Epigenetics in hepatocellular carcinoma development and therapy: The tip of the iceberg

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Summary
Hepatocellular carcinoma (HCC) is a deadly tumour whose causative agents are generally well known, but whose pathogenesis remains poorly understood. Nevertheless, key genetic alterations are emerging from a heterogeneous molecular landscape, providing information on the tumorigenic process from initiation to progression. Among these molecular alterations, those that affect epigenetic processes are increasingly recognised as contributing to carcinogenesis from preneoplastic stages. The epigenetic machinery regulates gene expression through intertwined and partially characterised circuits involving chromatin remodelers, covalent DNA and histone modifications, and dedicated proteins reading these modifications. In this review, we summarise recent findings on HCC epigenetics, focusing mainly on changes in DNA and histone modifications and their carcinogenic implications. We also discuss the potential drugs that target epigenetic mechanisms for HCC treatment, either alone or in combination with current therapies, including immunotherapies.

Introduction
Hepatocellular carcinoma (HCC) is the most frequent primary malignancy of the liver, ranking fourth among cancer-related causes of death worldwide.1,2 The World Health Organization projections estimate that by 2030 more than 1 million individuals will die from liver cancer per year.1 The World Health Organization projections estimate that by 2030 more than 1 million individuals will die from liver cancer per year. The prognosis of HCC remains poor, with a 5-year survival rate of just 18%, highlighting the limitations of available treatments.2 When detected early, HCCs are amenable to locoregional therapies and surgery, however the recurrence rate 5-years post-resection is about 70%.1 Systemic therapies are used for patients diagnosed at more advanced stages. HCC is very resistant to conventional chemotherapies,4 but targeted agents such as the multikinase inhibitors sorafenib, lenvatinib, regorafenib and cabozantinib, as well as monoclonal antibodies like ramucirumab (which targets vascular endothelial growth factor [VEGF] receptor 2), confer some survival benefit.1,4,5,6 Immuno-therapy is also being actively tested in HCC, and immune checkpoint inhibitors (ICIs), which are antibodies that block the programmed cell death protein 1 (PD-1)/programmed cell death ligand-1 (PD-L1) pathway, or the cytotoxic T-lymphocyte-associated protein 4 (CTLA-4) pathway, have shown clinical activity.5,7 Moreover, a recent study showed very promising effects in patients with unresectable HCC that were treated with antibodies targeting PD-L1 and VEGF.8 Despite these advances, the very high mortality rate seen in patients with HCC clearly indicates that the efficacy of systemic therapies needs to be improved. To this end, combination strategies including locoregional approaches, targeted agents and ICIs are of interest, with some combinations being actively investigated.1,9 Nonetheless, when exploring systemic therapies for advanced HCC it is important to bear in mind that this type of tumour usually develops on a background of chronic liver injury, inflammation, fibrosis and cirrhosis, in an organ with impaired metabolic function that renders patients more susceptible to hepatic and systemic toxicities.1,5 Chronic liver injury leading to HCC development is mainly caused by HBV and HCV infections, long-lasting alcohol abuse and non-alcoholic fatty liver disease (NAFLD).2,3,5,10 With the systematic implementation of HBV vaccination and the advent of effective anti-HCV therapies, it is likely that NAFLD will become the dominant cause of HCC in the coming years.2

Development of effective therapies for any cancer relies to a great extent on a deep understanding of the tumour’s molecular and cellular biology. Such knowledge may enable the identification (and targeting) of key tumour driver genes, as well as providing biomarkers for prognostic scoring and for the selection of potential responders to molecular therapies.5 Generating a precise molecular portrait and classification of HCC is not an easy task. Factors such as the aetiology of the liver disease, the stage of cancer progression, the molecular heterogeneity between different nodules, and even within the same tumoural mass, have hindered the genomic characterisation of...
HCC. Nevertheless, over the past decade, next generation sequencing technologies have been used to identify the most frequently recurring mutations, DNA copy number alterations and associated changes in gene expression that contribute to hepatocarcinogenesis.\(^\text{12–19}\) Such studies also enabled the classification of HCCs into different molecular subgroups with associated biological and clinical phenotypes.\(^\text{3,36}\) The most frequent (95% of tumours) and earliest genetic alteration found in the gradual process of hepatocarcinogenesis is the aberrant expression of telomerase reverse transcriptase (TERT). Uprogulation of TERT is due to promoter mutations (40–60%), gene amplification, translocation, and also to viral insertion in HBV-related HCCs.\(^\text{17}\) The second most frequent mutations activate CTNNB1 (~30%), the gene coding for β-catenin, or inactivate the tumour protein TP53 (~30%). Other members of the WNT/β-catenin pathway, such as AXIN1, AXIN2, ZNRF3, and APC are also recurrently mutated and inactivated in HCC.\(^\text{14,16}\) Mutations in epigenetic modifiers are also frequent in HCC, and altogether they can be found in up to 50% of tumours.\(^\text{12,20}\) Recurrent inactivating mutations were found in ARID1A and ARID2,\(^\text{21,22}\) key components of the chromatin remodelling complex SWI/SNF, which has tumour suppressor activity.\(^\text{23}\) Mutations in histone modifying enzymes were initially described for members of the KMT2 family, such as KMT2D, KMT2B, and KMT2C.\(^\text{12,19,22,24,25}\) However, more recently, the list of epigenetic modifiers mutated in HCC has been significantly expanded.\(^\text{26}\) DNA copy number alterations were also frequently found, and included deletions of tumour suppressor genes (like PTEN and RB1) and negative cell cycle regulators (such as CDKN2A and CDKN2B).\(^\text{14,19,22,25,27}\) As well as amplifications in promitogenic genes like FGFI9 and CCND1.\(^\text{27,28}\) This impressive wealth of knowledge has indeed exposed a number of oncogene addiction loops that drive HCC progression.\(^\text{29}\) However, the most frequently mutated genes identified, such as TERT, CTNNB1 and TP53, are very difficult to target pharmacologically, or are believed to be undruggable.\(^\text{30}\) While the search for effective inhibitors of key drivers like TERT must continue, exploring new strategies and targets to quell HCC growth is crucial.

