

Association of Single Nucleotide Polymorphisms in the Eosinophil Peroxidase Gene with Allergic Rhinitis in the Czech Population

Barbara Hrdlickova · Lydie Izakovicova-Holla

Department of Pathological Physiology, Faculty of Medicine, Masaryk University, Brno, Czech Republic

Key Words

Allergic rhinitis · Eosinophil peroxidase gene · Single nucleotide polymorphisms

Abstract

Background: Eosinophil peroxidase (EPO) gene codes a cationic protein released from the specific granules of activated eosinophils. Eosinophil granulocytes play a central role in the protection of organisms against parasites. They are also regarded as key effector cells in allergic inflammation. We attempted to determine the polymorphisms in the EPO gene typical for the Czech population and to analyze their associations with allergic rhinitis and its intermediary phenotypes. **Methods:** We sequenced all 12 exons of the EPO gene, and selected variants were subsequently analyzed by polymerase chain reaction and restriction fragment length polymorphism methods in a case-control study comprising a total of 613 subjects (319 controls and 294 patients with rhinitis). **Results:** In total, 5 polymorphisms (–1710T/C and –1710T/CTCC, 2649T/C, 3097A/G and 3979A/G) were found in the EPO gene. Polymorphisms 2649T/C and 3097A/G were in complete linkage disequilibrium ($D' = 1$ for both groups), and both of them were in a strong disequilibrium with the 3979A/G variant ($D' = 0.801$ for controls, $D' = 0.848$ for rhinitis). Consequently, these 3 polymorphisms were studied in association with the allergic phenotype. In a single locus analysis, only 3979A/G single nucleotide polymorphism was

marginally significantly associated with rhinitis ($p = 0.030$, $p_{\text{corr}} > 0.05$). This polymorphism also showed a marginal association with total serum IgE levels ($\log_e$ IgE, mean $\pm$ SD: genotypes GG = 2.60 ± 1.20 ; GA = 2.47 ± 1.88 ; AA = 2.38 ± 1.49 ; $p < 0.05$). Furthermore, significant differences in haplotype frequencies between patients and healthy subjects were observed ($p < 0.05$). **Conclusions:** Our study supports the hypothesis that genetic variability in the EPO gene may contribute to the susceptibility to allergic rhinitis (or related phenotypes) in the Czech population.

Copyright © 2009 S. Karger AG, Basel

Introduction

Allergic rhinitis is an inflammatory disease of the nasal mucosa induced by an immunoglobulin E (IgE)-mediated reaction in allergen-sensitized subjects. It is a common disease worldwide affecting at least 10–40% of the population, and its prevalence is increasing [1]. Clinically, this disease manifests as nasal itching (pruritus), sneezing, rhinorrhea (runny nose) and congestion. It is classified as ‘intermittent’ or ‘persistent’, depending on the duration of the symptoms, and as ‘mild’ or ‘moderate/severe’ depending on the severity of symptoms and on the quality of life outcomes [2]. Rhinitis has a negative impact on quality of life and generates important direct as well as indirect economic costs [3]. There is clear evidence

Table 1. Clinical characteristics of the studied subjects

Characteristics	Controls (n = 319)	Rhinitis patients (n = 294)
Age, years	41.0 ± 14.8	34.0 ± 12.3*
Sex, males/females	169/150	184/140
Smoking (no/yes), %	69.8/30.2	84.7/15.3*
Serum log IgE	1.92 ± 1.74	2.57 ± 1.42*
Rhinitis only	0	40 (13.6)
Rhinitis and asthma	0	148 (50.3)
Rhinitis and dermatitis	0	9 (3.1)
Rhinitis, asthma and dermatitis	0	97 (33.0)
Rhinitis, intermittent	0	204 (69.4)
Rhinitis, persistent	0	90 (30.6)
FVC ^a , % predicted	ND	94.2 ± 12.6
FEV ₁ ^a , % predicted	ND	93.9 ± 14.3
FEV ₁ /FVC ^a , %	ND	86.1 ± 22.4

Data are expressed as means ± SD, unless stated otherwise. Figures in parentheses are percentages. ND = Not determined.

^a Spirometry was performed in rhinitics only.

* $p < 0.001$ (controls vs. rhinitics).

supporting the concept that allergic diseases including rhinitis are influenced by genetic predisposition and environmental exposure [4]. Several regions in the human genome probably involved in the development of allergic reaction [5–7] and polymorphisms of many candidate genes associated with clinical expression of these diseases have already been described [8–11]. However, the characterization of susceptibility markers in discriminating an ‘allergic individual’ from the general population has not yet been achieved, and the value of how this genetic insight leads to the recognition of specific subtypes of these disorders still needs to be confirmed.

