# Polymorphisms of STAT-6, STAT-4 and IFN-γ genes and the risk of asthma in Chinese population

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**Summary**

*Background:* Asthma is a complex disease resulting from multiple gene–gene and gene–environment interactions. Study on gene–gene interactions could provide insight into the pathophysiologic mechanisms of the disease.

*Objectives:* We investigated the single nucleotide polymorphisms and interactions among three different loci in three candidate genes (STAT-6 G2964A, STAT-4 T90089C and IFN-γ T874A) in 95 Chinese asthmatic subjects and 95 matched controls to determine the possible associations with asthma.

*Methods:* Genotyping of the gene polymorphisms was performed by means of PCR-SSCP analysis. Genotype–phenotype associations were examined in dominant and recessive genetic models using logistic regression. The method of multifactor dimensionality reduction was used to analyze gene–gene interactions.

*Results:* No statistically significant difference was found in the distribution of the STAT-6 G2964A polymorphisms between asthmatic patients and controls in this case–control study. The STAT-4 T90089C polymorphisms were significantly associated with asthma in the dominant model ($p = 0.007$). As for the IFN-γ T874A, the significant associations were found in both dominant model ($p = 0.004$) and recessive model ($p = 0.006$). A significant gene–gene interaction was found among STAT-6, STAT-4 and IFN-γ on the risk of asthma. In the best 3-locus model, the odds ratio for the high-risk to the low-risk group was 6.9 (95% CI, 3.5–13.7; $p < 0.0001$).

*Conclusions:* Our findings suggest that STAT-4 T90089C and IFN-γ T874A polymorphisms might be the genetic factors for the risk of asthma in the Chinese population. In addition, the significant interactions among STAT-6 G2964A, STAT-4 T90089C and IFN-γ T874A may increase an individual's susceptibility and contribute to the pathogenesis of asthma.

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## Introduction

Asthma is one of the most common respiratory diseases caused by acute and chronic bronchial inflammation that result in variable airway obstruction.[1,2] The inflammatory response of asthma is tightly associated with an immune disorder characterized by IgE production and an imbalance between T helper cell 1 (Th1) and T helper cell 2 (Th2), both of which have a strong genetic component.[3–5]

The signal transducer and activator of transcription 6 (STAT-6) is critical for effects mediated by IL-4, including induction of Th2 and IgE responses, and antigen-induced airway inflammation and hyperresponsiveness.[6–9] A few groups have already studied the association between the polymorphisms in the 3′ untranslated region of the STAT-6 gene and asthma.[10–13] However, the results of these studies are not consistent. The signal transducer and activator of transcription 4 (STAT-4) is known to be essential for mediating responses of IL-12 in lymphocytes and for regulating the differentiation of T helper cells.[8,14,15] IFN-$\gamma$, a representative TH1 cytokine, also plays an important role in the induction of immune-mediated inflammatory responses of asthma.[16–19] However, thus far, little is known about the associations of the STAT-4 and IFN-$\gamma$ gene polymorphisms with asthma.

In this study, we investigated the single nucleotide polymorphisms of three genes [G2964A in 3′ untranslated region (the exon 23) of STAT-6, T90089C in intron 11 of STAT-4 and T874A in intron 1 of IFN-$\gamma$] in asthmatic patients and controls in a Chinese population to determine the possible associations between any of these three genes and asthma. Gene–gene interactions were also examined with recently developed multifactor dimensionality reduction (MDR) software.[20–22]

## Materials and methods

### Study population

One hundred and eighty unrelated subjects (95 asthmatic subjects and 95 healthy controls) from Chongqing in China were examined. The mean age was 39 years (range: 22–75 years). There were 50 men and 45 women in two groups. Each asthmatic subject was diagnosed by a specialist physician. The subjects meeting the following criteria for asthma were recruited: (1) at least two symptoms consistent with asthma (cough, wheeze, and shortness of breath); (2) a variation of $\geqslant$20% in diurnal peak expiratory flow (PEF) recording (reference to maximal value); (3) an increase of $\geqslant$15% in PEF or forced expiratory volume of 1 s (FEV$_1$) with a $\beta_2$-agonist; (4) plasma total IgE levels were elevated, and specific IgE was positive. Plasma total IgE levels were detected by ELISA kit (Biotinge, Beijing, China). Specific IgE was detected by Allergy Screen test kit (Arlington Scientific, Utah, USA), which provides a qualitative in vitro assay for human antibodies of the IgE class to seven different inhalation allergens. We selected controls who met the following criteria: (1) healthy subjects matching on age, sex and residential region with asthmatic subjects; (2) no history of allergic disease; (3) normal levels of plasma total IgE and negative for specific IgE. Both patients and controls were ethnic Chinese Han. Informed consent was obtained from all subjects.

