Association of Vitamin D Binding protein gene polymorphism with serum 25-hydroxy Vitamin D levels in COPD

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Abstract

Background: Vitamin D deficiency is associated with several diseases including Chronic Obstructive Pulmonary Disease (COPD).Vitamin D binding protein (DBP) is the major carrier for vitamin D in plasma. Objectives To determine whether vitamin D binding protein gene (GC) polymorphism is associated with COPD and its severity and to correlate the GC genotypes with Serum 25-hydroxy vitamin D (25-OHD) levels. Study Design Fifty COPD patients and fifty healthy controls sampled from north Indian population were genotyped for GC polymorphism(rs7041 & rs4588) by Restriction fragment length polymorphism method and genotypes were correlated with disease severity and serum 25-OHD levels. Results GC 1F allele frequency was increased significantly in COPD patients compared to healthy controls (30% versus 15%,chi square=6.76,p value=0.03).GC2 allele frequency was lower in COPD patients than controls (29% versus 39%).GC genotype 1F- 1F was found to contribute to the risk of developing COPD (OR=5.6, 95% CI=0.91-34.57) and GC 2 was found to be a protective allele in COPD patients (OR=0.4, 95% CI=0.10-2.16). Median serum 25-OHD levels (ng/ml) in COPD patients was significantly low compared to healthy controls (13.65 versus 23.45, p value-0.045). III & IV GOLD class staging (Global Initiative for Obstructive Lung Disease) COPD patients exhibited deficient median Serum 25-OHD levels (<20ng/ml).The geometric means of 25-OHD were compared among genotypes, being lowest in GC 1F-1F individuals. Conclusion Vitamin D deficiency is more common among COPD patients. GC polymorphism is significantly associated with susceptibility to COPD and serum 25-OHD levels. This data suggests adequate Vitamin D supplementation in COPD patients at risk.

KEYWORDS: DBP, GC, GOLD, COPD

Abbreviations: GC: Globulin gene, DBP-Vitamin D Binding protein, GOLD: Global Initiative for Obstructive Lung Disease, COPD :Chronic obstructive pulmonary disease

INTRODUCTION

Chronic obstructive pulmonary disease is an important cause of mortality and morbidity worldwide. WHO estimates, COPD will be the 5th most prevalent disease and 3rd most common cause of death worldwide by 2020[1]. Prevalence of COPD among Indians on average is about 5%. COPD is defined as an inflammatory respiratory disease and is

characterized by a progressive and incompletely reversible airflow obstruction. Although the major risk factor for COPD is tobacco smoking, only approximately 15% of smokers develop symptomatic airflow obstruction.[2] This variation in response to cigarette smoke in combination with familial aggregation of chronic obstructive respiratory diseasesuggests that there is role of genetic component in COPD development.[3-8]Numerous genes has been identified as risk factors for COPD.[9,10]

VDBP gene polymorphism

One gene that has been implicated in COPD is Gc-globulin (GC) gene, also known as vitamin D binding protein gene(VDBP).[11]GC is located on chromosome 4q11-q13, approximately 42kb in size,[12] part of a gene cluster that includes albumin and α – fetoprotein genes and mainly expressed in liver.[13]GC is highly polymorphic with three common Gc alleles (haplotypes), Gc1F, Gc1S, Gc2, by their respective single nucleotide polymorphism in rs7041 (Asp416Glu, T/G) and rs4588 (Thr420Lys, C/A) as follows: Gc1F, T and C; Gc1S, G and C; and Gc2, T and A, at single-nucleotide Polymorphism rs7041 and rs4588 respectively. The corresponding combined genotypes are GC 1S-1S, 1S-1F, 1F-1F, 2-1S, 2-1F, and 2-2.[14]Post-translational carbohydrate modifications leads to different Gc-globulin genotypes.[15] Thesepolymorphisms affect protein function.GC2 carriersexhibited reduced macrophage function because of least ability ofGC2 to be converted to macrophage-activating factor. COPD includes airway inflammation and the role of GC in macrophage activation and neutrophil chemotaxis led to studies of genetic associations of GC inCOPD.[16]

