ORIGINAL ARTICLE

Association of ADAM33 gene S1 and S2 transmembrane domain polymorphisms in COPD from South-Indian population

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Abstract *Background:* Chronic obstructive pulmonary disease (COPD) is defined as a disease characterised by partially reversible and progressive airflow limitation associated with an abnormal inflammatory response of the lung with systemic manifestation. COPD is influenced by both environmental and genetic factors. ADAM33 (a disintegrin and metalloproteinase 33) has been one of the most exciting candidate genes for asthma and it was first associated with the disease in Caucasian populations. Recently, ADAM33 was shown to be associated with excessive decline of lung function and COPD. The aim of the study was to investigate the association of ADAM33 – S1 and S2 polymorphisms with COPD.

Subjects and methods: A total of 150 COPD patients attending the Department of Pulmonary Medicine, Government Chest Hospital, Erragadda, Hyderabad, India and 200 healthy control subjects were considered for the present study. A standard PCR–RFLP method was carried out for genotyping of ADAM33 S1-A/G and S2-C/G polymorphisms in all the participants.

Results: Genotypic distribution of the control and patient groups was compared with values predicted by the Hardy–Weinberg equilibrium, odds ratios (OR) and their respective 95% confidence intervals were used to measure the strength of association between ADAM33 S1 (A/G) and S2 (C/G) gene polymorphisms and COPD. The genotypic frequencies of ADAM33 gene S1 (A/G) polymorphism were found to be AA/AG/GG – 36%, 56%, 8% in controls and 5.33%, 10.66%, 84% in COPD cases, respectively. Genotypic frequencies for S2 (C/G) polymorphism were found to be CC/CG/GG – 14.47%, 78.20%, 5.92% in controls and 4%, 8% and 88% in COPD cases,

respectively. There is a significant difference in distribution of genotypes and alleles of ADAM33 S1 and S2 gene polymorphisms between the two groups.

Conclusion: The present study suggests that the ADAM33 S1 and S2 gene promoter polymorphisms can be the major genetic predisposing factors in the aetiology of COPD.

1. Introduction

Chronic obstructive pulmonary disease (COPD) is defined as a disease characterised by partially reversible and progressive airflow limitation that is usually associated with an abnormal inflammatory response of the lung with systemic manifestation [1,2]. Most of the patients with COPD show airway hyper-responsiveness (AHR), an exaggerated airway response to non-specific stimuli resulting in airway obstruction. The severity of AHR is positively correlated with inflammation in lung tissue and increased number of CD8 cells in bronchial epithelium of COPD lung [3]. COPD ranks 3rd as a global cause of death and shows a worldwide increase in both morbidity and mortality. It becomes the second leading cause of death worldwide by 2030 (WHO). The overall prevalence of COPD is 4.36%. The prevalence amongst males and females was 5.32% and 3.41%, respectively. The prevalence was found to be increasing with an increase in age. There is a wide variation in the incidence of COPD across the various registries in India.

Pathophysiology of COPD is a multifactorial process with a complex inflammatory cell profile including eosinophils, monocytes, neutrophils and lymphocytes. Along with the inflammatory profile, increased levels of cytokines such as IL-6, IL-8 [4], TNF-α and VEGF in stable COPD patients are seen suggesting their key role in the pathogenesis of COPD [5]. In contrast to asthma, the inflammatory mediators involved in COPD are now apparent that many lipid mediators, inflammatory peptides, reactive oxygen and nitrogen species (ROS), chemokines, cytokines and growth factors are involved in orchestrating the complex inflammatory process that results in small airway fibrosis and alveolar destruction [6]. It is well documented that environmental and genetic determinants and their interaction play a vital role in the development of COPD [7]. Amongst various environmental factors, long term tobacco smoke is the significant one. However, all the smokers may not suffer from COPD, denoting that some genetic factors are contributing in the etiopathogenesis of COPD. The importance of proteases in extracellular matrix (ECM) and their imbalance in the genesis of pulmonary emphysema is generally accepted. In particular, MMPs manifest along with inflammatory cytokines in COPD and receive relatively little attention. Genomic approaches have investigated multiple candidate genes and SNPs, with inconsistent results [8]. Thus, genetic factors appear to predispose some individuals with tobacco exposure for development of this respiratory disease. Research on the genetic basis for COPD is therefore still required.

