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# Genetic Basis for Adverse Events Following Smallpox Vaccination

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## Abstract

**Background—**Although vaccinia immunization is highly effective in preventing smallpox, post-vaccination reactions are common. Identifying genetic factors associated with AEs might allow screening before vaccinia administration and provide a rational basis for the development of improved vaccine candidates.

**Methods—**Two independent clinical trials in healthy, vaccinia-naïve adult volunteers were conducted with the Aventis Pasteur smallpox vaccine (APSV). Volunteers were assessed repeatedly for local and systemic AEs to vaccine and were genotyped using the same panel of 1442 single-nucleotide polymorphisms (SNPs).

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**Results—**In the first study, thirty-six SNPs in 26 genes were associated with systemic AEs (p-value ≤ 0.05). In the second study, only those SNPs associated with AEs in the first sample were tested. In the final analysis, three SNPs were associated consistently with AEs in both studies. A nonsynonymous SNP in methylenetetrahydrofolate reductase (*MTHFR*) was associated with AE risk in both trials (odds ratio [OR]; 95% confidence interval [CI]); p-value [p]): (OR=2.3; CI=1.1–5.2; p=0.04) and (OR=4.1; CI=1.4–11.4; p<0.01). Two SNPs in the interferon regulatory factor 1 (*IRF1*) gene were associated with AE risk in both sample sets: (OR=3.2; CI=1.1–9.8; p=0.03) and (OR=3.0; CI=1.1–8.3; p=0.03).

**Conclusions—**Genetic polymorphisms in an enzyme previously associated with adverse reactions to a variety of pharmacologic agents (*MTHFR*) and an immunological transcription factor (*IRF1*) were associated with AEs after smallpox vaccination in two independent study samples. These findings highlight common genetic variants with promising clinical significance that merit further investigation.

### Keywords

adverse events; vaccination; smallpox; genetics; epidemiology

## INTRODUCTION

Although reactions following inoculation with vaccinia virus were common in the recent population-wide vaccination programs [1], the biological basis for these adverse events (AEs) is not well understood. The performance of two independent clinical studies of a single vaccinia vaccine at our study site afforded us the unique opportunity to assess genetic factors that might predict systemic AEs. All of the vaccinia-naïve subjects enrolled developed pock formation at the vaccination site, and a subset experienced systemic reactions including fever, rash or regional lymphadenopathy. Since poxviruses have evolved multiple mechanisms to evade host immune responses, such as targeting of primary innate immunity and manipulating intracellular signal transduction pathways [2], we questioned whether subjects encountering AEs exhibited unique genetic polymorphisms in these pathways that made them more susceptible to these reactions.

In earlier studies, we characterized humoral and cellular immune responses and outlined patterns of systemic cytokine expression following smallpox vaccination [3–8]. In the current report, we utilized data collected during two independent studies to identify stable genetic factors associated with AEs. Since many genetic association studies fail to replicate during subsequent studies, we sought to repeat the assessment on an additional study group [9,10]. Independent replication of the results of our first study with the second strengthens the plausibility of these genetic associations. An identical panel of candidate single-nucleotide polymorphisms (SNPs) was evaluated in each of the studies. Subjects with systemic AEs including fever, lymphadenopathy, or generalized acneiform rash, were compared with those who did not experience these reactions. For both studies, the data were genotypes at 1442 SNPs across at least 386 candidate genes. This investigation provides important preliminary findings in two independent data sets addressing the contribution of common genetic variants to a complex clinical phenotype, which also bears substantial importance with respect to public health.

## METHODS

### Study Subjects

Vaccines, study subjects, and study design for both of the clinical trials have been described previously in detail. Both trials were conducted at Vanderbilt University in the NIH-funded

Vaccine and Treatment Evaluation Unit (VTEU) [4,8,11]. The first study [7] enrolled 85 healthy vaccinia-naïve adults in genotyping studies and the second study [11] also enrolled 46 healthy vaccinia-naïve adults. In both studies, individuals were asked to self-identify ethnic background. Both studies complied with the Internal Review Board policies of Vanderbilt and the NIH, and written consent was obtained for all individuals.

## Clinical Assessments

For both studies, the same team of trained physicians and nurses used the same forms to obtain medical history and to record local and systemic AEs after vaccination. Subjects were examined at regular intervals (days 3–5, 6–8, 9–11, 12–15, and 26–30 after vaccination). Local and systemic AEs were recorded. Subjects with an oral temperature of greater than 38.3 °C anytime during the study, generalized skin eruptions on non-contiguous areas to the site of vaccination [11], or enlarged or tender regional lymph nodes associated with vaccination were defined as those experiencing systemic AEs.

