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Is There an Association Between *NOD2* Gene Polymorphisms and Chronic Obstructive Pulmonary Disease Progression?

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ABSTRACT Chronic obstructive pulmonary disease (COPD) is characterized by persistent airflow limitation associated with an increased chronic inflammation. Recent studies suggest that innate immune system receptors may be involved in this enhanced response as observed in COPD. The aim of this study is to investigate the correlation between the nucleotide-binding and oligomerization domain 2 *(NOD2)* polymorphisms and development, severity and progression of COPD in the Turkish population. Three *NOD2* polymorphisms were genotyped using polymerase chain reaction-restriction fragment length polymorphism (PCR-RFLP) and real time PCR analysis involving 168 patients and 100 healthy controls. No statistical difference was observed between the case and the control groups for genotype and allele frequencies of the rs2066844, rs1077861 and rs3135500 polymorphisms. Contrary to the available literature, annual forced expiratory volume in one second (FEV1) decline was statistically greater in GG carriers of the rs3135500 polymorphism than in AA carriers (p=0.048). The *NOD2* gene rs3135500 polymorphism may be related progression of COPD in the Turkish population.

INTRODUCTION

COPD is a common and treatable disease characterized by persistent respiratory symptoms and air flow limitation, resulting from combination of small airway disease and the destruction of lung parenchyma (GOLD 2017). These structural changes in the lung and airways are associated with chronic inflammation (Sayan and Mossman 2016; GOLD 2017). Smoking is a major risk factor for COPD. However, not all habitual smokers develop COPD, indicating the importance of genetic factors on development of this chronic disease (Bosse 2012; Pabst et al. 2013). The genetic background of COPD is also supported by family studies (Mccloskey et al. 2001; Nakamura 2011).

The human body continuously fights against large numbers of microorganisms. The initial response to infectious agents in this fight is given by the innate immune system. The innate immune system genes regulate the delicate balance between the stimulation and suppression of the immune response to foreign materials in order to maintain homeostasis in various body surfaces, including the lungs. The disruption of this balance can cause serious infectious diseases, chronic inflammation, and autoimmune diseases (Correa et al. 2012).

After the detection of pathogen-associated molecular patterns (PAMPs) or damage-asso-

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ciated molecular patterns (DAMPs), a group of pattern recognition receptors (PRRs) trigger an innate immune response. The *NOD2* protein is one of the *NOD* like receptors (NLRs) that is a member of the main intracellular PRR families in the innate immune system. The members of this family consist of three domains: (1) The N terminal caspase recruitment domain (CARD) which induces recruitment and activation of downstream effector molecules, (2) the central NOD or NACHT domain of which is responsible for self-oligomerization and NLR activity, and (3) C-terminal leucine-rich repeats (LRRs) which interact with ligands as well as modulate NLR activity (Correa et al. 2012; Philpott et al. 2014; Wang et al. 2017; Zhu and Cao 2017).

NOD2 senses muramyl dipeptide (MDP) present in both gram negative and gram positive bacterial peptidoglycan, and responds to bacterial infections via the stimulation of nuclear factor κβ (NF-κβ) and the mitogen-activated protein kinase (MAPK) pathway (Philpott et al. 2014; Al Nabhani et al. 2017). An impairment in *NOD2* function alters the protective immune response and causes dysbiosis. In order to cope with this problem, other pathways become activated, and chronic inflammation arises (Philpott et al. 2014; Becker et al. 2015). The *NOD2* gene that encodes 1040 amino acid *NOD2* protein has been mapped to chromosome 16q12, and contains 12 exons (Omrane et al. 2014). *NOD2* variants have been related to various inflammatory diseases such as Crohn's disease, Blau syndrome, early-onset sarcoidosis, Yao syndrome (*NOD2*-Associated Autoinflammatory Disease), graft-versus-host disease (GVHD), rheumatoid arthritis, allergic rhinitis, atherosclerosis, Behcet disease, and Asthma (Hamzaoui et al. 2012; Hu et al. 2013; Yao 2013; Jaskula et al. 2014; Franca et al. 2015; Wilson et al. 2016; Zhao et al. 2016; Al Nabhani et al. 2017; Ni et al. 2017; Yao and Shen 2017).

Some studies have suggested that the innate immune system plays a crucial role in the development and progression of COPD (Kinose et al. 2012; Apostolou et al. 2016; Fan et al. 2016; Sayan and Mossman 2016; Di Stefano et al. 2017; Ishii et al. 2017; Liu et al. 2017; Pinkerton et al. 2017). Alongside this, COPD exacerbations are generally caused by bacterial and viral infections (Kinose et al. 2012; Leissinger et al. 2014; Kinose et al. 2016; Tan et al. 2017). In the light of this, impaired *NOD2* function resulting in aberrant inflammation may be associated with the development, severity, progression, and exacerbation of COPD. However, there the present research assessing this association between *NOD2* and COPD is limited, and their results are contradictory (Kinose et al. 2012; Kinose et al. 2016; Di Stefano et al. 2017). In the current study, three *NOD2* variants have been chosen and investigated their association with COPD. Among these variants the missense R702W polymorphism, which is located in exon 4 of the *NOD2* gene, has been strongly associated with CD (Correa et al. 2012; Heresbach et al. 2004). The Rs1077861 and rs3135500 polymorphisms are located in intron 10 and 3' UTR regions of *NOD2* gene, respectively. It has been reported that rs3135500 polymorphism is within miRNA binding sites (Ahangari et al. 2014). For this reason, the rs3135500 polymorphism may be functional. There is a study arguing that rs1077861 may influence the development and progression of COPD (Kinose et al. 2012).