Vitamin D is mainly transported by DBP, a multifunctional plasma protein, which binds several diverse ligands.[17] Different GC genotypes bind vitamin D with different affinities, which may have an impact on serum 25-OHD levels. Serum levels of both 25-OHD and 1,25-OHD vary according to GC genotype, even within racial groups.[18]
In the process of understanding the complex pathogenesis of COPD, many new molecular pathways and therapeutic targets have been identified. The role of the vitamin D in lung disease including chronic obstructive pulmonary disease (COPD) has been studied.[19]Vitamin D deficiency is highly prevalent in chronic illnesses including cancers, cardiovascular disease and autoimmune disorders.[20,21]So we assessed the combined effects of two polymorphisms coding 416th and 420th amino acid in the Gc gene (rs4588 and rs7041), composing the phenotypic alleles Gc1S, Gc1F, and Gc2 on chronic obstructive pulmonary disease risk and compared it with serum 25(OH)D status in North Indian population.

MATERIALS AND METHODS

Present study was approved by Institutional ethical committee of Maulana Azad medical college & associated hospitals, New Delhi. Peripheral blood samples from 50 symptomatic COPD patients during their routine follow-up at the outpatient clinic and 50 gender-age matched healthy controls were collected. Patients who had exacerbation in past 4 weeks before sampling, those diagnosed as asthma or other respiratory diseases affecting pulmonary function and those on Vitamin D supplementation were excluded. From all consenting patients, demographic variables, pulmonary function assessment, occupational and smoking history were collected.

25-OHD serum levels

Serum 25-OHD levels were estimated by Electrochemiluminescence assay (cobas e 601 analyzer, roche diagnostics, ELECSYS 2010), taken normal Vitamin D reference range as 20-80 ng/ml.

Genotype analysis

A venous blood sample from each participant was drawn into an EDTA vial and genomic DNA was extracted by following manufacturer's protocol of DNA sure blood mini kit (Nucleo-pore Genetix brand). PCR was performed in a final volume of 50uL containing 50 µg genomic DNA, 5 µL of 10X polymerase buffer (200mMTris-HCl, pH 8.0; 500mMKCl), 1.5mMMgCl, 10mMdNTPs, 1.5 U of Taq polymerase (Genei, Bangalore) and 25 pmol/L of forward primer (5'TAATGAGCAAATGAAAGA AG3')[11]and a downstream primer downstream primer (5'ACC TCC TCT TTA CTG TGA TT3').[22] The primers produce an amplified region of 388 base pairs (bp). PCR program was started with an initial denaturation at 95°C for 5 minutes, followed by 40 cycles of denaturation at 95°C for 30 s, annealing at 54°C for 45 s, extension at 72°C for 45 s, and completed with a final elongation step at 72°C for 5 minutes. The expected 388 bp product was then digested by the fast digest restriction enzymes Hae III and Sty I (Fermentas) at 37°C for 15min. The variants were assessed as follows .The HaeIII enzyme produces cut bands of 295 and93 bp if the 1S allele is present. The Sty I enzyme cuts the Gc2 alleleproducing bands of 304 and 84 bp. The 1F variant remains uncut inboth enzyme digests. The digested DNA fragments were then resolvedon a 2.0% agarose gel stained with ethidium bromide.**(Figure 1)**

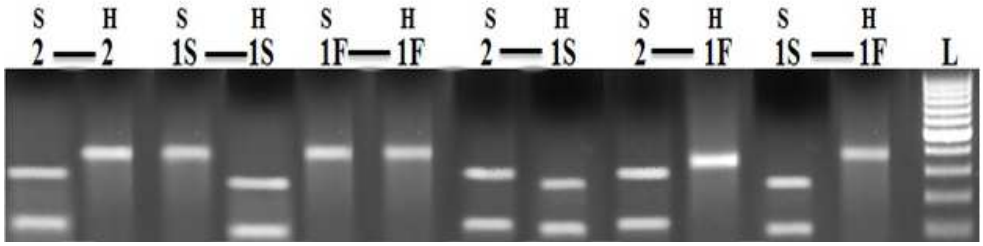


Figure 1: Restriction fragment length polymorphism analysis of six genotypes of Gc-globulin.

