Hypo-methylation in HBV integration regions aids non-invasive surveillance to hepatocellular carcinoma by low-pass genome-wide bisulfite sequencing

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## Abstract

## Circulating cell-free DNA (cfDNA) methylation has been demonstrated to be a promising approach for non-invasive cancer diagnosis. However, the low-level of cfDNA and high cost of whole genome bisulfite sequencing (WGBS) significantly hinders the clinical implementation of a methylation-based cfDNA early detection biomarker. Here we found a significant enrichment of cfDNA in intergenic and repeat regions, especially in previously reported HBV integration sites, as a feature of cfDNA and the bias of cfDNA release. Moreover, methylation profiles nearby hepatitis B virus (HBV) integration sites were found to enhance the prediction performance. Then, we proposed a novel method in which we utilized long-range methylation around HBV integration regions in low-pass WGBS data (~5 million reads) generated from cfDNA to detect methylation changes, and applied the method to investigate dynamic methylation changes in cfDNA from blood samples of patients with hepatitis, cirrhosis, early and advanced hepatocellular carcinoma (HCC). Models based on DNA methylation within 5kb flanking HBV integration sites and certain candidate regions exhibited powerful discrimination ability as quantified by receiver operating characteristic (ROC) curves. The results demonstrate the hypomethylation around viral integration sites aids low-pass cfDNA WGBS to serve as an non-invasive approach for early HCC detection in hepatitis B patients, and inspire future efforts on tumor surveillance for oncovirus with integration activity.

## Introduction

Liver cancer is the fourth cause of cancer-related mortality worldwide. In the United States, liver cancer death rate increased 43% from 7.2 to 10.3 per 100,000 between 2000-2016 (1, 2). Hepatocellular carcinoma (HCC), the most frequent form of primary liver cancer, generally develops in patients with chronic liver disease due to hepatitis B virus (HBV), hepatitis C virus (HCV), alcohol abuse or non-alcoholic fatty liver disease (3, 4). Chronic inflammation, fibrosis, and aberrant hepatocyte regeneration favor a series of genetic and epigenetic events that culminate in hepatocyte malignant transformation. Hepatocarcinogenesis is a complex and poorly-understood multistep process that includes the histological transition from regenerative nodules in the context of cirrhosis, through dysplastic nodules and ultimately HCC (5-7). The high risk of HCC development in patients with cirrhosis (i.e., 2-7% annual risk) justifies the recommendation of biannual HCC surveillance with abdominal ultrasound (US) with or without serum alpha-fetoprotein (AFP) in patients at high-risk (8). Non-randomized studies suggest that early HCC detection increases the odds to receive a curative treatment and increase survival. However, the sensitivity of US and AFP is 63% to detect early stage HCC (9), which underscores the need for improved early detection tools.

Circulating cell-free DNA (cfDNA) are small double-stranded DNA fragments (10) found in plasma, urine, saliva, cerebrospinal fluid (CSF) and other body fluids (11) originating of cell apoptosis and necrosis (12). In many settings, analyses of cfDNA can be regarded as a way to perform a “liquid biopsy”, which have been produced promising results for genetic testing (13, 14), early cancer detection (15, 16) and prognosis prediction (17, 18). Apoptotic and necrotic tumor cells can release cfDNA into the peripheral blood, which reflects tumor-related genetic features, including cfDNA fragment size (cfDNAsize) (19), mutations, copy number aberrations and epigenetic changes (17). Meanwhile, cfDNA also carries tissues-specific information which provides promising abilities for tissue-of-origin mapping (20-24). As such, cfDNA could be used as an important biomarker in clinical settings. There are different technologies to investigate methylation changes in cfDNA, including scRRBS (20) and cfMeDIPseq (23). A number of studies have focused on cfDNA as a potential source of novel early detection biomarkers in HCC based on DNA methylation (25-30). Multiple studies have focused on the use of cfDNA methylation in cancer diagnosis in the areas of specific biomarkers (26, 30), hypo-methylation (25) and tissue of origin (27-29). Single cytosine measurement and high accuracy have enabled whole genome bisulfite sequencing (WGBS) to become the gold standard in DNA methylation analysis (31). One challenge in detecting cell-free circulating DNA (cfDNA) in plasma is the minor fraction of cfDNA amidst the background of total circulating DNA. This is particularly true in patients with early stage cancers and in the minimal residual disease setting, which benefits from deep sequencing producing a more sensitive indicator for early cancer detection and surveillance (27, 29, 32). That said, low depth sequencing in high sample sizes is a cost-effective strategy for cohort studies (33). Utilizing reduced sequencing volume, low-pass sequencing and correspondingly low sequencing cost will be crucial to facilitate an easier clinical deployment of DNA methylation-based surveillance tools. Meanwhile, the epigenetic patterns of HBV integration regions, one of the most important features of HCC, have never been investigated in cfDNA based diagnosis system.

In this study, we investigated of cfDNA methylation profiling at low-pass WGBS and the performance of HCC prediction. We systemically collected the most comprehensive HBV integration sites (N=6,072) and explored the DNA methylation state around HBV integration regions of HCC patients. We evaluated the minimum sequencing depth for long-range methylation around reported HBV integration sites and provided the landscapes of low-pass WGBS in healthy individuals, cirrhosis, hepatitis and HCC patients. Finally, we proposed DNA methylation around HBV-integration regions carry utility to predict HCC from non-HCC samples.

