Short Communications

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Distribution and Clinical Significance of Hepatitis B virus A1762T/G1764A Double Mutation in Chronic Hepatitis B Patients: A Cross-Sectional Study

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Abstract

Background: Chronic hepatitis B (CHB) is well-known as a major risk for liver cirrhosis and hepatocellular carcinoma (HCC). The A1762T/G1764A double mutation in the hepatitis B virus genome affects the production of HBe antigen and is established as a predictive marker for progression to HCC. Thus, this study aimed to investigate the prevalence and clinical significance of the mutation in Thai CHB patients. **Methods:** A cross-sectional study was conducted in 78 Thai CHB patients who were assessed for hepatitis B profiles, HBsAg, HBeAg and anti-HBeAg, transaminitis, liver fibrosis defined by FIB-4 (FIB-4) score and AST to platelet ratio index (APRI), alpha-fetoprotein (AFP) and active hepatitis B status. HBV A1762T/G1764A mutation was examined by SYBR Green I Real-time PCR. Chi-square and Mann-Whiney U tests were performed to determine the association between the mutation and variables. **Results:** The prevalence of patients infected with the A1762T/G1764A mutation was 44.9%. The mutation was associated with HBeAg status (p=0.027) and HBsAg levels (p=0.008), transaminitis (p=0.011), and active hepatitis B (p=0.037), but not liver fibrosis markers, FIB-4 score and APRI, and AFP. Binary logistic regression identified the mutation as a predictive factor of active hepatitis B (OR 3.5, 95%CI, 1.1-11.3, p=0.037). Patients infected with the mutant exhibited significantly higher levels of HBsAg (p=0.011) and HBV viral load (p=0.047), but lower levels of HBeAg (p=0.12) than those infected with the wild-type HBV. **Conclusion:** The data indicate the high prevalence of the A1762T/G1764A mutation and its significant association with the severity of Thai CHB patients and the HBV mutation is proposed as a predictive marker of active hepatitis B status in CHB patients.

Keywords: A1762T/G1764A mutation- Chronic hepatitis B- transaminitis- liver fibrosis

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Introduction

Hepatitis B virus (HBV) infection is a major health issue, with an estimation of 296 million people living with chronic hepatitis B and 820,000 deaths from liver cirrhosis and primary hepatocellular carcinoma (HCC) in 2019. The burden of HBV infection is apparently high in the South-East Asia region with 18 million people chronically infected [1]. In Thailand, the pooled prevalence of chronic hepatitis B (CHB) was 5.1% (95% CI 4.3-6.0%), which would estimate the number of CHB as high as 3 million [2]. Despite Thailand's achievement on hepatitis B control since 2019, challenges remain especially in the implementation of national programs to prevent CHB complications.

Chronic persistent HBV infection has been known as a serious risk factor for HCC [3, 4]. Patients with either hepatitis B e antigen (HBeAg)-positive or negative phase, who are unable to suppress HBV replication, develop liver inflammation and fibrosis, leading to liver cirrhosis and HCC. CHB is a dynamic disease with variable clinical courses and is currently categorized by the American Association of the Study of Liver Diseases (AASLD) into three phases including the immune tolerant phase, immune active CHB, and inactive CHB [5]. Progression of CHB is known to be influenced by various factors including genetic variability of HBV. Many mutations in HBV genes caused by the unsuccessful and prolonged HBV immune clearance process have been reported [6, 7]. Among the hot spot HBV mutations, the basal core promoter (BCP) A1762T/G1764A double mutation is involved in HBeAg-negative HBV infection [8, 9] and

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several lines of evidence support the A1762T/G1764A mutation as a predictive marker for the progression to HCC [10-12]. While the identification of BCP mutants may support strategies in the proper management of CHB [7], the effect of A1762T/G1764A double mutation on clinical outcomes of CHB requires clarification in different settings. Thus, this cross-sectional study aimed to investigate the distribution of A1762T/G1764A double mutation and its clinical relevance in Thai CHB patients.

Materials and Methods

Study population

Blood samples and clinical data previously collected from the patients attending Thammasat University Hospital from 2012 to 2013 were recruited based on inclusion and exclusion criteria as follows. The samples from patients aged 20 years or older with positivity for hepatitis B surface antigen (HBsAg) for at least 6 months and with clinical data availability were included. The samples were excluded according to positivity for anti-hepatitis C virus test, poor quality and/or insufficient volume of blood samples, and incomplete clinical and laboratory data. The study protocol was approved by the Human Research Committee, Thammasat University (COA No. 144/2562).

