INTRODUCTION

Chronic obstructive pulmonary disease (COPD) is a serious and expanding cause of global mortality and morbidity with 3.2 million deaths in the year 2015, ranking it as the fourth commonest cause of mortality.

Aim: Data about polymorphism in tumor necrosis factor-alpha (TNF-α) and its serum levels in chronic obstructive pulmonary disease (COPD) are conflicting. We aimed to evaluate the association of TNF-α-308 G > A polymorphism in patients with COPD in Kashmir (North India), a high burden area and also determined the serum TNF-α levels in these patients.

Materials and Methods: One hundred spirometrically confirmed COPD patients and 163 controls resident from Kashmir valley (North India) were recruited. Genotyping of the promoter region of TNF-α was carried out using polymerase chain reaction-restriction fragment length polymorphism. The serum TNF-α was quantified using the Cytometric Bead Array flex system by flow cytometry. Results were subjected to appropriate statistical treatment and P < 0.05 was considered statistically significant.

Results: Ninety-one COPD patients (91%) had G/G (wild homozygous) genotype and nine patients (9%) had G/A (heterozygous) genotype. Among the control population, 150 (92%) had G/G genotype and 13 (8%) had G/A genotype. The variant allele “A” was not detected in either of the two groups. Serum levels of TNF-α were significantly higher in patients compared to control group (8.0 ± 10.1 pg/ml vs. 3.3 ± 0.42 pg/ml, respectively, P = 0.0001).

Conclusion: While serum levels of TNF-α are higher in COPD patients compared to the controls, there was no difference in the prevalence of TNF-α-308 polymorphism in the ethnic Kashmiri population with COPD.

KEY WORDS: Chronic obstructive pulmonary disease, polymorphism, tumor necrosis factor-alpha

ABSTRACT

A case-control study of tumor necrosis factor-alpha promoter polymorphism and its serum levels in patients with chronic obstructive pulmonary disease in Kashmir, North India

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worldwide. COPD is marked by continuous irreversible airflow restriction, which is associated with an unusual inflammatory reaction of lungs to toxic gases or particles. Although cigarette smoking is considered to be the most important environmental risk factor for developing COPD, only 10%–15% of cigarette smokers develop acute impairment related to COPD. Regardless of the large amount of published work, the elements determining disease progression and vulnerability remain poorly understood.

The prevalence of COPD has increased by 44.3% globally in the past 25 years. India shoulders a disproportionate burden of COPD, and the number of cases of COPD in India increased from 28.1 million in 1990 to 55.3 million in 2016. In a recent Burden of lung disease study, we have reported the prevalence of chronic airflow limitation in Kashmir to be 17.3% in males and 14.8% in females. The disease is punctuated with exacerbations and we have recently reported that acute exacerbations of COPD are associated with substantial cost to the patient and the exchequer.

Genetic predisposition has been implicated in the causation of COPD, and even as the exact nature of the genetic impact remains unclear, evidence suggests that inflammation of large and small airways as a reaction of exposure to inhaled gases/particles is essential in the pathogenesis of COPD. Deficiency of alpha-1 antitrypsin is recognized as one of the prominent genetic risk factors for COPD; however, such cases are reported to represent just 1%–2%. Various studies have been performed to recognize genetic factors and till date almost 25 unique candidate genes have been shown to be variably associated with COPD.

Tumour necrosis factor-α (TNF-α) is a pleiotropic pro-inflammatory cytokine mainly released by activated macrophages. TNF-α plays a vital role in various cellular processes including proliferation, inflammation, differentiation, apoptosis, etc. A biallelic single-nucleotide polymorphism in the promoter region at position 308 upstream of the transcription start site of TNF-α where nucleotide guanine (G) is substituted by adenine (A). Even as a large number of studies have been carried out to study the relationship of COPD risk and TNF-α polymorphism, the findings have been conflicting. A meta-analysis of 24 investigations demonstrated that TNF-α-308 polymorphism is related with an increased hazard of COPD in the Asian population; however, this association is not significant among Caucasians. Several investigations have found elevated levels of TNF-α in bronchial biopsies, induced sputum, and bronchoalveolar lavage fluids of COPD patients compared to healthy smokers. In an experiment on mice models, overproduction of TNF-α led to inflammation and pulmonary emphysema and is assumed to cause 70% of emphysema and inflammation-induced due to cigarette smoke.

The Kashmir region of North India has reported a high burden of COPD. The population also has a distinct ethnic character as reported earlier by us with the genetic admixture characterized by mixing of the European and North Indian DNA. While there is a high prevalence of smoking in the studied populations and also a high level of exposure to biomass fuels exists, an inquiry into the possible genetic determinants leading to a high burden of COPD is mandated. The present study has been designed to study the association of TNF-α polymorphism in COPD patients and also determine any alterations of the serum levels of TNF-α among such patients.

