Investigation of the genetic background of childhood asthma and allergy

PhD Thesis Outline

Lili Erika Készné Fodor

Semmelweis University
Doctoral School of Molecular Medicine

Supervisor:
Dr. Csaba Szalai, Professor, DSc.

Official reviewers:
Krisztina Vellainé Takács, PhD
Dr. Otília Menyhárt, PhD

Budapest
2017

1. Introduction

Asthma is a chronic inflammatory respiratory disease influenced by a wide range of environmental and genetic factors. It is characterized by airflow obstruction due to smooth muscle constrictions and airway inflammation with symptoms such as coughing, wheezing, tightness in the chest, bronchoconstriction and airway hyperresponsiveness that may remit spontaneously or upon treatment.

It is well-known, that apoptosis is a key feature in the pathomechanism of asthma. The balance between cell apoptosis and survival depends on the control and maintenance of different regulatory elements and pathways. Baculoviral IAP repeat containing 5 (BIRC5), also called survivin is an important anti-apoptotic member of the IAP family. Recently, it has been shown that BIRC5 has additional roles in inflammatory mechanisms and disorders, such as asthma. Furthermore, our research group has found several important aspects of BIRC5 in asthma. Furthermore, our group's previous results have shown that the gene expression of FERM-domain containing 6 (*FRMD6*) is significantly decreased in both the OVA-induced mouse model, as well as asthmatic patients compared to controls. FRMD6 is the upstream activator of the Hippo signalling pathway, which also regulates the expression of several proteins, such as BIRC5. The Hippo pathway is highly conserved from *Drosophila melanogaster* to mammals and regulates organ size via promoting apoptosis and inhibiting cell proliferation in the embryonic stages of development. Besides embryonic tissues, where YAP1 plays an important role in, for example, lung maturation and postnatal airway homeostasis, it is widely expressed in respiratory epithelial cells of the embryonic and mature lung, where the Hippo/YAP1 signalling regulates epithelial cell proliferation and differentiation.

Processes such as cytokine production, inflammatory cell infiltration to the lungs, injury to epithelial cells or apoptosis all play an important role in the development and severity of asthma. Structural changes in the airway walls due to both neovascularization and angiogenesis are also key aspects of asthma. The Tie2 receptor is an important player in angiogenesis. The *Tie2* mRNA and protein are abundantly

expressed in the lungs and the associated pathway also has an important role in the development and function of the eye. Recently, variations in the *TEK* gene, encoding Tie2, have been found associated with asthma.

Our research group has previously developed an ovalbumin-induced mouse model of asthma and have carried out a whole genome gene expression microarray analysis on different healthy and asthmatic mouse groups. We have chosen 60 genes based on the results of this study to further analyse.

2. Objectives

Our goal was to study the genetic and genomic background and the pathogenesis of childhood asthma and its associated phenotypes.

1. Investigation of the role of the Hippo signalling pathway in asthma
    - Evaluating the differences in gene expressions of seven Hippo pathway genes between asthmatics and healthy subjects based on previous results
    - Assess relationship of polymorphisms spanning the whole of *YAP1* gene and asthma and its phenotypes by estimation of allele frequencies between asthma patients and healthy controls. Moreover, gaining further associations through the haplotype analysis of our data and a more extensive Bayesian statistical analysis
    - Comparing FRMD6, BIRC5 and YAP1 protein levels in induced sputum samples from asthmatics and controls in order to evaluate role of Hippo signalling pathway in asthma through protein expression.
    - Investigating HeLa cells *in vitro* upon BIRC5 antagonist, YM155 treatment in order to find functional roles for Hippo signalling pathway components, BIRC5, YAP1 and FRMD6
2. Investigation of the role of angiopoietin receptor Tie-2 in asthma and its phenotypes
    - Assessing the incidence of different comorbidities of asthma within our study population.
    - Evaluating tagSNPs of Tie2, encoded by the *TEK* gene, in asthmatic and control subjects to find biomarkers for asthma susceptibility.
3. Investigation of associations between 60 previously identified genes and asthma

- Following variation selection, identification of associations by estimation of allele frequencies between asthma patients and healthy controls.

