**Genome-wide methylation profiling of different stages of HBV-related HCC development in plasma cell-free DNA reveals potential biomarkers for early detection and high-risk monitoring of HCC**

Yangxing Zhao1,6, Feng Xue2,6, Jinfeng Sun 3,6, Shicheng Guo 4,6, Hongyu Zhang 1, Zhenfeng Zhang1, Bijun Qiu 2, Jun Gu1, Wei Wang1, Yinghua He1, Ning Tang1, Xiaoyu Zhou5, Qiang Xia2, Jian Yu1

1 State Key Laboratory of Oncogenes and Related Genes, Shanghai Cancer Institute,Renji Hospital, Shanghai Jiao Tong University School of Medicine, Shanghai, China.

2 Department of Liver Surgery, Ren Ji Hospital, School of Medicine, Shanghai Jiao Tong University, Shanghai, China.

3 Zhongshan Hospital, Fudan University, Shanghai, China.

4 MOE Key Laboratory of Contemporary Anthropology and State Key Laboratory of Genetic Engineering, School of Life Sciences and Institutes of Biomedical Sciences, Fudan University, Shanghai, China

5 Key Laboratory of Contraceptive Drugs and Devices of NPFPC, Shanghai Institute of Planned Parenthood Research, Shanghai, China.

6 These authors contributed equally to this work

Correspondence:

Yu Jian, State Key Laboratory of Oncogenes and Related Genes, Shanghai Cancer Institute,Renji Hospital, Shanghai Jiao Tong University School of Medicine. LN 2200/25, Xietu Road, Shanghai 200032, P.R. China.

Xia Qiang, Department of Liver Surgery, Ren Ji Hospital, School of Medicine, Shanghai Jiao Tong University. No. 1630, Dongfang Road, Shanghai 200032, P.R. China.

Email:yujian@shsci.org, xiaqiang@medmail.com.cn

ACKNOWLEDGEMENTS

This work was supported by grant from the National Natural Science Foundation of China(81372368),Shanghai Municipal Commission of Health and Family Planning Foundation (20134055), the State Key Laboratory of Oncogenes and Related Genes Foundation (No. 90-10-01, 91-11-01, 91-13-10)

Conflicts of interest: None

Running title: Plasma DNA methylation pattern in HCC

Total number: Text pages: 13

Tables: 1

Figures: 5

Supporting information: SI1 to SI3,Supplementary Figure S1-S2,Supplementary tables ( S1-18)

**Abstract**

**Background:** An important model of HCC development that has been described in southeast Asia includes the transition from chronic hepatitis B infection (CHB) to liver cirrhosis (LC) and, finally, to HCC. The genome-wide methylation profiling of plasma cell-free DNA (cfDNA) has not been carried out to date for the assessment of HCC development. **Methods:**Using MethylCap-seq, we analyzed genome-wide cfDNA methylation profiles by separately pooling healthy control (HC), CHB, LC and HCC samples, and independently validating the library data for the tissue DNA and cfDNA by MSP, qMSP and Multiplex-BSP-seq. **Results:**The dynamic features of cfDNA methylation coincided with the natural course of HCC development. Data mining revealed the presence of 240, 272 and 286 differentially methylated genes (DMGs) corresponding with the early, middle and late stages of HCC progression, respectively. The validation of the DNA and cfDNA results in independent tissues identified 3 DMGs, including ZNF300, SLC22A20 and SHISA7, that show potential for distinguishing CHB from LC and LC from HCC. ROC values ranged from 0.65 to 0.80, and OR values ranged from 5.18 to 14.2. **Conclusions:**Our data have revealed highly dynamic cfDNA methylation profiles in support of HBV-related HCC development. We have identified a panel of DMGs that are predictive for the early, middle and late stages of HCC development, which are potential markers for the early detection of HCC and the screening of high-risk populations.

