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Review

DNA methylation markers in the detection of hepatocellular carcinoma

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Abstract Hepatocellular carcinoma (HCC) is the most common primary liver malignancy and has a poor prognosis. Epigenetic modification has been shown to be deregulated during HCC development by dramatically impacting the differentiation, proliferation, and function of cells. One important epigenetic modification is DNA methylation during which methyl groups are added to cytosines without changing the DNA sequence itself. Studies found that methylated DNA markers can be specific for detection of HCC. On the basis of these findings, the utility of methylated DNA markers as novel biomarkers for early-stage HCC has been measured in blood, and indeed superior sensitivity and specificity have been found in several studies when compared to current surveillance methods. However, a variety of factors currently limit the immediate application of these exciting biomarkers. In this review, we provide a detailed rationalisation of the approach and basis for the use of methylation biomarkers for HCC detection and summarise recent studies on methylated DNA markers in HCC focusing on the importance of the aetiological cause of liver disease in the mechanisms leading to cancer.

1. Introduction

Liver cancer is one of the most prevalent human tumours and contributes significantly to the global cancer burden, with an estimated 810,000 deaths per year [1]. Over 80% of all liver cancers are classified as hepatocellular carcinoma (HCC). HCC typically arises in the background of cirrhosis, although up to 20% of cases can develop in non-cirrhotic livers [2]. Infections with the hepatitis B virus (HBV) or hepatitis C virus (HCV) are major causes of HCC worldwide. Although two large clinical cohorts showed that with the advent of

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new therapies, the incidences of HBV- and HCV-related HCC have significantly reduced [3,4], the lifetime risks of HCC in patients with chronic HBV and HCV are still high [1,5,6], especially in those who have been diagnosed with cirrhosis [7]. In recent years, the rapidly rising incidence of metabolic syndrome in many countries has shifted the aetiology of HCC away from viral hepatitis towards non-alcoholic fatty liver disease (NAFLD) [8]. In addition, the number of patients with alcoholic liver disease (ALD)-related HCC has also increased across continents and cirrhosis secondary to alcohol intake is the dominant factor in the development of HCC in many parts of the world, including Central and Eastern Europe [9]. Other potential risk factors that contribute to hepatocarcinogenesis include aflatoxin B1, tobacco, and rare genetic mutations [10]. The stratification of HCC is largely based on the Barcelona Clinic Liver Cancer (BCLC) scoring system. Early-stage HCC is usually referred to BCLC 0/A stage [11]. The prognosis of HCC is driven by tumour stage, with curative options, patients have a more than 70% survival rate for early-stage HCC, while for symptomatic advanced-stage cases treated with systemic therapies, the survival rate only achieved 1–1.5 years [12,13]. Hence improved identification of early-stage HCC and prediction of those who are going to develop HCC is urgently needed. Currently, the surveillance of HCC in clinical practice relies on ultrasonography and alfa-fetoprotein (AFP). Given the poor sensitivity of AFP when used alone in surveillance of HCC (41–65%) [14], ultrasonography is recommended by most hepatological societies (European Association for the Study of the Liver (EASL), The American Association for the Study of Liver Diseases (AASLD), and The Asian Pacific Association for the Study of the Liver (APASL)) with or without AFP. However, the sensitivity of ultrasonography for detecting early-stage HCC is only 47%, and when combined with AFP, this increases to just 63% [15]. For this reason, the search for more sensitive blood biomarkers to detect early-stage HCC is an active field of research. Indeed, a large number of candidate markers are currently being studied, including protein markers (i.e. glypican-3, Golgi protein 73, AKR1B10), inflammatory markers (interleukins and angiogenic factors), and microRNAs (i.e. microRNA-122 and microRNA-21) [16]. Some of these markers are combined in algorithms consisting of gender, age, AFP-L3, AFP, and Des-Carboxy-Prothrombin (DCP), called the GALAD score, which seems to have a better profile in predicting the probability of developing HCC in patients with chronic liver disease, but has not been validated across the globe [17].

Other candidate biomarkers for the early detection of HCC are specific methylated genes detected in cell-free DNA (cfDNA) isolated from blood (Fig. 1). In general, cfDNA is derived from dead cells, such as leucocytes, but in patients with cancer, a fraction can also be released by the tumour itself into the circulation as a result of necrosis or apoptosis of tumour cells. This circulating tumour-derived DNA (ctDNA) can be analysed for methylation patterns of genes that are specific for the tumour, and not found in healthy cells. This approach holds great promise because of the advances in the field to determine DNA methylation from cfDNA by highly sensitive techniques in a minimally invasive manner using plasma or serum [18]. Indeed, a comparison of genome-wide profiles in cfDNA has revealed hypermethylated or hypomethylated genes that were

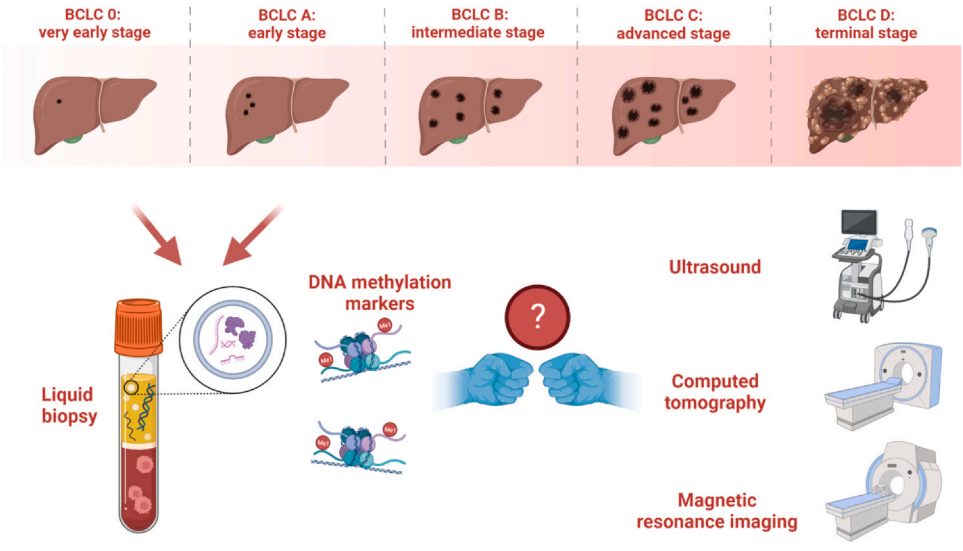

Fig. 1. Techniques for HCC detection. Early-stage HCC refers to patients diagnosed with BCLC 0 or A stage. However, current techniques, including imaging do not perform well for the diagnosis of early-stage HCC. Liquid biopsy holds great promise since tumour-related products can be found and extracted from blood, such as DNA methylation markers (DMMs) using circulating tumour DNA. BCLC, Barcelona Clinic Liver Cancer; HCC, hepatocellular carcinoma.

