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Original article

Associations of glutathione S-transferase P1, M1, and environmental tobacco smoke with wheezing illness in school children

Background: Polymorphisms at the glutathione S-transferase (GST) were associated with asthma-related phenotypes. We hypothesized that the GSTP1 and GSTM1 genotypes could modify the effects of household environmental tobacco smoke (ETS) on childhood wheezing illness.
Methods: We conducted a case-control study comprised of 216 lifetime wheezing children and 185 nonwheezing controls, all of whom were selected from 2524 fourth- to ninth-grade school children in southern Taiwan.
Results: Homozygous GSTP1 Ile-105 was significantly associated with current wheezing (OR = 1.78, 95% CI 1.04–3.12), but insignificantly associated with ever wheezing (OR = 1.26, 95% CI 0.82–1.94). The risks of ever or current wheezing on GSTM1 null genotype were positive but not statistically significant. Although household ETS exposure was not associated with wheezing illness, after excluding subjects having *in utero* ETS or active smoking habits, the adverse effects of household ETS exposure differed significantly by GSTP1-105 genotypes. In children without any ETS exposure at home, GSTP1 Ile-105 homozygosity was significantly related to increased risks for both ever wheezing (OR = 2.29, 95% CI 1.17–4.49) and current wheezing (OR = 4.86, 95% CI 1.86–12.70). In children with household ETS exposure, the risks of wheezing illness did not increase for those carrying two GSTP1 Ile-105 alleles. Children carrying any GSTP1 Val-105 allele were at a significantly greater risk of both ever and current wheezing when exposed to ETS, with a clear dose-response relationship to the number of smokers at home.
Conclusion: Household ETS exposure is a modifiable cause of wheezing illness in a genetically susceptible subpopulation.

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Key words: children; environmental tobacco smoke; gene–environmental interaction; GSTP1 polymorphism; wheezing.

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The prevalence of childhood asthma/wheezing has been reported as increasing around the world (1–4). Although the rapid increase suggests a role for environmental factors in the etiology of this evolving epidemic, it is clear that genetic factors also influence its occurrence (5, 6). It is equally believed that the increase in childhood wheezing illness is likely to involve changes in specific environmental exposures among the genetically susceptible population.

Exposure to environmental tobacco smoke (ETS) is common amongst children and causes substantial morbidity. Estimates of population attributable risk for household ETS exposure in children range from 9% for asthma prevalence to 25% for hospital admissions because of lower respiratory symptoms (7). The contributions of ETS exposure to a wide spectrum of respiratory illnesses in children have been reported (7–11). Although household ETS exposure is suggested to be a risk factor for childhood wheezing illness, only a portion of the exposed children developed symptoms. This suggests that some individuals are more susceptible to the effects of ETS and it is likely that these differences are caused by genetic differences in the population.

The presence of inflammation in the airway is an important biochemical feature of asthma/wheezing. Studies have shown that individuals with lowered antioxidant capacity are at increased risks of asthma (12). Inability to detoxify reactive oxygen species (ROS) would perpetuate inflammatory processes in the lung, activate bronchoconstrictor mechanisms, and precipitate respiratory symptoms. Tobacco smoke consists of a complex mixture of gases and particles including more than 4000 different chemicals (13). The metabolism and detoxification of tobacco smoke components are essential mechanisms to minimize the toxic effects of ETS. In the human organism, glutathione S-transferases (GSTs) are potentially involved in many detoxification processes (14). We have reported that GSTP1 and GSTM1 polymorphisms are predictors for childhood asthma (15, 16). Previous international studies have also suggested that GSTM1 and *in utero* ETS exposure had significant interactive effects on respiratory symptoms (17, 18). Although

GSTP1 and GSTM1 have the potential to explain a substantial portion of the prevalence of wheezing illness, our results were not stratified by ETS exposure and it can be speculated that a strong overall exposure to household ETS may have existed.

In Taiwan, it was reported that approximately 60% children under the age of 17 were exposed to household ETS (19), and therefore an estimated 3.3 million children were at risk for adverse health effects from this exposure. In this report, we compared the genotypic distribution of GSTP1 and GSTM1 genes in a case-control study nested within our previous 'International Study of Asthma and Allergies in Childhood' (ISAAC) questionnaire survey in Taiwanese school children (20). We also examined the association of wheezing illness with GSTP1 and GSTM1 gene polymorphisms and household ETS exposure, and evaluated their interactions.