## Molecular methods

Anticoagulated peripheral blood was obtained from all subjects. DNA was extracted from blood leucocytes. Genotyping of the gene polymorphisms was performed by PCR-SSCP analysis. PCR primers were designed to amplify a fragment containing each variant. Primers for STAT-6 G2964A variant: forward, 5′-GGAGCCAATCCACTCCTTCC-3′; and reverse, 5′-CAGACTCCTCCTATGCTCCC-3′. Primers for IFN-$\gamma$ T874A variant: forward, 5′-gctgttataattatagctgtc-3′; and reverse, 5′-TCA ACAAAGCTGATACTCCA-3′. Primers for STAT-4 T90089C variant: forward, 5′-TGTGGAAACTTTGTT-GCTGCTCT-3′; and reverse, 5′-GCCAAACTTACCAAATGTC-GAAAT-3′.

PCR reactions were performed in a total volume of 50 µl containing 50 ng of DNA, each dNTP at 125 µmol, 2 U of Pfu DNA polymerase (Promega, Wisconsin, USA), buffer, and 10 pmol of forward and reverse primer. The cycle conditions were 95 °C for 5 min, and then 40 cycles of 95 °C for 30 s, 60 °C (STAT-6) or 58 °C (STAT-4) or 53 °C (IFN-$\gamma$) for 30 s, and 72 °C for 30 s, with a final extension step of 7 min at 72 °C, in a MyCycler Thermal Cycler (Bio-Rad, California, USA). After PCR, fragment lengths were determined by agarose gel eletrophoresis.

The amplified products were fractionated by electrophoresis on polyacrylamide gels at 20 °C for 2 h in a Mini-PROTEAN 3 Cell (Bio-Rad, California, USA). The gels were visualized by silver staining. Samples from two known homozygotic individuals and one heterozygotic individual, as confirmed by sequencing, were included in each reaction.

## Statistical analysis

Allele frequencies were estimated using the gene-counting method. The $\chi^2$ goodness-of-fit test was used to examine the Hardy–Weinberg equilibrium. Genotype–phenotype associations were examined with dominant and recessive genetic models. Three genotypes were collapsed into two groups (e.g. STAT-6 G2964A, dominant model: AA+GA versus AA; recessive model: GG versus GA+AA). The associations were evaluated using logistic regression. Odds ratios (OR) for the risk of asthma and their 95% confidence intervals (CI) were calculated. All $p$-values are two-tailed. Logistic regression analysis was also performed to confirm the results from MDR analyses. A $p$-value of $<0.05$ was considered statistically significant. All above-mentioned calculations were performed with SPSS version 12.0.1 software.

MDR was used to determine the gene–gene interactions.[20–22] This method includes a combined cross-validation/permutation-testing procedure that minimizes false-positive results.[23] For each combination of a pool of genetic polymorphisms, cross-validation consistency (CVC) and testing accuracy were calculated. Cross-validation divides the data into a training set and a testing set. With 10-crossabilityold cross-validation, the data are divided into 10 equal parts, and the model is developed on 9/10 of the data (training set) and then tested on 1/10 of the remaining data (testing set). This is repeated for each possible 9/10

and 1/10 of the data, and the resulting 10 testing accuracies are averaged. CVC is a measure of how many times out of 10 divisions of the data that MDR found the same best model. Permutation testing was performed to assess the probability of obtaining a testing accuracy as large or larger than observed in the original data given the null hypothesis of no association is true. This is carried out by randomizing the case–control labels 1000 times and repeating the MDR analysis on each randomized dataset. This process yields an empirical distribution of testing accuracies under the null hypothesis that is in turn used to calculate a $p$-value. The model was statistically significant when the $p$-value derived from the permutation test was 0.05 or less. We used CVC, testing accuracy and statistical significance to select the best model. The best model should be significant and have a maximum CVC and a higher testing accuracy. The specific high- and low-risk genotypes in the model were also determined by MDR. The high-risk genotypes for asthma were defined as if the ratio of the number of patients to control subjects was equal to or greater than the threshold of 1.0. Whereas low-risk groups were defined as if the threshold was lower than 1.0. The MDR analysis was carried out using version 1.0.0 of the open-source MDR software package that is freely available (http://www.epistasis.org).

