Association of Tumor Necrosis Factor Alpha and Lymphotoxin Alpha Gene Polymorphisms with the Presence of Chronic Obstructive Pulmonary Disease

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ABSTRAK

Tujuan: *menganalisis terjadinya penyakit paru obstruktif kronik dan hubungannya dengan polimorfisme gen TNFα pada posisi -308 dan -238 dan gen LTα pada posisi +252.* ***Metode:*** *penelitian potong lintang dengan membandingkan genetik sekelompok orang pasien PPOK dan sekelompok orang yang tidak memiliki PPOK dengan riwayat merokok yang sama. Penelitian dilakukan mulai Januari 2011 sampai Maret 2012 di Poliklinik Paru RS Adam Malik, RS. Pirngadi, RS. Tembakau Deli, RS. Siti Hajar Medan dan beberapa Puskesmas di kota Medan. Pemeriksaan faal paru dilakukan dengan menggunakan spirometri dan analisis gen dengan teknik PCR-RFLP.* ***Hasil:*** *227 orang memenuhi kriteria inklusi dan dilakukan penyetaraan umur. Riwayat merokok sebanyak 186 orang (93 orang sebagai kelompok kasus dan 93 orang sebagai kelompok kontrol). Untuk polimorfisme -308G/A gen TNFα dijumpai genotip GA dan AA sebanyak 18 pada subyek PPOK dan 33 pada subyek non-PPOK. Sedangkan untuk genotip GG sebanyak 75 pada subyek dengan PPOK dan 60 pada non-PPOK, rasio odds 0,436 (95% IK 0,224 - 0,850; p=0,014). Polimorfisme -238G/A gen TNFα dijumpai genotip GA dan AA sebanyak 8 pada PPOK dan 4 pada non-PPOK. Sedangkan untuk genotip GG sebanyak 85 pada PPOK dan 89 pada non-PPOK dengan rasio odds 2,094 (95% CI 0,608 - 7,211; p=0,241). Polimorfisme +252A/Ggen LTα dijumpai genotip GA dan GG sebanyak 55 pada PPOK dan 60 pada non-PPOK. Sedangkan untuk genotip AA sebanyak 38 pada PPOK dan 33 pada non-PPOK, rasio odds 1,256 (95% CI 0,694 – 2,272; p=0,450).* ***Kesimpulan:*** *polimorfisme gen TNFα pada posisi -308 terbukti sebagai faktor protektif terjadinya PPOK. Polimorfisme gen TNFα pada posisi -238 dan gen LTα pada posisi +252 tidak terbukti berhubungan dengan kejadian PPOK.*

Kata kunci: *penyakit paru obstruktif kronik, gen TNFα, gen LTα, polimorfisme.*

ABSTRACT

Aim: *to analyze the occurrence of chronic obstructive pulmonary disease (COPD) and its association with the polymorphisms of -308G/A, -238G/A of TNFα gene and +252A/G LTα gene polymorphism in smokers.* ***Methods:*** *cross-sectional study, comparing the genetic group of people who have COPD and who do not have COPD with the same smoking history. The study was conducted from January 2011 to March 2012 at several health centers; such as Adam Malik Hospital, Pirngadi Hospital, Tembakau Deli Hospital, Siti Hajar Hospital in*

Medan and several health centers in the city of Medan. Examination of lung function was done using spirometry and the existence of genetic polymorhisms in the TNFα and LTα gene was performed using polymerase chain reaction-restriction fragment length polymorphisms (PCR-RFLP). ***Results:*** *of the total of 227 samples that met inclusion criteria and after equalizing the age and history of smoking, 186 subjects were enrolled, of which, 93 people as COPD group and 93 people as non-COPD group. Analysis of the association between the -308G/A polymorphism with the COPD revealed the odds ratio (OR) 0.436 (95% CI 0.224-0.850, p=0.014). Polymorphism at -238G/A of the TNFα gene showed the OR 2.094 (95% CI 0.608 - 7.211, p=0.241). Polymorphism at +252A/G LTα gene showed OR 1.256 (95% CI 0.694 - 2.272; p=0.450). The findings indicated that polymorphism at -308G showed a protective factor whereas the -238G/A of the TNFα and +252A/G of the LTα genes did not show any significant association with the COPD.* ***Conclusion:*** *polymorphism -308 TNFα gene shown to be a protective factor for the occurrence of COPD. Polymorphism -238 TNFα gene and +252 LTα gene did not show any significant association with COPD.*

