RESEARCH NOTE

PRESENCE OF HEPATITIS B VIRUS IN PARAFFIN-EMBEDDED LIVER TISSUES OF PATIENTS WITH HEPATOCELLULAR CARCINOMA

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Abstract. There are limited virological data regarding hepatitis B virus in liver tissues of hepatocellular carcinoma (HCC) patients. The study examined correlation between virological data and HCC status in 76 HCC patients classified into HBV infection status, genotype, mutation, and HBV DNA and covalently closed circular DNA (cccDNA) copies/cell. Genotype C was the most prevalent (70%); deletion of *preS1* was present in 43% and novel C1673G/A and C1773T/A in 62% of region 3 mutations of HBV samples sequenced. Stage of liver cell differentiation stage, serological status of HCC patients and HBV genotype were strongly associated with *preS* deletion (p-value <0.001). Functional nature of these findings awaits further investigation.

Keywords: hepatitis B virus, hepatocellular carcinoma, liver cell differentiation stage, liver tissue, mutation

INTRODUCTION

Seropositivity for hepatitis B virus (HBV) has been found in 4% of the Thai population (Suwannakarn *et al*, 2008), and a majority of the cases progress to hepatocellular carcinoma (HCC) (Tangkijvanich *et al*, 1999). Screening of HBV patients at risk for HCC could be an appropriate approach for early detection (Wanich *et al*, 2016).

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In chronic HBV-infected patients, molecular characteristics of viral nucleotide variations and genotypes influence clinical outcome and provide valuable information to physicians monitoring patients receiving antiviral drug treatment (Rezanezhadi *et al*, 2019). However, data on HBV molecular characteristics, genotypes and role of intrahepatic covalently closed circular DNA (cccDNA) in HCC are limited (Datta *et al*, 2014). The study was to provide such information in a small cohort of HCC patients in Thailand. This information will increase knowledge of HCC progression and improve patient treatment in the future.

MATERIALS AND METHODS

Study samples

Paraffin-embedded liver tissues (n = 76) of HCC patients (>15 years of age) admitted to King Chulalongkorn Memorial Hospital, Bangkok, Thailand (2006 - 2012) were examined. HCC grading (well, moderately and poorly differentiated HCC) was classified by pathologists according to WHO criteria (Nagtegaal *et al*, 2020).

The research protocol was approved by the Faculty of Medicine, Chulalongkorn University Ethics Committee (no.456/54).

Serological and biochemical analyses

Serological and biochemical data were obtained from the Central Laboratory, King Chulalongkorn Memorial Hospital. All analyses were performed using automated instrument (Roche Diagnostic, Mannheim, Germany).

Molecular characterization of HBV specimens

DNA was extracted from paraffin-embedded liver tissues as previously described (Abe *et al*, 1998). In brief, paraffin-embedded tissue was incubated with xylene at 60°C for 10 minutes, liquid phase removed and pellet washed twice with 1 ml of absolute ethanol. Then, the issue pellet was dried in a desiccator (Concentrator Plus, Eppendorf, Hamburg, Germany) and DNA extracted with 500 µl aliquot of lysis buffer (10 mM Tris-HCl pH 8.0, 0.1 M ethylenediaminetetraacetic acid and 0.5% SDS) containing 1 mg proteinase K at 60°C for five hours. Lysate was subsequently extracted with phenol/chloroform/isoamyl alcohol solution and DNA precipitated by addition of a solution of isopropanol containing 20 µg/µl glycogen and 3M sodium acetate and dissolved in 30 µl of diethylpyrocarbonate (DEPC)-treated water.

Nested PCR was performed to determine the presence of HBV DNA in liver tissues as previously described (Abe *et al*, 1998). In short, first round reaction mixture consisted of 12 µl of distilled water, 10 µl of 2.5X PerfectTaq™ Plus MasterMix (5 PRIME GmbH), 0.5 µl each of 25 µM MD24 and of 25 µM MD26 primers (Table 1) and 4 µl of DNA solution. For the second round reaction, the reaction mixture was as described above except primers HBx1 and HBx2 (Table 1) and 4 µl of the first round reaction solution. In both first and second rounds of amplification, thermocycling was performed in a MasterCycler Personal PCR machine (Eppendorf, Hamburg, Germany) as follows: 94°C for 3 minutes; 40 cycles of 94°C for 30 seconds, 55°C of 30 seconds and 72°C for 90 seconds; and a final step of 72°C for 7 minutes. Amplicons from the second round reaction were separated by 2% agarose gel-electrophoresis, stained with ethidium bromide, purified from gel and directly sequenced (First BASE Lab Sdn Bhd, Selangor Darul Ehsan, Malaysia).

