Molecular Perturbations in Cholangiocarcinoma: Is it Time for Precision Medicine?

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PREFACE

Cholangiocarcinoma (CCA) is the most lethal type of primary liver tumors (PLCs), responsible for 20% of liver-related deaths. Persistent increases in incidence and mortality rates have been observed over the past decades. The median survival of CCA is only 6 to 8 months, and the 5-year survival rate has remained at 10% since the 1980's. Importantly, the incidence of CCA is estimated to continue to increase the next 10 years, which could be speculated as a cause of an aberrant rearrangement of the hepatic metabolism owing changes in our lifestyles. The rise in mortality reflects the limited treatment options. Failure of current chemotherapy to extend median survival beyond 1 year highlights the extensive innate and rapidly acquired chemoresistance of these tumors, though the underlying molecular perturbations remain opaque.

Genomic heterogeneity is a hallmark of CCA. As a consequence, drug resistance is a major concern in the clinical management of these patients with more than 50% risk of recurrence after surgery. At the time of diagnosis 70% to 90% of patients present locally advanced and metastatic disease thus; curative surgical resection is not an option. Notably, a significant proportion of the mutational landscape in CCA comprises recurrent mutations in epigenetic regulators, implying extensive epi- and genome-wide consequences arising from mutant isoform activities. In this chapter, we will focus on understanding the mechanisms that contribute to the risk of CCA and leverage molecular data to elucidate markers, predictive factors of risk that may impact our current clinical management of cholangiocarcinoma. Importantly, we will emphasize advanced CCA and discuss if the molecular make of these tumors are different from resected cholangiocarcinoma. The current efforts in utilizing genome-based characterization and patient stratification to direct clinical decisions predominantly implicate patients with resectable disease.

Genetic alteration and putative risk factors in cholangiocarcinoma

Data on the inherited risk factors modulating genetic susceptibility to CCA is scarce. This might be due to the rarity and complexity of the disease, which renders collection of large, well-powered cohorts of patients troublesome. Nevertheless, a whole-genome sequencing (WGS) study of the Icelandic population recently identified a potential CCA-risk gene[1]. In brief, this consortium sequenced the genomes of 2,636 Icelanders identifying 1.5 million insertions and deletions (InDels) and 20 million single nucleotide polymorphisms (SNPs)[1]. The analysis demonstrated that several mutations in the *ABCB4* gene, which encodes for the hepatobiliary phosphatidylcholine translocase, increase the odds ratio of developing liver disease. Interestingly, the study showed the association between *ABCB4* mutations and an increased risk of bile duct cancers[1]. Of note, so far patients with rare *ABCB4* mutations are known to develop progressive familial intrahepatic cholestasis type 3

(PFIC3) in childhood or a milder phenotype (low phospholipid associated-cholelithiasis (LPAC) syndrome[2,3]) in adulthood. LPAC syndrome is defined by early-onset cholelithiasis (< 40 years of age), concurrent gallbladder, bile duct and/or intrahepatic cholesterol gallstones, and recurrence of biliary symptoms after cholecystectomy[3,4]. The Icelandic GWAS found that the common *ABCB4* variants might be determinants of cholestasis of pregnancy, liver cirrhosis, and hepatobiliary cancer[5]. These observations are in concordance with another GWAS of 1,042 Indian patients with gallbladder cancer (GBC), including 1,709 controls, which identified significant associations between SNPs in *ABCB4* and the risk of GBC. Although, the *Abcb4*-knockout mouse, which is deficient for the orthologous murine gene develops hepatocellular cancer (HCC), and not CCA, the above GWAS findings highlight the effects of genetic variants in hepatobiliary transporters on the development of CCA. Indeed, ABCB4 regulates the biliary concentration of phosphatidylcholine, whereas the levels of other bile compounds (namely sterols and bile salts) are determined by the secretion (efflux) rates of the corresponding ATP-dependent canalicular (basolateral) transporters of hepatocytes[6-8]. For example, the ATP-binding cassette, subfamily B member 11 (ABCB11) represents the bile salt export pump (BSEP). Mutations in *ABCB11* gene cause progressive familial intrahepatic cholestasis type 2 (PFIC2) with decreased bile acid secretion, accumulation of bile salts in the liver parenchyma, and liver injury. PFIC2 manifests typically within the first six months of life with pruritus and jaundice as well as progressive fibrosis, which in most cases results in liver cirrhosis within the first two years of life. Interestingly, in adulthood, the risk for developing hepatobiliary cancers is also increased. Indeed, 15% of patients with BSEP-deficiency might develop HCC or CCA[9,10]. However, a clear pathogenesis of this association has not been fully elucidated.