Objectives

The aim of this study was to investigate the correlation between *NOD2* gene polymorphisms and the development, severity, progression and exacerbation of COPD within the population of Turkey.

METHODOLOGY

Subjects

The present study was approved by the local ethics committee of Afyon Kocatepe University (2012/8-65) and conducted in accordance with the Helsinki declaration. Signed informed consent was obtained from all participants before recruitment. A total of 168 male COPD patients and 100 healthy unrelated male smokers who were at least 45 years of age and who had been smoking for at least 10 pack years were included in the study. The diagnosis of COPD was made or confirmed in accordance with the Global Initiative for Chronic Obstructive Lung Disease guidelines (GOLD) criteria (GOLD 2017), as based on pulmonary function tests (PFTs) [post-bronchodilator FEV1/forced vital capacity (FVC) <70%], clinical evaluation, and past smoking history. Patients who have moderate, severe and very severe airflow obstruction were

enrolled in the study. When patients were included in the study they were all in a stable period. Individuals with existing lung diseases, inflammatory diseases (other than COPD in the patient group), and malign diseases were excluded from the study group. Venous blood samples were taken and PFT was performed. Patients were classified according to their severity of airflow obstruction (moderate, severe, very severe), GOLD stages (A-D), frequency of exacerbation, and family history. Non-frequent exacerbators were defined as patients with less than two exacerbations, and who had no prior history of hospitalization. Frequent exacerbators were defined as having two or more exacerbations, or having been hospitalized at least once due to COPD exacerbations over the past year.

PFT

A clinical spirometer (ZAN 300 USB System) was used for all PFTs. All subjects were oriented about the maneuvers by the same laboratory technician before initiating the test. A maximal expiratory flow maneuver was performed by each subject at least three times while sitting. FEV_1 and FVC were measured and then the FEV_1/FVC was calculated. A second PFT was performed on 125 COPD patients one year after the initial PFT in order to calculate the annual FEV1 (L) decline.

DNA Extraction and Genotyping

Genomic DNA was isolated from peripheral blood samples using a high pure PCR template preparation kit (Roche Diagnostics, Mannheim, Germany), in accordance with the manufacturer's instructions. PCR-RFLP analysis was performed for the R702W polymorphism. PCR reactions were carried out using a pair of forward primer 52 –CTTCCTGGCAGGGCTGTTGTC -32 and reverse primer 52 - CATGCACGCTCTTGGCCTCAC -32 (Thermo Scientific, USA). The PCR protocol was 95 °C for 5 min, followed by 35 cycles of 95 °C for 1 min, 60 °C for 1 min, 72 °C for 2 min, and a final extension at 72 °C for 7 min. All PCR products were digested with an Hpa II restriction enzyme (Thermo Scientific, USA). Real-time PCR and melting curve analysis were performed using the LightCycler® 480 instrument (Roche Diagnostics, Mannheim, Germany), using appropriate primers and probs (TIB-MolBiol, Berlin, Germany) for the genotyping of the rs1077861 and rs3135500 polymorphisms.

Statistical Analysis

Statistical analysis was carried out using SPSS 18.0 statistical software (SPSS Inc., Chicago, IL, USA). A Chi-square test was used to evaluate the deviation of genotypic distribution of the rs1077861 and rs3135500 polymorphisms from the Hardy–Weinberg equilibrium for the patient and control groups. Differences in baseline characteristics were evaluated using the Mann-Whitney U and Pearson's chi-square test. Genotype and allele frequencies between the groups were compared using the Fisher's Exact and Pearson Chi-Square Tests. Backward Stepwise Logistic Regression Analysis was used to examine the relationship between the rs1077861 and rs3135500 polymorphisms and COPD susceptibility. The effects of polymorphisms and the frequency of exacerbations on annual FEV1 decline were tested using the Kruskal-Wallis and Mann–Whitney U tests, respectively. Spearman's Rho Correlation Coefficient was calculated in order to determine whether or not there was a correlation between pack years of cigarette smoking and annual FEV1 decline. The Kruskal-Wallis and Mann–Whitney U tests were used to compare annual FEV1 decline among different genotypes of polymorphisms, as well as between the frequent and non-frequent exacerbators. *P* values less than 0.05 were accepted as statistically significant.

