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Genetic Profiling of Intrahepatic Cholangiocarcinoma

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Abstract

Purpose of review—Intrahepatic cholangiocarcinoma (ICC) is a treatment-refractory disease with a dismal outcome. Limited success in the clinical management and a persistent increase in the incidence world-wide have made ICC one of the most lethal and fastest growing malignancies. However, recent advancements in genome-wide technologies combined with the application of integrative multidimensional analytical approaches have begun to provide both detailed insight into the underlying biological traits of ICC and identified new therapeutic opportunities.

Recent findings—In comparison with other cancers genomic studies of ICC have been limited. We and others have recently procured large cohorts of ICC patients intended for genome-wide analyses. In our study samples from ICC patients were obtained from three cancer centers and subjected to integrated genetic and genomic analyses. We provided new insights into both pathogenesis and optimal treatment options demonstrating the presence of unique subclasses of patients, based partly on *KRAS* mutations and increased levels of receptor tyrosine kinase signaling. The group of patients with the worst prognosis was characterized by transcriptional enrichment of genes regulating inflammation and proteasome activities, suggesting a combination of tyrosine kinase inhibitors and anti-inflammatory drugs as a new therapeutic option for these patients.

Summary—We have critically examined the progress in genome-wide studies of ICC including genetic profiling, transcriptomics and epigenomics. Current limitations in applying these technologies to archival samples and the insufficient access to fresh-frozen material are partly the cause of the delayed implementation of the omics-based investigations of ICC compared to other hepatobiliary diseases. Thus, selected candidate *single* gene studies will also be discussed.

Keywords

Cholangiocarcinoma (CCA); intrahepatic cholangiocarcinoma (ICC); extrahepatic cholangiocarcinoma (ECC)

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Conflicts of interest

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INTRODUCTION

Cholangiocarcinoma (CCA) is a heterogeneous malignancy which arises in the epithelia of the intra- and extrahepatic biliary ducts. ICC is classified as a peripheral tumor of interlobular bile ducts whereas tumors designated hilar are generally considered extrahepatic and originate from the main hepatic ducts or at the bifurcation of the common hepatic duct. The main difference between peripheral and hilar tumors is in the clinical presentation and gross appearance which is likely a result of late diagnosis. Recent genomic data suggests that these tumor subtypes are less distinct than the underlying biological variances which classify patients according to overall survival and early tumor recurrence [1**]. Although recent molecular insight into ICC has improved, the understanding of genetic mechanisms involved in the development of ICC is still insufficient.

GENETIC ALTERATIONS IN INTRAHEPATIC CHOLANGIOCARCINOMA

Genetic analyses of ICC are still few and often limited to select genes. A number of studies analyzing combined hepatocellular-cholangiocarcinoma have attempted to assess the provocative hypothesis that ICC is, similar to hepatocellular carcinoma (HCC), derived from a common precursor cell [2–5]. Comparison of chromosomal imbalance, i.e. copy number gains and losses, suggests that ICC and HCC are closely related and that they share major genomic aberrations and abnormalities in molecular pathways involved in neoplastic transformation.

1. Chromosomal instability – Copy number variations

Few comparative genomics hybridization (CGH) studies on ICC were performed during the past decade [6–10*]. Unfortunately, in several studies where the authors have investigated copy number variations (CNVs) in biliary tract cancers, the analyses were merged between subtypes such as intrahepatic and extrahepatic tumors [11] or even including gallbladders [12*–14], making an accurate interpretation difficult. We have reviewed 5 studies where data for 98 typical ICCs were investigated separately [6–10*]. In our meta-analysis of ICC, CGH revealed frequent copy number losses on chromosomal arm 1p, 4q, 8p, 9p, 17p and 18q while copy number gains were found on chromosome 1q, 5p, 7p, 8q, 17q and 20q in at least 3 studies with greater than 20% overall change (Table 1). Although we did identify common CNVs, the complexity of the karyotype revealed by the degree of genomic imbalance differed substantially among the 5 studies. Whereas the rigid parameters of our meta-analysis clearly do limit the data, the common CNVs identified for ICC may be of significance. It is also noteworthy to indicate that recent advancements in genomics, the limited number of cases analyzed in each study and the ethnic diversity (3 studies were performed in Korea [7–9*], one in Hong Kong [10*] and one in Germany [6*]) do warrant a more detailed analysis. In contrast, a meta-analysis of 31 CGH studies performed in 2005 examined a total of 785 cases of HCC [15]. Interestingly, 7 of 9 loci identified with significant CNV in HCC showed similar gains on 1q, 8q and 17q and losses on 4q, 8p, 13q and 17p common with ICC. These results suggest that HCC and ICC are genomically closely related.

