The Association Between Glutathione S-Transferase P1, M1 Polymorphisms and Asthma in Taiwanese Schoolchildren*

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Study objectives: **Genetic polymorphisms in the glutathione S-transferase P1 gene (GSTP1) and the glutathione S-transferase M1 gene (GSTM1) have been implicated as risk factors for asthma. However, their roles in asthma pathogenesis and the interaction between these two genes have not been extensively investigated. This study, therefore, examined the relationship among GSTP1 and GSTM1 genotypes and childhood asthma, and evaluated their gene-gene interactions.**

Setting: **The population from three southern Taiwan communities of a 2001 national survey.**

Subjects and methods: **Two hundred sixty-six fourth-grade to ninth-grade schoolchildren were recruited for oral mucosa samplings based on questionnaire information. Polymerase chain reaction-based assays were performed to determine GSTP1 and GSTM1 genotypes among asthmatic subjects and nonasthmatic control subjects. Multiple logistic regression was used to adjust for potential confounding factors.**

Results: **All of the participants were homozygous at the GSTP1 Ala-114 locus. After controlling for age, sex, and atopic eczema, compared with participants carrying any Val-105 allele, children who were homozygotic for GSTP1 Ile-105 had a significantly increased risk of physician-diagnosed asthma (adjusted odds ratio [adjOR], 1.94; 95% confidence interval [CI], 1.08 to 3.59). A positive risk for childhood asthma was also noted on the GSTM1 null genotype but did not reach statistical significance (adjOR, 1.37; 95% CI, 0.80 to 2.38). Among children with GSTM1 present genotypes, GSTP1-105 polymorphisms were associated with the increased risk of asthma. However, the reduced and statistically insignificant asthma risk was observed among those with GSTM1 null genotype.**

Conclusions: **We concluded that GSTP1-105 was a predictor for childhood asthma, whereas GSTM1 polymorphism might modify the risk. Our study also suggested a competitive effect for homozygous GSTP1 Ile-105 and GSTM1 null genotypes on childhood asthma.**

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Key words: asthma; children; glutathione S-transferase M1; glutathione S-transferase P1 gene; polymorphism

Abbreviations: adjOR = adjusted odds ratio; BHR = bronchial hyperresponsiveness; CI = confidence interval; GST = glutathione S-transferase; GSTM1 = glutathione S-transferase M1 gene; GSTP1 = glutathione S-transferase P1 gene; OR = odds ratio; ROS = reactive oxygen species

Asthma is the single most common chronic childhood disease in developed nations.[1] Current studies[2,3] indicate that many regions of the human genome containing susceptibility genes are associated with various asthmatic phenotypes. It is also thought that the inheritance of asthma does not follow a simple monogenic pattern.[4,5] Genome-wide search studies[2,3] also have demonstrated that many candidate regions contribute to asthma. In the can-

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didate gene approach, pathways involved in illness pathogenesis are defined, and genes with functional polymorphic variants that operate in the pathways are identified.[6]

The presence of inflammation in the airway is an important biochemical feature of asthma. Oxidative stress, with the formation of reactive oxygen species (ROS), is the key component of inflammation.[7] Studies[8] have shown that individuals with lowered antioxidant capacity are at an increased risk of asthma. The inability to detoxify ROS, including lipid and DNA hydroperoxides, should perpetuate the inflammatory process in the lung, activate bronchoconstrictor mechanisms, and precipitate asthmatic symptoms. Members of the glutathione S-transferase (GST) supergene family are critical for protecting cells from ROS. They are known as phase II xenobiotic detoxifying enzymes that conjugate reactive intermediates with glutathione to produce less reactive water-soluble compounds[9,10] and can also influence the synthesis of eicosanoid-like mediators via the modulation of ROS levels.[11] Because oxidative stress plays a role in the pathogenesis of asthma, and the GST superfamily is important, we hypothesize that polymorphisms of GST genes functioning in antioxidant pathways are determinants of asthma development.

Several members of the GST superfamily, notably the glutathione S-transferase P1 (GSTP1) gene and the glutathione S-transferase M1 (GSTM1) gene, are expressed in the respiratory tract and have common functional variant alleles.[9–11] Some association studies[12–15] have suggested that the individual with the GSTP1 Ile-105/Ile-105 genotype is at a higher risk to develop asthma, atopy, or allergic response, but results of investigations of lung function growth in schoolchildren have been inconsistent.[16] Several negative studies[12,14] have also been found with regard to the association between GSTM1 polymorphisms and respiratory illness. These conflicting results may reflect heterogeneity in ethnic groups and phenotypic definition, as well as the considerable complexity of asthma. Although GSTP1 and GSTM1 have the potential to explain a substantial portion of asthma occurrence at the population level, their roles in asthma pathogenesis have not been extensively investigated.

