

## RESEARCH ARTICLE

# Hepatitis B Virus Genetic Variation and TP53 R249S Mutation in Patients with Hepatocellular Carcinoma in Thailand

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### Abstract

Chronic hepatitis B virus (HBV) infection and dietary exposure to aflatoxin B1 (AFB1) are major risk factors for hepatocellular carcinoma (HCC). The aim of this study was to evaluate the role of HBV genetic variation and the R249S mutation of the p53 gene, a marker of AFB1-induced HCC, in Thai patients chronically infected with HBV. Sixty-five patients with and 89 patients without HCC were included. Viral mutations and R249S mutation were characterized by direct sequencing and restriction fragment length polymorphism (RFLP) in serum samples, respectively. The prevalences of T1753C/A/G and A1762T/G1764A mutations in the basal core promotor (BCP) region were significantly higher in the HCC group compared to the non-HCC group. R249S mutation was detected in 6.2% and 3.4% of the HCC and non-HCC groups, respectively, which was not significantly different. By multiple logistic regression analysis, the presence of A1762T/G1764A mutations was independently associated with the risk of HCC in Thai patients.

**Keywords:** Hepatitis B - aflatoxin - mutation - hepatocellular carcinoma - Thailand

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### Introduction

Hepatocellular carcinoma (HCC) is a major public health problem worldwide, accounting for at least 500,000 new cases diagnosed annually. The prevalence of HCC is geographically variable, with the highest frequencies observed in sub-Saharan Africa and Southeast Asia where hepatitis B virus (HBV) is endemic (Gao et al., 2012). HBV, a member of the family Hepadnaviridae, is a partially double-stranded DNA virus that contains four overlapping open reading frames (ORFs) encoding the surface, core, polymerase and X genes. The virus shows remarkable genetic variability and is currently classified into at least eight genotypes (genotypes A-H). HBV also has a high mutation rate resulting from its lacking of proofreading capacity during the viral replication (Kay et al., 2007). The well-known naturally occurring HBV variants include the precore (PC) stop codon mutation (G1896A) and double mutations in the basal core promotor (BCP) region (A1762T/G1764A). Several studies have showed that A1762T/G1764A have higher risks of liver cirrhosis and HCC (Wai et al., 2004). However, BCP mutations are also frequently found among patients without HCC. Apart from these variants, other mutations such as T1753C/A/G in the BCP region, C1653T in the enhancer II region (EnhII) and pre-S mutations/deletions have been recognized as being associated with

the outcome of chronic HBV infection, including HCC development (Chotiyaputta et al., 2009).

In addition to chronic HBV infections, dietary exposure to aflatoxin B1 (AFB1) is an important risk factor for HCC development (Yu et al., 2004). AFB1 is a mycotoxin produced by *Aspergillus flavus* and *Aspergillus parasiticus*, which contaminates staple foods during storage under conditions that promote fungal growth and toxin production. The molecular hallmark of AFB1-induced HCC is a missense mutation at codon 249 in the p53 tumor suppressor gene (TP53), AGG to AGT, leading to a substitution of an arginine for a serine (R249S mutation) (Wild et al., 2009). This mutation is extremely high in HCC in areas with high prevalence of chronic HBV infection and AFB1 exposure such as Qidong, China. In contrast, such mutation is uncommon in regions with low or negligible levels of AFB1 exposure such as the United States and Japan. Recent studies have shown that R249S mutation is detectable in free DNA extracted from serum or plasma of patients with HCC, which is identical to those detected in tumor tissues and can be served as a biomarker of AFB1 exposure (Gouas et al., 2009). The prevalence of this mutation in the serum of Thai patients with HCC and its interaction with HBV mutations are uncertain. Thus, the aim of this study was to assess the role of HBV genetic variation, R249S mutation, and their interactions in HCC risk in Thai patients with chronic HBV infection.

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## Materials and Methods

### Patients

Serum samples obtained from patients with HBV-related HCC and positive for HBV DNA were randomly selected from a pool of patients with chronic liver disease who were seen and followed-up at King Chulalongkorn Memorial Hospital (Bangkok, Thailand) between January 2009 and December 2012. The diagnosis of HCC was based on typical imaging studies and/or histology (fine needle aspiration or surgical resection) according to American Association for the Study of Liver Diseases (AASLD) guideline (Bruix et al., 2005). Diagnostic criteria of HCC by imaging modalities were based on reports of focal lesions with hyperattenuation at the arterial phase and hypoattenuation at the portal phase in dynamic CT or MRI. In cases without typical imaging features liver biopsy was performed to confirm the diagnosis of HCC.

