The role of genetic variations and gene-environment interactions in childhood asthma

Ph.D. theses

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Introduction

Asthma is a multifactorial disease influenced by complex interactions between multiple genetic and environmental factors. Linkage and association studies have already revealed several asthma genes and loci, however, at some of these regions, it is still not clear which gene or genetic variant is responsible for the development of asthma and its endophenotypes.

Many lines of evidence suggest that one of the most important causative factors of the critical increase in asthma incidence and prevalence rates is the raised exposure to environmental pollutants and airborne allergens. Traffic-related air pollutants such as nitrogen dioxide, particulate matter and ozone have been shown to increase the risk of asthma as well as exacerbate symptoms in patients with already existing airway disease, leading to the production of free radicals and enhancing the inflammatory response. Thus, genes involved in the oxidative stress response are candidates for susceptibility to the respiratory effects of air-pollutants. The oxidative stress response is centrally mediated by the redox-sensitive transcription factor, NFE2L2 (nuclear factor-erythroid-derived 2-like 2) and its negative regulator protein KEAP1 (Kelch-like ECH-associated protein 1). Low levels of oxidative stress following exposure to a pollution episode leads to the liberation of NFE2L2 from its repressor KEAP1 activating more than 200 genes which encode many antioxidant or phase II detoxifying enzymes and related stress-response proteins. The protective role of NFE2L2 is widely evident not only in oxidative processes but in inflammatory disorders also. In an ovalbumin-challenged asthmatic mouse model, Nfe2l2-deficiency resulted in increased airway inflammation, airway hyperreactivity and an elevated level of Th2 cytokines indicating the critical role of Nfe2l2 in asthma pathogenesis.

The observed increase in the prevalence rates of allergic diseases may also be caused by the decreased numbers and frequencies of early childhood infections and also by lower exposure rates of different microbial products. At the same time, airway infections may play an important role in the asthma pathomechanism and the development of asthma exacerbations; however the interaction between the infections and the disease is modified by several factors. One of these factors are the genetic variants of molecules implicated in the asthma pathomechanism, which can be protective or predisposing according to the studied population or the duration/extent of microbial exposure.

Different environmental triggers in the presence of susceptible genetic background induce several, partly overlapping mechanisms, which finally lead to the formation of airway inflammation. Apoptotic processes have been widely recognized as key factors in the asthmatic inflammation. Studies showed that after rhinovirus infections, the epithelial cells of the asthmatic lungs were unable to enter into apoptosis with the consequence that the replicating virus caused cytopathic cell death with extensive virus shedding. Recent studies suggest that accumulation of eosinophils in bronchial tissue is related to the dysregulation of apoptosis and eosinophil clearance. Furthermore, it has also been shown that reduced eosinophil apoptosis correlates with asthma severity. BIRC5 (also known as survivin) is an important member of the inhibitor of apoptosis protein family. Its impact in blocking apoptosis has already been widely demonstrated in the literature. Studies investigating the molecular basis of the anti-apoptotic function of BIRC5 have shown that it not only inhibits the caspase-dependent and independent apoptotic pathways, but also accelerates cell proliferation. Under normal conditions BIRC5 is highly expressed in fetal tissues but is barely detectable in the majority of terminally differentiated adult tissues. Besides its well-known role in the development and progression of common cancers, recent studies have been suggesting that survivin may also be implicated in inflammatory processes and in the pathogenesis of asthma. In our previously completed whole genome gene-expression microarray analysis of OVA-induced mouse model of asthma we found that Birc5 mRNA was expressed at elevated level after allergen exposure. Our observation was confirmed by other groups, and additionally, they found that increased Birc5 mRNA and protein levels in eosinophils recovered by bronchoalveolar lavage were in strong correlation with elevated eosinophil counts. On this basis we supposed that BIRC5 played an important role in the pathogenesis of asthma.