Although primarily the immune response in allergic rhinitis is mediated by IgE-triggering degranulation of mast cells, the development of allergic inflammation involves many other cells, e.g. eosinophils and Th2 lymphocytes [12, 13]. The Th2 cells seem to be the initiators of the process, whereas the eosinophils and mast cells are the major effector cells. The activation of mast cells leads to the release of histamine and the other mediators responsible for an acute allergic reaction. The eosinophil activation induces an extracellular release of numerous granular proteins, such as eosinophil cationic protein, major basic protein and peroxidase [14, 15]. They play a role in the development of subacute and chronic symptoms of allergy and have been implicated

as final effector molecules in eosinophil-mediated tissue damage.

Eosinophil peroxidase (EPO) is a cationic heme protein composed of 2 subunits, heavy chain ($M_r = 50\text{--}57$ kDa) and light chain ($M_r = 10\text{--}15$ kDa) [16, 17]. It is a member of the haloperoxidase family that catalyzes the peroxidative oxidation of halides and pseudohalides. It means that in the presence of peroxide and bromide, it can catalyze the formation of hypobromous acid and the highly reactive singlet oxygen [18]. These agents may contribute to inflammatory tissue injury similarly as in bronchial asthma or rhinitis [19–22]. The gene for EPO is located on chromosome 17q23.1 and is composed of 12 exons. To date, the variability of this gene has been examined mainly in hereditary EPO deficiency [23–25] and only in 2 Japanese studies of cedar pollinosis [26, 27]. We attempted to determine the polymorphisms in the EPO gene typical for the Czech population and to analyze their associations with allergic rhinitis or its related phenotypes.

Methods

Subjects

This case-control study comprised 613 unrelated Czech subjects living in the South Moravia region. The demographic characteristics of rhinitis patients and healthy controls are shown in table 1. All subjects filled in a detailed questionnaire considering lifetime symptoms suggestive of asthma, rhinoconjunctivitis and atopic eczema/dermatitis syndrome extended with additional questions regarding symptoms and therapy as well as other diseases. A total of 294 patients with clinically manifested allergic rhinitis – only rhinitis ($n = 40$), rhinitis and asthma ($n = 148$), rhinitis and dermatitis ($n = 9$) and a combination of all 3 manifestations ($n = 97$) – were studied. Diagnosis of allergic rhinitis was based on the coordination between a typical history of allergic symptoms which include watery secretion from the nose, nasal obstruction, nasal mucosa itching and sneezing that are reversible spontaneously or after therapy (according to the criteria of the Allergic Rhinitis and Its Impact on Asthma Initiative) as well as on a diagnostic test for IgE-mediated allergy. Concomitant asthma diagnosis was based on the criteria of the Global Initiative for Asthma 2002 which include the following symptoms: nonproductive cough, wheezing, breathlessness, reversibility airway obstruction and bronchial hyperactivity. The diagnosis of atopic dermatitis was defined according to the diagnostic criteria first described by Hanifin and Rajka [28] in 1980.

Atopy was defined as the presence of a positive skin test reaction (≥ 3 mm greater than the reaction to saline) to 1 or more of the common allergens (house dust mite, common mixed grass and tree pollens, mixed molds, cat and dog dander, together with histamine and normal saline as a positive and a negative control, respectively) and/or raised specific serum IgE levels (>0.35 kU/l by the AlaSTAT test, DPC Biermann, Bad Nauheim, Germany,

Table 2. List of primer pairs used for PCR

EPO gene region	Primer	Annealing temperature, °C	Product size bp
1–3	5'-CTGGAGGAAGTGAGAGGTCGG-3' 5'-TCTCCTCCCAGCACCCCAA-3'	64.5	918
4–5	5'-TTGGGGTGCTGGGAGGAGA-3' 5'-TGCGTGACTCTGGGCTGTG-3'	60	539
4–5 RFLP	5'-GCCAGCTCAGGTCTGTGCA-3' 5'-GGCTGTGTTTTACTCCGTGCA-3'	58	475
6 (= 6 RFLP)	5'-GGAATGAGAGCAGGAGGTGGC-3' 5'-ACCTCTCAAACACACACGGCG-3'	65	565
7	5'-CTGGGTGAGTCAAGGAGGGC-3' 5'-CACATCCCACCATTGCTTCCC-3'	65	530
8–9	5'-GGAGGGTGCGATGAGGAG-3' 5'-CTGGGGAGATATTAGCAGTGGC-3'	63	1,043
10	5'-CCAATCTGTGCTGCTCACAT-3' 5'-CAAAGCTGGGAGAAGAGGTG-3'	55	238
11	5'-GGGTACTGTCTTACTTGTGTCT-3' 5'-CCTGGGTCAAGTGTCTCTCTCC-3'	64	757
12	5'-GTAATTCTCTCCCCAGCCTT-3' 5'-TAAGAGGACTCAGCCATCCA-3'	55	339

