Mutations in the HBV PreS/S gene related to hepatocellular carcinoma in Vietnamese chronic HBV-infected patients

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Abstract

Background

Chronic hepatitis B virus (CHB) infection is a major health problem and leading cause of hepatocellular carcinoma (HCC) worldwide. Several point and deletion mutations on the PreS/S gene have been intensively considered associated with HCC. This study aimed to describe the characteristics of HBV PreS/S mutations in Vietnamese CHB-infected patients and their association with HCC.

Methods

This cross-sectional study was conducted from 02/2020 to 03/2021, recruited Vietnamese CHB-infected patients with HBV-DNA >3 log10 copies/mL and successful PreS/S gene sequencing. Mutations were detected by direct Sanger sequencing.

Results

247 CHB-infected patients were recruited, characterized by 68.8% males, 54.7% HBV genotype B, 57.5% HBeAg positive, 23.1% fibrosis score ≥F3 and 19.8% HCC. 61.8% amino acid replacements were detected throughout the PreS1/PreS2/S genes. The most common point-mutations included N/H51Y/T/S/Q/P (30.4%), V68T/S/I (44.9%), T/N87S/T/P (46.2%) on PreS1 gene; T125S/N/P (30.8%), I150T (42.5%) on PreS2 gene; S53L (37.7%), A184V/G (39.3%), S210K/N/R/S (39.3%) on S gene. The rates of case(s) with any point-mutation on the Major Hydrophobic Region (MHR) and the "a" determinant region were 63.6% and 39.7%, respectively. Most of S point-mutations were presented with low rates such as T47A/E/V/K (9.3%), P120S/T (8.5%), G145R (2%). On multivariable analysis, males (OR = 4.51, 95%CI 1.78–11.4, p = 0.001), age ≥40 (OR = 5.5, 95%CI 2.06–14.68, p = 0.001), W4P/R/Y on PreS1 (OR = 11.56, 95%CI 1.99–67.05, p = 0.006) and 4 S point-mutations as: T47A/E/V/K (OR = 3.67, 95%CI 1.19–11.29, p = 0.023), P120S/T (OR = 3.38, 95%
Conclusions

We detected 61% amino acid changes on PreS/S regions in Vietnamese CHB patients. One point-mutation at amino acid 4 on PreS1 gene and 4 point-mutations at amino acids 47, 120, 174, and 203 on S gene were associated with HCC. Further investigations are recommended to further clarify the relationship and interaction between mutations in HBV genome and HCC progression.

Introduction

Chronic HBV (CHB) infection affects 296 million people worldwide in 2019 [1], and has been considered as a major global health problem. CHB infection is the leading cause of liver cirrhosis and contributes over 50% of hepatocellular carcinoma (HCC) [2]. Vietnam locates in the HBV-high-prevalence area in Asia [3] which has the high incidence of HBV related-HCC [2]. It was reported that 62.3% of HCC cases and 81.3% of advanced HCC cases in Vietnam were HBV infected [4, 5].

HBV belongs to the Hepadnaviridae family with an incomplete double strand DNA genome, which carries 4 overlapped open reading frames, coding for 4 proteins PreS/S, pre-Core/Core, Pol, and X. HBsAg proteins are tran
csripted from PreS/S open reading frame that consists of 3 surface proteins: Small (S), Medium (M) and Large (L). The S protein that drives the releasing of viral particles consists of 227 amino acids (aa). The M protein that enriches the secretion of virion contains an extra N-terminal extension of 55 aa. The L protein that is involved in the interaction with core particles in the packaging of virion at the endoplasmic reticulum (ER) has a further N-terminal extension of 108 or 119 aa—depending on genotypes [6]. In the absence of L protein, S protein is secreted alone as noninfectious subviral particles. L protein can suppress the subviral particle secretion depending on the L/S protein ratio. During natural HBV infection subviral particles outnumber virions by a factor of 1000:1. The principal epitopes of HBsAg mainly locate in the “a” determinant (aa 124–147) in the major hydrophilic region (MHR) induces the neutralized B cell responses.

Mutations in PreS/S gene result in deletion of surface proteins or synthesis of varieties of truncated proteins. Especially, mutations on the C-terminal region (aa 179–226) of S gene contribute in retention of HBsAg within the hepatocytes ER [7, 8], activate multiple oncogenic signal pathways, promote the growth of hepatocytes and eventually lead to HCC development. Multiple scientific evidences had proved PreS mutations as prediction markers for HCC development and recurrence of HBV-related HCC [9–12]. The relationship between PreS/S gene mutations and HCC were studied distinctly on the PreS region [13]. Mutations at T53C, PreS deletions, PreS2 start codon, C7A, A2962G, C2964A and C3116T in the PreS region have been proved that significantly increase risk of HCC [14, 15]. Wang et al. (2006) had concluded that the retention of L antigens from the PreS mutants caused ER stress, induced oxidative DNA damage, and resulted in genomic instability. The L antigens from the PreS mutants are attributed to the upregulation cyclooxygenase-2 and cyclin A, and promotion of cell cycle and hepatocytes proliferation [16].

Mutations on the S gene in Vietnamese CHB patients had been described in the earliest study since 2012. Dunford et al. (n = 187) had reported a rate of 31% cases with point-

Funding: This study was funded for the PreS/S gene Sequencing by the grant of research from the University of Medicine and Pharmacy at Ho Chi Minh city. The funders had no role in study design, data collection and analysis, decision to publish, or preparation of the manuscript.

Competing interests: The authors have declared that no competing interests exist.
mutation in the immunodominant ‘a’ region, especially two major vaccine escape mutations with minor rates as G145A/R (2.2%) and P120L/Q/S/T (5.3%) had been detected [17]. The mutation of PreS deletion with a rate of 20% was reported from Matsuo et al. (2017) in Vietnamese CHB-infected patients [18]. Bui TTT et al. (2017) described more concretely about point-mutations (N38E 71.9%, N38K 71.1%, A60V/E 100% on the PreS1 region, L126T/S 77% on the PreS2 and N3S 27.4% on the S region) [19]. In a multicenter study on 660 patients from China, Korea and Vietnam, Kim et al. (2018) [20] had reported 237 amino acid mutations in the MHR on the S region. There was not any report on mutations and their association with HCC on the PreS/S gene in Vietnamese patients.

In this study, we described the characteristics of HBV PreS/S gene mutations in Vietnamese patient with CHB and analyzed the relations of these mutations with HCC.

**Materials and methods**

**Study design and population**

The cross-sectional study had been conducted at the Hepatology Clinic of University Medical Center (UMC), Ho Chi Minh city from February 2020 to March 2021. There were 293 male and female CHB patients participated in this study, who met the inclusion criteria of being older than 18 years old, had HBsAg positive more than 6 months, no previous nucleos(t)ide analogues treatment (NAs) and HBV DNA >3 log_{10} copies/mL. Their serum samples were extracted from 4 mL blood, stored in -80 celcius degree for PreS/S gene sequencing. Serum samples of 247 patients that had been successfuly sequenced the HBV PreS/S gene were analyzed for the final results. Among them, there were 212 CHB patients whose serum samples had been stored during 2014–2016 and 35 CHB patients were recruited in 2020–2021.

**Variables and measurements**

Personal characteristics, times from diagnosis of CHB infection and HBV markers were collected from the hospital electronic database. HCC was defined for cases with tumor detected on abdominal ultrasound, serum alpha-fetoprotein (AFP) >20 ng/mL and was confirmed on abdominal CT scan with focal lesions with early arterial phase enhancement and rapid "wash-out" in venous phase [21]. Cirrhosis was defined as having signs of portal hypertension (splenomegaly, ascites, vascular collaterals on abdominal wall, esophageal varices or portal hypertensive gastropathy on gastroscopy) and signs of insufficiency of liver function (palmar erythema, vascular spiders, low concentration of albumin (<35 g/dL), elevation of the international normalized ratio (INR >1.1), thrombocytopenia (<160,000/mm³)) as well as irregularity of hepatic surface on ultrasonography or F3 on Metavir score using Acoustic radiation force impulse (ARFI) measurement [22].

HBsAg quantification (Elecsys HBsAgII Quant-Roche kit), HBeAg (Cobas-Roche kit) and HBV DNA quantification (using the AccuPid HBV Quantification kit (KT-Biotech)) with limits of detection ≥300 copies/mL, linear range: 300 to 10⁸ copies/mL were performed at the University Medical Center laboratory. HBV genotype (B or C) was determined based on the sequence of S gene.

