Genetic Polymorphism of Prostanoid Receptors, *CRTH2* and *TBXA2R* in Aspirin Hypersensitivity

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- ABSTRACT -

Genetic Polymorphism of Prostanoid Receptors, *CRTH2* and *TBXA2R* in Aspirin Hypersensitivity

Background and Objective: Aspirin ingestion can induce a wide range of clinically recognized allergic reactions, including aspirin intolerant asthma (AIA), also called aspirin exacerbated respiratory disease (AERD), aspirin-intolerant urticaria/angioedema (AIU), chronic rhinitis, and anaphylaxis. We studied two major aspirin hypersensitivity, AIA and AIAU in an association with prostanoids receptor *CRTH2* and *TBXA2R*. The human *CRTH2* gene (official name *GPR44*) encodes a G protein-coupled chemoattractant receptor molecule which is expressed on Th2 cells including other allergy related cells like eosinophils, basophils and monocytes. Its importance in inflammatory cell activation and recruitment in vitro and in vivo has been continuously reported in several studies. Considering the fact that eosinophilic infiltration is more pronounced in AIA patients than it is in ATA patients, we hypothesized that activation of eosinophils via dysregulation of the *CRTH2* gene may play an important role in AIA and may be an important marker of this entity. Therefore, this case control study was designed to determine if variation in the *CRTH2* gene confers elevated risk for developing AIA.

Human Thromboxane A2 (TBXA2), is another eicosanoid product induced by cyclooxygenase may be as important mediator in aspirin hypersensitivity as it can induce bronchoconstriction and bronchohyperresponsiveness. TBXA2 exerts its action by interacting with the G protein-coupled thromboxane A2 receptor (*TBXA2R*).

Based on previous association of *TBXA2R* with asthma and atopy, we investigated whether

genetic polymorphisms of the *TBXA2R* gene are associated with AIAU phenotype through a case-control of three study subjects.

Materials and Methods: The three studies groups 107 patients with AIA, 115 patients with ATA, and 133 normal healthy controls (NC) were recruited from Ajou University Hospital, Suwon, Korea. We genotyped two polymorphisms of *CRTH2* located in front of exon-2 (upstream of the ATG) using a primer extension method and the SNAPshot ddNTP primer extension kit. (Applied Biosystems, Foster City, CA, USA). In case of *TBXA2R* gene polymorphism, three subject groups (167 patients with AIAU, 316 patients with AICU, and 265 patients with normal controls) were enrolled at Ajou University Hospital in Suwon, Korea. Two polymorphisms of *TBXA2R (*-4684T>C and 795 T>C) were genotyped using a primer extension method and the SNAPshot ddNTP primer extension kit (Applied Biosystems, Foster City, CA, USA). The functional effect of polymorphism of *CRTH2* and *TBXA2R* were analyzed by luciferase reporter assay, electrophoretic mobility shift assay and ELISA.

Results: AIA patients had significantly higher serum eotaxin-2 levels than did those with ATA ($p=0.034$). A significant difference in the genotype frequencies of *CRTH2* -466T>C was detected between AIA and ATA patients ($p<0.05$). The serum eotaxin-2 level was significantly higher in AIA patients carrying the TT genotype than those with the CT and CC type ($p<0.05$). In vitro functional study demonstrated that the -466T allele had lower luciferase activity ($p<0.001$) and lower mRNA expression with higher production of eotaxin-2 ($p=0.003$) in human lung epithelial cells. EMSA showed that *CRTH2* -466T produced a

specific band with a higher affinity than *CRTH2* -466C had. AIA patients exhibited a significantly higher prevalence of paranasal sinusitis and nasal polyps compared to ATA patients ($p<0.001$ and $p<0.001$, respectively). The serum eotaxin-2 level was significantly higher in AIA patients than in ATA patients ($p=0.034$) in our study, while no difference was noted in the eotaxin-1 level.

Furthermore, the genotype frequency of *TBXA2R* -4684T>C was significantly different AIAU and NC on both co-dominant and recessive analysis models. Specifically, AIAU patients showed a significantly higher frequency of the homozygous TT genotype of *TBXA2R* -4684T>C compared to NC ($p=0.015$). In addition, there was significant association of atopy status according to *TBXA2R* -4684T>C polymorphism. AIAU patients carrying TT genotype showed significantly higher atopy status compared to CT or CC type ($p=0.013$) but not in AICU patients ($P>0.05$). In vitro functional study demonstrated that the -4684T allele had lower luciferase activity ($p<0.001$) in HMC-1 cells. EMSA showed that *TBXA2R* -4684T produced a specific band with a higher affinity than *TBXA2R* -4684C. There was a significant difference between AIAU vs. NC with respect to serum Thromboxane B2 production (TXB2). AIAU patients showed a significantly lower TXB2 production compared to patients with NC ($p=0.016$).

Conclusion: These study suggest that genetic variability of prostanoid receptors *CRTH2* and *TBXA2R* may be associated with aspirin hypersensitivity, in particular *CRTH2* –466T allele showed higher frequency and increased serum and cellular eotaxin-2 production through lowering of CRTH2 expression in AIA and *TBXA2R* -4684T allele showed higher

frequency and lowered TXB2 production through lowering of TBXA2R expression in AIAU.

Key words: aspirin hypersensitivity, respiratory disease, acute urticaria, genetic polymorphism, eosinophils, eotaxin, thromboxane.

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ABBREVIATIONS

AIA; aspirin exacerbated respiratory disease

CRTH2; chemoattractant receptor molecule expressed in Th2 cells

ATA; aspirin tolerant asthma

ASA; acetyl salicylic acid

AIU; aspirin intolerant urticaria

AIAU;aspirin intolerant acute urticaria

AICU; aspirin intolerant chronic urticaria

LTs; leukotrienes

TBXA2R; thromboxane A2 receptor

TXA2;thromboxane A2 metabolite

TXB2;thromboxane B2 metabolite

PGD2; Prostaglandin D2

LTRA; Leukotriene antagonist

FEV1; Forced expiratory volume 1

SNP; Single nucleotide polymorphism

DNA; Deoxyribonuclear acid

EMSA; Electrophoretic mobility shift assay

Part-I

Genetic Polymorphism of *CRTH2* in Aspirin Intolerant Asthma

I.INTRODUCTION

Acetylsalicylic acid (ASA)/nonsteroidal anti-inflammatory drugs (NSAIDs) are commonly prescribed drugs; in particular, the use of aspirin is widespread because of its multifunctional activity, including the prevention of stroke and myocardial infarction. Most patients with asthma, rhinitis, and urticaria can tolerate aspirin; however, some report that their symptoms are exacerbated by aspirin. Aspirin ingestion can induce a wide range of clinically recognized allergic reactions, including aspirin intolerant asthma (AIA), also called aspirin exacerbated respiratory disease (AERD), aspirin-intolerant urticaria/angioedema (AIU), chronic rhinitis, and anaphylaxis.

AIA is a distinct clinical syndrome affecting 10-20% of adult asthmatic patients in whom ingestion of ASA/NSAID causes bronchoconstriction (Jenkins et al., 2004) and is highly associated with rhinosinusitis and nasal polyp (Loewe et al., 1985). The incidence of aspirin hypersensitivity in the general population ranges from 0.6 to 2.5%; however, the incidence in adult asthmatics ranges from 4.3 to 11% (8.8% in Finland, 4.3% in Poland, and 10.5% in Australia) (Hedman et al., 1999; Vally et al., 2002; Kasper et al., 2003). In terms of pathogenesis, altered eicosanoid metabolism is thought to be responsible for aspirin intolerance, including AIA and AIU. It is known that the pathogenesis of AIA is caused by arachidonic acid metabolites such as leukotrienes, prostaglandins and thromboxane. Most reports related to AIA emphasize the role of cysteinyl LTs in AIA. AIA induces moderate to severe asthma in which symptoms are aggravated by exposure to aspirin or NSAIDs. Affected patients frequently have chronic rhinosinusitis and nasal polyposis with persistent upper and lower airway inflammation and marked eosinophilia (Nasser et al., 1996). The

eosinophil count in AIA patients is 4 times higher than that seen in aspirin tolerant asthma (ATA) patients and 15 times higher than that seen in other asthmatic patients (Sampson et al., 1997). Moreover, eotaxin-2 levels are higher and nasal polyp tissues are significantly more common in AIA, indicating that eotaxin-2 is the major chemokine activating eosinophils in AIA patients (Olze et al., 2006).

PGD2, a major prostanoid produced by allergen-activated mast cells, is the important mediator in the pathogenesis of eosinophilic airway inflammation via its receptor, a chemoattractant receptor molecule expressed on Th2 cells (*CRTH2*). The human *CRTH2* gene (officially *GPR44*) encodes a G protein-coupled chemoattractant receptor that is expressed on Th2 cells, including other allergy-related cells like eosinophils, basophils, and monocytes (Nagata and Hirai, 2003; Spik et al.,2005; Luster and Tager, 2004). Its importance in inflammatory cell activation and recruitment in vitro and in vivo has been reported in several studies (Gervais et al., 2001; Bohm et al., 2004; Sanding., 2006). CRTH2 also mediates chemotaxis of Th2 lymphocytes, eosinophils, and basophils towards PGD2 (Hirai et al., 2001). There have been evidences to suggest that genetic alteration of *CRTH2* is related to allergic asthma in African-American (Huang et al., 2004) Japanese (Maeda et al., 2007), Chinese (Hsu et al., 2002; Wang et al., 2009) and Taiwan (Huang et al., 2005) populations. There has been no published data addressing the role of the *CRTH2* gene in AIA pathogenesis.

Studies have shown that the PGD2 concentration in AIA patients is significantly higher than that in ATA patients (Higashi et al., 2002). In addition, CRTH2 receptor activation by PGD2 released at the site of inflammation is essential in the pathogenesis of eosinophilic airway

inflammation. CRTH2 is expressed on the surface of eosinophils and has been shown to mediate PGD2-induced eosinophil migration in vitro (Gervais et al., 2001; Hirai et al., 2001). Three members of the eotaxin family have the potential to activate eosinophils *in vitro* and to increase susceptibility to asthma (Patel et al., 1997; White et al., 1997). However, an investigation has yet to be performed for the detection of eotaxin involved in the development of aspirin hypersensitivity. Considering the fact that eosinophilic infiltration is more pronounced in AIA patients than it is in ATA patients, we hypothesized that activation of eosinophils via dysregulation of the *CRTH2* gene may play an important role in AIA and may be an important marker of this entity. Therefore, this case control study was designed to determine if variation in the *CRTH2* gene confers elevated risk for developing AIA.

Furthermore, the functional significance of the two gene polymorphism, *CRTH2* -466T>C and *TBXA2R* -4684T>C polymorphisms were investigated using luciferase reporter assay, electrophoretic mobility shift assay (EMSA) and ELISA for production of cytokines.

II. MATERIALS AND METHODS

A. Study subjects and phenotyping

The three studies groups—107 patients with AIA, 115 patients with ATA, and 133 normal healthy controls (NC)—were recruited from Ajou University Hospital, Suwon, Korea. The diagnosis of AIA was based on a positive response to a lysine-acetyl salicylic acid (ASA) bronchoprovocative test, which was performed on all study subjects according to a previously described method (Park, 1995). Changes in forced expiratory volume in 1 sec (FEV1) were followed up for 7 hr following the final dose of the aspirin challenge. The ASA-induced change (%) in FEV1 was calculated as the percentage of post-challenge FEV1 relative to the pre-challenge FEV1 and was defined as positive if the FEV1 level declined more than 20% from baseline.

Methacholine bronchial challenge tests were performed as described previously (Kim et al., 2006). NC was chosen from the general population using a screening questionnaire, which had to indicate no history of respiratory symptoms or aspirin hypersensitivity. All NC subjects also exhibited an FEV1 greater than 80% of the predicted value, provocation concentration (PC20) of methacholine greater than 25 mg/ml, and normal findings on simple chest radiograms. Atopy was defined as one or more positive reactions on a skin prick test using 12 common aeroallergens (Bencard, Brendford, UK), with histamine and saline controls. To measure specific IgE, venous blood was collected from the antecubital vein, allowed to clot for 1-3 hours at 4 degrees, and centrifuged at 1500 g for 10 minutes at 4 degrees. The serum was aspirated, separated, and stored in aliquots at -20 degree until analysis was performed. Serum total IgE was measured using the UniCAP system (Phadia,

Valinge, Sweden), according to the manufacturer's instructions. The threshold cutoff value for a specific IgE level was 0.35 KU/L, as measured by UniCAP.

The presence of rhinosinusitis and nasal polyps was determined using paranasal sinus x-ray and rhinoscopy. Serum eotaxin-1 and eotaxin-2 were measured in the serum using a quantitative sandwich enzyme immunoassay kit (ELISA) (R&D Systems, Inc, Minneapolis, MN, USA). Informed consent was obtained from all subjects. The institutional review board of Ajou University Hospital (Suwon, Korea) approved the study.

B. SNP identification and genotyping

Forty healthy Korean volunteers were used for SNP identification. Genomic DNA was prepared from peripheral blood samples using the Puregene DNA purification kit (Gentra, Minneapolis, MN, USA) according to the manufacturer's protocol. We genotyped two polymorphisms of CRTH2 located in front of exon-2 (upstream of the ATG) using a primer extension method and the SNAPshot ddNTP primer extension kit. (Applied Biosystems, Foster City, CA, USA). The primer information has been described in table 1.

