

Association Between Tumor Necrosis Factor- α -308G/A Polymorphism and Chronic Obstructive Pulmonary Disease in Patients of the University of Santo Tomas Hospital

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Abstract

Introduction: Chronic obstructive pulmonary disease (COPD) has been associated with enhanced inflammatory response to noxious particles and irritants. Tumor necrosis factor- α (TNF- α) has been observed to be present in elevated levels in COPD patients. Up-regulation of TNF- α production may be a result of mutations in the gene complex coding for its production.

Objective: The main objective of this study is to determine an association between the COPD and TNF- α gene polymorphism in patients of the University of Santo Tomas Hospital (USTH).

Methods: The occurrence of TNF- α -308G/A gene polymorphism was examined in patients diagnosed with COPD at the USTH. The recruited participants underwent spirometry likewise COPD assessment test scores were determined. Blood samples were collected for genotyping. Recorded data were then statistically analysed.

Results: Forty-nine percent of the total number of participants were diagnosed with COPD (FEV1/FVC <

70) while 51% were part of the control group (FEV1/FVC > 70). Frequencies of the rare allele (A) were found to be higher (0.11) in the control group compared to the patient group (0.04). Participants with smoking history are less likely to develop COPD when carrying the heterozygous genotype G/A (OR 0.10, 95%CI 0.01–0.79, $p=0.021$). Within the overall participant population, occurrence of the rare allele 'A' was higher in the control group (0.12) compared to the patient group (0.7). Heterozygous (G/A) genotype is less likely to have COPD (OR 0.29, 95%CI 0.07–1.21) though disease-SNP polymorphism relationship did not have strong statistical association ($p=0.08$).

Conclusion: TNF- α -308G/A gene polymorphism is associated with the development and pathogenesis of COPD. Smokers bearing the heterozygous rare G/A allele are less likely to develop the disease as compared to their counterparts bearing the wild type allele. Genotypes containing the rare 'A' allele (G/A and A/A) are less likely to develop the COPD.

Keywords: COPD, Tumor Necrosis Factor- α , Genetics, Biomarkers

Introduction

Chronic obstructive pulmonary disease or COPD, is a chronic inflammatory disease of the airways that hinders the rate of expiration of air to the point that an individual is unable to release all used air before taking another breath. COPD diagnosis is considered in any patient exhibiting cough, sputum production, or dyspnea and/or history of exposure to risk factors for the disease.¹ Spirometry result of post-bronchodilator FEV1 < 80% of the predicted value with an FEV1/FVC < 70% confirms the presence of COPD.¹ Chronic inflammation that occurs in cases of COPD has been associated with different factors such as environmental exposure to noxious particles and oxidative stress.² Another factor that may contribute to inflammation during COPD is the deregulation of Tumor Necrosis Factor- α (TNF- α)

production.

Studies have delved into the possible usage of genetic biomarkers that may be involved in the development of COPD. TNF- α was found to induce the late-phase airway hyper-responsiveness and airway inflammation through cytosolic phospholipase A2 activation. Elevated levels of TNF- α in COPD patients may result from gene polymorphisms that up-regulate its production of the cytokine.³ The relationship, however, of TNF- α gene polymorphism and TNF- α production has not been established yet.⁴ Studies on the different TNF- α gene polymorphisms at locations -862C/A, -856C/T, -574G/A, -308G/A, -238G/A and +70(addition of C) have failed to show direct association to TNF- α production.⁵ It has been linked to numerous inflammatory diseases such as Behçet's disease,⁶ Crohn's Disease,⁴ rheumatic heart disease,⁷ and inflammatory bowel disease.⁸

Studies conducted on transgenic mice showed that overexpression of the TNF- α gene resulted in the development of general lung inflammation resulting in airway obstruction.⁹ Another study using TNF- α receptor knockout mice (TNF- α RKO mice) have shown differences in the inflammatory response of the lungs to cigarette smoke. TNF- α RKO mice have exhibited no changes in the gene expression of TNF- α , neutrophil and macrophage chemo-attractant. Moreover, an experiment using 129 pure mice known to produce low