3. Methods

Participants of sputum induction and gene expression measurements

The gene expression analysis was done using the induced sputum of 18 asthmatic patients and 10 healthy controls. All subjects completed a detailed, pre-edited questionnaire based on the ISAAC Phase Three Questionnaire. The recent Global Initiative for Asthma guidelines (www.ginasthma.org) were used to diagnose asthma by a respiratory medicine specialist. Healthy volunteers with no previous history of asthma or any airway conditions comprised the control group. The participants' sex, age, smoking habits and allergic statuses were compared between cases and controls and between severity groups, but no statistical significance was found.

Characteristics of Participants of Genotyping Analysis

The genotyping analysis of YAP1 SNPs included 1233 unrelated individuals, out of which 522 were asthmatic children (mean age±SD: 10.20±5.31 years; 328 males and 194 females) and 711 healthy controls (mean age±SD: 14.0±11.2; 429 males and 282 females). Further, the genotyping analysis of TEK SNPs included 1189 unrelated individuals, out of which 435 were asthmatic children (mean age±SD: 10.3 ± 4.4 years; 269 males and 166 females) and 754 healthy controls (mean age±SD: 13.8±11.8 years; 453 males and 297 females). Moreover, the genotyping analysis of 60 previously identified genes included 671 individuals, out of which 311 were asthmatic children (mean age±SD: 10.6 ± 4.8 years; 203 males and 18 females) and 360 healthy controls (mean age±SD: 21.7 ± 13.9 years;181 males and 179 females). Different types of asthma were defined in patients. Asthma was divided into allergic and non-allergic asthma subtypes. If asthma was not provoked by allergens, but allergy was also present, allergy types were, nonetheless, marked. In allergic patients depending on the types and quantities of allergens, subgroups of indoor, outdoor, or inhalative allergic phenotypes have been designated. Asthma was categorized as exercise-induced asthma when the asthma exacerbation was provoked by exercise in the medical history of the patients. If the onset of asthma or the asthma exacerbations have been associated with an infection

related acute respiratory illness the asthma was classified as viral-induced asthma. Non-atopic patients with viral-induced asthma phenotype composed the non-allergic asthma subgroup. The samples from the control children were collected at the Orthopaedic Department in the Budai Children's Hospital or at the Urological Department in the Heim Pál Hospital, both in Budapest. None of the controls had any symptoms of asthma or airway conditions, nor any need for medication.

A written informed consent was signed by all patients or by their parent/guardian. The study was carried out according to the Declaration of Helsinki and was approved by the Ethics Committee of the Hungarian Medical Research Council.

Sputum induction

Induced sputum was used for gene expression assays and Western blot analysis. After differential cell count by a specialist, cells were stored on lysis buffer at -80°C until use**.**

DNA isolation

Genomic DNA was isolated from whole blood samples of 1233 individuals using the QIAamp blood DNA midi kit (Qiagen, Maryland, USA) or the iPrep PureLink gDNA Blood Kit on iPrep Purification Instrument (Invitrogen, Carslbad, CA, USA) starting out from 1 ml whole blood.

RNA Isolation and cDNA transcription

RNA was isolated successfully from the induced sputum samples of 18 patients and 10 control subjects with the Qiagen Mini RNeasy Kit according to the manufacturer's instruction (Qiagen, Maryland, USA).

Gene expression with TaqMan

Real-time quantitative PCR was performed on *LATS1*, *LATS2*, *MST1*, *MST2*, *SAV1*, *YAP1*, *TAZ* and *β-actin* genes using 7900HT Fast Real-Time PCR System

(Thermo Fisher Scientific, Waltham, MA USA) according to the manufacturer's instructions. *β-actin* was used as an endogenous control and all results were normalized to it using the delta delta Ct method.

