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GENOME-WIDE ANALYSIS OF CORE PROMOTER REGION CYTOSINE-PHOSPHATE-GUANINE ISLANDS HYPERMETHYLATION PROFILES IN CHRONIC HEPATITIS B VIRUS PATIENTS IN SOUTH AFRICA

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ABSTRACT

Purpose: Hepatitis B virus (HBV) infection causes epigenetic changes through DNA hypermethylation. HBV integrates into the human genome thereby facilitating viral persistence. Infected hepatocytes express DNA methyltransferases that hypermethylate and inactivate integrated HBV genome inserts. This defence mechanism will methylate adjacent host DNA; this may result in the methylation of tumour suppressor genes and cell-cycle regulators leading to hepatocyte injury and malignancy. The aim of this study was to investigate for the presence of genome-wide promoter region cytosine-phosphate-guanine islands hypermethylation in HBV infected patients.

Materials and methods: Affymetrix Human Promoter 1.0R microarray chip was used for microarray analysis of DNA obtained from liver tissue of 17 HBV-infected patients, 2 normal liver histopathology controls, 7 hepatocellular carcinoma (HCC), 2 from liver tissue surrounding HCC lesions and 4 from patients with autoimmune hepatitis. Microarray data was analysed using Partek Genomic Suite focusing on the core promoter region within 100bp from transcription start site. Partek Pathway was used for biological pathway analysis. The discovered hypermethylation patterns were validated using bisulfite DNA sequencing in an additional set of 59 samples.
Results: HBV infected patients had significant hypermethylation compared to other study groups. Genes controlling hepatic inflammation, tissue regeneration and carcinogenesis were significantly affected ($P<0.01$). Age over 40 years, cirrhosis, hepatic inflammation and HBV e antigen status correlated with hypermethylation ($P<0.01$). On bisulfite DNA sequencing, *Cyclin D3*gene promoter was particularly highly methylated. This was associated with HBV e antigen positive status, high viral load and genotype D ($P=0.01$).

Conclusions: HBV-induced epigenetic changes were widespread, affecting genes controlling cell growth, differentiation, proliferation and apoptosis. These changes may explain the development of hepatic inflammation, fibrosis and malignancy observed in patients with chronic HBV infection.

INTRODUCTION

Chronic hepatitis B virus (HBV) is a major public health problem with more than 400 million people infected globally. The majority of HBV-infected individuals reside in the developing world, particularly sub-Saharan Africa. HBV infection contributes to more than one million deaths annually from liver cirrhosis and hepatocellular carcinoma (HCC), the third leading cause of cancer-related deaths worldwide. [1-4] The HBV genome is a small, relaxed circular molecule, 3.2 kb in size, containing four partially overlapping open reading frames, the *hepatitis Bx*(*HBx*) gene being the smallest. [4]It is postulated that over repetitive cycles of replication during chronic infection of hepatocytes, the HBV genome integrates into the human genome resulting in the accumulation of epigenetic alterations, particularly DNA methylation.Methylation is the attachment of a methyl group to cytosine within a

cytosine-phosphate-guanine (CpG) dinucleotide in the DNA sequence through the action of DNA methyltransferases. This typically alters gene expression. DNA methylation changes play an important role in liver tumorigenesis. [4-6] During HBV infection, the expression of DNA methyltransferases is high as viral inserts and neighbouring genomic DNA are methylated to suppress viral replication. This may alter the transcription of host genes. [5,6,7] The contribution of HBV-induced methylation changes in causing liver disease is not well understood. Previous studies showed that HBV DNA integration may result in the unintended and inappropriate methylation of adjacent host cytosine-phosphate-guanine (CpG) island promoter regions. [6-8] It has been reported that methylation of both host and viral DNA is attributable to productive HBV replication, and this correlates with alteration in gene transcription involving host tumour suppressor genes, accounting for the clinical phenotype of HBV-related carcinogenesis. [6,8-10] In this study, we hypothesised that HBV DNA methylation may result in the unintended and inappropriate methylation of adjacent host CpG island promoter regions. Methylation of CpG islands within gene promoter regions could alter gene transcription, accounting for the clinical phenotype of liver disease seen in HBV infection. To test our hypothesis, we investigated for the presence of genome-wide core promoter region CpG island hypermethylation in HBV-infected patients using microarray and bisulfite DNA sequencing techniques.

MATERIALS AND METHODS

Patient samples

Samples were collected for a microarray gene discovery study and for validation studies using bisulfite DNA sequencing techniques. Formalin fixed paraffin embedded (FFPE) tissue samples from patients undergoing liver biopsies as part of their clinical management during 2008 - 2010 were retrieved from the archives of the Anatomical Pathology Service, University of Cape Town, Groote Schuur Hospital, Cape Town, South Africa. FFPE samples were used to enable access to HCC samples from the same timeline for microarray work. The histology of the samples was scored using the Ishak histological activity index. Samples were categorised as cirrhotic if the fibrosis score was ≥ 5/6 and assessed as having significant hepatic inflammation if the inflammation grade score was ≥ 7/18.

For the microarray discovery study we used 32 samples from patients who had routine percutaneous liver biopsies or surgical excision of HCC. Seventeen had HBV infection, 4 AIH, 2 normal liver histopathology assessments (normal-control), 7 HCC and 2 from tissue neighbouring HCC lesions (control-HCC) (Figure 1A). All samples had immunohistochemistry staining for HBeAg and HBV core antigen. Normal-controls were patients biopsied for a hepatitis illness that subsequently resolved, had normal liver histopathology assessment, negative viral and autoimmune studies and no other identifiable cause of chronic liver disease. They were discharged from the clinical service after more than 2 years of follow-up with normal clinical parameters.

For the validation work, DNA was extracted from peripheral blood of HBV infected patients and normal controls. Validation using bisulfite DNA sequencing was done on several gene promoter regions and in the highly methylated *CCND3* gene promoter region (Figure 1B). The presence of HBeAg was determined by Elecsys HBeAg Kit (Roche Diagnostics, Germany) and HBV surface antigen by AxSYMHBsAg (V2) (Abbott Diagnostics, Germany). The institution's human research ethics committee approved the study (Ethics Approval Number: 247/2010).

DNA extraction and quality control

Genomic DNA from FFPE tissue was isolated using the AllPrep® DNA/RNA FFPE extraction Kit (Catalogue #80234; Qiagen, USA). Genomic DNA from peripheral blood was extracted using the Wizard® Genomic DNA Purification Kit (Catalogue #1125; Promega, USA). DNA quality was assessed using the Nanodrop ND-1000 UV-Vis Spectrophotometer (Thermo Scientific, USA).

