Significant Association of ADORA3 Genetic Polymorphisms with Aspirin-Intolerant Urticaria in a Korean Population

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Major in Molecular Medicine

Department of Biomedical Sciences

The Graduate School, Ajou University

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입학한 것이 엊그제 같은데 벌써 졸업을 앞두고 있습니다. 그 동안 실험실이라는 울타리 안에서 제 꿈과 목표를 향해 열심히 매진해 왔고, 또 그 안에서 추억도 많이 만들 수 있었습니다. 지금의 남은주, 그리고 또 앞으로의 남은주가 있도록 도와주신 분들이 참 많습니다. 이 글을 읽고 계신 교수님들, 그리고 여러 선생님들과 친구들, 가족 모두들에게 감사의 마음을 전하고자 2 년여 동안의 결실인 작지만 소중한 제 석사논문을 드립니다.

먼저 좋은 주제로 흥미 있는 연구를 할 수 있게 해주시고 지도해주신 박해심 교수님께 진심으로 감사의 말씀을 드립니다. 그리고 심사위원이신 최용준 교수님과 박상면 교수님께도 감사 드립니다. 또한 실험적으로 저에게 많은 가르침을 주셨던 김승현 선생님께도 감사의 말씀 드립니다. 지금까지 배운 것들을 토대로 더욱 발전하는 남은주가 되겠습니다.

제가 이 연구분야에 관심을 갖고 전공을 하게 도와주신 김귀진 선배님과 오정미 선생님, 따뜻한 가르침에 감사 드립니다. 그리고 잘 모르던 제가 이렇게 하나의 논문을 완성할 수 있었던 것은 실험실 선생님들의 가르침이 있었기 때문입니다. 김승현 선생님, 양은미 선생님, 강영미 선생님, 김설화 선생님, 최윤숙 선생님, 서근정 선생님께 실험을 많이 배웠습니다. 그리고 이현영 선생님과 조보영 선생님께서 가르쳐주신 통계부분도 너무 감사 드립니다.

실험실의 왕고이신 까칠은미 양은미 선생님, 옆에 앉아 진정한 아줌마의 모습을 보여주시는 Nami 선생님, 침착하게 많이 먹는다고 신기해하시는 연변총각 김성호 선생님, 수줍게 웃으시는 이햇님 선생님, 귀여운 사투리로 친절하게 말씀하시는 이유진 선생님, 차근차근 지적해주시는 조보영 선생님, 멀리서도 한번에 알 수 있게 큰 웃음 지으시는 이현영 선생님, 그리고 새롭게 배워가느라 바쁜 혜정이와 진경이, 항상 밝으시고 귀여우신 예비 아줌마 전자영 선생님, 보기랑 다르게 말도 많고 재미있으신 김건영 오빠까지 실험실 멤버들 모두 잊지 못할거에요-♥

식품과학을 전공하였던 제가 다른 분야에서 힘들어도 포기하지 않고 연구에 매진할 수 있었던 것은 아마도 학부 지도교수님이셨던 이근택 교수님, “사람은 여러 종류가 있는데 네가 대기만성형이 아니겠나 싶다”라는 말씀을 하셔서 제가 꾸준히 노력할 수 있었던 것 같습니다. 매년 Home Coming Day 로 선후배의 만남을 갖게 하시고 조언을 아끼지 않으시는 이근택 교수님 진심으로 감사 드립니다. 또한 식품포장학 실험실 식구들, 특히 힘들 때 고민 들어주시고 격려 해주신 준재 오빠와 의민 오빠에게 고맙다는 말 전하고 싶습니다.

대학생활 “예끔만아” 동아리 동기로 만난 순기와 무용이, 너희들과 만든 대학시절 추억을 생각하며 힘든 시간을 견딜 수 있었어. 지금은 흩어져 장교로서 나라에 이바지하는 너희들이 자랑스럽다. 이라크까지 다녀온 순기, 이제 왔으니 자주 만나야지~ 나름 다소곳이 종이 접었었는데..ㅋㅋ 그리고 식품과 진국멤버 미옥이와 진희! 항상 변함없는 너희를 만나고 나면 그 동안 쌓였던 스트레스가 한방에 풀려~ 역시 진국들..ㅋ

집에 자주 가진 못하지만 갈 때마다 꼭 술 한잔씩 하며 사는 얘기 들려주는 우리 패밀리 영미, 영희, 창재 너희가 있어 힘들어도 웃음 지을 수 있다. 올해에는 꼭 여행가자궁!~

제게 많은 도움을 준 또 한 사람이 있습니다. 실험적인 부분 말고도 여러 부분에서 생각을 공유할 수 있었고, 또한 많이 배울 수 있었습니다. 이제는 제가 존경하고 싶은 사람이 되었습니다. 평생을 함께 공부하고 취미를 공유하며 살 수 있게 된 discussion mate 정현민..씨? 힘든 일들을 함께 겪어나가는 과정에서 우리 사이의 믿음과 신뢰가 쌓여갈 수 있었고 확신할 수 있었습니다. 평소에는 오빠가 하는 말에 장난만 치고 내 고집만 피우는 것 같겠지만 제 인생의 큰 변환점을 만들어 준 정현민씨, 너무 고맙고 존경합니다♥

마지막으로 무엇보다도 저에게 큰 힘이 되어준 사랑하는 우리 가족이 있었습니다, 타지에서 힘들어도 날 항상 믿어주고 걱정해주었던 엄마 아빠, 최고가 아니더라도 최선을 다하는 법을 가르쳐주셨습니다. 언제나 내가 잘 되라고 기도해주는 착한 언니, 내색은 안 해도 항상 고마워하고 있어. 아마 내가 지금까지 잘 해 왔던 건 언니의 기도 때문이었는지도 몰라. 또 내가 세상 사는 법을 모른다며 한숨 쉬는 귀여운 동생 희수. 내가 너무 잘 웃어서 나랑 얘기하면 개그 욕심이 난다구..ㅋㅋ 나이가 들어도 나한테는 너의 재롱이 귀엽기만 할꺼야. 엄마, 아빠, 언니, 희수.. 사랑합니다.

저 남은주는 앞으로도 계속 노력하는 사람이 되겠습니다. 화려하지 않아도 오랫동안 빛나는 사회의 빛이 되겠습니다. 앞으로도 지켜봐 주시고 격려해 주세요. 감사합니다.

- ABSTRACT -

Significant Association of ADORA3 Genetic Polymorphisms with Aspirin-Intolerant Urticaria in a Korean Population.

Introduction Aspirin-intolerant urticaria (AIU) presents with the immediate onset of urticaria after aspirin ingestion persisting for 1 to more than 6 weeks depending upon the severity. Adenosine is a potent and ubiquitous signaling nucleoside that is generated in response to cellular stress and damage and is therefore increased during episodes of tissue hypoxia and inflammation.

Material and Methods To investigate the biological role of adenosine A3 receptor (ADORA3) in the pathogenesis of AIU, we performed an SNP haplotype-based case-control association study in 373 AIU patients including 180 aspirin-intolerant chronic urticaria (AICU), 193 aspirin-intolerant acute urticaria (AIAU), and 178 normal controls (NC) based on the Korean population. The functional effects of genetic polymorphisms in ADORA3 were analyzed by luciferase reporter assay, electrophoretic mobility shift assay (EMSA), and real-time PCR for the mRNA expression in PBMC of AIU patients.

Results The haplotype [$T_{-1050}C_{-564}$] frequency of ADORA3 gene showed significantly higher in AIAU group than in AICU and NC groups ($p = 0.047$, for AIAU vs. AICU; $p = 0.005$, for

AIAU vs. NC). The transcriptional activity of -564 C allele was higher than T allele, and EMSA finding showed that NF-κB bound more tightly to the C allele sequence than to the T allele sequence by EMSA. Moreover, in PBMC obtained from AIU patients, mRNA expression of ADORA3 in patients with haplotype [$T_{-1050}C_{-564}$] showed significantly higher than those without it in dominant model ($p = 0.033$).

Conclusion These results indicate that the haplotype [$T_{-1050}C_{-564}$] of ADORA3 could affect the clinical presentation of AIAU.

Keywords: ADORA3, adenosine, adenosine A3 receptor, aspirin-hypersensitivity, urticaria, Genetic polymorphism, SNP

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ABBREVIATION

SNP : single nucleotide polymorphism

AIU : aspirin-intolerant urticaria

AIA : aspirin- intolerant asthma

AIAU : aspirin-intolerant acute urticaria

AICU : aspirin-intolerant chronic urticaria

NC : normal control

ADA : adenosine deaminase

ADORA1 : adenosine A1 receptor

ADORA2a : adenosine A2a receptor

ADORA2b : adenosine A2b receptor

ADORA3 : adenosine A3 receptor

MPO : myeloperoxidase

MAF: minor allele frequency

OCT-1: octamer-binding transcription factor-1

Ⅰ. INTRODUCTION

Aspirin, acetylsalicylic acid (ASA), is a salicylate drug, is one of the most widely used medications in the world. The aspirin and other nonsteroidal anti-inflammatory drugs (NSAIDs) can induce several allergic reactions such as aspirin-intolerant asthma, urticaria/angioedema, or anaphylaxis. The prevalence of aspirin hypersensitivity in a general population have been reported to range from 10 % to 20 % of all asthmatic individuals and 20 % to 30 % of chronic urticaria patients (Grattan 2003; Lewis *et al.* 1983; Stevenson 2004). Urticaria is usually classified as acute or chronic depending on whether wheals occur repeatedly for less or more than 6 weeks, respectively. The ingestion of aspirin can induce two types of aspirin-induced urticaria (AIU); aspirin-intolerant acute urticaria (AIAU) and aspirin-intolerant chronic urticaria (AICU) (Kozel and Sabroe 2004; Palikhe *et al.* 2008). However, the knowledge about the molecular genetic mechanisms of AIU is still limited.

Adenosine is a potent and ubiquitous signaling nucleoside that is generated in response to cellular stress, therefore increased during episodes of tissue hypoxia and inflammation. The biological mechanism of adenosine is well known. It is degraded by adenosine deaminase (ADA) to inosine or recycled to AMP by adenosine kinase. The actions of adenosine are mediated by four adenosine receptor subtypes belonging to G protein-coupled receptors including adenosine A1 receptor (ADORA1), adenosine A2a receptor (ADORA2a), adenosine A2b receptor (ADORA2b), adenosine A3 receptor (ADORA3). ADORA1 and ADORA3 couple to G_i proteins that inhibit adenylyl cyclase while activating multiple other signaling pathways via the release of βγ subunits, whereas ADORA2a and ADORA2b

couple to G_s proteins that activate adenylyl cyclase resulting in formation of cAMP (Linden 2001).

