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EPIGENOME DYSREGULATION IN CHOLANGIOCARCINOMA

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ABSTRACT

Epigenomics is a fast-evolving field of research that has lately attracted considerable interest, mainly due to the reversibility of epigenetic marks. Clinically, among solid tumors, the field is still limited. In cholangiocarcinoma (CCA) it is well known that the epigenetic landscape is deregulated both during carcinogenesis and disease progression as a consequence of aberrant mechanisms leading to genome instability. In this article, we will briefly review the molecular alterations that have been described in the transformation of normal cholangiocytes into malignant derivatives, focusing on the role of non-coding RNA (ncRNA) interactions, DNA methylation, post-translational modifications (PTMs) of histones and chromatin remodeling complexes.

1. INTRODUCTION

Increasing incidence and mortality rates of cholangiocarcinoma (CCA) [1, 2] may potentially reflect an atypically high mutational burden shared by these malignancies in the biliary tract, which clinical intervention has thus far failed to identify [3]. It is therefore unanticipated that whole-exome [4] and whole-genome [5] sequencing approaches have revealed only intermediate global mutation rates in this cancer type [4, 6]. As such, the genetic alteration frequency is insufficient to entirely explain current clinical observations (such as the innate chemoresistance and rapid disease progression characteristic of CCA) or genomic observations (such as tumor subgroups characterized by grossly different transcriptomic profiles [4, 7, 8]). One substantial contributor to the missing oncogenic virulence could certainly involve epigenetics, heritable alterations in gene expression independent of DNA sequence changes, and epigenome dysregulation. This hypothesis is bolstered by the recurrent detection of mutations in epigenetic regulators (*IDH1/2*, *KMT2C*, *ARID1A*, *BAP1*), aberrations which likely have substantial (epi)genome-wide consequences.

Epigenetic information is transmitted trans-generationally through a variety of biochemical modifications and processes, including non-coding RNAs (ncRNAs), DNA and RNA modifications, histone post translational modifications (PTMs) and chromatin remodeling. The cholangiocyte epigenome constitutes a unique combinatorial state of these epigenetic marks, a minority of which are responsible for maintenance of biliary epithelial cell identity (and therefore, should remain constant) and a majority which fluctuate to control gene expression in response to endogenous and exogenous stimuli. Cholangiocarcinogenesis, like other neoplastic processes, is accompanied by gross epigenomic insult affecting almost all levels of epigenetic regulation. Such individual epigenetic alterations, or 'epimutations' [9], are becoming increasingly appreciated as potential pro-oncogenic lesions functionally synonymous to genetic mutations, though verification of true 'epi-driver' status remains elusive.

Comprehensive resolution of the origin(s) and perpetuation mechanism(s) of epimutations has not yet been achieved. One obvious contribution arises from classic genetic alterations (mutation and structural) of epigenetic enzymes, a phenomenon recurrently reported across all categories of epigenome regulators in CCA (**Figure 1**). However, given the widespread epigenomic insult in CCA that is emerging from the limited epigenome-wide association studies (EWAS) to date [10, 11], it is clear that other mechanisms (likely, epigenetic regulation of epigenetic regulators, rate-limiting enzymatic cofactors) also fuel the evolution of

CCA epigenomes. An enhanced understanding of these epimutational processes and their downstream consequences holds significant translational potential to improve both diagnostic and prognostic tools, as well as putative therapeutic strategies.

Accordingly, in this review we critically assess our current understanding of the processes that drive epigenome dysregulation in CCA, as well as their immediate and downstream biological consequences. Epimutations are broadly separated into ncRNA alterations, DNA methylation aberrations and chromatin perturbations. Additionally, we establish a rational framework to guide future design and execution of EWAS for CCA to optimize the potential for translational and clinical success.

2. NON-CODING RNAs IN CHOLANGIOCARCINOGENESIS

Non-coding RNAs (ncRNAs) are endogenous RNA molecules which are not translated into proteins and have emerged as key transcriptome regulators in many different cellular pathways and systems. ncRNAs are grouped into two broad subclasses according to their number of nucleotides (nt), small ncRNAs (100-200 nt) and long ncRNAs (>200 nt). Small ncRNAs mainly act as translational repressors and include microRNAs (miRs), piwiRNAs (piRNAs), small interfering RNAs (siRNAs) and small nucleolar RNAs (snoRNAs). On the other hand, long ncRNAs (lncRNAs) are generally involved in gene silencing. As such, this category of ncRNAs has lately emerged as new players in the malignant transformation of cholangiocytes [12], being able to act as oncogenes or tumor suppressor genes (TSGs) and, therefore, representing potentially valuable tools as predictive biomarkers or as therapeutic targets.

2.1 MicroRNA (miR) dysregulation in cholangiocarcinoma

MiRs are, so far, the best studied ncRNAs. Initially discovered in 1993 [13], these highly conserved, single-stranded 19-25 nt ncRNAs function as genomic rheostats that exert transcriptional control and impact translation by affecting initiation and mRNA destabilization of their multiple target genes [14]. Thus, miRs have a significant role in diverse fundamental cellular processes such as cell differentiation, proliferation, migration, cell cycle control and apoptosis. Through their capacity to regulate various genes, and consequentially multiple pathways, miRs are significant contributors to disease heterogeneity, a property which suggests translational potential for patient stratification, as well as confounding drug response [15]. Preliminary studies attempting to clarify the contributory pathogenic role of miRs in CCA have mainly focused on single candidate miRs. Accordingly, different studies have reported that miR-21 is upregulated in CCA patient tissue [16, 17], as well as in diverse CCA cell lines [18]. High levels of miR-21 have also been detected in serum of CCA patients [19]. Induced overexpression of miR-21 *in vitro* can lead to downregulation of the MMP inhibitor RECK (Reversion-inducing cysteine-rich protein with Kazal motifs) subsequently increasing proliferation, invasion and migration [17] and tumor growth [20]; PTPN14 (protein tyrosine phosphatase non-receptor type 14), regulating invasion and metastasis [17]; and tumor suppressor PTEN, causing chemoresistance in CCA cell lines [18]. Moreover, deregulation of the miR-200 family (enclosing 5 members divided in two clusters, miR-200a, miR-200b and miR-429 in chromosome 1; miR-200c and miR-141 in chromosome 12) contributes to the early stages of metastasis by epithelial-to-mesenchymal (EMT) transition. [21]. Although various studies have shown that expression of miR-200

family members is downregulated in biliary tract cancers compared to normal tissues, leading to EMT activation and cancer cell invasion [22, 23], miR-141 and miR-200b were previously reported to be significantly overexpressed in malignant cholangiocytes and further contribute to tumor growth and gemcitabine resistance [18]. These confounding results reflect both their ability to act as oncomiRs or tumor suppressors, as well as suggesting that certain miRs can retain dual roles depending on the cellular context. Further, miRs like miR-34a [24], miR-204 [25, 26], miR-214 [27] and miR-221 [28] have also been reported to regulate EMT in CCA, thus representing potential therapeutic targets for preventing post-operative recurrence. Other miRs involved in CCA development and progression are summarized in **Table 1**.

The expression of miRs can be controlled at both the transcriptional and post-transcriptional level. Also, endogenous (hormones, cytokines) and exogenous (xenobiotics) compounds may affect miR regulation. Intrahepatic CCA (iCCA) has previously been classified into two different biological subtypes with markedly different outcomes (i.e., the 'Proliferation' and 'Inflammation' subsets) [8]. The main mediator of the latter tumor subset is the inflammation-associated cytokine Interleukin-6 (IL6), known to be overexpressed and to contribute to tumor growth in CCA. In this regard, IL-6-mediated hypermethylation of miR-370 leads to overexpression of the oncogene *MAP3K8* [29]. In addition, IL-6 has been shown to upregulate the expression of DNA methyltransferase 1 (DNMT1) and epigenetically alter the expression of *miR-148a* [30] and *miR-152* [30], both of which can bind and regulate DNMT1. The connection between miR and epigenetic modifications of genes has also been reported in extrahepatic perihilar CCA (pCCA), where miR-373 is downregulated and negatively regulates methyl-CpG-binding domain protein 2 (MBD2), which in turn hampers the methylation-mediated tumor suppressor *RASSF1A* [31, 32]. miR-200 family members (miR-200a, miR-200b and miR-429), which share a common promoter, have been found to be hypomethylated in CCA [33]. As a consequence, subsequent miR upregulation results in significant downregulation of the miR-target genes, including tumor suppressors *DLC1*, *FBXW7* and *CDH6* [33].

