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Short title: cytokines polymorphisms in asthma

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Summary

Asthma is a chronic airway inflammatory disease associated with airway hyperresponsiveness which affects subjects with genetic predisposition. It has been reported an association between some polymorphisms in various cytokine genes and asthma. Most of them are single nucleotide polymorphisms (SNPs). These polymorphisms are detected in the protein coding sequence or in the promoter region thus influencing cytokine production. We investigated the involvement of SNPs mapping in 5 cytokine genes in mild to severe asthmatics from Italian Caucasians. The frequency of alleles and genotypes, relatively to 10 allelic specificities of the cytokine genes, was defined in 57 asthmatics and in 124 control subjects by a Polymerase Chain Reaction-Sequence Specific Primer method.

TNF-α–308A and TNF-α –238A allele frequencies were higher in asthmatics than in controls ($p<0.001$). Significant differences in the frequency of IL-4 –590T allele and of IL-4Rα +1902A allele were also detected in asthmatics in comparison with controls ($p<0.001$ and $p=0.005$, respectively). Similarly, IL1α -889C allele was present in 84.1% of asthmatics and in 70.2% of controls ($p=0.013$). Furthermore, the IL-4Rα +1902A/A and IL-1α -889C/C homozygous conditions and the TNF-α -308G/A, TNF-α -238G/A, IL-4 -590T/C and IL-10 -1082G/A heterozygous conditions were significantly associated with asthma ($p<0.05$). ACA haplotype of IL-10 was observed only in asthmatic patients. This study reports, for the first time, the frequency of 10 different single nucleotide polymorphisms in 5 cytokine genes in the Italian Caucasians. Furthermore, we also indicate that in our population some single nucleotide polymorphisms are associated with mild to severe bronchial asthma.

Introduction

According to recent literature, there is a clear association between polymorphisms in cytokine genes and different diseases such as transplant rejections, autoimmune diseases, vascular diseases and asthma (1, 2). Asthma is a chronic inflammatory disease of the bronchial wall (3) associated with airway hyperresponsiveness and its development seems to be dependent on the interaction between genetic predisposition and exposure to specific environmental stimuli, such as allergens, viruses and pollutants (4). The role of cytokines in modulating and directing the immune reaction and the inflammatory response of the airways is crucial in the development, progression and exacerbations of bronchial asthma (5).

Tumour necrosis factor-alpha (TNF-α) is an important mediator in many cytokine-dependent inflammatory events. Data from literature indicate that TNF-α plays a role in the initiation of allergic asthmatic airway inflammation and in the generation of airway hyperresponsiveness. Polymorphisms in the 5' untranslated region of the TNF-α gene, in particular the polymorphism -308bp, have been associated with an increased risk of asthma and bronchial hyperresponsiveness (BHR) (6, 7). Currently, the significance of TNF-α -308 G/A polymorphism association to asthma in Caucasians is controversial (6, 7) probably due to limitations of the meta-analysis related to inclusion criteria of published data recruited in both studies; further studies with large group of population are requested in order to assess the effect of these polymorphisms on asthma susceptibility taking into consideration the potential effect of different genetic backgrounds and of different environmental factors in different ethnicities. Furthermore, increased serum levels of TNF-α have been shown in severe asthma in conjunction with elevated serum levels of IL-8 (8).

IL-4 polymorphisms have been associated with asthma and other allergy-related phenotypes, although ethnical differences have been observed. For example, it has been reported a correlation between genetic variants in the promoter region and asthma, although some of these correlations are still controversial (9).

IL-4 acts through the IL-4 receptor which consists of two subunits, the α chain (IL-4Rα) and the γ chain. Some SNPs localized in the coding region of the IL-4Rα gene, such as Q576R, have been associated with asthma and atopic phenotype (10). In addition, a recent study showed an association between the IL-4 promoter polymorphisms at positions -1098, -590, and -33 and IL-4Rα at position +1092 and asthma, and between the haplotypes of these polymorphisms and total IgE in asthmatic patients (11).