Aspirin promotes synthesis of anti-inflammatory eicosanoids as 15-epi-lipoxin A4 and breakdown of intracellular ATP, thereby releasing micromolar amounts of the potent adenosine into extracellular fluids (Cronstein *et al.* 1994). While aspirin and NSAIDs reduce inflammation through inhibition of COX and prostaglandin synthesis, they also produce anti-inflammatory effects that are mediated through adenosine metabolism and adenosine-receptor interactions (Cronstein *et al.* 1999a; b).

A previous study investigated genetic polymorphisms of adenosine-related genes including ADA, ADORA1, ADORA2a, ADORA2b, and ADORA3 in patients with aspirin-intolerant asthma (AIA) (Kim *et al.* 2008). There were significant differences between normal and patients with AIA in the ADORA1 SNP. In this study, we analyzed the genotypes and haplotypes of adenosine-related genes in patients including AIAU and AICU. Therefore, we determined genetic polymorphisms of ADORA3 gene and their association with the AIU through a case-control study of three groups classified as AIAU compared to AICU and normal healthy control (NC) in a Korean population. Furthermore, the functional effect of the ADORA3 gene was investigated using luciferase reporter assay, electrophoretic mobility shift assay (EMSA), mRNA expression of peripheral blood mononuclear cell (PBMC) of AIU patients, and neutrophil chemotaxis assay to clarify its function of the promoter polymorphisms.

Ⅱ. MATERIALS AND METHODS

A. Genetic association study of ADORA3

1. Subjects and phenotyping

Subjects enrolled from Ajou University Hospital in Suwon, Korea, were divided into 3 groups including180 aspirin-intolerant chronic urticaria (AICU), 193 aspirin-intolerant acute urticaria (AIAU) and 178 normal controls (NC). The baseline characteristics of the study subjects are presented in Table 1. There were no significant differences in mean age or sex distribution between the AIAU and AICU groups; however, the prevalence of atopy and concentration of serum total IgE were significantly higher in the patients with AIAU and AICU than NC groups ($p < 0.001$). And the serum level of myeloperoxidase (MPO) was significantly higher in AIAU (2.15 ± 0.64 ng/ml) than AICU (1.78 ± 0.42 ng/ml).

Table 1. Clinical characteristics of the study subjects

	AICU (n=180)	AIAU (n=193)	NC (n=178)	*p* -value		
				AICU vs AIAU	AICU vs NC	AIAU vs NC
Sex (male/total)	81/180 (45.0%)	95/193 (49.2%)	83/178 (46.6%)	0.468	0.832	0.678
Age (years) [a]	35.83±11.93	35.92±13.38	38.40±14.07	0.946	0.067	0.090
Atopy (presence/total)	92/137 (67.2%)	78/116 (67.2%)	8/87 (9.2%)	1.000	<0.001 [b]	<0.001 [b]
Serum total IgE (IU/ml) [a]	258.63±263.12	293.77±399.99	72.51±189.27	0.478	<0.001 [b]	<0.001 [b]
Log MPO level (ng/ml) [a]	1.78±0.42	2.15±0.64	1.92±0.08	0.002 [b]	0.558	0.049 [b]
IL-8 (pg/ml) [a]	45.47±139.71	77.04±261.70	47.44±35.16	0.300	0.937	0.528
IL-18 (pg/ml) [a]	193.29±153.25	214.93±152.97	259.67±281.78	0.460	0.376	0.577

[a] This value was presented as means ± SD. MPO, Myeloperoxidase; AIAU, aspirin-intolerant acute urticaria; AICU, aspirin-intolerant chronic urticaria; NC, normal healthy controls. [b] Values indicate significant *p* value. Each *p* value of 0.05 or less was considered to be significant.

2. Genotyping in ADORA3

Each single nucleotide polymorphism (SNP) was genotyped by a primer extension method, which was performed with the SnaPshot ddNTP primer extension kit (Applied Biosystems, CA, USA). Table 2 shows the amplifying and extension primers of adenosine-related genes

including ADA, ADORA1, ADORA2a, ADORA2b, and ADORA3 for SNP genotyping. The genomic DNA region scanning the polymorphic site was amplified using one phosphothiolated primer and one regular PCR primer. We used a computer program that is used to select and evaluate tagging SNPs based on the empirical patterns of linkage disequilibrium (LD). For this analysis, we used pair-wise tagging to choose SNPs that were correlated at r^2 equal to 0.80 or greater with all other SNPs in a LD block.

3. Statistical analysis

Differences in genotype frequency between patients and controls were analyzed using a chi-square test and by calculating the odd ratio (OR) with a 95 % confidence interval (CI). Haplotypes of ADORA3 gene were analyzed using Haploview version 2.0 based on the EM algorithm (Barrett *et al.* 2005).

Linkage disequilibrium among loci was measured using the absolute value of Lewontin's *D'* correlated at r^2 equal to 0.80 or greater with all other SNPs in a LD block (Hedrick 1987). Differences of clinical characteristics among groups were examined using Student's *t*-test (for continuous variables) or a chi-square test (for categorical variables) by Pearson Chi-Square or Linear-by-Linear method. All statistical analysis were completed using SPSS version 12.0.1 (SPSS Inc., Chicago, IL, USA) and a p-value < 0.05 was considered to be significant.

Table 2. Amplifying and extension primers of the adenosine-related genes for SNP genotyping.

ADA	-1591A>C	Left	TGTAAAACGACGGCCAGTCCCCTTGTTTATGGGTTT
		Right	CAGGAAACAGCTATGACCGCGTTTCAAGCGATTTT
		Extenstion	GTCAGGTGTTCGAGACCAGCCTGGC C/A AACATGGTGAAACCCCGTCTCTACT
	-1491T>C	Left	TGTAAAACGACGGCCAGTCCCCTTGTTTATGGGTTT
		Right	CAGGAAACAGCTATGACCGCGTTTCAAGCGATTTT
		Extenstion	CTACTCGGGACGCTGAGGCCGAGAA C/T TGCTTAAAATCCAGGAGGTGGAGGT
	-1130T>A	Left	TGTAAAACGACGGCCAGTTGTGGGCGCCTATAATC
		Right	CAGGAAACAGCTATGACCTGCAGAATGTTCACACCA
		Extenstion	ATAAATAAATAAATAAATAAATAAA T/A AACCTGTACCCGCGTGTTATTTCCC
	V178V	Left	TGTAAAACGACGGCCAGTGACAGCAGGCAGGTAGG
		Right	CAGGAAACAGCTATGACCCAGAACTCAGGAGACACCA
		Extenstion	GAAGTACCAGCAG CAGACCGTGGT A/G GCCATTGACCTGGCTGGAGATGAGA
ADORA1	-39494C>T	Left	CAGGAAACAGCTATGACCGAATGGAGGAGGGAAAAC
		Right	TGTAAAACGACGGCCAGTCAGGGTATGGACAGGTCA
		Extenstion	ACTGAGAACCCACTCTGTGTCAGTT C/T CCTGGCTGGGACCTGACCTGGGGCA
	-39128G>A	Left	CAGGAAACAGCTATGACCGACCCTCTTGGTCTGAGC
		Right	TGTAAAACGACGGCCAGTGCAAAATCAGCAGGGACT
		Extenstion	TCTCTCAGAGCCGAGCTGTGGAGAC G/A CCTCATCTAGAGGCTTGGTCCTCCT
	-38242C>T	Left	CAGGAAACAGCTATGACCCATGCAAATCAGCATTGA
		Right	TGTAAAACGACGGCCAGTTCTTTTCATCTCTCCAGCA
		Extenstion	CACTAGTCCACCTTCTTCCTCCACA T/C ATGGGGAAATGGAGGCCCAGAGAAG
	A102A	Left	CAGGAAACAGCTATGACCAGTGGGACACATCACAGG
		Right	TGTAAAACGACGGCCAGTCTGGCTTACTTGGGGTCT
		Extenstion	CCATCCTGGCCCTGCTGGCAATTGC T/G GTGGACCGCTACCTCCGGGTCAAGA
	1278C>A	Left	CAGGAAACAGCTATGACCCCCTCATCCTCTTCCTCT
		Right	TGTAAAACGACGGCCAGTCTCCTCTGCAGTCTCAGC
		Extenstion	CCTGACCATCCCATGAGCAGTCCAG A/C GCTTCAGGGCTGGGCAGGTCCTGGG
	1405C>T	Left	CAGGAAACAGCTATGACCGAGCAGTCCAGMGCTTCA
		Right	TGTAAAACGACGGCCAGTCGAGTTGGCATGTCTAGTG
		Extenstion	TTGTCTTAGATGTTGGTGGTGCAGC C/T CCAGGACCAAGCTTAAGGAGAGGAG
	1627G>T	Left	CAGGAAACAGCTATGACCGAGCAGTCCAGMGCTTCA
		Right	TGTAAAACGACGGCCAGTCGAGTTGGCATGTCTAGTG
		Extenstion	GCCCTGGGCCAGCCCAGGATTGTAC G/T TGGGAGAGGCAGAAAGGGTAGGTTC
	1794T>-	Left	CAGGAAACAGCTATGACCGAGCAGTCCAGMGCTTCA
		Right	TGTAAAACGACGGCCAGTCGAGTTGGCATGTCTAGTG
		Extenstion	GCCCTGAGCTTTCCGGGGAGGAGCC T/- TGGAGTGTAATTACCTGTCATCTGG
ADORA2a	-1751A>C	Left	TGTAAAACGACGGCCAGTCATGGACAATGGGGTAGA
		Right	CAGGAAACAGCTATGACCGCTGACACCAGGAATGAC
		Extenstion	CCCCAGGCAGGTGGTGGCGGCTGGC A/C ACACACTCATAGGGCCCCATGAGGG
	Y361Y	Left	TGTAAAACGACGGCCAGTGCCCCTACACATCATCAA
		Right	CAGGAAACAGCTATGACCGCTCCCTTCTCTCAGTCC
		Extenstion	ACCCTGAGCGGAGGCCCAATGGCTA T/C GCCCTGGGGCTGGTGAGTGGAGGGA
	2134T>A	Left	TGTAAAACGACGGCCAGTTCACGTTGGGAGAAGAGA
		Right	CAGGAAACAGCTATGACCCTGCACATACGCACACAC
		Extenstion	AATGGCGTCTGAGTTCGTTTCCTAC T/A CCATAGCTAGGCCTGTGCACACATA
ADORA3	-2288A>G	Left	TGTAAAACGACGGCCAGTGAGGCACATGACCAAGAG
		Right	CAGGAAACAGCTATGACCCTAGGAGGCAGCACTCTG
		Extenstion	GCGGTAGGAATGGGCAAATGAAGTG A/G CCTTCTGCCCCAGCCTCTCTGTCCT
	-1369C>T	Left	TGTAAAACGACGGCCAGTCCTATGTACTGGGGTAGGG
		Right	CAGGAAACAGCTATGACCGTGCTTCTCAGCACCATC
		Extenstion	GAGGCGTCTCCCTTATGCCCCACTC T/C GAAGTGTTTGTTAGTAAACACCAGA
	-1144A>T	Left	TGTAAAACGACGGCCAGTGCCATTGTTGTTACTGCTG
		Right	CAGGAAACAGCTATGACCAGTTGACGCTTTGCTGAG
		Extenstion	TGAACCAAGTGGGTCCCCAAATAAC A/T ATGGCGTGCAAGTGTCTGGTTCCCA
	-1050G>T	Left	TGTAAAACGACGGCCAGTGCCATTGTTGTTACTGCTG
		Right	CAGGAAACAGCTATGACCAGTTGACGCTTTGCTGAG
		Extenstion	TTCCCAGACAGTCGCCTGTTCCTGC G/T GGGATGGGGCTGAGGCTTGGGGAAT
	-581A>G	Left	TGTAAAACGACGGCCAGTGGCTAAGCAGGTGTGATG
		Right	CAGGAAACAGCTATGACCTCACAGGTGGGTCACTTC
		Extenstion	GCTAAGCTGGCAGAAAGATTGCATA G/A TCAGTGCTTCCAGCTCTGCTCCCAC
	-564C>T	Left	TGTAAAACGACGGCCAGTGGCTAAGCAGGTGTGATG
		Right	CAGGAAACAGCTATGACCTCACAGGTGGGTCACTTC
		Extenstion	ATTGCATAGTCAGTGCTTCCAGCTC T/C GCTCCCACCTGATCCTGCACTGTCC
	A299A	Left	TGTAAAACGACGGCCAGTTCATCAACTGCATCATCTAC
		Right	CAGGAAACAGCTATGACCTTCAGGCTCCATGACTTA
		Extenstion	AAACCTACCTTTTGATCCTCAAAGC T/C TGTGTGGTCTGCCATCCCTCTGATT
	1146T>C	Left	TGTAAAACGACGGCCAGTTCATCAACTGCATCATCTAC
		Right	CAGGAAACAGCTATGACCTTCAGGCTCCATGACTTA
		Extenstion	CTCTCTTCCTCCACTTCATTTTTCC C/T TTGTCCTTTCTCTCTAATTCAGTGT

