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Tumor necrosis factor alpha promoter -238 *TNF-α* (G/A) locus polymorphism and the risk of Chronic Bronchitis

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ABSTRACT

Objective -Chronic bronchitis (CB) represent a classic phenotype of COPD having different genetic determinants. The overall prevalence of CB in India was found to be 3.49%. *Methods*- We assessed *TNF-alpha* polymorphism -238 *(*G/A) and risk of Chronic Bronchitis using PCR-RFLP. We studied 10 cases with chronic bronchitis and 10 healthy individuals. *Results*. *GA* genotype was found to be associated significantly with the increased risk of Chronic Bronchitis (*p, 0.5*); also *G allele* was displayed high risk of CB (76.9%) in our study population. In our study we also found elevated levels of *IFN-gamma* and *8 oxo-dG* and decreased level of *TNF-alpha* in CB patients. *Conclusion*. Our study indicated, the presence of *GA genotype* being a risk factor in the development of CB in Central Indian Population .

INTRODUCTION

Chronic Bronchitis with air-flow obstruction is known as Chronic Obstructive Pulmonary Disorder. Cigarette smoke is a most common cause of chronic bronchitis which contains $10^{14}10^{16}$ free radicals/puff.10-20% of chronic heavy smokers will have COPD [1]. There has been found a strong relationship between smoking and chronic bronchitis demonstrated in the heavy smokers (>25 cigarettes per day) [2]. Cigarette smoke induces an inflammatory response by heterogeneous population of neutrophils, macrophages and T lymphocytes (predominantly CD8+ cells). Macrophages and neutrophils are the two major inflammatory cells within the lung that release various proteolytic enzyme leading to tissue destruction and airway enlargement. Activated macrophages release various inflammatory proteins, such as cytokines, chemokines and proteolytic enzymes. *TNF-α* is one of the acute pro-inflammatory cytokines secreted by macrophages and plays a central role in inducing the recruitment of inflammatory cells to the lung in a variety of pulmonary diseases [3]. *TNF-α* plays a critical role in both tissue destruction and damage recovery and may initiate an inflammatory cascade consisting of other inflammatory cytokines [4]. Elevated levels of *TNF-* are found in bronchoalveolar lavage fluid (BAL) [5], bronchial biopsies [6] and induced sputum [7] from patients with chronic bronchitis/COPD, suggesting that *TNF-alfa* may contribute to the airway remodelling and altered smooth muscle cell function (i.e. airway hyper responsiveness) found in COPD [8,9].

TNFα gene polymorphism - *238 G/A* and *308G/A* is the change in the nucleotide bases guanine into adenine in position -238 and -308. These changes will result in changes in the transcription process that influence *TNFα.* And based on the position, polymorphisms affect only the velocity of protein synthesis [8]. Enhanced levels of *TNF-α* have been detected in sputum and in circulation of COPD patients, indicating that this cytokine is involved in both the local and systemic inflammation present in COPD [10]. Oxidative stress due to smoking is said to be the main etiological factor causing COPD [11]. The best studied nucleobase modification includes 8-oxo- 7,8-dihydro-2′-deoxyguanosine *(8-oxo dG).*

Interferon-gamma (IFN-gamma) is also a proinflammatory cytokine which is produced by Th1 lymphocytes and is an essential component of the host immune responses to pathogens, including viruses [12]. *IFN-gamma* is a low molecular weight (17,000-k dalton) peptide which seems to have a remarkably broad range of biological and immunologic effects, including antiviral action, growth regulation, and immune modulation. These effects are shared by other lymphokines and cytokines such as interleukin 1 *(IL-1),* interferon alpha *(IFN-alpha)* and *TNF-beta.* [13]. It exerts greater immunomodulatory activity, including

activation of macrophages, than the other interferons, it exerts greater lytic effects than the other interferons, it potentiates the actions of other interferons. *IFN-* inhibits intracellular microorganisms other than viruses (e.g., rickettsia). The only gene for interferon gamma is found on chromosome 12 [14].

