TUMOR NECROSIS FACTOR - α AND TRANSFORMING GROWTH FACTOR - β1 POLYMORPHISMS IN BRONCHIAL ASTHMA

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ABSTRACT

***BACKGROUND:** Bronchial asthma is a complex genetic disorder regulated by the release of cytokines and inflammatory mediators. Tumor necrosis factor alpha (TNF-α) and transforming growth factor beta (TGF-β1) cytokines play pivotal roles in the inflammatory response of the airways. Differential production of these two cytokines is associated with allelic variations in the transcriptional regulatory region of these genes. **AIMS:** The objective of the present study was to investigate G-308A TNF-α and C-509T TGF-β1 polymorphisms for their association with Bronchial Asthma. **MATERIALS AND METHODS:** DNA isolated from 123 asthmatics and 100 normal healthy controls were screened for these polymorphisms using the amplification refractory mutation system-polymerase chain reaction (ARMS-PCR) methods, developed in our laboratory. **RESULTS:** Significant allelic association was observed between G-308A TNF-α polymorphism and asthma (P = 0.031) while no association was observed with C-509T TGF-β1 polymorphism (P = 0.207). Further sub-grouping based on either allergic response or family history failed to reveal any statistical significance among the groups or with controls. The interaction between these polymorphisms revealed statistically significant association between the high producer genotype alleles of TNF-α and TGF-β (A/T) and asthma (P = 0.016). **CONCLUSIONS:** The present study reports, for the first time, the role of two polymorphisms, in concert, for their association with asthma in an Indian population. Our study supports the findings that the G-308A TNF-α promoter polymorphism is a risk factor for asthma and furthermore suggests that the patients with high producer alleles for TNF-α (-308) and TGF-β (-509) have the highest risk of getting this disease in the Punjabi population.*

***Key words:** Asthma, polymorphism TNF-α, TGF-β1*

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INTRODUCTION

Asthma is a chronic inflammatory disease of the airways characterized by recurrent episodes of airway obstruction and wheezing. Genome-wide scan and candidate gene studies have identified the role of genetic

factors in the pathophysiology of this complex disorder.[1] TNF-α, a pro-inflammatory cytokine, leading to smooth muscle contraction and activation of inflammatory cells during the late phase of inflammation[2] and TGF-β1, a pro-fibrotic cytokine, promoting the accumulation of extra cellular matrix (ECM) proteins,[3] are two important candidate genes targeted in disease association studies in asthma. Raised levels of both TNF-α and TGF-β1 have been observed in the asthmatic airways and bronchoalveolar lavage of asthmatics.[4,5] Differences between the levels of these secreted cytokines are under genetic control and are attributed to the presence of single nucleotide polymorphisms (SNPs), G-308A and C-509T, in the transcriptional regulatory region of TNF-α and TGF-β1 cytokines, respectively.[2,6] Studies conducted on these polymorphisms in different populations exhibited conflicting results; a few reporting positive[7,8] while others show no relationship.[9,10] Cytokines act in a network and have the ability to modulate, modify or substitute each others' function.[11] The present study is the first report of its kind from an Indian population, analyzing the joint role of G-308A TNF-α and C-509T TGF-β1 polymorphisms in the pathophysiology of asthma.

MATERIALS AND METHODS

Study population

Asthma diagnosis was based on international consensus reports on diagnosis and treatment of asthma[12] and physicians' recommendations. All patients with clinical symptoms of asthma i.e. dyspnea, cough and wheezing, with bronchodilator response to Salbutamol (5 mg/mL), as indicated by >15% increase in forced expiratory volume, were selected for the present study. Informed consent was taken from all the individuals participating in the study. A total of 223 blood samples were collected in disodium-EDTA, of which 123 samples were from asthmatic patients visiting Rai Bahadur Sir Gujjarmal and Kesradevi Tuberculosis Sanatorium, Amritsar and Sri Guru Ram Das Institute of Medical Sciences and Research, Amritsar, and 100 were matched normal healthy controls for sex, age and ethnic group with no history of asthma and allergic diseases. Of the 123 patients, 83 and 40 subjects were physician diagnosed allergic and non-allergic asthmatic cases, respectively. The family history for asthma was present in 54 patients while the remaining 69 cases were without such history.

