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COPD

SERPINA1 Hepatocyte-Specific Promoter Polymorphism Associate with Chronic Obstructive Pulmonary Disease in a Study of Kashmiri Ancestry Individuals

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Abstract

Purpose Different mutations in coding and non-coding sequences of the *SERPINA1* gene have been implicated in the pathogenesis of COPD. However, − 10T/C mutation in the hepatocyte-directed promoter region has not been associated with COPD pathogenesis so far. Here, we report an increased frequency of − 10C genotype that is associated with decreased levels of serum alpha1-antitrypsin (α1AT) in COPD patients.

Methods The quantification of serum α1AT was done by ELISA, the phenol–chloroform method was used for DNA extraction, PCR products were directly sequenced. The *IBM SPSS Statistics v21* software was used for statistical analyses of the data.

Results The mean serum α1AT level was found to be 1.203+0.239 and 3.162+0.160 g/L in COPD cases and in control, respectively. The − 10C allele is associated with an increased risk of COPD [OR, 3.50 (95%CI, 1.86-6.58); $p < 0.001$]. The combined variant genotype (TT+CC) was significantly found associated with an increased risk of COPD [OR, 3.20 (95% CI, 1.47-6.96); $p = 0.003$]. A significant association of the family history with COPD (overall p value= 0.0331) suggests that genetics may play an important role in the pathogenesis of COPD.

Conclusion The polymorphism associated with hepatocyte-specific promoter region (− 10T/C) is likely to be associated with the pathogenesis of COPD. It is quite possible that the change of the base in the hepatocyte-specific promoter of the *SERPINA1* gene can modulate its strength, thereby driving the reduced expression of α1AT.

Keywords COPD: Chronic obstructive pulmonary disease · *SERPIN1A*: Serine protease inhibitor 1A · α1AT: Alpha1-antitrypsin

Introduction

Chronic obstructive pulmonary disease (COPD) is a broad term used to describe progressive debilitating lung diseases that include chronic bronchitis, emphysema, refractory (non-reversible) asthma, and some types of bronchiectasis. According to the official American Thoracic Society/European Respiratory Society, it is defined as a preventable and treatable disease state characterized by airflow limitation (increased breathlessness) that is not fully reversible [1]. In accordance with the estimates of Global Burden of Disease investigations, It is the third leading cause of mortality worldwide including in United States where 12.6 million individuals have been diagnosed with COPD [2, 3]. In Kashmir-India, the prevalence of spirometrically diagnosed COPD is 17% in males

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and 15% in females aged ≥ 40 years [4]. Several environmental and genetic factors have been implicated in the pathogenesis of COPD. Severe alpha1-antitrypsin (α1AT) deficency is a well known genetic risk factor for the early onset of COPD. α1AT is a 52 kDa metastable glycoprotein protein coded by a serine protease inhibitor *(SERPINA1)* gene located on chromosome 14q32.1 [5]. α1AT is an abundant antiprotease in the circulation that targets and subsequently deactivates neutrophil elastase through the mouse-trap mechanism [6]. The Exon 1A and 1B of the *SERPINA1* gene comprise elements explicitly meant for macrophage-driven transcription and exon 1C harbors the promoter essential for hepatocyte-directed transcription of the *SERPINA1* gene (Fig. 1). So, the transcriptional regulation is controlled by two different elements within the *SERPINA1* gene and a number of modulation factors that include hepatocyte-specific factors, TNFα, IL1β, and IL6 [7]. The *SERPINA1* gene is inherited in an autosomal codominant fashion and is highly polymorphic with more than 125 single nucleotide polymorphisms reported so far [8]. The pathogenic mutations in the coding region of the *SERPINA1* gene like Z (Glu342Lys), S (Glu264Val) and null mutations have been extensively investigated and implicated in the pathogenesis of COPD [9, 10].

The Z-variant of α1AT (Glu342Lys) is highly susceptible to aggregation in the endoplasmic reticulum (ER) of hepatocytes, creates an ER stress, and thereby leads to the development of cirrhosis, hepatic fibrosis, and hepatocellular carcinoma through gain of function and emphysema via loss of its function [11]. However, no work has been done so far to bridge the gap between the *SERPINA1* promoter SNPs and COPD. In this study, we identified − 10T/C single nucleotide polymorphism (SNP) in the hepatocyte-specific promoter present in exon 1C of the *SERPINA1* gene in the COPD patients in Kashmir valley. This SNP was previously submitted to NCBI gene bank under accession number rs11558258. However, no work has been carried out to understand the possible association of exon 1C − 10T > C SNP with the development of COPD.

