 (2.9)	[6, 7]
	CCND1	6/202 (3.0)	[6, 16]
Homozygous deletion			
Cell cycle control	*CDKN2A*	31/202 (15.3)	[6, 7, 16]
	CDKN2B	28/202 (13.9)	[6, 7, 16]

*The percentage has been calculated by considering the number of samples presenting the copy number variation over the total number of samples analyzed in all cohorts (discovery and prevalence set of samples). Abbreviations: HGF, hepatocyte growth factor; c-MET, hepatocyte growth factor receptor; FGF, fibroblast growth factor; CKD6, cyclin-dependent kinase 6; CCND1, cyclin D1; CDKN2A, cyclin-dependent kinase inhibitor 2A; CDKN2B, cyclin-dependent kinase inhibitor 2B.

AHCYL1, *FGFR2-BHCC1* and *FGFR2-PPHLN1* fusions have been proven *in vitro* and *in vivo* [19]. *FGFR2* fusions were detected exclusively in iCCA, rarely in colorectal cancer (1/149 in Arai et al.) and HCC (0/100 in Daniela S et al. and 1/96 in Arai et al.) and none in gastric cancer (0/212 in Arai et al.), indicating a relative specificity of *FGFR2* fusions in iCCA [19, 42]. In contrast, the majority of HCC cases involved in aberrant FGF signaling were characteristic of *FGF19* amplification, which drove carcinogenesis via persistent activation of FGF19/FGFR4 signaling rather than FGFR2 signaling [43]. Selective FGFR kinase inhibitors (BGJ398 and PD173074) successfully inhibited the oncogenic activity of *FGFR2* gene fusions *in vitro* and might represent as a potential therapeutic target in iCCA [19]. A multi-center, open label, single arm phase II study evaluating anti-tumor activity of BGJ398 in advanced or metastatic cholangiocarcinoma patients with *FGFR* genetic alterations is ongoing (NCT02150967). A phase I/II study of ARQ 087, which preferentially inhibits FGFR family, has also been launched in advanced solid tumors with *FGFR* alterations (NCT01752920). Regorafenib, which inhibits multiple kinases including *FGFR2*, has been approved for treatment of partial metastatic colorectal carcinoma and its clinical implication in iCCAs has been under way (NCT02115542).

Besides *FGFR2* fusions, c-ros oncogene 1 receptor tyrosine kinase (*ROS1*) fusions have been identified in 8.7% of iCCAs [44]. *ROS1* encodes an orphan receptor tyrosine kinase related to anaplastic lymphoma kinase (ALK) and can be continuously activated by chromosomal rearrangement. *FIG-ROS* identified in iCCA patients was validated as a potent oncogene in an orthotopic allograft mouse iCCA model, and could accelerate tumor onset and form an aggressive and metastatic subtype with cooperation of *Kras* G12D and mutant *p53* [45]. Crizotinib, a small-molecule tyrosine kinase inhibitor of ALK, ROS1 and hepatocyte growth factor receptor (HGFR, also known as c-MET), has showed marked antitumor activity in patients with advanced *ROS1*-rearranged non-small-cell lung cancer (NSCLC) regardless of the type of *ROS1* rearrangement [46]. Ceritinib (LDK378) with similar mechanism to crizotinib is being investigated about its therapeutic effect on ROS1 and/or ALK over-expressed advanced iCCAs (NCT02374489) as well as tumors with *ROS1* or *ALK* aberrations (NCT02186821). Novel gene fusions including *RABGAP1L-NTRK1* have also been reported with clinical implication currently undefined [7].

Copy number variations in iCCA

Recurrent focal amplifications or deletions in iCCA based on NGS were summarized in **Table 2** [6, 7, 16]. Daniela el al. identified 5 regions with high-level amplification which affected 23% (30/128) of iCCAs [16]. One such amplification at 11q13.2 including 6 genes, for example, *CCND1, FGF* family members, and oral cancer overexpressed 1 (*ORAOV1*), was identified in 4% of iCCAs [16]. Notably, overexpression of ORAOV1 was also found in HCC with 11q13 high-level amplification, suggesting that this oncogene could possibly be a common driver in liver carcinogenesis [47]. *MET* and *FGF* amplifications have also been described in iCCA, wherein *MET* amplification has been associated with poor clinical outcome [6, 7, 48]. A phase II study of cabozantinib, which blocks c-MET and vascular endothelial growth factor receptor 2 (VEGFR2) signaling pathways, has been under investigation about its effect on patients with advanced iCCA after progression on systemic therapy (NCT01954745). Patients with *FGF* or *MET* amplifications may predict sensitivity to their inhibitors respectively, which may serve as biomarkers for precision medi-