For determination of HBV genotype and *PreS* mutation, semi-nested PCR was performed and analyzed as described above except in the first round reaction PreS1 F+ and PreS1 R primers and in the second round reaction PreS1 F+ and PreS1 R2 primers (Table 1) were used. Nucleotide sequences of *PreS* region were edited using a Chromas LITE version 2.01 (Technelysium, Queensland, Australia) and sub-genotypes were identified using the virus genotyping tool on the National Center for Biotechnology Information (NCBI) webpage (available on: https://www.ncbi.nlm.nih.gov/projects/genotyping/formpage.cgi).

Mutations in basal core promoter (BCP)/precore/core region were determined by semi-nested PCR as described above except in the first round reaction FMD and Ci1 primers and in the second round reaction FMD and PC1 primers (Table 1) were used. Sequences were aligned and compared using a Clustal X program version 2.0.10 (UCD Conway Institute of Biomolecular and Biomedical Research, Dublin, Ireland).

Quantitative (q)PCR assay

One μl aliquot of DNA solution prepared as described above was used to quantify HBV DNA, cccDNA, and β-globin DNA (internal reference) concentrations using SYBr Green I qPCR method (Payungporn *et al*, 2004; He *et al*, 2002; Shadrina *et al*, 2007). A calibration curve of 10^2-10^9 copies/μl was constructed from pGem-T Easy Vector (Promega, Madison, WI) carrying a partial *β-globin* sequence (Shadrina *et al*, 2007), HBV *preS1/preS2* (nt 475-2814) (Payungporn *et al*, 2004), or cccDNA fragment (nt 1662-1922) (He *et al*, 2002).

For HBV quantification, a 12.7-μl reaction mixture contained 5 μl of distilled water, 5 μl of 2.5X PerfectTaq™ Plus MasterMix (5 PRIME GmbH, Hamburg, Germany), 0.5 μl of 25 μM Mg^{2+}, 0.5 μl each of 25 μM PreS1 F+ and PreS1 R2 primers (Table 1), 0.2 μl 10X SYBr Green I (Invitrogen, Carlsbad, CA), and 1 μl of DNA solution. Thermocycling was performed in a LightCyclerTM Nano Real-time PCR system (Roche, Basel, Switzerland) as follows: 95°C for 900 seconds; 40 cycles of 95°C for 15 seconds, 60°C or 25 seconds and 72°C for 15 seconds. Fluorescence was measured for 15 seconds at 82°C after each amplification cycle. Melting curve analysis was carried out from 67°C to 95°C with acquisition

Table 1
Primers used in the study.

Primer name	Primer sequence	nt position
β-globin F	5′-GTGCACCTGACTCCTGAGGAGA-3′	nt 1615-1636
β-globin R	5′-CCTTGATACCAACCTGC CAG-3′	nt 1716-1696
cccHBV F	5′-ACTCTTGGACTCTCAGCAATG-3′	nt 1662-1682
cccHBV R	5′-CTTTATACGGGTCAATGTCCA-3′	nt 1922-1902
Ci1	5′-TTCCGGAGACTCTAA GGCC-3′	nt 2038-2020
FMD	5′-GCATGGAGACCACCGTGAAC-3′	nt 1606-1625
HBx1	5′-GTCCCCTTCTTCATCTGCCGT-3′	nt 1487-1507
HBx2	5′-ACGTGCAGAGGTGAAGCGAAG-3′	nt 1604-1584
MD24	5′-TGCCAACTGGATCCTTCGCGGGACGTCCTT-3 \′	nt 1392-1421
MD26	5′-GTTCACGGTGGTCTCCATG-3′	nt 1625-1607
PC1	5′-GGAAAGAAGTCAGAAGGC-3′	nt 1973-1957
PreS1 F+	5′-GGGTCACCATATTCTTGGGAAC-3′	nt 2814-2835
PreS1 R+	5′-GAACTGGAGCCACCAGCAGG-3′	nt 56-75
PreS1 R2	5′-CCTGAGCCTGAGGGCTCCAC-3′	nt 3094-3075

of fluorescence signal at 0.5°C intervals.

