

The interaction of the polymorphisms in transporter of antigen peptides (TAP) and lymphotoxin α (LT- α) genes and atopic diseases in the Czech population

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Summary

Background Transporter antigen peptide gene (TAP) products are involved in antigen processing. These genes, inducible by interferon γ , as well as lymphotoxin α (LT- α), are located in the HLA region. Their involvement in immune response regulation makes them candidate atopy susceptibility genes.

Objective This study investigates a possible association between previously identified polymorphisms within the TAP-1 and LT- α genes and clinically manifested atopic diseases in the Czech population.

Methods Caucasian subjects of Czech nationality ($n = 427$) were included in our study. We examined 184 healthy controls and 243 patients with histories of atopic asthma, allergic rhinitis and atopic dermatitis or their combinations. We used the amplification refractory mutation system polymerase chain reaction to determine TAP-1 gene polymorphisms. LT- α genotypes were determined by PCR and restriction analysis by NcoI.

Results No significant differences were found in allele or genotype frequencies of the LT- α gene, as well as in polymorphisms for Val $\rightarrow$ Ile at codon 333 and Gly $\rightarrow$ Asp at codon 637 in the TAP-1 gene between controls and patients. However, analysis of the concurrence of the double genotypes of the TAP-1 polymorphism at codon 333 and the LT- α genes showed differences between controls and atopic patients ($P < 0.02$).

Conclusion Several reports have indicated that different HLA products and genes may be risk factors for or protective factors against the development of atopy. We report no association between polymorphisms in the LT- α and TAP-1 genes alone and atopic diseases in the central Europe Caucasian (Czech) population, but there was an interesting interaction between the TAP333 and LT- α polymorphisms.

Keywords: TAP, LT- α , asthma, atopy, polymorphism, gene

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Introduction

Atopy is a heterogeneous state, determined by genetic and environmental interaction, which is characterized by the predisposition to respond to environmental allergens with hyperproduction of the immunoglobulin E (IgE) antibodies [1].

Atopic diseases are characterized by immunoregulatory abnormalities, including increased IgE production and decreased CD8⁺ suppressor/cytotoxic T lymphocytes, features consistent with a shift to a Th2-dominant cytokine profile [2]. Cytotoxic T lymphocytes recognize peptides presented by major histocompatibility complex (MHC) class I molecules [3,4]. Translocation of the resulting peptides across the membrane of the endoplasmatic reticulum (ER), where they bind to class I molecules, is

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mediated by the MHC-encoded transporters associated with antigen presentation (TAP) [5].

A more recent study [6] has shown that specialized antigen-presenting cells (macrophages, dendritic cells) are able to feed exogenous proteins into the proteolytic machinery in the cytosol [7]. Exogenous antigens can also be presented by HLA class I molecules, and recognized by CD8⁺ T lymphocytes. Thus TAP gene products can be important for antigen presentation, and because these genes, inducible by interferon γ , are located in the HLA II region, they make candidate atopy susceptibility genes [8].

Atopy refers to the predisposition to develop a group of inflammatory diseases. Tumor necrosis factors (TNFs) are potent proinflammatory cytokines involved in asthma and allergy [9]. The lymphotoxin α (LT- α or tumour necrosis factor beta (TNF- β)) gene is closely linked to the tumour necrosis factor alpha (TNF- α or cachectin) gene between the HLA-B and DR genes on chromosome 6p.2 [10]. Analysis of allelic associations between the TNF- α /LT- α and other polymorphic MHC loci uncovered several strong linkage disequilibria, but the associations between the particular TNF alleles were not significantly higher than those between the TNFs and other MHC loci [11].

Increased TNF- α levels have been detected in both sputa and bronchoalveolar lavage fluid from asthmatic patients [12] and they show constitutional variation in their secretion levels, which are linked to molecular polymorphisms located within and around the TNF loci (a biallelic polymorphism in the promoter region of the TNF- α , and a polymorphism within the first intron of the LT- α gene) [13]. In contrast to TNF- α , which is expressed mainly by macrophages, LT- α is expressed and released by lymphocytes [14]. Worm et al. [15] showed that LT- α plays a potentially significant role during B cell proliferation and IgE synthesis. Besides the role of TNFs in inflammation, some studies have shown association of LT- α with atopy [16] or with bronchial hyper-reactivity, which is one of the pathophysiological characteristics of bronchial asthma [17].

