

# Association between host TNF- $\alpha$ , TGF- $\beta$ 1, p53 polymorphisms, HBV X gene mutation, HBV viral load and the progression of HBV-associated chronic liver disease in Indonesian patients

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**Abstract.** In developing countries, including Indonesia, there is a high mortality rate associated with the progression of hepatitis B virus (HBV)-associated chronic liver disease (CLD). The pathogenesis of HBV infection is influenced by viral and host factors. To determine potential associations between these factors, host single nucleotide polymorphisms (SNPs) on TNF- $\alpha$ , TGF- $\beta$ 1 and p53, HBV X gene mutation and HBV viral load were investigated in patients with HBV-associated CLD in Surabaya, Indonesia. Sera were collected from 87 CLD patients with HBV infection. TNF- $\alpha$ , TGF- $\beta$ 1 and p53 SNPs were genotyped by PCR restriction fragment length polymorphism. The HBV X gene was sequenced and compared with reference strains to determine mutations and the viral load was measured using reverse transcription-quantitative PCR. In Indonesian patients, no association between TNF- $\alpha$ , TGF- $\beta$ 1 and p53 SNPs and CLD or X gene mutation were identified. A total of 23% (20/87) of samples had HBV X gene mutations, including ten substitution types, one deletion and one insertion. Multinomial regression analysis revealed that the K130M/V131I mutations were correlated with CLD

progression (OR, 7.629; 95% CI, 1.578-36.884). Significant differences in viral load were found in HBV-infected patients who had X gene mutations, such as R87W/G, I127L/T/N/S and K130M/V131I mutations ( $P < 0.05$ ). The presence of K130M and V131I mutations may be predictive for the progression of HBV-associated CLD in Indonesia.

## Introduction

Hepatitis B virus (HBV) infection is a major health problem worldwide, especially in developing countries (1,2). The WHO stated that in 2015, ~257 million people were living with an HBV infection and 887,000 people died from this condition, mostly from chronic liver disease (CLD) complications, such as liver cirrhosis (LC) and hepatocellular carcinoma (HCC) (3). The progression of HBV infection can be influenced by several factors, including viral, host and environmental ones. Variations in host genes are associated with the integration of mutated HBV genes that alter host genes and cause CLD (4,5).

TNF- $\alpha$  is a multifunctional cytokine that regulates inflammatory reactions and plays an important role in the pathogenesis of liver disease, infectious diseases and inflammation (5). The single-nucleotide polymorphism (SNP)-238 G/A and -308 G/A are SNPs of TNF- $\alpha$  gene promoters that have been a focus of several previous works, and were reported to affect TNF- $\alpha$  production at the transcriptional level (6,7). Another cytokine that contributes to the progression of HBV infection is TGF- $\beta$ 1. The SNP-509 C/T in the TGF- $\beta$ 1 promoter is reported to be associated with changes in the plasma TGF- $\beta$ 1 concentration (8-10). Genes that play a role in controlling the cell cycle, such as p53, also play an important role in the pathogenesis of CLD (11,12). The SNP Arg72Pro on the p53 gene disrupts the stability and function of p53 by inhibiting the cell cycle and apoptosis (12-14).

Interactions between host and viral factors are known to play an important role in the pathogenesis of HBV infection (15,16). Mutated HBV X protein (HBx) increases the

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**Abbreviations:** BCP, basal core promoter; CLD, chronic liver disease; HBV, hepatitis B virus; SNP, single nucleotide polymorphism

**Key words:** chronic liver disease, hepatitis B virus, host genetic polymorphism, X gene mutation

progression and metastasis of cancer cells (17,18). When the HBx mutant is formed, there is a transformation increase that continuously affects the host genome (19). Specifically, the integration of HBx into the host genome leads to the upregulation of certain important genes, including NF- $\kappa$ B, TNF- $\alpha$  and TGF- $\beta$ 1, and the downregulation of p53 (20).

