

Full Length Research Paper

Alpha 1 antitrypsin gene: A case-control study in chronic obstructive pulmonary disease

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Accepted 17 November, 2011

Alpha-1 antitrypsin (AAT) deficiency is an inherited disorder that causes low levels of, or no AAT in the blood. The most common illness in adults with AAT deficiency is lung disease during the third and fourth decades of life. Most commonly, it is associated with chronic obstructive pulmonary disease (COPD). Mutations in the protease inhibitor (PI) gene, located on chromosome 14, are associated with this genetic disorder. The Z protein is due to a single amino acid substitution of 342 glutamine lysine. Although cigarette smoking is the main environmental risk factor, only about 15% of smokers develop clinically significant disease suggesting other influences on disease expression. The study included hospital based age and sex matched 100 cases of COPD and 100 controls without COPD recruited from Christian Medical Centre, Jorhat, Assam. These cases were recruited from February 2009 to December 2009. Subjects were included in the COPD group on the basis of lung function test. DNA extraction was done by DNA extraction kit and amplification for AAT gene was done by site directed mutagenesis polymerase chain reaction (PCR) method as described by Tazellar et al. (1992). We found that smoking was the prior cause of COPD. A1AT deficiency is not prevalent in our population subset but certain other genes could be the attributable factor for COPD.

Key words: Alpha 1-antitrypsin, chronic obstructive pulmonary disorder (COPD).

INTRODUCTION

One of the most significant breakthroughs in the field of chronic obstructive pulmonary disease (COPD), in the past 30 years was the discovery of a close association between an inherited deficiency of a protein in the blood called the Alpha-1- antitrypsin (AAT). It is the only known genetic disorder that leads to COPD. AAT deficiency

accounts for less than one percent of COPD in USA (ATS, 1995). This deficiency is an autosomal hereditary disorder in which there is low level of AAT in serum and lung, with a high risk of development of panlobular emphysema in the third to fifth decade. The enzyme is synthesized and secreted by the hepatocytes and to a lesser extent by the mononuclear phagocytes, and then is released in to the blood from which it then diffuses in to the lungs (Crystal et al., 1990). AAT is a glycoprotein coded for by a single gene on chromosome 14; it is a serine protease inhibitor with primary function of inhabiting neutrophil elastase. Emphysema results from an imbalance between the neutrophil elastase in the lung and the anti-elastase. While the former has the capability of destroying elastin and other tissue components, the

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Abbreviations: AAT or A1AT, Alpha-1-antitrypsin; COPD, chronic obstructive pulmonary disease; FEV1, forced expiratory volume in one second; FVC, forced vital capacity.

later is responsible for protecting the lung from elastase. This concept is known as the “elastase –anti elastase balance hypothesis of emphysema.” The concept has been proved both in humans and animal experiments. According to the theory, either an excess of protease or a deficiency in the amount of functional activity of anti-protease or both can lead to the development of emphysema.

At least 75 alleles of the AAT gene have been identified (Guidelines for the approach to the patient with severe hereditary AAT deficiency, 1995) and categorized in to the protease inhibitor (PI) system.

The normal and deficient AAT alleles are designed from A to Z on the basis of their electrophoretic mobility. The families of normal AAT alleles are referred to as M (M1, M2, and M3) and are found in approximately 90% of the population. The most deficient allele associated with emphysema is the Z allele. The AAT phenotype, therefore, is made up of the two parental alleles and is referred to as Pi phenotype. AAT variants are inherited as co-dominant alleles. The most common phenotype is PiMM (PiM2M3) and the most common deficient phenotype associated with a high risk for the disease is PiZZ. The specific mutations responsible for many forms of AAT deficiency have been identified. The abnormal Z allele is associated with replacement of glutamic acid by lysine at position 342 as a result of a single base mutation from GAG to AAG. This substitution results in alteration of the three dimensional configuration of the molecule, thus it aggregates in the rough endoplasmic reticulum of the hepatocyte and consequently a decreased secretion of AAT occurs from the liver to about 15% of the normal (Weinberger et al., 1993; Crystal et al., 1990).