**PreS/S mutation analyzed by sanger sequencing**

PreS/S mutations were analyzed at Center for Molecular Biomedicine of University of Medicine and Pharmacy (UMP) at Ho Chi Minh City. HBV DNA was extracted from serum using the GeneJet™ Viral DNA and RNA Purification kit (Thermo Fisher Scientific, Waltham, MA, USA) according to the manufacturer’s protocol. Two sets of overlapping primers were then
used to amplify the whole PreS/S region of HBV with TaKaRa Taq\textsuperscript{TM} HotStart Polymerase (Takara Bio, Shiga, Japan). Primers for the PreS1/PreS2 region were: FA2-L (5’ - TTGAGAGAAGTCCACCACGAG-3’) and FA2-R (5’ - GCCGTGCGAGAATCTCAAT-3’); S region were FA3-L (5’ - CTGCTGGTGCTCCAGTT-3’); FA3-R (5’ - GCCATGAACTTGGCGA-3’). PCR involved initial denaturation at 98˚C for 3 min followed by 45 cycles of 98˚C for 10 sec, 58˚C for 30 sec, and 72˚C for 60 sec, with a final elongation of 72˚C for 2 min. PCR products were checked for size and purity using 1.5% agarose gel electrophoresis. PCR product was purified enzymatically using the ExoSAP-IT\textsuperscript{TM} PCR Product Cleanup Reagent (Thermo Scientific, Waltham, MA) to remove excess primers and dNTPs before Sanger sequencing using the BigDye Terminator v3.1 Kit and the ABI 3500 Genetic Analyzer (Applied Biosystems, Foster City, CA). PCR fragment was sequenced and analyzed in both directions. The sequences were compared to the reference sequence of genotype B (GenBank_AB073846) and genotype C (GenBank_X04615) using the CLC Main Workbench software (Qiagen, Germany).

Statistics

The SPSS 25.0 software was used to analyze the data. Percentage was used to present the rates of point-mutations, the rates of possessing at least one mutation and the rates of genes deletion on each region. The Chi-square test (or Fisher exact test) was used to compare the distributions of mutations among groups with or without HCC. Multivariable analysis with logistic regression was used to find out factors related to HCC. Two-side p value of <0.05 was considered statistically significant.

Ethics considerations

The study was done based on the Declaration of Helsinki. Stored serum samples and all variables included in the study was approved by the Ethics Committee of the University of Medicine and Pharmacy at Ho Chi Minh City (Reference number: 119/HDDD). Informed consents were obtained from all participants prior to the study.

Results

Characteristics of the study population

The study included 247 CHB patients, 68.8% were males, 57.9% were older than 40 years of age and older. 57.5% were positive with HBeAg marker, 83% had HBV DNA $\geq 5 \log_{10}$ copies/mL, 54.7% genotype B. 23.1% were with liver fibrosis and 19.8% were with HCC (Table 1).

Table 1. Characteristics of the study population (n = 247).

| Characteristics           | n (%) |
|---------------------------|-------|
| Sex (male)                | 170 (68.8) |
| Age group $\geq$40        | 143 (57.9) |
| HBeAg positive            | 142 (57.5) |
| HBV DNA $\geq$5 ($\log_{10}$ copies/mL) | 205 (83) |
| Genotype B (n = 245)      | 135 (54.7) |
| Fibrosis $\geq$F3         | 57 (23.1) |
| HCC                       | 49 (19.8) |

https://doi.org/10.1371/journal.pone.0266134.t001
Characteristics of the detected mutations on the PreS1, PreS2 and S genes in overall population and the HCC subgroups

There were 248/401 (61.8%) amino acid replacements that were detected throughout the PreS1/PreS2/S genes on the overall populations. In the PreS1 region, 57.1% replacements (68/119) were found. The mutations with a rate over 30% were N51Y/T/S/Q/P 30.4%, V68T/S/I 44.9%, and T/N87S/T/P 46.2%; from 10 to <30% were Q10K/H/R (16.2%), H48Y/N (13.8%), E/D54A/N (25.1%), I/N56H/W (25.9%), K57Q/K (25.1%), A60V (25.5%), D/A62S/T (25.1%), G73S/N (24.3%), and V95A (24.3%); and from 5 to <10% were G35R/K (8.5%), and I84V/M/L (7.7%). These above mutations were not differently distributed among non-HCC and HCC group. Interestingly, most of amino acid changes (54/68) in the preS1 were presented with the rates <5%. Among them, 4 point-mutations W4P/R/Y, S5L/T, A90T/S/G, and L108V/I were observed with the significantly higher rates in the HCC group compared to non-HCC group: W4P/R/Y (12.2% vs 2%, p = 0.005), S5L/T (6.1% vs 1%, p = 0.055), A90T/S/G (6.1% vs 0.5%, p = 0.026) and L108V/I (6.1% vs 0.5%, p = 0.026) (Table 2). In the PreS2 region, 41/55 (74.5%) amino acid changes were detected. The point-mutations with the rates of over 30% were T125S/N/P (30.8%), I150T (42.5%), and V158A (36%); from 10 to <30% of population were M120V/I (11.3%) and F141V/L/I (11.3%); and from 5 to <10% of population were Q121R/K (5.3%), T164I/D/S (6.1%), and F165S/L (5.7%). As same as the PreS1 region, most of amino acid changes on the PreS2 region (34/41 sites) were presented at a rate <5% (Table 3). Only F141V/L/I was found with a significantly higher rate in the HCC group (18.4% vs 9.6%, p = 0.08). The PreS1 deletion was detected in 27.5% (68 patients), and the PreS2 deletion was observed in 16.2% (40 patients) (Tables 2 and 3). In the S region, 61.2% amino acid changes were detected (139/227). The rate of cases with at least one point-mutation detected on the "a" determinant region (aa 124–148) was 39.7% and on the MHR region (aa 100–160) was 63.6%. The HCC group had significant higher rate of possessing ≥1 point-mutation on the MHR region (79.6% vs 59.6%, p = 0.009). The point-mutations on the S gene that owned the rates of >30% of the population were: S53L (37.7%), A184V/G (39.3%), and S210K/N/R/S (39.3%); from 15 to <30% were L21S (29.1%), G44E/V (18.6%), I126T/N/S (21.1%), and M198I/M (18.2%); and from 5 to <15% were V14A/G/Q (10.1%), N40S/K (6.9%), T47A/E/V/K (9.3%), P/L49R/H (5.7%), P62Q/L (9.7%), C76Y/T/W (10.5%), Y100C/F (5.3%), P120S/T (8.5%), R122K (8.9%), M133T/S/L/I (7.7%), Y161F/S (10.1%), T189I (5.3%), S204R/N (10.1%), I208T/S (5.7%), L213I/M (7.3%), and V224A (11.7%). Half of the amino acid changes (116/227) on the S region had the detection rates <5% and most of them were not related to HCC. Exceptionally, 13 S mutations presented the higher distributions (p <0.1) in the HCC group: F20S (8.2% vs 1%, p = 0.015), D33G (4.1% vs 0%, p = 0.039), (T47A/E/V/K (18.4% vs 7.1%, p = 0.025), R79H (6.1% vs 0%, p = 0.007), L88P (4.1% vs 0%, p = 0.039), P120S/T (on the MHR region, 16.3% vs 6.6%, p = 0.042), G145R (6.1% vs 1%, p = 0.055), S174N (6.1% vs 0.5%, p = 0.026), V190A (6.1% vs 0.5%, p = 0.026), P203R (8.2% vs 2%, p = 0.052), Y206H/F/C (6.1% vs 1.5%, p = 0.094), L209V/S/G (6.1% vs 1%, p = 0.055) and F212Y/L/C (8.2% vs 0.5%, p = 0.006), (Table 4).

The point-mutations on the Pres1/Pres2/S genes related to HCC–multivariable analysis

Nineteen point-mutations that distributed differently (p <0.1) among the HCC and non-HCC groups were checked to remove their interactions using multivariable analysis (Tables 2–4). Six point-mutations remained related to HCC. They were one mutation on the PreS1 region: W4P/R/Y (OR = 5.48, 95%CI 1.32–22.83) and 5 mutations on the S region: F20S (OR = 9.72,
Table 2. Distribution of point and deletion mutations on the PreS1 gene (n = 247).

