VDBP Gene Polymorphism in COPD

Sadan Soyyigit[1], Onur Baykara[2], Nur Buyru[2], Muzeyyen Erk[1]

[1] Istanbul University, Cerrahpasa Medical Faculty, Department of Chest Diseases, Istanbul, Turkey

[2] Istanbul University, Cerrahpasa Medical Faculty, Department of Medical Biology, Istanbul, Turkey

Abstract
Chronic obstructive pulmonary disease (COPD) is a disease which genetic and environmental factors play an important role in COPD development. VDBP (Vitamin D Binding Protein) gene might be responsible for COPD development. The purpose of this study to investigate the possible effect of VDBP on development of COPD.
We studied VDBP genotypes in 75 COPD patients, 36 smoker healthy subjects and 19 non-smoker healthy subjects with PCR-RFLP method to analyze the association between VDBP and COPD. In our study, the most frequently seen genotype was 1S-2 with a frequency of 32% in COPD group; and 1F-1S genotype with a frequency of 33.3% in smoker healthy group. There was no significant difference between these genotypes. In our study groups, we did not find any 2-2 genotype in non-smoker healthy subject group while it has been found 4% and 8% in COPD and healthy smoker subject groups, respectively. Although we did not find any significant difference for protective effect of the 2 allele on the disease or susceptibility of 1F allele to COPD formation in COPD patients and smoker healthy subjects (p: 0.346, p: 0.249, respectively), the only significant difference was between the patient and the healthy non-smoker groups with 1S-1S genotype (p=0.032). Our results do not show any evidence that indicate a protective or susceptibility effect of VDBP 2 and 1F alleles in development of COPD, respectively but results indicate that having the 1S-1S genotype may be associated with the etiology of COPD.

Key Words*:* COPD, VDBP, genetic polymorphism, allele frequency

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Corresponding Author: Nur BUYRU, Department of Medical Biology Cerrahpasa Medical Faculty, Istanbul University
E-mail: nbuyru@yahoo.com Phone: +90-212-414 30 00 ext. 22656

Introduction

Chronic obstructive pulmonary disease (COPD) is a disease of increasing public health importance around the world. COPD is charactarized by chronic airflow limitation and a range of pathological changes in the lung. The airflow limitation is usually progressive and associated with an abnormal inflammatory response of the lung to noxious particles or gases. Although the most critical factor of acquiring COPD is smoking [1], only 15 to 20% of chronic smokers get this disease [2, 3]. This indicates the presence of additional genetic or environmental factors which contribute to the development of COPD. Environmental factors that have been associated with COPD include viral infections, especially during childhood, air pollution and most commonly smoking. The genetic risk factor that is best documented is a severe hereditery deficiency of alfa-1 antitrypsin [4].

A hereditary basis for COPD was first suggested in 1845 [5], but this observation remained essentially uninvestigated until the mid-twentieth century. At that time, several investigators have shown increased prevalence of COPD in relatives of cases, compared to relatives the prevalence of COPD in controls [6-8]. Studies on twins also suggest a genetic basis in the development of COPD [9-11].

Among the candidate genes that have been studied in COPD are genes that effect the production of proteases and antiproteases, genes that modulate the metabolism of toxic substances in cigarette smoke, genes involved in mucociliary clearance and airway hyperactivity and genes that influence inflammatory mediators [12-13].

Genes that are involved in inflammatory processes include Tumor Necrosis Factor-α, immunoglobulins, and Vitamin D Binding Protein (VDBP) [12-13].

VDBP is a 55kDa protein secreted by the liver which is able to bind Vitamin $D_{1,}$ extracellular actin and endotoxin. VDBP is also known to undergo conversion to a potent macrophage-activating factor [14]. Thus, VDBP could exert an important effect on the intensity of the inflammatory reaction. The gene for VDBP is located on chromosome 4q11-q13 [15] and two common substitutions in exon 11 result in three possible isoforms, termed 1F, 1S and 2 [16]. Recent data indicate that the allele frequencies vary considerably among populations.