In order to investigate the association between the genetic polymorphisms of GSTP1 and GSTM1 and childhood asthma, based on questionnaire information, we recruited schoolchildren for genotype determination from our previous Taiwanese population.[17] We also examined the association of childhood asthma with these gene polymorphisms and evaluated their gene-gene interactions.

Materials and Methods

Study Population and Subjects

Between February and June 2001, we conducted a national, cross-sectional, school-based survey for respiratory diseases and symptoms in middle-school and elementary-school children. The study protocol has been described previously.[17] Briefly, the standard International Study of Asthma and Allergies in Childhood-Chinese version questionnaire was taken home by students and answered by parents. Stratified sampling by grade was applied in each school, and classroom incentives but not individual incentives were used to encourage participation. In total, we investigated 35,036 children from 22 elementary and 22 middle schools, and the overall response rate was 92.8%. In June 2001, we conducted the present study focusing on the 2,853 fourth-grade to ninth-grade schoolchildren who completed the questionnaire survey and resided in three southern Taiwan communities. The study protocol was approved by the Institutional Review Board at our university hospital, and it complied with the principles outlined in the Helsinki Declaration.[18]

Definition of Diseases by Questionnaire

The definition of asthmatic subjects was determined by a positive response to the question, "Has a physician ever diagnosed your child as having asthma?" Nonasthmatic control subjects were defined as those without physician-diagnosed asthma, reporting no nocturnal dyspnea associated with wheezing (from the video questionnaire), and not ever having dyspnea with wheezing (from the parental questionnaire). Atopic eczema was defined as the presence of itching skin eruption in the cubital, posterior popliteal, neck, periauricle, or eyebrow areas for ≥ 6 months and a diagnosis of atopic eczema by a physician.

Based on the criteria established from questionnaire information, we randomly selected 10% of the children without asthma and all of the children with physician-diagnosed asthma for the present study. Two hundred sixty-six subjects completed oral mucosa samplings and pulmonary function tests. All of the selected children were lifelong nonsmokers and were of the same ethnic origin. Standardized pulmonary function tests were conducted with equipment that met American Thoracic Society criteria (model 2130; SensorMedics; Yorba Linda, CA),[19] and the maneuvers were performed in a standardized manner.[20] Every procedure was executed by the same group of field workers in a double-blinded manner. Methacholine challenge tests were performed on subjects who met the following criteria[21]: (1) had a baseline FEV_1 of $\geq 70\%$ of the predicted value; (2) had no viral infection or common flu within at least the preceding 2 weeks; (3) had used no medications or herbal drugs within the week preceding the study; and (4) had the consent of a parent. After excluding those who terminated tests because of obvious wheezing or dyspnea before a 20% decline in FEV_1 was reached, 224 subjects completed the methacholine challenge test. Table 1 provides the demographic and clinical characteristics of the study population.

GSTP1 and GSTM1 Polymorphism Genotyping

Cotton swabs containing oral mucosa were collected and were immediately maintained at −80°C throughout the transfer and storage. Genomic DNA was isolated using the phenol/chloroform extraction method previously described[22] with some modification. In brief, the cotton swabs were directly immersed in 300 μL of cell lysis buffer (50 mmol/L Tris-HCl, 1 mmol/L ethylenediaminetetraacetic acid, and 0.1 mol/L NaCl [pH 8.0]) containing

Table 1—*Demographic, Phenotypic, and Genotypic Characteristics of Study Subjects

Categories	Asthmatic Subjects (n = 82)	Nonasthmatic Subjects (n = 184)	p Value
Age, yr	11.9 ± 1.5	12.2 ± 1.7	NS†
Sex			
Male	44 (53.7)	90 (48.9)	NS
Female	38 (46.3)	94 (51.1)	
Atopic eczema			
No	72 (87.8)	179 (97.3)	0.002
Yes	10 (12.2)	5 (2.7)	
FEV_1, % predicted	94.6 ± 13.4	99.6 ± 10.6	0.01†
FEV_1/FVC, %	85.0 ± 12.4	90.1 ± 9.6	< 0.001†
BHR‡			
Negative	25 (41.7)	133 (81.1)	< 0.001
Positive	35 (58.3)	31 (18.9)	
GSTP1-105			
Ile-Ile	62 (75.6)	112 (60.9)	NS
Ile-Val	18 (22.0)	64 (34.8)	
Val-Val	2 (2.4)	8 (4.4)	
GSTP1–114			
Ala-Ala	82 (100.0)	184 (100.0)	NS
GSTM1			
Null	49 (59.8)	97 (52.7)	NS
A	13 (15.9)	35 (19.0)	
B	18 (22.0)	44 (23.9)	
AB	2 (2.4)	8 (4.4)	

*Values given as mean ± SD or No. (%), unless otherwise indicated. NS = not significant.