Epidemiological studies indicate that a threshold value of 11 α M or about 35% of the average normal level is sufficient to protect the lungs. It follows therefore, that individuals who are at greater risk are PiZZ homozygotes, the null homozygotes, and, occasionally PiSZ heterozygotes. Severe AAT deficiency lead to premature emphysema of the panacinar type with more severe affection at the bases, and is often associated with chronic bronchitis and occasionally with bronchiectasis (Snider et al., 1989). Individuals with a PiZZ phenotype who smoke cigarettes are at increased risk, therefore becoming symptomatic earlier with dyspnea occurring at a median age of about 40 years. Smokers with AAT deficiency and COPD will have a life expectancy that is approximately 10 years less than the nonsmokers with this condition. Severity of lung disease varies considerably. Patients, who are detected on population surveys, only live longer to the age of 80 or 90 years. Airflow obstruction occurs more in men. Other risk factors are asthma, recurrent respiratory infections and familial factors (Silverman et al., 1989).

Many Indian studies have tried to examine the role of AAT deficiency in the causation of COPD and is summarized by Malik et al. (1977). The heterozygote state

(intermediate) was found to be 10.3 to 23.3% and homozygous (severe) state in 2.8 to 20% of cases of COPD. The present study was undertaken at Jorhat, Assam, to screen the A1AT gene for carriers of ZZ allele, in individuals with COPD against controls.

Study design

The study included hospital based age and sex matched, a 100 cases of COPD and 100 controls without COPD recruited from Christian Medical Centre, Jorhat, Assam. These cases were recruited from February 2009 to December 2009. Data was abstracted from the medical records of participants regarding the diagnosis of COPD based on pre and post bronchodilator spirometry, radiological examination, history of smoking and occupational exposure. Informed consent was taken from all the individuals participating in the study. The study was carried out at CSIR (NEIST), Jorhat after ethical clearance from Institutional Ethics Committee NEIST, Jorhat.

MATERIALS AND METHODS

Diagnosis and inclusion criteria of subjects

Altogether, 200 individuals (134 males and 66 females) were enrolled in our study. Spirometry was conducted with Spirometer (Model Schiller, USA) for each subject and the data for forced vital capacity (FVC) and forced expiratory volume in one second/forced vital capacity (FEV1/FVC) were recorded. Inclusion criteria comprises of the following symptoms: chronic airway symptoms and signs such as coughing, breathlessness, wheezing and chronic airway obstruction defined as FEV1/FVC of 70% and below, and an FVC of 80% of the predicted value. At the baseline, pre-bronchodilator spirometry was done for all the subjects. Then we tested for bronchodilator reversibility where each individual was first administered with four puffs of Metered dose inhaler (100 μ g/puff) of Salbutamol. They were allowed to rest for 15 mins and post-bronchodilator spirometry was performed. Irreversible airflow limitation was confirmed when FEV1 was less than 12%. These persons were included in symptomatic COPD group. Prior to spirometry, we performed radiological examinations of these patients to rule out the existence of any structural lung disease. The criteria for selection of asymptomatic (Non-COPD) subjects were on the basis of non-existence of respiratory symptoms. The spirometry of these subjects was above 80% of the predicted value. Based on survey data and lung function analysis, the subjects were sorted as:

- (a) Non-COPD (Smokers, n = 62 and non-smokers, n = 38)
- (b) COPD (Smokers, n = 78 and non-smokers, n = 22).

Blood sample collection

An amount of 3 cc of whole blood was drawn from each individual by venipuncture and stored in ethylene diamine tetra-acetic acid (EDTA) vials. The vials were stored at 4°C until DNA extraction.

DNA extraction and PCR amplification

Genomic DNA was extracted from whole blood using GeNei™

whole blood DNA extraction kit, Bangalore Genei, India. PCR amplification for carriers of ZZ allele for AAT gene was done by site directed mutagenesis PCR method as described by Tazellar et al. (1992) with slight modifications. Subsequently, restriction digestion was done to distinguish the normal MM allele from the mutant ZZ allele.

