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## Association of Tumor Necrosis Factor Alpha 308 G/A Polymorphism with Asthma in Pakistani Population

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### ABSTRACT

Asthma is a chronic inflammatory and remodeling disorder of the airways, in which many cells, cellular elements, and cytokines play important roles. The role of tumor necrosis factor- $\alpha$  (*TNF- $\alpha$* ) in asthma is unclear in Pakistani population. The aim of this study was to assess the relationship between *TNF- $\alpha$*  -308 polymorphism and asthma.

Polymorphism of *TNF- $\alpha$*  (G-308-A locus; rs 1800629) in 329 asthmatic patients and 151 healthy controls was evaluated. DNA was prepared from blood samples of cases and controls. Samples were genotyped for *TNF- $\alpha$*  308 G/A polymorphism.

There was no significant difference in the frequency of GG (OR 1.049 with 95% CI 0.68-1.63) and GA (OR 0.987 with 95% CI 0.64-1.53) genotypes of *TNF- $\alpha$* -308. The AA genotype was absent in cases and only one AA genotype was observed in the controls.

The genetic polymorphism of *TNF- $\alpha$*  does not seem to be associated with asthma in Pakistani population.

**Keywords:** Asthma; Pakistani population; TNF alpha 308

### INTRODUCTION

Asthma is an obstructive inflammatory disease of the airways caused by a combination of genetic and environmental factors.<sup>1,2</sup> Acute inflammation of the bronchial tubes occurs during asthma attacks.

The bronchial tubes become swollen and narrowed

and mucus is secreted into the tubes from glands in the walls of the tubes. Swelling of the tubes and their plugging with mucus make it difficult to breathe. Airway inflammation is a hallmark of asthma and is caused by the release of cytokines and mediators from a variety of cells. When swelling of the bronchial tubes and excessive mucus production cause cough and wheezing and shortness of breath, the anti-inflammatory medications can reduce these symptoms by reducing inflammation in the bronchial tubes. The *tumor necrosis factor (TNF)- $\alpha$*  gene is located on the short arm of chromosome 6 within the major

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histocompatibility complex, where genetic alterations in the *TNF- $\alpha$*  locus are now known to be involved directly in high *TNF- $\alpha$*  production.<sup>3</sup> *TNF- $\alpha$*  is a cytokine believed to play a central role in airway inflammation and increased bronchial hyperresponsiveness.<sup>4,5</sup> The expression of *TNF- $\alpha$*  is upregulated in asthmatic individuals, as shown by its increased secretion in the airways and higher levels in bronchoalveolar lavage (BAL) fluid of symptomatic subjects.<sup>6-8</sup> One of the polymorphisms that directly affects *TNF- $\alpha$*  expression is located at nucleotide position -308. Interestingly, a G/A substitution at position -308 in the promoter of the *TNF- $\alpha$*  gene has been associated with increased *TNF- $\alpha$*  production in vitro.<sup>9</sup> Although several studies have reported an association of the *TNF- $\alpha$*  -308 G/A polymorphism with asthma,<sup>10-13</sup> other studies reject this association.<sup>14, 15</sup> This polymorphism has been positively associated with clinical symptoms of asthma and self-reported asthma.<sup>16</sup> However, other studies have observed equivocal or inverse associations between the *TNF- $\alpha$*  -308 G/A polymorphism and asthma. Considering the evidence supporting a pathogenic role of *TNF- $\alpha$*  in asthma, the conflicting results in different studies and the influence of the promoter polymorphisms on *TNF- $\alpha$*  production, we performed a case-control study of 480 individuals to analyze whether the *TNF- $\alpha$*  -308 G/A polymorphisms were associated with asthma susceptibility and severity in a group of individuals from Pakistan.

## MATERIALS AND METHODS

### Subjects

A total of three hundred twenty-nine asthmatic patients and one hundred and fifty-one controls were recruited for the present study. The patients included 182 female and 147 male asthmatic patients with a mean (SD) age of 40 (16) years (range: 11-90 years). These were recruited from outpatient respiratory clinics at Rawalpindi, Islamabad and Lahore, Pakistan. As far as the ethnicity of the cases and controls is concerned, most of them were Punjabi and all the subjects were ethnically matched for this study. To be eligible for inclusion in the study, patients had to have asthma as defined by the Global Initiative for Asthma with minor modifications.<sup>17</sup> At least 1 symptom of asthma was enough for asthma diagnosis, including cough,

wheezing, breathlessness, and chest tightness. On physical examination, extensive wheezing was found in both lungs of the patients. Only on the basis of physical examination, the asthma cases were diagnosed by the pulmonologists and no other biochemical tests were conducted for patients. In addition, 151 healthy non asthmatic control individuals including 68 female and 83 male subjects with a mean (SD) age of 30.8<sup>17</sup> years, range (2-66 years) with no personal or family history of asthma or other allergic diseases were selected from the same area from where patients were recruited. All control subjects had no respiratory symptoms. Moreover, controls were matched for sex and ethnicity with the patients. Patients and healthy individuals were genetically unrelated. The ethical review committee of the parent organization approved this project (ERC-08-01). The project was also approved by Pir Mehr Ali Shah Arid Agriculture University Rawalpindi. Written informed consent was obtained from all participants.

