Thromboxane A_2 receptor +795T>C and chemoattractant receptor-homologous molecule expressed on Th2 cells -466T>C gene polymorphisms in patients with aspirin-exacerbated respiratory disease

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Abstract. It is well known that aspirin-exacerbated respiratory disease (AERD) is more common in women than in men, however, whether gene polymorphisms of the thromboxane A_2 receptor (TBXA2R) and chemoattractant receptor-homologous molecules expressed on Th2 cells (CRTH2) are associated with the susceptibility of AERD remains unknown. In this study, we examined the gene polymorphisms in a Japanese population. DNA specimens were obtained from the following three groups: 96 patients with AERD, 500 patients with aspirin-tolerant asthma (ATA) and 100 normal controls. The target DNA sequence of each gene was amplified, and an allelic discrimination assay for single nucleotide polymorphisms relating to expression of each gene was carried out. The frequencies of the CC/CT genotype of TBXA2R +795T>C were higher than those of the TT genotype in AERD patients compared to ATA patients (P=0.015). In female AERD patients, but not in males, frequencies of the CC/CT genotype were higher than those of the TT genotype of TBXA2R +795T>C compared to female ATA patients (P=0.013). Also, frequencies of the TT genotype of CRTH2 -466T>C were higher than those of the CC/CT genotype in AERD patients compared to ATA patients (P=0.034). In female AERD patients, but not in male, frequencies of the TT genotype were higher than those of the CC/CT genotype of CRTH2 -466T>C in AERD patients compared to female ATA patients (P=0.046). Based on our investigations, no significant relationship was found between the genotype and the clinical characteristics according to these gene polymorphisms in AERD patients. Our results suggest that an association between the TBXA2R and CRTH2 gene polymorphisms with AERD may exist in the Japanese population.

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Introduction

Aspirin-exacerbated respiratory disease (AERD), also known as aspirin-intolerant asthma, is a clinical syndrome characterized by aspirin hypersensitivity and severe asthmatic attacks after taking aspirin and/or non-steroidal anti-inflammatory drugs (NSAIDs). The pathogenesis of AERD has been suggested to be caused by arachidonic acid metabolites, such as leukotrienes (LTs) (1). The inhibitory action of aspirin and NSAIDs on cyclooxygenase (COX) activity may cause diversion to the 5-lipoxygenase pathway, leading to the overproduction of cysteinyl LTs (2). Therefore, genetic association studies of LT-related genes have been undertaken to explore the genetic determinants of AERD. LTC_4 synthase promoter polymorphism has been associated with AERD (3,4). Furthermore, the genetic polymorphisms of 5-lipoxygenase promoter (5) and cysteinyl LT receptor 1 promoter (6) have been shown to influence the susceptibility to AERD as risk factors. However, conflicting results have been reported (7,8). Notably, a recent study on Japanese asthmatics demonstrated that prostaglandin D_2 (PGD_2) was overproduced during aspirin-intolerant bronchoconstriction, and the urinary concentrations of LTE_4 and metabolites of PGD_2 correlatively increased during the reaction (9), indicating possible involvement of other arachidonic acid metabolites in AERD, such as thromboxane A_2 ($TBXA_2$) and PGD_2.

$TBXA_2$ and PGD_2, which are prostanoids induced by COX, may be important mediators in AERD. $TBXA_2$ exerts its action, such as bronchoconstriction and bronchial hyperresponsiveness (10,11), by interacting with the G protein-

coupled $TBXA_2$ receptor (TBXA2R). On the other hand, PGD_2, a major prostanoid of activated mast cells, plays an important role in allergic asthma (12) by interacting with the G protein-coupled receptor for PGD_2, a human chemoattractant receptor expressed on type 2 helper T cells (CRTH2).

The TBXA2R gene exists on chromosome 19p13.3, and conflicting results about the genetic alteration of TBXA2R in the involvement of asthma have been reported in a Korean population. Namely, Shin *et al* (13) showed a positive association between the TBXA2R polymorphism and the development of atopy and asthma. On the other hand, Kim *et al* (14) showed that the TBXA2R polymorphism was not associated with asthma susceptibility and the clinical parameters of asthma. In a Japanese population, Unoki *et al* (15) found that the synonymous +924T>C polymorphism in the TBXA2R gene was associated with a diagnosis of asthma in adult asthmatic patients, but not in children. However, their claim of an involvement of TBXA2R in Japanese asthmatics does not seem to be substantiated by their data (16,17).

