

Genotype Distribution of Vitamin D Receptor Polymorphisms among Indonesian Patients with Chronic Hepatitis B

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Abstract

Background: Chronic hepatitis B is a necro-inflammatory of the liver parenchyma caused by hepatitis B virus (HBV) infection leading to liver cirrhosis and hepatocellular carcinoma (HCC). Genetic variants including single nucleotide polymorphisms (SNPs) within genes regulating immune response may contribute to the progression of chronic hepatitis B (CHB) infection. This study aimed to examine the genotype distribution of vitamin D receptor (VDR) polymorphism among patients with CHB infection and to study its association with the development of cirrhosis and hepatoma.

Methods: This cross-sectional study analysed 75 CHB patients, consisting of 36 CHB patients without cirrhosis, 25 CHB patients with cirrhosis, and 14 CHB patients with hepatoma. VDR polymorphism was examined using the Amplification Refractory Mutation System Polymerase Chain Reaction (ARMS-PCR) method.

Results: Alanine aminotransferase (ALT) and alpha fetoprotein (AFP) levels did not show any significant differences between study groups, but albumin levels in CHB patients with cirrhosis and hepatoma were significantly lower than CHB patients without cirrhosis ($p < 0.05$). In contrast, the bilirubin levels in CHB patients with cirrhosis was higher than in CHB patients' cirrhosis. The most common genotypes of VDR polymorphisms were Ff (57.3%), TT (72%), aa (48%) and bb (74.7%) for Fok1, Taq1, Apa1 and Bsm1 respectively. There was no significant different in the genotype distribution of VDR polymorphism between CHB patients without cirrhosis and CHB with cirrhosis or hepatoma.

Conclusions: This study suggest that VDR gene polymorphism may not contribute to the progression of CHB infection.

Keywords: Cirrhosis, Hepatitis B, Hepatoma, Polymorphism, Vitamin D Receptor.

Introduction

Hepatitis B virus (HBV) infection is a major health problem affecting >250 million people globally and causes 880 000 death annually (1). The prevalence of hepatitis B surface antigen (HBsAg) in Indonesia is 7.1%, indicating a moderate endemicity for HBV infection. HBV infection that occurs during adults generally

trigger an effective immune response, resulting in resolution of acute infection (2). However, 95% of HBV exposure during perinatal will result in chronic HBV infection. Chronic hepatitis B (CHB) confers a substantial risk for liver cirrhosis and hepatocellular carcinoma (HCC), the latest is the second-leading cause of cancer-related death worldwide. Recently, accumulating evidence

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based on single nucleotide polymorphisms (SNPs) have suggested the important role of host genetic factors on the risk for liver cirrhosis and HCC among patients with CHB (3,4).

Hepatitis B virus is known as a non-cytopathic virus, which means that the inflammatory process and liver damage is not due to viral invasion but depend on the complex interaction between viral replication and host immune response (5). The adaptive immune responses governed by T-cell responses have been crucial in the persistence of chronic HBV infection. It has been shown that patients chronically infected with HBV demonstrated impaired T-responses (6–8).

Vitamin D is a fat soluble hormone that has important roles as immune modulator. Epidemiological studies suggest association between vitamin D deficiency and higher incidence of autoimmune diseases (9), cancer (10), metabolic (11) and inflammatory diseases including infection with HBV (12,13). The cellular actions of vitamin D are mediated by vitamin D receptors (VDR), a superfamily of nuclear transcription factor that is expressed in many cell types including various immune cells such as antigen-presenting cells, T cells, B cells and monocytes (14).

Vitamin D receptor gene is located on chromosome 12q12-q14. Previous studies have reported several SNPs within VDR gene that may affect its function in regulating the expression of target genes. Vitamin D receptors gene polymorphisms have been reported to be associated with diseases such as rheumatoid arthritis (15), tuberculosis (16), Graves' disease (6) and cancer (17). This study aimed to examine the distribution of genotype and allele of VDR polymorphisms among patients with CHB and analyse its association with risk for liver cirrhosis and hepatoma.