Double stranded DNA targets generation

Double stranded DNA targets for microarray analysis were prepared using methods described by Huang and Perry for human genome-wide DNA methylation analysis. [11] Briefly, genomic DNA was first digested with a four-base TTAA cutter, *MseI*(Catalogue #R0525S; New England Biolabs, USA), an enzyme that restricts bulk DNA into fragments of less than 200bp, but leaves larger GC-rich CpG islands fragments relatively intact. These GC-rich fragments were ligated to H12 (5′-TAA TCC CTC GGA-3′) and H24 (5′-AGG CAA CTG TGC TAT CCG AGG GAT-3′) linkers and then PCR amplified to check for successful linker ligation. The PCR products were purified using the QIAquick PCR Purification Kit® (Catalogue #28104; Qiagen, USA) and sequentially digested with two methylation-sensitive endonucleases *BstUI*and *HhaI*(Catalogue #R0518S[*BstUI*] and #R0139L [*HhaI*]; New England Biolabs, USA), purified again and PCR amplified. The PCR mix incorporated dUTP for replacement of dTTP during amplification, and to enable DNA fragmentation with Uracil-DNA glycosylase.

DNA targets hybridisation, labelling, staining and microarray scanning

Amplified double stranded DNA targets were fragmented to a size less than 200bp to enable hybridisation, and then labelled with biotin (Figure S2). Biotinylated double-stranded DNA targets were hybridised to the AffymetrixGeneChip® Human Promoter 1.0R array using the manufacturer's protocol (Catalogue #900775; Affymetrix, USA).

The Human Promoter 1.0R array has more than 4.6 million probes per array tilled over 25,500 human promoter regions. After hybridisation, the arrays were stained and washed on the AffymetrixGenechip® Fluidics Station 450 (Affymetrix, USA). The staining reagents contain the fluorescent molecule streptavidin-phycoerythrin, which binds to biotin. The chips were scanned with the AffymetrixGenechip® Scanner 3000 7G (Affymetrix, USA) which records the distribution of signals in the arrays, and generates array BitMap images and raw data files for analysis.

Microarray data processing and analysis

The workflow used to analyse the microarray data is outlined in Figure S3. The stages of analysis were: raw data importation into Partek Genomic Suite Software® 6.0 (Missouri, USA), statistical analysis by sampling groups, determination of methylated sites and biological pathway analysis using gene ontology terms. The human genome19 assembly was used to analyse the probe data. [12] Data was processed using the robust multichip averaging algorithm, which performs $\log_2$ transformation,

background correction and quantile normalization to remove any technical biological batch effects. PCA was done as part of quality control and to identify potential outliers that could significantly affect data analysis (Figure S4). Model-based analysis of tiling-arrays (MAT) algorithm was used to analyse the probe signals for significant differences that could have biological effects. It scores regions for chromatin immunoprecipitation enrichment, calculates robust P-value cut-offs and false discovery rates. Positive MAT scores represent hypermethylation and negative scores hypomethylation relative to a comparison group.

To compare enriched regions in the different groups, an ANOVA for the categories of HBV, AIH, HCC, control-HCC and normal controls was created with-values ≤ 0.05 considered significant and positive T-statistic values when comparing groups, signifying higher average signal intensity. ANOVA analysis identified enriched regions that were differentially methylated between groups and the MAT algorithm assessed degree of methylation as quantified by the MAT scores.

To identify the methylated regions, enriched regions were annotated against human genome 19 transcripts (Affymetrix U133_Plus_2_na24.hg19.-transcript.csv file). Hypermethylation of promoter CpG island regions in HBV cases compared to normal-controls, AIH, HCC and control-HCC samples was analysed. To determine whether methylation was associated with any known risk factors for chronic liver disease, analysis by HBeAg status, age above 40 years, presence of inflammation and cirrhosis was also performed.

This report focuses on significantly methylated sites in the core promoter region within 100bp upstream of the transcription start site, with high MAT scores and cut-off P-values ≤ 0.05. Methylation changes at these sites were expected to significantly affect gene transcription and therefore initiate potentially disease causing cellular processes. We report only on the upstream methylation findings.

Pathway analysis

Pathway analysis using gene ontology terms was used to determine the biological significance of identified genes. Genes were tested using Fisher's exact test, analysis being restricted to pathways with more than 2 genes, with high enrichment scores and P<0.001.

Microarray methylation data validation with bisulfite DNA sequencing

The methylation profiles identified on microarray analysis were validated using bisulfite DNA sequencing. Based on the functional groups of identified genes, 11 promoter CpG island regions were chosen for the validation work. The genes were selected due to the high methylation rates of their promoter regions in HBV cases when compared to normal-control, AIH or HCC cases on microarray data analysis. Bisulfite DNA conversion was done using the methods developed by Frommer and Clarke, recently modified by Zhang. [13-15]The primers used to target the desired regions were designed with Applied Biosystems Methyl Primer Express Software® v1.0 (Catalogue #4376041; Applied Biosystems, USA) using the algorithm. The PCR products were subcloned into a pGEM®-T Easy vector (Catalogue #A1380; Promega, USA) which has a single 3'-terminal thymidine that overhangs at both ends of the cloning site, ensuring highly efficient insertions. A minimum of 15 clones was sequenced for each gene promoter region.

Statistical analysis of bisulfite DNA sequencing data

The sequencing results were extracted in FASTA format and analysed using the biological sequence alignment editor, BioEdit. [16] This created a ClustalW multiple alignment of the gene promoter region sequence downloaded from Ensembl human genome 19 assembly [11] and the bisulfite converted sequence of positive clones. Patients' demographic and clinical variables were analysed using STATA 13 (Texas, USA). Categorical binary data were summarised as counts and percentages, ordinal numerical data as median and inter-quantile values, and continuous numerical data as means and standard deviations. The investigation of possible associations between independent categorical variables was performed using the Pearson Chi-Squared test and Fisher's exact test where appropriate. A two-sided P-value of < 0.05 was considered significant.

RESULTS

Principal component analysis

Sources of variation in the microarray data were investigated. From the principal component analysis (PCA), technical biological batch effects were found to be a source of variation. This was addressed using background correction and removal of batch effects through robust multichip averaging quantile normalization, resulting in a clustering pattern with easier identification and analysis of samples.

Clinical and pathological characteristics of microarray cohort

The microarray study included 32 individuals; mean age of 36.8 years, 62.5% male, 40.6% cirrhotic and 62.5% were HBV infected (Table I). The age at diagnosis was not significantly different. The prevalence of cirrhosis was similar and 29.4% of the HBV patients were HBV e antigen (HBVeAg) positive. Patients with autoimmune hepatitis (AIH) had significantly higher serum alanine aminotransferase (ALT) levels (P=0.03).

Methylation profiles of promoter CpG islands in HBV infection

Genome-wide methylation profiles of patients with HBV infection compared to normal controls, AIH and HCC patients was analysed using Human Promoter 1.0R array. Figure 2 shows hypermethylation profiles of gene promoter regions by chromosomes.

