Association of Tumor Necrosis Factor-α and Interleukin-10 Promoter Gene Polymorphisms with Chronic Hepatitis B Natural History Progression in Indonesian Population

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Background

Hepatitis B virus (HBV) infection affected approximately 3.9% of world population (291.9 million people) and 6.8% of Indonesian population (17.6 million people)[1], causing 887,000 deaths annually[2]. **Chronic hepatitis B** (CHB) infection is defined as the presence of serum hepatitis B surface antigen (HBsAg) for more than six months. Infections during perinatal or childhood are more likely to develop CHB (90% in perinatal and 20-30% in childhood) when the **immune system** is considered as immature, compared to immunocompetent person infected during adulthood (<1%)[3, 4, 5]. Based on the host and virus serological markers, the **natural history** of CHB can be categorized into 4 phases: immune tolerant (**IT**), immune clearance (**IC**), low replicative state (**LR**), and hepatitis B e antigen (HBeAg) negative immune reactivation (**ENH**)[6]. Regardless of the clear classification, CHB progression is a complex process with often **unpredictable outcomes**, which varies from one person to another. And even after 5 decades of HBV research[7], the host-virus interactions that affect the progression of CHB phases are still not well explored and explained.

The fact that **immune system** or **host-virus interaction** at the onset of infection is hypothesized to plays a role in the occurrence of CHB, can also be logically applied into the progression of CHB. Gene promoter of interleukin-10 (**IL-10**) and tumor necrosis factor (**TNF**)-**α** single nucleotide polymorphisms (**SNPs**) have been known to affect HBV susceptibility and prognosis of the patients[8, 9, 10, 11, 12, 13]. However, **none of the previous study** ever analyze the association of these SNPs with the progression of CHB natural history phase.

Aim of Study

This study aims to determine the **association** of the host factors, **cytokines** IL-10 and TNF-α gene promoter **polymorphisms** with the progression of chronic hepatitis B based on its **natural history** phase in Indonesian population.

Methods

- **249 patients of chronic hepatitis B** (mean age: 42±13.267; male/female: 182/67) were enrolled in this study. We determined the CHB natural history phase of patients based on the status of HBeAg, HBV DNA level and ALT level, according to AASLD 2018 Hepatitis B Guidance (2018).
- TNF-α -308G/A and IL-10 -592A/C SNPs of patients were detected using polymerase chain reaction with restriction fragment length polymorphism (**PCR-RFLP**). **Sanger sequencing** were performed as verification to genotyping result. **Hardy Weinberg Equilibrium** calculation were performed using chi-square goodness of fit test to determine the presence of genotyping error.
- **Chi-square test of independence** were performed to all available and complete variables compared to the CHB natural history phase, to determine the **covariates** to be included in logistic regression analysis. The association of SNPs with CHB progression were presented in **odds ratio** (OR) value with **95% confidence interval** (CI) using **ordinal logistic regression analysis** with age as covariates. All statistical tests were performed using the Statistical Program of Social Sciences (SPSS25.0 for Windows, SPSS, Chicago, IL) and R version 3.5.3.

Discussion

Despite the insignificant difference of both heterozygous and mutant genotypes compared to normal IL-10 -592 genotype as reference, there is a significant difference between the heterozygous and mutant genotypes in the progression of chronic hepatitis B natural history. **These results are in line with previous studies**, where IL-10 -592A/C (heterozygous) was highly associated with higher level of acute liver failure[8], the occurrence of cirrhosis and HCC[9, 10], and increased susceptibility to more persistent infection[11, 12, 13] compared to IL-10 -592C/C genotype (mutant). Therefore, we need to be **cautious** with the presence of **heterozygous** and **mutant** genotype of **IL-10 -592** in CHB patients.

This finding gives **new approach** and **more specific perspective** in the association of IL-10 SNP with the progression of CHB. However, further study with larger sample size is still needed.