ADA, adenosine deaminase; ADORA1, adenosine A1 receptor; ADORA2a, adenosine A2a receptor; ADORA3, adenosine A3 receptor.

B. Functional study for ADORA3 genetic polymorphisms

1. Cell culture

THP-1 (human monocyte leukemia cell line) cells were cultured in RPMI 1640 medium supplemented with 10 % heat-inactivated FBS (Gibco/BRL), 100 U/ml penicillin, 25 mM Hepes, and 100 mg/ml streptomycin in a humidified atmosphere with 5 % carbon dioxide (CO_2) at 37 ℃. HMC-1 (Human mast cell line) cells were provided by Kyung-Hee University (Seoul, Korea). They were cultured and maintained with IMDM (Invitrogen, Carlsbad, CA, USA) medium supplemented with 10 % FBS (Invitrogen), 100 U/ml penicillin, 25 mM HEPES, and 100 mg/ml streptomycin in an incubator at 37 ℃ and 5 % CO_2.

2. Reverse transcription-polymerase chain reaction (RT-PCR) analysis of ADORA3 expression in various cell lines

The reverse transcription reaction was performed at 42 ℃ for 1hr from 2 μg of total RNA extracted with TRI-reagent (Sigma). For amplification of human β-actin we used the primers 5'-TCCTTCTGCATCCTGTCGGC and 5'-CAAGAGATGGCCACGGCTGC. The PCR conditions included heating to 95 ℃ for 5 min, 22 cycles of 95 ℃ for 30s, 56 ℃ for 30s, and 72 ℃ for 30s. Forward primer 5'-ATACTTGCGGGTCAAGCTTAC-3' and reverse primer 5'-AAGGACTTAGCCGTCTTGAAC-3' for ADORA3 amplification were used. The reverse-transcription reaction was performed by heating to 95 ℃ for 5 min, 40 cycles of 95 ℃ for 30s, 56 ℃ for 30s, and 72 ℃ for 30s.

3. Plasmid construction and luciferase reporter assay

(A) Preparation of transgenic constructs

The 1277 bp fragments of the human ADORA3 gene promoter was prepared by PCR amplification of haplotype *ht1* [$T_{-1050}C_{-564}$], *ht2* [$G_{-1050}T_{-564}$], *ht3* [$G_{-1050}C_{-564}$], and *ht4* [$T_{-1050}T_{-564}$] human genomic DNA as a templat using a pair of primers (forward primer, 5'-CAGAACGCCATTGTTGTTACTGC-3'; reverse primer 5'-TAGCTCTCGCCAGACGTCTTC-3'). Each of the PCR products was gel purified with agarose-gel purification kit (iNtRON, Deajeon, Korea), ligased into TOPO vector (Invitrogen). And then this plasmid DNAs cut with *Kpn Ⅰ* and *Nhe Ⅰ* restriction enzymes (Takara, Shuzo, Japan) and ligated into a *Kpn Ⅰ – Nhe Ⅰ* site of the pGL3-Basic luciferase reporter vector (Promega, Madeson, WI, USA) using T4 DNA ligase (Elpis Biotech, Korea). All constructs were verified by direct sequencing. And the plasmid DNAs were prepared from these constructs using the Endo Free Plasmid Maxi kit (Qiagen, Hilden, Germany) and quantities and integrity were analyzed by using UV spectrometry and agarose-gel electrophoresis.

(B) Transient transfection and luciferase activity

Human monocyte leukemia cells, THP-1, and human mast cells, HMC-1, were transfected by using microporator MP-100 (Digital bio tech. Seoul, Korea) and PMK-1096 (Digital bio tech) according to the manufacturer's instruction. Briefly, 24 well plates were filled with 500 μl of culture medium with 10 % FBS without antibiotics, and pre-incubated in a humidified 37 ℃ and 5 % CO_2 incubator. 2 ~ 5 x 10^5 cells of THP-1 or HMC-1, ADORA3 plasmid

DNA 1 μg and Renilla plasmid DNA 5 ng were resuspended in 10 μl Solution R. Microporation tube was filled with 3 ml Solution E. The cell and DNA mixtures pipette and were shoked once by the microporator pipette into the pipette station. Cells were incubated a further 24 hr. Cells were then lysated with 100 μl of 1 X passive lysis buffer (Promega) per well. Twenty microliters were 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 after co-transfection of both ADORA3 construct and of Renilla control vector into the cell line.

4. Effect of a transcription factor, OCT-1, on ADORA3 promoter activity by co-transfection

A 2.2 kb fragment of the CDS of the human OCT-1 gene (NM_002697.2) was prepared by PCR amplification using human cDNA as a template. The forward primer sequence was 5'- CAA**GGTACC**ATGAACAATCCGTCAGAAACC -3' (the boldface characters indicate a *Kpn I* site) and the reverse primer sequence was 5'-CAA**CTCGAG**TCACTGTGCCTTGGAGG-3' (the boldface characters indicate an *Xho I* site). Amplification conditions involved an initial denaturation for 5 min at 95 ℃ followed by 35 cycles of 30 sec at 95 ℃, 30 sec at 58 ℃, and 1 min at 72 ℃ with a final 10 min extension at 72 ℃. Each of the PCR products was separately subcloned into *Kpn I* - *Xho I* in the pcDNA3 vector (Invitrogen).

THP-1 cells were cultured in RPMI 1640 medium supplemented with 10 % heat-inactivated FBS (Gibco/BRL), 100 U/ml penicillin, 25 mM Hepes, and 100 mg/ml streptomycin in a humidified atmosphere with 5 % carbon dioxide (CO_2) at 37 ℃. THP-1 cells were transfected by using microphorator MP-100 (Digital bio tech) and PMK-1096 (Digital bio tech) according to the above described method. Briefly, 5 x 10^5cells of THP-1 was transfected with ADORA3 plasmid DNA 1 μg, OCT-1 50 ng, 300 ng, and Renilla plasmid DNA 5 ng. 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 after co-transfection of both ADORA3 construct and of Renilla control vector into the cell line.

5. Nuclear extracts preparation and electrophoretic mobility shift assay (EMSA)

(A) Preparation of nuclear protein extracts

THP-1 cells were washed with ice-cold PBS and resuspended in ice-cold buffer A [10 mM HEPES (pH 7.9), 10 mM KCl, 0.1 mM EDTA, 1 mM DTT, 1 mM PMSF, 1 mM $NaNO_3$, 1 mM NaF, and complete protease inhibitor Cocktail (Roche, Basel, Switzerland)]. The cells were incubated on ice for 10 min and for an additional 15 min with 0.5 % Nonidet P-40. After centrifuging at 6,000 x g for 1 min, the pellet was resuspended in extract buffer [20 mM HEPES (pH 7.9), 400 mM KCl, 4.5 mM $MgCl_2$, 0.2 mM EDTA, 1 mM DTT, 1 mM PMSF, 1 mM $NaNO_3$, 1 mM NaF and complete protease inhibitor cocktail (Roche)] and incubated on ice for 1 hr and centrifuged 10,000 x g for 10 min. After the addition of 15 % glycerol, the protein concentration was determined using the Bradford's method. Oligonucleotieds and their complementary strands were designed and purchased as gel

purified (Bioneer, Deajeon, Korea).

(B) Electrophoretic Mobility Shift Assays (EMSA)

Complementary oligonucleotides were annealed to each other to generate double-stranded probes by incubation at 95 ℃ for 5 min and successive gradual cooling to 37 ℃. We used the probes as illustrated in figure 4. NF-κB oligonucleotide (5′-AGTTGAGGGGACTTTCCCAGG-3′) was labeled as previously described (Mukherjee *et al.* 2008) . The double-stranded DNA probes were radiolabeled at their 5'-ends with DNA polymerase Ⅰ large (Klenow) fragment (Promega) and [α -^{32}P] dCTP. The reaction mixture (10 μl) containing 3 μg of nuclear extract, 10 mM HEPES (pH 7.9), 500 ng of poly (dI-dC), 1 mM $MgCl_2$, 35 mM NaCl, 1 mM DTT, 1 mM $NaNO_3$, 1 mM NaF and 5 % glycerol were preincubated on ice in the absence of the probe for 20 min and incubated for additional 30 min at ice with the 0.2 pmol of radiolabeled probe. The reaction mixtures were then subjected to electrophoresis with a native 6 % polyacrylamide gel at 130 V for 3 hr in 0.5 X TBE buffer (45 mM Tris-borate, pH 8.0, 45 mM boric acid, 1 mM EDTA). For competition experiments, unlabeled blunt-ended competitor oligonucleotides were added to the binding reaction mixtures before the addition of the radiolabeled oligonucleotide probe. The gels were dried and radio activity was detected using a FLA 7000 (Fuji Photo Film, Tokyo, Japan).