Materials and Methods

Study Subjects

A total of 230 subjects (110 COPD patients and 120 healthy individuals, all ethnic Kashmiri population) were enrolled in the Government Chest Disease Hospital (GCDH), Srinagar, Jammu and Kashmir, India from November 2014–July 2015. The study was conducted in accordance with the Declaration of Helsinki principles. An official ethical clearance for this study was obtained from the institutional ethics committee (IEC) of the Government Medical College (GMC), Srinagar and all subjects gave informed and written consent to participate in the study. Approximately, 3 mL of venous blood was taken from the healthy individuals as well as from the patients (age ≥ 40 years), who were diagnosed with COPD, according to the criteria of Global Initiative for Chronic Obstructive Lung Disease, 2014 revised guidelines.

The criteria for case inclusion were: (i) expectoration, cough, and/or wheezes for more than three months in a year (ii) FEV1/FVC < 70% and FEV1 < 80% on pulmonary function test (PFT) before the administration of bronchodilator drugs like salbutamol.

The patients with lung malignancy, active respiratory tract infection, history of bronchial asthma, interstitial lung disease, nephropathy, seasonal flu, allergy, and tuberculosis indicated by chest radiograph and high resolution computed tomography scan were excluded from the present study. The control group included 105 healthy individuals who had no history or prior diagnosis of COPD or any other serious disease. They were recruited during the same time period and from the same geographic area and from whom venous blood was collected and used as a control for the present study. The control group also included general population based subjects in addition to hospital-based controls. The control group subjects were matched to the case group subjects individually for age (± 5 years), sex, place of residence (rural/urban), smoking habit, and ethnicity in order to minimize the confounding effect of these various relevant factors. Both the subject groups chosen for this investigation were strictly ethnic Kashmiris.

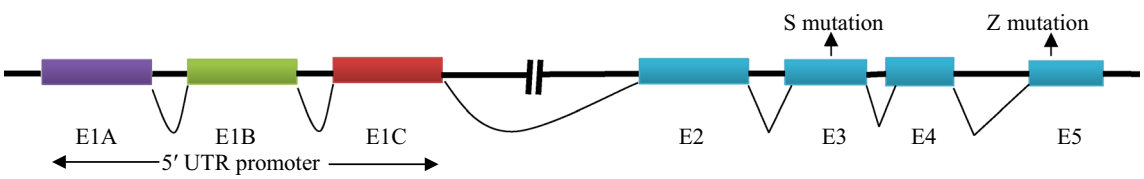

Fig. 1 Genomic organization of the *SERPINA1* gene. Exon 1 has three subregions namely as E1A, E1B, and E1C. The E1C harbors hepatocyte-specific promoter. E2, E3, E4, and E5 are the four exons that codes for α1AT

Data Collection

The data significant to the present study comprising all the COPD patients including their different clinicopathological parameters, demographic variables, and the environmental factors were acquired and assessed from the personal interview with the patients and/or their caretakers. The data collected included age, sex, place of residence, ethnicity, smoking habit, hepatological dysfunction (obstructive jaundice), and the family history of COPD.

The appropriate data were also obtained for each of the recruited controls generally through personal interviews that included some of the parameters mentioned above. All the patients, healthy controls and/or their guardians were accordingly informed about the present study and their inclination to take part in this study was acknowledged using a predesigned questionnaire.

Quantification of serum α1AT by enzyme-linked immunosorbent assay (ELISA)

The serum samples separated from the clotted blood obtained from both the subjects (70 cases and 105 subjects) were used to first estimate the level of serum α1AT by ELISA strictly as per the method described in *Abcam's* kit. The absorbance was measured at 410 nm using a spectrophotometer (*Epoch™ Microplate Spectrophotometer*).

