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Role of CYPA1 Gene Polymorphism with Oxidative Status in COPD

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Abstract**:** *Chronic Obstructive Pulmonary Disease (COPD) is a complex disease which involves the influence of genes and envinormental factors. Oxidative stress plays an vital role in the development of COPD. Pathogenesis of COPD is linked to genes which cause variation in oxidant/antioxidant imbalance of lungs. In this study plasma MDA and Nitric oxide were estimated by Spectrophotometric analysis. CYPA1 gene polymorphism was evaluated by PCR-RFLP in 250 COPD patients with an equal number of control subjects from Southern part of India. Appropriate statistical analysis with SPSS 19 tool was applied for the study. Significant increase in levels of plasma Malonialdehyde and Nitric oxide in COPD subjects (4.73 ± 3.57) (p<0.001) were observed (2.71 ± 1.55) (p<0.001) compared to controls (1.34±0.65) and (1.12 ± 0.69. There was a significant association of 'C' allele of CYPA1 polymorphism (p =0.03, OR = 1.32, CI =(1.022-1.724). Further study also reveals significant correlation of CC genotype with elevated levels of oxidative markers in COPD subjects compared to controls. Association of CYPA1 gene polymorphism in COPD can be determined in other population studies. By monitoring the oxidative status, COPD patients can be treated to reduce stress and to minimize COPD risk developed by cigarette smoking.*

Keywords: *Oxidative stress, Malondialdehyde (Lipid peroxidation), Nitric oxide, CYPA1 gene.*

1. Introduction:

Chronic Obstructive Pulmonary Disease (COPD) is characterized by airflow limitation in lungs and it is not fully reversible. It is a preventable and treatable disease with some significant extrapulmonary effects that may contribute to the severity in individual patients. The GOLD (Global intiative for obstructive lung disease) have shown the characteristic feature of COPD is associated with an abnormal inflammatory response of the lung to noxious particles or gases where the airflow limitation is usually progressive (GOLD 2006).

The airflow is blocked in COPD, caused by inflammation in the airway either by (obstructive bronchiolitis) and parenchymal destruction (emphysema), this related combinations differ from individual to individual. It has a different natural history and not all individuals follow the same features. The percussion of COPD on an individual patient depends on the severity of symptoms, systemic effects and co morbidities, or the patient may not have sufficient airflow.

COPD is the major cause of illness worldwide and it is increasing substantially resulting in socioeconomic burden. In the year 1990 COPD was ranked sixth cause of death and according to Global Burden of Disease Study, it will be ranked as the third most leading cause of death world wide by 2020. The morbidity, and mortality of COPD differ from other countries and among different groups within countries. The common risk factor for COPD is tobacco smoking, occupational dusts and exposure to chemicals are the only important inhalation exposures where COPD develops on their own. In few countries, air pollution and other biomass fuels has also been identified as a COPD risk. The burden of COPD is expected to increase in the coming years due to continued exposure to COPD risk factors (GOLD 2006). A study conducted on COPD from India with out spirometric observations had shown that 12 million people were affected by this disease (Sundeep *et al*., 2012) and recent study also shows that 7% of adults , 6% to 7% of non-smokers and 14 % of smokers show the prevalence rate of COPD from southern part of India (Arvind 2012).

Oxidative stress play an vital role in the pathogenesis of various lung disorders, either by causing direct injury to lungs and also by involving the process of inflammation at molecular level. Oxidant/antioxidant imbalance occurs in COPD and in smokers (Rahman *et al*., 1996). Free radicals released from cigarette smoke can trigger the structural changes in lipid perioxidation, which can impair the function of membrane and the activity of its receptor and enzymes disturbing the membrane permeability. MDA levels and their end products have been broadly studied as a marker in

pulmonary, systemic oxidative stress and in the pathogenesis of COPD (Halliwell and Chirico, 1993).