| PreS1 (aa 1–119) | Overall population n (%) | HCC n (%) | p<sup>a</sup> |
|-------------------|---------------------------|-----------|--------------|
|                   | Yes (n = 49) | No (n = 198) |               |
| Point-mutations   |             |            |               |
| G2R/G             | 1 (0.4)     | 1 (0.5)    | 1            |
| W4P/R/Y           | 10 (4)      | 6 (12.2)   | 4 (2.0)      | 0.003<sup>b</sup> |
| S5L/T             | 5 (2)       | 3 (6.1)    | 2 (1.0)      | 0.055<sup>b</sup> |
| S6A               | 3 (1.2)     | 1 (2.0)    | 2 (1.0)      | 0.49 |
| K7N               | 1 (0.4)     | 0          | 1 (0.5)      | 1   |
| P8T               | 1 (0.4)     | 1 (2.0)    | 0            | 0.2 |
| Q10K/H/R          | 40 (16.2)   | 8 (16.3)   | 32 (16.2)    | 0.98 |
| T14I/T            | 1 (0.4)     | 0          | 1 (0.5)      | 1   |
| S157/A            | 4 (1.6)     | 1 (2.0)    | 3 (1.5)      | 1   |
| P19S              | 1 (0.4)     | 0          | 1 (0.5)      | 1   |
| F25L              | 1 (0.4)     | 1 (2.0)    | 0            | 0.2 |
| D27G/S            | 33 (13.4)   | 6 (12.2)   | 27 (13.6)    | 0.8  |
| I31T              | 1 (0.4)     | 1 (2.0)    | 0            | 0.2 |
| P32L              | 3 (1.2)     | 0          | 3 (1.5)      | 1   |
| A33F/L            | 2 (0.8)     | 1 (2.0)    | 1 (0.5)      | 0.36 |
| F34Y              | 1 (0.4)     | 0          | 1 (0.5)      | 1   |
| G35R/K            | 21 (8.5)    | 3 (6.1)    | 18 (9.1)     | 0.78 |
| S38T              | 1 (0.4)     | 0          | 1 (0.5)      | 1   |
| N/E39K/G/D/A      | 11 (4.5)    | 2 (4.1)    | 9 (4.5)      | 1   |
| N40Y/T            | 2 (0.8)     | 0          | 2 (1.0)      | 1   |
| D42N              | 1 (0.4)     | 0          | 1 (0.5)      | 1   |
| D44H/N            | 3 (1.2)     | 0          | 3 (1.5)      | 1   |
| L45R/F            | 10 (4.0)    | 3 (6.1)    | 7 (3.5)      | 0.42 |
| H48Y/N            | 34 (13.8)   | 3 (6.1)    | 31 (15.7)    | 0.08 |
| D50N/E            | 2 (0.8)     | 1 (2.0)    | 1 (0.5)      | 0.36 |
| N/H51Y/T/S/Q/P    | 75 (30.4)   | 15 (30.6)  | 60 (30.3)    | 0.97 |
| E/D54A/N          | 62 (25.1)   | 9 (18.4)   | 53 (26.8)    | 0.23 |
| A55S              | 1 (0.4)     | 0          | 1 (0.5)      | 1   |
| I/N56H/W          | 64 (25.9)   | 14 (18.6)  | 50 (25.3)    | 0.64 |
| K57Q/K            | 62 (25.1)   | 13 (26.5)  | 49 (24.7)    | 0.80 |
| G59A              | 1 (0.4)     | 1 (2.0)    | 0            | 0.2 |
| A60V              | 63 (25.5)   | 14 (28.6)  | 49 (24.7)    | 0.58 |
| D/A62S/T          | 62 (25.1)   | 15 (30.6)  | 47 (23.7)    | 0.32 |
| P65T              | 1 (0.4)     | 0          | 1 (0.5)      | 1   |
| F67V/L            | 2 (0.8)     | 0          | 2 (1.0)      | 1   |
| V68T/S/I          | 111 (44.9)  | 16 (32.7)  | 95 (48)      | 0.053<sup>*</sup> |
| P70S              | 1 (0.4)     | 0          | 1 (0.5)      | 1   |
| G73S/N            | 60 (24.3)   | 11 (22.4)  | 49 (24.7)    | 0.74 |
| L74M/V            | 6 (2.4)     | 1 (2.0)    | 5 (2.5)      | 1   |
| L75V/M            | 5 (2.0)     | 0          | 5 (2.5)      | 0.59 |
| W77R/G            | 5 (2.0)     | 1 (2.0)    | 4 (2.0)      | 1   |
| S78N              | 3 (1.2)     | 1 (2.0)    | 2 (1.0)      | 0.49 |
| Q80L/H            | 3 (1.2)     | 1 (2.0)    | 2 (1.0)      | 0.49 |
| A81T              | 3 (1.2)     | 1 (2.0)    | 2 (1.0)      | 0.49 |
| Q82L              | 1 (0.4)     | 0          | 1 (0.5)      | 1   |

(Continued)
Table 2. (Continued)

| PreS1 (aa 1–119) | Overall population n (%) | HCC n (%) | p* |
|-------------------|---------------------------|-----------|----|
|                   | Yes (n = 49) | No (n = 198) |    |
| I84V/M/L          | 19 (7.7) | 3 (6.1) | 16 (8.1) | 0.77 |
| L85I/H/F          | 3 (1.2) | 1 (2.0) | 2 (1.0) | 0.49 |
| T86A/S            | 6 (2.4) | 2 (4.1) | 4 (2.0) | 0.34 |
| T/N87S/T/P        | 114 (46.2) | 18 (36.7) | 96 (48.5) | 0.14 |
| V88I/M/L          | 5 (2) | 2 (4.1) | 3 (1.5) | 0.26 |
| P98R              | 1 (0.4) | 0 | 1 (0.5) | 1 |
| A90T/S/G          | 4 (1.6) | 3 (6.1) | 1 (0.5) | 0.026^b |
| A91T/P            | 8 (3.2) | 1 (2.0) | 7 (3.5) | 1 |
| P92L              | 1 (0.4) | 0 | 1 (0.5) | 1 |
| P94T/S            | 5 (2.0) | 2 (4.1) | 3 (1.5) | 0.26 |
| V95A              | 60 (24.3) | 12 (24.5) | 48 (24.2) | 0.97 |
| T97I/T/A          | 4 (1.6) | 0 | 4 (2.0) | 0.59 |
| N98T/K/I          | 3 (1.2) | 0 | 3 (1.5) | 1 |
| S101T/L           | 2 (0.8) | 0 | 2 (1.0) | 1 |
| G102R/K           | 2 (0.8) | 0 | 2 (1.0) | 1 |
| R103T             | 1 (0.4) | 0 | 1 (0.5) | 1 |
| Q104R/K           | 4 (1.6) | 2 (4.1) | 2 (1.0) | 0.18 |
| L108V/I           | 4 (1.6) | 3 (6.1) | 1 (0.5) | 0.026^b |
| S109T             | 11 (4.5) | 2 (4.1) | 9 (4.5) | 1 |
| R113T             | 1 (0.4) | 0 | 1 (0.5) | 1 |
| D114E             | 1 (0.4) | 0 | 1 (0.5) | 1 |
| T115S/C           | 6 (2.4) | 0 | 6 (3.0) | 0.6 |
| Q118L             | 1 (0.4) | 0 | 1 (0.5) | 1 |
| A119V             | 1 (0/4) | 0 | 1 (0.5) | 1 |
| PreS1 deletion    | 68 (27.5%) | 15 (30.6) | 53 (26.8) | 0.59 |

percentage by column, ^ Chi-square test, b Fisher Exact test.

https://doi.org/10.1371/journal.pone.0266134.t002

95%CI 1.55–61.06, T47A/E/V/K (OR = 2.91, 95%CI 1.04–8.13), P120S/T (OR = 4.26, 95%CI 1.58–11.52), S174N (OR = 18.21, 95%CI 1.77–187.65), and P203R (OR = 9.72, 95%CI 1.55–61.06) (Table 5).

Personal and viral characteristics were also evaluated for their confounding effects on the correlation between mutations and HCC. Five basal characters that had different distributions in HCC and non-HCC groups were male (83.7% vs 65.2%, p = 0.012), age group >40 (85.7% vs 51%, p < 0.001), HBeAg negative (61.2% vs 37.9%, p = 0.003), HBV DNA <5 log10copies/mL (46.9% vs 9.6%, p < 0.001), and liver fibrosis of >F3 (38.8% vs 19.2%, p = 0.004) (Table 5).

The final multivariable analysis for related factors to HCC had included gender, age group, HBeAg marker and 6 mutations (PreS1 W4P/R/Y and five S point-mutations as F20S, T47A/E/V/K, P120S/T, S174N, P203R) on Table 5. The result found eight variables that composed of males (OR = 4.51, 95%CI 1.78–11.4, p = 0.001), age >40 (OR = 5.5, 95%CI 2.06–14.68, p = 0.001), HBeAg negative (OR = 2.46, 95%CI 1.1–5.53, p = 0.029) and 5 point-mutations such as W4P/R/Y (OR = 11.56, 95%CI 1.99–67.05, p = 0.006), T47A/E/V/K (OR = 3.67, 95%CI 1.19–11.29, p = 0.023), P120S/T (OR = 3.38, 95%CI 1.09–10.49, p = 0.035), S174N (OR = 29.73, 95%CI 2.12–417.07, p = 0.012) and P203S (OR = 8.45, 95%CI 1.43–50.06, p = 0.019) had significant relations with HCC (Table 6). S174N (S) had the highest OR (29.73) with positive predictive value (PPV), negative predictive value (NPV), sensitivity (SEN),...
### Table 3. Distribution of point and deletion mutations on the PreS2 gene (n = 247).