Sample Preparation and DNA Extraction

A volume of 2 mL of blood was put in an ethylene diaminetetraacetic acid (EDTA)-coated vials and 1 mL in clot-activator vials (purple and red capped tubes; *ADS Hitech Polymers, India)*. The blood samples present in EDTA-coated vials were stored at − 80 °C until further use. The blood samples in clot-activator vials were immediately processed and the serums were stored at − 80 °C until further use. The phenol–chloroform method was used to extract the genomic DNA from the white blood cells of the venous blood [15]. The qualitative and the quantitative assessments of the extracted genomic DNA samples were carried out by using *Nandrop*™. The samples that had 260/280 ratio in between 1.80–1.90 were used in subsequent experiments and those with a ratio below 1.80–1.90 were processed further till requisite purity was obtained. The qualities of the genomic DNAs were assessed by running it in 0.8% agarose gel in 1X Tris-acetate-EDTA (1X TAE) running buffer.

Polymerase Chain Reaction of Exon 1C of the *SERPINA1* Gene

The amplification of the 676 bp fragment that includes 210 bp exon 1B and 104 bp exon 1C promoter of the *SERPINA1* gene was carried out in a total volume of 50 µL. The total volume (50 µL) included 150 ng of genomic DNA, 1.25U *Taq* DNA polymerase with lX Standard *Taq* reaction buffer (*New England Biolabs*), 200 µM deoxynucleotide triphosphate mix (*New England Biolabs, UK*); 0.2 µM forward and revere oligonucleotide primers (*Integrated DNA Technologies, India*) and nuclease-protease free water (*Qiagen, Germany*).

The PCR conditions used for the amplification were as follows; initial denaturation at 95 °C for 5 min followed by 30 cycles of denaturation at 95 °C for 30 s; annealing at 61 °C for 30 s, extension at 72 °C for 1 min followed by a final extension step at 72 °C for about 10 min. The oligonucleotide primers used for the amplification of the promoter-1B/C were as follows: 5′-CCATCAAGAGGGTGT TTGTGT-3′ (Forward) and 5′-CGGATACCCACTCCACAA C-3′ (Reverse). The desired PCR product was 676 bp in size that includes 210 bp of exon 1C, 104 bp of exon IC, and additional 362 bp (flanked upstream and downstream of the target sequence) to increase the size and for sequencing purpose. The Fig. 2 represents successful PCR amplicons ran in 0.8% agarose gel.

Genotyping

All the amplified samples were sent to *Scigenom private limited, Cochin, Kerala, India* for sequencing. The sequencing report revealed the presence of − 10T/C (rs11558258) SNP in the hepatocyte-specific promoter region present in exon 1C of the *SERPINA1* gene (Fig. 3). This SNP is present 10 bp upstream of the start codon (ATG) of the *SERPINA1* gene. The transcription of the *SERPINA1* gene in hepatocytes occurs − 49 bp upstream of the ATG as shown in Fig. 3a and c. The substitution of T to C is highlighted with a yellow color in the representative chromatogram of a COPD patient in Fig. 3b.

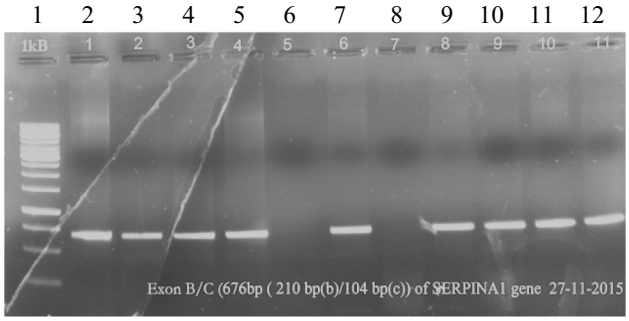

Fig. 2 PCR amplification of 676 bp region that includes hepatocyte-specific *SERPINA1* gene promoter 1C of 210 bp in size. Lane 1 shows 1kB DNA marker. Lane 2–5 shows 676 bp amplified fragment from the DNA of COPD cases. Lane 7 and 9–12 shows 676 bp amplified fragment from the DNA of healthy controls