## Results

### DNA methylation around HBV integration sites mirrors the hypo-methylation of HCC patients

In order to explore methylation profiles in cell-free based WGBS data, we conducted a pilot study with 5 cfDNA samples using relative high-depth WGBS: one healthy individual (D1), one patient with chronic hepatitis (D2), one patient with cirrhosis (D3) and 2 HCC patients (D4 and D5 of before and after surgery). The final read count equated to a mean of 58 million (M) reads per sample (**Table S1**). The average DNA methylation across the genome (Methylgenome) was much lower in the HCC patient (D4; 53.56%) compared to healthy individual, chronic hepatitis and cirrhosis (74.76%, 75.64% and 75.13%; **Fig S1A**; **Table S1**). We found the genome distribution of CpGs in WGBS data tended to be located at intronic, intergenic and repeat regions (**Fig 1A**).

Next, we identified differentially methylated CpGs (DMCs) and differentially methylated gene (DMGs) with cell-free WGBS data between HCC patient and healthy individual. On average, each cfDNA sample had 7,274,674 CpGs with sequencing depth over 5 reads (**Table S1**). In total, we identified 2,670 DMCs in HCC patient compared to healthy individual (**Table S2**), of which 99.8% were hypo-methylated in HCC patients. These hypo-DMCs clearly separated HCC patient from healthy individual, patient with chronic hepatitis, cirrhosis and HCC patient after surgery (**Figure 1B**). Among hypo-DMCs, 174 DMCs (6.5% of 2,670 DMC) were located in promoter or gene body of 77 genes. In particular, *SENP5* gene had six significantly hypo-methylated DMCs with consistently high sequencing coverage across all the five individuals (an average of 295 reads; **Fig 1B** and **Fig S1B**). Intriguingly, all six DMCs that we found in intron 2 of *SENP5* were located near previously reported HBV integration sites in HCC (**Fig 1C**) (34).

We found that 80% of DMCs of HCC patients were located within repeat regions (**Fig 1D**). Considering that repeat regions are a known target for HBV integration (35, 36), we analyzed the location of DMCs relative to reported HBV integration sites (34, 37-42). Totally, we collected 6,072 HBV integration sites from published researches (**Table S3**). Among the 2,670 DMCs observed in HCC patient, 21 completely overlapped with the HBV integration sites, including one in *SENP5*. Additionally, 26.8% of the DMCs were located within a 100bp region either upstream or downstream of integration sites, and 73.9% of DMCs were within 5Kbp (**Fig 1D**). Overall, these DMCs were more enriched in HBV integration sites compared to promoter and gene coding regions (**Fig 1E**, P < 2.2x10-16, Fisher’s exact test).

Although cell-free DNA were observed to be more likely to locate at HBV integration sites (**Fig 1A**, Fisher’s exact test), DMCs have higher enrichment in HBV integration sites compared to the whole cfDNA background (**Fig 1A**; **Fig 1E**). With above findings, we further examined whether DNA methylation levels around HBV integration regions could represent the hypo-methylation of HCC genome and be used in optimization of prediction model for HCC. In HCC tumor tissues and paired buffy coat samples in a previous study (25), the hypomethalytion near the HBV integration sites were observed in both tumor and buffy coat, and the closer to integration sites, the lower methylation levels. Methylation levels were further reduced in tumor tissue, especially within 100bp region near these sites (**Fig 1F and Fig S2**). We calculated the average methylation level of the CpGs within 100bp region nearby HBV integration sites (MethylHBV) in each tissue sample, as the indicator for methylation level **(Methods)**. Although MethylHBV was lower than average methylation level across the genome (Methylgenome) in both buffy coat and tumor tissue, tumor tissue samples had a significantly smaller MethylHBV compared with buffy coat (P = 8.8x10-5, t test; **Fig S3**). Particularly, MethylHBV was significantly lower than Methylgenome in tumor tissue samples (P = 8.8x10-5, t test; **Fig 1G**), which supports DNA methylation around HBV integration sites as a more sensitive indicator to detect HCC compared to average methylation level across the genome.

### Hypomethalytion of regions near HBV integration sites effectively detected by a low-pass sequencing strategy in cell-free WGBS data

Considering the dispersive and limited genomic regions represented by cfDNA fragments, particularly in patients with early stage HCC, long-range methylation around HBV integration sites (MethylHBV5K) was applied to measure the methylation status of cfDNA in the five cfDNA samples at high-depth sequencing volume (each composed of approximately 58M reads). As expected, MethylHBV5K was much lower in the HCC patient (49.85%) compared to healthy individual, chronic hepatitis and cirrhosis (72.72%, 71.58% and 71.92%; **Table S1**; **Fig S1A**). To determine the effective small sequencing depth, we randomly sampled 1M to 10M mappable reads from each sequencing dataset and calculated permuted MethylHBV5K respectively (**Methods**). As predicted, when we used more sequencing reads, permuted MethylHBV5K was closer to the value calculated using total sequencing reads. The correlation coefficient between the methylation level from low-pass WGBS and total WGBS data saturates when using 5M or more reads (**Fig 2A, Fig S4**). The correlation coefficient at permuted regions between 5M resampling reads and all sequencing reads was above 0.77 (Pearson’s correlation coefficient, **Fig 2, Fig S4**), and methylation level remained consistent after resampling 100-times (CV is 3.8%, 4.5%, 2.4%, 3.0%, 5.1% for D1, D2, D3, D4 and D5, respectively, **Fig S4**). In summary, we demonstrate that 5M mappable reads without redundancy in low-pass WGBS is a reliable approach to evaluate the methylation level of cfDNA samples in the long-range mode.