In asthma the allelic variants of some cytokines, such as TNF-α and IL-4, seem to be clinically relevant. The aim of the present study was to evaluate the role of polymorphisms in genes coding asthma-related cytokines in mild to severe asthmatics among Italian Caucasians.

Materials and Methods

Subject characteristics

A total of 57 unrelated Italian Caucasians with asthma were studied (Table I). The asthmatic subjects were stratified according to the GINA criteria (12). All asthmatics were non smokers and nobody was affected by chronic obstructive pulmonary disease (COPD). Positivity to the skin prick test (wheal diameter ≥3 mm greater than saline control) to a panel of 14 standard aeroallergens (ALK Abelló; Hørsholm, Denmark) was used to define allergic status in all recruited subjects. As control group, we used 124 healthy Italian subjects (bone marrow donors).

Forced expiratory volume in 1 second (FEV_1) was measured by spirometry (Med Graphics, Pulmonary Function System 1070 series 2, Med Graphics Corp., St. Paul, MN). Normal lung function was defined if spirometric parameters ranged in the normal reference intervals (13). Fractional nitric oxide (NO) concentration in exhaled breath (FeNO) was measured by a chemiluminescence analyser (Ecomedics CLD88, Eco Physics AG, Duernten, Switzerland) according to the ATS/ERS recommendations (14).

The study, conformed to the Declaration of Helsinki, was approved by the local Ethics Committee (A.O.U. San Luigi Hospital: n. of protocol 35/2009), and written informed consent was obtained from each subject.

DNA Extraction

DNA was purified from 200 μl of peripheral blood samples with EDTA as anticoagulant, using the Capture Column kit (Gentra Systems, Ste 110 Plymouth, UK), a special purification matrix, according to the manufacturer's procedure. Concentration and purity of DNA were determined by spectrophotometric method (Eppendorf

Biophotometer, Eppendorf AG, Hamburg, Germany). DNA concentration was adjusted to 50-100 ng/µl while purity (ratio A_{260}/A_{280}) was 1.6-1.8.

Gene polymorphism analysis

Polymerase chain reaction with sequence specific primers (PCR-SSP) assay (Heidelberg kit, from the Institute of Immunology, University of Heidelberg, Heidelberg, Germany) (15) was used to assess the following cytokine polymorphisms: IL-1α –889T→C, TNF-α –308G→A and –238G→A, IL-4 –1098T→G, –590T→C and –33T→C, IL-10 –1082G→A, –819C→T and –592C→A and IL-4Rα +1902G→A. The PCR amplification was carried out using a thermocycler GeneAmp PCR System 9700 (Perkin Elmer Inc. Waltham, MA 02451 USA) under the following conditions: initial denaturation at 94°C for 2 minutes followed by 10 cycles of denaturation at 94°C for 10 seconds, annealing and extension at 65°C for 1 minute. The next 20 cycles included 10 seconds of denaturation at 94°C, 50 seconds of annealing at 61°C and 30 seconds of extension at 72°C. Briefly, PCR-SSP typing by the Heidelberg kit consisted of PCR primer mixes aliquoted in well PCR trays. Master mix, which was supplied along with the reagents and consisted of $MgCl_2$, buffer, dNTPs and glycerol, was mixed with 50-100 ng/µl DNA and 16.5 U Taq polymerase (Applied Biosystems, Foster City, CA, USA). The internal control primer pairs generate a 440 bp amplification fragment (a part of C reactive protein gene) for IL-1α, TNF-α, IL-4Rα genes, and a 90 bp fragment (a part of the β-globin gene) for IL-4, IL-10 genes. The PCR products were visualized by 2% agarose gel electrophoresis with 0,625 mg/ml ethidium bromide.

The identification of haplotypes GCC, ACC, ATA, ACA and ATC of the IL-10 gene was performed by the Protrans Cytokines 2 Cyclerplate System REF 200 096

(PROTRANS, Medizinische Diagnostische Produkte Gmbh, Hockenheim, Germany) according to the manufacturer's procedure.

Statistical Analysis

The distribution of each variable was checked using the Shapiro–Wilk W test.

Age and FeNO and FEV_1 (% Pred) values were reported as median with inter-quartile ranges and as mean with standard deviation (SD) respectively.