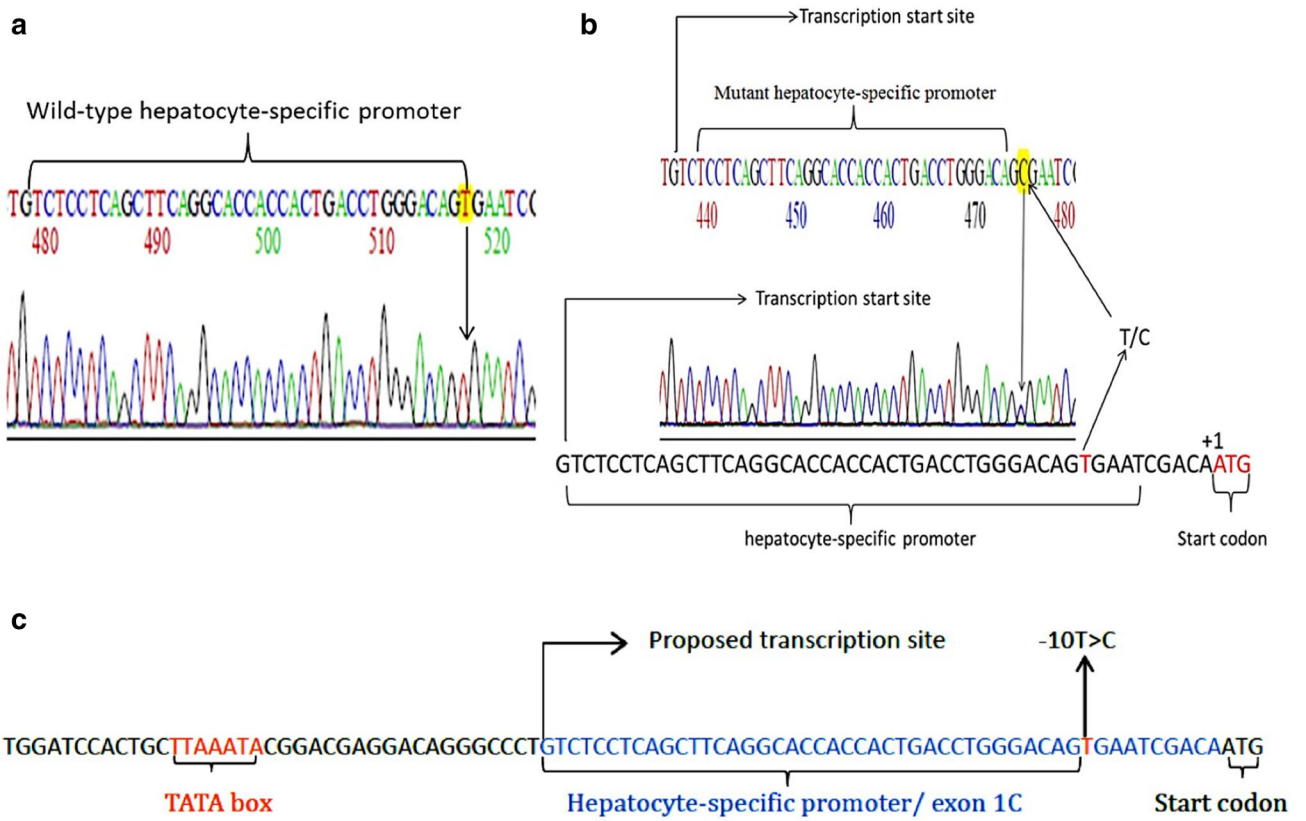

Fig. 3 Representative chromatograph showing the T > C substitution at − 10 bp upstream of the start codon of α1AT. This region encompasses the hepatocyte-specific promoter to drive the expression of α1AT in hepatocytes. **a** Representative chromatogram of a COPD patient showing wild type hepatocyte-specific promoter region. No change in base that is "T" is highlighted with a yellow color. **b** Representative chromatogram of a COPD patient showing mutant hepatocyte-specific promoter region. The transcription start site, hepatocyte-specific promoter, and start codon (highlighted with red color) is shown accordingly. **c** Sequence encompassing the 5′ untranslated region that includes the proposed hepatocyte-directed transcription site for α1AT. Transcription of α1AT in hepatocytes start at 49 bp upstream of the start codon. The substitution of T > C is at − 10 bp upstream of the start codon shown in red color. The TATA box sequence is shown in red color