**MATERIALS AND METHODS**

**Study population**

A case–control study was conducted during the years 2016–2019 in Sheri Kashmir Institute of Medical Sciences (SKIMS), an 850-bedded tertiary care cum referral center located in Srinagar, the summer capital of the northern Indian state of Jammu and Kashmir. A total of 100 patients (68 males, median age = 70 years) with spirometrically defined COPD and 163 controls (with normal spirometry, 111 males, median age = 65 years) were randomly selected and included in the study. COPD was defined according to the global initiative for chronic obstructive lung disease with a postbronchodilator forced expiratory volume in 1 sec/forced vital capacity ratio of <0.7. The inclusion criteria were: Patients suffering from COPD and confirmed by spirometry, patients aged >40 years of age and who had at least smoked one cigarette/day for 1 year. A current smoker was defined as a person who had smoked 100 cigarettes in his/her lifetime and who currently smokes. A nonsmoker was defined as a person who had smoked <100 cigarettes in his/her lifetime. The criteria for exclusion from the study among COPD group were patients with lung cancer, asthma, or pulmonary tuberculosis.

**Data collection**

A structured questionnaire was designed to collect the patient information. The information pertinent to the investigation concerning all the patients including demographic information, smoking history, clinical parameters, blood investigation reports, and spirometry data was obtained from the patients’ medical records.

**Sample preparation and genomic DNA extraction**

A volume of 5–6 ml of venous blood was obtained by phlebotomy, of which 2 ml was transferred in ethylenediaminetetraacetic acid vacutainers for genotyping and the remaining was put into a serum clot activator vial for cytokine serum measurement. The blood samples for genotyping were stored at −80°C until processing and analysis. Genomic DNA was extracted from blood using QIAGEN blood mini kit (Qiagen, Germany) using the manufacturer’s instructions. Extracted DNA samples were stored at −20°C until used. The quantitative and
The determination of serum tumor necrosis factor-α level
The serum concentration of TNF-α was determined using a flow cytometry-based array (BD) following the manufacturer’s protocol. Briefly, 100 µl of serum sample from each group was incubated using capture beads against TNF-α for 1 h at room temperature. It was followed by incubation using detection reagent for 2 h at room temperature. The beads were centrifuged at 200 g for 5 min at room temperature and resuspended in 200 µL of wash buffer. Data acquisition was performed on BD fluorescence-activated cell sorting verse and analyzed using the FCAP Array software (Soft Flow Hungary Ltd). Concentration was obtained using a standard curve generated using recombinant TNF-α provided in the kit, following the above-mentioned protocol.

Statistical analysis
SPSS software (version 21.0, SSPS Inc., Chicago, USA) was used for data analyses. For each categorical variable, percentage, and numbers were presented along with median and interquartile range. Demographic characteristics and parameters were compared using the student’s t-test or Mann–Whitney U-test for the continuous variables, where appropriate. The allelic distribution and comparison of genotyping between patients and controls were analyzed by the Chi-square test. Differences were considered statistically significant when P < 0.05.

RESULTS
The patient group (n = 100) constituted of 68 males and 32 females. The mean age among the cases was 68.3 years (media n = 70 years; interquartile range [IQR] 64.5–75 years), whereas the controls consisted of 111 males and 52 females with a mean age of 63.8 years (media n = 65 years IQR 58–70 years). With respect to the smoking status, the patient group included 85 smokers and 15 nonsmokers. Of the 85 smokers, 12 patients were exclusive cigarette smokers, 48 were exclusive hookah smokers, and 25 were both cigarette and hookah smokers. There was no significant difference in the pack-year history among cases and controls (20.4 vs. 18.7, P = 0.445). Of the 32 (32%) females recruited in the COPD group, 13 reported exposures to the biomass fuel for at least 10–20 years, mostly for domestic cooking purposes. The characteristics of the case group and the control group are summarized in Table 1.

The genotype frequencies of TNF-α-308G >A polymorphism among the cases and controls were found to be in concurrence with Hardy–Weinberg equilibrium (cases: χ² = 0.22; P = 0.63 and controls; χ² = 0.281; P = 0.595). Ninety-one COPD patients (91%) had G/G (wild homozygous) genotype.
and nine patients (9%) had G/A (heterozygous) genotype. Among the control population, 150 (92%) had G/G genotype and 13 (8%) had G/A genotype. A/A (variant) genotype was not determined in any of the groups and hence was absent in the study population. The overall association of TNF-α-308 polymorphism with the risk of developing COPD was determined to be statistically nonsignificant \( (P > 0.05) \) [Table 2]. The frequency of the wild type “G” allele was similar in controls and cases (96% vs. 95.5%) and the frequency of the variant allele “A” was common in cases than in controls (4.5% vs. 3.9%).