The CRTH2 gene exists on chromosome 11q13, and genetic alteration of CRTH2 has been associated with allergic asthma in African-American and Chinese populations (18). However, no association has been found between any polymorphisms or haplotypes in the CRTH2 gene and asthma in the Japanese population (19).

Investigations into the association between AERD susceptibility and prostanoid gene polymorphisms in a Korean population have shown that, among three single-nucleotide polymorphisms (SNPs) of the TBXA2R gene investigated, the +795T>C polymorphism was only associated with AERD susceptibility (20,21) and the -466T>C polymorphism of the CRTH2 gene was associated with AERD (22). However, there has been no published data addressing the role of TBXA2R and CRTH2 gene polymorphisms in Japanese patients with AERD. This is the first report of TBXA2R +795T>C and CRTH2 -466T>C polymorphisms in Japanese patients with AERD.

Materials and methods

Subjects. This study was performed with the approval of the Institutional Ethics Committee of the Gunma Institute for Allergy and Asthma, Gunma Hospital for Allergic and Respiratory Diseases, and written informed consent was obtained from each individual before the study commenced.

All subjects were Japanese non-smokers, recruited from the outpatient clinic of the Gunma Hospital for Allergic and Respiratory Diseases, Department of Respiratory Medicine, Dokkyo Medical University Koshigaya Hospital, Yukawa Clinic of Internal Medicine, and the Hiroshima Allergy and Respiratory Clinic (Japan). Smoking habits were ascertained by means of a questionnaire. Characteristics of the study population are shown in Table I.

Diagnosis of bronchial asthma was confirmed using the Global Initiative for Asthma guidelines. All patients presented clinical symptoms that met the criteria for asthma, such as cough, wheeze and shortness of breath, and they were diagnosed by experienced pulmonologists. Forced expiratory volume in one second (FEV_1) was measured with a spirometer, and airway reversibility was defined as a >12% and >200 ml increase in volume in the first second of forced expiration from baseline after inhalation of short-acting β_2-adrenergic bronchodilators. The diagnosis of AERD was made on the basis of either a positive result on the lysine-aspirin challenge test (4) or an apparent history of more than one self-reported episode of bronchial response to aspirin or NSAID ingestion. The provocation test could not be applied to the subjects who did not give written informed consent, mainly because of the risk of significant occult disease, although the likelihood of a severe reaction was considered very low. Aspirin-tolerant asthma (ATA) was defined as bronchial asthma with no history of NSAID-induced asthma attacks. Non-smoking subjects with no history of bronchial asthma or other respiratory symptoms were selected from healthy volunteers who visited our clinic for annual routine physical examinations, and comprised the normal controls. The serum levels of total IgE were measured by the CAP system (Phadia, Uppsala, Sweden). Serum specific IgE antibodies were measured, and the patients with positive radioallergosorbent test scores for house dust mite were classified as atopy. The total eosinophil count was measured in peripheral blood using a flow cytometer (Coulter Maxm; Beckman-Coulter Inc., Fullerton, CA, USA).

Table I. Clinical characteristics of the study subjects[a].

	AERD	ATA	NC
No. of subjects	96	500	100
Mean age (years)	51.4±13.4	49.7±13.6	47.1±13.6
Male gender[b], n (%)	26 (27.1)	205 (41.0)	34 (34.0)
FEV_1 (% predicted)	73.4±11.6	76.0±25.3	NA
Total serum IgE (IU/ml)[c]	202.5±233.0	505.3±676.6	NA
Eosinophil (cells/μl)[d]	712.2±818.0	382.5±337.9	NA
Atopy[e], n (%)	21 (21.9)	240 (48.0)	NA

[a]Data are presented as the means ± SD or n (%). [b]P=0.010 for AERD patients vs. ATA patients by the Chi-square test. [c]P<0.001 for AERD patients vs. ATA patients by the Welch's t-test. [d]P<0.001 for AERD patients vs. ATA patients by the Welch's t-test. [e]P<0.001 for AERD patients vs. ATA patients by the Chi-square test.