Statistical Analyses

The frequencies of genotypes and alleles for the − 10T/C SNP located in hepatocyte-specific promoter 1C of the *SERPINA1* gene under investigation were achieved by direct counting. The numbers and percentages were calculated and presented for each of the categorical variables. The conditional logistic regression analysis was carried out to determine the unadjusted and adjusted odds ratios (ORs) and corresponding 95% confidence intervals (CIs) to assess the possible association of the relevant SNP genotypes with COPD risk and to assess the possible gene-environment interactions, if applicable. The more common homozygous genotype was used as the baseline (reference) in the conditional logistic regression that was adjusted for the known risk factors like gender, age, and smoking habit and also with the place of residence in order to eliminate the possible confounding (third) variables. The correlation between the genotypes and the clinicopathological parameters, demographic variables and environmental factors including smoking habit within the case group was analyzed by Fisher exact test. A two sided probability value of 5% ($p \leq 0.05$) was considered statistically significant for all types of analyses. All statistical analyses were performed using *IBM SPSS Statistics v21* software.

Results

Standard Characteristics of Study Participants

110 confirmed COPD cases were diagnosed in the ethnic Kashmiri population comprising of males and females strictly on the basis of GOLD guidelines from November 2014–July 2015. We further scrutinized the patients on different grounds including additional necessary evaluations like PFT, stage of COPD etc. We excluded 40 COPD patients, who were suffering from multiple diseases

including malignancy and particularly those who had received bronchodilators before undergoing PFT. We also collected venous blood from 120 healthy controls including those that visited the same hospital during the same time period for a routine checkup and were subsequently diagnosed with a seasonal cough. We excluded 15 healthy controls, who were suffering from inflammation (increased C-reactive protein), acute respiratory infection, seasonal flu, post-surgical patients and individuals not belonging to Kashmir Valley. The serum of the 70 confirmed COPD cases and 105 controls were subjected to ELISA for the quantification of α1AT levels.

The concentration of serum α1AT in all the samples was determined by ELISA. α1AT concentration was found to be in the range of 0.48–1.59 g/L in COPD cases and 2.62–3.35 g/L in control samples, respectively as shown in Fig. 4a. The mean serum α1AT level in COPD cases was found to be 1.20 ± 0.23 and 3.16 ± 0.16 g/L in controls, respectively (Fig. 4b). Although, the mean serum α1AT level was in the normal range (1.0–3.6 g/L) in COPD cases but was found to be 2.6 times less than the controls.

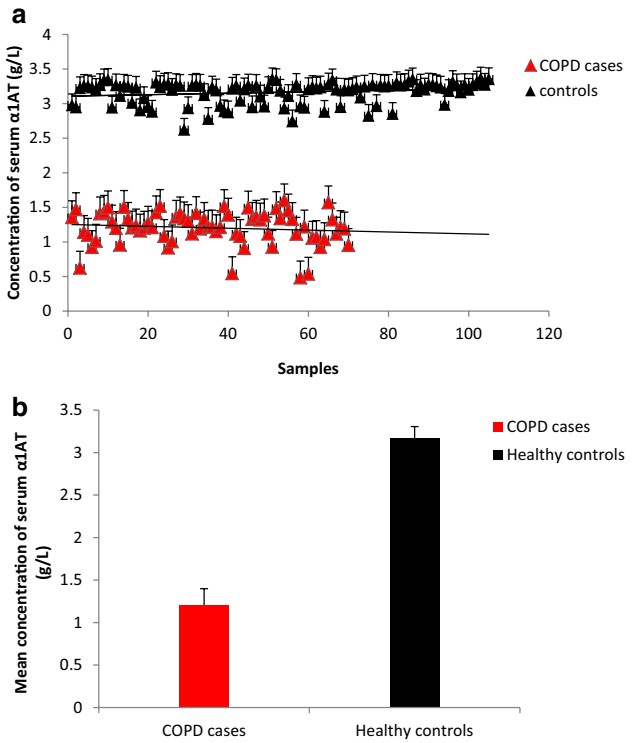

Fig. 4 **a**. The scatter plot showing the distribution of serum α1AT level in 70 COPD cases (Red) and 105 healthy controls (Black). The α1AT concentration was found to be in the range of 0.48–1.59 g/L in cases of COPD cases and 2.62–3.35 g/L in case of controls. The normal serum α1AT levels are in between 1.0 ± 3.6 g/L. **b**. Bar graph representing the mean concentration of serum α1AT (mean ± SE) in COPD cases (Red) and healthy controls (Black). The mean serum α1AT level in COPD cases was found to be 1.20 ± 0.23 g/L and 3.16 ± 0.16 g/L in controls, respectively. α1AT serum level in cases was found to be 2.6 times less than the controls

