

Polymorphism of the eosinophil cationic protein-gene is related to the expression of allergic symptoms

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Summary

Background We have found a polymorphism in the ECP (eosinophil cationic protein)-gene at position 434 according to GenBank accession number NM 002935. This polymorphism would cause the change of the amino acid arginine (base at position 434 is G) at position 97 to threonine (base at position 434 is C).

Objective To investigate the prevalence of the ECP-polymorphism and to screen for disease associations.

Methods DNA of 209 medical students and 76 asthmatic subjects was analysed. The 434 genotype in the ECP-gene was detected by cleavage of the amplified DNA sequence with the restriction enzyme *Pst*I and analysis of the cleaved product by agarose gel electrophoresis.

Results The prevalences of the polymorphism in the student population were 53%, 39% and 8% for the 434GG, the 434GC and the 434CC genotype, respectively, with allele frequencies of 72% (434G) and 28% (434C). Subjects reporting allergy had a higher prevalence of the 434G allele than non-allergic subjects ($P=0.0056$). Of the students who were Phadiatop-positive and had allergic symptoms, 79% had the 434GG genotype, whereas the 434GC and 434CC genotypes were present in 82% of those who did not express allergic symptoms ($P<0.001$). Among the 76 patients with asthma, patients with allergic asthma had a significantly higher proportion of 434GG compared with patients with non-allergic asthma ($P=0.04$). None of the 18 subjects of the two groups with the 434CC genotype had allergy.

Conclusion The 434(G > C) polymorphism in the ECP-gene is related to the development of allergic symptoms, suggesting a central role for the ECP molecule in the process.

Keywords allergy, asthma, DNA, ECP, eosinophil, gene polymorphism

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Introduction

The eosinophil granulocyte is an inflammatory cell, which is supposed to be important in parasite defence and in wound healing [1]. The pathophysiological role of the eosinophil has mostly been related to its presence and activation in allergic disease such as asthma [2]. The activity of the eosinophils is probably to a large extent determined by their secretion of the basic proteins, ECP (eosinophil cationic protein), EPO (eosinophil peroxidase), EPX (eosinophil protein x) and MBP (major basic protein) [3]. The biological activities of these proteins are many but one activity seems to be of particular interest. This is the cytotoxic activity of the molecules and their capacity to kill almost any cell, mammalian or non-mammalian. In this regard the eosinophil cationic protein seems to be one of the most potent. High levels of ECP are measured in body fluids of allergic and other inflammatory diseases as a sign of the involvement of the eosinophil in the process and the involvement of ECP [4]. In a recent study we found that ECP purified from a

large group of blood donors showed a molecular heterogeneity, which was not entirely explained by differences in glycosylations of the molecule (Trulsson et al., data to be published). We also showed that only one of the three purified variants of ECP had any biological activity with respect to cytotoxicity.

In order to test the hypothesis that part of this molecular and functional heterogeneity actually might be a consequence of the presence of genetic variants of ECP we started a screening programme on the DNA sequence of the ECP-gene. This screening resulted in the identification of base polymorphisms in the ECP-gene at several locations. One of the polymorphisms (434(G > C), GenBank accession number AF441205) caused the change of the arginine at position 97 to the amino acid threonine (Byström et al., data to be published). During the completion of this study another group also found the polymorphism [5]. The aim of this study was to investigate the prevalence in the population of the gene polymorphism 434 (G > C). In the first preliminary results we found a significant relationship between the polymorphism and the occurrence of allergy based on questionnaires. Therefore, the second aim of the study was to test the hypothesis of such a relationship further in a prospective study on hospital-admitted and doctor's diagnosed allergic and non-allergic asthma.

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Subjects

Two hundred and twenty students were asked to participate in the study. The students were informed about the purpose of the DNA analysis of their blood. Ninety-five per cent of these students gave their written informed consent. The cross-sectional population therefore consisted of 209 medical students, 91 women and 118 men, from whom blood was taken routinely as part of their course in clinical chemistry. All students filled in a health declaration in which they indicated, among other things, whether they suffered from any chronic disease such as allergy. For 16 students no information about allergy was given. Based on the questionnaire from the remaining 193 students, 142 students (74%) indicated that they did not suffer from allergy, whereas 51 students (26%) indicated that they suffered from allergy. The atopic status, i.e. the presence in blood of IgE-antibodies to allergens, was investigated in 117 of the 209 students (56%) by means of UniCAP™ Phadiatop® for common inhalation allergens (Pharmacia Diagnostics AB, Uppsala, Sweden).

