

## Original Article

# The -590C/T *IL4* single-nucleotide polymorphism as a genetic factor of atopic allergy

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**Abstract:** Elevated IgE levels in individuals with asthma, allergic rhinitis, and atopic dermatitis represents a situation in that increased IL4 production seems to occur because of the genetic component of the disease. In this study, one-hundred two matched-pairs of allergic and non-allergic individuals were phenotyped for total serum IgE level using enzyme-linked immunosorbent assay (ELISA). Atopic status was defined by serum IgE concentration  $\geq 100$  IU/mL. The -590C/T *IL4* (rs2243250) was screened by polymerase chain reaction-restriction fragment-length polymorphism (PCR-RFLP) analysis. An association between the *IL4* -590 TT genotype and levels of IgE was confirmed in the study population (ANOVA  $p=0.017$ ). Furthermore, the *IL4* T allele was significantly increased in allergic (0.299) compared with non-allergic subjects (0.172) (OR=2.060, 95% CI=1.285-3.301,  $\chi^2$  uncorrected  $p=0.002$ ) at total serum IgE cut-off of 100 IU/mL. A significant relationship between *IL4* -590 TT genotype and very high IgE levels ( $>1000$  IU/mL) (OR=3.968, 95% CI=1.499-10.5,  $\chi^2$  uncorrected  $p=0.01624$ ) was also established. The -590C/T *IL4* polymorphism is a potential risk factor to and correlates with atopic allergy.

**Key words:** Atopy, allergy, IL-4, single-nucleotide polymorphism, SNP, total IgE

## Introduction

The atopic triad of asthma, allergic rhinitis and atopic dermatitis is among the commonest causes of chronic illness and forms a part of the epidemiological shift towards increased prevalence in disease epidemic of the 21<sup>st</sup> century [1]. In the Philippines, 1 in 10 children have asthma and 32.5% of Filipino children aged 13 to 14 years old have allergic rhinitis and 7.1% have atopic dermatitis [2]. The etiology of these allergic disorders is complex and caused by largely uncharacterized interaction between genes and environmental factors. Atopic allergy is characterized by an increased production of IgE antibody against common environmental allergens. Cross-linking of the mast cell-bound IgE upon exposure to the allergen leads into inflammatory reactions in the airways, nasal cavity, and the integument that clinically manifest as symptoms of the allergic triad. Majority of the phenotypic symptoms of allergy have been defined by either binary response outcomes, i.e. physician's diagnosis

and questionnaire, or 'intermediate' traits like skin-prick-test and serum total and specific IgE levels [3]. The genetic components of allergy, however, are still currently under study with the use of genomewide or candidate gene linkage analysis and association, interaction, and functional studies.

Linkage and positional cloning studies in several populations revealed a number of chromosomal locations that are co-inherited with allergy. The most commonly reported among these markers are in the 5q, 12q, 13q, and 19q chromosomes [4, 5, 6, 7, 8, 9, 10]. Chromosome 5q31-33 has been a priced zone for most association study because it harbours the 160-kb region of the cytokine cluster that codes for several inflammatory molecules involved in the pathogenesis of allergy [11]. Interleukin-4 (IL-4) is one of the principal cytokines linked to elevated serum IgE levels or atopy and thus, susceptibility to allergy [12]. It is central to the development of the Th2 phenotypes that induce immunoglobulin  $\epsilon$  isotype

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switching and secretion of IgE, regulation of Fc $\epsilon$  receptor and vascular cell adhesion molecule-1 (VCAM-1) expressions, and promote transmigration of effector cells [13, 14, 15]. These cellular and molecular events conspire to bring about the observed symptoms in asthma and other allergies.

The approximately 9-kb, four-exon IL4 gene is separated from the IL13 by a 12,500-bp intergene segment [16, 17]. The IL4 promoter region which extends to about 500 bp from the "TATA"-like sequence [18] houses five sequence variants, four of which are rare and two has no association with atopy or asthma [19]. The most common IL4 promoter variant is the single nucleotide polymorphism (SNP) – 590C/T (NCBI Entrez SNP rs2243250) previously described to be involved in functional gene modification [20]. This SNP is found to be associated with elevated IgE levels, asthma pathogenesis, lower forced expiratory volume (FEV<sub>1</sub>) values, atopic dermatitis, allergic rhinitis, childhood asthma and severity in several studies; however some failed to confirm these associations [21].