Aims

I had the following aims during my work:

1. **To study the interactive effects of genetic and environmental factors on asthma development**
   - To analyze if common polymorphisms in the genes of *CCR5* and *RANTES* modify the susceptibility to *Mycoplasma pneumoniae* infection or the asthma risk, or both.
   - To discover the interactive effects of genetic variations in the master regulator elements of oxidative stress response, *NFE2L2* and *KEAP1*, and concentrations of NO_2 on asthma risk.

2. **To carry out a partial genome-screening on the 11q13 and 14q22 asthma susceptibility regions**
   - Because this method results in multiple data, our goal is to evaluate our database not only by frequentist but bayesian statistical methods also, which has the potential to detect interactions of studied genetic variants impacting the phenotype.
   - To compare the gene expression levels of genes found to be relevant in asthma in the SNP analysis in sputum samples of healthy and asthma populations.

3. **To study the association of antiapopototic *BIRC5* gene and asthma**
   - To explore whether BIRC5 expression is detectable in human sputum samples and to compare its level between healthy controls and asthma patients.
   - To analyze the potential relationship between genetic variations in the regulatory regions of *BIRC5* gene and asthma pathomechanism.

Methods

Subjects

Cases participated in association analyses were recruited from the Budai Children's Hospital and from the Heim Pal Hospital, Budapest. All the asthmatic children had specialist physician-diagnosed asthma. The control children were randomly selected from outpatients from the Orthopaedic Department in the Budai Children's Hospital, or from the Urological Department of Heim Pal Hospital, Budapest.
The adult controls were healthy blood donors and recruited by the Hungarian National Blood Transfusion Service, Budapest. In case of patients participated in induced sputum analyses, asthma was diagnosed by respiratory medicine specialist according to the recent Global Initiative for Asthma guidelines. According to their lung function data, they were divided into mild and moderate to severe groups. Ten patients regularly used inhaled corticosteroid (ICS). Asthma control was evaluated by the Hungarian translation of the Asthma Control Test™. Healthy controls were recruited from the staff and students of the participating Hungarian Universities. They had normal lung function and had no history of respiratory diseases.

DNA isolation, genotyping methods

Genomic DNA was isolated from whole blood samples using iPrep PureLink gDNA Blood Kit and Qiagen DNA Blood Mini kit. *RANTES* -403 G/A was genotyped by PCR-RFLP method. Genotyping of *CCR5* was carried out by using DNA amplification with PCR using the CCR5-specific primer pair that flanks the 32-bp deletion and is separated in 2% agarose gel. On the 11q12.2-q13.1 és 14q22.1-q22.3 chromosomal regions 144 SNPs out of 145 were genotyped using 48- and 12plex genotyping assays on GenomeLab SNPstream genotyping platform (Beckman Coulter). In addition, SNP rs545659 was genotyped using TaqMan SNP Genotyping Assay (Applied Biosystems) on an Applied Biosystems 7900 Real Time PCR System as per instructions of the manufacturer. The SNPs in the regulatory regions of *BIRC5*, *NFE2L2* és *KEAP1* genes were genotyped using the Sequenom iPLEX Gold MassARRAY technology at the McGill University and Génome Québec Innovation Centre, Montréal, Canada.

Detection of laboratory values

MP-specific antibodies were determined from serum by using SeroMP-IgA and IgG EIA kits. Total serum IgE levels and specific IgE levels to more than 100 allergens were determined by using the Pharmacia CAP System. Serum IgE levels were classified as normal or high according to the following age-specific reference ranges (kU/litre): 0-1 year, <15; 1-5 year, < 60; 5-10 year, < 90; adult, < 100, and specific IgE levels were considered positive in those having detectable allergen-specific IgE (>0.35 kU/L). The eosinophil cell counts were measured by Coulter MAXM Analyser. A value of 1 to 6 percent

eosinophils was considered a normal relative range and normal range of absolute eosinophil count was considered between 0.05 and 0.200 G/l.