We next sought to evaluate the ability of low-pass WGBS of cfDNA to discriminate the patients with different liver diseases. We conducted low-pass WGBS to the circulating cfDNA which are from 54 individuals, including 17 HCC (3 early stage HCC, 5 advanced HCC and 9 HCC patients after surgery; 16 were HBsAg positive and 1 was anti-HBs positive), 17 with cirrhosis (14 from HBV, 1 from NASH, 1 from alcohol and 1 cryptogenic cirrhosis), 17 with hepatitis B and 3 healthy volunteers (**Table S4**). On average, 10.2M mappable reads were obtained (IQR=6.3M, **Table S5**). The cfDNA fragment size (cfDNAsize)in HCC samples were significantly shorter than non-HCC samples (P=0.003, Wilcoxon rank sum test), consistent with recent observation (19). Particularly, cfDNAsize in advanced HCC group were much shorter than those in healthy individuals (P < 2.2x10-16, Wilcoxon rank sum test; **Fig 3A)**, and the size seemed to decrease along with liver disease progression (**Fig 3A)**. As expected, the distribution of CpGs captured by low-pass WGBS also tended to be located at intergenic and repeat regions. Moreover, CpGs in low-pass WGBS had much higher enrichment score of regions around reported HBV integration sites than high-depth WGBS datasets (**Fig 3B, Fig 1A**). To figure out the enrichment at repeat regions is a feature of cfDNA or artifacts of WGBS, we randomly extracted 10M single reads from published high-depth cfDNA WGBS datasets (25), including 58 cfDNA samples and 30 tissue samples as well as analyzed region enrichment score. Overrepresentation of regions around reported HBV integration sites was also observed in these datasets (**Fig 3C, Fig S5**). Strikingly, compared to tumor tissue and buffy coat, cfDNA samples were less enriched in functional elements (CpG island, promoter and exon) and more enriched in intergenic, repeat regions and HBV integration regions in both randomly 10M reads and high-depth data. (randomly 10M reads in **Fig 3C**; high-depth reads in **Fig S5**), suggesting this enrichment is a feature of cfDNA and the bias of cfDNA release.

Using our low-pass WGBS datasets, we explored whether DNA methylation in HBV integration regions could mirror the hypo-methylation profiles of cfDNA from HCC patients and the potential for early HCC detection. According to MethylHBV5K , the advanced HCC patients showed significantly hypo-methylation level compared to healthy individuals (<66.1%; P = 0.03, Wilcoxon rank sum test; **Fig 3D**; **Table S5)**. However, for early stage HCC patients, this methylation level was relatively higher, ranging from 68.5% to 72.3%. As expected, after surgery, most HCC patients (8/9) demonstrated similar cfDNA methylation levels to healthy individuals and patients with chronic hepatitis or cirrhosis. Nevertheless, one (P45) out of the nine HCC patients exhibited a lower methylation after surgery (63.97%, **Fig 3D**; **Table S5**), and died two months later due to tumor recurrence, suggesting that there were micro-metastasis with tumor cells in that individual. Additionally, a negative correlation was observed between MethylHBV5K and alpha-fetoprotein (AFP) levels (Pearson’s correlation coefficient = -0.59, P = 5.9 x10-6; **Fig 5D-E**). Besides, MethylHBV5K seemed to have no difference among healthy individuals, patients with chronic hepatitis and cirrhosis (P >0.1, Wilcoxon rank sum test). We also included one patient with acute hepatitis B in the hepatitis group and found that MethylHBV5K from this patient was similar to patients with chronic hepatitis (**Fig 3D**; **Table S4**).

### DNA methylation around HBV integration regions enhance HCC prediction

We evaluated MethylHBV5K by their differentiation ability to HCC from non-HCC cfDNA samples using receiver operating characteristic (ROC) curves based on a logistic regression model by five-fold cross-validation. MethylHBV5K showed the distinguish ability of HCC from non-HCC with AUC=0.85. We also applied random forest based feature selection to identify the potential high-performance biomarkers **(Methods)**. Top 5 regions were identified in distinguishing patients from healthy individuals (chr13: 19442162-20713822; chr1: 10121993-12279387; chr10: 11149668-13266296; chr10: 38027603-39151628; chr10: 84035111-85772043). All our cfDNA samples had these regions well sequenced, with the minimum amount of sequencing reads at 1,991 (**Table S6**). Their methylation levels were significantly lower in either early stage or advanced HCC patients than in healthy individuals, and demonstrated obvious decreasing tendency along with disease progression (**Fig 4A; Table S6**). Further investigation showed the prediction model using region 1, 2 and 5 could reach better performance for HCC patients (AUC>0.85; **Fig 4B**). All these prediction models exhibited improved discrimination performance compared to clinical variables (ALT, AST, Tbil, AFP) (**Fig S6A**).

To validate our findings, we applied this method in an independent cohort in a previous study (25). This cfDNA cohort was comprised of 32 healthy individuals and 26 HCC patients with early stage (BCLC stage is A or B) based on single-end bisulfite sequencing. To achieve similar sequencing depth, we randomly sampled 10M reads from each plasma sample. All the important features identified in above model showed significantly decreasing methylation in early stage HCC compared to healthy individuals (P<0.001, Wilcoxon rank sum test; **Fig 4C**; **Table S7**). Particularly, the above established prediction model demonstrated better performance of in HCC detection than genome-wide hypomethylation analysis in the paper (AUC=0.93; **Fig 4D**). With all the 58 cfDNA samples, region5 was still the best HCC indicator (AUC=0.918, **Fig S6B**). Moreover, the combination of multiple features provided improved prediction for HCC. When combing region1, region2, region3 region5 all together in the prediction model, it provided the best prediction performance with AUC=0.954 (**Fig 4E**).

