

Amino acid variants in Surfactant protein D are not associated with bronchial asthma

Krueger M, Puthothu B, Gropp E, Heinze J, Braun S, Heinzmann A. Amino acid variants in Surfactant protein D are not associated with bronchial asthma.

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Surfactant protein D (SFTPD) belongs to the family of collectins and is part of the innate immune system. Thereby it plays an important role in the defense of various pathogens. Besides it is involved in the development of acute and chronic inflammation of the lung. Levels of SFTPD are elevated in serum and alveolar lavage of asthmatic patients. As SFTPD binds and neutralizes common allergens like house dust mites it is especially important in allergic asthma. Three common amino acid variants have been identified in SFTPD and association of the first variant has been described to severe infection with respiratory syncytial virus. Furthermore the functional impact of all three amino acid variants has been demonstrated. Due to its function *SFTPD* represents an ideal candidate gene for bronchial asthma and we were interested whether the polymorphisms were in association with asthma in children. The three polymorphisms leading to amino acid exchanges (Met11Thr, Ala160Thr, and Ser270 Thr) were typed by restriction fragment length polymorphisms in 322 asthmatic children and 270 controls. Association analyses were performed by Armitage's trend test. In addition haplotypes were calculated by FASTEHPLUS and FAMHAP. None of the polymorphisms was in association with bronchial asthma. Haplotype analyses revealed four major haplotypes all of which were evenly distributed between the populations. We conclude from our data that functional amino acid variants in SFTPD do not play a major role in the genetic pre-disposition to bronchial asthma in children.

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Key words: asthma; genetics; pediatrics; polymorphisms; association; Surfactant D

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Accepted 5 October 2005

During the last years increasing attention has been paid to Surfactant D (SFTPD) and its potential role in the development of bronchial asthma in human and mice (see Ref. 1 for a recent review). SFTPD is secreted by type-II alveolar cells and belongs together with mannose-binding lectin and Surfactant Protein A to the family of collectins. It is part of the innate immune system and takes part in the defense to various pathogens like bacteria, viruses, and fungi (2). As SFTPD is mainly expressed in lungs it is especially important for the immune reaction to inhaled pathogens.

The involvement of SFTPD in the pathophysiology of bronchial asthma has been highlighted by the following studies: Levels of SFTPD are significantly elevated in serum

and alveolar lavage of allergic asthmatic patients (3, 4); same results were obtained in three different murine models of asthma. Consecutively serum levels of SFTPD have been proposed as a new biomarker to predict the extent of airway inflammation (1).

In addition, *SFTPD* knockout mice develop a higher extent of pro-inflammatory reactions in the lungs than wild type mice (5), whereas mice over expressing SFTPD show an attenuated bronchial hyper-responsiveness in response to allergic sensitization (6). Both studies imply strongly a protective role of SFTPD on allergic airway inflammation. Finally, human SFTPD can bind to house dust mite antigen and thus directly inhibits the binding of allergen-specific IgE to this allergen (7).

The gene coding for SFTPD is located in a gene cluster with further Surfactant proteins, *SFTPA1* and *SFTPA2*, on chromosome 10. Three amino acid variants have been identified in SFTPD (8–10). The first amino acid variant Met11Thr has been found in association with severe respiratory syncytial virus infection (9). Furthermore an association of a *SFTPD* allele with tuberculosis has been described in a Mexican population (11). These studies further underline the important role of SFTPD in the defense of viral and bacterial infections of the lungs.

A very recent study has investigated the functional impact of the three amino acid variants in SFTPD (12). Thr/Thr11 was shown to influence the oligomerization and thus the function of the molecule. Furthermore, the variant was associated with serum concentration of SFTPD.

Due to the important role of SFTPD in bronchial asthma and the proven functional impact of SFTPD variants *SFTPD* represent an ideal candidate gene for asthma. Therefore the aim of this study was to test the three common amino acid variants in SFTPD for association with bronchial asthma.

Material and methods

Subjects

Three hundred and twenty-two children with asthma (aged 5–18 yr) were recruited from the southwestern part of Germany between July 2000 and January 2005. An extended medical history was recorded including occurrence and duration of wheezing symptoms, previous and acute medications, severity of previous asthma attacks, previous allergic rhinitis or conjunctivitis, atopic dermatitis, and any family history of allergic diseases.

The diagnosis was based on a clear-cut history of asthmatic symptoms, the use of anti-asthmatic medication and the presence of bronchial hyper-reactivity. The anti asthmatic drugs included typical betamimetika like salbutamol and standard corticosteroids used in asthma treatment like budesonide. Bronchial hyper-reactivity was defined as a fall in forced respiratory volume in 1 s (FEV1) by at least 15% in histamine testing or exercise provocation. Only Caucasians were included.