Not only genetic variants in the hepatobiliary transporters have been implicated in the increased risk of developing CCA. The pathogenesis of CCA remains unclear but it has for long been appreciated that patients with primary sclerosing cholangitis (PSC) are at increased risk of developing CCA. PSC is a rare (prevalence 1:10.000) inflammatory disease of the bile ducts. Hence, it is plausible that genetic variants that are associated with an increased risk of developing PSC might modulate the risk of CCA itself. Also, this risk may be in the absence of chronic inflammation of bile ducts. As such, Krawczyk *et al.*[11] analysed a specific SNP (rs3197999) in the *MST1* gene in a cohort of European patients with CCA. This variant was previously associated with an increased risk of PSC in a GWAS study[12]. The variant allele (rs3197999) is a missense mutation that results in p.R689C amino acid substitution within the β-chain of MSP (MSPβ). The MSP/RON signalling axis is involved in several aspects of cancer-relevant and cellular processes, such as chemotaxis, innate immunity and macrophage activation. Given this data, the rs3197999 variant was analysed in a cohort of 223 CCA patients (including three with PSC-CCA) and in 355 cancer- and PSC-free controls[11]. Interestingly, the

cancer group departed from Hardy-Weinberg equilibrium (p=0.022) and exhibited a trend for rs3197999 [A] overrepresentation (31% vs. 26%: p=0.10). Homozygous rs3197999 [AA] carriers showed significantly increased overall (OR=1.97; p=0.023) and PSC-unrelated biliary tract cancer risk (OR=1.84; p=0.044), as compared to the homozygous carriers of the common *MST*-1 allele. The association was the most pronounced in patients with extrahepatic tumors and proved to be significant in multivariate models (p<0.05), validating the [AA] genotype as an independent CCA risk factor, indicating a possible modulation of inflammatory responses and/or altered MSP/RON signalling[11]. Chaiteerakij *et al.*[13] have investigated the consequences of other selected inflammation-modulating SNPs on the risk of developing CCA and patient's survival. In this case-control analysis[13], a total of 370 patients with CCA and 740 matched healthy controls were included. The authors selected eighteen variants in nine genes. Although two of the selected variants (rs2143417 and rs689466) in cyclooxygenase 2 (COX2) were associated with the risk of CCA in the discovery cohort (P=0.0003 and P=0.005, respectively), these associations failed to reach significance in the validation cohort (P>0.05), making the results difficult to interpret. In turn, Fingas *et al.*[14] analysed, the association between the G protein subunit-β 3 (*GNB3*) 825C>T, B-cell-lymphoma-2 (*Bcl2*) 938C>A, and myeloid cell leukemia-1 (*Mcl1*) 386C>G, genetic variants and their associated clinical outcomes in the setting of CCA. This analysis[14] was based on a cohort of 40 adult Caucasian patients with extrahepatic CCA (eCCA) and 40 age- and sex-matched healthy controls. Their analysis showed that carriers of *GNB3* 825C>T SNP have a longer overall survival as compared to the carriers with the T allele[14]. Other variants, enhancer of zeste homolog 2 (EZH2) (rs24179546[15]), nuclear factor (erythroid derived 2)-like 2 (NRF2)[16] or alpha1-antitrypsin (α1AT) deficiency Z heterozygosity[17], have been linked to increased risk of CCA. However, most of these studies were performed in single cohorts and are still awaiting validation in large groups of patients with CCA. Indeed, based on the relative rarity of CCA most of the collected cohorts lack the power to significantly detect the risk variant and later to replicate the genetic findings. Secondly, larger cohorts with available germline DNA are also required to analyze the genetic background in different CCA subtypes. Overcoming these limitations is one of the aims of the European Network for the Study of Cholangiocarcinoma (ENS-CCA), which is participating in the international CCA GWAS study currently underway. Although, we currently lack common genetic variants that substantially show an increased risk of developing CCA, genetic analyses might already be incorporated in the clinical management of patients with PSC, who are at-risk of developing CCA. Of note, it is recommended that patients with PSC should undergo regular surveillance and assessment of the serum marker Carbohydrate antigen 19-9 (CA19-9) to facilitate the early detection of the CCA. Interestingly, it has been shown that genetic variants of fucosyltransferases 2 and 3 (FUT2/3) might modulate the serum levels of CA19-9.

