Association of GSTM1 & HMOX-1 Gene Polymorphisms in COPD: A study from South Indian Population

Ashrafunnisa Begum1, A. Venkateshwari2, N Balakrishna3, A. Jyothy4

1,2,4 Institute of Genetics and Hospital for Genetic Diseases Osmania University, Begumpet, Hyderabad – 500016, India
3National Institute of Nutrition Tarnaka Hyderabad-India

Abstract: The major risk factor for COPD is smoking. The pathology involves the imbalance of oxidant/antioxidant mechanism. As smoking enhances, the Oxidative stress increases thereby decreasing the antioxidants capacity in airways causing damage to normal lung function. The aim of the present study was to evaluate the role of (Glutathione GSTM1 & T1) and HMOX-1 gene polymorphism in COPD and control subjects from Telangana state of India. GSTM1 and T1 gene polymorphism was evaluated by Multiplex polymerase chain reaction and HMOX-1 by ARMS-PCR in 250 COPD with equal number of control subjects. Appropriate statistics using students ‘t’ test, Fischer exact test, chi-square with Hardy Weinberg equilibrium were applied for the analysis. There was a significant association of GSTM1 null genotype with the disease ($\chi^2= 18.82; p=0.0001; OR=2.21; CI=1.543-3.18$). Significant association was also found between the GC genotypes of HMOX-1 gene in COPD patients ($\chi^2= 3.73; p=0.05; OR=1.52; (CI=0.9927-2.352$) compared with controls. However further studies from other populations are needed to confirm this results.

Keywords: Chronic Obstructive Pulmonary Disease (COPD), Glutathione Transferase - µ1 (GSTM1), Glutathione tranferase T1 (GSTT1) and Heemooxygenase -1 (HMOX-1).
oxidative stress [11]. The inducible HO-1, is active at high concentrations of heme and at times of physiological stress. A high number of (GT)n repeats have been reported to reduce HMOX1 inducibility by ROS due to cigarette smoke, resulting in the development of COPD [12]. The studies on SNPs in the anti-oxidant genes like GSTM1, GSTT1 and HMOX-1, have been associated with an accelerated decline of lung function in COPD [13 & 14].

2. Material and Methods

2.1 Study design

The study was approved by Institutional ethical committee. A total of two hundred and fifty (n=250) COPD cases were taken from Government Chest Hospital, Irranuma, Hyderabad which is one of the reputed hospitals in Andhra Pradesh, where patients from different socioeconomic strata are referred. The cases which were diagnosed by spirometry, chest X-ray and confirmed by pulmonologists were considered for the study. Special case proformas and consent forms have been collected with the detailed case histories and written consent from the cases willing to be recruited for the study. Emphasis was given for the details of the epidemiological variables like age, sex, hypertension, diabetes, height and weight for measuring body mass index (BMI) family history of any disease, addictions such as smoking, alcohol and other clinical symptoms like cough, fever, exacerbations etc for determining the risk factors. Cases with associated conditions such as tuberculosis, asthma, ischemic heart disease, malignancy, liver cirrhosis, systemic infection, patients suffering from lung cancer or any other lung infection and patients with any major surgery of lungs are completely excluded. The study included (n=250) control subjects blood samples which have been collected with clinically healthy, without any addictions and free of overt disease from the same geographic background with similar socioeconomic status.

2.2 Extraction of DNA and Genotyping:

DNA samples were stored in -80 °C for molecular studies. Approximately 2ml of whole blood was used for DNA extraction using Qiagen Kit method and by salting out method by Lahari et.al 1982 [15]. In order to detect deletion of the GSTM1 and T1 genes, multiplex PCR was performed. The PCR conditions consisted of an initial single cycle of 10 min at 95°C followed by 35 cycles of 30 s at 94°C, 20 s at 52°C and 5 sec at 72°C. The products of the multiplex PCR (GSTM1 215 bp, GSTT1 480 bp and Albumin gene fragment used as internal positive control 350 bp band were separated. For both GST genes individually, subjects were classified as having either a non-null or null (homozgyous deletion) genotype [16]. HMOX-1 gene of -19 G/C was done by ARMS-PCR at 60°, 60 sec of annealing showing 241 bp following the method of Ewelina synowiec et al 2011[17].