Genotyping -308 site in TNF-α gene and -509 site in TGF-β1 gene

DNA was extracted from collected blood samples by the modified inorganic method[13] and quantified following standard spectrophotometric analysis. G-308A TNF-α promoter polymorphism was studied using the amplification refractory mutation system-polymerase chain reaction (ARMS-PCR) method.[14] Similarly an ARMS-PCR method was developed to study C-509T TGF-β1 polymorphism. Briefly, two complementary reactions were established for each allele consisting of target DNA, allele specific ARMS primer (ARI or AR2) and the common primer (CF). A 349 base pair region in the TGF-β1 promoter was targeted for amplification. The sequences of primers used in the study are AR1 5' AAGGGGCAACAGGACACCTGGG 3', AR2 5' AAGGGGCAACAGGACACCTGGA 3' and CF 5' CTACGGCGTGGAGTGCTGAG 3'. To assist

in the genotyping of C-509T polymorphism, the penultimate base in the primers AR1 and AR2 was mutated from A to G. The optimized reaction conditions consisted of 40 ng of genomic DNA in a reaction volume of 30 µl containing 0.16 µM of each primer, 30 µM of each dNTP, 10 mM Tris-HCl (pH 9.0), 1.5 mM $MgCl_2$, 50 mM KCl, 0.01% gelatin and 0.3 U of Taq DNA polymerase (Bangalore Genei, Bangalore). Amplification was carried out for 35 cycles, each cycle consisting of denaturation at 94°C for 30 s, annealing at 61°C for 20 s, extension at 72°C for 20 s and finally a 3 min extension at 72°C.

The PCR products for both G-308A TNF-α and C-509T TGF-β1 polymorphisms were analyzed in 2% agarose gel and visualized following ethidium bromide staining. A known positive and negative control was included in each batch of amplification. The PCR products were validated by DNA sequencing (data not shown).

Statistical analysis

Allele frequencies between groups were compared using 2x2 contingency tables; Chi-squared (χ^2) statistics and their confidence intervals were determined while calculating the odds ratio. Statistical significance was defined at the standard 5% level.

RESULTS

In this study, 223 subjects were screened for G-308A TNF-α and C-509T TGF-β1 polymorphisms using ARMS-PCR methods. In all there were 123 asthmatic (55 males and 68 females, mean age with standard deviation 33.72 ± 17.78 years, range 4-80 years); and 100 controls (43 males and 57 females, mean age with standard deviation 35.62 ± 14.84 yrs, range 7-80 years). Their genotypic and allelic distributions are shown in Table 1. All these distributions were found to be in Hardy-Weinberg equilibrium. The allelic distribution of the G-308A TNF-α polymorphism was significantly different between asthmatics and control subjects (P = 0.031). However, no statistically significant difference was observed in either genotypic or allelic distribution in C-509T TGF-β1 polymorphism (P = 0.207). The asthmatic patients were further sub grouped as allergic and non allergic ones and also on the basis of their family history. The distribution of genotypes and alleles for both the studied polymorphisms in the former group [Table 2] revealed that non allergic asthmatic patients differed from the controls in their distribution of G-308A TNF-α count (P = 0.02; OR = 2.33; CI = 1.11-4.90) while allergic asthmatics failed to show any such difference. Unlike TNF-α polymorphism, TGF β1 failed to reveal any statistically significant differences between the two groups (P = 0.68). However, the asthmatic patients with and without family history also failed to exhibit any statistically significantly differences in both G-308A TNF-α and C-509T TGF-β1 polymorphisms (P = 0.91 and P = 0.56, respectively) [Table 3].