SNP Selection and Genotyping with Competitive Allele-Specific PCR

SNPs were selected from *YAP1* gene using UCSC Genome browser. Preferably, promoter, missense or UTR SNPs were chosen where MAF in the Caucasian population was higher than 0.1, which was confirmed by HapMap. *TEK* gene SNPs were chosen based on the paper of Ghosh et al. (Ghosh et al. 2016). KBioscience Competitive Allele-Specific PCR (KASP) version 4.0 genotyping assays were used (LGC Genomics, Berlin, Germany) to genotype fourteen SNPs on the *YAP1* gene and three SNPs on the *TEK* gene (Table 3) according to the manufacturer's instructions.

SNP Selection and Genotyping with Sequenom iPLEX Gold MassARRAY of Previous Mouse Model Based Asthma Investigation, and Statistical Analyses

SNP selection was done based on a previous study of our group on OVA induced mice where gene expression analysis was done by Agilent Whole Mouse Genome Oligo Microarray 4 x 44 K chip (GSE11911). Based on the microarray results our group has chosen 60 genes with a statistically significant change and a possible role in human asthma and allergy. From these genes 90 SNPs have been chosen by using UCSC Genome Browser. To genotype the selected SNPs the Sequenom iPLEX MassARRAY technology was used at the McGill University and Genome Quebec Innovation Centre, in Montreal, Canada.

Cell culturing

HeLa cells were cultured according to the manufacturer's advice and were harvested and lysed in RIPA lysis and extraction buffer (Thermo Fisher Scientific, Waltham, MA USA) for use as positive control during Western blot analysis. Separately, HeLa cells were cultured on six-well plates to be treated with BIRC5

inhibitor, Sepantronium Bromide (YM155) (Selleck Chemicals, Munich, Germany). Cells were treated with 1000nM, 100nM, 10nM, 1nM, 0nM YM155 or 1% dimethyl sulfoxide. After 48h incubation cells were harvested. 50% of cells/well were treated with RIPA lysis and extraction buffer (Thermo Fisher Scientific, Waltham, MA USA) for further use as protein and 50% of cells were treated with RLT RNeasy Lysis Buffer (Qiagen, Maryland, USA) for gene expression analysis.

Western blot analysis

Western blot analysis was carried out on induced sputum samples with YAP1, FRMD6 and GAPDH proteins.

Bioinformatics

For sputum analysis, normalized gene-expression levels of *LATS1*, *LATS2*, *MST1*, *MST2*, *SAV1*, *YAP1*, *TAZ* and *β-actin* genes were compared by Mann–Whitney *U* test or Kruskal–Wallis test, when appropriate. Further, Fisher's exact test, Spearman non-parametric test were also used for statistical analysis. *YAP1* and *TEK* SNP allele frequencies were estimated by allele counting and tested for deviation from HWE by the software program DeFinetti between cases and control subjects. Differences were considered to be significant when $p < 0.05$. SNP data were analysed using SPSS version 22 (SPSS Inc., Chicago, IL, USA) software. Logistic regression analyses adjusted for age and gender were used to evaluate the association between *YAP1* genotypes and asthma and its phenotypes. For *YAP1* SNPs multiple comparisons were corrected for using the Benjamini-Hochberg correction, and a new significance level of p=0.004 with the FDR < 6.5% was estimated. Haplotype analysis was carried out with the Haploview 4.2 program (Broad Institute of MIT, Cambridge, MA, USA). For *TEK* SNPs multiple comparisons were corrected for using the Benjamini-Hochberg correction, and a new significance level of P=0.008 with the FDR < 4.5% was estimated.