To examine the role of *R249S* mutation and molecular virological factors in the development of HCC, patients with chronic hepatitis B were randomly selected as controls. These patients visited our clinic every 4–6 months during the same period of recruitment of the study and none had liver cancer development during follow-up. None of the patients enrolled in this study had a history of hepatitis C virus (HCV) infection or human immunodeficiency virus (HIV) co-infection. In addition, none of the patients had a history of heavy alcoholic drinking, or received any antiviral therapy when the serum sample was obtained. All patients were informed about the purposes of the study, and subsequently gave their written informed consent. Serum samples were collected from each patient at the time of their evaluation and frozen at  $-70^{\circ}\text{C}$  until use. The study was approved by the Ethics Committee, Faculty of Medicine, Chulalongkorn University.

### Serological and virological assays

Sera tested for hepatitis B s antigen (HBsAg) was determined using commercially available ELISA assay (Abbott Laboratories, Chicago, IL). Serum HBV DNA level was quantified using a commercial kit (Amplicor HBV Monitor; Roche Diagnostics, Tokyo, Japan). The detection range of this assay was 2.4 to 8.7 log IU/mL.

### HBV DNA preparation, amplification, and direct sequencing

HBV DNA was extracted from 100  $\mu\text{l}$  serum sample by incubation in lysis buffer (10 mM Tris-HCl pH 8.0, 0.1 M EDTA pH 8.0, 0.5% SDS and 20 mg/mL proteinase K) and phenol–chloroform–isoamyl alcohol extraction. The DNA pellet was resuspended in 30  $\mu\text{l}$  sterile distilled water and subjected to amplification of the *pre-S1/pre-S2/S* and *EnhII/BCP/PC* regions by polymerase chain reaction (PCR). The *pre-S1/pre-S2/S* regions were amplified using the primers PreS1F+: 5'-GGGTCACCATAT TCT TGGG AAC-3' [forward primer, nucleotide (nt.) 2814 to 2835] and R5: 5'-AGCCAAAAGACCCACAATTC-3' (reverse primer, nt. 1015 to 995). The *EnhII/BCP/PC* regions were amplified using the primers X101:5'-TCTGTGCCTTCTCATCTG-3' (forward primer, nt.1552 to 1569) and CORE2:

5'-CCCACCTTATGAGTCCAAGG-3' (reverse primer, nt. 2476-2457).

Briefly, the reaction mixture comprised 2  $\mu\text{l}$  resuspended DNA, 0.5  $\mu\text{l}$  of 25 mmol of each primer, 10  $\mu\text{l}$  of 2.5X MasterMix® (Eppendorf, Hamburg, Germany) and sterile distilled water to a final volume of 25  $\mu\text{l}$  reaction. The reaction was performed in a PCR thermocycler (Eppendorf, Hamburg, Germany) with the initial denaturation at  $94^{\circ}\text{C}$  for 3 min, followed by 35 cycles at  $94^{\circ}\text{C}$  for 30 s (denaturing), at  $55^{\circ}\text{C}$  for 30 s (annealing), at  $72^{\circ}\text{C}$  for 1 min (extension) and concluded by a final 7 min extension at  $72^{\circ}\text{C}$ . The PCR products were segregated by 2% agarose gel electrophoresis. The PCR products were extracted from the agarose gel using the Perfectprep® Gel cleanup kit (Eppendorf, Hamburg, Germany). The sequencing reaction was performed using the AmpliTaq™ DNA Polymerase FS dye terminator from the ABIPRISM™ BigDye™ Terminator Cycle Sequencing Ready Reaction kit (Perkin-Elmer Applied Biosystems Division, Foster City, CA) according to the manufacturer's specification. Nucleotide sequences were edited and assembled using SEQMAN (LASERGENE program package, DNASTAR) and aligned with CLUSTALX (version 1.83) program as previously described (Suwannakarn et al., 2008).

### HBV genotyping

For the genotyping, phylogenetic analysis based on *pre-S1/pre-S2/S* and *preC/C* regions was performed using Molecular Evolutionary Genetics Analysis (MEGA) software version 4.0 (The Biodesign Institute, Tempe, AZ) as previously described (Sa-Nguanmoo et al., 2010).