In this study we investigated a possible association of two genes (namely molecular variants Ile 333 Val and Asp 637 Gly of the TAP-1 gene and the bi-allelic NcoI RFLP in the first intron of the LT- α gene), previously recognized as candidate genes for atopy including asthma bronchiale [9,18,19], with atopic diseases in the Czech population (homogenous central European Caucasians). Therefore, the aim of our study was to determine if the reported associations with atopic diseases in other populations also occur in Czech atopic patients.

Materials and methods

Caucasian subjects of Czech nationality ($n = 427$) were

included in this case-control study. Full details of this study have been published elsewhere [20]. Probands were selected using a detailed questionnaire modified from the American Thoracic Society respiratory questionnaire [21] regarding lifetime symptoms suggestive of asthma, rhinitis and dermatitis with additional questions on symptoms and therapy as well as on other diseases. The questionnaires were filled in by physicians regarding the patients' anamneses and data available in the documentation. Phenotype status was assigned without previous knowledge of genotypes by two investigators independently.

Unrelated, ethnically matched, controls were Czech Caucasians. This group included 186 subjects (101 men and 85 women), aged 37.2 ± 15.4 years (mean $\pm$ SD) without symptoms of any atopic, lung and skin diseases. They had no familial history of asthma/atopy. They were recruited from general practitioners' surgeries.

We studied 244 patients (126 men and 118 women), aged 30.7 ± 17.0 years (mean $\pm$ SD) with clinical histories of atopic asthma, allergic rhinitis and atopic dermatitis or their combinations. All subjects had clinical features of atopic diseases and they had received the appropriate treatment of care for the diseases. From 244 patients, 28 individuals had atopic asthma, 60 had allergic rhinitis, eight had atopic dermatitis, 75 had both asthma and rhinitis, seven had asthma and dermatitis, nine had rhinitis and dermatitis and 57 had all three diseases. The patients were recruited from the Department of Clinical Immunology and Allergology, St Anne's Teaching Hospital, Brno, and from allergologists' surgeries. All specialists used the same diagnostic criteria.

The patients were interviewed to obtain information on upper and lower airways symptoms, description of asthma attacks and/or symptoms of skin damage. They were also asked about seasonal variations, the frequency of the symptoms and their exposure to precipitants, such as dust, exercise, cold air and infections. Detailed data on their smoking habits and the home and occupational environments (passive smoking, indoor allergens, irritant exposure and occupational exposures) were collected.

Phenotype analysis

The diagnosis of atopy was based on several criteria, including clinical signs of atopic disease and the presence of one or more of the three following criteria:

- Positive skin prick tests (greater than or equal to 3 mm above negative control) to one or more common aeroallergens. These were house dust mites (*Dermatophagoides pteronyssinus*, *Dermatophagoides farinum*), common grass and tree pollen mixtures (see Appendix 1), animal fur extracts (cat and dog dandruff), and common moulds (*Alternaria*, *Cladosporium* and *Aspergillus*). Histamine was

used as a positive control and physiological solution as a negative control. We used standardized test procedures and a set of commercially available allergens routinely used in practice in our country (Sevapharm, Prague, Czech Republic).

- Raised specific serum IgE levels (> 0.35 kU/L, by Ala-stat, DPC-Biermann, Bad-Nauhiem, Germany) produced in response to one or more of the following common antigens: *D. pteronyssinus*, mixed epithelia from cats, dogs, horses and cows, mixed pollens and mixed moulds.

- Raised total serum IgE levels above normal values (greater than 150 IU/mL in non-smoking adults, by a nephelometric test (Boehringer, Mannheim, Germany) as described previously [20]. The total serum IgE levels in our atopic patients were 306 ± 349 IU/mL (mean $\pm$ SD).