## Discussion

In summary, we found cfDNA prefer enriched in intergenic, repeat regions and previously reported HBV integration regions indicating a non-random feature of cfDNA releasing from solid tissues. Furthermore, we demonstrated long-range of DNA methylation around HBV integration regions was a sensitive indicator to detect HCC compared to average methylation level across the genome. Hypomethylation of these regions are independent of integration events, which make them either suitable for the occurrence of viral integration, or ensure the transcription activity of integration sites recently attracting a lot of interests (43). We demonstrate that DNA methylation around HBV integration regions could serve as HCC detection biomarkers. We also demonstrated DNA methylation around HBV integration regions reflected genome-wide demethylation changes from non-tumoral tissues to HCC and could be used as a low-cost approach detecting minimal tumoral residual disease after surgical resection. In summary, our study provided a novel low-cost HCC diagnosis strategy in which HBV integration regions were employed, and this strategy will also be promising for similar attempts in a lot of oncovirus also known to have integration ability during infection (44).

Patients with chronic liver disease are at risk of HCC development, highest among those with cirrhosis. Professional societies recommend HCC surveillance in those patients at high risk who will benefit from early diagnosis so they might receive curative therapies. The recommended strategy for surveillance includes abdominal ultrasound with or without alpha-fetoprotein (AFP) every six months. However, image examination required special equipment (the ultrasound machine) and trained personnel to perform and interpret the study, potential barriers especially considering the large population of patients with HBV infection in China. Ultrasound is also operator dependent. Therefore, there is an unmet clinical need for new non-invasive diagnostic tests that is not operator dependent, such as liquid biopsy using circulating tumor cells (45). Unfortunately, The European Association for the Study of the Liver did not recommend the use of any existing tumor markers such as AFP and L3 fraction for HCC surveillance due to their suboptimal performance for early detection, and in the prior version of the American Association for the Liver Diseases, AFP was felt to lack both sensitivity or specificity for early detection of HCC. Subjects at highest risk for HCC are those with chronic hepatitis and advanced fibrosis; hepatic inflammation can result in elevation of AFP and up to 30% of HCC was non-AFP producing. Current study found a strong negative correlation between MethylHBV5k and AFP levels. However, unlike AFP, the MethylHBV5k level was not affected by the presence of inflammation, hence making it a more specific tumor marker. Currently new blood-based measurements are commonly compared with AFP, which had already been shown to have inadequate sensitivity and specificity, hence we believe future comparison should be between new biomarkers and ultrasound for early detection of HCC. Although WGBS of cfDNA has been shown effective for cancer detection (28), the cost of cfDNA WGBS in cancer patients is one of challenges for wide application. In this paper, we explored the cfDNA methylome of hepatitis, cirrhosis and HCC patients and examined the feasibility of HCC detection using low-pass WGBS. We demonstrated the measurement of DNA methylation around HBV integration regions could be applied in low-pass cell free WGBS at 5-million reads to reflect liver disease status of chronic hepatitis, cirrhosis and HCC. Moreover, DNA hypomethylation in HBV integration regions was shown promising results as a potential biomarker for early HCC detection.

Previous studies have been shown that the fragmentation process of cfDNA is not random (46, 47). Our results show low-pass WGBS for cfDNA tended to capture fragments from repeat regions and HBV integration sites. Due to open chromatin regions are easily degraded, fragments from open chromatin regions (promoter and gene coding regions) were less likely to be detected in cfDNA. When decreasing the sequencing volume, overrepresentation of genomic repeat regions and HBV integration regions were observed in cfDNA. This suggests that the signal from these regions could remain given adequate sequencing depth in low-pass WGBS. Since HBV integrations tend to localize at repeat regions, DMCs of advanced HCC patient were also enriched in previously reported HBV integration sites.

We adopted an approach focusing on regions from HBV integration sites as surrogate regions for plasma hypomethylation analysis in HCC patients. Although we chose HBV integration sites as the indicator, it does not necessarily indicate that the analysis is only suitable for patients with HBV infection. In our sample set, we also included three patients without HBV infection (P1, P18 and P19; **Table S2**). While HBV integrations carried by dominant tumor clones are likely to have some specific DNA molecular features (26, 48-50), we also demonstrated that methylation changes in HBV integration regions may be common in HCC and independent of HBV infection. Interestingly, we found hypomethylation in HBV integration regions have higher sensitivity for HCC diagnosis. For example, one chronic hepatitis patient, P14, had the MethylHBV5k at 69.5%, the methylation level of region5 at 72.4% and an abnormal AFP level (141.9 ng/ml). The corresponding P14 blood sample was initially labeled as chronic hepatitis since he was a follow-up patient with chronic HBV infection; however, he was diagnosed with HCC in this examination and died 8 months later. Therefore, it is plausible that the patient had significant circulating tumor cells at the time of sample collection since his AFP was also significantly elevated. Except P14, the sample from a chronic hepatitis patient, P2, showed that the methylation level of region5 was 70.7% and the MethylHBV5k was 68.5%. Using the sample from a clinical visit 6 months following the initial sample collection, the methylation level of region5 increased to 73.92%, whereas the MethylHBV5k increased to 71.34%. This patient had no detected HCC in follow-up. As a predictor of HCC, the most challenging aspect is to determine appropriate cutoffs for disease status, which necessitates large sample sizes in future studies. Nevertheless, our study successfully illustrated that it is necessary to monitor the patients with suspicious methylation changes in cfDNA according to multiple indicators, combining their prognostic signals to improve accuracy. We compared our strategy with genome-wide hypomethylation analysis in a published dataset, and our strategy had improved classification performance compared to the genome-wide hypomethylation analysis used in the original publication (25). Moreover, the calculation of methylation in these regionsdoes not rely on a reference panel of healthy individuals, and is thus independent of either sequencing quality and inclusion criteria of the reference panel.