The hospitalized group of patients consisted of 97 patients with suspected asthma. Forty-nine of these patients had a final diagnosis of allergic asthma and 27 a final diagnosis of non-allergic asthma. Twenty-one patients did not fulfil the criteria of asthma according to the criteria of the American Thoracic Society [6]. The patients were informed by the doctor of the purpose of the study and blood for DNA analysis was drawn after oral consent. A patient was classified as having allergic asthma if he/she fulfilled the criteria of asthma given above in addition to having a positive skin prick test and/or a positive *in vitro* testing by means of the Pharmacia UniCAP™ system for 10 common allergens. One patient with clinical reaction to rabbit was classified as allergic asthma. This patient, however, was not tested by skin prick test or *in vitro* test to confirm this reactivity. The ethics committee at The Medical Faculty, Uppsala University, approved the study.

Methods

DNA preparation

For DNA preparations, 200 µL EDTA-blood was used, as has been described [7], with minor modifications. The blood was mixed with 500 µL 10 mM Tris-HCl, 0.1 mM EDTA (pH 8.0) and centrifuged for 10 s at 15 000 *g* and the supernatant was discarded. To lyse all red blood cells this procedure was repeated three additional times. The preparations were resuspended in 100 µL Proteinase K-buffer (50 mM KCl, 20 mM Tris-HCl (pH 8.3), 2.5 mM MgCl₂, 0.5% Tween 20, 100 µg/mL Proteinase (K)) and incubated at 56 °C for 2 h. Subsequently the samples were heated to 95 °C for 10 min to inactivate the proteases. DNA concentration and purity was measured at 260 and 280 nm in a spectrophotometer, SPECTRAMax™ 250 (Molecular Devices, Sunnyvale, CA, USA).

PCR

A total of 100 ng DNA was used in a 50-µL PCR reaction containing 1 U Taq DNA polymerase and buffer from Invitrogen (Groningen, the Netherlands), 1.5 mM MgCl₂, 0.2 mM dNTP, and 0.4 µM of each primer. The primer pair

used was the 5' primer, 5'-gtgtgtcataaccgagaccgtag-3' (bp 495–519) and the 3' primer, 5'-ggacagttgctgataccagagtag-3' (bp 1138–1114) amplifying a 644-bp fragment according to GenBank accession number X16545. The fragment contained the entire protein coding part of the ECP-gene. The PCR reactions were subjected to PCR in a thermo cycler (Idaho Technology Inc., Idaho Falls, ID, USA) with the following PCR profile; 30 cycles of 96 °C for 30 s, 51 °C for 30 s and 74 °C for 1 min. This profile was followed by extension for 5 min at 74 °C. Five microlitres of the PCR reactions were visualized on a 1% agarose gel.

Endonuclease restriction digestion

Seventeen microlitres of the PCR product were incubated with 10 U *Pst*I in an appropriate digestion buffer (Invitrogen). The samples were digested overnight and subsequently analysed on a 1.5% agarose gel containing ethidium bromide. The DNA of the subjects with the 434GG genotype of the ECP-gene was completely cleaved and gave rise to two distinct bands, whereas DNA of subjects with the 434CC genotype of the ECP-gene was left uncleaved, giving rise to only one band. In heterozygous subjects with the 434GC genotype *Pst*I cleavage of the ECP-gene gave rise to three bands.

Statistics

Fisher's exact test and chi-squared test were used to estimate significant differences between the proportions of the different ECP variants. Statistica for Windows vs. 5.5 (Statsoft, Tulsa, OK, USA) was used for all calculations.

Results

The prevalences of the 434(G > C) polymorphism among the medical students were 53% being homozygous for the 434GG genotype, 39% being heterozygous (434GC) and 8% being homozygous for the 434CC genotype.

Table 1(a) shows that among those students who on the questionnaire indicated that they were allergic the prevalence of the 434GG genotype of the ECP-gene is more common than among those who did not indicate allergy on the questionnaire ($P = 0.002$). The corresponding allele frequencies are shown in Table 1(b) and show a significant disproportion ($P = 0.0056$) in the occurrence of the two alleles among subjects reporting allergy and those who did not report allergy. None of the

Table 1. (a) The prevalence of ECP 434 genotype among 193 students with reported allergy or no allergy ($P = 0.002$, χ^2 -test). (b) The allele frequencies of 434G and 434C of the ECP-gene in students with reported allergy or no allergy ($P = 0.0056$, χ^2 -test)

	Allergy	No allergy
(a)		
434GG	33 (65%)	67 (47%)
434GC	18 (35%)	59 (42%)
434CC	0	16 (11%)
(b)		
Allele 434G	84 (82%)	193 (68%)
Allele 434C	18 (18%)	91 (32%)