Association Analysis of the *SERPINA1*-exon 1C − 10T > C SNP

The frequencies of the genotypes of the *SERPINA1*-exon 1C − 10T/C SNP for both the case and the control groups are listed in Table 1. The more common TT genotype of *SERPINA1*-exon 1C was less frequent among the case group [68.57% (48/70)] compared to the control group [87.61% (92/105)].The frequency of the heterozygous genotype (TC) in the case group [15.71% (11/70)] was higher than the control group [8.57% (09/105)]. The variant genotype (CC) also showed a higher frequency in the COPD cases [15.71% (11/70)] in comparison to the control group [3.80% (04/105)].

Furthermore, the frequency of the more common − 10T allele was found to be 76.43% (107/140) among the COPD cases and 91.91% (193/210) among the control group. The frequency of the less common − 10C allele was found to be 23.57% (33/140) among the COPD cases and 8.09% (17/210) in the control group (Fig. 5). The frequency of the combined variant genotype (TC + CC) in the COPD cases was found to be 31.42% (22/70) and 12.38% (13/105) in the control group [Table 1]. The overall association between the *SERPINA1*-exon 1C − 10 T/C SNP and the modulation of COPD risk was found to be significant ($p = 0.0047$). The variant genotype (CC) was found significantly associated with an increased risk of COPD [OR 5.30 (95%CI 1.62–17.47); $p = 0.004$]. Furthermore, the less common − 10C allele showed an overall significant associations with COPD risk ($p < 0.0001$). The − 10C allele was associated with an increased risk of COPD [OR 3.50 (95%CI 1.86–6.58); $p < 0.001$]. The combined variant genotype (TC + CC) also showed an overall significant association with COPD risk ($p = 0.002$).The combined variant genotype (TC + CC) was significantly associated with an increased risk of COPD [OR 3.20 (95% CI 1.47–6.96); $p = 0.003$] (Table 1). The numbers and the frequencies of the subsets of various characteristics of the case group subjects under study i.e., gender, dwelling, smoking status, hemoptysis, hepatological dysfunction (obstructive jaundice) and family history for − 10T/C SNP are listed in Table 2. A significant association of obstructive jaundice with COPD was observed (overall p value, 0.0264). A significant association of the family history with COPD (overall p value, 0.0331) suggests that genetics may play an important role in the pathogenesis of COPD. No association of COPD with gender, dwelling, smoking status, hemoptysis, and PFT (spirometry) was seen ($p = 0.3616$, $p = 0.2176$, $p = 0.2431$, $p = 0.0839$, and $p = 0.2176$).

Table 1 The *SERPINA1* exon 1C − 10T/C single nucleotide polymorphism genotype frequency distributions among COPD cases and healthy controls and risk of COPD

Genotype	COPD cases ($N=70$)[a]	Controls ($N=105$)[a]	OR (95%CI); p value[b]	Adjusted OR[c] (95% CI); p value[b]	χ^2; Pearson p value (overall)[b,d]
TT	48 (68.57%)	92 (87.61%)	**1.0 (Referent)**	**1.0 (Referent)**	10.72 $p=0.0047$
TC	11 (15.71%)	09 (08.57%)	2.34 (0.91–6.04) $p=0.085$	2.36 (0.91–6.06) $p=0.085$	
CC	11 (15.71%)	04 (03.80%)	**5.27 (1.59–17.43) $p=0.004$**	**5.32 (1.62–17.83) $p=0.004$**	
T/C + C/C	22 (31.42%)	13 (12.38%)	**3.24 (1.50–7.00) $p=0.003$**	**3.18 (1.43–6.89) $p=0.003$**	**9.524 $p=0.0020$**
Allele					
T	107 (76.43%)	193 (91.91%)	**1.0 (Referent)**		
C	33 (23.57%)	17 (08.09%)	**3.50 (1.86–6.58) $p<0.0001$**	–	**16.43 $p=<0.0001$**