One emerging approach for the treatment of solid tumours comes from the field of epigenetics.\(^\text{30,31}\) Epigenetics refers to heritable traits not attributable to changes in DNA sequence that can control chromatin structure and the accessibility of the transcriptional machinery to DNA, thereby modulating gene expression.\(^\text{32}\) These mechanisms are thus fundamental for the maintenance of cell identity, but are also heavily implicated in development, stem cell renewal, genome integrity and proliferation.\(^\text{32–24}\) Their deregulation is central to pathogenesis, including tumorigenesis,\(^\text{35}\) impacting on all hallmarks of cancer.\(^\text{35}\) Multiple pathways are involved in chromatin dynamics and epigenetic gene regulation, including DNA methylation, ATP-dependent nucleosome remodelling, the introduction of histone variants, post-translational modifications (PTMs) of histones, and non-coding RNAs (ncRNAs).\(^\text{30,32}\) Contrary to genetic mutations, epigenetic mechanisms such as covalent modifications of DNA and histones are highly flexible and dynamic, involving reversible enzymatic reactions and specific protein-protein interactions, which make them amenable to pharmacological intervention.\(^\text{36}\) Epigenetic alterations involving the chromatin remodelling machinery and ncRNAs have recently been reviewed elsewhere.\(^\text{37–39}\) Herein, we review basic epigenetic mechanisms and the role of their dysregulation on hepatocarcinogenesis, focusing on DNA methylation and histone PTMs. We also highlight emerging strategies for the molecular targeting of epigenetic mechanisms with so-called “epidrugs” in HCC treatment and prevention.

**Key points**
- The term epigenetics defines somatic heritable differences in the genome not attributable to changes in the primary sequence of DNA. In a multicellular organism, epigenetic mechanisms establish cellular identity out of a common genome.
- Epigenetic mechanisms regulate chromatin conformation, nucleosome positioning and DNA wrapping around nucleosomes, modulating the interaction of the transcriptional machinery with genes, and thus controlling their expression.
- The epigenetic effectors that influence the structure and function of chromatin include chromatin-remodelling complexes, DNA methylation/demethylation enzymes, histone modification enzymes, histone marks readers and non-coding RNAs.
- Chromatin structure, DNA methylation and covalent histone modification patterns are altered in cancer, including hepatocarcinogenesis, and contribute to malignancy from its early stages. Mutations and changes in the expression of epigenetic effectors underlie these alterations.
- DNA methylation and covalent histone modifications are reversible enzymatic reactions amenable to pharmacological intervention with small-molecule inhibitors, epidrugs. Epidrugs are promising therapeutic agents that counteract tumour hallmarks and potentiate the response to chemotherapy, targeted therapy and immunotherapy in hepatocellular carcinoma.

**Writing, erasing and reading epigenetic marks on chromatin**

Nuclear DNA needs to be tightly packed, but it also needs to be accessible in a specific and regulated manner to allow for essential processes such as replication, repair and transcription. Efficient packaging of DNA in chromatin is mediated by its interaction with histones and the formation of nucleosomes. In nucleosomes, DNA is wrapped around 2 copies of each of the 4 core histones: H2A, H2B, H3 and H4, and outside the nucleosome the linker histone H1 facilitates further compaction in higher order chromatin structures.\(^\text{40}\) Nucleosomes are highly dynamic, they can slide along the DNA, fully or partially disassemble and their histone components may be replaced by sequence variants.\(^\text{40}\) The promoters and enhancers of transcriptionally active genes present reduced nucleosome abundance, which allows the recruitment of the transcriptional machinery and regulatory factors. Compact (repressed) and open (active) chromatin topologies, as found in heterochromatin and euchromatin, respectively, can be dictated by interrelated covalent epigenetic marks deposited on DNA and histones.\(^\text{41,42}\) DNA methylation mostly occurs on the 5' carbon of cytosine residues (5mC) in CpG dinucleotides. Methylation of CpGs in the so-called CpG islands (CGIs), present in about 70% of human gene promoters, has been widely associated with a closed chromatin conformation and inhibition of transcription initiation (Fig. 1).\(^\text{43}\) However, it has also been observed that CGI methylation in the transcribed regions of genes, i.e. gene bodies, increases gene expression.\(^\text{44}\) The list of histone PTMs is extensive and includes acetylation, methylation, phosphorylation, ubiquitinylation, sumoylation, ADP-ribosylation, neddylation, succinylation, crotonylation and butyrylation, among others.\(^\text{45,46}\) The best characterised modifications take place on amino acids present in the N-terminal tail regions of histones that protrude from the nucleosome surface.
Fig. 1. Chromatin conformation and regulation of gene expression by epigenetic effectors acting on DNA and histones: epigenetic writers, erasers and readers. PHD, plant homeodomain.
in the regulation of gene expression. This was initially shown to occur between DNA CpG methylation and histone deacetylation, leading to chromatin condensation and gene repression.54 This notion has been exponentially extended over the past years, and now we recognise extensive hierarchical relationships between DNA methylation and chromatin marks. Just to mention a few, there is an inverse correlation between DNA methylation and H3K4me2/3 and H3K79me3 levels in active gene promoters, while a positive association has been demonstrated between H3K36me3 and DNA methylation in the bodies of actively expressed genes. Conversely, gene expression and the presence of H3K27me3 and H4K20me3 levels in gene promoters is also negatively associated. High H3K9me3 and H4K20me3 levels in gene promoters is also associated with DNA methylation and gene repression.41,42,53 These interactions can operate in both directions and be mutually reinforcing, as described between DNMTs and H3K9 HMTs in gene repression and heterochromatin formation.54