### Detection of *R249S* in serum

Serum specimens were subjected for the amplification of exon 7 of the *TP53* gene by nested-PCR. The first PCR reaction consisted of 0.5  $\mu\text{L}$  of DNA template, 10  $\mu\text{L}$  of PerfectTaq plus MasterMix Kit (5 prime GmbH, Hamburg, Germany), 1.25 mM outer forward primer (*TP53-OS*: 5'-CTTGCCACAGGTCTCCCAA-3'), 1.25 mM outer reverse primer (*TP53-OAS*: 5'-AGGGGTCAGAGGCAAGCAGA-3'), and distilled water to final volume as 25  $\mu\text{L}$ . The second PCR reaction was performed using nested primers, 1.25 mM inner forward primer (*TP53-IS*: 5'-AGGCGCACTGGCCTCATCTT-3'), 1.25 mM inner reverse primer (*TP53-IAS*: 5'-TGTGCAGGGTGGCAAGTGGC-3') and 0.2  $\mu\text{L}$  of first PCR product as DNA template. The PCR cycles was initially denatured at  $94^{\circ}\text{C}$  for 3 min, followed by 40 cycles at  $94^{\circ}\text{C}$  for 18 s (denaturing), at  $50^{\circ}\text{C}$  for 21 s (annealing), at  $72^{\circ}\text{C}$  for 1.30 min (extension) and concluded by final 10 min extension at  $72^{\circ}\text{C}$ . The PCR-amplified products were examined by 2% agarose gel electrophoresis then stained with ethidium bromide and visualized under UV light. The size of the final PCR fragments were 237 bp (first PCR) and 177 bp (second PCR), respectively.

*TP53* mutation was analyzed by restriction fragment length polymorphism (RFLP) using a modified method described by Szymanska et al. (2004). Briefly, 15  $\mu\text{L}$  of second PCR product was digested by *HaeIII* restriction endonuclease which recognized sequence CCGG and

encompassed the 249 codon (AGG). The total 20  $\mu$ L RFLP reaction mixture comprised of 1 unit of *Hae*III (New England BioLabs inc, Ipswich, MA), 2  $\mu$ L of 10X Buffer4 (New England BioLabs inc, Ipswich, MA) and sterile distilled water. The mixture was incubated at 37°C overnight and segregated by 3% agarose gel electrophoresis. The PCR product of wild-type DNA generated 3 bands of 12, 24 and 116 bp, whereas in mutant, in which the restriction site was destroyed, yielded only two band of 12 and 153 bp. Sample with positive RFLP for *R249S* were further confirmed by direct sequencing.

#### Statistical analysis

Data were presented as percentage, mean and standard deviation. Comparisons between groups were analyzed by the  $\chi^2$  or Fisher's exact test for categorical variables and by the Mann-Whitney test or Student's t test when appropriate for quantitative variables. Multiple logistic regression analysis was used to assess the influence of each factor on the risk of HCC development. *P* values below 0.05 were considered statistically significant. Data were analyzed by using the SPSS software for Windows 17.0 (SPSS Inc., Chicago, IL).

## Results

The clinical features of all patients in this study are showed in Table 1. There were 65 patients with HCC and 89 patients in the control group. The mean age of patients with HCC (58.1 $\pm$ 12.0 years) was significantly higher than that of the controls (41.3 $\pm$ 8.8 years) (*p*<0.001). In addition, the prevalence of HBV genotype C was significantly higher in the HCC group than in the control group. However, there was no significant difference between groups in respect to sex, and HBV DNA levels.

*Pre-S1* mutations/deletions were detected in 27.3% and 19.6% samples of patients with HCC and controls, respectively. The prevalence of *pre-S2* mutations/deletions among the HCC and control groups was 21.8% and 25%, respectively. There was no significant difference in the prevalence of such mutations/deletions between the two groups. Also, there was no significant difference between groups regarding *S* start codon mutation. However, patients with HCC had significantly lower frequency of *S* deletion compared with the controls (23.6% and 44.6%, respectively, *p*=0.019) (Table 1).

Base on direct sequencing of EnhII/BCP/PC regions, mutational spots were found at nt 1653, 1753, 1762, 1764, 1896 and 1899. Patients with HCC had higher frequencies of T1753A/G/C mutations compared with the controls (59.1% and 35.5%, respectively, *p*=0.008). Moreover, patients with HCC had higher frequencies of A1762T/G1764A mutations compared with the controls (88.6% and 68.4%, respectively, *p*=0.003). In contrast, G1896A mutation was less frequently detected in patients with HCC than in the controls (34.1% and 65.8%, respectively, *p*=0.003). No significant difference between groups was found in respect to C1653T and G1899A mutations (Table 1).