Clinical data and laboratory investigation

General and clinical data were obtained from medical records including age, gender, and medication. Laboratory data were also collected including hepatitis B serologic profile, hepatitis B surface antigen (HBsAg), hepatitis B envelope antigen (HBeAg), anti-hepatitis B envelope (Anti-HBe), hepatitis B viral load (HBV viral load), aspartate aminotransferase (AST), alanine aminotransferase (ALT), alpha-fetoprotein (AFP) and platelet count. Infections with human immunodeficiency virus (HIV) and hepatitis C (HCV) were screened by anti-HIV and anti-HCV tests. HBV genotyping data were obtained by using in-house SYBR Green I Real-time polymerase chain reaction (Real-time PCR) and melting point analysis. Several markers for assessing liver abnormality in this patient group were performed including, levels of AST, ALT and AFP, transaminitis, fibrosis-4 (FIB-4) score, AST-to-platelet ratio index (APRI) as described previously [13]. In this study, active hepatitis B was defined as HBV DNA levels > 20,000 IU/mL and ALT values > 2 x ULN (40 U/L), while inactive or mild hepatitis B was defined as HBV DNA levels ≤ 20,000 IU/mL and ALT values ≤ 2 x ULN [14].

Detection of hepatitis B virus A1762T/G1764A mutation by SYBR Green I Real-time PCR

HBV A1762/G1764A double mutation was detected by SYBR Green I Real-time PCR and melting curve analysis as previously developed [15]. Briefly, HBV DNA was extracted from plasma samples using Neucleospin Blood ready-made extraction kits (MACHEREY-NAGEL GmbH & Co.KG, Germany) and the concentration of the extracted DNA was determined by NanoDrop One Microvolume UV-Vis spectrophotometers (Thermo Fisher Scientific, USA) at a wavelength of 260 nm and then stored at -20 °C before further use.

The extracted DNA together with mutant and wild-type plasmid vector controls (positive control), sterile distilled water (negative control), and normal human DNA (negative controls) were amplified using specific primers for A1762/G1764A wild-type and mutant, Wt_F1: 5' GCATGGAGAACAACGTGA 3', Wt_R1: 5' CTCCCAGTACAAAGACCT 3', Mut_F1: 5' GGAGGAGATTAGGTTAATGA 3' and Mut_R1: 5' CATGAACATGAGATGATTAGG 3'. A total volume of 20 uL of each reaction consisted of 10 uL iTaqTM Universal-SYBR Green Supermix (Bio-Rad Laboratories Inc., USA) at a concentration of 5 pmole/uL, 0.5 uL each forward and reverse primer, 5 uL distilled water, and 4 uL DNA. All ingredients were reacted 40 cycles by CFX96Touch Real-time PCR machine (Bio-Rad Laboratories Inc., USA), each cycle consisting of an initiation step at 95 °C for 3 min, denaturation at 95 °C for 10 sec, annealing at 55 °C for 20 sec, and extension at 72 °C for 30 sec. The melting curve was then analyzed by Bio-Rad CFX Manager program. Interpretations of positive results were as follows. Any sample reacting with wild-type strain-specific primers at the Cq value < 35 cycles and giving the Tm value between 80.0-81.5 °C was classified as a wild-type strain, whereas that reacting with mutant strain-specific primers at the Cq value < 35 cycles and giving the Tm value between 76.5-77.0 °C, was identified as a mutant strain. A sample reacting with either wild-type or mutant strain-specific primers at the Cq value 10 cycles or ≥ 1.3 times greater than another, was interpreted as a wild-type strain.

Statistical data analysis

The prevalence of HBV A1762/G1764A mutation in chronic hepatitis B patients was analyzed by descriptive statistics and presented as percentages. Chi-square and Fisher's exact tests were used to examine the association of HBV A1762/G1764A mutation with categorical variables and the Mann-Whitney U test was used to analyze those with continuous variables. Logistic regression to determine independent predictors for HBV A1762/G1764A mutation and active CHB. P values > 0.05 were considered statistically significant. The PASW Statistic 18 software (SPSS Inc.) was used for statistical analysis.