Materials and Methods

Study subjects

This study examined 75 CHB patients who were recruited from the Internal Medicine Department of the Arifin Achmad Hospital, Riau Province between March-September 2019. Of these 75 patients, 36 subjects were CHB patients without cirrhosis, 25 subjects were CHB patients with

cirrhosis and 14 subjects were CHB patients with hepatoma. Chronic hepatitis B was diagnosed based on HBsAg positivity for at least 6 months. Cirrhosis was diagnosed based on imaging (transient elastography and/or ultrasonography) and clinical signs, including oesophageal varices and ascites. Hepatoma was determined by computed tomography and/or magnetic resonance imaging and α -fetoprotein (AFP) levels. Written informed consent was obtained from all study participants after receiving adequate information about the study. The study protocol was approved by the Ethical Committee of Medical and Health Research, Faculty of Medicine, Universitas Riau in accordance with the Helsinki Declaration. The inclusion criteria were CHB patients aged equal or more than 18 years while the exclusion criteria were co-infection with other virus infection including hepatitis C virus and hepatitis D virus infection. Levels of serum ALT, albumin, and bilirubin were measured using commercial kits.

Genotyping of vitamin D receptor (VDR) polymorphisms

Ten mL of venous blood was withdrawn from each study participants and immediately stored in ethylenediaminetetraceticacid (EDTA) tubes. The blood was then centrifuged at 2000 g for 5 minutes and the plasma stored at -80 °C while the packed blood cells were stored at -20 °C for DNA extraction. Genomic DNA was extracted using Wizard® Genomic DNA Purification kit (Promega Inc., Madison, WI, USA) according manufacturer's protocol. The VDR FokI T>C (rs 10735810), BsmI C>T (rs 1544410), ApaI G>T (rs7975232), and TaqI C>T (rs 731236) single nucleotide polymorphisms (SNPs) were determined by the Amplification Refractory Mutation System Polymerase Chain Reaction (ARMS-PCR) method using primers designed by Jafari et al (18). as presented in Table 1. Each sample was amplified twice using one specific allele (wild-type or mutant) and one common primer. The PCR reaction was carried out in a total volume of 25 μ L using GoTaq® Green Master Mix (Promega Inc., Madison, WI, USA) containing GoTaq® reaction buffer (pH 8.5), 200 μ M dNTP, 1.5 mM of MgCl₂ and 5 μ L of genomic DNA. The thermal cycler conditions

were as follows: initial denaturation at 95 °C for 2 minutes, 29 cycles of denaturation at 95 °C for 25 seconds, annealing at 58 °C for 30 seconds and extension at 72 °C for 1 minute; and final extension at 72 °C for 5 minutes. PCR products

were subjected to electrophoresis on 2% agarose gel containing GelRed® Nucleic Acid Gel stain (Biotium, Fremont, CA, USA) submerged in 1x Tris Acetate EDTA (TAE) buffer at 75 volts for 40 minutes. PCR fragments were visualised in a UV GelDoc™ (Bio-Rad, USA).

Table 1. Primers used to detect VDR polymorphisms (26).

Primer sequences	Annealing temperature (°C)	Amplicon size (bp)
FokI (rs10735810)		
FokI/F: 5'-TGGCCGCCATTGCCTCCG 3'	62	
FokI/f: 5'- TGGCCGCCATTGCCTCCA 3'	60	77
FokI/C: 5'- AGCTGGCCCTGGCACTGA 3'	60	
BsmI (rs1544410)		
BsmI/B: 5'-AGCCTGAGTACTGGAATGT-3'	60	
BsmI/b:5'- AGCCTGAGTACTGGAATGC-3'	62	534
BsmI/C:5'- GGGAGGGAGTTAGGCACC-3'	60	
TaqI (rs731236)		
TaqI/B:5'- CAGGACGCCGCGCTGATT-3'	62	
TaqI/b:5'- CAGGACGCCGCGCTGATC-3'5'	60	148
TaqI/C:5'- CCTCATTGAGGCTGCGCAG-3'	62	
Apal (rs7975232)		
Apal/A:5'- TGGGATTGAGCAGTGAGGT-3'	58	
Apal/a:5'- TGGGATTGAGCAGTGAGGG-3'	60	229
Apal/C:5'- CCTCATTGAGGCTGCGCAG-3'	62	
<i>Internal Control</i>		
DRBI-F 5'- TGCCAAGTGGAGCACCCAA 3'	60	729
DRBI-R 5'-GCATCTTGCTCTGTGCAGAT 3'	60	

