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Genetic and biochemical markers in Emphysema

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Abstract

Aim of the present study to evaluate the Chronic Obstructive Pulmonary Disease (COPD), also known as chronic obstructive airway disease (COAD), is a group of diseases characterized by the pathological limitation of airflow in the airway that is not fully reversible. Emphysema is a multifactorial disorder and involves interplay of genetic, inflammatory and oxidative factors during its progression. The study has the following objectives. To identify the polymorphic variants of TNF α and MMP3 with the disease. To understand the role of oxidants (Nitrate/Nitrite) and antioxidant (Ceruloplasmin) in the etiology of emphysema. To evaluate the role of the inflammatory marker (hs-CRP) in emphysema. It is most often due to tobacco smoking but can be due to other airborne irritants such as coal dust, asbestos, or solvents as well as congenital conditions such as alpha-1-antitrypsin deficiency. It is the 4th leading cause of death in the U.S.

Key words: Emphysema, Biochemical markers, PCR, Clinical studies, TNF α and MMP3 genes.

INTRODUCTION

A person can live for weeks without food and a few days without water but only a few minutes without oxygen. Every cell in the body needs a constant supply of oxygen to produce energy to grow, repair or replace itself, and maintain vital functions. The oxygen must be provided to the cells in a way that they can use. It must be brought into the body as air that is cleaned, cooled or heated, humidified, and delivered in the right amounts. The respiratory system is the body's link to this supply of life-giving oxygen. A person at rest breathes about 6 liters of air a minute. Heavy exercise can increase the amount to over 75 liters per minute. During an 8-hour work day of moderate activity, the amount of air breathed may be as much as 8.5 m^3 (300 cubic feet). The skin, with its surface area of approximately 1.9 m^2 (20 sq. ft.) is commonly thought to have the greatest exposure to air of any body part. However, in reality the lungs have the greatest exposure, with a surface area exposed to air of 28 m^2 (300 sq. ft.) at rest and up to 93 m^2 (1,000 sq. ft.) during a deep breath.

COPD is a leading cause of chronic morbidity and mortality and should be a major public health concern. Reliable COPD prevalence data are lacking for many parts of the world, despite the frequency of major risk factors for COPD, such as cigarette smoking, use of biomass fuels, and air pollution. Approximately 15% of smokers will acquire COPD [1]. The World Health Organization (WHO) estimates that there are approximately 1.1 billion smokers in the world or approximately one third of the global population 15 years old [2]. The use of biomass fuel, such as wood for cooking, increases the risk of COPD by three to four times [3], and may be an important contributor to COPD prevalence for some parts of the world, particularly in developing countries and rural areas [4]. Air pollution increases the prevalence of COPD by an estimated 2% for each 10g increase in particulate matter [5]. The WHO has published data placing the world-wide prevalence of COPD at 0.8% [6]. Other reports place the prevalence of COPD substantially higher, at approximately 4 to 6% in the US [7].

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Studies of lung or bronchial biopsies and induced sputum have shown evidence of lung inflammation in all cigarette smokers. Studies of bronchial biopsy specimens from patients with mild to moderate COPD show an increase in inflammatory cell infiltration in the central airways, compared with nonsmokers or smokers who have not developed the disease [8]. Cigarette smoking is known to increase circulating neutrophil leukocyte count and to cause sequestration of neutrophils in the lung capillaries [9] by decreasing their deformability. Cigarette smoke activates macrophages to release inflammatory mediators, including tumor necrosis factor α, IL-8 and other CXC chemokines, monocyte chemotactic peptide-1, leukotriene B4, and reactive oxygen species. There is an increased number of dendritic cells in the airways and alveolar walls of smokers [10].

Abnormalities of skeletal muscle in COPD are due to increased work of breathing, inactivity, systemic inflammation, malnutrition, blood-gas abnormalities and impaired oxygen delivery, electrolyte imbalances, drugs, and comorbid states [11, 12]. Respiratory muscles are overloaded due to increased work of breathing [13, 14] and limb muscles are underloaded due to inactivity [15]. Bronchoalveolar lavage fluid of smokers and nonsmokers contains significant concentrations of ceruloplasmin, the major serum inhibitor of lipid peroxidation, with limited superoxide dismutase activity. This suggested that ceruloplasmin may protect the lower respiratory tract against oxidant(s) in cigarette smoke and air pollutants.