Results

General and clinical characteristics of hepatitis B patients recruited in this study were demonstrated in Table 1. A total of 78 chronic hepatitis B patients with HBsAg positivity for at least 6 months, 49 male (62.8%) and 29 female (37.2%) with a median age of 48 [range, 20-87] years were included. The negative rate for HBeAg was 61.6% (45/73) and the positive rate of anti-HBeAg was 62.2% (23/37) respectively. There were 67 out of 73 patients (91.8%) with detectable HBV DNA with a median of 72,800 [range, 35-640,000,000 copies/mL.]. Prevalence of HBV A1762/G1764A wild-type, mutant, and mixed infection were 55.1% (43/78), 43.6% (34/78), and 1.3% (1/73) respectively. With regards to 53 patients with the

Table 1. Characteristics and Clinical Features of Chronic Hepatitis B Patients According to HBV A1762T/G1764A Mutation (n=78)

Characteristics	All patients	Infection with		P
	N (%)	Wild-type (n = 43)	A1762T/G1764A mutant (n = 35)	
Age (years)				
< 40	28 (35.9)	19 (44.2)	9 (25.7)	
≥ 40	50 (64.1)	24 (55.8)	26 (74.3)	0.091
Gender				
Male	49 (62.8)	26 (60.5)	23 (65.7)	
Female	29 (37.2)	17 (39.5)	12 (34.3)	0.633
HBeAg status (n = 73)				
Positive	28 (38.4)	20 (50.0)	8 (24.2)	
Negative	45 (61.6)	20 (50.0)	25 (75.8)	0.024*
HBsAg levels (S/CO)(n = 72)				
≤ 2,000	32 (44.4)	23 (59.0)	9 (27.3)	
> 2,000	40 (55.6)	16 (41.0)	24 (72.7)	0.007*
HBV Viral load (IU/mL)(n = 73)				
≤ 2,000	30 (41.1)	20 (51.3)	10 (29.4)	
> 2,000	43 (58.9)	19 (48.7)	24 (70.6)	0.058
AST (U/L)				
≤ 40 U/L	42 (53.8)	29 (67.4)	13 (37.1)	
> 40 U/L	36 (46.2)	14 (32.6)	22 (62.9)	0.008*
ALT (U/L)				
≤ 40 U/L	22 (28.2)	16 (37.2)	6 (17.1)	
> 40 U/L	56 (71.8)	27 (62.8)	29 (82.9)	0.05
Transaminitis				
AST and/or ALT ≤ ULN (40 U/L)	18 (23.1)	15 (34.9)	3 (8.6)	
AST and/or ALT > ULN (40 U/L)	60 (76.9)	28 (65.1)	32 (91.4)	0.006*
Thrombocytopenia (/uL) (n = 73)				
< 150 X 103 /uL	21 (28.8)	10 (25.6)	11 (32.4)	
≥ 150 X 103 /uL	52 (71.2)	29 (74.4)	23 (67.6)	0.527
FIB-4 score (n=74)				
≤ 1.45	38 (51.4)	21 (53.8)	17 (48.6)	
> 1.45	36 (48.6)	18 (46.2)	18 (51.4)	0.65
APRI (n=74)				
≤ 0.5	32 (43.2)	21 (53.8)	11 (31.4)	
> 0.5	42 (56.8)	18 (46.2)	24 (68.6)	0.052
AFP (ng/mL) (n = 54)				
< 15	46 (85.2)	24 (88.9)	22 (81.5)	
≥ 15	8 (14.8)	3 (11.1)	5 (18.5)	0.444
Chronic hepatitis B status				
Inactive/mild hepatitis B	60 (76.9)	37 (86.0)	23 (65.7)	
Active hepatitis B	18 (23.1)	6 (14.0)	12 (34.3)	0.034*
Current hepatitis B therapy (n=77)				
No treatment	59 (76.6)	27 (64.3)	32 (91.4)	
Anti-viral drug treatment	18 (23.4)	15 (35.7)	3 (8.6)	0.005*

P values were calculated by Chi-square test. Some variables had missing data and n is given in parentheses. * Data are shown as P value < 0.05, AST: aspartate aminotransferase; ALT, alanine aminotransferase; FIB-4, fibrosis-4 score; APRI, AST to platelet ratio index; AFP, Alpha-fetoprotein.