2. Prevalent mutations identified in single gene studies

The first high-coverage genome sequencing project of ICC, e.g. whole genome or exome analyses, has not been performed. Most of the studies investigating genetic variation in ICC have focused on a specific gene, or at most a few genes. A project of this magnitude promises to identify and achieve a detailed map of the common and rare genetic variants to attain an understanding of the underlying genetic traits which result in the development of CCA. Insight into the role of genetic variants may improve the selection of therapy and, thus, avoid drug-resistance. A few common mutations in genes such as *KRAS, BRAF, EGFR, PIK3CA and TP53* were investigated in CCA; however, clinical outcome of the disease has not improved.

Among the pathways involved in the pathogenesis of ICC, the ErbB-family of receptors are perhaps affected the most [16]. The most notably are the aberrant regulation of ErbB2 and the epidermal growth factor receptor (EGFR) signaling. Whereas no mutations have been reported in *ERBB2*, increased expression and DNA amplification was reported ranging from 0 to 73% in tumors [16–18]. EGFR-targeted therapies may show promise in the treatment of a subclass of ICCs. Mutations in *EGFR* are rare events and we did not detect any in our cohort, however, in the subclass of patients with poor survival we observe a significant overexpression of EGFR and its downstream pathways [1**]. Activating mutations in *EGFR* were described in two small studies in 3/22 (13.6%) and 3/15 (20%) of cases [19–20]. Activation of EGFR triggers the activation of downstream RAS/RAF/MEK/ERK and PI3K/PTEN/AKT, two major cell survival pathways. Hotspot activating missense mutations in genes downstream to EGFR, such as, *BRAF* (0–22%) [21–22] and *PIK3CA* (9–32%) [23–24] are rarely described in ICC.

Gain of function mutations in KRAS downstream of EGFR represents one of the most frequent mutations found in ICC (8–54%) [25–28] as compared to 25% in our cohort of peripheral and hilar-type tumors [1**]. When classified by tumor site, 17% of peripheral-type CCAs were positive for mutations in *KRAS* with the most frequent alteration in codon12. Importantly, the incidence of mutations was higher in the hilar-type tumors (53%) [1**, 29]. It is noteworthy that *KRAS* mutations were detected at a higher incidence with increasing tumor stage (stage I, 8%; stage II, 15%; stage III, 31%; stage IV, 46%) [24], however, confirmation in an independent cohort is needed. Mutations in *KRAS* were detected in 30% of bile from patients with primary sclerosing cholangitis, suggesting that it is an early event contributing to the malignant transformation of cholangiocytes [30]. Although *KRAS* is established as a causative somatic mutation, we were unable to determine *KRAS* as an independent prognostic factor within our cohort [1**]. Integrating the *KRAS* mutational status with our prognostic gene classifier grouped all patients with mutated *KRAS* amid patients with poor prognosis.

Another pathway involved in ICC carcinogenesis which is frequently inactivated and associated with loss of heterozygosity is the central cell cycle regulator p53. More than 90 different mutations have been described in *TP53* of which most are associated with exposure to chemical carcinogens [31], such as thorotrast [32]. A review of 10 studies which included 229 cases found a total of 21% (49 patients) with mutations in *TP53*, ranging from 23% in

Asia, 14% in Europe and 26% in the USA [33]. However, there is a great deal of variability in the reported cases for most genetic alterations. *TP53* is no exception with 14–77% of cases positive reported in single studies [31, 33]. Other genes such as *CTNNB1* (8%), APC (13%), *AXIN1* (41%) and *CDH1* (11%) have occasionally been reported [34–35].

ALTERATIONS IN THE EPIGENOME IN THE PROCESS OF CHOLANGIOCARCINOGENESIS

Recent technological advances have brought epigenetics into the omics-age by the introduction of array-based and deep sequencing methods, highlighting the importance of the epigenome, including DNA CpG methylation, histone modifications and non-coding RNA species, in the process of human carcinogenesis. Only limited comprehensive epigenomic studies have been published on ICC and data on aberrant CpG promoter methylation in the regulation of ICC has largely been centered on individual genes (reviewed in [36*–39*]). This review provides an overview of select key genes frequently hypermethylated and microRNAs identified by miR-profiles in ICC.

1. Promoter hypermethylation in cholangiocarcinoma

Aberrant epigenetic regulation such as promoter hypermethylation was demonstrated in numerous important cancer-associated genes in ICC (a comprehensive list can be found in [36*–37*]). Studies profiling these modifications to established prognostic and predictive gene signatures attempting to predict the therapeutic potential of agents that target the cancer epigenome [40*] have not yet been investigated in ICC. Recently, a study in a large ICC cohort (n=102) associated with liver fluke infection was published demonstrating promoter hypermethylation in a handful of target genes compared to adjacent tissue [41]. Unfortunately, the authors only investigate 26 selected loci using traditional methodology rather than applying readily available genome-wide array-based technology.