Methods

Subject selection

In 2001, we conducted a national, cross-sectional, school-based survey for respiratory diseases and symptoms in middle- and elementary-school children. The study protocol has been described previously (20). Briefly, the standard ISAAC-Chinese version questionnaire was taken home by students and answered by parents. Some information concerning basic demography, residential environmental factors, and history of family atopic diseases was also collected from the questionnaire. In June 2001, we conducted the present genetic case-control study focusing on the 2853 fourth- 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 (21).

The definition of ever wheezing was determined by a positive response to the question, 'Has your child ever had wheeze or whistling in the chest at any time in the past when he/she did not have a cold or the flu?' In this group, those who reported attacks in the past 12 months were identified as current wheezing. Nonwheezing controls were defined as those reporting not ever having dyspnea with wheezing (from the parental questionnaire), no nocturnal dyspnea associated with wheezing (from the video questionnaire), and without physician-diagnosed asthma. After excluding questionnaires with unanswered questions, we found only 1.5% and 1.0% of subjects to have *in utero* ETS and active smoking habits in our population. Because of sample size limitation for stratification analysis, we excluded subjects with any of these two kinds of tobacco smoke exposure at the study entry. Based on criteria established from questionnaire information and parental informed consent, we randomly selected 50% of the children with ever wheezing and 10% of the nonwheezing controls for oral mucosa sampling, with the response rate 93.7%. All of the selected subjects were of the same ethnic origin. Table 1 provides the demographic characteristics of the study population.

Table 1. Demographic and the selected characteristics of the study population among fourth- to ninth-grade school children in Taiwan, 2001

	Participants genotyped			All eligible participants	
Categories	Ever wheezing (n = 216)	Current wheezing* (n = 110)	Controls (n = 185)	Ever wheezing (n = 441)	Controls (n = 2083)
Age (years)	12.0 ± 1.6	11.8 ± 1.6	12.1 ± 1.8	11.8 ± 1.7	12.1 ± 1.8
Sex					
Boys	120 (55.6)	59 (53.6)	88 (47.6)	255 (57.8)	942 (45.2)
Girls	96 (44.4)	51 (46.4)	97 (52.4)	186 (42.2)	1141 (54.8)
Parental atopy†					
Yes	92 (42.6)	43 (39.1)	45 (24.3)	189 (42.9)	520 (25.0)
No	124 (57.4)	67 (60.9)	140 (75.7)	252 (57.1)	1563 (75.0)
Parental education level (years)					
<10	50 (23.2)	30 (27.3)	59 (31.9)	127 (28.8)	747 (35.9)
10–12	87 (40.3)	40 (36.4)	75 (40.5)	169 (38.3)	853 (41.0)
≥13	79 (36.6)	40 (36.4)	51 (27.6)	145 (32.9)	483 (23.2)
Number of smokers at home					
0	97 (44.9)	48 (43.6)	81 (43.8)	200 (45.4)	909 (43.6)
1	102 (47.2)	52 (47.3)	89 (48.1)	197 (44.7)	955 (45.9)
≥2	17 (7.9)	10 (9.1)	15 (8.1)	44 (9.9)	219 (10.5)

Results were shown as mean ± SD or n (%).
We excluded samples with *in utero* environmental tobacco smoke or active smoking habits in this table.
*Based on the symptoms for the past 12 months.
†Defined as presence of paternal or maternal asthma, allergic rhinitis, or atopic eczema.

Tobacco smoke exposure

Exposure to household ETS and exposure to maternal smoking *in utero* were characterized using the responses from the questionnaire completed by parents. Household ETS was defined as daily smoking inside the house by anyone living there. The number of smokers at home was determined by the question, 'In general, how many smokers were there in your child's home environment during the past 12 months?' *In utero* exposure to maternal smoking was assigned by responses to the question 'Did your child's biological mother smoke while she was pregnant with your child?' Information regarding active smoking exposure was obtained from the children themselves. Participants with active smoking habits were defined by positive responses to the question, 'Have you ever had active smoking habit in your lifetime?'