The primers were designed to amplify the genomic region of a particular SNP. PCR was performed with 10 ng of genomic DNA. A 762-bp fragment of CRTH2 was scanned for polymorphisms by direct sequencing. Two polymorphic sites, -466T→C (rs 634681) and SNP -129C→A (rs 41407444), were identified infront of exon -2 upstream of the ATG region.

Table 1. Amplifying and extension primers of the *CRTH2* gene for SNP genotyping

Gene	Locus (SNP ID)		Primers
CRTH2	- 466T>C (rs 634681)	PCR Forward Primer	GATGCTGTGGGAAGGGGC
		PCR Reverse Primer	AGAGCACTGGCGGGTGAA
		Genotyping Primer	TCTCCATCGCCCAGGCCAGCAGAAT
CRTH2	-129C>A (rs 41407444)	PCR Forward Primer	TAAATGACTTGCCCAAGCA
		PCR Reverse Primer	GACCCTGCCCAGGAGATG
		Genotyping Primer	CCAATGTGACCCACGAATATATTGG

C. Statistical analysis

Differences in the mean values of phenotypic characteristics among AIA patients were compared using the t-test. Differences in genotype frequency between the two groups were examined using the chi-square test, and three logical regression models (co dominant, dominant, recessive) were used after accounting for age and sex covariates. Eotaxin-2 level according to severity of AIA was measured by one-way ANOVA-test. Dual luciferase assay and in vitro production of eotaxin-2 was measured by independent-t test. The level of statistical significance was set at $p<0.05$.

D. In vivo functional study of CRTH2 -466T>C by measurement of eotaxin-1 and eotaxin-2 using enzyme-linked immunosorbent assay

Serum eotaxin-1 and eotaxin-2 were further analyzed according to *CRTH2* -466T>C gene polymorphisms. The minimum detectable doses of eotaxin-1 and eotaxin-2 were 6.25 and 2.5 pg/ml respectively. Values were considered to be zero below this limit for the purpose of statistical analysis.

E. Preparation of the *CRTH2* construct and dual luciferase assay

A 762-bp fragment of the human *CRTH2* gene was prepared by PCR amplification from either -466C homozygous or -466T homozygous human genomic DNA as a template (forward primer, 5′-CTGTTTATGGTCGGCACA-3′, reverse primer, 5′-ATGACTCCATTCTCCACCA-3′). Each of the PCR products was gel-purified with an agarose-gel purification kit (iNtRON, Deajeon, Korea) and then ligated into the TA vector (Invitrogen). Next, the plasmid was digested with *Kpn*I and *Xho*I restriction enzymes (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 the Endo Free Plasmid Maxi Kit (Qiagen, Hilden, Germany). Plasmid DNA concentration and purity were assessed using UV spectrophotometry and agarose gel electrophoresis.

Human A549 alveolar type II epithelium-like cells (ATCC CCL-185) were purchased from the American Type Culture Collection and grown in RPMI 1640 medium (GIBCO) supplemented with 10% fetal bovine serum, 100 U/ml of penicillin G sodium, and 100 μg/ml of streptomycin sulphate (penicillin-streptomycin, GIBCO), and cultured at 37° C in 5% CO_2 in a humidified incubator. We seeded $2X10^5$ cells in each well of 12 plates for 1 day prior to transfection to maintain the confluency of cells at 60-70%.

The next day, the cell-containing media was replaced with serum-free RPMI media after washing with PBS. Reporter plasmid DNA of CRTH2 (1 μg) and Renilla plasmid DNA (5 ng) were resuspended and further mixed with 5 μl of lipofectamine transfection reagent (Invitrogen) dissolved in serum-free RPMI. They were then incubated for 30 minutes at

room temperature, after which 100 μl of each solution was dropped in the 12-well cell plates. Plates were incubated for 24 h.

HMC-1, Jurkat-T cells and HL-60 cells were transfected using microporator 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 RPMI for Jurkat-T cells or IMDM for HL-60 cells and HMC-1 cells in culture medium containing 10% fetal bovine serum (FBS) without antibiotics. Plates were preincubated in a humidified incubator at 37° C with 5% CO_2. Jurkat-T cells (2×10^5) and HL-60 and HMC-1 cells (2×10^5) reporter plasmid DNA of CRTH2 (1 μg), as well as Renilla plasmid DNA (5 ng), were resuspended in 10 μl of Solution R. Co-transfection with GATA-3 was carried out with reporter plasmid DNA (1 μg; CRTH2 promoter 0.7 μg, GATA-3 0.3 μg) and Renilla plasmid DNA (5 ng).The incorporation tube was filled with 3 ml of Solution E. The cell and DNA mixtures were inserted into the microporator pipette station. HMC-1 cells were shocked using 1750 V/20mA/plus number 1, Jurkat-T cells were shocked using 1380 V/30 mA/plus number 1, and HL-60 cells were shocked using 1350V/35mA/plus number 1. The samples were transferred to the preincubated 24-well plate. Plates were incubated for 24 h.

Cells were then lysed with 100 μl of passive lysis buffer (Promega) per well. From the 100-μl lysate, a 20-μl aliquot was assayed for luciferase activity using a Dual-Luciferase Reporter Assay Kit (Promega) and Clarity (Bio-Tek, Winooski, VT, USA). The pGL3-control (Promega) and the promoter-less 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 both the

reporter construct and the Renilla control vector into the cell line.

F. Nuclear extract preparation and electrophoretic mobility shift assay

A549 and HMC-1 cells were washed with ice-cold phosphate-buffered saline before being suspended in ice-cold hypoosmotic buffer (10 mM HEPES, 2 mM $MgCl_2$, 0.1 mM EDTA, 10 mM KCl, 1 mM DTT, 1 mM PMSF, 1 mM $NaVO_3$, 10 mM NaF, and 1X protease inhibitors). The cells were incubated on ice for 10 min, after which 0.2% of Nonidet P-40 was added and the cells were incubated for 15 min again. Centrifugation was performed at 15,000 g for 30 seconds, and the pellet was resuspended in a 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 $NaVO_3$, 10 mM NaF, and 1X protease inhibitor. The mixture was incubated on ice for 30 minutes. Centrifugation was carried out at 15,000 g for 30 minutes at 4° C. The supernatant, which contained the nuclear protein, was collected and subjected to the Bradford assay to measure the concentration of protein. Five micrograms of nuclear protein was aliquoted and stored at -80° C until use. Double-stranded oligonucleotides for CRTH2 -466T>C (-466C: 5'-CCAGCAGAATCCTCTTGCCCTA-3' -466T: CCAGCAGAATTCTCTTGCCCTA) and the nonspecific competitor SP-1 were used as probes for the electrophoretic mobility shift assay (EMSA). The double-stranded oligonucleotides were annealed by incubation at 95° C for 5 min, followed by gradual cooling at room temperature. The double-stranded DNA probes were radiolabeled at their 5' ends with DNA polymerase I (Klenow; Promega) and (α-^{32}P) CTP. The reaction mixture of 10 μl was prepared containing 5 μg of nuclear protein, 10 mM HEPES, 8 ng of polydidC, 1 mM $MgCl_2$, 30 mM KCl, 1 mM DTT, 100 mM $NaVO_3$, 10 mM NaF, and 10 mM 50%

glycerol and preincubated on ice for 20 min, then incubated for 30 min on ice in the presence of 0.2 pmol of radiolabeled probe. The reaction mixture was then subjected to electrophoresis on a native 6% polyacrylamide gel. A competition experiment was performed using unlabeled blunt-ended competitor oligonuleotides added to the binding reaction mixtures, prior to the addition of radiolabeled oligonucleotide probes. The gels were dried, and radioactivity was detected using a FLA3000 scanner (Fuji Photo Film, Tokyo, Japan).

G. Preparation of *CRTH2*-coding sequence (CDS) (-466T or -466C) and in vitro functional study of eotaxin-2 production in A549 cells and mRNA expression

To measure in vitro production of eotaxin-2 according to *CRTH2* gene polymorphisms, a 2082-bp fragment of the *CRTH2* gene was constructed in the pGL3 basic vector, including the coding regions (CDS) and the 762-bp fragment of CRTH2. The CRTH2 CDS in pcDNA3.1+ was purchased from the Missouri S & T cDNA resource center. A construct containing 762 bp fragments was digested with Hind III and Xba I and ligated with similarly digested CRTH2 CDS.

A549 cells ($5X10^5$) were seeded in six well plates one day before transfection. In order to induce overexpression of CRTH2 in A549 cells, transfections were carried out in serum-free medium using 5 μl of lipofectamine transfection reagent, 2 μg of CRTH2 (2082 bp fragment of *CRTH2* gene) of each construct, and 300 ng of EGFP. After 5 hours of transfection, cells were treated with FBS and cultured for 24 hours so they could express the transfected *CRTH2* gene. The next day, cell-containing media was replaced with serum-free RPMI media and stimulated with 10 ng of IL-4 for 24 hours. Supernatant was collected to measure

eotaxin-2 from overexpressed cells using specific ELISA (R&D Systems, Minneapolis, MN, USA), and further readings were measured using a multi-detection microplate reader (Bio tek, Instruments, Inc, Synergy^{TM} HT, Vermont). Eotaxin-2 was also measured from A549 cells, A549 cells followed by IL-4 stimulation and pGL3 basic vector. Cells were further harvested to measure EGFP fluorescence for transfection normalization. The fluorescence signal from EGFP-transfected A549 cells was measured in each construct and normalized with the obtained eotaxin-2 level.

Total RNA was extracted from cells using easy-blue reagent (Intron Biotechnology, Korea), according to the manufacturer's instructions. cDNA was synthesized, confirmed, and normalized in concentration using RT-PCR with beta actin, with 5'-TCCTTCTGCATCCTGTCGGC-3' as a forward primer and 5'-CAAGAGATGGCCACGGCTGC-3' as a reverse primer. CRTH2-specific primers used for RT-PCR were 5'-CTGCCCTTCTTCACCTACTTCT-3' (forward) and 5'-GCAGCACATTGTAGTAGCACAT-3' (reverse).

III. RESULTS

A. Clinical characteristics of the *CRTH2* gene study subjects

The mean ages of AIA patients were significantly higher than those of the ATA and NC patients ($p=0.03$ and $p<0.001$, respectively). The atopy rate was significantly lower in AIA patients than it was in ATA patients ($p=0.017$), but significantly higher than it was in NC patients ($p<0.05$). The percentage fall in forced expiratory volume (FEV1) after lysine ASA inhalation was significantly greater in AIA patients than it was in ATA patients ($p<0.001$). AIA patients exhibited a significantly higher prevalence of paranasal sinusitis and nasal polyps compared to ATA patients ($p<0.001$ and $p<0.001$, respectively). The serum eotaxin-2 level was significantly higher in AIA patients than it was in ATA patients ($p=0.034$) in our study (Table 3), while no difference was noted in the eotaxin-1 level.

Characteristics	AIA (n=107)	ATA (n=115)	NC (n=133)	p value	
				AIA vs ATA	AIA vs NC
Age (year)	44.59±13.24	40.44±14.82	34.88±14.818	**0.03**	**<0.001**
Sex (Male/total)	39/106 (36.8%)	60/115 (52.2%)	55/133 (41.4%)	0.168	0.511
Atopy (presence/total)	53/106 (50.0%)	72/115 (62.6%)	25/87 (28.7%)	**0.017**	**<0.001**
Asthma duration (year)	6.94±6.07	5.32±6.18	NA	**0.054**	NA
FEV1 (%)	82.63±23.75	87.07±22.96	NA	0.163	NA
Fall_FEV1(%)	22.25±10.61	7.24±3.99	NA	**<0.001**	NA
Log PC20 methacholine (mg/ml)	0.66±0.68	0.43±0.69	NA	**<0.001**	NA
Serum Log tIgE(IU/ml)	2.16±0.60	2.21±0.68	1.66±0.58	0.589	0.082
Paranasalsinusitis (presence/total)	52/99 (52.5%)	7/114(5.3%)	0/3 (0%)	**<0.001**	NA
Nasalpolyp (presence/total)	55/102 (53.9%)	3/114 (2.6%)	NA	**<0.001**	NA
Eotaxin 1 (pg/ml)	90.78±8.32/49	96.77±6.85/66	NA	0.577	NA
Eotaxin 2 (pg/ml)	1183.95±156.44/51	815.46±66.50/66	NA	**0.034**	NA

Logistic regression analysis was applied to control for age and sex as covariables.q; Minor allele frequency. AIA, aspirin intolerant asthu
NC, normal controls; ATA, Aspirin- tolerant asthma; N, number of patients; NA, not applicable. Values in bold indicate significant P v.
The value was presented as mean ±S.D except eotaxin1 and eotaxin 2, which was presented in S.E

B. Genotype and allele frequencies of the *CRTH2* gene with the AIA phenotype

We screened two SNPs according to *CRTH2* genetic polymorphisms (-466T>C and -129C>A) and selected *CRTH2* -466T>C SNP for further study. In case of -129C>A, data was not shown as distributions of the different genotypes were not homogenous. Majority of AIA patients (93.4%) had homozygous CC genotype and remaining 6.6% had heterozygous CA type. Similarly, 93.9% of ATA patients had CC genotype and remaining 6.1% had CA type. There were no minor homozygous genotype, AA, in our study population in case of -129C>A.