3. Statistics

Demographic and clinical characteristics were evaluated by using student t- test. Chi square test was used for comparing genotype frequencies. The polymorphisms were tested for Hardy Weinberg equilibrium using SNP stats and SNP analyzer web-based tool. The interaction between gene variants associated with COPD were evaluated using the Open EP16 software (Open Epi Version 2.3.1 from department of Epidemiology, Rollins school of Public Health, Emory University, Atlanta, GA 30322, USA). Genotypic frequencies were calculated according to the number of different genotypes observed and the total number of genotypes examined. Statistical significance was defined as p<0.05.

4. Results

4.1 Demographic and clinical characteristics

In the present study patients were in the age group of 25-85 years with a mean age of 59.32 ±10.29 while the healthy controls were in the age group of 24-84 yrs with a mean age of 41.79 ± 15.76. There were 239 (95.6%) males and 11(4.4%) females in the patient group with an equal number of male and female controls. The BMI values in COPD patients ranged from 15 to 36.70 with mean of 19.66 ± 4.90 and in the control group it ranged from 16.2 to 30.8 with a mean of 28.35 ± 8.04. Out of 250 COPD patients 28 (11.2%) had the previous history of diabetes and 35 (14.0%) had hypertension, 197 (68%) were smokers, 45 (29.6%) were ex-smokers and 8(3.2%) were non-smokers. The mean and SD pack years of smoking among the COPD smokers was 88.20 ± 19.76 and in ex-smokers it was 75.80 ± 15.26. The study included 47 (18.8%) of COPD patients with the habit of drinking alcohol. Based upon spirometric readings of FEV1/FVC and GOLD classification, 73 (29.2%) were mild cases, 48(19.2%) were moderate cases, 121(48.4%) were severe cases and 8(3.2%) were very severe cases (Table 1). Most of the patients with severe and very severe category showed symptoms of acute exacerbations compare to mild and moderate cases.

<table>
<thead>
<tr>
<th>Parameters</th>
<th>Controls</th>
<th>COPD</th>
<th>p Value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Number of subjects</td>
<td>250</td>
<td>250</td>
<td>NA</td>
</tr>
<tr>
<td>Males n (%)</td>
<td>239(95.6)</td>
<td>239(95.6)</td>
<td>NA</td>
</tr>
<tr>
<td>Females n (%)</td>
<td>11(4.4)</td>
<td>11 (4.4)</td>
<td>NA</td>
</tr>
<tr>
<td>Age (Mean ± SD)</td>
<td>58.92±13.39</td>
<td>59.32±10.29</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>BMI, Kg/m2</td>
<td>28.35±8.04</td>
<td>19.66±4.90</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>Alcoholics (%)</td>
<td>47(18.8)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Diabetes mellitus n(%)</td>
<td>28(11.2)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Hypertension n(%)</td>
<td>35(14.0)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Current smokers(%)</td>
<td>197(78.8)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Ex-smokers(%)</td>
<td>45(18.0)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Non-Smokers (%)</td>
<td>08(3.2)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Pack years(Mean±SD)</td>
<td>88.2±19.76</td>
<td>75.8±15.26</td>
<td></td>
</tr>
<tr>
<td>Smokers</td>
<td>75(29.2)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Ex-smokers</td>
<td>48(19.2)</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

Table 1: Demographic and clinical characteristics of controls and COPD patients
II Moderate n(%) (FEV1 < 80) 121(48.4)
III Severe n(%) (FEV1< 50) 08(3.2)
IV Very Severe n(%) (FEV1<30)

*p< 0.05* indicates significant difference. COPD, Chronic obstructive pulmonary disease; NA, not associated; SD standard deviation; BMI, body mass index; FEV1, forced expiratory volume in 1s.