**Keywords:**

Plasma, cell-free DNA, HBV, HCC development, genome-wide, DNA methylation

Introduction

Human hepatocellular carcinoma (HCC) is one of the most common primary liver malignancies and is ranked fifth in incidence and third in mortality among common solid tumors worldwide1,2. The highest incidences are observed in eastern Asia, where a model of multi-stage carcinogenesis developing from chronic HBV virus infection (CHB) to HBV-related liver cirrhosis (LC) and finally to HBV-related HCC (HBV-HCC) has been suggested by both epidemiologic data and laboratory investigations3. Three mechanisms are involved in HBV-related carcinogenesis, including the following: (1) the interruption of proper liver cell proliferation and viability by HBV viral proteins, and HBx in particular; (2) changes in gene function and instability, which are induced by the insertion of viral DNA into the host genome; and (3) genomic and epigenomic injuries resulting from liver cell inflammation that are induced by immune T cells targeting the HBV virus 4.

Even in the early stages of chronic hepatitis B infection, DNA methylation appears to be altered, and some of these aberrant methylation patterns could overlap with those that are associated with HCC. Some studies have revealed that the host cell modifies the foreign HBV DNA by methylation and histone acetylation to hinder viral RNA replication and protein expression as defense mechanisms against viral invasion. However, the enhanced expression of the host DMNT also leads to the methylation of its own CGI, particularly those that are located in the promoter regions of tumor suppressor genes (TSG) 5. Studies have also shown that HBV products, including the HBx protein, may manipulate DNMT1 to inactivate some TSGs, , 6.

Therefore, the establishment of DNA methylation profiles at the whole genome level, including those from healthy control (HC) livers and CHB, LC and HCC samples, will provide fundamental information with regard to the common or unique DNA methylation patterns that occur during different stages of HCC development and may even facilitate the identification of those genes that are involved in this process.

To date, some studies have reported DNAm profiles of HCC tumor/adjacent tissues using Illumina arrays7-9. These studies have differed in sample size, technology used and major etiologic cause (i.e., HBV, HCV or alcohol). However, relatively little is currently known with regard to DNA methylation alterations that occur at the early stages of HCC development; for example, those that are involved in the processes of CHB and LC. Additionally, genome-wide methylation profiling has not been reported for these two pathologic models to date. Moreover, the invasive procedure of tissue sampling limits the utility of this procedure for epidemiologic studies. In fact, CHB and LC samples are usually used as controls in genomic methylation analyses of HCC or in-gene locus-specific CGI studies10.In contrast, blood sampling is superior to that of tissue because it is minimally invasive, easy to obtain and can be consecutively applied as serial samples in clinical practice. Research shows that only a tiny amount of cell-free DNA (cfDNA) exists in peripheral blood circulation in healthy individuals, but it substantially increases in quantity during the development of HCC11. the accurate identification of primary tumor genomic methylation patterns in plasma cfDNA has recently been confirmed by a study of esophageal cancer12.

In the present study, we have conducted a genome-wide screening analysis to detect aberrant methylation events in plasma cfDNA using the MethylCap-seq method and observed some distinct DNAm alterations that were associated with the CHB, LC and HCC developmental stages (Figure 1).

**Materials and Methods**

**Patient samples**

With informed consent and the approval of the Medical Institutional Review Board of Renji Hospital, Shanghai Jiao Tong University, patient specimens were collected between 2010 and 2012. The criteria used to recruit clinical study subject refers to supporting information 1. Three separate sets of patient samples were obtained for the establishment of the genome-wide methylation profiles of the plasma cfDNA by MethylCap-seq, for screening and identifying candidate differential methylation regions (DMRs) in tissue DNA by MSP/qMSP and for validating candidate DMRs in plasma cfDNA by Multiplex-BSP-seq. The first set of samples included 31 HC, 30 CHB, 27 LC and 29 HCC in addition to 26 non-small cell lung cancer (NSCLC) samples, which were included as a control to represent a malignancy from a separate organ. The second set of samples included 10 HC, 29 LC and 33 HCC. The HC liver samples were obtained from biopsy tissues, from which other common liver pathological changes were excluded. The LC sample was also obtained from biopsy tissue, and the HCC sample was confirmed via histological examination by a pathologist. The third set of samples included 37 HC, 36 CHB, 40 LC and 47 HCC. All of the patients were HBV-positive with the exception of the NSCLC patient. No significant differences were found between the study groups in terms of age or gender. The blood samples were stored at 4℃ before plasma separation, and the tissue samples were stored at -80℃ until DNA extraction. Further detailed clinical information is provided in Table 1.