RESULTS

The baseline characteristics of the patient and the control groups are outlined in Table 1. The study group comprised of 168 male COPD patients and 100 healthy male controls. The mean age was significantly different between the cases (64 ± 8.55) and the controls (60 ± 7.96) ($p<0.001$). The number of ex-smokers and current smokers were 138 (82.1%) and 30 (17.9%), in the cases, 73 (73%) and 27 (27%) in the controls ($p=0.077$). The smoking history (pack years) of the cases and controls were 47.6 ± 33.8 and 37.4 ± 22.3, respectively ($p= 0.059$). There were 114 (68.3%) frequent exacerbators and 53 (31.7%) nonfrequent exacerbators in the case group. According to the GOLD stage, the number of moderate, severe and very severe patients were

Table 1: The baseline characteristics of the case and the control groups

	Cases (N=168) Mean (SD) or n (%)	*Controls (N=100) Mean (SD) or n (%)*	*p value*
Age (years)	64 ± 8.55	60 ± 7.96	<0.001
Smoking History (Pack years)	47.6 ± 33.80	37.4 ± 22.30	0.059
Initial FEV1*(L)	1.37 ± 0.58		
Recent FEV1 (L)	1.15 ± 0.56	2.95 ± 0.48	<0.001
Initial FEV1 (%)	47 ± 18.79		
Recent FEV1 (%)	40.7 ± 17.86	97 ± 11.34	<0.001
FEV1/FEVC**(%)	60.6 ± 7.02	85.50 ± 8.08	<0.001
FEF 25-75*** (%)	35 ± 17.31	100.80 ± 26.49	<0.001
SPO2**** (%)	93 ± 6.00	97 ± 1.20	<0.001
Smoking Status			
Ex-smokers	138 (82.1)	73 (73)	0.077
Current smokers	30 (17.9)	27 (27)	
Exacerbation Frequency			
Frequent exacerbators	114 (68.3)		
Nonfrequent exacerbators	53 (31.7)		
Stage (GOLD)			
Moderate (FEV1: 50%–79%)	50 (29.9)		
Severe (FEV1: 30%–49%)	64 (38.3)		
Very severe (FEV1 < 30%)	53 (31.7)		
Stage (GOLD)			
A	52 (31.1)		
B	10 (6)		
C	64 (38.3)		
D	41 (24.6)		

*FEV1, forced expiratory volume in 1 s
** FVC, forced vital capacity
***FEF 25-75, forced expiratory flow at 25-75% of the pulmonary volume
****SPO2, oxygen saturation

50 (29.9%), 64 (38.3%) and 53 (31.7%). The number distribution of A, B, C and D stage patients were 52 (31.1%), 10 (6%), 64 (38.3%), and 41 (24.6%), respectively. The mean FEV1/FEVC in the case and the control groups were 60.6 ± 7.02 and 85.5 ± 8.08, respectively (p<0.001) (Table 1).

The rs1077861 and rs3135500 polymorphisms were tested for the Hardy-Weinberg equilibrium in the patient and control groups. Since there was no individual who had the TT genotype of the R702W polymorphism, and as only one patient had the CT genotype, we did not test this polymorphism for the Hardy-Weinberg equilibrium. The researchers found that the genotype frequencies for rs3135500 were consistent with the Hardy-Weinberg equilibrium in both the patient (p=0.74) and the control groups (p=0.11). In addition, the genotype frequencies of the rs1077861 polymorphism were found in the Hardy-Weinberg equilibrium for the control group (p=0.052), but not for the patient group (p=0.003) (Table 2).

The distribution of allele and genotype frequencies for all polymorphisms in the patient and the control groups were demonstrated in Table 3. The TT genotype of the R702W polymorphism was not found in any of the 268 subjects; whereas the CT genotype was found in only one subject in the patient group, and not found in the control group. The frequencies of the C and T alleles were 99.7 percent and 0.3 percent within the patient group respectively. There was no significant difference between the patient and control groups in terms of genotype (p=0.683) and allele (p=1) frequencies for the R702W polymorphism (Table 3).

There were no significant differences between the rs1077861 genotypes in the COPD group (TT 27.4%, TA 60.1%, AA 12.5%) and the control group (TT 26%, TA 59%, AA 15%, p=0.840). Similarly, the allele frequencies were not different for the patients (T 57.4% vs A 42.6%) and the controls (T 55.5% vs A 44.5%, p=0.661) (Table 3).

The genotype and allele frequencies of rs3135500 polymorphism were not different for COPD and the control groups. The distribution of genotype frequencies was 43.5 percent for GG, 44 percent for GA, 12.5 percent for AA in COPD group and 37 percent for GG, 41 percent for GA, and 22 percent for AA in control group

Table 2: Results of Hardy–Weinberg Equilibrium for rs1077861 and rs3135500 polymorphisms

			Frequency			
Rs1077861	*Genotype*	*N*	*Observed*	*Expected*	χ^2	*p for HWE*
	T T	46	0.27	0.55		
Cases	T A	101	0.6	0.82	8.86	0.003
	A A	21	0.13	0.3		
	T T	26	0.26	0.31		
Controls	T A	59	0.59	0.49	3.78	0.052
	A A	15	0.15	0.2		
Rs3135500						
	G G	73	0.44	0.72		
Cases	G A	74	0.44	0.76	0.11	0.74
	A A	21	0.12	0.2		
	G G	37	0.37	0.33		
Controls	G A	41	0.41	0.49	2.6	0.11
	A A	22	0.22	0.18		

Table 3: Genotype and allele distributions of polymorphisms

Genotypes Rs2066844	*Cases N (%)*	*Controls N (%)*	*p value*
C C	167 (99.4)	100 (100)	0.683
C T	1 (0.6)	0 (0)	
T T	0 (0)	0 (0)	
Alleles			
C	335 (99.7)	200 (100)	1.00
T	1 (0.3)	0 (0)	
Rs1077861			
T T	46 (27.4)	26 (26)	0.840
T A	101 (60.1)	59 (59)	
A A	21 (12.5)	15 (15)	
Alleles			
T	193(57.4)	111 (55.5)	0.661
A	143(42.6)	89 (44.5)	
Rs3135500			
G G	73 (43.5)	37 (37)	0.117
G A	74 (44)	41 (41)	
A A	21 (12.5)	22 (22)	
Alleles			
G	220 (65.5)	115 (57.5)	0.065
A	116 (34.5)	85 (42.5)	

(p=0.117). The frequencies of G and A alleles were found to be 65.5 percent, 34.5 percent in COPD group, and 57.5 percent and 42.5 percent in the controls, respectively (p=0.065) (Table 3).