Based on this information, Wannhof *et al.*[18] incorporated the FUT2/3 variants in the analysis of 433 individuals with PSC, including 41 patients who had progressed to PSC-CCA[18]. Based on the genetic variants of FUT2/3, the authors calculated an optimal cut-off of CA19-9 associated with the risk of developing CCA. Overall, the inclusion of the FUT2/3 SNP-adjusted cut-off significantly improved the sensitivity of CA19-9 in detecting PSC-CCA cases, and have resulted in a 42.9% reduced risk of false positive[18].

Genomic aberrations and patient classification: Impact on clinical management

To date, genomic characteristics[19,20] and stratification[21,22] of CCA patients have been analysed in several studies based on high-throughput genomics. For example, Jiao *et al.* performed WES of patients with intrahepatic CCA (iCCA, n=32) and detected inactivating mutations in chromatin remodelling genes (for example *ARID1A, PBRM1 and BAP1*)[23]. In turn, Nakamura *et al.*[20] reported the presence of mutations in the oncogenes *KRAS, PIK3CA, NRAS, GNAS,* and *ERBB2*. In the latter study, a comprehensive exome and transcriptome analysis was performed on individuals with iCCA (n=145), eCCA (perihilar/pCCA and distal (dCCA) cases) (n=86) as well as GBC (n=29). Interestingly, around 40% of cases with biliary cancer proved to have alterations in putative driver genes. For example, the PKA gene fusions were specifically found in the eCCA, whereas FGFR2 gene fusions were detected in the intrahepatic cases. Likewise, *ERBB3* and *EGFR* mutations were detectable only in the setting of GBC[20]. Interestingly, alterations in the *TERT* promoter was not found in patients with eCCA, whereas it was common in patients with GBC as well as detected in one patient with iCCA. These results allude to a different genetic composition of the biliary cancers depending on their anatomical localization and thus, emphasize the need of including genomic analyses of the tumor samples when making clinical decisions.

Integrative analysis of 149 samples of iCCA[22] allowed identification of 2 unique subclasses: the `inflammation class´ and the `proliferation class´ with markedly different activation of signaling pathways. For example, in the `proliferation class´, the activation of RAS and MET oncogenic pathways, mutations in *KRAS* and *BRAF* as well as expression of genes that were previously associated with worse outcome in patients with HCC[22] may render the use of drugs approved for the therapy of HCC as possible therapeutic options in patients with iCCA[24]. Targeted sequencing on 153 biliary cancers (70 iCCA, 57 eCCAs and 26 GBCs) demonstrated putative driver-gene mutations in most cases (118/153), however, the genetic profiles differed significantly based on the localization of the tumor type[25]. Overall, *KRAS, TP53, ARID1A, IDH1/2, PBRM1, BAP1,* and *PIK3CA* genes were the most frequently altered whereas mutations in *TP53* proved to be independent determinants of survival[25]. Based on the tumor localization different genetic profiles were detected with *RAS*

mutations being the most common in dCCA. The above-mentioned genomic diversity in CCA might be one of the major reasons for the lack of effective therapies. Indeed, based on the localization of the tumor, different pathways seem to be involved and clinically they need to be tackled different. As such, Nepal *et al.*[26] recently investigated genome-wide data obtained from 496 iCCA patients. From these analyses, the team elucidated unique mutational signatures, co-mutational spectra, deregulated signaling pathways, structural alterations and DNA methylation aberrations associated with each patient subgroup. To test the clinical implications of the different onco-genetic programs, they utilized a drug repositioning approach and screened a library of 525 drugs in patient-matched cell models. These findings uncovered the potential of individual mutations to induce substantial downstream molecular heterogeneity which in turn could facilitate prediction of therapeutic sensitivities for CCA patients using standard targeted genotyping. Indeed, the potential involvement of inherited CCA predisposition which might be modulated by exogenous risk factors render the whole picture even more complex. For these reasons, large and integrated studies will be necessary in the future to bring us closer to personalized diagnostics and therapy in patients with different subtypes of CCA.

The molecular make up of advanced cholangiocarcinoma: Is it the same as resected tumors?

