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Original article

Ambient ozone modifies the effect of tumor necrosis factor G-308A on bronchitic symptoms among children with asthma

Background: Tumor necrosis factor (TNF)-α has a recognized role in respiratory pathophysiology. One genetic variant (G-308A) in the promoter region affecting the expression of this cytokine may contribute to airway inflammatory diseases, but the studies on bronchitic symptoms were still inconclusive. Because ozone produces oxidative stress, increased airway TNF, and inflammation, the associations of the TNF-308 polymorphism with bronchitic symptoms may vary by ambient ozone exposure.
Methods: We studied associations of TNF-308 genotype with bronchitic symptoms among asthmatic children in Children's Health Study. The association of TNF G-308A polymorphism with bronchitic symptoms was investigated and we also determined whether the associations vary with ambient ozone exposure.
Results: Asthmatic children with TNF-308 GG genotype had a significantly reduced risk of bronchitic symptoms with low-ozone exposure (adjusted OR: 0.53; 95% CI: 0.31–0.91). The risk was not reduced in children living in high-ozone communities (adjusted OR: 1.42; 95% CI: 0.75–2.70). This difference in genotypic effects between low- and high-ozone environments was statistically significant among asthmatics (P for interaction $= 0.01$), but insignificant among nonasthmatic children.
Conclusion: Our findings suggest a role of gene–environmental interactions on the occurrence of bronchitic symptoms among children with asthma.

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Key words: bronchitic symptoms; children; gene–environmental interaction; tumor necrosis factor.

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Tumor necrosis factor (TNF)-α plays a central role in the initiation of airway inflammation (1, 2). In addition to pro-inflammatory properties, evidence from *in vitro* studies has shown that TNF-α can promote tracheal smooth muscle proliferation (3) and alter smooth muscle function (4). In humans, it was proved that TNF-α could induce airway neutrophil infiltration, increase airway hyperreactivity, and thereby develop respiratory symptoms (5). Elevated TNF-α was also found in bronchoalveolar lavage fluid (6) and bronchial biopsies (7) from patients with chronic bronchitis.

The expression of TNF-α is regulated at the transcriptional level (8, 9), and differences in TNF-α production may partly be determined at the genetic level. It has been demonstrated in transfection studies that the TNF-308 genotype may influence mRNA expression and affect TNF levels (9–11). Although the TNF region is highly polymorphic with a complex pattern of long-range linkage disequilibrium, studies of respiratory conditions have focused on the G-308A single nucleotide polymorphism because of its reported associations with airway inflammatory diseases, including various phenotypes of bronchitis (12, 13). The studies have been inconsistent in the direction of effects and some have found no significant association (14, 15). A number of methodological issues may contribute to these inconsistencies among studies. Variation in bronchitic symptoms may also reflect differences in the magnitude of airway inflammatory response, which depends on exposures that produce oxidative stress, airway antioxidant defenses, and TNF responses. Differences in genetic background and environmental exposures that interact with TNF could also contribute to differences in results among studies.

Ozone, a common air pollutant that has been associated with airway hyperreactivity, is a strong oxidant that induces airway inflammatory responses (16–18). Limited evidence indicates that ozone may modify the effect of the TNF G-308A polymorphism on respiratory health (19). The level of oxidative stress produced by ozone contributes to TNF-mediated inflammation; however, the level of oxidative stress is likely to depend on airway antioxidant defenses and ozone levels.

To further investigate the role of TNF on the occurrence of bronchitic symptoms, we examined the associations of TNF G-308A polymorphism at entry into the Children's Health Study (CHS) (20, 21). We also

investigated the variation in associations of this gene polymorphism with outcome in high and low ambient ozone environments.

Methods

Study design

The design and methods of CHS and the participants contributing to the current analysis have been described previously (20–22). Briefly, the CHS recruited 4th-, 7th-, and 10th-grade students from schools in 12 southern California communities in 1993 and recruited a second group of 4th-grade students from the same schools in 1996. Information on demographics, respiratory risk factors, household exposures, asthma, and bronchitic symptoms outcomes was collected by questionnaire completed at study entry by the parents or guardians of children. An air pollution monitoring network was established in 1994, and levels were measured continuously in each of the 12 communities included in the CHS. Children and a sample of their parents provided buccal cells as the DNA resource for genotyping several years after study entry. The study protocol was approved by the institutional review board for human studies at the University of Southern California, and written, informed consent was provided by a parent or legal guardian for all participants.