Bayesian statistical analysis was carried out by the research group of dr. Péter Antal at the Budapest University of Technology and Economics in the Department of

Measurement and Information Systems. Earlier, our research groups have together developed an alternative, systems biological statistical method, named Bayesian network based Bayesian multilevel analysis of relevance (BN-BMLA). Bayesian networks offer a rich language for genetic association studies, because they exhaustively and exactly represent the strongly relevant variables and their interactions through the Markov Blanket Set and Markov Blanket Graph features and they are able to evaluate multiple targets. Furthermore, this Bayesian global relevance analysis method provides posteriors, which are direct statements about hypotheses, thus it can also be used to construct probabilistic data analytic knowledge bases in genetic association studies to support complex querying, off-line meta-analysis, and fusion with background knowledge. In this study 29 SNPs all in the *YAP1*, *FRMD6* and *BIRC5* genes (previously genotyped by others with the same methods and on the same populations) were involved in the BN-BMLA analysis.

4. Results

Evaluating the differences in gene expressions of seven Hippo pathway genes between asthmatics and healthy subjects based on previous results

The expression of all genes could be detected in both cases and controls. The mean gene expression level of YAP1 was slightly lower in asthmatic than in control patients (p=0.032). There were no other deviations in this respect. During the correlation studies, we found a significant and positive correlation between YAP1 mRNA level and the sputum bronchial epithelial cells (r=0.575, p=0.003).

Assess relationship of polymorphisms spanning the whole of *YAP1* gene and asthma and its phenotypes by estimation of allele frequencies between asthma patients and healthy controls. Moreover, gaining further associations through the haplotype analysis of our data and a more extensive Bayesian statistical analysis

There was no significant association with any of the SNPs and asthma susceptibility, allergic status, inhalative, outdoor, indoor allergies, allergic and non-allergic asthma, comorbidities of rhinitis and conjunctivitis or serum IgE and eosinophil levels. However, SNP rs2846836 was significantly associated with exercise-induced asthma (OR=2.1 [1.3-3.4], p=0.004, power=0.83). Additionally, distribution of genotypes of SNP rs11225138 showed a significant difference between GINA 1-2 and GINA 3-4 statuses in a dominant model (OR=2.8 [1.4-5.6], p=0.003, power=0.83).

Based on the results after genotyping 29 SNPs in YAP1, FRMD6 and BIRC5 genes the a posteriori probabilities of relevance between the variables with respect to target variables were calculated by BN-BMLA. As expected, e.g. eosinophil levels and allergic conjunctivitis are highly relevant to allergic rhinitis. In the case of genetic variations, no direct SNP-SNP or gene-gene interactions were found. The most relevant association was between rs9671722 in the FRMD6 gene and exercise-induced asthma with a posterior probability of strong relevance of 0.99. The most likely subgraph suggests a direct relevance of rs9671722 to exercise-induced asthma, while another SNP (rs3751464) of the FRMD6 gene was found to be directly relevant to allergic

rhinitis and transitively associated through allergic rhinitis with exercise-induced asthma.

In order to find more evidence for the associations, we also conducted haplotype analyses. There is a significant difference between patients of GINA 2 and GINA 3 when we compared the frequencies of a haplotype formed by the rare alleles of SNPs rs1426398 and rs11225138, where the frequency of TC haplotype was more prevalent in GINA 3 than in GINA 2 (28% vs. 8%; p=10-7). Furthermore, the CA haplotype from SNPs rs11225138 and rs1426394, also showed a significant difference when patients in the two GINA statuses were compared (26% vs. 7%, p=10-7).

Comparing FRMD6, BIRC5 and YAP1 protein levels in induced sputum samples from asthmatics and controls in order to evaluate role of Hippo signalling pathway in asthma through protein expression

The signal for the FRMD6 protein could be detected in all sputum samples from both asthmatic and control patients. Unfortunately, the BIRC5 protein could not be detected in any of the healthy or asthmatic samples. Interestingly, however, the YAP1 protein could not be detected in the sputum samples of the healthy controls, it was well-seen in the sputum samples of the mild asthmatics (GINA 1,2) and was also absent from the sputum of severe (GINA 3,4) asthmatics.

