**Long-Region Hypomethylation in HBV Integration Regions Enhance HCC Non-invasive Surveillance by Low-pass Whole Genome-wide Bisulfite Sequencing.**

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## Abstract

Circulating cell-free DNA methylation has been demonstrated to be a promising strategy for non-invasive cancer diagnosis. However, low-level of cell-free DNA in plasma limits whole genome bisulfite sequencing (WGBS) sequencing depth and subsequent biomarker identification. Here we demonstrate long-region hypomethylation (LRM) in low-pass WGBS data (<5-million reads) provide high sensitivity and specificity surveillance to hepatocellular carcinoma (HCC). We applied our WGBS approach and demonstrated DNA methylation abnormalities in HCC occurred in the HBV integration regions. These findings reflect the stage of hepatitis and cirrhosis thereby providing a suitable surrogate for methylation level estimation in plasma cfDNA analysis of liver diseases.

## Introduction

Circulating cell-free DNA (cfDNA) are small double-stranded DNA fragments(1) found in plasma, urine, and other body fluids(2), originating from cell apoptosis and necrosis(3). In many settings, analyses of cfDNA can be regarded as a way to perform a “liquid biopsy”, which have demonstrated the potential not only for cancer diagnosis and prognosis (4, 5), but also for identifying patients with premalignant states, inflammatory conditions or traumas (1). Apoptotic and necrotic tumor cells can release cfDNA into the peripheral blood, which can reflect the tumor-related genetic features, including mutations, copy number aberrations and epigenetic changes(4). As such, cfDNA represents important biomarkers of oncogenesis. However, genome-wide methylation assays require large amounts of input DNA—conventional WGBS require microgram input and reduced respresentation bisulfite sequencing (RRBS) requires 30ng of DNA input which is often approaching the maximum level of the cfDNA detected (or detectable) in human blood. In order to effectively investigate cfDNA, several novel methods have been proposed, such as scRRBS(6) and cfMeDIPseq(7).

Hepatocellular carcinoma (HCC) is the third leading cause of cancer-related death worldwide, and is the seventh most common cause in the U.S. Genetic, epigenetic and environmental (e.g. HBV and HCV infection, aflatoxin) interactions play important roles in hepatocarcinogenesis etiology and HCC progression (8, 9). HBV integration causes genomic instability and abnormal proliferation of hepatocytes which accompany epigenome dysfunction, especially perturbations in DNA methylation (10, 11). Furthermore, abnormal DNA methylation status of other epigenetic regulators such as miRNA (12), lncRNA(13) can play a role in promoting carcinogenesis. Cirrhosis from any etiology is a major risk factor for HCC. Hepatocarcinogenesis is a complicated and poorly-understood multistep process starting with chronic hepatitis, leading to fibrotic deposition(14), cirrhosis and eventually hepatocellular carcinoma in some patients (10, 15, 16). Characterizing the HCC stage and precancerous liver pathophysiologies using changes in DNA methylation promises to be an efficacious application of epigenetic biomarkers for non-invasive diagnosis (17-19). A number of studies have focused on cancer diagnosis of cfDNA methylation, such as the potential specific biomarkers (18, 20), pervasive hypo methylation (21) and tissue of origin (22-24). Previous attempts using WGBS for cancer diagnosis require deep sequencing (22, 24) which currently limits the wide-scale application in a clinical setting. Reducing the sequencing volume, low-pass sequencing and correspondingly low sequencing cost will be important bridge between basic research results and clinical translation.

Chronic hepatitis B is a major cause of chronic liver disease and HCC worldwide. World Health Organization estimated 257 million people in the world are infected with hepatitis B and account for over 80% of virus-associated HCC. In this study, we evaluated the performance of low-pass whole genome bisulfite sequencing (WGBS) in cfDNA methylation profiling to identify the lowest sequencing depth for long-range methylation measurement. Applying the approach to comparing patients at different stages of liver diseases (hepatitis, cirrhosis and HCC), low-pass WGBS at 5-millilion sequencing reads was able to detect the hypo-methylation profiles of plasma cfDNA from patients with liver diseases. Additionally, we demonstrate that the diagnostic performance of low-pass WGBS assessing the methylation status of HBV integration regions substantially exceeded the performance of traditional hypo-methylation biomarkers.

## Results

### Efficacy of low pass sequencing strategy illustrated by re-sampling reads from cell-free WGBS data

In order to identify the impact of sequencing depth on methylation profiles in cell-free based WGBS data, 5 samples were analyzed: 1 healthy individual (H1), 1 hepatitis patient (P1), 1 cirrhosis patient (P2) and 2 HCC patients (P3 and P4 of before and after surgery). The final read count equated to a mean of 58 million (M) read per sample (**Supplementary Table 1**). The average methylation across the genome was much lower in HCC patient (P3; 53.56%) compared to healthy individual, hepatitis and cirrhosis (74.76%, 75.13 and 75.64%; **Supplementary Table 1**). In the study, long range methylation (LRM) was applied to measure the methylation status of cfDNA. To identify the optimal region size of LRM, we divided the HCC genome (P3) into 500-Kb, 1-Mb, 1.5-Mb, 2-Mb and 2.5-Mb, respectively. For each region size, we calculated the average methylation level for each region across the genome. Then the percentage of regions with hypo-methylation (corresponding bin in P3 is less than -0.2 compared to healthy individual; method) was calculated in P3. The percentage of hypo-methylated regions was largest at the size of 2-Mb (**Figure S1; Supplementary Table 1**). So the LRM for all 1,382 autosomal 2-Mb regions were used for global methylation level calculation (Method).

