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## DNA methylation in cell plasticity and malignant transformation in liver diseases

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### Abstract

The liver possesses extraordinary regenerative capacity mainly attributable to the ability of hepatocytes (HCs) and biliary epithelial cells (BECs) to self-replicate. This ability is left over from their bipotent parent cell, the hepatoblast, during development. When this innate regeneration is compromised due to the absence of proliferative parenchymal cells, such as during cirrhosis, HCs and BEC can transdifferentiate; thus, adding another layer of complexity to the process of liver repair. In addition, dysregulated lineage maintenance in these two cell populations has been shown to promote malignant growth in experimental conditions. Here, malignant transformation, driven in part by insufficient maintenance of lineage reprogramming, contributes to end-stage liver disease. Epigenetic changes are key drivers for cell fate decisions as well as transformation by finetuning overall transcription and gene expression. In this review, we address how altered DNA methylation contributes to the initiation and progression of hepatic cell fate conversion and cancer formation. We also discussed the diagnostic and therapeutic potential of targeting DNA methylation in liver cancer, its current limitations, and what future research is necessary to facilitate its contribution to clinical translation.

### Keywords

Liver progenitor cell; cholangiocarcinoma; hepatocellular carcinoma; transdifferentiation; epigenetics; cellular reprogramming

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Conflict of Interest Statement

None of the authors have any interests to declare related to this study.

## Introduction

The liver is a central organ that maintains systemic tissue homeostasis by governing diverse metabolic functions including nutrient metabolism, detoxification and production/secretion of various enzymes (Monga 2015, Russell and Monga 2018). These numerous and complex tasks are facilitated by a single epithelial cell type, the “hepatocyte (HC)”, which accounts for more than 90% of the liver parenchyma. Aside from HCs, another parenchymal epithelial cell in the liver is the cholangiocyte (biliary epithelial cell, BEC), accounting for 5–7% of the total liver parenchyma. BECs accept HC-produced bile acid and transport it to the intestine through the common bile duct (Ko, Russell et al. 2019). Combined, these two epithelial cell populations account for more than 95% of hepatic parenchymal cells.

Although HCs and BECs have a dichotomy in morphology, size, function, and transcriptome in the adult liver; moreover, both are derived from a bi-potent parent cell called a “hepatoblast (HB)” during liver development. Additionally, both cell types contribute to liver regeneration primarily by self-replication as shown in the mammalian 2/3 partial hepatectomy (PHx) model (Hu and Monga 2021, Michalopoulos and Bhushan 2021). The entire mechanism is tightly orchestrated by numerous signaling pathways along with epigenetic regulators to initiate and halt parenchymal cell replication (Michalopoulos 2017, Macchi and Sadler 2020). These transient, but strictly controlled, transcriptional reprogramming events allocate limited energy to cell cycle responses and/or basal metabolic roles in the repopulating HCs (Arechederra, Berasain et al. 2020).

Unlike the 2/3 PHx model, chemical/genetic insults result in dying parenchymal cells in the regenerating tissue microenvironment. This leads to a completely different compensatory response in the liver. While intact HCs and BECs initiate self-replication (similar to the 2/3 PHx model), duct-like cells, which express BEC markers, are activated and subsequently proliferate and expand from the peri-portal region. This phenomenon results in the activation and expansion of bipotent liver progenitor cells (LPCs) generating both HCs and BECs (Ko, Russell et al. 2019, Gadd, Aleksieva et al. 2020). Unless differentiated into adult cells, active LPCs promote inflammation and stromal growth by secreting various cytokines and chemokines. Indeed, the degree of LPC activity has been demonstrated to correlate positively with liver disease severity in mouse and human (Ko, Russell et al. 2019). In transdifferentiation between HCs and BECs, epigenetic regulators determine which genes to turn on or off during regeneration and results in chromatin packing as either eu- or hetero-chromatin (Arechederra, Berasain et al. 2020, Aloia 2021, Basu and Tiwari 2021). Therefore, modulating chromatin condensation is critical for distributing transcription machinery accessibility and, as a result, transcriptional activation.

Theoretically, transdifferentiation into functional HCs and BECs are considered to only occur in end-stage cirrhotic liver when the liver has lost the ability to regenerate (Raven, Lu et al. 2017, Schaub, Huppert et al. 2018, Ko, Russell et al. 2019, Russell, Lu et al. 2019, Gadd, Aleksieva et al. 2020). Indeed, cirrhosis is counted as irreversible; 10–20% of patients progress to liver cancer without transplantation. In the cirrhotic liver, dying HCs and BECs have strong stromal expansion with irreversible fibrosis, inflammation, and continuous injury; all of which may contribute to an oncogenic microenvironment.

Here, cell fate changes via chromatin structure and epigenome remodeling can potentially increase risk for malignant transformation (Sandhu, Shire et al. 2008, Nebbioso, Tambaro et al. 2018). In this regard, the Zender<sup>26</sup> and Loude<sup>27</sup> groups have demonstrated HC transformation into different lineage fates via distinct epigenetic reprogramming can contribute to oncogenic transformation in the liver. Particularly, experimentally proven HC-derived cholangiocarcinoma (CCA, malignant bile duct cancer), LPC-derived hepatocellular carcinoma (HCC, malignant HC cancer), and mixed ICC/HCC indicate simultaneous cellular identity reprogramming and malignant transformation (Sandhu, Shire et al. 2008, Aloia 2021, Hu, Molina et al. 2022, Ko, Kim et al. 2022). Here, identifying epigenetic regulators will be key to reduce malignant transformation of activated/primed LPCs.

In the cirrhotic liver, lineage conversion and malignant transformation are both dependent on a delicate/pathologic imbalance of transcriptional activation and repression. This balance is regulated by both transcription machinery and epigenetic modifiers. First, chromatin remodelers modify the nucleosome to close and/or expose gene loci of transcription regulation complexes. Next, a variety of repressors, enhancers, and activators are recruited to perform the necessary duty to execute lineage commitment (Greenberg and Bourc'his 2019, Macchi and Sadler 2020, Martinez-Redondo and Izpisua Belmonte 2020, Aloia 2021, Basu and Tiwari 2021). In general, epigenetic regulation for chromatin remodeling consists of 1) post-translational histone modifications such as acetylation, methylation, phosphorylation, amination, etc; 2) exchange of core histones with histone variants; 3) action of various non-coding RNAs; and 4) DNA methylation of CpG islands. These mechanisms directly impact cell fate determination. Of these, DNA methylation is among the most studied and is carried out by a single enzyme family called DNA methyltransferases (DNMTs) (Sandhu, Shire et al. 2008, Greenberg and Bourc'his 2019). DNMTs have well-known transcriptional repressor functions; these enzymes typically repress tumor suppressors and/or senescence inducers during cancer development. Additionally, DNMTs are also reported to regulate the expression of fate commitment genes. Furthermore, in addition to cell-intrinsic effects, modulation of DNMTs are also implicated in the tumor microenvironment, particularly with regards to immune responses (Segovia, San Jose-Eneriz et al. 2019, Zhang, Yang et al. 2020, Hu, Liu et al. 2021).

Overall, delineating epigenetic mechanisms for cell plasticity and subsequent malignant transformation would be fundamental since preventative modulation of key elements might reverse the irreversible pathologic progression of end-stage liver diseases into a treatable stage. In this review, we will focus on the role of DNA methylation, one of the key epigenetic transcription regulators, in these two distinct but crucial cellular events. The clinical implications of altering DNA methylation in human liver cancer will also be discussed from diverse perspectives.

## Basics of DNA methylation

DNA methylation is an epigenetic modification catalyzed by DNA methyltransferases, which add a methyl group to the 5'-carbon of the pyrimidine ring of cytosine. 5-methylcytosine (5mC) is mainly found in the palindromic CpG dinucleotides (Zemach, McDaniel et al. 2010). The high-density of CpG regions are called "CpG islands" (CGIs).

DNA methylations are commonly localized in intergenic regions, transposons, or gene bodies; however, CpG islands are located at the promoter regions and are enriched in over two-thirds of mammalian promoters (Larsen, Gundersen et al. 1992, Deaton and Bird 2011). Although CGIs at promoters are hot spots for DNA methylation, there are two types of unmethylated CGI promoters, which are regulated by histone modification: 1) transcriptionally activated CGI promoters are enriched in trimethylated histone H3 Lys4 (H3K4me3) (Piunti and Shilatifard 2016), and 2) transcriptionally inactive CGI promoters show enrichment of Polycomb repressive complex 2 (PRC2)-mediated trimethylated histone H3 Lys27 (H3K27me3), which are subject to reactivate gene expression response to environmental stress (Marasca, Bodega et al. 2018). Figure 1A illustrates the domain structure of DNMTs and figure 2 depicts three distinct DNA methylation processes, enzymes involved, and transcriptional consequences. DNMT family consists of DNMT1, DNMT2, DNMT3A, DNMT3B, and DNMT3L in the human genome (Fig. 1A). DNMT1, DNMT3A, and DNMT3B are canonical DNMT enzymes that show methyltransferase activity. However, DNMT2 and DNMT3L are non-canonical DNMT enzymes with a lack of activity because of the loss of or truncated catalytic domain.

There are three types of regulatory processes in DNA methylation: 1) *de novo* DNA methylation, 2) maintenance of symmetrical methylation at replication foci, and 3) demethylation which are driven by different enzymes with distinct molecular structures (Fig. 2). DNMT3A/B are essential enzymes for *de novo* methylation that is largely guided by histone tails. Structurally, DNMT3 contains three functional domains: Pro-Trp-Trp-Pro (PWWP) domain; an ATRX-DNMT3L-DNMT3A (ADD) domain; and a C-terminal catalytic domain. The PWWP domain can bind to H3K36me3 that is enriched in the gene body of actively transcribed genes; this regulates the balance of transcription and DNA methylation in the gene bodies (Ge, Pu et al. 2004, Wagner and Carpenter 2012). The PWWP point mutation impairs the restriction of extra methylation by blocking DNMT3 anchoring to the H3K36 regions (Heyn, Logan et al. 2019, Sendzikaite, Hanna et al. 2019). The ADD domain binds to H3K4 and is repulsed by H3K4me3, a histone modification present in active promoters (Ooi, Qiu et al. 2007, Otani, Nankumo et al. 2009, Zhang, Jurkowska et al. 2010). Once the ADD domain binds to H3K4, the catalytic domain at C-terminus, the MTase domain, is released and catalyzes the DNA methylation (Guo, Wang et al. 2015). The unbound ADD domain can interact with the MTase domain and cause the autoinhibition of DNMT3 enzymes. The binding of ADD and PWWP to modified histone tails is critical for DNMT3 to identify specific target regions which is critical for toggling *de novo* methylation on and off. In DNMT3A/B-mediated *de novo* methylation, catalytically inactive DNMT3L is required for methyltransferase activity via stabilizing DNMT3A (Veland, Lu et al. 2019); specific inhibition of DNMT3L impairs overall *de novo* methylation activity (Jia, Jurkowska et al. 2007).