As mentioned, epigenetic marks are reversible and can be removed by a group of dedicated enzymes, collectively named epigenetic erasers (Fig. 1). Regarding DNA demethylation, this process can occur through 2 mechanisms. First, the lack of maintenance methylation during DNA replication, which is mainly carried out by DNMT1 in complex with the UHRF1 adaptor protein.55 This can result in the passive dilution of 5mC. More recently, a replication-independent process involving enzymatic demethylation is performed by 2 different families of enzymes involved in the timely recruitment of epigenetic writers and readers (Fig. 1). Regarding DNA demethylation, this was initially shown to occur between DNA CpG methylation and histone deacetylation, leading to chromatin condensation and gene repression.54 This notion has been exponentially extended over the past years, and now we recognise extensive hierarchical relationships between DNA methylation and chromatin marks. Just to mention a few, there is an inverse correlation between DNA methylation and H3K4me2/3 and H3K79me3 levels in active gene promoters, while a positive association has been demonstrated between H3K36me3 and DNA methylation in the bodies of actively expressed genes. Conversely, gene expression and the presence of H3K27me3 and H4K20me3 levels in gene promoters is also negatively associated. High H3K9me3 and H4K20me3 levels in gene promoters is also associated with DNA methylation and gene repression.41,42,53 These interactions can operate in both directions and be mutually reinforcing, as described between DNMTs and H3K9 HMTs in gene repression and heterochromatin formation.54

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| Epigenetic modifiers | Major modified/recongised site | Family | Examples |
|----------------------|--------------------------------|--------|----------|
| Writers              |                                |        |          |
| DNA methyltransferases (DNMTs) | CpG (Met, 5mC) | DNMT1 DNMT3 | DNMT1, DNMT3/3B |
| Histone methyltransferases (HMTs): lnsine (KMTs) | H3 K4/K9/K27/K36/K79 (Met) | SUV39 SET1/2 EZH PRDMs | G9a/KMT1C MLL1/KMT2A |
| Histone methyltransferases (HMTs): protein arginine (PRMTs) | H3 R2/R8/R17 (Met) | PRMT | SETD1A EZH2/KMT6 |
| Histone acetyltransferases (HATs) | H3 K9/K14/K56 (Ac) | GNAT MYST CBP/p300 | PRMT1 PRMT4/CARM1 |
| Serine-Threonine and Tyrosine kinases | Ser (P) | Haspin MSK CKII |
| Erasers              |                                |        |          |
| DNA demethylation system | CpG (Met, 5mC) | LSD/KDM1A-B JARID/KDM2-8 | TET TDG/BER |
| Histone demethylases (HDMS/KDMs) | H3 K4/K9/K27/K36/K79 H4 K20 | HDAC I-IV | KDM1A/LSD1 KDM4/JMJD2 |
| Histone deacetylases (HDACs) | H3 K9/K14 | HDAC1 | KDM5/RBP2 |
| Readers              |                                |        |          |
| MBD-containing proteins | Methylated DNA (Methyl-CpG) | MeCP2 MBDs 1–6 SETDB1/2 | |
| Chromo domain-containing proteins | Methylated H3 K4/K9/K27/K36 | CHD1 HPI | |
| Tudor domain-containing proteins | Methylated H3 K4/K9/K20/K36 | UHRF1 | |
| MBT-containing proteins | Methylated H3 K4/K9/K27/K36 | SMRT MBTD1 | |
| PHD-containing proteins | Acetylated H3 K14 Methylated H3 K4/K9 | TIDH KMT2D | |
| Bromodomain (BRD)-containing proteins | Acetylated H3 K14 Acetylated H4 K5/K8/K16 | GCN5 BRD4 | |
| Yeats domain-containing proteins | Acetylated H3 K9 | PCAF (HAT) | |

5mC, 5-methyl-cytosine; Ac, acetylation; MBD, methyl-CpG binding domain; MBT, malignant brain tumour; Me, methylation; PHD, plant homeodomain.

Table 1. Epigenetic writers, erasers and readers: target residues in DNA and histones, and representative examples.}

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metabolism. As described, most epigenetic writers and erasers utilise metabolites such as oxygen, ATP, SAM, NAD+, acetyl-CoA, FAD and α-KG as cofactors or substrates. Fluctuations in the levels of these metabolites have been shown to impinge on the enzymatic activities of epigenetic modifiers. Moreover, elevated levels of other types of cellular metabolites such as the ketone body β-hydroxybutyrate, or the tricarboxylic acid cycle intermediates succinate and fumarate, can inhibit the activities of HDACs and α-KG-dependent enzymes (TETs, KDMs), respectively. Therefore, changes in the levels of metabolic substrates and inhibitors may effectively modulate the epigenome, while in turn epigenetic mechanisms control essential metabolic pathways and participate in their alterations in cancer. This link between metabolism and epigenetics may be particularly relevant for a prominent metabolic organ like the liver.

In order to translate the epigenetic marks deposited on DNA and histones into functional responses, these modifications must first be recognised. This is mediated by a third group of proteins called epigenetic readers (Table 1), endowed with specialised binding domains for specific covalent modifications. Readers are often found as part of multimeric complexes in association with writers and erasers, enabling the dynamic integration of signals that modulate chromatin conformation. The best characterised epigenetic readers are those that recognise 5mC, acetyl-lysines and methyl-lysines, while there is less information available for other modifications, such as Ser- and Thr-phosphorylation (Fig. 1). The methyl-CpG-binding proteins recognise 5mC through their methyl-CpG binding domain. This is a family with 11 members, of which MeCP2 was the first characterised. The presence of MeCP2 is mainly associated with transcriptional inhibition via recruitment of corepressor complexes, like those encompassing HDAC or H3K9 HMT activities, to methylated regions of DNA. Interestingly, interactions between MeCP2 and 5mC can also protect these residues from binding and oxidation by TET enzymes. Histone methylation readers have been well-characterised, and include a broad variety of proteins that contain different types of methyl-lysine binding domains such as chromodomains, tudor domains, plant homeodomain (PHD) fingers, PWWP motifs, WD40 repeat domains, bromo-adjacent homology domains, or malignant brain tumour domains, among others (Table 1). These domains discriminate between specific lysine residues and the degree of their methylation, and can be found on many different protein types. For instance, H3K4me2 and H3K4me3 can be recognised by chromatin remodelers, such as the enzyme CHD1 through its 2 chromodomains, while H3K4me3 is bound by the general transcription factor TFIIID through a PHD domain-containing subunit, contributing to enhanced preinitiation complex formation. Repressive marks such as H3K9me3 can be recognised by the adaptor protein UHRF1 through a tandem tudor domain, or by a chromodomain present in heterochromatin protein 1 (HP1), leading to chromatin condensation. Several protein domains can recognise and bind acetylated histones. These domains are classified into 3 major categories: bromodomain (BRD), PHD finger and Yeats domains (Table 1). Based on structural similarities, BRD-containing proteins can be subdivided into 8 families, which display different affinities for specific histone acetylation marks, whereas PHD fingers are more flexible, recognising multiple acetylated histones but also methylation marks. Proteins containing the Yeats domain recognise several acetylation marks, nonetheless they have recently been shown to efficiently bind crotonylated lysine residues. Interestingly, many of the proteins encompassing these histone acetylation reader domains are chromatin remodelling/modifying enzymes, or can recruit these activities to chromatin. Just to name a few, the HAT PCAF encompasses a BRD domain that recognises acetylated H3K14 (H3K14ac) and the HMT KMT2D displays a PHD finger targeting H4K16ac, while the Yeats domain-containing AF9 protein can recruit the HMT DOT1 to chromatin regions displaying the H3K9ac mark. Together, these examples illustrate the extremely complex and intertwined nature of epigenetic regulatory mechanisms.