H=cut by HaeIII restriction enzyme. S=cut by Sty I restriction enzyme. A cut 295 base pair (bp) lower band in lane H of the sample indicates the presence of a Gc1S allele; whereas a noncut (388 bp) upper band may either be a Gc1F or Gc2 allele. A cut band (304 bp) in lane S indicates a Gc2 allele. uncut (388 bp) band, demonstrates the genotype 1F-1F.

Statistical Analysis

Statistical analysis was performed using SPSS 16.0 software. Assessment of the correlations between genetic carrier status and GC polymorphism was carried out using the Chi-Squared or Fisher Exact test. GC variants and risk of COPD were estimated by computing the odds ratios (ORs), risk ratio (RR) and risk difference (RD) with 95% confidence intervals (CIs) from multivariate logistic regression analysis. Kolmogorov-Smirnov test (KS-test) was used to calculate Mean±SD and allele frequencies among cases as well as controls were evaluated by using the Chi –square Hardy-Weinberg equilibrium test. ANOVA was used to compare geometric means of serum 25-OHD

values among GC genotypes. Kruskal Wallis test was applied to compare median 25 OHD levels in different stages of COPD. A p value < 0.05 was considered significant.

RESULTS

Case-Controls genotype distribution

As shown in Table 1, there were no significant differences in the distributions of sex and age between patients and controls, including 38 males and 12 females of age in <45 group (4%) and >45 group (96.0%) with Mean ±SD in cases of 55.86±7.42 (range, 40-70Years) and controls of 55.82±7.89(range,41-70 Years). Median Vitamin D levels between cases and controls (13.65 versus 23.45 ng/ml) were compared using Mann whitney U test (p value = 0.045).

Table 1.Baseline Characteristics of the Study Population.

VARIABLES	COPD PATIENTS (n=50)	HEALTHYCONTROLS (n=50)
Gender		
Males	38(76)	38(76)
Females	12(24)	12(24)
Age Group (Years)		
<45	2(4)	2(4)
≥45	48(96)	48(96)
Age(Mean±SD)	55.86±7.42	55.82±7.89
Smoking Status		
Non Smoker	12 (24)	
Smokers	38(76)	
Pack years(Mean±SD)	23.61±18.1	
GOLDClass Staging		
I	10(20)	
II	11(22)	
III	15(30)	
IV	14(28)	
Median25-OHD Level (ng/ml)	13.65(range,4–67.7)	23.45(range,4- 92)

Case-Controls genotype distribution

The allele frequencyof GC gene among COPD cases and controls were presented in **table 2**. The frequency of the three alleles (GC1F, GC1S and GC2) in COPD patients were 0.30, 0.41, 0.29 and in control group were 0.15, 0.46, 0.39 respectively. The observed allelic frequencies of GC (1F,1S and 2) were statistically different among patients and healthy controls (Chi square =6.76,df=2, p= 0.03). The proportion of GC1F allele frequency was significantly higher in COPD patients than in the control subjects (30 % versus 15%). The proportion of GC2 allele frequency was significantly higher in control groups than in COPD patients (39% versus 29%).