†Calculated by unpaired Student *t* test; all other p values were calculated with Pearson χ^2 test.

‡Provocative dose of methacholine < 4.7 mg causing a 20% fall in FEV_1. Numbers of subjects were not added up to total N because of some circumstances described in the text.

2% sodium dodecyl sulfate and 20 μg/mL proteinase K in a 1.5-mL microcentrifuge tube. After incubation overnight at 55°C, the swabs were discarded, and the DNA in the supernatants was purified by phenol/chloroform extraction and then precipitated with ethanol.

GSTP1 gene variants are caused by base-pair transitions at nucleotides + 313 and + 341. We adapted previous studies to detect these polymorphisms by polymerase chain reaction.[12,13] The 50-μL polymerase chain reaction mixture containing 10 mmol/L Tris-HCl (pH 8.4), 50 mmol/L KCl, 1.5 mmol/L $MgCl_2$, 0.1% Triton X-100, 200 μmol/L deoxyribonucleoside triphosphate, 0.2 μmol/L each primer, 2 U Taq DNA polymerase, and 20 ng of genomic DNA was amplified by 40 cycling reactions composed of 95°C denaturation for 30 s, 60°C annealing for 1 min, and 72°C elongation for 1 min. GSTM1 A, B, A/B, and null genotypes were identified with allele-specific primers to exon 7.[23] All of the assays were performed by workers who were unaware of the clinical status of individual subjects, and genotype assignments were based on two consistent experimental results. About 15% of randomly selected samples were directly sequenced, and all of them were concordant with the initial genotyping results.

Statistical Analysis

The Pearson χ^2 test and unpaired Student *t* test were applied to analyze the difference between two groups. Multiple logistic regression models were used to control for potential confounders, and to evaluate the associations between the risks of asthma and genetic polymorphisms. Crude odds ratios (ORs) and adjusted ORs (adjORs) with 95% confidence intervals (CIs) were shown. Statistical significance was set at a p value of < 0.05 based on a two-sided calculation.

The GSTM1 null genotypes are homozygous for the null allele, and the reference group consists of subjects heterozygous or homozygous for the GSTM1 wild-type alleles. To assess the presence of gene-gene interaction between the GSTP1 and GSTM1 polymorphisms, we compared the risk of asthma for subjects of joint exposure to that of subjects who were Ile-Val or Val-Val for GSTP1-105 and null allele for GSTM1. The joint effects of GSTM1 (null and present) and GSTP1-105 genotypes (Ile-Ile homozygous and carrier with any Val allele) were assessed using four mutually exclusive levels.

Results

Because of a 100% DNA extraction rate in oral mucosa samples, our study was finally composed of 82 physician-diagnosed asthmatic subjects and 184 control subjects who were asthma-free from birth. Table 1 presents the demographic, lung function, and results of methacholine challenge tests by study groups. Nonasthmatic children had a lower proportion of atopic eczema than did the asthma group. As expected, children with asthma had decreased baseline FEV_1/FVC, a lower percentage of predicted FEV_1, and also included a higher proportion of bronchial hyperresponsiveness (BHR).

Table 1 also shows the genotypic frequencies for the GSTP1 and GSTM1 polymorphisms in the two study groups. In each group, the frequency of the GSTP1-105 genotype was consistent with the Hardy-Weinberg equilibrium. All of the participants were homozygous at the GSTP1 Ala-114 locus. The frequency of the GSTP1 Ile-105/Ile-105 genotype was increased in asthmatic children compared with nonasthmatic control subjects but did not reach statistical significance (Table 1, Fig 1). Because the frequency of homozygosity at the GSTP1 Val-105 locus was relatively low, we combined the Ile-105/Val-105 and Val-105/Val-105 genotypes in the subsequent analyses.

