

ORIGINAL ARTICLE

Association of thromboxane A2 receptor (*TBXA2R*) gene polymorphism in patients with aspirin-intolerant acute urticaria

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Summary

Background The thromboxane A2 receptor (*TBXA2R*) is a potent broncho- and vaso-constrictor and is associated with leukotriene synthesis. Polymorphisms in the *TBXA2R* gene have been linked to atopy, asthma, and atopic dermatitis. This study evaluated the association between genetic *TBXA2R* variants and the development of acetyl salicylic acid (ASA)-intolerant acute urticaria (AIAU).

Methods AIAU patients ($n = 167$), ASA-intolerant chronic urticaria (AICU) patients ($n = 149$), and healthy controls (NC) ($n = 265$) were included. All patients were enrolled at Ajou University Hospital in Suwon, Korea. Two *TBXA2R* polymorphisms ($-4684T > C$ and $795T > C$) were genotyped by primer extension using a SNAPshot ddNTP primer extension kit. Luciferase activity was measured using a dual-luciferase reporter assay kit. An electrophoretic mobility shift assay (EMSA) was performed using a nuclear extract from a human mast cell line (HMC-1). **Results** Genetic association data demonstrated that compared with NC subjects, AIAU patients had a significantly higher frequency of the homozygous TT genotype of *TBXA2R* $-4684T > C$ ($P = 0.005$, $P_{\text{corr}} = 0.03$). No differences were identified between the AICU and the NC groups. Luciferase activity, reflecting promoter activity, was significantly lower with the *TBXA2R* $-4684T$ -containing construct than with the $-4684C$ -containing construct ($P < 0.001$); the activity decreased further upon co-transfection with ETS-like gene transcription factor-1 (ELK-1) ($P = 0.012$). EMSA revealed that the $-4684T$ allele produced a specific shifted band, with a greater affinity than that produced by the $-4684C$ allele.

Conclusion and clinical relevance These results suggest that the *TBXA2R* $-4684T$ allele may be associated with lower *TBXA2R* expression, which may contribute to the development of the AIAU phenotype.

Keywords ASA-intolerant acute urticaria, polymorphism, thromboxane A2 receptor gene, thromboxane A2

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Introduction

Aspirin ingestion can induce a wide range of clinically recognized allergic reactions, including aspirin-exacerbated respiratory disease (AERD), acetyl salicylic acid (ASA)-intolerant urticaria (AIU), chronic rhinitis, and anaphylaxis. There are two types of AIU: ASA-intolerant acute urticaria (AIAU), which occurs immediately after aspirin ingestion and usually resolves in 1–2 weeks [1], and ASA-intolerant chronic urticaria (AICU) [2]. The pathogenic mechanisms of AIU remain unclear. Genetic epidemiology studies in a Korean population demonstrated that the clinical demographics of AIU and AERD patients differ markedly. AIU patients had high rates of

atopy and increased total IgE levels, whereas AERD patients were non-atopic, with greater numbers of activated eosinophils in the upper and lower airways. Recent studies have identified the high-affinity IgE receptor (*FCER1A*) and histamine *N*-methyltransferase (*HNMT*) genes as genetic determinants of AIU [3, 4]. In general, AICU has a longer duration and more severe symptoms; however, major differences in the pathogenic mechanisms between the two types of AIU are not yet understood.

Histamine release from activated mast cells [5] and activation of neutrophils have been demonstrated in AIAU patients, in association with elevated IL-8 and IL-18 levels [6]. Leukotriene overproduction may contribute to the disease pathogenesis [7]. In an earlier study, the frequency

of the leukotriene C4 (LTC₄) synthase gene –444C allele was significantly higher in AIU patients than in chronic idiopathic urticaria patients [8]. However, cysteinyl leukotriene 1 receptor and leukotriene-related gene polymorphisms did not differ significantly between AIU and chronic idiopathic urticaria patients in previous studies by our group [9].

Human thromboxane A₂ (TBXA₂), a major eicosanoid product of the cyclooxygenase pathway, can induce bronchoconstriction and bronchial hyperresponsiveness. It may also be an important mediator of aspirin hypersensitivity [10–12] and may stimulate human airway smooth muscle cell proliferation [13]. TBXA₂ interacts with G protein-coupled TBXA₂ receptor (TBXA₂R) to act as a potent vasoconstrictor and stimulator of platelet aggregation. In addition, TBXA₂ induces acetylcholine release and airway microvascular leakage [11]. Aspirin blocks TBXA₂ production, as has been observed in cerebral infarction [14].

