IV. RESULTS

4.1 Patients Clinical Presentation

Emphysematous bullae and marked vascular changes in a patient’s X-ray radiography is shown in Fig. 12 (Page No. 61). Physical examination revealed a prolonged expiratory effort, grunting on inspiration and wheezing in upright and forward leaning position and a prominent barrel chest.

Following factors were analyzed among the COPD patients in the present study:

4.1.1 Age-Specific Distribution of COPD Patients and Controls

The patients ranging from 5 to 80 years were grouped into different age groups. The highest percentage distribution (39.13%) was found in the age group of 50 to 65 years and almost equal distribution (35.26%) in the age group of 35 to 50 years. Less than 15% of patients formed the age group of 65-80 years, less than 10% were found in the age group of 20-35 years and less than 2% were in the age group of 5-20 years (Fig. 13, Page No.62).

The controls ranged from 20 to 70 years and were grouped into different age groups. The highest percentage distribution (42.7%) was found in the age group of 50 to 65 years followed by age group of 35 to 50 years (31.8%).
Fig. 12. Chest X-Ray film of a COPD patient showing marked vascular changes (1) and emphysematous bullae (2) (Courtesy: Government Hospital for Thoracic Medicine, Tambaram Sanatorium, Chennai, Tamilnadu, India)
Fig. 13. Age-Specific Percentage Distribution of COPD patients (n= 207)
The percentage distribution in other age groups were 14.5% in 20-35 years age group and 7% in 65-80 years age group. The lowest percentage was found in the age group of 5-20 years (4%) (Fig. 14, Page No. 64).

4.1.2 Percentage Distribution of Clinical Symptoms in Different Age Groups of COPD Patients

The major symptoms identified were dyspnea, sputum production and wheezing (Fig. 15, Page No. 64). Of these patients, 69% were reported to exhibit exertional dyspnea; 45% with cough and chronic sputum and 33% were presented with a symptom of wheezing. Symptoms reported also included regular cough, cough 4-6 times daily and phlegm production (Table 7, Page No. 65).

The prevalence of clinical symptoms in different age groups is provided in Fig. 16, Page No. 66. In all the age groups, dyspnea was the commonest symptom identified followed by sputum production. In the age group of 50 to 65 years wheezing was the second most common symptom followed by cough and sputum production.

4.1.3 Smoking and Smoking Years

In the present study, 139 COPD patients (67.14%) were reported to be current smokers with a long smoking history. There were 50 ex-smokers (24.15%) who have not smoked for more than one year. 18 (8.69%) patients were reported as non-smokers (Fig. 17, Page No. 67).
Fig. 14. Age-Specific Percentage Distribution of Normal controls (n=207)

Fig. 15. Major Clinical Symptoms Identified in COPD Patients (n = 207)
Table 7  The Frequency of Clinical Respiratory Symptoms in COPD Patients (n= 207)

<table>
<thead>
<tr>
<th>Respiratory symptoms</th>
<th>No. of Respondents</th>
<th>Percentage (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Usually have a cough</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Yes</td>
<td>135</td>
<td>65.0</td>
</tr>
<tr>
<td>No</td>
<td>72</td>
<td>34.0</td>
</tr>
<tr>
<td>Usually have a cough 4-6 times</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Yes</td>
<td>120</td>
<td>57.0</td>
</tr>
<tr>
<td>No</td>
<td>87</td>
<td>42.0</td>
</tr>
<tr>
<td>Usually bring up phlegm while coughing</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Yes</td>
<td>169</td>
<td>81.6</td>
</tr>
<tr>
<td>No</td>
<td>38</td>
<td>18.3</td>
</tr>
</tbody>
</table>
Fig. 16. Distribution of Clinical Symptoms in Different Age Groups of COPD Patients (n = 207)
Fig. 17. Smoking Nature of COPD patients (in %) (n= 207)
The smoking patients were grouped into different groups according to the duration of smoking in years. The percent distribution of smoking years is shown in Fig. 18 (Page No. 69). Smoking periods for 20 to 30 years were reported by majority of the patients (77%), a duration for 30 to 40 years by 9.3%, less than 10 years by 7% and about 2% each in the group of 10-20, 40-50 and >50 years of smoking.

4.1.4 Occupational Exposures

The details regarding the patients’ occupation were collected and analyzed for the presence of exposure to any chemicals or dusts. Majority of the patients reported as daily-wage workers in farms. Two of the patients worked in rice mills.

4.1.5 Socio-economic Status

The socio-economic background of the study population was analyzed. Majority of them reported to earn their livelihood on a daily basis with a very low income.