B) The GSTM1/GSTT1 NON NULL/NULL Polymorphisms
The GSTM1 polymorphism in COPD showed 52.4% of null genotype and 47.6% of non null genotype where as in the control subjects the frequency of null genotype was 33.2% and non null genotype was 66.8% (Table # 2). There was a significant association of GSTM1 null genotype with COPD ($\chi^2 = 18.82; p=0.0001; OR=2.21; 95\% CI=1.543-3.18$). (Table # 3). In case of GSTT1 polymorphism in COPD, the null and non null genotype frequencies were 41.2% and 58.8% respectively compared to 44.0% and 56.0% in controls. There was no significant association of GSTT1 genotypes with COPD . (Figure 1 & 2).

Table 2: Distribution Of Genotypes Frequencies Of GSTM1/GSTT1 in COPD and Controls

<table>
<thead>
<tr>
<th>GSTM1 &amp; GSTT1</th>
<th>COPD patients (n=250)</th>
<th>Controls (n=250)</th>
</tr>
</thead>
<tbody>
<tr>
<td>NULL Genotypes</td>
<td>n</td>
<td>%</td>
</tr>
<tr>
<td>GSTM1</td>
<td></td>
<td></td>
</tr>
<tr>
<td>NON NULL</td>
<td>131</td>
<td>52.4</td>
</tr>
<tr>
<td>GSTT1</td>
<td></td>
<td></td>
</tr>
<tr>
<td>NULL</td>
<td>103</td>
<td>41.2</td>
</tr>
<tr>
<td>NON NULL</td>
<td>147</td>
<td>58.8</td>
</tr>
</tbody>
</table>

Table 3: Comparison Of Genotypes Of GSTM1/GSTT1 In COPD and Controls

<table>
<thead>
<tr>
<th>Genotype/Allele</th>
<th>$\chi^2$</th>
<th>p-Value</th>
<th>Odds Ratio</th>
<th>95% CI</th>
</tr>
</thead>
<tbody>
<tr>
<td>GSTM1 NULL/NULL</td>
<td>18.82</td>
<td>0.0001*</td>
<td>2.21</td>
<td>1.543-3.18*</td>
</tr>
<tr>
<td>GSTT1 NULL/NULL</td>
<td>0.49</td>
<td>0.425</td>
<td>0.88</td>
<td>0.625-1.271</td>
</tr>
</tbody>
</table>

*p <0.05 indicates significant difference; $\chi^2$ = chi square CI= confidence interval

Figure 1: Graphical Representation of Genotypes of GSTM1 & GSTT1 In The Study Subjects.

Table 4: Distribution of Genotypes and Allele Frequencies of HMOX1 -19G >C Polymorphism in COPD and Control

<table>
<thead>
<tr>
<th>HMOX1 -19 G/C Genotypes</th>
<th>COPD (n=250)</th>
<th>Controls (n=250)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Genotypes/Alleles</td>
<td>n</td>
<td>%</td>
</tr>
<tr>
<td>GG</td>
<td>54</td>
<td>21.6</td>
</tr>
<tr>
<td>GC</td>
<td>136</td>
<td>54.4</td>
</tr>
<tr>
<td>CC</td>
<td>60</td>
<td>23.0</td>
</tr>
<tr>
<td>G</td>
<td>244</td>
<td>48.8</td>
</tr>
<tr>
<td>C</td>
<td>236</td>
<td>31.2</td>
</tr>
</tbody>
</table>

Figure 2: Gel Picture Showing GSTM1 & GSTT1 Gene Polymorphism.
**Table 5:** Comparison of Genotypes And Alleles of HMOX1-19G → C In COPD and Controls

<table>
<thead>
<tr>
<th>Genotype/Allele</th>
<th>χ²</th>
<th>p-Value</th>
<th>Odds Ratio</th>
<th>95% CI</th>
</tr>
</thead>
<tbody>
<tr>
<td>GG vs GC+CC</td>
<td>0.71</td>
<td>0.39</td>
<td>0.83 (0.551-1.266)</td>
<td></td>
</tr>
<tr>
<td>GC vs GG+CC</td>
<td>3.73</td>
<td>0.05*</td>
<td>1.52 (1.092-2.132)*</td>
<td></td>
</tr>
<tr>
<td>CC vs GC+GG</td>
<td>0.87</td>
<td>0.34</td>
<td>1.23 (0.8015-1.871)</td>
<td></td>
</tr>
<tr>
<td>C vs G</td>
<td>1.29</td>
<td>0.25</td>
<td>0.88 (0.675-1.11)</td>
<td></td>
</tr>
<tr>
<td>G vs C</td>
<td>1.15</td>
<td></td>
<td>1.15 (0.9012-1.48)</td>
<td></td>
</tr>
</tbody>
</table>