Conclusion

- Overall, SNPs IL-10 -592 and TNF-α -308 are **not good predictors** toward the progression of CHB natural history phase, due to:
  1. The low frequency of heterozygous and mutant genotype of TNF-α -308
  2. No significant difference between heterozygous and mutant genotype of IL-10 -592 with the normal genotype as reference (p>0.05), **even though the effect of IL-10 -592 SNPs toward the progression of CHB natural history can be predicted with ordinal logistic regression model and can be ranked**.
- Since the odds of **IL-10 -592 heterozygous genotype** to develop advanced CHB phase was **2.385 times** (p<0.05) times higher than **mutant genotype** (in line with several previous studies), we still need to be to be **cautious** with the presence of this genotype in CHB patients.
- Further study with **larger sample size** will be needed to confirm this finding.

Results

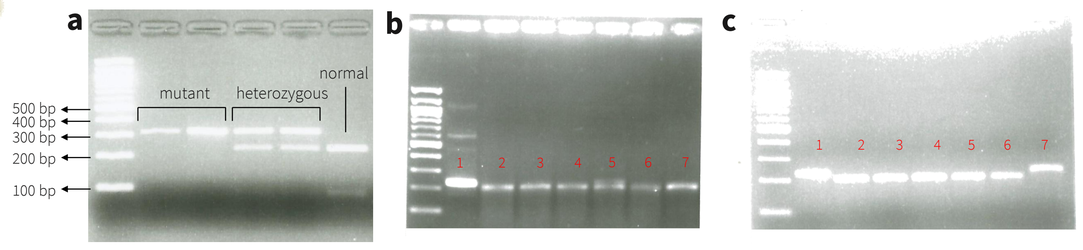

Figure 1. Visualization of IL-10 -592 & TNF-α -308 Restriction Products with Agarose Gel Electrophoresis
a. IL-10 -592 PCR products restriction with *Rsa*I restriction enzyme produced 3 kinds of cleavage patterns, normal genotype with cleaved band (AA), heterozygous genotype with 2 bands (AC), and mutant genotype with uncleaved band.
b. TNF-α -308 PCR products restriction with *Nco*I restriction enzyme, control in lane 1, cleaved products in lane 2, 3, 4, 6 and 7 showing normal genotype (GG), and 2 bands in lane 5 showing heterozygous genotype (GA).
c. Lane 7 showing the uncleaved band of TNF-α -308 mutant genotype (AA).

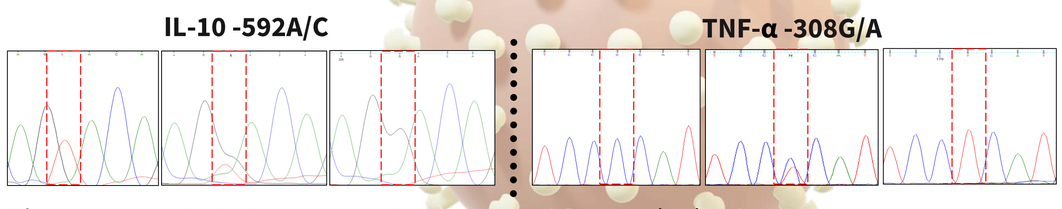

Figure 2. IL-10 -592 & TNF-α -308 Genotyping Results by Sequencing
Verification of IL-10 -592 and TNF-α -308 PCR-RFLP results by Sanger sequencing were performed to reduce the probability of genotyping error. From left to right, normal genotype, heterozygous genotype, and mutant genotype.

Table 1. IL-10 -592 & TNF-α -308 Genotype Distribution in CHB Patients

Genotype Frequency of IL-10 -592			Phase										HWE
			IT		IC		LR		ENH		Total		X2
IL-10 -592	Normal	(AA)	41	(29.5%)	14	(10.1%)	45	(32.4%)	39	(28.1%)	139	(100.0%)	0,342494
	Heterozygous	(AC)	20	(22.5%)	8	(9.0%)	36	(36.0%)	25	(28.1%)	89	(100.0%)	
	Mutant	(CC)	6	(28.6%)	5	(23.8%)	7	(33.3%)	3	(14.3%)	21	(100.0%)	
	Total		67	(26.9%)	27	(10.8%)	88	(35.3%)	67	(26.9%)	249	(100.0%)	