The allele and genotype frequencies were calculated by direct gene counting. The total was divided by the number of chromosomes in order to obtain allele and by the number of subjects to obtain genotype frequencies.

For comparisons between two groups, a Mann-Whitney U test was used for non-normally distributed quantitative data (i.e. age and FeNO), whereas a Student t test was used for normally distributed quantitative data [i.e. FEV_1 (% Pred)]. For comparisons among more than two groups, a Kruskall-Wallis test followed by Bonferroni's correction or ANOVA followed by Sheffè test were used when appropriate.

A bivariate analysis was performed and the comparison of frequency between asthmatics and controls was made calculating the chi-square test or the Fisher's exact test in case of expected frequencies less than five and reporting the bivariate odds ratio (OR) and 95% confidence interval (CI). Calculations were made with the chi-square-test by Epistat Statistical Package, for MS-DOS. All tests were two-tailed.

Results

The characteristics of the asthmatic and control subjects are reported in Table I.

In the three groups of asthmatics the atopic subjects were 18 out of 26, 11 out of 14 and 8 out of 17 for mild, moderate and severe respectively.

The male-to-female ratio was similar in the three groups of asthmatics. FEV_1 (% Pred) ($p=0.027$) and FeNO ($p=0.017$) were significantly different among the three groups of mild, moderate and severe asthmatics. As expected FEV_1 (% Pred) became progressively lower in mild, moderate and severe asthmatics ($p<0.05$ for each comparison). In addition, lower FeNO levels were found in severe asthmatics as compared to mild asthmatics ($p<0.05$). A significant longer disease duration was found in severe asthmatics compared to mild asthmatics ($p<0.01$).

Comparisons between atopic and non atopic patients did not reveal any significant difference in the analyzed parameters.

The allele and genotype frequencies for all patients and control subjects are indicated in Table II and III respectively. As shown in Table II, statistical analysis, based on the detected frequencies, showed that the alleles TNF-α –308A, TNF-α –238A, IL-4 –590T, IL-4Rα +1902A and IL-1α -889C are significantly associated with mild to severe bronchial asthma in comparison with the control group. Differently, allele IL-10 -819T seems to be associated with a lower frequency in asthmatics.

The IL-4Rα +1902A/A and IL-1α -889C/C homozygotes and the TNF-α -308G/A, TNF-α -238G/A, IL-4 -590T/C and IL-10 -1082G/A heterozygotes genotypes were significantly more frequent in asthmatics compared to the control group (Table III). On the contrary, the IL-4Rα +1902G/A and IL-1α –889T/C heterozygotes genotypes were less frequent in asthmatics as compared to controls (Table III).

Finally, GCC, ACC, ATA and ATC haplotypes of IL-10 gene do not seem to be associated with asthma in our population (Table IV). On the other hand, ACA haplotype of IL-10 was observed only in asthmatic patients (4.9%) (Table IV).

We did not find any significant correlation between FEV_1 or FeNO and the alleles, genotypes or IL-10 haplotypes that showed a significant difference between asthmatics and controls (data not shown).

Discussion

This is the first study that investigates the role of polymorphisms in multiple cytokine genes in asthma among Italian Caucasians.

Our results suggest that TNF-α –308A, TNF-α –238A, IL-4 -590T, IL-4Rα +1902A and IL-1α –889C alleles are associated with mild to severe asthma in adults. Furthermore, the IL-4Rα +1902A/A and IL-1α -889C/C homozygotes and the TNF-α -308G/A, TNF-α -238G/A, IL-4 –590T/C and IL-10 -1082G/A heterozygotes genotypes are significantly more frequent in asthmatics compared to the control group.

TNF-α

Our data indicate that the frequency of TNF-α -308A allele, which is the less frequent allele in the general population, is higher in asthmatics than in normal individuals. These findings are in agreement with several independent studies (6, 7, 16), but on the light of other negative studies (6, 7, 17) the effects of this genetic variant in asthma are still controversial. Discrepancies among these studies could be due to the small sample sizes or to a gene able to affect asthma phenotypes in *linkage disequilibrium* with TNF-α (18). Nevertheless, our data are supported by the observation that the TNF-α -308A allele is associated with elevated TNF-α transcriptional activity and this is not surprising because the -308 polymorphism is localized in the promoter region (19). Increased levels of TNF-α have been demonstrated to cause an increase in airway hyper-reactivity by increased airway smooth muscle responsiveness and/or producing a cascade of inflammatory responses with the release of mediators (20).

