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Single Nucleotide Polymorphism in the Promoter Region of Interleukin-4 and Risk of Asthma: TT Homozygotes May Have High Propensity of Developing Chronic Airway Remodeling

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ABSTRACT

Background: In view of a key role played by Interleukin-4 (IL-4) in the molecular mechanisms leading to the development of asthma and its complications like chronic airway remodeling, a few reports have attempted to investigate a possible role of SNP's in the promoter region to predict genetic risk. As it is associated with quantitative variations in IL-4 gene expression and may serve as an important candidate gene for asthma risk prediction.
Objective: To investigate the polymorphic variations, at -590 (C/T) in the promoter region of IL-4 gene.
Methods: 50 consecutively selected cases of asthma (36 females and 14 males) were recruited and genotyping for SNP at -590 C/T was done by PCR-RFLP method on the genomic DNA extracted from blood samples. Blood samples from 50 randomly selected healthy subjects served as controls. Mean values of Absolute Eosinophil Count and total serum IgE between the genotypes were compared by student's unpaired *t* test. Odds-ratio analysis was carried out to determine risk of developing asthma.
Results: Odds ratio (OR) analysis revealed two-and-a-half folds increased risk of asthma for individuals with TT genotype compared to those with CC genotype. A decreased frequency of CC genotype in patients (28%) compared to controls (58%) is indicative of protective nature of CC genotype. AEC counts and total serum IgE levels were significantly higher in TT compared to CC and CT genotypes.
Conclusion: It is concluded that individuals with TT and CT genotypes (overproducers of IL-4) have high risk of developing asthma and high vulnerability to develop chronic airway remodeling.

Key Words: Asthma, IL-4 gene, SNP at -590 C/T, Serum IgE, Eosinophil counts.

INTRODUCTION

Atopy refers to the genetic tendency to develop allergic diseases such as allergic rhinitis, asthma and atopic dermatitis. It is generally associated with raised immune responses predominantly of IgE type to common allergens, especially inhaled and food allergens. [1, 2]

Understanding molecular mechanisms and genetic predisposition to asthma assumes significance in view of rising incidence of respiratory allergy. Worldwide, asthma cases are increasing at a rate of 50 per cent every decade, with asthma affecting 3.5-20% of the population in any country. [3]

The chronic allergic inflammatory response in asthma is triggered by inhaled allergens and is dependent upon the activation of Th2 pathway of activation and release of an important cytokine IL-4. [4] Interleukin-4 binds to its specific receptor on B-lymphocytes and triggers the synthesis of allergen specific IgE response. [5] Interleukin-5(IL-5) is another cytokine released from activated Th2 lymphocytes which selectively induces synthesis of eosinophils. In chronic asthmatics, with repeated episodes of bronchospasm, eosinophils get accumulated leading to airway remodeling.

As for the role of genetic susceptibility in asthma is concerned, both conventional studies in the past and single nucleotide polymorphisms (SNPs) in more recent studies have established the role of genetic predisposition. Multiple genes are implicated in the causation of asthma and allergies establishing the multifactorial nature of these diseases. [6-11]

In view of its central role in Th2 response against allergens, IL-4 gene has been considered as a prospective candidate gene for assessment of risk; SNPs in IL-4 gene, particularly in the promoter region are evaluated. Some of the previous studies [8, 9, 12, 13] have investigated for a possible association of an SNP(C/T) in the promoter region at -590. However, the limitations of such studies were deviation from Hardy-Weinberg Equilibrium in the genotypic frequencies of control subjects. Moreover there was paucity of information on mean IgE levels, absolute eosinophil counts and spirometric findings in the three genotypic groups (TT, CT, CC) of patients and controls in those studies.

The objective of the present study is to compare allelic and genotypic frequencies at SNP-590 C/T not only between asthma patients and controls but also to compare mean IgE levels, mean eosinophil counts and FEV_1values between genotypes in the patients' group. Further, the cases were categorized according to severity of disease based onFEV_1values.

In view of the above aspects the present study is a more comprehensive one and provides relatively important clinical information about severity of asthma in TT and CT genotype patients compared to possibly a milder condition in CC patients.