reported to be specific for a tumour [19], and, therefore, potentially discriminative between early-stage HCC and cirrhosis [20]. Exploration of the details of these markers, referred to as DNA methylation markers (DMMs) in the detection of early-stage HCC and the heterogeneity between different liver diseases may be applicable and highly relevant for the surveillance of HCC. In this review, we provide an exhaustive description of these markers as well as their differences across variable causes of HCC.

2. Mechanisms of DNA methylation and role in tumourigenesis

Genomic DNA can be modified, resulting in modulation of transcriptional regulation. This process is called epigenetic modification, and includes methylation of specific nucleobases in DNA. During DNA methylation, a methyl group provided by S-adenosylmethionine is covalently coupled to DNA on the fifth position of cytosine (5-mC) [21,22]. This cytosine methylation reaction is mediated by various DNA methyltransferases (DNMT): DNMT1, DNMT3a, and DNMT3b are involved in inducing or maintaining global cytosine methylation [23]. Under normal circumstances, DNA methylation is observed on cytosine-guanine dinucleotides (CpGs) dispersed randomly throughout the DNA sequence, and the promotor and first exon regions are virtually unmethylated [24,25]. In contrast, in cancerous cells, this is the opposite, with relatively high methylation rates observed in the promotor and first exon regions (Fig. 2) [25].

DNA methylation at the promoter regions has major consequences since it modulates transcriptional regulation leading to changes in gene expression. How DNA methylation contributes to inhibit gene expression still remains unclear. Studies have shown that DNA methylation generates a physical barrier for some transcriptional factors, thereby preventing access to promoter-binding sites [23]. DNA methylation has also been shown to induce gene suppression by a mechanism associated with methyl-CpG-binding domain proteins. After the interaction of these domain proteins with methylated CpGs, repressive epigenetic modification enzymes are recruited, resulting in chromatin condensation and gene silencing [26–28]. In general, DNA methylation of the gene promoter is regarded as an initiation step for establishing an inactive transcriptional status. However, some studies recently described that DNA methylation at the promotor site does not always lead to gene suppression. For example, a differentially methylated region containing 34 CpGs was identified in the distal part of the telomerase reverse transcriptase (TERT) promoter, and hypermethylation of this region was associated with increased TERT expression [29].

In cancer, except for the hypermethylation of CpG islands in the transcriptional regulatory region, the genome of the tumour is characterised by an overall tumour-wide DNA hypomethylation, including hypomethylation of repetitive DNA sequences and demethylation of coding regions and introns. 5-mC can be actively reversed to unmodified cytosine through ten-eleven translocation (TET)-dioxygenase-mediated

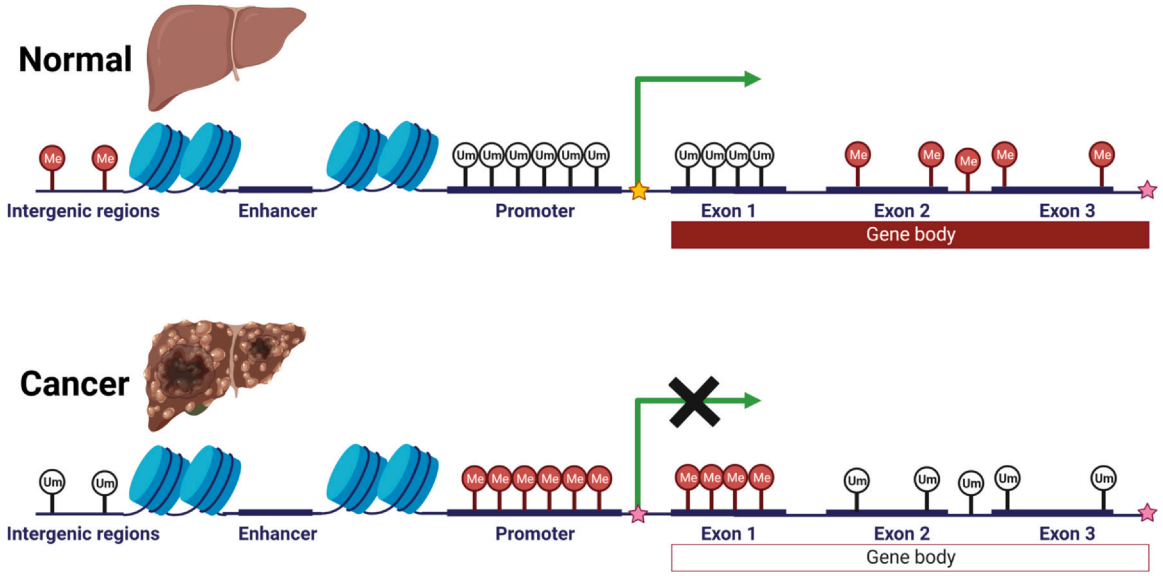

Fig. 2. The differences in DNA methylation status in normal and cancerous HCC tissue. The CpG sites are regions of DNA with repetitive cytosines and guanines in a linear sequence of bases along 5′ to 3′ direction. In mammals, CpG sites are dispersed throughout the genome or accumulated to form CpG islands near the transcriptional regulatory regions. Under normal circumstances, the promoter and first exon regions are virtually unmethylated, while other regions, such as gene bodies, intergenic regions, and repeated elements, are methylated. In contrast, in cancer, relatively high methylation rates are observed in the promotor and first exon regions, and methylation of those regions will inhibit the interaction of transcription factors in these regions. CpGs, cytosine-guanine dinucleotides; HCC, hepatocellular carcinoma.