Table 2. Allelic frequency in COPD cases and controls

Allele	COPD cases n(%)	Controls n(%)	Chi square	df	P value
GC 1F	30 (30.0)	15 (15.0)	6.76	2	0.03
GC 1S	41 (41.0)	46 (46.0)			
GC 2	29 (29.0)	39 (39.0)			

Genotype and COPD risk

An unconditional logistic regression was used to estimate association between the genotypes and risk of COPD **(Table 3).** It was found that an increased risk of COPD was associated with the GC 1F allele in an allele dosage-dependent manner. Compared to the 1S-1S genotype, odds ratio, risk ratio and risk difference for the homozygous 1F-1F genotypes were [OR=5.6(0.91-34.57); RR=2.6(0.75-8.98); RD=40.2(4.4-75.9); p value=0.04], suggesting a possible dominant role of this polymorphism with 5.6fold increased risk. GC 2-2 genotype was found to be protective [OR=0.4(0.10-2.16); RR=0.8 (0.55-1.20); RD= -14.7(-42.8-13.3)] in COPD.

Table 3.Odds Ratio to assess association between various GC Genotypes in COPD

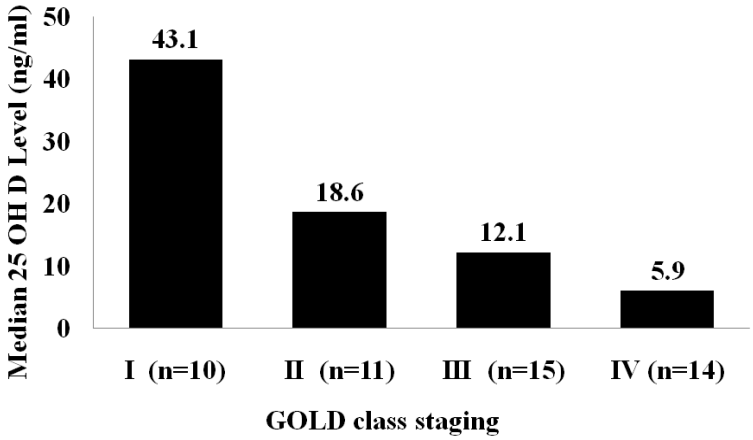
Genotypes	COPD cases n(%)	Controls n(%)	OR(95% CI)	RR(95% CI)	RD(95% CI)	P value
1S-1S	8 (16)	15 (30)	1	1		
1S-1F	10 (20)	6 (12)	3.1 (0.82-11.78)	1.7 (0.86-3.50)	27.7 (-2.9-58.4)	0.08
1F-1F	6 (12)	2 (4)	5.6 (0.91-34.57)	2.6 (0.75-8.98)	40.2 (4.4-75.9)	0.04
1S-2	15 (30)	10 (20)	2.8 (0.87-9.0)	1.6 (0.92-2.87)	25.2 (-2.1-52.6)	0.08
1F-2	8 (16)	5 (10)	3.0 (0.7-12.3)	1.7 (0.80-3.58)	26.8 (-6.1-59.6)	0.12
2-2	3 (6)	12 (24)	0.4 (0.10-2.16)	0.8 (0.55-1.20)	-14.7 (-42.8-13.3)	0.33

OR-Odds Ratio; RR-Risk Ratio; RD-Risk Difference

25-OHD serum levels in different GOLD class staging

Median values of 25-OHD were found to be decreasing as GOLD class staging increases with a significant p value as shown in **figure 2**.Notably, all patients with GOLD stage 3 and 4 exhibited 25- OHD levels <20 ng/ml.

Figure 2. Median values of 25 OH D level in different stages of COPD



25-OHD levels among various GC genotypes

Geometric means of serum 25-OHD among 1F-1F , 1S-1S, 1S-1F, 2-1F, 2-1S and 2-2 in COPD cases were 4.97, 22.21, 11.99, 9.25, 22.66, 59.83 ng/ml and in controls were 4, 25.47, 11.85, 9.44,18,81, 59.15 ng/ml respectively being lowest in 1F-1F individuals as shown in **Table.4**

Table 4 :25-OHD levels among various GC genotypes.