While *de novo* DNA methylation can take place in any CpG context across the genome, symmetrical (both strands of DNA) CpG methylation is only maintained during DNA replication by the enzyme DNMT1 in collaboration with another multidomain protein, the E3 ubiquitin-protein ligase UHRF1. Except for the conserved catalytic domain at the C-terminus, like other DNMTs, DNMT1 has unique domains which are specifically required for the maintenance of symmetrical DNA methylation (Chen and Zhang 2020):

1) DNMT1-associated protein 1 (DMAP1) binding domain, 2) a proliferating cell nuclear antigen (PCNA) binding domain, 3) a replication foci-targeting sequence (RFTS) domain, 4) a CXXC domain, and 5) two Bromo-adjacent homology (BAH) domains (Li and Zhang 2014). Each domain has a specific role and interaction partners. DMAP1 binding domain can interact with the transcriptional repressor DMAP1 and histone deacetylase 2 (HDAC2) (Rountree, Bachman et al. 2000). The PCNA binding domain and RFTS domain contribute to localizing DNMT1 to replication foci during the S phase (Chuang, Ian et al. 1997, Nishiyama, Yamaguchi et al. 2013, Qin, Wolf et al. 2015, Ishiyama, Nishiyama et al. 2017). RFTS also provides DNMT1 autoinhibitory activity by sequestering in the enzyme's catalytic domain (Song, Rechkoblit et al. 2011, Takeshita, Suetake et al. 2011, Ishiyama, Nishiyama et al. 2017). The CXXC domain can bind to unmethylated CpGs (Pradhan, Esteve et al. 2008, Song, Rechkoblit et al. 2011). Lastly, BAH domains are involved in the localization of DNMT1 to the replication foci (Yarychkivska, Shahabuddin et al. 2018). Despite having its own DNA methyltransferase activity, UHRF1 is required for DNMT1 to retain methyltransferase function in replicating cells (Bostick, Kim et al. 2007, Sharif, Muto et al. 2007). UHRF1 is a multidomain protein that consists of a ubiquitin-like (UBL) domain, a tandem TUDOR-PHD (TTD-PHD) domain, a SET-and RING-associated (SRA) domain, and a really interesting new gene (RING) domain (Xie and Qian 2018). The TTD-PHD domains contribute to the symmetrical methylation of hemimethylated DNA through binding to H3R2 and H3K9me2 or me3 (Nady, Lemak et al. 2011, Arita, Isogai et al. 2012, Rothbart, Krajewski et al. 2012, Rothbart, Dickson et al. 2013). Importantly, the SRA domain of UHRF1 confers specific binding to DNMT1 for hemimethylated DNA at the replication foci during S-phase (Bostick, Kim et al. 2007, Sharif, Muto et al. 2007, Arita, Ariyoshi et al. 2008, Avvakumov, Walker et al. 2008, Hashimoto, Horton et al. 2008). When the SRA domain binds to hemimethylated DNA this induces allosteric changes in the RING domain, thereby activating its E3 ligase activity. This results in the accumulation of mono-ubiquitin at the H3K14, H3K18, and H3K23 (Nishiyama, Yamaguchi et al. 2013, Qin, Wolf et al. 2015); hence, generating the specific binding site for the RFTS domain of DNMT1. Consequently, the emergence of the binding site prevents the RFTS domain from masking the active site of DNMT1's catalytic domain, together diminishing the autoinhibitory effect of DNMT1 (Ishiyama, Nishiyama et al. 2017). Furthermore, it has been discovered that UHRF1 can directly recruit DNMT1 to chromatin through the UBL domain, implying the importance of the role of UHRF1 in DNMT1-dependent DNA methylation (Li, Wang et al. 2018). Therefore, chemical/genetic inhibition of DNMT1, or UHRF1, similarly impairs the maintenance of methylation status in replicating cells thereby provoking malignant transformation in various cells (Mudbhary, Hoshida et al. 2014, Kong, Chen et al. 2019). As aforementioned, once specific CpG regions are methylated, transcription of target genes is repressed. This occurs in tandem with diverse chromatin remodelers such as lymphocyte-specific helicase (LSH), H3K9 methyltransferase, histone deacetylases (Fuks, Burgers et al. 2000, Dennis, Fan et al. 2001, Fuks, Burgers et al. 2001, Deplus, Brenner et al. 2002, Esteve, Chin et al. 2006, Epsztejn-Litman, Feldman et al. 2008, Myant, Termanis et al. 2011, Tao, Xi et al. 2011), methyl-CpG-binding domain (MBD) <sup>6</sup> and MeCP2 (Ren, Horton et al. 2018). The detailed molecular process behind methylated DNA-mediated transcriptional repression is thoroughly described in other reviews (Greenberg and Bourc'his 2019).

Given the importance of rapid and tight regulation of transcription, like other epigenetic regulations, DNA methylation is a reversible molecular event triggered by TET proteins. TET is a family of methylcytosine dioxygenases involved in the processes of oxidative demethylation of 5mC. TET enzymes catalyze the oxidation of 5mC to 5hmC, which is progressively oxidized to 5-formylcytosine (5fC) and 5-carboxylcytosine (5caC) (Kriaucionis and Heintz 2009, Tahiliani, Koh et al. 2009, Ito, D'Alessio et al. 2010, He, Li et al. 2011, Ito, Shen et al. 2011, Wu and Zhang 2014). TET family consists of TET1, TET2, and TET3 (Fig. 1B). The catalytic domain at the C-terminus of TET consists of a double-stranded  $\beta$ -helix (DSBH) domain and a cysteine-rich domain (Pastor, Aravind et al. 2013). In addition, the full-length of TET1 and TET3 contain a CXXC domain, which binds to unmethylated CpG dinucleotides (Xu, Xu et al. 2012). This confers the specificity of TETs to bind CGIs (Xu, Wu et al. 2011). However, other TET proteins which lack the CXXC domain such as TET1s, TET2, TET3o, or TET3s, need additional factors to be recruited to chromatin. Therefore, DNA demethylation can occur by multiple facets such as DNA replication-dependent dilution of DNA methylation (Howell, Bestor et al. 2001), and also TET enzymatic activity.

In baseline liver, DNMT1 expression is strongly detected in all CD45<sup>+</sup> immune cells, confirming important roles for hepatic immune response (Wang, Malnassy et al. 2021). While all HCs showed faint DNMT1 expression, a subset of BECs express DNMT1; in contrast, DNMT1 is massively overexpressed in ICC cells, implying the crucial contribution in malignant transformation (Hu, Molina et al. 2022). In murine liver injury models, hepatic expression of *DNMTs* and *TETs* are heterogeneous based on the injury types and disease stage. This indicates rapid and dynamic alterations of 5mC/5hmC in the liver are responsive to various chronic injury conditions (Pogribny, Tryndyak et al. 2009, Booth, Branco et al. 2012, Page, Paoli et al. 2016). Therefore, understanding DNA methylation in each cell type of the liver in a context-dependent manner would be necessary to understand the insight of respective enzymes in the diseased liver.

## DNA methylation in liver cell plasticity

During HC-to-BEC trans-differentiation, and vice versa, origin cells revert to LPC status; transiently express HB genes; downregulate original lineage-specific genes; and acquire lineage commitment of destination cell fate. Table 1 summarizes currently available liver cell plasticity models with experimental strategies and roles for epigenetic regulators on fate conversion. This process is conducted in collaboration with diverse histone modifiers and transcription regulator complexes. Specifically, these modulators hinder the expression of genes involved in lineage fate conversion and repress HB/LPC-related genes. Since DNA methylation-mediated gene silencing takes place postnatally during liver development (Reizel, Sabag et al. 2018) for cell-lineage-specific genes, the reprogramming of DNA methylation is necessary for cell fate conversion in the adult liver. Figure 3 depicted the roles of epigenetic regulators including DNMTs and TETs during liver cell fate conversion in animal models.

### BEC-to-HC transdifferentiation

There are several reports on the role of DNA methylation in the setting of BEC-to-HC fate conversion. *Aloia et al.* recently showed that the global level of 5hmC in ductal cells in the liver following DDC diet was increased through TET1-mediated demethylation (Aloia, McKie et al. 2019). Additionally, they revealed that BEC-specific *Tet1* elimination impairs BEC-derived HC repopulation in the mouse. They observed that BEC-specific *Tet1* deletion significantly diminished initial ductal reaction (DR) and subsequent BEC/LPC-derived HC repopulation. By analyzing TET1-specific peaks, they identified demethylated TSS regions of mTOR, ErbB, MAPK, and Hippo-YAP1 target genes with respective change to expression. Interestingly, these targets have been reported as critical regulators in mouse and/or zebrafish BEC (LPC)-to-HC conversion models. While *Fxr-mTor-Akt* axis is required for the survival of LPCs (Jung, Kim et al. 2021), *EGFR (receptor for ErbB)-MAPK-Sox9* (So, Kim et al. 2021) and *YAP1-Notch-Sox9* (Yimlamai, Christodoulou et al. 2014) axis are BEC/LPC lineage-specific for ducts to become HCs. However, other repressors such as HDACs and/or DNMTs may diminish liver developmental genes and biliary lineage genes at a later stage. Along this line, the *Shin* group described the critical roles of *Hdac1/Lsd1* in murine and zebrafish BEC-to-HEC transition through epigenetically repressing *sox9* transcription, a crucial BEC/LPC lineage commitment gene (Ko, Russell et al. 2019). Recently, *He et al.* demonstrated *Dnmt1* contributes to the BEC-to-HC differentiation by repressing *p53* in the zebrafish model (table 1) (He, Zhou et al. 2022). Although *p53* is a well-known DNMT1 target in a variety of cancer cells, this study demonstrated that a 20-fold higher dose of DNMT inhibitor (DNMTi, 100 mM Azacytidine) may cause chemical DNA damage leading to *p53* expression in regenerating liver (Mudbhary, Hoshida et al. 2014). Another caveat of this report entails that changes to the methylome are restricted to the gene body region, but not around the *p53* TSS region (Aloia, McKie et al. 2019). This is not common for DNMT1 activity and needs to be carefully repeated in the mammalian system. Therefore, the comprehensive investigation of all DNMT functions in liver cell plasticity and the identification of their direct targets will be fundamental studies.

### HC-to-BEC transdifferentiation

In the context of HC-to-BEC conversion, understanding regulatory mechanisms is not fully elucidated. Since DNA methylation permits murine HC-driven ICC through the repression of HC commitment genes (Hu, Molina et al. 2022), DNMTs may contribute to fate conversion of HC into the biliary lineage. An important study on HC-to-BEC reprogramming by *Merrell et al.* observed a link between chromatin accessibility and transcriptional regulation (Merrell, Peng et al. 2021). Here, open chromatin regions in hepatocytes showed an enrichment of binding sites for important regulators of HC identity, including HNF4 $\alpha$ , C/EBP $\alpha/\beta$ , and FOXA. Counter to this, open chromatin regions in BECs and reprogrammed cells showed the enrichment of binding sites for important factors of biliary identity (TEAD, and HNF1 $\beta$ ). Comprehensively, during the HC-to-BEC reprogramming, 63% of BEC-specific open chromatin peaks were newly opened, and 61% of HC-specific open chromatin peaks were closed in the reprogrammed cells. This suggests significant changes in chromatin accessibility are strongly correlated to the changes in the transcriptional network during HC-to-BEC reprogramming. Hence, this study emphasized that epigenetic regulation plays a crucial role in the massive transcriptional change

during the reprogramming from HC-to-BEC. This outlines the crucial roles of diverse transcriptional repressors, such as DNMTs, in this setting to be elucidated by future studies.

## DNA methylation in liver cancer

Aberrant DNA methylation has been widely recognized as a key feature of human liver cancer (Kulis and Esteller 2010, Jusakul, Cutcutache et al. 2017, Nakaoka, Saito et al. 2017, O'Rourke, Lafuente-Barquero et al. 2019, Dhayat and Yang 2020, Izykowska 2020). A growing body of evidence implicates modulating DNA methylation in altering tumor growth (Baylin 2005, Kulis and Esteller 2010, Dhayat and Yang 2020) and cellular lineage commitment in the liver (Cheedipudi, Genolet et al. 2014, Moris, Pina et al. 2016, Folguera-Blasco, Cuyas et al. 2018). Table 2 lists publications that discuss the impact of DNA methylation modulation in various murine liver cancer models.

## DNA Methylation and background etiologies leading to HCC

Progressing from chronic disease to neoplasia is a complex biological process. In the liver, chronic fibrosis is preceded by cirrhosis which in turn leads to HCC. However, the onset of carcinogenesis in the liver is complicated further by underlying pathologies that lead to primary HCC. These underlying pathologies tend to result in specific mutations in genes (e.g., *TERT* promoter, *CTNNB1*, *TP53*, *AXIN1*, *ARID1A*, etc.) that promote cell survival, growth thus disease pathogenesis (Nault and Zucman-Rossi 2016, Harding, Zhu et al. 2019, Rebouissou and Nault 2020, Paradis and Zucman-Rossi 2022). However, whether specific chronic liver pathologies result in specific mutational signatures is still being studied.

Cirrhosis results from the liver's attempts at repair during chronic disease, regardless of etiology, leading to hepatocytes becoming senescent (Rudolph, Chang et al. 2000). Hepatocytes that avoid this contribute to HCC pathogenesis (Farazi, Glickman et al. 2003). Here, studies have found, via methylome sequencing, several genes can become either hypermethylated or hypomethylated; thus, corresponding to disease pathogenesis which has been summarized elsewhere (Villanueva, Portela et al. 2015, Martinez-Quetglas, Pinyol et al. 2016, Cancer Genome Atlas Research Network. Electronic address and Cancer Genome Atlas Research 2017, Rebouissou and Nault 2020). DNMT enzyme expression regulating these methylation events is known to be dysregulated during HCC pathogenesis (Saito, Kanai et al. 2001, Saito, Kanai et al. 2003, Mudbhary, Hoshida et al. 2014, Barcena-Varela, Caruso et al. 2019, Bayo, Fiore et al. 2019). Here, DNMTs can play a role in silencing tumor suppressor genes and genes that maintain hepatocyte differentiation (Fernandez-Barrena, Arechederra et al. 2020, Rebouissou and Nault 2020). Therefore, the link between DNMT dysregulation and pathogenesis of HCC is strongly defined and a broader understanding of this mechanism can open the door to new therapeutic approaches.