We recently showed an increase in serum levels of TNF-α in severe asthma patients derived from the same population of this study (8). Finally, it has been shown that

children with the TNF-α -308A allele under home dampness exposure had an increased risk on asthma-related health outcomes (21).

In our samples set the frequency of the TNF-α -238A polymorphism was higher in asthmatic group compared to control group. This allele has been associated with an increased childhood asthma risk in children with non-smoking parents (22). This polymorphism maps in the promoter, but unlike the TNF-α -308 one, it does not seem to have a direct effect on gene expression, although this region (-280 to -172) contains a strong repressor site (23). However, the TNF-α -238A genetic variant may be in *linkage disequilibrium* with a functional polymorphism, either within the TNF-α gene or another gene within the MHC, that influences the TNF production.

IL-4

Our results indicate that frequency of the T base at position -590 of the IL-4 gene promoter region is higher among asthmatic patients compared to control subjects. The IL4 -590T allele, which is present in approximately 27% of Caucasians, has been associated with increased gene transcription and with higher levels of *in vivo* IgE (11). The IL4 -590T polymorphism has been related to asthma in different populations, to atopy and decreased pulmonary function (9, 11). It has been associated with low values of forced expiratory volume in 1 second (FEV_1) in a Caucasian population with asthma (24) and has been reported that the IL4 -590T allele influences asthma severity (9). However, the role of this polymorphism in asthma is still controversial because some authors did not find any correlation between this variant and asthma (25). On the basis of this observation we can speculate that the differences of the literature data regarding this polymorphism and asthma could be due, at least partially, to the different levels of

asthma severity among the analyzed patients. Noteworthy, 31 of the 57 patients collected in our study were moderate-severe asthmatics.

IL-4Rα

As reported by multiple studies in different populations (9-11), we found that the IL-4Rα +1902A polymorphism is associated with asthma in Italian population. On the other hand, other studies failed to detect a correlation between this genetic variant and asthma (26). The IL-4Rα +1902A polymorphism produces the aminoacid substitution from glutamine to arginine at codon 576 (Q576R) in the cytoplasmatic domain of the receptor. The combination of this genetic variant with the IL-4Rα I75V polymorphism has been reported to enhance CD23 induction in response to IL-4 in B-lymphocytes isolated from allergic patients and to be associated with allergic asthma (27). We speculate that the discrepancy between studies that found or did not an association of this polymorphism with asthma is due to a selection of subjects bearing or not the I75V polymorphism in addition to the Q576R genetic variant. A study in Chinese children strengthen this hypothesis demonstrating by haplotype analysis that the G-A and A-A haplotypes consisting of IL-4Rα I75V and IL-4Rα Q576R are significantly associated with asthma; in addition the authors showed significant gene-gene interactions among six genes including IL-4Rα I75V and IL-4Rα Q576R that may increase an individual's susceptibility to asthma and contribute to the pathogenesis of asthma (28).

IL-1α

We observed that the frequency of IL1α -889C polymorphism was higher in asthmatic patients as compared to controls. In addition, we detected that the carriers of CC genotype at the IL1α -889 position were more frequent in patients than in controls.

Unfortunately, the correlation between this genotype and asthma disease is poorly documented in literature.