Genotype Frequency of TNF-α -308			Phase										HWE
			IT		IC		LR		ENH		Total		X2
TNF-α -308	Normal	(GG)	36	(23.1%)	14	(9.0%)	65	(41.7%)	41	(26.3%)	156	(100.0%)	0,133878
	Heterozygous	(GA)	0	(0.0%)	1	(25.0%)	2	(50.0%)	1	(25.0%)	4	(100.0%)	
	Mutant	(AA)	1	(50.0%)	1	(50.0%)	0	(0.0%)	0	(0.0%)	2	(100.0%)	
	Total		37	(22.8%)	16	(9.9%)	67	(41.4%)	42	(25.9%)	162	(100.0%)	

Of the 249 patients, 67 patients were in IT phase, 27 patients in IC, 88 patients in LR, and 67 patients in ENH. Genotype distribution of both IL-10 -592 and TNF-α -308 are in Hardy-Weinberg Equilibrium (HWE) with p-value>0.05. Mutant and heterozygous genotypes of TNF-α -308 are rare and thus cannot be further analyzed.

Table 2. Ordinal Logistic Regression Analysis of IL-10 -592 Genotype

	Regression Coefficient	Std. Error	Wald	p-value	OR	2.5%	97.5%
Variables							
Age	0,04399	0,00935	22,13526381	<0,001	1,045	1,0262	1,0646
IL-10 -592 AA	0,59597	0,41881	2,024951901	0,155	1,814	0,8006	4,1713
IL-10 -592 AC	0,86922	0,43507	3,991546063	0,046	2,385	1,0203	5,6615
IL-10 -592 CC	REF	REF	REF	REF	1	REF	REF

	Regression Coefficient	Std. Error	Wald	p-value	OR	2.5%	97.5%
Variables							
Age	0,04399	0,00935	22,13526381	<0,001	1,045	1,0262	1,0646
IL-10 -592 AA	REF	REF	REF	REF	REF	REF	REF
IL-10 -592 AC	0,27320	0,24924	1,201505892	0,273	1,31421	0,80695	2,146093
IL-10 -592 CC	-0,59604	0,41881	2,025427612	0,155	0,55099	0,2397	1,2489

There was no significant difference between IL-10 -592 genotype AA (reference), AC, and CC with CHB progression (p>0.05). However, the odds of genotype AC to develop advanced CHB phase was 2.385 (95% CI: 1.02-5.66, Wald x^2 = 3.991, p=0.046) times higher compared to CC.

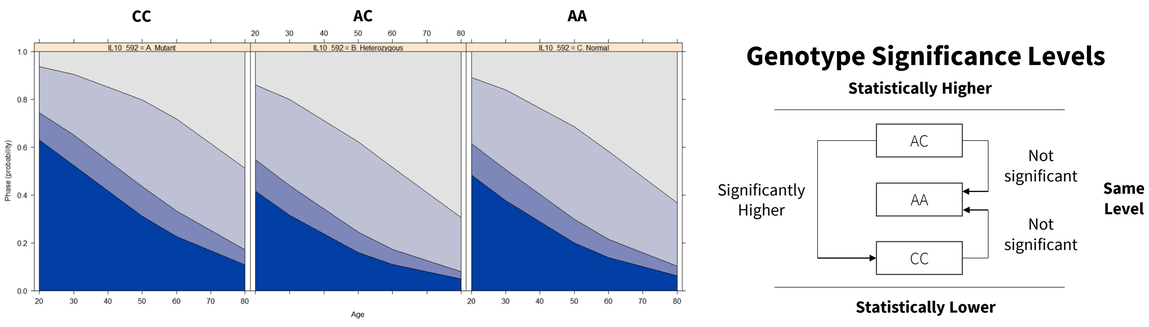

Figure 3. Visualization of IL-10 -592 Genotype Ordinal Logistic Regression Analysis [14]
Based on the surface area, the probability of someone to be classified in IT phase is higher in IL-10 -592 mutant genotype than normal and heterozygous. For ENH phase, the probability is higher in heterozygous than normal and mutant genotype. IL-10 -592 genotypes with higher probability to develop advanced CHB phase, from highest to lowest probability, are heterozygous (AC), normal (AA), and mutant (CC).

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