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Full Length Research paper

Lung function decline: Screening of alpha-1 antitrypsin gene in a population exposed to coal dust

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Alpha-1 antitrypsin (AAT) deficiency is an inherited disorder that causes low levels of, or no alpha-1 antitrypsin in the blood. Most commonly, it is associated with chronic obstructive pulmonary disease (COPD). COPD includes chronic bronchitis and emphysema chronic bronchitis - inflammation of the lining of the bronchial tubes emphysema - permanent destruction of the alveoli. Mutations in the 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 to lysine .Chronic respiratory diseases have a pre-eminent role in the health conditions of people residing near coalmine areas with implications for morbidity and excess mortality from specific causes. We screened 412 individuals (COPDs and Non-COPDs) for carriers of deficient ZZ allele of AAT gene at the coal mine site, Assam. DNA extraction was done by standard phenol chloroform method and amplification for Alpha-1-antitrypsin gene was done by site directed mutagenesis PCR method. Coal dust exposure was a potential factor in development of COPD. AAT deficiency was not found to be present in our study population.

Key words: COPD, A1AT gene, Coal Dust, ZZ type, air pollution.

INTRODUCTION

A widely accepted definition from Global Initiative for Obstructive Lung Disease (GOLD) defines Chronic Obstructive Pulmonary disease (COPD) as “a disease state characterized by airflow limitation that is not fully reversible. The airflow limitation is usually both progressive and associated with an abnormal inflammatory response of the lungs to noxious particles or gases” (Pauwels et al., 2001). One of the most significant breakthroughs in the field of 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 α1- antitrypsin (AAT). It is the only known genetic disorder that leads to COPD. AAT deficiency accounts for less than 1% of COPD in USA (ATS, 1995). Alpha -1- Antitrypsin deficiency is one of the mostcommon serious hereditary disorders in the world, because it effects all major racial subgroups, and there is an estimated 120.5 million carriers and deficient subjects in the 58 countries surveyed worldwide (de Seres, 2002). Alpha -1-Antitrypsin (AAT) is a 52 kDa alpha-1-glycoprotein, composed of 394 amino acid residues and three asparagines-linked complex carbohydrate side chains (Brantly et al,, 1988). It is produced mainly by hepatocytes and secreted into the blood, where it acts as a circulating serine protease inhibitor whose principal substrate is neutrophil elastase (NE) (Pierce, 1988). The AAT gene is located on the long arm of chromosome 14, has been mapped to chromosome 14q31 - 32.3 (Byth et al, 1994). The normal gene is designated PiM and about 100 normal and defective genetic variant is recognizable

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List of Abbreviations: COPD: Chronic Obstructive Pulmonary Disease; **A1AT/AAT:** Alpha 1 Antitrypsin; **GOLD:** Global Initiative for Obstructive Lung Disease; **DALY(s):** Disability Adjusted Life Year(s); **GBDS:** Global Burden of Disease Study; **ATS:** American Thoracic Society; **API:** Antiprotease Inhibitor; **OR**: Odds Ratio; **FEV1/FVC**: Forced Expiratory Volume in one Second/Forced Vital Capacity.

are recognizable by isoelectric focusing (IEF) (WHO, 1996). Intermediate and severe alpha -1-antitrypsin deficiency is almost entirely caused by the Z and S alleles as opposed to the wild-type M allele in the alpha -1-antitrypsin gene: individuals with six different genotypes, ZZ, SZ, MZ, SS, MS, and MM, have relative plasma alpha -1-antitrypsin concentrations of almost 16, 51, 83, 93, 97, and 100% respectively (Brantly et al., 1991). The families of normal α_1-antitrypsin alleles is referred to as M (M1, M2, and M3) and are found in approximately 90% of the population. The most deficiency allele associated with emphysema is the Z allele. The specific mutations responsible for many forms of α_1-antitrypsin deficiency have been identified. The abnormal Z allele is associated with replacement glutamic acid by lysine at position 342 as a result of a single base mutation from GAG to AAG (Nukiwa et al.,1986).This substitution results in alteration of the three dimensional configuration of the molecule.