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levels of TNF- α have been found to exhibit no inflammatory response to smoke inhalation.¹⁰ Further studies of Churg (2004) estimated that the TNF- α driven process accounts for 70% of the airspace lining enlargement and majority of the inflammatory cell influx.¹¹ Elevated levels of TNF- α have been found in the sputum and blood of COPD patients.¹² Studies performed on a Taiwanese population showed a relationship between chronic bronchitis and TNF- α gene polymorphism.¹³ Similar studies conducted on Japanese¹² and Caucasian populations¹⁴ have also shown a positive association between TNF- α gene polymorphisms and COPD. In a meta-analysis on six (-1031C/T, -863C/A, -857C/T, -237G/A, -308G/A and +487G/A) different polymorphisms of the TNF- α gene the -308 minor allele showed a higher odds ratio of being associated with COPD and FEV1 lowering. It also showed that studies on the -308G/A polymorphism of TNF- α have shown the strongest association to COPD compared to the other polymorphisms.¹⁵ Currently, no association studies have been performed in the Philippines regarding the relationship between TNF- α polymorphism and COPD.

According to the estimates of the Department of Health (2006), chronic obstructive pulmonary disorder is the leading cause of morbidity and seventh cause of mortality in the Philippines. This highlights the need for an early screening and intervention tool for COPD to improve the quality of life of patients.

Therefore, it is the aim of this study to determine the relationship between COPD and TNF- α -308G/A gene polymorphism on selected Filipino population from University of Santo Tomas Hospital (USTH). Specifically, the study was guided by the following objectives: 1) to isolate genomic DNA from blood sample collected from the USTH – Center for Respiratory Medicine, 2) determine the allele and genotype frequency of TNF- α -308G/A gene polymorphism COPD patients, 3) associate the TNF- α -308G/A gene polymorphism with the recognized standard of COPD diagnosis which is FEV1/FVC ratio of less than 0.70, and 4) ascertain a correlation between phenotypes and the genotype frequencies of the polymorphism.

This research studies the correlation and association of TNF- α -308G/A gene polymorphism and the development and severity of symptoms in COPD patients with major premises as follows: 1) the presence of TNF- α -308G/A gene polymorphism would result in an increase of severity and faster development of symptoms of COPD, and 2) there is a correlation between the frequency of TNF- α -308G/A gene polymorphism and the type of COPD (symptomatic/asymptomatic) the patient develops.

Materials and Methods

This is a case control study wherein patients diagnosed with COPD were compared to those who have no COPD based on the presence or the absence of TNF- α .

Patient Recruitment

Participants were recruited from the USTH – Center for

Respiratory Medicine through their attending physician. Consent of the participants in the study was obtained. Consent forms were available in both English and Filipino. All participants recruited were above 18 years of age. Individuals diagnosed to have asthma were not included in the study. A total of 123 participants have been recruited for the study.

Phenotyping

Participants underwent spirometry and measurement of their forced vital capacity (FVC) and forced expiratory volume in one second (FEV1) were taken. The ratio (FEV1/FVC) between the two measurements was calculated. Patients with a score of less than or equal to 0.7 FEV1/FVC were classified under the patient group while those scoring higher than 0.7 were assigned to the control group. Participants were also be given COPD assessment test to assess the manifestation or presence of COPD symptoms. General information, such as smoking history, age and gender were collected. Phenotyping of the participants was done by the medical arm of the study.

Sample Collection

The source of genetic material used in the study was blood specimens. Samples were collected by a registered medical technologist or a licensed physician. Samples extracted from the participants were immediately frozen to preserve them for future use and analysis. Samples were turned over to the genetics arm of the project without disclosing spirometry and COPD assessment test scores of the sources of the samples. This would eliminate bias in the results.