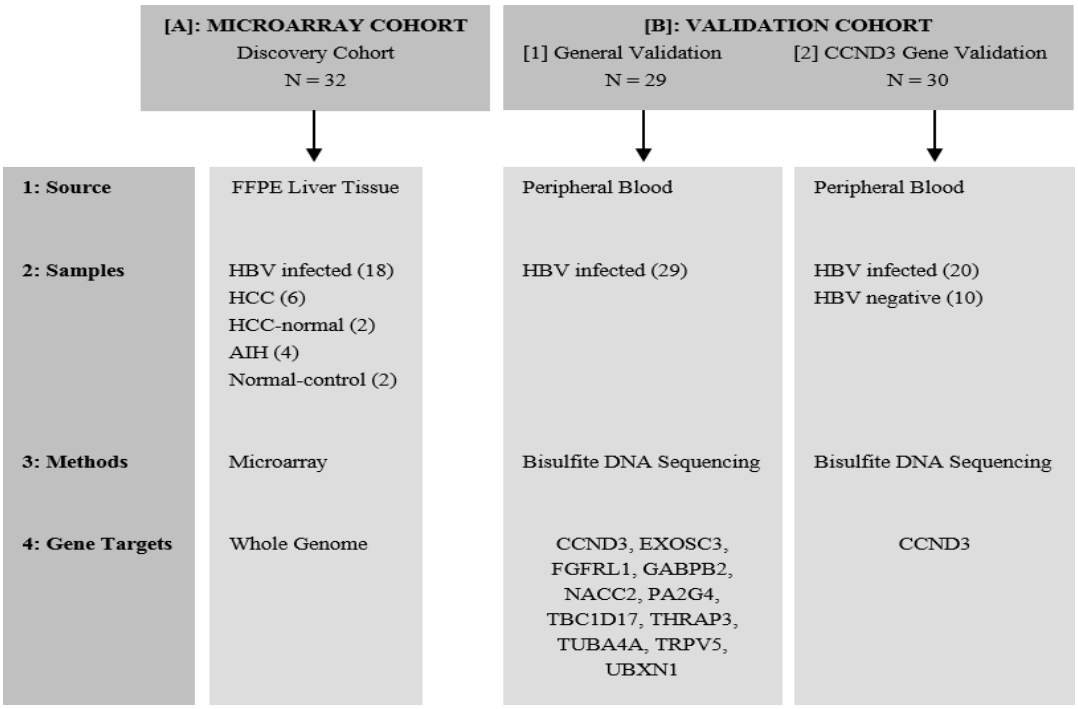

Figure 1: Outline of cohorts used in the microarray and bisulfite DNA sequencing validation studies. Twenty-nine DNA samples extracted from the blood samples of HBV patients were used to validate for the presence of CpG island methylation in 11 gene promoter regions identified through microarray analysis. Thirty DNA samples extracted from 20 HBV-infected patients and 10 HBV-negative individuals were used to determine methylation of *CCND3* gene promoter region.

Abbreviations: CCND3 - cyclin D3, EXOSC3 - exosome component 3, FFPE - formalin fixed paraffin embedded, FRFRL1 - fibroblast growth factor receptor-like 1, GABPB2 - GA binding protein transcription factor beta subunit 2, NACC2 - NACC family member 2, BEN and BTP (POZ) domain containing, PA2G4 - proliferation associated 2 G4, TBC1D17 - TBC1 domain family member 17, THRAP3 - thyroid receptor associated protein 3, TUBA4A - tubulin alpha 4A, TRPV5 - transient receptor potential cation channel, subfamily V member 5, UBXN1 - UBX domain protein 1.

Table I: Clinical and pathological characteristics of the microarray study cohort

	All Samples	**Normal-Controls**	**HBV**	**AIH**	**HCC**	**Control-HCC**	**P-value**
	N = 32	N = 2	N = 17	N = 4	N = 7	N = 2	
Mean (SD)							
Age in years	36.8 (15.4)	*15; 56	34.2 (13.1)	37.5 (9.9)	43.5 (19.9)	*48; 67	N/S
Median (IQR)							
ALT (0 - 40 u/L)	115.5 (30.5 - 202.5)	*162; 20	96 (30 - 217)	331.5 (212 - 377.5)	55 (26 - 124)	*12; 134	0.03^{K}
αFP (0 - 7 u/L)	2.6 (1.7 - 3.45)	*2; 1	3 (2.2 – 3.5)	2.6 (1.7 - 3.5)	3 (1 - 148508)	*300000; 2.6	N/A
Log_{10} viral DNA	3.7 (1-5.75)**	N/A	3.8 (2.3 - 6)	N/A	0	N/A	N/A
Number (%)							
Male	20 (62.5)	1 (50%)	12 (70.6)	0	5 (71.4)	2(100)	0.02^{P}
Inflammation (HAI≥7/18)	7 (21.9)	0	4 (23.5)	3 (75)	0	0	0.05^{H}
Cirrhosis (HAI ≥ 5/6)	13 (40.6)	N/A	8 (47.1)	2 (50)	2 (28.6)	2 (50)	N/S
HBV infected	20 (62.5)	0	17 (100)	0	2 (28.6)	1 (50%)	N/A
HBeAg positive**	5 (25)	N/A	5 (29.4)	N/A	0	0	N/A

Abbreviations: αFP - alpha fetoprotein; AIH - autoimmune hepatitis; ALT - alanine aminotransferase; DNA - deoxyribonucleic acid; HAI - histology activity index score; HBeAg - hepatitis B virus e antigen; HBV - hepatitis B virus; HCC - hepatocellular carcinoma; IQR - inter-quantile range; N– number; N/A - not applicable; N/S - not significant; SD – standard deviation; u/L - international unit per litre; % - percentage; * Raw data presented as N = 2; ** = HBV infected cases only; P = 0.03^{k} - AIH compared to HCC; P = 0.02^{P}- AIH compared to HBV; P = 0.05^{H} - AIH compared to all other

All gene promoters hypermethylated in HBV-infection group compared to normal controls (Figure 2A). Hypermethylation occurred in all chromosomes with peaks in chromosomes 2, 3, 5, 9, 11, 17, 19 and X (>3 genes per chromosome). Further analysis was restricted to genes with more than 5 promoter CpG island regions significantly hypermethylated within 100bp upstream of transcription start site in HBV cases compared to each study group. HBV samples were highly methylated in chromosome 9 compared to normal controls (Figure 2B), and in chromosomes 16 and 17 compared to HCC (Figure 2F).

Statistically significant (P<0.001) hypermethylated profiles of 31 promoter CpG islands located within a wide variety of genes were observed when HBV infection group was compared to normal controls, AIH and HCC groups (Table II). Hypermethylation profiles of the identified gene promoter regions across the compared groups covers mostly chromosome 9, 11 and 17 (Table II).

In order to determine the hypermethylation profiles associated with the risk factors such HBeAg positive status, age above 40 years, cirrhosis and hepatic inflammation at liver biopsy, we further conducted an

analysis between these risk factors and normal controls. About 29 additional hypermethylated promoter CpG islands of various gene promoter regions located mostly in chromosomes 3, 11 and 19 with statistical significance of P<0.001 were revealed (Table III).

Table IV illustrates a wide spectrum of hypermethylated gene promoter regions selected from different groups illustrated previously (Tables II and III) when HBV infection group was compared to normal controls and other liver disease groups including AIH and HCC. These genes were selected based on their biological functions, which when altered via HBV-induced hypermethylation may lead to severe HBV-related disease. The gene functional groups included cyclin dependent kinases, growth factors, regulators of hepatic metabolism, tumour suppressors, transcription regulators, splicing of mRNA, vitamin D receptor binding, ubiquitination and pseudogenes (Table IV).