Statistical analyses

Categorical data are presented as proportion while numerical variable is presented as mean \pm SD (min-max). Chi-square test was used to examine group differences for genotype and allele frequencies of VDR polymorphisms. $p < 0.05$ was considered statistically significant. Statistical analyses were performed with SPSS statistic version 22 (IBM Corp., Armonk, NY, USA) for Mac.

Results

Demographic and clinical characteristic of study subjects

The demographic characteristics of study population are presented in Table 2. The mean age of study subjects was 45.37 ± 13.26 years (18-73 years). Subjects consisted of 45 (60%) males and 30 (40%) females. Table 3 shows the comparison of liver function between CHB, cirrhosis and hepatoma. The mean ALT levels did not differ significantly between CHB,

cirrhosis and hepatoma patients. However, albumin levels in CHB patients with cirrhosis (3.1 ± 0.7 g/dL) and CHB with hepatoma (3.6 ± 0.5 g/dL) were significantly lower than those with CHB ($p < 0.05$). In contrast, albumin levels in CHB patients with cirrhosis were higher than CHB patients (2.9 ± 3.7 vs. 1.5 ± 3.3 mg/mL, $p < 0.05$).

VDR polymorphism

Genotype distribution of VDR polymorphisms among study subjects is presented in Table 4. Of four VDR polymorphisms, bb (74.7%), aa (48%), TT (72%) and Ff (57.3%) were the most prevalent genotypes for BsmI, Apal, TaqI, and FokI, respectively. Tables 5 and 6 show the genotype and allele frequencies of VDR polymorphisms among patients with CHB. We found no significant difference in the genotype and allele frequencies between CHB, cirrhosis and hepatoma patients.

Table 2. Characteristic of study subjects.

Variable	N= 75	%
Age		
Mean±SD (years)	45.37±13.26	
Min-max (years)	18-73	
Sex		
Male	45	60
Female	30	40
Diagnosis		
CHB without cirrhosis	36	48
CHB with Cirrhosis	25	33.3
CHB with hepatoma	14	18.7

CHB=Chronic hepatitis B

Table 3. Comparison of liver function tests among patients with CHB.

Variable	CHB	Cirrhosis	Hepatoma
ALT (mean±SD, IU/L)	76.3±195.7	53.4±43.1	49.4±57.3
Albumin (mean±SD, g/dL)	4.2±0.7	3.1±0.7*	3.6±0.5*
Bilirubin (mean±SD, mg/dL)	1.5±3.3	2.9±3.7*	1.3±1.4

*p< 0.05 vs. CHB using One-way ANOVA test.

Table 4. Genotypic distribution of VDR polymorphisms among chronic hepatitis B patients.

Genotype	N	%
BsmI		
BB	5	6.7
Bb	14	18.7
bb	56	74.7
Apal		
AA	12	16
Aa	27	36
aa	36	48
TaqI		
TT	54	72
Tt	4	5.3
tt	17	22.7
FokI		
FF	24	32
Ff	43	57.3
ff	8	10.7

Table 5. VDR genotype frequencies among patients with chronic hepatitis B patients.