Atopic asthma was defined according to the American Thoracic Society criteria [20]. The diagnosis of asthma was based on the presence of one or more asthmatic symptoms such as recurrent cough, wheezing, dyspnoea and airway reversibility and/or bronchial hyper-responsiveness. All patients were in a stable phase of the disease at the moment of medical examination. At presentation the total number of asthmatic patients were distributed relatively evenly between two severity categories, with 55% being in the mild group and 45% being in the moderate group. The treatment resulted in normal values of the pulmonary function test.

Atopic dermatitis was defined according to the major and minor diagnostic criteria initially proposed by Hanifin and Rajka in 1980 (for diagnosis, three or more major criteria and three or more minor criteria must be present) [22].

Subjects with allergic rhinitis had atopy and the symptoms of either hay fever or a whole year's rhinitis, as confirmed by a specialist.

All the subjects gave written informed consent to participation in the study. The study was approved by the Committee for the Ethics of Medical Experiment on Human Subjects, Medical Faculty, Masaryk University, Brno.

Genotype identification

The genomic DNA was isolated from peripheral blood leucocytes by a standard method using proteinase K digestion of cells according to Sambrook *et al.* [23].

Detection of the bi-allelic *NcoI* polymorphism in intron 1 of the LT- α (TNF- β) gene

The bi-allelic *NcoI* polymorphism in intron 1 of the LT- α gene was examined by a polymerase chain reaction by the method of Stüber *et al.* [24]. PCR products were digested with *NcoI* at 37 °C for 3 h. The digested fragments were separated by electrophoresis in 2% agarose gel and

visualized with ethidium bromide. The homozygous B₂ allele of the LT- α gene appears as a non-digested single 782 bp band and the homozygous B₁ allele as being digested into 586 bp and 196 bp. Heterozygotes had all three bands: 782, 586 and 196 bp.

Detection of the TAP-1 polymorphism by the amplification refractory mutation system polymerase chain reaction (ARMS PCR)

Polymorphism within the TAP-1 gene has been characterized by Collonna *et al.* [25], who identified two polymorphic residues: Val $\rightarrow$ Ile at position 333 and Gly $\rightarrow$ Asp at position 637. In our study we used the ARMS PCR technique [26] to determine the TAP-1 polymorphism in Caucasians of Czech nationality. In our method, a product amplified by two-allele-non-specific flanking oligonucleotides is used as an internal control. To analyse the single polymorphic site of the TAP-1 gene, four oligonucleotides were included in a single reaction mix. PCR products were separated on 2% agarose gel and visualized with ethidium bromide.

TAP-1 at position 333 – product size: Control: 533 bp; Ile-333: 241 bp, Val-333: 352 bp.

TAP-1 at position 637 – product size: Control: 429 bp; Asp-637: 307 bp, Gly-637: 180 bp.

Four possible common alleles of the TAP-1 were obtained:

- TAP-1-A (Ile at position 333 (b) and Asp at position 637 (c)).
- TAP-1-B (Val at position 333 (a) and Gly at position 637 (d)).
- TAP-1-C (Val at position 333 (a) and Asp at position 637 (c)).
- TAP-1-D (Ile at position 333 (b) and Gly at position 637 (d)).

Statistical analysis

Allelic frequencies were calculated from the observed numbers of genotypes. The significance of differences in allelic frequencies between each of the two groups was determined by Fisher's exact test. Except for allelic frequencies, the χ^2 test was used for all the other statistical analyses. Contingency table analysis, odds ratio (OR), 95% confidence intervals and significance values were estimated with the use of the program package Statistica v 3.0 (Statsoft Inc., Tulsa, OK, USA).

Corrections for multiple comparisons were performed and only values of $P < 0.05$ were considered to be significant.