The genotype distribution of the two polymorphisms did not depart significantly from Hardy-Weinberg equilibrium ($p>0.05$). The genotype frequency of *CRTH2* -466T>C was significantly different between AIA and ATA patients on both the co-dominant and recessive analysis models (Table 4). Specifically, AIA patients showed a significantly higher frequency of the homozygous TT genotype of *CRTH2* -466T>C compared to ATA patients ($p<0.05$). In addition, there was no significant association among clinical parameters such as atopy, FEV1, log PC20 methacholine, or rhinosinusitis in this polymorphism in AIA patients. No differences were noted in other SNPs of *CRTH2* gene.

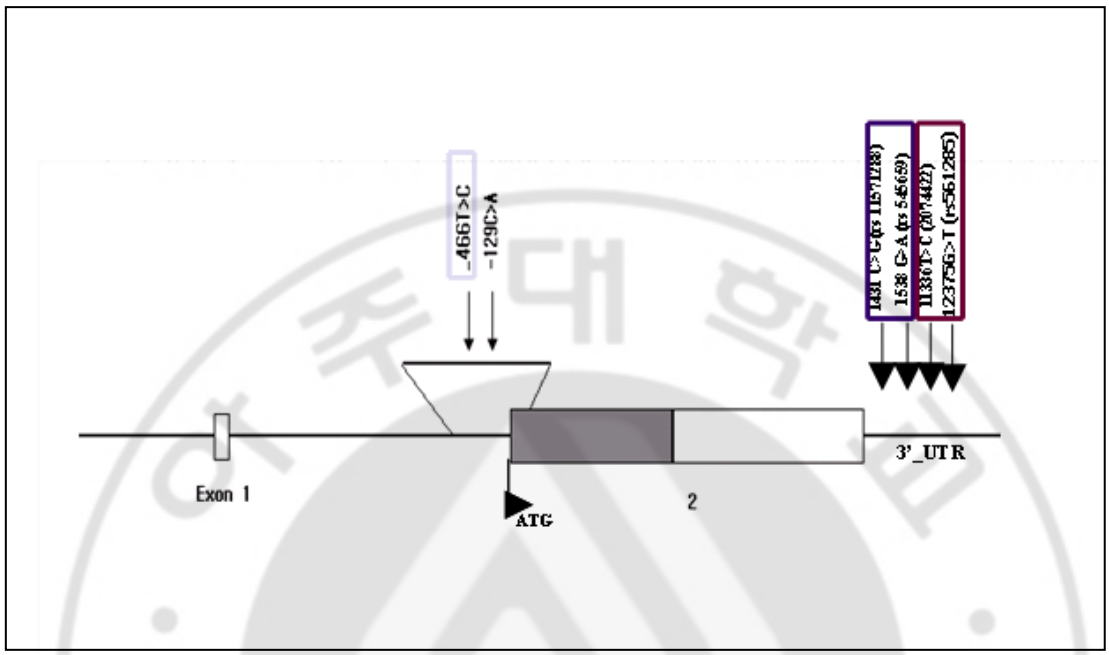

Fig. 1. Schematic representation of *CRTH2* gene. Gene map and SNPs of *CRTH2* gene.

1431C>G (rs11571288) and 1538G>A (rs 545659) SNPs located in 3'-UTR region: No association in Korean population. Target SNP: -466T>C (rs 634681), -129C>A (rs 41407444)

Table 4. Allele and genotype frequencies of the CRTH2 gene polymorphism in study subjects

Loci	Genotype	AIA (N=107)	ATA (N=115)	NC (N=133)	p value AIA vs ATA	p value AIA vs NC
-466T>C	TT	57 (53.8%)	46 (40.0%)	60 (45.1%)	**0.044**	0.278
	CT	43 (40.6%)	60 (52.2%)	62 (46.6%)	0.416	0.261
	CC	7 (5.6%)	9 (7.8%)	11 (8.3%)	**0.037**	0.444
	q	0.259	0.339	0.316	0.055	0.298

*Each P value was calculated with co-dominant, dominant, and recessive models. Logistic regression analysis was applied to control for age and sex as covariables.q; Minor allele frequency, AERD, aspirin exacerbated respiratory disease; NC, normal controls; ATA, Aspirin- tolerant asthma; N, number of patients; NA, not applicable. Values in bold indicate significant P value.

C. Eotaxin level according to the *CRTH2* gene polymorphism

There was a significant difference between AIA and ATA with respect to serum eotaxin-2 levels, according to *CRTH2* gene polymorphisms. AIA patients carrying the homozygous TT genotype of *CRTH2* -466T>C showed a significantly higher eotaxin-2 level compared to patients with CT and CC genotypes ($p<0.05$) (Table 5).

Table 5. The comparison of serum eotaxin level in AIA patients according to *CRTH2* gene polymorphism

	CRTH2 -466T>C		
	TT	CT or CC	p value
Eotaxin 1	92.14± 12.37/25	92.88± 15.72/16	0.971
Eotaxin 2	1513± 275.72/25	854.44± 161.08/18	**0.046**

The value was presented as mean ±S.E

Values in bold indicate significant *P* value.

D. Eotaxin-2 level according to severity of AIA patients based on classification of FEV1 (%)

Severity of AIA patients were divided into three groups based on FEV1 level (Table 6). Severe AIA is defined as group I having less than 50% of FEV1 in percentage. Moderate AIA is defined as group II having 51-79% of FEV1 and mild is defined as group II having > 80% of FEV1 (%). There was a significant difference between severe AIA and mild with

respect to serum eotaxin-2 levels. Severe AIA patients in our study subjects showed significantly higher eotaxin-2 level compared to patients with mild group ($p<0.001$). Moderate AIA showed significantly higher eotaxin-2 level compared to patients with mild group ($p=0.044$) as shown in (Fig. 2.)

Table 6. Classification of AIA according to severity based on FEV1 level

Types of Group	Severity of disease
Group I: <50%	Severe AIA
Group II: 51-79%	Moderate AIA
Group III: > 80%	Mild AIA

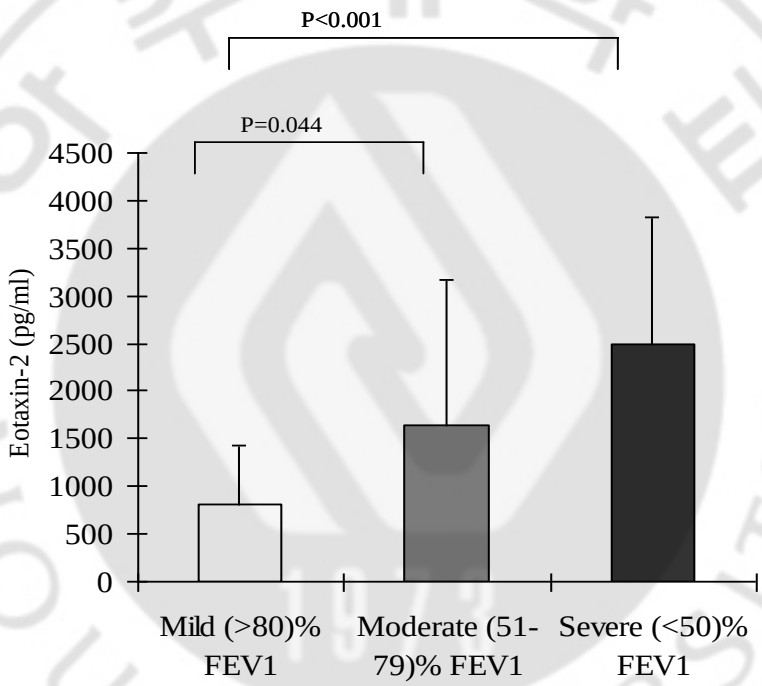

Fig. 2. Eotaxin-2 (pg/ml) level according to severity of AIA patients based on classification of FEV1(%). The p value was determined using one-way ANOVA- test.

E. Endogenous expression of *CRTH2* from various cell lines

We checked CRTH2 endogenous gene expression in different cell lines epithelial cell line (Bease-2B), human bronchial epithelial cell line (A549), human acute monocytic leukemic cell line (THP-1), human leukemic monocyte lymphoma cell line (U937), human epithelial carcinoma cell line (Hela), human mast cell line (HMC-1) and human promyelocytic leukemic cells (HL-60). (Fig. 3).

Fig. 3. *CRTH2* mRNA expression in different cell lines. Normalization of different cDNA was performed using beta actin. 1:Bease-2B (Human bronchus epithelial cell); 2:A549 (Human lung epithelial cell); 3:THP-1 (Human monocyte leukemia cell); 4:U937 (Human monocytes); 5:Hela (Human adenocarcinoma cell); 6:HMC-1 (Human mast cell); 7:HL-60 (Human Myeloblastic cell).

F. Dual luciferase activity of *CRTH2* -466T>C polymorphism

To determine if the *CRTH2* -466T>C polymorphism is associated with altered promoter activity, a construct composed of the CRTH2 sequence and a luciferase reporter gene were

transfected into the A549 cell line. Reporter activities were compared between constructs containing either *CRTH2* -466T or *CRTH2* -466C in the gene. Luciferase activity was enhanced significantly in the construct containing *CRTH2* -466C when compared to the -466T containing construct in A549 cells (p<0.001, Fig. 4b). Additional transfection experiments were performed in the HL-60, Jurkat-T cell and HMC-1 cell lines; significantly higher activity was also noted in the *CRTH2* -466C construct compared to the *CRTH2* -466T construct (p=0.024, p<0.001, p<0.001 Figs. 4c, 4d and 4e, respectively). Furthermore, a survey of a transcription factor database conducted using the consite program (http://asp.ii.uib.no:8090/cgi-bin/CONSITE/consite/) suggested that the -466T→C polymorphism generates a GATA-3 transcription factor-binding motif. The co-transfection experiment with GATA-3 containing the *CRTH2* -466T and the -466C alleles demonstrated that the *CRTH2* -466T allele produced a greater increase in induction of luciferase activity compared to the *CRTH2* -466C allele (P < 0.001) as shown in (Fig. 5).

G. Effects of *CRTH2* -466T>C gene polymorphisms on transcriptional activity

To determine if the *CRTH2* -466T>C locus serves as a transcription factor binding site, we performed EMSA using nuclear extract from two different cells (A549 and HMC-1 cells) with double-stranded oligonucleotide probes corresponding to -466T>C. We observed that -466T produced a specific band having higher affinity than that produced by -466C. This was not visible in the presence of the nonlabeled -466T probe as a competitor (Fig. 6).

Therefore, we checked the involvement of GATA-3 and carried out a competitive binding assay using two unlabeled probes (GATA-3 and SP-1) as competitors and found that -466T

and -466C both disappeared in presence of GATA-3, but not in the presence of SP-1 (data not shown). This finding indicates that GATA-3 may be a CRTH2-specific transcription factor only.

H. CRTH2 expression and in vitro production of eotaxin-2 from *CRTH2* transfected A549 cells

Given the increased in vivo levels of eotaxin-2 in the serum of AIA patients, we studied the *in vitro* production of eotaxin-2 by CRTH2 over expressed cells from constructs containing the CRTH2 sequence, including the coding sequence (CDS) region. This was performed in A549 cells stimulated for 24 hours with IL-4, which is the essential cytokine for the synthesis of eotaxin-2 in bronchial epithelial cells. When we studied eotaxin-2 production according to *CRTH2* -466T>C gene polymorphisms, we found that the CRTH2 construct containing the T allele produced more eotaxin-2 compared to the C allele ($p=0.003$, Fig. 7).

To determine if the *CRTH2* -466T>C polymorphism is associated with differing mRNA expression, we transfected a construct composed of the CRTH2 sequence along with the CDS region into the A549 cell line, followed by IL-4 stimulation. We noted lower mRNA expression of the CRTH2 -466T allele compared to the -466C allele (Figs. 8a, 8b).