Matrix metalloproteinases (MMPs) are Zn^{+2} dependent endopeptidases secreted in extracellular matrix (ECM) of lungs. Most MMPs are secreted as latent pro-enzymes and need to be activated by proteolytic conversion [9]. They generally do not act alone but interact with other types of proteases and trigger all the other cellular components. The observations of Wright et al. (2008), demonstrated that instilled elastases could produce emphysema in experimental animals and the inflammatory cells and proteases play a role in COPD [41]. It involves multiple processes including abnormal inflammation, protease–antiprotease imbalance, oxidant and antioxidant imbalance and ECM destruction [10]. They play an important role in the mechanism of the destruction of ECM involving cross-linking of elastin and collagen fibrils. MMPs and ADAMs are proteolytic enzymes that degrade the matrix components both in normal physiological states and in abnormal pathological processes [11].

A disintegrin and metalloproteinase 33 (ADAM33), is a membrane anchored metalloproteinase that has a role in cell fusion, adhesion, signalling and proteolysis. It maps on human chromosome 20p13 encoding an open reading frame of 2442 bp consisting of 22 exons. It is expressed in lung fibroblasts, heart and bronchial smooth muscle cells [12]. ADAM33 is a gene of putative interest for COPD as it was primarily associated with susceptibility for asthma and airway hyper-responsiveness (AHR) [13,14]. It is furthermore associated with accelerated decline in pulmonary function in the general and asthma population [15]. ADAM molecules are members of type I transmembrane zymogen glycoproteins containing an N-terminal secretion signal domain, pro-catalytic, disintegrin, cysteine-rich, EGF-like transmembrane domain and a cytoplasmic (C-terminal) domain. Its complex organisation involves eight domains that facilitate its participation in many cellular processes. The autocatalytic removal of pro-domain region is the activation signal for ADAM proteins, which are presumed to have a critical role in cell adhesion, proliferation differentiation, signalling, apoptosis and inflammatory responses [16,17]. Overall substrate specificity and function of ADAM33 gene are yet to be known. It is mainly involved in tissue remodelling, a physiological process intricately related to airway inflammation, hyper-responsiveness and airway obstruction [18].

Garlisi and colleagues (2003) demonstrated that α2-macroglobulin, an important member of pulmonary defence system is cleaved by an active proteinase ADAM33 [20]. These results suggested that ADAM33 is involved in the pathogenesis of airway obstruction with affecting tissue remodelling, a physiological process intricately related to airway inflammation [19,20]. In a Dutch cohort, S1 and S2 polymorphisms were examined along with other SNPs affecting the course of asthma and have been shown to influence the decline of forced expiratory volume (FEV1)/forced vital capacity (FVC) ratio in lung function [21,22]. In a German study the associations between SNPs in ADAM33 showed an annual decline in FEV1 in

cigarette smokers when compared to controls [23]. It is suggested to play a role in the airway remodelling of the lungs [24]. Genetic linkage analysis and association studies of families with asthma across diverse ethnic back-grounds support a relationship between ADAM33 polymorphisms and asthma phenotypes expressed in AHR and other respiratory disorders [25–28]. The mouse orthologue of ADAM33 also lies in the region linked to AHR [14]. Selective expression of the ADAM33 mRNA and its protein in adult bronchial smooth muscle and human embryonic bronchi and surrounding mesenchymal cells indicate specific roles of the gene in the observed phenotypes of AHR and airway remodelling and there are limited studies in relation to COPD [14,29,30]. There is selective expression of ADAM33 in lung mesenchymocytes suggesting that alterations in its activity may underlie abnormalities in the function of airway smooth muscle cells and fibroblasts linked to BHR and airway remodelling [31]. One possible explanation is that ADAM33 may serve as a cell surface 'sheddase' to release growth factors such as TGF-β and modify cell-surface receptor expression to induce the proliferation of airway mesenchymocytes [32]. It was observed that individuals with a genetic predisposition for a higher number of CD8 cells were more susceptible to a further increase in CD8 cells, which might finally result in airflow limitation [33]. Despite the above notion, negative associations between ADAM33 polymorphisms with asthma and other respiratory disorders have been reported from ethnically different populations yielding inconsistent results [3,34]. Hence, the present study was aimed at understanding the role of the transmembrane domain polymorphisms S1 and S2 of ADAM33 in the pathophysiology of COPD.