There is still an incomplete understanding of the interactions of SNPs on the TNF- $\alpha$ , TGF- $\beta$ 1 and p53 genes in CLD patients with chronic HBV infection. In the present study, to obtain a deeper understanding of the pathogenesis of CLD, we investigated the associations between these principal SNPs, HBV X gene mutation and viral load in patients with chronic HBV infection.

## Materials and methods

**Sampling.** This work involved a cross-sectional study using 87 blood samples collected from patients with chronic HBV at the inpatient/outpatient clinics of the Department of Internal Medicine, Dr. Soetomo General Hospital (Surabaya, Indonesia) between September 2016-May 2017 with a mean age of  $47.0 \pm 12.5$  years (64 male and 23 female). Inclusion criteria were as follows: CLD with chronic HBV infection; >16 years; willing to participate as research subjects; conscious; not in an emergency state; never been vaccinated for HBV; and HBV infection <10 years. Exclusion criteria were as follows: Co-infection with hepatitis C virus or HIV; and undergoing immunosuppressant therapy. This study received approval from the Ethics Committee of Dr. Soetomo General Hospital (approval no. 0949/KEPK/II/2019). Written informed consent was provided by the participants, in accordance with the Declaration of Helsinki. Patients were classified by CLD stage: (i) Chronic hepatitis (CH); (ii) LC; and (iii) HCC. The classification was determined by a hepatologist. CH was diagnosed based on positive HBsAg for >6 months without any LC/HCC specific clinical features, laboratory and radiology manifestation. LC was diagnosed based on clinical features of portal hypertension, liver stiffness by fibroscan and abnormal liver morphology by ultrasound. HCC was diagnosed based on clinical manifestation,  $\alpha$ -fetoprotein  $\geq 200$  ng/ml and HCC-typical ultrasound findings. Alanine aminotransferase (ALT), aspartate aminotransferase (AST), platelet and HBeAg were obtained from medical records.

**Genomic DNA extraction.** Host DNA was extracted from peripheral blood mononuclear cell isolated from blood samples and viral DNA was extracted from 100  $\mu$ l serum using QIAamp DNA Extraction kit (cat. no. 51104; Qiagen, Inc.) in accordance with the manufacturer's instructions.

**HBV load measurement.** Viral load was assessed in the extracted viral DNA from all 87 blood samples using a Taqman quantitative (q)PCR method for absolute quantification by the CFX96™ Real-Time PCR Detection system (Bio-Rad Laboratories, Inc.) with the Master Mix Real-Time PCR iTaq Universal Probes Supermix (cat. no. 1725130; Bio-Rad Laboratories, Inc.) in accordance with the manufacturer's instructions. The probes used was HBSP1 (FAM-5'-CAG AGTCTAGACTCGTGGTGGACTTC-3'-TAMRA). Primers included: SF1 forward, 5'-CACATCAGGATTCCTAGG ACC-3' and SR1 reverse, 5'-GGTGAGTGATTGGAGGGT

TG-3'. qPCR was performed with thermocycling condition as follows: 50°C for 2 min, 95°C for 10 min, followed by 53 cycles of 95°C for 20 sec and 60°C for 1 min (21). Data reading was performed by Biorad CFX Maestro software version number 4.1.2433.1219 (Bio-Rad Laboratories, Inc.).

**PCR restriction fragment length polymorphism (RFLP) of TNF- $\alpha$ , TGF- $\beta$ 1 and p53 on host genome.** PCR was carried out on host genome using PCR Master Mix Solution (cat. no. 25027; iNtRON® Biotechnology, Inc.). The primers, thermocycling conditions and restriction enzymes used are listed in Table I. All PCR products were assessed by 2% agarose gel electrophoresis under UV light. All restriction enzyme incubation steps were carried out overnight at 37°C, except for *Bst*UI for which 60°C were applied.