Given the marked reduction of viral infection-related liver diseases and the explosive increase in obesity, non-alcoholic fatty liver disease (NAFLD) and non-alcoholic steatohepatitis (NASH) are the most prevalent etiology progressing to HCC (Byrne and Targher 2015, Lazarus, Mark et al. 2022). NAFLD/NASH is one of among multiple pathologies that are categorized as dysregulated metabolic syndromes, and is progressively increasing in incidence in western countries like the United States (Byrne and Targher 2015,



Lazarus, Mark et al. 2022). Broadly, NAFLD is a precursor to oxidative damage and inflammation that results in HCC(Lazarus, Mark et al. 2022). Interestingly, several studies have identified specific patterns of methylation with regards to NASH or NAFLD related HCCs(Kuramoto, Arai et al. 2017, Kurokawa, Yoneda et al. 2022). For example, *PPAR $\gamma$*  hypermethylation was detected in NAFLD patients indicating its potential utility for HCC surveillance(Hardy, Zeybel et al. 2017). Also, studies have found that genes like *PPAR $\alpha/\delta$*  or *TGF $\beta$ 1* can demonstrate either hyper- or hypomethylation, respectively, depending on NAFLD disease severity(Zeybel, Hardy et al. 2015). *PPAR $\alpha$ /PPAR $\delta$*  and *TGF $\beta$ 1* are anti- and pro-fibrosis genes, respectively, so their differential methylation is important to disease progression. Specifically with regards to NASH driven HCC, Kuramoto et al. identified hypomethylation/overexpression of genes *ACOT7*, *FOXN3*, *MAML3*, *PCNT*, *PMPCA*, *RASA2*, *SPG7* and *WHSC1*; and hypermethylation/reduced expression of genes *RF4*, *DHX36*, *FLCN*, *FYTTD1*, *MICAL3*, *MRPS24*, *NQO2*, *PKP4*, *PLAG1*, *PROSER3*, *TRAPPC10*, *WDR6*, *ZC3H14* and *ZZEF1*. Of the targets identified, MAML3 was discussed as a potential regulator of the Wnt/ $\beta$ -catenin pathway and may be responsible for driving NASH towards oncogenesis(Alves-Guerra, Ronchini et al. 2007). Clearly, NAFLD-related HCC produces a unique epigenetic landscape that clinicians can potentially utilize regarding disease surveillance and diagnosis.

Alcohol-related liver disease (ARLD) also results in robust fibrosis and cirrhosis leading to oxidative damage. While ARLD can result in changes to the methylation landscape, these alterations are more likely associated with overall cirrhosis(Hlady, Tiedemann et al. 2014, Habash, Sehrawat et al. 2022). However, one recent study found hypomethylation in the *FKBP5* gene specifically in ARLD patient tissues(Kusumanchi, Liang et al. 2021). The FKBP5 protein regulates *Yap1/Tead1* expression of the cytokine CXCL1 and potentiates inflammation in the underlying fibrosis. Also, *PPAR $\alpha/\delta$*  and *TGF $\beta$ 1* are hypermethylated, while *Collagen1A1* is hypomethylated in ARLD tissues compared to normal livers(Zeybel, Hardy et al. 2015). While *PPAR $\alpha/\delta$*  are also hypermethylated in NAFLD patients, the overall degree of methylation in ARLD patients compared to NAFLD patients is significantly lower(Zeybel, Hardy et al. 2015). Thus, the ARLD methylome may be unique enough from NAFLD to distinguish when complexed together with current diagnostic approaches.

Classically, chronic viral HBV/HCV infection has been implicated in alternating the methylation landscape that progressively results in HCC(Nagashio, Arai et al. 2011, Um, Kim et al. 2011, Nishida, Kudo et al. 2012, Hlady, Tiedemann et al. 2014, Kuramoto, Arai et al. 2017, Wijetunga, Pascual et al. 2017, Hama, Totoki et al. 2018, Kurokawa, Yoneda et al. 2022). With regards to DNA methylation, HBV infection is associated with a progressive increase in methylation events during disease pathogenesis from early dysplasia to HCC in CpG loci(Um, Kim et al. 2011). For example, *APC* and *RASSF1A* demonstrate hypermethylation from HBV associated cirrhosis at all stages to progressed HCC. While hypermethylation of the tumor suppressor RASSF1A correlates with disease progression, the authors indicate that increased methylation of APC, a regulator of the Wnt/ $\beta$ -catenin pathway, is counterintuitive and most likely non-specific. In contrast, hypermethylation of the tumor suppressor, *PI6* is only observed in neoplastic stages(Um, Kim et al. 2011). This indicates that as HBV related HCC progresses the capacity of neoplastic cells to

enter the cell cycle increases as *P16* is silenced. Meanwhile, *SPRY2*, *ERK*, *GNMT*, and *PTEN* do not demonstrate differential methylation indicating their importance in the natural selection, survival, and proliferation of neoplastic cells (Um, Kim et al. 2011). On the other hand, HCV infection has been observed to have a stronger role in altering methylation patterns in cirrhotic patients compared to other etiologies which enhances the progression to HCC (Hlady, Tiedemann et al. 2014). One study found that methylation in common tumor suppressor genes (e.g., *HIC1*, *GSTP1*, *SOCS1*, *RASSF1*, *CDKN2A*, *APC*, *RUNX3*, and *PRDM2*) in HCV-infected patients was associated with shorter time to development of HCC, and the number of methylated genes in HCV patients were found to be an independent risk factor for developing HCC (Nishida, Kudo et al. 2012). Other groups have gone on to find unique epigenetic patterning comparing cirrhotic and HCC patients with chronic hepatitis which has the potential for differentiating patient risk for HCC development (Kurokawa, Yoneda et al. 2022).

Together, multiple environmental factors can influence the epigenetic landscape in the liver. These events have been demonstrated to potentiate pathogenesis during chronic liver injury leading up to oncogenic transformation. Understanding how each particular etiology (e.g., viral, chemical, etc.) specifically alters the methylome in the liver can provide information for HCC surveillance. The aberrant methylation may inform what personalized therapeutics patients can be treated with for preventive measures ultimately preventing an increasing burden on both patients and the healthcare system.

### **Common epigenetic landscape characteristics regarding methylation and HCC**

Recent studies have investigated whether the epigenetic landscape among HCC patients is consistent. What is consistent, however, is that underlying etiologies provide for unique epigenetic fingerprinting for HCC patients (Nishida, Kudo et al. 2012, Hlady, Tiedemann et al. 2014, Kuramoto, Arai et al. 2017, von Felden, Garcia-Lezana et al. 2020, Kurokawa, Yoneda et al. 2022). Taking into consideration the background etiologies discussed, the methylation status of HCC samples has been relatively thoroughly investigated (Villanueva, Portela et al. 2015, Cancer Genome Atlas Research Network. Electronic address and Cancer Genome Atlas Research 2017, Hama, Totoki et al. 2018, Kurokawa, Yoneda et al. 2022). As noted earlier, enhanced methylation is associated with reduced expression of tumor suppressor genes in HCC pathogenesis. Genes regulating cell cycle, in addition to those that regulate epithelial-to-mesenchymal transition, tend to be targets of hypermethylation (Fernandez-Barrena, Arechederra et al. 2020, Kurokawa, Yoneda et al. 2022). Recently, Zinc Finger Proteins have been identified as targets of hypermethylation, and can potentially differentiate between pre-malignant cirrhosis and early stage HCCs (Goncalves, Goncalves-Reis et al. 2022, Sun, Gan et al. 2022). With regards to disease progression, Kurokawa et al. identified *HNF4A*, *FABP1*, and *SGK1* can become hypermethylated during premalignant fibrosis, but become hypomethylated once progressed to HCC. This study demonstrates the complex dynamics of methylation status and disease pathogenesis which should not be taken for granted.

Studies have identified that hypomethylation can result in enhanced gene expression and potential genetic instability (Shen, Wang et al. 2013, Villanueva, Portela et al. 2015,

Hama, Totoki et al. 2018). Some hypomethylated regions may even pertain to specific transcriptional machinery such as *C/ebpβ*(Xiong, Wu et al. 2019), or members of the homeobox transcription factor family(Goncalves, Goncalves-Reis et al. 2022), or oncogenes like *Ras*(Maryam and Idrees 2018). In addition, recent studies have investigated the role of hypomethylation of Repetitive Elements (Res) in hepatocellular carcinogenesis. Specifically, investigators have consistently found that hypomethylation of the RE Long Interspersed Nuclear Elements 1 (LINE-1) is associated with genomic instability and worse overall prognosis(Baba, Yagi et al. 2018, Anwar, Hasemeier et al. 2019, Zheng, Hlady et al. 2019). LINE-1 elements compose of nearly 17% of the human genome and have the capacity to act at high frequency as retrotransposons if hypomethylated(de Koning, Gu et al. 2011, Lee, Iskow et al. 2012). Inadvertent hypomethylation of LINE-1 elements during carcinogenesis in the liver has the capacity to promote chromosomal dysregulation and promote oncogenic transformation. Studying how these, and other transposable elements, become activated in the background of chronic liver disease can lead to increased understanding of HCC oncogenesis and potential new therapeutic approaches.

### Common DNMT and other enzymes potentially mutated in HCCs

Dysregulated expression of DNMT enzymes is known to result in oncogenesis. Increased expression of DNMT1 has been observed in HCC patient tissues(Saito, Kanai et al. 2003, Mudbhary, Hoshida et al. 2014, Barcena-Varela, Caruso et al. 2019). This potentially leads to enhanced methylation and silencing of tumor suppressor genes. Additionally, other studies have identified that DNMT3a and DNMT3b are also upregulated in HCC patients(Saito, Kanai et al. 2001). One study analyzed HCC patient samples from TCGA data and found many patients have at least one mutation in genes that contribute to editing of the epigenome(Bayo, Fiore et al. 2019). Nearly 50% of these patients demonstrated upregulation in genes that contribute to changes in the epigenome while 20% were downregulated when comparing normal tissue to HCC tissues(Bayo, Fiore et al. 2019). Considering how methylation status can confer disease pathogenesis it is crucial to understand how the expression of enzymes responsible for changes to the epigenome is altered during pre-malignancy to late-stage disease. This information will provide a better understanding of the role of these molecules in HCC development and progression.

### DNA methylation in CCA pathogenesis (Fig. 4)

There have been extensive studies linking inflammation and the development of CCA via the persistent secretion of inflammatory cytokines(Wu, Yiang et al. 2018). Among various inflammatory cytokines, interleukin 6 (IL-6) expression is correlated with regulating tumor suppressor gene expression, including *Dnmt1* *in vitro* and *in vivo* (Wehbe, Henson et al. 2006, Isomoto, Mott et al. 2007, Meng, Wehbe-Janek et al. 2008, Isomoto 2009, Braconi, Huang et al. 2010). These studies revealed that sustained *IL6/STAT3* signaling induces *Dnmt1*-mediated hypermethylation at the promoter regions of tumor suppressor genes such as *p53*, provoking BEC transformation into CCA, reflecting the well-described mitogenic role of IL6/STAT3 stimulation in CCA cells. Given the importance of inflammatory signatures in the transcriptome-based classification of human CCA(Sia, Hoshida et al. 2013), comprehensive investigations of response to immune checkpoint inhibitor (ICI) along

with DNMTi and/or IL6 inhibitor in IL6/STAT3-*Dnmt1* activated CCA cluster would be informative.

The most frequently observed hypermethylated regions in CCA are representative tumor suppressors such as *p16INK4a*(Braconi, Huang et al. 2010), *FoxM1*(Cao, Liu et al. 2020, Pogribna, Koonce et al. 2020), *TP53*(Zhang, Chen et al. 2018, Li, Wang et al. 2019) or *PTEN*(Kumar, Raeman et al. 2018, Zhang, Liu et al. 2018), which typically induce death/senescence of malignant cells. Hypermethylation of these cell cycle inhibitors results in unstoppable oncogenic proliferation in various cancer including CCA. Importantly, hypomethylation of *p16INK4a* has been demonstrated in more than 80% of clinical CCA. Pharmacologic inhibition of CDK4, a direct binding target of p16, suppresses CCA growth, especially when combined with either an mTOR inhibitor(Song, Liu et al. 2019) or FAK inhibitor(Zeng, Zhou et al. 2022), suggesting a potentially relevant combination therapy with precision medical approaches. Thus, a substantial investigation of targeting these genes as broad-spectrum supportive therapy in tandem with targeted therapy would be fundamental.