Questionnaire

A history of asthma for each child was assessed on the basis of the report in the baseline questionnaire. Children were considered to have a history of asthma, if there was a 'yes' answer to the question 'Has a doctor ever diagnosed this child as having asthma?' The primary outcome of interest in this study was the period of prevalence of bronchitic symptoms, defined as having any one of the following: (i) one or more episodes of bronchitis (defined by the question 'How many times in the past 12 months did your child have bronchitis?'); (ii) chronic cough (defined by a 'yes' answer to the question 'During the past 12 months, has this child had a cough first thing in the morning that lasted for as long as 3 months in a row?' or to the question 'During the past 12 months, has this child had a cough at other times of the day that lasted for as much as 3 months in a row?'); or (iii) chronic phlegm (defined by a 'yes' answer to the question 'Other than with colds, does this child usually seem congested in the chest or bring up phlegm?').

In the baseline questionnaire, we also obtained information on children's sex, age, grade, ethnicity, parental education level, insurance coverage, and many residential risk factors, such as second-hand smoke, pet ownership, gas stove, gas heat, and carpet use at home.

Air pollution data

The California Air Resources Board helped establish the air pollution monitoring network and helped collect the air pollution data from this network for us in the study. Each station measured average hourly levels of ozone. We computed the annual average of the ozone levels obtained from 10:00 a.m. to 6:00 p.m. (the 8-h daytime average) in each community from 1994 to 1996. Community-level ozone exposures were grouped into low and high subcategories using a 50-ppb cut off. The mean annual ambient ozone level was 39.2 ppb in low-ozone communities and 56.5 ppb in high-ozone communities.

DNA collection and genotyping

Buccal cells were collected as a source of genomic DNA from consenting study subjects (23). Genomic DNA was isolated from buccal mucosal cells using Purgene DNA isolation kit (Gentra Systems, Minneapolis, MN, USA). The G to A transition polymorphism at position -308 for human TNF was identified by polymerase chain reaction (PCR), and allelic discrimination assays were performed on an ABI Prism 7700 Sequence Detector (Applied Biosystems, Foster City, CA, USA). Standard setup and operation for the allelic discrimination assay were used as detailed by the ABI Prism 7700 Sequence Detection System user's manual. The QuantiTect Probe PCR kit (Qiagen, Valencia, CA, USA) was used for PCR reactions. Primers and fluorescent probes were designed by using Primer Express applications-based primer design software (Applied Biosystems). The forward primer for human TNF-308 was 5′-GAAATGGAGGCAATAGGTTTTGA-3′, and the reverse primer was 5′-GTAGGACCCTGGAGGCTGAAC-3′. The minor groove-binding probe for the G allele was 5′-CCGTCCCCATGCC-3′, and the minor groove-binding probe for the A allele was 5′-CCGTCCTCATGCC-3′ (Applied Biosystems). Ten percent of the samples was selected as quality controls and each genotype was validated by using PCR and restriction fragment length polymorphism methods (12).

Statistical analysis

Unconditional logistic regression was used to estimate the association of the TNF-308 genotype with bronchitic symptoms. Genetic models were chosen based on available biological evidence from mechanistic and association studies. For TNF-308, we grouped genotypes into GG *vs* AG or AA because we hypothesized that the GG genotype is protective due to reduced inflammatory response. Confounding by *a priori* identified covariates was assessed by examining changes in TNF-effect estimates from the models with and without the potential confounder.

To investigate whether ambient ozone level modulated the association of TNF-308 genotype with bronchitic symptoms, we fitted stratified models to the children in communities with lower or higher ozone levels. Odds ratios (ORs) with 95% confidence intervals (CI) were calculated with or without adjustment for potential confounders. In assessing interaction, we compared the risk of bronchitic symptoms for subjects in each category of joint exposure to that of subjects who were GG genotype and resided in low-ozone communities. Individual and joint associations were estimated using indicator variables created for each category, omitting the hypothesized low–low risk reference group. All tests assumed a two-sided alternative hypothesis and a 0.05 significance level. The analyses were conducted using SAS software version 9.1 (SAS Institute, Cary, NC, USA).