2. Subjects and methods

A total of 150 patients (male:female 142:08) with COPD evaluated in the Department of Pulmonary Medicine, Government Chest Hospital, Hyderabad, Telangana, India from October 2012 to December 2014 were enroled for study with their written informed consent and adhered to the tenets of the Declaration of Helsinki (JAMA 1997;277:925–926). The clinical diagnosis was made according to the GOLD (The Global Initiative for Chronic Obstructive Lung Disease) guidelines of the ATS/ERS protocol by an expert pulmonologist.

A total of 200 healthy individuals matched for age and sex formed the control group. The subjects were included under the study with their written informed consent. The study was approved by the ethics committee of Institute of Genetics and Hospital for Genetic Diseases, Hyderabad. None of the controls had any history of COPD, bronchitis, bronchiectasis, bronchial asthma, AHR, pneumothorax, lung cancer, allergic broncho-pulmonary diseases and family history of COPD.

2.1. Genotyping determination of ADAM33 S1 and S2 by PCR–RFLP assay

The genomic DNA extracted from venous blood was collected in EDTA tubes using the standard Phenol–Chloroform method [38]. The genotyping of ADAM33 S1 and S2 gene polymorphisms was performed using PCR–RFLP assay using designed forward and reverse primers [Bioserve, India], respectively.

Gene name and fragment position	Forward and reverse primers
ADAM33 S1	F: 5′-TGTGCAGGCTGAAAGTATGC-3′
	R: 5′-AGAGCTCTGAGGAGGGGAAC-3′
ADAM33 S2	F: 5′-GACTGGGAGGCCACTGGA-3′
	R: 5′-CGCAGACCATGACACCTTCCT-3′

The amplified PCR products of S1 polymorphism 304 bp were digested with HinfI (Fermentas, USA) and 172 bp and 132 bp restriction digested fragments were obtained (see Fig. 1). The amplified PCR products of S2 polymorphism 328 bp were digested with HinpI (Fermentas, USA) and 262 bp and 66 bp restriction digested fragments were obtained, by incubating at 37 °C overnight and separating the polymorphic fragments on 3% agarose gel indicating the gene polymorphisms at S1 and S2 polymorphic sites, respectively (see Figs. 1 and 2).

2.2. Statistical analysis

Hardy–Weinberg equilibrium was tested for the gene polymorphisms. The allele and genotype frequencies amongst cases and controls were compared by Chi-square test and odds ratio with 95% CI. Two-tailed p-value of < 0.05 was considered significant using Open Epi 6 software (Open Epi version 2.3.1 from Department of Epidemiology, Rollins School of Public Health, Emory University, Atlanta, GA 30322, USA).

3. Results

The cohort is subdivided into four subgroups based on GOLD guidelines staging mild (Stage I), moderate (Stage II), severe (Stage III), and very severe (Stage IV) to assess the influence of genetic variation with the disease progression. The 4 subgroups were comprised of 25(16.6%), 23(15.3%), 95(63.3%) and 07(4.6%) patients, respectively. A total of 200 asymptomatic age and sex matched controls were included in the present study. The demographic characteristics such as age, sex, addictions like smoking and alcohol consumption along with GOLD staging were noted based on the standard proforma [36].

The demographic and clinical data of the samples and healthy controls under study are presented in Table 1. The gender-wise and age distribution of patients and control subjects revealed no significant difference. The proportion of smokers in COPD patients (92%) was significantly higher than the healthy controls. The proportion of alcoholics in COPD patients (90%) was significantly higher than the healthy controls.