Base on RFLP and direct sequencing, *R249S* mutation was present in 6.2% serum samples of the HCC group,

**Table 1. Demographic and Characteristics of Patients with or without HCC**

Characteristics	HCC group (n=65)	Non-HCC group (n=89)	<i>p</i>
Age (years)	58.1 $\pm$ 12.0	41.3 $\pm$ 8.8	<0.001
Sex			0.376
Male	53(81.5)	66(74.2)	
Female	12(18.5)	23(25.8)	
HBV genotype			0.003
B	8(12.3)	17 (19.1)	
C	48(73.8)	42 (47.2)	
Unknown	9(13.9)	30(33.7)	
HBV DNA level (Log IU/mL)	5.4 $\pm$ 1.0	5.3 $\pm$ 1.0	0.505
<i>Pre-S1/pre-S2/S</i> mutations/deletions			
PCR positive	55(84.6)	56(62.9)	
<i>Pre-S1</i> start codon mutation	10(18.2)	11(19.6)	1
<i>Pre-S1</i> deletion	5(9.1)	0(0)	0.07
<i>Pre-S2</i> start codon mutation	6(10.9)	5(8.9)	0.895
<i>Pre-S2</i> deletion	6(10.9)	9(16.1)	0.435
<i>S</i> start codon mutation	5(9.1)	7(12.5)	0.676
<i>S</i> deletion	13(23.6)	25(44.6)	0.019
EnhII/BCP/PC mutations			
PCR positive	44(67.7)	76(85.4)	
C1653T	9(20.5)	11(14.5)	0.575
T1753A/G/C	26(59.1)	27(35.5)	0.008
A1762T/G1764A	39(88.6)	52(68.4)	0.003
Precore start codon mutation	3(6.8)	6(7.9)	1
G1896A	15(34.1)	50(65.8)	0.003
G1899A	12(27.3)	12(15.8)	0.12
<i>TP53 (R249S)</i> mutation			0.669
Wild type	61 (93.8)	86 (96.6)	
<i>R249S</i> mutant	4(6.2)	3(3.4)	

\*Data were expressed as mean $\pm$ SD, no (%)

which was slightly higher than that found in the control group (3.4%), but did not reach statistical significance. Two of patients with HCC contained both A1762T/G1764A and *R249S* mutations in their sera and the median age of HCC diagnosis in these patients was 52.5 $\pm$ 9.2 years compared with 58.2 $\pm$ 12.1 years for the other HCC cases (*p*=0.510).

To determine the independent contribution of clinical and virological factors to the development of HCC, multiple logistic regression analysis was performed by using the significant factors identified in the univariate analysis. The significant factor associated with HCC development by multivariate analysis was A1762T/G1764A mutations (odds ratio: 6.51, 95% confidence interval: 1.41-30.16, *p*=0.017).

## Discussion

Viral genetic variations such as genotypes and mutations may play important roles in causing different disease profiles in chronic HBV infection, including HCC development. Moreover, epidemiological studies in high prevalent areas have showed significant interactions between chronic HBV infection and dietary AFB1 in relation to HCC risk. To better understand the contributions of virus-chemical interactions to hepatocarcinogenesis, data from various ethnic groups and countries are needed for comparison. To this end, this case-control study was

conducted to assess the role of HBV genetic variations, including genotypes and mutations, *R249S* mutation in the serum as a marker of mutagenesis by AFB1 and their interactions in HCC risk.

In agreement with previous data, our study showed that HBV genotypes B and C are the two common genotypes in Thailand. We found that the prevalence of genotype C was significantly higher among patients with HCC than those without liver cancer. In fact, several studies reported that genotype C was related to a higher risk of HCC development as compared to genotype B, although such association was not confirmed by other studies (Kim et al., 2011). Additionally, we found significantly higher frequencies of A1762T/G1764A and T1753A/G/C mutations in the HCC group than in controls, which are consistent with previous reports (Chen et al., 2008; Yuen et al., 2008). In fact, T1753A/G/C mutations are usually present along with the existence of A1762T/G1764A mutations. Previous data also demonstrated that T1753A/G/C mutations occurred later than the double BCP mutations in the natural course of chronic HBV infection (Takahashi et al., 1999). Regarding *pre-S* deletion, it has been shown in several reports that such mutant is an important risk factor of HCC (Chen et al., 2006; Choi et al., 2007). In vitro studies have also showed that intracellular accumulation of *pre-S* mutant proteins can modify HBV protein expression, induce ER stress and increased DNA damage (Hsieh et al., 2004). However, *pre-S* deletion was not considered to be a significant factor associated with HCC in this study.

