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Association of Tumor Necrosis Factor Alpha (TNFα) Gene Polymorphism with the Presence of Chronic Obstructive Pulmonary Disease

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Abstract

Background: Only 10-20% of chronic heavy smokers will develop into COPD. This indicates a different susceptibility to damages caused by smoking and that may be linked to genetic factors. The purpose of this study was to analyze the occurrence of COPD and its association with the polymorphisms; -308 G/A and -238G/A gene polymorphism in smokers. **Methods:** Case-control study, comparing the genetic group of people who have COPD (cases) and a group of people who doesn't have COPD (control) with the same smoking history. Examination of lung function was done using spirometry and the existence of genetic polymorhism in the TNFα gene was performed using Polymerase Chain Reaction - Restriction Fragment Length Polymorphisms (PCR-RFLP). **Results:** From 186 subjects were enrolled, 93 people as the case group and 93 people as the control group. Analysis of the association between the -308G/A polymorphism with the COPD revealed the Odds Ratio (OR) of 0.436 (CI:0.224 - 0.850, and *p*:0.014). Polymorphism at -238G/A of the TNFα gene showed the OR of 2.094 (CI:0.608 to 7.211 and *p*:0.241).**Conclusions:** Polymorphism -308 TNFα gene shown to be a protective factor for the occurrence of COPD. Polymorphism -238 TNFα gene did not show any significant association with COPD.

Keywords: Chronic Obstructive Pulmonary Disease, TNFα gene, Polymorphism

1. 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(1,2). However, only 10-20% of chronic heavy smokers will have COPD(3). This indicates different susceptibility to damage due to smoking is likely related to genetic factors(4). 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 in position -238 and -308. These changes will result inchanges in the transcription process that influence TNFα. And based on the position, polymorphisms affect only the velocity of protein synthesis(5). This study aims to analyze the occurrence of COPD based on the role of TNFα gene polymorphisms in position -308 and -238.

2. Methods

Case-control study, comparing a group of people who suffer from COPD (case) and a group of people who do not suffer from COPD (control) with the same smoking history. The study was conducted from January 2011to 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 Comitte Medical Faculty University Of North Sumatera approval on 8 Februari 2011 (No.47/KOMET/FK USU/2011, informed Consent (IC) written was required). Population target in this study are patients who suffer COPD, whereas the accessible population are COPD patients seeking treatment at study locations. The case group are patients with COPD who meet the inclusion criteria and exclusion criteria. The inclusion criteria: Patients with COPD who are already confirmed by spirometry test: $FEV_1/FVC<70\%$, that be measured 15 minutes after given two sprays of salbutamol in metered dose inhaler with spacer, severity of COPD according to GOLD: mild-very heavy, age ≥ 40 years, male, current smokers or ex-smokers with smoking history of ≥ 200 Brinkman index (BI). Exclusion criteria: Patients with asthma, pulmonary tuberculosis or other lung diseases.

The control group are adult smokers without COPD who meet inclusion and exclusion criteria. Inclusion criteria: normal pulmonary function which have been confirmed by spirometry test: $FEV_1/FVC>70\%$, age $\geq$ 40years, male, active smokers or ex-smokers with smoking history of >200 BI. Exclusion criteria: Patients with lung diseases such as tuberculosis, chronic bronchitis or other lung diseases, and history of family member with COPD. Venous blood was collected from the median cubital vein by using a 3cc syringe, and conducted further DNA isolation of genes analyzed by using PCR-RFLP method.

2.1. PCR-RFLP of -308 TNFα Gene

TNFα -308 genotype (rs1800629) was analyzed with PCR-RFLP method (Polymerase Chain Reaction-Restriction Fragment Length Polymorphism) by using primers for the PCR amplification of the gene by 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'-TCATCTGGAGGAAGC GGTA-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 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 is given additional denaturation time 94^0C for 5 minutes and at the end of the reaction is given additional elongation time 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 the addition of denaturation time at early reaction 94^0C for 5 min and the addition of the elongation of 72^0C for 5 minutes. Composition of the solution in the second phase of the PCR reaction is the same as the first stage of the reaction, except primers used is ATF-2 and ATR-2 with DNA of the first stage result as the template is as much as 1µL.