Genotyping of TBXA2R and CRTH2 polymorphisms. DNA in the specimens obtained by rubbing buccal mucosa with a cotton swab was extracted using QIAamp 96 DNA blood kits (Qiagen, Hilden, Germany). The target DNA sequence of TBXA2R +795T>C was amplified using a set of primers (forward 5'-GAGTGGACCCTGGATCTCAA-3'; reverse 5'-CCACGCGCAAGTAGATGAG-3'). The target DNA sequence of CRTH2 -466T>C was amplified using a set of primers (forward 5'-GAGCTGCATGGAGGATCTGT-3'; reverse 5'-AGGACTCCTTTTTCCCATCC-3'). Allelic discrimination assay for SNPs relating to the expression of TBXA2R +795T>C and CRTH2 -466T>C (rs11085026 and rs634681, respectively) was carried out using the previously

Table II. Genotype frequencies of the TBXA2R +795T>C and CRTH2 -466T>C gene in each group.

SNP loci	Genotype			Allele frequency	Allele frequency		HWE
	TT	CT	CC		P-value	P-value	P-value
TBXA2R +797T>C							
AERD	38 (39.6%)	47 (49.0%)	11 (11.4%)	0.359	**0.025**	0.541	0.535
ATA	264 (52.8%)	193 (38.6%)	43 (8.6%)	0.279	-	0.146	0.364
NC	42 (42.0%)	50 (50.0%)	8 (8.0%)	0.330	0.146	-	0.191
CRTH2 -466T>C							
AERD	46 (47.9%)	46 (47.9%)	4 (4.2%)	0.281	**0.009**	0.890	0.070
ATA	184 (36.8%)	251 (50.2%)	65 (13.0%)	0.381	-	**0.004**	0.151
NC	49 (49.0%)	47 (47.0%)	4 (4.0%)	0.275	**0.004**	-	0.074

Minor C alleles of each gene in patients with AERD were compared to those in patients with ATA and in control subjects by means of the Chi-square test. Values in bold indicate significant P-value. HWE, Hardy-Weinberg equilibrium.

described SNP detection system, sequence-specific thermal-elution chromatography (23,24). All subjects and researchers remained unaware of the genotype until the final analysis.

Statistical analysis. Data are presented as the means ± SD or n (%) of observations, unless stated otherwise. Differences in the mean value of the phenotypic characteristics within the groups were compared using either the ANOVA test or the t-test, and qualitative data were compared with the Chi-square test. Allele frequencies were estimated using the gene counting method. Significant departures of genotype frequency from the Hardy-Weinberg equilibrium at each SNP were tested with the Chi-square analysis. Differences in minor allele C frequency of both TBXA2R +795T>C and CRTH2 -466T>C in AERD patients were compared to those in ATA patients and control subjects by means of the Chi-square test. Each gene polymorphism related to the asthma phenotype was examined by multivariable logistic regression models with adjustment for covariates, namely with the asthma phenotype as dependent variable and independent variables, including age (continuous value), gender (male, 0, female, 1), two alternative genotype models that were combined homozygous CC and heterozygous CT genotype group and homozygous TT genotype. In addition, subgroup analyses with gender of the multivariable logistic regression analysis were performed, and the interactions between gender and the genotype were tested using the Wald statistic. Statistical analyses were undertaken using SPSS for Windows version 17 (SPSS Inc., Chicago, IL, USA). P-values of <0.05 were considered to be significant.

Results

Subject characteristics. The clinical characteristics of the subjects are summarized in Table I. The number of female patients with AERD was significantly higher than that of ATA (P=0.010). However there was no significant difference between AERD and ATA patients in terms of age and FEV_1 (% predicted). The levels of total serum IgE in AERD patients were significantly lower than those in ATA patients (P<0.001). AERD patients had a higher peripheral total eosinophil count compared to ATA patients (P<0.001). There was a significant difference in prevalence of atopy between AERD and ATA patients (P<0.001).