Although confounding factors might interfere with study results, in our population, the ORs did not change substantially after we controlled for age, sex, and history of atopic eczema. In the multiple regression model, homozygous GSTP1 Ile-105 was significantly associated with physician-diagnosed asthma (adjOR, 1.94; 95% CI, 1.08 to 3.59; p = 0.029) [Table 2]. In our series, the GSTM1 null genotype was overrepresented in the asthma group. The risk of childhood asthma on GSTM1 null genotype was positive but, nevertheless, failed to reach statistical significance (adjOR, 1.37; 95% CI, 0.80 to 2.38) [Table 2, Fig 1].

We conducted an additional analysis examining

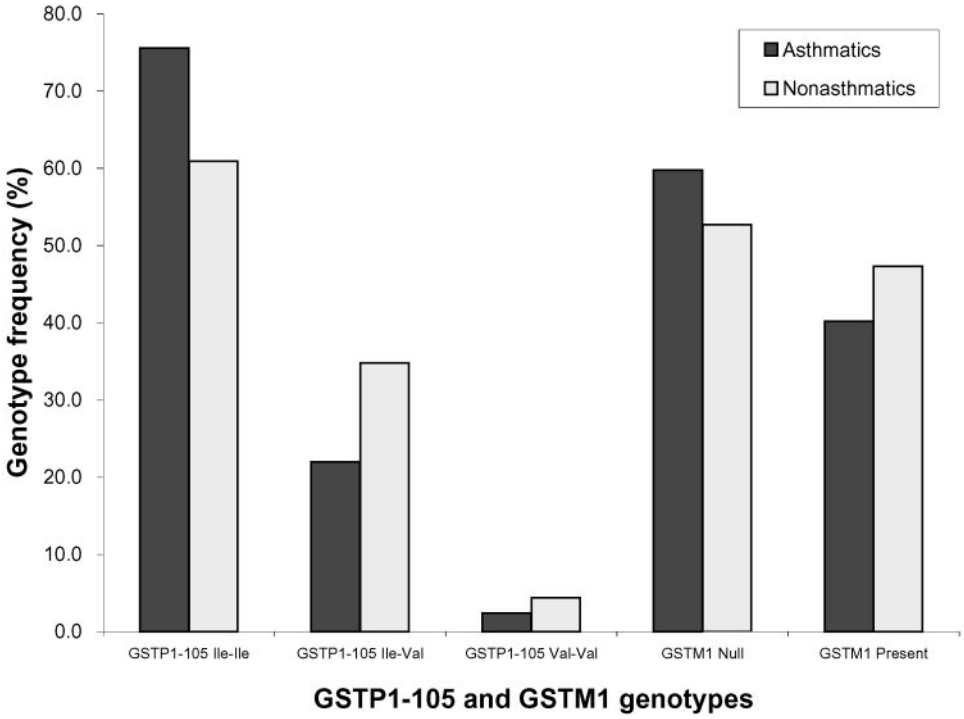

FIGURE 1. Genotype frequency of GSTP1-105 and GSTM1 among asthmatic and nonasthmatic subjects.

the interaction between these two genes on the risk of childhood asthma (Table 2). Because the joint effects were reduced, there were competitive effects for the homozygous GSTP1 Ile-105 genotype and GSTM1 null genotype on childhood asthma. We also examined the distribution of GSTP1-105 gene polymorphisms in asthmatic children and control subjects by GSTM1 genotypes (null and present) [Table 3]. Using carriers with any GSTP1 Val-105 allele as a reference category, among those with GSTM1 present genotypes, children with homozygous GSTP1 Ile-105 were at a significantly increased risk of asthma. The reduced and statistically insignificant asthma risk was observed among those who were of the GSTM1 null genotype.

DISCUSSION

In the present study, we tested for an association between markers in two candidate genes, GSTP1 and GSTM1, on the risk of childhood asthma. After controlling for age, sex, and atopic eczema, children who were homozygotic for GSTP1 Ile-105 had a significantly increased risk of physician-diagnosed asthma. The risk of asthma was positively associated

Table 2—*Association of GSTP1-105 and GSTM1 Genotypes With Asthma Risk**

Genotypes	Crude OR	95% CI	p Value	adjOR†	95% CI	p Value
GSTP1-105						
Ile-Val or Val-Val	1			1		
Ile-Ile	1.99	1.13–3.64	0.021	1.94	1.08–3.59	0.029
GSTM1						
Present	1			1		
Null	1.33	0.79–2.27	NS	1.37	0.80–2.38	NS
GSTP1-105/GSTM1						
Ile-Val or Val-Val/present	1			1		
Ile-Val or Val-Val/null	2.21	0.79–6.82	NS	2.33	0.82–7.32	NS
Ile-Ile/present	3.03	1.20–8.79	0.027	3.03	1.17–8.97	0.031
Ile-Ile/null	3.40	1.38–9.70	0.013	3.43	1.37–9.94	0.013

*See Table 1 for abbreviation not used in the text.
†ORs were adjusted by multiple logistic regression for age, sex, and history of atopic eczema.