availability of HBV genotype data, rates of infection with genotypes B, C, and mixed B and C genotypes were 25.0% (14/53), 73.2% (41/56), and 1.8% (1/56) respectively. Of the 72 patients, there were 4 patients coinfected with HIV

Table 2. Medians of Clinical Features of Chronic Hepatitis B Patients Stratified by HBV A1762T/G1764A Mutation (n = 78)

Characteristics	Chronic hepatitis B infection with		p
	Wild-type (n= 43)	*A1762T/G1764A* Mutant (n=35)	
HBsAg (S/CO) (n=72)	1,536.7 (53.0-5,860.0)	3,409.9 (14.0-6,097.0)	0.011*
HBeAg (S/CO) (n= 73)	1.0 (0.2-1876.4)	0.3 (0-1633.5)	0.012*
HBV viral load (copies/mL) (n= 67)	17,300.0 (46-640,000,000)	757,000.0 (2,206-640,000,000)	0.047*
AST (U/L)	42 (25-2,038)	68.0 (21-582)	0.076
ALT (U/L)	30 (9-1651)	49 (11-965)	0.05
APRI (n= 74)	0.43 (0.10-15.12)	0.73 (0.01-17.23)	0.184
FIB-4 score = (n= 74)	1.18 (0.33-15.80)	1.60 (0.02-27.84)	0.223
AFP (ng/mL) (n=54)	2.71 (1.00-266.00)	4.25 (1.00-1,210.00)	0.076

Data are shown as median (range). Some variables had missing data and n is given in parentheses. *Data are shown as P value < 0.05, AST, aspartate aminotransferase; ALT, alanine aminotransferase; FIB-4, fibrosis-4 score; APRI, AST to platelet ratio index; AFP, Alpha-fetoprotein.

(5.6%). Hepatitis B patients were evaluated for active hepatitis B and liver abnormality. There were 18 out of 78 (23.4%) patients with active hepatitis B. The rate of patients having transaminitis was 76.9% (60/78), while those having significant liver fibrosis, assessed by FIB-4 score >1.45 and APRI > 0.5, were 48.6% (36/74) and 58% (42/74) consequently. Out of 54 hepatitis B patients, there were 8 patients (14.8%) having higher AFP levels than 15 ng/mL. In this study group, there were only 18 patients (23.4%) currently on hepatitis B medication.

In his study, the effect of HBV A1762/G1764A double mutation on clinical features of chronic hepatitis B patients was examined by Chi-square test (Table 1). Characteristics of hepatitis B patients infected with wild-type (n = 43) and HBV A1762T/G1764A mutant HBV (n= 35), including age, gender, HBV viral load, ALT levels, thrombocytopenia, FIB-4score, and AFP levels were similar (p > 0.05). Apparently, the A1762/G1764A mutation was significantly associated with HBeAg status (p = 0.024), HBsAg levels (p = 0.007), and an active phase of CHB (p = 0.034). The mutation was also significantly associated with abnormal levels of AST (p = 0.008) and transaminitis (p = 0.006). The data also indicated a trend of association with abnormal ALT levels (p = 0.050). However, there was no significant association of the mutation with liver fibrosis assessed by FIB-4 score and APRI, as well as abnormal AFP levels (p > 0.05). Notably, there was a significant difference in the medication of patients with A1762/G1764A mutant compared to those with wild-type HBV (p = 0.005).

Consistently, the analysis by Mann-Whitney U test (Table 2) indicated significantly higher levels of HBsAg and HBV viral load (p = 0.011 and p = 0.047 respectively) and lower levels of HBeAg (p = 0.012) in patients with mutant than those with wild-type HBV. Although ALT levels were likely to be higher in the patients infected with the mutant than wild-type HBV (p = 0.05), the levels of AST, FIB-4 score, APRI, and AFP in the patients with the mutant HBV were similar to those with wild-type HBV (p > 0.05). Binary logistic regression analysis (Table 3) identified viral factors including HBeAg negativity, HBsAg > 2,000 S/CO, and active hepatitis B, but not HBV viral load > 2,000 IU/mL, as independent predictive factors for the HBV A1762/G1764A mutation (OR 3.1, 95%CI 1.1-8.6, p = 0.027, OR 3.8 95%CI 1.4-10.4, p = 0.008, OR 3.4, 95% CI 1.1-11.27, p = 0.037, respectively). While abnormal levels of liver enzymes, defined as transaminitis, were predictive for the HBV mutation (OR 5.7, 95%CI 1.5-21.8, p = 0.011), those of liver fibrosis markers, FIB-4 score and APRI, and liver cancer marker AFP were not significantly associated with the HBV mutation (p > 0.05). The data indicated associations of the HBV A1762/G1764A double mutation with clinical features involving viral production and abnormal levels of liver enzymes, but