To date, limited large-scale miRNome studies are available in sufficiently well-characterized CCA patient cohorts, including subsequent patient-matched integrative '-omics' functional investigations. Palumbo *et al.* [34] recently performed a functional high-throughput miR mimic screen with a library covering 316 different miRs. The study was performed using the human extrahepatic bile duct CCA cell line (TFK1), and designed to elucidate miRs regulating CCA cell proliferation (reduced cell growth of 50% was considered as a positive hit). MiR-410 was the top suppressor of growth out of the 21 miRs identified to regulated CCA cell growth [34]. Concurrently, Lin and colleagues [35] used small RNA sequencing to determine the miR expression profiles in 24 human CCAs. In total, 3 members of a common miR cluster (let-7c, miR-99a and miR125b) were found to be downregulated in the tumors, which aberrant regulation further was associated with the activation of the IL-6/STAT3 signaling pathway, reinforcing the key role this signaling axis plays in cholangiocarcinogenesis [36].

2.3 Long non-coding RNAs (lncRNAs) in cholangiocarcinoma

Long non-coding RNAs (lncRNAs) are known to play important regulatory roles in development and progression of different types of cancer. The length of these non-protein-coding molecules (>200nt) enables them to form secondary or tertiary structures, and themselves function as RNA sequences. However, little is known about the role of lncRNAs and their downstream mechanisms in the pathogenesis

of cholangiocarcinoma (**Figure 2**). It has been reported that some lncRNAs play an important function in the regulation of inflammatory response pathways stimulated by viral and/or fluke infections, as well as oxidative stress [37]. This is the case of H19 and HULC, which in CCA are both activated by oxidative stress and target IL-6 and CXCR4 respectively, subsequently promoting cell migration and invasion [37]. Interestingly, the biological mechanism(s) underlying this event involves the 'miR sponging' phenomenon. A mechanism, in which lncRNAs possess the ability to affect the miR activity through direct binding and therefore impede miR binding to its usual target (miR-target gene). Thus, lncRNA (H19) has been shown to 'sponge' let-7a/let-7b, subsequently increasing the expression level of its target IL-6 [38]. Additionally, HULC can bind miR-372 and miR-373, which are suggested to target CXCR4. As such, overexpression of IL-6 and CXCR4 in CCA cells consequently results in a chronic inflammatory response, promoting progression of the tumor. This regulatory mechanism highlights the importance of the lncRNA-miR-mediated crosstalk. Another example of sponging involves the lncRNA MALAT1 (Metastasis-associated lung adenocarcinoma transcript 1), miR-204 and its target CXCR4, which through this interaction promotes proliferation, invasion and migration of human pCCA [39]. Interestingly, the nuclear paraspeckle assembly transcript 1 (NEAT-1) lncRNA has recently been reported to have a pivotal role in modulating CCA drug sensitivity [40]. This study emphasized how reduced mRNA expression of the chromatin modulator *BAP1* (BRCA-1 associated protein-1) enhances sensitivity to gemcitabine in the CCA cell line (KMBC). Additionally, in an attempt to identify *BAP1*-regulated candidate lncRNAs with a role in controlling CCA drug response, an inverse correlation was observed between *BAP1* and NEAT-1 expression. This demonstrates that NEAT-1 acts as a functional downstream target of *BAP1*, and how their interplay may modulate drug responses. Nevertheless, the direct mechanism by which NEAT-1 modulates *BAP1* control still remains unknown. As such, the *BAP1* expression level varies substantial between different CCA cell lines. Recently, the mitochondrial enzyme encoded by *CPS1* and its lncRNA (*CPS1-IT1*) were shown to promote proliferation of iCCA cells [41], suggesting a putative role of this lncRNA as a potential diagnostic and prognostic biomarker in iCCA. Also, a recent study determined that the lncRNA (*AFAP1-AS1*) promotes growth and metastasis of CCA cells [42]. As such, *AFAP1-AS1* deficient cells gave rise to smaller xenograft tumors compared to control. In addition, the study demonstrated an elevated expression level of *AFAP1-AS1* found both in CCA tissues and cell lines compared to matched adjacent non-tumoral tissue as well as normal biliary epithelial cells, respectively.

High throughput methodologies have revealed an unexpectedly large number of abundant lncRNAs by far surpassing the miRNome. Furthermore, experimental evidence increasingly suggests the presence of network interactions (sponging) between lncRNA and microRNAs [43]. Transcriptomic profiling of 77 iCCAs and their adjacent non-malignant tissues was performed using lncRNA and mRNA microarrays [44]. This study defined more than 5100 lncRNAs and 6500 mRNAs as differentially expressed between paired tumor and adjacent noncancerous tissues. Further co-expression modules identified a total of 290 significant lncRNA-mRNA target gene pairs. Within this list, 6 gene pairs were previously reported to be associated with tumorigenesis in other cancers. Validation of these lncRNA-mRNA modules by qRT-PCR showed that four pairs (RNA42085-SULF1, RNA47504-KDM8, RNA58630-PCSK6 and RNA40057-CYP2D6) were positively correlated in iCCA tissues. As such, these pairs may have prognostic significance in CCA, i.e., patients with low levels of *PCSK6* and *CYP2D6* were found to have a reduced overall survival. Another recent analysis of the lncRNA landscape was performed in 34 iCCA patients, including their matched adjacent non-cancerous tissues and samples obtained from 4 healthy individuals [45]. Whereas, targeted qRT-PCR analyses were performed on all samples only 4 samples were used for lncRNA-mRNA expression profiles. In this study, a

total of 2716 lncRNAs and 883 mRNAs were found to be differentially expressed, with 142 putative lncRNA-mRNA pairs comprising co-modular signaling networks. Though current results are limited in scope, taken together, these studies reinforce the premise that the lncRNA landscape in CCA may contribute to disease pathogenesis and possibly drug resistance. Nevertheless, to attribute any concrete biological or clinical roles to lncRNAs or lncRNA-mRNA modules, larger and well characterized cohorts are needed.

3. DNA METHYLATION IN CHOLANGIOCARCINOGENESIS

3.1 DNA methylome dysregulation in cholangiocarcinoma

CpG dinucleotide methylation is by far the most prevalent DNA modification in the human genome. DNA methylation involves the S-adenosyl methionine (SAM)-dependent transfer of a methyl group onto cytosine to generate 5-methylcytosine (5mC). This fundamental epigenetic process is actively written by DNA methyltransferase enzymes (DNMTs) and erased by Ten-eleven translocation methylcytosine dioxygenases (TETs). Genomic distribution of CpG sites is non-random and can be categorized as CpG-rich regions or CpG islands (CGIs), CGI borders (CpG shores and shelves, 2kb and 4kb up- and downstream of CGIs, respectively) or CpG-depleted ('open sea'). These CpG-oriented domains co-localize with different regulatory elements to modulate transcription (promoters), facilitate long-range regulation (enhancers) and promote genomic stability (inter-genic elements, repetitive sequences). DNA methylation alterations are among the earliest molecular lesions to occur in tumorigenesis [46], displaying classic cancer hallmarks such as tumor suppressor promoter hypermethylation leading to transcriptional inactivation and, later in disease progression, global hypomethylation resulting in reactivation of endoparasitic sequences [47]. Characterization of the CCA methylome is still limited, though several classic epimutation mechanisms have been confirmed alongside some atypical features (**Figure 3**).

Given the functional diversity of presence or absence of CpG methylation sites at different regulatory regions, it is expected that modes of DNA methylation alteration are also non-uniform in CCA. Using a CGI-array (containing 237,000 probes), DNA hypomethylation events were found to be more common than hypermethylation (55.3% versus 44.7%) in 18 CCAs (mixed anatomical location), though hypermethylation events were more recurrent across patients (60% versus 40%) [11]. Suggestion of predominant hypomethylation conflicts with global 5mC quantification by LC-MS, which indicated comparable 5mC levels between a small sample set of CCA and surrounding normal [48]. This suggests the less CpG-rich regions of the genome, not covered by the CGI-array, may shift towards more methylated states or at least remain consistent in disease. Notably, 5-hydroxymethylcytosine (5hmC), the immediate downstream metabolite of 5mC during DNA demethylation, is significantly downregulated in iCCA, and is an indicator of poor prognosis [49]. Similar inter-chromosomal epimutational trends were observed with 94.8% X chromosome alterations comprising loss of methylation events, in comparison to predominant autosome hypermethylation. Evidence for sub-chromosomal regional differences has also been demonstrated, epitomized by the distal 30 megabase (Mb) region of chromosome 1. This region is commonly deleted in different cancers and contains many tumor suppressor genes. Intriguingly, gene bodies within this region in CCA are largely hypomethylated, suggesting substantial (though mechanistically unclear) transcriptional dysregulation.