Pulmonary function tests

Pulmonary function tests were performed using standard protocols. In addition exercise induced asthma was diagnosed by subjecting the

proband to physical exercise for 6 min under standardized conditions. The first spirometry and peak flow measurement were performed after 2–3 min and the second after 5–6 min. After 10 more minutes the children inhaled with salbutamol and a third spirometry and peak flow measurement were taken.

To test for bronchial hyper-reactivity inhalations with increasing, doubling concentrations of histamine were performed (from 0.125 to 8 mg/dl). Testing was stopped after a 15% fall in FEV1.

Control population

Two hundred and seventy randomly chosen probands were used as controls (aged 19–40 yr). They originate from the same area in the southwestern part of Germany and are all Caucasians. No medical history was taken and no medical testing was performed on controls.

Genotyping

DNA was extracted from peripheral blood leucocytes following standard protocols and column purified (DNA midikit, Qiagen, Germany).

Genotyping was performed by restriction fragment length polymorphism (RFLP). Met11Thr (**rs721917**) was typed using the primer pair 5'-CCCCATAGCAGAGGACAGAA-3' and 5'-CCAGGGTGCAAGCACTGGAC-3'. After PCR the product was digested with 1 U of HpyCH4 IV (New England Biolabs, Beverly, MA, USA) at 37°C over night and the fragments resolved on a 3% agarose gel. The PCR product is 238 bp in length, the wild type is not cut, the PCR product bearing the polymorphism is cut in 217 and 21 bp.

Ala 160 Thr (**rs2243639**) was typed using the primer pair 5'-CCCCACTTCTCTCTGACC-3' and 5'-CTGCTCACCTGCTGCCCCCG-3'. After PCR the product was digested with 1 U of BstU1 (New England Biolabs) at 60°C over night and the fragments resolved on a 3% agarose gel. The PCR product is 238 bp in length, the wild type is cut in 165 and 73 bp, the PCR product bearing the polymorphism is cut in 145, 73, and 20 bp.

Ser270Thr (**rs3088308**) was typed using the primer pair 5'-ACGGAGGCACAGCTGCTG-3' and 5'-GGAAAGCAGCCTCGTTCT-3'. After PCR the product was digested with 1 U of MnlI (Fermentas) at 37°C over night and the fragments resolved on a 4% NuSieve GTG agarose gel. The PCR product is 115 bp in length, the

wild type is cut in 54, 42, and 19 bp, the PCR product bearing the polymorphism is cut in 96 and 19 bp.

Sequencing

For each polymorphism three controls (homozygous wild type, heterozygous, and homozygous mutation) were sequenced by the dideoxy chain termination method (13) using the Big Dye Terminator cycle sequencing kit on an ABI 310 sequencer (Applied Biosystems, Foster City, CA, USA). All the following studies included these reference individuals.

Statistical analysis

An association analysis, based on the case-control design, was performed for each SNP using Armitage's trend test. This test takes into account the individuals' genotypes rather than just the alleles, following the guidelines given by Sasieni (14) as implemented in the program Finetti (Thomas F. Wienker, unpublished data; <http://ihg.gsf.de/cgi-bin/hw/hwa1.pl> and <http://ihg.gsf.de/linkage/download/finetti.zip>). Hardy-Weinberg equilibrium (HWE) was calculated for each polymorphism in both populations using also the program Finetti. In addition to analyses based on single polymorphisms, we performed haplotype frequency estimations by using the programs FASTEHPLUS (15) and FAMHAP (16). The extent of linkage disequilibrium between the polymorphisms has been calculated using the Arlequin program.

Approval

The collection of blood and the experimental procedures were approved by the Ethical Commission of the University of Freiburg. A statement of informed consent was signed by all participants or in the case of children signed by their parents.

Results

Genotyping

The three polymorphisms leading to an amino acid exchange in SFTPD were genotyped in 322 asthmatic children and 270 controls. The genotype distribution and the allelic frequencies of the mutant allele are given in Table 1. The frequencies of all three polymorphisms are in accordance to previous published studies (9). All but one polymorphism was in HWE in both populations as calculated by the Finetti program (see also Table 1). Thus a systematic error in genotyping seems unlikely.

Association studies

The results of the association analysis by the Armitage's trend test are given in Table 1. A $p < 0.05$ was regarded as significant for association with asthma. None of the polymorphisms showed an association with bronchial asthma.