Table 3—*Association of GSTP1-105 Genotypes With Asthma Risk, Stratified by GSTM1 Genotype

Genotypes	Crude OR	95% CI	p Value	adjOR†	95% CI	p Value
GSTM1 present						
GSTP1-105						
Ile-Val or Val-Val	1			1		
Ile-Ile	3.03	1.20–8.79	0.027	2.99	1.13–8.99	0.036
GSTM1 null						
GSTP1-105						
Ile-Val or Val-Val	1			1		
Ile-Ile	1.54	0.74–3.31	NS	1.45	0.69–3.16	NS

*See Table 1 for abbreviation not used in the text.
†ORs were adjusted by multiple logistic regression for age, sex, and history of atopic eczema.

with the GSTM1 null genotype but did not reach statistical significance. Among children with GSTM1 present genotypes, GSTP1-105 polymorphisms were significantly associated with the increased risk of asthma. Among children with the GSTM1 null genotype, the reduced and statistically insignificant asthma risk was observed on GSTP1-105 polymorphisms. Our study suggested a competitive effect for homozygous GSTP1 Ile-105 and GSTM1 null genotypes on childhood asthma.

Age, sex, ethic factors, and smoking habits are thought to contribute to the occurrence of childhood asthma.[17] We minimized interference from these confounders by recruiting lifelong nonsmokers of similar age and approximately evenly divided by sex. The ethnicity of all of the subjects was Asian Taiwanese, and we assumed them to be a rather homogenous population. In this study, asthma was noted to be associated with a history of atopic eczema (Table 1), which was used as a surrogate for individual atopic characteristics. These risk factors were controlled by regression models in our additional analyses.

For complex diseases with modest genetic effects, association studies[24,25] can play critical roles in the evaluation of candidate genes. Asthma is a polygenic disorder in which several candidate genes are involved that may modify the severity of inflammation. Excess ROS is the proximal event leading to inflammation, cell death, and subsequent airway remodeling among individuals with inadequate defenses, which is the key step in asthma pathogenesis. The measurement of long-term levels of oxidative stress is not currently feasible for large epidemiologic studies. Genetic variation may provide a useful method to classify long-term levels of oxidative stress.

The GST superfamily enzyme product plays an important role in asthma and wheezing occurrence, because xenobiotic metabolism and antioxidant pathways are involved in asthma pathogenesis.[6] In the human lung epithelium, the GSTP1 gene contributes > 90% of GST-derived enzyme activity.[26] Our results support the hypothesis that the individual ability to detoxify ROS and their products, determined by the polymorphism in GSTP1, contributes to the development of childhood asthma. This view is also supported by studies[8,27] showing that individuals with reduced antioxidant capacity are at an increased risk of allergic asthma and that a decreased intake of antioxidants is associated with the expression of asthma-related phenotypes. Because GSTP1 is strongly expressed in the respiratory epithelium and is the dominant GST in the lung, our data also showed that a variation in GSTP1 function had larger effects on asthma than did GSTM1 (Table 2).

BHR may be modulated by ROS levels through their ability to regulate eicosanoid production by stimulating the release of arachidonic acid.[28] GSTP1 maps on chromosome 11q13, which was suggested as a candidate region for asthma and BHR in some linkage studies.[29] In the Children's Health Study conducted in the United States, Gilliland et al[14] used the incidence rate of school absences as the outcome variable and reported that children with homozygous GSTP1 Ile-105 allele were at a higher risk for multiple symptomatic respiratory illnesses. However, from the same population, they found that children with a homozygous GSTP1 Val-105 allele had significantly larger deficits in lung function growth.[16] In the United Kingdom, the homozygous GSTP1 Ile-105 genotype, compared with the Ile-105/Val-105 type, was noted[12] to be associated with a nearly threefold higher risk of asthma. When compared with the Val-105/Val-105 genotype, the risk of asthma rose to ninefold. In our data, the homozygous GSTP1 Ile-105 allele also significantly contributed to physician-diagnosed asthma in childhood, with a 1.94-fold risk (Table 2). Variations in ethnicity and in phenotypic definition could in part explain our relatively lower risk.

