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Research Report

DETECTION OF TUMOR NECROSIS FACTOR-α (TNF-α) GENE PROMOTERS POLYMORPHISM AMONG LIVER CIRRHOSIS PATIENTS WITH CHRONIC HEPATITIS B VIRUS (HBV) INFECTION IN SURABAYA, INDONESIA

Citrawati Dyah Kencono Wungu[1,α], Mochamad Amin[2], S. Eriaty N. Ruslan[2], Priyo Budi Purwono[3], Ulfa Kholili[4], Poernomo Boedi Setiawan[4], Maria Inge Lusida[2,3], Soetjipto[1,2], Retno Handajani[1,2]

[1] Department of Medical Biochemistry, Medical Faculty of Universitas Airlangga, Surabaya
[2] *Institute of Tropical Disease*, Universitas Airlangga, Surabaya
[3] Department of Medical Microbiology, Medical Faculty of Universitas Airlangga, Surabaya
[4] Department of Internal Medicine, Medical Faculty of Universitas Airlangga - Dr. Soetomo General Hospital, Surabaya
[α] Corresponding author: cicit.biokimia@gmail.com

ABSTRACT

Polymorphisms in TNF-α gene promoter region are known of its role in the production of TNF-α which may influences the pathogenesis of liver disease. SNPs in positions 238 and 308 of TNF-α gene promoters may affect the production of these cytokines. This study was aimed to detect Single Nucleotide Polymorphism (SNP) on -238 and -308 positions in the TNF-α gene promoter among liver cirrhosis patients with HBV infection in Surabaya, Indonesia. This was descriptive exploratory research with cross sectional study design using serum liver cirrhosis patients with HBV infection in Endoscopy Outpatient Clinic Dr. Soetomo General Hospital, Surabaya from April-May 2017. SNPs at -238 and -308 on TNF-α gene promoter (rs361525 and rs1800629 respectively) were detected using Polymerase Chain Reaction-Restriction Fragment Length Polymorphism (PCR-RFLP) with primers specific for the TNF-α promoter region and restriction enzymes NcoI and MspI. The genotypes of TNF-α gene promoter were assessed according to the length of the fragments produced in RFLP. Serum TNF-α levels was measured by commercial ELISA. In this study, as much as 149 positive HBsAg patients was found in Endoscopy Outpatient Clinic, Dr. Soetomo General Hospital, Surabaya. From those amount, as much as 30 liver cirrhosis patients with positive HBsAg were obtained. From 2/30 (6.7%) patients showed the GA heterozygote SNP either position -238 or -308. No patient had the AA genotype. Median blood TNF-α level in women (38 ng / L) was higher than in men (33 ng / L). TNF-α levels in patients with GA heterozygote genotype at -238 and -308 in this research was not different than wild-type (GG genotype). Among patients with liver cirrhosis due to chronic HBV infection in Surabaya, Indonesia, Surabaya, we found GA polymorphisms the TNF-α promoter gene at positions -238 and -308 in 6.7% patients, and did not find homozygous AA polymorphisms. Further studies including larger numbers of patients from various ethnic backgrounds in Indonesia are needed to provide robust data on TNF-α gene promoter polymorphisms and their role in the pathogenesis of liver cirrhosis with HBV infection in this country.

Keywords: *Liver Cirrhosis, Hepatitis B Virus, SNP, TNF-α, PCR-RFLP*

ABSTRAK

Polimorfisme pada promotor gen TNF-α diketahui berperan pada produksi TNF-α yang selanjutnya berperan dalam patogenesis penyakit hepar, penyakit infeksi, serta inflamasi. SNP gen TNF, terutama pada posisi 238 dan 308 dari promotor gen TNF-α telah diidentifikasi dapat memengaruhi produksi sitokin tersebut. Penelitian ini merupakan penelitian pendahuluan yang dilakukan untuk mendeteksi Single Nucleotide Polymorphism (SNP) promotor gen TNF-α posisi -238 dan -308 dari sampel penderita sirosis hati dengan infeksi VHB di Poli Endoskopi RSUD Dr. Soetomo, Surabaya. Jenis penelitian ini adalah penelitian deskriptif eksploratif laboratorik dengan rancangan penelitian cross sectional study yang mengambil sampel pasien penderita sirosis hati dengan infeksi VHB di Poli Endoskopi RSUD Dr. Soetomo Surabaya dari bulan April-Mei 2017. SNP posisi -238 dan -308 (rs361525 dan rs1800629) promotor gen TNF-α dideteksi menggunakan teknik Polymerase Chain Reaction-Restriction Fragment Length Polymorphism (PCR-RFLP)