### Epigenetic alterations in hepatocarcinogenesis

#### DNA methylation

Alterations in DNA methylation, both genome-wide hypomethylation and region-specific hypermethylation, frequently occur in tumours and are among the most consistent epigenetic changes observed during multistage carcinogenesis. This also holds true for HCC, where changes in DNA methylation are already observed in the livers of patients with preneoplastic conditions, such as chronic hepatitis and cirrhosis of different aetiologies, including HCV/HBV infection, chronic alcohol consumption and, as more recently described, NAFLD. Furthermore, alterations in DNA methylation markedly increase during the progression from cirrhosis to early and more advanced neoplastic lesions, and many of them are preserved in fully developed HCCs. Importantly, the methylation changes observed in non-tumoural liver tissues from patients with chronic hepatitis and cirrhosis may have prognostic value for HCC development or recurrence. These findings already indicate a causal relationship between epigenetic alterations and liver carcinogenesis. Early studies did not observe significant differences in overall changes to DNA methylation in HCCs from patients with different liver disease aetiologies. However, later works implementing more sensitive technologies identified different patterns of altered DNA methylation between HCV, HBV and NAFLD-related HCCs, as well as during cirrhotic stages in alcohol or HCV-related carcinogenesis. DNA hypomethylation in HCC occurs in repeated DNA sequences, intergenic regions and in CpG sites away from CGIs, the so-called CpG shores, CpG shelves and “open sea” regions. Like in other tumours, DNA hypomethylation was initially associated with genomic instability in HCC. More recently, frequent mutations and rearrangements have been confirmed to occur in inactive chromatin regions specifically hypomethylated in HCCs. Very interestingly, a genome-wide hypomethylation pattern at transcriptional enhancers has also been reported recently. This was illustrated by the recurrent hypomethylation at the enhancer of C/EBPβ, resulting in the reactivation of its enhancer RNA (eRNA) and gene overexpression, leading to increased tumourigenesis. Other examples of genes – with possible tumour-promoting effects – that are hypomethylated and overexpressed in HCC are summarised in Table 2. Together, these observations provide further mechanistic links explaining the role of DNA hypomethylation in hepatocarcinogenesis.

DNA hypermethylation at preneoplastic stages and in HCC tissues occurs predominantly in promoter-associated CGIs and cis-regulatory elements, and correlates with reduced gene expression. Moreover, this epigenetic alteration precedes the appearance of chromosomal instability during hepatocarcinogenesis. Hypermethylated and downregulated genes
found in hepatocarcinogenesis include well-known tumour suppressor genes (TSGs), regulators of cell signalling, proliferation, survival and metastasis, such as SOCS1, HHIP, SFRP2, APC, RASSF1, CDKN1A, CDKN2B, CDKN2A and CDH1, among others (Fig. 2 and Table 2).39,82 Similar findings were made for metabolic genes like MAT1A, FBP1 and CPS1, involved in SAM synthesis, gluconeogenesis and the urea cycle, respectively, whose repression may contribute to pro-oncogenic metabolic reprogramming.83–85 Interestingly, hypermethylation of CGIs located in the gene bodies of bona fide oncogenes has recently been reported in experimental HCC, and this modification was consistently associated with their transcriptional activation.86 Of note, the hypermethylated and transcriptionally active regions in gene bodies seem to be more prone to mutagenic and rearrangement events, at least in HBV-related HCC.79 The strong association between DNA hypermethylation and hepatocarcinogenesis enabled the identification of a DNA-methylation signature in HCC tissues that could predict survival.78 More recently, the existence of a CGI methylator phenotype (CIMP), a biological phenomenon characterised by a subset of concurrently hypermethylated genes, has been identified in HCC tissues. Patients with higher CIMP scores were shown to have worse clinical outcomes, and robust CIMP-based diagnostic and prognostic models were developed based on few CIMP-associated genes.87,88