**Analysis of TNF- $\alpha$ , TGF- $\beta$ 1 and p53 SNPs.** SNPs were identified via agarose gel (3.5% for TNF- $\alpha$  SNP, 3% for TGF- $\beta$ 1 SNP and 2% for p53 SNP) electrophoresis analysis based on the following criteria: (i) In the presence of an SNP at position -238 on the TNF- $\alpha$  promoter, a 152-bp band (A allele) is visible, while 133 and 19 bp fragments are identified for the corresponding wild type (G allele). (ii) With an SNP at position -308 on the TNF- $\alpha$  promoter, a 107-bp band (A allele) is visible, while for the corresponding wild type (G allele) 87 and 20 bp fragments are identified (22). (iii) For an SNP at position -509 on the TGF- $\beta$ 1 gene, a 120-bp band (T allele) is visible, while the corresponding wild type (C allele) shows 74 and 46 bp fragments (23). (iv) The Arg72Pro SNP on the p53 gene shows a 396-bp band (C allele), while the corresponding wild type (G allele) displays 231 and 165 bp fragments (24).

**Analysis of HBV X gene.** Nested PCR on the HBV X gene was performed on the previously extracted viral genome following the work of Lee *et al* (25), as shown in Table I. It was performed using PCR Master Mix Solution (cat. no. 25027; iNtRON® Biotechnology, Inc.), following the procedure in the manufacturer's instructions. PCR products were visualized on 2% agarose gels. DNA sequencing was performed using an ABI Prism 310 Genetic Analyzer (PerkinElmer, Inc.). The sequenced nucleotides were compared with a reference strain nucleotide sequence that had previously been published in GenBank [accession no., EF473977 (26), AB219430 (27) and D23678 (28)] using Clone Manager 9 (Scientific & Educational Software).

**Statistical analysis.** Statistical analyses were performed using SPSS 23 (SPSS, Inc.). Data are presented as the mean  $\pm$  SD. Analysis was repeated at least twice for each subject.  $\chi^2$ , Mann-Whitney U, Kruskal Wallis with Dunn's post hoc test, or one-way ANOVA with LSD post hoc tests were performed to assess significance, depending on variable. Multinomial regression analysis was performed to assess correlations.  $P < 0.05$  following a two-tailed analysis was considered to indicate statistical significance.

## Results

**Patient characteristics.** For the 87 CLD patients included in this study, the age range was 16-72 years, with mean ages of  $45.0 \pm 14.0$ ,  $50.7 \pm 11.6$  and  $46.7 \pm 8.3$  years for patients with CH,

Table I. Primers and restriction enzymes used for host SNP and hepatitis B virus X gene detection.

SNP	Direction	Sequence (5'-3')	Amplicon (bp)	Thermocycling conditions	Restriction enzyme
TNF- $\alpha$ -238	Forward	AGGCAATAGGTTTTGAGGG CCAT	107	94°C for 5 min; 40x 94°C for 30 sec, 60°C for 30 sec and 72°C for 40 sec; 72°C for 7 min	<i>MspI</i>
	Reverse	TCCTCCCTGCTCCGATTCCG			
TNF- $\alpha$ -308	Forward	AGAAGACCCCCCTCGGAACC	152	94°C for 5 min; 40x 94°C for 30 sec; 58.5°C for 30 sec and 72°C for 40 sec; 72°C for 7 min	<i>NcoI</i>
	Reverse	ATCTGGAGGAAGCGGTAGTG			
TGF- $\beta$ 1-509	Forward	GGAGAGCAATTCTTACAGGTG	120	94°C for 3 min; 30x 94°C for 30 sec, 60°C for 30 sec and 72°C for 60 sec; 72°C for 10 min	<i>DdeI</i>
	Reverse	TAGGAGAAGGAGGGTCTGTC			
p53 Arg 72 Pro	Forward	TCCCCCTTGCCGTCCCAA	396	94°C for 5 min; 30x 94°C for 30 sec, 55°C for 30 sec and 72°C for 30 sec; 72°C for 7 min	<i>BstUI</i>
	Reverse	CGTGCAAGTCACAGACTT			
X gene round 1	Forward	CATGCGTGGAACCTTTGTG	840	94°C for 5 min; 35x 94°C for 50 sec, 50°C for 50 sec and 72°C for 60 sec; 72°C for 7 min	-
	Reverse	CTTGCTKAGTGCTGTATGG			
X gene round 2	Forward	TCCTCTGCCGAT CCATACTG	684	94°C for 5 min; 35x 94°C for 30 sec, 55°C for 30 sec and 72°C for 45 sec; 72°C for 7 min	-
	Reverse	CAGAAGCTCCAAATTCTTTATA			

SNP, single nucleotide polymorphism.