Backward stepwise logistic regression analysis showed that there was no association between the polymorphisms and COPD susceptibility. On the other hand, it was observed that risk of developing COPD increased 2.72-fold in individuals aged 60 years and over, and 2.94-fold in individuals with a family history of COPD. Furthermore, one unit increase in pack years of smoking led to a 1.013-fold increase in risk for development of COPD according to the researchers' logistic regression model (Table 4).

The researchers also found that the rs3135500 GG genotype carriers had a significantly higher annual FEV1 decline than the AA genotype carriers (p=0.048). On the other hand, the annual FEV1 decline was not different among genotypes of rs1077861 polymorphism (p=0.14). Moreover, no differences were found in terms of the genotype and allele frequencies of the rs1077861 and rs3135500 polymorphisms among patient groups classified according to severity of airway obstruction, GOLD stages (A-D), frequency of exacerbation, and family history (Table 5).

DISCUSSION

COPD is a complex multifactorial disease, and it is thought that enhanced chronic inflammation plays a crucial role in the development and progression of the disease. Several environmental and genetic factors contributing to development of COPD have been identified. Both differences between racial and ethnic groups and familial aggregation point out significant genetic predisposition. The genes favoring this susceptibility to COPD include those encoding antioxidants, enzymes that metabolize xenobiotics, proteases, antiproteases, and proteins that mediate the inflammatory response (Molfino and Jeffery 2007; Rovina et al. 2013; Vijayan 2013).

NOD2 is an innate immune system receptor. It recognizes MDP and triggers host innate immune responses (Philpott et al. 2014; Al Nabhani et al. 2017). There are many studies that state that the *NOD2* gene is associated with in-

Table 4: Results of Backward Stepwise Logistic Regression Analysis

Step	*Indep Var*[*]	*β*	*SE*	*Wald*	*p value*	*OR*	*CI (95%)*
1	Age (60 years or over)	0.972	0.277	12.307	0.000	2.643	1.536-4.550
	PFH	1.035	0.334	9.609	0.002	2.816	1.463-5.420
	Rs1077861 (TT)			1.455	0.483		
	Rs1077861 (TA)	-0.244	0.351	0.484	0.487	0.783	0.394-1.559
	Rs1077861 (AA)	-0.597	0.496	1.452	0.228	0.550	0.208-1.454
	Rs3135500 (GG)			3.280	0.194		
	Rs3135500 (GA)	-0.164	0.311	0.278	0.598	0.849	0.461-1.561
	Rs3135500 (AA)	-0.780	0.433	3.242	0.072	0.849	0.461-1.561
	Smoking History (Pack years)	0.013	0.005	6.086	0.014	1.013	1.003-1.023
2	Age (60 years or over)	0.992	0.275	12.980	0.000	2.696	1.572-4.624
	PFH[**]	1.048	0.335	9.811	0.002	2.853	1.480-5.497
	Rs3135500 (GG)			2.281	0.320		
	Rs3135500 (GA)	-0.089	0.297	0.090	0.764	0.915	0.511-1.637
	Rs3135500 (AA)	-0.571	0.387	2.184	0.139	0.565	0.265-1.205
	Smoking History (Pack years)	0.013	0.005	6.145	0.013	1.013	1.003-1.023
3	Age (60 years or over)	1.001	0.274	13.340	0.000	2.722	1.590-4.657
	PFH	1.078	0.333	10.453	0.001	2.939	1.529-5.651
	Pack years	0.013	0.005	6.622	0.010	1.013	1.003-1.023

[*]Indep Var, independent variable
[**]PFH, positive family history.

flammation, infection, inflammatory diseases, and cancer (Hamzaoui et al. 2012; Chaput et al. 2013; Hu et al. 2013; Yao 2013; Jaskula et al. 2014; Franca et al. 2015; Wilson et al. 2016; Zhao et al. 2016; Al Nabhani et al. 2017; Yao and Shen 2017; Zhu and Cao 2017). Therefore, the deregulation of *NOD2* may affect the risk of COPD development, as well as the severity and progression of the disease.

In a recent paper, it was reported that the expression level of NOD2 gene was significantly upregulated in human airway smooth muscle cells (HASMC) of asthma patients when compared to non-asthmatic individuals. Various authors have claimed that NOD2 could be a potential diagnostic biomarker and a therapeutic option for asthma (Ni et al. 2017). There is only one paper in the literature investigating the association between the *NOD2* gene polymorphism and COPD. The results of this paper indicated that the *NOD2* gene rs1077861 polymorphism may influence the development and progression of COPD in Japanese subjects (Kinose et al. 2012).