The mechanisms involved in the dysregulation of DNA methylation in hepatocarcinogenesis are likely multifarious. Oxidative stress,89 as well as HCV and HBV infection, have been shown to alter normal DNA methylation patterns in hepatocytes.10,80,91 The expression of the enzymes involved in DNA methylation and demethylation, DNMTs and TETs, is known to be altered in hepatocarcinogenesis. In this context, DNMT1 and its epigenetic adaptor partner UHRF1 are upregulated in HCC tissues.
and associated with poor prognosis.\textsuperscript{92–94} Interestingly, the expression of DNMT1 and DNMT3a is already elevated in chronic hepatitis, and DNMT3a and DNMT3b are also overexpressed in HCCs.\textsuperscript{25} Regarding active DNA demethylation, the levels of 5hmC are consistently reduced in cirrhotic tissues and early HCC stages, remaining low in tumours and correlating with cancer progression.\textsuperscript{96–98} The significant depletion in 5hmC may be attributed in part to the fall in global 5mC levels, but also to the reduced expression and activity of TET enzymes observed in HCC tissues,\textsuperscript{99} where the concentrations of their substrate 5-KG are also markedly diminished.\textsuperscript{98} Impaired 5hmC turnover and DNA demethylation activity may contribute to the epigenetic repression of TSGs in HCC, as shown for SOCS1.\textsuperscript{100} However, the functional consequences of 5hmC depletion may still not be fully appreciated. Beyond being an intermediary in the DNA demethylation process, this epigenetic mark is normally enriched in the bodies of actively transcribed genes and enhancer elements; it can modify interactions between DNA and TFs, interfering with maintenance DNA methylation, as the DNMT1-UHRF1 complex does not recognise 5hmC.\textsuperscript{99}

### Histone PTMs

The dysregulation of epigenetic modifiers of histones and their role in hepatocarcinogenesis is being actively investigated (Fig. 2). There is some information on the expression of HATs in hepatocarcinogenesis. For instance, a recent study showed that upregulation of hMOF/KAT8 promoted microvascular invasion in HCCs.\textsuperscript{101} However, many works have reported the overexpression of class I HDACs, like HDAC1 and HDAC2, in HCC tissues and their association with mortality.\textsuperscript{102,103} Class II and class III HDACs, such as HDAC4, HDAC5, SIRT1, SIRT2 and SIRT7, have also been found upregulated in HCCs,\textsuperscript{104,105} and their correlation with tumour progression has been established in some cases.\textsuperscript{104,106,107} Emerging experimental evidence also supports the involvement of HDACs in NAFLD-related HCC development.\textsuperscript{108} The mechanisms underlying the dysregulation of HDAC expression in HCC are not fully understood, but a relevant role for specific miRNAs is clearly being elucidated.\textsuperscript{105,109} A plethora of mechanistic studies have demonstrated the involvement of HDACs in the pathogenesis of HCC. When overexpressed, these epigenetic modifiers display multifaceted pro-oncogenic effects, including the inhibition of TSG expression, activation of cell cycle progression, apoptosis evasion, adaptation to hypoxia and metabolic reprogramming.\textsuperscript{105,106,111} The molecular mechanisms by which HDACs cause carcinogenesis can be quite complex. For instance, HDAC8 upregulation contributes to insulin resistance in NAFLD progression and, in coordination with the HMT KMT6 (EZH2), epigenetically represses the expression of Wnt antagonists, enhancing cell proliferation in HCC.\textsuperscript{108}

Many recent studies demonstrate the altered expression of genes coding for both HMTs and KDMs in HCC tissues. While histone deacetylation is generally associated with repression of gene expression, the intricacies of the histone methylation code make the impact of its dysregulation far more complex. Within the HMT family, one of the best characterised enzymes is EZH2/KMT6, a component of the polycomb repressive complex 2 that mediates gene repression through H3K27 trimethylation.\textsuperscript{112} Increased EZH2 expression and H3K27me3 levels are found in HCC tissues, correlating with tumour aggressiveness.\textsuperscript{112} Several mechanisms have been reported to be involved in the pro-tumourigenic effects of EZH2, including the repression of Wnt pathway antagonists, the silencing of tumour suppressor miRNAs or the cooperation with cell cycle-related kinases to enhance androgen receptor signaling.\textsuperscript{113–115} Other HMTs upregulated in HCC and also associated with poor prognosis are SUV39H1/KMT1A, SETDB1/KMT1E and G9a/KMT1C, which target H3K9 and are mostly associated with gene repression.\textsuperscript{94,116–118} The overexpression of these HMTs results in enhanced growth and survival of HCC cells through multiple mechanisms, such as repression of TSG expression in coordination with promoter DNA hypermethylation, the adaptation to hypoxia and pro-oncogenic metabolic reprogramming, as demonstrated for G9a/KMT1C.\textsuperscript{94,118} Interestingly, a recent study identified the HMTs MLL1/KMT2A and MLL2/KMT2B as transcriptional targets of mutated TP53 (p53\textsuperscript{R2485}), and their pharmacological inhibition abrogated p53\textsuperscript{R2485}-driven HCC cell growth.\textsuperscript{119} There are also numerous examples of the dysregulation of KDM enzymes in HCC tissues and their association with enhanced tumour progression. For instance, increased KDM3A expression was associated with tumour recurrence after resection,\textsuperscript{119} while the upregulation of LSD1/KDM1, KDM5B, KDM6B, KDM4B and KDM2A has been related to tumour aggressiveness and poor prognosis.\textsuperscript{120–124} The transcriptional programmes influenced by the overexpression of KDMs are complex to elucidate. The activity of these enzymes may have differential effects on gene expression depending on the specific histone residue on which they act. For example, LSD1/KDM1 and KDM4B, both upregulated in HCC, can remove repressive H3K9 methyl marks, while concomitantly, LSD1/KDM1 and KDM4B can eliminate H3K4 and H3K36 active methyl marks, respectively.\textsuperscript{124} Nevertheless, several pro-tumorigenic mechanisms triggered by KDMs in HCC have been elucidated, including the promotion of stem cell-like traits by KDM6B and KDM2A,\textsuperscript{122,124} and the maintenance of glycolytic metabolism, stemness and drug resistance by LSD1/KDM1.\textsuperscript{125,126}

The role of dysregulated histone PTM readers in hepatocarcinogenesis is increasingly being recognised. This is clearly illustrated by BRD-containing protein 4 (BRD4), which was originally shown to be overexpressed in HCC tissues – derived from patients with a poor prognosis – wherein it promoted epithelial-mesenchymal transition.\textsuperscript{127} BRD4 recognises H3K27ac marks in chromatin, and H3K27ac is highly enriched in large clusters of enhancers, called super-enhancers (SE), that synergistically drive gene expression. Interestingly, many of the H3K27ac-marked SEs in HCC cells were associated with well-known oncogenes, and the presence of BRD4 was necessary for the expression of these SE-driven oncoproteins.\textsuperscript{128}