## Results

### Asthma and individual polymorphisms

The genotypes of all three polymorphisms in three genes were distributed according to Hardy–Weinberg equilibrium ($p > 0.05$) in the control group. The frequencies of the genotypes are listed in Table 1. In the same table, the results of the association analysis using the dominant and recessive genetic models are given. No statistically significant difference was found in the distribution of the STAT-6 G2964A polymorphisms between asthmatic patients and controls. However, The STAT-4 T90089C polymorphisms were significantly associated with asthma in the dominant model

($p = 0.007$). As for the IFN-$\gamma$ T874A polymorphisms, the significant associations were found in both dominant model ($p = 0.004$) and recessive model ($p = 0.006$).

### Gene–gene interactions analysis

Table 2 shows the results of CVC, training accuracy and testing accuracy obtained from MDR analysis of the data. The 3-locus model was significant and had a maximum CVC and a higher testing accuracy. This 3-locus model consisted of the G2964A in STAT-6, T90089C in STAT-4 and T874A in IFN-$\gamma$. The combinations of low- and high-risk groups were classified in this model (Fig. 1). The OR for the high-risk to the low-risk group was 6.9 (95% CI, 3.5–13.7; $p < 0.0001$). A significant interaction among G2964A in STAT-6, T90089C in STAT-4 and T874A in IFN-$\gamma$ on the risk of asthma was also confirmed by logistic regression analysis ($p = 0.04$).

## Discussion

Studies on association between the polymorphisms of the STAT-6 gene and asthma have recently been reported. Gao et al.[11] found there was a strong association between G2964A polymorphisms and mild atopic asthma in a

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**Table 2** Summary of MDR results.

| Model | Training accuracy | Testing accuracy | $p$-value* | CVC[†] |
|---|---|---|---|---|
| STAT-6 and STAT-4 | 0.6497 | 0.5579 | 0.0547 | 9/10 |
| STAT-6, STAT-4 and IFN-$\gamma$ | 0.7175 | 0.5947 | 0.0107 | 10/10 |

*1000-fold permutation test.
[†]Cross-validation consistency.

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**Table 1** Distribution of genotypes in asthmatic patients and controls.

| Polymorphisms | Asthmatic patients ($n = 95$) (%) | Controls ($n = 95$) (%) | $p$-value* OR (95% CI)[†] |
|---|---|---|---|
| STAT-6 G2964A | | | 0.26 |
| GG | 36.8 | 40.0 | 2.067 (0.98–4.37) |
| GA | 37.9 | 44.2 | 1.18 (0.64–2.19) |
| AA | **25.3** | 15.8 | |
| STAT-4 T90089C | | | 0.05 |
| TT | 33.7 | 38.9 | 1.59 (1.14–2.22) |
| TC | 29.5 | 40.0 | 1.39 (0.74–2.60) |
| CC | 36.8 | 21.1 | |
| IFN-$\gamma$ T874A | | | 0.006 |
| TT | 35.8 | 55.8 | 3.14 (1.45–6.80) |
| TA | 35.8 | 31.6 | 2.30 (1.28–4.13) |
| AA | 28.4 | 12.6 | |

[†]OR (95% CI) for the risk of asthma were obtained by logistic regression; the top OR is for dominant genetic model and the bottom for recessive model.
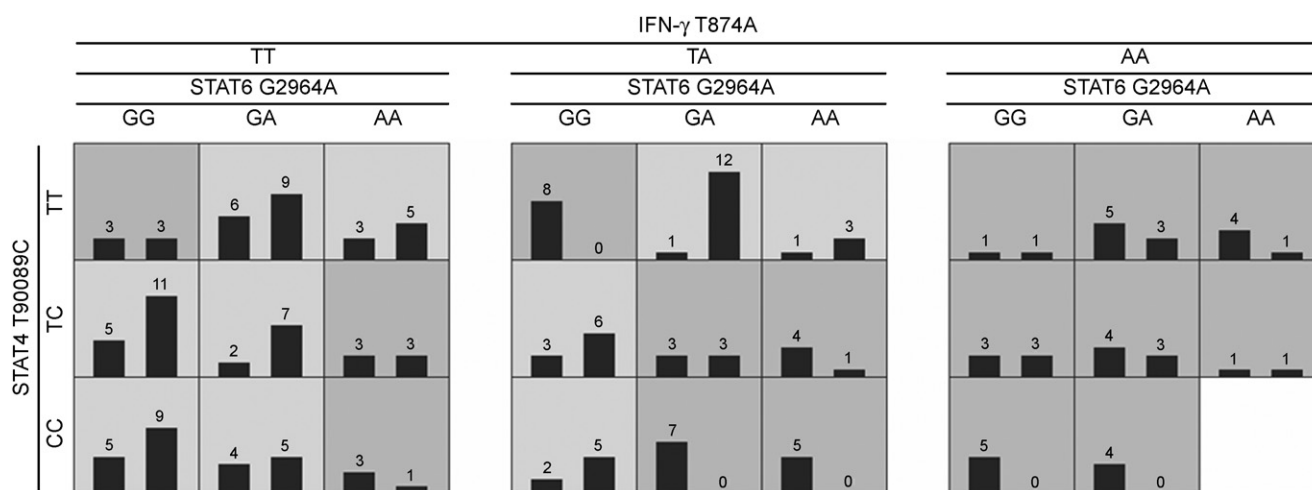*$\chi^2$ test.