dengan primer yang spesifik untuk daerah promotor gen TNF-α dan enzim restriksi endonuklease NcoI dan MspI. Selanjutnya dilakukan penentuan genotipe promotor gen TNF-α sesuai dengan panjang fragmen yang dihasilkan pada RFLP. Kadar TNF-α serum diukur dengan menggunakan metode ELISA. Dalam penelitian ini, didapatkan sebanyak 149 penderita dengan HBsAg positif di Poli Endoskopi RSUD Dr. Soetomo Surabaya. Dari jumlah tersebut, didapatkan sebanyak 30 penderita sirosis hati dengan HBsAg positif. Dari 30 serum sampel penelitian ini, didapatkan sebanyak 6,67% menunjukkan genotipe SNP heterozigot GA untuk promotor gen TNF-α baik posisi -238 maupun -308. Tidak ditemukan genotipe AA pada penelitian ini. Median kadar TNF-α pada pasien wanita (38 ng/L) lebih tinggi dibandingkan dengan laki-laki (33 ng/L). Kadar TNF-α pada pasien dengan genotipe SNP heterozigot GA pada posisi -238 dan -308 tidak berbeda secara signifikan dibandingkan dengan wild-type (genotipe GG). Pada penderita sirosis hati dengan infeksi VHB di Poli Endoskopi RSUD Dr. Soetomo, Surabaya ditemukan SNP heterozigot GA (6,67%) dan tidak ditemukan SNP homozigot AA promotor gen TNF-α (-238 dan -308). Studi lebih lanjut pada berbagai ras diperlukan untuk memberikan data yang jelas mengenai SNP promotor gen TNF-α pada sirosis hati dengan infeksi VHB.

Kata kunci: *Sirosis hati, Virus Hepatitis B, SNP, TNF-α, PCR-RFLP*

INTRODUCTION

About 240 million people of the world are infected with chronic HBV and about 600,000 people die each year from diseases related to HBV infection and Hepatocellular Carcinoma (HCC)(1). The 5-year cumulative incidence of cirrhosis ranges from 8–20% in untreated chronic HBV patients and, among those with cirrhosis, the 5-year cumulative risk of hepatic decompensation is 20%[2].

Although it is not fully understood, there are several factors suspected to affect the progression of HBV infection, including viral factors, environmental factors, and host genetic factors[3]. From these various factors, research on host factors has begun to be widely developed to understand the difference in progression and outcome of HBV infection in each patient[4].

Under conditions of chronic HBV infection, Th1 cytokines primarily played by TNF-*α* play a dominant role, especially in the immune clearance, inactive carrier, and reactivation phases[5]. Chronic inflammation and hepatic infiltration of leukocytes increase the production of cytokines including TNF-α that trigger cell death thus increasing hepatic damage. High production of TNF-α can cause liver fibrosis through upregulation of TIMP-1 and prevent apoptosis of hepatocytes[6].

Polymorphisms in TNF-α promoter genes are known to play a role in the pathogenesis of liver disease, infectious diseases, and inflammation[3]. Several previous studies have identified the presence of multiple Single Nucleotide Polymorphisms (SNPs) in the TNF gene group. TNF-α promoter polymorphisms can affect the transcriptional rate and, consequently, TNF-α cytokine production. G nucleotide transition to A in the promoter position -238 and -308 is known to affect the production of TNF-α[7,8].