IL-10

In our study the IL-10 polymorphisms at positions -819C→T and -592C→A and the GCC, ACC, ATA, and ATC haplotypes were not related to asthma; on the contrary, the IL-10 -1082G→A polymorphism showed a significant association to asthma. Negative results are in accordance with a previous report (29). Indeed other studies reported weak relations between IL-10 polymorphisms and asthma: the IL-10 -592C→A genetic variant has been weakly associated with asthma disease (30); the IL-10 -592 A/C and the -819 T/C polymorphisms, the ATA and the ACC haplotypes were shown to be related to serum eosinophil cationic protein (ECP) in asthmatic Korean children (31). The GCC haplotype has been found to be related to high IgE levels and FEV_1 in asthmatic patients (32). About IL-10 –1082 G/A polymorphism, a positive association of this SNP and ATA haplotype with asthma has been reported in North Indian population (33) and with airway hyperresponsiveness in Korean children (31). The IL-10 –1082 G/A polymorphism was also associated with both atopic and non-atopic asthma in Egyptian children and heterozigosity or homozigosity for the risk allele of the -1082 A/G was significantly associated with increased total IgE but not IL-10 serum levels (34). Furthermore, in our studied population, ACA haplotype was observed only in asthmatic patients. The discrepancies of results reported in literature could be explained by the small size of samples set analyzed, different allelic distribution among different populations, *linkage disequilibrium* or by differences of specificity and sensibility of techniques used for polymorphisms detection.

Finally, the IL-10 -819T allele and the IL-4Rα +1902GA and IL-1α –889TC heterozygotes genotypes resulted more frequent in the control group as compared to asthmatics; unfortunately, the correlation between these conditions and a protective role against asthma disease is, to our knowledge, poorly documented in literature.

The present study is in line with a recent paper that showed in Iranian population an higher frequency of TNF-α and other cytokines polymorphisms in asthmatics compared to controls (35). In our study we did not find associations between cytokine polymorphisms and severity, pulmonary function or FeNO levels but in the latter paper the authors showed that low producer of TNF-α (-308GG) genotype, and TNF-α (-308G) and IL-6 (-174C) alleles were associated with reduced FEF25–75 but no significant association was observed between cytokine polymorphisms and the amount of asthma control. These data suggest that asthma is a multifactorial disease dependent on genetic predisposition, environmental, inflammatory and non-inflammatory factors that interacts in a complex biological response in relation to clinical expression which characterizes the different clinical-biological phenotyping in asthma (36).

In summary, although previous studies have investigated the role of polymorphisms in few cytokine genes among Italian asthmatic patients, this is the first study that describes the allele and genotype frequencies of polymorphisms in several cytokine genes in mild to severe Italian asthmatics, suggesting that the TNF-α –308A, TNF-α –238A, IL-4 –590T, IL-4Rα +1902A and IL-1α -889C polymorphisms are positively associated with clinically relevant asthma.

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Table I. Clinical parameters of recruited patients

	Control	**All Asthmatics**	**Severe Asthmatics**	**Moderate Asthmatics**	**Mild Asthmatics**	**Skin test positive**	**Skin test negative**
Number of subjects	124	57	17	14	26	37	20
Male-to-female ratio	1.29	0.6	0.7	0.8	0.5	0.5	0.8
Median age (years) (LQ-UQ)	43.5 (27.0-52.5)	46.0 (39.0-57.5)	49.0 (45.0-60.5)	48.0 (39.0-61.0)	43.5 (32.5-53.0)	45.0 (36.5-57.5)	48.5 (45.0-57.0)
Mean FEV_1 (%Pred) (SD)	98.0 (7.2)	73.7 (21.8)	46.5 (7.24)	69.5 (5.62)	93.8[§] (8.9)	76.1 (22.7)	69.3 (19.7)
Median FeNO (ppb) (LQ-UQ)	11.1 (10.2-12.0)	25.8 (16.8-39.7)	17.3 (11.2-31.5)	23.5 (15.3-39.2)	36.6[†] (25.4-46.0)	32.9 (21.5-40.8)	24.0 (13.9-36.1)
Median asthma duration (years) (LQ-UQ)	-	22.0 (13.5-31.5)	36.0[‡] (20.5-40.5)	26.5 (13.0-39.5)	19.5 (9.5-29.5)	23.0 (17.5-33.5)	18.0 (12.0-33.0)

[§]p=0.027 among the three asthmatic groups;
[†]p=0.017 among the three asthmatic groups;
[‡]p<0.01 severe versus mild asthmatics.