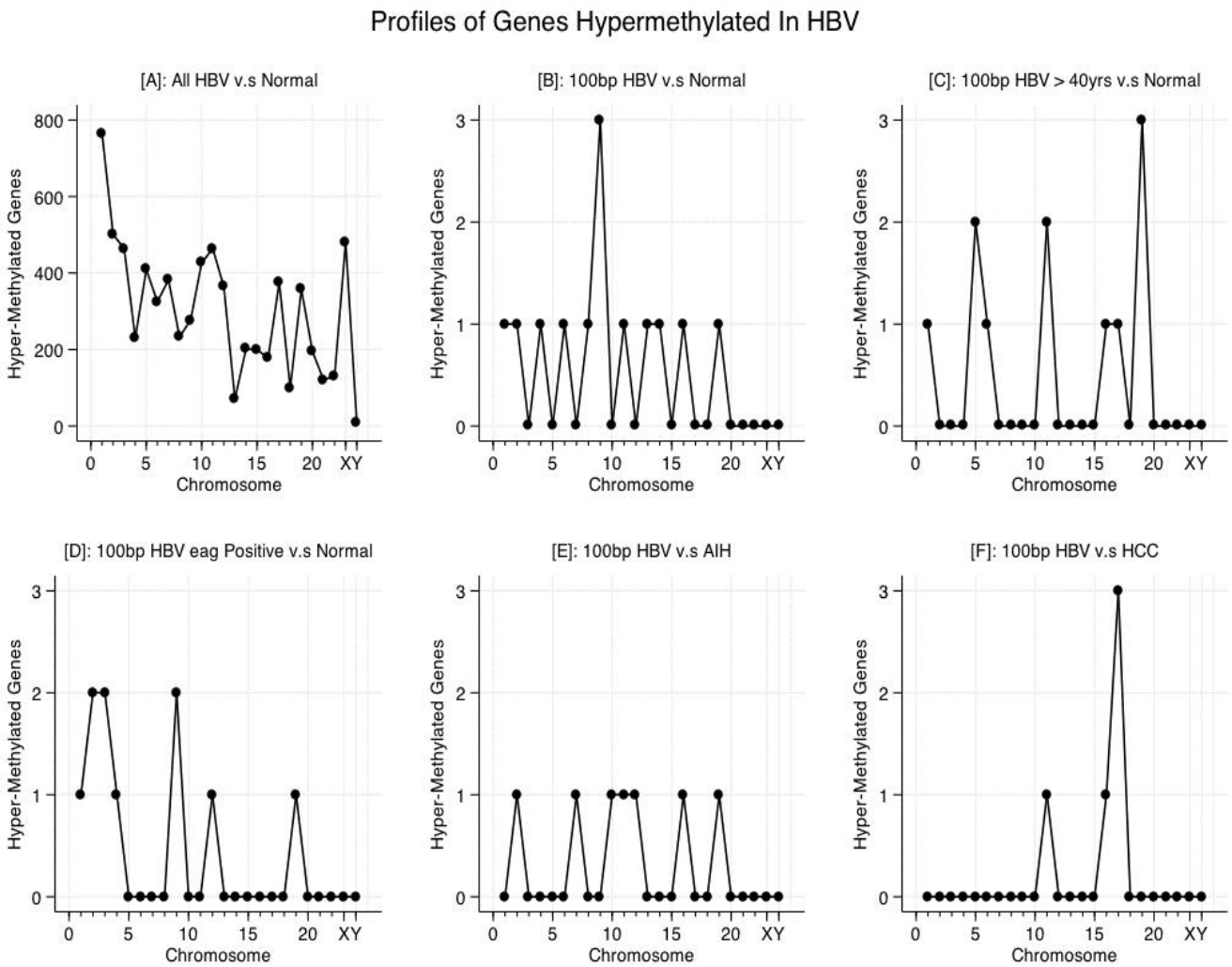

Figure 2: Profile by chromosome of hypermethylated gene promoter regions within 100bp of TSS. The promoter region hypermethylation within the chromosomes was obtained when HBV patients were compared to control study groups: All HBV vs. Normal, 100bp HBV vs. Normal, 100bp HBV > 40 years vs. Normal, 100bp HBeAg Positive vs. Normal, 100bp HBV vs. AIH, and 100bp HBV vs. HCC. **Abbreviations**: AIH - autoimmune hepatitis, bp- base pair, HBV - hepatitis B virus, HBeAg -hepatitis B virus e antigen, HCC - hepatocellular carcinoma, TSS - transcription start site.

Table II: Hypermethylation profiles of gene promoter CpG islands in HBV patients compared to normal controls and other liver disease groups

Gene Title	Gene Name	Transcript ID	Cytoband	MAT Score	Distance to TSS	P-Value
Hypermethylated in Hepatitis B Virus Compared to Normal-Controls						
LNX2	Ligand of numb-protein X 2	NM_153371	13q12.2	2.2629	-21	0.0074
NAIF1	Nuclear apoptosis inducing factor 1	NM_197956	9q34.11	2.2016	-26	0.0096
GABPB2	GA binding protein transcription factor beta subunit 2	NM_144618	1q21.3	2.3022	-57	0.0062
PTGR2	Prostaglandin reductase 2	NM_152444	14q24.3	2.2185	-60	0.0089
TUBA4A	Tubulin alpha 4a	NM_024463	2q35	2.1953	-60	0.0099
EXOSC3	Exosome component 3	NM_016042	9p13.2	2.2505	-84	0.0078
IL11RA	Interleukin 11 receptor, alpha	NM_001142784	9p13.3	2.4618	-86	0.0029
CCND3	Cyclin D3	NM_001136126	6p21.1	2.2651	-100	0.0073
Hypermethylated in Hepatitis B Virus Compared to Autoimmune Hepatitis						
PTOV1	Prostate tumour overexpressed gene 1	NM_017432	19q13.33	3.2297	-41	0.0014
TRIP12	Thyroid hormone receptor interactor 12	NM_004238	2q36.3	3.0035	-54	0.0034

TRPV5	Transient receptor potential cation channel, subfamily V, member 5	NM_019841	7q34	2.9971	-67	0.0035
Hypermethylated in Hepatitis B Virus Compared to HCC						
FAM106C	Family with sequence similarity 106, member C	NR_026810	17p11.2	3.0209	-11	0.0066
PRSS41	Protease serine 41	NM_001135086	16p13.3	4.3179	-17	0.0002
CCDC144B	Coiled-coil domain containing 144B (pseudogene)	NR_036647	17p11.2	3.2691	-35	0.0033
KRT42P	Keratin 42 pseudogene	NM_033415	17q21.2	3.2956	-43	0.0031
Hypermethylated in Hepatitis B Virus Compared to Control-HCC						
RP1	Retinitis pigmentosa 1 (autosomal dominant)	NM_006269	8q12.1	3.0040	-21	0.0033
ALAS1	Aminolevulinate, delta-, synthase 1	NM_000688	3p21.2	2.7556	-26	0.0078
KRBA2	KRAB-A domain containing 2	NM_213597	17p13.1	2.7537	-30	0.0078
PATE3	Prostate and testis expressed 3	NM_001129883	11q24.2	3.1863	-44	0.0017
Abbreviations: HCC - hepatocellular carcinoma; ID - identity number of gene transcript; MAT - model based analysis of tilling array; TSS - transcription start site						