Nitric oxide acts as a free radical in various aspects of pulmonary function. As nitric oxide is produced, it is metabolized to nitrite and nitrate or combined with super oxide to form peroxy nitrite. So, the end products of nitric oxide in plasma are useful in measuring the production of NO in many diseases (Christian Hesslinger *et al.,* 2009). The airways when exposed to oxidative stress decreases the levels of antioxidants which not only enhance ROS mechanism but effect reactive nitrogen species (RNS) that effects the airways along with changes in pathological conditions. Both ROS and RNS damage the DNA, as a result structural changes are observed in lipids, proteins and carbohydrates which impair the cell function and increases the inflammation in COPD (Athanasios *et al*., 2013).

The genes like eNOS, CYP1A1, GSTM1 & T1 which act as oxidants are also shown to be involved in the pathogenesis of COPD (Molfino, 2004). CYP1A1 is a protein of cytochrome p450 family which plays a key role in activation of polycyclic aromatic hydrocarbons which are present in cigarette smoke and considered as carcinogenic. The role of CYP1A1 in the metabolism of tobacco is well established. Studies from literature have shown that CYP1A1 Ile-Val mutation in the heme-binding region resulted in a two-fold increase in microsomal enzymes activity (Landi *et al.,* 1994). The studies carried out on COPD from North India showed the significant association of CYP1A1 genes and their involvement in oxidative stress development in COPD (Arpana *et al.,* 2010).

Keeping the above literature in view, this study was aimed to observe the oxidant levels of MDA and Nitric oxide, evaluating the role of CYPA1 gene polymorphism in COPD and to check the influence of (MDA and Nitric oxide) levels with CYPA1 genotypes in COPD compared with controls subjects.

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 Hyderabad which is one of the reputed hospitals in Andhra Pradesh, where patients from different socioeconomic strata are referred. Subjects diagnosed with clinical symptoms of cough, sputum, persistent dyspnea, acute exacerbations and other COPD risk factors, confirmed with spirometry, chest X-rays and CT scans by the pulmonologists were included. Emphasis were given to the epidemiological variables like sex, age, body mass index (BMI), hypertension, diabetics, habits of smoking and drinking alcohol for determing the risk factors. COPD cases with a history of cigarette smoking (Smokers and Ex-smokers) were included for the study. Non-smokers without history of cigarette smoking are also included. 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.

6 to 8 ml of venous blood was collected from all the subjects. Plasma and serum samples were stored at -80 ^{0}C for further use. Approximately 2ml of whole blood was used for DNA extraction using salting out method (Lahari *et.al.,* 1982). The extracted plasma and DNA samples were stored in -80 ^{0}C for biochemical and molecular studies. Plasma Malondialdehyde (Lipid peroxidation) and nitric oxide (nitrite/nitrate) levels were estimated by (Gavino.*et.al*., 1981) and (Green, *et.al*., 1982) methods.

CYP1A1 T→C polymorphism was detected by PCR based Restriction Fragment Length Polymorphism (PCR-RFLP) (Hayashi *et al*., 1991). The PCR was carried out using specific primers of 10 µmol each in a 30µl volume with 10X buffer, 200 µmol dNTPs, 1.0U Taq DNA polymerase and 100ng of genomic DNA. After mixing all the contents, PCR tubes were kept in thermal cycler for a 3 step PCR with an intial denaturation at 95 for 5 minutes followed by cycling at 95^{0}C for 30seconds annealing for 60^{0}C at 60 seconds and extension at 72^{0}C for 60 seconds and a final extension at 72^{0}C for 5 minutes was carried out for about 30 cycles.

The obtained PCR product (340 bp) was digested with MSP I restriction enzyme (New England Biolabs, Beverly, MA,USA) at 37^0 C for 5 min. Gel electrophoreses using 3 % agarose with ethidium bromide staining was used. The UV documentation showed wild TT (340bp), heterozygous TC (340/200/140bp) and homozygous CC (200 and 140 bp) genotypes. (Figure 1).

2.2 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 different 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. Yate's correction (Yates 1934) was applied wherever necessary. The association of genotypes with the study markers in all subjects was evaluated by analysis of variance (ANOVA) Package for Social Sciences Software version 19(SPSS, Chicago, IL). Statistical significance was defined as $p<0.05$.