Target sequencing have already achieved certain progress in tumor detection, but genome wide characterization of methylation profiles is the promising direction to overcome the false negative errors due to tumor heterogeneity and optimize the genomic regions used for surrogating the methylation level changes specific to tumor patients, such as previously reported HBV integration sites in our observation. We believe low-pass WGBS will facilitate efforts using large sample size for novel solutions and finally improve the clinical implementation of methylation evaluation. Although we have found some stable methylation patterns using low-pass WGBS, these findings still need to be validated in larger studies. The low-coverage caused by the low-pass WGBS sequencing introduced analysis challenges, however, it may still have clinical utility in augmenting early detection of HCC. This study can serve as a platform to motivate further development of low-pass DNA methylation approaches to improve the accuracy of HCC diagnoses and surveillance. Subsequent larger studies will aid in the determination of accurate cutoff values for disease stages, especially for those with small tumors. Furthermore, we anticipate that blood samples from HCC patients at multiple time points hold strong utility in tracking disease progression.

## Materials and Methods

### Sample collection

All the blood samples of patients were collected from Beijing You’an Hospital. Healthy individuals enrolled by Beijing Institute of Genomics were collected as controls. The diagnosis of chronic hepatitis B was made according to the guidelines for the prevention and treatment of chronic hepatitis B: a 2015 update (51). We collected age, gender, HBV-status, tumor size and alanine aminotransferase (ALT) test, aspartate aminotransferase (AST) test, bilirubin test, alpha-fetoprotein (AFP) test and other related clinical information for related samples. Meanwhile, HCC patients were classified as early and late stage according to the Barcelona Clinic Liver Cancer staging system, considering A as early stage, C and D as late stage. The study protocol conformed to the ethical guidelines of the 1975 Declaration of Helsinki and was approved by the Ethics Committee of Beijing You’an Hospital and Beijing Institute of Genomics (IRB number 2016H005). An informed written consent was obtained from all patients and volunteers.

### Cell free DNA extraction

Ten microliters (ml) of whole blood was collected from each patient in Streck Cell-Free DNA BCT® tubes (Streck, Omaha, NE) and immediately shipped to Beijing Institute of Genomics. Upon arrival, the blood was collected in Streck BCT tubes were centrifuged at 3,000 × g for 15 minutes at 4°C within two hours. Subsequently, the plasma was transferred into a fresh microcentrifuge tube, followed by a 2nd centrifugation at 16,000 × g for 10 minutes at room temperature. Five ml of resultant plasma was used for cfDNA extraction using a QIAamp Circulating Nucleic Acid Kit (Qiagen, Valencia, CA). After extraction, total DNA was quantified using a Qubit dsDNAHS Assay kit (Life technologies, Grand Island, NY, USA). All DNA samples were stored at -80°C before sequencing library construction.

### Whole genome bisulfite sequencing and data processing

Using the TruSeq DNA Methylation Kit (Illumina Inc.) according to the manufacturers’ protocol. Total cfDNA (range from 0.5 ng to 88.7 ng) was used for sequencing library construction. Bisulfite conversion of cfDNA was performed using the EZ DNA Methylation-Gold Kit (Zymo Research) according to the instruction manual. During conversion, 0.5% methylated lambda DNA was included as a spike-in DNA control to estimate the conversion efficiency of unmodified cytosine. The sequencing libraries were then performed paired-end sequencing (2 × 100 bp) on an Illumina HiSeq 4000 (Illumina Inc., San Diego, CA, USA). The raw sequence data reported in this paper have been deposited in the Genome Sequence Archive (52) in BIG Data Center (53), Beijing Institute of Genomics (BIG), Chinese Academy of Sciences, under accession numbers CRA001537, CRA001537 that are publicly accessible at <http://bigd.big.ac.cn/gsa>.

After base calling, all paired-end fastq files were trimmed using cutadapt (v 1.8.3) (54) to removed adapter sequences and low quality bases with parameters ‘-q 15 --minimum-length 36’. HG19 reference genome was downloaded from ENSEMBL. Lambda genome was also included in the reference sequence for calculating bisulfite conversion rate. Filtered paired-end bisulfite sequencing data were mapped with Bismark (v0.14.5) (55) using with default parameters. After alignment, read duplicates were removed using the deduplicate\_bismark application included in the bismark software. Then the BAM files produced by Bismark were sorted using samtools (v0.1.19) and overlapping paired-end reads were clipped using ClipOverlap function of bamUtil (<https://github.com/statgen/bamUtil>) to prevent counting twice from the same observation. For each CpG, the methylation level was combined from both DNA strands and estimated as m/(m + u), where m was defined as the number of methylated cytosines and u was defined as the number of unmethylated cytosines. The number of methylated and unmethylated cytosines of 1 Mb regions were generated using R package methylKit. The average methylation level of each long range region was calculated as the total number of cytosines divided by the number of methylated cytosines.

### cfDNA fragment size determination and distribution

Unique reads with well alignments to human genome (hg19) were applied for cfDNA fragment size evaluation. The end positions and start positions were extracted to calculate the cfDNA size and the distribution were prepared for different samples. Wilcoxon rank sum test was applied to test the association between the median of cfDNAsize in HCC and non-HCC samples.

### The enrichment score in each genomic region

CpGs with depth over 5 were used for calculation of enrichment score. The enrichment score is defined as follows: is the number of DMC sites in the genomic element; where the expected value , is the number of DMC sites in the genome, is the number of CpG sites in the genomic element, and is the total number of CpG sites in the genome.

### Identification and annotation of the differentially methylated CpGs (DMCs) and genes (DMGs)

Differentially methylated CpGs (DMCs) were identified between HCC patient and healthy individual (D4 vs. D1). The identification of DMCs was generated using the R package methylKit (56). The significance of the DMCs departure between two groups was calculated with at least 5-fold coverage. P-value was adjusted for multiple testing with the method of Benjamini and Hochberg (57). The CpG sites were considered different between case and control if the Benjamini-Hochberg corrected P-value ≤ 0.05 and the methylation level difference was ≥ 0.2. Each DMCs was annotated for each RefSeq transcript obtained from ENSEMBL GRCh37. Promoters are defined as regions 2kb upstream from TSS for each RefSeq transcript. RepeatMasker annotations were obtained from UCSC Genome Browser (58). The HBV integration sites were extracted from previous reports (34, 37-42).