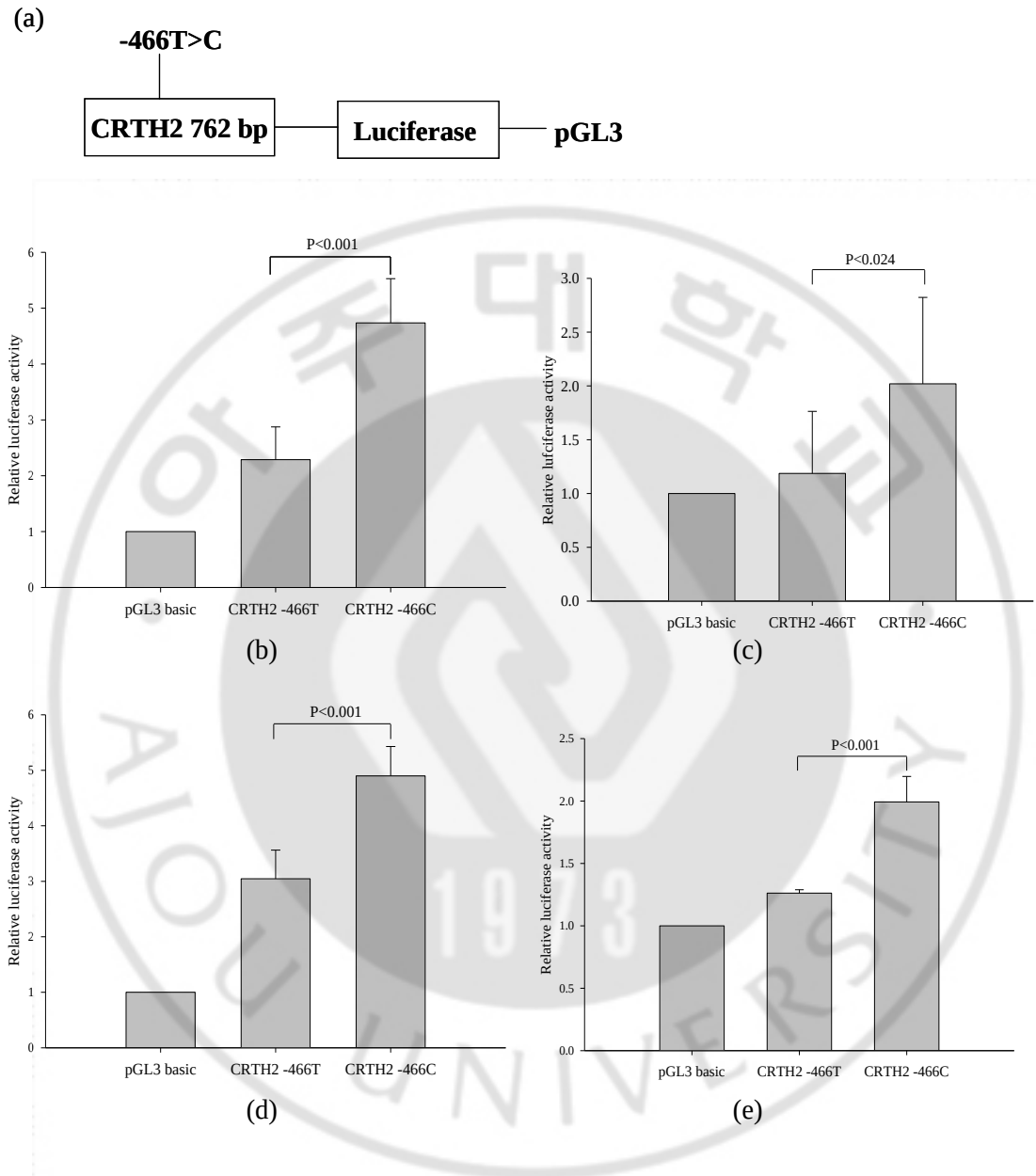

Fig. 4. Effects of the *CRTH2* -466T>C polymorphism on the transcription activity of human *CRTH2* gene. (a) Schematic representation of reporter CRTH2 (762 bp) gene construct upstream of exon 2. Datas are the mean values of independent experiments±S.D.

(b) Transfection of A549 cells. Luciferase activity assay was performed in three independent experiments (total, n=9). (c) Transfection of HL-60 cells. (d) Transfection of Jurkat-T cells. (e) Transfection of HMC-1 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 by independent-t test.

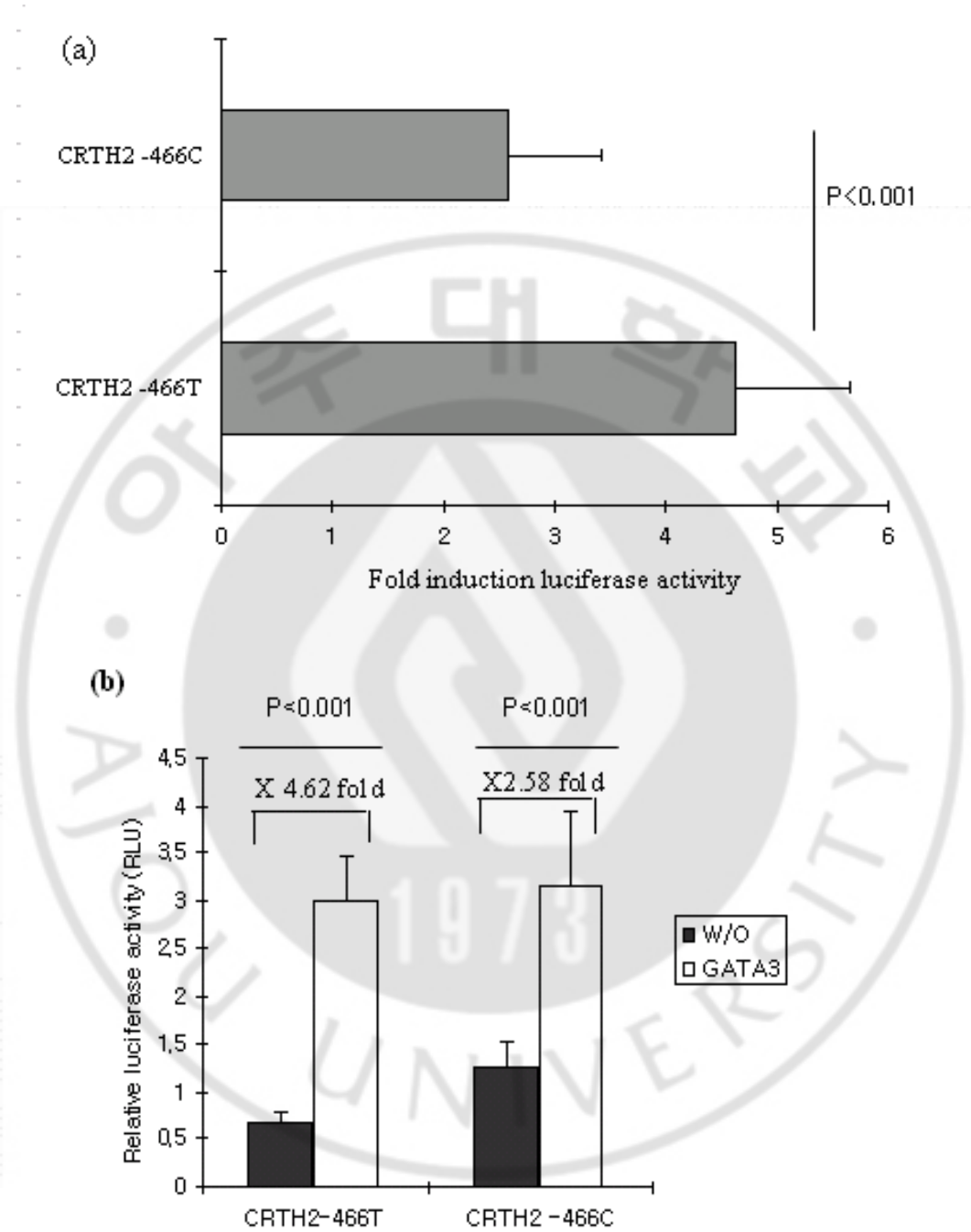

Fig. 5 Promoter activity of *CRTH2* gene cotransfected with GATA-3 in HMC1 cells. (a). The relative fold induction luciferase activity (RLU) is represented as the ratio of the RLU

(the relative luciferase activity is represented as the ratio of the activity to the luciferase activity in the cells transected with the promoterless basic vector, pGL3B) of *CRTH2* T and C allele. (b). The relative luciferase activity of *CRTH2* T and C allele cotransfected with and without GATA-3. Data are the mean ± SD values of three independent experiments in total, triplicated for each sample, n = 9). Statistical analysis was performed using an independent t-test.

(a)

-466T: CCAGCAGAAT**T**CTCTTGCCCTA

-466C: CCAGCAGAAT**C**CTCTTGCCCTA

SP1: ATTCGATCGGGGCGGGGCGAGC

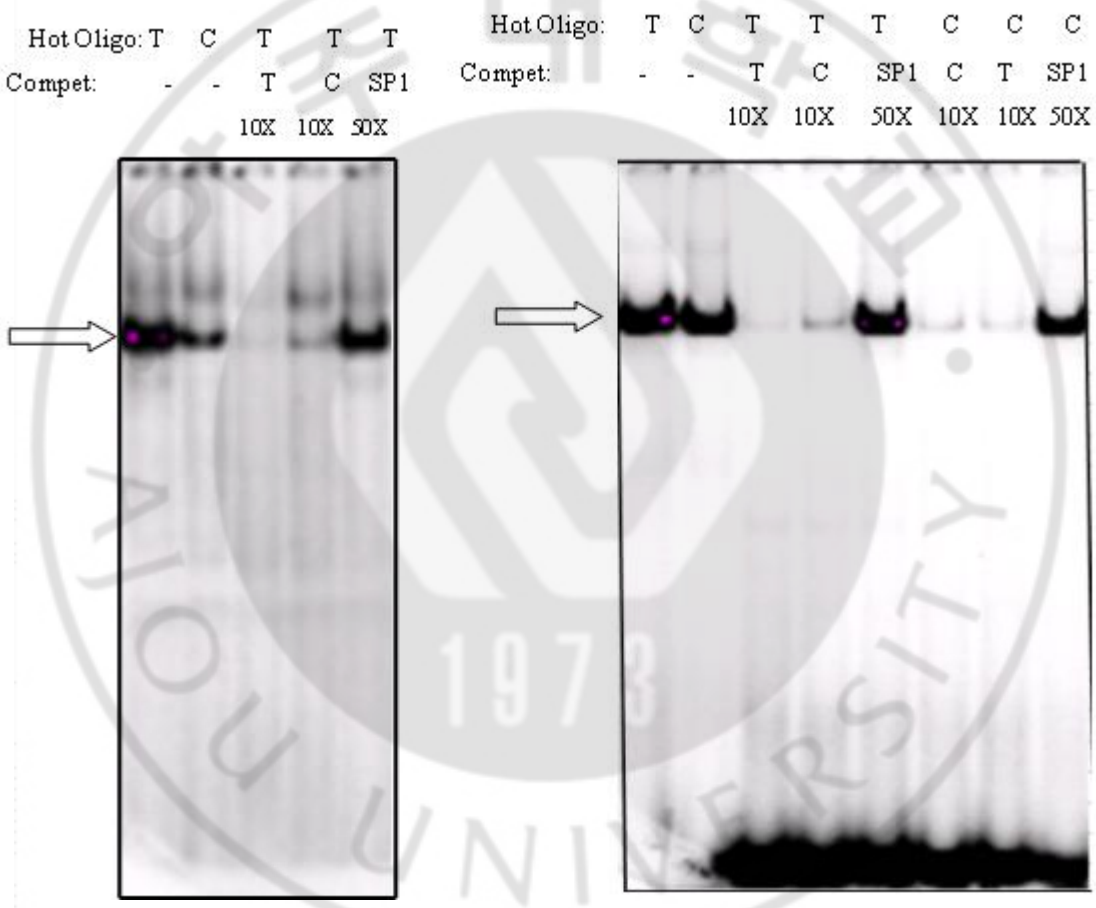

Fig. 6. Results of an electrophoretic mobility shift assay for the *CRTH2* -466T>C polymorphism using nuclear extracts obtained from A549 cells and HMC-1 cells. (a) Nucleotide sequences of oligonucleotides used as probes and competitors (-466T, -466C, SP-1). (b) Differential binding of a nuclear protein to -466 T/C alleles in A549cells. (c) Differential binding of a nuclear protein to -466T/C alleles in HMC-1 cells.

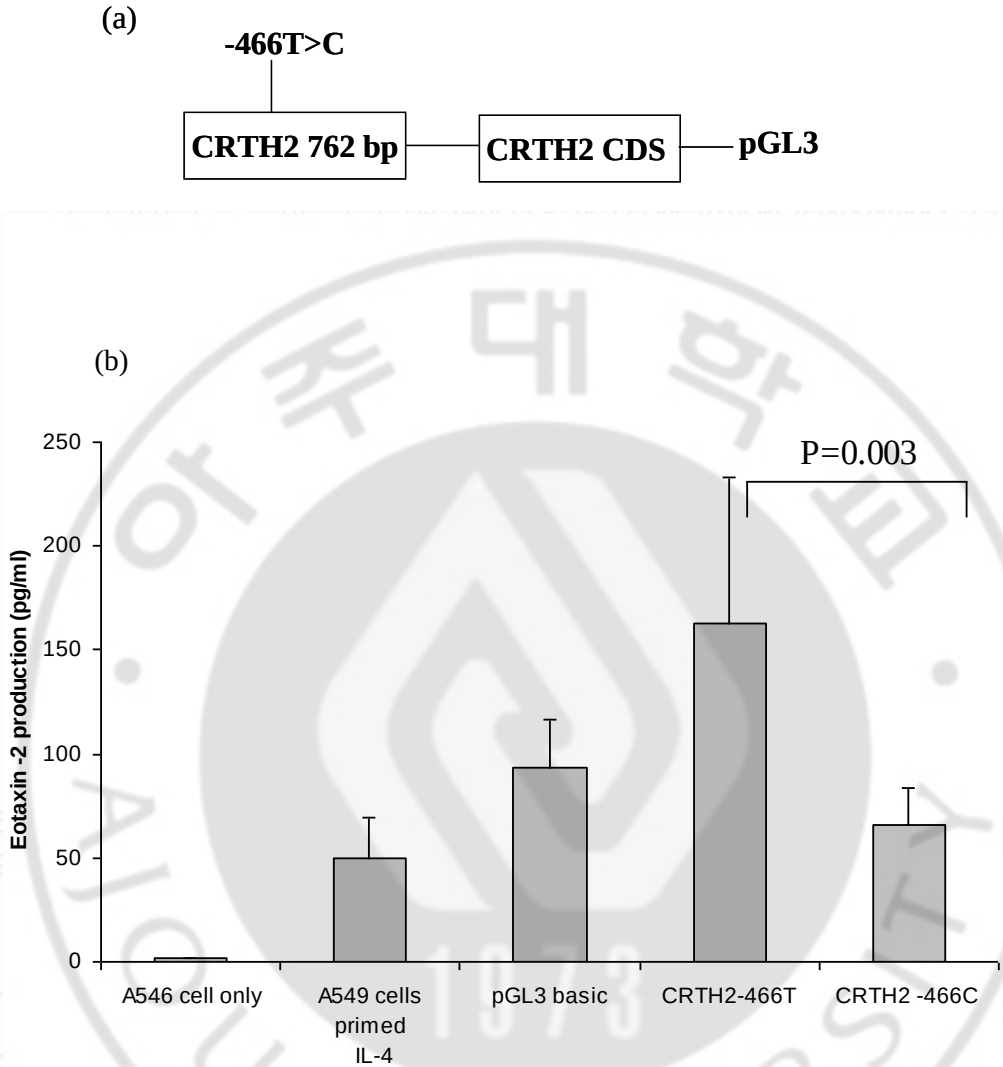

Fig. 7. In-vitro production of eotaxin-2 from *CRTH2* transfected in A549 cells. (a) Schematic representation of reporter *CRTH2* (762 bp) gene construct upstream of exon 2 with *CRTH2* CDS. (b) A549 cells were seeded in RPMI media for 24 hour followed by washing with PBS and transfected with pGL3 basic vector only and *CRTH2* -466T and -466C, incubated for 24 hours and stimulated with IL-4 (10ng) for 24 hours. Supernatant was collected and measured eotaxin-2 by ELISA. Data are the mean values of three independent experiments±S.D. (total, n=9). The p value was determined by independent-t test.

