

PEDIATRIC ASTHMA

Association of Hepatitis A Exposure and TIM-1 with Childhood Allergic Asthma

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Background. Hepatitis A virus (HAV) receptor (TIM-1) polymorphism plays an important role in asthma and autoimmune diseases. **Objective.** To analyze the association of TIM-1 polymorphism and HAV infection with childhood allergic asthma in Southwest China. **Methods.** TIM-1 exon 4 (157insMTTTPV) and two polymorphism loci, –416G>C and –1454G>A, in the HAV receptor promoter region were studied. Polymerase chain reaction (PCR) was used to test the genotypes of three polymorphism loci among 579 cases of asthma and 524 controls. The HAV infection status was determined in a case–control study with stratified analysis. **Results.** HAV exposure associated with childhood allergic asthma in the study population was compared with controls (odds ratio (OR) = 0.181, 95% confidence interval (CI) 0.126–0.260, $p < .001$). The –416G>C polymorphism was associated with asthma (OR = 1.384, 95% CI 1.148–1.669, $p < .001$), but the insertion variant 157delMTTTPV of exon 4 and the –1454G>A polymorphism were not. **Conclusion.** Our results indicated that the –416G>C polymorphism of the TIM-1 gene is associated with childhood allergic asthma, providing a better understanding of the pathogenesis of the allergic asthma among children aged below 15 years in Southwest China.

Keywords childhood allergic asthma, haplotype, hepatitis A virus, single-nucleotide polymorphism, T-cell immunoglobulin mucin

INTRODUCTION

Asthma, a complex disease of multiple etiologies including environmental factors and an obvious genetic element, is the most common chronic respiratory allergic disease in childhood (1, 2). In recent years, the prevalence of allergic asthma has increased worldwide, whereas the hepatitis A virus (HAV) infection rate has decreased (3–5). The negative epidemiologic correlation between HAV infection and allergic asthma implies that HAV infection may influence the incidence of asthma, but the mechanism involved is not well understood. McIntire *et al.* (6) discovered a T cell and airway phenotype regulator (Tapr) on mouse chromosome 11, which can regulate the T cell and airway phenotype; he also identified a novel gene family called TIM (T-cell immunoglobulin domain and mucin domain). TIM-1, TIM-2, and TIM-3 for *Homo sapiens* were found to be members of the TIM gene family located on chromosome 5q33.2 (7–9). This is also one of the major genetic susceptibility sites for asthma. TIM protein is a type-I transmembrane protein encoded by the TIM gene, including an N-terminal immunoglobulin V region (IgV) domain, a mucin-like domain, a transmembrane domain, and a cytoplasmic tail with a phosphorylation motif (10). The TIM gene family plays a critical role in the development of allergic disorders such as asthma and autoimmune diseases (11). TIM-1, the first molecule in the TIM family and the most important one, was first found in renal cells of the

African Green Monkey (12). In subsequent studies, it was confirmed to be the same molecule as the human HAV receptor, hHAVcr-1 (13), and the kidney injury molecule-1 (KIM-1) (14). TIM-1 is selectively expressed on activated CD4⁺ T cell surfaces, especially on Th2 cells, and regulates the Th2-type cell-mediated immune response (6, 8). In addition, it has an effect on macrophages involved in local inflammation (15). TIM-1, TIM-3, and TIM-4 serve as receptors for phosphatidylserine (PtdSer), and TIM-1 polymorphisms have been shown to be associated with the severity of HAV infection (16). Additionally, TIM-3 molecules are expressed mainly on the surfaces of Th1 cells, causing a negative effect on the regulation of those cells (17, 18). As the natural ligand of TIM-1, TIM-4 is exclusively expressed on the surfaces of antigen-presenting cells such as macrophages and dendritic cells. After binding to TIM-1, it stimulates the proliferation and differentiation of Th2 cells (19, 20). Therefore, the human TIM gene family is critical to Th1/Th2-mediated immunological regulation (7).

It has been reported that the effect of TIM-1 exon 4 (157insMTTTPV) on asthma is related to HAV infection (21). Moreover, Liu *et al.* found that one functional polymorphic locus, –416G>C, was related with bronchial asthma in the Chinese Han population (22). Recently, Mou *et al.* found that both –416G>C and –1454G>A were associated with allergic rhinitis in Chinese Han people (23). China is a developing country with rather poor sanitation conditions. The HAV exposure rate is fairly low in adolescents, but is almost 100% in adults. Nevertheless, the relationship between HAV exposure, TIM-1 polymorphism, and childhood allergic asthma is poorly understood.

