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Association between promoter IL-4 C 590-T polymorphism and asthma in Tunisian patients

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Abstract

Asthma is a common familial syndrome due to interacting genetic and environmental factors (1) (2). The aim of this study was to investigate the potential link between C590-T, the level of IL-4 and asthma prevalence in Tunisian patients (3) (4). A cohort of 50 subjects including 30 patients and 20 controls was analyzed , IL-4 concentrations were determined by ELISA test and IL-4 C590-T was determined by PCR –RFLP (Polymerase Chain Reaction – Restriction Fragment Length Polymorphism) method using BsmF enzyme(5). Serum levels of IL-4 were increased for asthmatic patients exceeding threshold (2pg/ml) reaching 2.665±0.52 for CT genotype and 2.185±0.8 for TT genotype compared to controls.

Our findings revealed that IL-4 C590-T SNP (Simple Nucleotide Polymorphism) showed a significant difference between asthmatic patients and controls, when comparing TT vs CC (OR 0, 10077. 95% CI: 0, 0285 – 0, 4068 P value 48.10^{-5}) and TT vs CT (OR 9, 2857. 95% CI: 2.4583 –35.0753 P value 48.10^{-5}) genotypes.

We also demonstrated that patients with heterozygous CT (63,3%) and homozygous TT (20%) showed significantly higher serum levels of IL-4, while wild genotype CC showed lower levels.

T allele seems to be associated with asthma especially CT combination (6) (7). This allele dominating in special population compared to others at genetic levels could be used as an indicator of genetic biomarkers inducing either pathogenesis or protection from the disease (8).

Key Words: C590-T, SNP (Simple Nucleotide Polymorphism), IL-4, Asthma, Tunisian patients

Introduction

Research on asthma has a long tradition for decades, one of the most popular ideas in asthma literatures is the idea that asthma is simply due to environmental factors. Recent theoretical researches have revealed that asthma is a common airway inflammatory disease (9), its pathogenesis is complex it results from several interactions between genetic predisposition and environmental factors (10) (11). Some 350 million people currently suffer from asthma; the asthma death rate is growing up reaching about 260.000 ill persons (12) (13).

To underline in depth asthma pathogenesis several studies axed on the identification of genes related to asthma (14). These approaches have been influential in the field because of specific molecular patterns that seem predicting asthma. Th2 lymphocyte play an important role in air way inflammation(15), subsequently more trials have been conducted targeting molecules related to Th2 cells such as IL-4, IL-13, IL-10 (16).

There are growing appeals for IL-4 effect in the field of asthma. IL-4 is a low molecular weight polypeptide of 129 AA. The protein is encoded by a gene on chromosome Chr 5q23- 31 (20). Il-4 plays a critical role in immune responses by acting as a growth factor for Th2 cells and inducing Ig class switching to IgE(18).

In the present study we analyzed genetic association, the relationship between IL-4 single nucleotide polymorphism SNP located on the promoter region and asthma. Polymorphism genes play a crucial role in cytokine production (19). The present study is among the first investigations carried on Tunisian asthmatic patients dealing with genotyping IL-4.

Material and methods

A total of 50 patients (30 asthmatics and 20 healthy patients) were recruited. Studies were approved by the Center Institutional

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Review Board. All patients were from asthma outpatient clinic of Pneumology department of Military Hospital of Tunisia. A short questionnaire was designed to ascertain the participants, all patients and controls were aged 5 to 75 years, 12 males and 18 females.

Each patient was drawn with 5ml venous blood; 3ml treated EDTA Anticoagulants serving to DNA extraction and genotyping. The remaining 2ml was collected in a common test tube the serum was extracted serving for ELISA test to detect IL-4 level.

After extraction from peripheral blood leukocytes using a QIAmp min Kit (Qiagen Tnc), DNA amplification was carried out by polymerase chain reaction (PCR)-restriction fragment length polymorphism (RFLP), genotyping one SNP C590-T in the promoter region of IL-4. Table 1

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Table 1: Primers of IL-4 C590T

PRIMER	SEQUENCE	
FORWARD	5′-ACTAGGCCTCACCTGATACG-3′	**C-590-T**
REVERSE	5′-GTTGTAATGCAGTCCTCCTG-3′	

The PCR conditions are summarized below on Table 2 and 3.