Chronic Obstructive Pulmonary Disease (COPD) is one of the leading causes of mortality. By 2020, it is expected to rise to the third position as a cause of death and at fifth position as the cause of disability adjusted life years (DALYs) as per projections made in Global Burden of Disease study (GBDS) (Jindal, 2006). Chronic irritation of the airways by inhaled substances such as cigarette smoke is the major known risk factor for COPD. However it has recently been estimated that 15 to 19% of COPD in smokers may be attributed to occupational exposures (Balmes et al., 2003; Hnizdo, 2002). Very less information on the disease is available from India. Screening of AAT deficiency in every community is essential for enhancing awareness of this disorder among health-care givers and the general public (WHO 1996, Carrell and Lomas, 1982) and also for planning health policy and financial medical resources and to their utilization by the scientific community, governments and the pharmaceutical industry. The diagnosis of this deficiency is relatively simple, but many population studies have indicated that AAT deficiency is under diagnosed and delay in its diagnosis is frequent (Mc Elvaney et al., 1997). The importance of dust exposure specifically, mineral dusts in the underground miners for the development of respiratory symptoms, airflow obstruction, and COPD has been well established (Becklake, 1989; Oxman et al., 1993).The present study was undertaken to screen the A1AT gene encoding the Antiprotease Inhibitor (API) protein, alpha 1 antitrypsin amongst the COPDs or non-COPDs indentified at open-cast coal mine site, Ledo.

MATERIALS AND METHODS

Study design

Prior to survey in the study site, we had conducted air analysis for parameters such as Respirable Suspended Particulate Matter (RSPM), SO_2 and NO_2 and we established that all these parameters were very high throughout the year 2009 and 2010 (Bhattacharjee, 2011) thus, indicating that the people living near the coal mine had a cumulative exposure to coal dust, SO_2 and NO_2. The individuals categorized as COPDs and non-COPDs had participated in an earlier study (Bhattacharjee, 2011) conducted by us in the vicinity of the open-cast coal mine area at Ledo, near Tirap in Assam (Latitude 27º13' to 27º23'N and Longitude 95º35' to 96'00E); with the help of doctors at the local Primary Health Centre during the period of January 2009 to December 2010. Our statistical population shows that these people have been living there for 11 to 35 years within this 1.5 km of the mine area. We drew 412 people randomly as the sample for our study irrespective of age, sex, and livelihood. The status of lung function was confirmed through pre and post –bronchodilator spirometry as described earlier (Bhattacharjee, 2011). Blood samples (3 ml) were collected with informed consent from each subject by standard venipuncture method and stored at 4ºC until DNA extraction. The study was carried out at CSIR (NEIST), Jorhat after ethical clearance from Institutional Ethics Committee NEIST, Jorhat. The authors declare that they have no conflicts of interest.

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 Alpha-1-antitrypsin gene was done by site directed mutagenesis PCR method as described by (Tazellar et al., 1992) with slight modifications using the primers thus highlighted:

Forward primer: ATAAGGCTGTGCTGACCATCGTC
Reverse primer: CTTTTCACCACTTAGGGTGGGTT

All amplifications were started in a 50 µl reaction volume containing 25 µM deoxynucleotide triphosphate (Bangalore GeNei, India), 2 mM $MgCl_2$ (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 94ºC for 10 min, amplification was carried out for 30 cycles, each cycle consisted of 2 min denaturation time at 94ºC, 2 min annealing time at 52ºC and 3 min extension time, at 64ºC followed by a final extension of 10 min at 72ºC. The PCR reaction was done in a thermal cycler (Applied Biosystems, USA, model 2720).

Restriction enzyme digestion and electrophoresis

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

Sequencing of A1AT gene

A few PCR products of COPD (smoker and non to smoker) group and non-COPD (smoker and non - smoker) group were sequenced at Vimta labs, Hyderabad, India. The DNA sequencing samples were processed using ABI 3130 (4 capillary) or 3730XI (96 capillary) electrophoresis instruments. We have used EMBOSS

Table 1. Demographic and lung function observations in the subjects participating in our study at open cast coalmine site (Ledo, Assam).