NOD2 gene variations have not been identified in genome-wide association studies (GWASs) of COPD (Cho et al. 2014). However most GWAS on COPD have been performed populations with European ancestry (Pillai et al. 2009; Cho et al. 2012). In light of this fact, that allele frequencies as well as environmental exposures may differ among different populations, and that generalizing these snp-trait associations to non-European populations may not prove to be correct each and every time. In addition, current COPD-associated variations explain only small fraction of disease heritability (Qiao et al. 2016). Therefore, the researchers think that it may be reasonable to investigate the relationship between COPD and different loci that may play a role in disease pathogenesis especially so in populations which have not been involved in GWAS, that is, Turkey.

In this study, the potential correlation between the *NOD2* gene rs2066844, rs1077861 and rs3135500 polymorphisms and development and severity of COPD in Turkish population was investigated. The researchers' results revealed that genotypic and allelic frequencies of R702W, rs1077861 and rs3135500 polymorphisms were not different between the patient and control groups; however, the annual FEV1 decline was higher in GG carriers of rs3135500 polymorphism than AA carriers.

The frequencies of C and T alleles for R702W polymorphism were 99.7 percent and 0.3 percent in the case group, and 100 percent and 0 percent in the control group, respectively (Table 3). When the researchers compared the minor allele (T) frequency of this polymorphism, their frequencies were lower in both the patient and the control groups than in other studies performed

Table 5: Genotype and allele frequencies of rs1077861 and rs3135500 polymorphisms among patient groups classified according to severity of airflow limitation, GOLD stages, exacerbation frequency and family history

	Severity of airflow limitation			*GOLD stages (A-D)*				*Exacerbation frequency (annual)*		*Family history*	
Rs1077861 Genotypes n (%)	*Moderate*	*Severe*	*Very Severe*	*A*	*B*	*C*	*D*	*Nonfrequent*	*Frequent*	*Yes*	*No*
TT	17 (34)	19 (29.7)	10 (18.9)	16 (30.8)	3 (30)	20 (31.3)	7 (17.1)	17 (32.1)	28 (24.6)	14 (25.9)	32 (28.1)
TA	30 (60)	34 (53.1)	36 (67.9)	30 (57.7)	5 (50)	36 (56.3)	29 (70.7)	28 (52.8)	73 (64)	35 (64.8)	66 (57.9)
AA	3 (6)	11 (17.2)	7 (13.2)	6 (11.5)	2 (20)	8 (12.5)	5 (12.2)	8 (15.1)	13 (11.4)	5 (9.3)	16 (14)
p value		0.185			0.703			0.387		0.60	
Alleles %											
T	64	56.3	52.8	59.6	55	59.4	52.4	58.5	56.6	58.3	57
A	36	43.8	47.2	40.4	45	40.6	47.6	41.5	43.4	41.7	43
p value		*0.252*			*0.733*			*0.742*		*.820*	
Rs3135500 Genotypes n (%)	*Moderate*	*Severe*	*Very Severe*	*A*	*B*	*C*	*D*	*Nonfrequent*	*Frequent*	*Yes*	*No*
GG	22 (44)	29 (45.3)	22 (41.5)	20 (38.5)	3 (30)	32 (50)	18 (43.9)	22 (41.5)	51 (44.7)	20 (37)	53 (46.5)
AG	22 (44)	23 (35.9)	28 (52.8)	24 (46.2)	6 (60)	22 (34.4)	21 (51.2)	25 (47.2)	49 (43)	26 (48.1)	48 (42.1)
AA	6 (12)	12 (18.8)	3 (5.7)	8 (15.4)	1 (10)	10 (15.6)	2 (4.9)	6 (11.3)	14 (12.3)	8 (14.8)	13 (11.4)
p value		0.202			0.359			0.879		0.497	
Alleles %											
G	66	63.3	67.9	61.5	60	67.2	69.5	65.1	66.2	67.5	61.1
A	34	36.7	32.1	38.5	40	32.8	30.5	34.9	33.8	32.5	38.9
p value		0.754			0.629			0.839		0.247	

in Turkish population. In one of these studies, the minor allele frequency in the controls and patients with sepsis was determined to be 1.6 percent and 5.5 percent respectively by Tekin et al. (2012). In another paper, Ozen et al. (2006) found that the R702W T allele frequencies were 1.9 percent, 1.4 percent, and 1.7 percent in controls, CD, ulcerative colitis patients, respectively. The T allele frequencies of R702W polymorphism in controls and CD patients were also investigated by Ince et al. (2007) and Uyar et al. (2006). These frequencies were 4.8 percent, 0.7 percent, and 0.5 percent, 0.9 percent, respectively. Minor allele frequencies of R702W polymorphism appear to be higher in European and American subjects and lower in Japanese, Chinese, and Korean subjects when compared to their Turkish counterparts (Serbati et al. 2014). The reason for this very low T allele frequency may be that the present study was done on either a small sample size, or that the patient carrying the CT genotype was of a different origin. The genotype and allele distributions of this polymorphism within the groups were not different in this study ($p>0.05$). Kinose et al. reported that none of the subjects in the COPD and the control groups carried the R702W polymorphism (Kinose et al. 2012). The researchers' results were similar to Kinose et al.'s findings in terms of the R702W polymorphism.