produced in response to 1 or more of the common allergens including *Dermatophagoides farinae*, grass pollens, animal danders and molds) and/or raised total serum IgE levels above normal values (>100 IU/ml in nonsmoking adults measured by the nephelometric test, Dade-Behring, Mannheim, Germany), as described previously [29].

The control group consisted of 319 healthy subjects who met the following criteria: (1) no prior or current physician diagnosis of allergic diseases (rhinitis and/or asthma and/or dermatitis), (2) no history of wheezing, shortness of breath and other symptoms of allergic diseases such as nasal and skin symptoms, (3) no use of antiallergic medications, and (4) absence of first-degree relatives with a history of allergic diseases.

The study was approved by the Committee for Ethics of the Faculty of Medicine, Masaryk University, and informed consent was obtained from all participants, in line with the Helsinki declaration before inclusion in the study.

DNA Isolation

Genomic DNA was isolated from peripheral blood by standard protocol (http://www.genome.ou.edu/protocol_book/protocol_partIII.html#III.H) and archived in the DNA bank at the Department of Pathological Physiology, Faculty of Medicine, Masaryk University, Brno, Czech Republic.

Polymerase Chain Reaction

In all reactions, polymerase chain reaction (PCR) was performed in 15- or 25- μ l volumes consisting of DNA 50 ng, dNTP

250 mM (Roche), MgCl₂ 25 mM (MBI Fermentas), 10 $\times$ buffer (BioTools), Taq polymerase 1 U/ μ l (BioTools) and 10 μ M of primers. The specific primers for exons 10 and 12 were designed with the program Primer 3 (http://www-genome.wi.mit.edu/cgi-bin/primer/primer3_www.cgi), and for the remaining exons as described previously [27] (table 2). The PCR products were resolved by electrophoresis on 2% agarose gel.

Heteroduplex Analysis

We analyzed PCR products of all 8 EPO regions (EPO1–3, EPO4–5, EPO6, EPO7, EPO8–9, EPO10, EPO11, EPO12) by heteroduplex analysis (double-strand conformation polymorphism analysis) using ethidium bromide for band visualization. Our set contained 60 samples (30 allergic patients and 30 healthy controls) for each region.

DNA Sequencing

We sequenced 3–5 samples from each EPO region according to the PCR-heteroduplex analysis results. In our laboratory, we used Sanger's DNA sequencing by Terminator Cycle Sequencing Kit + 33P Labeled (Amersham) and Thermo Sequenase Radiolabeled Terminator Cycle Sequencing Kit (USB Corporation) for regions EPO1–3, EPO4–5, EPO6, EPO7, EPO8–9, EPO10 and EPO11. EPO12 DNA sequencing was performed by genetic analyzer ABI PRISM 310 (Applied Biosystems) in the Core Laboratory, Department of Functional Genomics and Proteomics, Institute of Experimental Biology, Faculty of Science, Masaryk University, Brno, Czech Republic.

Table 3. List of fragment lengths for selected individual polymorphisms determined by PCR-RFLP

EPO region	Polymorphisms	Genotypes	Fragment lengths bp
EPO4–5	2649T/C	TT	272
		TC	272, 257, 15
		CC	257, 15
	3097A/G	AA	206
		AG	206, 189, 17
		GG	189, 17
EPO6	3979A/G	AA	556
		AG	556, 316, 240
		GG	316, 240

PCR-Restriction Fragment Length Polymorphism Analysis

PCR-restriction fragment length polymorphism (RFLP) methods were used to study 2 regions of the EPO gene: EPO4–5 [containing 2 polymorphisms in intron 3 (2649T/C) and intron 5 (3097A/G)] and EPO6 [1 polymorphism in exon 6 (3979A/G; Arg220Arg)].