To determine the effective sequencing depth in low pass WGBS of cfDNA, we randomly sampled 1M to 10M mappable reads from each sequencing dataset (each composed of approximately 58M reads) and calculated the average methylation level for each 2-Mb region (MethylLRM). During each extraction, we calculated MethylLRM forall 2-Mb regions, and adopted correlation coefficient to show their consistency with those based on total sequencing reads. For each sequencing depth, we repeated the random extraction for 100 times to examine the variation of correlation coefficient, and the difference (coefficient of variation, CV) among 100 values of correlation coefficient demonstrated sampling bias. The final results showed this CV was only around 4% (**Fig 1**). With increasing numbers of sequencing reads, the MethylLRM was closer to the value calculated using total sequencing reads (**Fig 1**).When sequencing depth increased from 1M to 10M reads, the correlation coefficient between the methylation level from low-pass WGBS and the raw WGBS data received saturation when using 5M or more. High correlation of MethylLRM was observed between 5M reads and all sequencing reads (R > 0.92, P < 2.2x10-16, Pearson’s correlation test, **Figure S2A-B**), and methylation level remained consistent during 100 resampling processes (CV is 0.72%, 0.11%, 1.09%, 0.13%, 0.38% for H1, P1, P2, P3 and P4, respectively, **Fig 1**). In summary, we demonstrated 5M mappable reads without redundancy in low pass WGBS were reliable to evaluate methylation level of cfDNA samples in the long-range mode.

### Methylation level of plasma cfDNA from hepatitis and cirrhosis patients resembles healthy individuals

Next, we applied low pass WGBS for plasma cfDNA samples from 54 individuals, including 17 HCC (4 early stage HCC, 4 advanced HCC and 9 HCC after surgery), 17 with hepatitis, 17 with cirrhosis and 3 healthy volunteers (**Supplementary Table 2**). On average, 10.2M mappable reads were obtained from each sample (IQR=6.3M, **Supplementary Table 3**). To evaluate the methylation levels in these samples, the LRM strategy was applied to define the hyper- or hypo-methylated LRM regions (Method), using MethylLRM in healthy individuals as the baseline level. The percentage of hyper- or hypo-methylated LRM regions is shown for each patient (**Fig 2; Supplementary Table 3**). Hepatitis and cirrhosis patients had similar cfDNA methylation levels compared with healthy individuals (**Fig 2**). However, we still identified few hyper- or hypo-methylated 2-Mb abnormal regions. Further, we found hyper-long methylated regions (hyper-LMRs) accounted for <3% of total 1382 autosomal LMRs (**Fig 2A**) while hypo-long methylated regions (hypo-LMRs) accounted from 0.0-20.04%, with only three patients exceeding 10% (**Fig 2B; Supplementary Table 3**). In early stage HCC patients, no hyper-methylated LMR were identified, however hypo-methylated LMRs accounted for 1.2% to 68.3%. In advanced HCC patients, also no hyper-methylated LMR were identified, and hypo-methylated LMR accounted for more than 65%. (**Fig 2; Supplementary Table 3**). As expected, after surgery, most HCC patients (8/9) demonstrated similar cfDNA methylation level to healthy individuals and patients with hepatitis or cirrhosis. Nevertheless, one (P45) out of nine HCC patients after surgery had a higher proportion of hypo-methylated regions (69.9%, **Fig 2B**; **Supplementary Table 3**), and died two months later due to tumor recurrence, suggesting that tumor cells remained in that individual. Our results demonstrate that LMR could serve as a dynamic biomarker reflecting the genome-wide demethylation process from normal tissues to HCC and hence could be used as a measure of surgical efficacy.