Table III: Hypermethylation profiles of gene promoter CpG islands in HBV patients at various stages of disease compared to normal controls

Gene Title	Gene Name	Transcript ID	Cytoband	MAT Score	Distance to TSS	P-Value
Hypermethylated in HBV e antigen positive patients compared to normal controls						
PCGF1	Polycomb group ring finger 1	NM_032673	2p13.1	2.1596	-42	0.0079
PA2G4	Proliferation associated 2G4	NM_006191	12q13.2	2.1286	-53	0.0089
FGFRL1	Fibroblast growth factor receptor-like 1	NM_001004358	4p16.3	2.3055	-55	0.0044
TUBA4A	Tubulin alpha 4A	NM_006000	2q35	2.1518	-96	0.0082
Hypermethylated in HBV patients with hepatic inflammation compared to normal controls						
NACC2	NACC family member 2, BEN and BTP (POZ) domain containing	NM_144653	3q34.3	2.7026	-25	0.0026
MIR527	MicroRNA-527	NR_030219	19q13.42	2.4586	-28	0.0064
NAIF1	Nuclear apoptosis inducing factor 1	NM_197956	9q34.11	2.363	-84	0.0093
Hypermethylated in HBV patients with cirrhosis compared to normal controls						
THRAP3	Thyroid hormone receptor associated protein 3	NM_005119	1p34.3	2.2761	-12	0.0079
UBXN1	UBX domain protein 1	NM_015853	11q12.3	2.3446	-28	0.0061
TUBA4A	Tubulin alpha 4a	NM_006000	2q35	2.2554	-60	0.0088
NAIF1	Nuclear apoptosis inducing factor 1	NM_197956	9q34.11	2.2422	-84	0.0094
Hypermethylated in HBV patients with cirrhosis compared to HBV patients without cirrhosis						
NPM2	Nucleophosmin/Nucleoplasmin 2	NM_182795	8p21.3	2.8722	-1	0.0029
SNORD48	Small nucleolar RNA C/D Box 48	NR_002745	6p21.33	3.1421	-15	0.0013
SCARNA10	Small Cajal body-specific RNA 10	NR_004387	12p13.31	3.1802	-21	0.0011
LAMA1	Laminin alpha 1	NM_005559	18p11.23	2.7098	-40	0.0052
Hypermethylated in HBV patients > 40 years compared to normal controls						
TBC1D17	TBC1 domain family, member 17	NM_024682	19q13.41	2.4194	-20	0.0079
MIR527	MicroRNA 527	NR_030219	19q13.42	2.6489	-28	0.0032
FANCE	Fanconi anaemia complementation group E	NM_021922	6p21.31	2.4768	-30	0.0032
PARD6A	Par-6 partitioning defective 6 homolog alpha	NM_016948	16q22.1	2.3921	-91	0.0088
SNORA52	Small nucleolar RNA, H/ACA Box 52	NR_002585	11p15.5	3.1180	-92	0.0004
Abbreviations: HBV - hepatitis B virus;ID - identity number of gene transcript; MAT - model based analysis of tilling array; TSS - transcription start site						

Table IV: Summary of genes with promoter region CpG island hypermethylation and their possible clinical significance in chronic HBV infection

Genes	Physiological Functions	Known Related Disease
A. Cyclin-dependent kinase		
CCND3	Liver regeneration; Wound healing process	Liver fibrosis; HCC suppression
PARD6A	Protein kinase C binding; Asymmetrical cell division	Defects in *Caenorhabditiselegans*
B. Growth factors		
FGFRL1	Negatively regulate cell growth; Tissue regeneration	Liver fibrosis; HCC
PARD6A	Transforming growth factor β receptor signalling pathway	Defects in *Caenorhabditiselegans*
C. Metabolism regulators		
PTGR2	Prostaglandin metabolism; Immune function	Autoimmune disease; Type 2 diabetes mellitus
TRPV5	Calcium and phosphorus metabolism	Rickets; Osteoporosis; Hyperparathyroidism
D. Tumour suppressors		
GABPB2	Cell adhesion; Immune function	Unknown
NAIF1	Induction of cell apoptosis	Gastric cancer
NACC2	Negatively regulates apoptosis	Unknown
PARD6A	Cell cycle arrest, proliferation and apoptosis	Metastatic carcinoma
PCGF1	Cell cycle progression and proliferation	B-cell lymphoma
PA2G4	Cell proliferation; Cell cycle arrest	Cancer
PARD6A	Cell cycle arrest, proliferation and apoptosis	Metastatic carcinoma
PATE3	Induces cell cycle arrest and apoptosis	Glioma cells
PCGF1	Cell cycle progression and proliferation	B-cell lymphoma
PTOV1	Enhances cell Proliferation	Prostate and bladder malignancies

PTGR2	Cell proliferation and apoptosis	Unknown
TBC1D17	Inhibits cell transformation and proliferation	Unknown
TUBA4A	Regulates cell adhesion	Unknown
E. Transcription regulators		
EXOSC3	Ribosomal transcription	Unknown
GABPB2	Initiates transcription via RNA polymerase 1	Gastric cancer; inflammation
PTOV1	DNA Replication, transcription	Prostate and bladder cancers
PA2G4	Negative regulation of transcription	Cancer
PCGF1	DNA transcription regulator	B-cell lymphoma
RRNP3	Initiates transcription via RNA polymerase II	Unknown
F. Regulation of RNA splicing		
EXOSC3	RNA and mRNA metabolic process	Unknown
SNORD48	snoRNA processing and binding	Cancer
THRAP3	Regulates mRNA processing	Hyperthyroidism; Hypercalcaemia
G. Genes encoding ubiquitin proteins		
UBXN1	E3 ubiquitin ligase activity	Breast cancer
LNX2	Histone H2A ubiquitination	Unknown
PA2G4	E3 ubiquitin ligase activity	Unknown
TRIP12	Histone H2A ubiquitination	Unknown
H. Vitamin D -related genes		
THRAP3	Vitamin D receptor binding	Hyperthyroidism; Hypercalcaemia
TRPV5	Transport vitamin D-induced calcium ion	Hyperparathyroidism
I. Pseudogenes		
RRNP3	Ribosomal transcription	Colorectal cancer
J. Genes that interact with HIV proteins		
THRAP3	Interact with HIV-1 Protease and Gag Proteins	HIV-1
TUBA4A	Interact with HIV-1 Tat and Rev Proteins	HIV-1

Microarray methylation data validation with bisulfite

DNA sequencing

Bisulfite DNA sequencing was done on HBV infected samples using 11 gene promoter regions chosen from the identified functional groups. The patients were young with normal ALT levels and median $\log_{10}$ HBV DNA of 3.4 (Table VA). In the samples analysed, the sequences that were consistently successfully cloned, sequenced and aligned with the reference gene sequence were in the genes: *CCND3*, *EXOSC3*, *FGFRL1*, *NACC2*, *THRAP3* and *TUBA4A*.