Sputum induction and analysis

The participants inhaled 4.5% saline solution generated by a De Vilbiss Nebulizer (Ultra-NebTm 2000 model 200HI) for 5 minutes after pre-treatment with 400 mg of inhaled salbutamol. Induction was performed three times and the pulmonary function was measured each time after the sputum induction. All portions that macroscopically appeared free of salivary contamination were selected. Samples were diluted with phosphate buffered saline containing 0.1% dithiotreitol, portions were agitated with a vortex and placed on a bench rocker for 30 minutes. Samples were filtered through a 40 mm Falcon cell strainer, and centrifuged at 1500 rpm for 10 minutes. The cell pellet was resuspended in 1 ml PBS and viability (Trypan blue exclusion method) was determined using Burker chamber. After differential cell count, cells were stocked on lyses buffer at 80ºC until use.

Lung function test, FENO measurement

Lung function was measured by spirometer (PDD-301/s) and fractional exhaled nitric oxide (FENO) measurements were carried out by NO sensor (NIOX MINO).

Determination of air pollution

As an indicator of local traffic related air pollution we used the concentrations of NO_2. Its level for the years preceding the onset of asthma was provided by the Reference Centre of the Hungarian Air Quality Network. Concentrations of NO_2 were measured by automatic stations located within 10 km of the residence of each study participant. When considering NO_2 concentration as a discrete parameter we regarded the concentration of 32 $\mu g/m^3$ as a threshold level, and all NO_2 concentrations below this threshold were classified as low and above as high. The threshold was determined at the median of the average NO_2 concentrations of the studied geographical locations.

RNA isolation, detection of quantity and quality

RNA was isolated with the Qiagen Mini RNeasy kit. Quantity of isolated RNA was determined by NanoDrop ND-1000 spectrometer, its quality was assessed using Agilent 2100 Bioanalyzer.

Reverse transcription and detection of mRNA expression

RNA was transcribed to cDNA with the High Capacity cDNA Reverse Transcription Kit (Applied Biosystems). Real-time quantitative PCR was performed using an ABI 7900HT Fast Real-Time PCR System and TaqMan Gene Expression Assays. Relative gene expression was determined by the comparative CT method (ddCT) with b-actin as the endogenous control.

Statistical analysis

Logistic regression analyses adjusted for age and gender were used to evaluate the associations between genotype data and asthma phenotype. Estimated haplotype frequency was calculated by Haploview 4.1. Linear regression models adjusted for age and gender were used to analyze the effect of the genetic background on dependent scale variables such as serum and sputum eosinophil levels and serum IgE. For sputum analysis, normalized gene expression levels were compared by Mann-Whitney U test or Kruskal-Wallis test, when appropriate. Contingency tables were analyzed by Fisher's exact test. Correlation studies were performed by Spearman non-parametric test. Multiple comparisons were corrected for using the Bonferroni method. The BN-BMLA (Bayesian network based Bayesian multilevel analysis of relevance) method was applied to our data by dr. Peter Antal and his collegues at the Department of Measurement and Information Systems, Budapest University of Technology and Economics.

Results

When studying the effects of *RANTES* -403G/A and *CCR5Δ32* polymorphisms and Mycoplasma pneumoniae infection on asthma risk we found that in the asthmatic group significantly more children had positive results for MP-specific antibodies than in the control group (31.1% vs 18.1%, p = 0.0009). The 2 groups did not differ in the distributions of genotypes and alleles for the *RANTES* -403G/A and *CCR5Δ32* polymorphisms. Allelic frequencies for the 2 polymorphisms were compared between MP-seropositive and MP-seronegative individuals to test whether the 2 polymorphisms influence the susceptibility to MPI. The allelic frequency of the *CCR5Δ32* allele was significantly higher among MP seropositive than among MP-seronegative children (18.0% vs 8.6%, respectively; p = 0.006). A similar ratio could be observed when children homozygous for the *CCR5Δ32* allele were considered (2.4% vs 1.3%). No significant difference between MP-seropositive and MP-seronegative individuals in the frequency of the *RANTES* -403G/A allele was revealed. With multiple logistic regression analyses, we detected that MPI was significantly associated with asthma (OR = 2.0; 95% CI = 1.3-3.1; p = 0.001) and atopy (OR = 1.7; 95% CI = 1.1-2.6; p = 0.01), and a significant association was found when MPI* *CCR5Δ32* interaction and asthma were considered (OR = 0.4; 95% CI = 0.2-0.7; p = 0.003). This result indicates that significantly less MP-seropositive asthmatic patients than MP-seropositive control subjects carry the *CCR5Δ32* allele (3.9% vs 12.7%, respectively), which suggests that carrying a defective CCR5 receptor might reduce the susceptibility to asthma in MP-infected children when comparing them with children with similar infection status but carrying only wild-type *CCR5*. No significant association was found when MPI**RANTES* -403A interaction and asthma, atopy, or asthma severity were considered.