3.1. Genotype analysis

Genotypic frequencies of ADAM33 gene S1 polymorphism (A/G) were found to be AA/AG/GG – 5.33%, 10.66%, 84% in COPD patients while they were found to be 36%, 56%, 8% in healthy controls, respectively. Genotypic frequencies for S2 polymorphism (C/G) were found to be CC/CG/GG – 4%, 8% and 88% in COPD patients while they were 14.47%, 78.20%, 5.92% in healthy controls, respectively.
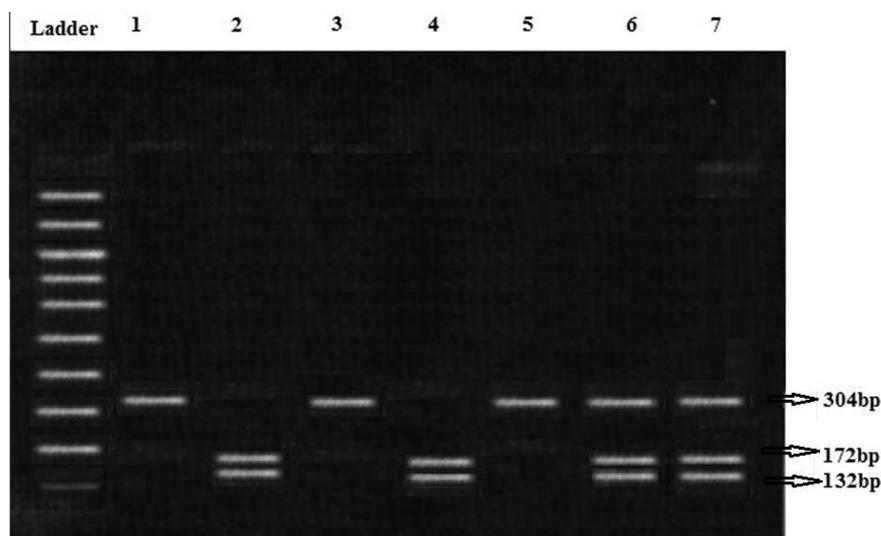
Figure 1 Agarose gel electrophoresis for RFLP–PCR product of ADAM33 S1 stained with ethidium bromide. 1st column – represents Ladder. Lanes 1, 3 and 5 represent homozygote AA genotype. Lanes 6 and 7 represent heterozygote AG genotype. Lanes 2 and 4 represent homozygote GG genotype.
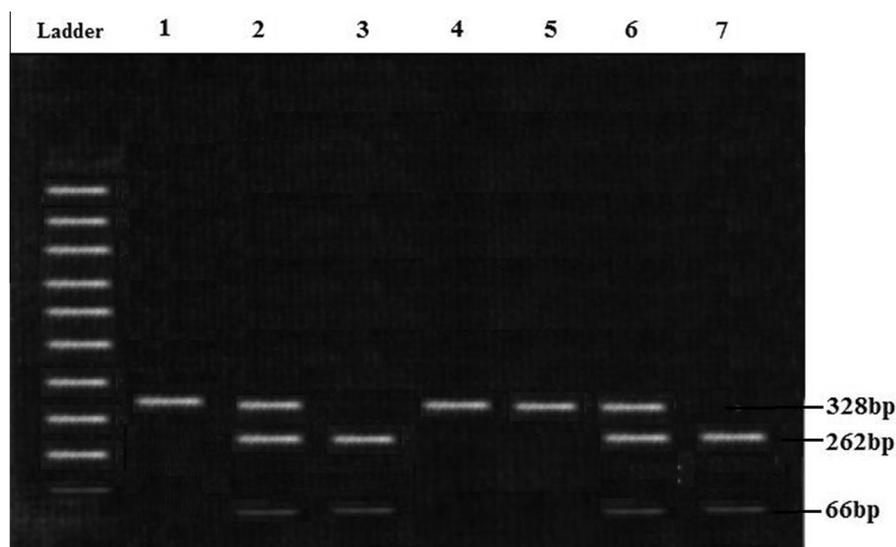

Figure 2 Agarose gel electrophoresis for RFLP–PCR product of ADAM33 S2 stained with ethidium bromide. 1st column – represents Ladder. Lanes 1, 4 and 5 represent homozygote CC genotype. Lanes 2 and 6 represent heterozygote AG genotype. Lanes 3 and 7 represent homozygote GG genotype.

When homozygous genotypes (AA in S1 and CC in S2) were taken as reference, it was found that frequencies for respective mutant alleles GG and GG genotypes in COPD patients were significantly associated with the disease. Genotype frequencies of ADAM33-S1-AG and GG genotypes along with mutant G allele were significantly associated with COPD compared to healthy subjects [OR = 0.402(0.296–0.547), $p \leqslant 0.001^{*}$]. Genotype frequencies of ADAM33-S2-AG and GG genotypes along with mutant G allele were significantly associated with COPD compared to healthy subjects [OR = 0.277 (0.202–0.380), $p < 0.001^{*}$]. The statistical analysis of genotypic data and allelic frequencies revealed a significant association of

S1 (A/G) and S2 (C/G) polymorphisms in patients when compared with controls, respectively (see Tables 1 and 2).