Table II. Characteristics of patients with chronic liver disease.

Characteristics	CH (n=45)	LC (n=27)	HCC (n=15)	Total (n=87)	P-value
Sex (male)	30 (66.7)	20 (74.7)	14 (93.0)	64 (73.6)	0.128
Age (years)	45.0 $\pm$ 14.0	50.7 $\pm$ 11.6	46.7 $\pm$ 8.3	47.0 $\pm$ 12.5	0.167
AST (U/l)	64.7 $\pm$ 95.2	91.8 $\pm$ 175.5	211.0 $\pm$ 204.1	98.3 $\pm$ 153.5	<0.001
ALT (U/l)	95.4 $\pm$ 107.7	125.5 $\pm$ 323.9	100.0 $\pm$ 71.9	105.5 $\pm$ 218.3	0.056
Platelet count/ $\mu$ l	196,711.1 $\pm$ 120,382.5	162,629.6 $\pm$ 82,014.1	238,333.3 $\pm$ 106,306.7	193,310.3 $\pm$ 10,929.1	0.098
HBeAg, positive	14 (31.1)	9 (33.3)	2 (13.3)	25 (28.7)	0.351

Data are presented as n (%) or the mean  $\pm$  standard deviation. Diagnosis of LC was based on clinical features of portal hypertension, liver stiffness by fibroscan and abnormal liver morphology by ultrasound. Diagnosis of HCC was based on clinical manifestation,  $\alpha$ -fetoprotein  $\geq$ 200 ng/ml and typical ultrasound findings. Statistical analysis was performed using two-way  $\chi^2$  for categorical variables and Kruskal Wallis followed by Dunn's or one-way ANOVA followed by LSD test for continuous variables. CH, chronic hepatitis; LC, liver cirrhosis; HCC, hepatocellular hepatoma.

LC and HCC, respectively. Male patients (73.6%) outnumbered females (26.4%), as shown in Table II. In this study, CLD patients were most often classed as CH (45/87; 51.7%), followed by LC (27/87; 31.0%) and HCC (15/87; 17.3%). Each CLD stage was dominated with male subjects (66.7, 74.7 and 93.0% for CH, LC and HCC, respectively). AST levels in

patients with HCC (211 $\pm$ 204.1 U/l) were significantly increased compared with those in patients with LC (91.8 $\pm$ 175.5 U/l) and CH (64.7 $\pm$ 95.2 U/l).

*Genotype distributions of TNF- $\alpha$ , TGF- $\beta$ 1 and p53 SNPs.*  
The genotype distributions of TNF- $\alpha$ , TGF- $\beta$ 1 and p53 SNPs

Table III. Distribution of host SNPs in patients with CLD.

SNP genotype	CLD stage			Total
	CH	LC	HCC	
TNF- $\alpha$ -238				
GG	44 (98.0)	24 (88.5)	15 (100)	83 (95.4)
GA	1 (2.0)	3 (11.5)	0	4 (4.6)
AA	0	0	0	0
Total	45 (100)	27 (100)	15 (100)	87 (100)
TGF- $\beta$ 1-509				
CC	10 (22.2)	5 (18.5)	3 (20.0)	18 (20.7)
TC	23 (51.1)	14 (51.9)	4 (26.7)	41 (47.1)
TT	12 (26.7)	8 (29.6)	8 (53.3)	28 (32.2)
Total	45 (100)	27 (100)	15 (100)	87 (100)
Arg72Pro				
CC	13 (28.9)	6 (22.2)	2 (13.3)	21 (24.2)
CG	23 (51.1)	16 (59.3)	10 (66.7)	49 (56.3)
GG	9 (20.0)	5 (18.5)	3 (20.0)	17 (19.5)
Total	45 (100)	27 (100)	15 (100)	87 (100)

Data are presented as n (%). CLD, chronic liver disease; SNP, single nucleotide polymorphism; CH, chronic hepatitis; LC, liver cirrhosis; HCC, hepatocellular hepatoma.