DNA Extraction

Extraction of the genetic material was done using Wizard Promega DNA extraction kit. First, addition of 900 μ l of cell lysis buffer to 300 μ l of the sample released the contents of the cell into the solution. The mixture was allowed to incubate for ten minutes to ensure complete lysis of all blood cells contained in the sample. Samples were then centrifuged at 13000xG to allow separation of heavy intracellular components from hemoglobin. The supernatant was subsequently discarded and 300 μ l of nuclei lysis buffer was added to the pellet. This would disrupt the nuclear membrane releasing the genetic material into the solution. The solution was then incubated at 37°C to remove pellet clumps that may have formed in the previous steps. RNase was then added to the solution to degrade any ribonucleic acid present. The solution was again incubated at 37°C for 15 minutes to allow maximum activity of the RNase. The tubes were then immersed in an ice bath to bring the temperature down prior to adding the protein precipitating solution (PPS). Addition of PPS would denature and subsequently precipitate the protein components of the solution and allow its separation from the genetic material via centrifugation. The resulting brown pellet was discarded and the supernatant was transferred to a tube containing

300 μ L of isopropanol, a DNA precipitating agent. The mixture was again spun and the supernatant was discarded. Ethanol was then added to the pellet to enhance the precipitation of the DNA. The mixture was again subjected to centrifugation and was air dried. A rehydrating solution was added to the DNA and was left to stand overnight at 4.0°C. Quality of the extracted DNA was assessed by using 1.0% agarose gel electrophoresis. Gel was made by heating a mixture of 1.05g of agarose and 35ml 1.0x TAE buffer until clear. The mixture was introduced into a caster and was allowed to stand until it solidified. Samples were loaded with EZ loading dye to enable tracking of the movement of the genomic DNA in the gel. The loading dye also increases the density of the DNA sample in order to prevent it from dispersing into the buffer. A DNA ladder was also loaded into the gel to serve as a basis for molecular size and concentration of the genomic DNA in the samples. Electrophoresis was run at 100 volts to about 50% of the total gel length. The gel was then stained in ethidium bromide solution for 20 minutes and was destained in sterile distilled water for five minutes. The gel was viewed under ultraviolet light and photo documentation was taken using the gel documentation software. Band intensities were compared to the DNA Ladder intensities to assess concentration of genomic DNA in each extract.

Polymerase Chain Reaction

Polymerase chain reaction was done to isolate a section of the gene of interest. The 5'-region of the TNF- α gene, positions -331 to 14, was amplified using gene specific primers.¹⁶ Primer sequences used were 5'-AGGCAATAGGTTTGAGGGCCAT-3' for the forward primer and 5'-GAGCGTCTGCTGGCTGGGTG-3' for the reverse.⁹ Each reaction, with a total volume of 25 μ L, contained 2.5 μ L 5x GoTaq Flexi buffer, 2 μ L 25mM MgCl₂, 0.2 μ L 5units μ L-1Taq pol, 0.25 μ L 0.1mM forward primer, 0.25 μ L 0.1mM reverse primer, 0.25 μ L 10mM dNTP's, and 6.05 μ L sterile nanopure water.

PCR was run under the following conditions as summarized in Table I.

Table I. PCR Profile

Initial Denaturation	94°C	3.0 mins.
Cycles: 35		
• Denaturation	94°C	1.0 min.
• Annealing	60°C	1.0 min.
• Extension	72°C	1.0 min.
Final Extension	72°C	5.0 mins
	4.0°C	∞

Restriction Enzyme Length Polymorphism

Restriction enzyme length polymorphism (RFLP) was used to determine the presence of the polymorphism in the amplified section of the DNA. NcoI restriction enzyme was used in the study. RFLP reactions consisted of 5 μ L of PCR

product and 0.5 μ L 20unit/ μ L of NcoI restriction enzyme. The mixture was then incubated at 37°C for two hours to allow the digestion of the PCR product. Restriction enzyme digests were then subjected to 3.0% agarose gel electrophoresis run at 100 volts to about 60% of the total gel length. The gel was then stained in ethidium bromide solution for 15 minutes. The gel was viewed under ultraviolet light and photo documentation was taken using the gel documentation software. Banding patterns were scored and were assessed for the presence of the polymorphism.