Results

Our study comprised 3593 participants with complete TNF-308 genotypic data from 12 southern California communities. Among the 548 asthmatic children in this subset of the CHS children, 234 (42.7%) had bronchitic symptoms in the year prior to study entry. Boys were significantly more likely to report symptoms (Table 1). Demographic characteristics, second-hand smoke at home, and residential factors were not significantly associated with bronchitic symptoms. Children with

Table 1. Distribution of demographic and other baseline characteristics by bronchitic symptoms among children with asthma

	N	% with symptoms	Total
Sex (*P* = 0.02)			
Boys	144	47.1	306
Girls	90	37.2	242
Age (*P* = 0.16)			
8–9	106	38.8	273
10–11	58	44.6	130
≥12	70	48.3	145
Ethnicity (*P* = 0.01)			
Non-Hispanic white	145	43.4	334
Hispanic white	65	47.1	138
African-American	5	14.3	35
Asian	4	28.6	14
Others	15	55.6	27
Parental education level (*P* = 0.82)			
Less than high school	22	46.8	47
High school	48	46.6	103
Technical school	115	41.4	278
College	24	42.9	56
Graduate training after college	25	39.1	64
Insurance (*P* = 0.26)			
No	26	50.0	52
Yes	208	41.9	496
Second-hand smoke at home* (*P* = 0.26)			
No	190	44.0	432
Yes	41	38.0	108
Pets ownership (*P* = 0.12)			
No	31	35.2	88
Yes	203	44.1	460
Gas stove use* (*P* = 0.33)			
No	53	46.9	113
Yes	180	41.8	431
Gas heat use* (*P* = 0.25)			
No	50	38.8	129
Yes	180	44.6	404
Carpet use* (*P* = 0.18)			
No	29	50.9	57
Yes	195	41.7	468
Life-long residence (*P* = 0.01)			
No	96	36.8	261
Yes	138	48.1	287
Ozone exposure (*P* = 0.63)			
High (≥50 ppb)	95	41.5	229
Low (<50 ppb)	139	43.6	319
TNF G-308A genotype (*P* = 0.24)			
GG	163	41.1	397
GA	66	48.5	136
AA	5	33.3	15

P-values were calculated from chi-square tests of homogeneity.
*A number of subjects do not add up to total *N* because of missing data.

symptoms (11.2 years; SD 2.3) were slightly older than children without symptoms (10.9 years; SD 2.2) ($P = 0.15$). Among asthmatic children, the overall allele frequencies of TNF-308 polymorphisms were G = 70.9%/A = 29.1% in bronchitic cases and G = 85.7%/A = 14.3% in nonbronchitic controls, and the alleles were in Hardy–Weinberg equilibrium in both groups.

Children who were homozygous for the TNF-308 G showed reduced risk for bronchitic symptoms [adjusted OR (aOR): 0.78; 95% CI: 0.63–0.95] (Table S1). When we assessed interaction for TNF-308 effects by asthma status, we could not find a significant result (*P* for interaction = 0.71). However, this genetic variant showed a similar but insignificant effect among asthmatic children (aOR: 0.81; 95% CI: 0.55–1.22) (Table 2). Asthmatic children with the GG genotype had a lower prevalence of bronchitic symptoms compared with children carrying at least one A allele (e.g. GA or AA).

We found that the protective effects of the GG genotype on bronchitic symptoms were stronger for children living in low-ozone communities than in high-ozone communities (Table 3). These OR did not change substantially after we controlled for age, sex, ethnicity, grade, second-hand smoke, and life-long residence. Compared with children who had the GA or AA genotypes, those with the GG genotype had a marked reduction of bronchitic symptoms in low-ozone communities (aOR: 0.53; 95% CI: 0.31–0.91); however, this reduction was not observed in children living in high-ozone communities (aOR: 1.42; 95% CI: 0.75–2.70). This difference in genotypic effects between low- and high-ozone environments was statistically significant (*P* for interaction = 0.01) (Table 3). We also assessed interactions for genetic effects by age, sex, ethnicity, or life-long residence, and none were statistically significant. However, in our analysis, the associations for TNF-308 GG genotype did not statistically differ between ambient ozone among nonasthmatic children (*P* for interaction = 0.47) (Table S2). By using dummy variables in each community, we could calculate betas of TNF-308 GG genotype on the occurrence of bronchitic symptoms among children with asthma. Adjusted community-specific betas were plotted against ambient ozone concentration, using weights proportional to the inverse variance (Fig. 1).