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Therefore, in this study, we aimed to analyze the association of TIM-1 polymorphism and HAV infection with childhood allergic asthma in Southwest China. For this purpose, we selected an exon 4 (157insMTTTVP) variation and two loci at promoter regions –416G>C and –1454G>A, and found that HAV exposure instead of exon 4 had a protective effect on childhood allergic asthma in a large case–control study. –416G>C was found to be related to childhood allergic asthma, but –1454G>A was not.

METHODS

Study Population

The subjects of our study were children aged between 2 and 15 years; among them, 579 were unrelated asthma patients (224 females, 353 males) with an average age of 7.2 years and 524 were healthy children without any allergic disorders such as asthma (304 females, 220 males) with an average age of 7.6 years. The serum HAV exposure level was determined by using HAV antibody ELISA test kit according to the manufacturer's instructions (Beijing Kewei Clinical Diagnostic Reagents Co., Ltd., Beijing, China). Only subjects without previous vaccinations against hepatitis A were recruited in this study. The asthma patients were admitted from the outpatient Pediatric Department of the Southwest Hospital of the Third Military Medical University, and the diagnosis of asthma was made according to the guidelines of the American Thoracic Society (24). The diagnosis met the following two medical criteria: first, wheezing rales on three or more lung examinations or a clinical history of gasping, shortness of breath, and respiratory distress, and second, gasping, decreased or relieved with anti-asthmatics (bronchodilators). The children in the control group were outpatients and inpatients of the Pediatric Department of the Southwest Hospital of the Third Military Medical University, and children in kindergarten or primary school who accepted physical examination. Two standards were adopted in the selection: first, control without asthma or any other allergic disorders, and second, control without any other chronic diseases; third, subjects were negative for SPT, total IgE, and EOS. The subjects were from Southwest China, and our study obtained written informed consent from both children and parents. The study was also approved by the Ethics Committee of the Third Military Medical University.

Genotyping

DNA extraction was performed with the E.Z.N.A.TM SE Blood DNA kit (D3471-02) (Omega, Norcross Georgia, USA), and polymerase chain reaction–restriction fragment length polymorphism (PCR-RFLP) was used to analyze exon 4 insertion loss variation and –416G>C and –1454G>A mutations in the promoter region. A 1000-series thermal cycling platform (Bio-Rad, CA, USA) was used to amplify DNA with KOD-plus-201 polymerase (TOYOBO, Tokyo, Japan). Referring to the sequence of human chromosome 5 (NT_023133.12) in the GenBank database, the following

primers were designed: exon 4 (157insMTTTVP) forward primer 5'-TCCAGCCTGAGGCTCTTGT-3', reverse primer 5'-ATGCTCATTGTTGTTGGAACAG-3'. Primers for –416G>C and –1454G>A were described previously (25). PCR was performed on 50 µl samples, including 50 ng DNA template, 0.2 mM dNTPs, 0.2 µM of each primer, 1 mM MgSO₄, 5 µl of 10× PCR buffer, and 1 U KOD-plus-DNA polymerase. The same volume of double-distilled water instead of DNA template was used as a negative control. The conditions for PCR were as follows: initial denaturation temperature of 94°C for 2 min and 30 cycles at 94°C for 15 s. The renaturation temperatures for –416G>C, –1454G>A, and exon 4 were 64°C, 60°C, and 58°C for 30 s each, 68°C for 60 s, and a final extension at 72°C for 5 min according to the manufacturer's instructions. The –416G>C, –1454G>A, and PCR products were digested by specific restriction enzymes (MspI for –1454G/A and TaqI for –416G/C, Fermentas, Maryland, USA), and the products were analyzed with a Gel-2000 gel imager (Bio-Rad, USA) after separation on 1.5% agarose gels stained with ethidium bromide. After native polyacrylamide gel electrophoresis (12%) and staining with ethidium bromide, exon 4 was analyzed by gel imaging (26).

Statistical Analysis

Hardy–Weinberg equilibrium tests were done both in the case and control groups with Arlequin software (<http://anthro.unige.ch/software/arlequin/>) based on a goodness-of-fit χ^2 test. The Fisher χ^2 test (SPSS 13.0) was used to analyze the statistical variance of alleles and genotypes between asthma and control groups. Risk odds ratios (ORs) and 95% confidence interval (CI) of asthma genetic polymorphisms were also analyzed. SHEsis software (<http://analysis.bio-x.cn/myAnalysis.php>) was used to conduct linkage disequilibrium analysis on single-nucleotide polymorphism (SNP) sites, and to construct haplotypes for the case–control study. Statistical differences between various groups were studied by one-way analyses, in which *p*-value <.05 was considered statistically significant.