Forward primer: ATAAGGCTGTGCTGACCATCGTC
Reverse primer: CTTTTCACCACTTAGGGTGGGT

All amplifications were started in a 50 µl reaction volume containing 25 µM deoxynucleotide triphosphate [dNTP (Bangalore GeNei, India)], 2 mM magnesium chloride (MgCl₂) (Bangalore GeNei, India), 20 pM of each primer synthesized by Sigma Aldrich (USA), 200 ng DNA, 2.5 U Taq polymerase (Sigma Aldrich, USA). An initial denaturation was carried out at 94°C for 10 min, amplification was carried out for 30 cycles, each cycle consisted of 2 min denaturation time at 94°C, 2 min annealing time at 52°C and 3 min extension time, at 64°C followed by a final extension of 10 min at 72°C. The PCR reaction was done in a thermal cycler (Applied Biosystems, USA, model 2720).

Restriction enzyme digestion and electrophoresis

10 µl of all the PCR products were then digested with Taq I (50 U) restriction enzyme (Sigma Aldrich, USA) diluted with 1x endonuclease buffer (Sigma Aldrich, USA) and volume made up to 20 µl. This digestion mixture was incubated at 65°C for 2 h according to manufacturer's instructions. Finally, the digested products were analysed in a 3% agarose gel (Amresco Superfine Resolution Grade, USA) in 89 mM Tris-borate buffer containing 1 mM EDTA, pH 8.3 at constant 120 V for 1 h.

Sequencing of AAT gene

The PCR products, one each from COPD smoker, non-COPD smoker, non-COPD non-smoker and COPD non-smoker, were sequenced at Vimta labs, Hyderabad, India. The DNA sequencing samples were processed using ABI 3130 (4 capillary) or 3730 XI (96 capillary) electrophoresis instruments. We have used EMBOSS needle (global) pairwise sequence alignment algorithm to find the mutation if any at the position Glu 342 GAG Lys → AAG of the sequence.

Statistical analyses

Statistical analysis was carried out using chi-square test for categorical variables and students t-test for continuous variables. Data were tabulated and classified as per the age, sex, smoking and lung function status. Chi-square test with Yates correction was applied to test significant difference in the number between smoker and non-smokers, males and females amongst COPD and non-COPD subjects. Unpaired t-test was applied to test for significant difference in smoking (pack-years), age, and lung function amongst the COPD and non-COPD subjects.

RESULTS

Demographic observations

Detailed observations in demographic variables are

shown in Table 1. The occurrence of COPD was significantly higher in male smokers [$X^2 = 2.87$, $p = 0.09$, OR = 1.84 (0.8 - 3.96)] than female smokers [$X^2 = 0.12$, $p = 0.72$, OR = 1.23 (0.34 - 4.61)]. There was no significant difference of age in the occurrence of COPD in smokers and non-smokers compared to non-COPD smokers and non-smokers. The lung function status was significantly different amongst COPDs and non-COPDs in both smoker [$t = 18.37$, $df = 138$, $p = 0.006$] and non-smoker group [$t = 13.59$, $df = 58$, $p = 0.001$].

PCR and restriction enzyme analysis

PCR amplification showed characteristic 179 bp band (Figure 1) indicating the presence of homozygous 'MM' type in all the samples. On restriction digestion, a band was observed at 157 bp in all the samples. As such, there was no ZZ mutation in these subjects in their A1AT gene. Since all the samples were homozygous 'MM' type, our data did not fit the 'Hardy-Weinberg equation'. Sequencing of the A1AT gene also agreed to the findings of PCR and restriction enzyme analysis. The sequences obtained were aligned with the mRNA of normal (MM type) ATT gene as mentioned in materials and methods. No difference was found in position Glu 342 GAG Lys AAG in COPD smoker, COPD non-smoker, non-COPD smoker and non-COPD non-smoker. Details of the sequences for various samples are shown in sequencing results in Figures 2 to 5.

