Interleukin-6 -174 promoter polymorphism and susceptibility to hepatitis B virus infection in Javanese individuals

A A Prasetyo1,2*, M Marwoto1 and W Monica1

1 A-IGIC (A-Infection, Genomic, Immunology & Cancer) Research Group, Sumber, Banjarsari, Surakarta, 57138, Indonesia
2 Department of Microbiology, Faculty of Medicine, Universitas Sebelas Maret, Jl. Ir. Sutami 36A, Surakarta, 57126, Indonesia

*afie.agp.la@gmail.com

Abstract. Hepatitis B virus infection is a significant risk factor for cirrhosis and hepatocellular carcinoma. Interleukin-6, a cytokine with broad pleiotropic actions, plays important role in cellular and humoral immune responses to viral infection. The -174 G/C polymorphisms of interleukin-6 is a frequent polymorphism and may determine the outcome of viral infection. To find out the genetic association between the -174 G/C polymorphism and susceptibility to hepatitis B virus infection in Javanese individuals, blood samples from Javanese hepatitis B virus-infected individuals and from Javanese healthy individuals confirmed by serological and molecular assays were evaluated. Genomic DNA was extracted from peripheral blood and the sequence specific primer-polymerase chain reaction method was applied for genotyping. The frequencies of genotypes C/C, C/G and G/G in hepatitis B virus-infected samples were 5.3%, 31.6%, 63.1% and in controls were 20%, 38%, and 42%, respectively. The frequencies of G allele in hepatitis B virus-infected samples was higher than that of healthy samples (OR 2.4, 95%CI: 1.289-4.459, p = 0.0057). These findings suggest that the G allele was associated with susceptibility to hepatitis B virus infection in Javanese individuals.

1. Introduction
Hepatitis B virus is considered to be a major public health problem worldwide [1]. Hepatitis B virus is characterized by a high degree of genetic heterogeneity because of the use of a reverse transcriptase during viral replication. The ten genotypes (A-J) that have been described so far further segregate into a number of subgenotypes which have distinct ethnic-geographic distribution [2]. Hepatitis B virus is transmitted by parenteral route, sexual and vertical transmission [3]. Infection with hepatitis B virus may lead to subclinical, acute, or chronic hepatitis [4]. The clinically present primarily with hepatitis B infection and can have many adverse effects or even be life-threatening at times, if not treated properly [5].

Hepatitis B virus is major causes of chronic liver diseases [6]. The prevalence of chronic hepatitis B virus infection is still more than 1% in many countries in the world although a vaccine against hepatitis B virus has been available since 1982 [7]. Chronic hepatitis B virus infection affects approximately 240 million people worldwide and remains a serious public health concern because its complete cure is impossible with current treatments. Covalently closed circular DNA (cccDNA) in the nucleus of infected cells cannot be eliminated by present therapeutics and may result in persistence and relapse [5, 8]. Chronically hepatitis B virus-infected individuals function as a reserve for sustained...
hepatitis B virus transmission and can lead to cirrhosis (may be asymptomatic in the beginning), hepatocellular carcinoma, and death in a significant portion of affected people [7, 9].

Interleukins are a group of immunomodulatory proteins that mediate a variety of immune reactions in the human body [10]. Interleukin-6 is a multifunctional key player cytokine in inflammation and the main factor for the induction of acute phase protein biosynthesis. Further to its central role in many aspects of the immune system, interleukin-6 regulates a variety of homeostatic processes. The complex signal transduction mechanism of interleukin-6 may help explain the pleiotropic nature of the proinflammatory cytokine [11]. Interleukin-6 signaling is unique because it can also occur via a soluble interleukin-6 receptor which allows for interleukin-6 signaling in tissues that do not normally express IL-6R through a process referred to as IL-6 trans-signaling [12]. As a pleiotropic proinflammatory cytokine, interleukin-6 modulating multiple functions of immune cells including T cells, dendritic cells, and macrophages, in innate and adaptive immune responses [13]. Interleukin-6 is responsible for the induction of hepatic acute-phase proteins, trafficking of acute and chronic inflammatory cells, differentiation of adaptive T cell responses, homeostatic regulation, and tissue regeneration [14].