### Calculation of average methylation level around HBV integaration sites

Average methylation level of the CpGs within the 100bp of the HBV integration sites (MethylHBV) was determined in tissue samples. All the CpGs with depth over 1 read were extracted. The average methylation level within the 100bp upstream or downstream of HBV integration sites (MethylHBV) was included in all the CpGs with depth over 1 read. This value was calculated as the number of the total number of methylated cytosines divided by the number of total cytosines within the 100bp of the HBV integration sites.

Long-range methylation around HBV integration sites (MethylHBV5K) was defined as the average methylation level of the CpGs within the 5kb of the reported HBV integration sites, calculating as the number of the total number of methylated cytosines divided by the number of total cytosines within the 5kb of the HBV integration sites.

### Randomly re-sampling lower reads from total WGBS data

Regions within 5kb of reported HBV integration sites were applied to measure the methylation status. Overlapping regions were merged to form a single region. A random sampling method was used to obtain low depth WGBS for 5 pilot WGBS of cell-free DNA. 1M to 10M read pairs (increasing by 1M step) was randomly extracted from each WGBS data set. In each iteration, we randomly permuted genomic regions of 5kb around the reported HBV integration sites using BEDTools shuffle (59). The average methylation level of permuted regions of this randomly sampled low-pass reads and the average methylation level of permuted regions of total sequencing reads were calculated. The permutation was repeated 100 times and a correlation coefficient was adopted to measure the consistency between low-pass resampling reads and those based on total sequencing reads. For each sequencing depth, we repeated the random extraction 10 times to examine the variation of the correlation coefficient, and the difference (coefficient of variation, CV) among 10 values of the correlation coefficient was used to assess dispersion in the sampling process.

### Feature selection based on HBV integration regions

Random forest based feature selection to identify the potential high-performance biomarkers was applied in order to support MethylHBV5k to have consistent performance in low-pass WGBS data and to solve the minor release of cfDNA and the lower sensitivity in early stage HCC. These regions should be long enough to be constantly detected at low-pass WGBS and could be suitable as markers of early stage HCC. For 6,072 regions flanking 5kb of HBV integration sites, Regions with depth over 10 reads in all the 54 cfDNA samples were selected (3,083), which were stable detected at low-pass sequencing. Then the neighbor regions were merged into one large region if their distance was less than 1Mb. At last, 144 candidate merged regions larger than 1Mb were selected and used for the feature selection procedure in healthy individuals and early stage HCC patients. Feature selection was conducted using the R package caret based on a random forest algorithm.

### Prediction analysis and receiver operating characteristics (ROC) curves

The AUCs measure the discrimination between HCC and non-HCC samples (healthy individuals, patients with chronic hepatitis and cirrhosis). AUC values calculated in our dataset were averaged AUC calculated across the the five-fold cross validation runs on the overall test dataset. The procedure is that the data including all the features were divided into 5 equal parts and each of them was set as the test dataset while the remaining as the training dataset. In the training stage, a logistic regression-based prediction model was used. Analysis of ROC curves was constructed using R package PredictABEL.

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**Authorship Contributions**

HZ and SG performed analyses, developed analysis methods and power calculations, interpreted results, and drafted the manuscript. PD enrolled patients and collected all the clinical information. CT and JK conducted sequencing experiments, WC and FJ processed the raw data and performed quality control. ZW collected and prepared tissue samples for sequencing analysis and collected results of clinical assays. RC and AV interpreted results, provided liver cancer and hepatology clinical expertise, reviewed and edited the manuscript. HD aided in the analyses and reviewed the manuscript. HD provided clinical advice and reviewed the manuscript. SJS provided analysis advice, aided in coordinating and supervised scientific activities, reviewed and edited the manuscript. DZ and CZ designed the study, supervised all experiments and analysis, provided molecular and cellular biology advice, reviewed and edited the manuscript.

**Disclosure of Conflicts of Interest**

The authors declare no conflict of interest.

**Data Availability**

The raw sequence data were deposited in the Genome Sequence Archive in BIG Data Center, Beijing Institute of Genomics (BIG), Chinese Academy of Sciences, under accession numbers CRA001537, CRA001537 that are publicly accessible at <http://bigd.big.ac.cn/gsa>.

**Code Availability**

All the related software and script were used in the manuscript are available through GitHub at <https://github.com/Shicheng-Guo/low-pass-WGBS/blob/master/readme.md>