DISCUSSION

Amongst many risk factors of COPD, the genetic deficiency of A1AT attributed to ZZ type is the best documented reasons (Carp and Janhoff, 1978). Phenotype M is the normal variant, phenotypes S and Z are the two most frequent abnormal variants (Hutchinson et al., 1998). Calculated values of PiZZ prevalence are approximately, 1:1000 to 1:45,000 in Western and Northern Europe, 1:45,000 to 1:10,000 in Central Europe; and 1: 10,000 to 1:90,000 in Eastern Europe and in Southernmost and Northern areas of the continent. In the White population of USA, Canada, New Zealand, PiZZ phenotype prevalence ranges from 1: 2000 to 1:7000 individuals (Andolfatto et al., 2003) which suggests that the frequency of ZZ carriers are quite low in different parts of the world. The data of prevalence of COPD in Asian countries is patchy and the disease burden is high. Studies on COPD in the Indian population are very limited. There are very less data on genetic epidemiologic studies of AAT deficiency in countries like India; and thus it is essential to ascertain whether populations are at risk or not. Considering these aspects, the prevalence of COPD in a subset of Indian population was studied by us and presence of normal homozygous MM allele in all the categories individuals that were studied. The frequency of

Table 1. Demographic observations and lung function status of the study population.

Variables	Study population			
	COPD smokers (N = 78)	COPD non-smokers (N = 22)	Non-COPD non-smokers (N = 38)	Non-COPD smokers (N = 62)
^a Male	56	16	26	36
^b Female	22	6	12	26
Sex ratio (F/M)	0.39	0.37	0.46	0.72
^c Smoking (pack years \pm S.D)	13.63 \pm 5.41			5.79 \pm 1.79
^d Mean age (\pm S.D)	43.47 \pm 10.49	37.59 \pm 5.52	39.94 \pm 5.71	34.01 \pm 8.45
^e FEV1/FVC (% predicted) (\pm S.D)	63.75 \pm 8.82	54.31 \pm 11.88	94.39 \pm 10.46	93.75 \pm 10.48
^f Post bronchodialator FEV1 (liters)	0.80 \pm 0.13	0.87 \pm 0.11		

^aCOPD Smokers vs. non COPD smokers* : $\chi^2 = 2.87$, $p = 0.09$, OR = 1.84 (0.86 - 3.96); ^aCOPD non Smokers vs. non COPD non smokers : $\chi^2 = 0.12$, $p = 0.72$, OR = 1.23 (0.34 - 4.61); ^bCOPD smokers vs. non COPD smokers* : $\chi^2 = 2.87$, $p = 0.09$, OR = 0.54 (0.25 - 1.17); ^bCOPD non smokers vs. non COPD non smokers : $\chi^2 = 0.12$, $p = 0.72$, OR = 0.81 (0.22 - 0.98); ^cCOPD smokers vs. non COPD smokers* : $t = 11.99$, $df = 138$, $p = 0.003$; ^dCOPD smokers vs. non COPD smokers : $t = 0.80$, $df = 138$, $p = 0.42$; ^dCOPD non smokers vs. non COPD non Smokers : $t = 1.55$, $df = 58$, $p = 0.12$; ^eCOPD smokers vs. non COPD smokers* : $t = 18.37$, $df = 138$, $p = 0.006$; ^eCOPD non smokers vs. non COPD non smokers* : $t = 13.59$, $df = 58$, $p = 0.001$.

*Statistically significant.