Interleukin-6-mediated cellular and humoral immune responses play a crucial role in determining the outcome of viral infection. In hepatitis B virus infection, interleukin-6 plays an important role in promoting lymphocytes responses that are essential for effective viral control [15]. The interleukin-6 -174 G/C polymorphism is a frequent polymorphism, that is in the upstream regulatory region of the interleukin-6 gene and affects interleukin-6 production. However, the data in the literature on the genetic association between the interleukin-6 -174 G/C polymorphism and some specific liver diseases are still controversial [16]. To find out the genetic association between the -174 G/C polymorphism and susceptibility to hepatitis B virus infection in Javanese individuals, our research group (A-IGIC/A-Infection, Genomics, Immunology & Cancer) evaluated the blood samples from Javanese hepatitis B virus-infected individuals and from Javanese healthy individuals.

2. Materials and methods

2.1. Samples origin
Since 2009, our research group has been conducting a molecular epidemiology study of human bloodborne pathogens by collecting epidemiological-clinical data and blood samples from high-risk communities in Central Java, Indonesia. For the present study, the blood samples collected in 2009-2012 were used.

2.2. Serological and molecular assays for infection status definition
All blood samples were subjected for serological and molecular assays to find out the human immunodeficiency virus, hepatitis B virus, hepatitis C virus, hepatitis D virus, Torque Teno virus, GB virus C, human T-cell lymphotropic viruses, and Toxoplasma gondii infection status as described previously [17-23]. Briefly, the subjects’ plasma was separated from whole blood with EDTA and subjected to the serological assays. Human immunodeficiency virus antibodies were detected using a Determine HIV-1/2 Kit (Abbott Diagnostics Japan, Tokyo, Japan). A SERATEC Hepatitis B Quick Test (Gesellschaft für Biotechnologische Forschung GmbH, Göttingen, Germany) was used to detect hepatitis B virus HBsAg. An Ortho HCV PA II (Ortho Diagnostics, Tokyo, Japan) and an HDV Ab ELISA (Diagnostic Automation, Calabasas, CA) were used to detect anti-hepatitis C virus and anti-hepatitis D virus antibodies, respectively. The MP Diagnostic HTLV-I/II ELISA 4.0 (MP Biomedicals, Singapore) was used to detect the anti-human T-cell lymphotropic viruses-1/2 antibodies. The DRG Toxoplasma gondii IgM Elisa Kit (DRG International, Springfield, NJ) was used to detect IgM anti-toxoplasma and a DRG Toxoplasma gondii IgG Elisa Kit (DRG International) was used to detect IgG anti-toxoplasma antibodies, respectively. All assays were performed according to the manufacturer’s instructions. All samples were tested at least in duplicate. The viral DNA and RNA were extracted from all plasma samples using the Quick-cfDNA™ Serum & Plasma (Zymo Research, Irvine, CA) and ZR Viral RNA
(Zymo Research), respectively, according to the manufacturer’s instructions. The nucleic acids were then aliquoted, and one aliquot was reverse-transcribed according to the SensiFAST cDNA Synthesis protocol (Bioline, London, UK). Molecular detection was performed by PCR using the MyFi Mix (Bioline). A portion of the human immunodeficiency virus 


gag gene and a portion of the pol gene were amplified by nested polymerase chain reaction. A portion of the hepatitis B virus 


HBsAg gene was amplified by nested polymerase chain reaction. A portion of the NS5B region and a portion of the 


E1-E2 region of the hepatitis C virus genome were amplified by nested polymerase chain reaction. Hepatitis D virus RNA was detected by polymerase chain reaction of a 400-nucleotide (nt)-long region. Torque Teno virus DNA was amplified by the nested polymerase chain reaction of a conserved region of open reading frame-2. A fragment from the 5’ non-coding region of GB virus C was detected by nested polymerase chain reaction. The long terminal repeats regions of the human T-cell lymphotropic viruses-1 genome and the long terminal repeats regions of the human T-cell lymphotropic viruses-2 genome were amplified using the nested polymerase chain reaction. Internal amplification controls were included to exclude any false-negative results. Corresponding positive controls and one negative control were included for each group. To prevent polymerase chain reaction contamination, the reagent preparation, sample processing, and polymerase chain reaction assays were performed in rooms separate from where the amplified products were analyzed. Aerosol-resistant pipette tips were used throughout the assays. The polymerase chain reaction products were subjected to electrophoresis in 3% agarose gels, stained with ethidium bromide, and visualized under ultraviolet illumination. The specificity was confirmed by sequencing the amplicons. All samples were tested at least in duplicate.