Genotype	CHB without cirrhosis (n= 36)	CHB with cirrhosis (n= 25)	CHB with hepatoma (n= 14)	P value
BsmI				
BB	2 (40%)	2 (40%)	1 (20%)	0.794
Bb	8 (57.1%)	5 (35.7%)	1 (7.1%)	
bb	26 (46.4%)	18 (32.1%)	12 (21.4%)	
Apal				
AA	5 (41.7%)	3 (25%)	4 (33.3%)	0.577
Aa	15 (55.6%)	8 (29.6%)	4 (14.8%)	
aa	16 (44.4%)	14 (38.9%)	6 (16.7%)	
TaqI				
TT	26 (48.1%)	18 (33.3%)	10 (18.5%)	0.765
Tt	3 (75%)	1 (25%)	0 (0%)	
tt	7 (41.2%)	6 (35.3%)	4 (23.5%)	
FokI				
FF	14 (58.3%)	8 (33.3%)	2 (8.3%)	0.468
Ff	18 (41.9%)	14 (32.6%)	11 (25.6%)	
ff	4 (50%)	3 (37.5%)	1 (12.5%)	

p< 0.05 vs. using Chi-square test.

Table 6. Allele frequencies of VDR polymorphisms among patients with CHB.

Allele	CHB (n= 36)	Cirrhosis (n= 25)	Hepatoma (n= 14)	p value
BsmI				
B	12 (50%)	9 (37.5%)	3 (12.5%)	0.685
b	60 (47.6%)	41 (32.5%)	25 (19.9%)	
Apal				
A	25 (49%)	14 (27.5%)	12 (23.5%)	0.407
a	47 (47.5%)	36 (36.4%)	16 (16.1%)	
TaqI				
T	55 (49.1%)	37 (33%)	20 (17.9%)	0.869
t	17 (44.7%)	13 (34.2%)	8 (21.1%)	
FokI				
F	46 (50.5%)	30 (32.9%)	15 (16.6%)	0.633
f	26 (44%)	20 (33.9%)	13 (22.1%)	

p< 0.05 vs. using *Chi-square* test.

Discussion

Patients with persistence HBV infection are at increased risk for developing liver cirrhosis and HCC, depending on viral and host factors (5). In recent years, several studies have shown that SNPs of VDR gene identified by allelic variation in restriction enzyme sites are associated with increased susceptibility of liver cirrhosis and liver cancer development among CHB patients in various global population. The present study analysed the genotype and allele frequencies of VDR polymorphisms among Indonesian patients with CHB. Our results showed that bb, aa, TT, and Ff genotype were the most frequent genotypes for BsmI, ApaI, TaqI, and FokI, respectively. Few studies have been conducted to examine the genotype distribution of VDR gene in Indonesian population. Setiabudiawan et al (19). reported that bb, AA, TT, and Ff/ff were the most prevalent genotype with regard to VDR gene, indicating that ApaI polymorphism has more genetic variation within Indonesian population than BsmI, TaqI, and FokI. Previous studies have reported different genotype distributions including BB, AA, TT, and Ff in Vietnamese (20) and Bb, Aa, TT, Ff in German population (21). This suggests that genetic variations within ApaI and BsmI sites exist between populations.

Furthermore, we found no significant difference in the distribution of VDR gene polymorphism between CHB patients without cirrhosis, with

cirrhosis and with hepatoma. There have been conflicting results regarding the association between VDR polymorphisms and the progression of HBV-related liver disease. Previous study demonstrated that FokI gene polymorphism (C>T) were linked to increased risk for HCC development in Egyptian population infected with HBV (22). Similarly, FokI polymorphism was significantly associated with risk for developing HCC in a Chinese population with chronic HBV infection (23) and showed better therapeutic response in CHB patients received pegylated interferons (PEG-IFNs) (24). A recent meta-analysis further supports the correlation of FokI polymorphism with HBV infection and liver disease progression (25). While a recent study reported that ApaI polymorphism was associated with the clinical picture and disease progression in Vietnamese population infected with HBV (20).