EPO4–5 polymorphisms (2649T/C, 3097A/G) were determined by a newly developed PCR technique with mismatch primers (table 2). The PCR product (478 bp) was digested by 2 enzymes simultaneously: *Alw44I* (*ApaLI*) and *NcoI* (both BioLabs). *NcoI* digestion resulted in 2 fragments of 272 and 206 bp, and at the same time, *Alw44I* digested the DNA only in the presence of polymorphism; a 15-bp fragment was created with the 2649T/C polymorphism, and the presence of the 3097A/G polymorphism produced a 17-bp fragment (table 3).

The analysis of the 3979 A/G (Arg220Arg) polymorphism in exon 6 of the EPO gene was performed by PCR-RFLP with the same primers as in the previously described PCR (table 2). *ApaI* digestion in the presence of polymorphism (allele G) resulted in 316- and 240-bp fragments. There was only one 556-bp fragment in the presence of allele A (table 3).

The PCR-RFLP products were resolved by electrophoresis, for EPO4–5 on 4% Metaphore (Cambrex Bioscience) and for EPO6 on 2% agarose (Serva) gel. All analyses were performed without prior knowledge of the subject's case/control status.

Statistics

Comparisons of the allele frequencies of selected EPO single nucleotide polymorphisms (SNPs) between the rhinitis patients and controls were carried out using the χ^2 test. The same analysis was also used to test for deviation of the genotype distribution from Hardy-Weinberg equilibrium and for comparison of differences in genotypes and genotype combinations among groups. Where appropriate, Bonferroni correction was applied to adjust the α level according to the number of independent comparisons to an overall value of 0.05. Adjusted p values are denoted as p_{corr} ; $p_{\text{corr}} < 0.05$ was considered significant.

Pairwise linkage disequilibrium among 3 common polymorphisms was measured by the Lewontin standardized disequilibrium

coefficient $|D'|$ [30] in both groups separately. Haplotype frequencies were calculated from the observed genotype frequencies assuming that during zygote formation, haplotypes combine randomly according to their frequencies in the set (the assumption being analogous to Hardy-Weinberg equilibrium). This assumption was verified by a reversed calculation of the expected genotype frequencies from the calculated haplotype frequencies. Differences between the patient and control groups were tested by a simulation using the Monte Carlo method. In this test, 10,000 random simulations were performed to generate haplotype distribution tables, and subsequently, tested for the frequency equal to, or more extreme than, the observed data. Empirical probabilities were transformed and tested with the χ^2 test.

The Kruskal-Wallis ANOVA test was used for the evaluation of total IgE levels. Contingency table analysis, odds ratios (ORs), 95% confidence intervals (95% CIs) and significance values were estimated with the use of the program package Statistica version 6.0 (Statsoft Inc., Tulsa, Okla., USA).

Results

A total of 294 patients (184 men and 140 women, aged 34.0 ± 12.3 years) with clinically manifested allergic rhinitis and 319 control subjects (169 men and 150 women, aged 41.0 ± 14.8 years) were enrolled in this study (table 1). The mean age of control subjects was significantly higher than that of the patients ($p < 0.001$). The significantly higher mean age of the controls compared with patients ($p < 0.001$) did not distort our findings seriously, contrary to the opposite situation (i.e. younger controls) where the possibility of the disease development could not be ruled out.

Smoking was significantly less common in the rhinitis group ($p < 0.001$) in which less than 15.3% persons smoked in comparison with more than a 30% representation of smokers in controls. As expected, total IgE levels were higher in patients with allergic rhinitis ($\log \text{IgE} = 2.57 \pm 1.42$) than in healthy controls ($\log \text{IgE} = 1.92 \pm 1.74$; $p < 0.001$).

All individuals were genotyped for EPO 2649T/C, 3097A/G and 3979A/G polymorphisms. Only in 2 cases of the 2649T/C and 3097A/G polymorphisms it was not possible to determine the genotype. Polymorphisms 2649T/C and 3097A/G were in complete linkage disequilibrium ($D' = 1.000$ for both groups) and both of them were in a strong disequilibrium with the 3979A/G variant ($D' = 0.801$ for controls, $D' = 0.848$ for rhinitics).