TBXA₂R gene polymorphism has been associated with atopy, asthma, and atopic dermatitis [15–18]. TBXA₂R and its ligand are important in the pathogenesis of airway inflammation and hyperresponsiveness, as shown in several previous studies [19–21]. Another study has reported the effects of TBXA₂R polymorphism on asthma-related phenotypes and responsiveness to leukotriene receptor antagonists in asthmatic children [22]. In addition, recent studies have demonstrated that TBXA₂ is a negative regulator of LTC₄ synthase production of leukotrienes, and other studies have suggested that LTC₄ synthase activity is elevated when TBXA₂ formation is prevented, leading to increased leukotriene biosynthesis and aggravated clinical symptoms [23, 24].

The TBXA₂R gene is located on chromosome 19p13.3, contains four exons with a total length of 15 kb, and codes for a protein of 343 amino acids [15]. Previously, we reported that TBXA₂R gene polymorphism was associated with bronchoconstrictive responses to lysine ASA inhalation in AERD patients [25]; however, no study has evaluated the role of TBXA₂R polymorphism in AIU pathogenesis. A clear understanding of the variability of TBXA₂R genetic polymorphisms and their associations with urticaria is essential for determining the regulatory role of TBXA₂R polymorphism. In this study, we investigated a TBXA₂R genetic polymorphism and its associations with the AIAU phenotype through a case-controlled study of three subject groups. Furthermore, the functional significance of the TBXA₂R –4684T>C polymorphism was investigated using a luciferase reporter assay and an electrophoretic mobility shift assay (EMSA).

Materials and methods

Study subjects and phenotyping

Three subject groups (167 AIAU patients, 149 AICU patients, and 265 normal controls) were enrolled at the

Ajou University Hospital in Suwon, Korea. AIU was defined by a positive result on an ASA oral provocation test, which was performed using 500 mg of ASA (Rhonal, KunWha Pharmaceutical Co., Seoul, Korea) as described previously [6]. Patients diagnosed with AIU were divided into two groups (AICU and AIAU) based on the duration of urticaria. Patients with cutaneous ASA hypersensitivity that resolved within 6 weeks were assigned to the AIAU group, and individuals with cutaneous symptoms that lasted longer than 6 weeks were assigned to the AICU group. Normal non-atopic controls with no family or personal history of allergic disease or a past history of ASA hypersensitivity were recruited from the general population.

Informed consent was obtained from all subjects, and the study was approved by the Ajou University Hospital Institutional Review Board. Skin prick tests were performed with 55 common aero-allergens (Bencard Co., West Sussex, UK). Atopy was defined as one or more positive reactions to common inhalant allergens. The total IgE concentration was measured using a UniCAP system (Pharmacia Diagnostics, Uppsala, Sweden), according to the manufacturer's instructions.

Single nucleotide polymorphisms identification and genotyping

To identify SNPs, two regions of the TBXA₂R gene (upstream of exon 1 and at exon 3) in samples from 40 healthy volunteers were sequenced using an ABI Prism 3100 DNA analyzer (Applied Biosystems, Foster City, CA, USA). We found one promoter SNP (TBXA₂R –4684T>C) and two exonic SNPs (924T>C and 795T>C) at exon 3. We selected 795T>C as a tagging SNP at exon 3 and genotyped the –4684T>C and 795 T>C TBXA₂R polymorphisms using primer extension and a SNAPshot ddNTP primer extension kit (Applied Biosystems). The primers for TBXA₂R –4684T>C included forward (F: 5'-ACC GTC TCT TTG CAG AGG-3'), reverse (R: 5'-AAG GTC AGC TTG TCC GGT-3'), and extension (5'-CAG GTC CCT AAC TCA AGG TTC TGT C-3') primers; for TBXA₂R 795T>C, the primers included forward (F: 5'-GAG TGG ACC CTG GAT CTC A-3'), reverse (R: 5'-GCG CTC TGT CCA CTT CCT-3'), and extension (5'-CGC AGC ACT GTC GGG C-3') primers.