Table 3. New target therapies under evaluation in clinical trials in iCCA*

Drug	Phase	Target	Trial enrichment	Primary endpoint
Elumetinib	II	MEK1/2	No	DCR
Everolimus	II	mTOR	No	DCR
MK2206	II	AKT	No	ORR
AG-120	I	IDH1	*IDH1* mutation	MTD
AG-221	I/II	IDH2	*IDH2* mutation	MTD, TTP
BGJ398	II	FGFR 1-4	*FGFR* alterations	ORR
ARQ 087	I/II	FGFR 1-4	*FGFR* alterations	Safety, TTP
Regorafenib	II	VEGFR1-3, TIE-2, MAPK, FGFR-1	No	OS
Crizotinib	II	ALK, ROS1, MET	ROS1 or ALK over-expression/genetic alterations	DCR
Cabozantinib	II	c-MET, VEGFR2	No	PFS

*The data were accessed in November 2015 on the ClinicalTrials.gov online database. Abbreviations: DCR, disease control rate; ORR, overall response rate; MTD, maximum-tolerated dose; TTP, time to progression; OS, overall survival; PFS, progression free survival.

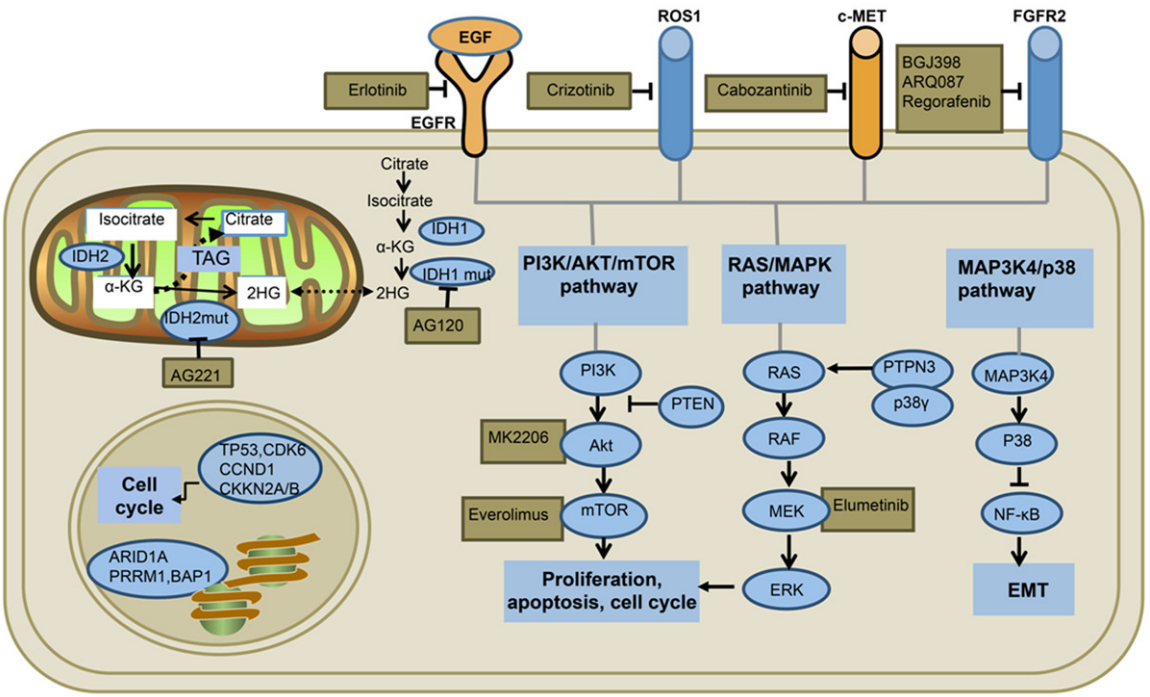

Figure 1. Recurrent genetic alterations and targeted therapies under investigation in iCCA. Genetic alterations in tyrosine kinase receptors and downstream RAS/MAPK, PI3K/AKT/mTOR effector casades result in aberrant modulation of proliferation, apoptosis and cell cycle. Newly identified mutations in MAP3K4 lead to EMT process in iCCA through p38/NF-kB signaling pathway. Mutated *IDH1/IDH2* result in the accumulation of oncogenic metabolite 2HG. Potential targeted therapies against the above alterations are currently under clinical trials. Recurrently mutated chromatin remodeling genes also present as druggable targets.

cine accordingly. Copy number alterations in chromosomal regions encoding genes related to MAPK, Wnt, TGF-β pathway were also noted via integrative analysis of transcriptional regulatory network (TRN) and CNV [49]. Focal deletions at 9p21.3, which harbored tumor suppressors *CDKN2A* and *CDKN2B,* were observed in 7%-18% of iCCAs [6, 7, 16]. Tumors with loss of *CDKN2A/CDKN2B* locus or with *CDK6* amplification may respond to Ckd4/6 inhibitors. Indeed, one of these agents palbociclib has presented a favorable outcome in patients with hormone-receptor-positive advanced breast cancer and deserves further

study in patients with iCCA [50]. Other copy number variations including copy number gain for chromosome 1 q, 7 p and loss for 6 q, 9 p, 3 p, 13 q, 14 q and 8 p were also identified in more than 20% of iCCAs [16].