The present work reported has been carried out with room for further developments. A large number of genes and biochemical factors have been found to be responsible for the onset and progression of emphysema. The involvement of only two genes and three factors has been shown here. Among the large number of polymorphisms seen in the TNF α and MMP3 genes only one each have been analyzed. Further work in this direction and in the quantification of the said gene products will aid in a better understanding of the disease and in the creation of better drugs against it.

MATERIALS AND METHODS

The subjects of the present study were 52 patients presenting Emphysema along with 34 sex – matched healthy controls. Blood samples were collected from the patients visiting the Government Chest Hospital, Erranuma, Hyderabad.

Collection of blood sample:

10ml of venous blood was drawn and 5 – 6ml of blood was collected in EDTA – vacationers, allowed to stand, centrifuged at 1500 rpm for 10 minutes for separation of plasma, which was collected in eppendorf tubes.3ml of blood was collected in vacationers, without anticoagulant, allowed to clot, centrifuged at 1500 rpm for 10 minutes for the separation of serum, which was collected in eppendorf tubes. All the eppendorf tubes were carefully and accurately labeled with the patient's code. All samples were stored at -20º C until further use.

Isolation of genomic DNA:

The process involved repeated washing of the blood sample with a low salt buffer called TKM1 and Triton X [isooctylphenoxypolyethene ethanol] which lyses the RBCs and weakens the WBCs membranes. Subsequent addition of a high salt buffer called TKM2 and 10% SDS lyses the WBCs, degrades proteins and releases DNA into the solution. 6M NaCl is added to precipitate the proteins and absolute ethanol is used to precipitate DNA. The DNA is washed with 70% ethanol and finally dissolved in TE buffer until further use [16].

Estimation of DNA:

The isolated DNA needs to be studied for its quality and quantity before using it in molecular biology experiments. Estimation is of two types,

1.Quantitative estimation, 2. Qualitative estimation

Nucleic acid (DNA & RNA) has maximum absorbance at 260ηm. One OD value (standard) corresponds approximately 50µg/ml of double stranded DNA, 40µg/ml of single stranded DNA/RNA and 20µg/ml of oligonucleotides.The ratio between the readings at 260nm and 280nm (OD 260/ 280) provides an estimate of the purity of nuclear acid. Pure preparations of DNA and RNA have a ratio of approximately 1.8 and 2.0 respectively. If the DNA is contaminated with protein, the ratio will be <1.8 and the ratio is >2.0 indicates that the DNA is contaminated with RNA.

Polymerase Chain Reaction:

The amplification Refractory Mutation System (ARMS) or Allele Specific Polymerase Chain Reaction (ASPCR) is useful in the analysis of mutations and a wide range of polymorphisms. The ARMS reaction relies on the absence 3' proofreading activity of Taq DNA Polymerase. ARMS assay comprises two PCR reactions using same target DNA .In both PCRs one common primer which anneals with invariant sequence on one side of the mutation to be detected and a second primer that is specific to mutant or normal allele are used. The allele specificity of these primers is confirmed by nucleotide sequence at 3'end. On electrophoresing the PCR products , a mutant sample will show a band only when mutant specific primer is used and a normal sample will show a bands only when wild type specific primer is used . When bands are detected with both the primers it indicates heterozygous.

Details of the primers used for the sequences amplified by PCR, along with the target band size and annealing temperatures were given in Table-1. After PCR, the products were analyzed by gel electrophoresis.

Estimation of serum C – Reactive Protein (CRP)

Table-1. Primers used for the sequences amplified by PCR along with the target band size and annealing termperatures

Genes and Primer sequences	Amplicon	Position	Genotype	Reference
TNF-α-308 5'TCTCGGTTTCTTCTCCAT CG3' – forward 5'ATAGGTTTTGAGGGGCATG**A**3' – reverse for allele A 5'ATAGGTTTTGAGGGGCATG**G**3' – reverse for allele G	183 bp	-308	G/A	[17]
MMP3 -1612 5'GATTACAGACATGGGTCACGGCAC3' – forward 5'AATCAGGACAAGACATGG**TTTTT**C3' – reverse for 5A 5'AATCAGGACAAGACATGG**TTTTTT**3' – reverse for 6A	179/ 180 bp	-1612	5A/6A	[18]

To determine the levels of human serum CRP by enzyme immunoassay, A set of standards is used to plot a standard curve from which the amount of CRP in patient samples and controls can be directly read.

Estimation of plasma Nitrate/Nitrite:

Plasma Nitrite/Nitrate is estimated by the method of Greiss.