Table 2. The prevalence of the ECP 434 genotype in relation to allergy among 46 Phadiatop-positive students ($P = 0.0001$, Fisher's exact test)

	Allergy	No allergy
434GG	23 (79%)	3 (18%)
434GC and 434CC	6 (21%)	14 (82%)

Table 3. (a) The prevalence of the ECP 434 genotype in 76 allergic or non-allergic asthmatic subjects ($P = 0.04$, χ^2 -test). (b) The allele frequencies of 434G and 434C of the ECP-gene in allergic or non-allergic asthmatic subjects ($P = 0.055$, χ^2 -test)

	Allergic asthma	Non-allergic asthma
(a)		
434GG	30 (61%)	11 (41%)
434GC	19 (39%)	14 (52%)
434CC	0	2 (7%)
(b)		
Allele 434G	79 (81%)	36 (67%)
Allele 434C	19 (19%)	18 (33%)

16 students who had the 434CC genotype indicated allergy on the questionnaire in spite of the fact that about 25% of all students indicated allergy ($P = 0.02$). There was no relationship between the ECP variants and the subject being atopic as measured by Phadiatop. Table 2 shows the distribution of the variants and allergy among the Phadiatop-positive students. The majority of those students who reported allergy had the genotype 434GG, whereas the majority of those students who reported no allergy were either heterozygous or homozygous for the 434CC genotype ($P = 0.0001$).

Among the asthmatic patients the prevalences of the ECP variants were similar to the student population, with 54% having 434GG, 43% being heterozygous and 3% having 434CC. However, allergic asthmatic subjects had higher prevalence of the 434GG and lower prevalence of the 434GC and 434CC genotypes than non-allergic asthmatics (Table 3a) ($P = 0.04$, χ^2 -test). The corresponding allele frequencies are shown in Table 3(b) and suggest a disproportion between allergic and non-allergic asthmatics ($P = 0.055$).

Discussion

We have shown that the ECP 434(G > C) polymorphism is common in the Swedish population. We have also shown that this polymorphism may be related to the development of allergic manifestations, as allergic subjects had a significantly higher prevalence of the homozygous 434G genotype of the ECP-gene and no subjects with reported allergic symptoms had the homozygous 434C genotype. These data indicate that the change in the amino acid 97 of the ECP molecule from an arginine to a threonine may be of major importance for the development of allergic symptoms and points to a central role of this molecule and the eosinophil in these processes. ECP is a molecule with many different biological activities, such as cytotoxicity, fibroblast activation, immunomodulation and mast cell activation [4]. Which of these functions are operative in the development

of allergic symptoms and affected by the amino acid change at position 97 is unknown, but is presently under investigation.

In the student group we related the genetic variants to self-reported symptoms of allergy and, although the students all had been studying medicine for 3 years, the perception of allergy varies between subjects. Therefore it was important to confirm the relationship to allergy in a group of patients with allergic disease in which experienced allergists had made an unbiased diagnosis. In this group of asthmatic subjects, the subjects with allergic asthma had a significantly higher prevalence of the 434GG genotype than the non-allergic asthmatics. Overall, however, the prevalence of asthma was not more common among those with the 434GG genotype, which indicates that this polymorphism is not predictive for the development of asthma, but for the development of allergy. This distinction is important and in keeping with the fact that eosinophils are predominantly found at increased numbers in the lungs of allergic asthmatics [2] and that elimination of eosinophils by antibodies to IL-5 [8] or by recombinant IL-12 [9] had little impact on the bronchial hyper-responsiveness.

The prevalence of the ECP variants was not related to the subject being atopic, but when the Phadiatop-positive students were studied separately the impact of the genetic variants for the development of allergic disease became even more striking. Thus, more than 80% of the students who were Phadiatop-positive, but had the 434GC or the 434CC genotype, did not report allergic manifestations. This contrasts with the fact that of those who reported allergic symptoms almost 80% had the homozygous 434G genotype. Converted into odds ratios the overall risk of developing allergy if one is Phadiatop-positive would be 117 (15–434) (95% CI), whereas this risk would increase to 240 (31–1900) if you have the 434GG genotype, but only 13.2 (1.46–121) if you have the 434GC genotype or 434CC genotype. Thus, being atopic and having the 434GG genotype, increases the risk of developing allergic manifestations by about 18 times. Indeed these calculations have to be interpreted cautiously given the low numbers on which these are based.

We conclude from this study that 434(G > C) polymorphism of the ECP-gene may be predictive of a person becoming allergic. This finding indicates a pivotal role of the ECP molecule for development of allergic symptoms.

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