COPD chronic obstructive pulmonary disease, *OR* odds ratio, *CI* confidence interval

ORs (95% CIs) were obtained from conditional logistic regression models

[a]*N* denotes the number of subjects or individuals

[b]The values in bold indicate significant results

[c]Adjusted ORs (95% CIs) were obtained in conditional logistic regression models when adjusted for age, gender, place of residence, and smoking status

[d]*P* values calculated using χ^2 tests

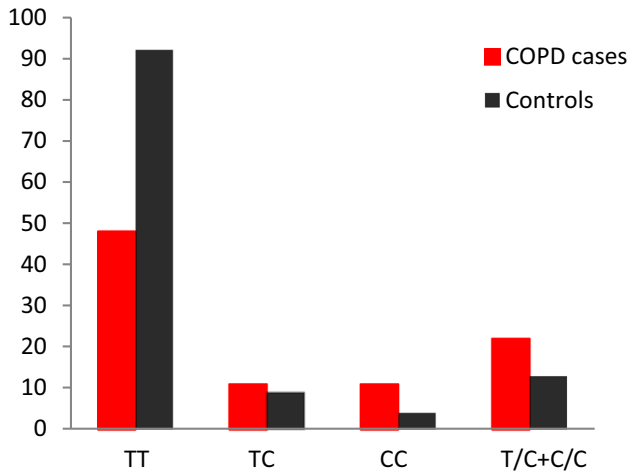

Fig. 5 − 10 T > C single nucleotide polymorphism frequency distributions among COPD cases and healthy controls

Discussion

We investigated the role of − 10T/C SNP present in the hepatocyte-specific promoter present in exon 1C of the *SERPINA1* gene as a potential COPD risk modulatory factor in a case–control study with 70 COPD cases and 105 controls. No work has been done till date to find an association between − 10T/C SNP and the pathogenesis of COPD. Therefore, we may fall short to substantiate our results furnished from this study. As far as our preliminary data are concerned, we found an association of − 10T/C SNP with an increased risk of COPD.

We observed an increased frequency of -10 C genotype in COPD cases compared to controls. Additionally, a significant association of obstructive jaundice with COPD was observed ($p=0.0264$). A significant association of the family history with COPD (overall p value, 0.0331) suggests that genetics may play an important role in the pathogenesis of COPD. In this study, we observed 2.6 times decreased levels of serum α1AT along with the increased frequency of − 10T/C SNP in COPD patients compared to control group in Kashmiri population. Although the mean serum α1AT levels were found within the normal range (1.0-3.6 g/L) but the mean serum α1AT levels in COPD cases was found to be 2.6 times less than the controls. In our study, we observed a mean of 3.16 g/L of serum α1AT in healthy controls. Therefore, one would argue that a mean of 1.20 g/L of α1AT in COPD patients cannot be considered as a normal level to deactivate the excessive production of proteases like neutrophil elastase from the neutrophil at the site of inflammation. Like most of the enzymes are replenished again, it is important here to mention that serum α1AT is itself deactivated after performing its job as a protease inhibitor. Therefore, the low amount of serum α1AT cannot suffice the need. The hepatocyte-specific promoter mutation can possibly have an impact on the decreased expression of the α1AT.

Table 2 Association of the *SERPINA1*-exon 1C − 10T/C single nucleotide polymorphism with various demographic variables, environmental factors, clinicopathological parameters, spirometry, and family history in COPD cases