IFN-gamma levels are raised in the airways of COPD patients [15-17] the number of *IFN-gamma*producing lymphocytes are increased in the lungs of COPD patients [18], and disease severity correlates with *IFN-gamma* production by CD8 cells [19]. This increase in *IFN-gamma* is not simply due to smoking, as CD8 cells from COPD patients release more *IFN-gamma* than those from current smokers without COPD [20]. Furthermore, viruses are a major cause of COPD exacerbations, and the levels of *IFN-gamma* are increased in COPD patients during virus-triggered exacerbations [21].Our study would throw light on *TNF haplotypes (*promoter -238 *TNF-α* G/A, rs361525 variants*)* contributing to increased risk in developing Chronic Bronchitis in a tertiary care hospital of central India. Additionally association of serum biomarkers *TNF-α*, *IFN-γ* and *8-oxo-dG* to genetic susceptibility would help in information regarding the immune status of study subjects.

METHODS

Subjects: Our study has been done on the Central Indian Population (Bhopal) suffering from Chronic Bronchitis (CB), visiting a tertiary care hospital in Bhopal. We selected 20 subjects with 10 cases and 10 controls. Our case subjects comprised of 10 patients suffering from CB (smokers and non-smokers both) and controls were the subjects with normal lung functioning (smokers and non-smokers both). Inclusion criteria for cases, subjects with chronic bronchitis; Exclusion criteria were subject with other lung related disorders; Control subjects with normal lung functioning and no episodes of chronic bronchitis.

Statistical analysis: Statistical Package for EndNote was used for statistical analysis. The Differences were considered to be significant when the *p*-value was ≤ 0.05.The relative risk associated with rare alleles was estimated as an odds ratio (OR) with a 95% confidence interval (CI).

Genotyping: Genomic DNA was isolated from blood by Phenol-Chloroform method (Sambrook and Russell 2001).Genomic DNA was amplified by Multiplex Polymerase Chain Reaction technique. We performed Multiplex PCR on thermal cycler (PTC-200, MJ Research) to determine the -238 *TNF-alpha* genotypes using one set of primer to amplify fragment of 231 bp. The primers used were: TNF--F:ATCTGGAGGAAGCGGTAGTG and TNF--R:AGAAGACCCCCCTCGGAACC. DNA (1.5ng) was amplified in 50µl of multiplex reaction mixture containing 40pmol/µl of each of the primers (Genei, USA) in a medium containing 50mM $MgCl_2$ (Invitrogen, USA), 10mM dNTPs (Genei, USA), 10X PCR Buffer (Invitrogen, USA) and 1U Taq DNA Polymerase (Genei, USA). The DNA was denatured at 95°C for 5 min, and temperature cycling was set at 94°C for 30 s, 58°C for 30 s, and 72°C for 1.5 min for 30 cycles, followed by a final extension at 72°C for 5 min. The size of the PCR product was 150 bp. To determine whether the samples contain nucleotide *A* and/or nucleotide *G* at position *-238 TNF-alpha*, DNA PCR product cutting was performed using *MspI* restriction enzyme having 5'-C CGG-3' recognition site. Either only one band appeared with the size of 150bp and homozygote *-238G (GG)* allele generated 2 bands with size of 130bp and 20bp. Heterozygous samples containing *allele G* and *A*, generating into 3 bands with the size of 150bp, 130bp and 20 bp.

Biomarkers: We used TNF-alfa, IFN-gamma and 8-oxo-dG as biomarkers for our study. These biomarkers are documented to be directly associated with oxidative stress hence also termed as oxidative stress biomarkers. Under stress conditions the level of these biomarkers vary in body fluids. Therefore, we performed Enzyme-linked Immuno Sorbant Assay (ELISA) for measuring the levels of oxidative stress markers using serum as sample. ELISA tests were performed using commercial kits on ELISA reader (BIO-RAD,Microplate reader, California, USA).

RESULTS

The association between *TNF Genotypes* and serum biomarkers (*TNF-, IFN-* and *8 oxo-dG*) to genetic susceptibility was studied in 13 cases (CB patients) and compared them with 12 healthy individuals in Central Indian Population.