Data on factors related to the incidence of liver cirrhosis in patients with chronic HBV infection in Indonesia are limited, eventhough this data is needed to understand the pathophysiology of liver cirrhosis development, so this study was conducted to detect the TNF-α gene promoter SNP in liver cirrhosis patients due to chronic HBV infection in Indonesia.

MATERIALS AND METHODS

Sampling. This research was a descriptive cross-sectional study and the samples were from liver cirrhosis patients with positive HBsAg who visited the Endoscopy Outpatient Clinic Dr. Soetomo General Hospital, Surabaya in April-May 2017.

The inclusion criterias of this study were: Chronic hepatitis B patients with a history of positive HBsAg ≥ 6 months, with ultrasound diagnosed results from an internist who showed cirrhosis of the liver, the willingness to participate in all study subjects and must sign informed consent, adult patients (š16 years), in conscious condition, and not in an emergency condition. Exclusion criterias in this study were: Patients coinfected with HCV or HIV, and received immunosuppressant therapy.

The study was conducted after obtaining approval from the Research Ethics Committee of Dr. Soetomo General Hospital, Surabaya. Blood collection was taken from cubital vein with 4 mL of blood put on a venoject tube with EDTA and 3 mL of blood put on a venoject tube without EDTA. Blood samples were then taken to the Laboratory of Hepatitis In Institute of Tropical Disease (ITD) of Universitas Airlangga for laboratory examination. Blood samples in the venoject tube with EDTA, plasma separation, PBMC isolation, and host genome extraction for PCR-RFLP examination were performed, while Blood samples in the venoject tube with EDTA, serum separation was performed for the TNF-α ELISA examination.

PBMC Isolation. After plasma separation, the remaining part of blood was performed PBMC isolation using Phosphate Buffer Saline (PBS) and Ficoll-Histopaque-10779. The obtained PBMC was then transferred into a 1.5 mL eppendorf tube and stored in the -30°C at freezer.

Examination of Serum TNF-α levels. Serum TNF-α was examined using diagnostic kit: Human TNF-α ELISA Kit with Cat. No. E0082Hu (Bioassay Technology Laboratory, China). ELISA was performed according to the procedures listed in the kit. Optical Density was measured using Microplate Reader: iMark (Biorad) S / N

12908. TNF-α serum level was calculated by using online software: elisaanalysis.com.

DNA Host Extraction. The DNA host was extracted using the QIAamp DNA Extraction kit (Qiagen, Inc., Hilden, Germany) with Cat.No.51104 using procedures in accordance to the kit. Controls were treated as the same as the sample.

PCR TNF-α Gene Promoters. A total of 5 µL DNA was used for amplification by PCR-RFLP technique, using a PCR 2x PCR Master mix solution (iNtRON®) kit with Ref No.25027. The PCR-RFLP process for TNF-α gene promoter was carried out using: forward: 5'- AGGCAATAGGTTTTGAGGGCCAT -3 'and reverse primers: 5'-TCCTCCCTGCTCCGATTCCG-3' to identify -238 SNP as well as the forward: 5'-AGAAGACCCCCCTCGGAACC-3 'and reverse primers: 5'- ATCTGGAGGAAGCGGTAGTG -3' to identify -308 SNP. Reaction mixture was made in 0.2 mL eppendorf tube with total volume of 50 µL for 1 sample. PCR was performed using the DNA thermal cycler: Applied Biosystem Veriti 96 Well. For -238 SNP, in the initial stage an initial denaturation was performed with 94°C for 5 min, followed by 40 PCR cycles in accordance to Jamil et al with modifications: denaturation at 94°C for 30 s, annealing at 60°C for 30 seconds and elongation at 72°C for 40 seconds. At the end of the process, the final extension was done at 72°C for 7 minutes. For -308 SNP, at the initial stage an initial denaturation was performed at 94°C for 5 minutes, was followed by 40 PCR cycles with the following details: denaturation at 94°C for 30 seconds, annealing at 58.5°C for 30 seconds and elongation at 72°C for 40 seconds. At the end of the process, the final extension was performed at 72°C for 7 minutes[9].