Moreover, in co-dominant, dominant and recessive models in Tables 2 and 3, the results showed that ADAM33 S1-GG and S2-GG alleles were conferring a significant increased risk for the disease in co-dominant and dominant models. In dominant model, when AA was taken as reference the frequency of S1-AG + GG in COPD patients was significantly higher than that in healthy controls [OR = 0.078(0.043–0.140), p-value $< 0.001^{*}$] and when CC was taken as reference the frequency of S2-CG + GG in COPD patients was significantly higher than that in healthy controls [OR = 0.043(0.021–

Table 1 Demographic and clinical data of COPD and control subjects.

S. no.	Characteristics		Patients	Controls
1	Total		(N = 150)	(N = 200)
2	Gender	Males	142(94.66)	172(86.0)
		Females	08(5.33)	28(14.0)
3	Median age (years)	>35	128(85.33)	132(66.0)
		≤35	22(14.66)	68(34.0)
5	Smoking	Smokers	138(92.0)	46(25.55)
		Non-smokers	12(8.0)	154(77.0)
6	Alcoholism	Alcoholics	135(90.0)	78(39.0)
		Non-alcoholics	15(10.0)	122(61.0)

Values in parentheses represent percentages N (%) compared to controls.

0.088), p-value < 0.001*] conferring 'G' allele as a predisposing factor for the disease respectively. As per International HAP Map consortium the present study revealed 28.55%, which could be due to the ethnic variation within different regions of South-Indian population. The ADAM33 gene frequency in COPD patients was not found to be in Hardy–Weinberg equilibrium at 55% level of significance. The gene frequency of control subjects was in Hardy–Weinberg equilibrium at 5% level of significance.

4. Discussion

COPD is a progressive airflow limitation disease due to obstructive bronchitis and emphysema and permanent destruction of alveoli resulting in pulmonary failure. To demonstrate the evidence for the association of genetic varia-

tions in the disease, two transmembrane domain SNPs of ADAM33 S1 and S2 were genotyped and analysed in South-Indian population by means of a case–control approach. However, from the data, it is observed that a significant distributive difference of the tested polymorphisms between COPD individuals with age and sex matched healthy controls is in accordance with the disease and its progression.

ADAM proteins are Zn^{+2}-dependent metalloproteinases with various biological functions, encompassing mainly signal transduction. Van Eerdewegh et al. (Nature 2002) [15] have identified ADAM33 as a susceptibility gene for asthma and demonstrated that ADAM33 is expressed in airway smooth muscle cells and lung fibroblasts. It may play a possible role in airway remodelling because of its high expression in epithelium, myofibroblasts or fibroblasts and airway smooth muscle cells (ASMCs) [37]. Because higher expression of ADAM33 is found in the fibroblast and smooth muscle cells of the lung, over or under expression of ADAM33 gene may result in alterations in airway remodelling and repair processes [35]. In consideration of these outcomes, the present study concentrated on the polymorphisms in ADAM33 gene and their role in COPD and its progression. However, in opposition to the other population results, our data suggest that the polymorphisms studied have an impact on COPD and its progression. This could be due to difference in the genetic background and ethnic variation.

SNPs located in functional domain of the ADAM33 gene contributed to transcription and expression of ADAM33 mRNA and proteins [39]. These expressed proteins finally influence the function of ADAM33 in the pathogenesis of AHR. S1 and S2 are transmembrane polymorphisms located in exon19 encoding the transmembrane domain, are predicted to influence anchoring ADAM33 proteins to the cell membrane. S2 encodes the synonymous exon (Gly717Gly)

Table 2 Frequency distribution of genotypes and of ADAM33 S1 (A/G) genotypes with risk of COPD: The AG and GG genotype frequencies was found to be high when compared to controls and p < 0.001* in co-dominant and dominant models indicating 'G' allele in heterozygous or homozygous form is conferring for the disease, whereas in recessive model p-value (p = 0.085) is not significant. The present study shows a significant association of G allele (**p < 0.001***) of **ADAM33 S1 (A/G)** transdomain polymorphism with COPD patients.