Table II. Involvement of different alleles in predisposition to asthma

	Cytokine	*% Controls*	*% Asthmatics*	*OR*	*95%CI*	*p*
– 308A	**TNF-α**	8.1	27.7	4.36	2.35-8.08	<0.0001
– 238A	**TNF-α**	5.3	17.0	3.69	1.75-7.78	0.0002
– 33T	**IL-4**	10.1	13.9	1.45	0.69-3.02	0.324
– 590T	**IL-4**	7.3	20.7	3.33	1.66-6.67	0.0004
– 1098G	**IL-4**	12.1	8.5	0.68	0.29-1.61	0.376
– 1098T	**IL-4**	87.9	87.8	1.47	0.62-3.50	0.376
+ 1902A	**IL-4Rα**	79	92.7	3.36	1.39-8.15	0.005
– 889C	**IL-1α**	70.2	84	2.26	1.18-4.33	0.013
– 1082A	**IL-10**	61	58.5	0.91	0.55-1.51	0.71
– 819T	**IL-10**	32	17	0.44	0.23-0.83	0.0099
– 592A	**IL-10**	27	22	0.76	0.42-1.38	0.36

P values refer to chi-square test, unless otherwise specificied; [#] Fisher's Exact test; OR: odds ratios; 95% CI: 95% Confidence Interval.

Table III. Involvement of different genotypes in predisposition to asthma

	Cytokine	*% Controls*	*% Asthmatics*	*OR*	*95%CI*	*p*
– 308AA	**TNF-α**	3.3	8.9	2.94	0.78-11.40	0.139#
– 308GA	**TNF-α**	9.7	37.5	5.60	2.51-12.52	<0.0001
– 238AA	**TNF-α**	1.7	0	0	-	1.000#
– 238GA	**TNF-α**	7.3	33.9	6.56	2.73-15.75	<0.0001
– 33TT	**IL-4**	2.5	0	0	-	0.572#
– 33CT	**IL-4**	15.3	28.6	2.21	0.97-5.06	0.06
– 590TT	**IL-4**	2.4	4.3	1.83	0.30-11.34	0.613#
– 590TC	**IL-4**	9.6	32.6	4.52	1.92-10.84	0.0003
– 1098GG	**IL-4**	0	0	-	-	-
–1098GT	**IL-4**	24.2	17.1	0.65	0.26-1.60	0.343
– 1098TT	**IL-4**	75.8	82.9	1.55	0.62-3.86	0.343
+ 1902AA	**IL-4Rα**	64.5	87.8	3.96	1.45-10.82	0.005
+ 1902GA	**IL-4Rα**	29	9.8	0.30	0.10-0.88	0.020
– 889CC	**IL-1α**	53.2	78	3.12	1.38-7.09	0.005

– 889TC	**IL-1α**	33.8	12.3	0.32	0.12-0.83	0.015
– 1082AA	**IL-10**	31.5	19.5	0.53	0.22-1.25	0.14
– 1082GA	**IL-10**	58.8	78	2.48	1.09-5.65	0.027
– 819TT	**IL-10**	12	2.4	0.18	0.02-1.42	0.12#
– 819CT	**IL-10**	39.5	29.3	0.70	0.35-1.38	0.300
– 592AA	**IL-10**	9	7.3	0.81	0.21-3.06	1.00#
– 592CA	**IL-10**	36.3	29.3	0.77	0.39-1.55	0.466

P values refer to chi-square test, unless otherwise specificied; # Fisher's Exact test; OR: odds ratios; 95% CI: 95% Confidence Interval.

Table IV. Involvement of IL-10 haplotypes in predisposition to asthma

IL-10 Haplotype	*% Normal*	*% Asthmatics*	*OR*	*95%CI*	*p*
GCC	39.1	41.5	1.10	0.66-1.83	0.71
ACC	29.1	35.4	1.34	0.79-2.27	0.28
ATA	27	17	0.56	0.29-1.05	0.07
ACA	0	4.9	nc	nc	0.004#
ATC	4.8	1.2	0.24	0.03-1.90	0.20#

P values in the table refer to chi-square test, unless otherwise specificied; # Fisher's Exact test; OR: odds ratios; 95% CI: 95% Confidence Interval; nc: not calculable.