MATERIALS AND METHODS

Selection of cases

Before initiation of the study Institutional Review Board (Ethics Committee) approval was obtained. A total of 50 patients visiting the out-patient of Pulmonology Department of Princess Esra Hospital (PEH), Owaisi Hospital and Research Centre (OHRC), Deccan College of Medical Sciences (DCMS) Hyderabad, were consecutively selected. Clinically confirmed cases of asthma were included. The laboratory tests used to confirm diagnosis were Chest X-ray, spirometry and supportive tests like Absolute Eosinophil Count and Serum Immunoglobulin E (IgE). The numbers of male and female patients were 14 and 36 respectively in the age range of 20-80 years. 50 normal non-asthmatic individuals in the age range 15-65 years served as controls in the study.

For IL-4 SNP -590C/T genotyping, 3 ml of venous blood was drawn aseptically in EDTA vacutainers and 2ml of clotted blood was taken in plain vacutainers to obtain the serum. Both serum and whole blood samples were stored at -20ºC until further use. Before withdrawing the blood an informed consent was obtained from each patient.

The inclusion criterion was spirometrically confirmed cases of asthma regardless of gender in the age range of 20-80 years. Cases suffering from other pulmonary

conditions like COPD and Tuberculosis were excluded.

DNA extraction and genotyping:

Genomic DNA was extracted from the blood samples according to rapid salting out method as described by Lahiri et al. (1991). Polymerase chain reaction (PCR) amplification was performed in a 25 µl reaction mixture that contained 1 µg of genomic DNA, 20 pmol of each primer and 12.5µl of Taq DNA master mix (2X Takara. Japan). The forward (F) 5′-ACTAGGCCTCACCTGATACG-3' and reverse (R) 5′-GTTGTAATGCAGTCCTCCTG-3′ primers for promoter region of IL-4 at SNP -590C/T were used to carry out the PCR in a thermal cycler (Bio-Rad T100model, USA). The thermal programming was as follows: an initial denaturation step of 5 minutes at 94°C, followed by 30 cycles of denaturation at 94°C for 1 minute, annealing at 58°C for 1 minute and extension at 72°C for 2 minutes and a final extension for 15 minutes at 72°C. As per the instructions of the supplier of enzyme for using restriction enzyme, the restriction digestion was performed in the 30µl volume containing 10µl PCR amplified product, 2U of BsmFIand appropriate buffers for restriction digestion enzyme (provided by the supplier). The mixture was incubated at 37ºC in a water bath for 16 hours. The restriction digestion products were subjected to Agarose gel electrophoresis and visualized in a Bio-Rad Gel DOC.

The enzyme digestion cleaved 252 bp fragment into two small fragments of 192 bp and 60 bp in the presence of wild-type sequence (C) and a single fragment of 252 bp when the T allele was present. The resulting genotypes were CC homozygotes (192 bp,60bp fragments), CT heterozygotes (252 bp,192 bp,60 bp) and a single 252 bp represents TT homozygote.

Serum IgE determination:

Quantitative measurement of serum IgE in patients' sera was done using standard antibody sandwich ELISA protocol (Calbiotech-USA).

25µl of patient's serum was loaded in each well coated with Streptavidin followed by 100µl of Biotin reagent to each well and incubated for 30 minutes at room temperature. The liquid was removed from the wells and all the wells were washed three times with 300 µl of 1X wash buffer and then 100µl of Enzyme Reagent was added. After 30 minutes of incubation, the liquid was removed and the wells were washed. TBM substrate in 100 µl was added to each well and incubated for 15 minutes. This was followed by addition of 50 µl of stop solution and the plate was shaken to mix the solutions. The absorbance was measured on ELISA reader at 450 nm within 15 minutes after adding stop solution.

RESULTS

Details of baseline characteristics of male and female patients reveal a significant difference in the mean age of male patients compared to that of the female patients. Female preponderance is evident from the gender ratio observed (36 females to 14 males). Statistically significant increase was recorded in the AEC values in males compared to females ($P<0.05$). Mean values of other asthma related parameters like Serum IgE, Forced expiratory volume in 1 second are also given in table-1.

Details of genotypic and allelic frequencies of SNP at -590C/T in fifty asthmatics and an equal number of healthy subjects are given in table-2. A significantly increased frequency of CT genotype 60% (30/50) was recorded in patients compared to 40% (20/50) in the controls. As for the TT homozygotes are concerned a frequency of 12% was recorded in asthmatics compared to a mere 2% in healthy subjects indicating that 'T' allele in both heterozygous and homozygous conditions predisposes to asthma. Contrary to this, there was a 30% reduction in the frequency of CC genotype in patients compared to controls (28% in asthmatics, 58% in

controls) indicating a protective nature of CC homozygotes.