| PreS2 (aa 120–174) | Overall population n (%) | HCC n (%) | p * |
|---------------------|--------------------------|-----------|-----|
|                     | Yes (n = 49)             | No (n = 198) |     |
| **Point-mutations** |                         |           |     |
| M120V/I             | 28 (11.3)                | 7 (14.3)  | 21 (10.6) | 0.47 |
| Q121R/K             | 13 (5.3)                 | 1 (2.0)   | 12 (6.1)  | 0.47 |
| W122R               | 3 (1.2)                  | 1 (2.0)   | 2 (1.0)   | 0.49 |
| N123T/K             | 3 (1.2)                  | 1 (2.0)   | 2 (1.0)   | 0.49 |
| S124T               | 8 (3.2)                  | 1 (2.0)   | 7 (3.5)   | 0.29 |
| T125S/N/P           | 76 (30.8)                | 12 (24.5) | 64 (32.3) | 0.29 |
| T126N/I/A           | 3 (1.2)                  | 1 (2.0)   | 2 (1.0)   | 0.49 |
| H128L               | 4 (1.6)                  | 1 (2.0)   | 3 (1.5)   | 1   |
| Q129K               | 1 (0.4)                  | 1 (2.0)   | 0         | 0.2 |
| A130T/N             | 12 (4.9)                 | 3 (6.1)   | 9 (4.5)   | 0.71|
| L/Q132I/H           | 5 (2.0)                  | 1 (2)     | 4 (2)     | 1   |
| D133N               | 1 (0.4)                  | 0         | 1 (0.5)   | 1   |
| P134H/T             | 2 (0.8)                  | 0         | 2 (1.0)   | 1   |
| R135K               | 2 (0.8)                  | 0         | 2 (1.0)   | 1   |
| V136A               | 1 (0.4)                  | 1 (2.0)   | 0         | 0.2 |
| R137K/Q             | 4 (1.6)                  | 1 (2.0)   | 3 (1.5)   | 1   |
| A138D               | 1 (0.4)                  | 1 (2.0)   | 0         | 0.2 |
| L139Q/P             | 2 (0.8)                  | 0         | 2 (1.0)   | 1   |
| Y140S/N/H/F/C       | 9 (3.6)                  | 2 (4.1)   | 7 (3.5)   | 1   |
| F141V/L/I           | 28 (11.3)                | 9 (18.4)  | 19 (9.6)  | 0.08|
| A143V               | 1 (0.4)                  | 0         | 1 (0.5)   | 1   |
| S146F               | 2 (0.8)                  | 0         | 2 (1.0)   | 1   |
| S147G               | 1 (0.4)                  | 0         | 1 (0.5)   | 1   |
| S148L               | 4 (1.6)                  | 0         | 4 (2.0)   | 0.59|
| G149E/K             | 7 (2.8)                  | 2 (4.1)   | 5 (2.3)   | 0.63|
| I150T               | 105 (42.5)               | 16 (32.7) | 89 (44.9)| 0.12|
| S152N               | 1 (0.4)                  | 1 (2.0)   | 0         | 0.2 |
| P153L               | 1 (0.4)                  | 0         | 1 (0.5)   | 1   |
| Q155P/H             | 3 (1.2)                  | 2 (4.1)   | 1 (0.5)   | 0.1 |
| N156T/I/S           | 7 (2.8)                  | 1 (2.0)   | 6 (3.0)   | 1   |
| T157S               | 2 (0.8)                  | 0         | 2 (1.0)   | 1   |
| V158A               | 89 (36.0)                | 18 (36.7) | 71 (35.9)| 0.91|
| A160T/P             | 5 (2.0)                  | 1 (2.0)   | 4 (2.0)   | 1   |
| I161T/L             | 4 (1.6)                  | 0         | 4 (2.0)   | 0.59|
| T164I/D/S           | 15 (6.1)                 | 3 (6.1)   | 12 (6.1)  | 1   |
| F165S/L             | 14 (5.7)                 | 3 (6.1)   | 11 (5.6)  | 1   |
| K167T               | 11 (4.5)                 | 2 (4.1)   | 9 (4.5)   | 1   |
| T168I               | 4 (1.6)                  | 0         | 4 (2.0)   | 0.59|
| V172A               | 3 (1.2)                  | 1 (2.0)   | 2 (1.0)   | 0.49|
| P173Q/L             | 4 (1.6)                  | 1 (2.0)   | 3 (1.5)   | 1   |
| N174S/K             | 2 (0.8)                  | 1 (2.0)   | 1 (0.5)   | 0.36|
| **PreS2 deletion** | 40 (16.2)                | 11 (22.4) | 29 (14.6) | 0.18|

percentage by column, * Chi-square test.

https://doi.org/10.1371/journal.pone.0266134.t003
Table 4. Distribution of point-mutations on the S gene (n = 247).

| Point-mutations | Overall population n (%) | HCC n (%) | p* |
|-----------------|---------------------------|-----------|----|
|                 | Yes (n = 49)              | No (n = 198)|    |
| E2G             | 1 (0.4)                   | 0         | 1  |
| N3S             | 1 (0.4)                   | 0         | 1  |
| T4I             | 1 (0.4)                   | 0         | 1  |
| A5T/S           | 11 (4.5)                  | 2 (4.1)   | 9 (4.5) | 1  |
| F8P             | 1 (0.4)                   | 0         | 1 (0.5) | 1  |
| L9P             | 3 (1.2)                   | 1 (2)     | 2 (1) | 0.49 |
| G10R            | 2 (0.8)                   | 1 (2.0)   | 1 (0.5) | 0.36 |
| P11H            | 1 (0.4)                   | 0         | 1 (0.5) | 1  |
| L13P/V          | 2 (0.8)                   | 0         | 2 (1.0) | 1  |
| V14A/G/Q        | 25 (10.1)                 | 5 (10.2)  | 20 (10.1) | 0.98 |
| L15S            | 4 (1.6)                   | 1 (2)     | 3 (1.5) | 1  |
| Q16P            | 2 (0.8)                   | 0         | 2 (1.0) | 1  |
| A17E            | 2 (0.8)                   | 0         | 2 (1.0) | 1  |
| G18R            | 2 (0.8)                   | 1 (2.0)   | 1 (0.5) | 0.36 |
| F19S            | 1 (0.4)                   | 1 (2)     | 0 | 0.20 |
| F20S            | 6 (2.4)                   | 4 (8.2)   | 2 (1.0) | 0.015b |
| L21S            | 72 (29.1)                 | 13 (26.5) | 59 (29.8) | 0.65 |
| L22W            | 1 (0.4)                   | 0         | 1 (0.5) | 1  |
| R24K            | 4 (1.6)                   | 0         | 4 (2.0) | 0.59 |
| I25V/A          | 4 (1.6)                   | 0         | 4 (2.0) | 0.59 |
| T27I            | 2 (0.8)                   | 0         | 2 (1.0) | 1  |
| L28T            | 1 (0.4)                   | 0         | 1 (0.5) | 1  |
| Q30R/K          | 5 (2.0)                   | 2 (4.1)   | 3 (1.5) | 0.26 |
| S31R            | 1 (0.4)                   | 1 (2)     | 0 | 0.20 |
| L32P            | 1 (0.4)                   | 0         | 1 (0.5) | 1  |
| D33G            | 2 (0.8)                   | 2 (4.1)   | 0 | 0.039b |
| S34L            | 1 (0.4)                   | 1 (2)     | 0 | 0.20 |
| W35STOP         | 1 (0.4)                   | 0         | 1 (0.5) | 1  |
| N40S/K          | 17 (6.9)                  | 2 (4.1)   | 15 (7.6) | 0.54 |
| F41S            | 2 (0.8)                   | 0         | 2 (1.0) | 1  |
| L42P/R          | 4 (1.6)                   | 1 (2)     | 3 (1.5) | 1  |
| G43K            | 1 (0.4)                   | 0         | 1 (0.5) | 1  |
| G44E/V          | 46 (18.6)                 | 13 (26.5) | 33 (16.7) | 0.11 |
| A45T/G/V        | 8 (3.2)                   | 3 (6.1)   | 5 (2.5) | 0.20 |
| P46H/L          | 4 (1.6)                   | 1 (2)     | 3 (1.5) | 1  |
| T47A/E/V/K      | 23 (9.3)                  | 9 (18.4)  | 14 (7.1) | 0.025b |
| C48S            | 2 (0.8)                   | 1 (2)     | 1 (0.5) | 0.36 |
| P/L49R/H        | 14 (5.7)                  | 2 (4.1)   | 12 (6.1) | 0.74 |
| Q51L            | 2 (0.8)                   | 0         | 2 (1.0) | 1  |
| S53L            | 93 (37.7)                 | 20 (40.8) | 73 (36.9) | 0.61 |
| S59N            | 1 (0.4)                   | 0         | 1 (0.5) | 1  |
| S61L            | 10 (4.0)                  | 2 (4.1)   | 8 (4.0) | 1  |
| P62Q/L          | 24 (9.7)                  | 6 (12.2)  | 18 (9.1) | 0.51 |
| C64Y            | 1 (0.4)                   | 0         | 1 (0.5) | 1  |
| P67Q            | 6 (2.4)                   | 1 (2)     | 5 (2.5) | 1  |

(Continued)
Table 4. (Continued)