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Between asthma cases and controls none of the studied SNPs in the regulatory regions of *NFE2L2* and *KEAP1* genes could withstand the correction for multiple testing. Studying the asthma endophenotypes, the strongest evidence for association was found between infection-induced asthma status and rs2588882 located in the 3' region of *NFE2L2,* as its minor allele was significantly less prevalent in the infection-induced asthmatic group (IIA) compared to non-infection-induced asthmatic (non-IIA) group (OR = 0.28, 95% CI = 0.13-0.60, p = 0.0005). The genotype associations were significant in both dominant and additive models. No associations

were seen for the markers in *KEAP1* with either risk of asthma, AA or IIA. The relation of the genotypes and NO_2 level was assessed within the asthma group. Our results show that the rare alleles of rs2588882 and rs6721961 (*NFE2L2* -617G/T) were significantly more prevalent in those children who lived in modestly polluted environment (in the dominant model, for rs2588882 OR (95% CI) = 0.43 (0.23-0.82), p = 0.01; for rs6721961 OR (95% CI) = 0.51 (0.29-0.90), p = 0.02, respectively). In line with the results of single SNP analyses, haplotype carrying the minor allele of both rs2588882 and rs6721961 were more prevalent within the low air pollution group (5.6% vs. 12.3%, OR = 2.31, 95% CI = 1.22-4.37, p = 0.0073).

By partial genome-screening of 11q12.2-q13.1 és 14q22.1-22.3 we genotyped 145 polymorphisms and analysed the genotype distributions of 102 of them by frequentist statistics and BN-BMLA (Bayesian network based Bayesian multilevel analysis of relevance) method. By the frequentist analyses we found significant associations between asthma and the rare allele of rs3751464 in the promoter of *FRMD6* (FERM domain containing 6) gene (OR = 1.43, 95% CI = 1.18-1.75, p = 0.0003), and the rare genotype (CC) of rs17831682 located in the 3' UTR region of PTGDR (Prostaglandin D receptor) (OR = 27.23, 95 % CI = 1.55-478.07, p = 0.00039). Besides, rs7928208 in the *PRPF19* gene provided an evidence for an association with asthma development before 6 years of age. Besides confirming the previous results, BN-BMLA method revealed 2 intronic polymorphisms in *PTGER2* (Prostaglandin E2 receptor) also. Rs569108, a missense SNP in *MS4A2*, the gene for the high affinity IgE receptor b subunit showed association with asthma when IgE and eosinophil level, allergic rhinitis and asthma as multiple targets were considered. Two SNPs (rs11231128 and rs11827029) in the *AHNAK* gene gave the strongest correlation with the phenotype asthma when the rhinitis status was known.