The allele and genotype distributions of all 3 polymorphisms in both groups are shown in table 3. The genotype frequencies were in Hardy-Weinberg equilibrium ($p > 0.05$, data not shown) and they did not differ between pa-

Table 4. Genotype distribution and allele frequencies of the EPO gene polymorphisms in allergic rhinitis patients and controls

EPO SNPs		Controls		Allergic rhinitis		p value	OR		
2649T/C	3097A/G	n	%	n	%		dominant model		recessive model
TT	AA	150	47.3	116	39.5	0.139	1.00	1.00	
TC	AG	129	40.7	140	47.6		1.40 (1.00–1.97)		1.00
CC	GG	38	12.0	38	12.9		1.29 (0.78–2.16)	1.38 (1.00–1.90)	1.09 (0.67–1.76)
T	A	429	67.7	372	63.3	0.106			
C	G	205	32.3	216	36.7				
3979A/G									
AA		106	33.2	75	25.5	0.084	1.00	1.00	
AG		153	48.0	150	51.0		1.39 (0.96–2.01)		1.00
GG		60	18.8	69	23.5		1.63 (1.03–2.56)	1.45 (1.02–2.06)	1.32 (0.90–1.95)
A		365	57.2	300	51.0	0.030			
G		273	42.8	288	49.0				

Figures in parentheses are 95% CIs. p value indicates the statistical significance for comparison of genotype and allele frequencies (χ^2 test).

Table 5. EPO genotype combinations in both groups

Genotype combinations 2649T/C, 3097A/G, 3979A/G	Control	Allergic rhinitis	p value ¹	OR ¹
TT AA AA	93 (29.3)	67 (22.8)	0.019	0.61 [0.40–0.92]
TT AA AG	54 (17.0)	38 (12.9)	0.039	0.59 [0.36–0.98]
TT AA GG	3 (1.0)	11 (3.7)	0.077	3.08 [0.83–11.40]
TC AG AA	13 (4.1)	7 (2.4)	0.099	0.45 [0.17–1.18]
TC AG AG	90 (28.4)	107 (36.4)	Wild	1.00
TC AG GG	26 (8.2)	26 (8.8)	0.579	0.84 [0.46–1.55]
CC GG AA	0 (0)	1 (0.3)	n.a.	n.a.
CC GG AG	7 (2.2)	5 (1.7)	0.394	0.60 [0.18–1.96]
CC GG GG	31 (9.8)	32 (11.0)	0.626	0.87 [0.49–1.53]
p level	p = 0.05, $p_{\text{corr}} > 0.05$		$p_{\text{corr}} > 0.05$ in all cases	
OR (95% CI)	1.44 (1.03–2.03) ²			

Figures in parentheses are percentages; figures in brackets are 95% CIs. Comparison of differences in genotype combinations among groups (χ^2 test). n.a. = Not applicable (small numbers).

¹ A combined genotype with the highest frequency (TC AG AG – the most frequent in the whole population – assigned as a wild genotype) was compared with all other genotypes.

² OR was calculated for TC AG AG versus the other genotypes.

tients and controls. However, the allele frequencies of the 3979A/G polymorphism in allergic rhinitis patients were marginally significantly different (table 4) from those in the controls group ($p = 0.030$, $p_{\text{corr}} > 0.05$).

This polymorphism also showed a marginal association with total serum IgE levels adjusted for age and sex that was the highest in GG homozygotes ($\log_e \text{IgE} = 2.60$

± 1.20), intermediate in heterozygotes (2.47 ± 1.88) and the lowest in wild-type (AA) homozygotes (2.38 ± 1.49) of the 3979A/G polymorphism ($p = 0.05$, $p_{\text{corr}} > 0.05$). The 2649T/C and 3097A/G variants showed the same but nonsignificant trend; the highest level was seen in CC and GG homozygotes (2.57 ± 1.18), intermediate in heterozygotes (2.49 ± 1.84) and the lowest in wild-type TT

Table 6. Frequencies of the EPO haplotypes in both groups

Haplotypes			Healthy subjects	Rhinitis patients	p level	OR
2649T/C	3097A/G	3979A/G				
C	A	A	0.5365	0.4817	0.0571	0.80 (0.72–1.04)
T	G	A	0.0361	0.0285	NS	0.79 (0.42–1.49)
C	A	G	0.1402	0.1509	NS	1.09 (0.79–1.50)
T	G	G	0.2873	0.3389	0.0304	1.27 (1.00–1.62)

Figures in parentheses are 95% CIs. NS = Not significant. $p < 0.05$, significant difference between cases and healthy controls. Bold values indicate the most significant differences in haplotype frequencies.

and AA homozygotes (2.42 ± 1.45 ; $p > 0.05$). Allergic rhinitics did not show any relationships among EPO variants and a number of other quantitative traits investigated in our study, including selected specific IgE levels and skin prick test responses to aeroallergens (data not shown).