Table IV. Hepatitis B virus X gene mutation profiles and its association to chronic liver disease progression.

Mutation	Samples with mutation (%)				Functional region affected	P-value
	CH (n=45)	LC (n=27)	HCC (n=15)	Total (n=87)		
T36P	0	3.7	0	1.2	B-cell epitope	0.483
L37I	0	3.7	0	1.2	B-cell epitope	0.483
P38S	2.2	0	0	1.2	B-cell epitope	1.000
S43P	6.7	0	13.3	5.8	B-cell epitope	0.147
A44V/T	2.2	3.7	6.7	3.4	B-cell epitope	0.748
P46S	2.2	3.7	0	2.3	B-cell epitope	1.000
H86P/R	2.2	7.4	0	3.4	Core promoter, EnhII	0.576
R87W/G	6.7	7.4	6.7	6.9	Core promoter, EnhII	1.000
H94Y	2.2	0	0	1.2	Box $\alpha$ , C/EBP, CCAAT/enhancing binding protein, core promoter, EnhII	1.000
L98I	2.2	3.7	6.7	3.4	Box $\alpha$ , C/EBP, CCAAT/enhancing binding protein, core promoter, EnhII	0.748
T105A	4.4	0	0	2.3	Core promoter, EnhII, BH3-like motif, T-cell epitope	1.000
L123S/V	2.2	0	6.7	2.3	BH3-like motif, core promoter, NRE	0.411
I127L/T/N/S	8.9	7.4	20	10.3	BH3-like motif, core promoter, NRE	0.461
Insertion (32 nt)	2.2	0	0	1.2	Box $\alpha$ , C/EBP, CCAAT/enhancing binding protein, core promoter, EnhII, BH3-like motif, T-cell epitope	1.000
Deletion (20 nt)	2.2	0	0	1.2	T-cell epitope, BH3-like motif, core promoter	1.000
K130M	4.4	25.9	26.7	14.9	BH3-like motif, core promoter	0.006
V131I	4.4	25.9	26.7	14.9	BH3-like motif, core promoter	0.006

Statistical analysis was performed using two-way  $\chi^2$  or Fisher's exact test. C/EBP, CCAAT/enhancer-binding protein; BH-3, Bcl-2 homology-3; NRE, negative regulatory element.



were determined by PCR-RFLP and are shown in Table III. No cases with the AA genotypes (homozygous minor-mutant type) on the TNF- $\alpha$  promoter (-238/-308) were identified. There was no difference in the distribution of genotypes or SNP alleles of TNF- $\alpha$ , TGF- $\beta$ 1 and p53 in different CLD groups ( $P>0.05$ ).

**Analysis of HBV X gene mutations.** Based on the multiple alignments, the main type of HBV X gene mutation identified was amino acid substitutions, as shown in Table IV. A total of 23% (20/87) of samples had HBV X gene mutations with ten types of previously reported substitution (29-32). There was one sample that showed both the 32-nucleotide insertion and the 20-nucleotide deletion. These types of mutation caused a shift in the nucleotide reading frame, resulting in the formation of a truncated HBx protein. X gene mutations were found in 17.8% (8/45) of patients with CH, 25.9% (7/27) patients with LC and 33.3% (5/15) patients with HCC. In addition to previously reported mutations, we also found five variants of the X gene located in the HBV functional region, including L37I, S43P, H86P/R, L98I and T105A, which have not been reported in previous studies and may be specific for Indonesian HBV.