Figure 1 represents the distribution of asthmatics and controls based on the presence or absence of high producing alleles (A/T) in TNF-α and TGF-β1 cytokines, respectively. The four groups i.e. A^+/T^+ (high TNF-α and high TGF-β1); A^+/T^- (high TNF-α and low TGF-β1); A^-/T^+ (low TNF-α and high TGF-β1) and A^-/T^- (low TNF-α and low TGF-β1) and their incidence in asthmatics and controls were

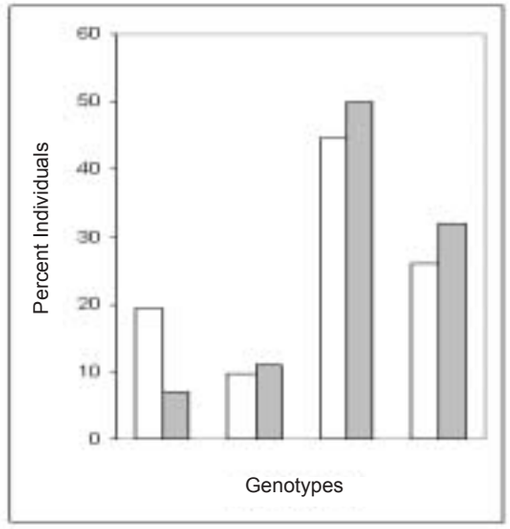

Figure 1: Distribution of the asthmatics (open bars) and normal controls (closed bars) based on TNF-α and TGF-β1genotypes.

Table 1: Distribution of Genotypes and Allele count of G-308A TNF-α and C-509T TGF-β1 polymorphisms in asthmatics and normal controls

G-308A TNF-α polymorphism

Population (n)	*Genotypes (%)*			*Allele Count (%)*	
	GG	*GA*	*AA*	*G Allele*	*A allele*
Asthmatics (123)	86 (69.9)	35 (28.5)	2 (0.02)	207 (84.1)	39 (15.8)
Controls (100)	82 (82.0)	18 (18.0)	0 (0.00)	182 (91.0)	18 (9.0)

χ^2 [degrees of freedom (df) = 1], (Asthmatic vs. Controls) = 4.65; P = 0.031, Odds ratio (OR) (Asthmatic vs. Controls) = 1.90, Confidence Intervals (CI) = 1.31-2.49.

C-509T TGF-β1 polymorphism

Population (n)	*Genotypes (%)*			*Allele Count (%)*	
	CC	*CT*	*TT*	*C Allele*	*T Allele*
Asthmatics (123)	44 (35.7)	55 (44.7)	24 (19.5)	143 (58.1)	103 (41.9)
Controls (100)	43 (43.0)	42 (42.0)	15 (15.0)	128 (64.0)	72 (36.0)

χ^2 (df = 1) (Asthmatic vs. Controls) = 1.59; P = 0.207; OR = 1.28; CI = 0.89-1.66.

Table 2: Distribution of Genotypes and Allele count of G-308A TNF-α and C-509T TGF-β1 polymorphisms in asthmatic subgroups and normal controls

G-308A TNF-α polymorphism

Population (n)	*Genotypes (%)*			*Allele count (%)*	
	GG	*GA*	*AA*	*G Allele*	*A allele*
Allergic Asthmatics (83)	60 (72.28)	23 (27.71)	0 (0.0)	143 (86.14)	23 (13.85)
Non-Allergic Asthmatics (40)	27 (67.50)	11 (27.50)	2 (5.00)	65 (81.25)	15 (18.75)
Controls (100)	82 (82.0)	18 (18.0)	00 (0.0)	182 (91.00)	18 (9.00)

χ^2 (df = 1), (Allergic vs. Controls) = 2.15; P = 0.14; OR = 1.63; CI = 0.85-3.13, χ^2 (df = 1), (Non-Allergic vs. Controls) = 5.22; P = 0.02; OR = 2.33; CI = 1.11-4.90, χ^2 (df = 1), (Allergic vs. Non-Allergic) = 0.99; P = 0.32; OR = 0.07; CI = 0.34-1.42

C-509T TGF-β1 polymorphism

Population (n)	*Genotypes (%)*			*Allele count (%)*	
	CC	*CT*	*TT*	*C Allele*	*T Allele*
Allergic Asthmatics (83)	30 (36.15)	38 (45.78)	15 (18.07)	98 (59.04)	68 (40.96)
Non-Allergic Asthmatics (40)	14 (35.00)	17 (42.50)	9 (22.50)	45 (56.25)	35 (43.75)
Controls (100)	43 (43.00)	42 (42.00)	15 (15.00)	128 (64.00)	72 (36.00)