Furthermore, the relationship between rhinitis and interaction of investigated variants was studied. A marginally significant association of the composite genotype (formed by 3 studied polymorphisms of the EPO gene) with allergic rhinitis was found ($p = 0.05$, $p_{\text{corr}} > 0.05$); patients with the EPO 2649/T/C, 3097/A/G and 3979/A/G genotypes had an almost 1.5 times increased risk of allergic rhinitis as compared with subjects without this combination (OR = 1.44, 95% CI 1.03–2.03; table 5).

Finally, when we combined multiple SNP sites and analyzed haplotypes, we observed only 4 haplotypes because all 3 SNPs in the EPO gene are in linkage disequilibrium with each other to various degrees. The frequencies of the EPO haplotypes are summarized in table 6. There was a significant difference ($p < 0.05$) between cases and healthy controls in the frequency of haplotypes. The risk of having allergic rhinitis was noted to increase in subjects positive for TGG (OR = 1.27, 95% CI 1.00–1.62) and to decrease in children with CAA (OR = 0.80, 95% CI 0.72–1.04) haplotypes.

Discussion

Allergic rhinitis, like other atopic diseases, is the result of an allergen-triggered early and late or chronic response mediated by a series of inflammatory cells and mediators. The known biological activity and contribution of eosinophils to allergic inflammation supports an important role of cationic proteins released from activated eosino-

phils: eosinophil cationic protein, eosinophil protein X, major basic protein and EPO. EPO is one of the most abundant proteins in eosinophils, comprising approximately 25% of the total protein mass of specific granule protein [31]. Increased levels of EPO were found in the bronchoalveolar lavage fluid of asthmatics 24 h after allergen provocation, but not after saline challenge, whereas in healthy subjects, no EPO was detectable in the bronchoalveolar lavage fluid. Interestingly, serum levels of EPO, but not the number of peripheral eosinophils, increased significantly in asthmatics [32].

These immunological studies have been supported by genetic research according to which EPO polymorphisms might be involved in the regulation of allergic processes. EPO, which maps to human chromosome 17q23.1, was recently identified to be a cedar pollinosis susceptibility gene [26, 27]. Our study was designed to investigate the involvement of the EPO polymorphisms in the pathogenesis of allergic rhinitis in a Czech population. To our knowledge, this is the first study to examine this gene as a susceptibility locus for nasal allergy in Caucasians. We performed a sufficiently powered case-control association study consisting of a total of 613 subjects. We screened a total of 12 exons of the EPO gene. A further 3 selected (from 5 found) SNPs were analyzed in detail and evaluated whether the genetic variants (alleles, genotypes and haplotypes) were associated with allergic rhinitis and related traits.

When comparing 294 rhinitics with 319 controls, a coding 3979A/G SNP in exon 6 at the EPO locus was marginally significantly associated with rhinitis. This finding is consistent with the results of a previous study, which reported an association between the same polymorphism and cedar pollinosis in a Japanese population of 40 families including 60 affected [27]. One year earlier, the same group of authors in a case-control study of 351

subjects (145 patients with pollinosis and 206 healthy controls) had shown a significantly higher frequency of the 358Leu allele (Pro358Leu variant in exon 7) of the EPO gene in patients than in healthy subjects with an OR of 6.92 and a 95% CI of 2.14–22.4, respectively [26]. According to these authors, this variant, associated with substitution of amino acid proline for leucine, might be involved in the hyperactivity of peroxidase, resulting in allergy, or might be in linkage disequilibrium with a true functional variant. However, exon 7 of the EPO gene was nonpolymorphic in our subjects, which can be a result of the ethnic differences in the gene structure.

In addition to targeting the disease status, we observed weak correlations between the 3979A/G polymorphism and rhinitis-related traits, such as the total IgE level. Patients with a G allele (and GG genotype) of this variant had higher total IgE levels compared with subjects with an A allele. The same but nonsignificant trend was also evident for both intronic variants. In contrast, no SNP was associated with the clinical severity of rhinitis, sensitization to the selected aeroallergens or eosinophilia.

However, individual SNPs do not exist in isolation, but are genetically linked, forming distinct haplotypes. As haplotype analyses may be of higher informative value to draw associations between phenotypes and genetic variation compared with SNPs [33], we inferred haplotypes in EPO from genotype data and performed a haplotype-based association analysis with rhinitis – for the first time to our knowledge – which revealed significant differences in the haplotype distributions between the study groups.