GSTP1 and M1 polymorphism genotyping

We collected oral mucosa samples from 401 school children and our DNA extraction rate was 100%. The procedures for oral mucosa collection and genomic DNA

isolation were described in detail previously (15, 16). The polymorphisms for GSTP1-105 and GSTM1 were identified by polymerase chain reaction (PCR)/restriction fragment length polymorphism (RFLP) methods (15). All assays were performed by workers 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

Unconditional logistic regression models were used to estimate the association of the GSTP1-105 and GSTM1 gene polymorphisms with lifetime and current wheezing in school children. To assess the presence of gene–environmental interactions between GSTP1-105, GSTM1 polymorphisms and household ETS, we compared the risk of wheezing illness for subjects in each category of joint exposure to that of subjects who were Val/Val or Ile/Val for the GST-105 or GSTM1 present polymorphisms, and who had no passive smoker at home. Individual and joint associations were estimated using indicator variables created for each category, omitting the hypothesized low–low risk category or reference group. For categorical variables with more than two categories, the interaction was evaluated using the likelihood ratio test (LRT), comparing the model with indicator variables for the cross-classified variables with a reduced model containing indicator variables for the main effects only. Within genotype category, a one degree of freedom trend test was used to evaluate the possible dose–response relationship across categories of the household ETS variables. All tests assumed a two-sided alternative hypothesis and a 0.05 significance level. All analyses were conducted using SAS software version 9.1 (SAS Institute, Cary, NC, USA).

Results

Our study finally comprised 216 participants with ever wheezing and 185 nonwheezing controls from three communities in southern Taiwan. Table 1 represents the demographic and household ETS exposure data. Children with wheezing illness were slightly younger, included a higher proportion of boys than did the controls, tended to have higher educated parents, and were more likely to have parental atopic histories. However, the distribution of household ETS exposure was not significantly different between cases and controls. Participants who had genotyping data had higher parental education level than did those without genotyping data (Table 1), and all of the other factors were almost identical between children with and without genotyping.

All participants had determined GSTP1-105 genotype but two had undetermined GSTM1 genotype. The overall allele frequencies of the GSTP1-105 polymorphisms were Ile = 83.3%/Val = 16.7% in children with ever wheezing and Ile = 80.0%/Val = 20.0% in nonwheezing controls. Children with wheezing illness were prone to have a higher percentage of the GSTP1-105 Ile/Ile genotype and a higher percentage of the GSTM1 null genotype than controls (Table 2). Because distributions of the GSTP1-105 Val/Val and Ile/Val genotypes between children with wheezing illness and controls were similar, and the frequency of homozygosity at the GSTP1 Val-105 locus was relatively low, we combined the GSTP1-105 Val/Val and Ile/Val genotypes as in dominant genetic models for the subsequent analyses. In multiple regression model adjusted for possible confounders, homozygous GSTP1 Ile-105 was significantly associated with current wheezing (OR 1.78, 95% CI 1.04–3.12), but insignificantly associated with ever wheezing (OR 1.26, 95% CI 0.82–1.94) (Table 2). In our series, the risk of ever or current wheezing on GSTM1 null genotype was positive but also failed to reach statistical significance.

Table 2. The association between GSTP1-105 and GSTM1 polymorphisms and wheezing illness

	Controls	Ever wheezing			Current wheezing		
	n (%)	*n* (%)	OR	95% CI	*n* (%)	OR	95% CI
GSTP1-105							
Ile/Ile	119 (64.3)	150 (69.4)	1.26	0.82–1.94	84 (76.4)	1.78	1.04–3.12
Ile/Val	58 (31.4)	60 (27.8) }	1.00		25 (22.7) }	1.00	
Val/Val	8 (4.3)	6 (2.8) }			1 (0.9) }		
GSTM1							
Null	99 (53.8)	127 (59.1)	1.21	0.80–1.82	64 (58.7)	1.21	0.74–2.00
Present	85 (46.2)	88 (40.9)	1.00		45 (41.3)	1.00	

OR, adjusted odds ratio; CI, confidence interval.
Models are adjusted by multiple logistic regression for age, sex, community of residence, parental atopic history, and parental education level.
Hardy–Weinberg equilibrium tests showed insignificance ($P > 0.05$) in each case and control group.