Key words: *chronic obstructive pulmonary disease, TNFα gene, LTα gene, polymorphism.*

INTRODUCTION

Chronic Obstructive Pulmonary Disease (COPD) will be the cause of illness and death worldwide. Based on WHO estimation, COPD will become the third leading cause of death in 2020. The causes are increasing number of smokers, fewer deaths from other diseases (heart and infection), and increasing life expectancy.[1] Smoking is an important risk factor in the decline in lung function, but only 10-20% of chronic heavy smokers will suffer from COPD. This indicates that different susceptibility to damage due to smoking is likely related to genetic factors.

Zhai[2] found a strong influence between genetics structure to the ratio of FEV1 value obtained from patients compared to the healthy non-smoker persons; moreover, the genetics itself is also influenced by smoking habit. Smoking habits will affect gene expression in lung function, but the mechanisms on how this happens in susceptible person are still not understood yet. COPD has been recently defined as a complex disorder derived from interactions of several genetics and environmental factors. The development of genetic variant identification technique is expected to reveal a new mechanism on COPD pathogenesis. A copy number variation (CNV) enables us to describe differences in gene sequence as well as the structures, which may contribute in explaining susceptibility of a person to COPD. A good understanding on genetic variation, gene expression and its regulation will allow us to reveal novel mechanism of COPD that can be applied in term of treatment.

Polymorphism or Single Nucleotide Polymorphism (SNP) is a nucleotide sequence variation or change in one of the nucleotide bases in the gene. TNFα gene polymorphism -238 G/A and -308G/A is the change in the nucleotide bases guanine into adenine at position of -238 and -308. LTα gene polymorphism +252 A/G is a change in the nucleotide bases adenine into guanine at position of +252. These changes will result in changes in transcription process that affect the protein production of TNFα and LTα. Based on the position, polymorphisms affect only the velocity of protein synthesis.[2-7]

Studies on TNFα and LTα gene polymorphisms in association with COPD patients in Indonesian population have not been performed. Therefore, our study was aimed to analyze the occurrence of COPD based on the role of TNFα gene polymorphisms at position of -308 and -238 and LTα gene at position of+252.

METHODS

Our study was a cross-sectional study comparing the genetics of a group of people who had suffered from COPD (case group) and a group of people who had not sufferred from COPD (control group) with the same smoking history. The study was conducted between January 2011 and March 2012 in several locations such as Lung Clinic at H.Adam Malik Hospital, Pirngadi Hospital, Tembakau Deli Hospital, Siti Hajar Hospital and several health

centers in Medan. Institutional Review Board (IRB) from Ethic Committee at Medical Faculty University Of North Sumatera had issued their approval on 8 Februari 2011 (No.47/KOMET/FK USU/2011, informed Consent (IC) written was required). The target population in this study was patients suffering from COPD; while the accessible population was COPD patients seeking treatment at study site. There were two groups in our study, i.e. the case group and control group. The case group included patients with COPD who fulfilled the inclusion criteria and exclusion criteria. The inclusion criteria were: Patients with COPD who had already been confirmed by spirometry test (VEP1/KVP <70% in 15 minutes after given two sprays of salbutamol in metered dose inhaler with spacer), the severity of illness was graded according to GOLD classification (mild to very heavy), age ≥40 years, male, current smokers or ex-smokers with smoking history of >200 Brinkman index (IB). The exclusion criteria were patients with asthma, pulmonary tuberculosis or other lung diseases.