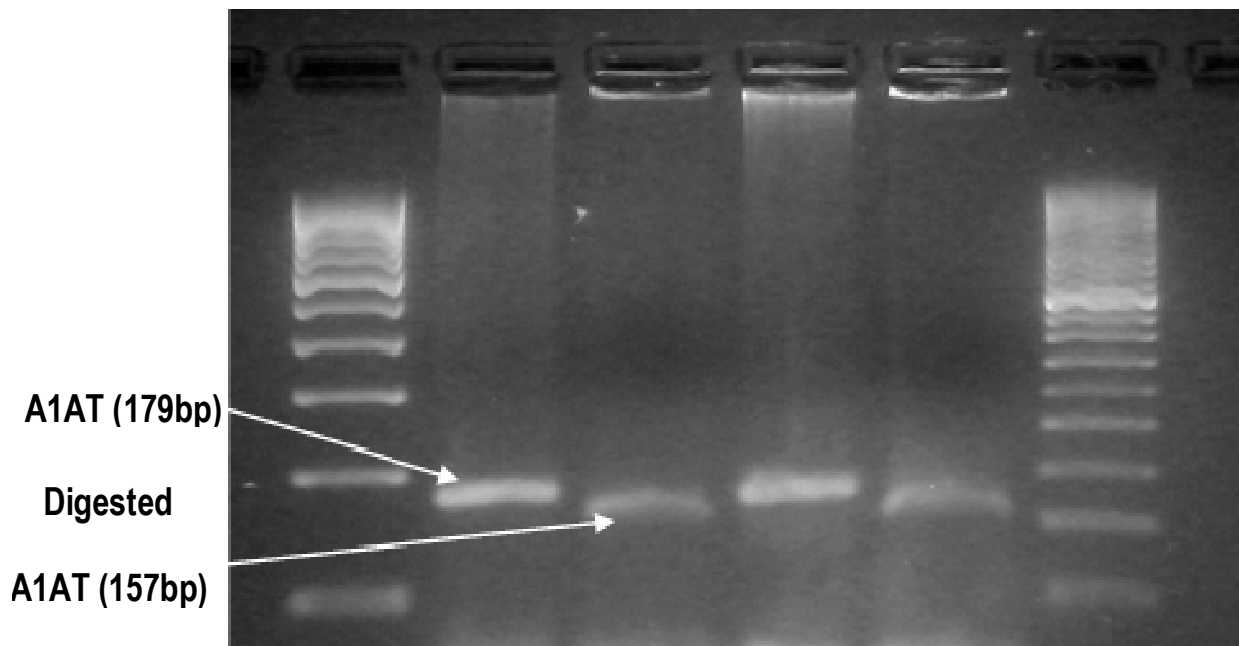


Figure 1. Detection of A1AT gene by site directed mutagenesis PCR method. Lanes 1, 2 A1AT gene 179 bp normal (MM type). The primers used to amplify the sequence that included the Z mutation site yielded a product of the correct size (179 bp) in all cases. Subsequently, PCR products were digested with *Taq* I enzyme. The normal fragment was 157 bp long.

ZZ carriers of A1AT gene is rare as already documented (Carp and Janhoff, 1978; Hutchinson et al., 1998 and Andolfatto et al., 2003) and we also did not find any ZZ carriers in our study population. The impression that AAT deficiency is a rare disease has resulted into infrequent orders for the tests of AAT deficiency even for many white patients who complain of allergy, asthma or pul-

monary problems. The findings on the prevalence of AAT deficiency worldwide are expected to affect the diagnosis of people with AAT deficiency by the medical community (Serres et al., 2003). In this study, we found that the lung function was significantly declined in both COPD smokers ($t = 18.37$, $df = 138$, $p = 0.006$) and COPD non-smokers ($t = 13.59$, $df = 58$, $p = 0.001$) compared to non-COPDs.

GGTGCATTCATCTTGCGCCCTTGATCATCATTAAAGAAGCAAAGGGTTTGTGAACTTGACCTCGGGGGGGATAGA
 CA
 TGGGTATGGCCTCTAAAAACATGGCCCCAGCAGCTTCAGTCCCTTTCTCGACGATGGTCAGCACAGCCTAAAAA



Figure 2. COPD smoker.