oxidation to 5-hydroxymethylcytosine (5-hmC) [30]. During tumourigenesis from benign cellular proliferation to invasive tumour cell growth, the degree of hypomethylation of genomic DNA increases [31]. As a result, there is a greater chance of undesired mitotic recombination. Transposable elements are then reactivated and can integrate at random sites in the genome, leading to mutagenesis and genomic instability [23,32–34]. In addition, studies have also found that changes in DNA methylation may lead to the development of mutations; and mutation rates tend to be higher at methylated CpGs than at non-methylated ones [35,36]. Hence, the frequencies of DNA hypermethylation and hypomethylation at certain regions are thought to increase the rate of tumourigenesis and progression of cancer [37,38], suggesting that the DNA methylation status of genes can act as predictive markers for cancer development and progression. It is within this mechanistically context that DMMs, as specific methylation sites within cancer are known, have shown significant promise and interest as a surveillance tool for HCC.

3. Techniques for the detection of DNA methylation

The methylation of DNA within certain genes cannot be determined by regular quantitative real-time polymerase chain reaction (qPCR) since the distinction between cytosine and methylated cytosine requires additional technical modifications. Therefore, approaches have been developed to discriminate between methylated and non-methylated cytosine. One such approach is based on bisulfite conversion of cytosine. During this chemical reaction, sodium bisulfite converts normal cytosine residues to uracil residues in DNA, while leaving methylated cytosine intact [39]. Following bisulfite treatment, the two converted strands of the DNA template are no longer complementary [40]. One major drawback of the method of bisulfite conversion is that high amounts of input DNA are lost due to degradation (sometimes up to 90%), which complicates the assay when the sample quantity is limited. Based on the bisulfite-converted sequences, several techniques were developed to determine the methylation status of genes, including whole genome bisulfite sequencing, reduced representative bisulfite sequencing, pyrosequencing, quantitative methylation-specific PCR, and microarray-based techniques [41]. A summary of these techniques, their requirements with respect to input DNA needed, and assay characteristics are presented in Table 1. Whole genome bisulfite sequencing is the gold standard for genome-wide measurements of DNA methylation levels. It can detect the methylation state of nearly every CpG site [42–44], but it is expensive and requires deep sequencing [41]. Compared to whole genome bisulfite sequencing, reduced representative bisulfite sequencing (RRBS) is relatively cost-effective since it targets CpG-rich regions while ignoring CpG-poor regions [42,45,46]. Recently, Van Paemel et al. reported on the application of RRBS on cfDNA with minute DNA input [47,48]. Pyrosequencing, on the other hand, is used for quantitative DNA methylation analysis and is suitable for both CpG-poor and CpG-rich regions. The main disadvantage of this method is that only relatively short regions (less than 350 bp) can be measured [49]. This can, however, be tackled by using more sequencing primers on one amplicon or by performing serial pyrosequencing [50]. Quantitative methylation-specific PCR is a PCR-based method that uses primers designed specifically for methylated and unmethylated alleles of a chosen region. This method is suitable for the evaluation of the methylation status of small numbers of specific genes, is relatively cheap, and data analysis is straightforward. However, standardisation of the PCR-based assays, including the design of optimal methylation-specific primers and probes, is important to avoid false positive or false negative results [50]. Microarray-based techniques can be used after the digestion of DNA with methylation-specific enzymes or bisulfite conversion [51]. One of the most used array-based platforms is the Illumina Infinium Human Methylation 450 Bead Chip array (Illumina HM450), which covers more than 485,000 CpG dinucleotides in 99% of known genes and 96% of CpG islands at a single base resolution [43,52,53]. This array has been used as a reference platform for DNA methylation in The Cancer Genome Atlas Consortium (TCGA), but it does require large amounts of DNA [54].

Other methods that do not require the bisulfite conversion step are also available. Some methods use methylation-specific enzymatic digestion of DNA [55,56], such as the recently described methylated DNA sequencing (MeD-seq), which uses LpnPI endonuclease, a novel methylation-dependent enzyme that specifically cuts 16 bp upstream and downstream from methylated CpG sites to generate 32 bp fragments [57,58]. Also, TET-assisted pyridine borane sequencing (TAPS) does not require bisulfite conversion. The method converts methylated cytosines into dihydrouracil and subsequently to thymine, and requires low input DNA requirement (about 1 ng DNA), and a high sensitivity and specificity have been reported [59,60]. Thus, these methods allow for genome-wide methylation profiling without the need for harsh bisulfite treatment. Also, enrichment-based techniques, such as methyl-DNA immunoprecipitation (MeDIP) and methyl-binding domain capture (MBDCap), do not need bisulfite conversion. MeDIP is based on the immunoprecipitation of single-stranded molecules containing methylated CpGs by using a monoclonal antibody specifically against 5-mC, while MBDCap captures double-stranded methylated DNA fragments by MBD-based proteins [61]. Both of them can be combined with next-generation sequencing [62–64]. These techniques are rather recent and novel, and further validation and standardisation are necessary before broad use.

Table 1
Current techniques for DNA methylation.

