Original Article

A disintegrin and metalloprotease 33 polymorphism association with COPD in long-term tobacco smokers of the ethnic Kashmiri population of India

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ABSTRACT

Background: Chronic obstructive pulmonary disease (COPD) is characterized by an interaction of various environmental influences especially cigarette smoking and genetic determinants. The prevalence of this disease is ever increasing and characterization of the genetic determinants of the disease has been undertaken globally. The 'A disintegrin and metalloprotease 33' (ADAM 33) gene is one candidate gene that has been studied. **Objective:** Our objective was to investigate whether single nucleotide polymorphisms in ADAM33 gene are associated with COPD in long-term tobacco smokers in the ethnic Kashmiri population of northern India. **Materials and Methods:** This was a randomized case–control study, which included 78 stable COPD (GOLD stage11-IV) patients, who were compared with 77 age- and sex-matched long-term tobacco smokers (>20 pack years) without any evidence of COPD. Polymorphic analysis for three single nucleotide polymorphisms (SNPs), (T1, T2, and Q1) of the *ADAM33* gene was done by polymerase chain reaction-restriction fragment length polymorphism (PCR-RFLP) followed by sequencing. The data were analyzed by descriptive statistics and comparative evaluation was done by parametric/non-parametric tests. **Results:** The analysis of the T1, T2, and Q1 SNPs, revealed that the frequencies of the T2GG, T1GG, and the Q1AG genotypes were significantly higher in patients with COPD in comparison with the controls ($P < 0.001$). Similarly, the T1G and T2G allele frequency was higher in the patients than in the controls ($p = 0.177$ and 0.43, respectively). **Conclusion:** Three SNPs of the *ADAM33* gene were significantly associated with COPD in the Kashmiri population of India. This study establishes the possible role of ADAM33 SNPS in the causation of COPD. Further studies across different geographical areas in the country will unravel the contribution of this gene in the causation of COPD in India.

KEY WORDS: A disintegrin and metalloprotease 33 polymorphism, chronic obstructive pulmonary disease, smoking

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INTRODUCTION

Chronic obstructive pulmonary disease (COPD) is a common disease, which is characterized by persistent airflow obstruction that is usually progressive and associated with an enhanced chronic inflammatory response in the airways and lungs to noxious particles and gases.[1] The global burden of disease estimates suggest that COPD will rise from the sixth to the third-most common cause of death by 2020.[2] COPD is recognized to be a major non-communicable disease in India with huge socioeconomic implications.[3] In a recent survey the prevalence of chronic bronchitis, considered a surrogate for COPD, in India was estimated to be 3.49%.[4] However, in a spirometry-based study in Kashmir we showed that the prevalence of COPD was found to be as high as 19% among participants >40 years of age.[5] Though cigarette smoking has been implicated as a risk factor in upto 80% of the patients,[6] the occurrence of COPD in non-smoking individuals point towards other factors leading to its genesis. In a recent Burden of Obstructive Lung Disease (BOLD) study in Kashmir,[5] non-smoking

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COPD contributed significantly to the total prevalence of COPD among the participants, and even as biomass fuels are considered an important contributor to this population,[7] the presence of the disorder in non-smoking males (less exposed to biomass fuels) points to other factors that could be responsible for the etiopathogenesis of the disorder in our population. A genetic basis for the development of COPD has been suggested based on its more than chance occurrence in first-degree relatives, who are smokers.[8-10] Many candidate genes have been suggested to play a role in the pathogenesis of COPD, secondary to their hypothesized roles in triggering proteolytic and inflammatory pathways, because of inhalation of cigarette smoke or other noxious particles. The candidate genes investigated in case–control genetic association studies have included genes involved in protease–antiprotease pathways, oxidant–antioxidant pathways, and inflammatory response to cigarette smoke.[11-14] Even as many studies are available that point towards the familial aggregation of COPD, severe alpha-1 antitrypsin (AAT) deficiency remains the only proven genetic risk factor for COPD.[15]