A total of 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, then incubated at 37^0C for 2 hours. PCR-RFLP was electrophoresized, obtained homozygote -308A allele which was cutted into 2 bands with the size of 208 pb and 231 pb and the homozygote allele -308G of which the cutting didn't occur, only one band appeared with a similar size to the size of the PCR product, 231 pb. While the heterozygous samples, which brought allele G and A, partly cutting occured and resulted in 3 bands with sizes of 231 pb, 208 pb and 23 pb.

2.2. PCR-RFLP of -238 TNFα Gene

TNFα -238 genotype (rs361525) was analyzed with PCR-RFLP method by using primers for the PCR amplification of the gene by ATC-TNFα−238G/A (rs361525), the primers were 5'ATCTGGAGGAAGCGGTAGTG-3' and 5'-AGAAGACCCCCCTCGGAACC-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), ATC-1 primer 40 pmol / µL (0,5 µL), the primary ATC-2 40 pmol / µL (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 was given additional denaturation time 94^0C for 5 minutes and at the end of the reaction is given additional elongation time 72^0C for 5 minutes. Positive TNFα gene PCR result 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 TNFαgene promoter, DNA PCR product cutting was performed using restriction enzymes MspI which possessed 5'-C˘CGG-3'recognition site. PCR-RFLP was electrophoresized, obtained homozygote allele -238A of which the cutting didn't occur, only one band appeared with a similar size to the size of the PCR product, 231 pb, and homozygote -238G allele was cutted into 2 bands with the size of 130pb and 20pb. While the heterozygous samples, which brought allele G and A, partly cutting occured and resulted in 3 bands with sizes of 150 pb, 130 pb and 20 pb.

3. Results

From 227 people examined, obtained the number of samples that met the inclusion criteria, exclusion and equalization of age and smoking history as much as186 people with details were 93 people as case group and 93 people as control group. The sample in case and control groups, respectively numbered 93 men (100%), and the largest age range was 50-59 years old (41.9% of the case group and 40.9% of the control group).

3.1. Analysis of TNFα -308 Gene Polymorphism

Table 2 shows the number of individuals who have allele G and A on TNFα -308 genotypes between cases and controls. G allele was fewer number of 145 in the control group (78%), while in the group of COPD cases was 158 (85%). Group of COPD cases had 28(15%) allele A and the control group was 41(22%). From the results obtained information that the number of allele A was less common in COPD cases compared with the control group.

3.2. Analysis of TNFα-238 Gene Polymorphism

Table 3 shows the number of individuals who have G or A allele in the genotype -238TNFα between cases and controls. G allele was at a number of 179 in control group (96.2%) and in the case of COPD which amounted to174 (93.5%). A allele in COPD cases was 12 (6.5%) and the control was equal to 7(3.8%).

3.3. Association of TNFα Gene Polymorphism with COPD

Table4 shows the -238G/A and -308G/A TNF gene polymorphism in COPD (cases) and controls. For -238G/A, GA and AA genotypes were found as many as 8 in cases and 4 in controls. As for the GG genotype, as many as 85 in cases and 89 in controls. If it's calculated statistically, obtained oods ratio (OR): 2.094 and the confidence level (confidence interval/CI): 0.608 to 7.211. P-value equals to 0.241 then this difference was not statistically significant. For -308 G/A genotypes polymorphism, GA and AA were found as many as18 in the case and 33 in the control. As for the GG genotype in 75 cases and 60 in controls. If it's calculated statistically, obtained oodsratio (OR): 0.436 and the confidence level (confidence interval/CI): 0.224 to 0.850. P value equals to 0.014 than this difference statistically significant.

4. Discussion

Bronchial smooth muscle, cell hypertrophy, inflammatory narrowing of peripheral airways and loss of elastic recoil may contribute to a different extent in certain individuals. Susceptibility to these processes may have differing genetic bases. A search for genes that increase susceptibility to airflow obstruction among smokers may have implications beyond the development of COPD[6].