Techniques	DNA input (Available data)	CpG sites coverage	cfDNA available	Pros	Cons	Reference
Whole genome bisulfite sequencing	• gDNA: 1–5 μg • FFPE DNA: 1–5 μg	95%	No	• High CpGs coverage	• Expensive • High DNA input	[42,44]
Reduced representative bisulfite sequencing	• gDNA: 0.2–1 μg • FFPE DNA: 0.5–1 μg	14.2%	No	• Targeted on high CpGs density regions	• Expensive • Low CpGs coverage	[42,45,46]
Cell-free reduced representation bisulfite sequencing	• cfDNA: 0.75–10 ng	10.6%	Yes	• Low threshold for DNA input	• Low CpGs coverage	[47,48]
Pyrosequencing	• gDNA: 0.25–1 μg	NA	Yes	• Available for both CpGs-rich and CpGs-poor regions	• Only short regions can be measured	[49,50]
Illumina HM450	• gDNA: 0.5–1 μg • serum DNA: 0.5 μg	1.7%	Yes	• Easy to use (pre-designed panel)	• Low CpGs coverage	[42,44,51,53]
Methylated DNA sequencing	• cfDNA: 10 ng	58.3%	Yes	• High CpGs coverage • Without harsh bisulfite conversion	NA	[57,58]
TET-assisted pyridine borane sequencing	• gDNA: 1 ng • cfDNA: 1 ng	Similar to WGBS	Yes	• High CpGs coverage • Without harsh bisulfite conversion	NA	[59,60]
Methyl-DNA immunoprecipitation sequencing	• gDNA: 50 ng • cfDNA: 1–10 ng	87.7%	Yes	• High CpGs coverage • Without harsh bisulfite conversion	• Low resolution (~150 bp)	[61–64]
Methyl-binding domain capture sequencing	• gDNA: 0.2–1 μg • FFPE DNA: 0.5–1 μg • cfDNA: 30 ng	17.7%	Yes	• Do not interact with 5hmC. • Without harsh bisulfite conversion	• Low resolution • Low CpGs coverage	[42,106]
Quantitative methylation-specific PCR	• cfDNA: 5 ng	NA	Yes	• Easy to apply in clinical practice • Cost-effective	• Harsh assay design and standardisations	[50,57]

cfDNA, cell-free DNA; cf-RRBS, cell-free reduced representation bisulfite sequencing; CpGs, cytosine-guanine dinucleotides; FFPE DNA, formalin-fixed paraffin-embedded DNA; genomic DNA, gDNA; Illumina HM450, Illumina Infinium Human Methylation 450 Bead Chip array; MeD-seq, methylated DNA sequencing; MeDIP-Seq, methyl-DNA immunoprecipitation sequencing; MBDCap-Seq, methyl-binding domain capture sequencing; qMSP, quantitative methylation-specific PCR; RRBS, reduced representative bisulfite sequencing; TAPS, TET-assisted pyridine borane sequencing; WGBS, whole genome bisulfite sequencing.

4. DNA methylation as a potential marker for cancer detection

Determination of the methylation status of a particular tumour type is usually performed by first evaluating genomic DNA from tumour biopsies or resections since relatively large amounts of tumour-derived DNA can be isolated. This approach will first provide candidate markers specific to the tumour. However, when the methylation status is intended to be assessed for screening purposes to detect HCC at an early stage this detection should be done in peripheral blood which is more reasonable as a screening technique. Therefore, the scientific community has attempted to identify DNA in blood that resembles that of a tumour. This is often referred to as a 'liquid biopsy'. Recently, Guo et al. and Moss et al. found that cfDNA in blood of patients reflects tumour tissue contributions that are in line with clinical findings [65,66]. Moreover, it has been demonstrated for various cancer types that ctDNA in the blood can be used in methylation studies for early-stage HCC. This ctDNA, is a highly variable fraction of cfDNA, ranging from 0.01% to more than 50% [67]. The major challenge for this is how to identify the small amounts of ctDNA out of total cell-free DNA in the blood. For the detection of ctDNA assays, the use of plasma is thought to be better than serum as the latter contains higher levels of genomic DNA generated mostly from leucocyte lysis that occurs during the clotting process when isolating serum [68]. In order to acquire enough ctDNA for analysis, a relatively high volume of plasma is needed [57]. Also, sensitive techniques, such as digital droplet PCR, can be applied, but the drawback of this assay is the low capability of multiplexing (identifying multiple targets at once). Indeed, current approaches require the identification of multiple DMMs for cancer detection, and it is, therefore, necessary to assess multiple methylation sites for better precision in detecting a tumour. Similarly, targeted sequencing-related techniques can allow the interrogation of multiple loci with high sensitivity through the use of methods that suppress background noise [69]. Therefore, ctDNA has been widely used in DNA methylation studies and showed encouraging results in the early detection of tumours, such as HCC [70–72], lung cancer [73], and colorectal cancer [74]. There are many advantages of using ctDNA from blood samples instead of relying on tumour tissue, but the most important one is the non-invasiveness of sample collection, which causes little discomfort for patients, and thus allows the possibility for more frequent screening using methylated DNA as blood biomarkers.

5. DNA methylation in HCC

5.1. DNA methylation markers in the diagnosis of early-stage HCC

Early detection is critical for HCC since more treatment options are available with clear beneficial effects on survival rates [75]. Xu et al. started the comparison of 401 DMMs generated by paired liver tissue DNA and plasma cfDNA from the same patients (n = 28) and subsequently validated this in cfDNA (n = 1933). The selected 10 DMMs yielded more than 80% sensitivity and 90% specificity for HCC (n = 1098) compared to healthy individuals (n = 835) in the following cohort. Also, these 10 DMMs showed a high area under the curve (AUC) of 0.97 in the HCC cohort and could differentiate patients with HCC from patients with other liver diseases (HBV, HCV, and NAFLD) [70]. A different study sequenced DNA from cirrhotic livers and HCC and identified different DMMs: Kisiel et al. identified a number of different DMMs, which were later validated on cfDNA from plasma of HCC patients (n = 95, 48% early-stage), cirrhosis patients and healthy individuals [20]. The study defined a six-marker DMMs panel consisting of HOXA1, EMX1, AK055957, ECE1, PFKP and CLEC11A normalised by B3GALT6, which showed a 95% overall HCC sensitivity and 92% specificity in detecting HCC. Importantly, this panel also detected more than 90% of early-stage HCC (n = 46, BCLC 0/A). As a follow-up of the study, 4 of the identified DMMs, HOXA1, EMX1, TSPYL5 and B3GALT6 were combined with the protein biomarkers AFP and AFP-L3 and tested on cfDNA from 135 HCC cases and 302 controls (87% cirrhosis). The panel achieved a sensitivity of 71% with a 90% specificity for early-stage HCC (n = 76) [76]. Subsequently, the same group improved the performance with only three DMMs (HOXA1, TSPYL5, and B3GALT6) in combination with AFP and gender on cfDNA from plasma, finding an overall 88% sensitivity for HCC detection in 156 cases compared to 245 controls (92% cirrhosis) as well as 82% sensitivity for early-stage HCC (n = 78) [77]. The patient population had diverse underlying liver diseases, with almost 85% ascribed to HCV, NAFLD or alcohol. Another study by Hernandez-Meza et al. demonstrated an increasing proportion of hypermethylated samples from cirrhotic tissue (< 1%), to dysplastic nodules (≥25%), and to early-stage HCC (≥50%) for four DMMs: TSPYL5, KCNA3, SPINT2 and LDHB [78]. Interestingly, TSPYL5 and SPINT2 were also hypermethylated in HCC tissues in the study of Kisiel et al. [20]. In addition, TSPYL5 also showed promising results in distinguishing HCC from paired non-tumour adjacent tissues in the same patients [79,80]. Recently, a multicenter study was performed on cfDNA from patients with HCC (n = 122, 37% early-stage) and controls (37% cirrhosis) using a prespecified diagnostic algorithm consisting of 28 DMMs. This test, known as HelioLiver Test, showed a sensitivity of 76% for HCC detection in patients with early-stage HCC [81]. Currently, a prospective clinical study launched by HelioGenomics on samples from patients with cirrhosis (n = 1600) is ongoing (NCT03694600). Besides DMMs, changes of 5-hmC seem specific to HCC compared to chronic liver