**Abbreviations**

HCC Hepatocellular Carcinoma

cfDNA Circulating cell-free DNA

cfDNAsize Fragment size of circulating cell-free DNA

MethyHBV5k Long-range methylation around HBV integaration sites

DMCs Differential Methylation CpGs

DMGs Differential Methylation Genes

HBV Hepatitis B virus

WGBS Whole Genome Bisulfite Sequencing

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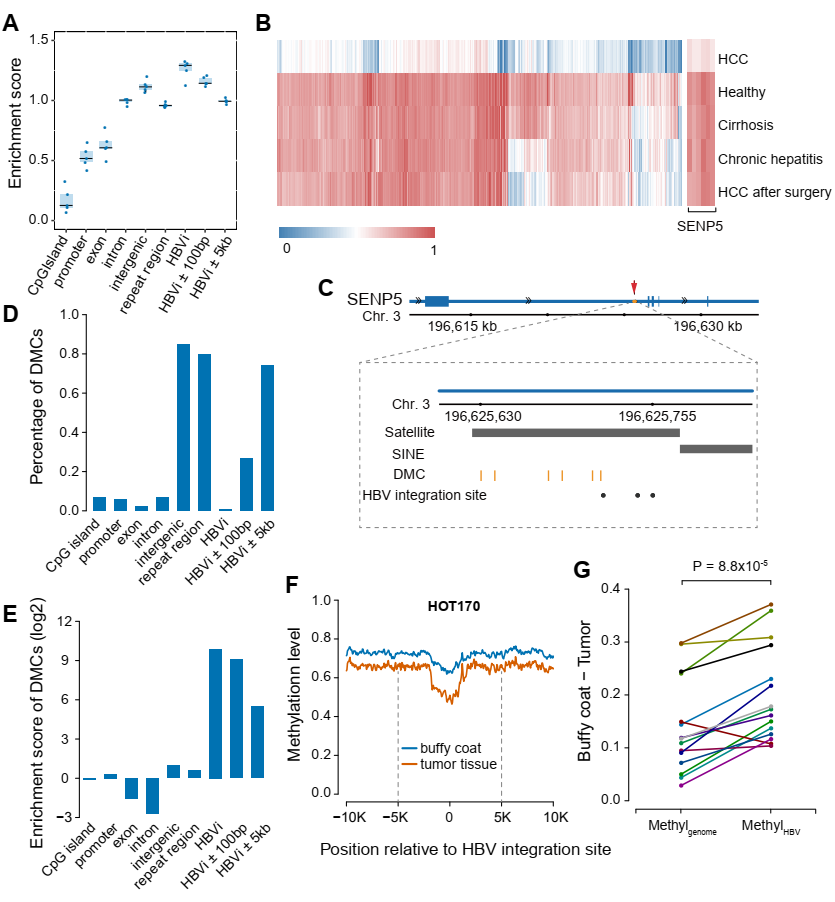
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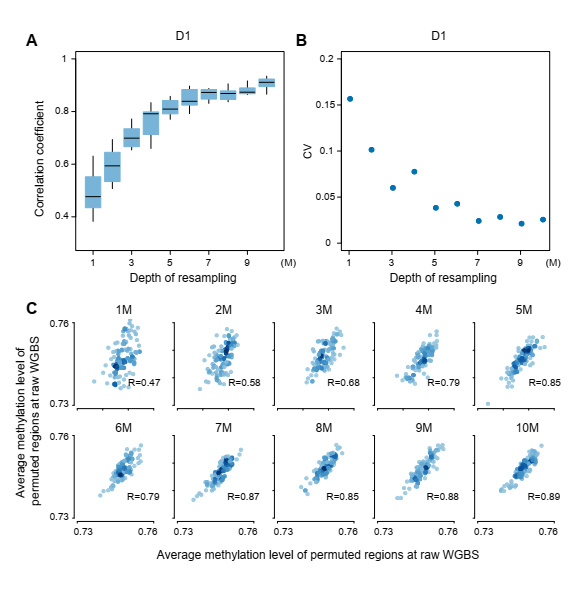
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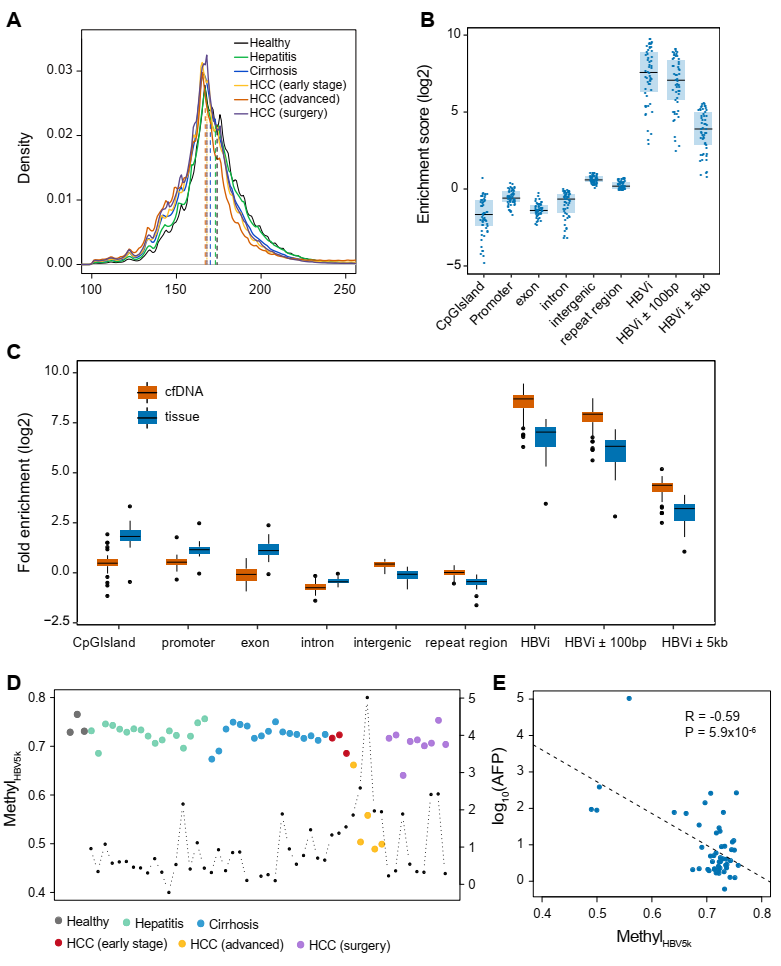
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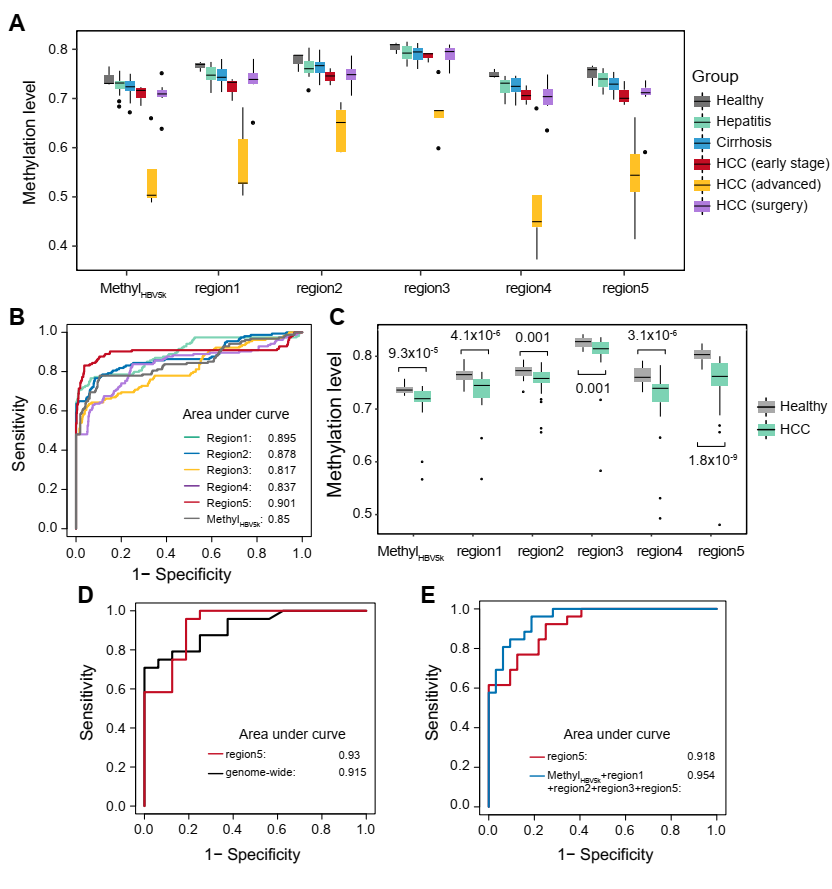
**Fig. 1. DNA methylation around HBV integration regions.** (A) The enrichment score of CpGs in different genomic elements in 5 pilot WGBS. HBVi represents for HBV integration site. (B) The heatmap displays the methylation level of DMCs between HCC patient and healthy individual in all the 5 individuals. (C) The locus of 6 DMCs and 3 reported HBV integration sites in intron 2 of SENP5. The black dots represent the HBV integration sites and the orange vertical lines represent the 6 DMCs. The black bar labels represent the locus of repeat marker in this region. (D) The percentage of DMCs located at different genomic elements and regions surrounding HBV integration sites. (E) The enrichment scores of DMCs at different genomic elements. (F) The average DNA methylation level profiles along 10 kb upstream and downstream of the HBV integration sites in buffy coat and tumor tissue of patient HOT170. The black dotted line represents 5kb upstream or downstream of HBV integration sites. (G) The difference between buffy coat and tumor tissue of average methylation level across the genome (Methylgenome) and average methylation level of the CpGs within the 100bp of HBV integration sites (MethylHBV)