These results are further confirmed by C and T allelic frequencies in test and control groups. While the frequency of 'T' allele was 42% in test group, it was only 22% in the controls. Contrary to this there was 20% reduction in the frequency of 'C' allele in the test group compared to controls (58% in patients and 78% in controls).

Odds ratio (OR) analysis of risk assessment in asthma patients (IL-4 SNP at -590 C/T) in which the protective nature of CC genotype was confirmed by comparing the proportion of CC homozygotes with 'T' allele containing genotypes (CT and TT). Nearly 70% reduction in the risk of asthma was observed in CC individuals (P≤0.004) (Table-3).

Table 1: Gender wise analysis of Baseline characteristics of Asthma patients

Parameter	Males Mean ±SD	Females Mean ±SD	Total Patients Mean ±SD	Normal range
Age	44.78*±17.66	39.61±12.65	42.19± 15.15	15-80
Gender (Number)	14/50	36/50	14:36	-
Absolute eosinophil count	346.35*±216.64	302.30±225.19	324.32 ±220.91	40-440cells/cumm
Serum IgE level	273.23±142.56	210.02±145.54	241.62 ± 144.05	Male= <250IU/ml Female = <175IU/ml
Forced expiratory volume in 1sec (FEV_1)	61.57±20.83	54.19±17.96	57.86 ± 19.37	≥70-100

*P< 0.05

Table:2 Genotypes and allelic frequencies of IL-4 Gene (SNP-C/T) in asthma patients and controls

Category	Number	Genotype			Allelic frequencies	
		CC No.(%)	CT No.(%)	TT No.(%)	"C"	"T"
Asthmatics	50	14 (28%)	30 (60%)*	6 (12%)	58	42**
Controls	50	29 (58%)	20 (40%)	1 (2%)	78	22

*P≤ 0.01 'T'allele vs. 'C' allele **P≤ 0.003

Table 3: Odds-ratio analysis for risk assessment in asthma patients (IL-4 SNP-590 C/T)

GENOTYPES COMPARED	ODDS RATIO	P- VALUE
CT versus CC	3.11 (CI: 1.32- 7.28)	P ≤ 0.01
TT+CT versus CC	0.28 (CI: 0.12- 0.64)	P ≤ 0.004
"T" allele versus "C" allele	2.56 (CI: 1.38- 4.76)	P ≤ 0.003

Table 4: Comparative analysis of means of asthma related parameters in relation to IL-4 genotypes at SNP-590C/T

GENOTYPE	NUMBER	ABSOLUTE EOSINOPHIL COUNT (AEC)	SERUM IgE LEVELS	FORCED EXPIRATORY VOLUME(FEV_1)
CC	14	265.78±105.87	180.40±149.66	57.35±21.20
CT	30	357.8±428.36*	228.53±139.69	57.4±18.36
TT	6	379.5±93.49*	334.10±133.83**	47.83±16.33

*AEC: CT vs. CC (P= 0.0001) TT vs. CC (P= 0.0356) TT vs. CT (P= 0.0016)

**IgE: TT vs. CC (P<0.044)

Table 5: Stratification of patients according to severity of disease (based on FEV_1 value) having different genotypes.

	GINA CLASSIFICATION OF ASTHMA SEVERITY	GENOTYPE		
		CC (14)	CT (30)	TT (06)
FORCED EXPIRATORY VOLUME IN ONE SECOND (FEV_1)	70-100 (Mild)	07(50%)	05(16.66%)	01(16.66%)
	60-69 (Moderate)	01(7.1%)	03(10%)	01(16.66%)
	50-59 (Moderately severe)	02(14.2%)	06(20%)	-
	35-49 (Severe)	01(7.1%)	06(20%)	03(50%)
	<35 (Very Severe)	03(21.4%)	10(33.33%)	01(16.66%)

Comparison of 'C' allele with 'T' allele by odds ratio in the test and control groups also revealed more than two and a half times increased risk for individuals with 'T' allele compared to those with 'C' allele (P=0.003). Risk assessment for CT

individuals when compared with those with CC genotype, revealed three- fold increased risk for individuals with CT genotypes (P≤ 0.01). It is inferred that individuals with CT and TT genotypes have significantly increased risk of developing asthma (Table-3).