Like other cancers, the most commonly described epimutations in CCA comprise promoter hypermethylation events of genes with tumor suppressor function. Binomial distribution of epigenome-wide hypermethylation events certainly suggest that these epimutations are not random but directed in CCA [11]. Epigenomic analysis of 18 CCA patients uncovered 65 distinct WNT pathway genes to harbor epimutations, predominantly targeting promoter and alternative promoter sites. Follow-up immunohistochemistry in a larger independent sample set confirmed significant transcriptional consequences of these epigenetic alterations for the WNT pathway, though such genes could be rescued *in vitro* by treatment with hypomethylating agent, 5-aza-2'-deoxycytidine [11]. In contrast, DNA hypomethylation is less well characterized in CCA. IMP3, a fetal oncoprotein that is restricted to expression during development, was found to be reactivated in iCCA [50]. Such transcriptional activation was mediated by gross promoter hypomethylation, highly recurrent (high immunohistochemical staining in 82% of patients) and independently associated with multiple adverse prognostic parameters (tumor volume, grade, metastasis and overall survival). DNA methylome alterations are not restricted to protein-coding genes and frequently target miRs in CCA [33], as discussed in the ncRNA section this review. Many of the epimutations described in CCA are not disease-specific and are found in other types of gastrointestinal cancers (including gastric, pancreatic and colorectal) [51]. Certain epimutations synergize with co-occurring mutations as indicated by the adverse outcome of *TP53* mutant CCA patients also presenting aberrant promoter hypermethylation of genes (*DAPK*, *p14*, *ASC*) involved in the mitochondrial apoptosis pathway [52]. Furthermore, certain loci are targeted by diverse perturbation mechanisms (mutational, epimutational or structural rearrangement) such as *RUNX3* promoter hypermethylation as well as 1p36.1 loss (containing *RUNX3*) in CCA, indicating universal tumor advantage by any perturbatory mechanism [53]. Integrating target gene data from different mechanisms may provide a novel method to in the future identify 'true' disease drivers, as well as likely increase the percentage of patients that may benefit from subsequent pathway-directed therapy.

Epigenetic modifications significantly govern differentiation states and, thus, cellular identity. It therefore logically follows that epimutations should target genes involved in differentiation during cholangiocarcinogenesis. Indeed, analysis of differentially methylated sites in CCA patient tissues has indicated an enrichment of epimutations in Polycomb Repressive Complex 2 (PRC2)-target genes [10]. One study remarkably found that 10% (97 out of 970) of aberrantly methylated CGIs mapped to *HOX* genes [54], further confirming preferential targeting of developmentally important loci. Focusing on the hepatobiliary system, *SOX17* is a highly specific marker of biliary differentiation and has emerged as a potent tumor suppressor in CCA [55]. Using a morphogen-driven *in vitro* differentiation system (from induced pluripotent stem cells to hepatic progenitors to induced cholangiocytes), *SOX17* was specifically identified as a factor induced exclusively in the progenitor to differentiated transition. *In vitro* knockdown of *SOX17* in normal cholangiocytes was sufficient to induce loss of biliary marker (CK7, CK19) expression, as well as increase proliferation and cancer-associated gene expression profiles, whereas overexpression in CCA cell lines had inversely complimentary effects. *In vivo*, *SOX17* overexpression effectively inhibited growth of pre-existing tumors and completely impaired tumor grafting when administered simultaneously. Mechanistically, *WNT3A* was elucidated to downregulate *SOX17* in a DNMT1-dependent manner, mirroring *SOX17* downregulation by promoter methylation in CCA patients. These findings highlight a unique case of tumor suppressive function that is tightly linked to biliary differentiation and is modulated by epimutations in cancer. Given the diversity of hypothesized cells of origin in CCA [56], it is likely that other differentiation-

associated epimutations exist and that these may vary between CCA patients arising from different cellular origins.

Akin to mutations, epimutations also appear to display temporal specificity. For example, promoter hypermethylation of *p16* has been well described in CCA but has also been detected in 45.6% primary sclerosing cholangitis (PSC) samples, a well-known predisposing risk factor for development of CCA, without any signs of malignant onset [57]. Similarly, this locus was also found to be hypermethylated in liver intraductal papillary neoplasia (IPN), a precursor lesion to CCA, and inversely correlated with gene expression [58]. Hepatitis C virus (HCV) core protein is capable of upregulating DNMT1, leading to suppression of miR-124 and induction of oncogenic SMYD3, linking early infection with pro-oncogenic effects [59]. In a series of matched extrahepatic CCA, biliary intraepithelial neoplasia (BiIN) and normal cystic ducts, 4 cancer-associated promoter hypermethylation events (*TMEFF2*, *HOXA1*, *NEUROG1*, *RUNX3*) were detected in pre-malignant lesions and retained in tumors, suggesting clonality of these epimutations [60]. Repetitive element analysis in these samples also revealed progressive hypomethylation of *SAT2* repeats from normal to BiIN and from BiIN to CCA, but *LINE1* hypomethylation exclusively occurred in BiIN to CCA transition. These findings begin to emphasize the early evolutionary dynamics of epimutations during cholangiocarcinogenesis. Little is known of these processes at the opposite end of the disease spectrum, however, during the transition from primary CCA to metastatic CCA. Promoter hypermethylation of *ITGA4* has been tentatively suggested as such, given its methylation status in 100% patients with lymph node metastasis in a modest study population [61]. Further clarification of the epimutational contribution to CCA dissemination is required given metastasis is the actual cause of death in >90% of cancer patients.

3.2 Origin(s) of DNA methylation alterations in cholangiocarcinoma

It is evident, even given the limited EWAS in CCA, that the DNA methylome undergoes widespread dysregulation with concomitant transcriptome destabilization. What lacks clarification, however, is the molecular origin(s) of these epigenomic insults. Two plausible scenarios currently exist: genetic (mutation and structural) alterations of DNA methylome regulators or epigenetic alterations of such epigenetic regulators.

Intrahepatic CCA is different among epithelial cancers in that approximately 10% of patients present tumors with mutations in *Isocitrate Dehydrogenase* (*IDH*) enzymes, *IDH1* and *IDH2* (otherwise mutated in tumors of mesenchymal and neuronal origins) [62]. Normally, *IDH1* and *IDH2* catalyze the conversion of isocitrate to α -ketoglutarate (α -KG) in the cytoplasm and mitochondria, respectively. When mutated, typically at hotspots R132 (*IDH1*) and R172 (*IDH2*), these enzymes acquire neomorphic activity and produce 2-hydroxyglutarate (2-HG). This oncometabolite severely impairs the normal function of multiple pathways, including DNA demethylation as mediated by TET2 [63]. The effects of these mutations are so pronounced in patient tumors that The Cancer Genome Atlas (TCGA) cholangiocarcinoma network has identified *IDH* mutant tumors as an independent subtype of biliary cancer [4]. *IDH* mutants have higher 5mC levels and lower 5hmC levels, indicative of DNA demethylation pathway dysfunction. Consequently, *IDH* mutant tumors are grossly hypermethylated in comparison to wild type tumors with DNA methylation alterations significantly enriched at promoters, depleted at gene bodies and predominantly targeting other epigenetic regulators [62]. This is further corroborated by observations of genes involved in chromatin regulation

being significantly downregulated in IDH mutant CCA [4], indicating a potential epigenomic 'snowball effect' between mutant IDH (functioning as an oncogene) and downregulated chromatin modifiers (functioning as tumor suppressors). Remarkably, however, IDH tumors did not on average have the greatest amount of DNA methylations alterations, re-emphasizing the unclear but significant contribution of other biomolecules outside DNA methylation pathways.