Intriguingly, recent studies revealed the essential roles of DNA methylation in HC transformation into iCCA cells. The Hippo-YAP1/TAZ signaling pathway plays a crucial role in the iCCA development, especially through epigenetic remodeling (Yimlamai, Christodoulou et al. 2014, Nishio, Sugimachi et al. 2016, Hyun, Al Abo et al. 2021, Song, Xu et al. 2021, Cigliano, Zhang et al. 2022, Li, Wu et al. 2022). Two recent studies reported DNA methylation is dysregulated under activated *Yap1/Taz-Tead* signaling in murine iCCA models (Hu, Molina et al. 2022, Wu, Mei et al. 2022). *Hu et al.* found that YAP1/TEAD activity is indispensable for the pathologic fate conversion of HC into biliary lineage by inducing DNMT1 expression in ICC cells. Importantly, they found that DNMT1 is preferentially localized to the promoter regions of HC lineage-specific genes regulated by HNF4 $\alpha$ , FOXA2, CEBP $\alpha/\beta$ , and HNF1 $\alpha$ ; thus, trans-differentiation into the biliary lineage is promoted and demonstrates the distinct roles of DNMT in HC-to-iCCA lineage reprogramming.

Recently, *Wu et al.* also described indispensable roles for *Tet1*-dependent DNA demethylation in YAP1-mediated HC-driven ICC formation(Wu, Mei et al. 2022). They revealed that TET1 directly binds to YAP1 target genes through the interaction with TEAD, thereby resulting in demethylation and a subsequent increase in expression of target genes. This effect only occurs at the distal intergenic regions which are enriched in the enhancer mark with *H3K4me1* rather than promoter regions. Taken together, not only is DNMT1-mediated DNA methylation of promoters contributes to HC identity loss, but this also results in the gain of BEC identity through *Tet1*-mediated DNA demethylation; all essential mechanisms in the context of *YAP1*-dependent HC-to-ICC transformation. Given that DNA methylation is diluted and lost during DNA replication without the maintenance of methylation by *Dnmt1*, the findings of *Hu et al.* suggest that *Dnmt1*-dependent methylation is essential for the transformed identity and the dysregulated cell cycle of cancer cells during their clonal expansion. However, to maintain DNA methylation on the newly replicated DNA in iCCA development, *de novo* methylation on the template DNA is a prerequisite. The mechanism by which *de novo* methylation is triggered at target regions remains

unknown. Thus, *Dnmt3a/b* may be elaborately regulated in a context-specific manner at the very early stages of HC-to-iCCA transformation.

Overall, DNA methylation may play heterogeneous roles in ICC based on the molecular drivers, stages, underlying etiologies and/or cellular origins. Given the tremendously heterogeneous methylome in clinical ICCs, it would be important to elucidate the distinct roles of DNA methylation regarding molecular targets and stages to consider FDA-approved DNMTi to appropriately test in potential responders in the clinic.

## Diagnostic and therapeutic potential of DNA methylation in liver cancer

Tables 3 and 4 summarize the literature on aberrant DNA methylation patterns in clinical HCC and CCA, respectively.

### Approaches to use DNA methylation as an HCC diagnostic

Epigenetics, especially regarding methylation, can provide a distinct differentiating fingerprint between patients, or more broadly etiological conditions. In fact, methylation status combined with advancements in technologies are sensitive enough to differentiate between cirrhosis and various HCC stages (Goncalves, Goncalves-Reis et al. 2022). Complexed with whole genome sequencing, identifying differentially methylated patterns can identify associations specific to early stages of HCC (von Felden, Garcia-Lezana et al. 2020, Goncalves, Goncalves-Reis et al. 2022, Kurokawa, Yoneda et al. 2022). Using differential methylation patterning can even now provide sufficient sensitivity and specificity in liquid biopsies regarding detection and even pathology site origin (Cohen, Javed et al. 2017). Studies that incorporate methylation for diagnosis or prediction can be powerful tools when combined with traditional methods for HCC surveillance. As this is the direction of several clinical trials ([NCT04856046](#), [NCT03694600](#), [NCT03804593](#)) it is promising that routine assessment of patient differential methylation may assist in overall surveillance and early detection for HCC.

Researchers and clinicians alike are optimistic about the potential of using methylome sequencing as a diagnostic, especially for the staging of HCC. This is demonstrated by the numerous clinical trials initiated and still ongoing which involve multiple modalities of testing for differential methylation. These studies have determined several methylated targets that can reach from 70% to 95% sensitivity and 89% to 92% specificity in detecting and staging HCC (Chalasan, Ramasubramanian et al. 2021, Goncalves, Goncalves-Reis et al. 2022). While promising, these studies acknowledge there are more gaps to be filled with regard to incorporating additional confounding factors such as sex, age, and race. Also, other studies are planning to incorporate differential alpha-fetoprotein levels together with differential methylation status to increase sensitivity and specificity.

Developing liquid biopsies are the most effective and approachable techniques that can provide availability to a large percentage of populations. A recent study demonstrated the specificity and sensitivity of distinct methylation patterns between cirrhosis and HCC patients when compared to normal samples (Goncalves, Goncalves-Reis et al. 2022). Here, the authors used a metadata approach combining the information from several datasets and

trained their algorithm such that they can not only differentiate between cirrhosis and HCC, but stage as well as specific target genes that may be the “canary in the coal mine” to determine progression from chronic disease to HCC. While using cell free DNA is the most approachable method for HCC surveillance(von Felden, Garcia-Lezana et al. 2020), these technologies require fine tuning prior to implementation in the clinic. To do this, clinical trials are needed to set standards which are currently being conducted in at least one trial that aims to determine the levels of methylation to prevent recurrence or metastasis in resectable HCC ([NCT04856046](#)).

Finally, on the direction of the future and cancer detection, [NCT05573217](#) is a new clinical trial that investigates cell-free methylation markers in liver cancer patients treated with systemic therapies to measure treatment response. Understanding how the epigenetic landscape shifts in HCC patients treated with systemic therapies is exciting as this information can potentially predict whether specific therapies are more effective than others, and when patients need to be shifted from one therapeutic intervention to another.

### Diagnostic significance of DNA methylation in clinical CCA

CCA still remains difficult to diagnose early and shows a poor survival rate with poor response to therapies(Sandhu, Shire et al. 2008, Limpaboon 2012). Like the other neoplasms, early detection is one of the most critical factors determining the prognosis of CCA patients. Often, CCA develops without underlying cirrhosis and lacks specific clinical signs, even compared to HCC. Thus, there is a dire need for the discovery of a more reliable, accurate, and effective prognostic strategy to detect the early stages of CCA. In this vein, detecting aberrant DNA methylation would be powerful since it is available in early CCA stages. These include intraductal papillary neoplasm of the liver/bile ducts (IPNL/B) and biliary intraepithelial neoplasia (BilIN), precancerous lesions of CCA and premalignant diseased liver. In addition, there are numerous reports demonstrating the efficacy of detecting hypermethylated targets using liver tissue or liquid biopsies, such as bile juice and serum, from CCA patients. According to tissue analysis, besides hypermethylated *p16INK4a*, several genes were suggested as suitable CCA biomarkers with significant sensitivity (>75%) and sensitivity (>90%) (e.g., CDO1, HPP1, SEMA3B, HOXA1 and SFRP1). While blood biopsy is the safest and most non-invasive source for hypermethylated biomarker detection, limited studies have been reported so far. Cell-free DNAs (cfDNAs) have been pursued as a biomarker to identify clinical CCAs since they can be released into circulating blood from tumor cells via metabolic processes (e.g., necrosis or apoptosis) and retain tumor-specific features (e.g., genetic mutations, epigenetic alterations, or chromosomal rearrangements(Warton and Samimi 2015, Lissa and Robles 2016, McAnena, Brown et al. 2017, Lu, Bi et al. 2018))(Mody, Kasi et al. 2019). Furthermore, recent reports provide evidence to support cfDNA as a prognostic biomarker(Wasenang, Chaiyarit et al. 2019, Yang, Ghos et al. 2021). *Wasenang et al.* found significant differences in DNA methylation levels of OPCML and HOXD9 from CCA patients(Wasenang, Chaiyarit et al. 2019) exhibiting 62.5% sensitivity and 100% specificity. Additionally, *Yang et al.* analyzed plasma samples from 53 eCCA and 117 control cohorts<sup>44</sup>. In this study, they determined nine markers with a sensitivity of 63%–86% and a specificity of 88%–98%: ZNF781, CYP26C1, RYR2, HOXA1, EMX1, ST8SIA1, PTGDR, PRKCB, and BMP3. Interestingly,

based on data sets from the Cancer Genome Atlas, all these markers were significantly hypermethylated and involved in cancer-related biological pathways, implying the efficacy of cfDNAs detecting CCA-specific hypermethylation as diagnostic. Altogether, numerous DNA methylation biomarkers have been suggested to improve the sensitivity of detecting CCA while specificity needs to be more carefully examined in larger patient cohorts with appropriate controls. Carefully considering specimens, such as those from HCC and PDAC, can provide a reliable and effective surveillance method for patients in premalignant stages at high-risk for developing CCA.

### Clinical trials using DNMT inhibitors in liver cancer

Fundamentally, the process of methylation leads to the silencing of genes which is counterintuitive to tumor progression. However, as discussed earlier, several groups have identified that tumor suppressor genes are the most likely genes to be methylated which in turn promotes tumor cell survival (Hlady, Tiedemann et al. 2014, Fernandez-Barrena, Arechederra et al. 2020, Goncalves, Goncalves-Reis et al. 2022, Kurokawa, Yoneda et al. 2022, Sun, Gan et al. 2022). In this direction, translational scientists have asked whether inhibiting DNMTs can reverse the methylation of tumor suppressor genes. In the liver specifically, DNMTi are rarely used for therapeutic purposes. Upon FDA approval, the DNMTi 5-azacytidine (Fig. 5A) under the trade name Vidaza has been used as a therapeutic for other malignancies for several years (Sekeres, Schuster et al. 2022). DNMT1 is inhibited by 5-azacytidine after its incorporation into DNA as a cytidine analog (Stresemann and Lyko 2008, Derissen, Beijnen et al. 2013, Kordella, Lamprianidou et al. 2021). With the incorporation of 5-azacytidine, the DNA structure is altered such that DNMT1 can no longer effectively methylate target DNA (Stresemann and Lyko 2008, McCabe, Brandes et al. 2009). 5-Azacytidine can be deactivated by cystine deaminase which is robustly expressed in the liver making the utilization of 5-azacytidine for liver pathologies, unfortunately, not very effective (Toh, Lim et al. 2019). Attempts to increase 5-azacytidine's bioavailability are ongoing, but some have derived analogs that increase the utility in the liver. 5-aza-4-thio-2-deoxycytidine (Aza-Tdc) is an example that has been implemented in the [NCT03366116](#) clinical trial to treat solid cancers which include the recruitment of patients with liver cancers. Aza-Tdc is a DNMT1 inhibitor by forming covalent bond with DNMT1 after Aza-Tdc is incorporated into the genome (Thottassery, Sambandam et al. 2014, Parker and Thottassery 2021) (Fig. 5B). With less off-target effects and increased specificity for DNMT1, Aza-Tdc is a promising drug that is understudied in the liver and can potentially contribute to broadening the therapeutic toolset (Thottassery, Sambandam et al. 2014, Parker and Thottassery 2021).

Guadecitabine, another 5-azacytidine analog, was developed to circumvent deactivation. It is converted into its bioactive metabolite, Decitabine, and is more stable in the liver since it is not readily deactivated by cystine deaminase (Bennett and Licht 2018) (Fig 5C). Decitabine has been used in a completed phase 2 clinical trial that aimed to treat colorectal metastasis to the liver ([NCT02316028](#)). During cell replication, Decitabine, similar to 5-azacytidine and other analogs, is incorporated into DNA and causes genome-wide hypomethylation which in turn is expected to reverse the suppressed expression of tumor suppressor genes (Derissen, Beijnen et al. 2013, Kordella, Lamprianidou et al. 2021).

Interestingly, pre-clinical studies demonstrated that Guadecitabine in cell lines can not only activate the expression of tumor suppressor genes, but also the expression of endogenous retroviruses (Liu, Zhang et al. 2018). Endogenous retroviruses are small pieces of genetic material that are normally silenced, and when reactivated can lead to a significant innate inflammatory response (Chiappinelli, Strissel et al. 2015, Roulois, Loo Yau et al. 2015, Liu, Ohtani et al. 2016, Stone, Chiappinelli et al. 2017). Thus, it is reasoned that DNMTi treatment may work in tandem with immune checkpoint inhibitors (ICIs). Since the current standard of care in HCC and many other malignancies has recently implemented ICIs, clinical trials have suggested combining ICIs with Guadecitabine (NCT03257761 and NCT01799083) (Liu, Zhang et al. 2018, Moufarrij, Srivastava et al. 2020). One Phase 1b trial combines the use of Guadecitabine and the PD-1 checkpoint inhibitor Durvalumab for primary HCC and other hepatobiliary malignancies (NCT03257761). While promising, minimal *in vivo* pre-clinical work in the liver has been conducted using Guadecitabine, thus a full understanding of the molecular mechanisms influenced by Guadecitabine is needed. Therefore, using Guadecitabine may not only reactivate the expression of tumor suppressor genes, but also induce an inflammatory response which in combination with checkpoint inhibitors may be a viable approach for patients.