In this study, a case group of atopic-allergic individuals and control group of non-atopic persons were characterized and genotyped for the -590C/T IL4 polymorphism. The association of the variant to total serum IgE level was determined to assess its clinical implication in the selected Filipino population.

### Materials and methods

#### *Study design, base, and subjects*

Case-control method was used in the study. The appropriate sample size and power of the study were determined using web-based programs QUANTO v1.2 [22] and Power for Association with Errors – PAWE-3D [23]. The sampling of subjects was done at the University of Santo Tomas Hospital. The study design, conduct of sampling, and experimental protocols were all approved by the Institutional Review Board of the hospital.

Subjects were initially screened using standardized questionnaires of the International Study of Asthma and Allergy in Childhood (ISAAC) and the International Primary Care Airways Group (IPAG). To further qualify in the study the following criteria were set: [1] naturally born Fili-

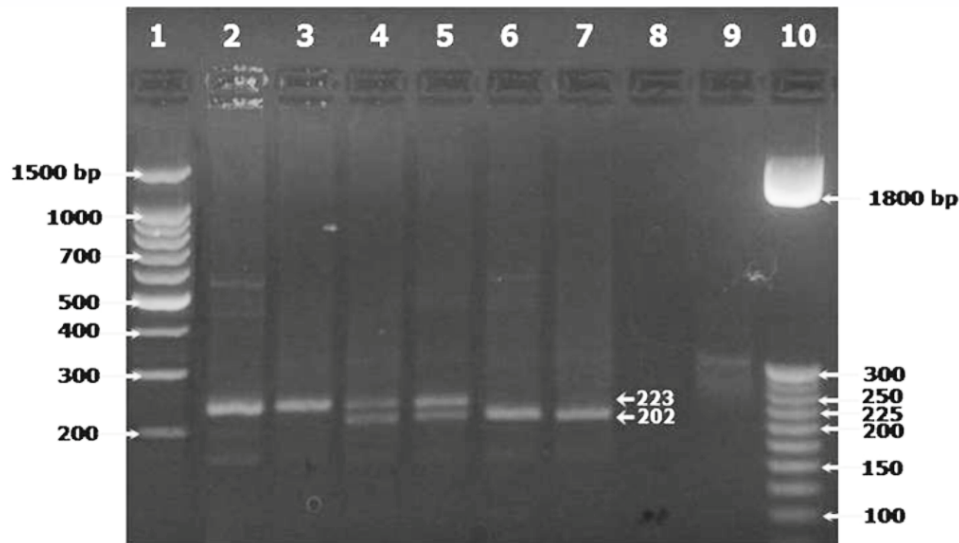
pino, [2] must be unrelated to any other individuals who have had already entered the study, [3] was born and living in Luzon Island, [4] aged 6 to 60 years old at the time of entry in the study, [5] with both parents also Filipinos, and [6] must not have any history of helminth infestation. Cases were conceptually defined in the study as individuals suffering from asthma, allergic rhinitis, or atopic dermatitis, either mild or severe, with total serum IgE level of  $\geq 100$  IU/mL [24, 25, 26] and thus atopic. Controls, on the other hand, were defined as non-atopic individuals (total serum IgE level of  $< 100$  IU/mL) without any history of allergy and neither do have immediate relatives with it. Cases and controls were matched-paired with their age ( $\pm 2$  years old), gender, and geographic location of residence.

#### *Clinical protocol and phenotyping*

Ten milliliters of blood were extracted by venipuncture from all the participants. Five milliliters of the whole blood were centrifuged for 5 minutes at 5000 rpm to isolate the serum. Total serum IgE was quantitated using standard antibody sandwich ELISA protocol. The concentration was expressed in IU/mL and was log-transformed to normalize the distribution.