**DNA and RNA extraction**

A peripheral venous blood sample was drawn into an EDTA tube for each subject, and the plasma sample was separated within 2 hours. The plasma was isolated by two-step centrifugation as described in reference13. The cfDNA was extracted from 800-µl aliquots of plasma using the Qiagen Blood DNA Kit (QIAGEN, Hilden, Germany). The tissue DNA was isolated from 100 mg of corresponding tissues using a conventional proteinase K/organic extraction method as previously described14. RNA extraction and reverse transcription were performed as described in reference15,16.

**Processing of MethylCap-seq**

To obtain adequate starting material for the MethylCap-seq library, 30 ng of plasma cfDNA from each participant were pooled for each group. Approximately 500 ng of DNA for each of the five four libraries (HC, CHB, LC, HCC and NSCLC) was processed for metylCap-seq library as previously described17.The resulting data were processed to generate genomic methylation profiles and then uploaded to a public database (Gene Expression Omnibus: GSE54961 (2014)).

**Processing of MethylCap-seq data**

We used the BWA alignment tool18 to map the libarary reads to the hg19 human genome reference assembly19. The PCR duplicates were removed with Picard; Samtools and Picard were used to convert, sort and index the aligned data. DMRs in the study groups for comparison with the HC were identified using two methods, MACS20  and BALM21, to increase the power of detection for the MethylCap-seq analysis. Methylation peaks (hypermethylated regions) were identified as previously described21. The refSeq genes (UCSC genes) and corresponding CpG islands (CGIs) were downloaded from the table browser of the UCSC database19. The BED files were manipulated with Bedtools22 in addition to assorted Perl scripts. Detailed analysis procedures provided in Supporting information 2.

**Screening with MSP and validation with qMSP**

The tissue DNA cfDNA were bisulfite converted using EpiTect kit (QIAGEN, Hilden, Germany). Methylation-specific PCR (MSP) primer pairs were listed in Supplementary Table S1). In total, 125 MSP were performed, and the results were visualized on gels. The MSP products were cloned by T-Vectors and verified by sequencing. The in vitro methylated DNA from the HepG2 cells was obtained using the CpG (M. SssI) methyltransferase (NEB) treatment and used as a positive control. Up to 33 gene targets were measured using methylation-specific real-time PCR (qMSP) as previously described23. Real-time PCR was conducted with the Rotor-Gene Q (Corbett, Mortlake, NSW). Target gene methylation was calculated using the following formula: 100 x methylated reaction/(unmethylated reaction+methylated reaction).

**Multiplex-BSP-seq**

The BSP regions were situated within the regions that were assessed by the qMSP analysis. The BSP primer pairs were listed in Supplementary Table S2). Degenerate primers were utilized in the promoter regions, in case where inevitably contained CpG sites. Multiplex-BSP-seq was performed to overcome the difficulty of scarcity of cfDNA. Detailed technique information was provided as Supplementary Figure S1and Supporting Information 3. The methylation level (%) that was measured at each individual CpG site was expressed as the percentage of methylated CpG vs. total CpG.

**Real-time RT-PCR**

Expression levels of selected genes were measured by real-time quantitative RT-PCR analysis. At least triplicate reactions were performed for each sample using a RealMasterMix (SYBR Green) kit (TianGen, Beijing, China) with gene-specific primers (Supplementary Table S3) on a Rotor Gene 6000 instrument (Corbett Life Science, Mortlake, NSW, 2137). RNA quantity was normalized to ACTB content, and gene expression was quantified according to the 2-ΔCt method.

**Statistical analyses.**

To evaluate the potential of the assay for discriminating between different disease stages, the data that were obtained for the HC, CHB, LC and HCC samples were used to calculated the optimal cut-off value using the most significant area under the receiver operating characteristic (ROC) curve (AUC). Univariate models were used to examine the association between gene CpG methylation and HCC stage. Multivariate models were then developed that adjusted for the most important covariates, including age and gender. The measurement data were analyzed using a one-way ANOVA. The sample means were compared using an unpaired t-test assuming unequal variances, and all of the tests were two-tailed. P values are shown for statistic significance judgment. All of the statistical analyses were performed using the SPSS statistical package (version 13.0, Chicago, IL, USA).