Ever since the identification of the A-disintegrin and metalloprotease 33 (ADAM 33) gene, by Van Erdgeweigh and co-workers, as a susceptibility gene for asthma and bronchial hyper-responsiveness,[16] it has been identified as an asthma susceptibility gene in ethnically diverse populations.[17-19] ADAM 33 is a transmembrane metalloproteinase belonging to a subgroup of a zinc-dependent metalloproteinase super-family comprising of over 30 members, which are structurally very complex.[20] The gene is expressed by lung fibroblasts and bronchial smooth muscle cells and is involved in cell adhesion, cell fusion, cell signaling, and proteolysis, by releasing various factors,[21] suggesting its potential role in pulmonary defenses and tissue remodeling. Some studies have linked polymorphisms in ADAM33 to airway hyper-responsiveness and airway inflammation in COPD, and to accelerated lung function decline.[22,23] As low lung function is associated with high mortality risk, particularly on account of COPD, it is important to study the genetic aspects, which increase the susceptibility to COPD and lung function decline.[5]

The present study was undertaken with this background, to ascertain the relationship between *ADAM 33* gene polymorphism and COPD in the ethnic Kashmiri population of India, which had a high prevalence of this disease.[5] This study might give an insight into the genetic basis of this disease and help in exploring newer treatment options in future.

MATERIALS AND METHODS

A prospective randomized case–control study was undertaken in the department of Pulmonary Medicine in Sher-i-Kashmir Institute of Medical Sciences, a tertiary care cum referral University Hospital, in Srinagar, Kashmir. The study subjects included 78 randomly selected, consenting diagnosed cases of COPD, who attended the hospital for routine follow-up, over a period of two years. Pulmonary function tests (PFT) were performed according to American Thoracic Society guidelines[24] and COPD was defined on the basis of a post bronchodilator FEV1/FVC ratio of less than 0.70 and FEV1 <80%. The patients were staged based on the FEV1/FVC ratio according to the GOLD stage.[25] Patients with age >40 years with GOLD stages II–IV, who had 20 or more pack years of smoking were included in the study.

The control group consisted of 77 healthy age- and sex-matched volunteers, documented to have normal PFTs. Healthy controls with a family history of COPD were excluded from the study. Patients with an expected survival of less than one year secondary to cancer or other chronic disease and patients who did not consent to be the part of the study were excluded. The demographic data, smoking history, examination findings, and basic laboratory data, including complete blood count, kidney function test, liver function tests, blood glucose; electrocardiogram, and chest skiagram were recorded in all participants.

A written informed consent was obtained from all the participants and the study was approved by the Ethical Committee of the Institute.

Genetic analysis

Genetic analysis was done for three SNPS (T1, T2, and Q1) of the ADAM 33 gene chosen [Figures 1-3] from the published data showing an association with excessive lung function decline and/or presence of COPD.[16,23] DNA extraction was performed according to the manufacturer's protocol for Qiagen DNA extraction kits (Qiagen, Hilden, NRW, Germany). The DNA content was quantified by spectrophotometric absorption. Polymerase chain reaction (PCR) was performed using an iCycler Thermal Cycler (Bio-Rad, Hercules, CA, USA). Amplifications were carried out and the PCR product was amplified using the specific forward and reverse primers. Primers were designed and selected using Primer 3, version 0.4.0 software. The primer sequence and annealing temperatures for the amplification for different SNPs are given in Table 1. Genotyping of AD-T1 A > G (2280091), AD-T2 A > G (2280090), and AD-Q1 A > G (612709) genotypes were determined by using the PCR-Restriction Fragment Length Polymorphism (RFLP) method. The enzymes and the digested products are given in Tables 1 and 2.

Statistical analysis

The data were analyzed by using the appropriate statistical tests, that is, descriptive statistics and comparative evaluation by parametric/non-parametric tests. The data was described as mean ± SD. Inter- and intra-group comparisons were done by using the students *t*-test. A *p* value of less than 0.05 was considered significant. Data analysis was performed using SPSS version 11.0 version.

RESULTS

The study consisted of 78 COPD patients who were matched for age and sex with 77 healthy controls. The mean age of the patients was 57.7 ± 8.8 years (range 40–80 years) and that of the controls was: 56.5 ± 9.3 (range 40–78 years). Males constituted 70% in the cases and 71.4% in the controls. There was a predominance of rural patients, who were matched for controls, (71.8% in cases and 76.6% in controls). The duration of smoking (pack years) was also similar in both cases and controls (32.6 ± 8.5 and 32.3 ± 8.0, respectively).