6. Quantitative Real-time RT-PCR in PBMC from AIU patients

Human peripheral blood samples were collected from patients with 20 AIU. The PBMC were isolated from the whole blood (10 ml) using Ficoll-Paque Plus (Amersham Biosciences, Sweden) gradient centrifugation and then washed with PBS twice.

After isolation of PBMC in AIU patients, the total RNA was isolated using the Trizol reagent according to manufacturer's instructions (Nalgene, USA). The reverse transcription reaction was performed at 42 ℃ for 1hr from 2μg of total RNA extracted. The real-time PCR conditions included heating to 95 ℃ for 10 min, and 40 cycle of 95 ℃ for 15s, 58 ℃ for 30s, and 72 ℃ for 33s using the 2 x SYBR Green PCR Master Mix (SYBR Green dye, AmpliTaq Gold, DNA polymerase, dNTP Mix, and optimized buffer components; Applied Biosystems). Forward primer 5'-ATACTTGCGGGTCAAGCTTAC-3' and reverse primer 5'-AAGGACTTAGCCGTCTTGAAC-3' for ADORA3 amplification were used.

7. Trans-well chemotaxis assay

Peripheral blood was obtained from 17 healthy human volunteers. Heparinized blood (10 ml) was mixed with 6 % (wt/vol) Dextran dissolved in 0.9 % NaCl solution and allowed to sedimentate for 1 hr at room temperature. The cells in the supernatant were separated with Ficoll gradient centrifugation. After RBC lysis, the human polymorphonuclear neutrophils (PMN) layer was collected and washed with Hanks' balanced salt solution (HBSS) and suspended at a concentration of 1×10^7 PMN/ml and used for experiments immediately. Assays were performed with Trans-well 24well plate containing a filter plate with a pore size of 3.0 μm (Corning Costar, Corning, NY). A suspension of 1×10^6 cells was added into the upper filter plate. Elastase activity of the lysed cell suspension in the lower well was used as

an indicator of the number of migrated cell as previously described (Chen *et al.* 2006). After stimulation for 1 hr at 37 ℃ with 1 nM N-formyl-methionyl-leucyl-phenylalanine (fMLP) as a chemoattractant, the upper filter plate was removed and the cells in the lower wells were collected. After removal of the remaining supernatants, we mixed cells with 160 μl of a buffer consisting of 50 mM Tris/Cl and 100 mM NaCl, pH 7.4 containing 0.05 % (v/v) Triton X-100. Enzymatic reactions were started by the addition of the elastase-specific chromogenic substrate N-methoxysuccinyl-Ala-Ala-Pro-Val-p-nitroanilide (Sigma) at a final concentration of 1 mM. After overnight reaction at room temperature, the change in optical density was measured at a wavelength of 405 nm. As a control, each set of experiments included wells containing HBSS with 10 % FBS or with the mock (DMSO) instead of fMLP in the lower well.

8. Statistical analysis

Results are expressed as mean ± SEM. The differences among groups were analyzed by Mann-Whitney *U* test and where appropriate, by ANOVA with Bonferroni test. Significance was assumed for p values < 0.05. All data analyses were performed with the SPSS 12.0.1 software package (SPSS Inc., Chicago, IL, USA).

Ⅲ. RESULTS

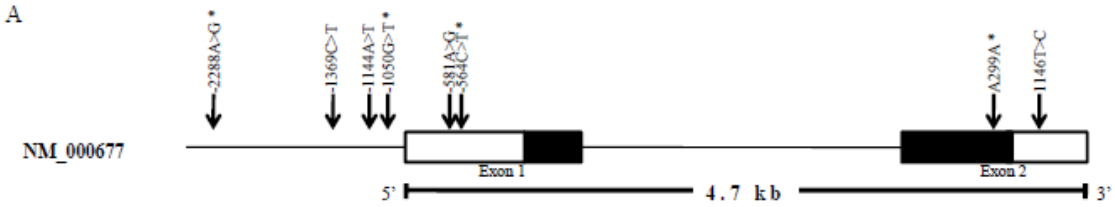
A. Genetic association study of ADORA3

1. Genetic association of ADORA3

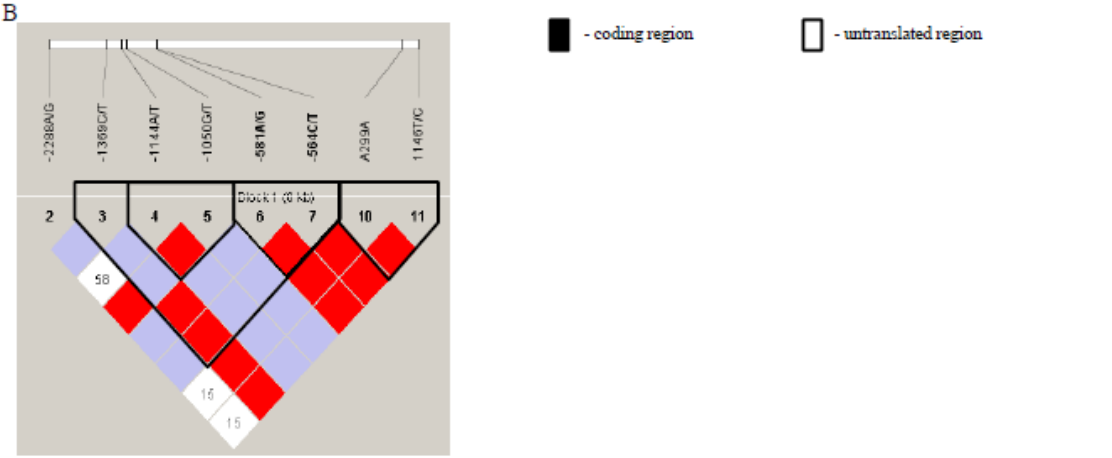
Previous study selected 13 tagging SNPs (ADA, -1130T>A, V178V; ADORA1, -38242C>T, A102A, 1278C>A, 1405C>T, 1627G>T; ADORA2a; -1751A>C, Y361Y; ADORA3; -2288A>G, -1050G>T, -564C>T, A299A) correlated at r^2 equal to 0.80 or greater with all other SNPs in a LD block and investigated whether these tagging SNPs were associated with the case-control groups. Figure 1 shows the gene map and LD block of ADORA3.

Among 13 tagging SNPs, two promoter polymorphisms, -2288A>G and -1050G>T, of ADORA3 gene showed a significant association with AIAU. Table 3 shows that -2288 A allele frequency was significantly lower in AIAU group than NC group (p = 0.013 in dominant analysis model). And -1050 T allele frequency was significantly higher in AIAU group than those with control groups (p = 0.015, for AIAU vs. AICU; p = 0.020, for AIAU vs. NC in recessive analysis model). It also shows the significant results in minor allele frequencies. However, no significant association was found in ADA, ADORA1, ADORA2a and ADORA2b genes (data not shown).

A



B



C

r^2 \ D'	-2288A>G	-1369C>T	-1144A>T	-1050G>T	-581A>G	-564C>T	A299A	1146T>C	
-2288A>G		1 (0.07,0.98)	0.587 (0.07,0.84)	1 (0.47,1)	1 (0.07,0.98)	1 (0.07,0.98)	0.157 (0.03,0.96)	0.157 (0.03,0.96)	
-1369C>T	0.122		1 (0.13,0.99)	1 (0.25,1)	1 (0.85,1)	1 (0.85,1)	1 (0.63,1)	1 (0.63,1)	
-1144A>T	0.187	0.226		1 (0.79,1)	1 (0.13,0.99)	1 (0.13,0.99)	1 (0.07,0.98)	1 (0.07,0.98)	
-1050G>T	0.454	0.270	0.837		1 (0.25,1)	1 (0.25,1)	1 (0.11,0.99)	1 (0.11,0.99)	
-581A>G	0.122	1.000	0.226	0.270		1 (0.85,1)	1 (0.63,1)	1 (0.63,1)	
-564C>T	0.122	1.000	0.226	0.270	1.000		1 (0.63,1)	1 (0.63,1)	
A299A	0.002	0.560	0.127	0.151	0.560	0.560		1 (0.8,1)	
1146T>C	0.002	0.560	0.127	0.151	0.560	0.560	1.000		

r^2 | |D'| (Low,High)

Fig.1. Gene map and haplotypes of ADORA3. A. Gene map of ADORA3 on chromosome 1p13.2 and the location of single-nucleotide polymorphisms (SNPs). The first base of the translation site is denoted as nucleotide 1. Asterisks indicate SNPs selected for large-scale genotype analysis of allele frequencies and locations and their linkage disequilibrium (LD). **B**. LD block of ADORA3. **C**. LD coefficients (*/D/* and r^2) among ADORA3 SNPs.

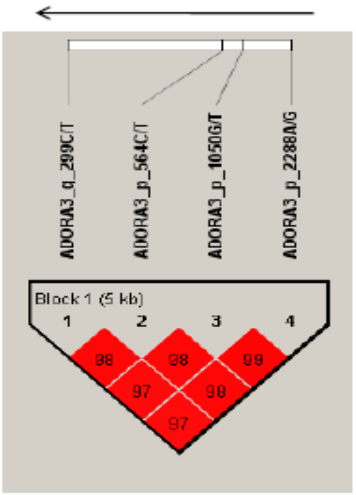
Table 3. The genotype distribution of ADORA3