Genotype	Controls (200) N (%)	Patients (150) N (%)	OR (95% CI)	p-Value
AA	108(54.0)	16(10.66)	1-Reference	
AG	41(20.5)	94(62.66)	0.060(0.034–0.122)	**< 0.001***
GG	51(25.5)	40(26.66)	0.188(0.096–0.368)	**< 0.001***
Dominant				
AA	108(54.0)	16(10.66)	1-Reference	
AG + GG	92(46.0)	174(89.34)	0.078(0.043–0.140)	**< 0.001***
Recessive				
AA + AG	149(74.5)	126(73.34)	1-Reference	
GG	51(25.5)	40(26.66)	1.078(0.669–1.737)	0.0851
Allele frequency				
A allele	257 (64.25)	126 (42.0)	1-Reference	
G allele	143 (35.50)	174 (58.0)	0.402(0.296–0.547)	**< 0.001***

There is a statistical difference in the distribution of genotypic and allelic frequencies between the patient and control groups when compared with different models.

* $p < 0.05$.

Table 3 Frequency distribution of genotypes and of ADAM33 S2 (C/G) genotypes with risk of COPD: The CG and GG genotype frequencies was found to be high when compared to controls and $p < 0.05$ in co-dominant and dominant models indicating 'G' allele in heterozygous or homozygous form is conferring for the disease, whereas in recessive model p-value ($p = 0.244$) is not significant. The present study shows a significant association of G allele (**$p < 0.001^*$**) of **ADAM33 S2 (C/G)** transdomain polymorphism with COPD patients. The present study depicts both the ADAM33 S1 and S2 transmembrane domain polymorphisms show significant association for the disease in the studied ethnic group.

Genotype	Controls (200)	Patients (150)	OR (95% CI)	p-Value
	N (%)	N (%)		
CC	124(62.0)	10(6.67)	1-Reference	
CG	32(16.0)	98(65.34)	0.026(0.012–0.056)	**< 0.001***
GG	44(22.0)	42(28.0)	0.084(0.039–0.182)	**< 0.001***
Dominant				
CC	124(62.0)	10(6.67)	1-Reference	
CG + GG	76(38.0)	140(93.33)	0.043(0.021–0.088)	**< 0.001***
Recessive				
CC + CG	156(78.0)	108(72.0)	1-Reference	
GG	44(22.0)	42(28.0)	0.725(0.449–1.180)	0.244
Allele frequency				
C allele	280(70.0)	118(39.34)	1-Reference	
G allele	120(30.0)	182(60.67)	0.277(0.202–0.380)	**< 0.001***

There is a statistical difference in the distribution of genotypic and allelic frequencies between the patient and control groups when compared with different models.

* $p < 0.05$.

which does not change amino acid sequence or missense, but may alter the mRNA folding, mRNA stability and translation process [41]. Further expression studies are warranted to reveal the influence of S2 variation to ADAM33 function. S1 SNP was associated with both excessive FEV1 decline in the general population and development of COPD. The S1 polymorphism causes an amino acid change (valine to isoleucine), but it is unknown whether this also modifies the structure of the protein [40]. Further, the ADAM33 protein may not be anchored correctly in the membrane and therefore may not be able to exert its function. The intracellular domain of ADAM33 is relatively short in comparison with its nearest homologues and is rich in prolines and has a putative SH3 binding site (PsWPLDP) affecting the function [35]. There is an association between ADAM33 and the severity of AHR in COPD. It is an important predictor in association with accelerated FEV1 decline for COPD mortality. The exact pathophysiology underlying AHR in the disease is unclear, but it is thought to result from an inflammatory process in the airways in addition to geometric changes owing to airway remodelling [41]. It is possible that ADAM33 has a role in both these processes thereby contributing to the severity of AHR and COPD and its progression.

5. Conclusion

In conclusion, our study is the first to reveal a significant genetic association of ADAM33 S1-A/G and S2-C/G polymorphic variants in the pathophysiology of COPD in the studied ethnic group.

Conflict of interest

The authors declare no conflict of interest.

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