Genotype frequencies of the TBXA2R +795T>C and the CRTH2 -466T>C gene in each group. The frequencies of the TBXA2R +795T>C and CRTH2 -466T>C genotype and the C minor allele in each group are shown in Table II. The genotype distribution fulfilled the Hardy-Weinberg equilibrium in each group. Frequency of the C minor allele of TBXA2R +795T>C in patients with AERD [frequency of allele (q) = 0.359] and control subjects (q=0.330) was similar, whereas the frequency in patients with ATA was decreased (q=0.279). Frequency of the C variant allele in patients with AERD was significantly higher than that in patients with ATA (P=0.025).

Frequency of the C minor allele of CRTH2 -466T>C in patients with AERD (q=0.281) and control subjects (q=0.275) was significantly lower than that in patients with ATA (P=0.009 in AERD patients and P=0.004 in control subjects). However, no significant difference in the allele frequency was found between AERD patients and control subjects.

Multivariable logistic regression analysis and subgroup analysis with gender of genotype of TBXA2R +795T>C and CRTH2 -466T>C. The results of the multivariable logistic regression analysis of the TBXA2R +795T>C and CRTH2 -466T>C genotype controlling age and gender in patients with AERD compared to those with ATA are shown in Table III-A. Frequencies of the combined homozygous CC and heterozygous CT genotype group of the TBXA2R +795T>C gene were significantly higher than those of the homozygous TT genotype in patients with AERD compared to those with ATA (P=0.015). The odds ratio (OR) of patients with AERD compared to those with ATA associated with the combined homozygous CC and heterozygous CT genotype group of the TBXA2R +795T>C gene to those with the homozygous TT genotype was 1.748 (95% CI 1.116-2.739). Frequencies of the homozygous TT genotype of CRTH2 -466T>C were significantly higher than those of the combined homozygous CC and heterozygous CT genotype group in patients with AERD

Table III. Multivariable logistic regression analysis and subgroup analysis with gender of genotype of the TBXA2R +795T>C and CRTH2 -466T>C gene in Japanese patients with AERD compared to those with ATA.

A, Multivariable logistic regression analysis

Genotype	OR (95% CI)	P-value
TBXA2R +797T>C		
TT	1.000	
CC/CT	1.748 (1.116-2.739)	**0.015**
CRTH2 -466T>C		
CC/CT	1.000	
TT	1.616 (1.037-2.518)	**0.034**

B, Subgroup analysis with gender

	Males		Females	
Genotype	OR (95% CI)	P-value	OR (95% CI)	P-value
TBXA2R +797T>C				
TT	1.000		1.000	
CC/CT	1.317 (0.577-3.007)	0.513	1.961 (1.150-3.346)	**0.013**
CRTH2 -466T>C				
CC/CT	1.000		1.000	
TT	1.405 (0.617-3.196)	0.418	1.712 (1.010-2.903)	**0.046**

Multivarible logistic regression analysis was applied for (A) age and gender, and (B) age as covariables. Values in bold indicate significant P-values.

Table IV. Comparison of the clinical characteristics according to TBXA2R +795T>C and CRTH2 -466T>C gene polymorphisms in Japanese patients with AERD.

SNP loci/genotype	FEV_1 (% predicted)	Total IgE (IU/ml)	Eosinopils (cells/μl)	Atopy (%)
TBXA2R +797T>C				
TT	72.2±11.6	237.2±316.1	809.2±1,144.7	10.4%
CC/CT	74.2±11.6	183.2±170.9	647.6±498.7	11.5%
P-value	0.414	0.321	0.442	0.394
CRTH2 -466T>C				
CC/CT	73.6±11.2	199.8±202.6	725.4±958.4	11.5%
TT	73.3±12.0	205.2±261.7	699.4±664.2	10.4%
P-value	0.924	0.918	0.884	0.975

Differences in the mean value of the phenotypic characteristics among the patients were compared by t-test, and qualitative data were compared by the Chi-square test.

compared to those with ATA (P=0.034). The OR of patients with AERD associated with the homozygous TT genotype to those associated with the combined homozygous CC and heterozygous CT genotype group compared to ATA was 1.616 (95% CI 1.037-2.518).