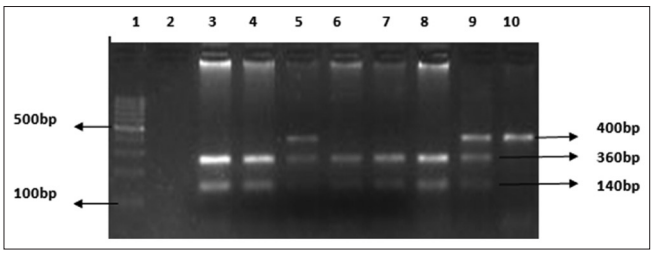

Figure 1: RFLP picture of ADAM 33 T1 SNP A/G after restriction digestion with NcoI (3%) agarose gel electrophoresis. Lane 1–100 bp DNA Marker. Lane 2-Blank. Lanes 5, 9 heterozygous AG (400 and 260 bp, 140 bp), Lanes 3, 4, 6, 7, 8 homozygous wild AA (260 bp, 140 bp), Lanes 10 homozygous GG

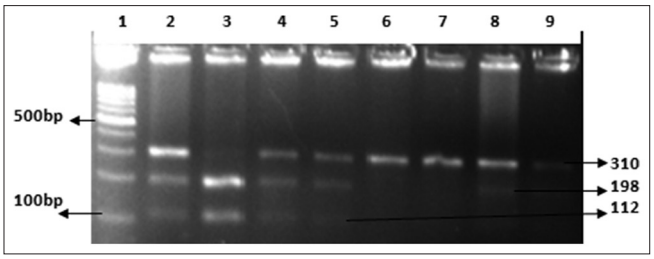

Figure 2: RFLP picture of ADAM 33 T2 SNP A/G after restriction digestion with HpyCH4III (3%) agarose gel electrophoresis. Lane 1: 100 bp DNA Marker. Lanes 6, 7 homozygous GG (312 bp). Lanes 2, 4, 5, 8, 9 heterozygous AG (310 and 198 bp, 112 bp), Lanes 3 homozygous AA (198 bp, 112 bp)

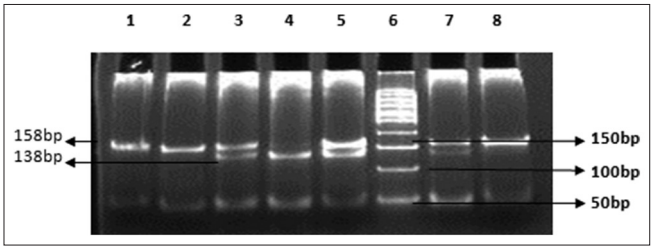

Figure 3: RFLP picture of ADAM 33 Q-1 SNP A/G after restriction digestion with BtsCI (20% PAGE) agarose gel electrophoresis. Lane 6: 50 bp DNA Marker. Lanes 3, 5, 7 heterozygous AG (158 and 138 bp, 20 bp), Lane 4 homozygous AA (138 bp, 20 bp), Lanes 1, 2, 8 homozygous GG (158 bp)

Comparing the genotype and allele frequencies in the two groups, the T2GG genotype, the T1GG genotype, and the Q-1AG genotype were significantly more frequent in the cases than in the controls ($P < 0.001$). However, T2 AA, T1AA, Q1AA, and Q1GG genotypes were found to be expressed more in the controls [Table 3].

The frequencies of alleles, T1G and T2G were higher in the patient group than in the control group. The frequency of alleles T1A and T2A was significantly higher in controls than in cases [Table 4].

DISCUSSION

The present case–control study is the first demonstration of an association between ADAM 33 polymorphisms and COPD in the ethnic Kashmiri population of India. In this association study, we genotyped 78 well-characterized COPD cases and 77 healthy controls, who were long-term tobacco smokers for the three SNPs (T1, T2 and Q-1) of the *ADAM33* gene. The analysis revealed that the SNPs were significantly associated with COPD patients.