| S (aa 1–227) | Overall population n (%) | HCC n (%) | p* |
|--------------|--------------------------|-----------|----|
|              | Yes (n = 49)             | No (n = 198) |    |
| I68T                     | 9 (3.6)                  | 2 (4.1)    | 1  |
| R73H                     | 1 (0.4)                  | 0          | 1  |
| W74S/L                  | 6 (2.4)                  | 0          | 1  |
| M75T                     | 1 (0.4)                  | 1 (2)      | 1  |
| C76Y/T/W                | 26 (10.5)                | 17 (14.3)  | 1  |
| L77R                     | 7 (2.8)                  | 3 (6.1)    | 1  |
| R79H                     | 3 (1.2)                  | 2 (6.1)    | 0  |
| I92T                     | 4 (1.6)                  | 0          | 1  |
| L95W                     | 5 (2.0)                  | 1 (2)      | 1  |
| V96G                     | 1 (0.4)                  | 1 (2)      | 1  |
| L98V                     | 2 (0.8)                  | 1 (2)      | 1  |
| Y100C/F                 | 13 (5.3)                 | 2 (4.1)    | 1  |
| Q101K/H/R               | 12 (4.9)                 | 2 (4.1)    | 1  |
| M103T                    | 2 (0.8)                  | 0          | 1  |
| I104W                    | 1 (0.4)                  | 0          | 1  |
| I110I/Q                 | 9 (3.6)                  | 2 (4.1)    | 1  |
| R112K                    | 1 (0.4)                  | 0          | 1  |
| T113N                    | 1 (0.4)                  | 0          | 1  |
| S114I/P/A               | 7 (2.8)                  | 3 (6.1)    | 1  |
| T115N                    | 1 (0.4)                  | 1 (2)      | 1  |
| T116N                    | 1 (0.4)                  | 0          | 1  |
| T118M                    | 1 (0.4)                  | 0          | 1  |
| P120S/T                 | 21 (8.5)                 | 8 (16.3)   | 1  |
| R122K                    | 22 (8.9)                 | 5 (10.2)   | 1  |
| T123A/N                 | 4 (1.6)                  | 1 (2)      | 1  |
| I126I/N/S               | 52 (21.1)                | 13 (26.5)  | 1  |
| P127T/A/S               | 12 (4.9)                 | 3 (6.1)    | 1  |
| A128Y                    | 2 (0.8)                  | 1 (2)      | 1  |
| Q129R/N/L/H             | 5 (2.0)                  | 2 (4.1)    | 1  |
| G130R                    | 1 (0.4)                  | 0          | 1  |
| T131N/S                 | 10 (4.0)                 | 2 (4.1)    | 1  |
| S132P                    | 2 (0.8)                  | 1 (2)      | 1  |
| M133T/S/L/I             | 19 (7.7)                 | 5 (10.2)   | 1  |
| F134Y/V/L/I             | 6 (2.4)                  | 2 (4.1)    | 1  |
| S136F                    | 1 (0.4)                  | 1 (2)      | 1  |
| T140I                    | 7 (2.8)                  | 1 (2)      | 1  |
| T143M                    | 3 (1.2)                  | 0          | 1  |
| D144E/A/D               | 2 (0.8)                  | 0          | 1  |
| G145R/A                 | 5 (2.0)                  | 3 (6.1)    | 2 (1) |
| N147S                    | 1 (0.4)                  | 1 (2)      | 0  |

(Continued)
Table 4. (Continued)

| S (aa 1–227) | Overall population n (%) | HCC n (%) | p* |
|--------------|--------------------------|-----------|---|
|              | Yes (n = 49)             | No (n = 198) |   |
| P151H        | 1 (0.4)                  | 0         | 1 |
| W156L        | 2 (0.8)                  | 1 (2)     | 0.36 |
| A159V        | 9 (3.6)                  | 3 (6.1)   | 0.39 |
| R160K        | 6 (2.4)                  | 2 (4.1)   | 0.34 |
| Y161F/S      | 25 (10.1)                | 4 (8.2)   | 0.79 |
| F162Y        | 1 (0.4)                  | 0         | 1 |
| Y163F        | 2 (0.8)                  | 0         | 1 |
| E164G        | 1 (0.4)                  | 1 (2.0)   | 0.36 |
| A166V/G      | 6 (2.4)                  | 1 (2.0)   | 1 |
| S167L        | 1 (0.4)                  | 0         | 1 |
| V168A        | 1 (0.4)                  | 0         | 1 |
| F170S        | 1 (0.4)                  | 1 (2)     | 0.20 |
| W172C        | 1 (0.4)                  | 1 (2)     | 0.20 |
| L173P        | 3 (1.2)                  | 2 (4.1)   | 0.10 |
| S174N        | 4 (1.6)                  | 3 (6.1)   | 0.026 |
| L175S        | 1 (0.4)                  | 0         | 1 |
| V177L        | 2 (0.8)                  | 0         | 1 |
| V180A        | 2 (0.8)                  | 1 (2.0)   | 0.36 |
| W182STOP     | 1 (0.4)                  | 0         | 1 |
| A184V/G      | 97 (39.3)                | 23 (46.9) | 0.22 |
| L186H        | 2 (0.8)                  | 0         | 1 |
| T189I        | 13 (5.3)                 | 2 (4.1)   | 11 (5.6)   | 1 |
| V190A        | 4 (1.6)                  | 3 (6.1)   | 0.026 |
| S193L        | 4 (1.6)                  | 0         | 4 (2.0)   | 0.59 |
| I195T        | 1 (0.4)                  | 1 (2)     | 0         | 0.20 |
| M198I/M      | 45 (18.2)                | 7 (14.3)  | 38 (19.2) | 0.43 |
| W199I/STOP   | 2 (0.8)                  | 1 (2)     | 1 (0.5)   | 0.36 |
| Y200F/W      | 12 (4.1)                 | 2 (4.1)   | 10 (5.1)  | 1 |
| P203R        | 8 (3.2)                  | 4 (8.2)   | 4 (2.0)   | 0.052 |
| S204R/N      | 25 (10.1)                | 7 (14.3)  | 18 (9.1)  | 0.28 |
| L205V        | 1 (0.4)                  | 1 (2)     | 0         | 0.20 |
| Y206H/F/C    | 6 (2.4)                  | 3 (6.1)   | 3 (1.5)   | 0.094 |
| N207S        | 1 (0.4)                  | 1 (2)     | 0         | 0.20 |
| I208T/S      | 14 (5.7)                 | 3 (6.1)   | 11 (5.6)  | 1 |
| L209V/S/G    | 5 (2.0)                  | 3 (6.1)   | 2 (1.0)   | 0.055 |
| S210K/N/R/S  | 97 (39.3)                | 22 (44.9) | 75 (37.9) | 0.42 |
| P211R        | 1 (0.4)                  | 0         | 1 (0.5)   | 1 |
| F212Y/L/C    | 5 (2.0)                  | 4 (8.2)   | 1 (0.5)   | 0.006 |
| L213I/M      | 18 (7.3)                 | 5 (10.2)  | 13 (6.6)  | 0.37 |
| L216STOP/Y   | 5 (2.0)                  | 2 (4.1)   | 3 (1.5)   | 0.26 |
| P217S/L      | 2 (0.8)                  | 1 (2)     | 1 (0.5)   | 0.36 |
| I218L        | 1 (0.4)                  | 0         | 1 (0.5)   | 1 |
| F220Y/L/C    | 7 (2.8)                  | 0         | 7 (3.5)   | 0.35 |
| C221Y/R      | 11 (4.5)                 | 1 (2)     | 10 (5.1)  | 0.70 |
| L222P        | 1 (0.4)                  | 0         | 1 (0.5)   | 1 |
| V224A        | 29 (11.7)                | 7 (14.3)  | 22 (11.1) | 0.62 |

(Continued)
specificity (SPE) for predicting HCC respectively, 75%, 81.1%, 6.1%, 99.5%. The predictive values of all other 4 point-mutations related to HCC were concretely described in S3 Table, based on our current available data.

**Discussion**

To the best of our knowledge, this investigation was one of the first studies on PreS/S gene mutations and their relation with HCC in Vietnamese CHB infected patients. The study population included CHB infected patients with HBV DNA >3 log_{10} copies/mL (for the higher chance of mutation detection) and had successful PreS/S sequencing (for better mutation description and analysis its correlation with HCC). The rate of mutations that were presented in this study therefore might be higher than that of the real HBV infected population in Vietnam. Our study sample composed of 54.7% genotype B, 57.5% HBeAg positive, 23.1% liver fibrosis of >F3, 83% HBV DNA >5 log_{10} copies/mL and especially 19.8% HCC accompanied. These special characteristics on the population were not only presented the variables that need to be adjusted for their confounding effects but also ensured the aim of detection of mutations and its relationships with HCC.

There were 61.8% amino acid replacements that were detected on the entire PreS/S gene. The rates of changes that were higher on the PreS2 and S gene (74.5% of 228 and 70% of 55 amino acid sites, respectively, versus 56.7% of 120 amino acid sites on the PreS1) revealed the high variability of these regions.

On the PreS1 region that consists of 119 amino acid, 57.1% amino acid replacements were detected with a wide range of mutation rates from 0.4% to 46.9%. However, 79.4% of these replacements (54/68) presented in less than 5% of population. These frequently observed

### Table 4. (Continued)

| S (aa 1–227) | Overall population n (%) | HCC n (%) | p* |
|--------------|--------------------------|-----------|----|
|              | Yes (n = 49) | No (n = 198) |    |
| Y225F        | 1 (0.4) | 1 (2) | 0 | 0.20 |
| I226M/T/S    | 5 (2.0) | 0 | 5 (2.5) | 0.59 |

| S Functional sequence (≥1 point-mutations) |
|------------------------------------------|
| MHR                                      |
| 157 (63.6) | 39 (79.6) | 118 (59.6) | 0.009 |
| “a” determinant                            |
| 98 (39.7) | 24 (49.0) | 74 (37.4) | 0.14 |

percentage by column. * Chi-square test, b Fisher exact test.

https://doi.org/10.1371/journal.pone.0266134.t004

### Table 5. Point-mutations related to HCC–multivariable analysis (n = 247).

| Mutation | OR (95%CI) | p |
|----------|------------|---|
| W4P/R/Y (PreS1) | 5.48 (1.32–22.83) | 0.019 |
| F20S (S) | 9.72 (1.55–61.06) | 0.015 |
| T47A/E/V/K (S) | 2.91 (1.04–8.13) | 0.042 |
| P120S/T (S) | 4.26 (1.58–11.52) | 0.004 |
| S174N (S) | 18.21 (1.77–187.65) | 0.015 |
| P203R (S) | 9.72 (1.55–61.06) | 0.016 |

The characteristics of these 6 point-mutations had been analysed and found that W4P/R/Y (PreS1) (p = 0.022) and T47A/E/V/K (S) (p<0.001) had significant higher rates on genotype C, P120S/T (S) had higher rates on genotype B (p<0.001), HBeAg (-) group (p = 0.019) and low HBV DNA group (<5 log_{10} copies/mL) (p = 0.013) (S1 Table).

https://doi.org/10.1371/journal.pone.0266134.t005
point-mutations were mostly not related to HCC. Contrarily, 4 point-mutations that belong to the low-rate group were related to HCC. They were W4P/R/Y and S5L/T (p = 0.055) on the NTCP region; A90T/S/G on the HSP70 region and L108V/I on the S promoter and B cell epitopes (Table 2).