## Identification of Genetic Polymorphisms

We used a previously described custom SNP panel based on the NCI SNP500 Cancer project [12]; specifically, this panel targets investigation of soluble factor mediators and signaling pathways, many of which have known immunological significance [13]. There is a heavy weighting towards non-synonymous SNPs in this panel (*i.e.*, those that result in an amino acid substitution). Genotyping for single nucleotide polymorphisms (SNPs) was performed using DNA amplified directly from EBV-transformed B cells generated from peripheral blood samples collected from each subject. Genotyping was performed at the Core Genotyping Facility of the National Cancer Institute (NCI) in Gaithersburg, MD. Genotypes were generated using the Illumina™ GoldenGate assay technology. Of the 1536 SNPs assayed, a total of 1442 genotypes passed quality control filters for both the first and second sample sets. A complete list of the SNPs examined in this study is found in Supplemental Table 1.

## Statistical Analysis

Demographic characteristics including age, gender, and race were compared between the first and second study using Student's t-test (for age) and two-sample tests of proportions (for AE status, gender, and race). Allele frequencies were estimated from the total number of copies of individual alleles divided by the number of all alleles in the sample, and compared between the two studies using a two-sample test of proportions. Deviations in the fitness for Hardy-Weinberg proportion were evaluated using the exact test described in Wigginton *et al* [14].

We chose a two-stage design for identifying and replicating genetic associations in the independent clinical trials. This study design was selected with the goal of minimizing Type I errors (false positives). For comparison, we also performed the genetic association analysis in a single pooled sample. In the first study, potential associations were tested between each of the 1442 SNPs passing quality control filters and the occurrence of AEs using logistic regression. For each SNP in the first sample set, we recorded the odds ratio estimate and p-value of the likelihood ratio test for a univariate logistic model. No correction for multiple comparisons was made in our first set, because we reserved the second study sample set for determination of probable true positives. In the second sample set, we tested only those SNPs having an AE-associated p-value $\leq 0.05$ in the first study. We considered a significant SNP association in the first study to have replicated if it met the following criteria in the second study: an odds ratio that consistently associated AE risk with the same genotypes and a p-value $\leq 0.05$. To obtain an empirical probability of meeting our replication criteria purely by chance, we generated 1,000 simulated data sets from both study sample sets by permuting case-control labels. An additional association with p-value 0.06 is discussed below because of its high biologic plausibility.

Patterns of linkage disequilibrium (LD) between replicated SNPs on the same chromosome were assessed using Haploview [15]. Haplotypes were inferred for SNPs in high LD using the iterative approach described in Lake *et al* [16]. The resulting haplotypes were tested for association with AEs using univariate logistic models. Statistical analyses and simulations were performed using R version 2.5.1, Stata version 9 (Stata Corp, College Station, TX), and Haploview version 3.32 [15,17,18].

## RESULTS

### Demographic Characteristics of Subjects Included in Genetic Analysis

In both studies, all participants were invited to donate genetic samples. In the first study, of the 148 vaccinia-naïve participants enrolled in the clinical trial, a total of 96 individuals gave consent for the genetic substudy. Of those 96 subjects with genetic data, 16 experienced *systemic* AEs following immunization. An additional 11 genotyped subjects who reported only a localized rash near the inoculation site were removed from the analysis to focus only on systemic AEs. The other 69 reporting no AEs were used as controls. Thus the first study included analysis of 85 subjects. In the second study, which included 48 vaccinia-naïve healthy adults, 46 gave consent for genotyping and were enrolled. Of the 46 individuals, 24 experienced systemic AEs.

Table 1 summarizes age, race, gender, and AE status decompositions of both studies. Table 1 also describes the results of the demographic comparisons between the first and second studies. As the table indicates, there was no statistical difference in age, gender, or race between the two study populations. In the first study, 40 (47%) individuals were male, 84 (99%) were white and 1 (1%) was Asian. In the second study, 27 (59%) individuals were male, 44 (96%) were white, 1 (2%) was black, and 1 (2%) was Asian.

