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Association study of mitochondrial genetic polymorphisms in asthmatic children

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ABSTRACT

It has been suggested that mitochondrial dysfunction plays a role in the pathogenesis of asthma. To test whether mitochondrial variants influence the risk of asthma, we analyzed 16,158 mtSNPs in a sample of 372 asthmatic children and 395 healthy children using the DNA pooling technique and genome wide association analysis. Stratified analysis by sex was performed to explain the differences observed between sexes in the etiology of asthma. Different variants were detected to be significant in the sample of girls and boys with the smallest adjusted *p* values being 1.4×10^{-09} (mt5295) and 3.6×10^{-12} (mt16158), respectively. Most of the significant locations found in boys are within the *CYB* gene and the non-coding region. For girls, most of the significant mtSNPs lie within NADH-dehydrogenase-subunits. The variants reported here have not previously been described in connection with asthma. Although further studies in other cohorts are needed to confirm these findings our study highlights the importance of the mitochondria among the factors that contribute to the risk of asthma.

1. Introduction

Asthma is the most common chronic inflammatory disease in childhood and its prevalence is still increasing worldwide (Anandan et al., 2010). Many environmental stimuli are known to further its development like, for example, exposition to cigarette smoke or certain allergens. While many studies have investigated the role of the nuclear genome and have identified more than one hundred susceptibility genes in recent years only very few studies have focused on the mitochondrial genome. Those have found significant associations of the mitochondrial haplogroup U with elevated total IgE levels and asthma (Raby et al., 2007; Zifa et al., 2012); and mitochondrial dysfunction to be associated with an increased allergic airway inflammation in mice (Aguilera-Aguirre et al., 2009).

It has also been observed that the risk of asthma for children of mothers with asthma is higher than for those of fathers with asthma (Lim et al., 2010). One explanation for the increased transmission through females may be an involvement of the mitochondrial genome which is predominantly maternally inherited.

Besides, mitochondria play a pivotal role in inflammatory processes. It is well known that asthma is characterized by a strong inflammation of the airways leading eventually to lung remodeling. Inflammation is triggered by exposure of the airway epithelial cells to oxidative stress — which is mediated by so-called reactive oxygen species (ROS) (Riedl and Nel, 2008). Endogenous ROS are mainly produced by mitochondria. Thus mitochondrial dysfunction can cause excess ROS and boost inflammation. Vice versa, excessive ROS can damage the mitochondria until apoptosis of bronchial epithelial cells occurs. In this way allergic inflammation of the bronchial epithelial cells can lead to dysfunction and remodeling of the airways during the course of asthma (Trian et al., 2007).

Given the mitochondria's important function in allergic airway inflammation and their possible role in the maternal inheritance of asthma we were interested in investigating whether mitochondrial single nucleotide polymorphisms (mtSNPs) are associated with asthma in children. So far, only a limited number of mtSNPs were investigated in the context of asthma (Raby et al., 2007; Zifa et al., 2012). Thus we resequenced the whole mitochondrial genome using microarrays with the DNA pooling method.

DNA pooling has been used as a cost-effective genotyping method for more than two decades. Several studies have established the accuracy of allele frequency estimates in pooled DNA on platforms

Abbreviations: bp, basepairs; Cyt b, cytochrome b; HVR1, Hypervariability Region 1; IgE, Immunoglobulin E; mtSNP, mitochondrial single nucleotide polymorphism; NADH, nicotinamide adenine dinucleotide; ND2, NADH dehydrogenase subunit 2; OXPHOS, oxidative phosphorylation; QC, quality control; ROS, reactive oxygen species; rRNA, Ribosomal Ribonucleic Acid; 16S RNA, mitochondrially encoded 16S RNA; SNP, single nucleotide polymorphism.

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allowing researchers to simultaneously analyze hundreds of thousands of genetic markers (Kirov et al., 2006; Meaburn et al., 2005). The relative efficiency of different platforms has also been compared (Macgregor et al., 2008). Boore (1999) demonstrated, both experimentally and theoretically, that pooling based GWA studies are effective at identifying major genetic contributions to diseases. In addition, many targeted resequencing applications utilizing pooling have been successful in recent years (Calvo et al., 2010; Momozawa et al., 2011).

In this study, we performed genetic association analyses to systematically examine the relationship between mtDNA variants and asthma in children.

2. Material and methods

2.1. Study groups

372 children with bronchial asthma (aged 5 to 18 years, 35% female, 65% male) were recruited between July 2000 and January 2009 at the Centre of Pediatrics and Adolescent Medicine, University of Freiburg. An extensive medical history was recorded in all children including previous occurrence and duration of wheezing symptoms, acute medications, severity of asthma attacks, symptoms of allergic rhinitis or conjunctivitis, atopic dermatitis, and any family history of allergic diseases. Recruitment criteria were as follows: a clear-cut history of asthmatic symptoms, regular use of anti-asthmatic medication, and presence of bronchial hyperreactivity. Bronchial hyperreactivity was defined as a fall in forced expiratory volume in 1 s by at least 15% in histamine testing or exercise provocation following a standard lung function testing procedure (Beasley, 1998). The protocol for clinical testing has been described in detail before (Heinzmann et al., 2004). The control sample was recruited in a pediatric doctor's office in the same geographical area and includes 395 children (aged 5 to 18 years, 44% female, 56% male). These children never suffered from allergic diseases, i.e., they were never diagnosed with bronchial asthma, allergic rhinoconjunctivitis, or atopic dermatitis. Blood and serum collection and experimental procedures were approved by the Ethical Committee of the University of Freiburg. A statement of informed consent was signed by the parents of all participating children.