## Concluding remarks

It is widely accepted that epigenetic alterations are critical in the onset and progression of various liver diseases, including liver cancer. Truly, differential DNA methylation is a delicate, two-way street in the liver that requires sensitive regulation. While there are beneficial consequences regarding specific cellular responses, such as proliferation and fate conversion that ultimately contribute to restoring normal liver function, this process can become dysregulated and result in tumorigenesis. Given the high prevalence of DNA hypermethylation in liver cancer, this field has grown significantly in the last decade, and technological advances have dramatically accelerated related research. Accordingly, these advances have led to the identification of many methylation targets that provide high sensitivity and specificity in liver cancer surveillance. While promising, since DNMTs/TETs lack specific binding motifs, other than CpG sequences, the full understanding of their function is complicated due to the lack of understanding of genetic tropism of DNA (de)methylation in the liver (Irizarry, Ladd-Acosta et al. 2009). Indeed, a recent study demonstrated the heterogeneity of DNA methylation located at the promoter and intergenic regions; thereby attributing diverse, distinct features of clinical CCAs (Jusakul, Cutcutache et al. 2017). These findings highlight the need for more comprehensive and unbiased complexed methylome and transcriptome analysis. As aforementioned, despite numerous publications reporting hypermethylation targets in liver cancer, upstream regulators triggering DNMT/TET expression or activity remain largely unknown. Furthermore, redundancy and crosstalk with other epigenetic transcription repressors such as HDACs should be comprehensively investigated. This is especially important in the context when DNMT/TET activity is inhibited as this may inform on the full therapeutic effect of DNMT inhibition in liver cancer. Since the anti-tumor effect of DNMT inhibition in liver cancer is minimal in both animal studies and clinical trials, a full understanding of epigenetic modifications should provide insight into how to improve



these approaches. Therefore, a substantial understanding of context-dependent upstream regulators, as well as the identification of functional methylation targets, capable of rescuing the effect of DNMT inhibition, will be essential for considering the use of FDA-approved DNMTi in liver cancer. Finally, using hypermethylation targets for disease surveillance and prediction is a promising discovery and will be fundamental for managing premalignant liver diseases and early-stage liver cancer.

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## Abbreviation

<b>HC</b>	hepatocyte
<b>BEC</b>	biliary epithelial cell
<b>HB</b>	hepatoblasts
<b>PHx</b>	hepatectomy
<b>LPC</b>	liver progenitor cell
<b>CCA</b>	cholangiocarcinoma
<b>HCC</b>	hepatocellular carcinoma
<b>ICC</b>	intrahepatic cholangiocarcinoma
<b>DNMT</b>	DNA methyltransferase
<b>5mC</b>	5-methylcytosine
<b>CGI</b>	CpG island
<b>H3K4me3</b>	trimethylated histone H3 Lys4
<b>PRC2</b>	Polycomb repressive complex 2
<b>H3K27me3</b>	trimethylated histone H3 Lys27
<b>PWWP</b>	Pro-Trp-Trp-Pro
<b>ADD</b>	ATRX-DNMT3L-DNMT3A
<b>H3K36me3</b>	trimethylated histone H3 Lys36
<b>MTase</b>	methyltransferase
<b>UHRF1</b>	Ubiquitin Like With PHD And Ring Finger Domains 1
<b>DMAP1</b>	DNMT1-associated protein 1
<b>PCNA</b>	proliferating cell nuclear antigen

<b>RFTS</b>	replication foci-targeting sequence
<b>BAH</b>	Bromo-adjacent homology
<b>HDAC2</b>	histone deacetylase 2
<b>UBL</b>	ubiquitin-like
<b>TTD-PHD</b>	tandem TUDOR-PHD
<b>SRA</b>	SET-and RING-associated
<b>RING</b>	really interesting new gene
<b>H3R2</b>	histone H3 Arg2
<b>H3K9me2</b>	dimethylated histone H3 Lys9
<b>H3K14</b>	histone H3 Lys14
<b>H3K18</b>	histone H3 Lys18
<b>H3K23</b>	histone H3 Lys 23
<b>LSH</b>	lymphocyte-specific helicase
<b>MBD</b>	methyl-CpG-binding domain
<b>MeCP2</b>	methyl-CpG binding protein 2
<b>TET</b>	ten-eleven translocation
<b>5hmC</b>	5-Hydroxymethylcytosine
<b>5fC</b>	5-formylcytosine
<b>5caC</b>	5-carboxylcytosine
<b>DSBH</b>	double-stranded $\beta$ -helix
<b>DDC</b>	3,5-Diethoxycarbonyl-1,4-Dihydrocollidine
<b>DR</b>	ductal reaction
<b>TSS</b>	transcription start site
<b>mTOR</b>	mammalian target of rapamycin
<b>MAPK</b>	mitogen-activated protein kinase
<b>YAP</b>	Yes1 Associated Transcriptional Regulator
<b>NOTCH</b>	Neurogenic locus notch homolog protein 1
<b>SOX9</b>	SRY-Box Transcription Factor 9
<b>LPC</b>	liver progenitor cell

<b>ERFR</b>	Epidermal Growth Factor Receptor
<b>FXR</b>	Farnesoid X receptor
<b>AKT</b>	AKT Serine/Threonine Kinase 1
<b>TP53</b>	Tumor protein P53
<b>HNF4A</b>	Hepatocyte Nuclear Factor 4 Alpha
<b>C/EBP</b>	CCAAT Enhancer Binding Protein Beta
<b>FOXA</b>	Forkhead Box A1
<b>TEAD</b>	TEA Domain Transcription Factor 1
<b>HNF1<math>\beta</math></b>	Hepatocyte nuclear factor-1beta
<b>TERT</b>	Telomerase Reverse Transcriptase
<b>CTNNB1</b>	Catenin Beta 1
<b>ARID1A</b>	AT-Rich Interaction Domain 1A
<b>NAFLD</b>	non-alcoholic fatty liver disease
<b>NASH</b>	non-alcoholic steatohepatitis
<b>PPAR</b>	Peroxisome Proliferator Activated Receptor
<b>TGF<math>\beta</math>1</b>	Transforming Growth Factor Beta 1
<b>ARLD</b>	Alcohol-related liver disease
<b>FKBP5</b>	FKBP Prolyl Isomerase 5
<b>CXCL1</b>	C-X-C Motif Chemokine Ligand 1
<b>HBV</b>	hepatitis B virus
<b>HCV</b>	hepatitis C virus
<b>LINE-1</b>	Long Interspersed Nuclear Elements 1
<b>RE</b>	Repetitive Element
<b>IL6</b>	Interleukin 6
<b>STAT3</b>	Signal Transducer And Activator Of Transcription 3
<b>ICI</b>	immune checkpoint inhibitor
<b>IPNL/B</b>	intraductal papillary neoplasm of the liver/bile ducts
<b>BIIN</b>	biliary intraepithelial neoplasia
<b>cfDNA</b>	cell-free DNAs

<b>PDAC</b>	Pancreatic ductal adenocarcinoma
<b>DNMTi</b>	DNMT inhibitors
<b>Aza-TdC</b>	5-aza-4 -thio-2 -deoxycytidine
<b>PD-1</b>	Programmed Cell Death 1
<b>MCD</b>	methionine- and choline-deficient
<b>TAA</b>	thioacetamide
<b>CDE</b>	choline-deficient ethionine-supplemented
<b>BDL</b>	bile duct ligation
<b>DAPM</b>	methylene dianiline
<b>CCL<sub>4</sub></b>	carbon tetrachloride
<b>NICD</b>	Notch intracellular domain
<b>Rbpj</b>	Recombination Signal Binding Protein For Immunoglobulin Kappa J Region
<b>IRES</b>	Internal ribosome entry site
<b>NTR</b>	nitroreductase
<b>Mtz</b>	Metronidazole
<b>CK19</b>	Keratin 19
<b>Alb</b>	Albumin
<b>BET</b>	bromodomain and extra-terminal domain
<b>HDTV1</b>	hydrodynamic tail vein injection
<b>AAV8</b>	adeno-associated virus serotype 8
<b>BDL</b>	bile duct ligation
<b>DEN</b>	diethylnitrosamine

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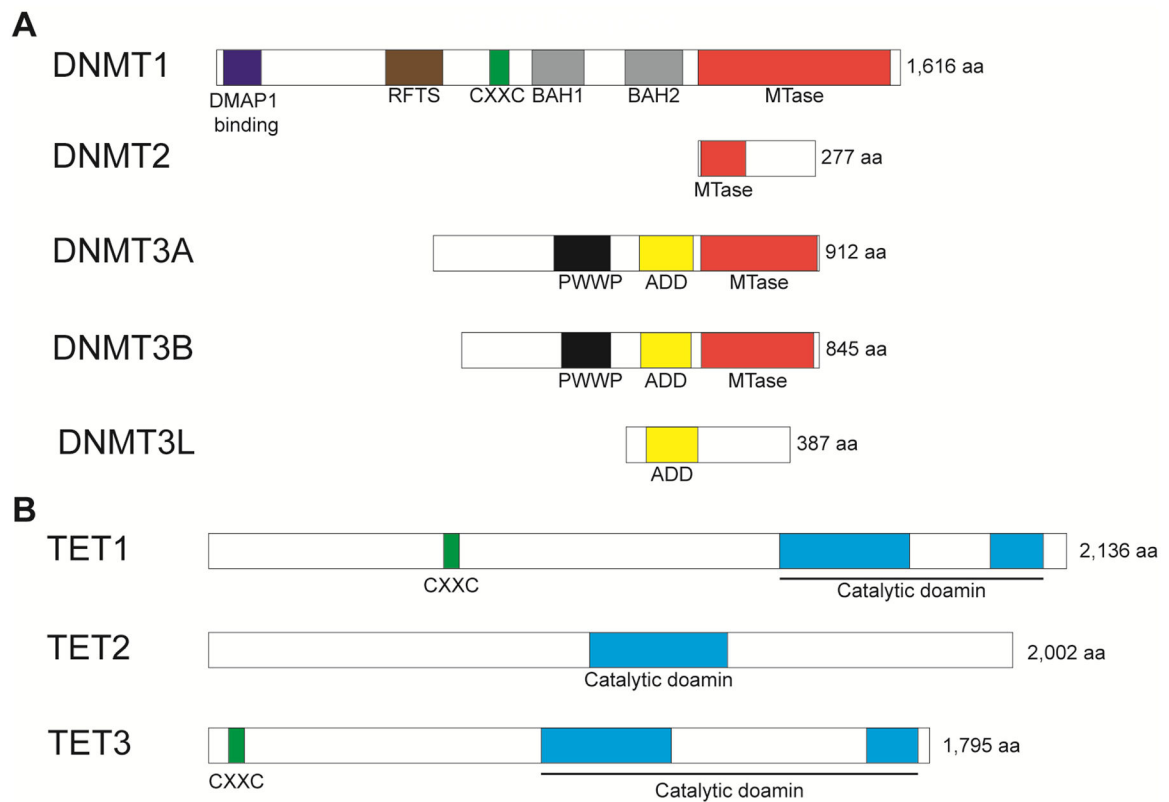