4.2 Mutation Analysis

Blood samples were collected and high molecular weight genomic DNA was isolated. The \textit{aat} gene exons of interest were amplified and SSCP analysis was carried out. Further confirmation of the genotypes was also done by restriction digestion analysis.
Fig. 18. Smoking years of the COPD patients (n= 207)
Genomic DNA isolated was found to be pure. OD_{260/280} ratio showed in the range of 1.8-2 indicating the absence of protein contamination. The agarose gel electrophoregram of the genomic DNA isolated from patients and control blood samples indicated high molecular weight DNA.

The normal variant PiM1 and the deficient variants PiS and PiZ are located on exon III and V respectively. PCR amplification of exon III and Exon V of \textit{aat} using primer sets RG13, RG14 and RG15, RG16 and RG11, RG12 produced amplicons of 98bp, 360bp and 110bp respectively. The amplicons spanned the region of interest in \textit{aat} gene. The results obtained are shown in Figs. 19-21 (Page No. 71-73) indicating the amplification of all the 3 segments and the yield was of expected sizes.

4.2.1 Single Strand Conformation Polymorphism

The amplification products were subjected to SSCP analysis. DNA bands were visualized by silver staining (Bassam \textit{et al.}, 1991; Peng \textit{et al.}, 1995; Wallace, 1997).

In the case of 110bp PCR product of exon V, the DNA separated into 3 distinct bands – the fast moving band represents the reannealed double stranded DNA (dsDNA) and the two slow moving bands represent the single stranded DNA (ssDNA) (Fig. 22, Page No. 74). The results clearly indicate that the bands are identical in all the samples. They did not show any mobility shifts suggesting absence of sequence variation in exon V fragments of patients and controls analyzed.
Fig. 19. 2.5% Agarose gel electrophoregram of PCR amplified product (110bp) of exon V (9967bp – 10076bp) of the *aat* gene using primers RG11 and RG12.

3 μl of DNA sample + 2 μl of loading dye electrophoresed @ 50V for 1 ½ hours

Lane 2 : 100bp ladder
Lane 1, 3-13 : PCR products (110bp)
Fig. 20. 2.5% Agarose gel electrophoreogram of PCR amplified product (98bp) of exon III (7655bp – 7752bp) of the aat gene using primers RG13 and RG14

3 μl of DNA sample + 2 μl of loading dye electrophoresed @ 50V for 1½ hours

Lane 2 : 100bp ladder
Lane 1, 3-6 : PCR products (98bp)
Fig. 21. 1% Agarose gel electrophoreogram of PCR amplified product (360bp) of exon III (7439bp – 7799bp) of the *aat* gene using primers RG15 and RG16

3 µl of DNA sample + 2 µl of loading dye electrophoresed @ 50 V for 2½ hours

Lane 1-2 : PCR products (360bp)
Lane 3 : 100bp ladder
Lane 4-6 : PCR products (360bp)
Fig. 22. Silver stained composite gel showing SSCP patterns of exon V (9967-10076) PCR amplified product (110bp). All bands show similar band pattern

* Lane 1-4: Denatured PCR amplicons of different COPD patients with no change in the band pattern
Lane 5: Denatured PCR amplicon of control sample with similar band pattern
Lane 6: Non denatured sample

* ssDNA – single stranded DNA
** dsDNA – double stranded DNA
In the case of 98bp PCR product of exon III, the SSCP pattern consisted of three bands of single stranded DNA and one band of dsDNA (Fig. 23, Page No. 76). There was no mobility shift suggesting the absence of sequence variations in exon III fragments in the patients and controls.

In the case of 360bp PCR product of exon III, the SSCP pattern consisted of three bands - one of double stranded DNA and two bands of two single stranded DNA (Fig. 24, Page No. 77). A mobility shift was observed in the SSCP pattern obtained from COPD patient sample No.176 (Fig. 25, Page No. 78). The rest of the patients and control samples exhibited identical mobility pattern that was different from that of the variant sample (No. 176).

4.2.2 Results of Restriction Fragment Length Analysis

Taq I digestion of 110bp PCR products amplified from exon V yields an 89bp and 21bp fragments for the M allele and 110bp fragment for the Z allele. The restricted products were subjected to composite gel electrophoresis. Fig. 26 (Page No. 79) represents the restriction digestion pattern on composite gel respectively. All the 110bp PCR samples were cleaved into 89bp and 21bp. No bands were seen in the 110bp region, indicating the absence of Z variants among the COPD patients and control samples. All samples therefore belong to M genotype.
Fig. 23. Silver stained composite gel showing SSCP patterns of exon III (7655bp – 7752bp) PCR amplified product (98bp). All bands show similar band pattern

Lane 1 : Non denatured sample
Lane 2-8 : Denatured PCR amplicons of different COPD patients with no change in the band pattern
Lane 9 : Denatured PCR amplicon of control with similar band pattern

* ssDNA – single stranded DNA
** dsDNA – double stranded DNA
Fig. 24. Silver stained composite gel showing SSCP patterns of exon III (7439-7799) PCR amplified product (360bp). All bands show similar band pattern