Since DNA methylation profiles are actively modulated in response to exogenous stimuli, it is logical to assume inter-cellular signaling mechanisms may direct methylome alterations. Accordingly, the inflammatory mediator IL-6 (which is overexpressed in CCA patients) and its signaling axis came to attention due to its capacity to attenuate the hypomethylating and subsequent cellular effects of 5-aza-2'-deoxycytidine treatment *in vitro* [64]. Epigenetic mechanisms sustain IL6 signaling in biliary tumors through promoter hypermethylation of its negative regulator *SOCS3*, thereby inhibiting the feedback loop [65]. Persistent IL-6 signaling in turn has pleiotropic effects including upregulation of cancer associated pathways [64], hypermethylation and downregulation of tumor suppressor genes [30], hypermethylation and downregulation of miRs (miR-148a, -152) [29] and, intriguingly, upregulation of *DNMT1* [29, 30]. Subsequently, *DNMT1* upregulation was shown to be due to IL6-associated downregulation of miRs that target the 3'-UTR of *DNMT1* through an elusive mechanism [30]. This is another exemplar case of epigenetic snowball effects in CCA, whereby epigenetically-perpetuated IL-6 signaling (via *SOCS3* hypermethylation) downregulates epigenetic regulators (miRs) leading to upregulation of the epigenetic regulator *DNMT1* and subsequent epigenetic modulation of tumor suppressor genes. Anecdotally, *DICER* also appears to be able to direct DNA methylation (specifically *SFRP1* promoter hypermethylation) through translocation to the nucleus, though the specific mechanism behind this is unknown [66].

3.3 Translational potential of the cholangiocarcinoma DNA methylome

Characterization of DNA methylation alterations in CCA to date has revealed that these events are recurrent and occur early during disease manifestation (including under predisposing conditions such as PSC and precursor lesions such as BiIN). These properties together indicate DNA methylation could be a useful biomarker tool for the early detection of CCA, arguably one of the biggest issues in the management of this disease given the low resectability rates (10-30%) of patients at time of diagnosis. *In vitro*, pharmacological rescue experiments using 5-aza-2'-deoxycytidine has led to the identification of a candidate 4-gene biomarker panel (*SFRP1*, *DCLK1*, *CDO1*, *ZSCAN18*) [67]. Targeted methylation analysis of these promoters correctly diagnosed tumor from normal controls with an impressive sensitivity of 87% and a specificity of 100%. These findings support the relevance of *in vitro* culture systems to understand molecular changes in patients, as well as highlight the predictive potential of epimutations in fresh frozen (FF) and formalin-fixed paraffin-embedded (FFPE) samples. Nonetheless, biopsies taken at biliary tract are highly invasive and non-invasive tests (liquid biopsies) would be preferred. While tissue-guided selection of a 2-gene panel (*SHOX2*, *SEPT9*) did correctly identify some tumors when quantified in plasma [68], its performance was significantly poorer than in tissue, suggesting that these two biomaterials are not directly comparable from a DNA methylation perspective. Rather, preliminary data suggests bile may be a more relevant biofluid to question. Biliary brush cytology is currently used to aid diagnosis of CCA but possesses several limitations, including difficulty in discriminating atypical reactive epithelia from true neoplasia, low cell recovery and cellular disintegration. By initially testing in tissue samples, Andersen and colleagues

identified an optimal 4-gene panel (*CDO1*, *CNRIP1*, *SEPT9*, *VIM*) for targeted methylation analysis in bile samples [69]. To compare the diagnostic ability of this novel method against routine brush cytology, DNA methylation analysis achieved sensitivities of 73% and 91% in both test and validation cohorts, whereas cytology achieved 58% and 63% (specificity was comparable across diagnostic tools). As such, DNA methylation-based biomarkers may serve useful when the liquid biopsy is biofluid (e.g., bile) in close proximity to the hepatobiliary system.

Similarly, DNA methylation alterations are attractive therapeutic targets since, unlike mutations, they are reversible. Debate still exists as to the existence of 'epi-drivers' [70], epigenetic alterations upon which tumors have developed a functional dependence and which function synonymous to classic mutational drivers. Nonetheless, traditional use of 5-aza-2'-deoxycytidine as a pharmacological rescue agent across many DNA methylation studies confirms the *in vitro* efficacy of such agents. Zebularine, a second-generation nucleoside analog, competitively binds DNMTs, leading to an overall reduction in DNMT levels, genome hypomethylation and active induction of apoptosis in CCA [71]. DNMTs are overexpressed in CCA patients, though the extent to which is highly variable, indicating that a responder signature may be required to identify patient subgroups that could benefit from DNA hypomethylating agents [72]. However, the relatively poor solid tissue bioavailability of these compounds alongside peak cytosine deaminase (a rate limiter of demethylating agents) concentrations within the liver brings the therapeutic potential of these compounds into question.

4. CHROMATIN DYNAMICS IN CHOLANGIOCARCINOGENESIS

The fundamental unit of chromatin organization, the nucleosome, is comprised of a histone octameric complex around which 146bp DNA is spooled. Through a variety of histone post translational modifications (PTMs) added and removed by specialized epigenetic enzymes, nuclear compaction is regulated to direct gene expression profiles, facilitate DNA repair and promote genome stability. Analogous to ncRNA and DNA methylation epimutations, histone PTMs also become dysregulated in cancer. Of the many PTMs identified, only two biochemical modifications (histone acetylation, histone methylation) have been evaluated in CCA across a limited number of studies.

Histone acetylation is written by histone acetyl transferase (HAT) enzymes and erased by histone deacetylase (HDAC) enzymes. Addition of acetyl groups onto the histone tail is a pro-euchromatin event, altering the tail charge from positive to neutral and promoting loosening of the nucleosomes, while removal results in nucleosome compaction (a pro-heterochromatin event). A variety of HDACs have been uncovered as overexpressed in CCA, including associations with adverse prognosis [73, 74]. Unsurprisingly, the effects of aberrant HDAC expression appear to be quite diverse. One of the characteristic features of malignant transformation of cholangiocytes involves the shortening and/or loss of ciliary appendages. This loss could be driven *in vitro* by forced overexpression of HDAC6 (also resulting in increased proliferation and anchorage-independent growth) and subsequently rescued *in vivo* through treatment with the HDAC inhibitor (HDACi) tubastatin [75]. *SPRR2A*, a gene involved in maintenance of epithelial barriers and wound repair, was found to be elevated in a single CCA cell line. This event resulted in deacetylation of P53 by p300 acetylation and increased HDAC1 expression, providing a histone-based link for attenuation of normal TP53 signaling [76]. HDAC4 was identified as overexpressed in CCA through downregulation of its negative

regulator miR-29a facilitated by TGF- β 1 signaling, resulting in increased metastatic characteristics *in vitro* [77]. A variety of compounds with HDACi activity, in particular Valproic Acid (VPA) and Vorinostat, have shown success in inhibiting CCA growth alone and in combination with chemotherapeutics *in vitro* [78, 79] and *in vivo* [80]. The downstream effects of HDACi treatment underpinning therapeutic responses remain poorly characterized, though it is in part mediated by TACC3 downregulation [74]. Nonetheless, in spite of such lacking molecular clarity, VPA appeared to display anti-neoplastic activity with manageable safety profiles in a small mixed cohort of pancreato-biliary patients (including CCA) [81] and Vorinostat *in vivo* effects appeared to be enhanced through delivery with nanoparticles [82]. A prospective phase 1b clinical trial (NCT02856568) will test the effects of treatment with Ricolinostat (HDAC6 inhibitor) in combination with standard gemcitabine and cisplatin in unresectable and metastatic CCA patients.

Unlike histone acetylation, the effects of histone methylation are varied dependent on the target amino acid. EZH2, the only histone methylation enzyme analyzed in CCA to date, is a histone methyltransferase specifically catalyzing the methylation of histone 3 at lysine 27 (H3K27me) mark. Acting from within the Polycomb Repressive Complex 2 (PRC2), H3K27me is associated with transcriptional repression. EZH2 is overexpressed in CCA and associated with poor prognosis in both intrahepatic and extrahepatic CCA [83]. Inhibition of EZH2, either through siRNA-mediated knockdown or the compound DZNep, substantially impairs tumor cellular properties *in vitro* (hyper-proliferation, evasion of apoptosis, efficient cell cycling), synergizes with gemcitabine treatment [83, 84] and slows tumor growth *in vivo*, in part through rescue of *RUNX3* expression [85].