From our study we found that *GA* genotype was significantly associated with increased risk of CB in our studied population. *GA* genotype was present among 46.15% of cases and 33.3% of controls. Homozygous genotype *GG* and *AA* were found 30.7%, 50% and 23.07%, 16.6% in cases and controls respectively. *GG* genotype was most common in our studied population (Table 1).

We found that *G allele* was associated with increased risk of CB (76.9%). Our result was not significant because p 0.05 (p=0.69). *Allele A* was found 69.2% and 50% in tests and controls respectively. The Central Indian Population may susceptible to CB due to *G Allele (Table2).*

TNF-alpha levels decreased in test subjects (0.49±0.44) as compared to control subjects (12.51±24.7). Enhanced levels of IFN-gamma were found in *GA* genotype in cases (0.75±0.24) alongwith 8 oxo-dG levels were found in *AA* genotype in cases (183.55±108.8) (Table 3).

TNF-alpha is a proinflammatory serum biomarker and their

Table 1. : Summarizes the genotypic distribution of studied population.

Genotypes	Controls	Tests	p- value	OR	95% CI
GA	4 (33.3%)	6(46.15%)	0.51	1.71	0.33-8.67
GG	6 (50%)	4 (30.7%)	0.33	0.44	0.08-2.27
AA	2 (16.6%)	3(23.07%)	0.69	1.50	0.20-10.99

Table 2. : Described allele distribution in test and control subjects.

Alleles	controls	cases	p-value	OR	95% CI
G	10 (83.3%)	10 (76.9%)	0.69	0.66	0.09-4.88
A	6 (50%)	9 (69.2%)	0.33	2.25	0.43-11.52

Table 3. : Describes the association of average concentration of biomarkers (TNF-alpha, IFN-gamma and 8 oxo-dG) with different genotypes.

Genotypes	avg. TNF-alpha (ng/ml)		avg. IFN-γ (IU/ml)		avg. 8 oxo dG (ng/ml)	
	cases	controls	cases	controls	cases	controls
GA	0.49±0.44	12.51±24.7	0.75±0.24	0.01±0.01	178.7±58.5	67.91±12.9
GG	0.40±0.62	2.33±5.04	0.57±0.4	0.39±0.2	174.91±59.5	63.35±20.4
AA	1.62+2.68	8.23+9.43	0.67+0.05	0.62+0.05	183.55+108.8	71.62+12.6

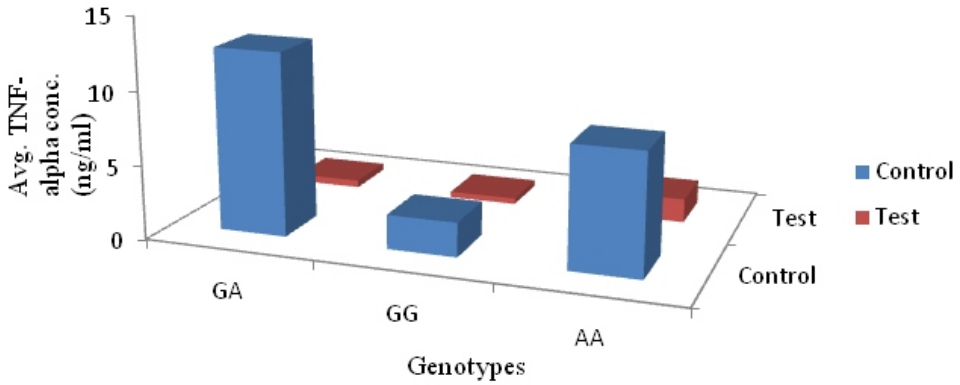

Fig 1. : Avg. TNF-alpha conc. (ng/ml) with different genotypes in test and control subjects.

serum level were measured through ELISA test (Fig. 2 and 3). TNF-alpha level was significantly decreased in test subjects as compared to control subjects. Its concentration in serum gives information about the increased production of oxidative stress in body. In our study TNF-alpha conc. was found to be lowered in test cases. This showed the activity of TNF-alpha decreases with increasing oxidative stress. From this, *GG* genotype was more susceptible to oxidative stress.