Detection of PCR Products with Electrophoresis. PCR product was examined by electrophoresis using 2% agarose gel which indicated the expected band, ie 107 bp for -308 SNP and 152 bp for -238 SNP. 100bp ladder marker, the negative control, and the samples were put into agarose gel. Electrophoresis results were visualized in the UV light and documented.

Incubation with Restriction Endonucleases. The PCR products of TNF-α gene promoter -238 was incubated with *MspI* restriction enzyme, while TNF-α gene promoter -308 was digested with *NcoI* restriction enzyme. Incubation with restriction enzyme used protocols from manufacturers (New England Biolabs) with a total reaction volume of 50 µL. Incubation was performed at 37°C overnight.

Detection of PCR-RFLP Products with Electrophoresis. PCR-RFLP products were examined using 3% agarose gel. 20bp and 100bp ladder markers, PCR-RFLP products from samples, as well as negative control were incorporated into agarose gel wells. Electrophoresis gel apparatus was run on 100 volts for approximately 25 minutes, then viewed under UV light and documented with Doc Printgraph Gel AE-6933FXCF.

SNP Analysis of TNF-α Gene Promotor. PCR-RFLP product of TNF-α gene at -238 region showed a fragment of 152 base pair (bp) if there is SNP (A allele) and fragment 132 and 20 bp if it is normal allele (G). If a band of 152 bp was found, the sample had homozygous AA allele. But, if the samples showed 152, 132, and 20 bp bands, the sample has GA heterozygous alleles. When two bands 132 and 20 bp were found, the sample has GG homozygous allele.

PCR-RFLP product of TNF-α gene at -308 region showed a fragment of 107 base pair (bp) if there is SNP (A allele), and fragment 87 and 20 bp if it has normal allele (G). When there was a 107 bp band in the sample, the sample has homozygous AA allele. While the samples were showed 107, 87, and 20 bp, the samples has GA heterozygous alleles. If the sample showed two bands 87 and 20 bp, the sample has GG homozygous allele.

RESULTS

In this study, as many as 149 positive HBsAg patients were cared for in the Endoscopy Outpatient Clinic, Dr. Soetomo General Hospital, Surabaya. They were further screened to meet the inclusion and exclusion criteria resulting in blood samples of 30 liver cirrhosis patients with positive HBsAg for more than 6 months

Table 1. Sex and age characteristics of the patients

Sex	Number of patients	Median age	Age distribution			
			≤ 40	41–50	51-60	>60
Male	24 (80%)	49	6	7	6	5
Female	6 (20%)	54	1	2	1	2
TOTAL	30 (100%)		7	9	7	7

Among the patients with cirrhosis of the liver with HBV infection in the Endoscopy Outpatient Clinic, Dr. Soetomo General Hospital, Surabaya in this study the youngest patient was 30 years old and the oldest patient was 71 years old. As shown at Table 1, male patients were dominating (80%), especially in the 41–50 years age range. Female patients in this study had a higher median with age than male, yet the number of female patients in this study was lower than male.

Table 2. Ethnicity of the patients and time diagnosed with cirrhosis

Sex	Ethnicity		Time diagnosed (years)		
	Javanese	Other	< 1	1–3	>3
Male	23	1	12	9	3
Female	6	0	4	2	0
TOTAL	29	1	16	11	3

As shown at Table 2, samples in this study were predominantly obtained from patients of Javanese ethnicity (97%). Only 1 male patient was non-Javanese (Batak). Most

patients, 16/29(53%), had also just recently been diagnosed with liver cirrhosis (<1 year).

After separation of PBMC and HBV DNA isolation, PCR was performed to amplify the TNF-α gene promoter of the -238 and -308 positions. All samples were showed positive PCR results. The result of PCR on TNF-α -238 region gene promoter gave positive result of 152 bp band (Figure 1), while the result of PCR on TNF-α -308 region gene promoter gave positive result of 107 bp band (Figure 2).