Frequencies of the 1F, 1S and 2 alleles are estimated as 0.19, 0.53 and 0.28, in the Caucasians [17] and 0.48, 0.27 and 0.24 in the Japanase population, respectively [18].

There are some studies in the literature investigating the association of VDBP polymorphisms with the development of COPD; however, contradictory results have been reported for COPD patients of different ethnicities or even within the same ethnic group [8, 17-21]. Individuals who have one or two copies of allele 2 have been shown to be protected against to COPD [17, 19, 20], whereas the 1F-1F genotype has been associated with increased risk [17-21].

In this study, we investigated the allele frequencies in the Turkish population and the association of VDBP gene polymorphism with the development of COPD in Turkish patients according to the smoking status.

Materials And Methods

Blood samples were collected from 75 patients (66 males and 9 females) with COPD (all smokers COPD group), 36 healthy smokers (C group) and 19 never-smokers (H group).

The confirmative diagnosis of COPD was based on the subject's medical history, physical examination and pulmonary function tests, using GOLD guidelines [22]. Pulmonary function tests were performed to determine FVC, FEV_1, FEV_1/FVC, TLC and DLCO. Patients with FEV_1 values $\geq$ 80% were excluded from this study. All patients had moderate-to-severe COPD according to the Global Initiative for Chronic Obstructive Lung Disease classification of severity [22].

Criteria for involving the study were as followed; smoking more than 10 packages of cigarette per year, postbronchiodilator values for FEV_1 < 80% and FEV_1/FVC < 70%,increase in FEV_1 is less than %12 and 200 mL according to basal value in reversibility test. COPD patients were classified according to the stage of the disease as moderate (50%≤FEV < 80%), severe (30%≤FEV_1<50%), very severe (<30%) [22]. Mild group is not involved in the study (FEV_1≥80%). Severe and very severe groups have been calculated together for statistical evaluation.

Healthy smoker group consists of the people who have applied to Cerrahpasa Medical Faculty Chest Diseases Department, Section of Quitting Smoking who smoke 10 or more packages of cigarette per year. Subjects were considered healthy smokers if they did not have COPD or other diseases, and their pulmonary function tests showed a FEV_1/FVC ratio > 70%.

Non-smoker group consists of the voluntary people who work in our hospital, with a FEV_1/FVC >70% ratio who do not have any lung disease such as COPD or asthma or any systemic disease and who are not exposed to passive smoking.

Genomic DNA for molecular analysis was isolated from peripheral blood samples by Proteinase K digestion and a salting-out with ammonium acetate. Polymerase chain reaction (PCR) to amplify a segment of the VDBP gene including the polymorphic site was performed with 10 pmol forward – 5' TAA TGA GCA AAT GAA AGA AG- 3' and reverse – 5'-AAT CAC AGT AAA GAG GAG GT- 3' primers in a mixture (25 μl) containing 0.2 mM dNTP, 10 mM Tris-HCl (pH:8.8), 50 mM KCl, 0.08 % Nonidet p40, 2.5 mM $MgCl_2$, 1,25 U Taq polymerase (MBI, Fermentas, Lithuania) and approximately 300 ng genomic DNA. The mixture was heated to 94^{o}C for 10 min and then subjected to 30 cycles of 94°C for 30 s, 56°C for 30 s and 72°C for 30 s. The final extension was carried out for 5 min at 72^{o}C.

VDBP genotypes were investigated by-PCR based digestion analysis with restriction enzymes *Hae III* and *Sty I* (Fermentas, Lithuania) at 37°C overnight. The PCR products were digested seperately. Digestion products were electrophoresed on a 2 % agarose gel in 0.5xTBE buffer at 120 V for 3 h. The genotypes were determined under UV illumination using a video gel documentation system (Vilber-Lourmat, Cedex, France) after staining with ethidium bromide. The size of the PCR product was 388 bp. Digestion by Hae III cuts the 1S allele at codon 416 into two bands of 295 bp and 93 bp, whereas Sty I digestion creates two bands of 304 bp and 84 bp in the presence of the C→A substitution at codon 480 for allele 2. Therefore, the PCR product from 1F homozygotes remains uncut by either of the enzymes (Figure 1).