The control group were adult smokers without COPD who met inclusion and exclusion criteria. The inclusion criteria were those with normal pulmonary function, which have been confirmed by spirometry test (VEP1/KVP >70%), age ≥40 years, male, active smokers or ex-smokers with smoking history of >200 IB. The exclusion criteria were patients with lung diseases such as tuberculosis, chronic bronchitis or other lung diseases, and history of family member with COPD. Venous blood was withdrawn from the median cubital vein by using a 3-cc syringe, and the blood was collected for further DNA gene isolation analyzed by using PCR-RFLP method.

PCR-RFLP of -308 TNFα Gene

The TNFα-308 genotype (rs1800629) was analyzed with the PCR-RFLP method (Polymerase Chain Reaction-Restriction Fragment Length Polymorphism) by using primers for the PCR gene amplification using ATF-3 primer (5'-GTTCCTTGG AAGCCAAGACT-3') and ATR-1 (5'-GTCAGGGGATGTGGCGTCT-3' in the first reaction stage and ATF-2 primer (5'-TGGAGGCAATAGGTTTTGAGGGCCAT -3' and ATR-2 (5'-TCATCTGGAGGAAGCG GTA-3') for the second PCR reaction. PCR was performed using a Perkin Elmer PCR model 9700 machine. PCR reaction volume was 50 μL containing the following ingredients: 10 X buffer solution (5 μL), 50 mM MgCl 2 (1.5 μL), 10 mM dNTPs (1 μL), ATF-3 primer 40 pmol/μL (0.5 μL), the primary ATR-1 40 pmol/mL (0.5 μL), the isolated DNA samples (5 μL) and 1 unit of Taq polymerase enzyme. PCR cycle condition in the first stage reaction in TNF gene fragment amplification was 940C for 30 sec, 58^0C for 30 sec and 72^0C for 1.5 min in 30 cycles. At the beginning of the reaction, an additional denaturation time 94^0C for 5 minutes was allocated and at the end of the reaction, there was additional elongation time for denaturation at 72^0C for 5 minutes. PCR cycle condition in the second stage TNF gene amplification reaction was 94^0C for 30 sec, 61^0C for 30 sec and 72^0C for 1 min as many as 40 cycles with additional denaturation time at early reaction of 94^0C for 5 min and additional elongation time of 72^0C for 5 minutes. Composition of the solution in the second phase of the PCR reaction was equal to the first stage of the reaction, except the used primers were ATF-2 and ATR-2 with DNA, which were the products of the first stage PCR. They were used as the template with the amount of 1μL.

About 5 μL product of the PCR second phase from each sample was mixed with 2 μL loading buffer and then inserted into the gel wells. In one line of wells, one well was included to place the marker DNA. Electrophoresis was run at a voltage of 100 volts (~ 50 mA) for approximately 40 minutes. Electrophoresis result was seen under ultra violet light (UV). Positive TNFα gene PCR result was indicated by the presence of DNA band with size 231 pb. To determine whether the samples brought nucleotide A and/or nucleotide G at position -308 TNFα gene promoter, DNA PCR product excision was performed using restriction enzymes NcoI which possessed 5'-CCATGG-3' recognition site. RFLP process was performed by adding 1 unit of restriction enzyme NcoI and 1 μL of the buffer solution NE buffer, 4 to 5 μL of PCR product and adding distilled water until 10 μL. All of them were

then incubated at 370C for 2 hours. The product of PCR-RFLP was electrophoresized resulting in homozygote -308A allele, which was cut into 2 bands with the size of 208 pb and 231 pb and the homozygote allele-308G of which without any cutting; therefore, there was only one band appeared with a similar size to the size of the PCR product, i.e. 231 pb. While for the heterozygous samples, which brought allele G and A, partial cutting occured and resulting in 3 bands with sizes of 231 pb, 208 pb and 23 pb.