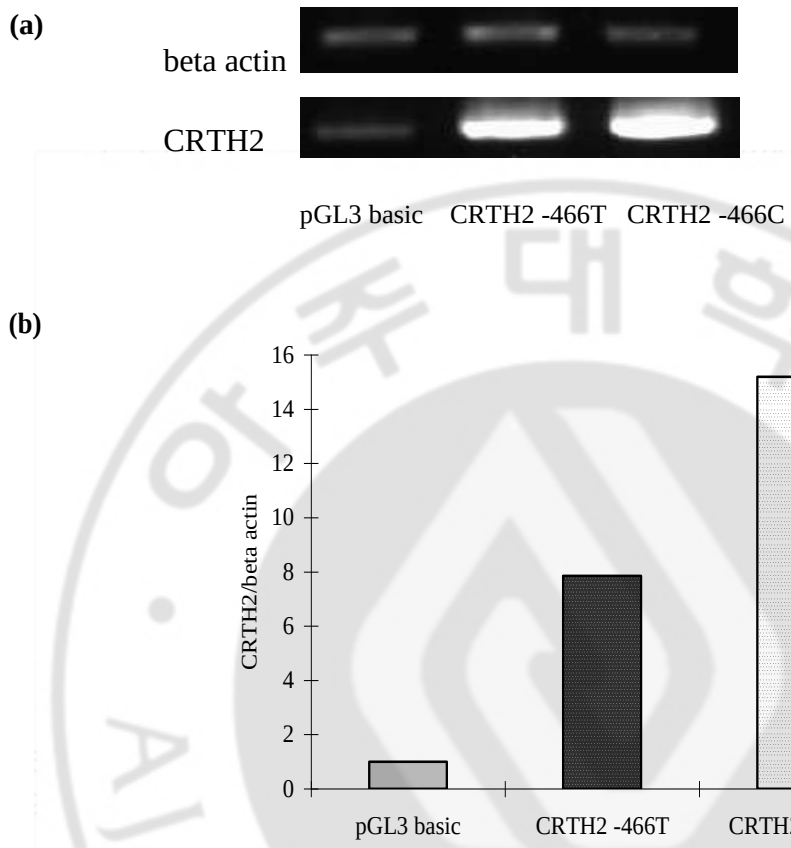

Fig. 8. (a) RT-PCR amplification of *CRTH2* -466T, -466C, pGL3 basic and b-actin mRNA from transfected A549 cells expressing -466T, -466C and pGL3 basic. Beta-actin was used for normalisation with pooled cDNA prepared from pGL3 basic, -466T and -466C transfected in A549 cells. (b) Quantitative expression of pGL3 basic, *CRTH2* -466T and *CRTH2* -466C after normalisation with beta-actin.

IV. DISCUSSION

Altered eicosanoid metabolism is the generally accepted mechanism behind AIA. However, the detailed mechanism has not yet been determined. Aspirin intolerance and the aspirin triad are usually accompanied by eosinophilia of the upper and lower airways. Chemotactic cytokines and the CC-chemokines regulated by activation, eotaxin, and eotaxin-2 stimulate and attract eosinophils to the site of inflammation Eosinophilic infiltration is a characteristic feature of the nasal polyps associated with AIA (Ogata et al., 1999). Eosinophilic nasal polyps are a rich source of eotaxin, especially eotaxin-2 (Olze et al., 2006). An active role has been postulated for the alveolar epithelium in eosinophilic inflammation in asthma, and this role is supported by previous studies (Sexton et al., 2001). Recent reports have suggested that hematopoietic-type PGD(2) synthase (h-PGDS) plays a proinflammatory role in the pathophysiology of chronic rhinosinusitis (Yamamoto et al., 2009). CRTH2, a receptor of PGD2, is known to be associated with eosinophilic inflammation. Therefore, we hypothesized that the eosinophil-associated gene *CRTH2* plays an important role in the pathogenesis of AIA by modulating the eotaxin level and leading to eosinophilic infiltration.

This is the first study to demonstrate a significant contribution of *CRTH2* gene polymorphisms in AIA patients. In this study, we noted a significant association between the *CRTH2* -466T>C polymorphism and the AIA phenotype and provided supporting evidence from in vitro and in vivo functional studies. We also found two SNPs 1431C>G (rs 11571288) and 1538G>A (rs 545659) in 3'-UTR region from ATG region but didn't find any significant association with AIA in Korean population (data not shown). Previous study has also reported regarding these SNPs as a negative association study in Japanese asthmatic

population (Maeda et al., 2007).

Previously, it was reported that eotaxin-2 was secreted to a greater degree in patients with ATA than it was in those with AIA (Min et al., 2005). In contrast, the higher serum eotaxin-2 levels noted in AIA in this study indicated that the eosinophilic chemokine eotaxin-2 is important in AIA. We also found significantly higher eotaxin-2 according to AIA severity based on FEV1 (%) level but not with duration of asthma. (data not shown). Furthermore, the higher frequency of TT and lower frequency of CC and CT seen in AIA indicate that the -466T allele may be the risk allele for this group. Similarly, the higher eotaxin-2 levels seen in the TT genotype led to increased susceptibility to AIA. The elevated levels may have been attributable to CRTH2 orchestrating eosinophilic inflammation through the production of eosinophil-activating cytokines and chemokines; in particular, eotaxin-2.

We performed in vitro functional studies using bronchial epithelial cells. We previously reported that CRTH2 endogenous expression was observed in HMC-1 cells, HL-60 cells, and A549 cells (Palikhe et al., 2009). In this study, *CRTH2* -466T showed decreased promoter activity, which may have led to decreased expression of CRTH2 in inflammatory cells. A possible explanation for the lower *CRTH2* promoter activity and expression seen in -466T compared to -466C is that down-regulation of CRTH2 expression occurs in the -466T type *CRTH2* gene polymorphism which may enhanced IL-5 production. Enhanced IL-5 production could provide a feasible explanation for enhanced eosinophil recruitment. Therefore, in *CRTH2* -466C allele, CRTH2 controls eosinophil recruitment through limitation of IL-5 production. CRTH2 mRNA levels have also been shown to inversely correlate with IL-5 and eotaxin levels (Yamamoto et al., 2009). In vivo studies have revealed

that mice deficient in CRTH2 have increased eosinophil recruitment and splenocyte IL-5 production in an asthma model, suggesting that CRTH2 may mediate anti-inflammatory signals (Chevalier et al., 2005). However, cross-regulation between IL-5 and CRTH2 is needed to further elucidate. CRTH2 expression in human Th2 cells has been shown to decrease upon CD3/CD28 stimulation (Tanaka et al., 2004), and CRTH2 expression in human eosinophils has been shown to decrease upon either PGD2 or IL-5 activation. Additionally, CRTH2 levels in lung tissue have been shown to decrease following antigen challenge in a murine model of asthma (Fujitani et al., 2002). Previous studies done in septic shock patients showed that eotaxin receptors were strongly linked to CRTH2 and noted low levels of CRTH2 in the patient groups (Venet et al., 2004). CRTH2 mRNA was lowered in radiologically more severe cases of chronic rhinosinusitis (Huang et al., 2004), a major feature of AIA. We suggest that the lowered expression of CRTH2 may be responsible for the acceleration of eosinophilic infiltration in AIA patients.

In addition, the EMSA result demonstrated that -466T-specific DNA binding was more pronounced and might have been more important in the transcriptional regulation of the *CRTH2* gene, compared to -466C. We previously shown that GATA-3 as a candidate transcription factor for T specific allele, however, specific band in both alleles disappeared with GATA-3 competitor indicates that GATA-3 is CRTH2 specific transcription factor rather than allele specific. GATA-3 cotransfection with the *CRTH2* -466T>C gene polymorphism in HMC-1 cells produced a greater increase in luciferase activity in the -466T allele than did polymorphisms in -466C (Palikhe et al., 2009). Therefore, GATA-3 may act as a positive regulator of the -466T allele.

We developed an *in vitro* model study for measuring eotaxin-2 production according to *CRTH2* gene polymorphisms. We noted significantly higher eotaxin-2 release from overexpressed *CRTH2* -466T compared to -466C in A549 cells, suggesting that -466T may be associated with eosinophilic infiltration causing AIA. Evidence suggests that the airway epithelium is a cytokine-stimulated source of eotaxin (Heiman et al., 2005). Our results further confirmed that IL-4 upregulated eotaxin-2 production by airway epithelial cells, an effect that was more pronounced in CRTH2 -466T transfected cells. Furthermore, CRTH2 -466T mRNA expression was decreased compared to -466C mRNA expression, as measured by RT-PCR.

The interaction of eosinophils and bronchial epithelial cells plays an important role in the upregulation of adhesion molecule on eosinophils and epithelial cells via a differential intracellular signaling pathway during allergic inflammation (Wong et al., 2006).. Therefore, eotaxin-2 derived from *CRTH2* -466T-expressing A549 cells plays an important pathogenic role in eosinophilic airway inflammation in AIA patients.

In conclusion, these findings suggest that the -466T>C polymorphism of the *CRTH2* gene is associated with AIA. Specifically, the *CRTH2* -466T allele showed higher frequency and increased serum and cellular eotaxin-2 production through the lowering of CRTH2 expression. This led to eosinophilic infiltration and likely contributed to the development of AIA.

Part-II

Genetic Polymorphism of *TBXA2R* in Aspirin Intolerant Acute Urticaria

I. INTRODUCTION

Aspirin intolerant acute urticaria (AIAU) presents with the immediate onset of urticaria after aspirin ingestion, which often resolves within 1-2 weeks (Grattan, 2003). Aspirin ingestion could aggravate chronic urticaria and this condition is defined as aspirin intolerant chronic urticaria (AICU) (Kim et al., 2006). However, knowledge of the pathogenic mechanism of AIU is limited as there is few published data comparing the clinical features and pathogenic mechanisms of AIAU and AICU. One major feature of urticaria is the release of histamine from activated mast cells, leading to raised, superficial, erythematous wheals and intense pruritus (Powell et al., 2007). Neutrophilic urticaria has been observed in cases of chronic cholinergic, cold urticaria and has been also demonstrated in AIAU, in association with elevated levels of IL-8 and IL-18 (Choi et al., 2008).

Human thromboxane A2 (TBXA2), an another eicosanoid product induced by cyclooxygenase may be an important mediator in aspirin hypersensitivity, as it can induce bronchoconstriction and bronchohyper-responsiveness (O'Byrne, 1989; Taylor et al., 1991; Devillier et al., 1997) and stimulate proliferation of human airway smooth muscle cells (Capra et al., 2003). TBXA2R exerts its actions by interacting with the G-protein-coupled TBXA2R receptor. TBXA2 is a potent vasoconstrictor and stimulator of platelet aggregation. It is an inducer of acetylcholine release and of airway micro vascular leakage (Devillier et al ., 1997). The mechanism of aspirin is through blocking the production of TBXA2 has been shown in cerebral infarction (Preston et al., 1981). *TBXA2R* gene polymorphism has been associated with atopy, asthma and atopic dermatitis (Unoki et al., 2000; Leung et al., 2002; Tanaka et al., 2002; Shin et al., 2003). Several studies have demonstrated important roles of

TXA2R and its ligand in the pathogenesis of bronchial inflammation and bronchial hyperresponsiveness (BHR) (Nagase et al., 1997; Shi et al., 1998; Rolin et al., 2006). Recent studies have shown that TBXA2 can be considered a negative regulator of LTC4 synthase activity, which is responsible for leukotriene production, and have also suggested that when TBXA2 formation is prevented, LTC4 synthase activity is elevated, leading to increased biosynthesis of leukotriene and clinical symptoms (Tornhamre et al., 1995; Tornhamre et al., 2002).