Variable	Study Population (coal mine area)			
	COPD smokers (N = 92)	COPD non-smokers (N = 194)	Non-COPD non-smokers (N = 84)	Non-COPD smokers (N = 42)
[a]Male	85	140	73	38
[b]Female	7	54	11	4
Sex ratio (F/M)	0.08	0.38	0.15	0.10
[c]Smoking (pack years ± S.D)	15.91± 7.62			16.21 ± 7.31
[d]Coal dust exposure (years ± S.D)	30.40 ± 13.76	30.03 ± 13.16	15.17 ± 7.2	15.16 ± 3.83
[e]Mean age (± S.D)	40.5 ± 10.58	35.53 ± 13.14	34.17 ± 12.84	41.0 ± 11.99
[f]FEV1/FVC(% predicted) (± S.D)	53.15 ± 8.93	56.68 ± 9.56	112.75 ± 16.97	107.78 ± 15.11
[g]post bronchodilator FEV1 (liters)	0.87 ± 0.09	0.85 ± 0.13		

Demographic observations and status of lung function of study subjects at open-cast coal mine , Ledo, Assam. X^2 test was done for categorical variables and unpaired t- test for continuous variables (Bhattacharjee, 2011). [a] COPD Smokers vs. Non COPD Smokers: $X^2 = 0.14$, p = 0.70, OR = 0.29 (1.28 to 5.27); [a] COPD Non Smokers vs. Non COPD Non Smokers*****: $X^2 = 7.08$, p = 0.007, OR = 0.39 (0.18 to 0.83); [b] COPD Smokers vs. Non COPD Smokers: $X^2 = 0.14$, p = 0.70, OR = 0.29 (1.28 to 5.27); [b] COPD Non Smokers vs. Non COPD Non Smokers***** : $X^2 = 7.08$, p =0.007, OR = 0.39 (0.18 to 0.83); [c] COPD Smokers vs. Non COPD Smokers: t = 0.21, df = 132, p = 0.8; [d] COPD Smokers vs. Non COPD Smokers*****: t = 7.03, df = 132, p = 0.009; [d] COPD Non Smokers vs. Non COPD Non Smokers*****: t = 9.71, df =276, p = 0.02; [e] COPD Smokers vs. Non COPD Smokers: t = 0.28, df = 132, p = 0.77; [e] COPD Non Smokers vs. Non COPD Non Smokers: t = 0.796, df = 276, p = 0.42; [f] COPD Smokers vs. Non COPD Smokers*****: t = 27.17, df = 132, p = 0.006; [f] COPD Non Smokers vs. Non COPD Non Smokers*****: t = 34.53, df = 276, p = 0.003; *****Statistically significant.

Needle (global) pairwise sequence alignment algorithm to find the mutation if any at the position Glu342 GAG → Lys AAG of the sequence.

Statistical analysis

Data were tabulated and classified as per the study variables mentioned in 'Material and Methods'. Chi-square test with Yates correction was applied to test significant difference in the number between smoker and non-smokers, males and females amongst symptomatic and asymptomatic subjects. Unpaired' test was applied to test for significant difference in continuous variables and X^2 test was used to test the significance in categorical variables.

RESULTS

Lung function, smoking status and sorting out of the subjects

The symptomatic smokers (n = 92) and non-smokers in the (n = 194) coal mine showed obstructive pattern of lung function in both males and females. In the coal mine site, amongst COPD non-smokers (n = 194) and Non-COPD non-smokers (n = 84), significantly, more number of symptomatic males [$\chi^2 = 7.08$, p = 0.007, OR = 0.39 (0.18 to 0.83)] and symptomatic females [$\chi^2 = 7.08$, p = 0.007, OR = 0.39 (0.18 to 0.83)] was recorded. Detailed observations on demographic variables are shown in Table 1 (Bhattacharjee, 2011).