**Results**

**Genome-wide methylation profiling with plasma cfDNA from different stages of HBV-related HCC development**

The data mining resulted in 37,610,900, 37,072,952, 35,016,215, 33,286,609 and 33,002,633 raw reads for the samples from the HC, CHB, LC, HCC, and NSCLC patients, respectively. When these were mapped to the hg19 genome reference, 19,083,142, 18,285,110, 16,389,041, 14,422,110 and 14,894,363 reads were obtained, respectively, with a mapping rate of approximately 50% (Figure 2A, Supplementary Table S4). Notably, very high background levels of methylation were observed for the HC plasma cfDNA.

**Association of cfDNA methylation disturbances with progressive stages of HCC development**

We used hierarchical clustering to trace the progression of methylation disturbances during the carcinogenic process for the HC, CHB, LC and HCC samples. The analysis revealed the closeness of aberrant DNA methylation patterns among defferent groups of samples (Figure 2B), suggesting the possible sequential direction of the development of epigenetic alterations for the HC, CHB, LC and HCC samples. Moreover, we estimated the relationships of the aberrant methylation patterns among the various stages of HCC development by the pairwise overlap of the peak bases from each stage, which revealed the similarities between HC and CHB in addition to LC and HCC (Supplementary Table S5). In addition, we investigated the DNA methylation densities surrounding the TSS, which is a crucial region affecting the gene expression of regulatory mechanisms, and found the accumulation of aberrant DNA methylation events as the disease stage progressed from HC to CHB to LC, and finally, to HCC (or NSCLC) (Figure 2C). These results suggest a stage-related stepwise process, in which the gene methylation regulatory elements were affected sequentially during the carcinogenesis process. Collectively, the above observations indicate that the aberrant methylation of plasma cfDNA occurs in a dynamic, progressive and stage-responsive manner during HCC development. This creates the basis for our further analyses of HCC progression-related DNAm abnormalities.

**Identification of differentially methylated loci among various stages of HCC development**

To obtain the DNA methylation peaks with high reliability, we used two methods, which included MACS and BALM. The peaks that were generated by these two methods were generally consistent (90%~97% coincidence). We performed our analysis using these peaks, which totaled 226,898, 257,344 282,203, 242,987 and 290,096 for the HC, CHB, LC, HCC and NSCLC groups, respectively (Supplementary Table S6).

Because our long-term aim was to establish methylation markers that can be applicable for the detection and monitoring of HCC development and progression, we were particularly interested in hypermethylated DNA alterations. To identify the stage-related DNA methylation disturbances, we investigated the alterations that occurred at each stage, including CHB, LC and HCC in addition to NSCLC, against the normal background levels of the HC. The different methylation peaks that were subsequently generated are referred to as the DMRs. The most abundant DMRs were observed in the “total DMR” category, reflecting all of the hypermethylation peaks that were elevated compared with the HC, (Figure 2D; Supplementary Table S6); in addition, the “gene-related DMR” and “CGI-associated DMR” categories could be isolated, and the category that included both the CGI and gene-related DMRs became the focus of our further analyses (Figure 2D, Supplementary Table S7).

The analysis of aberrantly methylated DMRs at different loci in association with the HCC developmental process also revealed that widespread genomic structural elements were affected, which included a variety of structural elements as defined by the UCSC (Figure 2E and Supplementary Table S8).

**Early-, middle- and late-stage DMRs and their related DMGs**

To further elucidate the cfDNA methylation alterations that occur during the CHB, LC and HCC stages, we compared the DMRs that were associated with each stage on a chromosomal distribution basis against those from the HC (Figure 3A). The analysis showed that CHB is characterized by sparse and mild hypermethylation alterations, suggesting that certain epigenetic disturbances that are produced by HBV begin as early as this stage. However, these hypermethylation alterations obviously increased during the transition from CHB to LC, indicating the occurrences of more extensive epigenetic disturbances. The HCC stage was characterized by substantial hypermethylation, and a similar degree of epigenetic change was also observed for NSCLC, suggesting that such alternations may be a features in association with various malignancies in late stages of carcinogenesis.