Kinose et al. reported that there were significant differences between the rs1077861 genotypes in the COPD group (TT 61%, TA 36%, AA 2%) and the control group (TT 76%, TA 22%, AA 2%, $p=0.036$), and that the rs1077861 A allele was significantly more frequent in the COPD group when compared to the controls (20% vs. 13%, $p=0.027$). In addition, this polymorphism was associated with a lower FEV1 percent predicted (57.2±1.8 for TT vs. 50.8±2.3 for TA/AA, $p=0.03$) in Japanese COPD patients. Another result of this study was that the rs3135499 polymorphism C allele, in close linkage with rs3135500 A allele, was associated with the tendency towards the COPD. The investigators suggested that the rs1077861 polymorphism may influence the development and progression of COPD (Kinose et al. 2012). In another paper, the T allele of rs1077861 polymorphism was associated with the decreased risk of developing asthma; and that the rs3135500 polymorphism A allele was associated with increased risk of this disease (Weidinger et al. 2005). A paper by Ahangari et al. showed that there was an association between the AA genotype of the rs3135500 polymorphism and colorectal cancer. On the contrary, Chaleshi et al reported that this polymorphism was not a genetic risk factor for the disease in Iranian subjects (Ahangari et al. 2014; Chaleshi et al. 2016).

To the best of the researchers' knowledge, the rs1077861 and rs3135500 polymorphisms were studied for the first time using Turkish subjects. The genotype frequencies of the rs1077861 polymorphism were deviated from Hardy-Weinberg equilibrium in the researchers patient group (Table 2). This deviation may be caused by excess of heterozygotes or ethnic heterogeneity. There were no significant differences between the cases and the controls in terms of the genotype and allele frequencies of the rs1077861 polymorphism (Table 3). Moreover, the annual FEV1 decline was not different among the genotypes of the rs1077861 polymorphism ($p=0.14$). The researchers' results were not consistent with those of Kinose et al. and Weidinger et al. for the rs1077861 polymorphism (Weidinger et al. 2005; Kinose et al. 2012). These results may be due to the higher minor allele frequency of this polymorphism in the Turkish study group due to ethnicity, the small sample size of the current study, and different environmental exposure factors among different populations.

The genotype frequencies of the rs3135500 polymorphism were not different for the COPD and control groups in the present study. Although the p value for allele frequencies were not statistically significant, it was close to a level of significance (Table 3). Nevertheless, there was no observable association between this polymorphism and risk of developing COPD in this study. On the other hand, the researchers' results indicated that the annual FEV1 decline was higher in carriers of the rs3135500 GG genotype ($p=0.048$). Kinose et al. and Weidinger et al. reported that A allele may be associated with tendency towards COPD and increased risk of asthma, respectively (Weidinger et al. 2005; Kinose et al. 2012). On the contrary, it is considered that GG genotype may be associated with progression of COPD in the present study. The researchers' findings for the rs3135500 polymorphism appear incompatible with the findings reported by Kinose et al. (2012) and Weidinger et al. (2005). However, they did not evaluate annual FEV1 decline. Another important contribution

of the present study to the literature is the determination of higher annual FEV1 decline in carriers of the rs3135500 GG genotype.

There were no significant differences for genotype and allele frequencies of the rs1077861 and rs3135500 polymorphisms among patient groups classified according to their severity of airflow obstruction, GOLD stages (A-D), frequency of exacerbation, and family history (Table 5).

It cannot be strongly claimed that there is an association between GG genotype of the rs3135500 polymorphism and higher FEV1 decline in this study as there was no association between this polymorphism and other parameters such as exacerbation frequency, disease development, and severity. Another reason is small sample size of the current study. Contrary to the researchers findings, the AA genotype of this polymorphism was associated with diseases in other studies. It may sometimes be possible that different alleles of the same polymorphism are associated with same the disease in different populations. There are a couple of explanations for this observation: i. This polymorphism may not be the source of the risk for that disease; ii. This polymorphism may be in linkage disequilibrium with a nearby mutation that is the source of the disease risk; and iii. It may be caused by different genetic backgrounds. These reasons yield the need for further studies on a larger sample size in order to verify the researchers' results.

CONCLUSION

Based on the results from this study, the researchers conclude that genotype and allele frequencies of the R702W, rs1077861 and rs3135500 polymorphisms are not risk factors for disease development, severity, or acute exacerbations; however, the rs3135500 polymorphism may be associated with the disease progression within Turkey's COPD population.

RECOMMENDATIONS

Further studies that can overcome present study's limitations (that is, a small sample size, short term follow up, and a lack of replication on an independent cohort) are needed to confirm the results.

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REFERENCES

Ahangari F, Salehi R, Salehi M, Khanahmad H 2014. A miRNA-binding site single nucleotide polymorphism in the 3'-UTR region of the NOD2 gene is associated with colorectal cancer. *Med Oncol,* 31: 173.

Al Nabhani Z, Dietrich G, Hugot JP, Barreau F 2017. Nod2: The intestinal gate keeper. *PLoS Pathog,* 13: e1006177.

Apostolou A, Kerenidi T, Michopoulos A, Gourgoulianis KI, Noutsias M et al. 2016. *Association Between TLR2/TLR4 Gene Polymorphisms and COPD Phenotype in a Greek Cohort*. Herz, (in press). Berlin/Germany: Springer Medizin Verlag Berlin

Becker C, Neurath MF, Wirtz S 2015. The intestinal microbiota in Inflammatory Bowel Disease. *ILAR J*, 56: 192-204.