### Typing of the *TNF- $\alpha$* -308 G/A Gene Polymorphism

A venous blood sample was obtained from each study participant, and genomic DNA was extracted from whole blood using standard phenol chloroform extraction protocol.<sup>18</sup> DNA analysis was carried out by polymerase chain reaction (PCR) using the conditions previously described by Verjan et al.,<sup>19</sup> with some modifications. Three primers were used for the allele-specific PCR: the 3' primer (position-144/-164:5'-TCTCGGTTTCTTCTCCATCG-3') was used in combination with either a 5' primer complementary to the TNFA1 allele (position -328/-308 G: 5'-ATAGGTTTTGAGGGGCATGG-3') or one that is complementary to the TNFA2 allele (position -320/-308A: 5'-ATAGGTTTTGAGGGGCATGA-3'). Allele-specific primers only differed in terms of their 3' terminal nucleotide. As an internal control, primers against  $\beta$ -globin (5' primer, 5'-ACACAAGTGTGTTCACTAGC; 3' primer, 5'-CAACTTCATCCACGTTTACC-3') were included in the reactions. Ten microliters of PCR reaction mixture was used, containing genomic DNA samples (250 ng), 200  $\mu$ mol/L dNTPs, 2 mMol/L MgCl<sub>2</sub>, 1  $\mu$ L 10X Taq DNA polymerase buffer, 1 unit of Taq DNA polymerase (Fermentas), 10 pmol of each test primer and 10 pmol of internal control primers.

## TNF Alpha 308 G/A Association in Pakistani Asthmatics

**Table 1. Genotypes,  $\chi^2$  and Odds Ratio (OR) of cases (Asthma) and controls for *TNF- $\alpha$* -308**

Genotype Table	Asthma patients n=329	Controls n=151	$\chi^2$	OR (95% CI)	P value
GG	88(27%)	39(26%)	0.05	1.049 (0.68-1.63)	0.823
GA	241(73%)	111(73%)	0	0.987 (0.64-1.53)	1
AA	0(0%)	1 (0.006%)	--	-- (--)	--

**Table 2. Alleles,  $\chi^2$  and Odds Ratio (OR) of cases (Asthma) and controls for *TNF alpha*-308**

Alleles	Asthma Cases n=329	Controls n=151	$\chi^2$	OR (95% CI)	P value
G	417	189	0.06	1.03 (0.78-1.37)	0.806
A	241	113	0.06	0.967 (0.73-1.28)	0.806

Reaction conditions used were as follows: 95°C for 5 minutes; 31 cycles of 95°C for 90 seconds, 61°C for 150 seconds, and 72°C for 60 seconds, with a final extension step of 72°C for 10 minutes. PCR products were electrophoresed on 2% agarose gels containing 0.5 mg/mL ethidium bromide at 120V for 45 minutes. The gels were visualized with a UV transilluminator imaging analyzer.

### Statistical Analysis

Data were analyzed using  $\chi^2$  test and odds ratio with 95% confidence interval. Statistical calculations were carried out using vassar stats online (<http://vassarstats.net/odds2x2.html>). Statistical significance was established at a value of  $p < 0.05$ .

### RESULTS

The genotype distribution of *TNF- $\alpha$*  -308 G/A polymorphism was not in Hardy-Weinberg equilibrium within each group. The frequency of genotypes and alleles of the *TNF- $\alpha$*  -308 G/A polymorphism in the asthmatic and non-asthmatic subjects are shown in Table 1. We found no significant differences in the prevalence of this polymorphism between patients and control subjects (Table 1 and 2).

### DISCUSSION

Previous studies have shown several linkages between asthma and different regions of the human genome.<sup>1</sup> Despite this, no specific polymorphisms in

candidate genes have been definitively implicated in the pathogenesis of the disease. The reason could be that individuals who have asthma might carry genes with differing propensity that determine the susceptibility to asthma and modify the severity of the disease depending on their interaction with each other and with the environmental milieu. Among the many genes involved in the induction of airway inflammation, gene encoding *TNF- $\alpha$*  is a potential candidate. Previous studies have shown an association between the *TNF- $\alpha$*  -308 G/A polymorphism and asthma.<sup>10-15</sup> In the present study, we have not found an association between the *TNF- $\alpha$*  -308 G/A polymorphism and asthma in patients compared with normal subjects. Our results suggest that the *TNF- $\alpha$*  -308 G/A allele could not be considered as a genetic factor for susceptibility to asthma in our studied population. The *TNF- $\alpha$* -308 AA and GA genotypes have been reported to be associated with susceptibility to rheumatic heart disease ( $p=0.012$ ; and  $p=0.046$ , respectively) while the GG genotype seemed to confer resistance ( $p=0.003$ ) in Pakistani population.<sup>20</sup> The associations with RHD may be due to the disease condition and due to auto immune status of RHD which is not the same in asthma cases.