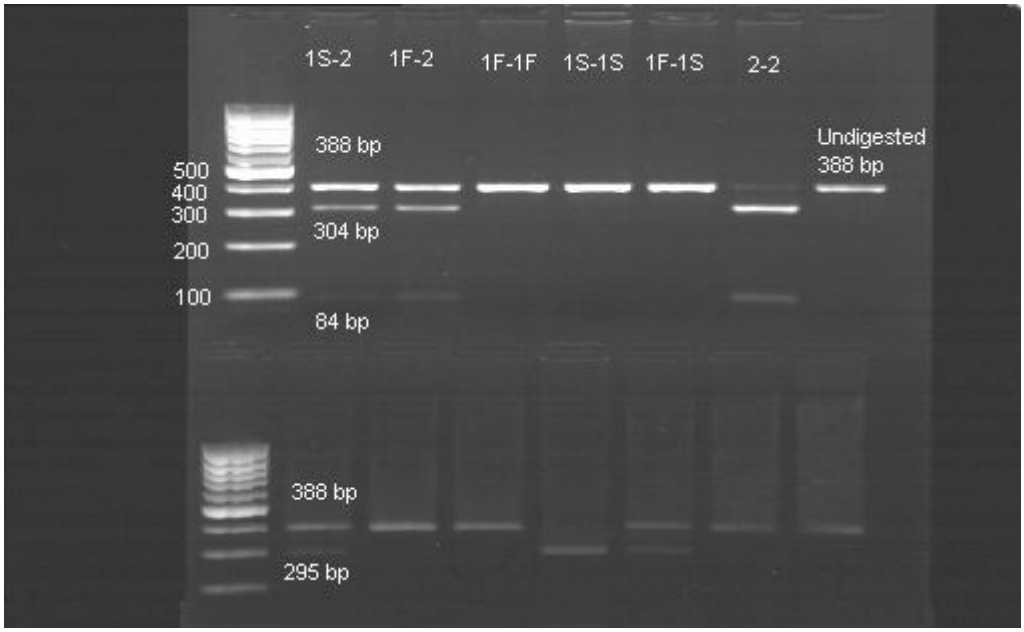

Figure 1: Visualization of samples digested with Sty I (upper lane) and Hae III (lower lane) on agarose gel electrophoresis. The size of the PCR product is 388 bp. Digestion by Hae III cuts the 1S allele into two bands of 295 bp and 93 bp, whereas Sty I digestion of allele 2 creates two bands of 304 bp and 84. Therefore, the PCR product from 1F homozygotes remains uncut by either of the enzymes.

The differences in allele distribution and allele frequencies among groups were evaluated by the Chi-squared and Fisher's exact tests. p values < 0.05 were considered to be statistically significant.

An explanatory statement about the study and the procedures have been made to all of the patients regulated through the ethical statements prepared by The Ethical Committee of Istanbul University Cerrahpasa Medical Faculty. The Committee has approved the study and the written informed consent has been obtained from all of the patients.

Results

A total of 130 subjects, including 75 COPD patients, 36 healthy smokers and 19 nonmokers as control subjects were studied. The mean age of the COPD patients was 64,4±11,0, male-female ratio was 66/9, number of packages smoked per year was 51,4±31,1 and the

FEV_1/FVC ratio was 50,8±11,3. The clinical characteristics of the COPD patients are shown in Table 1.

Table 1: Clinical characteristics of the COPD patients.