2.2. Preparation of DNA

Standard procedures (Qiagen Kit, Hilden, Germany) were followed in isolating DNA from blood and DNA samples were run on a 1% agarose gel to check their quality. Concentration and purity of DNA were measured by PicoGreen assays in duplicates. Only DNA that passed quality control (QC) was for the DNA pools which were created by grouping DNA samples according to case/control status and sex yielding a total of four DNA pools (pool 1: 129 asthmatic girls, pool 2: 243 asthmatic boys, pool 3: 173 healthy girls, pool 4: 222 healthy boys).

2.3. Microarray analyses

The Human Mitochondrial Resequencing Array 2.0 (Affymetrix, UK) was used for mtDNA resequencing. All experimental procedures were performed according to the manufacturer's instructions. GeneChip® Operating Software 1.4 and GeneChip® Sequence Analysis Software 4.0 (GSEQ) were used to read out the arrays. Each of the four DNA pools was measured on 10 microarrays (array replication).

2.4. Statistical method

The arrays produce measures of fluorescent intensity, corresponding to the four nucleic acid bases A, T, G and C. On the Affymetrix chip each base is measured twice, once on the forward and once on the reverse strand (strand replication). With ten replicates per pool there is a maximum number of $4 \times 2 \times 10 = 80$ intensity measures per pool.

The primary interest with DNA pool data is in testing the difference in allele frequency between case and control pools. While pooling offers a substantial reduction in genotyping cost most of the analytical tests relying on allele frequency estimates have undesirable statistical properties when applied to DNA pools (Visscher and Le Hellard, 2003). Essentially, the additional variance generated by pooling-specific errors must be appropriately taken into account.

Here we adapted the method for the analysis of large DNA pools proposed by Macgregor et al. (2006) to be suitable for mtSNPs. The method estimates the pooling-associated variance and uses it to construct a test statistic with desirable statistical properties. Given the structure of the genotyping data, a simple test statistic, such as the *t*-test, which ignores this structure, is unsatisfactory. As for the structure, we have multiple measurements for every SNP due to case and control samples, as well as strand and array replication. Using a statistical test based on a general linear mixed model (GLMM) allows us to take that structure into account.

Let the alleles of a given nuclear SNP be represented by two of the four possible base pairs denoted by *A* and *B*, for example. The response variable in the linear model is the estimate of the proportion of *A* alleles in the sample. The original method computes the proportion as $p = A / (A + B)$ where *A* and *B* are intensity measurements for the major and minor allele, respectively. We had to adjust the formula of the proportion to read $p = A / (A + B + C + D)$ because in the mitochondrial genome there are not only two but four possible alleles of which *A*, *B*, *C*, and *D* are the intensity measurements and *A* is the major or reference allele.

The idea is to compare the proportion of the reference allele between cases and controls. Let us denote the proportion from sample *i*, replicate *j*, and strand *l* by p_{ijl}. With *C* samples (cases and controls), *R* array replicates, and *S* strand measures the vector $p = (p_{ijl})$ will contain $C \times R \times S$ values per mtSNP. The model for a measure p_{ijl} is given by

$$p_{ijl} = c_i + r_j + s_l + e$$

where c_i, r_j, and s_l are mixed effects for case/control status, array replicate, and strand, respectively, and *e* is an error term.

A test statistic correcting for the pooling error and following a chi-square distribution was used to test for significant SNPs. The test statistic is:

$$T_{2-x} = T_{simple} \times \frac{\widetilde{V}}{\widetilde{V} + 2var\left(e_{pool-2}\right) + var(\hat{c}_x)}$$

with T_{simple} equal to d^2 / V, where *d* is the mean value of allele frequency in the case pool minus the mean value in the control pool, and *V tilde* is the binomial sampling variance. $var(e_{pool\_2})$ and $var(\hat{c}_x)$ are the two components of the pooling error — see Appendix 2 of Macgregor et al. (2006) for full details.

The method was applied to our data in three separate analyses, each time considering different pools. First, we compared cases and controls irrespective of sex (pools 1 and 2 versus pools 3 and 4). Then, the analysis was stratified by sex and cases and controls were separately compared within girls (pool 1 versus pool 3) and within boys (pool 2 versus pool 4). In each of the analyses Bonferroni correction was applied to correct for multiple comparisons and where the correction factor was derived from the number of mtSNPs and the number of analyses performed.

2.5. Sample size

A sample size with sufficient statistical power is critical to the success of genetic association studies to detect causal genes of human complex diseases. McRae et al. (2008) have compared the power for detecting causal genetic variants in the mitochondrial genome with that