### Differentially methylated CpGs (DMCs) and genes (DMGs) identified by low-pass cell-free WGBS

We identified DMCs and DMGs with low-pass cell-free WGBS data, even very limited CpGs were coverage by our assay. On average, each dataset for corresponding cfDNA sample had 61,018 CpGs with sequencing depth over 5 (**Method, Supplementary Table 3**). Totally, advanced HCC patients had 1,841 DMCsidentified (**Supplementary Table 4**), of which all the DMCs were hypo-methylated comparing to healthy individuals. Among those, 23 DMCs located in gene body of six genes: *HFM1, PMF1, PMF1-BGLAP, SENP5, SLCO5A1, REXO1L1P*. In the four early stage HCC patients, we identified 193 DMCs (**Supplementary Table 5**), of which 155 were in common with those observed in advanced HCC patients and 8 located within *PMF1* and *PMF1-BGLAP* (**Fig 3A; Table 1**). Relative high proportions of hypo-LMRs (>10%) were observed in one hepatitis and two cirrhosis patients (**Fig 2B**), indicating their high HCC risk. Totally, all four groups had 134 DMCs in common (**Fig 3A**), which suggested that methylation changes may occur in early stage of liver disease progression prior to HCC. **Table 1** displayed the promoter or the gene body with DMCs in the four comparisons. Particularly, *SENP5* gene had 7 significantly hypo-methylated DMCs with consistently high sequencing coverage across all individuals (149 reads, on average, **Supplementary Fig 3**, and **Fig 3B**). Intriguingly, all 7 DMCs that we found in intron 2 of *SENP5* were located near previously reported HBV integration sites in HCC (**Fig 3C**) (25).

### Over representing of DMCs in repeat regions and surrounding HBV integration sites

Genome feature distribution of CpGs illustrated that they tended to locate at intergenic region and repeat regions (Methods, **Supplementary Fig 4A**), and CpGs in repeat regions had much higher sequencing depth in this low pass sequencing strategy than those in other regions (P < 2.2x10-16, Wilcoxon rank sum test; **Supplementary Fig 4B**). On average, 64% of all these CpGs were in the repeat regions (**Supplementary Fig 4C**), and this percentage varied from 49% to 87% among individuals. Differential methylation analysis required the CpG sites having sequencing reads over 5 in all individual (**Method**), and qualified CpG were over represented in repeat regions. Finally, 91% of DMCs of advanced HCC patients located within repeat regions (**Fig 4A**). Considering repeat region is a known feature of HBV integration sites (26, 27), we subsequently analyzed the location of DMCs relative to reported HBV integration sites (25, 28-33). Among 1,841 DMCs in advanced HCC patients, twenty completely overlapped with the HBV integration sites, including two in *SENP5* (**Supplementary Table 8**). Meanwhile, 36% of DMCs located within 100bp region either upstream or downstream of integration sites, and 95.1% of DMCs within 5K regions (**Fig 4A**). Overall, these DMCs were more significantly enriched in HBV integration sites compared with promoter and gene body regions (**Fig 4B**).

In order to evaluate whether methylation levels of CpGs near HBV integration sites could mirror the hypo-methylation statutes of cfDNA from HCC patients. We analyzed CpGs with depth over 5 in all the 54 samples that within the 100 bp upstream or downstream of HBV integration sites and calculated the percentage of hypo-CpGs. We found these CpGs were significantly hypo-methylated in advanced HCC patients, with 53% to 59% of CpGs were hypo-DMCs (**Fig 4C; Supplementary Table 3**). Then, we incorporated all the CpGs in each samples and calculated the average methylation level of the CpGs within the 100bp of the reported HBV integration sites (**Supplementary Table 2)**, and the advanced HCC patients still showed significantly hypo-methylation level (<48.4%; **Fig 4D**; **Supplementary Table 3)**. However, for early stage HCC patients, this value was relatively higher, from 66.5% to 71%. We adopted the median of early stage HCC patients (68.83%) as a cutoff for the detection. Moreover, four patients with hepatitis or cirrhosis (P2, P14, P18, P19) showed slightly hypo-methylated with these sites. One hepatitis patient, P14 had the average methylation level at 67.4%, with abnormal AFP level (141.9 ng/ml; **Table 2**) and this patient was diagnosed with HCC later. Patient P19 was diagnosed as alcoholic cirrhosis, with the transaminase indicator and jaundice mildly abnormal at each of the follow-up time point. For patient P2 with chronic hepatitis and P18 with nash-related cirrhosis, both of them had no abnormal measurements observed and haven’t had tumor detected yet. Our results indicate hypomethylation in HBV integration regions could be a potential biomarkers to evaluate the risk of the transformation from hepatitis and cirrhosis to HCC in patients with chronic hepatitis B.

## Discussion

Chronic hepatitis B is a major cause of HCC worldwide. Professional societies recommend HCC surveillance in patients with CHB with abdominal imaging (usually ultrasound) with or without alfa-fetoprotein every 6 months. The goal is to diagnosis HCC at early stage which is curable. Unfortunately alfa-fetoprotein is neither sensitive nor specific, and there is a great need for a new non-invasive diagnostic test such as liquid biopsy for circulating tumor cell(34). Although WGBS of cell-free DNA has been proved effective for cancer detection, the cost of cfDNA WGBS in cancer patients is one of challenges for wide application. 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 long-range methylation could be applied in low-pass cell-free WGBS at 5-million reads to reflect liver disease status of hepatitis, cirrhosis and HCC. Meanwhile, DNA hypomethylation in HBV integration regions was proved to potential biomarkers for cancer progress prediction.