Epigenetic silencing by promoter hypermethylation is common in tumor suppressor genes: *p16^{INK4a}/CDKN2* (17–83%) [42–45]; *SOCS3* (62%) [46]; *RASSF1A* (31–69%) [10, 42, 45]; *p15* (54%) [45]; *CDH1* (17–49%) [42–43, 45]; *hMLH1* (19–45%) [45]; *APC* (27–47%) [42–43, 45]; *p14ARF* (19–30%) [42, 44–45] and *GSTP1* (15–31%) [42, 45] are some of the most frequent events reported in ICC.

ICC often arises in the background of chronic inflammation such as primary sclerosing cholangitis (PSC) which provides survival signals to the tumor (the contribution of the inflammatory microenvironment and epigenetics is reviewed in [47]). The cyclin-dependent kinase inhibitor p16 is frequently silenced by epigenetic modifications and was proposed as a prognostic marker associated with dismal outcome in patients with PSC [48]. Also, inflammatory signals (such as AKT) can trigger cytokine pathways (i.e. JAK/STAT) which in turn were shown to be involved in cholangiocarcinogenesis [49–50]. Indeed, a common target in ICC is the suppressor of cytokine signaling 3 (SOCS3) which is silenced by promoter hypermethylation [46]. Inhibition of SOCS3 results in a self-enhancing constitutive activation of the oncogenic pathway IL6/STAT3 with a subsequent autocrine feedback and paracrine signaling to the tumor environment [46, 49–51].

The frequency by which these targets are methylated in ICC is variable and the ability to utilize any or a group of silenced genes as predictors for outcome remains to be critically investigated. Since inconsistencies can be a result of limited study size, heterogeneous cohorts (i.e. mixed ICC and ECC cases), etiology and methodology, future studies applying epigenome-wide technology to a standardized ICC cohort will be needed to enhance our understanding of both the underlying biology and importance of epigenetic changes in ICC pathogenesis.

2. Profiling of non-coding RNAs

Non-coding RNAs include both large and small RNA species. A major group and the best characterized non-coding RNAs in ICC are microRNAs (miRs) which are involved in the epigenetic regulation of gene expression, transcriptional stability and translation. A number of studies have concentrated on investigating the biological significance and aberrant expression of miRs in ICC cell lines [52*–57], demonstrating a link between tumor growth, response to therapy and expression of inflammatory cytokines (reviewed in [38*]). Recently, Kawahigashi, Y. and colleagues [58] performed a comprehensive study by sequencing two ICC cell lines (HuCCT1 and MEC) and a normal intrahepatic bile duct epithelial cell line (HIBEpiC), providing the first profile of differently expressed miRs in ICC derived cells. A unique 27 miRs ICC signature was identified that included down-regulation of 8 miRs (i.e. miR-22, miR-125a, miR-127, miR-199a, miR-199a*, miR-214, miR-376a and miR-424) specific for normal bile duct epithelium. The authors suggested that these miRs may serve as biomarkers for ICC, however the functional and clinicopathological significance of this miR signature remains to be evaluated in clinical samples. In two recent studies miRs were profiled in ICC [53**, 59**]. Selaru and colleagues [59**] showed that expression level of miR-21 could distinguish ICC from normal bile ducts. Furthermore, increasing expression of miR-21 correlated with a decreased expression of programmed cell death 4 (PDCD4) and tissue inhibitor of metalloproteinase 3 (TIMP3), suggesting that miR-21 may act as an oncomir in ICC. However, 9 of the 23 samples in this study were of distal extrahepatic origin, making the interpretation of the data difficult. Another study profiling 27 ICCs reported 38 miRs differentially expressed in the tumors compared to 10 normal cholangiocytes [53**]. Interestingly, hierarchical clustering grouped the clinical samples into two clusters, distinguishing the patients according to the level of CA19-9, a serum secreted mucin-type glycoprotein which is commonly used as a biomarker for CCA and was shown to be associated with poor prognosis after surgical resection of ICC [60]. In addition, the status of vascular invasion was found to be significantly correlated with the two clusters. However, the potential prognostic importance of the 38 miRs signature remains to be evaluated in an independent cohort.