#### *Genotyping*

Genomic DNA was extracted from the whole blood using the WIZARD<sup>®</sup> Genomic DNA Purification Kit (Promega, Madison, WI, USA). Polymerase chain reaction – restriction enzyme fragment length polymorphism (PCR-RFLP) analysis was used as previously described [27] to amplify and analyze for the -590C/T IL4 polymorphism. Briefly, a 15- $\mu$ L reaction was prepared containing 2  $\mu$ L genomic DNA, 0.20 mM dNTPs (Promega, Madison, WI, USA), 1X PCR buffer (Roche Diagnostic Corporation, Basel, Switzerland), 1.50 mM MgCl<sub>2</sub>, 6 pmol of each primer, and 1.2U AmpliTaq Gold<sup>®</sup> DNA polymerase (Roche Diagnostic Corporation, Basel, Switzerland). Thermocycling condition (Gradient Palm-Cycler<sup>™</sup>, Corbett Research, Sydney, Australia) that was used include initial denaturation at 95°C for 12 mins; 35 cycles of denaturation at 95°C for 30s, annealing at 56°C for 2 min, and extension at 72°C for 40s followed by a final extension step at 72°C for 5 min.



**Figure 1.** Identification of the -590 Polymorphism of the 5' Flanking Region of the IL4 Gene by Restriction Fragment-Length Assay. Lane 1: 100-bp DNA ladder, Lanes 2 & 3: CC homozygote, Lanes 4 & 5: CT heterozygote, Lanes 6 & 7: TT homozygote, Lane 8: (-) control, Lane 9: actin amplification, and Lane 10: 25-bp DNA ladder.

The PCR product was digested with 5U of *AvaII* endonuclease (DCC-Bionet, Beams Biotech Co. Ltd., Korea) at 37°C for 5 hours. The digested DNA was separated on 3.5% agarose gel containing ethidium bromide at 60V for 1 hour. The assay yielded a single 223-bp band for the CC genotype; three bands of 21, 202, and 223 bp for CT heterozygotes; and two bands of 21 and 202 bp for TT homozygote (**Figure 1**). The samples were randomly genotyped and -590 *IL4* profiles were read independently by two individuals. The samples were re-genotyped if there was any disagreement concerning the genotype.

#### Statistical Analysis

Data were managed and analyzed using the SPSS v11.5 (SPSS Inc., Chicago, IL, USA) and StatXact (Cytel Inc., Cambridge, MA, USA) statistical packages and MS Excel (Microsoft Corp., Redmond, WA, USA). Hardy-Weinberg equilibrium was tested by Fisher Exact Test  $\chi^2$  analysis for a 2x2 table. Differences and association between or among variables in all the inferential statistics were considered significant when *p*-value is <0.05. Both parametric and non-parametric tests were done. To test for association,  $\chi^2$ , Fisher's, Fisher-Freeman-

Halton, Armitage, and Odds ratio tests were performed. Correction for multiple testing was not done in the analysis.

#### Results

##### Power analysis and demographics

A total of 102 matched-pairs were recruited in the study based on the minimum sample size of 100 pairs. QUANTO v1.2 and PAWE-3D calculations showed that the sample size, together with the specified study design, allele frequencies, hypothesis, inheritance model, prevalence of disease, significance, and allowable error rates; can give as high as 90% power and can detect variant allele frequency of at least 0.05 and genotype relative risk of  $\geq 1.8$  at 80% power.

The mean age of the 102 qualified case-control pairs was 25.99 $\pm$ 8.94 years with a range from 11 to 57 years. There were roughly equal proportions of male (48%) and female (52%) qualified subjects. Furthermore, the qualified subjects came from Metro Manila cities, municipalities, and the surrounding provinces populated mostly by the Tagalogs. In the atopic-allergic case group, 43% have

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**Table 1.** Genotype and Allele Frequencies of -590C/T *IL4* Polymorphism and their Association to the Risk of Atopic Allergy in a Filipino Population

Genotype/ Allele	Frequency		Odds ratio	95% Confidence Interval	Statistic value	p-value
	Cases	Controls				
CC	0.510	0.716	Baseline	-		
CT	0.382	0.225	1.886	1.202-2.960	2.816	0.004861*
TT	0.108	0.059	3.558	1.446-8.759		
CT + TT	0.490	0.284	2.420	1.356-4.321	9.110	0.003 <sup>§</sup>
CC + CT	0.892	0.941	0.517	0.184-1.456	1.604	0.205 <sup>¶</sup>
C	0.701	0.828				
T	0.299	0.172	2.060	1.285-3.301	9.208	0.002 <sup>Δ</sup>

\* Cochran-Armitage Trend test; Additive Model; <sup>§</sup>  $\chi^2$  test; CC as baseline; Dominant Model; <sup>¶</sup>  $\chi^2$  test; TT as baseline; Recessive Model; <sup>Δ</sup>  $\chi^2$  test; Multiplicative Model

asthma, 35% have allergic rhinitis, and 22% have atopic dermatitis.