### Genetic Associations with Adverse Events

A total of 36 SNPs (within 26 genes) that showed significant associations in the first study were tested for potential associations in the second study. Three variant genotypes were confirmed to be associated with AEs in the second study. These included one SNP in *MTHFR* ($p < 0.01$) and two SNPs in *IRF1* ($p = 0.03$). The strong significance of the association in the replication study suggested a high level of plausibility that the gene products were involved in the pathogenesis of the AEs. The results of our simulation study indicated that the probability of meeting our replication criteria (an odds ratio that consistently associated AE risk with the same genotypes and a p-value $\leq 0.05$) entirely by chance was $p < 0.001$. It is important to note that we also reanalyzed the data as a single pooled sample and found the same pattern of statistically significant associations. The statistical results that replicated in the second study are shown alongside those from the first study in Table 2.

Three SNPs in a third gene, *IL4*, had p-values equal to 0.06 in the second study. While not significant using a strict requirement for $p \leq 0.05$, we thought this association of great interest because of the prior biologic studies showing a central role for this cytokine in poxvirus biology [19–21]. Considering the reduced size of the second sample and the fact that the AE risk associated with variant genotypes was consistent across studies, these *IL4* SNPs warrant further study, because additional variants in linkage disequilibrium could also be associated with AE outcomes (Table 3).

The SNPs located in *IRF1* and *IL4* are located in the same chromosomal region (5q31.1), suggesting an indirect association with one or more functional variants in that region. Because of the close physical proximity of the associated variants in the two genes, Haploview [15] software was used to examine the patterns of LD among those variants in each sample. Figure

1 shows that the LD plots for SNPs in the two genes follow the same pattern in each study sample. While there is strong LD between SNPs within the two genes, there is little evidence for LD between the two genes, indicating that the associations for each gene are statistically separate signals.

This region of chromosome 5q31 contains discrete haplotype blocks [22]. Accordingly, haplotypes were inferred for AE-associated SNPs in *IRF1* (rs839 and rs9282763) and *IL4* (rs2070874, rs2243268, rs2243290). In both studies, two *IRF1* haplotypes accounted for all subjects. The common *IRF1* haplotype listed in Table 4 represented 71% of the first sample set and 63% of the second sample set. The rare *IRF1* haplotype was significantly associated with AEs in both studies ($p = 0.03$). Across both studies, two different three-SNP haplotypes in *IL4* accounted for 99% of subjects. The common *IL4* haplotype listed in Table 4 represented 78% of the first set and 87% of the second set. The rare *IL4* haplotype was significantly associated with risk of AEs in the first study ($p = 0.05$); the association was similar in the second study ($p = 0.06$).

## DISCUSSION

The candidate genes identified with the strongest association with AEs in both studies include a metabolism gene previously associated with adverse reactions to a variety of pharmacologic agents (*MTHFR*) and an immunological transcription factor (*IRF1*). The statistical results from these studies have strong biological plausibility and are in agreement with previous work on the immune response to poxviruses.

### MTHFR

A SNP in the 5,10-methylenetetrahydrofolate reductase (*MTHFR*) gene (rs1801133) was associated strongly with AE risk in both studies. This non-synonymous SNP in exon 5 causes an amino acid change from alanine to valine, and functional characterization of this SNP demonstrated that it is thermolabile and affects both the quantity and activity of the MTHFR enzyme [23]. The enzyme catalyzes the conversion of 5,10-methylenetetrahydrofolate to 5-methyltetrahydrofolate, which is a co-substrate for homocysteine remethylation to methionine. *MTHFR* function provides pools of methyl groups that are crucial for the control of DNA synthesis and repair mechanisms [24]. *MTHFR* is a key enzyme in homocysteine metabolism, which plays a major role in regulating endothelial function. It may be of interest in the future to examine the association of genetic variation in this gene with the rare cardiac events that occur after vaccination.

Genetic variation of *MTHFR* has been associated with a range of clinical outcomes, including altered cardiovascular function, organ transplantation, toxicity of immunosuppressive drugs, and systemic inflammation [25–28]. Elevated plasma homocysteine levels stimulate endothelial inflammatory responses, which could contribute to systemic AEs. Alternatively, since vaccination elicits immune responses involving the rapid proliferation of cells, demand for DNA synthesis metabolites would be elevated, and alterations in the level or activity of *MTHFR* enzyme may exert significant influence over this process.