Higher order regulation of nucleosomes and chromatin dynamics has additionally been implicated in CCA pathogenesis. Whole-exome and whole-genome sequencing has uncovered recurrent, frequently inactivating mutations in chromatin remodelers such as *ARID1A*, *ARID1B*, *BAP1* and *PBRM1* [86-88]. Chromatin modifiers are also targeted by alternative perturbation mechanisms, such as promoter hypermethylation and transcriptional silencing of *ARID1A* in *IDH* mutant tumors [4]. The functional implications of this impairment of chromatin remodeling in cancer cells require substantial clarification, though they do appear to act broadly as tumor suppressors (**Figure 4**). Indeed, functional testing through hepatocyte-targeted CRISPR screening *in vivo* confirmed positive selection of *ARID1A* mutations in cancer cells [89]. Specifically, *ARID1A* is a member of the SWI/SNF chromatin remodeling complex. *ARID1A* expression becomes lost in CCA, though its expression was still detected in pre-malignant lesions [90]. Similarly, another SWI/SNF complex member, *PBRM1*, displayed comparable patterns with normal expression in precursor lesions before becoming lost in CCA [91]. These findings indicate loss of expression of SWI/SNF members is a late event in cholangiocarcinogenesis and may underpin acquisition of the invasive cancer phenotype.

5. EXPERIMENTAL DESIGN & THE FUTURE OF CCA EWAS

Development of EWAS, particularly in oncology, has led to the retroactive identification of fundamental pitfalls in experimental approaches to identify true epimutational associations with a given phenotype [92, 93]. It is perhaps, therefore, relatively opportunistic that CCA epigenomics remains in its infancy and that careful study design may prospectively maximize the likelihood for detection of true disease-associated epigenetic alterations. Such issues identified to date include careful patient stratification, tissue

deconstruction of cell types and inference of association and/or causation (whether direct or reverse) (Figure 5).

Common population variables have been shown to influence DNA methylation profiles (and plausibly may affect other epigenome modifications). Unlike genetic information, DNA methylation profiles are temporal and have been proven to flux over time. Though CCA is typically a disease associated with advanced age, efforts should be made to design study populations with matched age profiles. Similarly, study populations should be controlled for ethnicity and disease etiology (e.g. HCV infection, alcohol exposure, fluke parasitism) as potential confounders, especially given the findings of alcohol- and HCV-associated DNA methylation signatures in hepatocellular carcinoma [94]. Gender distribution should also be considered. While sex chromosomes are typically excluded from DNA methylation analysis of mixed-sex disease (due to female X chromosome inactivation), many cancer-relevant genes are located on the X chromosome. Of note, Goeppert and colleagues identified gross DNA hypomethylation of the X chromosome in CCA that was demonstrated to be statistically independent of technical issues or sampling bias [11]. This suggests that sex chromosomes should not automatically be discredited in future CCA EWAS. Also, longitudinal sampling and epigenetic characterization should be pursued to characterize intra-individual epigenome evolution in response to disease progression and clinical intervention.

Given the intimate link between epigenomes and cellular identity, perhaps the biggest challenge in EWAS to date is the stratification of epigenetic signals based on their cellular origins and identification of biologically-relevant comparison points. CCA epigenetic studies have typically, like most other cancers, employed surrounding normal as the control to tumor. This has three primary limitations: (1) the majority of 'surrounding normal' tissues are cirrhotic and, therefore, will not have truly normal epigenomes; (2) even if surrounding tissue appears histologically normal, extensive field cancerization mediated by epigenetic field defects are common; and (3) cell subpopulation composition between tumor and control may significantly differ leading to spurious epimutation results, in particular in comparison of iCCA with its predominantly hepatocyte-derived surrounding normal liver tissue. Potential solutions to some of these issues include the use of appropriate donor tissues comprising truly normal cholangiocytes (potentially introducing inter-individual variability as a confounder) or extensive laser microdissection of samples. Alternatively, cellular deconvolution strategies should be incorporated into CCA EWAS. While this may be computed purely using statistical algorithms ('reference-free') [95], future establishment of reference cholangiocyte epigenomes ('reference-based'), alongside epigenomes of co-occurring cells (hepatocytes, fibroblasts, endothelial, immune), is highly warranted to accurately optimize these analyses to the hepatobiliary system.

Definitively proving the causative or consequential role of high confidence epimutations is notoriously difficult. Estimates of the extent of DNA methylation alterations driven by SNPs range from 22-80% [93], indicating a need for tight mutational controls in epigenomic studies. Similarly, discriminating epigenetic-driven transcriptomic changes from transcriptome-driven epigenetic changes supports matched epigenome and transcriptome profiling experiments. Such 'cross-omics' analysis was recently generated by the TCGA, highlighting *IDH* mutant-associated *ARID1A* promoter hypermethylation and subsequent transcriptional silencing [4]. Similar difficulties also exist at the phenotypic level in establishing whether epimutations are causative or a consequence of CCA. While certain epimutations have been tentatively proffered as 'epi-drivers' in liver cancer purely on the basis of recurrence, these are insufficient to address the

causation/consequence debate. Rather, functional epigenetics experiments involving the targeted modulation of epimutations followed by their rescue are required, technologies which are only in the early stages of development [96-98].

6. CONCLUSION

To date, CCA remains largely understudied from an epigenetics-perspective, likely due in part to its status as a rare orphan disease. Nonetheless, its unique epigenetically-orientated mutational profile indicates biliary tract malignancies may comprise ideal solid tumors to benefit from epigenetic biomarkers and, potentially, epigenome-targeted therapies enabled through continued CCA epigenome characterization. Many epigenetic modifications (for example, RNA modification or chromosome conformation states) have yet to be investigated and those which have (ncRNA, DNA methylation, histone PTMs, chromatin remodeling) require expansion and refinement to (epi)genome-wide scales, facilitated by universal EWAS guidelines and standards. Only then may epigenomic data be accurately allied with other genome readouts, heralding 'integromics'-driven personalized medicine for CCA patients.

FIGURE TEXT

Figure 1. Genomic alterations of epigenetic regulators in CCA patients. Plot depicting the frequency of mutation and structural alterations to epigenetic regulators in TCGA biliary tract cancer tissue samples. Data were assessed from whole-exome sequencing experiments conducted by TCGA CHOL consortium. Categories of epigenetic enzymes were mined from EpiFactors database (<http://epifactors.autosome.ru/>).

Figure 2. Schematic representation of pathways modulated by long non-coding RNAs in CCA cells. H19, HULC and MALAT1 are upregulated in CCA cells. LncRNA-miR sponging has been shown for these lncRNAs and miR-let-7a/let-7b, miR-372/miR-373 and miR-204, respectively. In this process, sponging has been demonstrated to increase the expression of inflammation-related genes, IL-6 and CXCR4, causing abnormal inflammatory state, which is involved in the pre-neoplastic onset and progression of the disease. Secondly, lncRNA (NEAT1) is upregulated in CCA cells when the level of its upstream regulator BAP1 is attenuated, thereby, enhancing sensitivity to gemcitabine treatment. Third, a high expression level of lncRNA AFAP1-AS1 promotes growth and metastasis of CCA cells. Also, increased expression of the mitochondrial enzyme encoded by the gene *CPS1* and its lncRNA (CPS1-IT1) have been shown to promote CCA proliferation. These data emphasize the potential of lncRNAs as diagnostic and prognostic markers as well as putative regulators of drug response in CCA.

Figure 3. DNA methylome dysregulation during cholangiocyte transformation. The malignant transformation of normal cholangiocytes is accompanied by gross insult to the DNA methylome. This is mediated through diverse mechanisms which impede DNA methylation catalysis, including mutations (e.g. IDH1, IDH2), epigenetic alterations (e.g. DNMT-targeting miRs) and autocrine factors (e.g. elevated IL-6

levels). The CCA methylome displays several hallmarks characteristic of cancer. These include (1) promoter DNA hypermethylation of genes with tumor suppressive functions, resulting in gene body hypomethylation and transcriptional silencing; (2) promoter hypomethylation of genes with oncogenic functions, leading to gene body hypermethylation and transcriptional activation; and (3) loss of methylation at intergenic regions, promoting reactivation of endoparasitic sequences and genomic instability. *5hmC*: 5-hydroxymethylcytosine. *DNMT*: DNA Methyltransferase. *TSG*: Tumor Suppressor Gene.