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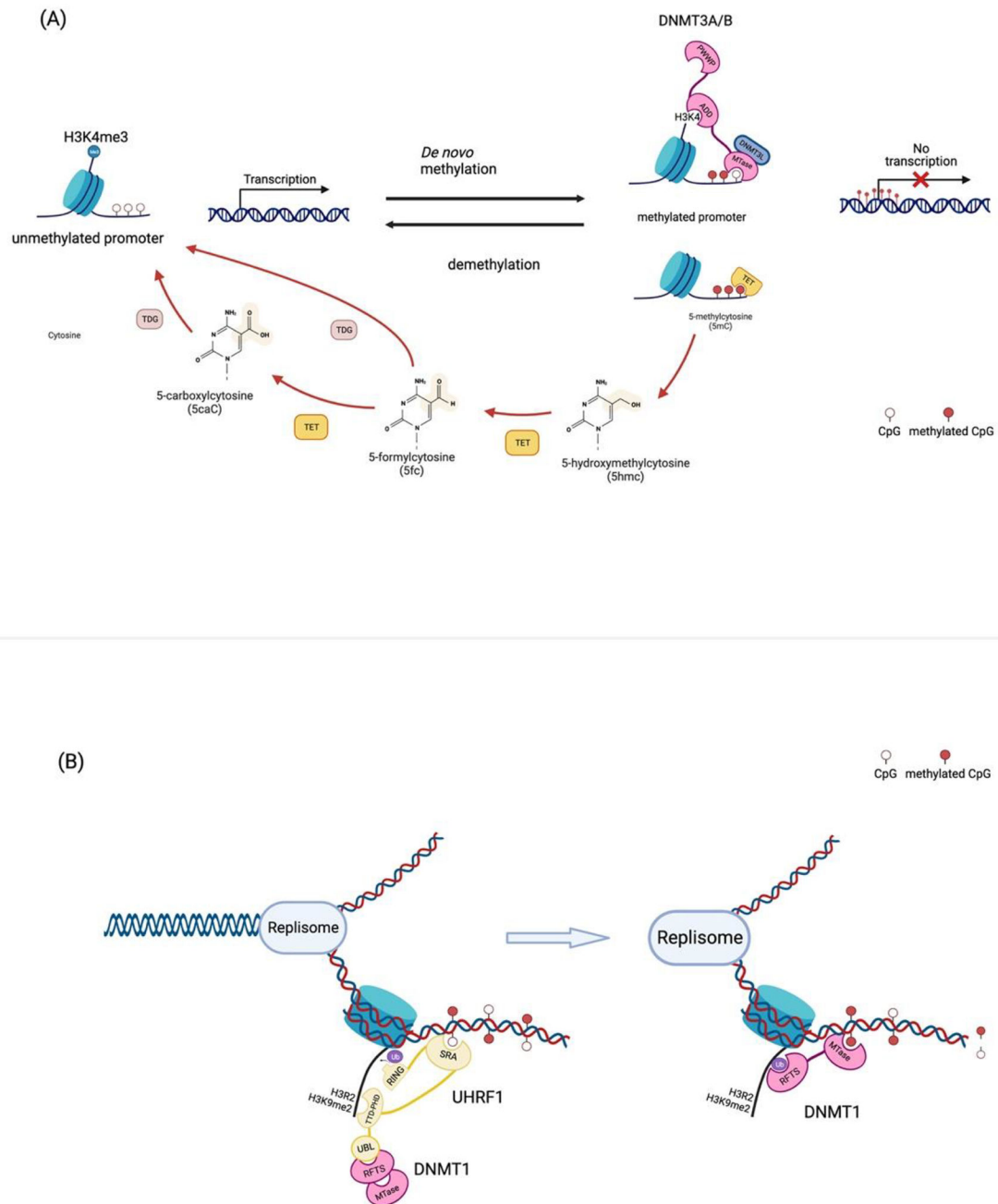
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**Figure 1. Domain structure of the human DNMT and TET family proteins.**

(A) Conserved domains of DNMTs showing DMAP1 binding, RFTS, CXXC, BAH, PWWP, ADD, and MTase domains. Canonical DNMTs, which are DNMT1, DNMT3A, and DNMT3B, retaining catalytic domain MTase, however, non-canonical DNMTs, which are DNMT2 and DNMT3L, showing truncated and loss of catalytic domain. (B) Conserved domains of TETs showing CXXC and catalytic domains.



**Figure 2. The machinery and mechanism of DNA methylation.**

(A) *de novo* DNA methylation and active demethylation at the promoter region. Once the methyl groups at H3K4, which marks active/poised promoters, are removed, ADD domain of DNMT3A/B binds to H3K4. This allows the methyltransferase domain to methylate 5-cytosine of DNA. The methylated CpG island of the promoter prevents transcriptional activation. The methylated cytosine (5mC) can be catalyzed by TETs. TETs progressively oxidize 5mC to 5hmC, 5fC, and 5caC. Thymine DNA glycosylase (TDG) also carries out 5fC and 5 acC oxidization via deamination. (B) DNA methylation maintenance. TTD-PHD domain of UHRF1 can recognize and bind to H3R2 and H3K9me2. Recruitment of UHRF1

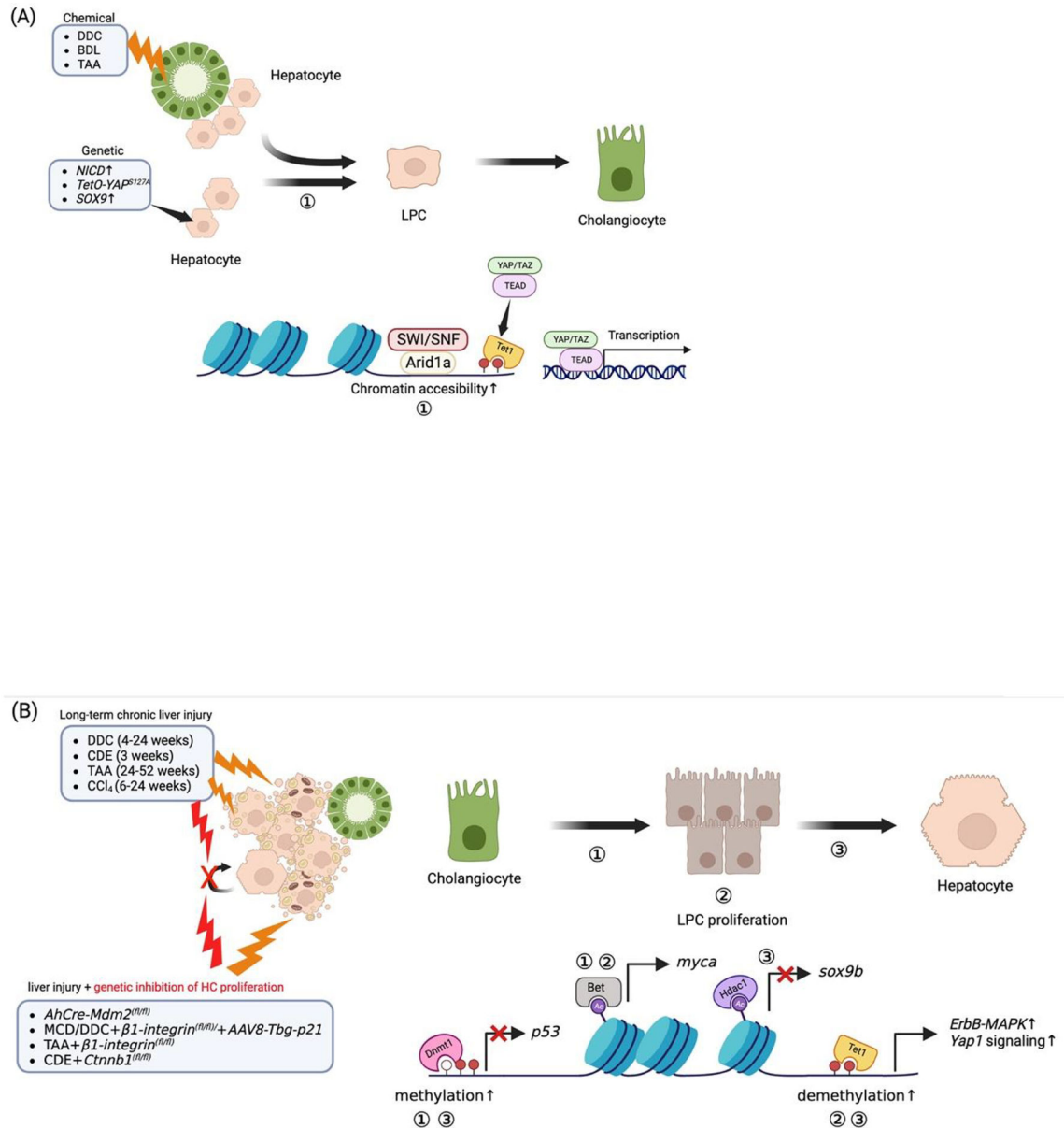
allows the SRA domain, which can recognize hemimethylated DNA, to the target site. This produces the RING domain that drives ubiquitination on the histone tail. This ubiquitination offers the docking site for RFTS of DNMT1. Binding of DNMT1 on the target site makes it possible that MTase methylates the hemimethylated DNA.

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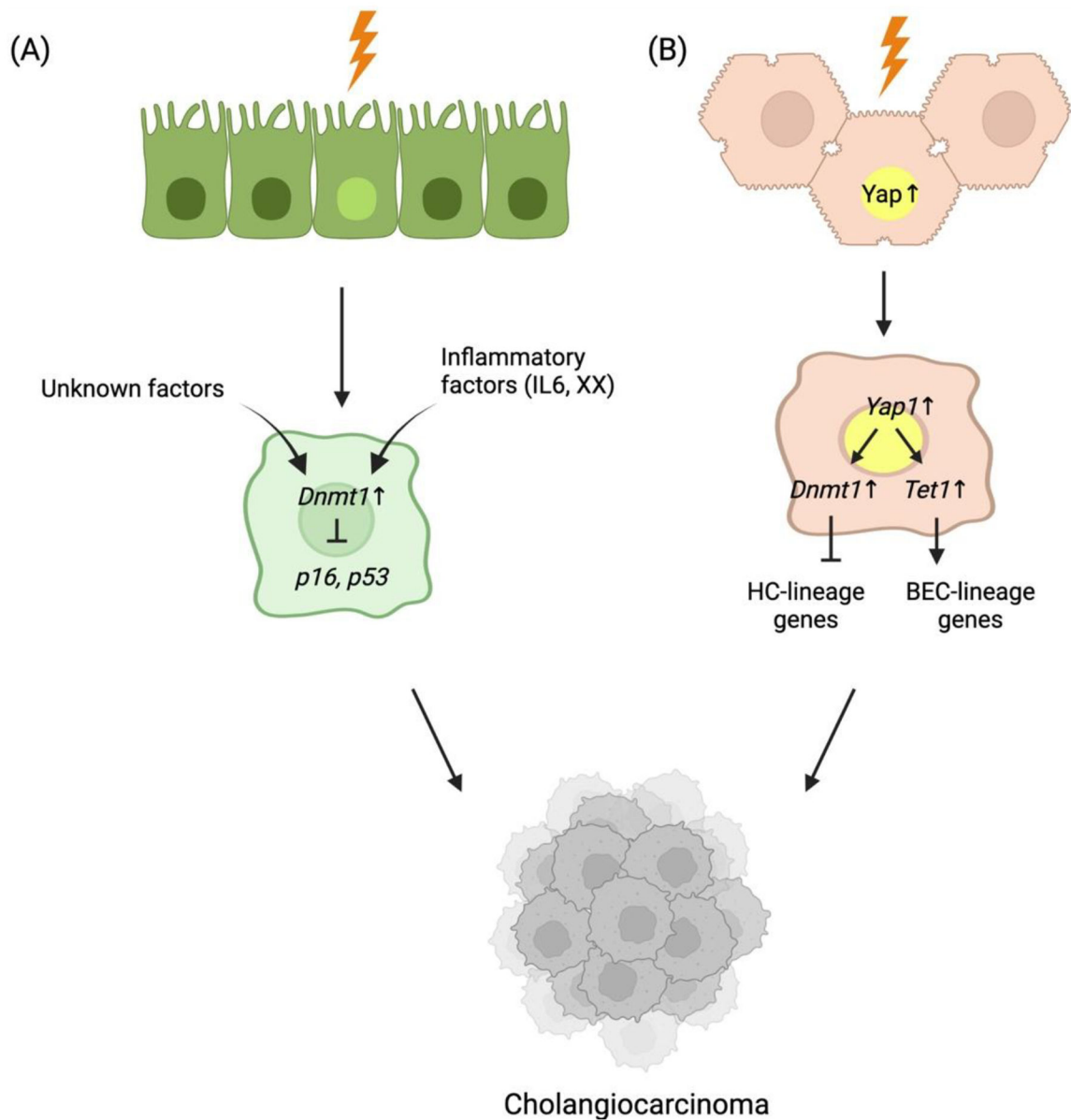
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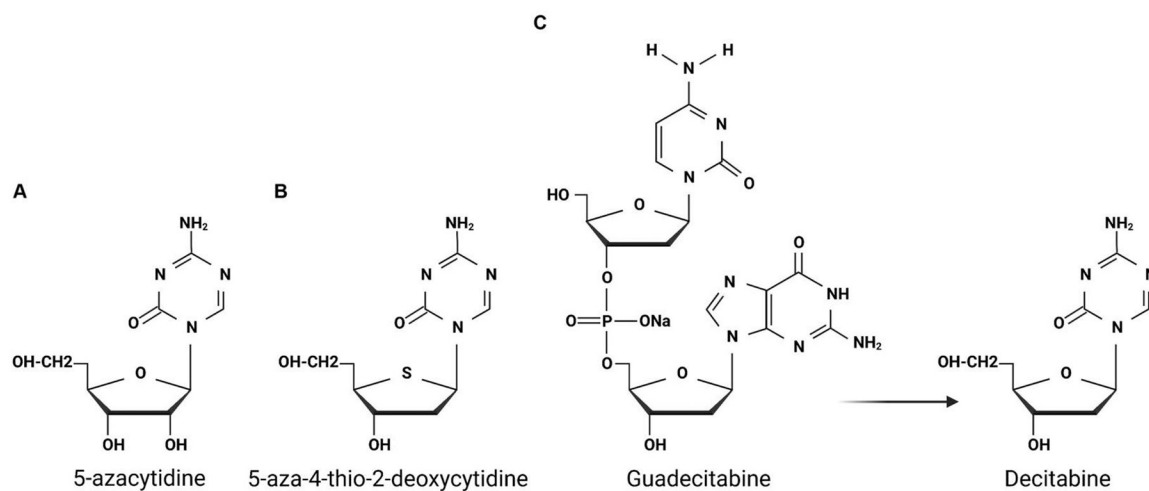