	Mean ± SD
Age	64,4±11,0
Sex (M/F)	66/9
Cigarette (package/year)	51,4±31,1
FVC (%)	76,4±18,2
FEV_1 (%)	49,0±17,9
FEV_1/FVC (%)	50,8±11,3
TLC (%)	98,8±18,7
RV/TLC (%)	51,4±10,7
DLCOAdj (%)	65,1±19,6
DLCO/VA (%)	78,0±28,0

Among whole patients, 40 of them were classified as moderate, 21 as severe and 14 as very severe.

In healthy smoker and non-smoker control groups the mean age was 50,5±35,2 and 35,2±9,5, respectively. Male/female ratio was 18/18 and 10/9, respectively. Number of packages smoked per year was 36,9±29,2 in healthy smoker controls.

The distribution of the six major genotypes in the patients and the control groups are as follows: 1S-1S genotype in patient group was 22.7%, whereas 22.3% in healthy smoker controls and 47.4% in healthy non-smoker controls. The results for 1F-1S was 22.7%, 33.3% and 15.8%, respectively. For 1F-1F; the results were 5.3%, 11.1% and 5.2%, respectively. 32%, 19.4% and 31.6%, respectively were the distribution ratios for 1S-2 group. In 1F-2 group, we found 13.3% and 5.6% while there was no result for healthy non-smoker group. Similar to this situation, in 2-2 group there was no result for for healthy non-smoker group whereas 4% for patient group and 8.3% for healthy smoker group were the ratios. First, we compared the patient vs. healthy smoker controls to find the statistical differences where $p<0.05$ was meant to be statistically significant. In the latter analysis, we compared the patient group vs. healthy non-smoker controls group to see the differences in two groups. The frequencies are not significantly different between the patient and control groups except one group, which is the 1S-1S genotype with a significant difference between the patient and the healthy non smoking group with a p value of 0.032. This may suggest that, having 1S-1S

genotype may have an important role in COPD. The distribution of the six major genotypes are shown in Table 2.

Table 2: Distribution of the six major genotypes in patient and control groups. The frequencies are not significantly different between the COPD patient and control groups except the 1S-1S patient and healthy non smoker group.

GENOTYPE	PATIENT GROUP n=75 (%)	HEALTHY SMOKER n=36 (%)	p	HEALTHY NON-SMOKER n= 19 (%)	p
1S-1S	17 (22.7)	8 (22.3)	0.958	9 (47.4)	0.032
1F-1S	17 (22.7)	12 (33.3)	0.231	3 (15.8)	0.755
1F-1F	4 (5.3)	4 (11.1)	0.434	1 (5.2)	1.000
1S-2	24 (32.0)	7 (19.4)	0.168	6 (31.6)	0.972
1F-2	10 (13.3)	2 (5.6)	0.331	0	0.204
2-2	3 (4.0)	3 (8.3)	0.387	0	1.000

The frequencies of the three alleles in COPD patients were also compared with the frequencies in the healthy smoking and non-smoking control groups. In the COPD patients, the frequencies of the 1S, 1F and 2 alleles were 0.5, 0.23 and 0.27, respectively. In the healthy

smoker and nonsmoker groups the frequencies were 0.49, 0.30 and 0.21 and 0.71, 0.13 and 0.16, respectively. The results are presented in Table 3.

Table 3: Allele frequencies of the genotypes in all groups. Allele 1S with a frequency of 0.020 between the COPD patient and healthy non-smoker group may be effective on disease development.

1S	75 (50)	35 (48.6)	0.846	27 (71)	0.020
1F	35 (23.3)	22 (30.5)	0.249	5 (13.2)	0.171
2	40 (26.7)	15 (20.9)	0.346	6 (15.8)	0.240

Our study revealed a significant difference in the prevalence of the allele 1S and 1S-1S genotype in the patients and nonsmoker groups. We did not find any significant differences among the patients and the control groups who had 1F or 2 alleles and correlating genotypes

(Table 2,3). The genotype frequencies were also compared between COPD stages. Moderate COPD patients were compared with severe and very severe COPD patients in one combined group to have a statistical validation. As shown in Table 4, we did not find any association between the COPD stages and VDBP genotype or allele frequencies. The differences of the genotype frequency between these three groups were not statistically significant.