Table 3. Binary Logistic Regression Analysis to Assess Associations of HBV A1762T/G1764A Mutation with Clinical Features in CHB Patients (n = 78)

Characteristics	Patients n	Crude OR (95% CI)	P
HBeAg negativity	73	3.1 (1.1-8.6)	0.027*
HBsAg > 2,000 S/CO	72	3.8 (1.4-10.4)	0.008*
HBV Viral load > 2,000 IU/mL	73	2.5 (1.0-6.7)	0.061
Active hepatitis B	78	3.4 (1.1-11.27)	0.037*
Transaminitis (AST and/or ALT > ULN (40 U/L))	78	5.7 (1.5-21.8)	0.011*
FIB-4 score > 1.45	74	1.2 (0.5-3.1)	0.651
APRI > 0.5	74	2.5 (1.0-6.6)	0.054
AFP ≥ 15 ng/mL	54	1.8 (0.4-8.5)	0.448

*Data are shown as P value < 0.05; OR, odds ratio; CI, confidence interval

Table 4. Risk Factors for Active Hepatitis B in HBV-infected Patients Analyzed by Binary Logistic Regression (n = 78)

Characteristics	Patients n (%)	Patients with active hepatitis B n (%)	Active hepatitis B	
			Crude OR (95% CI)	P
Gender (n = 78)				
Female	29 (37.2)	6 (37.5)	1	
Male	49 (62.8)	10 (62.5)	1.0 (0.3-3.1)	0.976
Age (n = 78)				
< 50	40 (51.3)	9 (56.3)	1	
≥ 50	38 (48.7)	7 (43.8)	0.8 (0.2-2.4)	0.656
HBsAg (S/CO) (n = 72)				
≤ 2,000	32 (44.4)	3 (21.4)	1	
> 2,000	40 (55.6)	11 (78.6)	3.7 (0.9-14.5)	0.064
HBeAg status (n = 73)				
Negative	45 (61.6)	6 (42.9)	1	
Positive	28 (38.4)	8 (57.1)	2.6 (0.8-8.5)	0.115
A1762T/G1764A mutation (n = 78)				
Wild-type	43 (55.1)	5 (31.3)	1	
Mutant	35 (44.9)	11 (68.8)	3.5 (1.1-11.3)	0.037*
Current therapy (n=77)				
Anti-viral drug treatment	18 (23.4)	2 (12.5)	1	
No treatment	59 (76.6)	14 (87.5)	0.4 (0.1-2.0)	0.26

Data are shown as p value < 0.05. Some variables had missing data and n is given in parentheses. OR, odds ratio; CI, confidence interval

not markers reflecting chronic liver damage including, FIB-4 score, APRI, and AFP levels.

Since active phases of hepatitis B classified according to the EASL2017 guideline is recommended for treatment in CHB regardless of fibrosis [14], clinical risk factors for active hepatitis B in this study group were examined (Table 4). Apparently, only the HBV A1762/G1764A mutation was identified as an independent risk factor for active hepatitis B (OR 3.5, 95% CI 1.1-11.3, p = 0.037), whereas other clinical parameters, gender, age, HBsAg levels, HBeAg status, and current therapy were not predictive (p > 0.05).

Discussion

This cross-sectional study demonstrated a high HBV A1762/G1764A double mutation prevalence in Thai CHB patients. Most patients presented detectable levels of HBV viral load with a relatively high HBV DNA median, and there was a high percentage of patients with HBeAg negativity in this study group. Consistent with the previous study [16], higher rates of HBV genotype C than that of HBV genotype B were reported. Evaluation of liver function demonstrated that most patients have elevated liver enzymes, AST, and ALT, and showed significant liver fibrosis, a sign of chronic liver disease, assessed by non-invasive markers, FIB-4 score, and APRI. Apparently, a relatively high percentage of active hepatitis B, defined as high levels of HBV DNA (> 20,000 IU/mL) and increased ALT values (2 x ULN), was observed. However, there was a low prevalence of patients with abnormal levels of liver cancer marker, AFP, and a low rate of patients who are on current medication especially those infected with the A1762/G1764A mutant (8.6%).