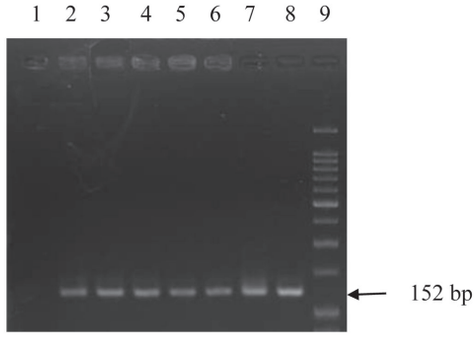

Figure 1. Example of electrophoresis product of PCR promoter gene TNF-α position -238
Description: lane 1: negative control; lane 2-8: samples with positive results; lane 9: marker

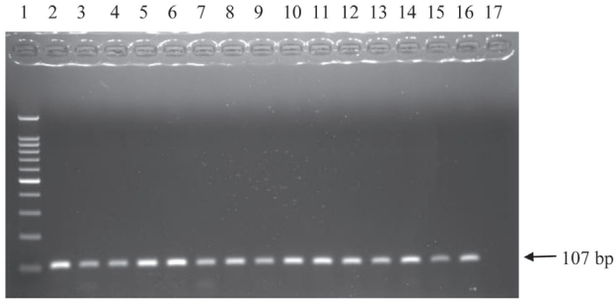

Figure 2. Example of electrophoresis product of PCR promoter gene TNF-α position -308
Description: lane 1: marker; lane 2-16: samples with positive results; lane 17: negative control

Furthermore, all samples were incubated with endonuclease restriction enzyme using *MspI* for -238 SNP, and *NcoI* for -308 SNP. Incubation was performed overnight at 37°C, then the RFLP product was visualized in 3% agarose gel. The determination of TNF-α gene promoter genotypes both in the -238 and -308 positions was based on the DNA fragment formed. In the PCR-RFLP position -238, the perfect cutting of *MspI* result that yielded the 132 and 20 bp fragments was showed the wild-type GG homozygous alleles, *MspI* partial cutting yielded three fragments 152, 132, and 20 bp were indicated GA heterozygotes, while intact 152 bp DNA fragments which was not digested with *MspI* indicated homozygous AA. As shown at Figure 3, in this study, we found samples with GG genotype (132 and 20 bp fragments) and GA genotype (152, 132, and 20 bp fragments), while no AA genotype was found.

At the -308 PCR-RFLP position, the perfect *NcoI* cutting yield of 87 and 20 bp fragments showed a wild-type GG homozygous allele, *NcoI* partial cutting yielded of three fragments 107, 87 and 20 bp were indicating GA heterozygotes, while intact 107 bp DNA fragments undigested with *NcoI* showing homozygous AA. As shown at Figure 4, in this study, we found samples with GG genotype (87 and 20 bp fragments) and GA genotype (107, 87, and 20 bp fragments), while no AA genotype was found.

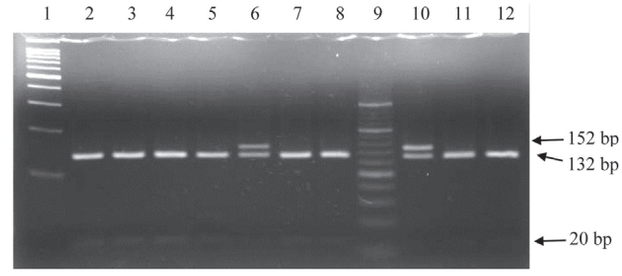

Figure 3. PCR-RFLP products of TNF-α promoter on -238 position
Description: Lane 2-5, 7, 8, 11 and 12: GG genotype with 2 fragments (132 bp and 20 bp); lane 6 and 10: GA genotype with 3 fragments (152 bp, 132 bp, and 20 bp); lane 1: DNA marker 100 bp; lane 9: DNA marker 20 bp