Investigating HeLa cells *in vitro* upon BIRC5 antagonist, YM155 treatment in order to find functional roles for Hippo signalling pathway components, BIRC5, YAP1 and FRMD6

After 48h incubation time, YM155 treatment seemed successful. As a result, in a Nikon light microscope we found that the higher the concentration of YM155 was, the less cells were found in a given well. DMSO and negative controls were similar in number of cells, therefore DMSO had no visible effect on the cells. After protein expression analysis with Western blot, none of the investigated proteins, BIRC5, FRMD6 or YAP1 could be detected.

Assessing the incidence of different comorbidities of asthma within our study population.

Altogether, 435 asthmatic children and 754 healthy controls participated in the genotyping study. Among the fully phenotyped 320 asthmatic patients 178 (55.6%) had allergic rhinitis and 100 (31.3%) had conjunctivitis. Among the rhinitis patients 98 (55.1%) also had conjunctivitis. And two patients had conjunctivitis without rhinitis.

Evaluating tagSNPs of Tie2, encoded by the *TEK* gene, in asthmatic and control subjects to find biomarkers for asthma susceptibility

We found no association between either of the two SNPs, rs581724 or rs7876024, and asthma. However, SNP rs581724 was significantly associated with allergic conjunctivitis in a recessive way (p=0.007; OR=2.4 (1.3-4.4)). More specifically the homozygote carriers of the rare allele (AA genotype) had a significantly increased risk of developing allergic conjunctivitis within the asthmatic population. The risk remained significant when the whole population without conjunctivitis was involved in the calculation (p = 0.003; OR = 2.1 (1.3-3.6)).

No other phenotypes (GINA status, viral- or exercise-induced asthma, allergic asthma, indoor, outdoor, inhalative allergies, IgE and absolute eosinophil levels, allergic rhinitis) were associated with either of the two SNPs in the statistical analysis.

Investigation of associations between 60 previously identified genes and asthma
Following variation selection, identification of associations by estimation of allele frequencies between asthma patients and healthy controls

Besides identifying 4 SNPs on two genes to differ significantly between cases and controls after frequentist statistical analysis which also included correction for multiple testing, which are rs2240572, rs2240571 and rs3735222 on *SCIN* and rs32588 on *PPARGC1B* we have identified two other polymorphisms that may be of interest in the elucidation of asthmatic mechanisms, that are SNP rs9862203 of *KLF15* (Kruppel Like Factor 15) and rs1508147 of *BIRC5*. Both SNPs show a significant deviation

between cases and controls on the Armitage's trend test, as well as in the difference of allele frequencies (rs9862203: p=0.04984, OR=1.293 for allele G; p=0.04359, respectively; rs1508147: p=0.04928, OR=1.26 for allele A; p=0.04148, respectively).

5. Conclusion

Our study provides additional evidences that the FRMD6/Hippo/YAP1 pathways might have a role in asthma and its different subtypes.

We showed that all investigated Hippo signalling pathway members were detectable in the induced sputum of both asthmatics and controls.

We showed that *YAP1* mRNA expression is significantly lower in asthmatics compared to controls and found also that the main source of *YAP1* in asthmatics may be the bronchial epithelial cells. Additionally, more correlations have been revealed between Hippo member gene expressions and various cell types, suggesting their source of origin.

We found genetic variations on the *YAP1* gene to be associated with exercise-induced asthma, and asthma severity. The latter result was also confirmed by the haplotype analysis. Of course, the genetic associations must be confirmed in independent populations.

The BN-BMLA revealed a direct relevance of SNP rs9671722 on the *FRMD6* gene to exercise-induced asthma, while another SNP, rs3751464 from the same gene was found to be directly relevant to allergic rhinitis and transitively associated through allergic rhinitis with exercise-induced asthma, suggesting an increased importance of the FRMD6/Hippo/YAP1 pathway in the pathogenesis of asthma and its associated phenotypes.