disease. Cai et al. performed genome-wide 5-hmC sequencing on cfDNA to develop a 32-gene diagnostic model, which exhibited high AUC (0.85) in the comparison between early-stage HCC and cirrhotic livers as well as chronic HBV livers [82].

To date, methylation of SEPT9 is the first and only blood-based methylation marker approved by the United States Food and Drug Administration (FDA) for colorectal cancer screening [83]. Interestingly, expression of SEPT9 was found to be decreased in HCC via promoter hypermethylation in a study of 304 HCC tissues compared to cirrhotic and normal liver tissues [84]. Also, the SEPT9 hypermethylation status exhibited an AUC of 0.86 in discriminating BCLC-A HCC from cirrhosis using plasma cfDNA in a multicenter study [85]. In line with this, using digital droplet PCR, it was recently demonstrated that the median copy numbers of methylated SEPT9 were higher (6.4 versus 2.0) in patients with HCC (n = 136) compared to chronic liver disease by cfDNA from plasma (Table 2) [86].

Despite the exciting data described above, numerous studies have demonstrated a high degree of heterogeneity among methylation patterns in patients with HCC. One of the most important reasons for this is the differences in major risk factors driving the development of HCC, such as HBV, HCV, alcohol and NAFLD. It has been shown that DNA methylation patterns are different in HCC based on the associated risk factors. Unsupervised hypermethylation clustering on DNA sequenced from 196 HCC tissues compared to adjacent normal tissues exhibited different methylation profiles between HBV- and HCV-related HCC [87]. PAX6 was more frequently hypermethylated in HCC associated to HCV (61%) and less methylated in HBV-related HCC (22%) [88]. Similarly, HCV-related HCC tissues showed higher methylation levels of p16 than HBV-related HCC [89], and methylguanine-methyltransferase (MGMT) was more methylated in alcohol-related HCC compared to non-alcohol-associated HCC [90]. Moreover, differences have been found across infectious and non-infectious causes of HCC. Two studies found that some DMMs are different in NAFLD-related HCC, showing lower methylation levels of TRIM4, PRC1, TUBA1B, WHSC1, MAML3, and higher methylation levels of FLCN and WDR6 compared to viral hepatitis-related HCC [91,92]. In addition to aetiology, age and geographical distribution have also been reported to influence the degree of methylation in HCC. HCC patients over the age of 60 show more hypomethylated LINE-1 in liver tissue compared to younger age groups (30% versus 11%) [90]. Also, HCC patients from Thailand exhibited different methylation levels as compared to HCC patients from France, with higher methylation levels in P14 (4.1 versus 0.7) and DOK1 (30.7 versus 18.1) as well as lower methylation levels in GNMT (8.7 versus 14.8) [90]. All of these speak of the complexities in defining specific DMMs for HCC detection that can encompass multiple HCC causes, and geo-demographic factors in a one-size fits all style.

5.2. DNA methylation markers in HBV-related HCC

As mentioned above, the methylation status of specific genes may not be similar between HCCs with distinct aetiological causes. However, only a few studies stratified HCC patients for specific etiologies. For HBV-related HCC, it has been demonstrated that several genes are hypermethylated in HBV-related HCC compared to cirrhosis and healthy individuals.

Lambert et al. performed pyrosequencing on genomic DNA from HBV-positive HCC tissues (n = 32) and found that the promoters of RASSF1A, GSTP1 and DOK1 were significantly hypermethylated with correspondingly decreased mRNA levels as compared to HBV-positive cirrhotic tissues with no HCC (n = 25) [90]. RASSF1A is one of the prototypical tumour-suppressor genes universally inactivated in human malignancies by a methylation-based mechanism [93]. GSTP1 has been suggested to play an important role in protecting cells against damage induced by carcinogens [94]. DOK1 is a member of a family of intracellular adaptor proteins that exhibits tumour-suppressive activity in both hematopoietic and non-hematopoietic malignancies [95]. Using methylation-related PCR techniques, the findings were confirmed for the promoter of RASSF1A and GSTP1 in HCC tissues [93,96]. Another study demonstrated that the methylation ratios of SFRP1 and SFRP5 in HBV-related HCC tissues were higher than in paired non-tumourous tissues [97]. Finally, it has been reported that E-cadherin has a relatively higher methylation ratio in HBsAg-positive HCC tissues compared to HBsAg-negative HCC tissues [98]. E-cadherin has been shown in multiple studies to be a key growth and invasion suppressor in cancer [99].