### Phenotype

The mean log total IgE (IU/mL) was  $3.11 \pm 0.54$  for the cases and  $1.70 \pm 0.23$  for the control group. A significant difference between the two means was obtained ( $t=27.649$ ,  $df=101$ ,  $p<0.05$ ). The atopic-allergic cases was also divided into severe and mild-moderate groups based on the cut-off at 1000IU/mL with the former having mean IgE level of  $3536.28 \pm 2147.37$  and the latter,  $439.33 \pm 270.07$ . Subgroup analysis for difference was likewise significant ( $t=-9.059$ ,  $df=100$ ,  $p<0.05$ ).

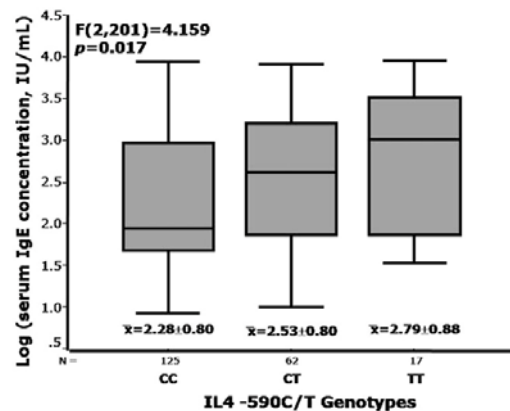
### Genotype distributions of -590C/T *IL4* polymorphism

The genotype distributions showed greater number of heterozygous and homozygous TT individuals in cases than in controls (**Table 1**). Wild type C allele and CC genotype, on the other hand, predominated in the control group.

**Table 1** also summarizes the statistical tests done to evaluate the association between the *IL4* SNP and the risk of atopic allergy. Significant associations were established in all but one model. Among the three genotypes, homozygote TT gave the highest significant odds of atopic allergy of about 3.6 fold for cases than for controls under the additive model (95% CI: 1.446-8.759, uncorrected  $p=0.005$ ). The  $\chi^2$  tests in the dominant ( $\chi^2=9.110$ , uncorrected  $p=0.003$ ) and multiplicative ( $\chi^2=9.208$ , uncorrected  $p=0.002$ ) models likewise indi-

cated the risks of developing atopic allergy for carriers of T allele at odds of 2.4 (95% CI: 1.356-4.321) and 2.1 (95% CI: 1.285-3.301), respectively. It can also be inferred from the allele counts that there was 63.5% probability of developing atopic allergy for an individual possessing the variant allele at the -590 position of the *IL4* gene. The non-significant result under the recessive model meant that two copies of the variant T allele may not be required for an increased risk.

### Association between total serum IgE level and -590C/T *IL4* polymorphism



**Figure 2.** Boxplots of Total Serum IgE Levels by -590 *IL4* Genotypes in a Filipino Population. Illustrated are the median values and interquartile distance.

To characterize the association between atopy phenotype and the -590C/T *IL4* SNP, the log total IgE was analyzed relative to each genotype (**Figure 2**). A significant between-group difference in mean log total IgE among the

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**Table 2.** Differences in the Pooled Serum Total IgE Levels among the -590 *IL4* Genotypes

Genotypes	n	log (serum IgE concentration, IU/mL)	t statistic	p-value
CC	125	2.28±0.80	2.622	0.009
CT + TT	79	2.58±0.82		
CC + CT	187	2.37±0.81	2.086	0.038
TT	17	2.79±0.88		

**Table 3.** Genotype and Allele Frequencies of -590C/T *IL4* Polymorphism and their Association to the Risk of Mild-Moderate and Severe Atopic Allergy in a Filipino Population

Atopy Status	Genotype	Frequencies		Odds Ratio	95% Confidence Interval	Statistic Value	p-value
		Cases	Controls				
Low IgE Level (<1000 IU/mL)	CC	0.525	0.716	Baseline	-	5.507	0.0637*
	CT	0.425	0.225	1.626	0.902-2.932		
	TT	0.050	0.059	2.645	0.814-8.595		
	CT + TT	0.475	0.284	2.278	1.070-4.846		
Very High IgE Level (>1000 IU/mL)	CC	0.500	0.716	Baseline	-	8.24	0.0162*
	CT	0.355	0.225	1.992	1.224-3.240		
	TT	0.145	0.059	3.986	1.499-10.5		
	CT + TT	0.500	0.284	2.517	1.304-4.860		