Chan and colleagues applied genome-wide pervasive hypomethylation in hepatocellular carcinoma detection and shown low sequencing depth of ~10 million reads was available for the cell-free detection for cancer (21), However, we found actually the sequencing depth could be decreased to ~5 million reads with long-range DNA methylation measurement. In our study, although we required 5M qualified reads for low-pass WGBS for 54 samples, there were 2 samples only having 3.6M read (**Supplementary Table 3**). The average correlation coefficient of 100 re-sampling were larger than 0.9 using 3M reads (**Fig 1**), and theoretically they were sufficient to evaluate the methylation level.

One limit of cfDNA detection is the application of early stage cancer, our attempt showed that the sensitivity of early stage HCC detection was much lower than advanced HCC. For early stage HCC patients, P35 and P36, both the proportion of hypo-LMRs (1.23% and 4.7%) and the average methylation level around HBV integration sites (70.48% and 71.48%) were similar to the healthy individuals and hepatitis patients. Both of these two patients had small tumor size (P35, 1.5cm; P36, less than 2.5cm, multiple; **Supplementary Table 2**). Another application of cfDNA methylation analysis is to evaluate the residual tumor or risk of tumor recurrence after surgery. We found the methylation level of cfDNA should resemble with those from healthy individuals or patients with other chronic liver diseases after complete HCC resection. We found one HCC patient after interventional therapy, P45, showed significantly hypomethylation of the CpGs near the HBV integration sites (60.87%), indicating presence of hepatic micro-metastasis. The patient died two months later in follow-up due to multiple and recurrent lesion. The medium follow up of the other 8 patients was x months without clinical evidence of HCC recurrence.

Previous studies have been shown that the fragmentation process of cell free DNA is not random (35, 36).Our results showed low-pass WGBS for cfDNA tended to capture fragments from repeat regions and HBV integration sites. More than 49% of CpGs located in the repeat regions and had a higher sequencing depth. When decreasing the sequencing volume, over representing of genomic repeat regions was observed in our data. This suggested that the repeat regions could remain adequate sequencing depth in low pass WGBS. Since HBV integrations tend to locate at repeat regions, DMCs of advanced HCC patients were also enriched in previously reported HBV integration sites. Notably, CpGs near the HBV integration sites were likely to have methylation levels reflecting hypo-methyation status of tumor genome in HCC patients, which can discern the HCC patients from patients in other stages of liver diseases.

We adopted 100 bp upstream and downstream of HBV integration sites as surrogate regions for plasma hypomethylation analysis in HCC patients. Although we chose HBV integration sites as the indicator, it did not mean the analysis was only suitable for patients with HBV infection. In our patients we also included 3 patients without HBV infection (P1, P18 and P19; **Supplementary Table 2**). This kind of regions may have some biological features suitable for HBV integrations, and here we also demonstrated their methylation changes may be common in HCC development independent of HBV infection. We found hypomethylation in HBV insertion regions in some how have higher sensitivity for HCC diagnosis. Take P14 as example, the proportion of LMRs was 3.47%, far below the median cutoff of HCC indication (**Table 2; Supplementary Table 3**). However, according to hypomethylation HBV integration indicator, average methylation level of regions around known HBV integration sites, the value was 67.4% for P14, only slightly lower than the up limit median cutoff for HCC. We followed up this patient and found him diagnosed as HCC within half a year. Another hepatitis patient, P2, the proportion of LMRs was 17.8%, and the average methylation level around HBV integration sites was 67.7%; while in the re-visit after half a year, the former value became only 1.1% and the latter one was 69%, seemingly free of HCC risk. For a HCC indicator, the most challenging part is to determine cutoffs for candidate diseases, which need a large sample size in further study. Nevertheless, our study successfully illustrated it is necessary to monitor the patients with suspicious methylation changes in cfDNA according to multiple indicators to combine their powers together.

Although we have found some stable pattern at low-pass WGBS, we still need to enlarge sample size to validate the sensitivity and specificity of this pattern to obtain more precise information for HCC diagnosis and surveillance. The most challenging part in future attempt is to set the cutoff for disease stage definition, and especially for those with small tumors. Furthermore, blood samples from HCC patients at multiple time points were also needed to trace the 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 was made according to the guidelines for the prevention and treatment of chronic hepatitis B: a 2015 update (37). 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 was classified by BCLC system (Ref). 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. An informed 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 transmitted to Beijing Institute of Genomics. Upon arrival, the blood 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 (XXX ng-XXX 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). All the sequencing data was deposited in the BIG Data Center (http://bigd.big.ac.cn/bioproject/) under the BioProject accession code XXXXXXXX.

After base calling, all paired-end fastq files were trimmed using cutadapt (v 1.8.3)(38) 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)(39) 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 (v 0.1.19) 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 mi/(mi + ui), where mi was defined as the number of methylated cytosines and ui 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 1-Mb region (Methyl1MB) was calculated as the total number of cytosines divided by the number of methylated cytosines.