Despite the accumulating data on epigenetic alterations in HCC that we have tried to summarise herein, the epigenetic map of human HCC is far from complete. A recent TCGA-based comprehensive study analysed the mutational status and expression of 90 histone epigenetic readers, writers and erasers in HCC tissues. The authors found that 75% of patients presented at least one somatic mutation in one of the epigenetic modifiers examined, while 20% had more than 5. Regarding gene expression, when epigenetic modifiers were analysed in aggregate, 43% were upregulated and 22% downregulated in HCC tissues vs. non-tumoural tissues.\textsuperscript{26}

### Targeting epigenetic mechanisms in HCC

As opposed to gene mutations, the inherent reversibility of epigenetic abnormalities makes them promising targets for small molecules (epidrugs) in cancer treatment. The first FDA-approved epidrugs were DNMT and HDAC inhibitors for the treatment of haematological malignancies.\textsuperscript{31,29,130} The
experimental and clinical experience gathered over the past years has shown us that: i) these specific but at the same time “globally acting” agents can reprogramme cancer stemness through their interaction with multiple genes and pathways, inhibiting cancer initiation and progression;129,131 ii) long-lasting effects can be achieved at low and less toxic doses of epidrugs;129,132 iii) the inhibitory effects of SGI-110 on HCC growth and its ability to increase immune recognition of tumour cells and synergise with sorafenib142,143 Decitabine was tested in patients with advanced HCC at low doses, showing favourable toxicity and signs of clinical benefit.144 However, 5-aza and decitabine have very short half-lives in vivo due to degradation by cytidine deaminase (CDA), which is abundant in the liver;39 therefore another second generation DNMTi consisting of 5-aza-2’-deoxycytidine (decitabine). Both can be incorporated into DNA, bind DNMT1 irreversibly and induce its degradation, leading to DNA demethylation. In addition, significant amounts of 5-aza can also be incorporated into RNA, also altering gene expression.130 In response to these agents TSG expression is reactivated and HCC cells partially recover their hepatocellular microenvironment (TME), both stromal and immune cells, contribute to carcinogenesis and can be targeted to enhance therapeutic efficacy. Indeed, recent evidence indicates that targeting different epigenetic regulators, including writers (DNMTs and HMTs), readers (BRDs) and erasers (HDACs, KDMs) can increase immune recognition of tumour cells and synergise with immunotherapy.135,136

A wide variety of epidrugs have been developed over the past years. Most of them are in use, or are undergoing clinical trials, for the treatment of haematologic malignancies (Table 3). However, these compounds are increasingly being experimentally and clinically tested in solid tumours, including HCC (Fig. 3 and Table 3). The effects of DNA methylation inhibitors (DNMTi) on HCC cells have been extensively studied. The first-generation DNMTi included 5-azacytidine (5-aza) and 5-aza-2’-deoxycytidine (decitabine). Both can be incorporated into DNA, bind DNMT1 irreversibly and induce its degradation, leading to DNA demethylation. In addition, significant amounts of 5-aza can also be incorporated into RNA, also altering gene expression.130 In response to these agents TSG expression is reactivated and HCC cells partially recover their hepatocellular differentiation, becoming less tumourigenic and more sensitive to sorafenib.142,143 Decitabine was tested in patients with advanced HCC at low doses, showing favourable toxicity and signs of clinical benefit.144 However, 5-aza and decitabine have very short half-lives in vivo due to degradation by cytidine deaminase (CDA), which is abundant in the liver;39 therefore improved DNMTIs have been developed. These include zebularine, an orally available and more stable DNMTi that is potentially less toxic as it does not incorporate into DNA, which has shown preclinical efficacy in a subclass of HCCs with a high degree of CpG methylation.145 Guadecitabine (SGI-110) is another second generation DNMTi consisting of 5-aza-2’-deoxycytidine linked to deoxyguanosine by a phosphodiester bond that is converted in vivo into decitabine; it is resistant to CDA and thus more stable.130 Preclinical studies have shown the inhibitory effects of SGI-110 on HCC growth and its ability to improve the antitumoral actions of sorafenib and

| Drug | Disease | Phase | Reference/Clinical trial number |
|------|---------|-------|---------------------------------|
| DNMTi | Azacitidine | CMML, AML, MDS | Clinical practice (137) |
|      | Decitabine | CMML, AML, MDS | Clinical practice (137) |
|      | Decitabine + Chemo- or immunotherapy | HCC | Phase I/II NCT01799083 |
|      | Guadecitabine (SGI-110) | AML | Phase III NCT029290008 |
|      | Guadecitabine (SGI-110) + sorafenib + oxaliplatin | HCC | Phase II NCT01752933 |
|      | Guadecitabine (SGI-110) + duralumab | HCC and biliopancreatic tumours | Phase I NCT03257761 |
|      | TdCyd (4’-thio-2’- deoxycytidine) | Advanced solid tumours | Phase I NCT02423057 |
| HDACi | Belinostat (PXD-101) | Relapsed or refractory PTCL | Clinical practice (138) |
|      | Belinostat (PXD-101) | HCC | Phase I/II NCT00321594 |
|      | Chidamide | PTCL | Phase II NCT02944812 |
|      | Panobinostat | Multiple myeloma | Clinical practice (139) |
|      | Quisinostat | Ovarian cancer | Phase II NCT02948075 |
|      | Resminostat + sorafenib | HCC | Phase I/II NCT00945449 |
|      | Romidepsin | CTCL | Clinical practice (140) |
|      | Vorinostat | CTCL | Clinical practice (141) |
| HMTi | MAK683 | DLBCL, NPC and other advanced solid tumours | Phase I/II NCT02900651 |
|      | Tazemetostat (EPZ-6438) | Refractory B cell (NHL) with EZH2 gene mutation | Phase I/II NCT03456726 |
| HDMi | GSK2879552 | Relapsed or refractory SCLC | Phase II NCT02034123 |
|      | INC8059872 | Advanced solid tumours and hematologic malignancies | Phase II NCT027212905 |
| BETi | BMS-986158 | Advanced solid tumours and hematologic malignancies | Phase I/II NCT02419417 |
|      | GS-5829 | Solid tumours, lymphoma | Phase I NCT02392611 |
|      | INC8057643 | Advanced solid tumours and hematologic malignancies | Phase I/II NCT02711137 |
|      | PF-06821497 | SCLC, DLBCL, CRPC, and FL | Phase I NCT03460977 |
|      | ZEN003694 | Triple negative breast cancer | Phase II NCT03901469 |