GSTM1 is present in the respiratory system, although its expression is highest in the liver.[30] A common homozygous deletion polymorphism of the

GSTM1 (null genotype) abolishes enzyme activity and may increase susceptibility to oxidative stress in airways. Our result showed a positive but insignificant risk for the GSTM1 null genotype on childhood asthma in the general population (Table 2). In Mexico, Romieu et al[31] found that children with the GSTM1 null genotype are more vulnerable to the effects of ozone on the small airways and might receive greater benefit from antioxidant supplementation. In the US Children's Health Study, Gilliland et al[16] found that children with the GSTM1 null genotype had significantly larger deficits in lung function growth. The authors also found that the GSTM1 genotype was unassociated with asthma and respiratory illnesses in the same population.[14,32] However, among the subpopulation with the GSTM1 null genotype rather than those with the GSTM1 present genotype, *in utero* exposure to maternal smoking was associated with an increased prevalence of many asthmatic phenotypes.[32]

Among children with the GSTM1 present genotype, the homozygous GSTP1 Ile-105 genotype was noted to be associated with a higher risk (2.99-fold) of physician-diagnosed asthma (Table 3). However, among children with the GSTM1 present genotype, the GSTP1 Ile-105 genotype was associated with a statistically insignificant asthma risk. To our knowledge, only one preliminary study has described the combination effect of GSTM1 and GSTP1 on allergic responses. Gilliland et al[15] used a human inhalation challenge model and found that people with both the GSTM1 null and GSTP1-105 Ile-Ile genotypes had significantly higher allergic responses to diesel exhaust particles than those with other genotypes combined. Although GSTP1 and GSTM1 are located on different chromosomes, probably because of having a common pathogenic pathway or using overlapping substrates, they revealed a competitive effect on childhood asthma in our study.

Results from our laboratory showed that the GSTP1 Ile-105 allele and GSTM1 null genotype seems to be higher in the Taiwanese population than in whites. The frequency of the GSTP1 Ile-105 allele in Western populations ranged from 61.6%,[14] to 64.3%,[12] to 68.4%,[13] to 72.3%,[33] respectively, throughout Britain, Mexico, America, and Northern Europe. Nearly 40% of the children had a GSTM1 null genotype in the white population.[16,31] In contrast, here we found that the frequency of the GSTP1 Ile-105 allele was around 78.3% (control subjects) to 86.6% (asthmatic subjects), and the GSTM1 null genotype was $> 50\%$ in the Taiwanese population. The higher frequency in the Taiwanese population would make the ORs of the GSTP1 Ile-105 homozygote or GSTM1 null genotype carriers for acquiring asthma lower than that of white populations. Using only questionnaire results rather than biomarkers would induce misclassification, which may have also decreased the ORs.

Questionnaires have been widely used to assess childhood asthma. In our study design, the history of physician-diagnosed asthma or dyspnea with wheezing was ascertained by questionnaires. Misclassification of asthma status may have arisen from imperfect recalls, variations in access to medical care, differences in diagnostic terminology, or delay in diagnosis. More than 99% of participants were covered by national health insurance in Taiwan, suggesting that bias from differential access to care is not possible. We lack data to assess the magnitude of the misclassification of asthma status from recall or medical practice. However, it is unlikely that our findings result from a spurious association that arose from variations in medical practice across the three communities. Because any recall bias would be independent of GST genotypes, this factor is unlikely to explain the association. We did not have genotyping data from all of the subjects, which made selection bias possible. However, there were no substantial differences between the characteristics of those with genotypes and those without genotypes (data not shown), and the DNA extraction rate of our population was 100%, indicating that any bias from the selection mechanism or availability of DNA is limited.

In summary, we found that the GSTP1 and GSTM1 genes might be important determinants to asthma development in schoolchildren, based on the functional biology of the genes. Probably through common pathogenic pathways or overlapping substrates, the GSTP1 and GSTM1 genes also showed a competitive effect on childhood asthma. This stresses the importance of the variation in detoxification ability of the GST superfamily that may be used as markers of individuals susceptible to inhalational insults. Our data suggest that childhood asthma is a complex disease associated with many genes, and susceptibility likely involves multiple gene-gene interactions. We think that additional studies on the other genotypic variants involved in oxidative stress response and asthma phenotypes are warranted.

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