There was no significant difference on the prevalence of bronchitic symptoms in high- and low-ozone communities (Table 1). In addition, we found no substantial differences in the effect of the GG genotype in asthmatic children in relation to exposure to the other monitored air

Table 2. Associations of TNF G-308A with bronchitic symptoms among children with asthma

		Unadjusted model	Mutually adjusted model
TNF-308	Ca/Co	cOR (95% CI)	aOR (95% CI)
G/A or A/A	71/80	1.00	1.00
G/G	163/234	0.79 (0.54–1.14)	0.81 (0.55–1.21)

Models were adjusted by sex, age, ethnicity, grade, second-hand smoke, and life-long residence.
Ca/Co, number of cases/number of controls; cOR, crude odds ratio; aOR, adjusted odds ratio; CI, confidence interval; TNF, tumor necrosis factor.

Table 3. Associations of TNF G-308A with bronchitic symptoms among children with asthma in lower and higher ozone communities*

	Unadjusted model				Mutually adjusted model			
	Low ozone		High ozone		Low ozone		High ozone	
TNF-308	Ca/Co	cOR (95% CI)	Ca/Co	cOR (95% CI)	Ca/Co	aOR (95% CI)	Ca/Co	aOR (95% CI)
G/A or A/A	48/38	1.00	23/42	1.00	48/38	1.00	23/42	1.00
G/G	91/142	0.51 (0.31–0.84)	72/92	1.43 (0.79–2.59)	91/142	0.53 (0.31–0.91)	72/92	1.42 (0.75–2.70)
P interaction		0.01				0.01		

Models were adjusted by sex, age, ethnicity, grade, second-hand smoke, life-long residence, and communities within each stratum.
Ca/Co, number of cases/number of controls; cOR, crude odds ratio; aOR, adjusted odds ratio; CI, confidence interval; TNF, tumor necrosis factor.
*Two ozone strata were defined as ≤ 50-ppb ozone average.

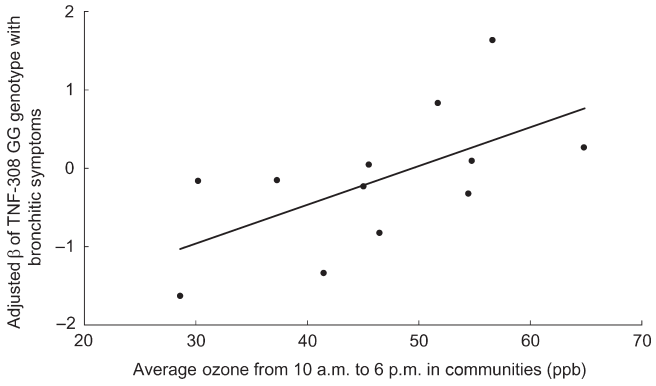

Figure 1. Ozone modifies the effect of TNF G-308A genotype on bronchitic symptoms among children with asthma.

pollutants in the CHS (PM_{10}, $PM_{2.5}$, NO_2, and acid vapor levels) or second-hand smoke exposure (data not shown). The variation in the protective effect of the GG genotype on bronchitic symptoms in high- and low-ozone communities was also similar in analyses restricted to the subgroup of lifetime residents (data not shown).

Discussion

The previous studies have reported that bronchitis occurrence may depend on antioxidant and inflammatory genes (12–15). We examined the relationships between the genotypic distribution of TNF G-308A polymorphism, ambient ozone exposure, and bronchitic symptoms among CHS schoolchildren. Among asthmatic children, we found that the TNF-308 GG genotype was protective for bronchitic symptoms but the protection depended on outdoor ozone levels in the community of residence. Children living in high-ozone communities were not protected from increased bronchitic symptoms by the GG genotype. Our findings suggest a role of gene–environmental interactions on the occurrence of bronchitic symptoms among children with asthma.