3. Results and Discusssion

3.1 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. The study included 47 (18.8%) of COPD patients with the habit of drinking alcohol. Out of 250 COPD patients 28 (11.2%) had the previous history of diabetes and 35 (14.0%) had hypertension, 197 (68%). 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. Lifetime pack-years of smoking was calculated as number of packs of cigarettes smoked per day multiplied by number of years of smoking. This study showed 197 (78.8) were smokers 45 (29.6%) , ex-smokers and 8(3.2%) 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 75.8 ± 15.26. 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 more exacerbations compare to mild and moderate cases.

Table.1. Demographic and clinical characteristics of Controls and COPD patients

PARAMETERS	CONTROLS N=250	COPD N=250	p Value
Males n (%)	239(95.6)	239(95.6)	NA
Females n (%)	11 (4.4)	11 (4.4)	NA
Age (Mean ± SD)	58.92±13.39	59.32±10.29	NA
BMI, Kg/m^2	28.354± 8.049	19.660 ± 4.990	<0.001
Alcoholics (%)		47(18.8)	
Diabetes mellitus n(%)		28(11.2)	
Hypertension n(%)		35(14.0)	
Current smokers(%)		197(78.8)	
Ex-smokers(%)		45(18.0)	
Non-Smokers (%)		08(3.2)	
Pack years(Mean±SD) Smokers Ex-smokers		 88.2 ± 19.768 75.8 ± 15.262	
GOLD stage: I/II/III/IV I Mild n (%) (FEV1>=80%) II Moderate n(%) (FEV1 < 80) III Severe n(%) (FEV1< 50) IV Very Severe n(%) (FEV1<30)		 73(29.2) 48(19.2) 121(48.4) 08(3.2)	

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

3.2 Oxidative status

The levels of lipid peroxidation end product i.e Malondialdehyde (MDA) and nitrite/nitrate (NOx) have been analysed in COPD patients and controls. The MDA and nitrite levels were found to be significantly high in COPD patients (4.73 ± 3.57) and (2.71 ± 1.55) when compared to healthy controls(1.34±0.65) and (1.12 ± 0.69) with (p<0.001).

3.3 CYP1A1 Polymorphism

CYPA1 genotype frequency analysis in COPD patients showed 36.8% of TT, 50.8% of TC, and 12.4 of CC genotypes (Table 2) where as in controls they were 44.8% of TT, 47.6% of TC and 7.6 % of CC genotypes. The C allele was found to be significantly associated with the disease [χ^2= 4.5; p=0.03; OR=1.32; 95% CI=1.022-1.724]. (Table 3).

Table 2. Distribution of genotypes and allele frequencies of CYPA1 -3801 in COPD and controlS

CYPA1-3801	COPD patient (n=250)		Controls (n=250)	
Genotypes /Alleles	n	%	n	%
TT	92	36.8	112	44.8
TC	127	50.8	119	47.6
CC	31	12.4	19	7.6
T	311	62.2	343	68.6
C	189	37.8	157	31.4

Table 3. Comparison of genotypes and alleles of CYPA1-3801 in COPD and controls

Genotype/Allele	χ^2	p-Value	Odds Ratio	95% CI
TT Vs TC+ CC	3.31	0.06	2.76	(2.088-3.669)
TC Vs TT+ CC	0.51	0.47	1.13	(0.800-1.614)
CCVs TC + TT	3.14	0.07	1.71	(0.940-3.123)
C Vs T	4.5	0.03	**1.32**	**(1.022-1.724)**
T Vs C			0.75	(0.57-0.978)