PCR-RFLP of -238 TNFα Gene

TNFα -238 genotype (rs361525) was analyzed with PCR-RFLP method using primers for PCR gene amplification, i.e. primer of ATC-TNFα−238G/A (rs361525), the primers were 5'ATCTGGAGGAAGCGGTAGTG-3' and 5'-AGAAGACCCCC CTCGGAACC-3'. PCR reaction volume was 50 µL containing the following ingredients: 10 X buffer solution (5 µL), 50 mM MgCl 2 (1.5 µL), 10 mM dNTPs (1 µL), 40 pmol/µL of ATC-1 primer (0.5 µL), 40 pmol/µL of ATC-2 primer (0.5 µL), the isolated DNA samples (5 µL) and 1 unit of Taq polymerase enzyme. PCR cycle condition in TNFα gene fragment amplification was 94^0C for 30 sec, 58^0C for 30 sec and 72^0C for 1.5 min in 30 cycles.At the beginning of the reaction, additional denaturation time was given at 940C for 5 minutes and at the end of the reaction, there was additional elongation time at 72^0C for 5 minutes. Positive electrophoresis results of TNFα gene PCR was indicated by the presence of DNA band with the size of 150 pb.

To determine whether the samples brought nucleotide A and/or nucleotide G at position -238 TNFα gene promoter, DNA PCR product cutting was performed using restriction enzymes MspI which had 5'-C˘CGG-3'recognition site. The product of PCR-RFLP was electrophoresized resulting in homozygote allele -238A of which without any cutting; therefore, there was only one band appeared with a similar size to the size of the PCR product, 231 pb, and homozygote -238G allele was cut into 2 bands with the size of 130pb and 20pb. While for the heterozygous samples, which brought allele G and A, partial cutting occured and resulting in 3 bands with sizes of 150 pb, 130 pb and 20 pb.

PCR-RFLP of +252 LTα Gene

LTα +252 genotype (rs909253) was analyzed with PCR-RFLP method using primers for PCR gene amplification. The primers were HTB-1 (5-CTG-ACT CTC CAT CTG TCA GT-3) and HTB-3 (5-TAC CAA TGA GGT GAG CAG CA-3). In the second reaction, the used primers were HTB-1 and HTB-2 (5-AGA CGT TCA GGT GGT GTC AT-3). Composition and condition of PCR amplification of LTα gene was performed using the same method for TNFα gene; however, there were some differences such as the use of HTB-1, HTB-2 and HTB-3 primers. Positive PCR result for LTα gene was indicated by the presence of DNA band with the size of 285 pb. To determine whether the samples brought nucleotide A and/or nucleotide G at position +252 LTα gene promoter, DNA PCR product cutting was performed using restriction enzymes NcoI which carried 5'-C˘CATGG-3' recognition site. For the homozygote allele +252A at which the cutting did not occur, there was only one band appeared with a similar size to the size of the PCR product, 285 pb and homozygote -238G allele was cut into 2 bands with sizes of 231pb and 54pb. While for the heterozygous samples, which brought allele G and A, partial cutting occured and resulting in 3 bands with sizes of 285 pb, 231 pb and 54 pb.

Data Analysis

The sample size of this study was calculated based on the study of Huang[6], that found a correlation of gen alleles TNF-alpha 308 between 19% of COPD patients and 2.4% of the control group with odds ratio of 11.1. We used the formula of case-control study for analytical unpaired comparative categorical data, applying the value of $\alpha = 0.05$ and $\beta = 0.2$ and resulting as much as 81 samples in each group; therefore, the total number of sample size was 162 subjects. Samples collecting procedure was conducted by consecutive sampling after obtaining approval from the Ethical Committee of Medical Faculty, University of North Sumatra.

Data analysis was performed using chi-square test. We used chi-square test to determine the association between gene polymorphisms of TNFα as well as LTα and FEV1 values. The magnitude of risk of COPD in the TNFα gene

polymorphism and LTα was stated in odds ratios (OR). If the OR >1, we could conclude that TNFα or LTα gene polymorphism is a risk factor for COPD. The analysis was then continued to determine the magnitude of risk factor, expressed in 95% confidence interval (CI). Statistical findings was considered to be significant when $p<0.05$.

RESULTS

Of 227 people who had been examined, we obtained eligible sample size of 186 subjects after being matched with age and history of smoking. In details, there were 93 subjects in the case group and in the control group. The subjects for the case and control group was respectively 93 male subjects (100%) with the eldest age range of 50-59 years (41.9% of the case group and 40.9% of the control group).