Provided that the introduction of DMRs occurred as a continuous, dynamic process during the different stages of HCC development, we observed the following 3 categories of DMR involvement: 1) those that were common to CHB, LC and HCC, which may play important roles in the process of HCC development from CHB to HCC; because they occurred as early as the CHB stage, we referred to them as early DMRs; 2) those that were common to both LC and HCC, which may be essential for the transition from LC to HCC and were called middle DMRs; and 3) those that were unique to HCC, which are most likely indispensible for sustaining the malignant status and were therefore called late DMRs. To identify the exact genes that are affected by the aforementioned early-, middle- and late-stage DMRs (the differentially methylated genes, DMGs), we used “CGI + gene” involvement as the selection criterion and generated 240 early-, 272 middle- and 286 late-stage DMGs (Figure 3 B, Supplementary Table S9, S10 and S11).

Recent studies have reported that tissue and cancer-specific DMRs occur more often within CGI shores, which are regions of relatively low CpG densities that flank traditional CGIs (up to 2 kb in distance), than within CGIs themselves, implying the importance of considering CGI shores in DNA methylome analyses24. Therefore, we generated 225 early-, 232 middle- and 259 late-stage DMGs according to CGI involvement (Supplementary Table S12, S13 and S14), while a much more expanded panel of 1,350 early-, 1,291 middle- and 968 late-stage DMGs were produced when CGI-shore involvement was taken into account (Figure 3C, Supplementary Table S15, S16 and S17). This represents an obvious trend that CGI-associated DMGs gradually increase along with HCC progression, while CGI-shore-associated DMGs decrease, possibly suggesting that the epigenetic effects that occur during the late stages of HCC development are associated with CGI themselves rather than CGI-shores.

**MSP screening, qMSP validation and gene expression of library revealed DMGs in independent tissue DNA**

To validate the accuracy of the plasma DNAm profile library, two techniques were employed to assess the tissue DNA. First, 125 DMR targets that possessed the top p-values were screened by MSP in 10 normal liver and 33 HCC (Supplementary Figure S2) tissue DNA samples, generating 33 targets that were informative of HCC. Subsequently, the informative targets were investigated by qMSP in the same tissues sets that were supplemented with 29 LC tissues. As expected, a substantial portion of the targets exhibited hypermethylation in the LC or HCC or both the LC and HCC. The typical results in selected genes are shown in Figure 4A and 4B, with the UCSC scheme of the gene information and their quantitative methylation statues in HC, LC and HCC. These results suggest that the altered methylation patterns that were observed in the plasma are consistent with the respective liver tissues.

In addition, the expression level of selected genes, ZNF300, SLC22A20 and SHISA7, were examined using real-time RT-PCR in liver tissue of HC, LC and HCC. There appeared a coincidence that hypermethylated ZNF300 showed expression downregulation along the progression of HC, LC and to HCC (p<0.05). However SLC22A20 and SHISA7 did not showed the hypermethylation coupled with gene silence pattern (Figure 4C) .

**Multiplex-BSP-seq validation of DMGs in independent plasma cfDNA**

To further confirm the cfDNA methylation levels, we used Multiplex-BSP-seq on the targets that were selected by qMSP in the independent plasma cfDNA samples. Of the 33 target genes that were studied, 22 were successfully amplified and deep-sequenced. The representative results are shown in Figure 5A. For each gene, the methylation levels of 7 to 10 individual CpG sites were investigated. GAPDH and KCNV1 exhibited relatively fixed methylation patterns for the 7 CpGs that were inspected, regardless of HCC developmental stage. In this study, we found that the methylation levels in some of the CpGs of 3 particular genes were closely related to the developmental stage of HCC and were therefore predictive of disease stage. These genes and their informative CpG sites included ZNF300 at CpG6, SLC22A20 at CpG3 and 5 and SHISA7 at CpG1, 5 and 6 (see Figure 5 B, C), which were able to distinguish between HC+CHB and LC+HCC with a lower cut-off value while distinguishing between HC+CHB+LC and HCC with a higher cut-off value of CpG methylation (Figure 5B). When these CpGs methylation measurements were subjected to multiple univariate logistic regression analyses, they showed strong or very strong associations with stage discrimination (OR: 5~14) in distinguishing between HC+CHB and LC+HCC or between HC+CHB+LC and HCC (Figure 5C). Taken collectively, Multiplex-BSP-seq using an independent set of plasma samples validated certain CpG methylation changes in a subset of genes that were revealed by MethylCap-seq during HCC development. This also suggests the important role of aberrant methylation in these CpG sites during HCC carcinogenesis.