Thromboxane A2 is short lived and very rapidly transformed non-enzymatically in aqueous solution to thromboxane B2 (TXB2) (Westlund et al., 1986). TXB2 is further metabolized enzymatically to a series of compounds, of which 11-dehydro-TXB2 is the major product found both in plasma and urine (Westlund et al., 1986). The dehydrogenase catalyzing 11-dehydro-TXB2 formation is tissue bound and widely expressed, with the highest occurrence being found in lung and kidney (Westlund et al., 1986). 11-dehydro-TXB2 has been used to monitor TxA2 production in vivo and in vitro because of its stability (Lupinetti et al., 1989).

Recently the other study has reported the effect of this gene polymorphism on asthma-related phenotype or responsiveness to leukotriene receptor antagonist (LTRA) in asthmatic children (Kim et al., 2008). The *TBXA2R* gene is located on chromosome 19p13.3, contains 4 exons with total length of 15 kb, and codes for a protein of 343 aminoacids (Unoki et al., 2000).

We previously reported that TBXA2R has been associated with aspirin intolerant asthma (Kim et al., 2005). However, there has been no study of the role of *TBXA2R* polymorphisms in the pathogenesis of AIAU. Therefore, elucidation of variability of genetic polymorphism

of *TBXA2R* in an association with urticaria will be essential to determine the regulatory role of it. In this study, we have investigated whether genetic polymorphisms of the *TBXA2R* gene are associated with AIAU phenotype through a case-control of three study subjects.

II. MATERIALS AND METHODS

A. Study subjects and phenotyping

Three subject groups (167 patients with AIAU, 316 patients with AICU, and 265 patients with normal controls) were enrolled at Ajou University Hospital in Suwon, Korea. AIU defined by a positive result on an ASA oral provocation test, which was performed using 500mg of ASA (Rhonal, KunWha Pharmaceutical Co., Seoul, Korea). Those patients diagnosed with AIU were divided into two groups, AICU and AIAU, according to the duration of urticaria. Patients shows cutaneous symptoms of ASA hypersensitivity were resolved within 6 weeks were of ASA ingestion were assigned to AIAU group, and those whose cutaneous symptoms lasted longer than 6 weeks were placed in the AICU groups. NCs who were non- atopic with no personal or family history of allergic disease and no past history of ASA hypersensitivity were recruited from the general population.

Informed consent was obtained from all subjects, and the Institutional Review Board of Ajou University Hospital approved the study. Skin prick tests were performed with 55 common aeroallergens (Bencard Co., West Sussex, UK), and atopy was defined as one or more positive reactions to common inhalant allergens. Total IgE concentrations were measured using UniCAP system (Pharmacia Diagnostics, Uppsala, Sweden) according to the manufacturer's instructions. Two thyroid autoantibodies, antithyroglobulin and antimicrosomal antibodies which are known to be associated with CU were detected using anti-Tg and anti-TPO radioimmunoassay (B·R·A·H·M·S AG, Hennigdorf, Germany), respectively. Selective antinuclear antibodies were detected by direct immunofluorescensce (Fluoro HEPANA test; Medical and Biologic Laboratories Co., Ltd., Nagoya, Japan).

B. SNP identification and genotyping

The *TBXA2R* region upstream of exon 1 and at exon 3 was sequenced to examine single nucleotide polymorphisms (SNPs) using the ABI Prism 3100 DNA analyzer (Applied Biosystems, Foster City, CA, USA). Two polymorphisms of *TBXA2R (*-4684T>C and 795 T>C) were genotyped using a primer extension method and the SNAPshot ddNTP primer extension kit (Applied Biosystems, Foster City, CA, USA). The primer information has been described in table 2 .

Table 2. Amplifying and extension primers of the *TBXA2R* gene for SNP genotyping

Gene	Locus (SNP ID)		Primers
TBXA2R	4684T/C (rs4807491)	PCR Forward Primer	ACCGTCTCTTTGCAGAGG
		PCR Reverse Primer	AAGGTCAGCTTGTCCGGT
		Genotyping Primer	CAGGTCCCTAACTCAAGGTTCTGTC
TBXA2R	795T>C	PCR Forward Primer	GAG TGG ACCCTGGATCTCA
		PCR Reverse Primer	GCG CTC TGT CCACTTCCT
		Genotyping Primer	CGCAGCACTGTCGGGC

C. Statistical analysis

Differences in the mean values of phenotypic characteristics among AIAU patients were compared using the t-test. Differences in genotype frequency between the two groups were examined using the chi-square test, and three logical regression models (co dominant,

dominant, recessive) were used after accounting for age and sex covariates. Dual luciferase assay was measured by independent-t test. Differences in TXB2 production in two groups were examined by Mann-Whitney test. All statistical analyses were performed using SPSS version 11 (SPSS Inc., Chicago, IL,USA). The level of statistical significance was set at $p<0.05$.

D. Preparation of the *TBXA2R* construct and dual luciferase assay

A 1082 bp fragment of the human *TBXA2R* gene was prepared by PCR amplification from either -4684C homozygous or -4684T homozygous human genomic DNA as a template (forward primer, 5′-CTGTTTATGGTCGGCACA-3′, reverse primer, 5′-ATGACTCCATTCTCCACCA-3′). Each of the PCR products was gel-purified with an agarose-gel purification kit (iNtRON, Deajeon, Korea) and then ligated into the TA vector (Invitrogen). Next, the plasmid was digested with *Kpn*I and *Xho*I restriction enzymes (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 the Endo Free Plasmid Maxi Kit (Qiagen, Hilden, Germany). Plasmid DNA concentration and purity were assessed using UV spectrophotometry and agarose gel electrophoresis.

Transfection protocol in A549 cells was similar with the previous one as described in CRTH2. Transfection was further repeated in U937 cells and HMC-1 cell line. HMC-1 will be transfected using microporator MP-100 (Digital Bio Technology, Seoul, Korea) and PMK-1096 (Digital Bio Technology), according to the manufacturer's instructions. Briefly, 24-well plates will be filled with 1000 μl IMDM in culture medium containing 10% fetal

bovine serum (FBS) without antibiotics. Plates will be incubated in a humidified incubator at 37° C with 5% CO_2. HMC-1 cells (2×10^5), reporter plasmid DNA of TBXA2R (1 μg), as well as Renilla plasmid DNA (5 ng), will be resuspended in 10 μl of Solution R. The incorporation tube will be filled with 3 ml of Solution E. The cell and DNA mixtures will be inserted into the microporator pipette station. HMC-1 cells will be shocked using 1750V/20mA/plus number 1. The samples will be transferred to the preincubated 24-well plate. Plates will be incubated for 24 h.

Cells will be then lysed with 100 μl of passive lysis buffer (Promega) per well. From the 100-μl lysate, a 20-μl aliquot will be assayed for luciferase activity using a Dual-Luciferase Reporter Assay Kit (Promega) and Clarity (Bio-Tek, Winooski, VT, USA). The pGL3-control (Promega) and the promoter-less pGL3-basic vectors will be used as positive and negative controls, respectively. Transfection efficiency will be determined by measuring Renilla activity using a Dual-Luciferase Reporter Assay Kit (Promega) after co-transfection of both the reporter construct and the Renilla control vector into the cell line.

E. Electrophoretic mobility shift assay

HMC-1 cells were washed with ice-cold phosphate-buffered saline before being suspended in ice-cold hypoosmotic buffer (10 mM HEPES, 2 mM $MgCl_2$, 0.1 mM EDTA, 10 mM KCl, 1 mM DTT, 1 mM PMSF, 1 mM $NaVO_3$, 10 mM NaF, and 1X protease inhibitors). The cells was incubated on ice for 10 min, after which 0.2% of Nonidet P-40 was added and the cells were incubated for 15 min again. Centrifugation was performed at 15,000 g for 30 seconds, and the pellet was resuspended in a 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 $NaVO_3$,

10 mM NaF, and 1X protease inhibitor. The mixture was incubated on ice for 30 minutes. Centrifugation was carried out at 15,000 g for 30 minutes at 4° C. The supernatant, which contained the nuclear protein, was collected and subjected to the Bradford assay to measure the concentration of protein. Five micrograms of nuclear protein was aliquoted and stored at -80° C until use. Double-stranded oligonucleotides for TBXA2R and the nonspecific competitor SP-1 were used as probes for the electrophoretic mobility shift assay (EMSA). The double-stranded oligonucleotides was annealed by incubation at 95° C for 5 min, followed by gradual cooling at room temperature. The double-stranded DNA probes was radiolabeled at their 5' ends with DNA polymerase I (Klenow; Promega) and (α-^{32}P) CTP. The reaction mixture of 10 µl was prepared containing 5 µg of nuclear protein, 10 mM HEPES, 8 ng of polydidC, 1 mM $MgCl_2$, 30 mM KCl, 1 mM DTT, 100 mM $NaVO_3$, 10 mM NaF, and 10 mM 50% glycerol and preincubated on ice for 20 min, then was incubated for 30 min on ice in the presence of 0.2 pmol of radiolabeled probe. The reaction mixture was then subjected to electrophoresis on a native 6% polyacrylamide gel. A competition experiment was performed using unlabeled blunt-ended competitor oligonuleotides added to the binding reaction mixtures, prior to the addition of radiolabeled oligonucleotide probes. The gels will be dried, and radioactivity will be detected using a FLA3000 scanner (Fuji Photo Film, Tokyo, Japan).

F. In vivo measurement of TXB2 production from sera of AIAU and NC

TXA2 is rapidly hydrolyzed non-enymatically to form TXB2, which is then quickly metabolized to urinary metabolites for clearance of kidneys. Because of the transient nature

of this compound, measurement of TXB2 metabolites in plasma may give more accurate estimates of in vivo TXA2 production. Therefore, sera of AIAU, AICU and NC were collected, diluted and measured TXA2 production using Thromboxane B2 Express EIA kit-Monoclonal (Cayman, Ann Arbor, MI). Further readings were measured using a multi-detection microplate reader (Bio tek, Instruments, Inc, Synergy™ HT, Vermont). Data were analyzed according to the procedure and methods as supplied by the kit.

III. RESULTS

A. Clinical characteristics of the *TBXA2R* study subjects in AIAU

The atopy rate and log serum total IgE were significantly higher in AIAU than it was in NC (P<0.001) however, those association were not found compared to AICU (P>0.05). AIAU patients did not show any significant difference in anti-thyroglobulin antibody, antimicrosomal antibody and anti nuclear antibody compared to AICU and NC (Table 7).

B. Genotype and allele frequencies of the *TBXA2R* gene with the AIAU phenotype

We screened two SNPs according to genetic polymorphisms (-4684T>C and 795T>C) and selected TBXA2R -4684 SNP for further study. Gene map and SNPs information have been shown in figure (Fig 9). The genotype frequency of TBXA2R -4684T>C was significantly different AIAU and NC on both co-dominant and recessive analysis models (Table 8). Specifically, AIAU patients showed a significantly higher frequency of the homozygous TT genotype of TBXA2R -4684T>C compared to NC (p=0.015). Similarly, AIAU patients carrying CT and CC genotype exhibited significantly lower frequency compared to NC (p=0.005). In addition, there was significant association of atopy status according to TBXA2R -4684T>C polymorphism. AIAU patients carrying TT genotype showed significantly higher atopy status compared to CT or CC type (p=0.013) but not in AICU patients (P>0.05) (Fig. 10).