\* Fisher-Freemant-Halton test; <sup>Δ</sup>  $\chi^2$  test

three genotypes was observed with the greatest difference seen between serum IgE levels of the wild-type CC and the variant homozygous TT ( $F(2,201)=4.159$ ,  $p=0.017$ ). Moreover, significant results were also found if log total IgE are grouped based on the dominant ( $t=2.622$ , uncorrected  $p=0.009$ ) and recessive ( $t=2.086$ ,  $p=0.038$ ) models and analyzed using t-test (Table 2). This further indicates that the high serum total IgE levels of the allergic subjects in the study were largely due to the presence of the T allele at the -590 position of the *IL4* gene.

Subgroup analysis of low and very high serum IgE level in association to -590C/T *IL4* gave results that bordered on statistical significance (Table 3). In this instance, there is an estimated 2.3 (95% CI: 1.070-4.846) to 4 (95% CI: 1.499-10.5)-fold greater odds of atopic allergy for carriers of one or two copies of T allele than for the C allele. The former then could possibly have modulated serum IgE levels whether the status of atopy was mild, moderate, or severe.

### Discussion

Atopy phenotype was clearly defined and deli-

imited in the study by utilizing both binary response and quantitative trait definitions. Only the sera of qualified allergic individuals in the initial screening were quantitated for total IgE. The selected candidate gene SNP was chosen based on prior association data, positional and linkage studies, functional, and pharmacological criteria [28].

The use of genetic epidemiological study design requires necessary measures to control confounders and bias during sampling of subjects and data analysis. The matched-pairing of cases and controls ensured that variables, like age, gender, and geographical location of residence, do not distort the measure of association. To minimize the likelihood of population stratification, the inclusion criteria of residency in Luzon Island was set in a hospital-based sampling. The genotype frequencies of the control group, however, failed to agree with the Hardy-Weinberg proportions. Thus, the multiplicative, dominant, and recessive models' measure of odds of disease might not be the true measure of association in the population. This was complemented with the use of Cochran-Armitage trend test for the additive model which is more conservative, does not

rely on assumptions of Hardy-Weinberg Equilibrium, and may be extended to account for population heterogeneity, if existing [29]. Furthermore, the deviation from Hardy-Weinberg proportion is likely to be statistical rather than methodological.

The observed number of control individuals with the variant T allele is close to that observed in a white [30] and American [31] populations. Moreover, the results gathered in this study showing that the -590T allele of *IL4* is a risk factor to the development of atopic allergy replicated the results in another American [20] and Japanese [32] populations. The -590C/T *IL4* SNP-allergy association is also consistent with the results of other similar association studies that utilized a different or modified study design [13, 33].

The significantly high serum total IgE levels among individuals carrying the T allele may be attributed to the presence of the SNP at the 5'-flanking region of the *IL4* gene. The -590C/T polymorphism is located in one of the unique binding sites for the nuclear factor of activated T cell (NF-AT) which plays an important role in the transcription of several cytokine genes. The SNP is situated within an inverted palindrome from -603 to -588 that could possibly explain the observed increase in the affinity of NFAT-1 dimers to the region and correspondingly the increased serum IgE levels and the occurrence of atopy [34]. It is also close, about 50 bp away, to a putative glucocorticoid response element (AGAACA) that can somewhat affect *IL-4* expression [20].

The reported inconsistencies between different populations might have resulted from racial differences in allele frequencies which probably brought by balancing selection or random mutation during the course of the population's adaptation to its environment. Although it was concluded that the *IL4* -590T allele may increase the likelihood of developing atopic allergy, the results cannot address whether the allele itself is in linkage disequilibrium with another loci that also predisposes a person to allergy. Furthermore, while significant associations were observed, these findings should be considered in conjunction with some of the limitations of the study which include the data not being adjusted for multiple testing and possible confounding due to the observed deviation from Hardy-Weinberg Equi-

librium.

In conclusion, the -590C/T *IL4* promoter polymorphism is a possible risk factor to the development of atopic allergy in the Filipino population. It may be directly involved in the overexpression of serum IgE levels in the selected Filipino allergic individuals. As this is an initial finding, replication in a larger Filipino population and stratification with polymorphisms located in other candidate genes are highly recommended.

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