Statistical Treatment

The sample population was tested for deviation from the Hardy-Weinberg equilibrium using SNPStat software. Associations of genotype and allele frequencies were tested for association with logistic regression to adjust possible effects of covariates and confounding variables such as smoking history and age.

Results

A total of 123 participants were included in the study with a mean age of 55.23 years. Patient group consisted of 60 individuals diagnosed with COPD. Control Group consisted of 63 individuals without COPD. A total of 62 participants had smoking history while 61 did not have any previous history of smoking (Table II).

Allele frequencies for the entire population are summarized in Table III. Higher occurrence of the rare type A-allele are observed in the control population (COPD=NO).

Participants with smoking history were tested separately for the polymorphism (Table IV). Heterozygous genotype had a higher occurrence in the control group (COPD=NO) compared to the patient group (Tables V and VI).

Logistic regression, adjusted for age and smoking history, was performed on the three genotypes following different models of inheritance. This enabled corrections of the possible confounding effects of lung physiology change due to age and smoking. Genotypes were tested using codominant, dominant, recessive and overdominant models of inheritance (Table VII).

Table II. Sample demographics

	Patient Group (FEV1/FVC > 0.70)	Control Group (FEV1/ FVC<=0.70)	All Subjects
Number of Participants	60	63	123
Gender			
Male	46	28	74
Female	14	35	49
Smoking History			
Smoker	35	27	62
Nonsmoker	25	36	61
Mean Age:	64.12	46.9	55.23

Table III. Allele frequencies for the entire participant pool

SNP allele frequencies (n=123)						
	All subjects		COPD=NO		COPD=YES	
Allele	Count	Proportion	Count	Proportion	Count	Proportion
G	227	0.92	112	0.89	115	0.96
A	19	0.08	14	0.11	5	0.04

Table IV. Allele Frequencies for the Participants with Smoking History

SNP allele frequencies (n=62)						
	All subjects		COPD=NO		COPD=YES	
Allele	Count	Proportion	Count	Proportion	Count	Proportion
G	115	0.93	41	0.85	74	0.97
A	9	0.07	7	0.15	2	0.03

Table V. Genotype frequencies of the entire participant population

SNP genotype frequencies (n=124)						
	All subjects		COPD=NO		COPD=YES	
Genotype	Count	Proportion	Count	Proportion	Count	Proportion
A/A	5	0.04	4	0.06	1	0.02
A/A	9	0.07	6	0.1	3	0.05
G/G	109	0.89	53	0.84	56	0.93
NA	1	--	1	--	0	--

Table VI. Genotype frequencies of the participants with smoking history.

SNP genotype frequencies (n=62)						
	All subjects		COPD=NO		COPD=YES	
Genotype	Count	Proportion	Count	Proportion	Count	Proportion
A/A	2	0.03	2	0.08	0	0
G/A	5	0.08	3	0.12	2	0.05
G/G	55	0.89	19	0.79	36	0.95

Discussion

The 123 participants of this study comprised mostly of males than females (60% versus 40%) with equal number of smokers and nonsmokers, as well as patients with and without COPD. Results showed that rare type allele "A" and heterozygous had higher occurrence in the control group compared to the patient group. Also, there was a strong tendency for dominant model inheritance of the genotypes to be associated with COPD.

Table VII. Logistic regression of genotypes versus COPD statistical significance (P<0.05), marginal significance (P<0.10)

SNP genotype frequencies (n=62)							
Model	Genotype	COPD=NO	COPD=YES	OR (95% CI)	p-value	AIC	BIC
Co-dominant	G/G	53 (84.1%)	55 (93.2%)	1.00	0.16	143	157
	G/A	6 (9.5%)	3 (5.1%)	0.30 (0.06-1.47)			
	A/A	4 (6.3%)	1 (1.7%)	0.21 (0.02-2.74)			
Dominant	G/G	53 (84.1%)	55 (93.2%)	1.00	0.56	141.1	152.3
	G/A-A/A	10 (15.9%)	4 (6.8%)	0.27 (0.07-1.09)			
Recessive	G/G-G/A	59 (93.7%)	58 (98.3%)	1.00	0.24	143.4	154.6
	A/A	4 (6.3%)	1 (1.7%)	0.24 (0.02-3.02)			
Over-dominant	G/G-A/A	57 (90.5%)	56 (94.9%)	1.00	0.14	142.6	153.8
	G/A	6 (9.5%)	3 (5.1%)	0.32 (0.07-1.54)			
Log-additive	--	--	--	0.39 (0.14-1.10)	0.06	141.2	152.4