2.3. Molecular detection for Interleukin-6 -174 G/C polymorphism status

The molecular detection techniques for interleukin-6 -174 G/C gene polymorphisms status were performed as described previously with minor modifications [24]. Briefly, genomic DNA was extracted from whole blood by using High Pure PCR Template Preparation Kit (Roche Life Science, Manheim, Germany) according to the manufacturer’s instructions. Molecular assay was performed by a sequence-specific primer-polymerase chain reaction using the MyFi Mix (Bioline). The genotypes were defined according to generated fragment patterns in the agarose gel electrophoresis analysis of polymerase chain reaction products. To exclude any false-negative results, the internal amplification controls were included in all assays. Corresponding positive controls and one negative control were included for each assay. The reagent preparation, sample processing, and polymerase chain reaction assays were performed in rooms separate from where the amplified products were analysed. Aerosol-resistant pipette tips were used throughout the assays. The polymerase chain reaction products were subjected to electrophoresis in 3% agarose gels, stained with ethidium bromide, and visualized under ultraviolet illumination. All samples were tested at least twice.

3. Results and discussion

Through 2012, 38 blood samples were found positive for hepatitis B virus but negative for other bloodborne pathogens (human immunodeficiency virus, hepatitis C virus, hepatitis D virus, Torque Teno virus, GB virus C, human T-cell lymphotropic viruses, and Toxoplasma gondii, respectively) by serological and molecular assays. The epidemiological data of the hepatitis B virus-infected individuals and the molecular characterization of the hepatitis B virus already published partially elsewhere [17]. All 38 blood samples then used for the present study and subjected for the molecular detection of interleukin-6 -174 G/C gene polymorphisms status. For control, 100 blood samples negative for all bloodborne pathogens (human immunodeficiency virus, hepatitis B virus, hepatitis C virus, hepatitis D virus, Torque Teno virus, GB virus C, human T-cell lymphotropic viruses, and Toxoplasma gondii, respectively) were randomly selected and then used for the molecular detection for interleukin-6 -174 G/C gene polymorphisms status (Table 1).
Table 1. The frequency of the interleukin-6 -174 G/C genotypes.

| Genotype | Case (%) | Control (%) |
|----------|----------|-------------|
| C/C | 5.3 (2/38) | 20.0 (20/100) |
| C/G | 31.6 (12/38) | 38.0 (38/100) |
| G/G | 63.1 (24/38) | 42.0 (42/100) |

Model of Inheritance

| Inheritance | Case (%) | Control (%) |
|-------------|----------|-------------|
| Dominant    | C/C | 5.3 (2/38) | 20.0 (20/100) |
|             | C/G + G/G | 94.7 (36/38) | 80.0 (80/100) |
|             | C/G + C/C | 36.8 (14/38) | 58.0 (58/100) |
|             | G/G | 63.1 (24/38) | 42 (42/100) |

| Co-dominant | G/G | 68.4 (26/38) | 62.0 (62/100) |

The presence of a G/C single nucleotide polymorphism at the promoter -174 of the interleukin-6 gene is related to the interleukin-6 gene transcription rate and to the control of circulating interleukin-6 levels. Subsequently, two phenotypes for this polymorphism were identified: the high-producer phenotype, including the -174 G/G and -174 G/C genotypes, characterized by higher circulating IL-6 levels; and the low-producer phenotype, including the -174 C/C genotype [16]. In the present study, the frequencies of G/G genotype in hepatitis B virus-infected samples was higher than that of healthy samples (OR 2.4, 95%CI: 1.097-5.111, p = 0.0282). Moreover, the frequencies of G allele in hepatitis B virus-infected samples was higher than that of healthy samples (OR 2.4, 95%CI: 1.289-4.459, p = 0.0057) (Table 1). Taken all data together, the G allele of the interleukin-6 -174 was associated with susceptibility to hepatitis B virus infection in Javanese individuals. However, the present study had several limitations. First, the sample size was limited. Second, we only assessed one polymorphism in the interleukin-6 gene; therefore, we cannot rule out that other polymorphisms or haplotypes in this gene might be implicated in the susceptibility to hepatitis B virus infection in Javanese individuals.