The coal dust exposure years also significantly differed amongst COPD smokers versus non-COPD smokers (t = 7.03, df = 132, p = 0.009); and COPD non-smokers versus Non-COPD non smokers (t = 9.71, df = 276, p = 0.02).

Genotyping and sequencing

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. There was no change in position of Glu342 GAG→Lys AAG (highlighted in yellow) of the sequences indicating normal MM type in all categories of the individuals studied (Samples 1 to 4).

DISCUSSION

Amongst many risk factors of COPD, the genetic deficiency of A1AT attributed to ZZ type is the best documented reasons (Carp, 1978). Phenotype M is the normal variant phenotypes, S and Z are the two most frequent abnormal variants (Hutchinson, 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 to1:7000 individuals (Andolfatto et al., 2003). In our population subset, we found that all the subjects were having the normal MM type (Figure 1) which was confirmed through site directed mutagenesis PCR and restriction digestion. Our investigation suggests

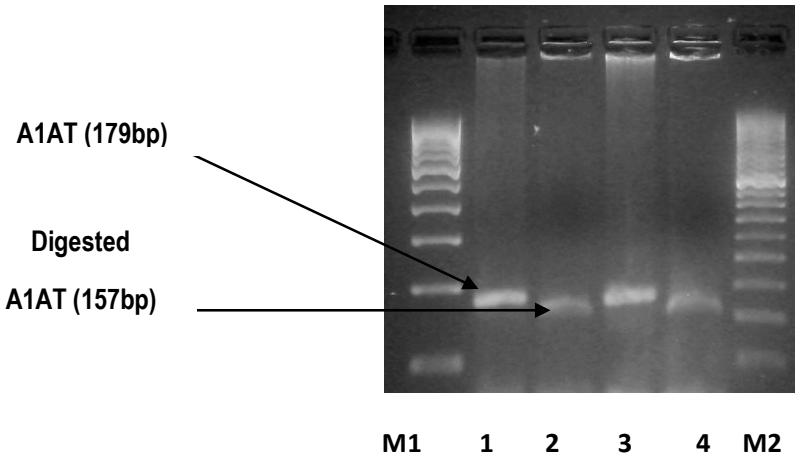

Figure 1. Detection of A1AT gene by site directed mutagenesis PCR method. Lanes 1, 2 A1AT gene179 bp normal (MM type). The primers used to amplify the sequence that included the Z mutation site yielded a product of the correct size (179 bp) in all cases. Lane 1, 2 (Non-COPD); lanes 3, 4 (COPD); M1 = 100bp ladder, M2 = 50bp ladder. Subsequently, PCR products were digested with *Taq* I enzyme. The normal fragment was 157 bp long. Lane 1, 2 (Non-COPD); lanes 3, 4 (COPD); M1 = 100bp ladder, M2 = 50 bp ladder.

TCCGCCATCAATATCGAGTTCTGGTCATCATTAAGAAGACAAAGGGTTTGTTGAACTTGACCTCGGG
GGGGATAGACATGGGTATGGCCTCTAAAAACATGGCCCCAGCAGCTTCAGTCCCTTTCTCGACGAGG
GTCAGCACAGCCTAAAAAA

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EMBOSS_001         1 -------TGCATAAGGCTGTGCTGACCATC-----GACGAGAAAGGG---
35
                           |..|||..| .||.|||..||||     ||.||.||||||
EMBOSS_001         1 TCCGCCATCAATATCG-AGTTCTGGTCATCATTAAGAAGACAAAGGGTTT
49

EMBOSS_001        36 ACTGAAGCTG--CTGGGGCCATGTTTTTAGAGGCCATACCCATGTCTATC
83
                     ..||||..||  ||.|||             ||..|||..||||..|||.
EMBOSS_001        50 GTTGAACTTGACCTCGGG-------------GGGGATAGACATGGGTATG
86