RESULTS

The allelomorphic gene and genotype distribution of three sites were determined in both the asthma and control groups according to Hardy–Weinberg equilibrium tests (all *p*-values were >.05). The results are shown in Table 1. The SNP C allelomorphic gene in the asthma group at the –416G>C site was less frequent than that in control group, which was statistically significant (*p* = .001, asthma = 68.7%, control = 75.2%). The frequencies of exon 4 (157insMTTTVP) insertion, and the A allelomorphic gene in the asthma group compared with that in the control group, were not statistically different (*p* = .085, asthma = 23.7%, control = 20.6%; *p* = .834, asthma = 16.0%, control = 15.7%). The results showed that the TIM-1 promoter –416G>C SNP was correlated with childhood allergic asthma in Southwest China (*p* = .002). Asthmatic children

TABLE 1.—Cross-tabulation analysis of the genotype and allele frequencies of TIM-1 polymorphisms with asthma.

Polymorphism	Genotype/allele	Asthma, <i>N</i> (%)	Control, <i>N</i> (%)	OR (95% CI)	<i>p</i> -Value
157insMTTVP	Deletion/deletion	335 (57.9)	332 (63.4)	1.000	.175
	Insertion/deletion	214 (37.0)	168 (32.1)	1.262 (0.891–1.625)	
	Insertion/insertion	30 (5.2)	24 (4.6)	1.239 (0.709–2.164)	
	Deletion	884 (76.3)	832 (79.4)	1.000	
	Insertion	274 (23.7)	216 (20.6)	1.194 (0.976–1.461)	
–416G>C	C/C	264 (45.6)	288 (55.0)	1.000	.002
	G/C	267 (46.1)	212 (40.5)	1.374 (1.075–1.757)	
	G/G	48 (8.3)	24 (4.6)	2.182 (1.300–3.661)	
	C	795 (68.7)	788 (75.2)	1.000	
	G	363 (31.4)	260 (24.8)	1.384 (1.148–1.669)	
–1454G>A	G/G	411 (71.0)	372 (71.0)	1.000	.787
	G/A	151 (26.1)	140 (26.7)	0.976 (0.746–1.278)	
	A/A	17 (2.9)	12 (2.3)	1.282 (0.604–2.720)	
	G	973 (84.0)	884 (84.4)	1.000	
	A	185 (16.0)	164 (15.7)	1.025 (0.815–1.289)	

TABLE 2.—Haplotype analysis in children with asthma.

Haplotype ^a	Asthma (%)	Control (%)	χ^2	OR (95% CI)	<i>p</i> -Value
C-del-G	50.84	58.05	11.266	0.747 (0.630–0.886)	.001
G-del-A	11.42	9.63	1.954	1.217 (0.924–1.599)	.162
G-del-G	11.59	7.46	10.932	1.632 (1.218–2.187)	.001
C-ins-G	14.47	11.23	5.279	1.344 (1.044–1.729)	.022
G-ins-G	7.12	7.61	0.175	0.934 (0.678–1.286)	.676

^aSequence of haplotype: –416G>C, exon 4 (157insMTTVP), and –1454G>A.

carrying the G allele had increased risk compared with those carrying the C allele (OR = 1.384, 95% CI 1.148–1.669). But, the TIM-1 exon 4 (157insMTTVP), promoter –1454G>A SNPs genotype, and allele were not correlated with allergic asthma ($p = .175$, $p = .787$). The Linkage disequilibrium was in weak LD of 0.12 between SNP exon 4 (157insMTTVP) and –416G>C, a moderate linkage disequilibrium of 0.489 between that and –1454G>A, and a moderate linkage disequilibrium of 0.553 between SNP –416G>C and –1454G>A. The analysis of haplotypes with frequencies larger than 5% is shown in Table 2. The frequency of the C-del-G haplotype in the asthma group was significantly lower than that in the control group ($p = .001$, OR = 0.747). In contrast, the frequency of the G-del-G and C-ins-G allele in the asthma group was obviously higher than that in the control group ($p = .001$, OR = 1.632 was less than $p = .022$, OR = 1.344).