Smoking itself is documented to be one of the risk factors for COPD and interestingly only 10 to 15% develop clinical symptoms of COPD (Hogg et al., 2004). There was a significant association of smoking towards

the occurrence of COPD [$\chi^2 = 6.10$, $p = 0.01$, $OR = 2.17(1.12- 4.25)$]. The COPD smokers were found to have higher number of pack years of smoking ($t = 11.99$, $df = 138$, $p = 0.003$); and were 2.1 times at higher risk to

AGTCCCCCATCACTCTTAAGTCCTTCTATCATCATTAATAAGACAAAGGGTTTGTGAACTTGACCTCG
 GGGGGGATAGACATGGGTATGGCCTCTAAAAACATGGCCCCAGCAGCTTCAGTCCCTTTCTCGACGAT
 GGTCAGCACAGCCTTAAAAACGCTC

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EMBOSS_001      1 -----TGCATAAGGCTGTGCTGACCATC-----GACGGAGAAA
32
                | |.|||||. | .|.|| |.|||| | |.|||.|||
EMBOSS_001      1 AGTCCCCCATCACT-CTTAAGTC-CTTCT-ATCATCATTAAGAAGGACAAA
47
EMBOSS_001      33 GGG---ACTGAAGCTG--CTGGGGCCATGTTTTTAGAGGCCATAACCCATG
77
                |||  ..|||||. || | |.|||| | |..|||. |||||
EMBOSS_001      48 GGGTTTGTGAACTTGACCTCGGG-----GGGGATAGACATG
84
EMBOSS_001      78 TCTATCCCCCCCAGGTTCAAGTTCAACAAA-----CCCTTTGTCTTCTT
121
                ..|||. ||| | |.|.||| | |..|||. |||||
EMBOSS_001      85 GGTATGGCC-----TCTAAAAACATGGCCCCAGCAGCTTC--
119
EMBOSS_001      122 AATGATTGAACAAAATACCAAGTCTCCCCTCTTCATGG--GAAAAGTGGT
169
                |||  |||| | || |. | |  |||  ||||
EMBOSS_001      120 -----AGT---CCCT-TTC-TCGACGA----TGGT
140
EMBOSS_001      170 GAATCCCACCC--AAAATAACTGCCTCTCGCTCCTCAACCCCTCCCCTC
217
                .|.|.|||. || | | | | | | | | | |
EMBOSS_001      141 -CAGCACAGCCTTAAAAA-----CGCTC-----
162
EMBOSS_001      218 CATCCCTGGCCCCCTCCCTGGATGACATTAAGAAGGGTTGAGCTGG      264
EMBOSS_001      163 -----
162

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Figure 3. COPD non-smoker.

develop COPD. Male COPD smokers were significantly 1.8 times at risk of developing COPD [$X^2 = 2.87$, $p = 0.09$, OR = 1.84(0.86 – 3.96)]. Amongst female smokers with COPD the association of smoking was also found to be significant [$X^2 = 2.87$, $p = 0.09$].

In the entire study, it was found that smoking was the prior cause of COPD and A1AT deficiency is not prevalent in the population subset. In addition to this, our study reveals that other genes besides A1AT (ZZ type) could be responsible for the prevalence of COPD.

CCGGGATCCGGACTTCCCTCCTTGATCATCATTAAAGAAGACAAAGGGTTTGTGAACTTGACCTCGGGGGGATA
 GACATGGGTATGGCCTCTAAAAACATGGCCCCAGCAGCTTCAGTCCCTTTCTCGACGATGGTCAGCACAGCCTTA
 TAAA