The dominant mutations in the basal core promoter (BCP) were K130M/V131I, as found in 12.6% (11/87) of CLD patients. These two mutations were observed as double mutation in one sample. There was an association between the K130M/V131I mutations and the progression of CLD ( $P<0.05$ ), but not for other X gene mutations ( $P>0.05$ ; Table IV). Multinomial regression analysis confirmed that K130M/V131I mutations were correlated with CLD progression (OR, 7.629; 95% CI, 1.578-36.884; Table V). No association of HBV X gene mutations with TNF- $\alpha$  (-238, -308), TGF- $\beta$ 1 or p53 gene SNPs were found in CLD patients with HBV infection (Fig. 1).

**HBV viral load measurement.** The mean viral load was the highest in LC patients ( $4.98\pm 4.37$  log copies/ml), followed by patients with HCC ( $3.49\pm 2.63$  log copies/ml) and patients with LC ( $2.95\pm 3.59$  log copies/ml). Analysis showed no significant differences in viral load depending on CLD stage ( $P>0.05$ ; Table VI). There were significant differences in viral load levels in HBV-infected patients who had X gene mutations, as well as the R87W/G, I127L/T/N/S and K130M/V131I mutation ( $P<0.05$ ).

## Discussion

In the present study, 87 patients from Surabaya, Indonesia, with HBV-associated CLD were enrolled. We found no significant differences in the distribution of genotypes or alleles of TNF- $\alpha$ -238 or -308 SNPs among the three CLD stages. Additionally, we found no cases with the AA genotype for these SNPs. This result was in line with reports from Banday *et al* (33) that showed that the frequency of A alleles in Asia is decreased compared with other regions. A similar study that was performed in China by Xu *et al* (6) also failed to identify patients with AA genotypes. The SNPs in TNF- $\alpha$  at positions -238 and -308 are often associated with various diseases, including severe inflammation, infection and malignancy (34). Research on SNPs

Table V. Multinomial regression analysis of the K130M/V131I mutation in chronic liver disease progression.

	Chi-square value	P-value	OR	95% CI
K130M/V131I	8.71	0.011	7.629	1.578-36.884

Table VI. Hepatitis B virus viral load in patients with X gene mutations.

Mutation	Viral load (log copies/ml)		P-value
	Median	IQR	
S43P			0.137
Mutation	5.74	1.90	
No mutation	2.50	6.95	
A44			0.063
Mutation	7.10	0.00	
No mutation	14.22	6.68	
R87W/G			0.029
Mutation	6.78	1.36	
No mutation	2.14	6.78	
I127L/T/N/S			0.035
Mutation	6.86	4.00	
No mutation	2.00	6.65	
K130M/V131I			0.004
Mutation	6.86	2.53	
No mutation	1.04	5.90	
X gene mutation			<0.001
Mutation	6.90	2.94	
No mutation	0.00	4.86	

Data are presented as the median with IQR. Statistical analysis was performed using Mann-Whitney U tests. IQR, interquartile range.

of the TNF- $\alpha$  promoter in patients with HBV infection has shown conflicting results regarding population- and ethnic-specificity (35) and some but not all studies showed a correlation between SNPs of TNF- $\alpha$  promoters and HBV infection (36-38).

In this study, we found no differences in the distribution of genotypes or alleles of TGF- $\beta$ 1 and p53 SNPs between patients in the CLD groups. The frequency of SNPs in patients with CLD was greater than occurrence of the wild type in this study. Previous studies reported different results regarding the TGF- $\beta$ 1-509 and Arg72Pro p53 SNPs among diverse populations. A meta-analysis conducted by Guo *et al* (39) showed that in an Asian population, the TGF- $\beta$ 1-509 SNP T allele was correlated with the incidence of HCC, but this was not observed in Caucasian and African participants. However, in a study conducted in China by Qi *et al* (40), the risk of HCC in patients with the TT genotype was decreased compared