On the PreS2 region that consists of 55 amino acids, 74.5% amino acid changes were detected with the mutation rates ranged from 0.4% to 42.5%. 82.9% amino acid changes (34/41) belong to the group of < 5% rates. Only F141V/L/I had the higher distribution in the HCC group (18.4% vs 9.6%, p = 0.08) (Table 3). Our finding seemed compatible with a report from Mun et al., who had found that F141L mutation strain increased the risk of HCC in HBV genotype C infected subjects [23]. They had also proved the enhanced cell cycling effects of F141L-expression cell lines through the doubling frequencies of colony-forming versus the wild types.

The PreS1 deletion (27.5%) and PreS2 deletion (16.2%) were equally distributed in the HCC and non-HCC groups (Table 4). The same rates of PreS deletion (20%) were reported from Matsuo et al. (2017) (on 5/21 Vietnamese CHB patients) [18] and from Choi et al. in Korean genotype C patients [24]. Literature reviews found mutations of these PreS genes effect on retaining of HBV inside the host’s cells and on malignant transforming of hepatic cells afterwards [7, 25–27]. More studies had specified on PreS1 deletion and HCC correlation (Zhang et al. (2017) [28], Choi et al. (2019) [29]).

On the S region, 61.2% amino acid changes were detected with the mutation rates range from 0.4% to 39.3%. We found mutations as Y100C/F, P120S/T, R122K, I126T/N/S, S132P, M133T/S/L/I, G145R (the vaccine escape mutant, 2%), Y200F/W and Y206H/F/C as same as that were reported from other studies (Hudu et al. (2015) [30], Kim et al. (2018) [20], Hazawa et al. (2018) [31], but amino acid change which had been described at amino acid 125 had not been detected in our study.

Moreover from our study, we often found the lower rates of amino acid changes compared to other studies such as from Bui TTT et al. (2017) (N38E 71.9%, N38K 71.1%, A60V/E 100% on the PreS1 region, L126T/S 77% on the PreS2 and N3S 27.4% on the S region) [19], from Kim et al. (2018) (K122R 69.3% on the S region compared to 8.9% R122K in our study) [20]. Inversely, we detected higher rates of S point-mutations as L21S (29.1%), S53L (37.7%), A184V/G (39.3%), S204R/N (10%) and S210K/N/R/S (39.3%), and also on the “a” determinant (39.7% cases with mutation, compared to 7% from Hudu’s group [30]. In spite of these lower and higher amino acid change rates, all of these mutations were found not related to HCC in our cross-sectional study. These differences in rates among studies could not only be explained by the distribution of genotype and by the varieties of subgroups in the study populations (such as the co-existence of HBsAg-AntiHBs status, nucleot(s)ide or immunoglobulin treatment, liver cirrhosis and HCC). Moreover, among our study population, antiHBs that had

| Variables | OR (95%CI) | P |
|-----------|------------|---|
| Sex (male) | 4.51 (1.78–11.4) | 0.001 |
| Age group (≥40) | 5.5 (2.06–14.68) | 0.001 |
| HBeAg negative | 2.46 (1.1–5.53) | 0.029 |
| W4P/R/Y (PreS1) | 11.56 (1.99–67.05) | 0.006 |
| T47A/E/V/K (S) | 3.67 (1.19–11.29) | 0.023 |
| P120S/T (S) | 3.38 (1.09–10.49) | 0.035 |
| S174N (S) | 29.73 (2.12–417.07) | 0.012 |
| P203R (S) | 8.45 (1.43–50.06) | 0.019 |

https://doi.org/10.1371/journal.pone.0266134.t006
been tested on 186 cases with clinical symptoms were tested antiHBs and had detected 37 cases (19.9%) with HBsAg-AntiHBs co-existence, higher than the rates of 3–5% in other investigation [32, 33]. Therefore, it was presumed that CHB patients with varieties of presentations had been included in our study and contributed to the difference in rates of point-mutations compared to other studies.

We also found a significant higher distribution of cases with mutation on the MHR region \((p = 0.009)\) in the HCC group, especially a higher rate of P120S/T. Outside of the MHR region we also detected higher rates of other 3 S mutations T47A/E/V/K, S174N (in the HLA II region) and P203S (in the HLA II region, the C-terminal domain) in the HCC group (Table 4). Hossini et al. (2019) had previously found the higher rate of P120T/S in HCC with cirrhosis group [34]. Qiao et al. (2017) had also reported that the N-glycosylation mutations on the MHR region accompanied with HBsAg-antiHBs co-existence was related to HCC [35]. Liu et al. (2013) further stated that the large N glycosylation of HBsAg antigen modulates HBsAg secretion, causes ER stress, expresses cell cycle and cell proliferation [36].

The mutant strains with amino acid changes at T or B cell epitopes on the PreS region can escape the immune surveillance that prolong the HBV infection. Mutants at specific regions of PreS/S genes may create premature stop codons, produce abnormal truncated proteins, disbalance the synthesis of surface proteins, result in retaining of HBV inside of the host cells, promote the endoplasmic reticulum stress pathway, cause DNA oxidative damage and genome instability, upregulate cell cycles and lead to malignant transforming of hepatic cells [7, 25–27]. The PreS/S mutant strains enhance cell cycle progression through the down-regulating effects on the p53 and p21 pathways; upregulate the cyclin-dependent kinase 4, cyclin A, hamper HBsAg secretion, increase cellular proliferation [8].

Many other concerns related to the mutation strains and its replacements on virion secretory defect (at amino acid 172 on S gene) (Warner et al. [37]), on cell proliferation and transformation effects (at amino acids 95, 182, and 216 on S gene) (Huang et al. 2014 [38]) or predisposition of the HCC development (at amino acids 69, 95, 182, 216, 210 on S gene) [8, 38]. However, all of these concerned point-mutations were not found related to HCC in our study.

By study on liver tissue of HCC patients (2008), Hatazawa et al. had detected 2 PreS mutations (W4R and A60V) and more other PreS amino acid replacements at codon 5, 30, 35, 5, 54, 77, 184, 98, 102, 118, 123 and 124 [31]. Chen et al. (2008) had also reported W4P/R and other changes at codons 7, 81 on the PreSI, and at codon 68 on the S region related to HCC [39]. Several years later, the significantly higher frequencies of 3 PreS mutations at codons 4, 60 and 125 in HCC patients were recorded by Yin et al. (2010) [40], Zhang et al. [28]. Interestingly in a longitudinal study, Zhang had also observed the increasing of quasi-species complexity and diversity of the HBV strains during the progression to HCC; He had specially stated that the majority of these mutations existed at least 10 years in advance of development of HCC [41]. Zhang et al. (2017) had also repeatedly reported significantly higher rates of PreS deletion and other PreS mutations at codons 4, 27 and 167 in the HCC group [28].

Scientific reviewed on point-mutations that related to HCC, we realized that there were big differences on the patterns and characteristics of the amino acid changes related to HCC between studies. These differences might originate from the structure of study populations, HBV genotypes, the large spectrum of amino acid changes along the PreS1/PreS2/S sequences, and the interactions between mutations.

The multivariable analysis was applied twice in our study. Firstly, to adjust interactions between 19 point-mutations that had showed higher rates on the HCC group and recognized 6 mutations which had higher risks of HCC (Table 5). Secondly, to adjust for the confounding effects of personal and viral factors (Table 6). The final findings had recognized 5 mutations.
Mutations in the HBV PreS/S gene related to HCC

(W4P/R/Y on the PreS1 region and T47A/E/V/K, P120S/T, S174N, P203R on the S region) that significantly related to HCC. The findings that related to the first 3 mutations that were in agreement with other published papers, except the P203R which had not been well reported. Salpini et al. (2017) had stated that P203Q and the combination of P203R and S210R hampered the HBsAg secretion and increased cellular proliferation. The correlation of the C-terminus P203Q (17.4% vs 1.0%, p = 0.004), S210R (34.8% vs 3.8%, p<0.001) and of their combination with HCC had been reported in genotype A and D CHB patients [8].