One of the viral proteins encoded by the HBV virus, the HBV X protein (HBx), has been shown to play a significant role in the development of HBV-related HCC, acting as a carcinogenic protein [100,101]. In HBx-positive HCC tissues, studies found that patients had significantly increased methylation ratios in the promoter of IGFBP-3 [102], and relatively lower mRNA expression of RASSF1A compared to HBx-negative HCC tissues [103]. In HBx-positive HCC cell lines, the activities of different DNMT were also altered [102]. Also, Qiu et al. investigated the expression of RASSF1A in seven cell lines and found that RASSF1A mRNA levels were reduced in the presence of HBx [103]. Similarly, the promoter activity and gene expression of IGFBP-3, GSTP1, and E-cadherin were decreased in HBx-positive cell lines compared to HBx-negative cell lines [102,104,105]. These *in vitro* results in HCC cell lines are in line with the data from HCC tissues, indicating the close correlation among HBV-related proteins, cancer-related genes and methylation in HBV-related HCC.

Only a few studies have specifically studied the differential methylation status in HBV-related HCC using

Table 2
Selection of relatively large studies of DMMs in early-stage HCC.

DMMs	Sources (Volume)	Testing samples (n)	Comments	Reference
10 DMMs	Plasma (1.5 mL)	• 1098 HCC • 835 normal individuals	• This model showed high AUC (> 0.94) for HCC detection compared to normal controls, which is better than AFP (0.82).	[70]
HOXA1, EMX1, AK055957, PFKP, CLEC11A, ECE1, and B3GALT6	Plasma (> 1 mL)	• 95 HCC (48% early-stage) • 51 cirrhosis without HCC • 98 controls without cirrhosis	• This panel detected 75% (n = 4) BCLC-0, and 93% (n = 42) BCLC-A HCC.	[20]
HOXA1, EMX1, TSPYL5, and B3GALT6	Plasma (3 mL)	• 135 HCC (56% early-stage) • 302 controls (87% cirrhosis)	• In combination with AFP and AFP-L3, this panel achieved 71% early-stage sensitivity for HCC, which is much higher than AFP, DCP and GALAD score.	[20,76]
HOXA1, TSPYL5, and B3GALT6	Plasma (5–6 mL)	• 156 HCC (50% early-stage) • 245 controls (92% cirrhosis)	• In combination with AFP and gender, this panel exhibited 82% sensitivity for early-stage HCC, better than AFP and GALAD score.	[20,77]
TSPYL5, SPINT2, KCNA3, and LDHB	Liver tissues	• 227 HCC (87% early-stage) • 8 dysplastic nodules • 139 cirrhosis • 16 healthy controls	• These four DMMs may be epigenetic gatekeeper candidates because of the increased proportion trend in the development of HCC with confirmed inverse correlation between DNA methylation and gene expression.	[78]
SEPT9	Plasma (3.5 mL)	• 98 HCC with cirrhosis (31% early-stage) • 191 cirrhosis without HCC	• To diagnose BCLC A HCC, the mSEPT9 test exhibited 64–88% sensitivity and 68–95% specificity.	[84,85]
SEPT9	Serum (0.4 mL)	• 136 HCC (45% early-stage) • 45 chronic liver disease • 80 healthy individuals	• The median copy numbers of methylated SEPT9 showed a dynamic trend from 0 in healthy control, 2.0 in chronic liver disease groups, to 6.4 in HCC groups.	[86]
HelioLiver Test (28 DMMs)	Plasma (NA)	• 122 HCC (37% early-stage) • 125 controls (37% cirrhosis)	• The algorithm exhibited 76% sensitivity for early-stage (stage I and II) HCC, better than AFP and GALAD score.	[81]
5-hmC diagnostic model	Plasma (5–10 mL)	• 809 HCC (27.1% early-stage) • 129 cirrhosis and chronic hepatitis B • 256 controls	• The model showed high AUC for early-stage HCC both in the training cohort (87.3%) and the validation cohort (84.6%); better than AFP (79.3% and 69.2%, respectively).	[82]

AFP, alfa-fetoprotein; BCLC, Barcelona clinic liver cancer; B3GALT6, beta-1,3-galactosyltransferase 6; CLEC11A, C-type lectin domain containing 11A; DCP, des-carboxy-prothrombin; DMMs, DNA methylation markers; EMX1, empty spiracles homeobox 1; ECE1, endothelin-converting enzyme 1; HCC, hepatocellular carcinoma; HOXA1, homeobox A1; KCNA3, potassium voltage-gated channel, shaker-related subfamily, member 3; LDHB, lactate dehydrogenase B; mSEPT9, methylated SEPT9; NA, not available; PFKP, phosphofructokinase; SPINT2, serine peptidase inhibitor, Kunitz type 2; SEPT9, septin 9 gene; TSPYL5, testis-specific Y-encoded-like protein 5; 5-hmC, 5-hydroxymethylcytosine.

cfDNA isolated from plasma. One study by Zhao et al. compared DMMs in cfDNA of 29 HBV-related HCC with 27 HBV-related cirrhosis, 30 chronic HBV patients and 30 healthy individuals, subsequently validating the results in DNA of 36 HCC tissues [106]. Three genes were identified that distinguished HCC from the other groups: ZNF300 (AUC 0.70), SHISA7 (AUC 0.72) and SLC22A20 (AUC 0.66). The prediction power for HCC of < 0.8 for each methylated gene was good but far from optimal. A study by Hu et al. using methylation-specific PCR on cfDNA observed significantly lower methylation frequencies of UBE2Q1 in HBV-related HCC patients (n = 20) compared to patients with cirrhosis (n = 40) [107]. In combination with different cut-off values of AFP, AUCs between 0.69 and 0.76 were reported in discriminating HCC from cirrhosis and chronic HBV infection. All these studies indicated the potential benefit of DMMs in identifying HCC in HBV-infected patients. However, larger studies addressing the specific status of the virus (immune active disease or controlled viremia) that could affect virus-related methylation rather than cancer-related methylation are needed.

