Association of CTLA4 and DEFB1 with Asthma Disease

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ABSTRACT

Asthma is a chronic inflammatory disorder of the airways, characterized by chronic airway inflammation and obstruction which is associated with recurrent episodes of breathlessness, wheezing, chest tightness, and coughing. It is mediated by T-helper 2 (TH2) cells and immunoglobulin E (IgE) in response to exposure of allergens. Two candidate genes - CTLA4 and DEFB1 are investigated. CTLA-4 is an important negative regulator of T cell activation, playing a major role in immunoregulation. DEFB1 produces defensin-β1, an antimicrobial peptide which inhibits TH2 cell differentiation. Two Single Nucleotide Polymorphisms (SNPs) were chosen for mass screening on 767 subjects via allele-specific Polymerase Chain Reaction (ASPCR). Statistical analysis was done to investigate the association with asthma and various interactions were examined via specified stratifications. None of the SNPs was found to be associated with asthma. However, when the subjects were stratified into gender specific, weak association is seen in CTLA4 with asthma. Gene-gene interaction between CTLA4 and DEFB1 showed association with asthma. This demonstrates the complexity of interactions involved in the pathogenesis of asthma.

INTRODUCTION

CTLA-4 (CD152) is expressed on the surface of the T cells after their activation (Jasek et al., 2006). Signaling through CTLA4 disrupt the balance of Th1/Th2, such that there is a higher proportion of Th2. Higher Th2 level is associated with allergic diseases like asthma (Oosterhout et al., 2004). In Vercelli’s review on asthma, CTLA4 has been studied extensively and been reported nine times having a positive association to asthma across different population.

Defensins are antimicrobial peptides that may take part in airway inflammation and hyperresponsiveness that plays an important role in innate immunity against infection. (Levy et al., 2005) It is also the most abundant cationic proteins in neutrophils. With respect to asthma, DEFB1 may contribute to airway inflammation via its chemo-attractant activity for dendritic cells, which is an important regulator of allergic immune responses (Duits et al., 2002; Prado-Montes de Oca et al., 2007).

Candidate gene studies have been studied in different populations across the world. In a recent review by Donata Vercelli, asthma susceptibility genes in overall can be grouped into four categories: genes associated with innate immunity and immunoregulation; genes associated with TH2-cell differentiation and effector functions; genes associated with epithelial biology and mucosal immunity; and genes associated with lung function, airway remodeling, and

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disease severity. In this research, gene associated with TH2-cell differentiation (CTLA4) and genes associated with epithelial biology and mucosal immunity (DEFB1) are focused.

**MATERIALS AND METHODS**

A total of 767 Chinese subjects in Singapore were included in this study. Ethnicity was self-declared by the individuals. The subjects were grouped into atopic asthmatic cases and control. Subjects are classified as case if they meet the following conditions: a) positive reactions to skin prick test; b) history of physician-diagnosed asthma.

**Collection of samples**

SPT was carried out on the interior of the subject’s forearm. The allergens includes dust mite *Dermatophagoides pteronyssinus*, dust mite *Blomia tropicalis*, fungus *Curvularia lunata* and oil palm pollen *Elaeis guineensis*. Histamine and saline were used as positive and negative control respectively. The positive result is determined by a presence of wheal diameter of at least three millimeters.

**Selection of Single Nucleotide Polymorphisms (SNPs)**

Single Nucleotide Polymorphisms (SNPs) are selected from LD block from an in-house sequencing database. The SNPs chosen for large scale screening in this study are CTLA4 -1147C/T, CTLA4 -1722A/G, DEFB1 -52A/G and DEFB1 692G/A. Genotyping is carried out using Allele Specific Polymerase Chain Reaction (ASPCR). The primers designed for ASPCR were either generated from WASP, a Web-based Allele Specific Primer designing tool, or manually generating using Gene runner version 3.01.

**Genotyping by ASPCR**

The PCR was performed in a 15µl reaction mixture containing 30ng of amplified DNA, 3µmol of each dNTP, 0.6U of Taq DNA polymerase (Fermentas, Glen Burnie, USA), 0.6X Taq buffer (750mM Tris-HCl pH 8.8 at 25°C, 200mM (NH₄)SO₄, 0.1% Tween 20), 30µmol of MgCl₂, and 3µmol of forward and reverse primers each. The PCR is done in MJR PTC-100 Thermal Cycler (GMI, Minnesota, USA) using the following condition: 95°C for 5 minutes as initial denaturing step, variable cycles (35-37 cycles) of 95°C for 30 seconds as denaturing step, variable temperatures (59°C - 63°C) for 30 seconds as annealing step and 72°C for 5 minutes as final extension step.

**Statistical Analysis**

Deviation from Hardy-Weinberg Equilibrium (HWE) was examined with chi-square test. z-score was calculated to evaluate allelic distributions, and chi-square goodness-of-fit test was used to analyze genotypic distributions. Fisher exact test was used when the number of subjects was five or less. Z-test was not performed if the total number of individuals involved in the comparison was less than 10. A p-value of 0.05 and below suggests significant differences.

**RESULTS AND DISCUSSION**

Both CTLA4 SNPs are in Hardy-Weinberg equilibrium. This shows that the genes are conserved through evolution. From the genotypic distribution, there is no significant difference
observed between the cases and controls. When the subjects are further stratified into gender-specific, there is some significance observed in the female subjects, suggesting that there is a gene-environment interaction. However, due to the skewed number of sample size towards the female, the weak significance could not be taken into account and hence, conclusion could not be drawn that there is an interaction between CTLA4 and gender which possibly give rise to association of the gene to disease.

As for DEFB1 SNPs, there is no significance difference observed in genotypic and allelic distribution comparison between cases and controls. It is noted that the allelic distribution is deviated from Hardy-Weinberg equilibrium, which is probably due to genetic drift in the population, gene flow, natural selection or mutation. The analysis shows that there is no association between DEFB1 with asthma. Then, the subjects are further stratified to gender to investigate whether there is a masking effect of gene-environment factor. However, result shows that there is no significant difference between the stratified cases and controls. Hence, there is no gene-gender interaction and no association between DEFB1 and asthma.

Further investigation was carried out on gene-gene interaction. There is an interaction between CTLA4 1722 and DEBF1 -52 observed (p-value=0.02). This shows that when these two genes interact, known as epistasis, the susceptibility for asthma disease increases. This finding is important such that although there is no strong association between the genes to the disease, the genes interacting with each other increases the susceptibility to the disease.

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