When studying the potential interactions between SNPs, we found relevant intragenic (in *PTGER2*), intrachromosomal (between *PTGER2* and *FRMD6*) and interchromosomal (between *PRPF19* and *FRMD6*) interactions as well. According to our results, the most significant SNP in this study is the rs3751464 in the *FRMD6* gene. It influences the asthma risk both alone and in interactions with other SNPs in *PRPF19* and *PTGER2* genes. In all the interactions with the minor rs3751464 TT genotype significantly increase the asthma risk, while interactions with the more frequent CC genotype decrease the asthma risk. We computed the a posteriori probability whether a variable is directly relevant or its association is only mediated by other variants or

through interactions with rhinitis. According to the calculated a posteriori values the majority of the observed associations is mediated rather by transitive than direct effects. We compared the gene expression levels of genes found to be relevant in asthma in the SNP analysis in sputum samples of 12 asthmatics and 9 controls using TaqMan Gene Expression Assays (*FRMD6, PTGDR, PTGER2, MS4A2, AHNAK, PRPF19, TXNDC16*). Sputum mRNA level of FRMD6 was significantly lower in the asthmatic patients compared to healthy controls with a fold change of 2.73 ($p=10^{-6}$). Moreover, the expression level of FRMD6 was consequently lower (in average with 1.52 fold) in the lungs of mice with OVA-induced allergic airway inflammation. No other gene showed statistically significant difference in this comparison.

To reveal the role of BIRC5 in asthma pathomechanism, first we validated our data generated from OVA-induced mouse model of asthma by real-time PCR and compared the BIRC5 mRNA level between sputum samples from healthy controls and asthma patients. The mean gene expression level of BIRC5 was significantly higher in induced sputum derived from asthmatic patients in comparison to healthy controls (p = 0.03). When studying the asthma severity subgroups defined by GINA guidelines, we found no differences between the mild and moderate-to-severe asthma groups. There was a significant relationship between sputum eosinophil percentages and BIRC5 mRNA levels (p = 0.02, r = 0.468). Gender, allergic status or smoking habits had no effect on BIRC5 mRNA expression. Consistent with previous publications, eosinophil ratios detected in sputum samples were significantly increased with increasing asthma severity. We found no correlation between BIRC5 mRNA expression levels and FENO, ICS dose or ACT total scores. There was a significant correlation between FENO levels and both eosinophil and neutrophil percentages in induced sputum (p= 0.006, r = 0.742 and p = 0.048, r = -0.58, respectively). In addition, we found a significant negative relationship between ACT score and eosinophil percentages (p = 0.048, r = -0.55). There was no correlation between the amount of ICS used and sputum eosinophil level (p = 0.1).

Analyzing the genotypes of *BIRC5* regulatory polymorphisms we found that the minor allele of rs8073903 and was significantly associated with increased risk of asthma (OR = 1.458, 95% CI = 1.126–1.889, p = 0.004). In addition, this association was more evident when only women were studied. In respect of asthma endophenotypes, the minor alleles of rs8073069 and rs8073903 were associated with and showed significantly higher predisposing effect for the

development of non-allergic asthma (OR = 2.010, 95% CI = 1.336–3.024, p = 0.001 for rs8073069 and OR = 1.622, 95% CI = 1.072–2.454, p = 0.022 for rs8073903). Moreover, consistent with the results observed between asthma cases and controls, these associations were also more prominent when studying only women. To determine the effects of the studied SNPs on serum eosinophil level, linear regression analysis was performed. Rs9904341 was found to be significantly correlated with both absolute and relative serum eosinophil counts (OR = 0.917, 95% CI = -0.145 to -0.026, p = 0.004 and OR = 0.262, 95% CI = -2.132 to -0.468, p = 0.002, respectively). No such correlation could be observed for serum IgE levels. To obtain more statistical evidence for the associations, we performed haplotype analyses. In the majority of the cases, the most significant results were observed when performing two-marker analyses with SNPs rs8073903 and rs8073069. Between asthma cases and controls, a strong significant difference was found for the haplotype consisting of the wild-type alleles (TG) of rs8073903 and rs8073069, as the haplotype was less frequent among the asthma patients compared with controls (57% versus 65%, p = 0.004). Further analyzing the asthma subgroups, we found that CC haplotype showed a very strong association with non-allergic asthma, as it was significantly more prevalent in those subjects than in other patients (46% versus 61%, p = 0.00005).