Preparation of the human thromboxane A₂ construct and dual luciferase assay

A 1082 bp fragment of the human TBXA₂R gene was prepared by PCR amplification using either –4684C homozygous or –4684T homozygous human genomic DNA as a template (forward primer, 5'-CTG TTT ATG GTC GGC ACA-3' and reverse primer, 5'-ATG ACT CCA TTC TCC ACC A-3'). Each PCR product was gel-purified using an agarose gel purification kit (iNtRON, Deajeon, Korea) and ligated into TA vector (Invitrogen, Deajeon, Korea).

The plasmid was digested with *KpnI* and *XhoI* (Takara, Shuzo, Japan) and ligated into a similarly digested pGL3-basic luciferase reporter vector (Promega, Madison, WI, USA) using T4 DNA ligase (iNtRON). All constructs were verified by direct sequencing. Plasmid DNAs were prepared from these constructs using an Endo Free Plasmid Maxi kit (Qiagen, Hilden, Germany). Plasmid DNA concentrations and purity were assessed using UV spectrophotometry and agarose gel electrophoresis.

Transfection was performed in human mast cell line (HMC)-1 cells and repeated in U937 and A549 cells. Detailed transfection protocols were described in our previous study [26]. HMC-1 was transfected using a microporator (model MP-100; Digital Bio Technology, Seoul, Korea) and PMK-1096 (Digital Bio Technology) according to the manufacturer's instructions. Briefly, 24-well plates were filled with 1000 µL of Iscove's modified Dulbecco's medium containing 10% fetal bovine serum without antibiotics. Plates were incubated in a humidified incubator at 37 °C under 5% CO₂. HMC-1 cells (2 × 10⁵), reporter plasmid DNA containing *TBXA2R* (1 µg), and Renilla plasmid DNA (5 ng) were suspended in Solution R (10 µL). The incorporation tube was filled with Solution E (3 mL). Cell and DNA mixtures were inserted into the microporator pipette station. HMC-1 cells were shocked using 1750 V/20 mA/plus number 1. The samples were transferred to pre-incubated 24-well plates and incubated for 24 h.

Cells were lysed with passive lysis buffer (Promega), and 100 µL of lysate was added to each well. A 20 µL aliquot from each well was assayed for luciferase activity using a dual-luciferase reporter assay kit (Promega) and a ClarityTM Luminescence microplate reader (BioTek, Winooski, VT, USA). The pGL3-control (Promega) and the promoterless pGL3-basic vectors were used as positive and negative controls, respectively. Transfection efficiency was determined by measuring Renilla activity using a dual-luciferase reporter assay kit (Promega) after co-transfection of the reporter construct and Renilla control vector.

Nuclear extract preparation and electrophoretic mobility shift assay

HMC-1 cells were washed with ice-cold phosphate-buffered saline before suspension in an ice-cold hypoosmotic buffer (10 mM HEPES, 2 mM MgCl₂, 0.1 mM EDTA, 10 mM KCl, 1 mM DTT, 1 mM PMSF, 1 mM NaVO₃, 10 mM NaF, and 1 × protease inhibitors). Cells were incubated on ice for 10 min, 0.2% Nonidet P-40 was added, and the cells were incubated again for 15 min. After centrifugation at 15 000 g for 30 s, the pellets were suspended in cold saline buffer containing 50 mM HEPES, 50 mM KCl, 300 mM NaCl, 0.1 mM EDTA, 10% glycerol, 1 mM DTT, 1 mM PMSF, 1 mM NaVO₃, 10 mM NaF, and 1 × protease inhibitor. The mixture was incubated on ice for 30 min. After centrifugation at 15 000 g for 30 min at 4 °C, the super-