We also examined the distribution of GSTP1-105 and GSTM1 gene polymorphisms in cases and controls by household ETS exposure. In children without any household ETS exposure, the frequency of GSTP1 Ile-105 homozygosity was significantly related to increased risks for both ever wheezing (OR 2.29, 95% CI 1.17–4.49) and current wheezing (OR 4.86, 95% CI 1.86–12.70) (Table 3). In children with ETS exposure at home, the risks of wheezing illness did not increase for children carrying two GSTP1 Ile-105 alleles. After adjustment for potential confounders, the tests for interaction between the GSTP1-105 genotype and household ETS exposure by LRT reached statistical significance both in ever wheezing and current wheezing. However, the analyses in GSTM1 did not show significant interactive effects (Table 3). Among the 216 children with ever wheezing, 73 subjects had been diagnosed with asthma by physicians. The reduced sample size for asthma made the present study a lower power to detect small effects.

Table 3. The association of GSTP1-105 and GSTM1 polymorphisms with wheezing illness among schoolchildren by household ETS exposure

	Without household ETS				With household ETS				
Gene polymorphisms	Cases	Controls	OR	95% CI	Cases	Controls	OR	95% CI	*P* for interaction*
Ever wheezing									
GSTP1-105									
Val/Val or Ile/Val	23	34	1.00		43	32	2.22	1.07–4.58	0.01
Ile/Ile	74	47	2.29	1.17–4.49	76	72	1.61	0.84–3.09	
GSTM1									
Present	41	33	1.00		47	52	0.78	0.42–1.47	0.28
Null	56	47	0.93	0.50–1.73	71	52	1.15	0.62–2.12	
Current wheezing									
GSTP1–105									
Val/Val or Ile/Val	7	34	1.00		19	32	3.81	1.34–10.84	0.004
Ile/Ile	41	47	4.86	1.86–12.70	43	72	3.30	1.28–8.50	
GSTM1									
Present	23	33	1.00		22	52	0.64	0.29–1.38	0.09
Null	25	47	0.74	0.34–1.57	39	52	1.14	0.55–2.34	

ETS, environmental tobacco smoke; OR, adjusted odds ratio; CI, confidence interval.
Models are adjusted by multiple logistic regression for age, sex, community of residence, parental atopic history, and parental education level.
*Calculated by likelihood ratio tests with d.f. = 1.

Table 4. The association of number of smokers at home with wheezing illness among schoolchildren by GSTP1-105 polymorphism

	GSTP1-105 Val/Val or Ile/Val				GSTP1-105 Ile/Ile				
No. of smokers at home	Cases	Controls	OR	95% CI	Cases	Controls	OR	95% CI	*P* for interaction*
Ever wheezing									
0	23	34	1.00		74	47	2.48	1.28–4.87	0.02
1	34	27	2.16	1.02–4.65	68	62	1.79	0.94–3.49	
≥2	9	5	2.97	0.87–11.15	8	10	1.22	0.39–3.76	
Current wheezing									
0	7	34	1.00		41	47	5.20	2.10–14.45	0.01
1	15	27	3.82	1.34–11.84	37	62	3.50	1.42–9.66	
≥2	4	5	5.35	1.03–27.66	6	10	3.26	0.83–13.00	

OR, adjusted odds ratio; CI, confidence interval.
Models are adjusted by multiple logistic regression for age, sex, community of residence, parental atopic history, and parental education level.
*Calculated by likelihood ratio tests with d.f. = 2.

Restricting the analyses to children without asthma (nonasthmatic wheezers) did not substantially alter the above findings (data not shown). We also checked interactions for genetic effects by sex, age, community of residence, parental atopic history, or parental education level, and none were statistically significant.

Although household ETS exposure was not associated with wheezing illness, in our study, the effect of ETS exposure differed significantly by GSTP1-105 genotype. Children who had any GSTP1 Val-105 allele were at the significantly greater risk for both ever wheezing and current wheezing when exposed to household ETS (Table 4). With increasing number of smokers at home (0, 1, ≥2), a borderline significant dose–response relationship was seen for ever wheezing (P trend = 0.04), and for current wheezing (P trend = 0.06). However, we observed no increase in risk of wheezing illness among children possessing the homozygous GSTP1 Ile-105 genotype (Table 4). The effects of household ETS exposure seem to be increasing among children with GSTM1 null genotype. However, we could not find a significant increasing risk for either ever wheezing (P trend = 0.62), or for current wheezing (P trend = 0.42).