AML, acute myeloid leukaemia; BETi, bromodomain and extra-terminal domain inhibitors; CMML, chronic myelomonocytic leukaemia; CRPC, castration-resistant prostate cancer; CTCL, cutaneous T cell lymphoma; DLBCL, diffuse large-B cell lymphoma; DNMTi, DNA methyltransferase inhibitor; FL, follicular lymphoma; HCC, hepatocellular carcinoma; HDACi, histone deacetylase inhibitor; HDMi, histone demethylase inhibitor; HMTi, histone methyltransferase inhibitor; MDS, myelodysplastic syndrome; NHL, non-Hodgkin lymphoma; NPC, nasopharyngeal carcinoma; PTCL, peripheral T cell lymphoma; SCLC, small cell lung cancer.
oxaliplatin. Interestingly, besides TSG reinduction, the antitumoral effects of SGI-110 may also be attributable to DNA demethylation in gene body regions and the downregulation of pro-tumorigenic gene expression, including the epigenetic regulator UHRF1 and the HMT KMT76 (EZH2). Moreover, SGI-110 robustly reactivated the expression of epigenetically silenced endogenous retroviruses in HCC cells, triggering an innate immune response that can be harnessed to improve ICI sensitivity in vivo. The results of clinical trials testing SGI-110 administered alone or in combination with other anti-tumoural or immunotherapeutic agents in patients with advanced HCC are awaited (Table 3).

HDAC inhibitors (HDACi) have also been approved and clinically evaluated, mostly for the treatment of haematological malignancies (Table 3). Their efficacy in experimental HCC has been demonstrated. For instance, the non-selective pan-HDACi panobinostat was found to inhibit HCC proliferation, induce apoptosis, reprogramme cancer cell metabolism and reduce tumour angiogenesis. Another pan-HDACi, belinostat, also showed experimental efficacy and its clinical performance in patients with unresectable HCC suggests some value as a second-line treatment. Interestingly, the combination of belinostat with ICIs increased their efficacy in an experimental model of HCC. Resminostat, an oral pan-HDACi, was tested in patients with advanced HCC who had previously progressed on sorafenib. The combined administration of resminostat with sorafenib showed clinical efficacy, indicating that this HDACi may restore sensitivity to sorafenib. Mechanistically, the ability of resminostat to induce the reversion of HCC cells’ stem-like properties may underlie the increased cytotoxic effects of the multikinase inhibitor. A recent study also provided a rationale for SIRT7 inhibition to increase the efficacy of immunotherapy (anti PD-L1) in mouse models, however potent and specific SIRT7 small molecule inhibitors still need to be synthesised.

The development of HMT and HDM inhibitors is also being actively pursued, with some compounds being clinically tested in haematological malignancies (Table 3). In the context of HCC, the KMT6/EZH2 inhibitor GSK126 has recently been shown to enhance natural killer (NK) cell-mediated HCC cell death through its ability to re-induce the expression of NK cell ligands in HCC cells. Another recent study demonstrated that combined administration of 5-aza and 3-deazaneplanocin A (a non-specific EZH2 inhibitor) enhanced intratumor T cell trafficking and improved the antineoplastic effects of ICIs in a model of subcutaneously implanted HCC cells. However, another recent report found that administration of decitabine and GSK126 resulted in an impaired antitumorigenic T cell response and increased growth of orthotopically implanted HCC cells. Although, targeting G9a/KMT1C with different selective inhibitors reactivated TSG expression in HCC cells and demonstrated antitumoral effects in both in vitro and in vivo HCC xenograft models.

Regarding KDM (HDM) targeting, inhibition of LSD-1/KDM1 with GSK2879552 could revert stem cell-like properties and re-sensitise HCC cells to sorafenib in vivo. More recently the JmjC KDM family inhibitor JIB-04 was shown to exert potent
antitumoral effects in an immunocompetent mouse model of inflammation and fibrosis-associated HCC.\\textsuperscript{35}

As mentioned, epigenetic mechanisms act in concert in the normal regulation of gene expression, but can also become dysregulated in a “coordinated” manner to drive disease progression.\\textsuperscript{41} This notion has led to the development of a novel set of drugs that combine dual inhibitory activities by acting on different epigenetic targets, or against an epigenetic and a non-epigenetic enzyme.\\textsuperscript{36} One of these agents is CM-272,\\textsuperscript{158} a new class of dual inhibitors of G9a/KMT1C and DNMT1, enzymes that coordinate to mediate TSG silencing and foster cancer growth.\\textsuperscript{159} CM-272 showed anti-HCC efficacy \\textit{in vitro} and \\textit{in vivo}, being able to restore the differentiated phenotype of HCC cells and to abate the pro-tumorigenic effects of the fibrogenic stroma.\\textsuperscript{34} CM-272 has been shown to potentiate the efficacy of ICIs in other solid tumours;\\textsuperscript{160} thus, evaluation of this combination in immunocompetent HCC models is warranted.