		AICU (N=180)	**AIAU** (N=193)	**NC** (N=178)	AICU vs AIAU *p* value	AICU vs AIAU OR (95% CI)	AICU vs NC *p* value	AICU vs NC OR (95% CI)	AIAU vs NC *p* value	AIAU vs NC OR (95% CI)
-2288 A > G [a]	AA	77 (42.8%)	72 (37.3%)	85 (47.8%)	0.155		0.192		0.006 [b]	
	AG	84 (46.7%)	92 (47.7%)	81 (45.5%)	0.218	0.668 (0.360~1.238)	0.260	1.631 (0.767~3.472)	0.013 [b]	2.445 (1.206~4.950)
	GG	19 (10.6%)	29 (15.0%)	12 (6.7%)	0.292	0.796 (0.525~1.205)	0.396	1.222 (0.806~1.855)	0.046 [b]	1.536 (1.015~2.326)
	q	0.339	0.389	0.295	0.171	0.806 (0.598~1.088)	0.228	1.225 (0.894~1.681)	0.008 [b]	1.520 (1.119~2.062)
-1050 G > T [a]	GG	60 (33.3%)	42 (21.8%)	58 (32.6%)	0.042 [b]		0.479		0.004 [b]	
	GT	84 (46.7%)	105 (54.4%)	94 (52.8%)	0.458	0.827 (0.506~1.350)	0.165	1.513 (0.872~2.625)	0.026 [b]	1.828 (1.074~3.115)
	TT	36 (20.0%)	46 (23.8%)	26 (14.6%)	0.015 [b]	0.556 (0.351~0.883)	0.911	0.967 (0.622~1.502)	0.020 [b]	1.739 (1.093~2.762)
	q	0.436	0.510	0.410	0.048 [b]	0.742 (0.556~0.990)	0.497	1.112 (0.827~1.497)	0.006 [b]	1.499 (1.121~2.004)
-564 C [a] > T	CC	87 (48.3%)	114 (59.1%)	94 (52.8%)	0.085		0.753		0.174	
	CT	79 (43.9%)	66 (34.2%)	67 (37.6%)	0.842	0.856 (0.391~1.875)	0.578	1.252 (0.597~2.624)	0.346	1.462 (0.689~3.104)
	TT	14 (7.8%)	13 (6.7%)	17 (9.6%)	0.061	0.663 (0.440~0.998)	0.462	0.855 (0.565~1.294)	0.250	1.290 (0.855~1.945)
	q	0.294	0.238	0.284	0.097	0.750 (0.541~1.039)	0.805	0.949 (0.687~1.311)	0.180	1.266 (0.911~1.758)
299 C [a] > T	CC	110 (61.1%)	128 (66.3%)	109 (61.2%)	0.501		0.528		0.207	
	CT	66 (36.7%)	58 (30.1%)	58 (32.6%)	0.545	1.656 (0.476~5.755)	0.070	2.898 (0.905~9.280)	0.335	1.750 (0.663~4.619)
	TT	4 (2.2%)	7 (3.6%)	11 (6.2%)	0.332	0.798 (0.523~1.218)	1.000	0.995 (0.650~1.522)	0.331	1.247 (0.816~1.905)
	q	0.206	0.187	0.225	0.520	0.886 (0.617~1.273)	0.585	1.120 (0.784~1.600)	0.204	1.264 (0.884~1.807)

Each *p* value was calculated with co-dominant, dominant and recessive model respectively. AICU, aspirin-intolerant chronic urticaria; AIAU, aspirin-tolerant acute urticaria; NC, normal controls. OR: odds ratio CI: confidence interval, q: minor allele frequency. [a] This allele is considered as a risk factor from odds ratio. [b] Values indicate significant *p* value. Each *p* value of 0.05 or less was considered to be significant.

2. Haplotype frequencies of the ADORA3 gene

We performed a haplotype association study of the four genetic polymorphisms in ADORA3 (-2288A>G, -1050G>T, -564C>T and A299A). The strong LD was observed among the four SNPs in this study subject population (figure 2). Table 4 shows the haplotype frequencies of these four SNPs. We can find that the haplotype [$G_{-2288}T_{-1050}C_{-564}C_{299}$] was significantly higher in AIAU group than NC group (p=0.008 in haplotype analysis model). For examining the promoter activity of ADORA3, we also performed a haplotype association study of two polymorphisms in ADORA3 promoter (-1050 G>T and -564 C>T). Table 5 shows the haplotype frequencies of these two SNPs. According to the results, the *ht1* [$T_{-1050}C_{-564}$] that considered both [$G_{-2288}T_{-1050}C_{-564}C_{299}$] and [$A_{-2288}T_{-1050}C_{-564}C_{299}$] is associated with increase risk (p = 0.047, odds ratio (OR) = 0.742, 95 % confidence interval (CI) = 0.555 ~ 0.990 for AICU vs. AIAU; p = 0.005, OR = 1.521, 95% CI = 1.136 ~ 2.036 for AIAU vs. NC).



L1	L2	D'	r^2
A299A	-564 C>T	0.983	0.667
A299A	-1050 G>T	0.976	0.211
A299A	-2288 A>G	0.974	0.135
-564 C>T	-1050 G>T	0.984	0.311
-564 C>T	-2288 A>G	0.983	0.200
-1050 G>T	-2288 A>G	0.991	0.632

Block 1

CCTG .348
CCGA .267
TTGA .205
CCTA .105
CTGA .070

Fig.2. Haplotypes and linkage disequilibrium (LD) coefficients (*/D/* and r^2) among four SNPs in ADORA3.

Table 4. The haplotype frequencies of four SNPs in ADORA3

	AICU	AIAU	NC	AICU vs AIAU		AICU vs NC		AIAU vs NC	
	(N = 360)	(N=386)	(N=356)	*p* value	OR (95% CI)	*p* value	OR (95% CI)	*p* value	OR (95% CI)
Haplotype 1 [GTCC]	125 (34.7%)	150 (38.9%)	105 (29.5%)	0.255	0.837 (0.621~1.128)	0.150	1.272 (0.928~1.742)	0.008[a]	1.519 (1.119~2.064)
Haplotype 2 [AGCC]	146 (40.6%)	147 (38.1%)	151 (42.4%)	0.500	1.109 (0.827~1.488)	0.649	0.926 (0.688~1.247)	0.232	0.835 (0.622~1.120)
Haplotype 3 [AGTT]	44 (12.2%)	42 (10.9%)	49 (13.8%)	0.569	1.140 (0.728~1.788)	0.579	0.872 (0.564~1.350)	0.263	0.765 (0.493~1.188)
Haplotype 4 [ATCC]	24 (6.7%)	33 (8.5%)	35 (9.8%)	0.408	0.764 (0.442~1.320)	0.136	0.655 (0.381~1.126)	0.611	0.857 (0.521~1.412)
Haplotype 5 [AGTC]	21 (5.8%)	12 (3.1%)	16 (4.5%)	0.077	1.931 (0.936~3.984)	0.500	1.316 (0.675~2.566)	0.341	0.682 (0.318~1.462)
Haplotype 6 [Others]	0 (0%)	2 (0.5%)	0 (0%)	0.500	NA	NA	NA	0.500	NA

AICU, aspirin-intolerant chronic urticaria; AIAU, aspirin-tolerant acute urticaria; NC, normal controls. OR: odds ratio CI: confidence interval, NA: not applicable. The order of haplotypes is -2288 A>G, -1050 G>T, -564 C>T, and A299A. [a] Value indicates significant *p* value. Each *p* value of 0.05 or less was considered to be significant

.

Table 5. The haplotype frequencies of two SNPs in ADORA3

	AICU	AIAU	NC	AICU vs AIAU		AICU vs NC		AIAU vs NC	
	(N = 360)	(N=382)	(N=354)	*p* value	OR (95% CI)	*p* value	OR (95% CI)	*p* value	OR (95% CI)
HT1[TC]	157 (43.6%)	195 (51.0%)	144 (40.7%)	0.047[a]	0.742 (0.555~0.990)	0.449	1.128 (0.838~1.518)	0.005[a]	1.521 (1.136~2.036)
HT2[GT]	106 (29.4%)	87 (22.8%)	101 (28.5%)	0.044[a]	1.415 (1.018~1.967)	0.805	1.045 (0.757~1.445)	0.076	0.739 (0.530~1.030)
HT3[GC]	97 (26.9%)	100 (26.2%)	109 (30.8%)	0.868	1.040 (0.751~1.441)	0.283	0.829 (0.599~1.146)	0.191	0.797 (0.578~1.099)

AICU, aspirin-intolerant chronic urticaria; AIAU, aspirin-tolerant acute urticaria; NC, normal controls. The order of haplotypes is -1050 G>T and -564 C>T. OR: odds ratio CI: confidence interval, NA: not applicable. [a] Values indicate significant p value. Each p value of 0.05 or less was considered to be significant.

B. Functional study for ADORA3 genetic polymorphisms

1. ADORA3 is abundantly expressed in myeloid cell lines.

We screened several cell lines to measure mRNA expression of ADORA3 gene using RT-PCR in various cell lines such as HL60 (human myeloblastic cell), THP-1 (human monocyte leukemia cell), bease2B (human bronchus epithelial cell), Hep3B (human hepatocellular carcinoma cell), A549 (human lung epithelial cell), U937 (human monocytes), HMC-1 (human mast cell), Hela (human cervical adenocarcinoma cell), KU 812 (human myeloblast cell) and Jurkat (human T cell leukemia). As illustrated in Figure 3, we detected abundant transcripts for ADORA3 in HL60 and THP-1 cDNA. This analysis suggests that ADORA3 transcript express predominantly in human myeloid cell lines.

2. Effects of the polymorphisms, -1050G>T and -564C>T, on transcriptional activity

To analyze effects of -1050G>T and -564C>T on ADORA3 promoter activity, four fragments of the ADORA3 promoter 1277 bp containing two SNPs, *ht1* [$T_{-1050}C_{-564}$], *ht2* [$G_{-1050}T_{-564}$], *ht3* [$G_{-1050}C_{-564}$], and *ht4* [$T_{-1050}T_{-564}$] were cloned into pGL3-basic with a firefly luciferase gene reporter. Firefly luciferase was quantified and normalized to renilla luciferase activity. The luciferase activity of *ht1*[$T_{-1050}C_{-564}$] and *ht3*[$G_{-1050}C_{-564}$] which have -564 C allele was increased compare to those of *ht2*[$G_{-1050}T_{-564}$] and *ht4*[$T_{-1050}T_{-564}$] which have -564 T allele in THP-1 cells and HMC-1 cells (figure 4).

A

Primer	sequence (5' to 3')	Accession Number	Size (bp)
Forward	ATACTTGCGGGTCAAGCTTAC	NM_000677	372
Reverse	AAGGACTTAGCCGTCTTGAAC		

B

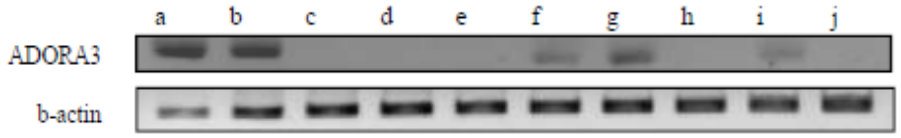


Fig.3. Primer pairs used for ADORA3 expression and expression of ADORA3 mRNA in various cell lines A. Primer pairs used for ADORA3 expression. **B.** Expression of ADORA3 mRNA in various cell lines. a: HL60 (human myeloblastic cell); b: THP-1 (human monocyte leukemia cell); c: bease2B (human bronchus epithelial cell); d: Hep3B (human hepatocellular carcinoma cell); e: A549 (human lung epithelial cell); f: U937 (human monocytes); g: HMC-1 (human mast cell); h: Hela (human adenocarcinoma cell); i: KU 812 (human myeloblast cell); j: Jurkat (human T lymphocytes)