Genetic heterogeneity in iCCA

Although agents targeting the above genetic alterations (summarized in **Table 3** and **Figure 1**) may bring out promising results, the genetic characteristics of iCCA have further been complicated by prominent genetic heterogeneity. In fact, the prevalence of the genetic alterations varied widely among studies, partly reflecting interpatient tumor heterogeneity, also known as population heterogeneity. This might be related to the specific context where a tumor grows as well as other host factors like germ line variants. Compared with iCCAs with normal liver, iCCAs with chronic advanced liver diseases (viral, alcoholic, autoimmune and cryptogenic pre-cirrhosis or cirrhosis) featured significantly lower overall mutation rates and lower mutation rates of *KRAS, MLH1, GNAS* but higher *EGFR* mutation rate [9]. And mutations in *PIK3CA, PTEN, CDKN2A* and *TP53* harbored exclusively in iCCAs with normal liver. Liver fluke infection by O. viverrini contributes to a major risk factor for iCCAs in endemic regions. Whole-exome sequencing analysis of mutation patterns in O. Viverrini-related and non-O. Viverrini-related iCCAs identified more frequent mutations of *BAP1, IDH1/2* in non-O-viverrini-related iCCAs, whereas *TP53, KRAS, SMAD4* were more frequently mutated in O. viverrini-related iCCAs [13]. Although approximately 70% of iCCA patients harbored at least one actionable alterations, no single gene was mutated in >25% of resected iCCA tissues, suggesting a need for broad-based mutational profiling in these patients for targeted therapy [5, 7, 19].

Despite marked population heterogeneity, intratumor subclonal heterogeneity makes the targeted therapy for iCCA even more complex [51-55]. Tumor progression is an evolutionary process, which leads to another two types of tumor heterogeneity -- spatial and temporal heterogeneity. Spatial heterogeneity exists among subclones separated across the different topograohic regions of the primary tumor (intratumoral heterogeneity) and the same metastasis (intrametastatic heteriogeneity) as well as among metastases (intermetastatic heterogeneity). A snapshot of a small subset of neoplastic cells at a given moment by single tumor biopsy results in an underestimation of the tumor genomic landscape and a possibly biased selection of targeted medicine [53]. Sequencing of multiple tumor sites at different time points and matching the therapy to the identified actionable targets will probably represent the best approach in iCCA as this malignancy is characterized by multifocal lesions even at diagnosis. Recently, Shi et al. applied multiregional whole-exome sequencing in a patient with synchronous two HCC and one iCCA as well as two postoperative recurrent tumors, which successfully delineated the clonality and heterogeneity [56]. Genotyping circulating tumor DNA (ctDNA) with the same genetic alterations to the tumor itself, also known as “liquid biopsy”, may represent an effective and less invasive strategy to track clonal evolution and monitor drug resistance during tumor progression [57].

Future prospects

The translation of NGS into oncology practice has promoted the development of precision medicine, which tailors anticancer therapy to each individual based on the genetic landscape. Compared with the traditional therapy focused on the primary site of origin of a tumor, precision medicine endeavors to identify actionable genetic alterations. There also comes a new and evolving form of clinical trial design, the so-called “basket trials” which assign patients to specific treatment arm based on the genetic findings of NGS regardless of histologic subtypes [58]. As to iCCA individuals, selection of an effective targeted therapy is much more challenging because of the presence of extremely marked genetic heterogeneity. Fortunately, NGS has offered an opportunity to identify candidate actionable mutations. But whether iCCA patients can benefit from tailored targeted therapy needs further clarification by clinical trials, especially those designed to provide treatment based on genetic findings. More recently, Adam et al. have proposed a new strategy for precision medicine, which additionally applies next-generation functional diagnostics, such as measuring tumor cell death from biopsies after *ex vivo* drug exposure [59]. As a result, functional testing by providing the response of patient cells without a prior knowledge of the mechanism of drug activity can complement NGS in identification of responders to the targeted therapy. However, its clinical

application needs further studies to assess the validity. Besides genetic heterogeneity, environmental risk factors, epithelial-to-stromal crosstalk, epigenetic alterations also contribute to the pathogenesis of iCCA and should be integrated to form a more individualized target approach.

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Declaration of conflict of interest

None.

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