The results of the association of HAV seropositivity with the asthma TIM-1 gene polymorphism are shown in Tables 3 and 4. The HAV exposure seemed to protect against asthma as the percentage of subjects with asthma was significantly lower in HAV-seropositive population compared with that in HAV-seronegative population. It is likely that the association observed for asthma could be confounded by HAV exposure. To avoid this possibility, the correlation between TIM-1 gene polymorphism and asthma was investigated among all subjects who were HAV seronegative. As shown in Table 4, the exon 4 (157insMTTVP) and –1454G>A in TIM-1 gene yielded no significant association with asthma in

TABLE 3.—Comparison of HAV serostatus in asthma and control groups.

HAV serostatus	Asthma	Control	OR (95% CI)	<i>p</i> -Value
HAV seronegative	536	363	1.00	<.01
HAV seropositive	43	161	0.181 (0.126–0.260)	

HAV-seronegative population. However, a statistically significant difference in the association between asthma and control was found in HAV-seronegative cohort with –416G>C. This discrepancy might be due to the limited number of cases included. Furthermore, TIM-1 gene polymorphism was not associated with asthma in HAV-seropositive population (Table 5).

DISCUSSION

Matricardi and his colleagues (27) have reported a negative correlation between HAV exposure and allergic diseases such as asthma, which has been verified by molecular genetics with epidemiological and empirical studies (9). Since then, the association of HAV exposure and TIM-1 gene polymorphism with asthma has attracted widespread interest. Many studies have revealed a protective effect of HAV exposure on asthma (3, 28), whereas others failed to confirm that observation (4, 29). At the 99th Dahelm Conference on Infection, Inflammation, and Chronic Inflammatory Disorders, Matricardi explained the

TABLE 4.—Relationship of HAV seronegativity with TIM-1 gene polymorphism.

Polymorphism	Genotype	Asthma, <i>N</i> (%)	Control, <i>N</i> (%)	OR (95% CI)	<i>p</i> -Value
157insMTTVP	Insertion/insertion	27 (5.0)	16 (4.4)	1.00	.399
	Insertion/deletion	198 (37.0)	120 (33.1)	0.978 (0.506–1.889)	
	Deletion/deletion	311 (58.0)	227 (62.5)	0.812 (0.427–1.542)	
–416G>C	G/G	42 (7.8)	11 (3.0)	1.00	<.01
	G/C	251 (46.8)	148 (40.8)	0.444 (0.222–0.889)	
	C/C	243 (45.3)	204 (56.2)	0.312 (0.157–0.622)	
–1454G>A	G/G	381 (71.1)	256 (70.5)	1.00	.971
	G/A	140 (26.1)	96 (26.4)	0.980 (0.723–1.328)	
	A/A	15 (2.8)	11 (3.0)	0.916 (0.414–2.027)	

TABLE 5.—Relationship of HAV seropositivity with Tim-1 gene polymorphism.

Polymorphism	Genotype	Asthma, <i>N</i> (%)	Control, <i>N</i> (%)	OR (95% CI)	<i>p</i> -Value
157insMTTVP	Insertion/insertion	3 (7.0)	8 (5.0)	1.00	.517
	Insertion/deletion	16 (37.2)	48 (29.8)	0.889 (0.210–3.761)	
	Deletion/deletion	24 (55.8)	105 (65.2)	0.610 (0.150–2.469)	
–416G>C	G/G	6 (14.0)	13 (8.1)	1.00	.499
	G/C	16 (37.2)	64 (39.8)	0.542 (0.178–1.646)	
	C/C	21 (48.8)	84 (52.2)	0.542 (0.184–1.594)	
–1454G>A	G/G	30 (69.8)	116 (72.0)	1.00	0.149
	G/A	11 (25.6)	44 (27.3)	0.967(0.446–2.094)	
	A/A	2 (4.7)	1 (0.6)	7.733(0.678–88.176)	