Our data demonstrated that the double BCP mutations were the only independent risk factor for the development of HCC. A prospective cohort of approximately 1600 high-risk individuals in Qidong, China, showed that the double BCP mutations were identified in 50% of HCC cases before cancer detection, suggesting that the variants might indicate a high potential risk for HCC (Kuang et al., 2004). It has also been reported that such mutations is usually associated with HBV genotype as their prevalence is higher in genotype C than genotype B (Kao et al., 2003). Although the mechanism of double BCP mutations in hepatocarcinogenesis remains uncertain, several hypotheses have been proposed. The dual BCP mutants may enhance viral replication by creating a specific transcription factor binding site or modulating the relative levels of *pre-core* and *core* RNAs (Hunt et al., 2000). Furthermore, the presence of the mutants may be associated with decreasing T-cell immune responses (Malmassari et al., 2007). In addition, mutations in the BCP region, which overlaps the coding sequence for the X gene, may result in amino acid changes in the X protein (Malmassari et al., 2007). For example, A1762T/G1764A mutations are corresponding to K130M/V131I in the X protein. These genomic variations could modify the oncogenic potential of the X protein and induce inactivation of *p53*-mediated apoptosis or impairment of DNA repair (Hussain et al., 2007).

The risk of HCC associated with AFB1 is mainly reported in countries with high prevalence of HBV infection. Both double BCP mutations and *R249S* mutation are considered to be surrogate markers of AFB1-induced

HCC in the context of chronic HBV infection. In fact, HCC containing *R249S* mutation can occur in the absence of significant recent AFB1 exposure, which may allow the detection of the mutant DNA in blood specimens several months ahead of clinical diagnosis of HCC (Jackson et al., 2003). In a case-control study in The Gambia, another area of high prevalence of HCC and common exposure to HBV and AFB1, it was reported that *R249S* mutation was detectable in control subjects or patients with cirrhosis, although with a significantly lower prevalence than in HCC patients (Kirk et al., 2005). A previous study conducted in northern Thailand reported the detection rates of A1762T/G1764A and *R249S* mutations in plasma of HCC patients in approximately 40% and 25%, respectively (Kuang et al., 2005). Recent data of case-control studies from Thailand also suggested that the development of HCC might be enhanced by the cooperation of chronic HBV infection and of AFB1 induced *R249S* mutation, particularly among HCC patients with young age and without pre-existing cirrhosis (Villar et al., 2012; Ortiz-Cuaran et al., 2013). Data from our cohort, however, did not support the interactions of HBV and AFB1 in promoting hepatocarcinogenesis, because the presence of *R249S* mutations in patients with or without HCC was rather similar. In addition, the mean age of HCC patients who had both A1762T/G1764A and *R249S* mutations did not significantly differ from that of the others.

It should be noted that the prevalence of *R249S* mutation in our study was relatively low compared to other reports from Thailand. The low prevalence of such mutant in this study also explained why there was no association between AFB1 exposure and HCC risk among chronic HBV carriers. The basis of this discrepancy between our study and the other reports was unclear but might be related to the following hypotheses. First, the method using RFLP assay might not be sensitive for detecting *R249S* mutation in some specimens containing trace amounts of the mutant DNA. In this regard, it has been shown that, at high blood levels of *R249S* mutation, RFLP provides good results in agreement with more sensitive methods such as short oligonucleotide mass analysis (SOMA) (Szymanska et al., 2009). At low levels, however, the signals generated by RFLP are below the detection threshold and might be responsible for the false-negative results. Thus, based on RFLP assay, serum specimens negative for *R249S* mutation might contain levels that were too low for detection by this method. Second, the presence and amount of *R249S* mutation in serum may fluctuate among individuals according to season, with a pattern differs from the well-known seasonal variations in exposure to AFB1 (Villar et al., 2011). In addition, individual exposure to AFB1 is likely to fluctuate among different geographic areas and ecological zones (Liu et al., 2010). Nonetheless, little research has been conducted on comparisons of the seasonal and geographical influences on AFB1 contamination in Thailand.

In conclusion, our case-control study demonstrated that the dual BCP mutations were an independent virological factor associated with the risk of HCC. Identification of these HBV mutants may be helpful for predicting the development of HCC. In contrast, our cohort failed to

identify an AFB1-associated risk for HCC. Further large-scale prospective studies using more sensitive methods for detecting R249S mutation are needed to elucidate these observations.

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