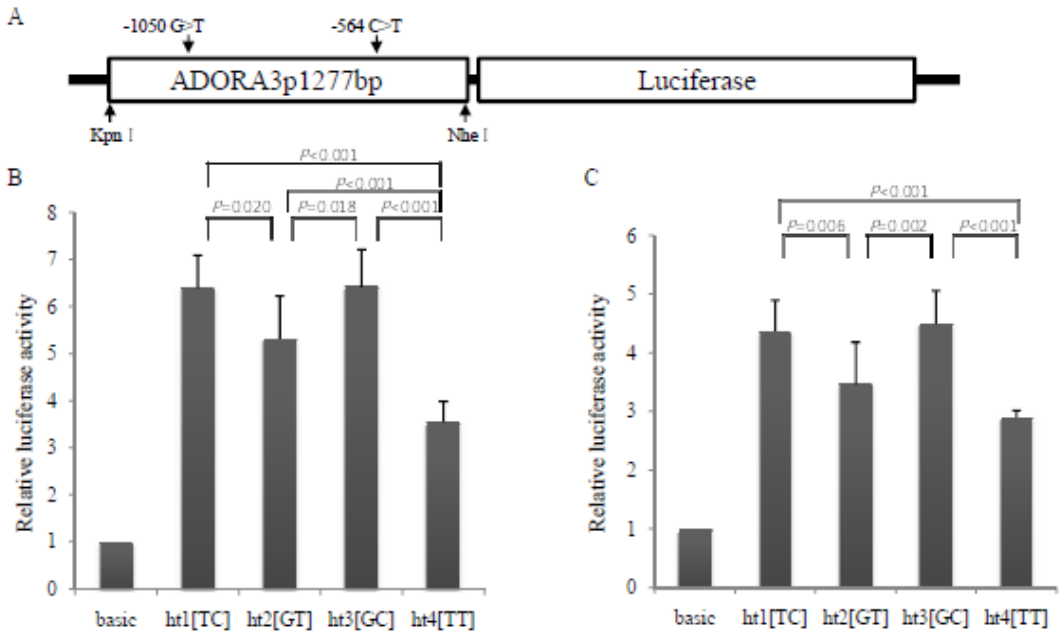


Fig.4. Effects of -1050 G>T and -564 C>T polymorphisms on the transcription activity of human ADORA3 promoter. A. Schematic representation of reporter gene constructs that contained the ADORA3 promoter region with the -1050 and -564 polymorphisms. **B**. Transfection of THP-1, human monocyte leukemia cell line. **C**. Transfection of HMC-1, human mast cell line. 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. Luciferase activity assay was performed in three independent experiments (in total, n = 9). Values represent mean ± SD of 9 times in each group. Statistical differences were evaluated with ANOVA and Bonferroni test.

3. Effect of a transcription factor, octamer-binding transcription factor-1 (OCT-1), on ADORA3 promoter activity by co-transfection

To examine whether the ADORA3 SNPs create any different transcription factor binding affinity, we searched transcription factor binding site (TFSEARCH, Searching Transcription Factor Binding Sites *ver*. 1.3). The web search result suggested that -564 C>T alters the ability of a transcription factor, OCT-1 to interact with this promoter region. However, there were no difference according to the -564C>T polymorphism (figure 5).

4. Electrophoretic mobility shift assay (EMSA)

To determine whether the -564 C>T polymorphism altered a transcription factor binding affinity, we performed an EMSA with nuclear extracts from THP-1 and HMC-1 using double-stranded oligonucleotide probes that corresponded to -564C>T (figure 6). The -564C probe produced a specific band that had a higher affinity than that produced by the -564T probe. Moreover, NF-κB bound more tightly to the C allele than T allele, which was replicated in HMC-1 cells (data not shown).

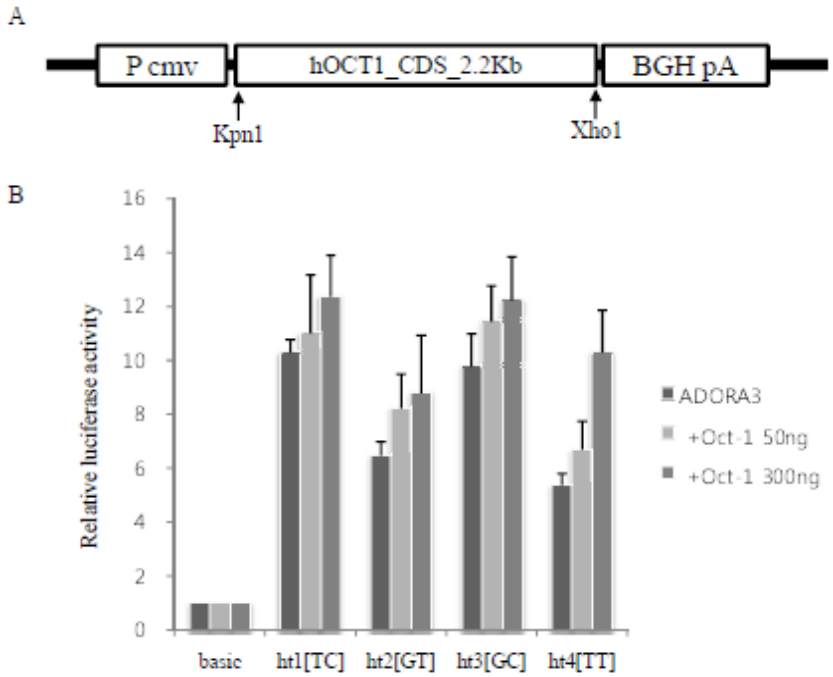


Fig.5. Effect of OCT-1 transcriptional regulation on the activity of human ADORA3 promoter A. Schematic representation of transcription factor, OCT-1, gene constructs cloned in pcDNA3 vector. **B.** Effect of OCT-1 transcriptional regulation activity of human ADORA3 promoter. Data are the mean values±SEM of independent experiments. 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.

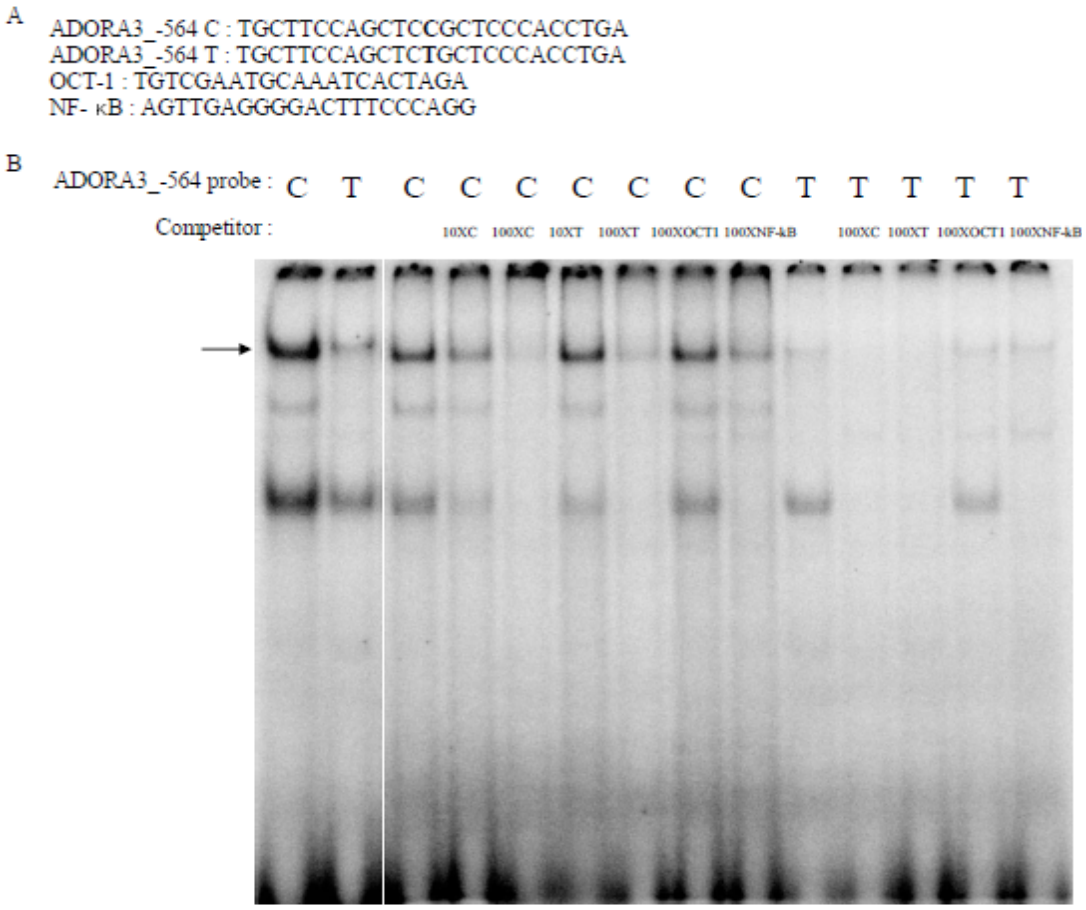


Fig.6. Electrophoretic mobility shift assay for ADORA3 -564C>T polymorphism using nuclear extracts obtained from THP-1 cells. A. Nucleotide sequences of oligonucleotides used as probes and competitors (-564 C, -564 T, OCT-1, and NF-κB). The boldface characters indicate -564 C>T. **B.** Differential binding of nuclear proteins to -564 C>T alleles, binding affinity of OCT-1 and NF-κB protein for ADORA3 promoter with either a C or T at position -564. Identification of the transcription factor binding to -564 probe using competitive binding assay. Competitive binding assay was performed with unlabeled OCT-1 and NF-κB probe. The amount of competitor was 10-fold molar excess than probe.

5. Real-time RT-PCR analysis of ADORA3 expression in Aspirin-Intolerant Urticaria (AIU)

The ADORA3 gene expression was detected by means of real-time PCR in purified PBMC from 20 AIU patients. Figure 7 shows that the mRNA expression of ADORA3 was significantly higher in the patients with *ht1*[$T_{-1050}C_{-564}$] ($p = 0.033$) and also with *ht1*[G_{-2288} $T_{-1050}C_{-564}C_{299}$] ($p = 0.056$) than others.