Bosse Y 2012. Updates on the COPD gene list. *Int J Chron Obstruct Pulmon Dis,* 7: 607-631.

Chaleshi V, Tajali R, Savabkar S, Zali N, Mojarad EN et al. 2016. Lack of association between NOD2 rs3135500 and IL12B rs1368439 microRNA binding site SNPs and Colorectal Cancer susceptibility in an Iranian population. *Microrna*, 5: 152-156.

Chaput C, Sander LE, Suttorp N, Opitz B 2013. NOD-like receptors in lung diseases. *Front Immunol,* 4: 393.

Cho MH, Castaldi PJ, Wan ES, Siedlinski M, Hersh CP et al. 2012. A genome-wide association study of COPD identifies a susceptibility locus on chromosome 19q13. *Hum Mol Genet,* 21: 947-957.

Cho MH, McDonald ML, Zhou X, Mattheisen M, Castaldi PJ et al. 2014. Risk loci for chronic obstructive pulmonary disease: A genome-wide association study and meta-analysis. *The Lancet Respiratory Medicine,* 2: 214-225.

Correa RG, Milutinovic S, Reed JC 2012. Roles of NOD1 (NLRC1) and NOD2 (NLRC2) in innate immunity and inflammatory diseases. *Biosci Rep,* 32: 597-608.

Di Stefano A, Ricciardolo FLM, Caramori G, Adcock IM, Chung KF et al. 2017. Bronchial inflammation and bacterial load in stable COPD is associated with TLR4 overexpression. *Eur Respir J,* 49: 1602006.

Fan VS, Gharib SA, Martin TR, Wurfel MM 2016. COPD disease severity and innate immune response to pathogen-associated molecular patterns. *Int J Chron Obstruct Pulmon Dis,* 11: 467-477.

Franca R, Vieira SM, Talbot J, Peres RS, Pinto LG et al. 2015. Expression and activity of NOD1 and NOD2/RIPK2 signalling in mononuclear cells from pa-

tients with rheumatoid arthritis. *Scand J Rheumatol,* 23: 1-5.

GOLD 2017. Global Strategy for the Diagnosis, Management and Prevention of COPD, Updated 2017. Global Initiative for Chronic Obstructive Lung Disease (GOLD).

Global Initiative for Chronic Obstructive Lung Disease (GOLD) 2017. Global Strategy for the Diagnosis, Management and Prevention of Chronic Obstructive Pulmonary disease (2017 Report). From < http://goldcopd.org.>

Hamzaoui K, Abid H, Berraies A, Ammar J, Hamzaoui A 2012. NOD2 is highly expressed in Behçet disease with pulmonary manifestations. *J Inflamm* (Lond), 9: 3.

Heresbach D, Gicquel-Douabin V, Birebent B, D'halluin PN, Heresbach-Le Berre N et al. 2004. NOD2/CARD15 gene polymorphisms in Crohn's disease. *Eur J Gastroenterol Hepatol,* 16: 55-62.

Hu S, You X, Cao P, Liu Z, Cui Y 2013. The expression of NOD1 and NOD2 and the regulation of glucocorticoids on them in allergic rhinitis. *Lin Chung Er Bi Yan Hou Tou Jing Wai Ke Za Zhi,* 27: 393-396.

Ince AT, Hatirnaz O, Ovünç O, Ozbek U 2007. 1007fs, G908R, R702W mutations and P268S, IVS8+158 polymorphisms of the CARD15 gene in Turkish inflammatory bowel disease patients and their relationship with disease-related surgery. *Dig Dis Sci,* 53: 1683-1692.

Ishii T, Hosoki K, Nikura Y, Yamashita N, Nagase T et al. 2017. IFN Regulatory Factor 3 potentiates Emphysematous aggravation by Lipopolysaccharide. *J Immunol,* 198: 3637-3649.

Jaskula E, Lange A, Kyrcz-Krzemien S, Markiewicz M, Dzierzak-Mietla M et al. 2014. NOD2/CARD15 single nucleotide polymorphism 13 (3020insC) is associated with risk of sepsis and single nucleotide polymorphism 8 (2104C>T) with herpes viruses reactivation in patients after allogeneic hematopoietic stem cell transplantation. *Biol Blood Marrow Transplant,* 20: 409-414.

Kinose D, Ogawa E, Hirota T, Ito I, Kudo M et al. 2012. A NOD2 gene polymorphism is associated with the prevalence and severity of chronic obstructive pulmonary disease in a Japanese population. *Respirology,* 17: 164-171.

Kinose D, Ogawa E, Kudo M, Marumo S, Kiyokawa H et al. 2016. Association of COPD exacerbation frequency with gene expression of pattern recognition receptors in inflammatory cells in induced sputum. *Clin Respir J,* 10: 11-21.

Leissinger M, Kulkarni R, Zemans RL, Downey GP, Jeyaseelan S 2014. Investigating the role of nucleotide-binding oligomerization domain-like receptors in bacterial lung infection. *Am J Respir Crit Care Med,* 189: 1461-1468.

Liu T, Wang FP, Wang G, Mao H 2017. Role of Neutrophil Extracellular traps in Asthma and Chronic Obstructive Pulmonary Disease. *Chin Med J (Engl),* 130: 730-736.