Table 2: PCR mix reaction for genotyping of IL-4 gene position −590 (C>T).

Component	Volume (µl)
Each primer (T or C allele + reverse)	1.5
DNA template	2
dMix	15
Taq polymerase	1.5

Table 3: PCR conditions for genotyping of IL-4 gene position −590 (C>T).

Steps	Temperature (°C)		Time
Denature template	94		5min
Initial denaturation	94		45sec
Annealing	60	35 cycles	45sec
Extension	72		50sec
Final extension	72		10min

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A blank amplification was always run to check for the presence of contamination. Primers and probes were obtained from NCBI database.

15µl PCR products were digested for 1 to 16 hours at 37°C with 1to 2µl of BsmFI 2µl of Tango Buffer for a final volume of 15µl.

Electrophreisis of 15µl digest product and DNA markers of various molecular weight were charged above an Agarose gel (3%agarose /1Xtbe gel).The electrophoresis gel was performed using

BioRad Wide mini Sub cell at 75V for 1hour. We visualized the reaction stained by 10µl BET.

The restriction digest was carried at 37°C rather than 65°C, optimal temperature for BsmFI activity on account of undesirable temperature for Taq DNA polymerase activity at 37°C. BsmFI activity upholds 50%of its efficacy.

Elisa test Enzyme Linked Immuno Sorbent Assay was adopted to detect the IL-4 level, the reagent were purchase from Invitrogen ThermoFisher SCIENTIFIC IL-4 Human ELISA Kit.

Microplate reader was provided by Biometra.

Statistical analysis

The data were normalized using Statistical differences for patients and controls were tested for Hardy Weinberg equilibrium using χ^2 test. Statistical analysis were performed using (EPi info) (V.7) and graph Prism (V.8.1) programs. To evaluate the difference of several biological settings between patients and controls we used ANOVA test. The significance level was set at 0, 05.

Results

The first set of questions aimed to analyze the effects of polymorphism on the incidence of asthma. A promoter polymorphism of IL-4 gene (C-590 T) was screened by PCR - RFLP in Tunisian patients (asthmatics and controls). Interestingly, the polymorphism was correlated with IL-4 serologic levels.

In summary, these results show that: at the end of cleavage by BsmFI of IL-4 gene (252 bp full length), PCR product display two fragments of 192 bp and 60 bp. Figure 1 illustrates this with examples of an homozygous for the wild type allele in lane 2, an heterozygous in lane 1 and 3, and a homozygote for the -590 C->T allele in lane 4. Under these electrophoresis

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conditions the 60 bp cleavage product is not visible. The polymorphism of IL4 −590 (C>T) showed three genotypes (CC, CT, and TT) by using the specific C, specific T, and reverse primers (Figure 1).