5.3. DNA methylation markers in HCV-related HCC

Specific methylation patterns have been observed in patients with HCV-related HCC as compared to other etiologies. A large clinical study looking at HCV-related HCC found dramatic differences in the expression levels of the DNA methyltransferases, with DNMT1, DNMT3A and DNMT3B showing a distinct pattern in patients with HCV genotype 1b and 3a compared to those with genotypes 5 and 6 [108]. Another study showed that the expression of DNMT1, DNMT3A and DNMT3B had an upward trend in blood from healthy individuals compared to chronic HCV infection, HCV-related cirrhosis, and finally to HCV-related HCC, suggesting increased hypermethylation with the progression of the disease stage. Expression of these markers also exhibited over 80% sensitivity in discriminating HCV-related HCC (n = 26) from cirrhosis (n = 45) [109]. Other studies have shown hypermethylation of RASSF1A and GSTP1 in HCC tissue of patients with HCV detected by pyrosequencing and methylation-specific PCR [90,110]. Multiple studies have found higher methylation ratios of p16 (79% versus 16%), APC (93% versus 20%) and RIZ1 (79% versus 9%) in HCV-related HCC as compared to paired non-tumour tissue [89,111]. Finally, SFRP2, a Wnt inhibitor, showed increased methylation levels in HCV-related HCC (n = 41) compared to cirrhosis, as detected by pyrosequencing on DNA from tissue [112].

Interestingly, HCV core protein influences the expression of SFRP1, RAR-β2 and E-cadherin through DNA methylation in HCC cell lines [113–115]. Also, higher levels of HCV-RNA have been associated with decreased methylation levels of MGMT [90], suggesting a direct correlation between viral activity and DNA methylation, which is exciting in terms of viral-related carcinogenesis but speaks of further detailing for biomarker specificity.

The use of cfDNA from plasma to monitor differences between HCV-related HCC and controls has also been conducted in a very limited fashion. Although it has been shown that higher cfDNA concentrations could be extracted from the plasma of HCV-positive HCC as compared to samples from cirrhosis and chronic HCV patients [116,117]. currently, only one study about specific DNA methylation on cfDNA in HCV-related HCC is available. The study found that in serum, the methylation ratio of p16 was higher in HCV-related HCC (n = 25, 92%) than in cirrhosis and chronic HCV samples [118], supporting the methylation data of p16 from HCC tissues [110].

5.4. DNA methylation markers in non-viral hepatitis-related HCC

Compared to viral hepatitis-related HCC, studies have found that DMMs in NAFLD- and ALD-related HCC are associated with alterations of metabolism-related pathways. Interestingly, chronic alcohol intake has been shown to impair retinoic acid homoeostasis [119], and studies have revealed retinol metabolism-related DMMs with decreased gene expression profiles in ALD-related HCC tissues [120]. This suggests that increased DNA methylation levels in response to alcohol use can regulate the expression of specific genes, and that these methylated markers may be involved in the development of ALD-related HCC. A study by Lambert et al. reported slightly higher methylation levels of MGMT in patients with viral hepatitis-related HCC with alcohol consumption as compared to a group without alcohol consumption (17% versus 11%) [90].

The available data on NAFLD-related HCC in one-carbon metabolism is mainly restricted to animal experiments and includes data on genes such as GNMT and MAT1A [121–123]. Only one study examined the methylation status of liver tissues obtained following partial hepatectomy, and compared NAFLD-related HCC (n = 22) with corresponding non-tumour tissues [91]. Six genes, including DCAF4L2, CKLF, UBE2C, TRIM4, PRC1 and TUBA1B, showed down-regulated methylation levels from normal liver tissues to paired non-tumourous tissues and to NAFLD-associated HCC tissue. Similarly, in mice fed with a high-fat diet, TUBB2B showed hypomethylation in the promoter region in correlation with an increased expression in NAFLD-related HCC [124]. However, more detailed studies for DMMs, especially in cfDNA, are needed in metabolic-related HCCs to grasp an understanding of the methylation-related mechanisms and potential biomarkers.