The power of our analysis has been insufficient to detect small effects, and the existence of a major genetic effect is highly unlikely. In our data, we could not find a significant main effect of TNF-308 GG polymorphism among asthmatic children (aOR: 0.81; 95% CI: 0.55–1.21) (Table 2). However, this genetic variant showed a similar but significant effect among 3045 nonasthmatic children (aOR: 0.77; 95% CI: 0.61–0.98). Our results are consistent with the previous Taiwanese study reporting that the allele G of the TNF-308 polymorphism is associated with decreased risk of chronic bronchitis (12). Nonetheless, some studies on the association of TNF-308 polymorphism with bronchitic symptoms have reported conflicting results (14, 15). Based on our findings, the inconsistency among studies may be due to differences in ambient ozone exposure among the populations studied. Although we did not directly study the mechanisms for the effects of the TNF-308 GG variant or the effects of ozone exposure on TNF-mediated airway inflammation, we speculate that TNF-308 GG genotype is associated with less intense inflammatory responses to oxidant stressors and that this reduced responsiveness results in reduced risk for bronchitic symptoms.

Although individuals may vary in genetic susceptibility to ambient air pollution, to our best knowledge, there are still limited epidemiologic studies concerning the different effects of TNF-α gene polymorphisms by environmental exposures. In the analyses of asthmatics, we found the risk of bronchitic symptoms decreased (aOR: 0.53; 95% CI: 0.31–0.91) in those with TNF-308 GG genotype under low-ozone exposure. The reduction was not observed in children living in high-ozone communities (aOR 1.42; 95% CI: 0.75–2.70) (Table 3). The slight changes of OR under high-ozone exposure were not statistically significant and should not be considered as real increases in the population. Meanwhile, our results provide an important demonstration of a gene–environmental interaction. Because different polymorphic forms of the TNF-308 gene have different effects on the airway inflammatory response of an individual, it is interesting to speculate that an ozone-induced airway injury might be different in children carrying different polymorphic forms of this gene.

Ozone, an ambient photochemical oxidant, is capable of producing pulmonary inflammation and injury (17,

18). Inhaled ozone might enhance the airway response to environmental stimuli, thereby inducing respiratory symptoms (24). Exposure to ozone results in the release of inflammatory cytokines, including TNF-α (25). Pretreatment of rats with antibody to TNF can reduce ozone-induced inflammation and lung damage, and acute ozone-induced airway hyperreactivity is reduced in TNF receptor-deficient mice (26). Oxidative stress is intimately involved in multiple inflammation processes that are regulated in part by cytokines such as TNF-α (27, 28). TNF-308 A allele may alter gene expression and amplify the intensity of the inflammatory response to oxidants, leading to increased risk of bronchitic symptoms in susceptible groups. The findings in this study suggest that the protective effects of the -308G on transcription are overcome by higher levels of oxidative stress.

Some demographic factors, such as age, sex, ethnicity, grade, and life-long residence, may contribute to the occurrence of bronchitic symptoms. Second-hand smoke also has the potential to confound the relationship between the TNF-308 polymorphisms and bronchitic symptoms. We minimized interference from these confounders by adjustment in regression models. Adjusting for pet ownership, gas stove, gas heat, and carpet use at home individually and in combination resulted in negligible changes in the associations. Exposure to second-hand smoke was assessed using questionnaire responses about household sources and was not validated by objective measurements such as cotinine levels. However, the validity of exposure estimates based on questionnaire responses has been investigated and found to provide reasonably valid estimates of exposure for adjustment for confounding (29, 30).

TNF region comprised fewer than 20 common haplotypes. The -308G variant occurs on two long-range haplotypes in populations with European ancestry, one of which is the most common haplotype in the linked region (31). Association studies that have examined the TNF loci haplotypes have been conducted and have found associations with haplotypes containing the -308G variant (32). Based on the available information on locus region linkage structure, the associations with the TNF-308 polymorphism reflect a comparison of haplotypes containing the -308G variant to the remaining haplotypes across the 45-kb region and indicate that the -308G variant or another variant on these haplotypes underlie the increased susceptibility for bronchitic symptoms and the loss of protection in high-ozone communities in the context of variant genotypes.