Estimation of Serum Ceruloplasmin:

Ceruloplasmin is an oxidase and has been termed as copper oxidase. It can catalyse the oxidation of some polyamines in its action on para – phenylenediamine was used by Ravin as a measure of the amount present in serum.

RESULTS

The results of the investigations carried out in Emphysema patients and controls are presented in the tables. All the subjects were examined clinically and information pertaining to age, sex, habits and health status were recorded in special case Proforma. Clinical examination was followed by a series of laboratory investigation for TNF-α (-308G/A) polymorphism, MMP3 (-1612 5A/6A) polymorphism, hs-CRP, nitrite/nitrate and serum ceruloplasmin levels.

Age & Sex:

In the present study Emphysema patients belonged to the age group of 38 – 80. The results were tabulated in table 2.

Table-2. Health status and Habits of patients and controls.

Parameters	Emphysema	Controls
Age (Mean ± SD)	60.5517 ± 9.3586	23.25 ± 4.2426
Sex	Male	Male
Smokers	47	0
Non-smokers	5	34
Alcoholics	15	0
Non-alcoholics	37	34
Vegetarians	8	4
Non-vegetarians	44	30

Habits/Smoking:

Out of the 52 patients, 47of them smoked. There are no smokers among controls.

Alcohol:

Out of the 52, only 15 were drinkers and the remaining were non-alcoholics. There are no alcoholics among controls.

Food habit:

Out of the 52 cases 44 were non-vegetarians and rest were vegetarians. Among controls, 30 were non-vegetarians and 4 were vegetarians. Table-2.

Tumour Necrosis Factor α polymorphism:

In emphysema patients, 3 (8.33%) were GG homozygotes, while only 1 (2.94%) among controls had this genotype. The number of GA heterozygotes was 32 (88.89%) among patients and 33 (97.06%) among controls. There was 1 (2.77%) AA homozygote among patients and none among controls.

The number of G alleles was 38 (52.78%) and A alleles was 34 (47.22%) among patients. Among controls the numbers were 35 (51.47%) and 33 (48.53%) respectively. (Tables 3 and 4).

Table-3. Distribution of TNF-α (-308G/A) genotypes in emphysema patients and controls.

Subjects	Number of cases	TNF α Genotypes		
		GG	GA	AA
Emphysema	36	3 (8.33%)	32 (88.89%)	1 (2.77%)
Controls	34	1 (2.94%)	33 (97.06%)	0

Table-4. Distribution of TNF-α (-308G/A) alleles in emphysema patients and controls.

Subjects	Number of cases	TNF α alleles	
		G	A
Emphysema	36	38 (52.78%)	34 (47.22%)
Controls	34	35 (51.47%)	33 (48.53%)

Tumour Necrosis Factor α polymorphism:

In emphysema patients, 3 (8.33%) were GG homozygotes, while only 1 (2.94%) among controls had this genotype. The number of GA heterozygotes was 32 (88.89%) among patients and 33 (97.06%) among controls. There was 1 (2.77%) AA homozygote among patients and none among controls. The number of G alleles was 38 (52.78%) and A alleles was 34 (47.22%) among patients. Among controls the numbers were 35 (51.47%) and 33 (48.53%) respectively. (Tables 3 and 4)

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Table-5. Distribution of MMP3 (-1612 5A/6A) genotypes in emphysema patients and controls.

Subjects	Number of cases	MMP3 Genotypes		
		5A/5A	5A/6A	6A/6A
Emphysema	36	2 (5.56%)	26 (72.22%)	8 (22.22%)
Controls	34	0	33 (97.06%)	1 (2.94%)

Table-6. Distribution of MMP3 (-1612 5A/6A) alleles in emphysema patients and controls.

Subjects	Number of cases	MMP3 alleles		
		5A		6A
Emphysema	36	30	(41.67%)	42 (58.33%)
Controls	34	33	(48.53%)	35 (51.47%)

Serum C Reactive Protein:

Emphysema patients showed a mean of 5.0425 ± 2.4886 µg/ml of serum CRP. The controls showed lower levels of 2.025 ± 0.6718 µg/ml. Mean levels of CRP in patients was almost twice that of controls. (Table-8).

Table-7. Association of TNF α and MMP3 genotypes in emphysema patients.