EMBOSS_001        84 CCC-------------CCCGAGGTCAAGTTCAACAAACCCTTTGTCTTCT
120
                     .||             |||.||  ||..||   ||..||||||.||
EMBOSS_001        87 GCCTCTAAAAACATGGCCCCAG--CAGCTT---CAGTCCCTTTCTC----
127

EMBOSS_001       121 TAATGATTGAACAAAATACCAAGTCTCCCCTCTTCATGGGAAAAGTGGTG
170
                                                      ||..|| |||
EMBOSS_001       128 ----------------------------------------GACGAG-GGT-
136

EMBOSS_001       171 AATCCCACCCAAAAATAACTGCCTCTCGCTCCTCAACCCCTCCCCTCCAT
220
                     .|.|.||.||.||||.|
EMBOSS_001       137 CAGCACAGCCTAAAAAA-------------------------------
153

EMBOSS_001       221 CCCTGGCCCCCTCCCTGGATGACATTAAAGAAGGGTTGAGCTGG    264

EMBOSS_001       154 ----------------------------------------------    153


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Sample 1. (COPD smoker).

GAGAAGGGGGGGACGCACCGCCCCTGTTTTTAGAGGCCATACCCATGTCTATCCCCCCCGAGGTCAAGTTCAACA
AACCCTTTGTCTTCTTAATGATTGACCAAAATACCAAGTCTCCCCTCTTCATGGGAAAAGTGGTGAATCCCACCCAA
ACATC

```
EMBOSS_001         27 GAGAA---AGGGACTGAAGCTGCTGGGGCCATGTTTTTAGAGGCCATACC
73
                      |||||   .|||||       ||...|.||.||||||||||||||||||
EMBOSS_001          1 GAGAAGGGGGGGAC-------GCACCGCCCCTGTTTTTAGAGGCCATACC
43

EMBOSS_001         74 CATGTCTATCCCCCCCGAGGTCAAGTTCAACAAACCCTTTGTCTTCTTAA
123
                      ||||||||||||||||||||||||||||||||||||||||||||||||||
EMBOSS_001         44 CATGTCTATCCCCCCCGAGGTCAAGTTCAACAAACCCTTTGTCTTCTTAA
93

EMBOSS_001        124 TGATTGAACAAAATACCAAGTCTCCCCTCTTCATGGGAAAAGTGGTGAAT
173
                      |||||||.||||||||||||||||||||||||||||||||||||||||||
EMBOSS_001         94 TGATTGACCAAAATACCAAGTCTCCCCTCTTCATGGGAAAAGTGGTGAAT
143

EMBOSS_001        174 CCCACCCAAAAAT    186
                      ||||||||||.||
EMBOSS_001        144 CCCACCCAAACAT    156


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Sample 2. (COPD non-smoker).

TGAGGTGAGTGAACCCCCCCAATGTTTTTAGAGGCCATACCCATGTCTATCCCCCCCGAGGTCAAGTTCAACAAAC
CCTTTGTCTTCTTAATGATTGAACAAAATACCAAGTCTCCCCTCTTCATGGGAAAAGTGGTGAATCCCACCCAAAAC
CCCGT

```
EMBOSS_001         27 GAGAAAGGGACTGAAGCTGCTGGGGCC-ATGTTTTTAGAGGCCATACCCA
75
                      |||   |.||.||||.|..|     || ||||||||||||||||||||
EMBOSS_001          2 GAG---GTGAGTGAACCCCC-----CCAATGTTTTTAGAGGCCATACCCA
43

EMBOSS_001         76 TGTCTATCCCCCCCGAGGTCAAGTTCAACAAACCCTTTGTCTTCTTAATG
125
                      |||||||||||||||||||||||||||||||||||||||||||||||||||
EMBOSS_001         44 TGTCTATCCCCCCCGAGGTCAAGTTCAACAAACCCTTTGTCTTCTTAATG
93

EMBOSS_001        126 ATTGAACAAAATACCAAGTCTCCCCTCTTCATGGGAAAAGTGGTGAATCC
175
                      |||||||||||||||||||||||||||||||||||||||||||||||||||
EMBOSS_001         94 ATTGAACAAAATACCAAGTCTCCCCTCTTCATGGGAAAAGTGGTGAATCC
143

EMBOSS_001        176 CACCCAAAA    184
                      |||||||||
EMBOSS_001        144 CACCCAAAA    152


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Sample 3. (Non-COPD smoker).