χ^2 (df = 1), (Allergic vs. Controls) = 0.94; p =0.33; OR = 1.23; CI = 0.81-1.88, χ^2 (df = 1), (Non-Allergic vs. Controls) = 1.45; P =0.23; OR = 1.38; CI = 0.82-2.34, χ^2 (df = 1), (Allergic vs. Non-Allergic) = 0.17; P =0.68; OR = 0.89; CI = 0.52-1.53

Table 3: Distribution of Genotypes and Allele count of G-308A TNF-α and C-509T TGF-β1 polymorphisms in asthmatic subgroups based on family history and normal controls

G-308A TNF-α polymorphism

Population (n)	*Genotypes (%)*			*Allele Count (%)*	
	GG	*GA*	*AA*	*G Allele*	*A allele*
Positive FH* (54)	38 (70.37)	15 (27.77)	1 (1.85)	91 (84.26)	17 (15.74)
Negative FH (69)	49 (71.01)	19 (27.54)	1 (1.45)	117 (84.78)	21 (15.22)
Controls (100)	82 (82.0)	18 (18.0)	0 (0.0)	182 (91.00)	18 (9.00)

χ^2 (df = 1), (Positive FH vs. Controls) = 3.16; p = 0.07; OR = 1.89; CI = 0.93-3.84, χ^2 (df = 1), (Negative FH vs. Controls) = 3.09; *P* = 0.08; OR = 1.81; CI = 0.93-3.55, χ^2 (df = 1), (Positive vs. Negative FH) = 0.01; *P* = 0.91; OR = 1.04; CI = 0.52-2.09

C-509T TGF-β1 polymorphism

Population (n)	*Genotypes (%)*			*Allele Count (%)*	
	CC	*CT*	*TT*	*C Allele*	*T Allele*
Positive FH (54)	21 (38.89)	23 (42.59)	10 (18.52)	65 (60.19)	43 (39.81)
Negative FH (69)	23 (33.33)	32 (46.38)	14 (20.29)	78 (56.52)	60 (43.48)
Controls (100)	43 (43.00)	42 (42.00)	15 (15.00)	128 (64.00)	72 (36.00)

χ^2 (df = 1), (Positive FH vs. Controls) = 0.44; p =0.51; OR = 1.18; CI = 0.73-1.90, χ^2 (df = 1), (Negative FH vs. Controls) = 1.91; *P* =0.17; OR = 1.37; CI = 0.88-2.13, χ^2 (df = 1), (Positive vs. Negative FH) = 0.33; p =0.56; OR = 0.86; CI = 0.52-1.43, *FH: Family History

24(7), 13(11), 55(50) and 31(32), respectively. A statistically significant difference in A^+/T^+ and A^-/T^- frequencies was observed between the asthmatic and control populations (*P* = 0.016); all other groups failed to show any statistically significant difference in their distribution.

DISCUSSION

Asthma is a complex genetic disease with a broad range of phenotypes distinguished by hyper responsiveness and airway inflammation to various extrinsic and intrinsic stimuli.[15] However, this definition has been modified with the availability of additional information regarding the role of immunological mechanisms underlying this disease; the new definition given by The Global Strategy for Asthma Management and Prevention report states “Asthma is a chronic inflammatory disease of the airways in which many cell types play a role, in particular mast cells, eosinophils and T lymphocytes”.[12] Quite clearly, more emphasis is now laid on the role of immunological mechanisms involved in the process of inflammation. Cytokines are cell signaling proteins playing a central role in immunological and inflammatory mechanisms by relaying the necessary instruction to their target cell via specific receptor(s) in an autocrine, paracrine and endocrine fashion.[15] Cytokines have been divided into two discrete groups based on their ability to cause inflammation: i) pro-inflammatory: TNF-α, IL-1, IL-6, and ii) anti-inflammatory: TGF-β1, IL-1Ra and IL-10.[16]

Given the diametrically opposite physiological role of proteins in these groups, two cytokines, TNF-α and TGF-β1 were short listed to study their association with asthma. The former is responsible for smooth muscle contraction in the early phase and helps in the influx of inflammatory cells during the late stages of inflammatory responses[2] in subjects with asthma, while the latter one is known to induce fibrosis and promote accumulation of ECM.[3] Raised levels of these cytokines

have been observed in asthmatic airways.[4,5] Collectively, these observations strongly suggest that an imbalance in their levels contributes to the pathogenesis of asthma. Thus, recent data suggesting inter-individual variability to synthesize TNF-α and TGF-β1 cytokines due to allelic variation within the regulatory regions of these genes[4,8] becomes important to evaluate the role of these polymorphisms in susceptibility to asthma.