TNF α Genotype	MMP3 Genotypes			
	5A/5A		5A/6A	6A/6A
GG	1	(2.78%)	2 (5.56%)	1 (2.78%)
GA	1	(2.78%)	24 (66.67%)	7 (19.44%)

Plasma Nitrate/Nitrite

In the present study, emphysema patients showed a mean of 3.76 ± 1.11 µmoles/ml while the controls gave a mean of 1.56 ± 0.34 µmoles/ml. Mean levels of nitrate/nitrite was almost twice that of controls (Table-9).

Table-8. Serum hs – C Reactive Protein levels in emphysema Patients and controls.

Subjects	Number of cases	hs - CRP levels (µg/mL) Mean ± SD
Emphysema	52	5.0425 ± 2.4886
Controls	34	2.025 ± 0.6718

Table-9. Plasma Nitrite/Nitrate levels in emphysema patients and controls.

Subjects	Number of cases	Nitrite/Nitrate levels (µmoles/mL) Mean ± SD
Emphysema	52	3.76 ± 1.11
Controls	34	1.56 ± 0.34

Serum Ceruloplasmin

The mean ± SD levels of serum ceruloplasmin in emphysema patients was found to be 22.8794 ± 12.6762 and those in controls was 24.5123 ± 5.7835. The results show that the levels of ceruloplasmin in patients are lesser than those of controls. (Table-10).

Table 10: Serum Ceruloplasmin levels in emphysema patients and controls.

Subjects	Number of cases	Ceruloplasmin levels (mg/dL) Mean ± SD
Emphysema	52	22.8794 ± 12.6762
Controls	34	24.5123 ± 5.7835

TNF-α and Ceruloplasmin levels:

Emphysema patients with genotype GG showed a mean ceruloplasmin level of 28.38 ± 5.92 mg/dL while those with the GA genotype showed a higher mean level of 33.36 ± 14.44 mg/dL (Table-11).

Table-11. Comparison of TNF alpha Genotypes and Ceruloplasmin, hs-CRP and Nitrite/Nitrate levels in emphysema patients.

TNF α Genotype	GG	GA
Ceruloplasmin levels (mg/dL)	28.38 ± 5.91	33.36 ± 14.43
C Reactive Protein (µg/mL)	3.70 ± 0.01	5.70 ± 2.31
Nitrate/Nitrite levels (µmoles/ml)	4.9 ± 1.02	3.13 ± 0.61

TNF-α and hs-CRP levels:

Patients with GA genotype showed a higher hs-CRP levels of 5.71 ± 2.31 µg/ml while those with GG genotype showed a lower level of 3.71 ± 0.02 µg/ml. (Table-11).

TNF-α and nitrate/nitrite levels:

Patients with genotype GG showed a higher level of 4.9 ± 1.02 µmoles/ml and those with genotype GA showed lower levels of 3.13 ± 0.61 µmoles/ml. (Table-10).

MMP3 and ceruloplasmin levels:

Emphysema patients with genotype 5A/5A showed a mean ceruloplasmin level of 14.745 ± 9.85 mg/dL while the heterozygotes showed a higher value of 27.83 ± 15.03 mg/dL and those with the 6A/6A genotype showed a higher mean level of 30.21 ± 4.45 mg/dL (Table-12).

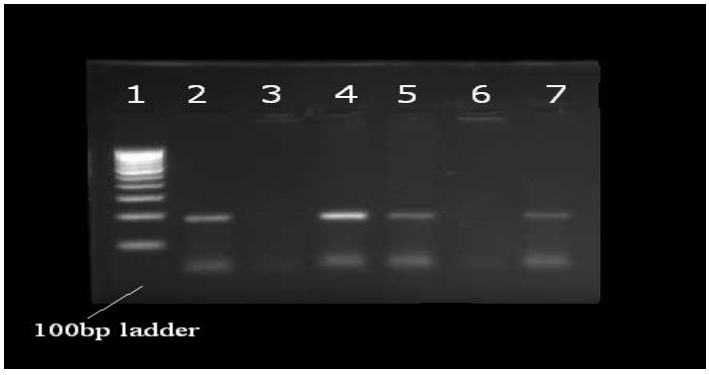
MMP3 and hs-CRP levels:

Patients with 5A/5A, 5A/6A and 6A/6A genotypes showed progressively increasing hs-CRP levels of 4.61 ± 2.58, 5.56 ± 2.42 and 6.91 ± 0.17 µg/ml respectively. (Table-12).

Table-12. Comparison of MMP3 genotypes and ceruloplasmin, hs-CRP and Nitrite/Nitrate levels in emphysema patients.