In 2002, Van Erdgeweigh and co-workers mapped the ADAM 33 (A distintegrin and metalloprotease 33)[16] gene polymorphism, as a suspected gene for asthma/bronchial hyper-responsiveness on chromosome 20p13, and since then, a number of studies have replicated the association of the ADAM 33 polymorphism with asthma in different populations.[19,26,27]

In addition to the association between asthma and the ADAM33 polymorphism, different alleles have been characterized, which are associated with an excessive decline in lung function.

In a cohort of 200 asthmatic patients followed over 20 years Jongepier *et al*. found that rare alleles of S2, T1, and T2 SNPs of ADAM33, were associated with an excessive decline in FEV1.[26] Simpson *et al*. also found an association of SNPs, F+1, M+1, T1, and T2 of ADAM 33 and impaired early life lung function in a population-based birth cohort of European children.[28] Similarly, Van-Dieman in a cohort

Table 1: Primer sequence of adam33 gene

Reference SNP ID	SNP name	Allele	Primer sequence	Annealing temp.	Amplicon size.(b.p)
2280091	T1	A/G	F-5'ACTCAAGGTGACTGGGTGC-3' R: 5'GAGGGCATGAGGCTCACTTG-3'	60^{0}C	400
2280090	T2	A/G	F: 5'-TTCTCAGGGTCTGGGAGAAA-3' R: 5'-GCCAACCTCCTGGACTCTTA-3'	60^{0}C	310
612709	Q-1	A/G	F: 5'-GGATTCAAACGGCAAGGAG-3' R: 5'-GTTCACCTAGATGGCCAGGA-3'	60^{0}C	158

SNPs: Single nucleotide polymorphisms

Table 2: Enzyme characteristics

SNP name	Enzyme name	Restriction site	Concentration
T1 (rs2280091)	Nco1 (fermentas)	5'..C↓CATGG..3' 3'.GGTAC↓C..5'	A: 140+260 G: 400
T2 (rs2280090)	HpyCH4III (fermentas)	5'ACN↓GT3'3'TG↓NCA 5'	A: 198+112 G: 310
Q-1 (rs612709)	BtsCI (fermentas)	5'GGATGNN↓3'3'CCTAC/NN 3'	A: 20+138 G: 158

SNPs: Single nucleotide polymorphisms

Table 3: Association between ADAM 33 genotypes and COPD

Genotype	Cases *N*=78 (%)	Control *N*=78 (%)	*P* value OR (CI 95%)
T1 SNP			
AA	32 (41)	43 (55.12)	0.09, 0.54 (0.2-1.07)
AG	38 (48.7%)	28 (35.8)	0.07, 1.87 (0.93-3.56)
GG	08 (10.25)	07 (8.97)	0.5, 1.53 (0.50-4.67)
T2 SNP			
GG	29 (37.17)	40 (51.28)	0.29, 0.66 (0.33-1.31)
AG	34 (43.59)	30 (38.4)	0.2, 1.51 (0.7-2.99)
AA	15 (19.23)	08 (10.02)	0.09 , 2.58 (0.96-6.9)
Q-1 SNP			
AA	14 (17.9)	23 (30.7)	0.005,0.29 (0.122-0.69)
AG	36 (46.2)	18 (23.0)	0.008, 3.42 (1.43-8.17)
GG	28 (35.9)	36 (46.15)	0.53, 1.33 (0.58-3.03)

SNP: Single nucleotide polymorphism, OR: Odds ratio, CI: Confidence interval, COPD: Chronic obstructive pulmonary disease

Table 4: Association between Allele frequency and COPD

Group	No	Genotype			Allele frequency		*P* value
		AA	AG	GG	A	G	
T1 SNP							
Patient	78	32	38	08	0.65	0.35	0.177
Control	78	43	28	07	0.73	0.27	
T2 SNP							
Patient	78	29	34	15	0.58	0.41	0.43
Control	78	40	30	08	0.70	0.29	
Q-1SNP							
Patient	78	14	36	28	0.41	0.59	0.9
Control	78	23	18	36	0.42	0.58	