4. Conclusions
The present finding suggests that the G allele was associated with susceptibility to hepatitis B virus infection in Javanese individuals. Larger studies and sequencing of the whole coding sequence of the interleukin-6 gene would provide additional valuable data.

Acknowledgement
This work was supported in part by grants from the Indonesian Ministry of Research, Technology and Higher Education 2018 (No. 089/ SP2H/ LT/ DRPM/ 2018) and PNBP UNS 2018 (No. 543/ UN27.21/ PP/ 2018).

References
[1] A Sauerbrei 2014 World J. Gastroenterol. 20 436-444
[2] G Zehender, E Ebranati, E Gabanelli, C Sorrentino, A Lo Presti, E Tanzi, M Ciccozzi and M Galli 2014 World J. Gastroenterol. 20 7622-7634
[3] L M Villar, H M Cruz, J R Barbosa, C S Bezerra, M M Portilho and P Scalioni Lde 2015 World J. Virol. 4 323-342
[4] A D Kosinska, J Liu, M Lu and M Roggendorf 2015 *Med. Microbiol. Immunol.* **204** 103-114
[5] F Akhtar and S Rehman 2018 *Cureus.* **10** e2077
[6] D H Sinn, E J Cho, J H Kim, D Y Kim, Y J Kim and M S Choi 2017 *Clin. Mol. Hepatol.* **23** 189-195
[7] G Ray 2017 *J. Clin. Transl. Hepatol.* **5** 277-296
[8] X Li, J Zhao, Q Yuan and N Xia 2017 *Viruses.* **9** E139
[9] L Ma, N R Alla, X Li, O A Mynbaev and Z Shi 2014 *Rev. Med. Virol.* **24** 396-406
[10] M Zhang, J Xu, X Bao, W Niu, L Wang, L Du, N Zhang and Y Sun 2017 *PLoS One* **12** e0169891
[11] U Hahn 2017 *Int. J. Mol. Sci.* **18** E2641
[12] S P Didion 2017 *Int. J. Mol. Sci.* **18** E2563 (2017)
[13] H Tsukamoto, K Fujieda, S Senju, T Ikeda, H Oshiumi and Y Nishimura 2018 *Cancer Sci.* **109** 523-530
[14] M Mesquida, B Molins, V Llorenç, M S de la Maza and A Adán 2017 *Autoimmun Rev.* **16** 1079-1089
[15] T Lan, L Chang, L Wu and Y F Yuan 2015 *J. Clin. Transl. Hepatol.* **3** 271-276
[16] L Giannitrapani, M Soresi, D Balasus, A Licata and G Montalto 2013 *World J. Gastroenterol.* **19** 2449-2455
[17] A A Prasetyo, P Dirghahayu, Y Sari, H Hudiyono and S Kageyama 2013 *J. Infect. Dev. Ctries.* **7** 453-467
[18] A A Prasetyo and K U N Zaini 2015 *Southeast Asian J. Trop. Med. Public Health.* **46** 662-668
[19] A A Prasetyo, M N Desyardi, J Tanamas, Suradi, Reviono, Harsini, S Kageyama, H Chikumi and E Shimizu 2015 *Intervirology.* **58** 57-68
[20] A A Prasetyo and R Sariyutun 2015 *Asian J. Microbiol. Biotechnol. Environ. Sci.* **17** 349-355
[21] A A Prasetyo and R Sariyutun 2015 *J. Teknol.* **77** 67-70
[22] A A Prasetyo, R Dharmawan, I Raharjo and Hudiyono 2016 *J. Global Infect. Dis.* **8** 75-81
[23] A A Prasetyo 2017 *AIP Conference Proceedings* **1788** 030100
[24] M Attar, M Mansoori and M Shahbazi 2017 *Asian Pac. J. Cancer Prev.* **18** 3025-3029