We found that YAP1 protein was only expressed in mild asthmatics, but neither in controls nor in severe asthmatics. It would be also interesting to reveal how exactly the activity of YAP1 protein is regulated in the airways of the asthmatic patients. If additional studies can confirm that the YAP1 associated pathways have a role in the regeneration processes in airway inflammations, these pathways can be potential novel therapeutic targets in asthma and other inflammatory airway diseases.

Although several lines of evidences indicate that the Tie2 pathway might have a role in asthma, the investigated variations in the *TEK* gene, which are associated with lower Tie2 expression, did not influence the susceptibility to the disease.

We found however, that the homozygote carriers of the rs581724 SNP had significantly increased risk to allergic conjunctivitis. If additional studies can confirm the role of the Tie2 pathway in allergic conjunctivitis, this can be a potential novel therapeutic target in the disease.

We investigated 90 SNPs on 60 genes chosen based on a previous study on OVA-induced mice. We have identified two SNPs, one of them, rs9862203 is an intronic SNP on the *KLF15* gene and the other, rs1508147 is a near-gene SNP on the 3' end of the *BIRC5* gene. Both of them showed a significant deviation between asthmatics and controls, but the significance was lost after multiple testing correction. Nonetheless, both SNPs and genes may have a role in the pathogenesis of asthma due to their functions. Both genes may play a role in asthma but their functional studies are needed to better understand asthma processes and to find new potential therapeutic targets.

6. Publications

Publications related to doctoral dissertation

1. **Fodor LE**, Gézsi A, Ungvári L, Semsei ÁF, Gál Z, Nagy A, Gálffy G, Tamási L, Kiss A, Antal P, Szalai C. Investigation of the Possible Role of the Hippo/YAP1 Pathway in Asthma and Allergy. Allergy Asthma Immunol Res. 2017 May;9(3):247-256. doi:10.4168/aair.2017.9.3.247 IF: 2.957

2. **Fodor LE**, Gézsi A, Gál Z, Nagy A, Kiss A, Bikov A, Szalai C. Variation in the TEK Gene is Not Associated with Asthma but with Allergic Conjunctivitis. Int J Immunogenet. 2018 Apr 18. doi: 10.1111/iji.12365. [Epub ahead of print] IF: 1.093

3. Temesi G, Virág V, Hadadi É, Ungvári I, **Fodor L E**, Bikov A, Nagy A, Gálffy G, Tamási L, Horváth I, Kiss A, Hullám G, Gézsi A, Sárközy P, Antal P, Buzás E, Szalai C. Novel genes in Human Asthma Based on a Mouse Model of Allergic Airway Inflammation and Human Investigations. Allergy Asthma Immunol Res. 2014;6(6):496-503. doi:10.4168/aair.2014.6.6.496. IF: 2.160

Publications not related to doctoral dissertation

1. Balla Bernadett, **Fodor Lili**, Lakatos Péter: Az obesitas genetikája c. fejezet. Klinikai obezitológia. Budapest: Semmelweis Kiadó, 2017. pp. 31-48.
ISBN:9789633313435

2. Sági, J. C., Kutszegi, N., Kelemen, A., **Fodor, L. E**., Gézsi, A., Kovács, G. T. Erdélyi, D. J., Szalai, C., Semsei, Á. F. Pharmacogenetics of anthracyclines. Pharmacogenomics 2016:17(9): 1075-1087. doi:10.2217/pgs-2016-0036 IF: 2.350

3. Sándor N, Schilling-Tóth B, Kis E, **Fodor L**, Mucsányi F, Sáfrány G, Hegyesi H. TP53inp1 Gene Is Implicated in Early Radiation Response in Human Fibroblast Cells. Piva T, ed. Int J Mol Sci. 2015;16(10):25450-25465. doi:10.3390/ijms161025450. IF: 3.226