**Fig. 2. Efficacy of a low-pass sequencing strategy illustrated by re-sampling reads from 5 pilot cfDNA WGBS data.** (A) The boxplot shows the correlation coefficient between resampling low-pass WGBS and total sequencing reads for 10 times from 1M to 10M. (B) The coefficient of variation (CV) for 10 correlation coefficient between resampling low-pass WGBS and total sequencing reads from 1M to 10M. (C) The correlation of average methylation level of permutated regions between different resampling reads and total sequencing reads at one resampling.



**Fig. 3. Landscape of plasma cfDNA in healthy individuals, hepatitis, cirrhosis and HCC patients.** (A) The distribution of cfDNA fragment size in the group of healthy, hepatitis, cirrhosis, early stage HCC, advanced HCC and HCC after surgery. The vertical dashed lines indicate the median values in all groups. (B) The enrichment scores of CpGs at different genomic elements and regions surrounding HBV integration sites of all the 54 cfDNA samples at low-pass WGBS. HBVi represents for HBV integration site. (C) The enrichment scores of CpGs at different genomic elements of cfDNA and tissue samples by randomly re-sampling 10M reads from published dataset. P values between cfDNA samples and tissue samples at CpG island, promoter, exon, intron, intergenic, repeat region, HBV integration site, HBVi ± 100bp, HBVi ± 5kb are 4.1x10-12, 7.6x10-12, 1.5x10-13, 4.9x10-8, 4.7x10-13, 2.1x10-12, 1.3x10-11, 9.2x10-12, 1.9x10-11, respectively. (D) Long-range methylation around HBV integration sites (MethylHBV5k) in all the 54 samples. The black dot represents for AFP level (log10) for the corresponding individual. (E) The correlation between AFP (log10) and MethylHBV5k.



**Fig. 4. Hypomethylation around HBV integration regions enhance HCC prediction.** (A) Boxplot displays MethylHBV5k and the methylation level of top 5 selected regions in all the samples. (B) Receiver operating characteristics (ROC) curve based on five-fold cross-validation for HCC detection by different indicators in discriminating HCC patients from individuals without HCC (healthy individuals, patients with hepatitis and cirrhosis). (C) The comparisons between healthy individuals and patients with early stage HCC using MethylHBV5k and the methylation level of top 5 selected regions in the validation dataset. (D) ROC curves for 16 healthy individuals and 24 HCC patients in the validation cohort using genome-wide hypomethylation analysis and region5. (E) ROC curves for HCC patient detection using all the healthy individuals and HCC patients in the validation cohort by the identified features.