Analysis of TNFα -308 Gene Polymorphism

Table 2 shows the number of individuals who had allele G and A on TNFα -308 genotypes in the case and control group. There was a fewer number of allele G in the control group, i.e. 145 subjects (78%); while in the case group with COPD, the number was 158 subjects (85%). The COPD case group had 28 subjects (15%) with allele A; while in the control group, there were 41 subjects (22%). Based on the results, we found that there was less number of allele A in COPD cases compared to the control group.

Table 1. Data characteristics

Variables	COPD	non-COPD
Sex male, n (%)	93 (100)	93 (100)
Age (years), n (%)		
- 40-49	3 (5.4)	5 (3.2)
- 50-59	38 (41.9)	39 (40.9)
- 60-69	31 (31.2)	29 (33.3)
- 70-79	15 (15.1)	14 (16.1)
- 80-89	6 (6.5)	6 (6.5)
Smoking habit (IB), n (%)		
- Mild (IB<200)	0 (0.0)	0 (0.0)
- Moderate (200≤IB<600)	47 (50.6)	44 (47.3)
- Heavy (IB≥600)	46 (49.4)	49 (52.7)
Gold stage (%FEV1), n (%)		
- GOLD I (FEV1≥80%)	1 (1.0)	
- GOLD II (50%≤FEV1<80%)	26 (28.0)	
- GOLD III (305≤FEV1<50%	47 (51.0)	
- GOLD IV (FEV1<30%)	19 (20.0)	

IB=daily number of cigarettes x years

Table 2. Allele -308 gene TNFα between COPD and non-COPD

	COPD n (%)	non-COPD n (%)
Allele A	28 (15.0)	41 (22.0)
Allele G	158 (85.0)	145 (78.0)
Total	186 (100.0)	186 (100.0)

Analysis of TNFα-238 Gene Polymorphism

Table 3 shows the number of individuals who had allele G or A of TNFα-238 genotype in the case and control group. Allele G was found in 179 subjects of the control group (96.2%); while in the COPD case group, we found 174 subjects (93.5%). Moreover, allele A was found in 12 subjects of the COPD case group (6.5%) and in the control group, there were 7 subjects (3.8%).

Analysis of LTα +252 Gene Polymorphism

Table 4 shows the number of individuals who had allele G or A at LTα +252 genotype in the case and control group. There were 179 subjects (96.2%) who had allele G in the control group; while in the COPD case group, the number was 174 subjects (93.5%). We found 12 subjects (6.5%) with allele A in the COPD case and the number in the control group was equal to 7 subjects (3.8%).

Table 3. Allele -238 gene TNFα between COPD and non-COPD

	COPD n (%)	non-COPD n (%)
Allele A	12 (6.5)	7 (3.8)
Allele G	174 (93.5)	179 (96.2)
Total	186 (100.0)	186 (100.0)

Table 4. Allele +252 gene TNFα between COPD and non-COPD

	COPD n (%)	non-COPD n (%)
Allele A	130 (70.0)	126 (67.0)
Allele G	56 (30.0)	60 (33.0)
Total	186 (100.0)	186 (100.0)

Table 5. Association of TNFα and LTα gene polymorphism with COPD

Genotypes	COPD	non-COPD	OR (95% CI)	p
-238 TNFα				
- GA - AA	8	4	2.09 (0.61-7.21)	0.24
- GG	85	89		
-308 TNFα				
- GA - AA	18	33	0.44 (0.22-0.85)	0.01
- GG	75	60		
+252 LTα				
- GA - GG	60	55	1.26 (0.69-2.27)	0.45
- AA	33	38		

The association of TNFα Gene Polymorphism and LTα with COPD

Table 5 shows the -238G/A, -308G/A TNF gene and +252 A/G gene LTα polymorphism in COPD (case) and control group. For -238G/A, GA and AA genotypes, we found as many as 8 subjects in the case group and 4 subjects in the control group. As for the GG genotype, there were as many as 85 subjects in the case group and 89 subjects in the control group. Using statistical calculation, we obtained odds ratio 2.094 (95% CI 0.608 to 7.211), however, the difference was not considered statistically significant. For -308G/A genotypes polymorphism, GA and AA genotypes, we found as many as 18 subjects in the case group and 33 subjects in the control group. As for the GG genotype, there were 75 subjects in the case group and 60 subjects in the control group. Statistical calculation revealed an odds ratio 0.436 (95% CI 0.224 to 0.850).