In a European-American study it was reported that asthmatics were more likely than controls to carry one or two copies of the *TNF- $\alpha$*  -308\*A allele: 30% of the cases, vs 22% of the controls, had one or more copies of *TNF- $\alpha$*  -308\*A ( $p=0.03$ ).<sup>21</sup> Other genetic epidemiologic studies, however, have been equivocal with regard to the relation between *TNF- $\alpha$*  -308\*A and

asthma. Our results contradict the recent meta-analysis showing the -308 G/A polymorphism in *TNF- $\alpha$*  gene to be associated with asthma risk.<sup>22</sup> In this meta-analysis study, subgroup analysis by ethnicity showed significant elevated risks for asthma to be associated with A allele carriers in Asians but not in whites.<sup>22</sup> These conflicting results may have arisen from a range of factors, such as sample size, racial/ethnic differences, study designs, environmental interactions, or even molecular/statistical analyses and the strictness of the asthmatic enrolment by definite diagnosis. This study has been done as a case-control study with a modest sample size. The size of our sample may have accounted for our inability to demonstrate a statistically significant difference in the frequency of the *TNF- $\alpha$*  -308 G/A polymorphism between patients and control subjects, and future studies including a larger number of patients may provide confirmation of our results. Nonetheless, the results observed here help to further establish that *TNF- $\alpha$*  -308 (or a nearby gene) is causally related to asthma. Furthermore, this relation may be more strongly linked in individuals with acute asthma and a family history of this phenotype.

Several factors may be influencing discrepancies observed in different studies. Firstly, if another variant in or near the *TNF- $\alpha$*  gene was the causal variant, the true association could easily be missed. It is feasible that the *TNF- $\alpha$*  gene variant is playing a role in asthma in cooperation with other gene variants exhibiting a more limited biology.

Secondly, several asthma association studies have shown inconsistent results in Caucasian versus Asian populations. This suggests that there are racial differences in genetic risk, but the current evidence is that genetic effects are usually consistent across human populations.<sup>23</sup> Small sample size, study design flaws, population stratification, genotyping error, and other biases may be more common reasons than true racial heterogeneity for the observed discrepancies between studies of genetic risks.<sup>24</sup> Specific environmental exposures such as smoking or allergen exposure are another confounding factor for a disease with a strong gene-environmental interaction in explaining the inconsistencies among observational studies.<sup>25,26</sup>

Given the biological regulation of *TNF- $\alpha$*  and its role in the inflammatory processes, it is perhaps surprising that the genetic influences on cytokine production have much influence on disease processes and their outcome. The associations between *TNF- $\alpha$*

genotype and disease are not absolute as suggested by different conflicting studies.<sup>10, 21, 27</sup> In a study on Caucasian children, it was found that carriers of the GG genotype of *TNF- $\alpha$*  G-308>A polymorphism were at an increased risk of having difficult asthma when compared with carriers of other genotypes of these polymorphisms.<sup>28</sup> Nevertheless, it is clear that the genetic regulation of *TNF- $\alpha$*  at sites of inflammation is important. Under circumstances where the release of *TNF- $\alpha$*  has been triggered the genetically endowed capacity for greater *TNF- $\alpha$*  production leads to more severe inflammatory reactions.

Other applications are readily foreseeable, so that genotyping for *TNF- $\alpha$*  polymorphisms is likely to be useful in many areas of medicine. For understanding our personal uniqueness in genomics, the analysis of different polymorphisms is likely to contribute to the management and optimisation of our health. Clearly this is a distant goal; equally true is the fact that we have already started the journey towards it. These results can be confirmed by increasing number of cases and controls and the results will be helpful in treatment of disease.

The number of asthma cases and controls is not same in this study this may be the reason that we have not got any positive association. As far as the age factor is considered, the controls are younger as compared to cases. These are the drawbacks in the present study which will be taken into consideration in future for sampling purpose.

Likewise *TNF- $\alpha$* -308, another SNP *TNF- $\alpha$* -238 has also some functional relevance to the disease but in most of the studies conducted primarily the -308 SNP is studied and found associated in different populations. Therefore, in the present study this SNP was included and in future the other SNP can also be evaluated in these asthmatic cases and controls.

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