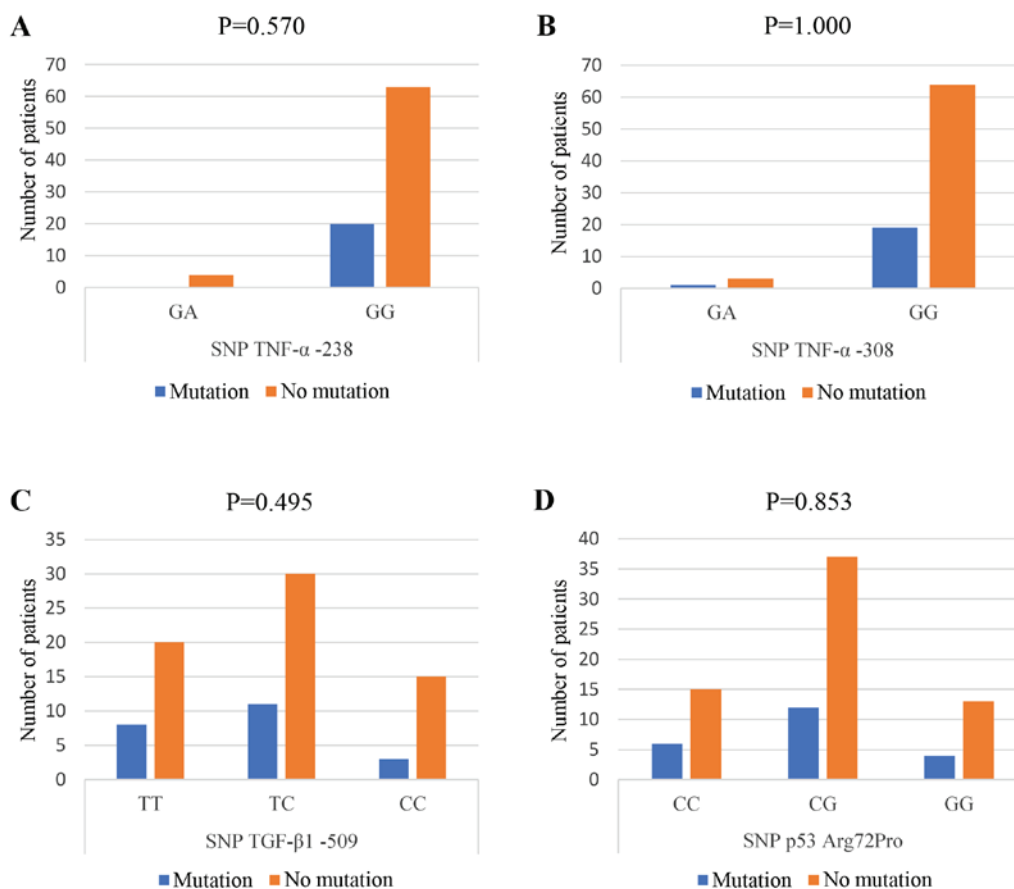


Figure 1. Distribution of hepatitis B virus X gene mutations and host SNPs in patients with chronic liver disease. (A) TNF- $\alpha$ -238, (B) TNF- $\alpha$ -308, (C) TGF- $\beta$ 1-509 and (D) p53 Arg72Pro SNPs.  $\chi^2$  test were used to assess significance. SNP, single nucleotide polymorphism.

with the CC genotype. Research conducted in Turkey by Sümbül *et al* (41) showed a correlation between the Arg72Pro SNP GG genotype and the incidence of HCC that was not observed for the normal population without HBV infection. This was not confirmed by research conducted in China by Cai *et al* (42) that found no significant differences between the Arg72Pro SNP in patients with HBV infection and healthy controls. In addition to studies focusing on CLD, studies on the association between the Arg72Pro p53 SNP and various cancers types have also shown controversial results (43-45). This is thought to be associated with the ethnic background of the patients (41).