GC Genotypes	Geometric means of 25-OHD levels in COPD cases (ng/ml)	Geometric means of 25-OHD levels in controls(ng/ml)
1S-1S	22.21	25.47
1S-1F	11.99	11.85
1F-1F	4.97	4.25
1S-2	22.66	18.81
1F-2	9.25	9.44
2-2	59.83	59.15

Discussion

Several association studies between GC polymorphism and COPD showed conflicting results[23-25]. To our knowledge this is the first study in Delhi population to show the variation of serum 25 OHD levels in relation to GC polymorphism in COPD patients. In this hospital based case control study, genotypic frequencies of GC 1F-1F , 1S-1S, 1S-1F, 2-1F, 2-1S and 2-2 (%) determined among controls were 4, 30, 12, 10, 20 and 24 % respectively which is consistent with frequencies determined by Kamboh and colleagues in Delhi population.[26]Many previous studies confirmed that the frequency of the GC 1F allele was higher in the COPD group.[11,23-25]Ishii and colleagues showed in the Japanese population that the proportion of GC 1F homozygotes was significantly higher in patients with COPD than in healthy control subjects.[27] Horne and colleagues suggestedthat the homozygous GC 1F allele increased the risk of developing COPD[24], which is compatible

with our study results (OR=5.6). Association between GC 1F allele and airway obstruction showed variable results[11,23,25]. The GC1S variant has not been associated with COPD in any racial group.[28,29] The GC2 variant appears protective in Caucasians[29,30] and we also reported a protective effect of GC 2 allele for COPD (OR=0.4) in our population. Mechanism by which Gc2 offers protection from COPD might be that this isoform is less able to get converted to macrophage activating factor, thus causes decrease in inflammation. COPD is a chronic airway inflammatory disease in which lung damage occurs by the release of toxic free radicals and proteases by neutrophils and macrophages. Different Gc globulin isoforms could determine the variability in lung parenchymal damage. The conversion of Gc to GcMAF (macrophage activating factor) by β-galactosidase and sialidase is a deglycosylation process. The Gc-2 protein has no glycosylated Lys at residue 420 and is unable to be converted to MAF.[17,31] This could explain the possibility that in homozygous Gc-2 individuals, cigarette smoking may cause less pulmonary inflammation because of reduced MAF activity.[11] Different glycosylation patterns in the Gc alleles may exhibit different function. In our study, COPD patients belonging to GOLD class staging II-IV exhibited deficient serum 25-OHD levels (<20 ng/ml), depicting that Vitamin D deficiency is prevalent in chronic illnesses like COPD and it correlates with severity of COPD and it warrants the need for vitamin D supplementation in COPD patiemts.

Different GC genotypes have varying serum levels of both 25-OHD and 1,25-OHD.[18] Our data showed a significant decrease of 25-OHD levels in GC 1F homozygous individuals. Vitamin D has been recently studied for its extra calcemic effects and it regulates more than 200 genes including genes for cellular proliferation differentiation and apoptosis.[33]In COPD patients, TNF-α increases MMP-9 from alveolar macrophages, while IL-10 reduces the ratio of MMP-9 to the MMP inhibitor, tissue inhibitor of metalloproteinase (TIMP)-1. The equilibrium of MMP-9 and TIMP-1 is crucial in airway remodelling. Vitamin D can inhibit TNF-α and enhance IL-10 in immune cells, thus reducing exacerbation attacks in COPD[34]. Strengths of our study is the consideration of serum 25(OH)D status. We selected known functional variants with potential effect on vitamin D metabolism, however our findings on COPD risk need verification in further studies with more sample size.

In conclusion, this study showed a protective effect of GC 2-2 genotype in COPD. Noncalcemic effects of vitamin D is in rise, giving an insight into many new roles. Vitamin D deficiency is more prevalent in chronic illnesses. A potential benefit of vitamin D supplementation on noncalcemic view in patients with severe COPD, has not been explored. Randomized control trials with optimal dose of vitamin D supplementation should therefore be strongly encouraged. COPD is a suitable disease for such studies because it often integrates many comorbidities including repetitive infections, persistent inflammation, muscular dysfunction, all domains that vitamin D may affect.

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