Table 4: Genotype frequencies according to the stages of patients. Severe and very severe COPD patients were calculated together for a valid statistical calculation.

Genotype	Moderate COPD n=40	Severe and very severe COPD n=35	p=
1S-1S	11	6	0.408
1F-1S	9	8	0.334
1F-1F	1	3	1.000
1S-2	12	12	0.742
1F-2	6	4	0.805
2-2	1	2	0.591

Discussion

Variability in the susceptibility to develop COPD is related to both genetic and environmental factors. Cigarette smoking is the major risk factor for COPD [1, 23]. However, it is estimated that only 15-20% of chronic heavy smokers develop symptomatic COPD [2, 3, 24, 25]. Numerous epidemiological studies also provide compelling evidence that genetic factors influence the development of COPD [26]. Current theories suggest that inflammation and oxidative stress induced by smoking may lead to a proteolytic imbalance and progressive lung structural derangement. Therefore disease susceptibility is controlled by inherited variations in genes involved in antiproteolysis, metabolism of toxic substances in the cigarette smoke and the efficiency of mucociliary clearance in the lung. A variety of studies have examined candidate gene loci with association studies, comparing the distribution of variants in genes hypothesized to be involved in the development of COPD in patients and control subjects [27].

One important candidate gene that is involved in the inflammatory process is VDBP. Numerous isoforms of VDBP have been identified by isoelectric focusing. Two common

substitutions in exon 11 of the gene result in three possible isoforms termed 1F, 1S and 2 [16]. It has been reported that individuals homozygous for allele 2 are significantly underrepresented among COPD patients, illustrating a protective role for allele 2 in COPD [17]. It has also been shown that the genotypes which contain allele 2 (1F-2, 1S-2 and 2-2) had a protective effect [19, 20]. However, this observation has not been confirmed by a subsequent study [18]. In accordance with this study, we did not find an association between the genotype containing allele 2 and susceptibility to COPD. In our study the frequency of allele 2 in the control group is slightly lower than reported by Schellenberg et al. [17] and Ishii et al. [18] for the Caucasian and Japanase population, respectively. The incidence of allele 2 carrying genotypes and allele 2 frequencies were similar in both COPD patients and the control groups. In addition, Horne et al. [19] have shown a significantly increased risk for COPD in 1F homozygous individuals and Ishii et al. [18] confirmed this result in the Japanese population. Our finding is discordant with these results since we did not observe a difference in the frequency of VDBP genotypes between the patients and controls except 1S-1S genotype.

As a result, we have not found any evidence that differences in the VDBP gene 1F and 2 alleles could confer a genetic predisposition to COPD except the allele 1S. 1S allele and 1S-1S genotype were significantly more frequent in non-smoker controls. These results indicate that 1 S allele in homozygous state may be protective against to COPD in smoking people. Therefore, 1S allele may have a role in detoxification of substances which is found in smoke. This may be related to the posttranslational modification of VDBP. It has been shown that macrophage activating factor is formed from VDBP by modification of an oligosaccharide side chain [14]. It has also been reported that isoforms of VDBP have different deglycosylation pathways [14]. Therefore, in addition to the possibility that VDBP gene polymorphism modulates neutrophil and monocyte chemotaxis, the difference in glycosylated forms of 1F, 1S and 2 isoforms may also effect activation of macrophages. Recently, Hautamaki et al [27] reported that its metalloproteinase plays a key role in the pathogenesis of emphysema. This report supports the hypothesis that the glycosylation of VDBP may have a role in susceptibity to COPD.

In conclusion, our results do not support previous studies which indicate a protective or susceptibility effect of VDBP 2 and 1F alleles in development of COPD, respectively; as we found a complete new result which is, having 1S-1S genotype may have protective effect on

development of COPD in non-smokers whilst having the allele 1S together with other alleles may not be enough for development of the disease.

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