inconsistent results with three models (30). TIM-1 insertion loss variation at exon 4 and SNP at the promoter region may be related to the incidence and development of asthma. However, because of differences in research methods, environmental factors, and patient groups, the results of various studies were inconsistent. Chae *et al.* reported a case–control study of exon 4 variation (5383_5397del, 5509_5511delCAA) in a Korean population, and found that TIM-1 was significantly related to rheumatoid arthritis (RA) and atopic dermatitis (31). But, the two loci were not found to be correlated with allergic asthma or allergic rhinitis in Chinese Han, Korean, and other populations (23, 32–34). McIntire *et al.* reported that inserting six amino acids in exon 4 (157insMTTVP) had a protective effect on asthma in HAV antibody-positive individuals (21). But, Gao *et al.* argued that the protective effect on asthma did not depend on the status of HAV (35). Noguchi indicated that exon 4 insertion (157insMTTVP) had no relationship with allergic asthma. No studies were done on the association of HAV exposure with that gene because the exposure rate of HAV in adolescents was very low (34). These reports indicated that the protective effects of exon 4 gene variation and HAV exposure on asthma may be different because of differences in ethnicities and sanitation conditions. Besides, the 5383_5397del variation site of Tim-1 exon 4 is suggested to be associated with atopic dermatitis susceptibility in Korean population (36). Wu *et al.* showed that TIM-1 gene is associated with elevated serum total IgE levels, particularly in individuals with atopic asthma (37). Page *et al.* demonstrated that genetic variants in the ligand for TIM-1 and TIM-4 contribute to the presentation of childhood atopic dermatitis

(37). Many of the studies define their patients differently, and based on this, the results may differ (e.g., all asthma vs allergic asthma, vs atopy that may or may not include eczema).

The negative epidemiologic correlation between HAV infection and allergic asthma indicates that HAV infection might influence the incidence of asthma. But, the specific mechanism is not clear. A hygiene hypothesis has been put forward, reasonably explaining the epidemiologic negative relation between HAV infection and allergic asthma (30, 38), indicating an immune response equilibrium state between Th1 and Th2 (39). The present case–control study on childhood asthma in Southwest China is consistent with the hygiene hypothesis. A possible mechanism is that HAV preferentially combines with TIM-1 expressed in Th2 after it invades the body. This interferes with the binding of TIM-1 with its ligand, blocking induction of Th2 caused by the combination of TIM-1 and its ligand. This inhibits the activation of Th2 and facilitates the Th1 differentiation signal, accelerating the growth of Th1 (6). Umetsu (20) and Sonar (40) showed the potential therapeutic effect of TIM-1 antibody interference on asthma and other immunological diseases in a mouse model.

The human HAV receptor (HAVCR1) is expressed on the surface of liver cells and enterocytes and mediates the whole process of HAV entry into cells (41). TIM-1 participates in the activation and polarization of CD4⁺ T cells and adjusts the production of Th2 cytokines (20). The polymorphisms of TIM-1 might be affected by the selection of hepatitis A and asthma (42). Therefore, allergic asthma may be related to HAV infection through TIM-1 molecules, which may involve TIM-1 polymorphism.

Insertion loss variation of TIM-1 at the mucin domain occurs in both humans and mice. HAV infection can decrease the incidence of allergic diseases in adult individuals with TIM-1 exon 4, 157insMTTTPV. But, from case-control studies on African Americans and genealogy, Gao *et al.* (35) suggested that the protection of exon 4 insertion variation (157insMTTTPV) on asthma was not related with HAV infection. Later studies on Chinese, Korean, and Japanese populations did not reveal any protective effect of TIM-1 exon 4 insertion loss variation on asthma, nor was any relation with HAV infection found (21, 32, 42). The differences in geographic location and race might have led to differing frequencies of alleles. Gao *et al.* pointed out that the frequencies of 157insMTTTPV variation in African Americans, European Caucasians, and Koreans were 64%, 58%, and 13.5%, respectively (35). The frequency of 157insMTTTPV variation found in Southwest Chinese children was 23.7%, which is similar to that found in Koreans, but quite different from that found in African Americans and European Caucasians. This suggests that exon 4 insertion loss variation (157insMTTTPV) in Africans and Europeans might be related with allergic asthma, but not in Asians. This study did not reveal any association between exon 4 insertion loss variation (157insMTTTPV) and HAV infection.

We found that the haplotype constructed at SNP -416G>C was also related with childhood allergic asthma. Liu *et al.* (22) found that one functional polymorphic locus, -416G>C, was related with bronchial asthma, and replacement of -416G>C could enhance the transcriptional activity of TIM-1. Mou *et al.* (23) found that both -416G>C and -1454G>A were related with allergic rhinitis in Chinese Han people. But Liu *et al.* (22) and the present study did not observe any relationship between -1454G>A and allergic asthma. The differences in these results might be due to different ethnic and genetic backgrounds, small sample sizes, and few analyzed sites.

CONCLUSIONS

Despite differences in specific polymorphisms in previous studies, our results are in accordance with some of the previous reports. These findings confirm that the TIM-1 gene is indeed associated with childhood allergic asthma.

DECLARATION OF INTEREST

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