There are a number of limitations to this study. The present study is limited by the fact that rhinitis is a heterogeneous group of different phenotypes. Importantly, this limitation could lead to a reduced ability to identify genetic risk factors but would not account for significant positive associations. Second, the case-control approach used is generally quite vulnerable to the population stratification, e.g. due to the different ethnic origin. However, the present sample is exclusively of Czech Caucasian origin, restricted to the limited geographical area populated by a quite homogeneous population. Moreover, a previous Japanese study that found a significant association of this EPO 3979A/G variant used a different approach – a family study, with similar results.

In summary, we conclude that the association between EPO polymorphisms and allergic rhinitis found by us supports previous findings of the role of EPO gene variants in cedar pollinosis [26, 27]. However, the functional relationship of ascertained associations still needs clarification. Thus, further genetic and functional studies are needed to elucidate the role of EPO polymorphisms in the molecular mechanisms underlying.

Acknowledgements

We thank Dr. H. Melicharova, J. Teturova, A. Pazdirkova, V. Izakovic, I. Nemcova, L. Siskova, O. Rybnicek, B. Novotna, D. Kindlova, D. Buckova and M. Schüller for their help with the recruitment of patients, and A. Stejskalova for the technical collaboration. The work was supported by grant 310/06/0827 of the Czech Science Foundation.

References

- Bachert C, van Cauwenberge P, Olbrecht J, van Schoor J: Prevalence, classification and perception of allergic and nonallergic rhinitis in Belgium. *Allergy* 2006;61:693–698.
- Bousquet J, van Cauwenberge P, Khaltaev N; Aria Workshop Group; World Health Organization: Allergic rhinitis and its impact on asthma. *J Allergy Clin Immunol* 2001; 108(suppl 5):S147–S334.
- Beasley R: Worldwide variation in prevalence of symptoms of asthma, allergic rhinoconjunctivitis, and atopic eczema: the International Study of Asthma and Allergies in Childhood/ISAAC/Steering Committee. *Lancet* 1998;351:1225–1232.
- Wang DY: Risk factors of allergic rhinitis: genetic or environmental? *Ther Clin Risk Manag* 2005;1:115–123.
- Malerba G, Pignatti PF: A review of asthma genetics: gene expression studies and recent candidates. *J Appl Genet* 2005;46:93–104.
- Brasch-Andersen C, Haagerup A, Børghlum AD, Vestbo J, Kruse TA: Highly significant linkage to chromosome 3q13.31 for rhinitis and related allergic diseases. *J Med Genet* 2006;43:e10.
- Haagerup A, Bjerke T, Schøitz PO, Binderup HG, Dahl R, Kruse TA: Allergic rhinitis – a total genome-scan for susceptibility genes suggests a locus on chromosome 4q24–q27. *Eur J Hum Genet* 2001;9:945–952.
- Halonen M, Stern DA: Predicting the course of asthma. *Allergy Asthma Proc* 2006;27: 328–331.
- Malerba G, Lindgren CM, Xumerle L, Kiviluoma P, Trabetti E, Laitinen T, Galavotti R, Pescollerung L, Boner AL, Kere J, Pignatti PF: Chromosome 7p linkage and GPR154 gene association in Italian families with allergic asthma. *Clin Exp Allergy* 2007;37:83–89.
- Kim KR, Cho SH, Choi SJ, Jeong JH, Lee SH, Park CW, Tae K: TAP1 and TAP2 gene polymorphisms in Korean patients with allergic rhinitis. *J Korean Med Sci* 2007;22:825–831.
- Garcia-Martin E, Garcia-Menaya J, Sanchez B, Martinez C, Rosendo R, Agundez JA: Polymorphisms of histamine-metabolizing enzymes and clinical manifestations of asthma and allergic rhinitis. *Clin Exp Allergy* 2007;37:1175–1182.