### Identification of the optimal region size of long range methylation (LRM)

The HCC genome was divided into 500-Kb, 1-Mb, 1.5Mb, 2-Mb and 2.5-Mb, respectively. For each size, the average methylation level for each region from autosome were calculated. The hypo-methylated region were identified as methylation level difference larger than 0.2 compared to the corresponding region in heathy individual. Then the percentage of hypo-methylated regions across the genome was calculated. The largest percentage of hypo-methylated region size was selected as the optimal size of LRM

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

A randomly sampling method was used to obtain low depth WGBS for 5 medium WGBS of cell-free DNA. (a) 1M to 10M read (increasing by 1M step) was randomly extracted from each medium WGBS data set. (b) For each re-sampling, the average methylation level for each 2-Mb region (MethylLRM) from autosome were calculated and pearson correlation coefficient was used to show the correlation of all the autosomal MethylLRM between this re-sampling reads and total WGBS reads. This process was repeated for 100 times. (c) For each re-sampling, coefficient of variation (CV) for correlation coefficient was calculated across 100 randomly re-sampling to examine the variability of 100 extraction.

### Identification of hyper-LRMs and hypo-LRMs

We adopted the method of Chan et al.(21) to define the hyper- or hypo- MethylLRM compared to the healthy ref group. Only autosomes were included in this analysis. A 2-Mb region of a sample was defined as hyper- or hypo-methylated if its average methylation level was at least 3 SDs above or below the mean of the corresponding region of the healthy individuals. Then the number and percentage of hyper- or hypo- MethylLRM within the genome was calculated.

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

The identification of DMCs was generated using R package methylKit. The significance of the DMCs between case (hepatitis, cirrhosis, early stage HCC, advanced HCC) group and healthy group was performed by logistic regression test with at least 5-fold coverage. P-value was adjusted for multiple testing with the method of Hochberg and Benjamini. The CpG sites were considered differentially, 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.

### The enrichment score in each genomic region

The enrichment score for CpGs or DMCs was calculated by the following formula:The enrichment scorein the genomic element = log2 (# DMCsin the genomic element/# expected). # expected was computed as: # DMCsin the genome × # CpG sitesin the genomic element/# total CpG sitesin the genome. # means the number of sites.

### DNA methylation of CpGs near the HBV integration sites in hepatitis, cirrhosis and HCC

Identification of hypo-CpGs within the 100 bp upstream or downstream of HBV integration sites. The HBV integration sites were extracted from previous reports (25, 28-33).We extracted CpG within the 100 bp upstream or downstream of HBV integration sites. Only autosomal CpGs and CpGs with depth over 5 in all the 54 samples were included in the hypo-CpGs analysis. Similar to the identification of hypo-Methyl1MB, a CpG of a sample was defined as hypo-methylated if its methylation level was 3 SDs or more below the mean of the corresponding CpGs of the healthy individuals. Then the percentage of hypo-CpGs was calculated. Average methylation level of the CpGs within the 100bp of the HBV integration sites. For each sample, the average methylation level of CpGs within the 100 bp upstream or downstream of HBV integration sites was included in all the CpGs. This value was calculated as the number of the total number of cytosines divided by the number of methylated cytosines within the 100bp of the HBV integration sites.

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**Authorship Contributions**

SG performed analyses, interpreted results, designed the functional experiments, and aided in drafting the manuscript. SJ conducted molecular and cell biology experiments. NE interpreted results, provided hematology and pathway expertise, reviewed and edited the manuscript. MM aided in the analyses and reviewed the manuscript. MW, YM and WW provided clinical and biochemistry advice and aided in drafting the manuscript. MM aided with the experimental design and analyses. ZY performed initial genetic and statistical analyses, performed data management and reviewed the manuscript. BO implemented and refined the phenotyping algorithms. TK and JJ aided in the regulatory paperwork and reviewed the manuscript. RS performed data management tasks. JJM provided clinical advice and reviewed the manuscript. JKM supervised the management of biological samples for genotyping and reviewed the manuscript. LJ reviewed the manuscript and provided general scientific advice. JAS provided molecular and cellular biology advice, clinical advice, reviewed and edited the manuscript. JW supervised the functional experiments, reviewed the manuscript and provided biological advice. SJS designed the study, supervised the genetic analyses, developed phenotyping algorithms, developed analysis methods and power calculations, interpreted results and aided in drafting and editing the manuscript.

**Disclosure of Conflicts of Interest**

The authors declare no conflict of interest.

**Abbreviations**

LRM Long-Region Methylation

LRLM Long-Region Hypo-methylation

LRHM Long-Region Hyper-methylation

HCC Hepatocellular Carcinoma

DMCs Differential Methylation CpGs

DMGs Differential Methylation Genes

HBV Hepatitis B virus

HCV Hepatitis C virus

GWBS Genome-wide Bisulfite Sequencing

RRBS [Reduced Representation Bisulfite Sequencing](https://en.wikipedia.org/wiki/Reduced_representation_bisulfite_sequencing)

cfDNA Circulating cell-free DNA

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## Figure Legends

**Fig. 1. The efficiency of re-sampling sequencing depth for low pass WGBS.** Left of the figure showed the correlation coefficient between re-sampling low pass WGBS and medium WGBS for 100 times from 1M to 10M. Right of the figure showed the coefficient of variation (CV) for 100 correlation coefficient between re-sampling low pass WGBS and medium WGBS from 1M to 10M

**Fig. 2 Whole genome-wide changed methylation of all the patients.** (A) The percentage of hyper-methylated 2-Mb regions in chronic hepatitis, cirrhosis and HCC patients. (B) The percentage of hypo-methylated 2-Mb regions in chronic hepatitis, cirrhosis and HCC patients.