**Figure 1** Distribution of high-risk (dark shading) and low-risk (light shading) combinations associated with asthma in the significant gene–gene models using MDR analysis. The number of asthmatic patients (left bar in boxes) and matched controls (right bar in boxes) is shown for each combination. Boxes were labeled as high-risk if the ratio of the number of patients to controls met or exceeded the threshold of 1.0.

Japanese population. However, this association was not replicated in later studies on Japanese populations.[12,13] Similar negative associations were found in both a British population[11] and a Caucasian sib-pair study.[10] In the present study, no significant association was found between asthma and the G2964A polymorphisms in a Chinese population. Hence, the biologic importance of described polymorphisms in STAT-6 remains unclear. Apparently both ethnic and clinical differences in study populations may add to the complexity of the results.

STAT-4 spans 120 kb on chromosome 2q32.2–q32.3.[24] This region has been linked to atopy susceptibility in previous genome-wide scans.[25] In previous study, Pykäläinen et al.[26] found no associations of STAT-4 T90089C polymorphisms with asthma in a Finnish population. However, our study demonstrated that the STAT-4 T90089C polymorphisms were associated with asthma in the dominant genetic model. Our results might be partly confirmed by the recent study of Park et al.,[27] which showed a significant association of STAT-4 T90089C with the increased production of specific IgE in Korea asthmatic patients.

We firstly conducted a case–control study to investigate the association of the IFN-γ T874A polymorphisms with asthma and found that these polymorphisms were significantly associated with asthma in both dominant and recessive model. It has been reported that IFN-γ production was genetically controlled. IFN-γ T874A polymorphisms have been considered directly influencing the level of IFN-γ production.[28] The genotypes AA, TA and TT of IFN-γ 874 locus were thought to confer three different phenotypes: low, intermediate and high producers of IFN-γ, respectively.[29,30] Therefore, by regulating the level of IFN-γ production, IFN-γ T874A polymorphisms may play an important role in pathophysiologic mechanisms and be a useful marker of asthma phenotypes.

As for a multifactorial disease, it is clear that asthma is not caused by a single genetic risk factor. Gene–gene interactions could provide insight into the pathophysiologic

mechanisms of this disease. Traditionally, the identification and characterization of gene–gene interactions have been limited by the lack of powerful statistical methods and large sample size.[31] MDR method, overcoming some of the limitations, is a promising new approach for detecting and characterizing gene-to-gene interactions in case–control studies.[22] Therefore, the gene–gene interactions were analyzed with MDR in this study. Our results suggest that the susceptibility to asthma in the Chinese population involves gene–gene interactions among STAT-6, STAT-4 and IFN-γ. This result was confirmed by traditional logistic regression analysis. The immune disorders characterized by an imbalance between Th1 and Th2 play a central role on the inflammatory response in asthma.[3] The coexistence of these polymorphisms probably interacts to regulate Th1/Th2 differentiation. It is therefore possible that different polymorphisms in STAT-6, STAT-4 and IFN-γ contribute to the complex regulation of asthma phenotypes.

In summary, our findings suggest that STAT-4 T90089C and IFN-γ T874A polymorphisms might be the genetic factors for the risk of asthma in the Chinese population. In addition, the significant interactions among STAT-6 G2964A, STAT-4 T90089C and IFN-γ T874A may increase an individual's susceptibility. A limitation of our study is the relatively small sample size. The contributory role of genetic variants of STAT-6, STAT-4 and IFN-γ genes in asthma is in our opinion still open to debate. Additional genetic and epidemiological studies as well as functional analysis are required to fully elucidate the role of these interesting gene polymorphisms in the development of asthma.

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