Table III-B shows the results of the subgroup analyses with gender of the TBXA2R +795T>C and CRTH2 -466T>C genotype. The positive association between the asthma phenotype and the genotype of the TBXA2R +795T>C gene was present in females (OR=1.961; 95% CI 1.150-3.346), but not in males (OR=1.317; 95% CI 0.577-3.007). In female patients with AERD, frequencies of the combined homozygous CC and heterozygous CT genotype group of the TBXA2R +795T>C gene were significantly higher than those of the homozygous TT genotype compared to those with female ATA (P=0.013). The interactions between gender and the genotype were not significant (P=0.706). Moreover, the positive association between the asthma phenotype and the genotype of the CRTH2 -466T>C gene was observed in females (OR=1.712; 95% CI 1.010-2.903), but not in males (OR=1.405; 95% CI

0.617-3.196). In female patients with AERD, frequencies of the homozygous TT genotype of the CRTH2 -466T>C gene were significantly higher than those of the combined homozygous CC and heterozygous CT genotype group compared to those with female ATA (P=0.046). The interactions between gender and the genotype were not significant (P=0.430).

Clinical characteristics according to the TBXA2R +795T>C and CRTH2 -466T>C gene polymorphisms in AERD patients. Comparison of the clinical characteristics in AERD patients according to the TBXA2R +795T>C and CRTH2 -466T>C gene polymorphisms showed that FEV_1 (% predicted), the level of total serum IgE, the total count of peripheral eosinophils and the prevalence of atopy were not different between the polymorphisms in each gene (Table IV).

Discussion

AERD is known to be associated with higher female incidence, less atopic tendency and a more severe clinical course. The possible role of arachidonic acid metabolites, such as LTs, in the pathogenesis of AERD has also been suggested (1). We recently reported that the Arg16Gly β_2-adrenergic receptor gene polymorphism in AERD is different from that in ATA (24). In the present study, we extended our investigations to explore the genetic determinations of AERD.

The role of TBXA2, one of the prostanoids induced by COX, in asthma has been reported to be evident through its induction of bronchoconstriction and airway hyperreactivity (10,11). PGD_2, a major prostanoid of activated mast cells, has also been shown to play an important role in allergic asthma (12) by acting as a chemoattractant for Th2 lymphocytes, eosinophils and basophils (25).

Conflicting results about the genetic alteration of TBXA2R in the involvement of asthma have been reported in an Asian population (13-15). Genetic alteration of CRTH2 has been correlated to allergic asthma in African-American and Chinese populations (18), but not in a Japanese population (19). On the other hand, recent reports have shown a positive association of TBXA2R and CRTH2 polymorphisms with AERD susceptibility in a Korean population (20-22), and Akahoshi *et al* (26) showed that the -1933T>C SNP in the promoter region of TBX21 was associated with AERD in a Japanese population. However, to our knowledge, there has been no published data addressing the role of TBXA2R and CRTH2 gene polymorphisms in Japanese patients with AERD, and this is the first study to examine the relevance of genetic polymorphisms in these prostanoid receptors.

First, we investigated the allele frequencies of the genotypes of TBXA2R +795T>C and CRTH2 -466T>C in the three groups (patients with AERD, patients with ATA and normal controls). The genotype distribution fulfills the Hardy-Weinberg equilibrium in each group. Frequency of the C minor allele of TBXA2R +795T>C in patients with AERD was significantly higher than that in patients with ATA, whereas frequency of the C minor allele of CRTH2 -466T>C in patients with AERD was significantly lower than that in patients with ATA.

We then investigated the genotype frequencies of TBXA2R +795T>C and CRTH2 -466T>C in the three groups. Frequencies of the combined homozygous CC and the heterozygous CT genotype group of the TBXA2R +795T>C gene were significantly higher than those of the homozygous TT genotype in patients with AERD compared to those with ATA. The positive association between the asthma phenotype and the genotype of the TBXA2R +795T>C gene was present in females, but not in males. In female patients with AERD, frequencies of the combined homozygous CC and heterozygous CT genotype group of the TBXA2R +795T>C gene were significantly higher than those of the homozygous TT genotype, compared to those with female ATA. Also, frequencies of the homozygous TT genotype of CRTH2 -466T>C were significantly higher than those of the combined homozygous CC and heterozygous CT genotype group in patients with AERD, compared to those with ATA. The positive association between the asthma phenotype and the genotype of the CRTH2 -466T>C gene was also present in females, but not in males. In female patients with AERD, frequencies of the homozygous TT genotype of the CRTH2 -466T>C gene were significantly higher than those of the combined homozygous CC and heterozygous CT genotype group, compared to those with female ATA (OR=1.712; 95% CI 1.010-2.903, P=0.046).