Figure 4. SWI/SNF remodeling complex dysfunction in cholangiocarcinogenesis. Cholangiocarcinogenesis is a multi-step process involving the degeneration of normal bile ducts to hyper-proliferative pre-malignant lesions *in situ* (e.g. BiIN, IPN), followed by acquisition of an invasive phenotype to form primary adenocarcinoma. SWI/SNF is a multi-protein chromatin remodeling complex responsible for directed repositioning of nucleosomes, enabling efficient DNA repair, transcriptional homeostasis and genome stability. Recurrent mutations in SWI/SNF complex members, *ARID1A* and *PBRM1*, have been detected in CCA patients and have been specifically identified to occur after the development of carcinoma *in situ*. The broad consequences of such late mutational events (likely DNA misrepair/damage, transcriptome dysregulation and genomic instability) remain to be characterized in CCA and, given their timing during disease pathogenesis, SWI/SNF dysfunction may contribute to manifestation of the invasive phenotype. *BiIN*: Biliary Intraepithelial Neoplasia. *IPN*: Intra-ductal Papillary Neoplasia. *SWI/SNF*: SWItch/Sucrose Non-Fermentable.

Figure 5: A rational framework for conducting EWAS in CCA. Expansion of epigenetic analyses from locus-specific to (epi)genome-wide scales presents new challenges for experimental design. These include careful study population stratification to avoid potential confounding factors (age, gender, hepatitis infection, alcohol exposure, NAFLD/NASH), cellular deconvolution of epigenetic signals (using reference-based or reference-free statistical algorithms, microdissection) and cautious inference of causation or indirect causation (through matched 'omic' controls and functional editing of epimutations). Prospective consideration of such limitations is warranted in CCA to maximize likelihood for successful translation of findings into clinical trials. *EWAS*: Epigenome-Wide Association Study. *NAFLD*: Non-Alcoholic Fatty Liver Disease. *NASH*: Non-Alcoholic Steatohepatitis.