IFN-gamma conc. elevated in test subjects as compared to control and increased with increasing oxidative stress (Fig.4 and 5) . We compared the level of IFN-gamma with different genotypes (fig.4 and 5). The levels of IFN-gamma were elevated in *GA* genotype (0.75±0.24) than *GG* (0.57±0.4) and *AA* (0.67±0.05) *genotypes* in test subjects. From this we can conclude that *GA genotype* of our studied population were more susceptible to CB than *GG and AA genotype.*

The level of serum 8 oxo-dG were measured through ELISA in tests and controls both (Fig.6 and 7). 8 oxo-dG is an oxidative stress biomarker and its increased level showed more oxidative DNA damage in an organism. We compared the level of 8 oxo-dG between different genotypes. Increased concentration was found in *AA genotype* (183.55±108.8) in case subjects, which showed that cases having *AA genotype* were more susceptible to DNA damage than *GA* (178.7±58.5) and *GG* (174.91±59.5) genotypes.

DISCUSSION

Huang *et.al.*1997 [23], reported that greater TNF- production is associated with a higher risk of developing chronic bronchitis, both in smokers or non-smokers. TNF- production increased at the site of inflammation. They studied TNF-1 and TNF-2 gene polymorphisms in CB patients in European population. This stresses the importance of TNF- in inflammatory processes that

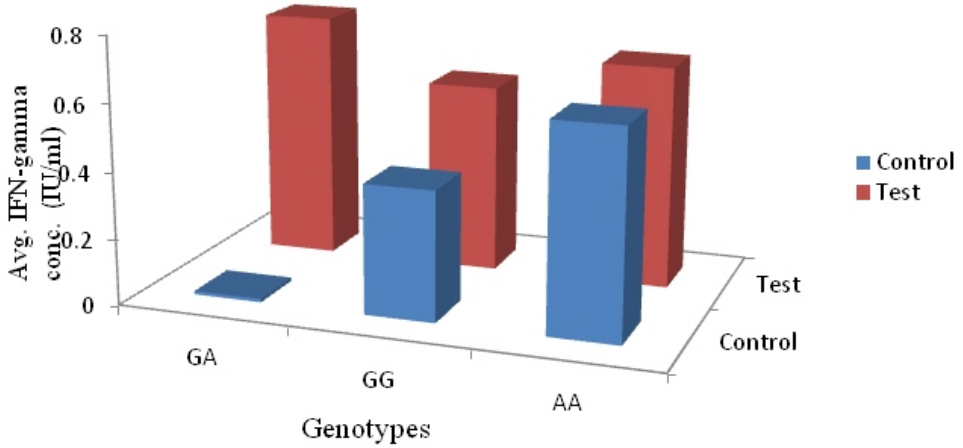

Fig 5. : Avg. IFN-gamma conc.(IU/ml) with different genotypes in test and control subjects.

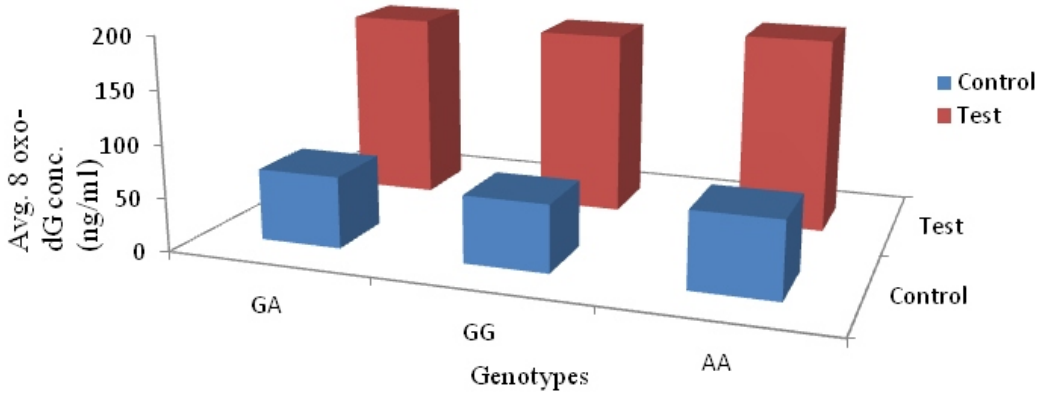

Fig 7. : Average 8 oxo- dG concentration with different genotypes in test and control subjects.