Seventy to eighty percent of CCA patients present at an advanced stage and are not amenable to surgical intervention[27,28]. A great effort is directed toward the development of novel therapeutics for these patients. The hope of a personalized approach lies in the ability to use therapeutics specifically designed to act against a molecular target that drives tumor growth. However, the main challenge in advanced CCA personalized treatment is developing a targeted therapy against the molecular drivers of the disease, whilst the knowledge of CCA molecular landscape is limited to small resected tumors. Would the molecular targets identified in the early stage be expressed in the advanced disease and, above all, would they still represent the main lethal drivers of tumor progression? The lack of systemic large genomic studies performed in advanced CCA limits the knowledge to provide appropriate answers to these questions. Recent findings suggest that there may be minimal driver gene mutations heterogeneity in untreated advanced cancer[29]. However, it is recognized and experimentally verified that tumors evolve under the pressure of systemic therapy[30,31], thus making the knowledge of the molecular landscape in advanced CCA even more compelling with the introduction of adjuvant chemotherapies[32].

The shortage of molecular data on advanced CCA is caused by the paucity of tissue available. CCA are often diagnosed with cytology or small biopsies which do not enable a comprehensive and full molecular and genomic characterization. Feasibility studies on targeted captured sequencing in

gastrointestinal cancers within routine clinical practice have shown that sequencing may be successful only in a minority of CCA patients (26% in advanced CCA versus >50% advanced colorectal cancers)[33]. In addition, success has often been limited to iCCA narrowing the appreciation of genomic differences between different subtypes in the advanced setting. To date, two reports are available on the genetic characterization of advanced CCA by targeted sequencing, while no data are available on whole genome analyses as well as on transcriptomic landmarks. Ahn *et al.* pursued the first targeted Next Generation Sequencing (NGS) study in formalin-fixed-paraffin-embedded tissues from chemotherapy-naïve advanced biliary cancer patients, including 142 iCCA and 31 eCCA[34]. GBC and ampullary cancers were included but represented less than 5% of the whole series. The study covered the entire coding sequence of 236 cancer-related genes with an averaged depth greater than 250x. Unfortunately, 25% of cases represented stage I-II disease, as the analysis was performed on archival tissue available from resections. The genes most frequently altered were *CDKN2A* (29%), *TP53* (28%) and *KRAS* (22%), followed by *ARID1A* and *IDH1* (13%), *FGFR2* (12%), *PI3KCA* (10%), *SMAD4* (190%), *PBRM1* (10%). The genes involved were the same identified in studies performed in resected CCA. However, each tumor had a median of 3 actionable mutations, with a trend toward an increased number of mutations in advanced tumors compared to early stages. In the cohort of 86 patients with advanced disease who underwent palliative first line chemotherapy no individual gene mutations were predictive factors of response to chemotherapy. However, loss of function mutations in *CDKN2A* and *TP53* were significantly associated to worse overall survival. Whether this is related to the prevalence of these mutations in this cohort remains to be addressed. *ARID1A* mutations, in presence of mutations in *TP53* or *CDKN2*A, were associated to a more chemosensitive phenotype to platinum regimen likely through its role in DNA damage, but further studies are warranted to validate these findings. More recently data on the advanced biliary cancer cohort of the MOSCATO 01 trial have been released. MOSCATO 01 was a prospective clinical trial which evaluated the benefit of incorporation of genomic analyses in the selection of systemic therapy for advanced cancer patients[35]. Among 1035 patients enrolled in MOSCATO 01, 4% had advanced biliary cancers (N=43) with 67% being iCCA. In this case molecular analysis was completed for 79% of patients[36]. The high rate of success is likely to be related to the clinical trial frame with on-purpose research biopsies, the collection of fresh frozen tissue and the prevalence of iCCA in the series. If the genes mutated in advanced CCA reflected those identified in the early stage disease (*TP53* 26%; *RAS* 24%; *IDH* 18%; *FGFR 1/2* 16%; *EGFR* and *ERBB* 16%, *CDKN2* 16%, *PTEN* 14%, *PI3KCA* 10%, and *MDM2* 10%), it is interesting to note that multiple molecular alterations were detected in 87% of the samples with a median of 3 molecular alterations per patients. These data are interesting when they are compared to the molecular landscape of resected tumors in which the co-occurrence of two or