natants containing the nuclear proteins were collected. The protein concentrations were determined using the Bradford assay. Nuclear protein aliquots (5 µg) were stored at -80 °C until use. Double-stranded oligonucleotides for *TBXA2R* and the non-specific competitor SP-1 were used as probes for EMSA. The double-stranded oligonucleotides were annealed at 95 °C for 5 min and gradually cooled at room temperature. The double-stranded DNA probes were radiolabelled at their 5'-ends using DNA polymerase I (Klenow; Promega) and [α -³²P]-CTP. A 10 µL reaction mixture comprising nuclear protein (5 µg), 75.2 mM NaCl, 20 mM HEPES, 1 mM EDTA, 5% glycerol, 0.0504 mM MgCl₂, 5 mM DTT, 1 mM NaVO₃, 10 mM NaF, 400 ng of BSA, and 250 ng of poly(dI-dC) was pre-incubated on ice for 20 min. Radiolabelled probe (0.2 pmol) was added, and the reaction was incubated on ice for another 30 min. The reaction mixture was then subjected to electrophoresis in a native 6% polyacrylamide gel. A competition experiment was performed using unlabelled, blunt-ended competitor oligonucleotides added to the binding reaction before the addition of radiolabelled oligonucleotide. Gels were dried, and radioactivity was detected using an FLA3000 scanner (Fuji Photo Film, Tokyo, Japan).

Statistical analysis

Differences in the mean values of the phenotypic characteristics among patients were compared using Student's *t*-test (for continuous variables) or a chi-squared test (for categorical variables). Differences in the genotype frequency between the two groups were examined using a chi-squared test. A logistic regression recessive analysis model was used after adjustment for age and gender covariates, followed by a multiple comparison test. The dual luciferase assay was assessed using an independent *t*-test. All statistical analyses were performed using SPSS version 12.0 (SPSS Inc., Chicago, IL, USA). Statistical significance was established as $P \leq 0.05$.

Results

Clinical characteristics of acetyl salicylic acid-intolerant acute urticaria study subjects

The clinical characteristics of the study population are summarized in Table 1. The atopy rate and the total serum IgE level were significantly higher in the AIAU and AICU groups compared with the NC group ($P < 0.001$).

Genotype and allele frequencies of the TBXA2R gene

Based on chi-square analyses, the genotype distribution of the two *TBXA2R* polymorphisms did not differ significantly from Hardy-Weinberg equilibrium ($P > 0.05$).

Table 1. Clinical characteristics of the study subjects

	AIAU (n = 167)	AICU (n = 149)	NC (n = 265)	P-value		
				AIAU vs. AICU	AIAU vs. NC	AICU vs. NC
Sex (male/total)	77/167 (46.1%)	68/149 (45.6%)	122/265 (46%)	1	1	1
Age (years)	35.662±11.678/167	35.662±11.678/149	37.815±14.526/265	0.46	0.449	0.101
Atopy (presence/total)	88/132 (66.7%)	95/134 (70.9%)	15/138 (10.9%)	0.509	<0.001	<0.001
Log (serum total IgE, IU/mL)	5.019±1.19/105	5.21±1.077/111	3.577±1.324/43	0.812	<0.001	<0.001

Bold values indicate a significant *P*-value.

AIAU, aspirin-intolerant acute urticaria; AICU, aspirin-intolerant chronic urticaria; NC, normal control.

Table 2. Allele and genotype frequencies of *TBXA2R* gene polymorphisms

	AIAU (N = 167)	AICU (N = 149)	NC (N = 265)	AIAU vs. AICU	AICU vs. NC	AIAU vs. NC	<i>P</i> _{corr} [†]
TBXA2R –4684 T>C							
TT	63 (37.7%)	48 (32.2%)	66 (24.9%)				
CT or CC	104 (62.3%)	101 (67.8%)	199 (75.1%)	0.306*	0.11*	0.005*	0.03*
<i>q</i>	0.41	0.423	0.481	0.808	0.111	0.042	0.252
TBXA2R 795 T>C							
TT	63 (37.7%)	49 (32.9%)	96 (36.2%)				
CT or CC	104 (62.3%)	100 (67.1%)	169 (63.8%)	0.369*	0.494*	0.753*	1*
<i>q</i>	0.401	0.413	0.419	0.808	0.883	0.62	1

*This *P*-value was calculated using recessive model of analysis (TT vs. CT or CC). Bold values indicate a significant *P*-value. Each *P*-value of 0.05 or less was considered to be significant. Significant *P*-value was further tested using two SNP×one model×three comparisons = six test multiple comparisons, as indicated.

[†]*P*_{corr} for AIAU vs. NC.

AIAU, aspirin-intolerant acute urticaria; AICU, aspirin-intolerant chronic urticaria; NC, normal controls; *q*, minor allele frequency.