**Discussion**

In the present study, methylCap-seq was applied to establish genome-wide methylation profiles, in which a cross-sectional group of HC, CHB, LC and HCC patients were recruited for a process consequence model study. In addition, NSCLC was included as a non-liver malignancy group. Plasma cfDNA was collected from these individuals for the analyses. Because of the minute quantities of cfDNA that were obtained, MethylCap-seq analyses could not be performed individually, and instead, the cfDNA was pooled. In fact, sample pooling has been used in a number of -omic studies because it is cost-efficient and suitable for scarce sample models25,26.

In this experiment, we initially explored features of genome-wide DNA methylation profiling by cluster analyses and found similarities between the patterns of aberrant DNA methylation for the different HCC developmental stages, which resembled the natural course of development of this disease. Hence, our study model can be utilized to assess the patterns of DNA methylation alterations that occur in cfDNA that underlie HCC development. We further compared our plasma data, which indicated significant levels of hypermethylation in HCC relative to HC, with data that was based on tissue DNA. Shen *et al* analyzed HCC tumor and adjacent non-tumor tissues from 62 Taiwanese HCC cases using Illumina methylation arrays and found that 684 CpG sites in 550 genes were significantly hypermethylated in the tumor tissues9. In fact, 343 of the genes from that study were detected in our plasma HCC analysis (Supplementary Table S18) despite the fact that different analysis methods and different samples were used in both investigations. Therefore, these identical genes may serve as good resources from which to select plasma markers for HCC evaluations because they exhibit hypermethylation not only in the tissues but also in the cfDNA, and thus, the cfDNA methylation alterations parallel those of hepatic cells.

According to the accepted hypothesis, aberrant DNA methylation in association with carcinoma is characterized by genome-wide hypomethylation and clustered hypermethylation27,28. Thus, we analyzed the relationship between HCC stage-related DMRs and gene promoters and revealed that the DMRs were more abundant in the promoters during the advanced stages of HCC development than in the early stages, with the highest concentrations occurring in the promoter regions for HCC. In terms of HCC development progression, we found 240 early-, 272 middle- and 286 late-stage DMGs. These results indicate that HBV invasion led to hypermethylation in the genomic DNA of the hepatic cells, and some of these alterations could be maintained as late as the HCC stage, presumably because they are the important factors for HBV-related HCC development. In addition, these results also suggest that the alterations gradually accumulated rather than appearing all at once during the HBV-related HCC progression. It is possible that the late-stage DMGs are the driving factors underlying, or are merely consequences of, the hepatic cell malignant transformations that occur during HCC development.

The DNA methylation patterns that were revealed by our analysis may play important roles in HCC development and additionally may facilitate early tumor diagnosis, disease progression monitoring and the identification of high-risk individuals. To further elucidate these findings, we used a combination of Multiplex-BSP-seq, Illumina index adaptor techniques and deep sequencing to validate 33 DMG targets in approximately 160 cfDNA samples from four stages of HCC development, which resulted in the identification of 3 relevant genes (SHISA7, ZNF300 and SLC22A20) in association with 10 CpG methylation statuses that were informative of HBV-related HCC development. Similar to the methylation patterns that were observed in the MethylCap-seq analysis, Multiplex-BSP-seq also revealed the higher similarities of HC and CHB in addition to LC and HCC, suggesting close relationships between their pathogenic processes in terms of epigenetic involvement, with epigenetic changes occurring gradually from HC to CHB and more substantially from CHB to LC. The accumulation of alterations again slows in the transition to the final malignant state of HCC. The entire process is not likely to occur at a steady rate as indicated by this study.

Epigenetic plays its part through regulations on corresponding gene expressions. In our present study, the increasing hypermethylaton of gene ZNF300, during the HCC development, is coupled with gene down expression. However, the other two genes, SHISA7 and SLC22A20，failed to show this correlation. These result might suggest that only a subset hypermethed genes might play its role through methylation-gene silence pathway, while most of the other hypermethylated genes influence the tumor development by other alternative mechanisms.