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Figure 1

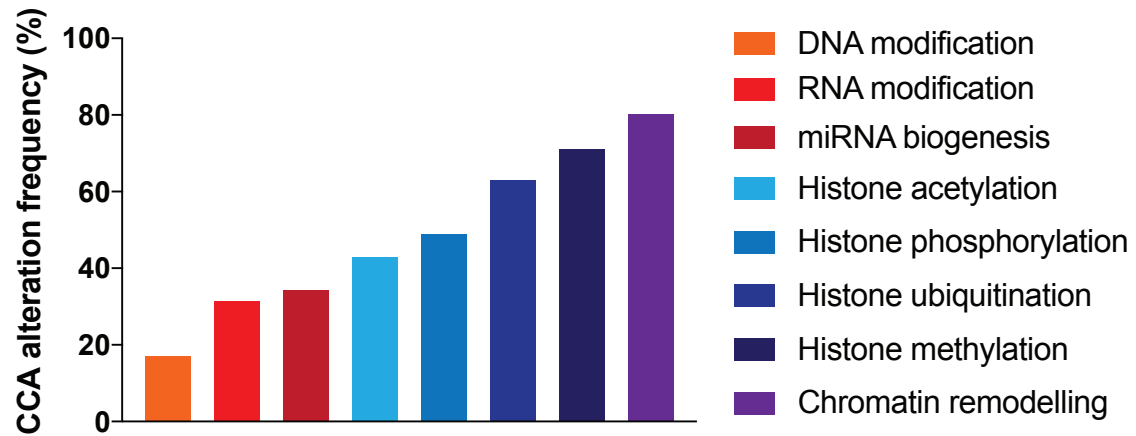


Figure 2
CCA cell

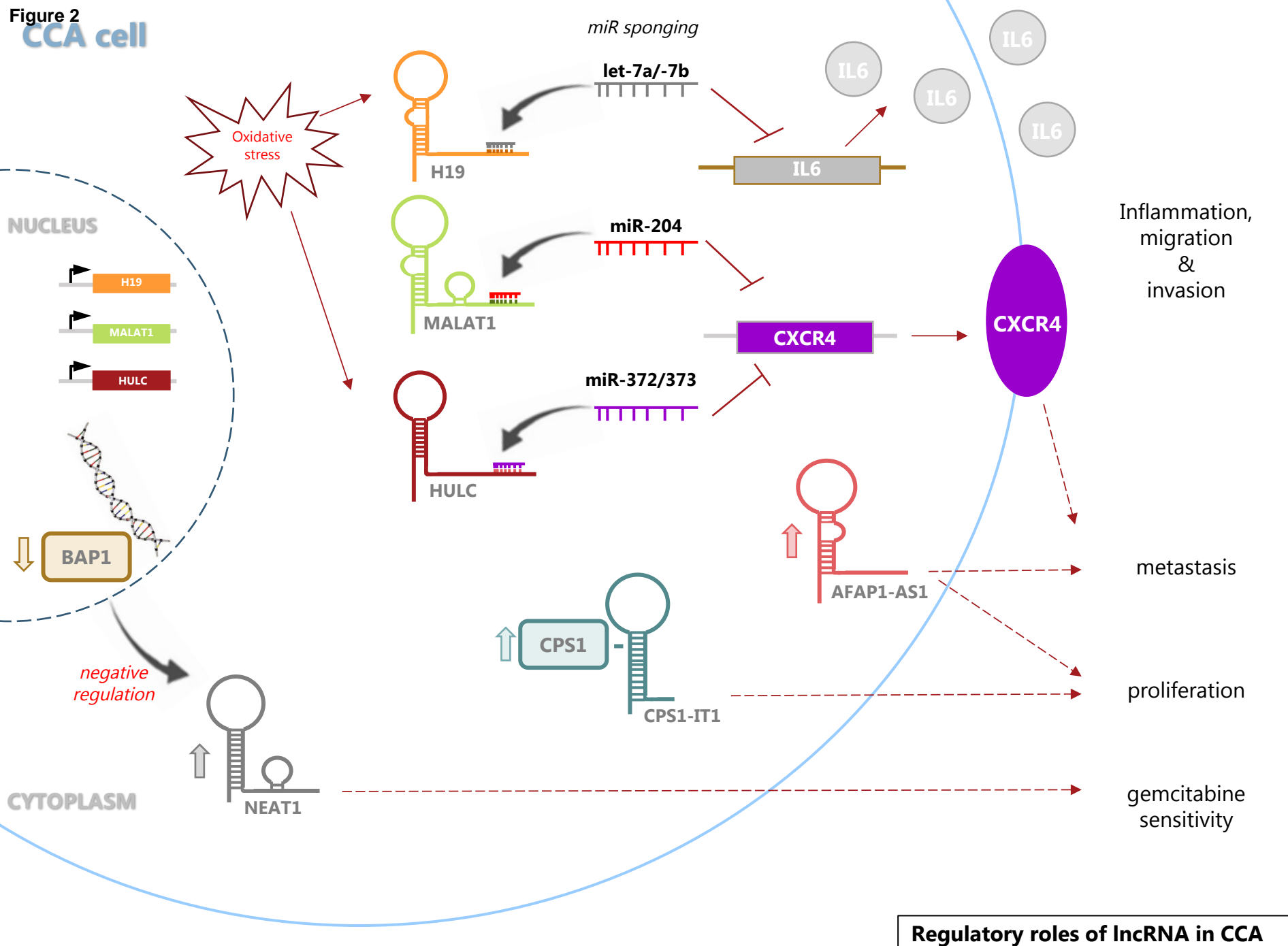


Figure 3

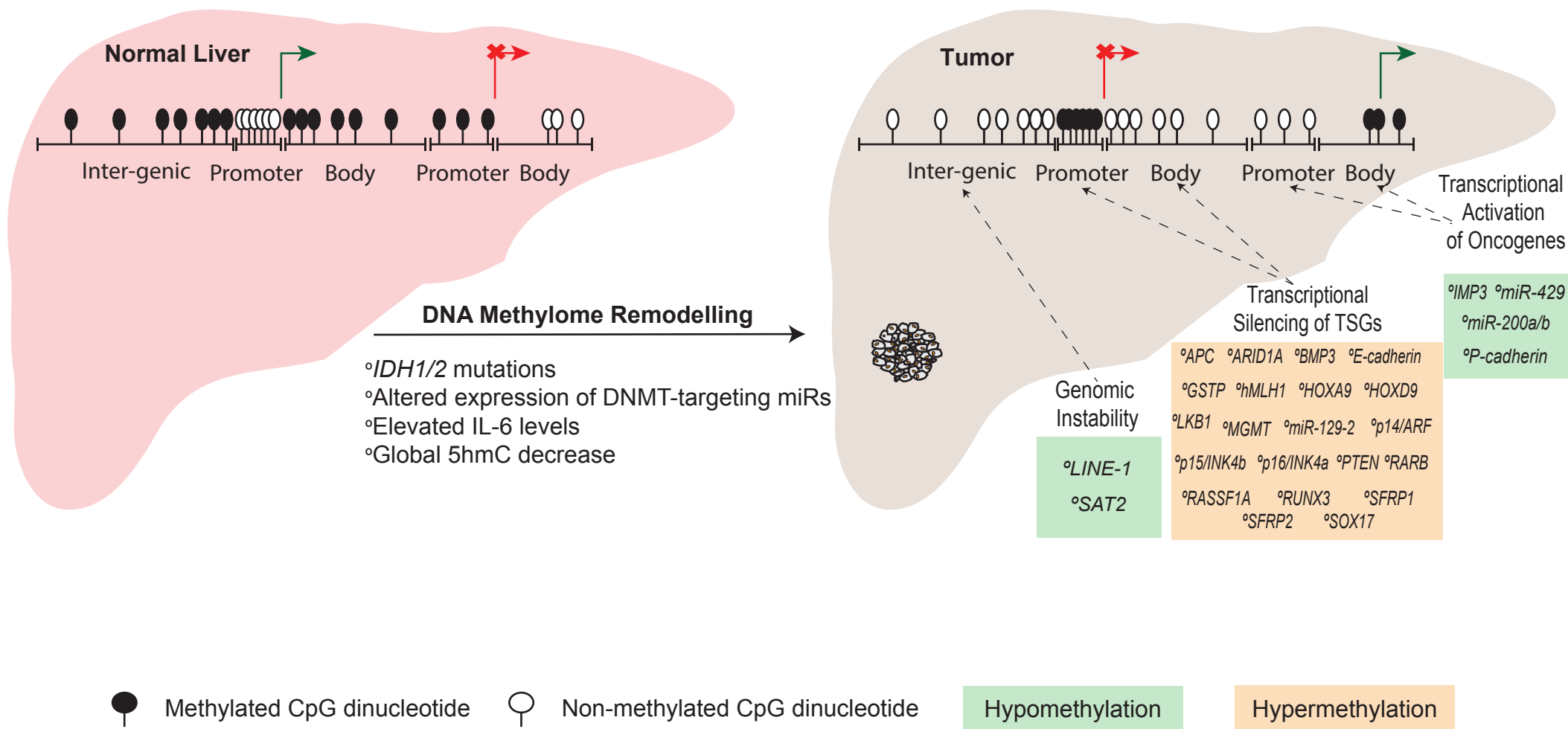


Figure 4

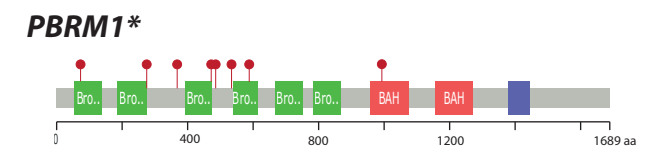
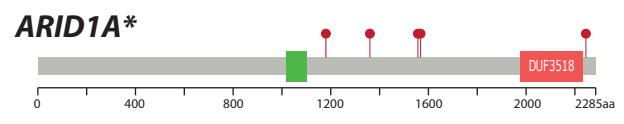
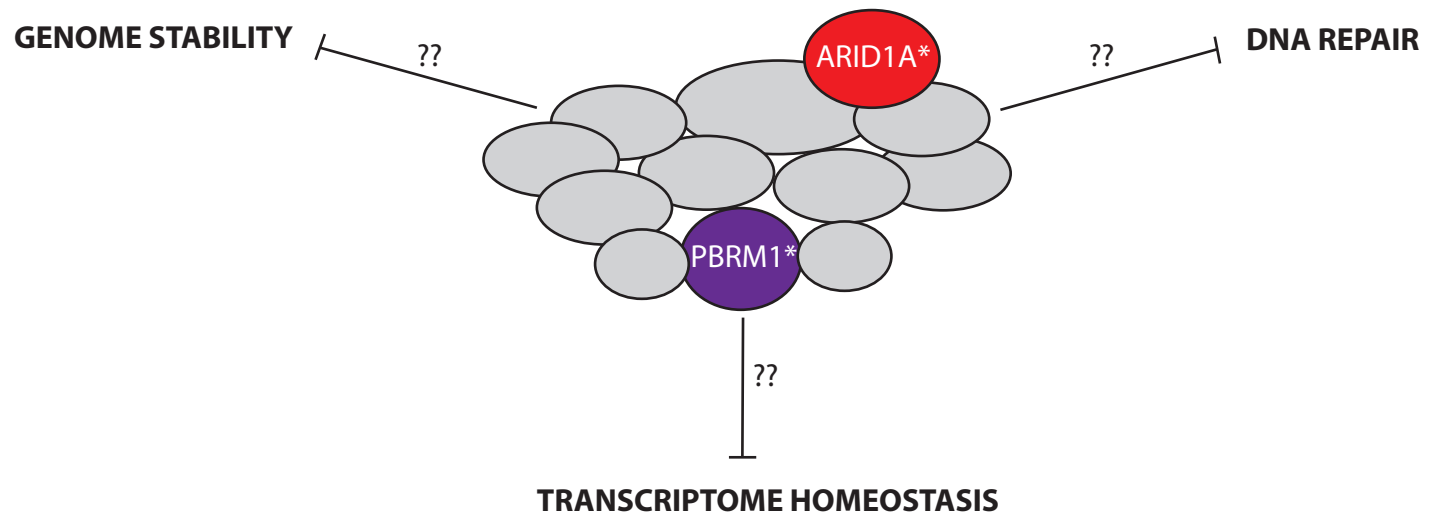
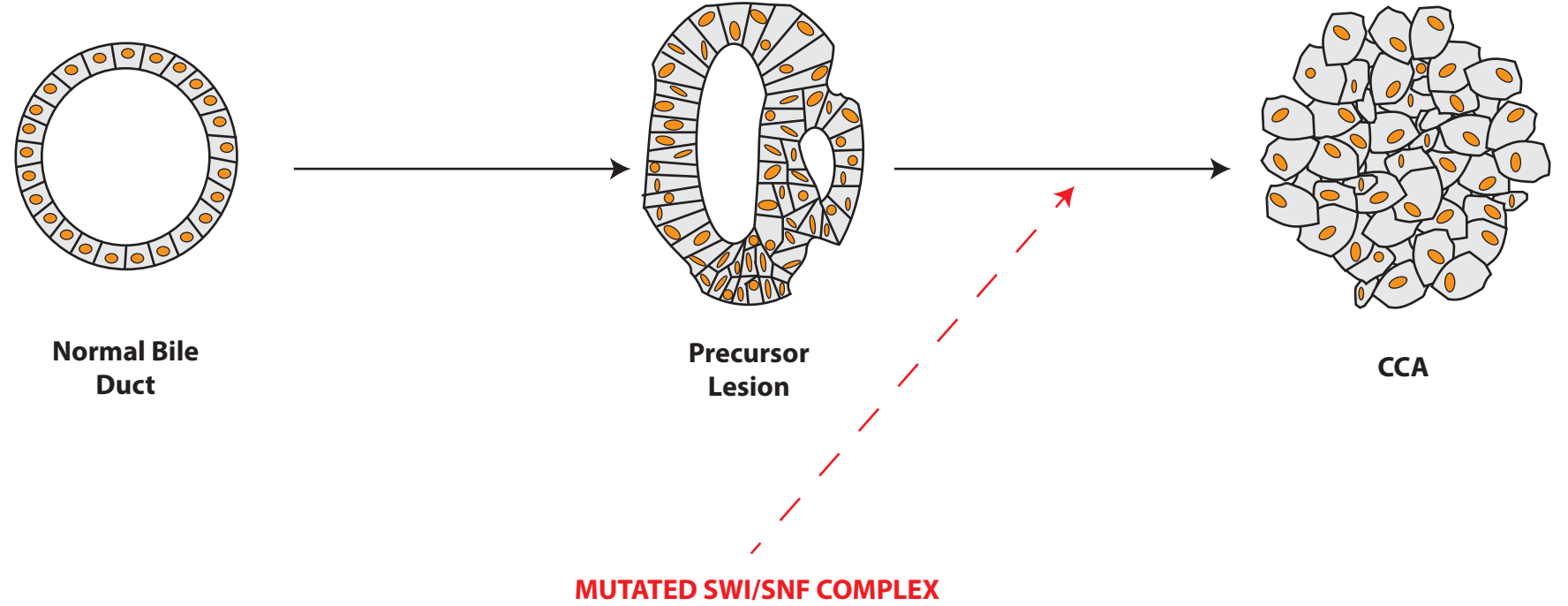