- 12 Holgate ST: Genetic and environmental interaction in allergy and asthma. *J Allergy Clin Immunol* 1999;104:1139–1146.
- 13 Busse WW, Rosenwasser LJ: Mechanisms of asthma. *J Allergy Clin Immunol* 2003;111(suppl 3):S799–S804.
- 14 Saeed W, Badar A, Hussain MM, Aslam M: Eosinophils and eosinophil products in asthma. *J Ayub Med Coll Abbottabad* 2002;14:49–55.
- 15 Venge P: Monitoring the allergic inflammation. *Allergy* 2004;59:26–32.
- 16 Sakamaki K, Tomonaga M, Tsukui K, Nagata S: Molecular cloning and characterization of a chromosomal gene for human eosinophil peroxidase. *J Biol Chem* 1989;264:16828–16836.
- 17 Ten RM, Pease LR, McKean DJ, Bell MP, Gleich GJ: Molecular cloning of the human eosinophil peroxidase. Evidence for the existence of a peroxidase multigene family. *J Exp Med* 1989;169:1757–1769.
- 18 Giembycz MA, Lindsay MA: Pharmacology of the eosinophil. *Pharmacol Rev* 1999;51:213–340.
- 19 Thomas EL, Bozeman PM, Jefferson MM, King CC: Oxidation of bromide by the human leukocyte enzymes myeloperoxidase and eosinophil peroxidase. Formation of bromamines. *J Biol Chem* 1995;270:2906–2913.
- 20 Wu W, Chen Y, d'Avignon A, Hazen SL: 3-Bromotyrosine and 3,5-dibromotyrosine are major products of protein oxidation by eosinophil peroxidase: potential markers for eosinophil-dependent tissue injury in vivo. *Biochemistry* 1999;38:3538–3548.
- 21 Wu W, Chen Y, Hazen SL: Eosinophil peroxidase nitrates protein tyrosyl residues. Implications for oxidative damage by nitrating intermediates in eosinophilic inflammatory disorders. *J Biol Chem* 1999;274:25933–25944.
- 22 Wu W, Samoszuk MK, Comhair SA, Thomassen MJ, Farver CF, Dweik RA, Kavuru MS, Erzurum SC, Hazen SL: Eosinophils generate brominating oxidants in allergen-induced asthma. *J Clin Invest* 2000;105:1455–1463.
- 23 Nakagawa T, Ikemoto T, Takeuchi T, Tanaka K, Tanigawa N, Yamamoto D, Shimizu A: Eosinophilic peroxidase deficiency: identification of a point mutation (D648N) and prediction of structural changes. *Hum Mutat* 2001;17:235–236.
- 24 Romano M, Dri P, Dadalt L, Patriarca P, Baralle FE: Biochemical and molecular characterization of hereditary myeloperoxidase deficiency. *Blood* 1997;90:4126–4134.
- 25 Romano M, Baralle FE, Patriarca P: Expression and characterization of recombinant human eosinophil peroxidase. Impact of the R286H substitution on the biosynthesis and activity of the enzyme. *Eur J Biochem* 2000;267:3704–3711.
- 26 Nakamura H, Miyagawa K, Ogino K, Endo T, Imai T, Ozasa K, Motohashi Y, Matsuzaki I, Sasahara S, Hatta K, Eboshida A: High contribution contrast between the genes of eosinophil peroxidase and IL-4 receptor α -chain in Japanese cedar pollinosis. *J Allergy Clin Immunol* 2003;112:1127–1131.
- 27 Nakamura H, Higashikawa F, Miyagawa K, Nobukuni Y, Endo T, Imai T, Ozasa K, Motohashi Y, Matsuzaki I, Sasahara S, Hatta K, Ogino K, Eboshida A: Association of single nucleotide polymorphisms in the eosinophil peroxidase gene with Japanese cedar pollinosis. *Int Arch Allergy Immunol* 2004;135:40–43.
- 28 Hanifin JM, Rajka G: Diagnostic features of atopic dermatitis. *Acta Derm Venereol Suppl (Stockh)* 1980;92:44–47.
- 29 Buckova D, Holla LI, Vasku A, Znojil V, Vacha J: Lack of association between atopic asthma and the tumor necrosis factor alpha-308 gene polymorphism in a Czech population. *J Invest Allergol Clin Immunol* 2002;12:192–197.
- 30 Lewontin RC: On measures of gametic disequilibrium. *Genetics* 1988;120:849–852.
- 31 Carlson MG, Peterson CG, Venge P: Human eosinophil peroxidase: purification and characterization. *J Immunol* 1985;134:1875–1879.
- 32 Erpenbeck VJ, Hohlfeld JM, Petschallies J, Eklund E, Paterson CG, Fabel H, Krug N: Local release of eosinophil peroxidase following segmental allergen provocation in asthma. *Clin Exp Allergy* 2003;33:331–336.
- 33 Stephens JC, Schneider JA, Tanguay DA, Choi J, Acharya T, Stanley SE, Jiang R, Messer CJ, Chew A, Han JH, Duan J, Carr JL, Lee MS, Koshy B, Kumar AM, Zhang G, Newell WR, Windemuth A, Xu C, Kalbfleisch TS, Shaner SL, Arnold K, Schulz V, Drysdale CM, Nandabalan K, Judson RS, Ruano G, Vovis GF: Haplotype variation and linkage disequilibrium in 313 human genes. *Science* 2001;293:489–493.