### Interferon regulatory factor-1

The interferon regulatory factor-1 (*IRF1*) gene is part of the immunological gene cluster on chromosome 5q31. We found two SNPs in *IRF1* that are significantly associated with AEs in both study samples. The *IRF1* gene encodes an important member of the interferon regulatory transcription factor (IRF) family. The IRF family regulates interferons and interferon-inducible genes. *IRF1* activates transcription of the Type I interferons α and β as well as genes induced

by the Type II interferon γ [29]. Many viruses target IRFs to evade host immune responses by binding to cellular IRFs and blocking transcriptional activation of IRF targets [30].

Polymorphisms in the gene coding for a transcription factor with such far-reaching effects as *IRF1* could have profound effects on the proper immune response and clearance of vaccinia virus. Mice deficient in interferon receptors are especially susceptible to vaccinia virus infection, suggesting an important role for these molecules in controlling vaccinia infection [31]. Vaccinia dedicates several host modifying genes to counteracting interferons. For example, the viral gene B18R encodes a protein that serves as a viral IFN-α/β binding protein that binds interferons from several species [32]. This protein also can bind to the cell surface after secretion, thus preventing host interferon from binding to cellular interferon receptors [33]. Although the SNPs identified in *IRF1* and *IL4* do not change amino acids in the encoded proteins, recent evidence suggests that synonymous SNPs, such as rs839, can alter regulation of mRNA or splice junctions [34,35]. It is also plausible that one or both SNPs are in LD with the causal variant not tested in this study.

## Interleukin-4

Genetic polymorphisms in this major cytokine gene involved in adaptive immunity to viruses also may be associated with AEs, however with a p-value of 0.06 in our relatively small replication study. We found three SNPs in *IL4* that may be associated with AEs in both studies. There was high intragenic LD ($r^2 > 0.9$) between the tested SNPs within each gene, *IRF1* and *IL4*, and haplotypes inferred separately for each of these genes mirrored the significant risk patterns of the SNPs observed individually. Thus, the fact that multiple SNPs in high LD were identified in regions of *IRF1* and *IL4* strongly suggest that there are additional markers in LD, several of which could functionally contribute to the risk for AEs.

The *IL4* gene encodes a pleiotropic cytokine produced by a variety of immune cells, especially activated T cells. *IL4* controls humoral immune responses, isotype switching, and suppression of cytotoxic T cell function and expansion. Thus, genetic polymorphisms related to inappropriate regulation of *IL4* expression and/or activity of IL-4 cytokine could be associated with over-stimulated inflammatory responses leading to the development of clinical AEs. Previous studies on the role of *IL4* in poxvirus pathogenesis have shown it to have a central role in altering the adaptive immune response. *IL4* over-expression during infection with recombinant poxviruses encoding *IL4* suppresses the induction of cytotoxic T cell activity by inhibiting CD8+ T cell proliferation, which increased the pathogenicity of such recombinant viruses even in previously immunized animals [36]. *IL4* also plays a role in preventing optimum innate immune responses to poxviruses. IL-4 secretion during vaccinia virus infection of individuals with atopic dermatitis alters the cytokine milieu, resulting in a block of production of the antimicrobial peptide LL-37, accounting in part for the increased risk of vaccinia virus infection in subjects with atopic dermatitis [37].

## Model of pathogenesis

Since the outcome of interest here was the aggregation of specific AEs, it is logical that more than one gene may be involved. The genes with variants for which we discovered an association with AEs are all potentially involved in pathways that are in line with our previously hypothesized mechanism of AEs involving excess stimulation of inflammatory pathways and the imbalance of tissue damage repair pathways. This model was developed from studies of circulating cytokines and relevant immunological effector cells [3–5]. For subjects experiencing AEs, vaccination appears to trigger an acute inflammatory response that is excessive. Antigen presentation to T cells in the dermis leads to the release of T-cell cytokines that trigger a cascade of cytokines and chemokines whose release enhances the inflammatory response by promoting the migration of monocytes into the lesion and their maturation into

macrophages and by further attracting T cells [38,39]. Taken together, these previous findings suggest that systemic AEs following smallpox vaccination may be consistent with low-grade macrophage activation syndrome caused by virus replication and vigorous tissue injury and repair.

There are limitations to this study. The subject numbers are small for a genetic association study of low-penetrance high-frequency alleles. The association of the *IL4* variations with AEs was weaker than that of the other genes. Nevertheless, findings of the same variants in two independent clinical trials, the high biologic plausibility of these associations in light of what is known about poxvirus biology, and the potential public health significance suggest the findings are of interest.