Table 1. Selected characteristics of normal and atopic subjects

Parameter analysed	Men		Women	
	Control	Atopic	Control	Atopic
Number of subjects	101 (54.3%)	126 (51.6%)	85 (45.7%)	118 (48.4%)
Average years ($\pm$ SD)	35.1 ($\pm$ 14.5)	27.0 ($\pm$ 16.2)	39.7 ($\pm$ 16.1)	34.6 ($\pm$ 17.1)
Positive smoking status	37 (37.8%)	14 (11.7%)	20 (24.5%)	14 (12.4%)

Results

The genotype distributions for all three polymorphisms were within Hardy–Weinberg equilibrium both in the atopic and control groups. Concurrence of the double genotypes of the LT- α a TAP 333 genes showed a marginal Hardy–Weinberg disequilibrium. The demographic data (age, sex and smoking status of the two groups) are shown in Table 1. The average age was slightly higher in the control group (both for men and women). Among smokers there was a higher proportion of healthy males (37.8%) than healthy females (24.5%). In the group of atopic patients we found lower proportions of smoking subjects (11.7% and 12.4%, respectively).

No significant differences in the allelic frequencies of the NcoI polymorphism of the LT- α gene were found in the patients with atopic diseases compared with the healthy controls. Allelic frequencies for Val→Ile at codon 333 and Gly→Asp at codon 637 in the TAP-1 gene were also similar in both the atopic patients and the controls (Table 2). The differences in allelic or genotype frequencies of these genes between the single clinical manifestations of atopic diseases (or between the two severity categories of asthmatic patients) were not significant (data not shown).

Analysis of the concurrence of the double genotypes of the LT- α and TAP 333 genes showed a marginally significant difference between the atopic patients and the control subjects ($P < 0.02$, $P_{\text{corr}} < 0.05$, Table 3, odds

ratio (OR) 2.70; confidence interval (CI) 1.15–6.34). No significant difference was found when comparing the concurrence of the double genotypes of the LT- α and TAP 637 genes ($P =$ not significant, data not shown).

Only the linkage disequilibrium in the TAP-1 gene between polymorphisms in codon 333 and 637 was found significant ($P < 0.01$, data not shown). Linkage disequilibrium between polymorphisms in the TAP-1 gene and the NcoI polymorphism in the LT- α gene were not found.

Discussion

Although recent research has helped in gaining new insights into the pathophysiology of asthma, the aetiology of this complex disease still remains obscure. Several studies have indicated that different HLA genes may be risk factors for or protective factors against the development of atopy [27], but the results are controversial.

The polymorphisms of the INF- γ -induced TAP genes, which are localized in the same region as genes for HLA class II antigens, could be related to the regulation of the immune response in atopic patients, especially at the level of antigen presentation of HLA class I. Ismail *et al.* [8] studied an association of the TAP gene polymorphisms with a predisposition to atopy in the Tunisian population. Allelic frequencies presented in this study were different in

Table 2. Allele frequencies of the LT- α and TAP-1 polymorphisms in atopic patients and controls

Polymorphic site	Variant	Allele frequency		<i>P</i>
		Atopic patients	Controls	
LT α NcoI	B ₂ →B ₁	0.707/0.293	0.734/0.266	> 0.05
TAP1333	a→b	0.153/0.847	0.183/0.817	> 0.05
TAP1637	c→d	0.869/0.131	0.852/0.148	> 0.05

Table 3. Concurrence of the double genotype (LT- α a TAP 333 polymorphisms) in percentage

LT- α TAP1333	B ₂ B ₂			B ₁ B ₂			B ₁ B ₁		
	aa	ab	bb	aa	ab	bb	aa	ab	bb
Healthy controls	2.8	19.7	34.5	0.7	4.9	27.5	0	4.9	4.9
Atopic patients	0.8	11.9	34.3	1.7	12.3	32.2	0	1.3	5.5

* $P < 0.02$ ($P_{\text{corr}} < 0.05$). The occurrence of double genotypes (B2B2aa through B1B1bb) were compared for cases and controls by means of χ^2 test. The P -value was corrected by Holm's method (P_{corr}).

comparison to those found in the Northern European Caucasian population [26]. The allelic frequencies in our study are in agreement with those found in this European population. In contrast to the results obtained by Ismail, we did not reveal a decline of a common allelic combination 'A' (bc) and a rise in the frequency of rare allele 'D' (bd) in an atopic population. The differences between the Tunisians and the Czech population may be related to differences in ethnic origin (European Caucasians vs. non-European subjects) and/or exposition to different environmental conditions including climatic ones (an industrial region in the temperate zone vs. an agricultural region in the subtropical zone).