The analysis of these 10 CpGs by ROC revealed that LC and HCC were able to be distinguished from HC and CHB when a lower methylation cut-off value was used, while HCC could be effectively discriminated from HC, CHB and LC when a higher methylation cut-off value was adopted. Univariate and multivariate logistic regression analyses indicated the strong association of these CpG methylation sites with LC and HCC, suggesting that these aberrant DNA methylations may represent risk factors for HCC development. The possibility that the DNA methylation patterns of single CpG bases may be useful indicators of carcinoma risk and development has been suggested by other researchers29

In summary, the present study, which is based on the four-stage progression model of HBV-related HCC development, has revealed stage-associated DNA methylation disturbances by MethylCap-seq analyses of cfDNA that could reflect their particular methylation patterns, providing further insight into the participation of epigenetic events in HCC development. Additionally, the aberrant methylation statuses of some of the candidate genes’ CpG sites may be effective tools for the early diagnosis, progression monitoring and high-risk population screening of HCC. We plan to expand our cross-sectional study and include a prospective longitudinal cohort to further validate the present findings.

**References**

(1) Bruix J, BoixL, Sala M, and Llovet JM. Focus on hepatocellular carcinoma. Cancer Cell.2004; 5 (3): 215–219.

(2) Dufour JF, Johnson P. Liver cancer: from molecular pathogenesis to new therapies: summary of the EASL single topic conference. J Hepatol.2010; 52(2): 296–304. doi: 10.1016/j.jhep.2009.11.010.

(3) Parkin DM, Bray FI, DevesaSS. Cancer burden in the year 2000. The global picture.Eur J Cancer. 2001; 37 Suppl 8: S4-S66.

(4) Gehring AJ, Ho ZZ, Tan AT, Aung MO, Lee KH, Tan KC, Lim SG, Bertoletti A. Profile of tumor antigen–specific CD8 T cells in patients with hepatitis B virus–related hepatocellular carcinoma. Gastroenterology. 2009; 137: 682–690.

(5) Vivekanandan P, Daniel HD, Kannangai R, Martinez-Murillo F, Torbenson M. Hepatitis B virus replication induces methylation of both host and viral DNA. J Virol. 2010; 84(9):4321-9. doi: 10.1128/JVI.02280-09

(6) Wei X, Xiang T, Ren G, Tan C, Liu R, Xu X, Wu Z. miR-101 is down-regulated by the hepatitis B virus x protein and induces aberrant DNA methylation by targeting DNA methyltransferase 3A. Cell Signal.2013; 25(2):439-46. doi: 10.1016/j.cellsig.2012.10.013.

(7) Gao W, Kondo Y, Shen L, Shimizu Y, Sano T, Yamao K. Variable DNA methylation patterns associated with progression of disease in hepatocellular carcinomas. Carcinogenesis.2008; 29(10):1901-1910.

(8) Ammerpohl O, Pratschke J, Schafmayer C, Haake A, Faber W, vonKampen O. Distinct DNA methylation patterns in cirrhotic liverand hepatocellular carcinoma. Int J Cancer. 2011; 130(6): 1319-28. doi: 10.1002/ijc.26136.

(9) Shen J, Wang S, Zhang YJ, Kappil M, Wu HC, Kibriya MG. Genome-wide DNA Methylation Profiles in Hepatocellular Carcinoma. HEPATOLOGY. 2012; 55(6):1799-1808.

(10) Um TH, Kim H, Oh BK, Kim MS, Kim KS, Jung G, Park YN. Aberrant CpG island hypermethylation in dysplastic nodules and early HCC of hepatitis B virus-related human multistep hepatocarcinogenesis. J Hepatol. 2011; 54(5):939-47.

(11) Ren N, Qin LX, Tu H, Liu YK, Zhang BH, Tang ZY. Quantitative Analysis of Circulating DNA Level in Plasma from Patients with Hepatocellular Carcinoma and Its Potential clinical value. Journal of Fudan University, Medical Science. 2005; 32(2): 134-138.

(12) Zhai R, Zhao Y, Su L, Cassidy L, Liu G, Christini DC. Genome-wide DNA methylation profiling of cell-free serum DNA in esophageal adenocarcinoma and barrett esophagus. Neoplasia.2012; 14(1): 29-33.

(13) Nygren AO, Dean J, Jensen TJ, Kruse S, Kwong W, Boom D. Quantification of Fetal DNA by Use of Methylation-Based DNA Discrimination. Clinical Chemistry.2010; 56: 1627–1635.