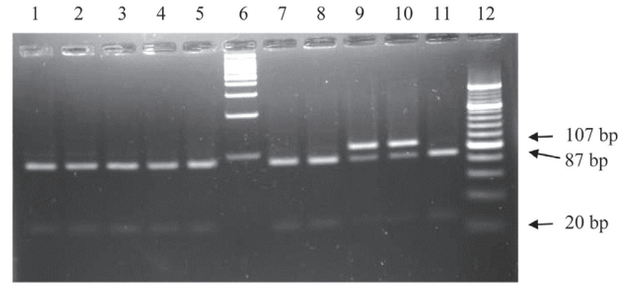

Figure 4. PCR-RFLP products of TNF-α promoter on -308 position
Description: Lane 1-5, 7, 8, and 11: GG genotype with 2 fragments (87 bp and 20 bp); lane 9 and 10: GA genotype with 3 fragments (107 bp, 87 bp, and 20 bp); lane 6: DNA marker 100 bp; lane 12: DNA marker 20 bp

From 30 patients in this study, 2 (6.7%) patients were showed GA heterozygote genotype on -238 TNF-α promoter SNP. No sample was found with AA homozygous SNP. The remaining 28 samples were showed genotype of wild-type GG. For -308 positions, 2 of 30 samples (6.7%) showed GA heterozygote SNP genotype. These were not the same patients that had GA at position -238. No sample was found with AA homozygous SNP genotype. The remaining 28 samples were showed a genotype of wild-type GG alleles. GA heterozygote samples for the -238 and -308 SNP were sampled with different numbers. No sample was found with two SNP-228 and -308 simultaneously in this study.

Table 3. TNF-α levels by sex in liver cirrhosis patients with HBV infection

Sex	Number of patients	Median (ng/L)	IQR
Male	24 (80%)	33	31,85
Female	6 (20%)	38	627,525

Table 4. TNF-α levels based on SNP genotype of TNF-α gene promoter in liver cirrhosis patients with HBV infection

Genotype	Median (ng/L)	IQR
-238 GG	35	30,51
GA	41	-
-308 GG	35	28,36
GA	30	-

In this study, higher levels of TNF-α were obtained in women than in men. In 1 sample, TNF-α level even reached 1118.78 ng/L (Table 3). Patients with liver cirrhosis with GG genotype for both -238 and -308 positions in this study had no different levels of TNF-α than GA genotypes (Table 4).

DISCUSSION

We were able to detect the presence of TNF-α promoter SNP -238 and -308 in patients with liver cirrhosis due to chronic HBV infection using PCR-RFLP. Tumor Necrosis Factor-α is a major cytokine in the inflammatory response to infection. TNF-α normally functions to activate cellular immunity and to provide protection against microbes, but excessive levels of this cytokine will result in severe tissue damage, septic shock and even death[10]. Schwabe[11] suggests that in liver, TNF-α can induce cell death as well as hepatocyte proliferation. In HBV infection, TNF-α levels tend to increase and are associated with inflammation, fibrosis, and hepatic damage. It is also said that TNF-α can be used as a predictor of liver inflammation[12].

It was found that most liver cirrhosis patients with HBV infection in this study was male (80%). This is consistent with the demographic data in previous studies suggesting that patients with cirrhosis of the liver due to chronic HBV infection are more frequent men than women[13–15]. This may due to a protective role of estrogen that is prevents damage to the liver because estrogen inhibits the proliferation of hepatic stellate cells and fibrogenesis that play an important role in the course of cirrhosis. In animal models with liver cirrhosis, estradiol administration leads to decreases in type I and III collagen and stellate cell proliferation[16].

Polymorphisms at TNF-α promoters -238 and -308 have been associated with various diseases associated with severe inflammation, infection, and malignancy. Researches on SNP of TNF-α gene promoters in patients with HBV infection reported conflicting results. A study in China showed a low A allele frequency, which was 4.6% for position -238 and 7.4% for position -308[17]. Another study in China also showed that no AA genotype found[18]. This distribution is different from that observed in Tunisia, where the GA and AA genotype frequencies in liver cirrhosis with HBV infection are quite high, ie 38.8% G/A and 44.5 A/A for -308 SNP, and 44.5% G/A and 33.3% A/A for -238 SNP[19]. A study in Turkey showed that AA genotype was found in healthy control individuals (6.7%), but none in hepatitis patients (0%).[20] TNF promoter polymorphism was known to be ethnic-specific, so each region might has unique distribution of TNF-α SNPs[21].