Limitations of this study include the relatively imprecise assessment of outcomes based on symptom reporting by questionnaire. However, these outcomes have been widely used in epidemiologic studies of children. Cough of 3 or more months duration, chronic phlegm, and a report of bronchitis in this study are suggestive of chronic, indolent asthma exacerbation, which would likely be remembered well. Symptoms reporting might also reflect repeated acute exacerbation, but acute bronchitis has been reported to have a marked impact on the quality of life, at least in adults, and to persist for several weeks, so such episodes also would be likely to be recalled well (33). Additionally, because outcomes were defined without the knowledge of genotype, differential misclassification of bronchitic symptoms by TNF-α genotype is probably not a major source of bias that accounts for our results. Therefore, although there is likely to be nondifferential misclassification of bronchitic symptoms, such misclassification would not account for the genetic associations we observed.

Migrating from one community to another could lead to misclassification of ozone exposure. However, errors in exposure assessment were likely to be random, which would not introduce a positive bias in the associations. In addition, ozone exposure assessment was based on ambient levels measured at monitoring stations in each community. We dichotomized exposure into high- and low-ozone communities to account for uncertainties in indoor levels and time–activity patterns of the study participants. Although these estimates have associated measurement error, it is likely that the groups provided substantial contrasts in average ozone exposure. We only observed an association with ozone in a susceptible subgroup. We previously reported that children playing three or more team sports in a high-ozone environment had increased risk for developing asthma (34). Because of sample size limitations, we were unable to consider team sports as an additional effect modifier in this genetic analysis.

In conclusion, the protective association of TNF-308 GG genotype in asthmatic children with low ozone exposures suggests that this relatively common genetic polymorphism, or haplotype marked by this polymorphism, plays a protective role of bronchitic symptoms depending on airway oxidative stress levels. Additional research is necessary to prove that the elimination of environmental exposures among genetically susceptible individuals could reduce rates of such symptoms.

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Supporting Information

Additional Supporting Information may be found in the online version of this article:

Table S1. Associations of TNF G-308A with bronchitic symptoms among CHS children.

Table S2. Associations of TNF G-308A with bronchitic symptoms among nonasthmatic CHS schoolchildren in lower and higher ozone communities.

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Table S1. Associations of TNF G-308A with bronchitic symptoms among CHS children

		Unadjusted model		Mutually adjusted model	
TNF-308	**Ca/Co**	**cOR**	**95% CI**	**aOR**	**95% CI**
G/A or A/A	187/691	1.00		1.00	
G/G	452/2263	0.73	(0.60-0.89)	0.78	(0.63-0.95)

Models were adjusted by sex, age, ethnicity, grade, second-hand smoke, and lifelong residence.
Ca/Co: number of cases/number of controls; cOR: crude odds ratio; aOR: adjusted odds ratio; CI: confidence interval; TNF: tumor necrosis factor.

Table S2. Associations of TNF G-308A with bronchitic symptoms among non-asthmatic CHS schoolchildren in lower and higher ozone communities*

	Unadjusted model						Mutually adjusted model					
	Low Ozone			High Ozone			Low Ozone			High Ozone		
TNF-308	**Ca/Co**	**cOR**	**95% CI**	**Ca/Co**	**cOR**	**95% CI**	**Ca/Co**	**aOR**	**95% CI**	**Ca/Co**	**aOR**	**95% CI**
G/A or A/A	68/321	1.00		48/290	1.00		68/321	1.00		48/290	1.00	
G/G	173/1181	0.61	(0.51-0.94)	116/848	0.83	(0.58-1.19)	173/1181	0.70	(0.51-0.96)	116/848	0.87	(0.60-1.26)
P interaction	0.54						0.47					

Models were adjusted by sex, age, ethnicity, grade, second-hand smoke, lifelong residence, and communities within each stratum.
CHS: Children's Health Study; TNF: tumor necrosis factor; Ca/Co: number of cases/number of controls; cOR: crude odds ratio; aOR: adjusted odds ratio; CI: confidence interval.
* Two ozone strata were defined as less than and greater than 50-ppb ozone average.