For cccDNA quantification, a 15-µl reaction mixture contained 6.0 µl distilled water, 6 µl of 2.5X PerfectTaq™ Plus MasterMix (5 PRIME GmbH), 0.3 µl of 25 µM Mg^{2+}, 0.75 µl each of 25 µM cccHBV F and 25 µM cccHBV R primers (Table 1), 0.24 µl of 10X SYBr Green I (Invitrogen, Carlsbad, CA), and 1 µl of DNA solution. Thermocycling and data collection were performed as described but consisted of 40 cycles of 95°C for 15 seconds, 59°C for 30 seconds and 72°C for 60 seconds; and melting curve analysis from 72°C to 95°C.

For β-*globin* quantification, a 10-µl reaction mixture contained 3.5 µl distilled water, 5 µl PerfectTaq™ Plus MasterMix (5 PRIME GmbH), 0.1 µl each of 25 µM β-*globin* F and 25 µM β-globin R primers (Table 1), 0.3 µl of 10X SYBr Green I (Invitrogen, Carlsbad, CA), and 1 µl of DNA solution. Thermocycling and data collection were performed as described above but with 40 cycles of 95°C for 10 seconds, 60°C for 15 seconds and 72°C for 20 seconds; fluorescence measurement for 20 seconds at 78°C; and melting curve analysis from 72°C to 95°C, with fluorescence acquisition at 0.1°C intervals.

Amounts of HBV and liver cccDNA are initially determined relative to that of β-*globin* internal reference and subsequently expressed as copy/cell.

RESULTS

Demographic and serological data

There is no statistical difference between each serologically classified group of HCC patients positive for HBV DNA in liver tissues as regards to gender and age (Table 2).

Molecular characteristics of HBV and cccDNA in liver tissues of HCC patients

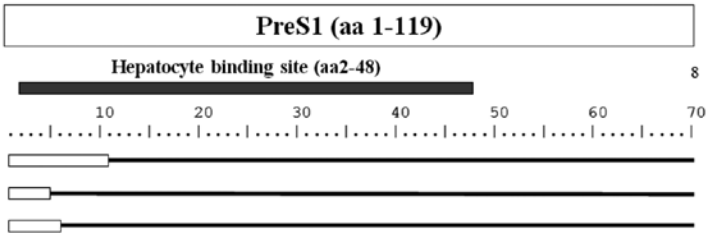
HCC tissues from 76 patients were positive for HBV DNA by nested PCR in 61% (52/76). Quantitation of intrahepatic HBV DNA and cccDNA by qPCR assay were 23% (12/52) and 4% (2/52), respectively.

Regions of interest in HBV genome were sequenced from amplicons generated by semi-nested PCR of DNA extracted from paraffin-embedded liver sections. Mutations in *preS1* region, C1673G/A in EnhII and C1773T/A in X gene, were predominant (Table 2). Genotypes were strongly associated with *preS* deletion (p-value <0.001), especially genotype A (p-value <0.001) and genotype C (p-value = 0.033), while *preS1* deletion was not present in genotype B (p-value = 0.045). The majority of deletion in PreS1 was that of aa 1-11 in hepatocyte binding region, with 62% in HBV-A (Fig 1).

Liver cell differentiation stage of HCC patients with liver positive HBV DNA was not correlated with age, HBV genotype or type of mutations (C1673G/A and C1773T/A) (Table 2). However, differentiation stage was correlated with serological findings (p-value < 0.001) and *preS1* deletion (p-value = 0.032), in particular well differentiated stage with *preS1* deletion (p-value = 0.002).

DISCUSSION

The study shows 55% of patients with anti-HBs positive serology still harbored HBV DNA including cccDNA in their liver. It is believed cccDNA remains within hepatocytes of patients and continues to accumulate in the nucleus of liver cell where it replicates the viral genome and generate virus transcripts (Bréchot *et al*, 2001). Wong *et al* (2006) reported cccDNA levels in tumor and non-tumor tissues of HBsAg-positive patients of 0.35 and



Deletion	n	Genotype A	Genotype C	*P*-value
nt 2848-2880	16	10 (62)	6 (38)	0.035
nt 2848-2862	2	0(0)	2(100)	0.476
nt 2848-2865	3	0(0)	3 (100)	0.214

Fig 1-Hepatitis B virus (HBV) *PreS1* deletion in patients with hepatocellular carcinoma (HCC) admitted to King Chulalongkorn Memorial Hospital, Bangkok, Thailand (2006 - 2012)

DNA samples extracted from paraffin-embedded liver tissues were amplified by semi-nested PCR using HBV *PreS1*-specific primers PreS1 F+ and PreS1 R (first round reaction) and PreS1 F+ and PreS1 R2 (second round reaction) (Table 1) and amplicons were directly sequenced.