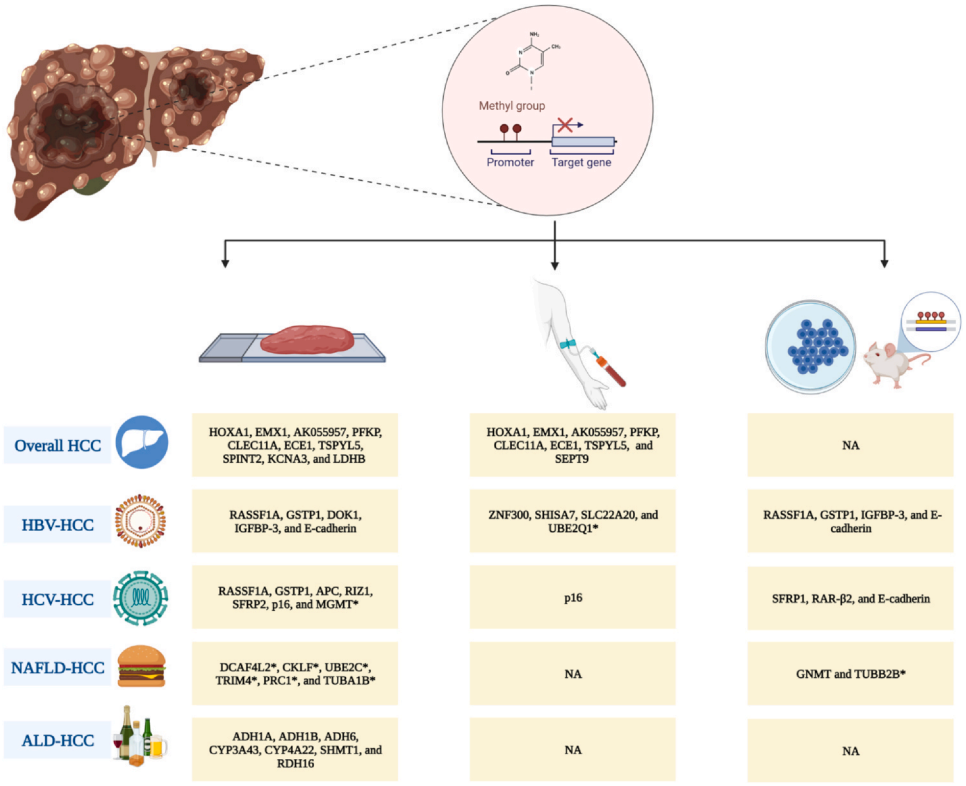

Fig. 3. A summary of the DNA methylation markers described in this review that may differentiate HCC from non-HCC samples. The currently available data for DMMs in HCC: the biomarkers presented may not necessarily be specific to only one aetiology-related HCC since most of the studies did not perform genome-wide methylation profiling. In an overall heterogeneous HCC population, HOXA1, EMX1, AK055957, PFKP, CLEC11A, ECE1, TSPYL5, SPINT2, KCNA3, LDHB, and B3GALT6 are methylated in tissues; HOXA1, EMX1, AK055957, PFKP, CLEC11A, ECE1, TSPYL5, SEPT9, and B3GALT6 are methylated in blood. In HBV-related HCC, RASSF1A, GSTP1, DOK1, IGFBP-3, and E-cadherin are methylated in tissues; ZNF300, UBE2Q1, SHISA7, and SLC22A20 are methylated in blood; RASSF1A, GSTP1, IGFBP-3, and E-cadherin are methylated in HCC cell lines. In HCV-related HCC, RASSF1A, GSTP1, APC, RIZ1, SFRP2, MGMT, and p16 are methylated in tissues; p16 is methylated in blood; SFRP1, RAR-β2, and E-cadherin are methylated in HCC cell lines. In NAFLD-related HCC, DCAF4L2, CKLF, UBE2C, TRIM4, PRC1, and TUBA1B are methylated in tissues; GNMT and TUBB2B are methylated in mice livers. In ALD-related HCC, ADH1A, ADH1B, ADH6, CYP3A43, CYP4A22, SHMT1, and RDH16 are methylated in tissues. APC, adenomatous polyposis coli; ADH1A, alcohol dehydrogenase type 1A gene; ADH1B, alcohol dehydrogenase 2; ADH6, alcohol dehydrogenase 6; ALD, alcoholic liver disease; CYP3A43, cytochrome P450 3A43; CYP4A22, cytochrome P450 4A22; CKLF, chemokine-like factor; DOK1, downstream of tyrosine kinases 1; DCAF4L2, DDB1-and Cul4-associated factor 4-like 2; DMMs, DNA methylation markers; GNMT, glycine N-methyltransferase; GSTP1, glutathione S-transferase pi 1; HCC, hepatocellular carcinoma; HBV, hepatitis B virus; HCV, hepatitis C virus; IGFBP-3, insulin-like growth factor binding protein-3; MGMT, O6-methylguanine-methyltransferase; NA, not available; NAFLD, non-alcoholic fatty liver disease; p16, cyclin-Dependent Kinase Inhibitor p16; PRC1, Polycomb repressive complex 1; RASSF1A, Ras associated domain family 1A; RAR-β2, retinoic acid receptor-beta2; RIZ1, retinoblastoma protein-interacting zinc-finger gene 1; RDH16, retinol dehydrogenase 16; SHISA7, shisa family member 7; SLC22A20, organic anion transporter; SFRP1, secreted frizzled-related protein 1; SFRP2, secreted frizzled-related protein 2; SHMT1, serine hydroxymethyltransferase 1; TRIM4, tripartite motif-containing 4; TUBA1B, tubulin alpha 1b; Tubb2b, tubulin, beta-2b; UBE2Q1, Ubiquitin-Conjugating Enzyme2 Q1; UBE2C, Ubiquitin conjugating enzyme E2C; ZNF300, zinc finger protein 300.*, hypomethylated.

6. Final remarks

Over the last decade, numerous studies have been conducted to discover and validate novel non-invasive, blood biomarkers that can be used for screening high-risk patient populations in order to diagnose HCC at early stages. As described in this review, altered DNA methylation has been shown to progress in a dynamic fashion from healthy individuals to cirrhosis patients to patients with HCC [78]. Numerous studies have described the altered methylation status of genes in liver tissues of HCC patients as compared to surrounding non-HCC tissues, and mechanistic studies have demonstrated the importance of altered methylation of genes in the tumourigenesis process. However, as can be concluded from Fig. 3, which summarises the various studies described in this review, different distinctive DMMs are found depending on the characteristics of the tumour and the type of clinical material examined.

Much effort has been invested in examining the applicability of DMMs as novel biomarkers, especially for early-stage HCC using cfDNA isolated from blood. The

findings of these studies are highly promising, and panels of different DMMs have been identified that perform equally well or better than other biomarkers, including AFP and the GALAD score, thereby opening the possibility to use liquid biopsy in early-stage HCC diagnosis. Some studies report AUC-values for these multi-marker panels in the range of 0.88–0.92 for early-stage HCC [76,77]. However, a number of important issues need to be resolved before DMMs can be broadly applied for HCC detection. One of the issues is related to the sensitivity of the assay, especially in early-stage HCC. At this stage, the tumour is small, and hence the contribution of ctDNA within cfDNA is quite limited. In addition, bisulfite conversion of DNA causes DNA degradation and loss, which, combined with the low starting point of ctDNA necessitates the use of high volumes of plasma for the assay in order to be above the lower limit of DMM detection. It has been reported that even with a blood draw of 10 mL, ctDNA was not always detected in very early-stage tumours [125]. Another issue that is highly relevant with respect to the identification of DMM as biomarkers is the heterogeneity of HCC as a result of genetic diversity, and as a result of the diversity of underlying liver diseases such as viral hepatitis, alcoholic and non-alcoholic liver disease. Moreover, the fact that HCC can arise in non-cirrhotic livers further complicates the use of a reliable and specific DMM panels. However, given the highly promising results that have been published to date and given the abundance of new technologies and methodologies that increase the sensitivity of the assays, it is highly likely that DMM detection in liquid biopsies will be one of the tools aiding in early-HCC detection in the years to come.

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CRediT authorship contribution statement

S.F., J.D. and A.B. conceptualization and writing. All authors have read and agreed to the published version of the manuscript.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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