This study shows that -238 TNFα gene polymorphism is not associated with the incidence of COPD, but for the -308G/A TNFα gene polymorphism, obtained result that the -308A allele is more common in normal individuals and significantly proven to be a protective factor for the development of COPD. Results of statistical analysis are: oods ratio 0.436 with confidence interval (CI): 0.224 to 0.850 and p-value: 0.014. Conclusion from the data analysis of this study is the polymorphism of -308 TNFα gene reduces the risk of COPD in smokers. COPD is likely to less occur by 0.436 times in smokers who have a TNF-308A gene allele. These results are not in accordance with previous studies that analyzed the association of TNFα gene polymorphisms with the incidence of COPD [7-16] . Likely due to differences in the definition of the phenotype, racial differences used in this study with previous research. As well as that reported by Molfino in 2004 on the effect of racial differences in the risk factors for COPD, that COPD is a disease involving genetic complex[17]. Therefore the risk of each different alleles become important in different populations because of differences in the populations or the differences in the form of exposure to environmental factors. Differences in phenotypes of COPD is likely due to differences in mutations or gene polymorphisms. Data research shows that it takes about genes and gene association analysis of the incidence of COPD in different populations. The population differences may be related to differences in specific genetic risk and environmental exposure. The difference in the results of this study and a study reported by Huang [10], one possibility is that the difference in the position of the promoter segment. PCR products generated in this study were different in DNA base pairs from the PCR products which were examined by Huang. In this study the resulted size was 231pb, whereas Huang obtained 345pb. TNFα promoter tip length of 1.5 κB place from the starting position of the gene transcription process (Transcription Start Site / TSS) as a regulator of TNFα production. The possibility that -308 TNFα gene polymorphism occurs in the study population leads to lower TNFα gene transcription process resulting in the production of TNFα as the inflammatory mediator is lowered. Another possibility is caused partly due to different study populations, different patient selection criteria which were COPD patients in this study, while Huang was using patients with Chronic Bronchitis, and the number of samples in this study are two-folds. However, we could not confirm this association between the polymorphism at position-308 of TNFαgene and COPD. Because the actual effect of thispolymorphism -308TNFa in vivohas not been clearly demonstrated in this study. Further research needs to be done on the gene expression of -308A TNFα allele in patients with COPD on levels of TNFα in patients blood with COPD and further analysis of the sequences around position -308 TNFα gene promoter and identification of proteins associated with DNA sequences covering position -308 needs to be done, so will provide useful information in an effort to characterize the gene promoter and its relation to protective factors to the incidence of COPD. A large number of genes and polymorphisms must first be identified, and an extremely large number ofsuch polymorphisms will need to be tested[18].

5. Conclusions

In summary, our results suggest that polymorphism of -308G/A TNFα gene was proven to be a protective factor of the development of COPD, and polymorphism of -238G/A TNFα gene did not show any association with the incidence of COPD.

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Table 1. Characteristic Data : Sex, Age, Smoking and GOLD Stage

Characteristics	**Control**		**Case**		
	Total	%	Total	%	p
1. Sex					
Male	93	100	93	100	
2. Age					
a. 40-49	5	3.2	3	5.4	0.961
b. 50-59	39	40.9	38	41.9	
c. 60-69	29	33.3	31	31.2	
d. 70-79	14	16.1	15	15.1	
e. 80-89	6	6.5	6	6.5	
3. Smoking Habit (BI)					
(BI = daily number of cigarettes x years					
Mild (IB < 200)	0	0	0	0	0.660
Moderate (200 < IB < 600)	44	47.3	47	50.6	
Heavy (IB > 600)	49	52.7	46	49.4	
4. GOLD Stage (%FEV_1)					
GOLD I ($FEV_1 \geq 80\%$)			1	1	
GOLD II ($50\% \leq FEV_1 < 80\%$)			26	28	
GOLD III ($30\% \leq FEV_1 < 50\%$)			47	51	
GOLD IV ($FEV_1 < 30\%$)			19	20	

Table 2. Allele -308 Gene TNFα Between Cases and Controls

	Case		**Control**	
	n= 93	%	n= 93	%
Allele A	28	15	41	22
Allele G	158	85	145	78
Total	186	100	186	100

Table 3. Allele -238 Gene TNFα Between Cases and Controls

	Case		**Control**	
	n= 93	%	n= 93	%
Allele A	12	6.5	7	3.8
Allele G	174	93.5	179	96.2
Total	186	100	186	100

Table 4. Association of TNFα Gene Polymorphism with COPD

Genotype	**Case**	**Control**	**OR**	**95%CI**	**P**
-238 TNFα					
GA - AA	8	4	2.094	0.608-7.211	0.241
GG	85	89			
-308 TNFα					
GA – AA	18	33	0.436	0.224-0.850	0.014
GG	75	60			