Highly-significant differences were recorded when mean absolute eosinophil count (AEC) values were compared between CT and CC (P=0.0001) and TT vs. CT (P=0.0016) genotypes. Comparison of mean AEC counts between TT and CC homozygous patients also revealed a significantly higher mean in TT homozygotes (P=0.0356) (Table-4).

Comparison of means of total serum IgE levels between genotypes revealed lack of significant difference between patients with CC genotypes when compared with those of CT. However, when the mean of CC genotype was compared with that of TT homozygous patients, a significantly higher mean value was recorded (P=0.044) in TT homozygotes (Table-4).

Stratification of cases according to severity of disease was carried out (based on FEV_1 values) in patients having different genotypes (Table-5). It was noted that nearly 50% of patients with CC genotype were (according to GINA guidelines) mild, while, the proportion of mild cases in CT and TT groups were much lower. In contrast to this, nearly two-thirds of the patients of TT genotype were (according to GINA guidelines) severe, while in CC group only above 21% were in these categories.

DISCUSSION

It is believed that asthmatics with T-allele containing genotypes may respond to allergic episodes with significantly higher levels of IgE and significantly increased mean eosinophils when compared to those with the CC genotype because of a reported linkage between IL-4 and IL-5 gene. [6]

Asthmatics with TT homozygous genotype produce highest amount of IL-4 and are presumed to suffer from a relatively more severe form of asthma compared to those with CT and CC. Patients with CC genotype (having least levels of IL-4) are likely to manifest a relatively milder form of asthma. It is also likely that these patients may show a certain degree of relenting capability against pulmonary airway remodeling. It is hypothesized that asthma patients with CT genotypes may develop modest symptoms, but may show manifestations relatively akin to TT but not as mild as CC. [12]

The spirometric findings in the present study also support the above mentioned observation. It was found that mean FEV1 reduced significantly in TT patients (47.83± 16.33) compared to those of CC patients (57.35± 21.20). In another study, involving the same SNP in the promoter region (-589 C/T) the authors not only carried out genotyping but also compared the mean values of total serum IgE, absolute eosinophil counts and spirometric findings between TT and CT genotypes as well as TT and CC. However, statistical analyses carried out by these authors showed lack of significant difference between the mean values between genotypes (TT versus CT and TT versus CC) due to marginal differences between the means. [9]

It can be inferred that the present study provides clinically useful information on predisposition of patients with ‘T’- allele containing genotypes to this chronic debilitating disease. More importantly, this study suggests that the asthma patients with TT genotypes are the highly vulnerable group who may proceed to chronic airway remodeling.

Further analyses comparing allelic frequencies for T and C alleles between patients and controls also revealed that asthmatic patients carrying ‘T’ allele with highly significant eosinophil counts and serum-IgE levels showed an increased risk of allergic asthma.

Eosinophil counts assume importance not only as a diagnostic test but also in view of the observation that the number of accumulated eosinophils correspond to the

severity of asthmatic reaction in terms of late-phase hyper responsiveness. [14] Further, in the present study a relationship has been demonstrated between IgE levels and eosinophil counts in patients with atopic allergy (P values of AEC as shown in Table-4: CC vs. CT (P= 0.0001); CC vs. TT (P= 0.0356); CT vs. TT (P= 0.0016) as calculated by student's unpaired'-test). Di.Lorezo.et.al. [12] reported that there is an inter-relationship between total serum IgE, Eosinophil count and bronchial hyper-responsiveness suggesting that all three play an important role in the manifestations of asthma. Chemokines released from eosinophils contract bronchial smooth muscles, increase vascular permeability and induce airway hyper responsiveness. [15]

Thus, the presence and known functions of eosinophils suggest that the eosinophil is a major cellular participant in allergic asthma and a significant cellular factor in the development of allergic airway disease and in asthma airway remodeling particularly in late phase allergic airway disease. [16] Trials are underway to use monoclonal antibody to Interleukin-5 in order to inactivate its biological functions vis-a-vis induction of eosinophils, a cardinal cell type which enhances late phase airway hyper-responsiveness contributing to fibrosis and airway remodeling.

It is inferred that the presence of eosinophils in abundance in the lungs is required for the release of a variety of chemokine and fibrogenic factors like transforming-growth factor (TGF-β) which contribute to the process of chronic airway remodelling. [17]

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Conflicts of interest:

The authors do not have any conflicts of interest.

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