Vitamin D is an important immune modulator by binding with vitamin D receptors (VDR). VDR is a member of intranuclear receptor family that acts as a transcription factor. Several cells of the immune system such as dendritic cells, macrophages, T and B lymphocytes are known to express VDR on the cell membrane. Vitamin D has been reported to modulate the activity of > 900 genes (14). These data further strengthen the role of vitamin D in the pathogenesis of malignant diseases, metabolic

diseases and infections, including HBV infection.

The gene encoding vitamin D receptor is located on chromosome 12 and consists of 9 exons. There are four major polymorphic sites within the VDR gene: intron 8 consists BsmI (rs1544410) and ApaI (rs7975232) polymorphisms, exon 9 contains TaqI (rs731236) polymorphism, which is created by a silent T to C substitution, and exon 2 has FokI (rs10735810) polymorphism, producing a protein variant with three additional amino acids at the amino terminus resulted from an alternative translation initiation (21).

To our knowledge, this is the first study to investigate the association between VDR gene

polymorphism and HBV infection within Indonesian population. The limitation of this study was the small size of study subjects enrolled and lack of clinical data related to cirrhosis and HCC severity. So, further studies with larger samples are required to confirm the role of VDR gene polymorphisms on the risk for liver cirrhosis and HCC among CHB patients.

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References

1. World Health Organization. Global Hepatitis Report, 2017 [Internet]. WHO. 2017. 62 p. Available from: <https://apps.who.int/iris/handle/10665/255016>.
2. H Muljono D. Epidemiology of Hepatitis B and C in Republic of Indonesia. *Euroasian J Hepatogastroenterol*. 2017;7(1):55-59.
3. Zhang Z, Wang C, Liu Z, Zou G, Li J, Lu M. Host Genetic Determinants of Hepatitis B Virus Infection. *Front Genet*. 2019;10:696.
4. Zhang J, Liu L, Lin Z, Ji X, Pi L, Lin X, et al. SNP-SNP and SNP-environment interactions of potentially functional HOTAIR SNPs modify the risk of hepatocellular carcinoma. *Mol Carcinog*. 2019;58(5):633-642.
5. Trepo C, Chan HL, Lok A. Hepatitis B virus infection. *Lancet*. 2014;384(9959):2053-63.
6. Meng S, He S, Jiang W, Xiao L, Li D, Xu J, et al. Genetic susceptibility to autoimmune thyroid diseases in a Chinese Han population: Role of vitamin D receptor gene polymorphisms. *Ann Endocrinol (Paris)*. 2015;76(6):684-9.
7. Meng Z, Chen Y, Lu M. Advances in Targeting the Innate and Adaptive Immune Systems to Cure Chronic Hepatitis B Virus Infection. *Front Immunol*. 2019;10:3127.
8. Yang PL, Althage A, Chung J, Maier H, Wieland S, Isogawa M, et al. Immune effectors required for hepatitis B virus clearance. *Proc Natl Acad Sci U S A*. 2010;107(2):798-802.
9. Bizzaro G, Antico A, Fortunato A, Bizzaro N. Vitamin D and Autoimmune Diseases: Is Vitamin D Receptor (VDR) Polymorphism the Culprit?. *Isr Med Assoc J*. 2017;19(7):438-443.
10. Mondul AM, Weinstein SJ, Layne TM, Albanes D. Vitamin D and Cancer Risk and Mortality: State of the Science, Gaps, and Challenges. *Epidemiol Rev*. 2017;39(1):28-48.
11. Zhai N, Bidares R, Makoui MH, Aslani S, Mohammadi P, Razi B, et al. Vitamin D receptor gene polymorphisms and the risk of the type 1 diabetes: a meta-regression and updated meta-analysis. *BMC Endocr Disord*. 2020;20:121.
12. Farnik H, Bojunga J, Berger A, Allwinn R, Waidmann O, Kronenberger B, et al. Low vitamin D serum concentration is associated with high levels of hepatitis B virus replication in chronically infected patients. *Hepatology*. 2013;58(4):1270-6.
13. Gao W, Wang R, Wang X, Wu H, Wang Y, Lu X, et al. Vitamin D serum levels and receptor genetic polymorphisms are associated with hepatitis B virus and HIV infections and IFN- λ levels. *Biomark Med*. 2017;11(9):733-740.
14. Sassi F, Tamone C, D'amelio P. Vitamin D: Nutrient, hormone, and immunomodulator. *Nutrients*. 2018;10(11):1656.
15. Tizaoui K, Hamzaoui K. Association between VDR polymorphisms and rheumatoid arthritis disease: Systematic review and updated