The most hypermethylated, in the regions analysed, were *CCND3* (100%), *TUBA4A* (18%) and *NACC2* (5.6%) (Table VA.1) while cloning of *TBC1D17*, *TRPV5* and *UBXN1* gene promoter regions was not successful in the samples studied (Table VA.2).

ClustalW multiple sequence alignment comparing Ensembl human genome 19 assembly gene promoter sequences and DNA sequences amplified from positive colonies demonstrated the presence of hypermethylation, confirming the microarray methylation data findings. Hypermethylation in *CCND3* and *TUBA4A* gene promoter regions is shown in Figure 3. The highly methylated *CCND3* gene region was further validated in HBV patients and normal controls (Table VB). Only HBV samples were hypermethylated, particularly in HBeAg positive (P=0.001) and HBV genotype D patients (P=0.005) who also had significantly higher HBV DNA levels (P=0.006) but with similar age, alanine aminotransferase and albumin levels (Table VB.2)

Table V:Clinical and gene promoter CpG island methylation profiles of cohorts used for bisulfite DNA sequencing validation work

A: GENERAL VALIDATION STUDY COHORT							
	Age in years	ALT u/L	Log_{10} HBV DNA	HBV eAg$^+$			
	Median (IQR)	Median (IQR)	Median (IQR)	N (%)			
All Cases (N = 11)	33 (26-33)	23 (21 - 33)	3.4 (2.6 - 8.3)	5 (45.5)			
1. Genes Successfully Cloned and Aligned (Cytosine Methylation Percentage Present):							
CCND3 (100)	TUBA4A (18)	NACC2 (5.6)	THRAP3 (4.8)				
FGFRL1 (3.1)	EXOSC3 (0.2)	GABPB2 (0)	PA2G4 (0)				
2. Failed Cloning:							
TBC1D17	TRPV5	UBXN1					

B: *CCND3* GENE VALIDATION STUDY COHORT							
1. Experimental Data:	Whole Cohort N = 30	HBeAg$^-$ N = 10	HBeAg$^+$ N = 10	Controls N = 10			
Number (%) Bisulfite Conversion	30 (100)	10 (100)	10 (100)	10 (100)			
Cloned in Vector	18 (60)	8 (80)	7 (70)	3 (30)			
Successful Sequencing	17 (56.7)	7 (70)	7 (70)	3 (30)			
2. HBV Infected Cohort:	Whole Cohort N = 14	HBeAg$^-$ N = 7	HBeAg$^+$ N = 7	P Value	Genotype A N = 8	Genotype D N = 6	P Value
Male Gender [N, (%)]	11 (78.6)	5 (71.5)	6 (85.7)	NS	6 (75)	5 (83)	0.6
Median (IQR)Age (years)	22.3 (18 - 45)	21.1 (18 - 45)	24.7 (18 - 45)	0.7	20.3 (18.5 - 34)	25.3 (23 - 45)	0.3
HBV DNA Log_{10}	8.2 (5.2 - 8.3)	5.2 (3.9 - 16.9)	8.3 (8.2 - 8.5)	0.02	5.2 (4.6 - 5.2)	8.3 (8.2 - 8.5)	0.006
Methylation Percentage	50 (40 - 80)	40 (20 - 40)	80 (80 - 80)	0.001	40 (30 - 80)	80 (80 - 80)	0.005
αFP (u/L)	2.5 (2.1 - 3.3)	2.5 (1.3 - 2.8)	3 (2.4 - 43.6)	0.2	2.5 (1.7 - 3.1)	2.7 (2.4 - 3.5)	0.7
Albumin (35 - 50g/L)	44.5 (42 - 46)	45 (40 - 46)	42 (44 - 49)	0.7	44.5 (11 - 45.5)	45 (43 - 49)	0.4

ALT (0 - 40u/L)	42.5 (27 - 108)	32 (21 - 45)	67 (37 - 271)	0.09	36 (24 - 146.5)	60.5 (37 - 108)	0.4
INR (<1.2)	1.02 (0.9 - 1.1)	0.96 (0.9 - 1.1)	1.1 (1 - 1.35)	0.09	0.98 (0.92 - 1.1)	1.19 (1 - 1.4)	0.07
Platelets (150-400 x10^9/L)	239 (212 - 254)	237 (212 - 244)	254 (204 - 40)	0.6	240 (219 - 303)	244 (204 - 254)	0.8
Abbreviations: % - percentage;, αFP - alpha fetoprotein; ALT - alanine aminotransferase; INR - international normalised ratio; IQR - inter quantile range; HBeAg$^-$ - HBV e antigen negative; HBeAg $^+$- HBV e antigen positive							