more actionable lesions is present only in 30% of cases[37], and probably provide the bases for understanding the failure of targeted therapies in advanced CCA. It is noteworthy to observe that targeted therapies have given limited benefits also in cases of highly selected sub-populations, as in the case of FGFR2 inhibitors in FGFR2-fused iCCA: the response rate of only 18% suggests that progression of these advanced CCA is driven by multiple forces most of which are still unknown[38]. A better knowledge of the interplay between multiple pathways in promoting tumor progression and drug resistance in the advance setting is essential for the development of *ad-hoc* treatment combinations and adaptive therapies that enable a long-term control of the disease. In line with this hypothesis multiregional sequencing studies have recently shown that parallel evolution and chromosomal alteration can shape spatial heterogeneity and promote branch diversity in iCCA[39]. The availability of tissue from the primary and the recurred tumor in one case allowed Dong *et al* to assess the temporal evolution of iCCA[39]. Multiple mutational clusters were present at a sub-clonal occurrence in more than one area in the recurrent tissue, indicating a polyclonal metastatic seeding pattern in CCA (**Figure 1**). We can then speculate that two or more primary clones can be responsible for metastatic progression, either because a synergistic cooperation between the clusters may prove beneficial in the evolutionary dynamics, or because an early colonization may remodel the microenvironment to facilitate colonization of further clones. Interestingly the number of clonal mutations was the same between the primary and the recurred tumors, but the number of sub-clonal mutations was lower in the recurrence suggesting that only the fittest clones can develop and give rise to advanced disease making the molecular profile of advanced tumors different from their matched primary. In addition, new oncogenic events can occur in the metastatic tumors that contribute to a different profile. Interestingly, Dong *et al* observed new mutations in the recurrence which were not present in the primary tumor and were known to be associated to chemo-resistance[39].

Taken altogether these data underline the importance of understanding the molecular landscape of advanced CCA in order to be able to develop novel effective therapeutic strategies. Two different strategies could be implemented to overcome the issues related to lack of tissues from advanced CCA: 1) establishment of primary cell lines, and 2) liquid biopsies. Generation of primary cell lines from advanced biliary cancers was shown to be feasible through generation of 2D cell lines or 3D organoid models[39-41]; they will have the advantage of enabling expansion of tumor cells and achievement of cell purity for a comprehensive characterization of the molecular make-up of tumor cells, even though their representation of the intra-patient heterogeneity will be limited by sampling bias. Liquid biopsy may represent a promising technology to identify the clones that drive the progression of the tumor and the process of metastasis; however, given the limited number of

circulating tumor cells in CCA, the studies are likely to be limited to the analysis of the mutational profile through the assessment of circulating free DNA.

Epigenetic deregulation of cholangiocarcinoma. Clinical implications

In recent years, it has become apparent that genetic alterations may not fully explain the rapid progression and high chemoresistance of CCA[42,43]. Epigenetic perturbations may play an important role in these processes and have, therefore, received increasing attention. In addition, epigenetic alterations have been proposed to function as oncogenic drivers and constitutional epimutations have been proposed to be the missing link of cancer heritability[44,45]. Supporting this hypothesis, a multitude of epigenetic alterations, including DNA methylation, histone post-translational modifications, chromatin remodeling and non-coding RNA have been identified in CCA. Interestingly, these different epigenetic pathways are interconnected resulting in alterations of multiple epigenetic factors during cholangiocarcinogenesis[46-49]. Here, we give an overview of recent findings and assess the potential clinical implications of targeting epigenetic alterations.

Aberrant methylation status in cholangiocarcinoma

DNA methylation is a major epigenetic mark with important roles in gene regulation during normal development and cancer[50]. Thereby, genomic DNA is mainly methylated at CpG dinucleotides by DNA methyltransferases (DNMTs) and de-methylation is carried out by Ten-eleven translocation methylcytosine dioxygenases (TETs). Interestingly, frequent genetic alterations of epigenetic key players have been observed in CCA implicating specific epigenetic processes in cholangiocarcinogenesis[51]. Deletion of or mutation in genes encoding the chromatin remodeling enzymes BAP1, ARID1A (AT-rich interactive domain-containing protein1A) and PBRM1, or IDH (isocitrate dehydrogenase) gain-of-function mutations are the most common alterations perturbing the epigenetic landscape of iCCA[19,52,53]. The tumor suppressor BAP1 is a deubiquitinase which participates in chromatin remodeling, whereas, PBRM1 and ARID1A are both subunits of the chromatin remodeling complexes SWI/SNF[19]. However, the inactivation of these chromatin remodelers by mutation makes it difficult or may make it even impossible to reactivate them. Therefore, it will be crucial to better understand the downstream signaling events induced by inactivation of BAP1, PBRM1 and ARID1A hopefully leading to specific treatment regimens for CCA patients with inactivation of these chromatin remodelers.