MMP3 Genotype	5A/5A	5A/6A	6A/6A
Ceruloplasmin levels (mg/dL)	14.7 ± 9.8	27.83 ± 15.02	30.21 ± 4.45
C Reactive Protein (µg/mL)	4.60 ± 2.5	5.55 ± 2.41	6.90 ± 0.17
Nitrate/Nitrite levels (µmoles/ml)	3.01 ± 0.1	3.53 ± 0.91	2.8 ± 0.14

Picture-1. TNF-α (-308) polymorphic forms

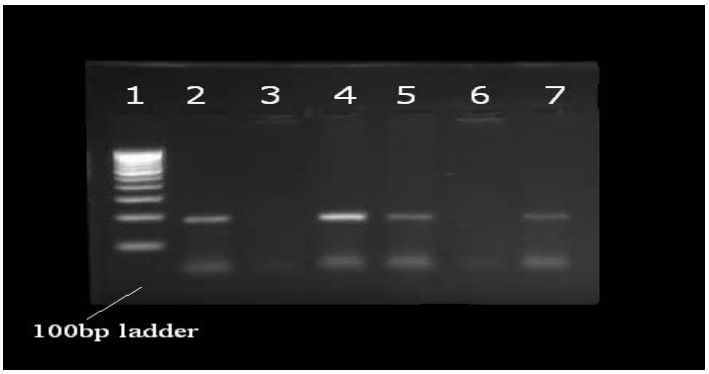


Lane 1: 100bp DNA ladder;
Lanes 2 and 3: Homozygous GG;
Lanes 4 and 5: Heterozygous GA;
Lanes 6 and 7: Homozygous AA;

MMP3 and nitrate/nitrite levels:

Patients with genotype 5A/5A showed a level of 3.01 ± 0.11 µmoles/ml and those with genotype 5A/6A showed levels of 3.53 ± 0.91 µmoles/ml. Patients with 6A/6A genotype showed levels of 2.8 ± 0.14 µmoles/ml. (Table-12).

Picture 2: MMP3 (-1612) polymorphic forms



Lane 1: 100bp DNA ladder;
Lanes 2 and 3: Homozygous GG;
Lanes 4 and 5: Heterozygous GA;
Lanes 6 and 7: Homozygous AA;

Discussion

COPD is an internationally important cause of morbidity and mortality. It is the only common cause of death in the United States that has increased over the last 30 years [19, 20]. COPD is a complex inflammatory disease that involves many different types of inflammatory and structural cells, all of which have the capacity to release multiple inflammatory mediators [21]. The present study demonstrates that there is almost no difference in frequency of the A and G alleles in patients (47% and 53%) with emphysema and control (48% and 51%) subjects. This is in accordance with the work done [22], who found no significant difference in frequency of the A and G alleles in COPD patients and controls. In vivo TNF- converting activity is brought about by certain metalloproteases [23]. TNF α induces the production of MMP3 in degeneration of intervertebral discs [24]. MMPs are a family of enzymes that degrade the ECM [25, 26]. MMP-9, also called gelatinase B, has been proposed to play arole in the development of emphysema and is involved in the digestion of extracellular matrix components such as gelatin, collagens (IV, V, XI, XVII), and elastin [27, 28]. MMP12$^{-/-}$ (knockout) mice showed resistance to cigarette – smoke induced emphysema [29, 30]. *In vitro* promoter activity as well as *in vivo* gene expression of the 5A variant is about 2–4-fold higher than that of the 6A allele [31, 32]. We observed reduced levels of CRP (4.6µg/ml) and ceruloplasmin (14.7mg/dL) in patients with 5A genotype compared with

those having 6A genotype. There seems to be no known connection between the MMP3 genotypes and nitrate/nitrite levels (3, 3.5 and 2.8µmoles/ml for 5A/5A, 5A/6A and 6A/6A MMP3 genotypes).

Our findings are preliminary and we hypothesize that MMP3 and TNF α polymorphisms and their association with inflammatory and oxidative markers may contribute to emphysema initiation and progression.

Conclusion

Though this study is preliminary, we can conclude that there exists an inverse relationship between the levels of oxidants (nitrate/nitrite) and antioxidants (ceruloplasmin) in emphysema. Also, true to its inflammatory nature, the disorder shows high levels of the acute phase protein (CRP). As far as the gene polymorphisms are concerned, we find that the results vary according to the ethnicity of the population and that expression studies in combination with polymorphic analyses would help us associate these factors with the disease better.

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Competing interests

The authors have declared that no competing interests exist.

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