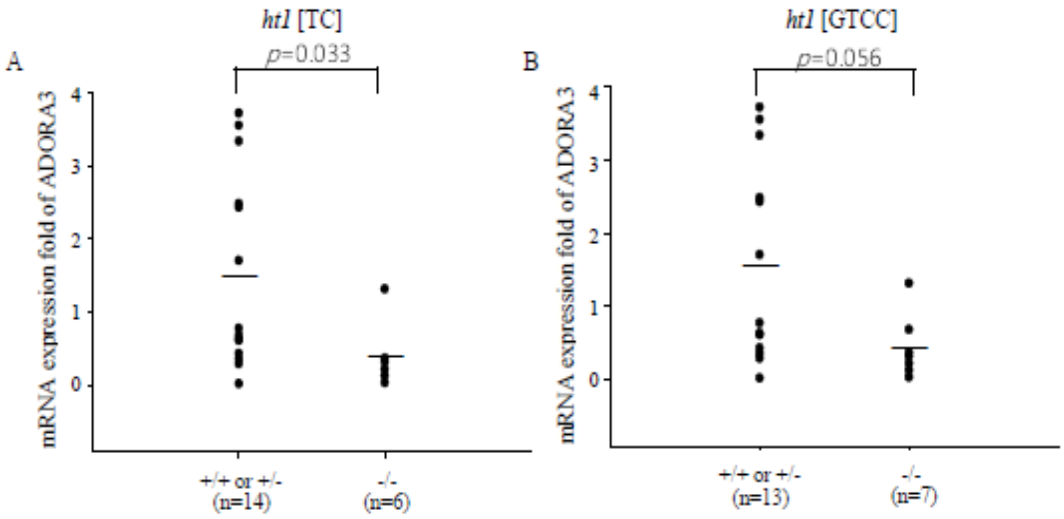


Fig.7. ADORA3 mRNA expression in PBMC of aspirin-intolerant urticaria (AIU). Comparison of ADORA3 mRNA expression level with *ht1* [$T_{-1050}C_{-564}$](**A**) and with *ht1* [$G_{-2288}T_{-1050}C_{-564}C_{299}$](**B**) than others. Statistical analysis was done in dominant model. Statistical differences were evaluated with Mann-Whitney *U* test. Horizontal bars indicate median values.

6. Trans-well chemotaxis assay

To investigate whether the genetic polymorphisms in ADORA3 promoter region associate

with chemotactic effects in PMN, we performed a trans-well assay in 17 normal control group. Figure 8 shows that the neutrophil chemotactic activity significantly (p = 0.019) increased in the people with *ht3*[$G_{-1050}C_{-564}$] (and *ht2*[$A_{-2288}G_{-1050}C_{-564}C_{299}$] was the same result) of ADORA3 compare to others. It further suggested a special role of -1050 G allele for ADORA3 in normal control group.

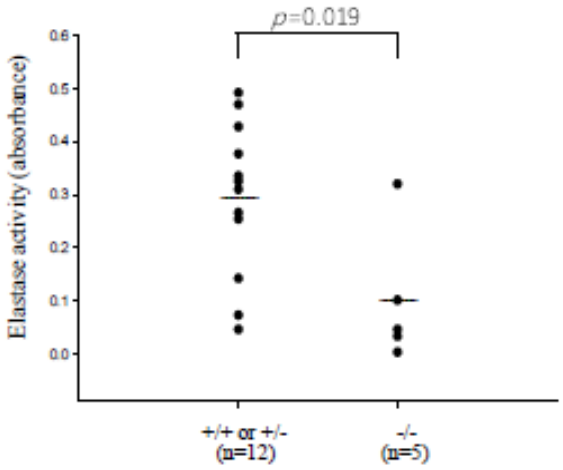


Fig.8. Role of *ht3*[$G_{-1050}C_{-564}$] in neutrophil migration in normal control group. Trans-well assays with human neutrophils in upper wells seperated from lower wells containing 1 nM fMLP by a filter with 3-μm pore size were used to assess chemotaxis. Statistical analysis was done in dominant model. Statistical analysis was done in dominant model. Statistical differences were evaluated with Mann-Whitney *U* test. Horizontal bars indicate median values.

Ⅳ. DISCUSSION

This is the first study to demonstrate a significant association between genetic polymorphisms of ADORA3 and the pathology of acute and chronic in AIU patients. Our results suggest that the promoter polymorphisms of human ADORA3 gene may play an important role in the development of acute inflammation related with aspirin hypersensitivity.

Urticaria is associated with perivascular infiltration of leukocytes into the lesions. Although mast cell-derived chemical mediators are considered to play crucial roles in the infiltration of leukocytes as well as in the dermal edema, other mechanisms for the leukocyte infiltration have not been well defined. In this study we investigated the effect of genetic polymorphisms of adenosine-related genes in the pathogenesis of AIU by an SNP haplotype-based case-control association study. In clinical characteristics of subjects, the serum MPO level, a marker of neutrophil activation in serum (Bochenska-Marciniak *et al.* 2003) was significantly higher in the AIAU than AICU group. Previous study suggested that serum MPO levels from AIU patients were in correlation with the levels of IL-8 and IL-18 (Choi *et al.* 2008). Therefore, we speculated that neutrophil activation is involved in AIU and it may play a pathogenic role in AIAU.

ADORA3 located on chromosome 1p13.2 is the most recently discovered among adenosine receptors. Expression of ADORA3 mRNA is regulated by many transcription factor binding motifs (Atkinson *et al.* 1997; Murrison *et al.* 1996) (figure 9) and has been also found in various cell types including lung mast cells, monocytes, lymphocytes, and PBMC (Fozard *et al.* 1996; Thorn and Jarvis 1996). However, marked differences exist in expression levels within and among species. In rats and guinea-pigs the activation of

ADORA3 induces mast cell degranulation in response to allergens although this finding was not continued human mast cells (Walker *et al.* 1997). In our results, we showed that ADORA3 is abundantly expressed in myeloid cell lines. Chen, Y et al reported that human PMN and human myeloblastic cell line, HL60, express predominantly ADORA2a and ADORA3 receptors. Adenosine is an important endogenous regulator of neutrophil function that can modulate neutrophil activity by interacting with the ADORA3 on the cell surface of PMN (Chen *et al.* 2006). Until now, however, discrepancy between anti- and pro-inflammatory effects induced by ADORA3 has been observed in many cells of the immune system and in a variety of inflammatory conditions (Gessi *et al.* 2008). In PMNs, ADORA2a is linked to G_s protein and suppress PMN responses by cAMP/PKA pathway. But ADORA3 through PMN chemotaxis up-regulate PMN responses by stimulating PI3K pathway which linked to G_i protein (Hammarberg *et al.* 2004; Varani *et al.* 2005). Released ATP at the leading edge of PMN is rapidly converted to adenosine allows a second stage of signal amplification through controlling migration speed by distributed ADORA3 at leading edge (Chen *et al.* 2006). Moreover, in PMN from ADORA3 knockout mice, interference with adenosine signaling significantly reduced migration speed.

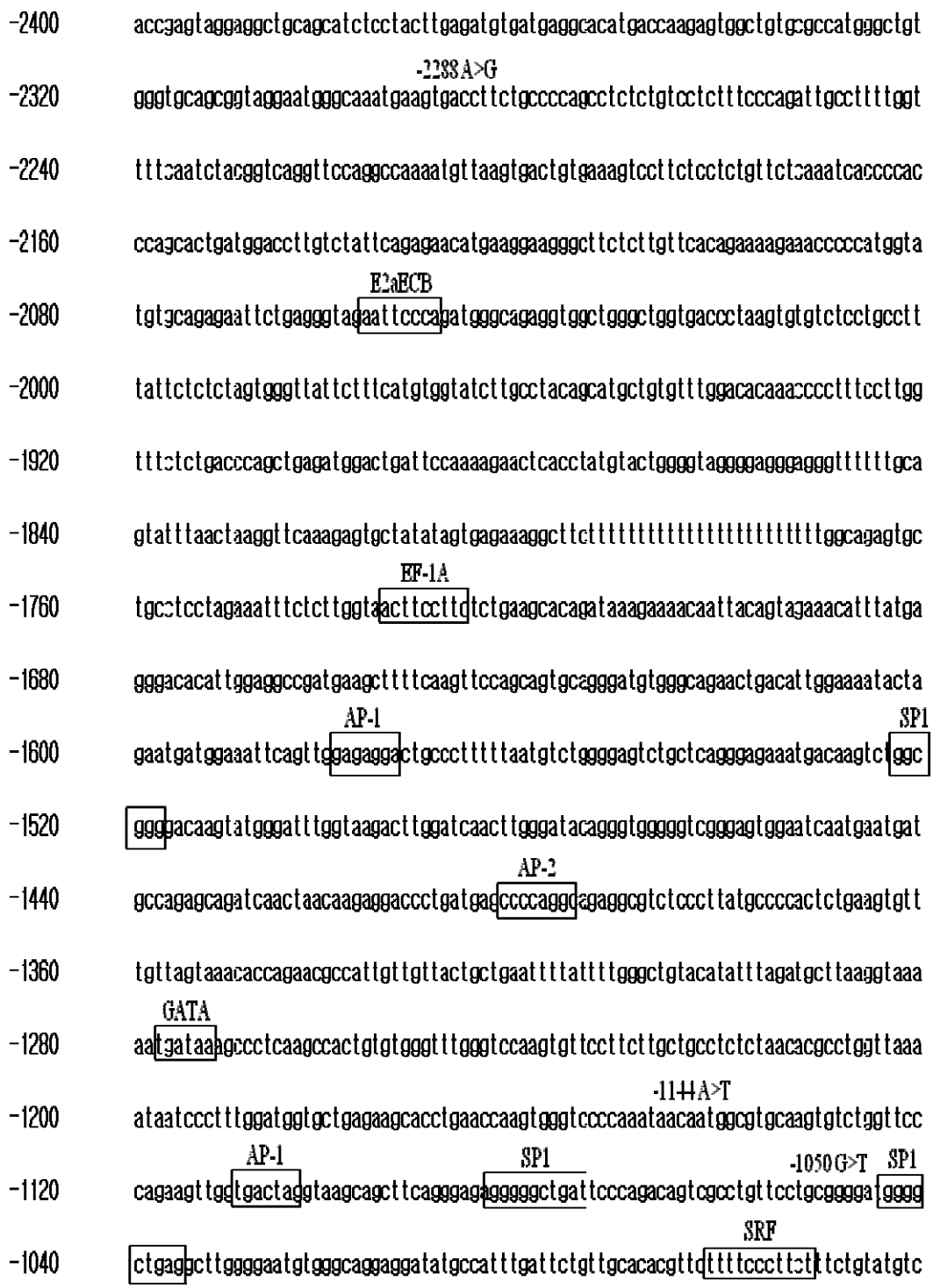
Several recent reports described ADORA3 up-regulation in a variety of cancerous disease (Madi *et al.* 2004; Merighi *et al.* 2003; Schlotzer-Schrehardt *et al.* 2005). Interestingly, leukocytes of rheumatoid arthritis patients have been found to over-express ADORA3, suggesting that increased ADORA3 expression could be related to the accumulation of PMN in inflamed sites (Madi *et al.* 2007). Moreover, a high level of ADORA3 expression was noted in PBMC from a patient with colon cancer, and returned to normal level after tumor removal (Gessi *et al.* 2004). The authors proposed that the evaluation of ADORA3 protein

may be useful for the prognosis and diagnosis of human malignancy. And examination of neutrophil ADORA3 expression could play a potential role in the screening of high-risk individuals or in the follow-up of patients after surgical resection.