Pharmacological targeting of epigenetic readers is also an area of active research, and studies on haematological malignancies are also pioneering the field (Table 3).\\textsuperscript{161} As mentioned, BRD4 and H3K27ac marks are known to be increased in HCC.\\textsuperscript{128,162} Targeting the H3K27ac reader BRD4 with the inhibitor JQ-1 reduced HCC cell growth and survival,\\textsuperscript{36,128} and most interestingly it prevented non-alcoholic steatohepatitis-associated experimental HCC development.\\textsuperscript{162} Besides disrupting tumour-intrinsic oncogenic pathways, interference with BRD4 in myeloid-derived suppressor cells inhibits the development of fibrosis-associated experimental HCC, and enhances ICI efficacy.\\textsuperscript{163} Together, these findings highlight the potential of preventing/treating HCC by targeting epigenetic mechanisms in the fibrotic stroma. Indeed, fibrogenesis is a key contributor to HCC development, and dysregulation of epigenetic circuits is central to liver fibrogenic cell activation and HCC progression.\\textsuperscript{31,164,165} Pharmacological interference with such circuits is therefore emerging as an alternative strategy to halt liver fibrosis, and ultimately HCC development, as shown for BRD4 inhibitors,\\textsuperscript{166} and more recently for the dual G9a/DNMT inhibitor CM-272.\\textsuperscript{167}

\textbf{Conclusions}

Our understanding of epigenetics has increased significantly over the last 2 decades. We are now aware of its importance for the regulation of cell function and its involvement in cancer development. However, we have also learned that the complexity of epigenetic regulatory circuits is enormous, and we have barely begun to scratch the surface of the iceberg. Although not covered here because of space limitations, epigenetic modifications such as HMTs, KDMs and HDACs may also act on multiple protein targets, besides histones, with important regulatory consequences.\\textsuperscript{30,129} Moreover, a given epigenetic modifier, such as G9a/KMT1C, can behave as a coactivator or corepressor of the transcription of different target genes in the same cell;\\textsuperscript{168} while others like EZH2/KMT6 may act as gene repressors or as transcriptional activators depending on the tumour type where they are overexpressed.\\textsuperscript{30} It is therefore critical to unravel all the dimensions of the epigenetic machinery to fully understand their pathophysiological implications. Given the magnitude of the task, the implementation of artificial intelligence tools for the molecular analyses of clinical samples and relevant experimental models appears essential.

Despite our still limited knowledge, current evidence suggests that epigenetic therapies also hold promise for the treatment of HCC, particularly in combination with other chemotherapeutics or with ICIs. However, there are still important general issues that need to be addressed when epigenetic drugs are considered as combination partners in cancer therapy. One relevant aspect is the potential systemic toxicity of these compounds. In general, combinations with chemotherapeutic agents or ICIs have been well tolerated.\\textsuperscript{169} Nevertheless, adverse reactions to epidrugs may emerge due to their pleiotropic effects on gene expression, their activity on non-histone targets, or their interaction with non-tumoural cells.\\textsuperscript{31,170} The specificity issues of epigenetic drugs are difficult to harness, as the diversity of their biological effects depends not only on the quality of their chemical design, but mostly on the complex functional interactions among their targets, as previously discussed. Accumulating evidence indicates that epidrug toxicity may be averted or attenuated by modifying the administration strategies. Importantly, it should be considered that dosing epigenetic drugs below their maximum tolerated dose can still result in full pharmacodynamic effects.\\textsuperscript{171,172} Additionally, in combination regimens, selection of treatment sequence and schedule (i.e. concomitant administration, sequential administration, intermittent dosing, epigenetic priming, etc.) may not only reduce toxicity but also leverage synergies and overcome intrinsic and acquired resistance.\\textsuperscript{169} The selection of adequate dosages and schedules may be particularly important in combinations between epidrugs and ICIs, as pro-tumorigenic effects have been recorded for both types of agents in experimental and clinical settings.\\textsuperscript{160,173} Therefore, administration strategies still need to be thoroughly addressed in carefully designed clinical studies. Another determinant for the optimal application of epigenetic therapies is the availability of epigenetic biomarkers. Ideally, molecular biomarkers would enable patient selection and stratification, as well as the prediction of therapeutic response. However, the diversity and dynamism of epigenetic marks make the development of biomarkers for heterogeneous tumours such as HCC particularly challenging. So far, the identification of mutations in epigenetic genes, and the detection of CpG methylation at specific loci constitute the most successful epigenetic biomarkers in cancer.\\textsuperscript{31} All in all, extracting the full potential of epidrugs for HCC treatment will require a precision-medicine approach, involving multidisciplinary cooperation and \textit{ad hoc} trial designs. Nevertheless, the potential reward is clearly worth the effort.

\textbf{Abbreviations}

5acC, 5-acetylcysteine; 5fC, 5-formylcytosine; 5hmC, 5-hydroxymethylcytosine; 5mC, 5-methylcytosine; Acetyl-CoA, acetyl coenzyme A; α-KG, α-ketoglutarate; BER, base excision repair; BRD, bromodomain; CDA, cytokine deaminase; CGI, CpG island; CIMP, CGI methylator phenotype; CTLA-4, cytotoxic T-lymphocyte-associated protein 4; DNMT, DNA methyltransferase; DNMT1, DNMT inhibitor; FAD, flavin adenine dinucleotide; HAT, histone acetyltransferases; HCC, hepatocellular carcinoma; HDAC, histone deacetylase; HDACi, HDAC inhibitor; HDM, histone demethylase; HMT, histone methyltransferase; KMT, lysine methyltransferase; LSD/KDM, lysine specific demethylases; NAFLD, non-alcoholic fatty liver disease; ncRNAs, non-coding RNAs; NK, natural killer; NPC, nasopharyngeal carcinoma; PD1, programmed cell death protein 1; PD-L1, programmed cell death ligand-1; PHD, plant homeodomain; PTM,
post-translational modification; SAM, S-adenosyl-L-methionine; TDG, thymidine-DNA-glycosylase; TERT, telomerase reverse transcriptase; TET, ten-eleven translocation; TME, tumour microenvironment; TSG, tumour suppressor gene; UHRF1, ubiquitin like with PHD and ring finger domains 1; VEGF, vascular endothelial growth factor.

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Conflict of interest

The authors declare no conflict of interest.

Please refer to the accompanying ICMJE disclosure forms for further details.

Authors’ contributions

All authors systematically revised the literature and revised the manuscript. CB and MAA supervised, drafted and reviewed the manuscript.

Supplementary data

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