The large number of previous association studies examining the LT- α polymorphism in these diseases reflect an interest in the role of the TNFs genes (TNF- α and/or LT- α) in atopic diseases. Some genetic studies have revealed an association of the B2 allele (NcoI polymorphism of the first intron of LT- α) with bronchial asthma in spite of the absence of atopy [9,19,28]. Although the LT- α polymorphism has been correlated in other populations, this is the first time that such an association was studied in a Czech group. The results from the analysis in this study do not suggest a major role for the LT- α polymorphism alone in atopic diseases in the Czech Caucasian population. This concurs with Castro's findings [29] in Spanish atopics but differs from other populations that showed an increased risk for atopic diseases.

There is an interesting (but only marginally significant, after correction for multiple comparisons) concurrence of the TAP-1 polymorphisms in codon 333 with the NcoI polymorphism in first intron of the LT- α (Table 3), because the difference between the cases and the controls was caused by the excess of double heterozygotes in the atopics in comparison with the controls. This phenomenon resembles an inversion of the 'effect of heterosis'; it may be due to the disproportion of our genetic background and the altered conditions of the environment. Moreover, in the case of atopic diseases as well as other common diseases, such effects can be expected as a result of the genetic and environmental imbalance in an important part of the population.

Since the HLA loci were not tested in our study, a possibility cannot be excluded that the observed effect (concurrence of the TAP-1 polymorphisms in codon 333 with the NcoI polymorphism in the first intron of the LT- α) is not caused by 'gene interaction' but could be haplotype-related. We have proved a difference in occurrence between patients and controls in the double heterozygotes only without this being expressed in any of the homozygotes (Table 3). Since the polymorphisms on the two genes behave as being capable of forming statistically random combinations (the χ^2 test did not prove a

non-random distribution), an association with the haplotype effect of these two genes does not seem to be found. It is well known, however, that there exist strong linkage disequilibria between the genes for TNF- α /LT- α and other polymorphic loci of the HLA of all three classes [11,30,31], and hence, a possibility cannot be excluded that these genes are actually linked with some other genes of the HLA complex acting, considering asthma, just as their markers.

The discrepancy between the risk alleles of the above-mentioned genes, comparing other studies [8,9,19] and the present work, is intriguing and may be attributable to HLA effects on antigen recognition in different environments as well as being population dependent. Our results suggest that 'genetic interaction' of the investigated genes may be important in the pathogenesis of atopic diseases. Replication of our findings among other populations will be of use in determining whether the relationships between these risk factors and the development of atopic diseases can be generalized.

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Appendix 1

The grass and tree pollen mixtures were used as follows:

- Pollinare mixtum: *Agrostis alba*, *Alopecurus pratensis*, *Anthoxanthum odoratum*, *Apera spica-venti*, *Arrhenatherum elatius*, *Avenastrum* sp., *Dactylis glomerata*, *Festuca rubra*, *Holcus lanatus*, *Lolium* sp., *Phleum pratense*, *Poa pratensis*, *Secale cereale*, *Trisetum flavescens*, *Plantago lanceolata*.
- Mixture of grass I: *Arrhenatherum elatius*, *Dactylis glomerata*, *Festuca rubra*, *Lolium* sp., *Phleum pratense*, *Secale cereale*.
- Mixture of grass II: *Argopyrum repens*, *Bromus erectus*, *Calamagrostis epigeios*, *Deschampsia flexuosa*.
- Early spring mixture: *Alnus glutinosa*, *Betula pendula*, *Carpinus betulus*, *Corylus avellana*, *Fraxinus excelsior*, *Salix caprea*.
- Late spring mixture: *Taraxacum officinale*, *Sambucus nigra*, *Urtica dioica*.
- Autumn mixture of pollen: *Artemisia absinthium*, *A. vulgaris*, *Solidago canadensis*.
- Artemissia mixtum: *Artemissia absynth*, *A. vulgaris*.