Patients with COPD had significantly higher serum levels of TNF-α as compared to the controls \( (8.0 \pm 10.1 \text{ ng/ml vs. 3.3} \pm 0.42, P = 0.0001) \) [Figure 1]. When the serum TNF-α levels between GA and GG genotypes of cases versus controls were compared, we found that mean serum TNF-α levels were higher in both GA and GG genotype of cases as compared to controls [Figure 2], but the trend did not reach statistical significance. There was no statistical correlation between BMI and TNF-α when compared among cases and controls.

**DISCUSSION**

COPD is represented by chronic inflammation and oxidative stress. Cigarette smoke, air pollution, and reactive oxygen species (generated by inflammatory cells) are considered to be the source of oxidative stress emanated through inhaled noxious gases/particles. Increasing evidence propose a contributing role of genetic factors in COPD pathogenesis. TNF-α-308 polymorphism has been extensively studied for its possible role in various cancers and diseases.

Our data reveal that there was no difference in the frequency of G/G and G/A genotype among COPD patients and control group in the ethnic Kashmiri population. A/A, the variant genotype of TNF-α-308 was nonexistent in the study population. The frequency of -308A allele in our study was marginally higher in patients than in controls (4.5 vs. 3.9%) which is in concordance with another study that was conducted in North India by Shukla et al., wherein they reported the presence of -308A allele to be higher in COPD patients than controls. There are a few case–control studies examining the relationship of TNF-α polymorphisms and the risk of COPD, yet most of them exhibited no relationship of TNF-α-308 G/A with COPD. The frequencies of the TNF-α-308 G/A polymorphism in this investigation were congruous with the reports previously demonstrated in other studies.

A meta-analysis by Zhan et al. revealed that there was a significant association of TNF-α-308 polymorphism with an increasing risk of COPD, and the -308A allele emerged as an important risk factor in COPD development among Asian population but not in Caucasian population. However, we did not find the -308A allele in any of our patients or controls. Characteristic ethnic background could have contributed to this finding which requires further corroboration by further studies.

Cigarette smoking is considered as a major etiological factor for the development and advancement of COPD. The risk of developing COPD among individuals with a comparative history of smoking might be unique owing to the genetic susceptibility and/or different lifestyle. Despite having a similar pack-year history of smoking among cases and controls, TNF-α-308 gene polymorphism was not found to be a significant factor in the association of COPD in our patient population.

Serum levels of TNF-α were significantly higher in COPD patients as compared to the control population.
population \( P = 0.0001 \). Our results are in concordance with the outcomes reported by El-Shimy et al.\[36\]. Various studies that have been conducted previously\[23-26\] have also demonstrated elevated levels of TNF-\(\alpha\) in serum samples of COPD patients as compared to the healthy counterparts. A recent study by Singh et al.\[37\] reported an elevated serum TNF-\(\alpha\) levels in COPD patients when matched against healthy controls. Mitra et al.\[38\] studied 30 tobacco smoking COPD patients and 20 tobacco smoking non-COPD patients and found statistically significant difference in TNF-\(\alpha\) concentration among the study groups. In the present study, even though the mean serum concentrations of TNF-\(\alpha\) were higher in wild (GG) and heterozygous genotype (GA) among COPD patients than control population, the association was statistically nonsignificant.

To the best of our knowledge, this is the first study reported on the association of TNF-\(\alpha\)-308 polymorphism among COPD patients and controls from a similar ethnic background. The variant genotype was absent in both the study populations. Although the sample size was a limitation of the study, we believe that our results, the first for this unique ethnicity with a high COPD burden, would be useful for future investigations on larger samples regarding better understanding of the role of TNF-\(\alpha\) and possible association with other candidate genes identified in different populations across the globe.

**CONCLUSION**

From the present investigation, it is established that the TNF-\(\alpha\)-308G/A gene polymorphism is not associated with COPD. However, to ascertain whether TNF-\(\alpha\) plays a role in the development of COPD, other TNF-\(\alpha\) polymorphisms (\(-376G/A, +489G/A\) and \(-238G/A\)) need to be studied. The limitation of the study was the smaller sample size and the impact of TNF-\(\alpha\)-308G/A gene polymorphism in relation to the inflammatory processes and susceptibility of COPD needs to be explored in a larger cohort. The circulating serum levels of TNF-\(\alpha\) are significantly elevated in patients with COPD, which may be associated with the episodes of AECOPD and hence is not reliable to be used as a prognostic marker for COPD.

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**Conflicts of interest**

There are no conflicts of interest.

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