An integrative genomic analysis of CCA identified distinct IDH-mutant molecular profiles which define a distinct CCA subtype[43]. IDH mutations alone have been shown to be sufficient to induce a hypermethylator phenotype[54] and they tend to appear more frequently in recurrent iCCA with gene

expression traits of epithelial-mesenchymal transition[55]. However, an integrative analysis of genetic and epigenetic profiles revealed that a subgroup of CCA patients with high rate of *IDH* or *BAP1* mutation and CpG shore hypermethylation had better prognosis compared to other patient groups [56,57]. Thus, it is still under debate whether epigenetic alterations, caused by specific mutations of epigenetic modulators or by other mechanisms, may drive tumor development and progression.

Gain-of-function mutations of *IDH* occur in mutational hotspots affecting R132 of IDH1 and R172 of its mitochondrial isozyme IDH2[57]. Interestingly, *IDH* mutations seem to exclusively occur in iCCA but not in pCCA and dCCA[37]. The frequency of *IDH* mutations has been reported to differ between cohorts with 5%, 6.1%, 18.6% and 31% in a Chinese, Japanese, Italian and US American cohort, respectively[37,58,59]. Mechanistically, *IDH1* and *IDH2* gain-of-function mutations lead to the neomorphic production of the oncometabolite 2-hydroxyglutarate (2-HG) which impairs DNA demethylation by TET2[60,61]. This leads to hypermethylated CpG sites significantly enriched in CpG shores and upstream of transcription start sites predominantly targeting other epigenetic regulators [57]. Thus, the additional repression of epigenetic regulators by *IDH* mutation potentiates and potentially synergizes to induce tumorigenic effects. However, IDH-mutant CCA did not exhibit the largest average DNA methylation and share hypermethylation targets with glioblastomas, suggesting the contribution of additional specific factors outside the DNA methylation pathways[51,57].

Based on histology iCCAs can be subdivided into two groups: the bile duct-type which resembles eCCA with columnar cells with mucin production and the cholangiolar-type which recapitulates a small-duct iCCA morphological pattern with cell-rich tubuli formed by cuboidal cells without extracellular mucin[62]. It is likely that these two histological subtypes of iCCA have distinct cells-of-origin[62]. Consistent with the almost exclusive detection of *IDH* mutations in iCCA, cholangiolar-type iCCA show a higher frequency of *IDH* mutations compared to bile duct-type iCCA[37,62]. The distinct profiles of IDH-mutant iCCA suggest that this subgroup of iCCA patients may be ideal candidates for targeted therapies. In addition, the circulating oncometabolite 2-HG, resulting from IDH gain-of-function mutation, may be used as a surrogate biomarker for patients with IDH mutation[59]. In glioblastoma, *IDH* mutation decreased the levels of STAT1 and the accumulation of T cells in tumor sites suggesting a mechanism of immune evasion[63]. Targeted therapies for IDH mutant tumors are already in clinical trials and may alone or in combination with immunotherapies improve patient outcome[64,65].

However, besides cases with IDH-mutation additional CCA cases exhibit distinct DNA methylation profiles some of which have a larger average of altered DNA methylation compared to IDH-mutant CCA. Thus, multiple mechanisms may lead to distinct DNA methylation profiles. Recent studies in CCA suggest that promoter hypermethylation of tumor suppressor genes may be a key event of CCA

progression and the non-random binomial distribution of hypermethylation patterns suggests specific mechanism inducing these DNA methylation alterations[56,66,67]. The inactivation of tumor suppressive genes by hypermethylation was successfully reversed using DNMT inhibiting cytidine analogues, such as 5-aza-2'-deoxycytidine (decitabine), in vitro[66-68]. The cytidine analogues 5-aza-2'-deoxycytidine and 5-azacytidine received approval for the treatment of hematologic malignancies and have gained interest as priming agents in the treatment of solid tumors[69]. But 5-aza-2'-deoxycytidine has been reported to be mutagenic[70] and cytidine analogue chemotherapeutics are rapidly metabolized into inactive uridine counterparts by the enzyme cytidine deaminase (CDA) which is highly expressed in the liver[71,72]. Therefore, it may be difficult to reach adequate levels of cytidine analogue within the liver. Zebularine is a second-generation nucleoside analog with increased stability compared to 5-aza-2'-deoxycytidine and 5-azacytidine. The identification of CCA patients with a responder gene signature may aid in the identification of patients who may benefit from Zebularine treatment[73,74]. Thus, it will be important to identify potent DNMT inhibitors and a subgroup of CCA patient who will most like respond to DNMT inhibition.