Furthermore, the two polymorphisms were not in linkage disequilibrium ($|D'| = 0.34$, $r^2 = 0.077$). Therefore, no haplotypes were drawn for the two SNPs in the *TBXA2R* gene.

The genotype frequency of *TBXA2R* –4684T>C differed significantly between the AIAU and NC groups in the recessive model (Table 2); however, no significant difference was noted between the AICU and NC groups. AIAU patients demonstrated a significantly higher frequency of the *TBXA2R* –4684T>C homozygous TT genotype compared with NC subjects and the CT and CC genotypes occurred significantly less frequently in AIAU patients than in NC subjects (*P*=0.005). The significance of the relationship between the *TBXA2R* –4684T>C polymorphism and AIAU remained after a multiple comparison test (two SNP×one model×three comparisons), with *P*_{corr}=0.03 for AIAU vs. NC. Using power analysis and sample size (PASS, 2005), the power of this genetic association study was calculated to be 74% for detecting an effect size (*W*) of 0.1398 using 2 degrees of freedom with the chi-squared test, at a significance level (α) of 0.05000.

Effect of the *TBXA2R* –4684T>C polymorphism on promoter activity

To assess whether the *TBXA2R* –4684T>C polymorphism is associated with altered promoter activity, constructs

containing either *TBXA2R* –4684T or *TBXA2R* –4684C and a luciferase reporter gene were transfected into HMC-1 cells. Luciferase activity was significantly lower with the *TBXA2R* –4684T construct than with the –4684C construct (*P*<0.001; Fig. 1b). Further transfection experiments performed in A549 and U937 cells yielded similar results: significantly lower promoter activity with the *TBXA2R* –4684T construct compared with the –4684C construct in A549 (*P*=0.025; Fig. 1c) and U937 cells (*P*<0.001; Fig. 1d). To identify possible transcription factor-binding sites in this promoter region, transcription factor databases were searched using the CONSITE program (<http://asp.ii.uib.no:8090/cgi-bin/CONSITE/consite/>). As the *TBXA2R* –4684T>C polymorphism creates a potential ETS-like gene (ELK-1) transcription factor-binding site, we chose ELK-1 as a candidate transcription factor for co-transfection with the *TBXA2R* promoter constructs, to examine the effect of the *TBXA2R* polymorphism. While both the *TBXA2R* –4684T (*P*=0.012) and –4684C (*P*=0.041) alleles showed significantly decreased induction of luciferase activity with ELK-1 co-transfection (Fig. 2), the *TBXA2R* –4684T allele demonstrated a tendency towards a greater reduction in promoter activity compared with *TBXA2R* –4684C, although the difference in luciferase activity was not statistically significant (data not shown).