**Figure 3. Schematic of epigenetic regulations in hepatobiliary fate conversions in animal models.** Liver insults derived from chemicals and/or genetic modulation induce dedifferentiation of HC or BECs to multipotent LPCs. (A) In HC-to-BEC, the *Arid1a* complex binds and opens the chromatin, thereby increasing chromatin accessibility. This event allows *Yap1/Taz-Tead* complex to bind to their target genes. Hence, this event promotes the activation of *Yap1/Taz-Tead* signaling during HC dedifferentiation into LPC. (B) In BEC-to-HC, the epigenetic reader *BET* and *Dnmt1* positively regulate the LPC formation via the upregulation of *myca* and downregulation of *p53* (in zebrafish), respectively. *Bet-myca* and *Tet1*-mediated *ErbB-MAPK* and *Yap1* activation contribute to LPC proliferation and expansion. Subsequently, the epigenetic writer *Hdac1* suppresses *sox9b* expression contributing to HC lineage differentiation; a mechanism of HC differentiation that also involves *Tet1* and *Dnmt1*.





**Figure 4. Schematic of the regulatory mechanism of DNA methylation in CCA tumorigenesis.** Under chronic liver injury, HCs or BECs transform into cholangiocarcinoma. (A) under biliary injuries, inflammatory factors, such as IL-6 and/or other unknown factors induce *Dnmt1* expression. The increased Dnmt1 suppresses tumor suppressor genes, such as *p16* and *p53*, thereby contributing to tumorigenesis of CCA. (B) In HC, overactivation of *Yap1* induced by injuries in HC can trigger induction of *Dnmt1* and *Tet1* expression. Dnmt1 prevents the transcriptional activity of HC-lineage transcriptional factors, such as HNF4 $\alpha$  and FOXA2, ultimately diminishing the HC-lineage identity. In contrast, *Tet1* promotes the expression of biliary lineage-specific *Yap1* targets driving CCA tumorigenesis.



**Figure 5. Molecular structures of DNMTi used in the clinical setting.**

(A) Structures of 5-azacytidine and (B) 5-aza-4-thio-2-deoxycytidine. (C) Molecular structure of Guadecitabine and how it can be converted to its bioactive component, Decitabine.

**Table 1.**

Summary of liver cell plasticity models and effect of diverse epigenetic modulation

Animal	Conversion	Injury		Promoter for Lineage tracing	Epigenetic Factors
Mouse	HC→BEC	Chemical injury	DDC	<i>AAV8-TBG-Cre</i> <sup>(Yang, Zong et al. 2013)</sup>	<i>Arid1a</i> <sup>(Li, Yang et al. 2014)</sup>
				<i>Hnf4a-Dre<sup>ERT2</sup>;Sox9-Cre<sup>ERT2</sup></i> <sup>(Han, Wang et al. 2019)</sup>	
				<i>Alb-Cre<sup>ERT2</sup></i> <sup>(Han, Wang et al. 2019)</sup>	
				<i>Mxl1-Cre</i> <sup>(Nagahama, Sone et al. 2014)</sup>	
			BDL	<i>AAV8-Tbg-Cre</i> <sup>(Yang, Zong et al. 2013)</sup>	
				<i>Hnf4a-Dre<sup>ERT2</sup>;Sox9-Cre<sup>ERT2</sup></i> <sup>(Han, Wang et al. 2019)</sup>	
				<i>Alb-Cre<sup>ERT2</sup></i> <sup>(Han, Wang et al. 2019)</sup>	
				<i>Mxl1-Cre</i> <sup>(Nagahama, Sone et al. 2014)</sup>	
			DAPM	<i>Mxl1-Cre</i> <sup>(Nagahama, Sone et al. 2014)</sup>	
			TAA	<i>Mxl1-Cre</i> <sup>(Nagahama, Sone et al. 2014)</sup>	
	CCI <sub>4</sub>	<i>Alb-Cre</i> <sup>(Sekiya and Suzuki 2012)</sup>			
		<i>Mxl1-Cre</i> <sup>(Nagahama, Sone et al. 2014)</sup>			
	Genetic modulation	<i>NICD</i>	<i>AAV8-Tbg-Cre</i> <sup>(Yang, Zong et al. 2013)</sup>		
		<i>TetO-YAP<sup>S127A</sup></i> <i>TetO-YAP<sup>S127A</sup>;AAV8-Tbg-Cre-Rbpj<sup>(fl/fl)</sup></i>	<i>AAV-Tbg-Cre</i> <sup>(Yimlamai, Christodoulou et al. 2014)</sup>		
		<i>CAGGS-GFP-IRES-SOX9</i> <sup>(Yoshii, Shimata et al. 2022)</sup>			
	BEC→HC	Chemical injury	CCI <sub>4</sub> (6–24 weeks)	<i>OPN-Cre<sup>ERT2</sup></i> <sup>(manco, Clerbaux et al. 2019)</sup>	
			DDC (4–24 weeks) <sup>(Deng, Zhang et al. 2018)</sup> , (3 weeks) <sup>(Aloia, McKie et al. 2019)</sup>	<i>CK19-Cre<sup>ERT2</sup></i> <sup>(Deng, Zhang et al. 2018)</sup>	<i>Tetf</i> <sup>(Aloia, McKie et al. 2019)</sup>
			TAA (24–52 weeks)	<i>CK19-Cre<sup>ERT2</sup></i> <sup>(Deng, Zhang et al. 2018)</sup>	
			CDE (3 weeks) (Minnis-Lyons, Ferreira-Gonzalez et al. 2021), (10 days) (Ko, Choi et al. 2016)	<i>OPN-Cre<sup>ERT2</sup></i> <sup>(Minnis-Lyons, Ferreira-Gonzalez et al. 2021)</sup>	<i>Betf</i> <sup>(Ko, Choi et al. 2016)</sup>
		Chemical injury + Genetic modulation	<i>AbCre+Mdm2<sup>(fl/fl)</sup></i>	<i>CK19-Cre<sup>ERT2</sup></i> <sup>(Lu, Bird et al. 2015)</sup>	
MCD+ <i>AAV8-Tbg-p21</i>			<i>CK19-Cre<sup>ERT2</sup></i> <sup>(Minnis-Lyons, Ferreira-Gonzalez et al. 2021)</sup>		
DDC+ <i>AAV8-Tbg-Cre-β1-integrin<sup>(fl/fl)</sup></i> <sup>(Raven, Lu et al. 2017)</sup> / <i>AAV8-Tbg-p21</i> <sup>(Raven, Lu et al. 2017, Aloia, McKie et al. 2019)</sup>			<i>CK19-Cre<sup>ERT2</sup></i> <sup>(Raven, Lu et al. 2017)</sup>	<i>Tetf</i> <sup>(Aloia, McKie et al. 2019)</sup>	
MCD+ <i>AAV8-Tbg-Cre-β1-integrin<sup>(fl/fl)</sup></i> / <i>AAV8-Tbg-p21</i>			<i>CK19-Cre<sup>ERT2</sup></i> <sup>(Raven, Lu et al. 2017)</sup>		
TAA+ <i>AAV8-Tbg-Cre-β1-integrin<sup>(fl/fl)</sup></i> <sup>(Raven, Lu et al. 2017)</sup>			<i>CK19-Cre<sup>ERT2</sup></i> <sup>(Raven, Lu et al. 2017)</sup>		
CDE+ <i>AAV8-Tbg-Cre-Ctnnb1<sup>(fl/fl)</sup></i> <sup>(Ko, Russell et al. 2019, Russell, Lu et al. 2019)</sup>			<i>CK19-Cre<sup>ERT2</sup></i> <sup>(Russell, Lu et al. 2019)</sup>	<i>Hdacf</i> <sup>(Ko, Russell et al. 2019)</sup>	
Pharmacogenetic HC ablation		NTR/Mtz-driven pan-HC ablation	<i>fabp10a-Cre</i> <sup>(Choi, Ninov et al. 2014)</sup> , <i>Tp1-Cre</i> <sup>(Choi, Ninov et al. 2014, He, Lu et al. 2014, Huang, Chang et al. 2014)</sup>	<i>Betf</i> <sup>(Ko, Choi et al. 2016)</sup>	
				<i>hdacf</i> <sup>(KO, Russell et al. 2019)</sup>	
				<i>dnmtf</i> <sup>(He Zhou et al. 2019)</sup>	
Zebrafish					

Animal	Conversion	Injury		Promoter for Lineage tracing	Epigenetic Fa

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Table 2.

Effect of DNA methylation modulation in murine liver cancer models

Cancer type	Model	Target	Manipulation	Effect on tumor	Mechanism	Reference
CCA	Orthotopic xenograft	<i>Tet1</i>	sh- <i>Tet1</i>	CCA growth↓	cell growth↓ and apoptosis↑	(Bai, Zhang et al. 2021)
	Orthotopic xenograft	<i>Dnmt1</i>	CM272	CCA growth↓	-	(Colyn, Barcena-Varela et al. 2021)
	<i>Jnk<sup>hep</sup></i> + DEN + CCl4			CCA-enriched genes↓	Metabolic reprogramming (carbohydrate/cholesterol metabolism)↓	
	<i>myrAkt+NICD</i> HDTV1	<i>Dnmt1</i>	Azacytidine	HC-to-BEC fate conversion↓, ICC formation↓	HC lineage-specific genes↑, downstream targets of <i>tp53</i> ↑	(Hu, Molina et al. 2022)
			sh- <i>Dnmt1</i>			
	<i>Akt+YAP1S127A</i> HDTV1	<i>Dnmt1</i>	Azacytidine	HC-to-BEC/ICC transformation↓		
<i>ApoE-rtTA-YAP &amp; Nf2<sup>-/-</sup></i>	<i>Tet1</i>	sh- <i>Tet1</i>	CCA proliferation↓, CCA tumorigenesis↓	<i>Yap1</i> target genes↓	(Wu, Mei et al. 2022)	
		Deletion of <i>Tet1</i>				
HCC	Xenograft	<i>Dnmt1</i>	CM272	HCC growth↓, angiogenesis↓	<i>G9a</i> expression↓, <i>HK2</i> ↓, <i>Fbp1</i> ↑, <i>Gnmt</i> ↑	(Barcena-Varela, Caruso et al. 2019)
		<i>Dnmt1</i>	Guadecitabine	HCC growth↓, angiogenesis↓	<i>Dab2ip</i> , <i>Dlec1</i> , <i>Gstp1</i> , <i>Rassf1</i> , <i>Runx3</i> , and <i>Socs1</i>	(Jueliger, Lyons et al. 2016)
		<i>Dnmt1</i>	Guadecitabine	HCC growth↓	-	(Kuang, El-Khoueiry et al. 2015)
		-	Decitabine + ACRBP-specific CTL	HCC growth↓, HCC apoptosis↑	-	(Ge, Zhang et al. 2021)
		<i>Tet1</i>	miR-29a↑→TET1↓	HCC growth↑, metastasis↑	<i>Socs1</i> ↓→ <i>Jak/Stat3</i> signaling↑→ <i>Mmp9</i> ↑	(Chen, Yin et al. 2017)

**Table 3**

Summary of aberrant DNA methylation in human HCC.