▭ represents deleted region; ▬ reprsents hepatocyte binding site; scale bar indcates amino acid position in *PreS1*.

A: HBV-A; B: HBV-B; n: number of samples.

Percent value is shown in parenthesis.

0.16 copy/cell respectively. This study confirms that cccDNA are still in the livers of patients. The concentration of cccDNA/cell in HCC group of this study is 0.51±0.61 copy/cell.

Although DNA isolated from formalin-fixed paraffin-embedded tissues is fragmented and difficult to genotype (Baak-Pablo *et al*, 2010), short HBV *preS1* region of HBV (nt 2848-3046) revealed HBV-C was predominant. Patients infected with HBV-C are more likely to rapidly develop cirrhosis and progress to HCC than those with HBV-B (McMahon, 2009). Approximately 43% of HBV DNA isolated from HCC patients' liver sections contained deletions in the 5′ region (majority being del nt2848-2880 and restricted to HBV-A and -C) and were associated with stage of liver cell differentiation stage of HCC patients. Deletions in HBV *preS1* are frequently found in HCC than in liver cirrhosis patients (Chen *et al*, 2012) and believed to lead to accumulation of S protein in liver cell causing host DNA damage, cell stress and HCC and cirrhosis progression (Hsieh *et al*, 2004). The majority of HBV *preS* mutations are deletions mutations of *preS1* coding region or of the start codon mutations of *preS2* (Garcia *et al*, 2009; Chen *et al*, 2012). In the present study, the deletions removed short segments (5-10 amino acids) of preS1 protein N-terminal sequence. As secretion of HBV subviral particle depends on S proteins (Garcia *et al*, 2009; Chen *et al*, 2012), such deletions would probably compromise HBV virion secretion. In addition, the deletions are located in the hepatocyte binding site, which might reduce viral infection, virion assembly and transport out of the hepatocyte (Ganem, 1991; Chen *et al*, 2012). However, Kao *et al* (2012) noted no association of functional preS1 variants with hepatocyte binding.

EnhII mutations such as C1653T/del are associated with HCC development (Kramvis and Kew, 1999; Tanaka *et al*, 2006; Liu *et al*, 2009), as are the other

Table 2
Demographic and virological data of patients with hepatocellular carcinoma (HCC) admitted to King Chulalongkorn Memorial Hospital, Bangkok, Thailand (2006 - 2012).