SNP: Single nucleotide polymorphism

of 1390 subjects in the general population, found that four SNPs (F+1, S1, S2, T2) were significantly associated with accelerated lung function decline.[23] Various other studies have also shown that various SNPs in ADAM33 were associated with reduced lung function and COPD included T1, ST+5, T2, Q1, S1, S2, V1, and V4.[22,29]

There are only few studies that have shown a relationship between ADAM 33 gene variation and COPD. In the Dutch general population, Van Diemen and colleagues genotyped SNPs in ADAM 33 and found that the subjects homozygous for the minor alleles of SNPs, S2 and Q1, and heterozygous for SNP S1, have an excessive annual decline in FEV1 compared to the wild-type. They also saw a significantly greater frequency of minor alleles of SNP F+1, S1, S2, and T2 in COPD subjects compared to the entire general population. This was the first study of its kind to show an association between ADAM33 and COPD.[23] Later, Gosman found an association between SNPs (ST+5, T1, T2, and S2) with airway hyper-responsiveness, higher number of sputum inflammatory cells, and CD8 cells in bronchial biopsies, indicating that the gene was associated with the pathophysiology of COPD.[22] Sadeghnejad, in his case–control study, also demonstrated the association of SNPs (Q-1, S1, S2, V1, V4) with COPD and lung function decline in long-term smokers, in the Caucasian population.[29] Similar observations were also made by Wang *et al*. in the Chinese Han population for four SNPs (T2G, T1G, S2C, and Q-1G alleles ($P < 0.001$).[30] In our study, we observed that compared to the wild allele, heterozygous AG and homozygous GG genotypes of T1 SNP, homozygous GG genotype of T2 SNP, and heterozygous AG genotype of the Q-1 SNP were distributed significantly in higher ($P < 0.05$) frequency among cases when compared to controls; suggesting the increased susceptibility among carriers of these genotypes to develop COPD.

In a recent study, Jun-lung Xiao *et al*. found an association of four SNPs (V4, T2, T1, and S1) in long-term smoking COPD patients in the Tibetan population of China,[31] which was again comparable to our study. We also observed that the homozygous AA genotype of the T1 and T2 SNPs and the GG genotype of Q-1 were significantly higher in the controls pointing toward a possible protective role in the development of COPD. We were able to replicate the study by Wang *et al*. for the association of SNPs with COPD, but the other studies differed in terms of a positive association with all the SNPs.[16,23,29] Some studies showed no association of the T1 and T2 SNPs, as was also shown in our study, but they demonstrated this association in other SNPs.[16]An association of the q-1 SNP of ADAM33 and COPD was shown in these studies.[16,23,29]

T1 and T2 SNPs are located in exon 19 (which included an SH3 domain and a phosphorylation site) of the cytoplasmic tail, which could affect signaling. The SNP Q-1 located in the intron immediately before exon 16, containing an epidermal growth factor (EGF) domain,[32] which is important in lung morphogenesis.

These inconsistencies in replication could be on account of population heterogeneity, as observed by some studies.[19,33] Thus, differences in the haplotype structure or even in the occurrence of SNPs may exist between ethnicities, which have not yet been investigated sufficiently for ADAM33, but which are known to exist for a number of other genes.

Differences in the study populations in terms of genes, by unknown environment interactions may also explain some of the observed discrepancies in replication, as has been suggested to be the case with other genes being inconsistently replicated.[34] In addition, ethnicity-specific differences with environmental or genetic risk factors may account for the observed differences.[35]

CONCLUSION

There are many studies[26,27] that show a relationship of asthma with ADAM 33 SNPs, but to date only few studies[22,29]have shown an association of COPD with

ADAM33. In these studies, some common SNPs are associated with both the diseases, suggesting that they contribute to accelerated airway dysfunction in various disease progressions. However, some specific SNPs are only associated with one disease. This phenomenon is consistent with the pathogenetic mechanisms of the two diseases. At present, the mechanistic roles of the disease-associated SNPs have yet to be elucidated, especially in the context of the pathophysiology of asthma and COPD.

There are some potential limitations of the study, as the number of patients studied was small and we could include only few SNPs in our study. Genome-wide association studies are needed to fully elucidate the potential candidate genes implicated in the genesis of COPD in our population.

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