Methylation	Regulator	Target	Biologic function (regulation)	Sensitivity (%)	Specificity (%)	# of samples	Effect on tumor	Reference
Hypo	–	<i>CEBPB</i>	transcription factor, HC proliferation↑	$P < 2.2e-16$	–	33	Proliferation↑	(Xiong, Wu et al., 2019)
	–	<i>IFNGR2</i>	IFN- $\gamma$ pathway		–		HBV viraemia, Redox	
	–	<i>SLC45A4</i>	sucrose transport		–		homeostasis	
	–	<i>LITAF</i>	transcription factor, NF $\kappa$ B signaling		–			
	–	<i>SRC</i>	Regulation of telomere maintenance hippo signaling		–		–	
	–	<i>CCL20</i>	cell-cell signaling, inflammatory response	$P = 1.70e-14$		62	–	(Shen et al., 2012)
Hyper	–	<i>HOXA1</i>	transcription factor	71	90	135	–	(Chalasanani et al., 2021)
	–	<i>EMX1</i>	transcription factor					
	–	<i>TSPYL5</i>	cell proliferation, protein kinase B signaling, protein ubiquitination					
	–	<i>ZNF334</i>	transcription factor	88	–	25	Tumor suppressor	(Sun et al., 2022)
	–	<i>HIC1</i>	Wnt signaling↓, transcription, DNA damage response↑	64.8	93.6	177	HCC-related tumor suppressor genes	(Nishida et al., 2012)
	–	<i>GSTP1</i>	Apoptosis↓, MAPK signaling↓	75	91.9	177		(Nishida et al., 2012, Song et al., 2013)
	–	<i>SOCS1</i>	Regulatory T cell differentiation ↑	57.4	93.6	177		(Nishida et al., 2012)
	–	<i>RASSF1</i>	G1/S cell cycle transition, Ras signaling transduction	68.8	92.5	177		(Nishida et al., 2012, Um et al., 2011)
	–	<i>P16 (CDKN2A)</i>	G2/M cell cycle transition and cell-matrix adhesion	62.6	95.4	177		(Nishida et al., 2012, Shen et al., 2012, Song et al., 2013)
	–	<i>APC</i>	regulation of cell death↑, regulation of cell migration↑	84.1	93.1	177		(Nishida et al., 2012)
–	<i>RUNX3</i>	cell cycle↓	62.5	93.6	177			
–	<i>PRDM2</i>	histone methylation, transcription regulation	72.2	83.3	177			

Methylation	Regulator	Target	Biologic function (regulation)	Sensitivity (%)	Specificity (%)	# of samples	Effect on tumor	Reference
	DNMT1	<i>CDH1</i>	cell-cell adhesion↓ and cell migration↓	57.1	–	42	EMT↑, metastasis↑	(Lim et al., 2008)
	–	<i>DAB2IP</i>	angiogenesis↓, PI3K signaling↓	$P = 3.58e-17$	–	62	–	(Shen et al., 2012)
	–	<i>BMP4</i>	BMP signaling pathway	$P = 5.36e-16$	–			
	–	<i>ZFP41</i>	transcription factor	$P = 3.25e-15$	–			
	–	<i>ZNF154</i>	transcription factor	$P = 1.14e-12$	–			
	–	<i>ZNF540</i>	transcription factor	$P = 2.03e-12$	–			
	–	<i>ETS2</i>	transcription factor	$P < 2.2e-16$	–	33	–	(Xiong, Wu et al., 2019)
	–	<i>DACH1</i>	DNA biosynthesis↓, fibroblast proliferation↓	$P < 2.2e-16$	–		–	
	–	<i>SMPD3</i>	lipid metabolic process, cell cycle	$P < 0.001$	–	71	Tumor suppressor	(Revill et al., 2013)
	–	<i>NEFH</i>	microtubule cytoskeleton organization	$P < 0.001$	–			

**Table 4**

Summary of aberrant DNA methylation in human CCA.

Methylation	Regulator	Target	Biologic function (regulation)	Sensitivity (%)	Specificity (%)	# of samples	Effect on tumor	Reference
Hypo	–	<i>PCLAF</i>	cell cycles, DNA repair, DNA replication	$P < 0.05$	–	36	hOCT1↓	(Liu et al., 2022)
	–	<i>TTYH3</i>	chloride transport	$P < 0.05$	–	TCGA	CCA migration, invasion, and proliferation↑	(Xue et al., 2021)
	–	<i>MTHFD1</i>	nucleotide biosynthetic process, neutrophil homeostasis	–	–	102	–	(Moruzzi et al., 2017)
	–	<i>IMP3</i>	rRNA processing, ribosome biogenesis	82	100	72	–	(Gao et al., 2014)
	–	<i>LINE-1</i>	RNA-mediated transposition	$P < 0.001$	–	172	CCA differentiation, lymphatic invasion, and survival	(Jeong et al., 2017)
	–	<i>CDH3</i>	TGFβ2↓, <i>IGFR-WNT/β-CATENIN</i> pathway↑, cell-cell adhesion↑	51	–	59	CCA Prognosis	(Sakamoto et al., 2015)
	–	<i>EGFR</i>	PI3K/MAPK/JNK signaling↑, DNA replication/repair↑, apoptosis↓	44.6	–	65	–	(Limpaiboon et al., 2005)
Hyper	–	<i>14-3-3σ</i>	cyclin-dependent protein serine/threonine kinase activity	60	100	79	CCA Prognosis	(Wu et al., 2020)
	–	<i>HOXA5</i>	transcription factor	$P < 0.05/P < 0.01$	–	209/34	MXD1↓ → CCA↑	(Xiong et al., 2022)
	–	<i>PTEN</i>	<i>PI3K</i> inhibition	35	93	29	CCA Prognosis	(Sriraksaet al., 2011)
	–	<i>APOB</i>	lipid metabolism	$P < 0.05$	–	36	Survival	(Xu et al., 2021)
	–	<i>EFHD2</i>	Unknown, cadherin/metal ion binding	$P < 0.019$	–	118	Survival	(Peng, Meng, Sheng, & Gao, 2021)
	–	<i>PHYHIPL</i>	Unknown, protein binding	$P < 0.0001$	–	118	Survival	(Peng, Meng, Sheng, & Gao, 2021)
	–	<i>CNR1P1</i>	Cannabinoid Receptor signaling receptor activity	70	100	112/49	CCA migration, invasion, and proliferation↑ by inhibiting PKM2 activity	(Chen et al., 2021) (Andresen et al., 2015)



Methylation	Regulator	Target	Biologic function (regulation)	Sensitivity (%)	Specificity (%)	# of samples	Effect on tumor	Reference
	–	<i>EBF1</i>	transcription factor	75	–	72	CCA progression <sup>↑</sup>	(Armstrong et al., 2021)
	–	<i>miR-212-3p</i>	<i>MUC13</i> expression in CCA	100	–	26	<i>MUC13</i> <sup>↑</sup> → CCA <sup>↑</sup>	(Tiemin et al., 2020)
	<i>MGMT</i> <i>MGMT</i>	<i>p21,p27</i>	DNA methylation/repair/ligation/modification, apoptosis i	–	–	4	p21 <sup>↑</sup> , p27 <sup>↑</sup> ,CYCLINE1 <sup>↑</sup>	(Chen et al., 2020)
	–			33	100	72		(Yang, House, Guo, Herman, & Clark, 2005)
	–			11	100	79		(Lee, Kim, Jung, Yang, & Kang, 2002)
	–	<i>DLEC1</i>	cell proliferation	23	100	172	CCA proliferation	(Kim et al., 2019)
	–	<i>HTATIP2</i>	transcription factor	14		54	apoptosis <sup>↓</sup>	(Nanok, Jearanaikoon, Proungvitaya, & Limpaboon, 2018)
	–	<i>UCHL1</i>	proteolysis	57	–	54	Survival	(Nanok et al., 2018)
	<i>5-Aza</i>	<i>F2</i>	protein phosphorylation	–	–	228	Survival	(Chen et al., 2019)
	<i>5-Aza</i>	<i>AHSG</i>	inflammatory response and phagocytosis	–	–	228	Survival	(Chen et al., 2019)
	<i>5-Aza</i>	<i>ALDH8A1</i>	retinoic acid metabolism	–	–	228	Survival	(Chen et al., 2019)
	<i>5-Aza</i>	<i>SERPIND1</i>	peptidase activity	–	–	228	Survival	(Chen et al., 2019)
	<i>5-Aza</i>	<i>AGXT</i>	L-serine/ cysteine/ alanine/ glyoxylate metabolic process, NOTCH signaling pathway	–	–	228	Survival	(Chen et al., 2019)
	–	<i>CDOI</i>	L-cysteine catabolic process, taurine	76	92	81	CCA prognosis	(Nakamoto et al., 2018)
	–		biosynthetic process	77	100	39		(Andresen et al., 2012)
	–			77	98	49		(Andresen et al., 2015)
	<i>5-Aza</i>	<i>GATA5</i>	transcription factor and endodermal cell fate commitment	–	–	152	CCA growth <sup>↑</sup> and metastasis <sup>↑</sup> via <i>WNT/β-CATENIN</i> pathway	(Liu et al., 2018)
	–	<i>HOXA1</i>	transcription factor	89	100	9	–	(Prachayakul et al., 2017)

Methylation	Regulator	Target	Biologic function (regulation)	Sensitivity (%)	Specificity (%)	# of samples	Effect on tumor	Reference
–	–	<i>RASSF1A</i>	G1/S cell cycle transition, Ras signaling transduction	56	100	9	–	(Prachayakul et al., 2017)
–	–	<i>P16 (CDKN2A)</i>	G2/M cell cycle transition and cell-matrix adhesion	25	–	36	–	(Lee et al., 2002, Liu et al., 2012, Prachayakul et al., 2017, Tannapfel et al., 2000, Xiaofang, Kun, Shaoping, Zaiqiu, & Hailong, 2012)
–	–	<i>NEUROG1</i>	transcription factor; cell cycle and differentiation	100	100	9	–	(Prachayakul et al., 2017)
–	<i>DNMT1/5-Aza</i>	<i>SOX17</i>	transcription factor, <i>WNT/β-CATENIN</i> pathway↑, cell differentiation↑ and cell growth↑	P < 0.0001	–	48 + 37 + 6	CCA prognosis and survival	(Merino-Azpitarte et al., 2017)
–	–	miR-191/ <i>TP53</i>	tumor suppressor; cell growth↓ and DNA damage↑	76 68	– –	152	miR-191→ Tet1↓→ p53↑→ CCA↑	(Li et al., 2017)
–	–	<i>DCLK1</i>	cell differentiation and intracellular signal transduction	87	100	93	CCA prognosis	(Andresen et al., 2012)
–	–	<i>SFRP1</i>	apoptotic↑ and cell cycle↓	–	–	–	–	(Andresen et al., 2012)
–	–	<i>CNRIP1</i>	regulation of signaling receptor activity	–	–	–	–	(Andresen et al., 2015)
–	–	<i>VIM</i>	collagen biosynthetic process↑	–	–	–	–	(Andresen et al., 2015)
–	–	<i>PYCARD</i>	adaptive immune response↑, IL-8/IL-10 production↑	39	–	36	Survival	(Xiaofang et al., 2012)
–	–	<i>CCND2</i>	cell proliferation↑ and apoptosis↓	74	100	45	CCA proliferation	(Shin et al., 2012)
–	–	<i>CDH13</i>	cell-matrix adhesion↑, <i>Rho/Rac</i> pathway↑, <i>EGFR</i> pathway↑	–	–	–	–	–
–	–	<i>GRIN2B</i>	calcium-mediated signaling and glutamate	–	–	–	–	–

Methylation	Regulator	Target	Biologic function (regulation)	Sensitivity (%)	Specificity (%)	# of samples	Effect on tumor	Reference
	-	<i>TWIST1</i>	receptor signaling TNF ligand↑ PI3K signaling↑, cellular senescence↑					
	-	<i>RUNX3</i>	cell cycle↓	33	100	111	CCA proliferation	(Kim et al., 2007)
	-	<i>MLH1</i>	meiotic chromosome segregation, DNA repair	45	100	65	-	(Limpaiboon et al., 2005)
	-	<i>DAPK1</i>	defense response to tumor cell, positive regulation of autophagy and apoptosis	6	100	36	CCA autophagy and apoptosis	(Liu et al., 2007, Xiaofang et al., 2012)
	-			8	100	79		(Lee et al., 2002)
	-	<i>GSTP1</i>	negative regulation of apoptosis, negative regulation of MAP kinase/ERK1&2/NF-kB cascade	6	100	79	-	
	-	<i>CDH1</i>	cell-cell adhesion↓ and cell migration↓	22	100	79	CCA migration and invasion	(Lee et al., 2002)
	-			30	95	111		(Kim et al., 2007)