Of the various SNPs, the G-308A TNF-α and C-509T TGF-β1 polymorphisms have been associated with their differential production, A allele being the high producer of TNF-α[2] and T allele being the high producer of TGF-β1.[6] These two polymorphisms have been extensively studied amongst populations from developed nations and have yielded conflicting results. A study of G-308A TNF-α polymorphism on children from Western Australia revealed that the G allele was present at higher frequency in asthmatics as compared to controls,[4] while a recent study from California, USA, indicated that the A allele increases risk of asthma.[7] Additionally, a study on a Czech population found no association between this polymorphism and susceptibility to develop asthma.[10] Several other investigators also failed to find association between G-308A polymorphic alleles and asthma;[17,18] the results obtained for TGF-β1varied among different populations. A study on white Caucasians[3] observed an association between the C-509T TGF-β1 polymorphism and asthma severity and finds support from a recent study,[8] though another study conducted on the Czech population[9] reported no association between this polymorphism and asthma.

The genotypic and allelic distribution of G-308A TNF-α in our population presents a fairly interesting scenario [Table 1]. The allelic but not the genotypic count, in asthmatics, revealed a statistically significant difference from the controls (*P* = 0.031). In C-509T TGF-β1 polymorphism, the genotypic and allelic variations between the two groups are high but do not reach standard 5% level of significance. In order to find any selective association of TNF-α among the asthmatic patients, the patients were further subgrouped based on the broad etiology of the disease i.e., either allergic or non allergic asthmatic. Interestingly, the non allergic asthmatics revealed statistically significant difference in the allelic distribution from the controls; however, no significant differences were obtained with the allergic asthmatic subgroup or the controls [Table 2]. Given that the non allergic asthma in itself is a very heterogeneous phenotype it becomes very difficult to assign a precise reason for the observed differences. Furthermore, the TGF-β1 polymorphism failed to exhibit any statistically significant differences between the studied subgroups or with the controls. Another observation that the two polymorphisms failed to show any association based on the family history of patients is a little surprising [Table 3]. However, this finds support in a recent review which suggests that a large number of genes are associated with asthma or related phenotypes.[20]

Given that cytokines interact in a network, it is advantageous to consider the role of various polymorphic alleles of cytokines in concert, instead of considering them in isolation.[11] Accordingly, the asthmatic and the control populations were re-categorized into four

groups based on the presence or absence of high producing alleles of the two cytokines and it was observed that the A^+/T^+ group revealed statistically significant variation when compared to A^-/T^- group [Figure 1], while other groups failed to reveal any association, thereby lending support to the observation that individuals carrying high TNF-α and TGF-β1 producer alleles are at greater risk of developing asthma.

In a study from Pittsburg, the role of these two cytokines together was evaluated and it was reported that the genotypes for high production of TNF-α and low production of TGF-β1 were associated with an increased frequency of parental allergic rhinitis and asthma, although no significant interactions between TNF-α and TGF-β1 genotypes exist.[19] Our findings are distinct from the earlier report and also find support in the observation of other investigators reporting abnormally high levels of TGF-β1 in the airways of human asthmatics.[5,8] The plausible reason for this observation could be either the diverse ethnic background of the studied populations or the diverse etiologies that may contribute to the development of asthma, which might suggest that the physiological basis of these polymorphisms might have a broader role in the susceptibility to asthma. Furthermore, determination of the levels of these cytokines in serum or bronchoalveolar lavage fluid might prove helpful in better understanding of the role of these cytokines in asthma.

The present study reports, for the first time, the role of two polymorphisms, in concert, for their association with asthma in an Indian population, thereby suggesting that the patients with high producer alleles for TNF-α and TGF-β1 have the highest risk of getting this disease.

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