*p <0.05 indicates significant difference; χ² = chi square CI= confidence interval

**Figure 3:** Graphical Representation of Genotypes and Alleles of HMOX1 -19 G → C in Study Population

**Figure 4:** Gel Picture Showing HMOX1 -19 G C Gene Polymorphism

**5. Discussion**

We have investigated the demographic characteristics like age, BMI and genes such as GSTM1 null & HMOX-1 GC genotypes are the major risk factors associated with COPD when compared with controls. The results were also compared and checked with other population studies from Asia, China, Western and European countries [12 & 9]. Hence we report the association of GSTM1 null and HMOX-1 GC genotypes as genetic risk factors involved in COPD.

The Mean age ± SD of COPD cases was 59.32±10.29 yrs and in controls it was 41.79±15.79 yrs. Maximum number of COPD cases belonged to the age group of 55-65 yrs. In the patient group males were more prone to COPD in comparison with females (96.4%). This could be because of the habit of smoking and consumption of alcohol in males than in females in Indian population. The observations are in accordance with earlier reports [18 & 19]. In other populated countries females are more prone to COPD because of the high rates of alcohol consumption and smoking in these ethnic groups. In the present study, COPD females were from rural areas and were exposed to biomass fuel...
BMI is one of the major risk factor for COPD [22]. Results of the present study also showed that BMI values in COPD patients (19.6 ± 4.98) were significantly low when compared to their respective controls (28.35 ± 8.04) (p=0.001). This may be due to malnutrition or intake of low calorie diet or due to complete utilization of calories leading to heavy weight loss. This is in accordance with a previous study in Indian population where COPD patients had decreased BMI [23].

In the present study, among 250 COPD patients, 197 (68%) were smokers, 45 (29.6%), ex-smokers and 8(1.6%) were non-smokers. Studies from India have shown tobacco smoking to be responsible for over 82% of COPD cases [24]. Rani et al. [25] reported that the prevalence of smoking in South Indian males was significantly high (35.4%) compared to North Indians (23.9%). In smokers the risk for COPD is based upon the age, intake of cigarettes, pack years of smoking, present status of smoking. But not all smokers develop COPD which indicates that there is a possible risk of associated genetic factors for the susceptibility of the disease. [26]. The emerging consensus that pack years of smoking is the key predictive factor in the development of COPD is well supported [27]. This study also collected the data from patients regarding number of cigarettes smoked per day along with duration of cigarette smoking from the smokers and ex-smokers with COPD. The information obtained from the proforma of COPD non smokers showed that the patients were industrial workers, farmers, welders, washer-men and painters who were suffering with COPD for more than 2 years.

There are three main themes within the pathogenesis of COPD. The oxidant-antioxidant theory states that disparity between levels of harmful oxidants and protective antioxidants leads to oxidative stress, which in turn influences the actions of anti-proteases, and expression of proinflammatory mediators [28]. The protease-anti-protease theory suggests that there is an imbalance between proteases that digest elastin together with other components of the extra-cellular matrix and anti-proteases that protect against it [29]. Both of the above theories link to the third theory about the importance of inflammation in the pathogenesis of COPD [30,31]. Polymorphisms in genes relating to oxidants, proteases, antioxidants and inflammation have been found to relate to the presence of features of COPD suggesting reasons for the heterogeneity observed in clinical phenotype.