**Fig. 3. Differentially methylated CpGs (DMCs) identified in all the groups.** (A) Venn diagram showing the overlap of DMCs generated by 2 hypo-methylated chronic hepatitis patients, 1 hypo-methylated cirrhosis patient, 4 HCC patients and 2 hypo-methylated HCC patients after surgery compared to healthy individuals. (B) Boxplot displays the methylation level of 6 DMCs of SENP5 in 3 healthy individuals, 21 chronic hepatitis, 15 cirrhosis, 4 HCC and 12 HCC patients after surgery. (D) 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 in the bottom of the figure represent the locus of repeat marker in this region.

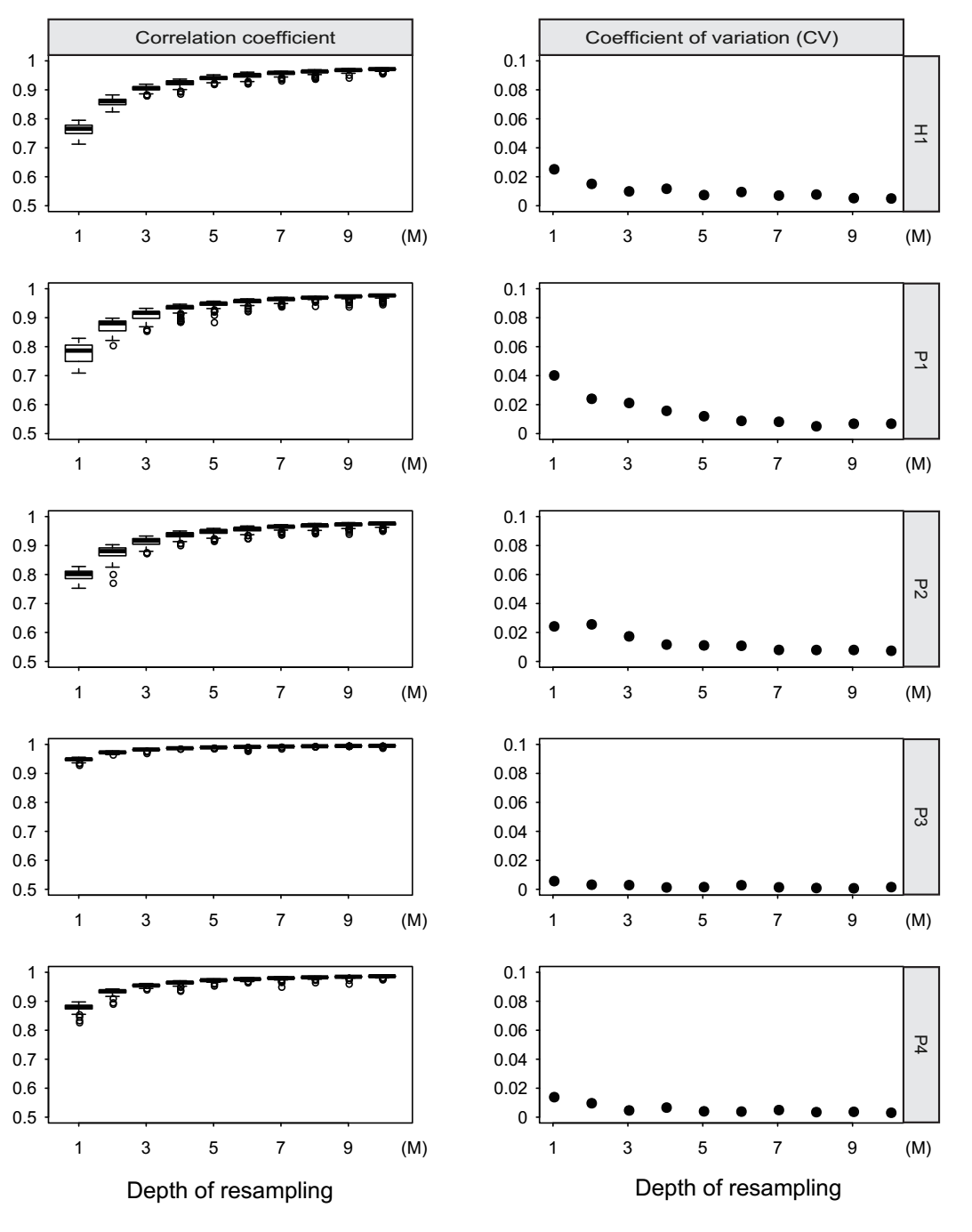
**Fig. 4. DMCs and CpGs are related to HBV integration sites.** (A) The percentage of DMCs located in different genomic elements and regions related to HBV integration sites. (B) The enrichment scores of DMCs in different genomic elements. (C) The heatmap display the methylation level of the CpGs located within 100 bp of the HBV integration sites in all the samples. (D) The average methylation level of the CpGs located within 100 bp of the HBV integration sites in all the samples. The red arrows showed the examples of CH18 patient