- meta-analysis of case-control studies. *Immunobiology*. 2015;220(6):807-16.
16. Salimi S, Farajian-Mashhadi F, Alavi-Naini R, Talebian G, Narooie-Nejad M. Association between vitamin D receptor polymorphisms and haplotypes with pulmonary tuberculosis. *Biomed Rep*. 2015;3(2):189-194.
 17. Gandini S, Gnagnarella P, Serrano D, Pasquali E, Raimondi S. Vitamin D receptor polymorphisms and cancer. *Adv Exp Med Biol*. 2014;810:69-105.
 18. Jafari M, Pirouzi A, Anoosheh S, Farnia P, Tajik N. Rapid and simultaneous detection of vitamin D receptor gene polymorphisms by a single ARMS-PCR assay. *Mol Diagn Ther*. 2014;18(1):97-103.
 19. Setiabudiawan B, Kartasasmita CB, Garna H, Parwati I, Maskoen AM. Polimorfisme FokI, BsmI, ApaI, dan TaqI Gen Reseptor Vitamin D pada Kejadian Tuberkulosis Anak. *MKB*. 2010;42:187.
 20. Hoan NX, Khuyen N, Giang DP, Binh MT, Toan NL, Anh DT, et al. Vitamin D receptor ApaI polymorphism associated with progression of liver disease in Vietnamese patients chronically infected with hepatitis B virus. *BMC Medical Genetics*. 2019;20:201.
 21. Ramos-Lopez E, Kurylowicz A, Bednarczuk T, Paunkovic J, Seidl C, Badenhop K. Vitamin D receptor polymorphisms are associated with Graves' disease in German and Polish but not in Serbian patients. *Thyroid*. 2005;15(10):1125-30.
 22. Mohammed MA, Shabana T, Sheta NM, Omar SAM. Vitamin D Receptor Gene Polymorphisms as a Predictive Risk Factor for Hepatocellular Carcinoma Development and Severity in Chronic Hepatitis B. *International Journal of Cancer Research*. 2017;13(1):26-35.
 23. Yao X, Zeng H, Zhang G, Zhou W, Yan Q, Dai L, et al. The associated ion between the VDR gene polymorphisms and susceptibility to hepatocellular carcinoma and the clinicopathological features in subjects infected with HBV. *Biomed Res Int*. 2013;2013:953974.
 24. Mostafa-Hedeab G, Sabry D, Mostafa Abdelaziz G, Ewaiss M, Adli N, Fathy W. Influence of Vitamin D Receptor Gene Polymorphisms on Response to Pegylated Interferon in Chronic Hepatitis B Egyptian Patients. *Rep Biochem Mol Biol*. 2018;6(2):186-196.
 25. He Q, Huang Y, Zhang L, Yan Y, Liu J, Song X, et al. Association between vitamin D receptor polymorphisms and hepatitis B virus infection susceptibility: A meta-analysis study. *Gene*. 2018;645:105-12.
 26. Tangoh D, Mahmood Y, Apinjohn T, Nyingchu R, Azhar A, Achidi E. A Rapid Multiplex ARMS-PCR Method for the Detection of Four Single Nucleotide Polymorphisms of the Vitamin D Receptor (VDR) Gene. *Imp J Interdiscip Res*. 2017;3(9):573-80.