Discussion

We examined the relationships between the genotypic distribution of the GSTP1 and GSTM1 polymorphisms, household ETS, and wheezing illness among fourth- to ninth-grade school children. After excluding subjects having *in utero* ETS or active smoking habits, we found that GSTP1-105 genotype significantly modified the effects of household ETS exposure on childhood wheezing illness. The adverse effects of household ETS on wheezing illness were largely restricted to children carrying any GSTP1 Val-105 allele. Our findings indicate that there are important adverse effects of household ETS exposure in a genetically susceptible group of children.

Age, sex, ethnic factors, active smoking habits, parental atopic history, and parental education level were believed to contribute to childhood asthma and wheezing illness (4, 20). We minimized interference from these confounders by recruiting lifelong nonsmokers of similar age at study entry, and adjusting potential confounders by regression models. *In utero* exposure to maternal smoking was also suggested to increase wheezing and asthma occurrence during childhood (9, 11). Because the prevalence of maternal smoking during pregnancy (1.5%) was too low for stratification analyses in our data, we excluded subjects with *in utero* exposure to maternal smoking and examined the effects of household ETS exposure independently. Exposure to other indoor residential factors, such as cockroaches, visible mold, water damage, and incense burning, were also considered in our survey. Adjustment for these factors resulted in only small changes in the effect estimates, and these covariates were not included in the final models.

The GST superfamily enzyme products play important roles in asthma and wheezing occurrence because xenobiotic metabolism and antioxidant pathways are involved in asthma pathogenesis (22). In human lung epithelium, the GSTP1 gene contributes more than 90% of GST-derived enzyme activity (23). Our results support the hypothesis that individual ability to detoxify ROS and their products, determined by polymorphism in GSTP1, contributes to the development of childhood wheezing illness. This view is also supported by studies showing that individuals with reduced antioxidant capacity are at increased risk of allergic asthma, and that decreased intake of antioxidants is associated with the expression of asthma-related phenotypes (12, 24). Because GSTP1 is strongly expressed in the respiratory epithelium and is the dominant GST in the lung, our data also showed that variation in GSTP1 function had larger effects on wheezing illness than did GSTM1 (Table 2).

Bronchial hyper-responsiveness (BHR) may be modulated by ROS levels through their ability to regulate eicosanoid production by stimulating the release of arachidonic acid (25). The GST genes are candidates for a role in BHR because the enzymes they encode modulate ROS levels (26). In the Children's Health Study conducted in USA, Gilliland et al. (27) used 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. In the UK, homozygous GSTP1 Ile-105 genotype, as compared with Ile-105/Val-105 type, was noted to be associated with a near threefold higher risk of asthma (28). When compared with Val-105/Val-105 genotype, the risk of asthma rose to ninefold. In our data, homozygous GSTP1 Ile-105 allele also contributed to wheezing illness in childhood, with risks of 1.26-fold in ever wheezing and 1.78-fold in current wheezing (Table 2). Variations in ethnicity and in phenotypic definition could in part explain our relatively lower risks.

A common homozygous deletion polymorphism of the GSTM1 gene (null genotype) abolishes enzyme activity and may increase susceptibility to oxidative stress in airways. Our results showed a positive but insignificant risk for GSTM1 null genotype on childhood wheezing illness (Table 2). In Russian and Turkish populations, researchers found that individuals without the GSTM1 gene were at higher risks to develop asthma/wheezing (29, 30). In Children's Health Study, Gilliland et al. (31) reported that children with GSTM1 null genotype had significantly larger deficits in lung function growth. They also found that among the GSTM1 null rather than GSTM1 present subpopulation, *in utero* exposure to maternal smoking was associated with increased prevalence of asthma and wheezing illnesses (17). In Germany, Kabesch et al. (18) also reported that GSTM1 had significant interactive effects with *in utero* and current ETS exposure on asthma-related phenotypes.