**Table 1. Genes with DMCs between liver disease patients and healthy individuals.**

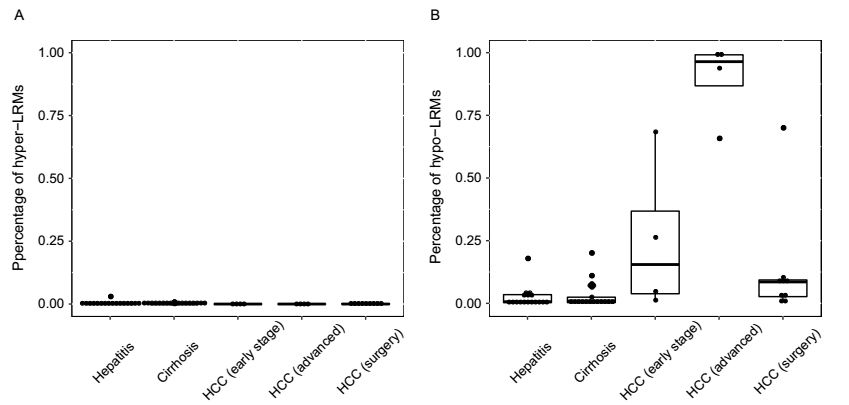
|  |  |  |  |  |  |
| --- | --- | --- | --- | --- | --- |
| **Chr** | **Start** | **End** | **CpG num** | **Position** | **Gene** |
| **Hepatitis vs. healthy** | | | | | |
| chr1 | 156186377 | 156186549 | 18 | gene body | PMF1 |
| chr1 | 156186377 | 156186549 | 18 | gene body | PMF1-BGLAP |
| chr1 | 91853073 | 91853096 | 3 | gene body | HFM1 |
| chr11 | 85195089 | 85195090 | 1 | gene body | DLG2 |
| **Cirrhosis vs. healthy** | | | | | |
| chr1 | 156186492 | 156186493 | 1 | gene body | PMF1 |
| chr1 | 156186492 | 156186493 | 1 | gene body | PMF1-BGLAP |
| chr8 | 70602451 | 70602487 | 2 | gene body | SLCO5A1 |
| **Early stage HCC vs. healthy** | | | | | |
| chr1 | 156186392 | 156186540 | 8 | gene body | PMF1 |
| chr1 | 156186392 | 156186540 | 8 | gene body | PMF1-BGLAP |
| **Advanced HCC vs. healthy** | | | | | |
| chr1 | 91852973 | 91852974 | 1 | gene body | HFM1 |
| chr1 | 156186410 | 156186506 | 12 | gene body | PMF1 |
| chr1 | 156186410 | 156186506 | 12 | gene body | PMF1-BGLAP |
| chr3 | 196625630 | 196625734 | 7 | gene body | SENP5 |
| chr8 | 70602381 | 70602487 | 2 | gene body | SLCO5A1 |
| chr8 | 86572360 | 86572383 | 2 | gene body | REXO1L1P |

**Table 2. The information of hepatitis and cirrhosis patients with lower methylation levels of CpGs located in the 100 bp of HBV integration sites**