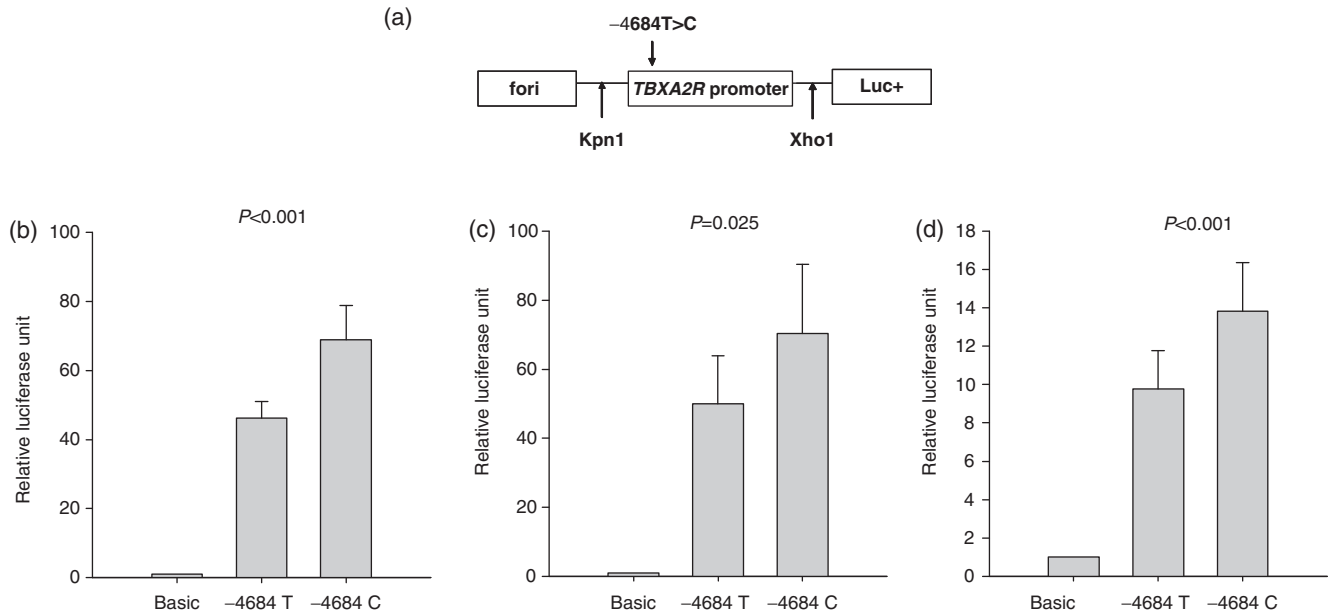


Fig. 1. Effects of the *TBXA2R* -4684 T>C polymorphism on the transcription activity of the human *TBXA2R* gene. (a) Schematic representation of reporter *TBXA2R* (1023 bp) gene construct in the promoter region. Data are the mean values of independent experiments $\pm$ SD. (b) Transfection of human mast cell line (HMC)-1 cells. Luciferase activity assay was performed in three independent experiments (total, $n = 9$). (c) Transfection of A549 cells. (d) Transfection of U937 cells. Relative luciferase activity is represented as the ratio of the activity to the luciferase activity in the cells transfected with the empty control vector pGL3 basic. The P -value was determined using an independent t -test.

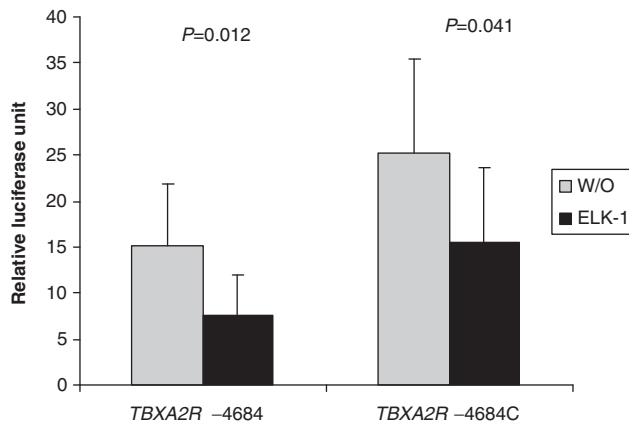


Fig. 2. Effect of ELK-1 transcription factor on *TBXA2R* -4684T>C promoter activity. Co-transfection of ELK-1 with *TBXA2R* -4684T>C was performed at a dose of 50 ng. Relative luciferase production by promoterless control vector, pGL3 basic. Each experiment was conducted in triplicate for each sample. P -value was determined using an independent t -test.

Effects of *TBXA2R* -4684T>C gene polymorphism on transcriptional activity

In an EMSA of nuclear extracts from HMC-1 cells using a double-stranded oligonucleotide, probes corresponding to *TBXA2R* -4684T>C, -4684T produced a specific band with a higher affinity than that produced by -4684C. The band disappeared in the presence of a non-labelled -4684T probe as a competitor, but not in the presence of

(a)
 -4684T: CAA GGT TCT GTC TGG CTC GGG TGT
 -4684C: CAA GGT TCT GTC CGG CTC GGG TGT
 SP1: ATT CGA TCG GGG CGG GGC GAG C
 NF- κ B: AGTTGAGGGGACTTTCCAGG

(b)
 Hot Oligo T C T T T T T T T C
 Competitor: - - T T C C SP1 NF C
 10X 100X 10X 100X 50X 50X 10X