In a recent study in Brazil, among patients with Hepatitis C (HCV) infection with mild fibrosis, a -308 A/A SNP was found in 1.9% of the cases, and a G/A SNP in 26.1%. In patients with severe fibrosis, -308 A/A SNP in 0.8% while G/A SNP was present 21% patients[22]. Genotype AA is a rare genotype compared to genotype GG and GA[23]. Also in this study the AA genotype was not found. It is said that the frequency of A alleles is much lower in Asia than in other regions of the world.[24]

For Indonesia itself, this is the first time that TNF-α promoter genes polymorphism was studied in hepatitis patients. However, there have been several studies on TNF-α promoter gene SNP (-238, -308) in patients with other diseases such as acne vulgaris[25], Chronic Obstructive Pulmonary Disease (COPD) [26], and Down Syndrome[27]. In these studies, the frequency of AA genotype was also very low, even undetectable in in certain cases. Also, the GA genotype was found in frequently. However, in all those studies, the level of TNF-α was not measured. The low frequency of SNP in these researches could be due to the influence of Indonesian race and ethnicity[27].

Several previous studies on TNF-α SNP in cirrhosis gave controversial results. Some studies were showed that the G-308 allele is associated with low levels of TNF-α both in vitro [28] and in vivo[29] However, some other studies did not support this phenomena[30–32]. Studies of SNP at position -238 have likewise produced conflicting results with regards to TNF-α levels. Some studies were showed that having an A allele at this position is associated with elevated levels of TNF-α[33], but other studies were showed that there is no relationship between the two[34]. In addition, studies have shown that A allele is associated with lower TNF-α levels[17,35]. The expression of TNF-α, just like other cytokines is tightly regulated both at the transcriptional and post-transcriptional level. The polymorphisms located within the regulatory regions of TNF-α have been reported to influence the expression and secretion of this cytokine[35]. In TNF-α -238 and -308 SNP, the presence of the A-allele increases the binding of transcription factor to the promoter region of TNF-α, thereby altering its expression[36]. Nonetheless, several studies in various infectious diseases have shown the importance of TNF G>A in disease susceptibility, albeit with currently unknown molecular mechanism[37].

In this study, patients with liver cirrhosis with the GG genotype both for the -238 and -308 positions actually had

levels of TNF-α comparable to those with the GA genotype. Despite the associations between TNF promoter SNPs and disease, a direct impact of TNF promoter polymorphisms upon TNF transcription has not been conclusively demonstrated.[35] It was also said that TNF-α expression may not be directly related to TNF-α promotor gene polymorphism, but requires further variation in adjacent genes. This is due to the location of the TNF-α gene close to the HLA allele. As a result, pathological conditions might be caused by the variation in a linked gene that regulates the expression of this cytokine rather than due to polymorphism within the TNF-α gene itself.[24] The presence of other polymorphisms in the TNF-α promoter region of the gene may also contribute to TNF-α expression[34]. Finally, contradictory results may also be due to ethnic differences.[17,28,34] Given that in Indonesia harbours various ethnic groups, there is a possibility of variation based on ethnic groups studied in Indonesia, so further research is needed on the subject.

CONCLUSION

In this pilot study, 2/30 of liver cirrhosis patients with chronic HBV infection in Dr. Soetomo General Hospital, Surabaya were GA heterozygote for the TNF-α promoters at positions -238 and -308. 2 patients with SNP at position -238 and 2 patients with SNP at position -308 were different samples. No genotype AA was found. TNF-α level in this study was found higher in women than men. TNF-α level in patients with GA heterozygote genotype at -238 and -308 TNF-α gene promoters in this study was not different than wild-type (GG). Different results might also be due to racial or ethnic differences in each study. Further studies including larger numbers of patients from various ethnicities are needed to provide solid evidence on the prevalence TNF-α promoters polymorphisms in liver cirrhosis patients with HBV infection in Indonesia, and their relationship with the expression of TNF-α.

CONFLICT OF INTEREST

The authors state that there is no conflict of interest in this study.

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