Imbalance of oxidants-antioxidants results in oxidative stress and release number of oxidants that destroy the antioxidant capacity. Various studies have proved the existence of oxidative burden in blood, breath, lung airspaces and in urine of smokers suffering with COPD. The oxidants released from cigarette smoke increase the oxidative stress along with increase in leukocytes with ROS in the blood and in airways of lungs. Smoking leads to oxidative stress that causes chronic inflammation of lungs [32]. Antioxidant enzymes increase the activity of cells and other antioxidant defences to reduce oxidative burden under physiological conditions. This is achieved by a variety of antioxidant defense systems that activate enzymes and the synthesis of antioxidants. ROS and RNS increase the oxidative stress and decrease the antioxidant levels [33]. Thus, decline in levels of antioxidant enzyme activity could lead either to higher oxidative stress or low levels of defense. Hence the present study was aimed at evaluating the role of antioxidant genes (GSTM1 & HMOX-1) that are shown to be associated with COPD in different population and to see whether these genes are involved in COPD from Indian population. So here we report the association of GSTM1 null and HMOX-1 GC in our population as the genetic risk factors in developing COPD.

Various studies have shown the involvement of Glutathione S-transferase µ (GSTM1) null genotype to be associated with COPD and lung cancer [34 & 35]. Few studies have reported that the function of GSTM1 is lost in null genotype leading to non-detoxification of aromatic hydrocarbons released from cigarette smoke, resulting in increase of inflammatory cells which damage the tissue of lungs with decline in lung function [36]. The frequency of mutant GSTM1- null genotype has also been reported to be significantly higher in mild/moderate types of COPD. GSTM1 polymorphism which is in accordance with earlier studies [37, 38, 39 & 40]. The evaluation of GSTT1 does not show any significance with the genotypes of COPD and the observations were similar to the studies of Yim et.al [41].

In the present study the frequency of GC genotype of HMOX-1 was found to be significantly associated in COPD patients when compared to control. This is the first study from India to show the HMOX-1 -19/GC (rs2071747) variant to be associated with COPD. Among the antioxidative enzymes HMOX1 is considered as protective gene for lungs from oxidative stress. Recently the role of microsatellite polymorphism of HMOX1 gene promoter has been reported for some human diseases. It was shown that longer (GT)n repeat was associated with angiographic restenosis after coronary stenting, lung adenocarcinoma and pneumonia [42, 43, 44]. There are very few studies that investigated on the role of antioxidant gene polymorphisms in COPD and one study had shown the nominal association (p = 0.015) between one intronic HMOX-1 SNP (rs2071749) and lung function decline [45]. Some studies did not show the association of HMOX-1 gene in lung associated diseases [46]. However the studies on HMOX-1 in relation with COPD from other populations are required to clarify these results.

6. Conclusion

This is the initial study carried out in Telangana region with COPD population. The demographic feature like age and BMI was found to be the risks factors associated with disease. This study also shows the male gender is more prone to COPD as smoking remains the main risk factor of the disease where as female gender are not habituated towards smoking in our country when compared with most western and European countries. This study reveals the relation of GSTM1 and HMOX-1 genes were found to be significantly associated with COPD. Hence it is clear that the role of genes also play an important role in the pathology of the disease. It is essential to have knowledge on genetic
contribution to COPD development and especially interaction of different candidate genes and GWAS (Genome wide association studies) from India would provide in better understanding the pathways involved in COPD pathogenesis.

7. Future Study

Further research will be focused on the role of oxidative genes involved in COPD. This study will help in early diagnosis and to discover new drugs to eradicate the habit of smoking and Exacerbations. Studies are also needed on a large sample size to bring about the knowledge and awareness of COPD among people living in the rural areas with Confirmational studies in other prospective cohorts will be of great importance.

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Author Profile

Ashrafunnisa Begum Research scholar (Genetics) Department of Cell Biology at Institute of Genetics and Hospital for Genetic Diseases Osmania University Begumpet Hyderabad Telangana India.

Dr. A. Venkateshwari presently working as Assistant professor, Department of Cell Biology at Institute of Genetics and Hospital for Genetic Diseases Osmania University Begumpet Hyderabad Telangana India.

Dr. N. Balakrishna presently working as statistician at National Institute of Nutrition Tarnaka, Hyderabad, Telangana India.

Prof. A. Jyothy at present acting as Director and Head Department of Cell Biology at Institute of Genetics and Hospital for Genetic Diseases Osmania University Begumpet Hyderabad, Telangana India.

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