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TGTTATCCCTCTTCGCCTCCTTATCCATCATTAAGAAGACAAAGGGTTTGTTGAACTTGACCTCGGGGGGGATAGA
CATGGGTATGGCCTCTAAAAACATGGCCCCAGCAGCTTCAGTCCCTTTCTCGACGATGGTCAGCACAGCCTTATAA
A

EMBOSS_001        13 TGCTGA-CCATC-----GACGAGAAAGGG---ACTGAAGCTG--CTGGGG
51
                     |.||.| |||||     ||.||.||||||   ..||||..||  ||.|||
EMBOSS_001        18 TCCTTATCCATCATTAAGAAGACAAAGGGTTTGTTGAACTTGACCTCGGG
67

EMBOSS_001        52 CCATGTTTTTAGAGGCCATACCCATGTCTATCCCC-------------CC
88
                                ||..|||..||||..|||..||              ||
EMBOSS_001        68 -------------GGGGATAGACATGGGTATGGCCTCTAAAAACATGGCC
104

EMBOSS_001        89 CGAGGTCAAGTTCAACAAACCCTTTGTC---TTCTTAATGAT--TGAACA
133
                     |.||  ||..||||           |||   ||||..|.|||  |.|.||
EMBOSS_001       105 CCAG--CAGCTTCA-----------GTCCCTTTCTCGACGATGGTCAGCA
141

EMBOSS_001       134 AA-----ATA    138
                     .|     |||
EMBOSS_001       142 CAGCCTTATA    151


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Sample 4. (Non-COPD non-smoker).

that A1AT deficiency is not prevalent in our population subset. As already documented, the ZZ allele is rarely present in most of the populations; the reasons for occurrence of COPD in our study subset could be attributed to other factors. We already established earlier, that the study area was considerably polluted due to high RSPM, SO_2 and NO_2 and also that there was increase incidence of COPD cases when the coal dust exposure years was more (Bhattacharjee, 2011). It was important to also screen the AAT gene for ZZ carriers and to observe whether the population was genetically susceptible to COPD, as in most parts of the world, the disease is under diagnosed (McEvlaney, 1997). A recent report by the World Health Organization (WHO) and the recent guidelines of the American Thoracic Society (ATS)/European Respiratory Society (ERS) for the management of COPD, has recommended the detection programmes of AAT deficiency (Anonymous, 1997; ATS/ERS statement, 2003). Several longitudinal, epidemiological and associative studies have established that acute episodes of atmospheric pollution causes increased risk of adverse pulmonary events (Dockery et al., 1993; Laden et al., 2006). Significantly, more number of symptomatic that is, people with COPD were recorded than asymptomatic that is, people without COPD in our study site ($p < 0.01$).

There are about 1.00 billion tonnes coal reserves estimated in the coal bearing zone of North-East (NE) India, which is 0.5% of the country's total reserve of about 200 billion tonnes (Chaoji, 2002). Pressure to increase coal mining is likely to intensify because of concerns about energy and power generation and exploration of natural resources. Increased coal demand may exacerbate negative health effects of coal mining activities including occupational health hazards of open-cast coal mining and community exposure (Scott et al., 2004; Coggon and Taylor, 1998).

Hitherto, we can interpret that the lung function decline in the population is mostly due to the effect of respirable mixed coal dust as per our earlier findings (Bhattacharjee, 2011) and there is no deficiency of alpha 1 antitrypsin in the population.

Moreover, COPD is a complex polygenic disease (Mehrotra et al, 2010). It is also possible that certain other genes might play a role and these genes need to be studied to find out whether individual susceptibility due to genetic factors or external risk factors such as smoking, occupational exposure etc are responsible for the disease.

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