χ^2 , Chi-square; CI, confidence interval, statistical significance of *p <0.05

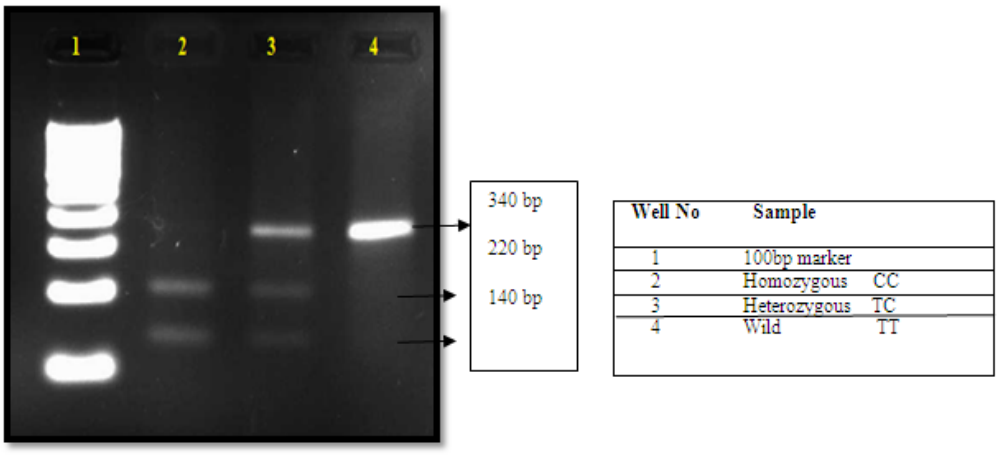

Figure 1. Gel Picture Showing CYP1A1- 3801, Polymorphism

3.4 CYP1A1 genotypes with plasma MDA levels in the study subjects

In the present study the CC, TC and TT genotypes showed significantly different levels of MDA in COPD cases (Table 4).

Table 4. Mean plasma MDA levels in the study subjects with CYP1A1 genotypes

Subjects	CC	TC	TT	F value	p value
COPD (n=250) Mean ± SD	4.78±1.42	4.24±1.20	3.18±1.22	28.24	**0.001**
CONTROLS(n=250) Mean ± SD	1.01±0.80	1.32±0.62	1.34±0.59	2.350	0.098

MDA, Malanioldehyde; COPD, Chronic obstructive pulmonary disease; CC, homozygous; TC heterozygous; TT wildtype; SD, standard deviation, statistical significance of *p <0.05

3.5 Plasma Nox levels in the study subjects with CYP1A1 genotypes.

There was a significant difference between the levels of NOx with different genotypes of CYP1A1 in the study population. (Table 5).

Table 5 Mean plasma Nox levels in the study subjects with CYP1A1 genotypes

Subjects	CC	TC	TT	F value	p value
COPD (n=250) Mean ± SD	2.45±1.20	1.45 ±0.44	2.06±1.93	10.594	**0.001**
CONTROLS (n=250) Mean ± SD	0.92±0.14	1.09±0.66	1.03±0.42	2.350	0.098

Nox, Nitric oxide; COPD, Chronic obstructive pulmonary disease; CC, homozygous; TC heterozygous; TT wild type, statistical significance of *p <0.05

The lungs along with respiratory system when exposed to smoking results in oxidative stress, injury and produces ROS along with lipid peroxidation leading to chronic inflammation (Morrison *et al.*, 1991). In the present study the mean levels of MDA were significantly high in COPD patients when compared to healthy controls. The end product of lipid production is Malondialdehyde (MDA), which gets increased in blood of COPD patients with severity. The high levels of MDA is associated with exposure to tobacco. The studies carried out with MDA levels in smokers and non-smokers, had observed elevated levels of MDA in smokers (Barnes and Celli 2009; Birgul and Isik 2007), and the studies did by Yessica *et al.,* noticed increase levels of MDA levels in all the four types of COPD compared to healthy controls (Yessica *et al.,* 2009; Schünemann et al., 1997). As far as the Indian studies is concerned the studies from north Indian population reports MDA levels gets increased in COPD (Arpana *et al.,* 2007).