Conclusions

Based on our results the following conclusions may be drawn:

When studying the effects of genotype-environment interactions on the asthma risk, we showed that children carrying the defective form of chemokine receptor CCR5 might have a higher susceptibility to chronic MPI when comparing them with children without this mutant allele, whereas infected children with the *CCR5Δ32* allele might have reduced risk of asthma development when comparing them with children with the same infection status but without the mutant allele. Our results showing that chronic MPI is associated with asthma confirms previous findings.

None of the investigated polymorphisms in *NFE2L2* and *KEAP1* genes were found to be more prevalent in the asthma group compared to controls, questioning the elemental effect of these SNPs on the development of asthma phenotype. However, when analyzing the presence of infection-induced exacerbations within the asthma group, genotypes carrying the variant alleles of rs2588882 and rs6721961 appeared to have a significant protective effect. Additionally, these SNPs showed significant differences between asthma cases that lived in highly or modestly polluted environment. These data show that polymorphisms in the *NFE2L2* gene may affect the risk of infection-induced asthma, and may alter the effects of air pollutants on asthma development.

By partial genome screening of 11q12.2-q13.1 and 14q22.1-22.3 genome regions, association between a SNP in *FRMD6* (rs3751464) 5'region and asthma risk was identified with both the frequentist and BN-BMLA methods. A haplotype in this gene also influenced the disease susceptibility, and the rs3751464 showed an influential role in interactions with other SNPs in this respect. Additionally, the expression level of the FRMD6 was consistently lower in the lungs of mice with allergic airway inflammation and it was significantly lower in human asthmatics compared with controls. We showed that SNPs in *AHNAK (*rs11231128) and *TXNDC16 (*rs1565970) genes influenced asthma risk in interaction with rhinitis. Strong and direct association was found between rs7928208 in the *PRPF19* gene and asthma and this SNP was also associated with asthma development before 6 years of age. Associations were confirmed

between SNPs in *PTGDR* (rs17831682), *PTGER2* (rs708502 and rs17197), and *MS4A2* (E237G, rs569108) genes and asthma. We showed that SNPs in the *PTGER2* influence asthma susceptibility in interactions or indirectly, and similarly, the association between a SNP in *MS4A2* and asthma is transitive. Our results clearly show the several advantageous features of the BN-BMLA method over the traditional frequentist methods generally used in gene association studies.

Conforming to our expectations based on previous allergic mouse model of asthma, we found that the mean BIRC5 expression level was significantly higher in the airways of patients with asthma than of healthy controls. We demonstrated that the eosinophil ratio in induced sputum of asthma patients was in correlation with the BIRC5 mRNA expression level. This observation supports previous data that describe the anti-apoptotic effect of survivin on eosinophil. Furthermore, consistent with other studies, we confirmed that better asthma control was significantly correlated with lower sputum eosinophil count. Also, we found significant correlation between sputum eosinophil levels and clinical severity. According to the results of our SNP association analysis two of our studied SNPs, rs8073903 and 8073069, both are located in the 5' region, showed significant associations with asthma. When examining the male and female subjects separately, we found that the observed associations were even more prominent among women. When studying the relationships between the SNPs and different asthma phenotypes, we observed more apparent predisposing effects of these polymorphisms in the non-allergic asthma subgroup. Furthermore, consistent with the results of genotyping analyses, haplotypes carrying the minor alleles of these SNPs were found to be associated with a significantly increased risk of asthma and non-allergic asthma. We found that individuals homozygous for the wild-type allele of rs9904341 had elevated absolute and relative eosinophil levels in their serum compared with those carrying either one or two copies of the rare allele. According to our data we propose that in eosinophils, the apoptotic process may be regulated by the anti-apoptotic protein BIRC5 that can be regarded as a new therapeutic target in eosinophilic asthma.

List of own publications

Publications summarized in the current work:

1. **Ungvári I**, Tölgyesi G, Semsei AF, Nagy A, Radosits K, Keszei M, Kozma GT, Falus A, Szalai C. CCR5 Delta 32 mutation, Mycoplasma pneumoniae infection, and asthma. J Allergy Clin Immunol. 2007 Jun;119(6):1545-7. IF: 8.115
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