Post-transcriptional modifications and non-coding RNA landscape

Non-coding RNAs (ncRNAs) are single stranded RNA molecules which are not translated into protein. NcRNAs can regulate multiple cellular pathways and they are divided into two subclasses based on their length: long ncRNA (lncRNA; >200nt) and small ncRNA (<200nt). Small ncRNAs are less than 200nt long and include microRNA (miRNA; 19-25nt), small interfering RNA (siRNA; 19-25nt), piwi-RNA (piRNA; 26-32nt) and small nucleolar RNA (snoRNA; >60nt). Of these ncRNAs, miRNAs have been studied most in cancer. The mainly function of miRNAs is the inhibition of protein translation, whereas, lncRNAs appear to exhibit diverse functions through forming secondary and tertiary structures regulating multiple cellular processes.

Depending on their target genes, miRNAs may function as oncogenes or tumor suppressors and miRNA profiling may be useful for patient stratification or to classify poorly differentiated tumors[47,75,76]. The first miRNAs and anti-miRs are now in clinical trials demonstrating that both, oncogenic and tumor suppressive miRNAs, may be successfully targeted. In CCA, most studies used a candidate approach focusing on a single miRNA. MiR-21 has been suggested to function as an oncogene and consistently miR-21 is upregulated in CCA[77], increases cell invasion[52] and decreased sensitivity to gemcitabine[78]. In addition, circulation miR-21 has been found to be increased in plasma of iCCA patients and together with miR-221 it has been proposed to be a non-invasive diagnostic marker for iCCA[79]. In contrast, a dual role of the miR-200 family (miR-200a, miR-200b, miR-429 in one cluster, and miR-200c and miR-141 in a second cluster) may exist. On one hand, miR-200c and

miR-141 are downregulated in CCA and induces epithelial-to-mesenchymal transition (EMT) and cell invasion[80]. On the other hand, miR-200a, miR-200b and miR-429 are hypomethylated and may target the tumor suppressor genes DLC1, FBXW7 and CDH6[49]. Thus, miR-200 family members may exhibit different functions depending on the cellular context or CCA subtype. The let-7 family members, let-7a[81-83], let-7b[81], let-7c[84], let-7d[83], let-7e[83] and let-7f[83], are downregulated in iCCA and have been shown to inhibit self-renewal capacity and subcutaneous cancer cell growth in vivo[84,85]. In concordance, inhibition of let-7a in bile duct-ligated mice increased intrahepatic bile duct mass and expression of nerve growth factor[86]. Deregulated expression of miRNAs may also lead to alteration in DNA methylation. MiR-191 expression is increased in iCCA promoting proliferation, invasion, and migration[87]. The DNA demethylase TET1 is a direct target of miR-191 and reduced TET1 expression in CCA increased DNA methylation at the TP53 gene transcription start site resulting in reduced p53 expression[87]. Although, these studies show that miRNAs may play key roles in cholangiocarcinogenesis, it is still unclear how these miRNAs may be exploited as therapeutic targets. Only few miRNAs have been confirmed to be deregulated in CCA by independent studies. This might be caused by the use of relatively small cohorts which differed greatly in etiology, CCA subtypes and ethnicity. Thus, larger cohorts are needed to identify relevant patient subgroups which may benefit from miRNA-based targeted therapies.