Regarding to the OR values of mutations on the final multivariable analysis, 2 S mutations including 23 cases of T47A/E/V/K (S) and 21 cases of P120S/T (S) had revealed three folds increase in HCC risk associated with reasonable confidential intervals. On the contrary, three remaining mutations had only been detected on small numbers of cases with especially high ORs and wide 95% confidential intervals including 3 cases of W4P/R/Y (preS1), OR 11.56 (1.99–67.05); 4 cases of S174N (S), OR 29.73 (2.12–417.07); and 8 cases of P203R (S), OR 8.45 (1.43–50.06) (Table 6). A small sample size of this study resulted in a wider confidence interval with a larger margin of error for these sporadic mutations. It was suggested that a tighter confidence interval with values closer to the actual OR would be obtained if the sample size was increased. We had calculated the predictive values of these 5 point-mutations and had all found the high SPE and NPV values, but all revealed modest SEN due to small number of cases. S174N (S) for instance had been observed in our study with the highest OR and relative high PPV, NPV and specificity (75%, 81.1% and 99.5%, respectively) but its sensitivity was only 6.1% (S3 Table). If possible, the deep sequencing technique with its higher sensitivity could either potentially increase detection rates or improve the SEN values of these low frequency point-mutations. However, we were unable to perform it this time due to a large cost associated with the technique. Further studies are recommended in continuing upon findings of this study in which the direct sequencing would be the best and compulsory technique for better recognizing point mutations at quasispecies levels.

Contrarily, frequent amino acid replacements in our study were detected in the widely known structural and functional sequences such as N51Y/T/S/Q (30.4%), V68T/S/I (44.9%) on the S promoter; T/N87S/T/P (46.2%) on the HSP 70 (heat shock protein) and T125S/N/P (30.8%) on the NBS region. These structures are concerned by their role on the structure and morphology of HBV, the dual topology of L proteins (HSP70), the CAD—Cytosolic anchorage determinant), the virion morphogenesis (NBS—The nucleocapside binding site) and the S RNA transcription (The S promoter and the CCAAT/CBF) [42]. At the cellular level, the mutations at these functional regions has been known to contribute to the production and secretion of surface proteins, on the intracellular retention of envelope proteins and on the endoplasmic reticulum (ER) stress [43]. However, these above point-mutations had equally distributed on the HCC and non-HCC group in our study. More longitudinal cohorts need to be continued apart from this population because the diseases and HCC outcomes need at least one or more decades to appear.

There were some HCC related factors that were not included in the multivariable analysis such as Basal core promoter mutations, history of vaccination, HBsAg-antiHBs co-existence status, HBV genotype, cirrhotic status. Also, the combination of mutations and their interactive effects had not yet been analyzed. Other limitations of our study were also rooted from the study population that was not large enough for the low-rate mutations. A wide spectrum of significant mutations on the 3 regions (PreS1, PreS2 and S) and the interactive effects between mutations that need to be concretely clarified.

Further larger investigation and observation longitudinal studies were in need to be done to describe and analyse the relation between PreS/S mutation and HCC.
Conclusions

61% amino acid changes with a broad range of mutation rates were detected on the PreS1/PreS2/S regions of chronic HBV infected patients. The W4P/R/Y (on preS1 region) and T47A/E/V/K, P120S/T, S174N and P203R (on S region) were found related to HCC. Further investigation included cohort studies are recommended to continue to further investigate the relation of mutations on the HBV genome and HCC outcome.

Supporting information

S1 Table. Distributions of 6 mutations related to HCC in groups of personal and HBV characteristics.

S2 Table. Distribution of personal characteristics and HBV viral markers in HCC and non HCC group (n = 247).

S3 Table. Predictive values of point-mutations related to HCC (n = 247).

S1 File.

Acknowledgments

We would like to acknowledge all patients who participated in this study. Special thanks all the medical health staff members of Hepatology Clinic of UMC and Center for Molecular Biomedicine of UMP at Ho Chi Minh city who attended in patient recruitment, blood sampling, storage, sequencing and interpreting the results.

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References

1. WHO. HBV fact sheet. 2019:https://www.who.int/news-room/fact-sheets/detail/hepatitis-b.
2. Sung H, Ferlay J, Siegel RL, Laversanne M, Soerjomataram I, Jemal A, et al. Global Cancer Statistics 2020: GLOBOCAN Estimates of Incidence and Mortality Worldwide for 36 Cancers in 185 Countries. CA Cancer J Clin. 2021; 71(3):209–49. Epub 2021/02/05. https://doi.org/10.3322/caac.21660 PMID: 33638336.
3. Schweitzer A, Horn J, Mikolajczyk RT, Krause G, Ott JJ. Estimations of worldwide prevalence of chronic hepatitis B virus infection: a systematic review of data published between 1965 and 2013. Lancet. 2015; 386(10003):1546–55. Epub 2015/08/02. https://doi.org/10.1016/S0140-6736(15)61412-X PMID: 26231459.
4. Nguyen-Dinh SH, Do A, Pham TND, Dao DY, Nguyen TN, Chen MS Jr. High burden of hepatocellular carcinoma and viral hepatitis in Southern and Central Vietnam: Experience of a large tertiary referral center, 2010 to 2016. World J Hepatol. 2018; 10(1):116–23. Epub 2018/02/06. https://doi.org/10.4254/wjh.v10.i1.116 PMID: 29399285; PubMed Central PMCID: PMC5787675.
5. Le VQ, Nguyen VH, Nguyen VH, Nguyen TL, Sudenga SL, Trinh LH, et al. Epidemiological Characteristics of Advanced Hepatocellular Carcinoma in the Northern Region of Vietnam. Cancer Control. 2019; 26(1):1073274819862793. Epub 2019/07/11. https://doi.org/10.1177/1073274819862793 PMID: 31290350; PubMed Central PMCID: PMC6620729.
6. Zhang X, Ding HG. Key role of hepatitis B virus mutation in chronic hepatitis B development to hepatocellular carcinoma. World J Hepatol. 2015; 7(9):1282–6. Epub 2015/05/29. https://doi.org/10.4254/wjh.v7.i9.1282 PMID: 26019744; PubMed Central PMCID: PMC4438503.
7. Pollicino T, Cacciolato I, Saffioti F, Raimondo G. Hepatitis B virus PreS/S gene variants: pathobiology and clinical implications. J Hepatol. 2014; 61(2):408–17. Epub 2014/05/08. https://doi.org/10.1016/j.jhep.2014.04.041 PMID: 24801416.
8. Salpini R, Surdo M, Warner N, Cortese MF, Colledge D, Coppe S, et al. Novel HBsAg mutations correlate with hepatocellular carcinoma, hamper HBsAg secretion and promote cell proliferation in vitro. Oncotarget. 2017; 8(9):15704–15. Epub 2017/02/06. https://doi.org/10.18632/oncotarget.14944 PMID: 28152517; PubMed Central PMCID: PMC5362517.
9. Chiao-Fang Teng H-CW, Ih-Jen Su, Long-Bin Jeng. Hepatitis B Virus Pre-S Mutants as Biomarkers and Targets for the Development and Recurrence of Hepatocellular Carcinoma. viruses. 2020; 12:945.
10. Li-Shuai Q, Yu-Yan C, Hai-Feng Z, Jin-Xia L, Cui-Hua L. Pre-S deletions of hepatitis B virus predict recurrence of hepatocellular carcinoma after curative resection. Medicine (Baltimore). 2017; 96(43):e8311. Epub 2017/10/27. https://doi.org/10.1097/MD.0000000000008311 PMID: 29069001; PubMed Central PMCID: PMC5671834.
11. Zhang Y, Huang J, Chen J, Yang K, Chen J, Xu L, et al. The mutation of hepatitis B virus and the prognosis of hepatocellular carcinoma after surgery: a pilot study. Cancer Manag Res. 2018; 10:599–611. Epub 2018/04/10. https://doi.org/10.2147/CMAR.S160047 PMID: 29628777; PubMed Central PMCID: PMC5877868.
12. Zhao ZM, Jin Y, Gan Y, Zhu Y, Chen TY, Wang JB, et al. Novel approach to identifying the hepatitis B virus pre-S deletions associated with hepatocellular carcinoma. World J Gastroenterol. 2014; 20(37):13573–81. Epub 2014/10/14. https://doi.org/10.3748/wjg.v20.i37.13573 PMID: 25309088; PubMed Central PMCID: PMC418909.
13. Chen BF. Hepatitis B virus pre-S/S variants in liver diseases. World J Gastroenterol. 2018; 24(14):1507–20. Epub 2018/04/18. https://doi.org/10.3748/wjg.v24.i14.1507 PMID: 29662289; PubMed Central PMCID: PMC5897855.
14. Liu S ZH, Gu C, Yin J, He Y, Xie J, Cao G. Associations between hepatitis B virus mutations and the risk of hepatocellular carcinoma: a meta-analysis. J Natl Cancer Inst. 2009; 101:1066–82. https://doi.org/10.1093/jnci/djp180 PMID: 19574418.
15. Qu LS, Liu JX, Liu TT, Shen XZ, Chen TY, Ni ZP, et al. Association of hepatitis B virus pre-S deletions with the development of hepatocellular carcinoma in Qidong, China. PLoS One. 2014; 9(5):e98257. Epub 2014/05/23. https://doi.org/10.1371/journal.pone.0098257 PMID: 24849936; PubMed Central PMCID: PMC4029943.
Jiang X, Chang L, Yan Y, Wang L. Paradoxical HBsAg and anti-HBs coexistence among Chronic HBV.

Zhang AY, Lai CL, Huang FY, Seto WK, Fung J, Wong DK, et al. Deep sequencing analysis of quasispecies in hepatitis B virus.