The results obtained in this study also indicates the higher levels of plasma NOx in COPD patients when compared to controls. The activity of NO/ONOO activity is shown to be increased in plasma and lung epithelial lining fluid of lungs in smokers with elevated nitrotyrosine levels (Petruzzelli *et al.,* 1997; Ichinose *et al*., 2000). There will be an decrease or increase in levels of nitric oxide which is observed in COPD as the nitric oxide combines with superoxide anions to form peroxynitrite leading to the increased levels of oxidative stress in COPD (Maziak *et al.,* 1998; Rutgers *et al.,*1998). As in our studies the patients with more exacerbations were observed than with stable COPD, which may be also a possible factor for increase in levels of plasma nitric oxide levels in COPD. This condition was also noted in other studies related with COPD exacerbations (Mario *et al.*, 2014).

Oxidative stress develops more rapidly in lungs in disease condition, as the levels of reactants increase in reactive oxygen species (ROS). Various studies have proved the existence of oxidative burden in blood, breathe, air spaces, and in urine of smokers suffering with COPD. The oxidants released from cigarette smoke increases the oxidative stress along with increase in leukocytes with ROS in the blood and in airways of lungs. (GOLD 2005).

Oxidative/antioxidative imbalance, inflammation, imbalance of protease/antiprotease and apoptosis are the important mechanisms involved in the pathology of COPD. Oxidative stress is the major process observed in COPD caused by direct injury to the respiratory tract or COPD with excaberations (MacNee 2005). The other reason for the cause of COPD is the deficiency of antioxidants which are also involved in the development of oxidative stress (MDA and Nitric oxide) in COPD. Imbalance of oxidant–antioxidant results in oxidative stress and release more number of oxidants that destroy the antioxidant capacity.

The discovery of alpha-1 antitrypsin (AAT) deficiency was a major factor in developing the protease-antiprotease hypothesis for COPD. Hence, it was natural for us to study other COPD susceptibility genes (oxidants-antioxidants) that would lead to similar novel insights into the causes of COPD. Among all the oxidant genes we emphasized to study on CYPA1 gene, as the literature from different populations, have suggested that genetic factors are also responsible for COPD risk and it is the major enzyme present in lungs which functions to detoxify the harmful substances released during smoking.

Polycyclic aromatic hydrocarbons in their intial phase are harmless in low doses. CYP enzymes, can become very toxic substances for the lungs upon bioactivation (Bartsch *et al*., 2000). Cytochromes P450 (CYP450) family consists of CYP1A1 and CYP1A2, which metabolize arylamines and polycyclic aromatic carbons (PAC). The function of these cytochromes is to detoxify the envinormenal pollutants and to bind to DNA molecule by formation of electrophilic metabolites (Wang *et al.*, 2003).

The association of CYP1A1 gene polymorphism with COPD has not been investigated from South Indian population. In the present study, the C allele of CYPA1 gene was associated significantly with COPD. A study carried out on COPD from North India has shown the significant association of CYP1A1 genes and their involvement in oxidative stress development in COPD (Arpana Vibhuti *et al*., 2010).

Further the CC genotype frequencies of CYPA1 gene were significantly correlated with MDA and NO levels. The levels were found to be high in COPD cases with CC genotype compared to controls. Elevated levels of these enzymes with the genotype could be due to activation of more rapidly acting polyaromatic hydrocarbons which are released from the cigarette smoke. The products released are toxic and cause damage to lungs leading to diseases like COPD or lung cancer (Crofts *et al.,* 1994; Kiyohara *et al*., 1996)

The activation of transcription factor NF-Kβ and activating protein -1 act as protector for lungs but during smoking in COPD, inactivation of antiproteases, apoptosis, epithelial and increase of neutrophils in the pulmonary vasculature with expression of genes associated with proinflammatory mediators which causes chromatin changes in air way remodelling effecting the genes associated with antioxidant expression. (Repine et al., 1991; Henricks and Nijkamp, 2001; Macnee 2001).

In conclusion, elevated plasma MDA, NOx levels and association of CYPA1 gene polymorphism in COPD subjects shows high oxidative stress due to smoking. By monitoring the oxidative status, the patients can be treated to reduce exacerbations, the risk of developing COPD and proper functioning of lung airways. However other studies and most possible remodeling studies on oxidative stress can help in better understanding the pathological traid involved in COPD.

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