Interestingly, miRNAs may themselves be targeted by lncRNA, thereby, inhibiting the miRNA's function. LncRNAs may act as regulatory factors by presenting `decoy´ binding sites which bind miRNAs leading in turn to reduced inhibition of the miRNA's targets and thus, functioning like a miRNA `sponge´[88]. The lncRNA H19 may bind let-7a which can no longer inhibit IL6 a potent antiapoptotic signaling mechanism in CCA[89-91]. Another example, of sponging is the lncRNA HULC which may bind miR-372 and miR-373 leading to increased expression of their target CXCR4 [91]. The lncRNA NEAT1 is a functional downstream target of BAP1 and negatively regulated by BAP1[92]. BAP1 expression is reduced in CCA inducing NEAT-1 expression and decreasing cytotoxicity to gemcitabine[92]. Although, the role of lncRNAs has received increasing attention during the last years and large numbers of lncRNAs have bene shown to be differentially expressed, only little is known about the function of lncRNAs. It is also crucial to analyze larger cohorts to better understand their function in CCA. A recent large-scale CRISPR-based screen assessing the function of ~17,000 lncRNAs in seven human cell lines found that the function of lncRNAs was highly cell type-specific, often limited to just one cell type[93]. Given the large heterogeneity of CCA between anatomical subtypes and different etiologies, it is highly likely that most lncRNAs are functional in a subset of CCA only. Thus, additional research is needed to understand the concrete function of lncRNAs in CCA patient subgroups and to potentially utilize or target lncRNAs in the clinic.

CONCLUDING REMARKS

Aside from being categorized by heterogeneous anatomic location, CCA is categorized based on histopathological analysis and by growth-type patterns as well. A prominent histological feature of CCA is the abundant desmoplasia (tumor stroma), which is a fibrogenic tissue completely surrounding and tangled into the tumor epithelia and constitutes myofibroblasts, immune cells and vessels. This is a milieu that supports active cross-talk between the stromal and epithelial tumor cells and plays a causal role in tumor onset, metastasis and the pronounced drug resistance. CCA is highly heterogeneous not only in initiation and location but in progression as well, making it difficult to categorize CCA into distinct molecular subtypes. It is apparent that our current approaches to CCA are lacking as evidenced by the continued poor survival rates and limited treatment options. For these reasons, it is critical that new and effective therapies be developed. Indeed, most patients are diagnosed at a stage with locally advanced disease or distal metastasis when 705 to 90% of the patients are ineligible for surgery. Even amongst patients, who undergo surgery, more than 50% of cases are at risk of developing recurrence within 12 months due to inadequate adjuvant therapy[94]. Therefore, understanding the causal biology of CCA metastasis is urgently needed.

A comprehensive and multi-layered understanding of the disease pathogenesis is fundamental in the development of novel therapeutic strategies. For example, the inception of epigenomic profiling technologies rapidly confirmed such modes of genetic regulation to be far more complex than traditional genomics, invoking new challenges in experimental design and interpretation. CCA lags behind the majority of cancers in epigenomics, though this may afford opportunity to prospectively in the future design robust molecular studies. We need to focus on genome-wide integromics for patient characterization, stratification and discovery of biomarkers to advance early diagnosis and precision therapy. Thus, to get to this stage in the clinical management of cholangiocarcinoma, it will be essential to further 1) elucidated the genome perturbations that dictate unique regulatory networks in primary and metastatic sites, 2) delineated synergistic drug repositioning and chemosensitization in CCA, 3) investigate the role of desmoplastic stromal cells in CCA tumor growth and resistance to treatment (for example the potential for immunotherapy targeting this niche), 4) comprehensively define the involvement of DNA methylation regions in transcriptional regulation and as driving factors in CCA, and capitalize on the potential of epigenome-based targeted therapy. Finally, we currently know surprisingly little about 5) the role of non-coding RNAs both as markers in CCA diagnosis and prognosis, and importantly also as regulators in drug responses.

FIGURE LEGEND

Figure 1. Schematic representation of the changes occurring in the molecular make-up of advanced CCA. (**A**) Little information is available on the molecular landscape of advanced CCA and on the evolutionary dynamics of these tumors. Based on evidence available so far, we can speculate that CCAs have polyclonal seeding potential where only the fittest clones survive. In addition, emergence of new clones induced by anti-cancer treatment has been detected in isolated cases of relapsed CCA which were not present in the primary tumor. (**B**) The differences in the molecular profile of primary and advanced cancers can be explained according two different hypotheses. There is evidence that isolated cancer cells with a specific phenotype can initially form a pre-metastatic niche, causing changes in the stroma that in turns induces molecular and phenotypic changes of the cancer cells that will populate the metastatic deposit. The poly-clonality of the metastatic deposit may alternatively be justified by a potential synergism of cancer clones that can give rise to a metastatic growth only when their oncogenic properties are combined.

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