McCloskey SC, Patel BD, Hinchliffe SJ, Reid ED, Wareham NJ et al. 2001. Siblings of patients with severe chronic obstructive pulmonary disease have a significant risk of airflow obstruction. *Am J Respir Crit Care Med,* 164: 1419-1424.

Molfino N, Jeffery P 2007. Chronic obstructive pulmonary disease: Histopathology, inflammation and potential therapies. *Pulmonary Pharmacology & Therapeutics,* 20: 462-472.

Nakamura H 2011. Genetics of COPD. *Allergol Int,* 60: 253-258.

Ni G, Chen Y, Wu F, Zhu P, Song L 2017. NOD2 promotes cell proliferation and inflammatory response by mediating expression of TSLP in human airway smooth muscle cells. *Cell Immunol,* 312: 35-41.

Omrane I, Mezlini A, Baroudi O, Stambouli N, Bougatef K et al. 2014. 3020insC NOD2/CARD15 polymorphism associated with treatment of colorectal cancer. *Medical Oncology,* 31: 954.

Ozen SC, Dagli U, Kiliç MY, Törüner M, Celik Y et al. 2006. NOD2/CARD15, NOD1/CARD4, and ICAM-1 gene polymorphisms in Turkish patients with inflammatory bowel disease. *J Gastroenterol,* 41: 304-310.

Pabst S, Bradler O, Gillissen A, Nickenig G, Skowasch D et al. 2013. Toll-like receptor 9 polymorphisms in sarcoidosis and chronic obstructive pulmonary disease. *Adv Exp Med Biol,* 756: 239-245.

Philpott DJ, Sorbara MT, Robertson SJ, Croitoru K, Girardin SE 2014. NOD proteins: Regulators of inflammation in health and disease. *Nat Rev Immunol,* 14: 9-23.

Pillai SG, Ge D, Zhu G, Kong X, Shianna KV et al. 2009. A genome-wide association study in chronic obstructive pulmonary disease (COPD): Identification of two major susceptibility loci. *PLoS Genet,* 5: e1000421.

Pinkerton JW, Kim RY, Robertson AA, Hirota JA, Wood LG et al. 2017. Inflammasomes in the lung. *Mol Immunol,* 86: 44-55.

Qiao D, Lange C, Beaty TH, Crapo JD, Barnes KC et al. 2016. Exome sequencing analysis in severe, early-onset chronic Obstructive Pulmonary Disease. *Am J Respir Crit Care Med,* 193: 1353-1363.

Rovina N, Koutsoukou A, Koulouris N 2013. Inflammation and immune response in COPD: Where do we stand? *Mediators of Inflammation,* 2013: 413735.

Sayan M, Mossman BT 2016. The NLRP3 inflammasome in pathogenic particle and fibre-associated lung inflammation and diseases. *Part Fibre Toxicol,* 13: 51.

Serbati N, Badre W, Diakite B, Nadifi S 2014. NOD2/CARD15 gene influences disease behaviour but not IBD susceptibility in a Moroccan population. *Turk J Gastroenterol,* 25: 122-128.

Tan DBA, Armitage J, Teo TH, Ong NE, Shin H et al. 2017. Elevated levels of circulating exosome in COPD patients are associated with systemic inflammation. *Respir Med,* (in press).

Tekin D, Dalgic N, Kayaalti Z, Soylemezoglu T, Diler B et al. 2012. Importance of NOD2/CARD15 gene variants for susceptibility to and outcome of sepsis in Turkish children. *Pediatric Critical Care Medicine,* 13: e73-e77.

Uyar FA, Over-Hamzaoðlu H, Türe F, Gül A, Tözün N et al. 2006. Distribution of common CARD15 variants in patients with sporadic Crohn's disease: Cases from Turkey. *Dig Dis Sci,* 51: 706-710.

Vijayan VK 2013. Chronic obstructive pulmonary disease. *Indian J Med Res,* 137: 251-269.

Wang W, Zhang Y, Yang L, Li H 2017. The innate immune signaling in cancer and cardiometabolic diseases: Friends or foes? *Cancer Lett,* 387: 46-60.

Weidinger S, Klopp N, Rümmler L, Wagenpfeil S, Baurecht HJ et al. 2005. Association of CARD15 polymorphisms with atopy-related traits in a population-based cohort of Caucasian adults. *Clin Exp Allergy,* 35: 866-872.

Wilson P, Larminie C, Smith R 2016. Literature mining, gene-set enrichment and pathway analysis for target identification in Behçet's disease. *Clin Exp Rheumatol,* 34: 101-110.

Yao Q 2013. Nucleotide-binding oligomerization domain containing 2: Structure, function, and diseases. *Seminars in Arthritis and Rheumatism,* 43: 125-130.

Yao Q, Shen B 2017. A systematic analysis of treatment and outcomes of NOD2-associated autoinflammatory disease. *Am J Med,* 130: 365.e13-365.e18.

Zhao X, Gu C, Yan C, Zhang X, Li Y et al. 2016. NALP3-inflammasome-related gene polymorphisms in patients with Prehypertension and Coronary Atherosclerosis. *Biomed Res Int,* 2016: 7395627.

Zhu H, Cao X 2017. NLR members in inflammation-associated carcinogenesis. *Cell Mol Immunol,* 14: 403-405.

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