Two investigations from Korea demonstrated that the TBXA2R +795T>C gene polymorphism was associated with AERD susceptibility (20,21). Namely, the first report (20) showed that AERD patients had a significantly higher frequency of the combined homozygous CC genotype and heterozygous CT genotype group of TBXA2R +795T>C compared to ATA patients, and the second (21) showed that the frequency of CC homozygotes of TBXA2R +795T>C was higher in AERD patients than in ATA patients. Furthermore, a recent investigation in a Korean population found that AERD patients exhibited a significantly higher frequency of the homozygous TT genotype of CRTH2 -466T>C compared to ATA patients (22), indicating that our findings may correspond to the results of the Korean study on the TBXA2R +795T>C and CRTH2 -466T>C gene polymorphism in AERD patients (20-22). However, neither of the reports from Korea showed the subgroup analyses with gender of the TBXA2R +795T>C and CRTH2 -466T>C gene polymorphism in the patients. In this study, we investigated the interactions between gender and the genotype of the asthma phenotype, and demonstrated the positive association between the asthma phenotype and the genotype of both the TBXA2R +795T>C and CRTH2 -466T>C gene in females, but not in males, patients in a Japanese population. It has been well known since the late 1960's that AERD is more commonly found in females than in males (27); our data suggest the possible role of the TBXA2R +795T>C and CRTH2 -466T>C gene polymorphisms in AERD.

Finally, we compared the clinical characteristics in AERD patients according to TBXA2R +795T>C and CRTH2 -466T>C gene polymorphisms. To the extent that the clinical characteristics of AERD were investigated, the value of FEV_1 (% predicted), the level of total serum IgE, the total count of peripheral eosinophil and the prevalence of atopy were not different between the polymorphisms in each gene.

The present study has certain limitations; first, the number of study subjects was limited, and, second, patients with AERD were diagnosed by experienced pulmonologists, and

the diagnosis of AERD was made on the basis of either a positive result on a lysine-aspirin challenge test (4) or an apparent history of more than one self-reported episode of bronchial response to aspirin or NSAID ingestion. The clinical history indicated that drug hypersensitivity appeared less than 24 h between administration of the suspected drug and the onset of reactive symptoms. Although placebo-controlled oral challenge tests are considered the most appropriate tests for drug hypersensitivity, the suspected drugs were not applied to the subjects in this study in a blinded test because of risk/benefit considerations and patients' refusal to give written informed consent, due to our safety policy of inpatient provocations only (28). ATA was defined as bronchial asthma with no history of NSAID-induced asthma attacks. Whenever possible, asthmatic patients with no history of aspirin and/or NSAID intolerance and with a negative response in the aspirin challenge test as well should be classified as having ATA. Ideally, everyone in the study should have had the challenge test to confirm aspirin intolerance. However, in the present study, the majority of the patients with AERD were recruited from outpatient clinics staffed with experienced Japanese pulmonologists.

In the present study, the relationship between the genotyping and clinical findings in patients with AERD were not demonstrated. Notably, an agonistic effect of indomethacin on a CRTH2 has been reported (29), which may lead to eosinophilic infiltration in AERD patients. Nevertheless, it is easy to speculate that a single genetic factor cannot explain the genetic background of AERD and, therefore, other factors conferring susceptibility of AERD remain to be identified.

In conclusion, we were the first to analyze TBXA2R +795T>C and CRTH2 -466T>C gene polymorphisms in Japanese patients with AERD, and suggest that TBXA2R +795T>C and CRTH2 -466T>C gene sequence variations may have a role in the development of AERD.

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