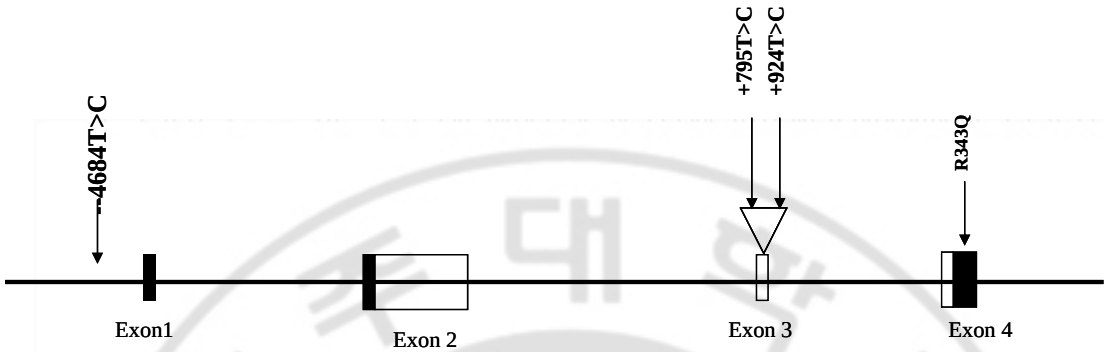

Fig. 9. Schematic representation of *TBXA2R* gene. Gene map and SNPs of *TBXA2R* gene.

				p value		
	AIAU (n=167)	AICU (n=316)	NC (n=265)	AIAU vs AICU	AIAU vs NC	AICU vs NC
Sex (Male/total)	77/167 (46.1%)	68/149 (45.6%)	122/265(46%)	1.000	1.000	1.000
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**
Anti-thyroglobulin antibody (positive/total)	NA	12/84 (14.6%)	NA	1.000	NA	NA
Antimicrosomal antibody (positive/total)	1/5 (20%)	8/82 (9.8%)	NA	0.429	NA	NA
Anti nuclear antibody (positive/total)	1.875±0.341/16	1.841±0.367/88	NA	1.000	NA	NA

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

Table 8.Allele and genotype frequency of TBXA2R gene polymorphism

		AICU (N=149)	AIAU (N=167)	NC (N=265)	AICU vs AIAU	AICU vs NC	AIAU vs NC
TBXA2R -4684T>C	TT	48 (32.2%)	63 (37.7%)	66 (24.9%)	NS	NS	**0.015**
	CT	76 (51%)	71 (42.5%)	143 (54%)	NS	NS	NS
	CC	25 (16.8%)	33 (19.8%)	56 (21.1%)	NS	NS	**0.005**
	q	0.423	0.410	0.481	NS	NS	**0.042**
TBXA2R 795T>C	TT	49 (32.9%)	63 (37.7%)	96 (36.2%)	NS	NS	NS
	CT	77 (51.7%)	74 (44.3%)	116 (43.8%)	NS	NS	NS
	CC	23 (15.4%)	30 (18%)	53 (20%)	NS	NS	NS
	q	0.413	0.401	0.419	NS	NS	NS

q minor allele frequency

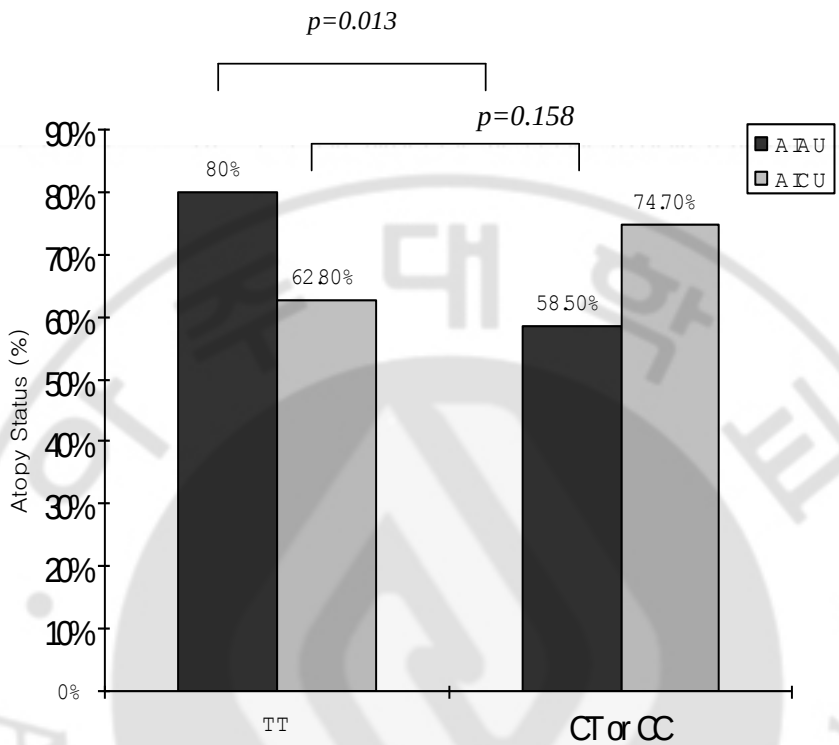

Fig. 10. Comparison of atopy status in AIAU, AICU and ATCU patients according to genotype of *TBXA2R* -4684T>C

C. Dual luciferase activity of *TBXA2R* -4684T>C polymorphism

Whether *TBXA2R* -4684T>C polymorphism is associated with altered promoter activity, a construct composed of the *TBXA2R* sequence and a luciferase reporter gene were transfected into the HMC1 cell line. Reporter activities were compared between constructs containing either *TBXA2R* -4684T or *TBXA2R* -4684C in the gene. Luciferase activity was enhanced significantly in the construct containing *TBXA2R* -4684C when compared to the -4684C containing construct ($p<0.001$, fig 11). Further transfection experiments were performed in the A549 and U937 cell lines; significantly higher activity was also noted in the *TBXA2R* -4684C construct compared to the *TBXA2R* -4684 T construct ($p=0.025$, $p<0.001$), figs 11b, 11c respectively). Transcription factor data base was conducted in search of suitable transcription factor using the consite programme. The co-transfection experiment with ELK-1 containing the *TBXA2R* -4684 T and -4684C alleles demonstrated that the *TBXA2R* -4684 T allele ($p=0.012$) and -4684C ($p=0.041$) produced significantly decrease in induction of luciferase activity (Fig 12). *TBXA2R* -4684T allele showed higher tendency to decrease promoter activity after being transfected with ELK-1 however, no significant difference in the fold induction luciferase activity was observed (Fig. 13).

D. *TBXA2R* -4684T>C gene polymorphisms on transcriptional activity

We performed EMSA using nuclear extract from HMC-1 cells with double-stranded oligonucleotide probes corresponding to -4684T>C. We observed that -4684T produced a specific band having higher affinity that that produced by -4684C. The band was disappeared

in the presence of the nonlabeled -4684T probe as a competitor but not in case of -4684C probe (Fig. 14). But in the presence of SP-1 and NF-kB, the band was not disappeared or did not go on competition assay.

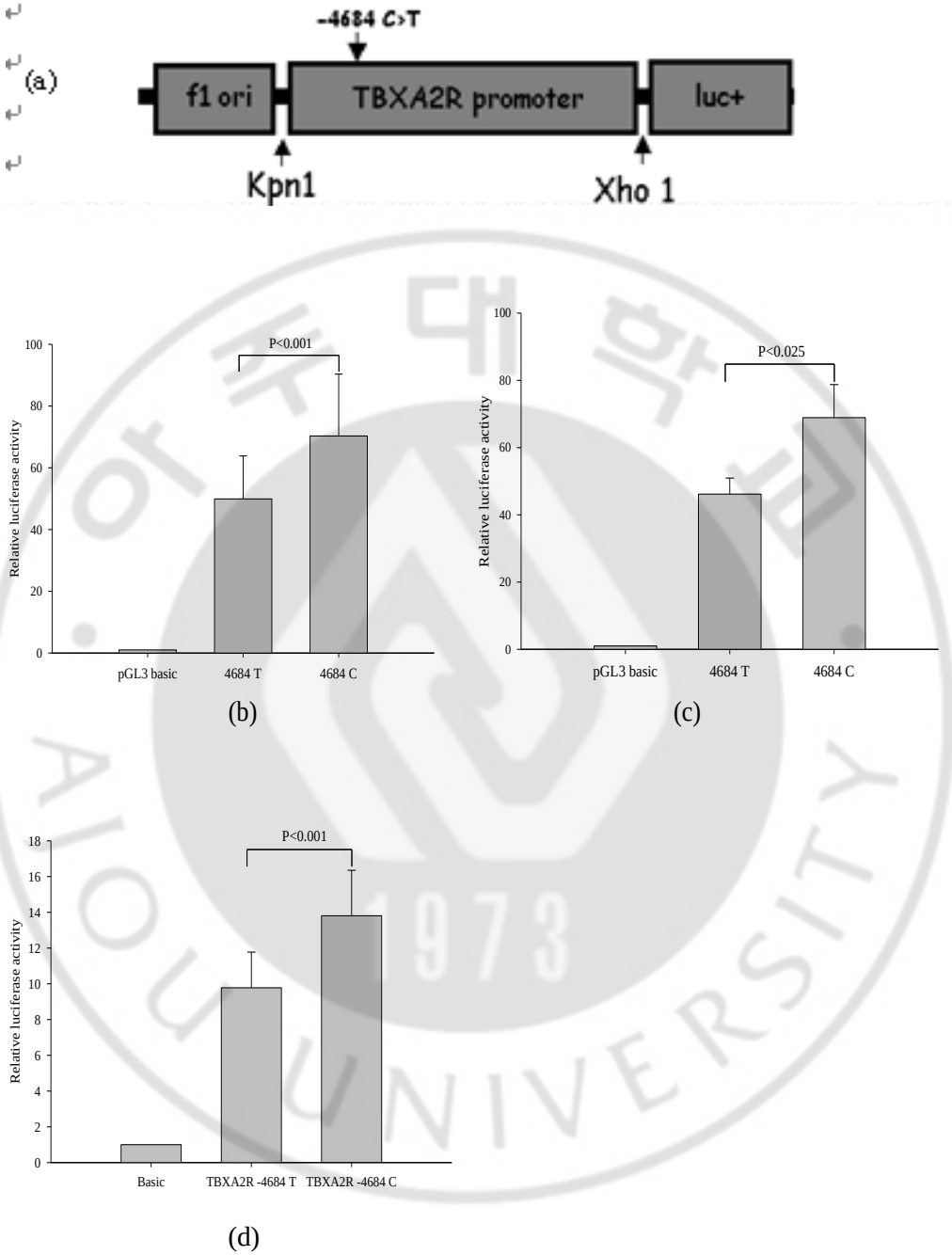

Fig.11. Effects of the *TBXA2R* -4684 T>C polymorphism on the transcription activity of human *TBXA2R* gene. (a) Schematic representation of reporter TBXA2R (1023 bp) gene construct in promoter region. Data are the mean values of independent experiments±S.D. (b) Transfection of HMC1 cells. Luciferase

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 transected with the empty control vector pGL3 basic. The p value was determined by independent-t test.

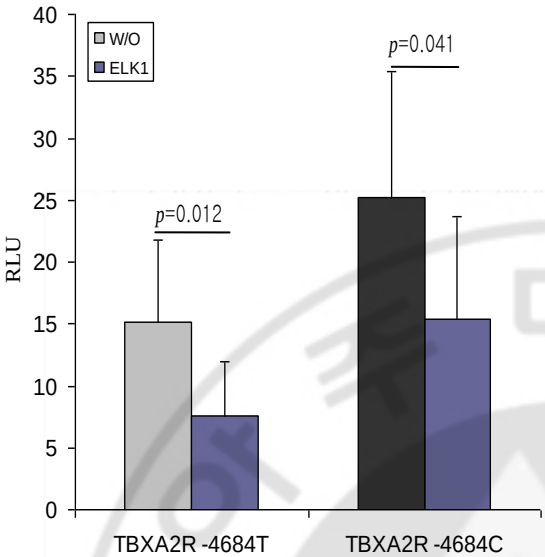

Fig. 12. Effect of ELK-1 Transcription factor on *TBXA2R* 4684T>C promoter activity. Cotransfection of ELK-1 with TBXA2R -4684T>C was performed at dose of 50 ng. Relative luciferase production by promoter less control vector, pGL3 basic. Each experiment was conducted in triplicate for each sample. P value was determined by independent- t test.

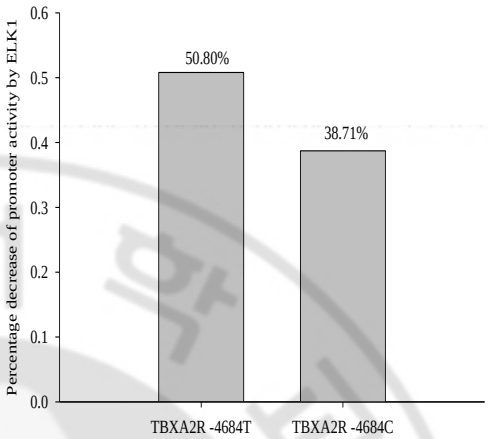

Fig. 13. Percentge decrease of promoter activity after co transfection with ELK1.

-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-kB: AGTTGAGGGGACTTTCCCAGG

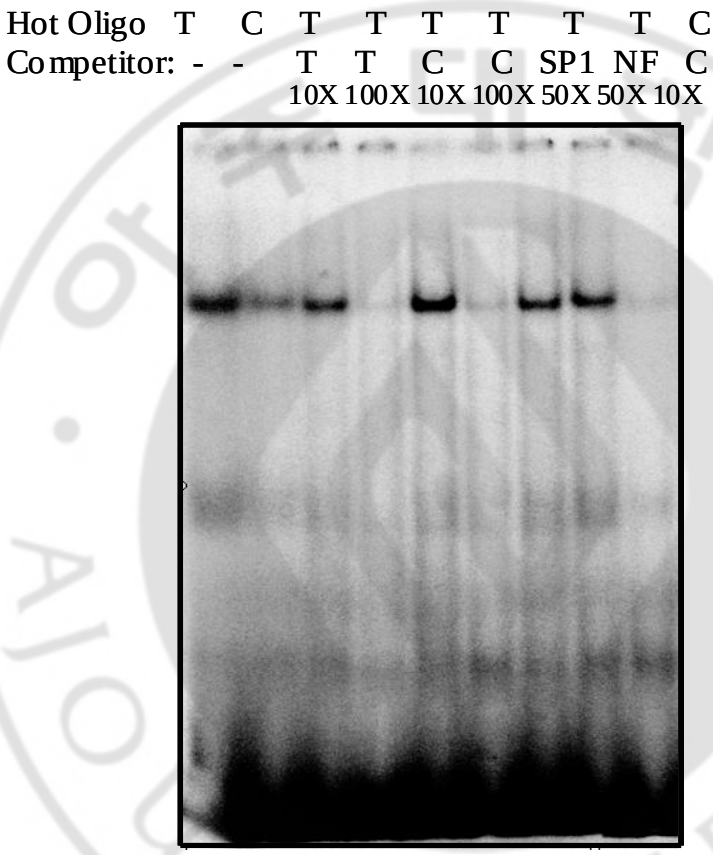

Fig. 14. Results of an electrophoretic mobility shift assay for the *TBXA2R* -4684T>C polymorphism using nuclear extracts obtained from HMC1 cells. (a) Nucleotide sequences of oligonucleotides used as probes and competitors (-4684T, -4684C, SP-1, NF-kB). (b) Differential binding of a nuclear protein to -4684 T/C alleles in HMC1cells.