may lead to tissue injury and also suggests that these polymorphisms may be used as a marker to identify individuals who are more susceptible to inhalational insults. In our study we found that *G allele* was associated with increased risk of CB (p=0.6900,OR=0.6667). A study conducted in Chinese Han population by Guo and others (2012), found same results. They found that the G allele of the TNF-alpha gene was more frequently detected in COPD patients (95%) versus control subjects (90%) (OR=1.97, 95% CI 1.21-3.21, p=0.0060). Tanaka and Co-workers ,2007 [25], found that the frequency of the TNF-308A allele was significantly higher in the cases than control (p 0.001, OR=11.1, 95% Confidence Interval 2.9-42.6). Keicho and others,1999 [26], in their study found that the *-308 A* allele was rare in normal Japanese population. In Caucasians, the frequency of the same allele is much higher (16-27%). Only one of the 76 Japanese patients and none of the 87 controls carried the *-308 A allele.*

In our study we found elevated levels of *IFN-gamma* and *8 oxo-dG* and decreased level of *TNF-alpha* in CB patients. A study conducted in the Caucasian population, to find serum *TNF-alpha* level in smokers and non-smokers in COPD patients between 43 healthy smokers and 19 healthy non-smokers. The serum levels of *TNF-alpha* were significantly higher in the smoker group than in the non-smoker group (p 0.05). They further compared the concentration of *TNF-alpha* in the serum of non-smokers and smokers with a daily exposure of less than 1 pack, the difference did not reach statistical significance (p=0.17) [27]. Our work is suggestive of an association of *TNF genotypes* and serum biomarkers (*TNF-alpha, IFN-gamma* and *8 oxo-dG*) with CB. On comparing CB patients with controls we found an association of *GA* genotype with CB (P value= 0.5). But further study with a larger sample size can throw some light on the association of *TNF genotypes* to CB. Various other studies have also reported the association of *TNF* polymorphism in development of CB.

A study done by Shukla and Co-workers,2012 [28] in North Indian population, revealed that the genotypic frequency of the heterozygous genotype in *TNF-alpha 308 G>A* polymorphism was higher in COPD patients (20.5%) as compared to the healthy controls (14.4%). In our study we found that *G allele* was associated with increased risk of CB (p=0.6900,OR=0.6667). A study conducted in Chinese Han population by Guo and others (2012), found same results. They found that the G allele of the TNF-alpha gene was more frequently detected in COPD patients (95%) versus [25], found that the frequency of the TNF-308A allele was significantly higher in the cases than control (p 0.001, OR=11.1, 95% Confidence Interval 2.9-42.6). Keicho and others ,1999 [26], in their study found that the *-308 A* allele was rare in normal Japanese population. In Caucasians, the frequency of the same allele is much higher (16-27%). Only one of the 76 Japanese patients and none of the 87 controls carried the *-308 A allele.*

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Limitations of the Study: The study has few limitations. First, our finding is based on small sample size observations; we cannot, therefore, exclude the possibility of confounding variables that may be associated with each of the exposures. Secondly, we compared CB patients with non CB patients and frequency of CB -238 *TNF-alpha* polymorphism (rs2569190) in each group was analyzed. Our control group may not truly represent to the whole local population as sample size was small.

CONCLUSION

From this study we can conclude that, the *GA genotype* of the *TNF-alpha* gene was found to be associated with the development of CB. In all cases which were suffered from CB, high concentration of *IFN-gamma, 8 oxo dG* and decreased concentrations of *TNF-alpha* were found as compared to control subjects. *GG* genotype was most commonly found in our population. Our study indicated, the presence of *GA genotype* might be a risk factor for the development of CB in Central Indian Population which was supported by elevated serum concentrations of *IFN-gamma* and *8 oxo-dG* while decreased levels of *TNF-alpha.* This study would provide new insight into CB development in other different population.

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