Hudu SA, Harmal NS, Saeed MI, Alshrari AS, Malik YA, Niazlin MT, et al. Naturally occurring hepatitis B surface antigen mutant variants in Malaysian blood donors and vaccinees.

Choi YM, Lee SY, Kim BJ. Naturally Occurring Hepatitis B Virus Mutations Leading to Endoplasmic Reticulum Stress and Their Contribution to the Progression of Hepatocellular Carcinoma.

Bui TTT, Tran TT, Nghiem MN, Rahman P, Tran TTT, Dinh MNH, et al. Molecular characterization of hepatitis B virus in Vietnam.

Kim HS, Chen X, Xu M, Yan C, Liu Y, Deng H, et al. Frequency of hepatitis B virus surface antigen variants (HBsAg) in hepatitis B virus genotype B and C infected East- and Southeast Asian patients: Detection by the Elecsys(R) HBsAg II assay.

Choi MS, Kim DY, Lee DH, Lee JH, Koh KC, Paik SW, et al. Clinical significance of pre-S mutations in patients with genotype C hepatitis B virus infection.

Matsuo J, Do SH, Yamamoto C, Nagashima S, Chuo C, Katayama K, et al. Clustering infection of hepatitis B virus genotype B4 among residents in Vietnam, and its genomic characters both intra- and extra-familiar.

Kim HC, Huang W, Lai MD, Su IJ. Hepatitis B virus pre-S mutants, endoplasmic reticulum stress and hepatocarcinogenesis. Cancer Sci. 2006; 97(8):683–8. Epub 2006/07/26. https://doi.org/10.1111/j.1349-7006.2006.00235.x PMID: 16863502.

Dunford L, Carr MJ, Dean J, Nguyen LT, Ta Thi TH, Nguyen BT, et al. A multicentric molecular analysis of hepatitis B and blood-borne virus coinfections in Viet Nam. PLoS One. 2012; 7(6):e39027. Epub 2012/06/22. https://doi.org/10.1371/journal.pone.0039027 PMID: 22720022; PubMed Central PMCID: PMC3374772.

Matsuo J, Do SH, Yamamoto C, Nagashima S, Chuo C, Katayama K, et al. Clustering infection of hepatitis B virus genotype B4 among residents in Vietnam, and its genomic characters both intra- and extra-familiar. PLoS One. 2012; 12(7):e0177248. Epub 2017/07/29. https://doi.org/10.1371/journal.pone.0177248 PMID: 28753615; PubMed Central PMCID: PMC5533320.

Kim HS, Chen X, Xu M, Yan C, Liu Y, Deng H, et al. Frequency of hepatitis B virus surface antigen variants (HBsAg) in hepatitis B virus genotype B and C infected East- and Southeast Asian patients: Detection by the Elecsys(R) HBsAg II assay. J Clin Virol. 2018; 103:48–56. Epub 2018/04/15. https://doi.org/10.1016/j.jcv.2018.04.005 PMID: 29655170.

Bialecki ES, Di Bisceglie AM. Diagnosis of hepatocellular carcinoma. HPB (Oxford). 2005; 7(1):26–34. https://doi.org/10.1080/13652893.2006.10784 PMID: 18333158.

Udell JA, Wang CS, Timouth J, FitzGerald JM, Ayas NT, Simel DL, et al. Does this patient with liver disease have cirrhosis? JAMA. 2012; 307(8):832–42. Epub 2012/02/24. https://doi.org/10.1001/jama.2012.186 PMID: 22357834.

Lin YT, Jeng LB, Chan WL, Su IJ. Hepatitis B Virus Pre-S Gene Deletions and Pre-S Deleted Proteins: Clinical and Molecular Implications in Hepatocellular Carcinoma. Int J Mol Sci. 2017; 52(9):1064–74. Epub 2017/03/30. https://doi.org/10.1007/s00535-017-1334-1 PMID: 28353014.

Choi YM, Lee SY, Kim BJ. Naturally Occurring Hepatitis B Virus Mutations Leading to Endoplasmic Reticulum Stress and Their Contribution to the Progression of Hepatocellular Carcinoma. Int J Mol Sci. 2019; 20(3). Epub 2019/02/02. https://doi.org/10.3390/ijms20030597 PMID: 30704071; PubMed Central PMCID: PMC6387469.

Hatazawa Y, Yano Y, Okada R, Tanahashi T, Hayashi H, Hirano H, et al. Quasispecies variant of pre-S/S gene in HBV-related hepatocellular carcinoma with HBs antigen positive and occult infection. Infect Agent Cancer. 2018; 13:7. Epub 2018/02/13. https://doi.org/10.1186/s13027-018-0179-4 PMID: 29434654; PubMed Central PMCID: PMC5797373.

Jiang X, Chang L, Yan Y, Wang L. Paradoxical HBsAg and anti-HBs coexistence among Chronic HBV Infections: Causes and Consequences. Int J Biol Sci. 2021; 17(4):1125–37. Epub 2021/04/20. https://doi.org/10.7150/ijbs.55724 PMID: 33867835; PubMed Central PMCID: PMC8040313.
33. Liu W, Hu T, Wang X, Chen Y, Huang M, Yuan C, et al. Coexistence of hepatitis B surface antigen and anti-HBs in Chinese chronic hepatitis B virus patients relating to genotype C and mutations in the S and P gene reverse transcriptase region. Arch Virol. 2012; 157(4):627–34. Epub 2012/01/10. https://doi.org/10.1007/s00705-011-1215-5 PMID: 22222283.

34. Hosseini SY, Sanaei N, Fattahi MR, Malek-Hosseini SA, Sarvari J. Association of HBsAg mutation patterns with hepatitis B infection outcome: Asymptomatic carriers versus HCC/cirrhotic patients. Ann Hepatol. 2019; 18(4):640–5. Epub 2019/05/21. https://doi.org/10.1016/j.aohep.2018.12.006 PMID: 31105017.

35. Qiao Y, Lu S, Xu Z, Li X, Zhang K, Liu Y, et al. Additional N-glycosylation mutation in the major hydrophilic region of hepatitis B virus S gene is a risk indicator for hepatocellular carcinoma occurrence in patients with coexistence of HBsAg/anti-HBs. Oncotarget. 2017; 8(37):61719–30. Epub 2017/10/06. https://doi.org/10.18632/oncotarget.18682 PMID: 28977899; PubMed Central PMCID: PMC5617459.

36. Liu W, Cao Y, Wang T, Xiang G, Lu J, Zhang J, et al. The N-Glycosylation Modification of LHBs (Large Surface Proteins of HBV) Effects on Endoplasmic Reticulum Stress, Cell Proliferation and its Secretion. Hepat Mon. 2013; 13(9):e12280. Epub 2013/10/06. https://doi.org/10.5812/hepatmon.12280 PMID: 24282423; PubMed Central PMCID: PMC3830522.

37. Warner N, Locarnini S. The antiviral drug selected hepatitis B virus rtA181T/sW172* mutant has a dominant negative secretion defect and alters the typical profile of viral rebound. Hepatology. 2008; 48(1):88–98. Epub 2008/06/10. https://doi.org/10.1002/hep.22295 PMID: 18537180.

38. Huang SF, Chen YT, Lee WC, Chang IC, Chiu YT, Chang Y, et al. Identification of transforming hepatitis B virus S gene nonsense mutations derived from freely replicative viruses in hepatocellular carcinoma. PLoS One. 2014; 9(2):e89753. Epub 2014/03/04. https://doi.org/10.1371/journal.pone.0089753 PMID: 24587012; PubMed Central PMCID: PMC3933656.

39. Chen CH, Changchien CS, Lee CM, Hung CH, Hu TH, Wang JH, et al. Combined mutations in pre-s/surface and core promoter/precore regions of hepatitis B virus increase the risk of hepatocellular carcinoma: a case-control study. J Infect Dis. 2008; 198(11):1634–42. Epub 2008/10/23. https://doi.org/10.1086/592990 PMID: 18939932.

40. Yin J, Xie J, Zhang H, Shen Q, Han L, Lu W, et al. Significant association of different preS mutations with hepatitis B-related cirrhosis or hepatocellular carcinoma. J Gastroenterol. 2010; 45(10):1063–71. Epub 2010/04/27. https://doi.org/10.1007/s00535-010-0253-1 PMID: 20419326.

41. Zhang AY, Lai CL, Huang FY, Seto WK, Fung J, Wong DK, et al. Evolutionary Changes of Hepatitis B Virus Pre-S Mutations Prior to Development of Hepatocellular Carcinoma. PLoS One. 2015; 10(9):e0139478. Epub 2015/10/01. https://doi.org/10.1371/journal.pone.0139478 PMID: 26421619; PubMed Central PMCID: PMC4589234.

42. Chen BF. Different pre-S deletion patterns and their association with hepatitis B virus genotypes. World J Gastroenterol. 2016; 22(35):8041–9. Epub 2016/09/28. https://doi.org/10.3748/wjg.v22.i35.8041 PMID: 27672298; PubMed Central PMCID: PMC5028817.

43. Pollicino T, Amaddeo G, Restuccia A, Raffa G, Aibrandi A, Cutroneo G, et al. Impact of hepatitis B virus (HBV) pre/S genomic variability on HBV surface antigen and HBV DNA serum levels. Hepatology. 2012; 56(2):434–43. Epub 2012/01/25. https://doi.org/10.1002/hep.25592 PMID: 22271491.