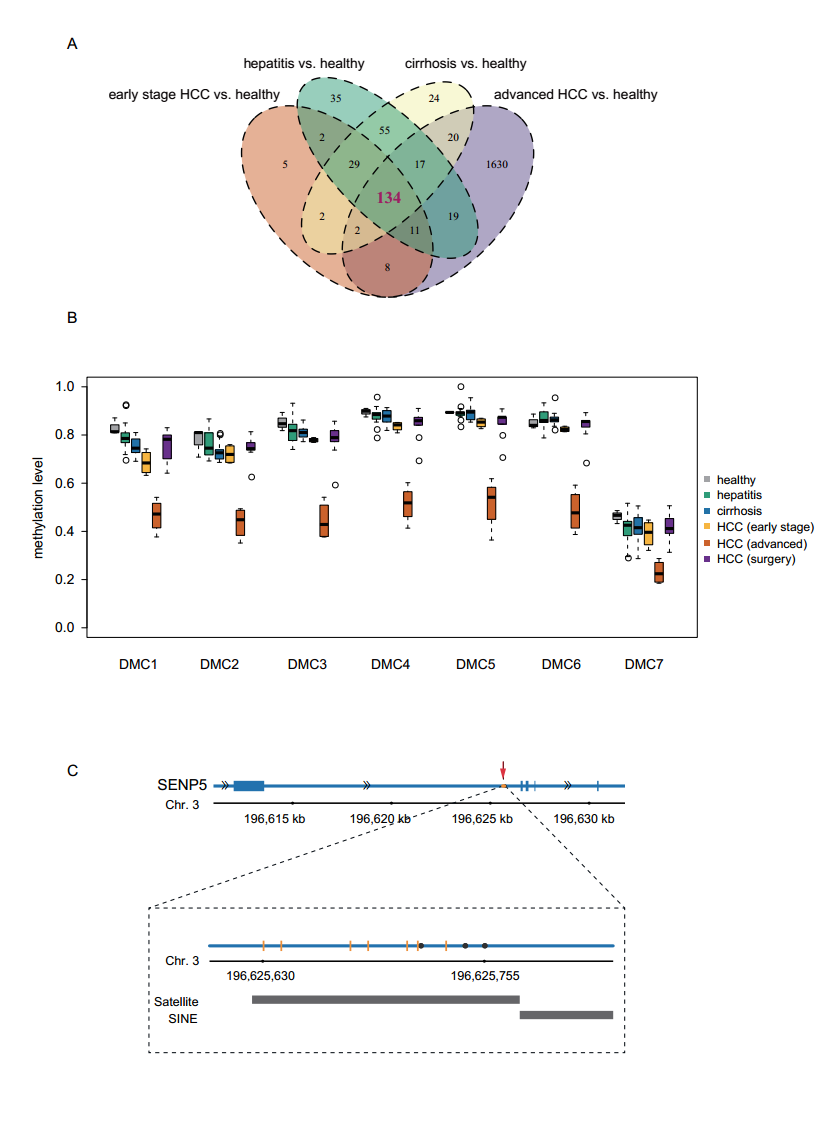
|  |  |  |  |
| --- | --- | --- | --- |
| **Patient** | **Disease** | **Percentage of hypo 1-Mb regions** | **average methylation level of DMS in the 100bp of HBV integration sites** |
| P2 | chronic hepatitis | 19.69% | 67.69% |
| P14 | cirrhosis | 6.40% | 67.39% |
| P18 | nash-related cirrhosis | 21.84% | 66.04% |
| P19 | alcoholic cirrhosis | 14.23% | 67.96% |



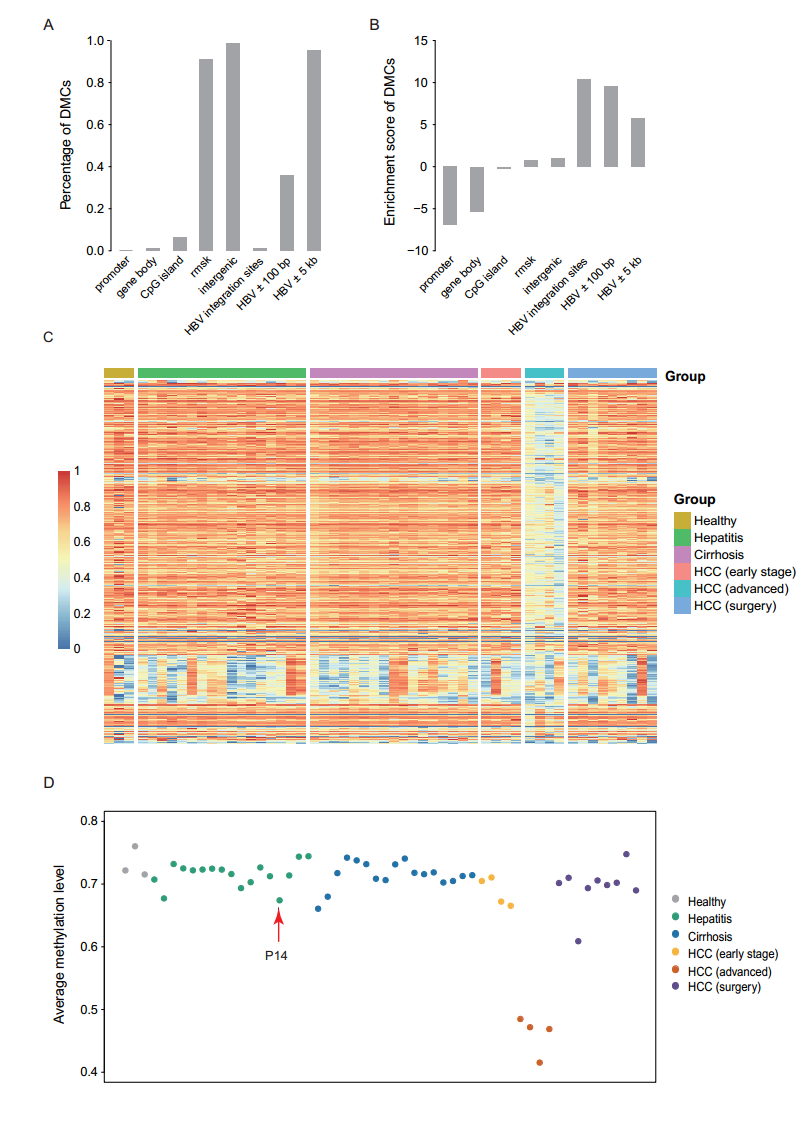
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