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EMBOSS_001      1  --TGCATAAGG-----CTGTGCTGACCATC-----GACGAGAAAGG
34
                .|.|||.|||      ||      |||.|||      |||.|||
EMBOSS_001      1  CCGGGATCCGGACTTCCCTCCT----TGATCATCATTAAAGAAAGACAAAGG
46
EMBOSS_001      35  G---ACTGAAGCTG--CTGGGGCCATGTTTTTAGAGGCCATACCCATGTC
79
                |  ..|||..|||  ||.|||      ||..|||..|||..
EMBOSS_001      47  GTTTGTGAACTTGACCTCGGG-----GGGGATAGACATGGG
83
EMBOSS_001      80  TATCCCC-----CCCAGGTCAAGTTCAACAAACCCTTTGTC
116
                |||.|||      |||.|||  ||..|||      |||
EMBOSS_001      84  TATGGCCTCTAAAAACATGGCCCCAG--CAGCTTCA-----GTC
120
EMBOSS_001     117  ---TTCTTAATGAT--TGAACAAA-----ATACCAAGTCTCCCCTTTCA
156
                |||..|.|||  |..|||.|      |||  ||
EMBOSS_001     121  CCTTTCTCGACGATGGTCAGCACAGCCTTATA--AA-----
154
EMBOSS_001     157  TGGGAAAAGTGGTGAATCCCACCCAAAATAACTGCCTCTCGCTCCTCAA
206
EMBOSS_001     155  -----
154
EMBOSS_001     207  CCCCTCCCCTCCATCCCTGGCCCCCTCCCTGGATGACATTAAAGAAGGGT
256
EMBOSS_001     155  -----
154
EMBOSS_001     257  TGAGCTGG      264
EMBOSS_001     155  -----      154

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Figure 4. Non-COPD smoker.

Moreover, COPD is polygenic and prevalence rates are different in different ethnic groups. Worldwide, the ZZ carriers are mostly found in the Caucasians of European ancestry (Povey, 1990). Most of the people studied by us are of the tribal community belonging to Mongoloid origin

and as such they are different from the rest of the Indian population. We suggest that further studies should be carried out to investigate individual susceptibility to COPD due to genetic factors or other environmental reasons such as air pollution, occupational exposure, etc.

CTCCTGTCATCTTGCGTCCTTGATCATCATTAAGAAGACAAAGGGTTTGTGAACTTGACCTCGGGGG
 GGATAGACATGGGTATGGCCTCTAAAAACATGGCCCCAGCAGCTTCAGTCCCTTTCTCGACGATGGTC
 AGCACAGCCTTATAAACGGAAC

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EMBOSS_001      1 ----TG-CATAAGGCTGTGC-----TGACCATC-----GACGAGAAAGGG
35
                || |||  || |||  |||.|||  ||.||.|||||
EMBOSS_001      1 CTCCTGTCAT----CT-TGCGTCCTTGATCATCATTAAGAAAGACAAAGGG
45

EMBOSS_001      36 ---ACTGAAGCTG--CTGGGGCCATGTTTTTAGAGGCCATACCCATGTCT
80
                ..||||..||  ||.|||  ||..|||.|||..|
EMBOSS_001      46 TTTGTTGAACTTGACCTCGGG-----GGGGATAGACATGGGT
82

EMBOSS_001      81 ATCCCC-----CCCGAGGTCAAGTTCAACAAACCCCTT-----
113
                ||..||  |||.||  ||..||  ||..|||||
EMBOSS_001      83 ATGGCCTCTAAAAACATGGCCCCAG--CAGCTT---CAGTCCCTTTCTCG
127

EMBOSS_001      114 -----GTCTTCTTAATGATTGAACAAAATACCAAGTCTCC
148
                |.|||||.|||.|  ||||
EMBOSS_001      128 ACGATGGTCAGCACAGCCTTATAAACG---GAAC-----
158

EMBOSS_001      149 CCTTTCATGGGAAAAGTGGTGAATCCCACCCAAAATAACTGCCTCTCG
198

EMBOSS_001      159 -----
158

EMBOSS_001      199 CTCCTCAACCCCTCCCCTCCATCCCTGGCCCCCTCCCTGGATGACATTAA
248

EMBOSS_001      159 -----
158

EMBOSS_001      249 AGAAGGGTTGAGCTGG      264

EMBOSS_001      159 -----      158
#-----
#-----
  
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Figure 5. Non-COPD non-smoker.

ACKNOWLEDGEMENT

The authors are thankful to CSIR, New Delhi, for financing the research work under Network Project. The author is also thankful to Miss Ola lyabo Olunike for proof reading the article for English.

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