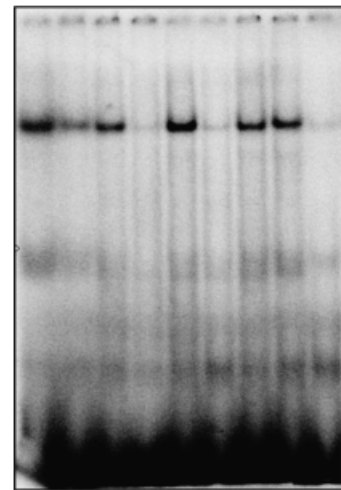


Fig. 3. Results of an electrophoretic mobility shift assay for the *TBXA2R* -4684T>C polymorphism using nuclear extracts obtained from human mast cell line (HMC)-1 cells. (a) Nucleotide sequences of oligonucleotides used as probes and competitors (-4684T, -4684C, SP-1, NF- κ B). (b) Differential binding of a nuclear protein to -4684 T/C alleles in HMC-1 cells.

a non-labelled –4684C probe (Fig. 3) or in the presence of the transcription factor SP-1 or NF- κ B.

Discussion

This is the first study to show a significant contribution of *TBXA2R* polymorphism to the pathogenesis of AIAU, demonstrating a regulatory role for the *TBXA2R* promoter polymorphism *TBXA2R* –4684 T>C in AIAU pathogenic mechanisms. Our results indicate that –4684TT is the risk-associated genotype of the *TBXA2R* –4684T>C promoter polymorphism, whereas the CC and CT genotypes have a protective role.

The mechanisms of aspirin hypersensitivity reactions, including AIAU, may be associated with abnormalities in eicosanoid metabolism. Although platelets have been identified as the principal source of TBXA2 in asthma patients [26], TBXA2 is also produced by other cell types, including mast cells [27]. Thromboxane biosynthesis is attenuated as a result of ASA-induced COX inhibition, leading to the overproduction of leukotrienes, especially cysteinyl leukotrienes, via the uncoupling of LTC4 synthase from its down-regulation by TBXA2R [24]. Leukotriene overproduction may also be potentiated by the suppression of PGE2-protective effects and the elevation of LTC4 synthase expression [28]. Leukotrienes are plausible factors for aspirin hypersensitivity. This proposed mechanism is supported by studies demonstrating an improvement in AIU after treatment with leukotriene receptor antagonists [29, 30].

Thus, with ASA exposure, a genetic polymorphism of *TBXA2R* may result in increased vasoconstriction and cellular recruitment in urticaria lesions of the skin in AIAU patients owing to the overproduction of leukotrienes. In this way, the *TBXA2R* polymorphism may be related to leukotriene-dependent phenotypes of AIAU, as described previously in AERD patients [25] and in asthmatic patients with exercise-induced bronchoconstriction [22]. The precise effects of the *TBXA2R* polymorphism on leukotriene or LTC4 synthase are yet to be evaluated.

We provided additional evidence of the effects of the *TBXA2R* polymorphism in *in vitro* and *in vivo* functional studies. The decreased promoter activity observed for *TBXA2R* –4684T compared with –4684C suggests the down-regulation of TBXA2R expression on inflammatory cells in the presence of the –4684T allele. Down-regulation of thromboxane receptor mRNA and protein expression via the activation of the MAPK, ERK1/2, and p38/NF- κ B pathways in vascular smooth muscle cells has been reported previously [31]. Additionally, MAPK activation has been associated with several allergic conditions. In the present study, EMSA demonstrated a more distinct and specific band with the –4684T probe compared with the –4684C probe, suggesting a factor important in the

transcriptional regulation of the *TBXA2R* gene. In co-transfection experiments in the present study, ELK-1 [32] had a suppressive effect on both alleles, but a more marked effect on the –4684T allele, suggesting a greater suppressor action towards the *TBXA2R* –4684T allele than the –4684C allele. Thus, the *TBXA2R* –4684T>C polymorphism may affect *TBXA2R* transcriptional activity by altering its binding affinity for a specific transcription factor. Further studies are needed to confirm this. Despite the genetic association and functional data provided by the present study, our limited sample sizes of three different study groups cannot be neglected. Replication is essential, but can be difficult because of differences in the study design, gene–environment interactions, and phenotype classification. The *TBXA2R* –4684T>C polymorphism must be examined using larger cohorts in different populations.

Our findings suggest that the –4684T>C polymorphism of the *TBXA2R* gene is associated with the AIAU phenotype. The *TBXA2R* –4684T allele demonstrated a higher frequency in AIAU patients and lower promoter activity, suggesting that it may contribute to the development of AIAU.

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