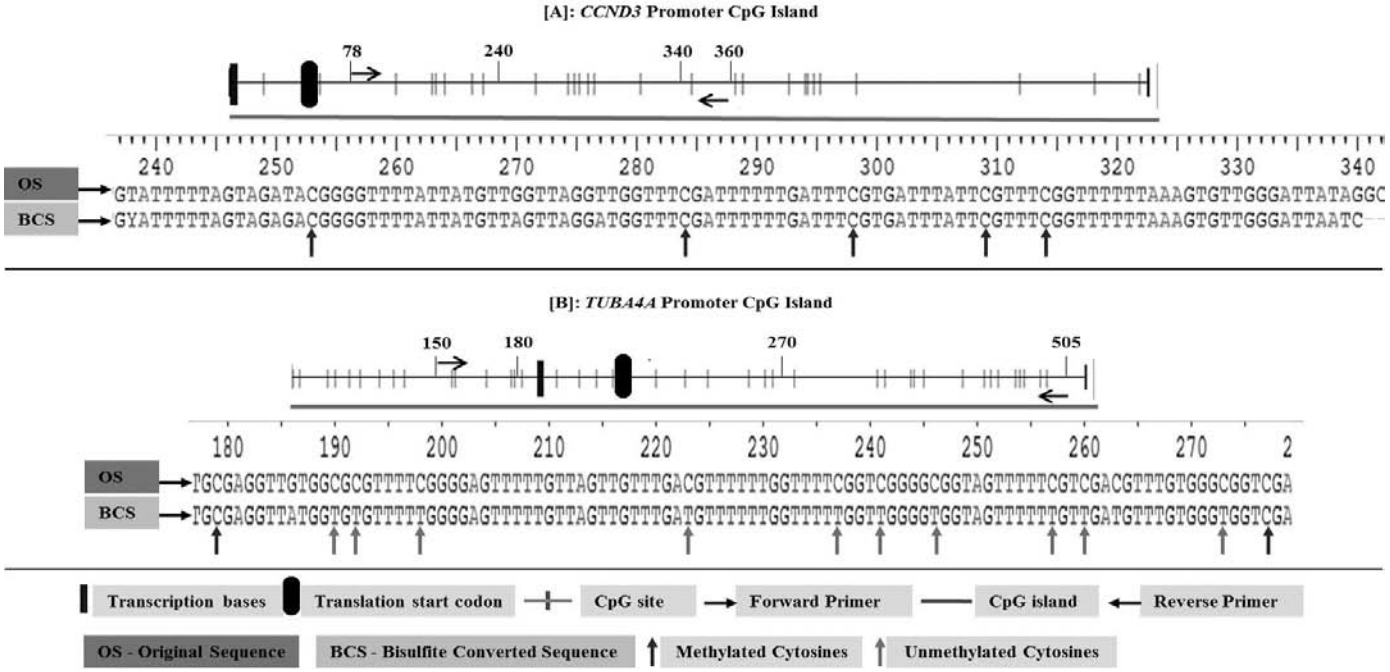

Figure 3:Methylation profiles of selected sections of bisulfite-DNA sequenced clones of gene promoter CpG island regions. (**A)***CCND3* and [**B**] *TUBA4A* gene promoter CpG island regions. After the bisulfite DNA conversion, the products were PCR amplified using primers specific to the target region. The PCR products were gel-purified and cloned into the pGEM®-T Easy vector. Clones with vector plus insert were PCR amplified and sequenced with M13 reverse primer. Methylation profile analysis was performed using trace file viewer like Chromas and sequence alignment of original and bisulfite converted sequences was done with ClustalW multiple alignment in BioEdit Sequence Alignment Editor. After bisulfite conversion, methylated cytosines remain unaffected and unmethylated cytosines were converted to uracils that appear as thymines in the DNA sequence. The ClustalW multiple alignment of *CCND3* gene promoter CpG island (chromatograph not shown) has no conversion of any cytosine to thymine, indicating complete methylation of the sequence shown. For *TUBA4A* gene promoter region, only 2 cytosines are methylated.

DISCUSSION

Genome-wide analysis using Human Promoter 1.0R array revealed HBV-related hypermethylation affecting more than 50 various gene promoter regions in patients with chronic HBV infection (see Tables II and III). Identified hypermethylation profiles covers gene promoter regions that are located mostly in chromosomes 2, 3, 5, 9, 11, 17 and 19, and this may be explained by an increased quantity of tumour suppressors and oncogenes associated with these chromosomes, which are prone to high methylation rate. [17] The identified gene promoter regions encode genes that are linked to cellular signalling pathways activated via transcription factors such as transforming growth factor beta (TGFβ), nuclear factor-kappa B and mitogen-activated protein kinases (MAPK) whose targets include inflammation and fibrosis related genes. [18, 19] The HBV-induced hypermethylation changes may potentially alter gene transcription and disrupt pathways involved in liver regeneration, causing inflammation, cirrhosis and cancer. [18-21]

The *FGFRL1* and *PARD6A* gene promoter regions were hypermethylated in HBeAg positive and 40 years older patients, respectively. Hypermethylation of these gene promoter regions may alter normal gene expression. This may contribute to the progression of HBV related liver disease by disrupting cell proliferation, polarity and migration through activation of TGFβ and Rho family GTP hydrolases, which modulate the availability of catenins and other essential adherens proteins necessary for creating cell-cell junctions needed during morphogenesis. The PARD6-atypical protein kinase complex is essential for epithelial morphogenesis, asymmetric cell division, and directed cell migration. This has been shown in breast cancer and other tissues where loss of normal function alters cell architecture, tissue remodelling and facilitates malignant transformation. [21,22] Altered gene function could increase the fibrogenic actions of TGFβ on hepatic stellate cells. [19,20] TGFβ also activates the MAPK pathway and Smad proteins, which regulate epithelial-to-mesenchymal cell transition, tumour invasion and metastasis, suggesting that these genes may contribute to HBV related HCC. [18-22]

Vitamin D receptor regulated gene promoter regions *TRPV5* and *THRAP3* were hypermethylated. *TRPV5* geneencodes for epithelial calcium selective channels that maintain the balance of extracellular calcium while *THRAP3* is a potent transactivator. [23] Methylation of these gene promoter regions would affect the physiology of vitamin D and its receptor, thereby contributing to abnormal cellular calcium signalling and the deleterious effect of HBx protein induced store operated calcium on hepatocyte proliferation and apoptosis. HBx protein alters cytosolic calcium signalling thereby changing the intracellular environment to favour viral propagation, capsid assembly and cell survival. [23,24] Store operated calcium stimulates viral replication by inhibiting antiviral immunity, and deregulating protein tyrosine kinase 2 and proto-oncogene tyrosine-protein kinase signalling pathways. This

deregulates apoptosis and cell proliferation, contributing to HBV pathogenesis and tumorigenesis. [23-25]

We identified hypermethylation of gene promoter regions encoding ubiquitin-related proteins LNX2 and TRIP12 that may be linked to liver disease through ubiquitination and protein degradation pathways activated by HBx protein via p53, Wnt and Notch signalling pathways. [26,27] Disruption of ubiquitination and proteosomal degradation pathways by HBx protein may be used for HBV evasion of host immune defences as reported in another oncogenic virus, Epstein-Barr virus (EBV). [28] Altering the proteosomal activity of infected cells facilitates viral entry, replication and survival thereby contributing to the risk of malignant transformation in hepatocytes. Methylation of ubiquitin-related genes is observed in malignancies including HCC and is associated with abnormal protein expression and function. [26,27] They are potential tumour markers for the early detection of malignancies.

Several tumour suppressor genes including *GABPB2*, *NACC2*, *NAIF1*, *PA2G4*, *PARD6A*, *PATE 3*, *PCGF1*, *PTOV1*, *PTGR2*, *TUBA4A* and *TBC1D17* were hypermethylated. These genes can be linked to liver disease through the activation of cAMP response element binding, Wnt/β-catenin, MAPK and mammalian target of rapamycin cellular signalling pathways [28]. These pathways, which are important regulators of cell growth and proliferation, are key HBxprotein targets. [27-30] HBV-induced hypermethylation of these genes could suppress transcription of genes critical for preventing tumorigenesis. *NACC2* and *NAIF1* genes repress the transcription of oncogenes, induce apoptosis and negatively regulate cell growth respectively. [31,32]Hypermethylation may aberrantly activate oncogenes, inhibit apoptosis and if combined with the transactivation properties of HBx protein, could cause hepatic inflammation, fibrosis and HCC. [33-35]

Hypermethylation of *TUBA4A, THRAP3* and *CCND3* gene promoters may help explain the more aggressive nature of liver disease observed in HBV and HIV-1 co-infection. *TUBA4A* gene is one of the alpha tubulin genes that form the major constituent of eukaryotic cytoskeleton microtubules and are important in cell division and movement. It has been shown that HIV-1transactivator of transcription (Tat) protein alters microtubule dynamics by interacting with tubulin alpha/beta dimers through a four-amino-acid subdomain of its conserved core region. In return, this enhances tubulin polymerisation leading to the activation of a mitochondria-dependent apoptotic pathway. [36-38] Apoptosis contributes to the massive depletion of $CD4^+$ T cells, which is the hallmark of HBV-HIV coinfection and subsequent progression to cirrhosis and liver cancer. Depletion of $CD4^+$ T cells in HBV-HIV coinfection has been suggested to be partly due to the transcriptional activation of Tat. [38,39] Given these evidences, we hypothesise that HBV-induced hypermethylation of *TUBA4A* promoter region may alter normal gene expression in such a way that it interferes with the function of Tat especially, contributing to aggressive progression and ultimately severe liver disease that have been observed in HBV-HIV co-infected patients.[40-42]