In the present study, we found the adverse effects of household ETS on wheezing illness were largely restricted to children carrying any GSTP1 Val-105 allele, with a clear dose–response relationship with increasing number of smokers at home (Tables 3 and 4). Because different polymorphic forms of the GSTP1 gene at position 105 have different effects on the detoxification ability of an individual, it is interesting to speculate that a tobacco smoke-induced airway injury might be different in children carrying different polymorphic forms of this gene. To the best of our knowledge, there is no previous study investigating the combination effect of GSTP1 genotypes and household ETS on childhood wheezing illness. The statistically suggested interactions in our data add to the plausibility of a biological interaction between GST enzymes and components of ETS in the detoxification process. In lung tissue of current smokers, Ryberg et al. (32) found that individuals with GSTP1 Val-105 allele have significantly higher levels of aromatic DNA adducts than those with Ile-105 allele. In a human inhalation challenge study, however, Gilliland et al. (33) found that people with GSTP1 Ile-105 homozygotes showed larger IgE and histamine increases in nasal lavage fluid after challenge with diesel exhaust particles and allergens. Although homozygous GSTP1 Ile-105 was positively associated with wheezing illness in our study, in children with ETS exposure at home, those carrying GSTP1-105 Val/Val or Ile/Val genotypes were at increased risks for ever wheezing (OR 1.34, 95% CI 0.75–2.41) and current wheezing (OR 1.18, 95% CI 0.57–2.41) after adjustment for confounders. This dipolar phenomenon might be explained by different pathogenic pathways under environmental stimulation. Besides the complexity of asthma pathogenesis, combination of all the above findings indicates that different polymorphic forms of the GSTP1 gene may contribute to different detoxification ability or allergic sensitization ability. Additional research is necessary to explore the different enzyme activity or

biomarkers after tobacco smoke exposure among genetically susceptible individuals.

Although not based on objective measurements such as cotinine levels, the exposure of household ETS in our study was assessed by self-administered parental questionnaire, which was shown to be a reasonably valid tool (34). In our data, household ETS exposure was not associated with childhood wheezing illness overall, consistent with a previous study conducted in southern Taiwan (35). We lack data to assess changes in time activity patterns of tobacco smoke at home. However, errors in exposure assessment were likely to be random, which would reduce the magnitude of association, but would not introduce a positive bias in the associations. Because any recall bias would be independent of genotypes, this factor is unlikely to explain the interaction between household ETS exposure and GST genotypes. In Taiwan, the ratio of current smoker/ex-smoker rates in adulthood is close to 7 (19), far higher than the ratio near to 1 in USA (36), indicating a particularly low rate of smoking cessation in Taiwanese adults, and current ETS exposure could somehow represent past ETS exposure at home. Notably, we could find similar effects of current household ETS exposure on ever wheezing and current wheezing from our data.

Our study has some limitations. We did not have genotyping data from all subjects, which made selection bias possible. However, participants with genotyping data included in this analysis did not differ from those without genotyping data on many demographic and residential environmental factors, but did show small differences in the proportion of parental education levels (Table 1). Children from families with higher education level were less likely to have moved and to be lost to follow-up. Because the differences in distribution were modest and are probably not associated with the genotypes, it is unlikely that selection of subjects biased the effect estimates in our results. Childhood wheezing illness was ascertained by parental-reported questionnaire, so misclassification of wheezing status may have arisen from imperfect parental recall of events. We lack data to assess the magnitude of misclassification of wheezing status from parental recall; however, it is unlikely that our findings result from a spurious association that arose from variations across the three communities or from smokers over-reporting wheezing in their children.

In summary, our results showed that household ETS exposure was a modifiable cause of childhood wheezing illness in a genetically susceptible subpopulation. We also found a significant gene–environmental interaction between the GSTP1 genotype and ETS exposure at home. This stresses the importance of the variation in detoxification or allergic sensitization ability of the GSTP1 genetic polymorphisms that may be used as markers of individuals susceptible to inhalational insults. Our data suggest that childhood wheezing illness is a complex disease associated with many genes, and interactions between genes and environmental factors. Additional genetic epidemiologic studies of sufficient size are needed to replicate and expand our findings to additional genes and pathogenic pathways.

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