TRANSCRIPTOMIC PROFILING OF ICC

Tumor infiltrating stroma is often a prominent characteristic of ICC, indicating that to capture the gene expression profile of the tumor epithelial, laser microdissection may be needed. The first comprehensive transcriptomic study applied this approach to 25 ICCs and identified a total of 473 differentially expressed genes between the tumor and non-malignant biliary epithelial cells [61]. Even though the authors established a *clean* gene signature in the

tumor epithelial compartment the stromal compartment was not investigated. Few studies have since been published analyzing the gene expression profiles in ICCs [62–63], associated with liver fluke infection [64–65] or anatomical site of the tumor [12]. We recently published a genetic and genomic study in a large cohort of 104 peripheral- and hilar-type tumors and 59 matched surrounding liver samples from Australia, Europe and USA, accompanied by a detailed analysis of the epithelial and stromal compartments from 23 laser-capture microdissected tumors [1**]. Our study provided new insights into the ICC pathogenesis and identified two patient categories by a 238-gene prognostic classifier which stratified the patients according to overall survival and early recurrence. We minimized the classifier to 36 genes which in combination with other molecular predictors (i.e. mutations, co-activation of multiple oncogenic pathways), improved the molecular classification and outcome prediction in the cohort. A detailed class comparison identified a total of four patient subclasses based on dependence of receptor tyrosine kinases (EGFR, ERBB2 and MET), activation of the mTOR pathway, increased proliferation and overrepresentation of gene involved in proteasomal activity and inflammation. These data suggested a novel therapeutic potential for dual-target tyrosine kinase inhibitors (e.g. lapatinib), either alone or in combination with proteasome inhibitors to improve therapeutic response of this currently treatment-refractory malignancy.

CONCLUSION

ICC is a rare malignancy, diagnosed at a frequency less than 1% of primary hepatic tumors. Genome-wide technologies have provided a detailed characterization of genetic variation, genomics, and epigenomics in ICC. This unprecedented capacity to study the underlying traits of a human disease such as ICC at the molecular level and to integrate these data at a multidimensional scale suggests that future advancements in clinical management may come from systems-based translational genomics. The realization of this goal, at least for ICC, may require extensive collaborative team efforts across institutions.

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KEY POINTS

- CCA is a rare disease with poor outcome, and increasing incidence worldwide. A limited number of genomic studies on CCA have been published during the past decade. Inadequate sample size and heterogeneous anatomical locations of CCA (intrahepatic, extrahepatic (hilar) and distal extrahepatic) frequently complicate interpretation of the data.
- Receptor tyrosine kinases have been shown to be potential drug-able targets in CCA. Detailed translational approaches are needed to study the efficacy of tyrosine kinase inhibitors in CCA either alone or in combination with other chemotherapeutics. This may warrant small proof of concept clinical trials.
- Application of translational system biology approaches, and utilizing multidimensional integrative techniques including whole genome sequencing promises to yield both deeper insights into the mechanism of CCA pathogenesis as well as providing novel therapeutic options for CCA patients.

Table 1

Percentage of involved ICC cases with CNV gain or loss

Chromosome arm	ICC	Studies (no)	Koo *et al.* [7] (Korea)	Wong *et al.* [10] (Hong Kong)	Lee *et al.* [8] (Korea)	Uhm *et al.* [9] (Korea)	Homayounfar *et al.* [6] (Germany)	Moinzadeh *et al.* [15] HCC (31 studies)
Cases (no.)	**98**	**5**	**11**	**13**	**33**	**19**	**22**	**785**
1p-	**28**	**3**			21	37	27	
3p-	53	2		38			68	
3q-	23	1					23	
4p-	36	1					36	
4q-	**35**	**4**		46	21	32	41	**34**
5q-	26	1				26		
6q-	69	2		46			91	
8p-	**33**	**3**		31		26	41	**38**
9p-	**40**	**3**		38		26	55	
9q-	36	1					36	
11p-	23	1					23	
11q-	23	1					23	
12q-	23	1					23	
13q-	45	1					45	26
14q-	43	2		31			55	
16q-	0	0						36
17p-	**40**	**3**	55	38		26		**32**
18q-	**31**	**3**		38		32	23	
19p-	32	1				32		
21q-	23	1					23	
1p+	23	1					23	
1q+	**40**	**3**		54	24		41	**57**
2q+	26	1				26		
3q+	37	2		46	27			
5p+	**28**	**3**	27		24	32		

Chromosome arm	ICC	Studies (no)	Koo *et al.* [7] (Korea)	Wong *et al.* [10] (Hong Kong)	Lee *et al.* [8] (Korea)	Uhm *et al.* [9] (Korea)	Homayounfar *et al.* [6] (Germany)	Moinzadeh *et al.* [15] HCC (31 studies)
Cases (no.)	**98**	**5**	**11**	**13**	**33**	**19**	**22**	**785**
5q+	27	2		31			23	
6p+	0	0						22
7p+	**28**	**3**	27			26	32	
7q+	36	1					36	
8p+	39	1			39			
8q+	**45**	**4**	36	54		58	32	**47**
11q+	37	2		31	42			
12p+	27	1					27	
12q+	23	1					23	
13q+	45	1	45					
15q+	37	2		38	36			
16p+	36	1			36			
17q+	**42**	**3**		38	61	26		**22**
18p+	39	1			39			
18q+	27	2	27			26		
19q+	26	1				26		
20q+	**45**	**3**	36	31	67			