THRAP3 protein binds to HIV integrase and interacts with helicase with zinc finger 2 protein to transcriptionally activate peroxisome proliferator activated receptor gamma (*PPARγ*) gene. [24,43,44] Disruption of *PPARγ*gene function, a key regulator of adipogenesis, glucose metabolism and insulin resistance, is central in the pathogenesis of HIV lipodystrophy and hepatic steatosis. [44] HBx protein induces hepatic steatosis through transcriptional activation of *PPARγ* gene, which predisposes hepatocytes to inflammation, fibrosis and cancer. [46] The combined effects of *THRAP3* gene promoter region hypermethylation and HBx protein may increase the risk of hepatic steatosis in HBV-HIV infection. HIV-1 Tat protein is a soluble neurotoxin that has been shown to interact with *CCND3* and other cell cycle proteins to inhibit cell growth and proliferation, contributing to the pathogenesis of HIV-induced neuropathies. [39]

High HBV viral load was associated with hypermethylation of *CCND3* and *PA2G4* gene promoter regions. These genes can be linked to liver disease through their ability to stimulate inflammation and fibrosis through MAPK/phosphatidylinositol 3 kinase signalling pathways. [46] MAPK induces the transcription of cyclins with the help of platelet-derived growth factor, which induces proliferation and fibrosis. Cirrhotic livers, HCC occurring in cirrhotic livers and breast cancer tissues have high *CCND3* gene expression, implying a significant role in cancer development. [46,47]

Bisulfite DNA sequencing analysis of *CCND3*, *EXOSC3*, *FGFRL1*, *NACC2*, *TUBA4A* and *THRAP3* gene promoters demonstrated hypermethylation in association with HBV infection, validating the microarray data. The *CCND3* gene promoter region was highly methylated and in this study, was associated with HBeAg positivity, high HBV viral load and genotype D. Several studies show that high viral loads and HBeAg positive status are associated with higher risk of HCC. [48] This may relate to the presence of more hypermethylation causing extensive gene promoter disruption. HBeAg related hypermethylation was prominent in chromosomes 2, 3 and 9, chromosomes with large amounts of oncogenes and tumour suppressor genes. [32,48,17] The role of the HBV genotype and antigen status in possibly influencing the sites and degree of DNA methylation in the human genome requires further study. In a cohort study from birth to age of 3 years, global DNA methylation quantification was shown to increase over time. [49] Cumulative DNA methylation is affected by various factors including heritable familial DNA methylation marks, birth weight, medication use, smoking and viral infections such as HBV that are known to trigger DNA methylation. [49-51] It could be argued that ideal comparisons could have been controls matched for age and some of the factors known to contribute to age-associated cumulative DNA methylation. Studies that specifically account for methylation associated with age and viral infections such as HBV, EBV and human papilloma virus are needed.

In this study, finding ideal “normal” tissue for control purposes was difficult. Although elevated ALT levels usually predict hepatic injury, it is important to note that it may also be elevated in other conditions such as thyroid disorders, celiac disease, and muscle disorders. [52,53] Notwithstanding abnormal elevated levels of ALT, liver biopsy tissues obtained from histopathologically normal liver tissues of patients used in this study were assessed normal as they had no evidence of chronic liver disease. Therefore, such normal controls served as ideal

"normal" controls to distinguish DNA methylation profiling triggered by liver related from non-liver related diseases. Liver biopsy is an invasive procedure, and is only performed when there is hepatic damage as predicted by abnormal elevated levels of liver enzymes to confirm the diagnosis and to determine the amount of damage. [54] Due to this limitation, the sample size for normal controls was also too small, and may have compromised the statistical analysis when HBV-infection group was compared to this control group. Although these limitations were counter-balanced with disease controls of AIH and non HBV-related HCC patients who had both biochemical and histopathological hepatitis, further study with adequate sample size is still required to confirm the results.

Peripheral blood derived DNA has been shown to accurately reflect tissue derived DNA microarray findings in direct tissue-blood matched comparisons. Using paired plasma and tissue DNA samples from the same patients with HCC, the concordance between plasma and tissue DNA methylation profiles was shown to be high, independent of viral infection status with a positive predictive value of plasma DNA of more than 90% for the genes tested. [55] We believe therefore that the use of plasma DNA in the validation studies does not weaken the strength of our findings. Also, in view of the increasing difficulty of obtaining HCC tissue as minimally invasive and non-invasive diagnostic methods are now highly specific for diagnosing HCC, the use of blood derived DNA will become the most feasible way of studying patients with proven or suspected HCC. The aim of restricting the data analysis for this report to 100bp upstream of TSS was to critically focus on the region with the highest density of CpG islands in most human genes. These CpG islands are normally unmethylated in their basal state to allow for transcription and would therefore be most prone to methylation related to the presence of viral inserts. [56] This analysis has specifically excluded the CpG island shores, which are regions of lower CpG density and are in close proximity (~2kb) to CpG islands. They will be important to study further as they are known to be associated with tissue-specific DNA methylation. [55,56] Methylation data from these regions could be important for understanding HBV associated HCC.

Although the genome-wide studies based on HBV-related methylation have been conductedpreviouslyin other HBV genotypes[8-10], none of the studies reported similar CpG island promoter regions hypermethylation profiles identified in the current study. Distinct methylation profiles compared to previous studies may be associated with underlying genetic background or variation, disease genotype, heterogeneous population of cells in tissues, disease severity (tumour) and different detection methods. [9] Another possible reason is that previous studies reported methylation profiles across the entire promoter region within 100 to 1000 bpupstream of the transcription start site. This may have excluded some methylation profileslocated in the core promoter region (within 100 bp upstream of the transcription start site), which was the main focus of our study. We intentionally restricted our analysis only on the core promoter region as it is the key and primary regulator of gene transcription. Nonetheless, our findings provide evidence supporting the hypothesis that chronic HBV infection causes significant whole genome hypermethylation of promoter regions of diverse genes that may result in the disruption of important biological processes such as hepatocyte growth, differentiation and apoptosis. The affected gene promoter regions may affect gene transcription, activating pathways that may cause hepatic inflammation, fibrosis and HBV-related hepatocarcinogenesis.

In conclusion, our study supported our hypothesis by showing the presence of core promoter region CpG island hypermethylation in the genome of HBV infected patients. This hypermethylationaffect a spectrum of various genes whose function can be directly linked to liver disease. Since methylation of gene promoter region is associated with alteration in gene expression [57], it is highly recommended that future studies investigate theexpression status of the hypermethylated genes in the same materials. New studies that will investigate integration of HBV genome around the hypermethylated genes are also needed. Such insights will broaden our understanding of some of the gene transcriptional alterations that contribute to the development of HBV induced liver injury and cancer. In addition, this will provide opportunities for revealing new biomarkers for diagnosis, prognosis and therapeutic development.

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