The present study found 12 types of X gene mutation, among which the K130M/V131I mutations were significantly correlated with CLD progression. These two mutations are located in the BCP, which overlaps with the X gene. The presence of these double mutations is thought to exacerbate the host's immune response, increase viral replication and modify the coding region of the X protein causing the progression of CLD (46). H94Y and I127L/T/N/S mutations have been associated to K130M/V131I in previous studies (47,48). The H94Y mutation causes changes in the  $\alpha$  box, an element strongly activating the EnhII/core promoter, and increase the binding affinity of the  $\alpha$  box and EnhII/core promoter (29,49). The rate of the P38S mutation was reported to be significantly higher in HCC than in asymptomatic carriers, but not significantly different from the rates in CH and LC patients (49). The rate of the T36P mutation was reported to be higher in HCC patients,

presumably due to its location at the B epitope, which affects the immune response (50).

No associations of HBV X gene mutations with TNF- $\alpha$  (-238 and -308), TGF- $\beta$ 1 and p53 SNPs were found in CLD patients. In previous studies, several SNPs associated with HBV X gene mutations were identified, particularly SNPs associated with HLA-DQ/DR (rs9272105 and rs9277535) (51,52). Research has also focused on the association between the SNP of the STAT3 gene (rs1053004) and EnhII/BCP/PC mutations that overlap with the X gene (53). However, to date, no studies have linked SNPs to other genes with HBV mutations, including TNF- $\alpha$  (-238 and -308), TGF- $\beta$ 1 and p53 gene SNPs, as it was shown in this study.

In this study, the mean viral load was highest in LC patients, followed by HCC and CH patients. Our results showed no significant differences in viral load between the CLD stages. Several studies have shown an association between HBV viral load and liver damage, and high viral load is often associated with severe liver damage (54,55). Although HBV viral load is often associated with the risk of HCC, a study conducted by Harkisoen *et al* (56) showed lower viral load in LC patients. Similarly, a study conducted by Tseng *et al* (57) showed no association between viral load and HCC risk. This may be caused by the presence of HBV DNA, which is integrated into hepatocytes more efficiently compared with serum (58,59). In addition, there are other influential factors for CLD progression to HCC, such as alcoholism, cigarette smoking and HBV genotypes (56,57).

There were significant differences in viral load levels between patients with X gene mutations, including R87W/G, I127L/T/N/S and K130M/V131I mutations. X gene mutations affect HBV viral load, as indicated by observations of a higher HBV replication rate when featuring multiple mutations. This high rate of HBV replication causes increased inflammation and viral invasion (29,49). However, to date, conflicting findings on the association between X gene mutations and HBV viral load have been obtained (60). A study conducted by Ghabeshi *et al* (61) showed no association between BCP mutations and viral load. This may be due to BCP mutations reducing viral secretion in the blood, but increasing viral load in the liver, which directly causes liver damage. These differences in results are influenced by differences in populations, such as HBV genotypes, HBeAg status and clinical conditions (60).

In this study, the presence of K130M/V131I mutations constituted an independent risk factor for CLD progression. X gene mutations, including R87W/G, I127L/T/N/S and K130M/V131I mutations, were correlated with HBV viral load. Additionally, we found no association between SNPs of the TNF- $\alpha$ , TGF- $\beta$ 1 and p53 genes with X gene mutation. While further studies are required to examine the clinical value of these results, the presence of K130M/V131I mutations may serve as a predictive marker for the progression of CLD in Indonesia and may also be applicable for other countries where chronic HBV infections are prevalent.

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## Availability of data and materials

All data generated in the present study are included in this article.

## Authors' contributions

This study was conducted and designed by MIL, SS and RH. CDKW, PBP, UK, UM and PBS performed sample collection. MA and SENR performed the laboratory experiments. CDKW analyzed the data and wrote the manuscript. All authors read and approved the final manuscript.

## Ethics approval and consent to participate

This study received approval from the Ethics Committee of Dr. Soetomo General Hospital (Surabaya, Indonesia) (approval no. 0949/KEPK/II/2019). Written informed consent for participation was obtained from each individual.

## Patient consent for publication

Not applicable.

## Competing interest

The authors declare that they have no competing interests.

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