This study indicated the effect of BCP A1762/G1764A mutation on viral production. There was a strong association of A1762/G1764A mutation with HBeAg status and a significantly lower level of HBeAg in patients infected with the mutant HBV. The result was consistent with the previous studies indicating a higher frequency of A1762/G1764A mutation in patients with HBeAg negativity [17] and this is potentially due to the fact that BCP/PC mutations reduce basal core promotor activities, leading to a loss of HBeAg production and progression to a severe form of CHB [18, 9, 19]. While the influence of A1762/G1764A mutation on HBeAg production is well-studied, a few studies demonstrated the effect of A1762/G1764A mutation on the capacity of viral replication. The in vivo experiments indicated that the mutated HBV exhibited higher levels of pregenomic RNA transcription and HBV DNA replication than the wild-type strain [9]. This is correlated with our analysis demonstrating a significant association of the mutation with HBsAg levels higher than 2,000 S/CO and higher medians of HBsAg and HBV viral load in patients infected with the mutant than those with the wild-type.

Since BCP A1762/G1764A mutation has previously been implicated in liver disease progression and proposed as a biomarker for HCC [10-12, 7], the effect of BCP A1762/G1764A mutation on the severity of liver disease was also examined. The data indicated an association of the mutation with the elevated liver enzyme demonstrated by transaminitis and the tentatively higher median levels of ALT in the patients infected with the HBV mutant. This may be a consequence of the higher viral production as demonstrated by HBV DNA and HBsAg levels in the

mutant HBV-infected patients. However, there was no correlation between the HBV mutation and long-term disease progression assessed by FIB-4 score and APRI, indicating no impact of the mutation on liver fibrosis in the studied group. However, it is important to note that most patients in this study were at the early stage of liver fibrosis with relatively low median levels of FIB-4 and APRI (Table 2). Consistently, there was a low number of patients with abnormal levels of AFP, a liver cancer marker, and no association between the HBV mutation and abnormal levels of AFP was observed. Therefore, long-term monitoring of patients with more advanced liver diseases is required.

According to the European Association for the Study of the Liver (EASL) 2017 clinical practice guidelines on hepatitis B virus infection [14], patients with HBV DNA > 20,000 IU/mL and ALT > 2 times the upper limit of normal (ULN), defined as active hepatitis B, should start treatment regardless of the degree of liver fibrosis. Our data indicated that active hepatitis B status was significantly associated with the A1762/G1764A mutation. The risk analysis of active hepatitis B in this studied group also suggested that only the infection with A1762/G1764A HBV mutant was predictive for active hepatitis B. The patients with the HBV mutant carried 3.5 times higher risk than those with the wild type, suggesting the BCP A1762/G1764A mutation as a biomarker for monitoring hepatitis B progression. The finding of A1762/G1764A mutation-associated active hepatitis B is consistent with the high prevalence of HCC observed in Thai chronic HBV patients [3].

This study has some limitations. The cross-sectional study design is unable to control some confounding factors. A limited number of subjects and missing values in some variables may limit statistical significance for the variable tested and further cohort studies in this patient group are required. In summary, this study reports the prevalence rate of HBV infection with BCP A1762/G1764A mutant in Thai CHB patients and demonstrates an impact of the mutation on hepatitis virus profiles, HBeAg, HBsAg as well as HBV DNA production, and severity of liver disease assessed by the elevated liver enzymes, but not liver fibrosis. Apparently, as a predictive factor of active hepatitis B, the detection of A1762/G1764A mutation could be used in hepatitis B management and a long-term prospective study on the influence of this HBV mutation is warranted.

Author Contribution Statement

C. A. contributed to funding acquisition, resources, supervision, study design, data analysis, manuscript preparation, review, and editing, K.C. and T. D. are responsible for funding acquisition, supervision, data collection and analysis, and manuscript preparation. T. S. participated in data collection and analysis. W. F. contributed to blood samples and clinical data collection, and D. P. contributed to statistical analysis.

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General

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Ethical Declaration

The study protocol was approved by the Human Ethics Committees Thammasat University (COA No. 144/2562)

Data Availability

The data supporting this study are available from the corresponding author upon reasonable request.

Conflict of Interest

The authors declare no conflict of interest.

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