Figure 5

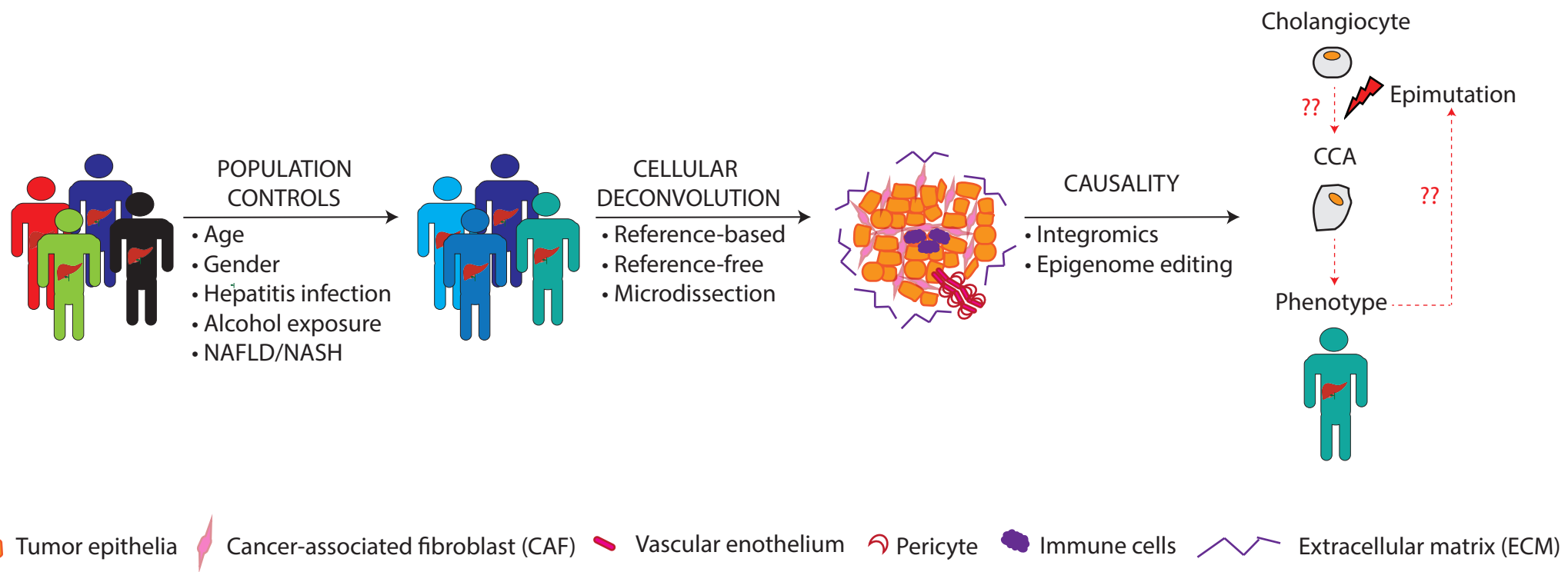


Table 1. MicroRNA expression in CCA, target genes and functions.

miR	expression	Target(s)	Role	Ref.
miR-21	↑	RECK, PTEN, PTPN14	Migration/invasion, proliferation	[18, 20, 99]
miR-421	↑	FXR	Migration/invasion, proliferation	[100]
miR-124	↓	SMYD3	Migration/invasion	[59]
miR-138	↓	RhoC	Migration/invasion, proliferation	[101]
miR-144	↓	LIS1	Migration/invasion, proliferation	[102]
miR-200b/c	↓	ROCK2; SUZ12	Migration/invasion; drug resistance	[23]
miR-376c	↓	GRB2	Migration	[103]
miR-605	↓	PSMD10/Gankyrin	Migration/invasion, apoptosis, inflammation	[104]
miR-494	↓	CDK6	Proliferation	[105]
miR-26a/b	↑	15-PGDH/HPGD	Proliferation	[106]
miR-141	↑	CLOCK	Proliferation, circadian rhythm	[18]
miR-200b	↑	PTPN12	Proliferation, drug resistance	[18]
miR-26a	↑	GSK-3β	Proliferation, colony formation	[107]
miR-31	↑	RASA1	Proliferation, apoptosis resistance	[108]
miR-214	↓	Twist	EMT	[27]
miR-200c	↓	NCAM1	EMT	[22]
miR-34a	↓	SMAD4	EMT	[24]
miR-204	↓	SNAI2, Bcl2	EMT	[25, 26]
miR-221	↑-↓	PTEN; PIK ₃ R1	EMT; drug resistance	[28, 109]
miR-17-92 cluster	↑	PTEN	EMT, migration/invasion, proliferation, cell cycle, inflammation	[110]
miR-let7a	↑	NF2	Drug resistance & survival	[38]
miR-320	↓	Mcl-1	Drug resistance	[26]
miR-205	↓	<i>To be clarified</i>	Drug resistance	[109]
miR-148a	↓	DNMT1	Proliferation, DNA methylation, poor prognosis	[30]
miR-152	↓	DNMT1	Proliferation, DNA methylation, poor prognosis	[30]

miR-370	↓	MAP3K8; WNT10B	Proliferation, DNA methylation	[29, 111]
miR-373	↓	MBD2	Epigenetic changes in DNA methylation	[31, 32]
miR-29b	↓	Mcl-1; PIK ₃ R1, MMP-2	Apoptosis; drug resistance	[109, 112]
miR-25	↑	TRIAL DR4	Apoptosis resistance	[113]
miR-410	↓	XIAP	Apoptosis resistance	[34]
miR-101	↓	COX-2, VEGF	Angiogenesis, tumor growth	[114]
miR-203	↓		Clinical relevance (poor prognosis)	[115]

Abbreviations: *RhoC*, Ras homolog gene family, member C; *LIS1*, platelet-activating factor acetylhydrolase isoform 1b; *NCAM1*, neural cell adhesion molecule 1; *15-PGDH*, 15-hydroxyprostaglandin dehydrogenase; *RASA1*, RAS p21 GTPase activating protein 1; *TRIAL DR4*, TNF-related apoptosis inducing ligand death receptor-4; *ROCK2*, Rho-associated protein kinase 2; *XIAP*, X-linked inhibitor of apoptosis protein; *PTEN*, Phosphatase and tensin homolog; *RECK*, Reversion-inducing-cysteine-rich protein with kazal motifs; *PTPN14*, Tyrosine-protein phosphatase non-receptor type 14; *PTPN12*, Tyrosine-protein phosphatase non-receptor type 12; *SMAD4*, SMAD family member 4; *FXR*, Farnesoid X receptor; *SMYD3*, SET and MYND domain-containing protein 3; *SUZ12*, Polycomb protein SUZ12; *SNAI2*, snail family transcriptional repressor 2; *Bcl2*, B-cell lymphoma 2; *PIK₃R1*, Phosphatidylinositol 3-kinase regulatory subunit alpha; *GRB2*, Growth factor receptor-bound protein 2; *PSMD10*, 26S proteasome non-ATPase regulatory subunit 10; *GSK-3β*, Glycogen synthase kinase 3 beta; *HPGD*, Hydroxyprostaglandin dehydrogenase 15-(NAD); *CLOCK*, Circadian Locomotor Output Cycles Kaput; *Mcl-1*, Induced myeloid leukemia cell differentiation protein; *MMP-2*, Matrix metalloproteinase-2; *COX-2*, Prostaglandin-endoperoxide synthase 2; *VEGF*, Vascular endothelial growth factor; *DNMT1*, DNA (cytosine-5)-methyltransferase 1; *MAP3K8*, Mitogen-activated protein kinase kinase kinase 8; *WNT10B*, Wnt Family Member 10B; *CDK6*, Cell division protein kinase 6; *MBD2*, Methyl-CpG-binding domain protein 2; *NF2*, Neurofibromin 2.

***Conflict of Interest Form**

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