Lane 1-8: Denatured PCR amplicons of different COPD patients with no change in the band pattern
Lane 9: Denatured PCR amplicon of control with similar band pattern

ssDNA – single stranded DNA
* dsDNA – double stranded DNA
Fig. 25. Silver stained composite gel showing SSCP patterns of exon III (7439bp – 7799bp) PCR amplified product (360bp)

Lane 1-4 : Denatured PCR amplicons of different COPD patients with no change in the band pattern
Lane 5  : Denatured amplicon of COPD patient (No. 176) with a deviant band pattern
Lane 6-9 : Denatured PCR amplicons of control samples with no change in the band pattern

ssDNA – single stranded DNA
* dsDNA – double stranded DNA
Fig. 26. Silver stained composite gel showing *Taq I* restriction digestion of *aat* exon V (9967bp – 10076bp) PCR product (110bp)

* Lane 1 : 110bp unrestricted product (*)
* Lane 2 : 100bp Ladder
* Lane 3-4 : 89bp Restricted products (**)
Taq I digestion of 98bp PCR product amplified from exon III yields 78bp and 20bp fragment for the M allele and is not digested by the enzyme and it remains as a 98bp fragment for the S allele. As observed by the restriction digestion gel patterns in Fig. 27 (Page No. 81) all the samples were restricted into 78bp and 20bp products. No bands were seen in the 98bp region indicating the absence of S variants among the COPD patients and healthy control samples. All the samples were genotyped to have M allele.

BstE II digestion of 360bp PCR product amplified from exon III yields 228, 83 and 49bp fragments for the M1 Val allele and 311 and 49bp fragments from M1 Ala allele. The results are given in Fig. 28 (Page No. 82) Upon restriction digestion the 360bp PCR fragments were cleaved into 228, 83 and 49bp products. The absence of 311bp band shows the lack of M1 Ala213 allele among the COPD patients and controls screened.

4.2.3 DNA Sequencing

The PCR amplified exon III fragment (360bp) from one patient (sample No: 176) exhibited mobility shift on SSCP gel. On BstE II digestion, this fragment was cleaved into 228bp, 89bp and 49bp products. This showed that the mobility shift was due to the sequence change and is likely a novel variant. This sample (SSCP variant) and two normal samples (with no mobility shifts) were subjected to DNA sequencing. The SSCP variant and the normal controls were sequenced using reverse primer RG 16.
Fig. 27. Silver stained composite gel showing Taq I restriction digestion *aat* exon III (7655bp – 7752bp) PCR product (98bp)

<table>
<thead>
<tr>
<th>Lane</th>
<th>Description</th>
</tr>
</thead>
<tbody>
<tr>
<td>1-2</td>
<td>78bp Restricted products (**)</td>
</tr>
<tr>
<td>3</td>
<td>100bp Ladder</td>
</tr>
<tr>
<td>4</td>
<td>98bp unrestricted products (*)</td>
</tr>
</tbody>
</table>
Fig. 28. Silver stained composite gel showing \textit{BstE II} restriction digestion of \textit{aat} exon III (7439bp – 7799bp) PCR products (360bp)

Lane 2 $^*$ : 311bp Unrestricted product (*)
Lane 3 : 100bp Ladder
Lane 1, 4-5 : Restricted products (228bp, 83bp and 49bp) (**)
The DNA sequences obtained is shown in the Fig. 29 (Page No. 84). These were compared with the genbank control data (http://www.ncbi.nlm.nih.gov). Only one base change was found to be significant. In controls (sample Nos. 82 and 152) at the 7489th base position of the aat exon III region, the base was ‘C’ where as in the variant (sample No 176) ‘T’ replaces ‘C’. (Cytosine replaced by Thymine). When compared to the Genbank data sequence at the 7489th position the base was ‘C’ thus confirming a base change in the sample No. 176.

Hence it can be concluded that:

1. Out of 2 control samples analyzed, the sample nos. 82 and 152 showed normal mobility pattern on SSCP gel and the DNA sequence was consistent with that of the Genbank data.

2. The patient sample no. 176 showed a mobility shift on SSCP analysis and a DNA sequence different from that of the Genbank data. The base change is at the 7489th nucleotide position of exon III (COPD patient sample no. 176).

3. At the 7489th base sequence of the aat exon III sense strand ‘Guanine’ has been replaced by ‘Adenine’. The codon change observed was AAG to AAA with the resultant amino acid change Lys201Lys.

4. This is a unique codon change reported for the first time.
Fig. 29. Chromatogram of the control sample (Nos. 82 and 152) and the variant sample (COPD Patient No. 176) with the ‘C’ to ‘T’ transition, i.e., G to A in sense strand

C - Normal base
T - Variant base