E. TXB2 production according to *TBXA2R* -4684T>C gene polymorphism

There was a significant difference between AIAU vs. NC with respect to serum TXB2 production, according to *TBXA2R* gene polymorphisms. AIAU patients showed a significantly lower TXB2 production compared to NC (p=0.016). (Fig 15).

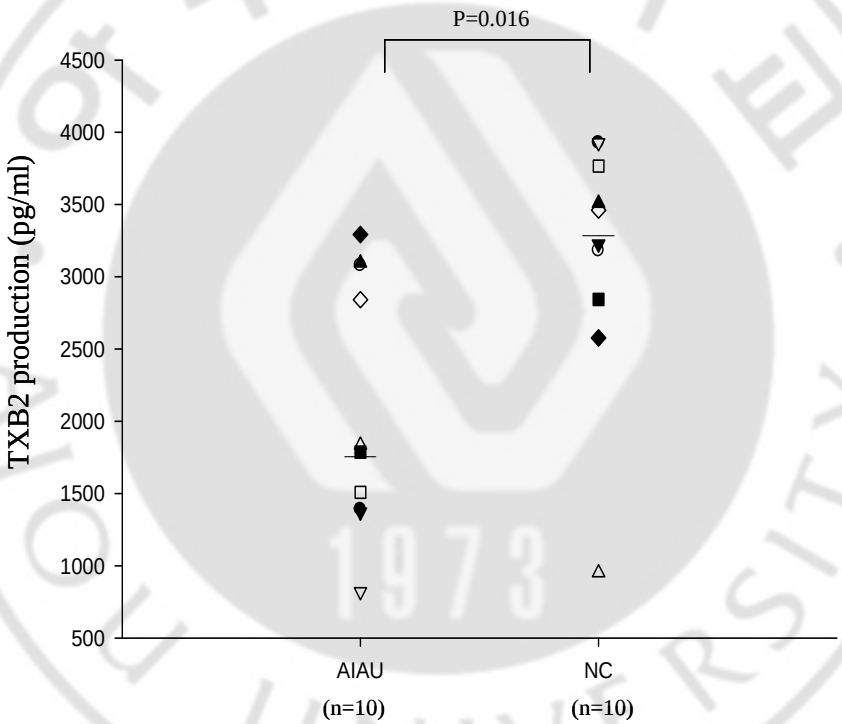

Fig. 15. Comparison of TXB2 production in the sera of AIAU and NC. P value was calculated from Mann- Whitney test.

IV. DISCUSSION

This is the first study to demonstrate a significant contribution of the *TBXA2R* polymorphisms in the pathogenic mechanisms of AIAU patients. Abnormalities in eicosanoid metabolism are the common thought in the mechanism of aspirin hypersensitivity including the AIAU pathogenesis. The detailed mechanism has not yet been still understood. It is a general thought that aspirin inhibits thromboxane production due to inhibition of the COX pathway. This decrease production of thromboxane could be related with overproduction of leukotriene related mediators such as cysteinyl LTs which are known to be the plausible factor for aspirin hypersensitivity. This study has been designed to determine a regulatory role of *TBXA2R* promoter polymorphism, *TBXA2R* -4684 T>C in the pathogenic mechanisms of AIAU. Although platelets have been indicated as the principal source of TXA2 in asthma (Lupinetti et al; 1989), this compound may also be produced by other relevant cell types, such as mast cells (Macchia et al;1995). Moreover, the involvement of *TBXA2R* -4684TT genotype in mast cell activation can be also speculated as this genotype showed higher tendency in serum total IgE level.

Furthermore, -4684 TT were observed to be the risk genotype of *TBXA2R* -4684T>C promoter polymorphism. In other hand, CC and CT genotypes of *TBXA2R* -4684T>C promoter polymorphism were considered to have a protective role in a Korean population. These results suggest that a genetic polymorphism of *TBXA2R* can increase vascular tone with exposure to ASA and increase susceptibility to develop AIAU. The key mechanism for increasing vascular tone can be explained by overproduction of LTs, which are known to be the result of suppression of protective effect of PGE2 and elevation of LTC4 synthase

expression. This is also supported by the studies showing an improvement in AIU after treatment with leukotriene receptor antagonists (Asero R;2000, Perez C;2001).

Besides genetic association result, we provided supporting evidences from *in vitro* and *in vivo* functional studies. In this study, *TBXA2R -4684T* showed decreased promoter activity, which may have led to decreased expression of TBXA2R on inflammatory cells. The previous study reported that down regulation of thromboxane receptor mRNA and protein expression via activation of MAPK, ERK1/2, p38/NF-kB pathway in vascular smooth muscle cells (Zhang et al., 2009). Activation of MAPK has been thought with several allergic and inflammatory diseases including urticaria.

In addition, EMSA showed -4684T allele specific band which underwent on self competition assay of T allele but not in -4684C allele indicating differential transcriptional binding between two alleles of *TBXA2R* gene polymorphism indicating that -4684T allele produced specific band more pronouncedly compared with -4684C allele. In this study, the role of ELK1 was found to have a suppressor action in both alleles, but it tended to be more significant in the -4684T allele indicating that ELK1 may have a more suppressor action in T allele than C allele as suggested in the previous study (Sharrocks, 2002).

TXA2 production was measured by TXB2 in sera from the patients between AIAU and NCs. They showed lower production of TXB2 in the AIAU group compared with NC groups. This is due to inhibition of TXB2 production by ASA and may be associated with attenuation of vasoconstriction effect of thromboxane, likely to enhance vascular tone and capillary leakage. The other explanation for decrease in TXB2 production may be associated with increased LTs production. Aspirin treatment can lead to increase biosynthesis of cysteinyl LTs under

conditions such as asthma, with ongoing formation of leukotrienes and thromboxane. Ongoing thromboxane production in the AIAU patients may constitute an intrinsic negative feedback loop, limiting the overproduction of Cys-LT under basal conditions. When thromboxane formation is prevented by aspirin, LTC4 synthase activity is elevated resulting to increased production of cys-LT and elevation of AIAU. This concept is parallel with the previous study that they also showed suppressed levels of urinary thromboxane metabolites (Christie et al;1991, Knapp et al;1992). Therefore, COX inhibition by ASA leads to attenuated thromboxane biosynthesis which then causes overproduction of LTs via uncoupling of LTC4 synthase which is downregulated by TBXA2R (Tornhamre et al., 2002).These findings suggest that TBXA2 and *TBXA2R* polymorphisms may be related to leukotriene-dependent phenotypes of AIAU, similarly as described previously in AIA (Kim et al., 2005) and asthma with exercise induced bronchoconstriction (Kim et al., 2008). Precise effects of *TBXA2R* polymorphism on leukotriene or LTC4 synthase gene polymorphism are not known clearly therefore, *TBXA2R* gene polymorphism may provide better results about genetic variability in an association with TXB2 production and leukotrienes overproduction if further studies are conducted in different ethnic group and population. Further studies would be helpful to investigate how this association is present in the AIAU phenotype, but absent in the AICU phenotype.

These findings suggest that the -4684T>C polymorphism of the *TBXA2R* gene is associated with the phenotype of AIAU. Specifically, the *TBXA2R* -4684T allele showed higher frequency, lower expression and production of TXB2 which could contributed to the development of AIAU.

V. CONCLUSION

This thesis deals with genetic association and functional study of prostanglandin and prostanoid gene polymorphism in aspirin hypersensitivity.

We demonstrated AIA patients exhibited higher prevalence of paranasal sinusitis, nasal polyps and serum eotaxin-2 level. *CRTH2* -466T allele had lower luciferase activity and lower mRNA expression with higher production of eotaxin-2 in human lung epithelial cells and may be associated with AIA in the Korean population.

Furthermore, AIAU patients showed a significantly higher frequency of the homozygous TT genotype of *TBXA2R* -4684T>C compared to NC. AIAU patients carrying TT genotype showed significantly higher atopy status compared to CT or CC type but not in AICU patients. In vitro functional study demonstrated that the -4684T allele had lower luciferase activity in HMC-1 cells. AIAU patients showed a significantly lower TXB2 production compared to NC and likely to contribute for development of AIAU.

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-국문요약-

아스피린 천식 과민성 질환에서의 프로스타노이드 수용체인 *CRTH2*와 *TBXA2R*의 유전자 다형성 연구

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연구배경 및 목적: 아스피린 복용은 일부 사람들의 경우 아스피린 과민성 천식, 아스피린 과민성 두드러기, 만성 비염, 아나필락시스 같은 다양한 알레르기 반응을 유발하기도 한다. 본 연구는 아스피린 과민성으로 나타나는 대표적인 두 질환인 과민성 천식과 아스피린 과민성 두드러기에서 프로스타글란딘 D2 수용체, CRTH2,와 프로스타노이드 수용체, TBXA2R 유전자들에 대한 유전체 연구이다. 아스피린 과민성 천식 환자에서 호산구 염증 및 유입이 아스피린 내성 천식 환자에 비해 두드러지게 나타남을 고려해 볼때, 프로스타글란딘 D2 수용체인 CRTH2 유전자의 유전자 다형성에 따른 기능적 변화를 통한 호산구의 활성화가 아스피린 과민성 천식의 병인기전에 중요한 역할을 담당할 것으로 가정하고 CRTH2 유전자의 유전자 다형성 조사 및 아스피린 과민성 천식과의 연관성 연구를 수행하였다. 더욱이, 천식 및 아토피와의 연관성이 보고된 TBXA2R 유전자의 경우, 그 유전자의 유전자 다형성에

따른 기능 변화가 아스피린 과민성 두드러기의 병인기전과 관련되는지를 유전자 연관성 연구로 조사하였다.

재료 및 방법: 본 연구는 아주대 병원에 내원한 107명의 아스피린 과민성 천식 환자, 115명의 아스피린 내성 천식 환자, 그리고 133명의 정상대조군을 대상으로 CRTH2 유전자의 유전자 다형성 조사 및 연관성 연구를 수행하였다. TBXA2R 유전자의 경우, 아주대 병원에 내원한 167명의 아스피린 과민성 급성 두드러기 환자, 316명의 아스피린 과민성 만성 두드러기 환자, 그리고 265명의 정상대조군을 대상으로 수행하였다. 두 유전자의 유전형은 SNAPshot ddNTP primer extention 키트를 사용하여 결정되었다. 두 유전자의 유전자 다형성에 따른 기능 변환 연구는 luciferase reporter assay와 electrophoretic mobility shift assay로 수행하였다.

결과: 아스피린 과민성 천식 환자군에서 아스피린 내성 천식 환자군에 비해 높은 eotaxin-2 생성이 관찰되었다 (*p=0.034*). 아스피린 과민성 천식 환자군과 아스피린 내성 천식 환자군에서 *CRTH2* -466T>C 유전자 다형성의 대립유전자 빈도의 차이가 통계적으로 유의하게 관찰되었다 ($p<0.05$). 또한, 아스피린 과민성 천식 환자군에서, *CRTH2* -466TT 유전자형을 가진 환자들의 경우 -466CT 또는 -466CC 유전자형을 가진 환자들 보다 혈청 eotaxin-2 생성이 높게 관찰되었다. 인간 폐 상피세포를 이용한 시험관내 기능 연구를 통해 -466T 대립유전자는 낮은 luciferase activity를 보였고(*p<0.001*), 낮은 mRNA 발현을 보이고, 반면 높은 eotaxin-2 생성이

관찰되었다(*p=0.003*). EMSA 실험을 통해, -466T 대립유전자에 -466C 대립유전자보다 더 높은 친화도를 가지고 결합하는 전사인자를 확인하였다.

TBXA2R -4684T>C 유전자 다형성의 대립유전자 빈도의 차이는 아스피린 과민성 급성 두드러기 환자군과 정상 대조군에서 통계적으로 유의하게 관찰되었다 (p= 0.015). 아스피린 과민성 급성 두드러기 환자군에서, TBXA2R -4684T>C 유전자 다형성은 아토피와 연관성(p= 0.013)을 보였지만, 아스피린 과민성 만성 두드러기 환자군에서는 아토피와 연관성을 보이지 않았다. 인간 비반 세포주인 HMC-1을 이용한 시험관내 기능 연구를 통해 TBXA2R -4684T 대립유전자는 낮은 luciferase activity를 보였고, EMSA 실험을 통해 -4684T 대립유전자에 -4684C 대립유전자보다 더 높은 친화도를 가지고 결합하는 전사인자를 확인하였다. 또한, 아스피린 과민성 급성 두드러기 환자들은 정상 대조군에 비해 낮은 thromboxane B2 생성을 보였다 (p=0.016).

결론: 본 연구를 통해, 프로스타글란딘 D2 수용체, CRTH2,와 프로스타노이드 수용체, TBXA2R 유전자들의 유전자 다형성들이 아스피린 과민성과 연관성이 있으며, 특히 CRTH2 유전자는 아스피린 과민성 천식과 TBXA2R 유전자는 아스피린 과민성 급성 두드러기와 연관성이 있음을 확인하였다.

핵심어: 아스피린 과민성, 천식, 급성 두드러기, 유전자 다형성, 호산구