For polymorphism of +252 A/G, GA and GG genotypes, we found as many as 60 subjects in the case group and 55 subjects in the control group. While for AA genotype, there were as many as 33 subjects in the case group and 38 subjects in the control group. We obtained an odds ratio 1.256 (95% CI 0.694 to 2.272).

DISCUSSION

Bronchial smooth muscle, cell hypertrophy, inflammatory process narrowing the peripheral airways and loss of elastic recoil may contribute to different extent in certain individuals. Susceptibility to these processes may have different genetic bases. A search for genes that increase susceptibility to airflow obstruction among smokers may have implications beyond the development of COPD.

Our study shows that -238 TNFα and +252 LTα genes polymorphisms are not associated with the incidence of COPD; however, for the -308G/A TNFα gene polymorphism, our results showed that -308A allele is more common in normal individuals and has been significantly proven to be a protective factor for the development of COPD. Based on the data analysis of our study, we conclude that polymorphism of -308 TNFα gene reduces the risk of COPD in smokers. COPD is also likely to occur less by 0.436 times in smokers who have a TNF-308A gene allele. However, these results are not in accordance with previous studies that have analyzed the association of TNFα gene polymorphisms with the incidence of COPD.[8-10] The differences are likely due to different definition of the phenotype and race. Therefore, the risk of each different alleles is essential in different population as there may be different form of exposure and environmental factors. Moreover, differences in COPD phenotypes are more likely due to differences in mutations or gene polymorphisms. Data of some studies shows that the association of genes and gene analysis with the incidence of COPD in different populations should be studied further. The popula tion differences may be related to differences in specific genetic risk and environmental exposure. There are different results between our study and the study reported by Huang.[5] It may be due to different position of the promoter segment.[11] PCR products generated

in our study were different in DNA base pairs from the PCR products which were examined by Huang. In our study, the result sized 231pb; while Huang obtained 345pb. TNFα promoter tip with 1.5 κB length was placed at the starting position of the gene transcription process (transcription start site/TSS) that served as a regulator of TNFα production.

It is possible that -308 TNFα gene polymorphism occurred in the population of our study leading to lower TNFα gene transcription process resulting in lower production of TNFα, which serves as inflammatory mediator. Another explanation is that the difference may be partially caused by different study populations and different criteria in selecting participants, in which we included COPD patients in our study; while Huang included patients with chronic bronchitis. Moreover, the number of sample size of our study is two-folds larger than the study conducted by Huang.[5]

Nevertheless, we still could not confirm the association of TNFα gene polymorphism at position -308 with COPD since the actual effect of the polymorphism in vivo has not been clearly demonstrated in our study. Further studies should be performed on the gene expression of -308A TNFα allele in patients with COPD, on the serum levels of TNFα in patients with COPD and the sequences around the -308 position of TNFα gene promoter as well as the identification of proteins associated with DNA sequences covering position. The studies are expected to provide useful information as an effort to characterize the gene promoter and its correlation to protective factors on the incidence of COPD. A large number of genes and polymorphisms must first be identified and tested.

The limitation of this study includes the fact that there were only two genes analyzed in our study and this number was too small compared to all genes involved in the pathogenesis of COPD. Another limitation is that the sample in our study included all Indonesian ethnics (Bataknese, Javanese, etc) and it was not limited to a specific ethnic group; therefore, the effect of racial or ethnicity on the incidence of COPD is still obscure.

CONCLUSION

In summary, our results suggest that polymorphism of -238G/A TNFα and +252A/G LTα genes do not show any association with the incidence of COPD and polymorphism of -308G/A TNFα gene has been proven to be a protective factor for the development of COPD.

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