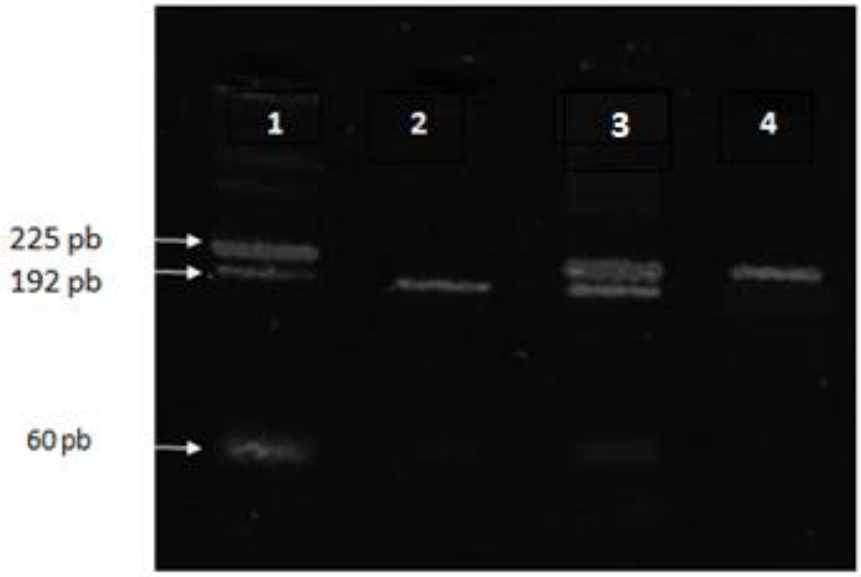

Figure 1: *BsmFI restriction endonuclease digestion of PCR products amplified of IL-4 gene sequence; 1 and 3= Heterozygous for the -590C/T allele (CT), 2= Homozygous for the wild type allele (CC) and 4= Homozygous for mutant -590C/T (TT).*

These results suggest that there is an association between polymorphism and asthma. Significant differences were found in the genotype distribution of SNP C-590T polymorphism between asthmatics patients and controls (83, 3% vs. 35%) respectively, Table4 shows an overview of statistical analysis.

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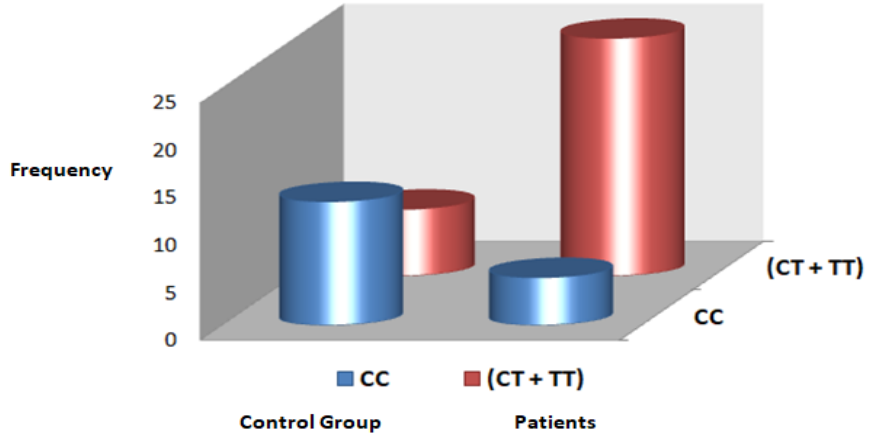
Table 4: Frequency distribution of IL4 −590 (C>T) genotypes in asthma patients and controls.

Gene	Genotype	Asthma patients number (%)	Controls number (%)	OR (95% CI)	P-value
IL-4 −590 (C>T)	CC	5 (16.6)	13 (65)	0.1077 (IC = 0.0285-0.4068)	48×10^{-5}
	CT	19 (63.3)	4(20)	9.2857 (IC = 2.4583-35.0753)	48×10^{-5}
	TT	6 (20)	3(15)	9.7879 (IC = 2.3317-41.0872)	2.05×10^{-3}

(OR = odds ratio, CI = confidence intervals).

182 The most striking result to emerge from the data is that C590T polymorphism was related to asthma in Tunisian patients with a high significance p< 0,005.Figure 2



186

187 **Figure 2:** *Phenotypic study of control and patients groups. The frequencies show that the C*

188 *allele is more frequent in controls compared to the T allele; however in asthmatic patients T*

189 *allele is more expressed. CT combination in the patient group is over-represented compared*

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to other genotypes (63%). The distribution of TT genotype was an approximate proportion between patients and controls; 15% in controls VS 20% for patients.

CT genotype was associated with a potential link with asthma (OR=9.2857; 95%IC: 2.4583-35.0753 p< 0,005). We found that there is no significant association between CC genotype the wild genotype, however T allele conveys a significantly increased risk of asthma which is consistent with other studies. Taken together the two mutant genotypes we observed a trend towards association with risk of asthma for both of CT and TT. We also detected an increased susceptibility of asthma for CT combination.

If we now turn to serologic analysis we measured IL-4 levels, we detected remarkably increased levels in asthmatic patients compared to controls.

As shown in table 5:

Table 5: Serum IL-4 concentration (pg/ml) in control and patient's groups.

	controls n=20	**Patients n=30**
Interleukine 4 [pg/ml]	0.978 ± 0.89	1.663±1.02
	p-value = 0.013	

The concentration of IL-4 was elevated in patient's group at an average of 1.663±1.02 pg/ml. While the control group it was normal for an average of 0.978 ± 0.89 pg/ml.