(14) Yu J, Zhang H, Ma Z, Lu W, Wang Y. Methylation profiling of twenty four genes and the concordant methylation behaviours of nineteen genes that may contribute to hepatocellular carcinogenesis. Cell Res. 2003; 13: 319–333.

(15) Doleshal M, Magotra AA, Choudhury B, Cannon BD, Labourier E, Szafranska AE. Evaluation and Validation of Total RNA extraction Methods for MicroRNA Expression Analyses in Formalin-Fixed, Paraffin-Embedded Tissue. Journal of Molecular Diagnostics.2008; 10(3): 203-211.

(16) Kim SJ, Kelly WK, Fu A, Haines K, Hoffman A, et al. Genome-wide methylation analysis identifies involvement of TNF-alpha mediated cancer pathways in prostate cancer. Cancer Lett. 2011; 302: 47–53.

(17) Zhao Y, Guo S, Sun J, Huang Z, Zhu T, Zhang H. Methylcap-Seq Reveals Novel DNA Methylation Markers for the Diagnosis and Recurrence Prediction of Bladder Cancer in a Chinese Population. PLoS ONE. 2012; 7(4): e35175.

(18) Li H, Durbin R. Fast and accurate short read alignment with Burrows-Wheeler transform. Bioinformatics. 2009; 25: 1754–1760.

(19) Kuhn RM, Haussler D, Kent WJ. The UCSC genome browser and associated tools. Briefings in bioinformatics. 2013; 14(2): 144-161. doi: 10.1093/bib/bbs038

(20) Zhang Y, Liu T, Meyer CA, Eeckhoute J, Johnson DS, Bernstein BE. Model-based analysis of ChIP-Seq (MACS). Genome Biol. 2008; 9: R137. doi:10.1186/gb-2008-9-9-r137

(21) [Lan X, Adams C, Landers M, Dudas M, Krissinger D, Mamellos G. High Resolution Detection and Analysis of CpG inucleotides Methylation Using MBD-Seq Technology. PLoS ONE. 2011; 6(7): e22226. doi:  10.1371/journal.pone.0022226](http://dx.doi.org/10.1371%2Fjournal.pone.0022226)

(22) Quinlan AR, Hall IM. BEDTools: a flexible suite of utilities for comparing genomic features. Bioinformatics. 2010; 26(6): 841-842. doi:10.1093/bioinformatics/btq033

(23) Lacopetta B, Grieu F, Philips M, Ruszkiewicz A, Moore J, Minamoto T. Methylation levels of LINE-1 repeats and CpG island loci are inversely related in normal colonic mucosa. Cancer Sci. 2007; 98(9): 454–1460.

(24) Doi A, Park IH, Wen B, Murakami P, Aryee MJ, Irizarry R (2009) Differential methylation of tissue- and cancer-specific CpG island shores distinguishes human induced pluripotent stem cells, embryonic stem cells and fibroblasts. Nat Genet. 2009; 41: 1350–1353. doi:10.1038/ng.471

(25)Jemal A, Bray F, Center MM, Ferlay J, Ward E, Forman D. Global cancer statistics. CA CANCER J CLIN. 2011; 61(2): 69– 90.

(26) Marcellin P, Gane E, Buti M, Afdhal N, Sievert W, Jacobson IM. Regression of cirrhosis during treatment with tenofovirdisoproxilfumarate for chronic hepatitis B: a 5-year open-label follow-up study. Lancet. 2012; 381(9865): 468-475.

(27)Gaudet F, Hodgson JG, Eden A, Jackson-Grusby L, Dausman J, Gray JW. Induction of tumors in mice by genomic hypomethylation. Science. 2003; 300(5618): 489-92.

(28) [Rakyan VK, Hildmann T, Novik KL, Lewin J, Tost J, Cox AV. DNA methylation profiling of the human major histocompatibility complex: a pilot study for the human epigenome project. PLoS Biol. 2004; 2(12): e405.](http://www.ncbi.nlm.nih.gov/pubmed/15550986)

(29) Fisel P, Kruck S, Winter S, Bedke J, Hennenlotter J, Nies AT. DNA Methylation of the SLC16A3 Promoter Regulates Expression of the Human Lactate Transporter MCT4 in Renal Cancer with Consequences for Clinical Outcome. Clin Cancer Res. 2013; 19 (18): 5170–81.