AIC Akaike Information Criterion

BIC: Bayesian Information Criterion

The participants with smoking history are less likely to develop COPD when carrying the heterozygous genotype G/A (OR 0.10, 95%CI 0.01-0.79, $p=0.021$). Within the total participant population, occurrence of the rare allele 'A' was higher in the control group (0.12) compared to the patient group (0.7). Heterozygous (G/A) genotype is less likely to have COPD (OR 0.29, 95%CI 0.07-1.21) though disease-SNP polymorphism relationship did not have strong statistical association ($p=0.08$). In the contrary, studies on chronic bronchitis have shown that TNF- α -308 gene polymorphism is involved in the inflammatory response characteristic of bronchitis. The allele frequency of TNF2 or the A allele is 5.1% in the school children, 2.4% in the control group, and 19% in the bronchitis group ($p, 0.01$). This means that individuals, smokers and nonsmokers alike, with such polymorphism have a higher risk of developing bronchitis.⁶ Another study conducted on a Japanese population shows that the frequency of the TNF2 allele of 16.5% in COPD patients as compared to 7.8% in the population control and 8.2% in the smokers without COPD. The same study determined the genotype frequency of TNF1/2 to be 21% in COPD patients compared to 9.3% in the control.¹⁶

Higher frequency of the rare type allele was observed in control group suggesting that possession of the allele may reduce the risk of developing COPD. Presence of the "A" allele has been previously found to affect the regulation of TNF cytokine production. The polymorphism, located at the promoter sequence of the gene complex, may cause abnormal expression of the TNF- α gene leading to anomalous levels of the TNF cytokine.¹² Studies performed

by Kroeger (1996) have shown that the -308 region has the ability to bind to certain transcription factors.¹⁴ Variations in such region may change its overall interaction with certain transcription factors leading to either increase or decrease of cytokine production. The actual mechanism of regulation of TNF cytokine production has not been established.⁴ It is more than likely that predisposition to COPD is not hinged on a single gene but an interaction between inflammation related genes.¹⁵ A meta-analysis showed multiple studies on Asian populations were positive while studies dealing with Caucasian populations were mostly negative for the association.¹⁶ This indicates that variations of gene to disease relationships may involve ethnicity related genes. Data from this study indicate the presence of a weak association between possession of the rare type allele and COPD. Such findings suggest that the TNF- α gene polymorphism may play a role in susceptibility to COPD.

Table VII showed that there was no statistically significant association among any of the inheritance models. Genotype frequencies also show a higher occurrence of the heterozygous G/A allele in the control groups. It can however be noted that dominant model inheritance of the genotypes has a strong tendency to be associated with COPD ($p = 0.056$). This indicates that presence of the "A" allele may confer reduce susceptibility to the disease regardless of the patients genotype.

The TNF- α 308*2 allele may not affect COPD susceptibility but could be in linkage disequilibrium with the true susceptibility allele in a closely linked gene. Hence, determinants between smokers susceptible to the development of COPD and those resistant to it cannot be explained by a single gene polymorphism.¹⁶

Conclusions and Recommendations

TNF- α -308G/A gene polymorphism is associated with the development and pathogenesis of COPD. Smokers bearing the heterozygous rare G/A allele are less likely to develop the disease as compared to their counterparts bearing the wild type allele. Genotypes containing the rare 'A' allele (G/A and A/A) are less likely to develop COPD. It is recommended that future studies that delve into COPD epidemiology and the like increase the number of participants to make differences in frequencies more pronounced. Matched case-control design is also recommended for this study.

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