Serum concentration of IL-4 in asthmatic patients exceeded the threshold reaching 2,66pg/ml however in controls levels were under the thresholds 0,4pg/ml. Figure3 below provides the results obtained from the preliminary analysis.

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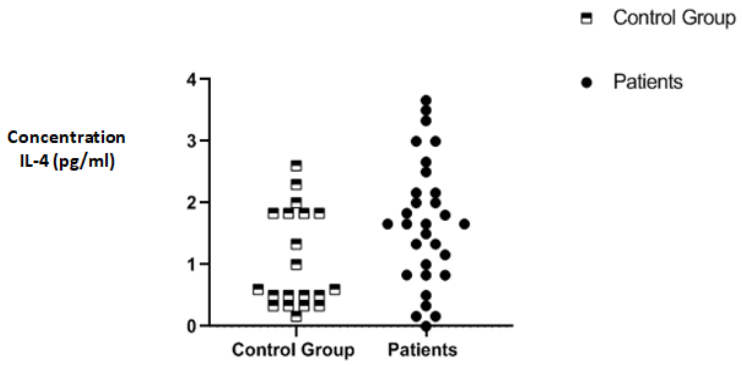

220

221 **Figure 3:** *IL-4 serum concentration (pg/ml) in control group and patients.*

222 The overall outcome of the survey is that
223 T allele promotes the development of
224 asthma. IL-4 is significantly over
225 expressed within asthmatic patients it
226 means that the immune dysfunction
227 contributes to the development and
228 progression of asthma.

229 **Discussion**

230 New trends in human genetics have
231 demonstrated that gene alterations play a
232 crucial role in asthmatic diseases (20)
233 Several reports have also shown that IL-4,
234 which is a Th2 cytokine, is a part of the
235 complex net work of allergic
236 inflammation, An initial objective of the
237 study was aimed on the fact that
238 substitution of C590T in IL-4 gene which
239 may affect asthma prevalence (9).

240 This study set out with the aim of assessing
241 the importance of IL-4 in asthma. In
242 accordance with previous studies the
243 current work have demonstrated that IL-4
244 polymorphism effects in a population help
245 to bring insight into pathogenesis of
246 asthma (22) (23). We have determined a
247 single nucleotide polymorphism SNP (C-
248 590 T) of IL-4 effects in Tunisian patients.

249 The most obvious finding to emerge from
250 the analysis is that there were differences
251 in genotype frequencies in the cohort study
252 of asthmatics and controls. An association
253 between C-590 T IL-4 polymorphism and
254 asthma has been found especially for

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mutated genotypes CT and TT, although this association is controversial (24). As shown in table 4 we have found that T allele is more expressed in asthmatic patients(25)(26).

The mutant TT presented higher frequency than the wild one CC and CT mutant showed the highest linkage with asthma incidence, the association of C and T allele seems to be the most implicated in the disease prevalence. The inconsistency between our findings and those of several previous studies may be at least partly explained by differences in the genetic backgrounds of the populations examined, definitions of asthma, and statistical power.(27)(28).

Also an increasing in IL-4 secretion has been reported in relation with T allele (29) (30), IL-4 increases throughout Th2 differentiation inducing inflammation and may be a crucial factor promoting asthma (31) (32).

In conclusion

This paper has argued that C-590T polymorphism in IL-4 gene promoter has an influence on asthma. It has also found that generally C-590 T polymorphism was correlated to IL-4 high serum levels.

This finding is described for the first time for Tunisian patients, Asthma risk in Tunisian patients is tightly linked to T allele CT and TT genotypes frequencies were higher than in controls. And CT combination plays a crucial role in the development of the disease. This could be an important biomarker predicting asthma. Since our sample size was relatively small and only one SNP of IL-4 were genotyped, association or linkage study in larger sample size and multiple loci are needed to clarify the inheritance role of IL-4 in the development of asthma. Further studies, which take these variables into account, will need to be undertaken.

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