The AIU patients are hypersensitive to aspirin and/or NSAIDs and may show excessive leukotriene production, similar with AIA patients, and increased vasopermeability and subsequent urticaria (Grattan 2003). Especially, Leukotriene B_4 plays pro-inflammatory mediator through its chemo-attractant properties for human PMN. Acute inflammation may be characterized by the rapid influx of PMNs into affected tissues. The mechanisms that control the migration of PMN determine the final rate at which PMN are dispatched in response to inflammation or infection (Nauseef 2007; Seely *et al.* 2003). Some patients with neutrophilic urticaria showed a shorter mean duration of the disease than did the other urticarial patients (Toppe *et al.* 1998). In the present study, *ht1*[$T_{-1050}C_{-564}$] of ADORA3 gene showed a significant association with AIAU group in haplotype frequencies. In vitro functional study, we also found that transcriptional activity of the -564 C allele was higher than T allele in the human monocyte leukemia cell line, THP-1, and human mast cell line, HMC-1. These effects suggesting that the -564 C allele may be leading to induction of ADORA3 mRNA expression. In addition, the -564 C allele also showed affected transcription factor binding in the promoter region of ADORA3 by EMSA. The transcription factor, NF-κB, bound more tightly to the C allele sequence than to the T allele sequence. Our present EMSA result demonstrated that a -564 C-specific DNA-binding protein is involved in the transcriptional regulation of the ADORA3 gene perhaps accounting for the differences in transcription. Thus, we performed the real-time PCR comparing of ADORA3 mRNA expression levels according to the promoter polymorphisms in PBMC from AIU patients.

The patients with *ht1*[$T_{-1050}C_{-564}$] tended to express higher mRNA levels than those with others. Recently, several studies supporting evidence of neutrophil involvement with NF-κB activity have been reported. Kim et al (Kim *et al.* 2002) suggest that NF-κB signaling may be primarily involved in and an important regulator of the neutrophil migration in the infected tissues. And another report said ADORA3 level is determined by inflammatory cytokines that control the level of the transcription factor NF-κB participating in the regulation of ADORA3 expression (Madi *et al.* 2007).

Furthermore, we also showed that the neutrophil chemotaxis activity according to the SNPs of ADORA3 was inducted at *ht3*[$G_{-1050}C_{-564}$] which is comparable to the findings of transcriptional promoter activity and mRNA expression levels in PBMC from AIU patients. This additional finding showed the different SNP pattern between in AIU patients and NC group. Even though there was no significant at transcription activity in -1050G>T, we found that the neutrophil chemotactic activity significantrly increased with *ht3*[$G_{-1050}C_{-564}$] using the trans-well assay in normal group. Further studies will be needed to compare to the level of neutrophil migration in AIU patients.


-2400
-2238A>G
-2320
-2240
-2160
E2aECB
-2080
-2000
-1920
-1840
EF-1A
-1760
-1680
AP-1
SP1
-1600
-1520
AP-2
-1440
-1360
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-1144A>T
-1200
AP-1
SP1
-1050G>T
SP1
-1120
SRF
-1040

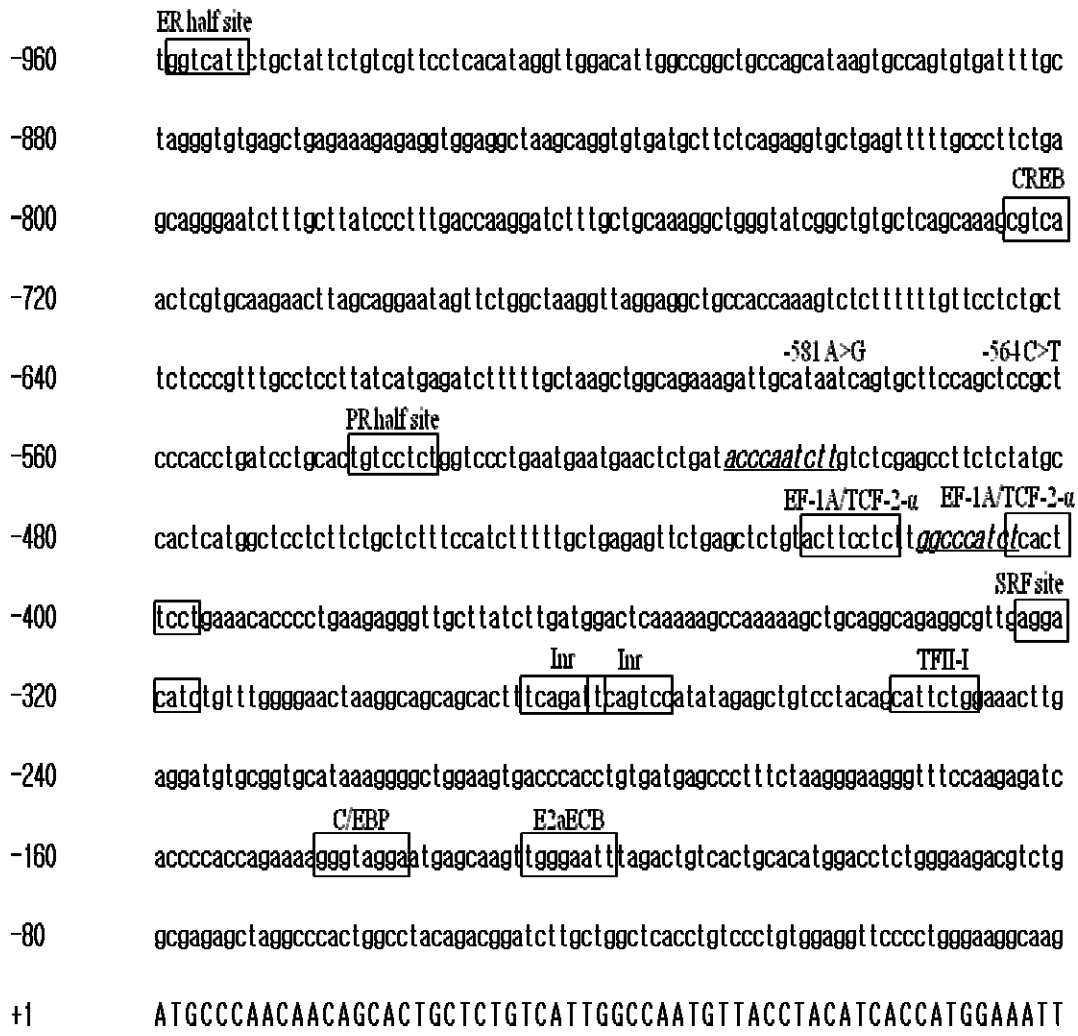

Fig.9. Sequence of the region upstream to the initiating ATG of the human ADORA3.

Potential transcription factor recognition motifs are boxed. Coding sequence is shown in uppercase letters. The genetic polymorphisms at ADORA3 gene promoter are highlighted in bold. Nucleotides are numbered with the A of the ATG translation initiation codon being +1. No CAAT or TATA boxes were identified in the 5' flanking region, however, two CAAT box like regions are present (shown underlined in italics). Putative transcription factor binding sites are boxed.

Ⅴ. CONCLUSION

These results suggest that the ADORA3 gene is a potentially important gene in AIAU development, as transcriptional activity and basal level of ADORA3 mRNA were found to be different according to ADORA3 promoter polymorphisms. Therefore, the results indicate that the *ht1*[$T_{-1050}C_{-564}$] of ADORA3 could increase susceptibility to AIAU in the Korean population.

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-국문요약-

아데노신 A3 수용체의 유전자 다형성과 아스피린 과민성 두드러기 환자의 연관성 연구

아주대학교 대학원 의생명과학과

남 은 주

(지도교수: 박 해 심)

연구배경 및 목적 아스피린 과민성 두드러기 (aspirin-intolerant urticaria, AIU)는 아스피린에 노출 시 두드러기의 악화를 나타낸다. 아데노신은 세포 내 스트레스와 손상에 반응하면서 생기는 강력하고 중요한 신호전달 뉴클레오시드로서 비만세포나 호중구 등 비 신호 염증세포의 표면에 있는 아데노신 수용체를 통해 주요 역할이 나타나고 아스피린 노출 후 그 양이 더 늘어날 수 있다. 본 연구는 아스피린 과민성 두드러기 환자의 발병에서 아데노신 관련 유전자들과의 관련성 여부를 연구하였다.

재료 및 방법 아스피린 과민증을 동반한 두드러기를 기간에 따라 두 군으로 나누어, 만성 두드러기 (aspirin-intolerant chronic urticaria, AICU) 환자 180 명, 아스피린 과민증을 동반한 급성 두드러기 (aspirin-intolerant acute

urticaria, AIAU) 환자 193 명 그리고 정상 대조군 (normal healthy control, NC) 178 명의 한국인을 대상으로 아데노신 A3 수용체의 유전자 다형성 (single nucleotide polymorphisms, SNPs) haplotype-based case-control 연구를 수행하였다. 아데노신 A3 수용체 (adenosine A3 receptor, ADORA3)의 유전자 다형성에 따른 기능 차이를 규명하기 위해 luciferase reporter assay 와 electrophoretic mobility shift assay (EMSA)를 수행하였고, 생체기능 연구로 아스피린 과민성 두드러기 환자의 유전자다형성에 따라 말초혈액 단핵구에서 ADORA3 mRNA 발현을 real-time PCR 로 비교하였으며, 정상대조군에서 유전자 다형성에 따른 호중구의 화학주성능 분석실험을 수행하였다.

결과 각 유전자의 linkage disequilibrium (LD) 블록의 패턴으로 tagging SNP 을 선정하여 AIU 환자에서의 연관성을 본 결과 ADORA3 유전자의 프로모터에 있는 -1050 G>T 와 -564 C>T 의 haplotype 인 [$T_{-1050}C_{-564}$]의 대립유전자 빈도가 AICU 와 정상 대조군에 비해 AIAU 에서 현저하게 높게 나타났다. THP-1 세포주와 HMC-1 세포주를 이용한 luciferase reporter assay 결과, -564 C 대립유전자를 가진 plasmid 일수록 프로모터 활성도가 유의하게 증가되어 있었다. 그리고 EMSA 실험에서는 -564 T 서열에 비해 C 서열에 염증 유전자들의 강력한 전사인자로 알려져 있는 NF-κB 가 결합함을 확인하였다. 또한 AIU 환자들의 말초혈액 단핵구에서 *ht1*[$T_{-1050}C_{-564}$]를 지닌 환자들에서 ADORA3 mRNA 발현이 유의하게 높았다. 하지만 정상 대조군에서

수행한 호중구의 화학주성능 분석실험에서는 *ht3*[$G_{-1050}C_{-564}$]를 지닌 사람의 화학주성능이 현저하게 높았다.

결론 상기 결과들은 ADORA3 *ht1*[$T_{-1050}C_{-564}$] 대립 유전자는 NF-κB 와의 상호작용에 의해 ADORA3 유전자의 발현을 증가시켜 AIAU 의 병인기전에 기여할 가능성을 시사한다.

핵심어: 아데노신 A3 수용체, 아데노신, 아스피린 과민성, 두드러기, 유전자 다형성