### Conclusions and Future Directions

These data present the rare opportunity to study two independent cohorts of smallpox vaccinees relating common genetic variation to the occurrence of post-vaccination AEs. Statistical analysis of the first study revealed potentially significant associations between SNPs in biologically interesting candidate genes. Of the AE-associated genes identified in the first study, two replicated in an independent study, with one additional candidate gene just beyond our statistical significance cut-off but with a high level of biologic plausibility. It is possible that our findings could be due to chance, but we avoided multiple testing issues by testing only the most promising results in the validation sample. While all SNPs were tested in the first study, only those SNPs significantly associated with AEs were tested in the second study, and our empirically derived probability of replication by chance alone was less than 0.1%. The association of SNPs in three genes across both studies and their biologically plausible connection with AEs lends credence to the reproducibility of these associations.

As with any statistical association, follow-up studies are needed to identify the particular genetic susceptibility variants and examine the functional consequences of polymorphisms in the AE-associated genes. Since we found multiple AE-associated SNPs in regions of *IRF1* and *IL4*, focused studies should be undertaken to characterize the genetic variability in these candidate regions. Indeed, haplotypes in *IRF* and *IL4* displayed altered susceptibility to a specific systemic AE (fever) after smallpox vaccination [40]. While the association of AEs with a non-synonymous polymorphism in the gene for *MTHFR* points toward functional significance of this SNP, fine mapping of this locus should determine whether this is indeed the case. For all three candidate genes, both follow-up replication and functional studies are needed to establish the plausibility of the association of common genetic polymorphisms with the hypothesized etiological pathways.

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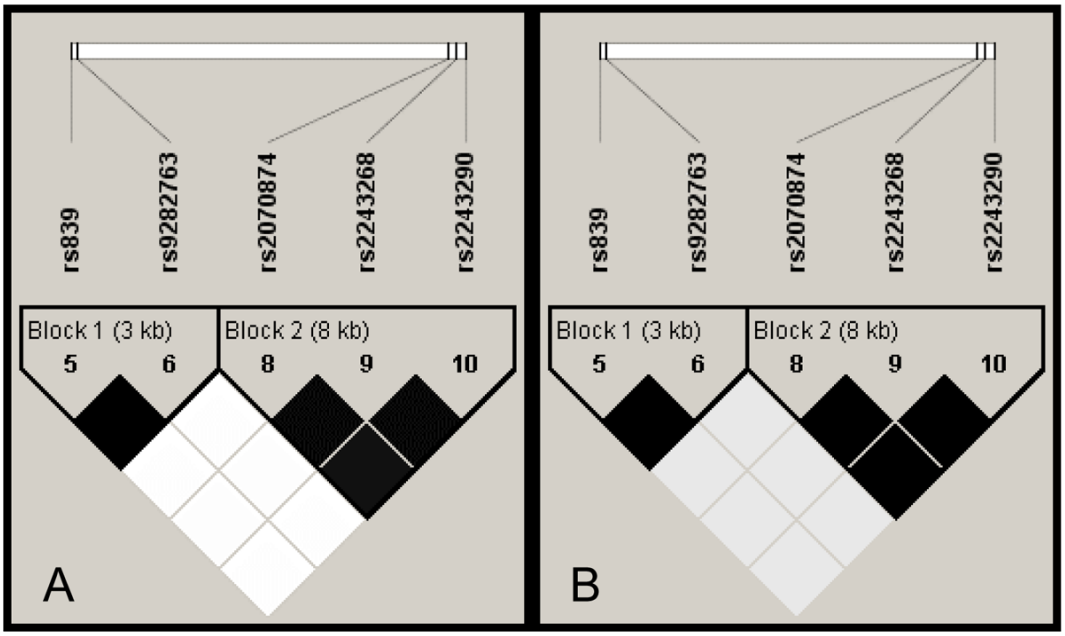


**Figure 1. Haploview plot of SNPs at chromosome 5q31.1**

Panel A =first study; panel B =second study. Squares are shaded to indicate strength of evidence for LD between the pairwise markers. Dark = strong evidence ($r^2 > 0.90$), light gray = weak evidence ($r^2 < 0.10$), white = no evidence ($r^2 < 0.0$). The same two LD blocks are apparent in both studies, encompassing SNPs in *IRF1* (rs839 and rs9282763) or *IL4* (rs2070874, rs2243268, and rs2243290).