Haplotype analyses and linkage disequilibrium

Haplotype analyses revealed four major haplotypes (see Table 2 for their frequencies in the asthmatic and control population). Analyses by the programs FASTEHPLUS and FAMHAP showed no significant difference in haplotype distribution between the populations ($p = 0.419$ and 0.3360 , respectively). Thus not even a specific combination of polymorphisms seems to have an effect on the development of asthma.

Table 2. Haplotype distribution in asthmatics and controls (1 = wild type, 2 = mutation).

Haplotype	Asthmatics	Controls
1 1 1	0.162903	0.167201
1 1 2	0.002226	0.000000
1 2 1	0.407218	0.376195
2 1 1	0.329058	0.385629
2 1 2	0.091021	0.064151
2 2 1	0.007573	0.006823

Table 1. Listed are the amino acid variants under investigation and the p values for association with bronchial asthma calculated by Armitage's trend test. In addition the frequency is given for the mutant allele (i.e., the frequency of Threonine for all three variants), the genotype distribution (in the following order: homozygous wild type, heterozygous and homozygous mutation) and the HWE as calculated by Finetti

Polymorphism	p Value	Asthmatics			Controls		
		Frequency	Genotypes	HWE	Frequency	Genotypes	HWE
Met11Thr	0.2935	0.427	112;136;66	0.0420	0.459	80;128;58	0.6136
Ala160Thr	0.2770	0.418	112;144;59	0.2944	0.382	99;132;37	0.5044
Ser270Thr	0.1261	0.092	265;49;5	0.1672	0.067	234;32;2	0.3327

All three polymorphisms were in very tight linkage disequilibrium as calculated by Arlequin ($p = 0.000000$ for all possible pairs) and therefore transmitted together.

Discussion

Despite the important role of SFTPD in the pathophysiology of bronchial asthma we did not find association of any of the three functional amino acid variants with the disease.

In addition to analyses of single polymorphisms we also conducted haplotype calculations to include additive effects of the polymorphisms on the genetics of asthma. Hereby four common and two very rare haplotypes (that is frequency less than 0.01) were revealed. All haplotypes were evenly distributed between both populations. Surprisingly the haplotype bearing the wild type for all three polymorphisms has a frequency of only 16%. That is, only about every sixth allele encodes the so-called wild type protein. However, as two polymorphisms are very common, this might just reflect confusion in nomenclature.

SFTPD is located about 300 kb centromeric of the genes coding for *SFTPA1* and *SFTPA2*. These genes have been found in association with neonatal respiratory distress syndrome in the Finish population (17, 18). Interestingly, there is a slight tendency to an increasing association towards the 3' end of *SFTPD* and thus towards *SFTPA1* and *SFTPA2*. As linkage disequilibrium has been shown to exist up to a distance of 800 kb (19, 20), this might be a hint that a disease causing polymorphism is located in *SFTPA1* or *SFTPA2*. Further studies to clarify this issue are currently underway.

In association studies the size of the study population is crucial in order to obtain enough statistical power. Our asthmatic population comprises about 320 children. This is a reasonable number to identify genes with a major effect on a complex genetic disease like bronchial asthma. In fact, in our study populations the power to find an association to asthma with a $p = 0.05$ assuming a relative risk of the variant of only 2 is 0.986 for 11Thr and 160Thr and 0.698 for 270Thr. However, we can't rule out the possibility that we have missed weak association.

Another important factor in such studies are the choice of suitable controls. We used randomly chosen controls. Thus it is likely that our control population includes some individuals who have bronchial asthma themselves. Thereby the possibility of a type II error – that is missing real association – will be enhanced. Some studies have started to use so called super-controls – i.e.,

controls, which have been tested negative for allergic phenotypes – to avoid this problem. However the selection of such super-controls will probably also include some selection bias. In addition, we have already found strong association of asthma with different genes using the same control population. Therefore we would expect to see at least weak association if any of the polymorphisms had a major effect on the development of asthma.

Finally, it might be possible that we missed the real disease causing polymorphism in *SFTPD*. As the gene has been repetitively searched for polymorphisms this seems unlikely. Furthermore all three polymorphisms are in very tight linkage disequilibrium spanning the whole gene. Therefore any missed polymorphism should be located in these haplotypes and thus indirectly included in the analyses.

In summary, we conclude from our data that functional amino acid variants in *SFTPD* do not play a major role in the development of bronchial asthma in German children.

Acknowledgments

This project was supported by a grant from the Deutsche Forschungsgemeinschaft (DFG HE 3123/3-1). The recruitment of asthmatic children was partially supported by a grant from the NIH (NIH R01 HL66533-01).

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