Characteristics	$N=70$	TT 48 (68.57%)	TC 11 (15.71%)	CC 11 (15.71%)	Odds ratio (class interval (CI); p value	$\chi 2$; p value (Overall)[a]
Gender						
Male	49 (64.28%)	36 (75.00%)	06 (54.54%)	07 (63.63%)	TC 2.50 (0.65–9.70); 0.2671	2.035 0.3616
Female	21 (35.71%)	12 (25.00%)	05 (45.45%)	04 (36.36%)	CC 1.71 (0.43–6.90); 0.4681	
Dwelling						
Rural	45 (57.14%)	34 (70.83%)	06 (54.54%)	05 (45.45%)	TC 2.02 (0.53–7.73); 0.3085	3.050 0.2176
Urban	25 (42.85%)	14 (29.16%)	05 (45.45%)	06 (54.54%)	CC 2.91 (0.76–11.13);0.1585	
Smoking status						
Ever	42 (41.42%)	32 (66.66%)	05 (45.45%)	05 (45.45%)	TC 2.40 (0.64–9.08); 0.2997	2.828 0.2431
Never	28 (58.57%)	16 (33.33%)	06 (54.54%)	06 (54.54%)	CC 2.40 (0.64–9.08); 0.2997	
Hemoptysis						
Yes	25 (74.28%)	13 (27.08%)	06 (54.54%)	06 (54.54%)	TC 0.31 (0.08–1.19); 0.1490	4.955 0.0839
No	45 (25.71%)	35 (72.91%)	05 (45.45%)	05 (45.45%)	CC 0.31 (0.08–1.19); 0.1490	
PFT[b]						
< 70%	45 (57.14%)	34 (70.83%)	06 (54.54%)	05 (45.45%)	TC 2.02(0.53–7.73); 0.3085	3.050 0.2176
> 70%	25 (42.85%)	14 (29.16%)	05 (45.45%)	06 (54.54%)	CC 2.91 (0.21–11.13); 0.1585	
Obstructive jaundice						
Yes	29 (42.85%)	15 (31.25%)	06 (54.54%)	08 (72.72%)	TC 0.38 (0.10–1.44); 0.1747	**7.270 0.0264**
No	41 (57.14%)	33 (68.75%)	05 (45.45%)	03 (27.27%)	CC 0.17 (0.04–0.74); **0.0166**	
Family history						
Yes	44 (62.85%)	35 (72.91%)	04 (36.36%)	05 (45.45%)	TC 0.21 (0.05–0.85); **0.0334**	**6.814 0.0331**
No	26 (37.14%)	13 (27.08%)	07 (63.63%)	06 (54.54%)	CC 0.31 (0.08–1.19); 0.1490	

[a]The values in bold indicate significant results

[b]Spirometry/ PFT (pulmonary function test) done before the salbutamol nebulization

RNA polymerase binds to the promoter region and drives the expression of a particular protein. It is quite possible that the change of the base in the hepatocyte promoter can modulate its strength, thereby driving the decreased expression of α1AT. Our study thus provides a possible link between decreased serum α1AT levels and exon 1C promoter mutation. However, due to the less sample size we cannot draw a generalized picture about the role of hepatocyte-specific promoter mutation (− 10T/C) in COPD. Large sample size is necessary to strengthen the data furnished by our study.

Conclusion

The overwhelming majority of investigations till date have tried to bridge the gap between various SNPs of the *SERPINA1* gene and the pathogenesis of COPD. We are first time reporting from Kashmir that − 10T/C SNP present in hepatocyte-specific promoter present in exon 1C of the *SERPINA1* gene may be associated with the decreased levels of circulating α1AT in COPD patients. However, no report, till date, has validated an association of − 10T/C SNP present in exon 1C of the *SERPINA1* gene with the COPD. We are unable to exactly explain how this SNP drives the decreased expression of circulating α1AT. The weak binding of RNA polymerase to exon 1C having − 10T/C SNP could be one of the plausible reasons however; promoter assay and techniques like real-time PCR could possibly unravel an underlying cause of the decreased expression of α1AT and could possibly substantiate our investigation.

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Author Contributions All authors have made substantial contributions: (1) The concept and design of the study was done by AB, YMH, KMF,

and NNS (2) the article was drafted critically for important intellectual content by Dr. KMF, Dr. NNS and AB (3) Dr. MKI, NH, MB, SB and MB prepared reagents for DNA extraction, (4) Statistical analysis was done by Dr. TRJ, and (5) Pulmonary function assistance by SSF and (6) final approval of the version by KMF, NNS, and AB.

Compliance with Ethical Standards

Conflict of interest All authors declared that they have no competing interests.

Ethical Approval This study is approved by the Ethics Committee of the Government Medical College Srinagar-India.

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