**Table 1**

Summary of AE status, age, gender, and race for both studies.

| Dataset | AE/nonAE | Age[a] | Gender (M/F) | Race (W/B/A)[b] |
|---|---|---|---|---|
| First study (*N* = 85) | 16/69 | 23.2 (3.9) | 40/45 | 84/0/1 |
| Second study (*N* = 46) | 24/22 | 24.2 (3.8) | 27/19 | 44/1/1 |
| [c] P-value of difference | < 0.01 | 0.15 | 0.20 | 0.25 |

[a] Mean (standard deviation)

[b] W = white, B = black, A = Asian

[c] Two-sided p-value for t-test (age) or two-sample test of proportions (AE/nonAE, gender, race)

**Table 2**

Genetic polymorphisms associated with AEs in both studies.

| Gene | SNP (rs#) | SNP Location (Base pair) [a] | Chromosomal Location | First Study | | Second Study | |
|---|---|---|---|---|---|---|---|
| | | | | Odds Ratio[b] | p-value ($X^2$)[b] | Odds Ratio[b] | p-value ($X^2$)[c] |
| *MTHFR* | 1801133 | 6393745 | 1p36.3 | 2.3 (1.1–5.2) | 0.04 | 4.1 (1.4–11.4) | < 0.01 |
| *IRF1* | 9282763 | 34237146 | 5q31.1 | 3.2 (1.1–9.8) | 0.03 | 3.0 (1.1–8.3) | 0.03 |
| | 839 | 34234139 | 5q31.1 | 3.2 (1.1–9.8) | 0.03 | 3.0 (1.1–8.3) | 0.03 |

[a] Base pair according to dbSNP (NCBI Human Genome Build 36.1).

[b] Estimated odds ratio (95% confidence interval)

[c] Likelihood ratio chi-square ($X^2$) test with one degree of freedom

**Table 3**

Distribution of genotypes at SNPs in *MTHFR*, *IRF1*, and *IL4*.

| Gene | SNP (rs #) | SNP Location (Base Pair) | Genotype | First Study Count (Percent) | Second Study Count (Percent) |
|---|---|---|---|---|---|
| ***MTHFR*** | 1801133 | 6393745 | CC | 36 (42) | 18 (39) |
| | | | CT | 39 (46) | 21 (46) |
| | | | TT | 10 (12) | 7 (15) |
| ***IRF1*** | 9282763 | 34237146 | AA | 39 (46) | 17 (37) |
| | | | AG | 43 (51) | 24 (52) |
| | | | GG | 3 (4) | 5 (11) |
| | 839 | 34234139 | GG | 39 (46) | 17 (37) |
| | | | AA | 43 (51) | 24 (52) |
| | | | AG | 3 (4) | 5 (11) |
| ***IL4*** | 2070874 | 34424723 | CC | 52 (62) | 34 (74) |
| | | | CT | 28 (33) | 12 (26) |
| | | | TT | 4 (5) | 0 (0) |
| | 2243268 | 34428976 | AA | 52 (62) | 34 (74) |
| | | | AC | 27 (32) | 12 (26) |
| | | | CC | 5 (6) | 0 (0) |
| | 2243290 | 34433182 | CC | 53 (62) | 34 (74) |
| | | | AA | 26 (31) | 12 (26) |
| | | | AC | 6 (7) | 0 (0) |

**Table 4**

Haplotypes inferred for AE-associated SNPs in *IRF1* (rs839 and rs9282763) and *IL4* (rs2070874, rs2243268, rs2243290).

| Gene | SNP (rs#) | Baseline Haplotype[a] | Risk Haplotype[b] | First Study | | Second Study | |
|---|---|---|---|---|---|---|---|
| | | | | Odds Ratio[c] | p-value ($X^2$) [d] | Odds Ratio[c] | p-value ($X^2$) [d] |
| ***IRF1*** | 9282763 | A | G | 3.2 (1.0–10.2) | 0.03 | 3.0 (1.0–9.0) | 0.03 |
| | 839 | G | A | | | | |
| ***IL4*** | 2070874 | C | T | 2.4 (1.0–5.7) | 0.05 | 3.8 (1.0–14.4) | 0.06 |
| | 2243268 | A | C | | | | |
| | 2243290 | C | A | | | | |

[a] Most common haplotype considering 2 SNPs in *IRF1* or 3 SNPs in *IL4*

[b] Rare (variant) haplotype considering 2 SNPs in *IRF1* or 3 SNPs in *IL4*

[c] Estimated odds ratio comparing risk haplotype to baseline haplotype (95% confidence interval)

[d] Likelihood ratio chi-square ($X^2$) test with one degree of freedom