Characteristic	Number (%) (n = 76)	HBV Number (%) (n =71)			HBV/HCV Number (%) (n = 5)	
		HBsAg+	anti-HBs+/ anti-HBC+	ND[a]	anti-HCV+/ anti-HBs+/ anti-HBc+	anti-HCV+/ HBsAg+
HCC patients	76 (100)	23 (32)	29 (41)	19 (27)	3 (60)	2 (40)
HBV DNA positive	52 (61)	20 (87)	16 (55)	13 (68)	1 (33)	2 (100)
Gender (M/F) (HBV DNA positive)	45(86)/7(14)	18(90)/2(10)	14(87)/2(13)	10(77)/3(23)	1(100)/0(0)	2(100)/0(0)
Age (years, mean ± SD)	58 ± 12	55 ± 12	61 ± 12	58 ± 14	52	57 ± 6
Genotyped	49 (64)	20 (87)	15 (52)	11 (58)	1 (33)	2 (100)
HBV-A	10 (20)	2 (10)	5 (33)	2 (18)	0 (0)	1 (50)
HBV-B	5 (10)	4 (20)	0 (0)	1 (9)	0 (0)	0 (0)
HBV-C	34 (70)	14 (70)	10 (67)	8 (73)	1 (100)	1 (50)
HBV DNA quantified	12 (23)	4 (17)	3 (10)	4 (21)	0 (0)	1 (50)
HBV copy/cell	0.10 ± 0.11	0.04 ± 0.06	0.10 ± 0.13	0.10 ± 0.17	0 (0)	0.02
cccDNA quantified	2 (4)	1 (4)	1 (3)	0 (0)	0 (0)	0 (0)
cccDNA copy/cell	0.51 ± 0.68	0.99	0.03	0	0	0
Region 2 sequenced	49 (64)	20 (87)	15 (52)	11 (58)	1 (33)	2
preS deletion	21 (43)	7 (35)	8 (53)	5 (45)	0 (0)	1 (50)
HBV-A	10 (48)	2 (29)	5 (62)	2 (40)	0 (0)	1 (100)
HBV-C	11 (52)	5 (71)	3 (38)	3 (60)	0 (0)	0 (0)
Region 3 mutation[b]	21 (28)	10 (43)	6 (21)	3 (16)	1 (33)	1
C1653T/deletion	3 (14)	3 (30)	0 (0)	0 (0)	0 (0)	0 (0)
C1673T/G/A[c]	13 (62)	7 (70)	3 (50)	1 (33)	1 (100)	1 (100)
A1762T	7 (33)	5 (50)	1 (17)	1 (33)	0 (0)	0 (0)
G1764A	8 (38)	5 (50)	2 (33)	1 (33)	0 (0)	0 (0)
C1773T/A[c]	13 (62)	5 (50)	5 (83)	3 (100)	0 (0)	0 (0)
HCC differentiation	43 (56)	19 (83)	15 (52)	7 (37)	1 (33)	1 (50)
Well	12 (28)	7 (37)	5 (33)	0 (0)	0 (0)	0 (0)
Moderate	19 (44)	8 (42)	5 (33)	5 (71)	0 (0)	1 (100)
Poor	12 (28)	4 (21)	5 (33)	2 (29)	1 (100)	0 (0)

[a]Data not available; [b]Includes multiple mutations in *cis*; [c]Novel mutation found in the study.

mutations such as V1753, T1762 and A1764 (Liu *et al*, 2006; Asim *et al*, 2010). In the present study, no differences were found among serological classified groups of HCC patients and these mutations. On the other hand, C1673G/A and C1773T/A were predominant.

C1673G/A is located in the nuclear receptor response element $(NRRE)_{enhII}$ region (nt 1663-1675) that is embedded in enhancer elements (Yu and Mertz, 2001; Ramière *et al*, 2008). Nuclear receptors (NRs) are members of a superfamily of transcription factors that bind to their respective NRREs and contribute to their functional activities (Tsai and O'Malley, 1994). Other NRREs situated in HBV *preC* promoter, such as $NRRE_{preC}$ and $NRRE_{enhI}$, can be bound by several NRs, whereas $NRRE_{enhII}$ is only bound by hepatocyte nuclear factor 4α, levels of which affect on synthesis of virus pregenomic RNA (Guo *et al*, 1993; Yu and Mertz, 2003). Transfected recombinant plasmid carrying a deletion of the entire enhancer II region expressed 10% of preC and pregenomic RNA as that of a plasmid carrying the wild type gene (Yu and Mertz, 2001). A point mutation in $NRRE_{enhII}$-binding HNF4α decreases rate of synthesis to 50% of that in the wild-type (Yu and Mertz, 2001).

C1773 is located in farnesoid X receptor alpha (FXRα) putative binding site 2 (Ramière *et al*, 2008). FXRα is an NR that regulates HBV core promoter activity and level of viral replication. FXRα increases synthesis of viral pregenomic RNA and DNA replication (Ramière *et al*, 2008). Mutations in both C1673 and C1773 were found in the present study and functional investigations are required to elucidate the roles of these mutations. C1673G/A and C1773T/A are also located in the X gene region, and X protein promotes cell differentiation, proliferation and apoptosis (Li *et al*, 2014). C1673G/A and C1773T/A are synonymous substitutions that do not change the encoded amino acid in the respective X protein region.

There is a correlation between serological data and liver cell differentiation stage in HCC patients. Both immune response of the host and virological factors may play key roles in this phenomenon (Xie, 2017) but the actual mechanisms remain unclear.

In conclusion, the study demonstrates screening for HBV DNA in liver tissues or liver biopsies provides useful information regarding virus genotypes and genome mutations, which should assist physicians in early detection and surveillance for hepatocellular carcinoma, which will be of benefit in reducing morbidity and mortality associated with liver disease progression and in managing patients' treatment.

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CONFLICTS OF INTEREST

The authors declare no conflicts of interest.

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