ASIAN PACIFIC JOURNAL OF ALLERGY AND IMMUNOLOGY (2008) 26: 205-211

Association between *ADAM33* Polymorphisms and Asthma in a Thai Population

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SUMMARY *ADAM33 (*A Disintegrin And Metalloprotease 33) is an asthma susceptibility gene found across several human populations**.** However, no information on *ADAM33* exists for Thai population. The objective of this study was to determine the association, if any, between *ADAM33* polymorphisms and asthma in Thai subjects. Genotyping revealed 8 single nucleotide polymorphisms (SNPs) within the 3′ region of the *ADAM33* gene among 200 asthmatics and 100 control subjects. Asthmatic subjects were further sub-categorized into high and low severity groups. Multiple genetic model statistic tests for single-marker and haplotype association were carried out. Differences in allele frequencies at the SNPs rs528557/S2, rs598418 and rs44707/ST+4 in asthmatics were statistically significant compared to controls. The SNP rs528557/S2 could also be linked to the low severity group and the SNPs rs598418 and rs44707/ST+4 with the high severity group. Two-SNP haplotype analysis at the SNPs rs528557/S2 and rs598418 revealed a significant association with asthma. This study in a Thai population confirmed a positive association between *ADAM33* polymorphisms and asthma susceptibility.

Asthma is a common chronic respiratory disease in which chronic inflammation leads to an airflow obstruction and bronchial hyperresponsiveness (BHR) and results in irreversible structural changes in airway remodeling.[1] The cause of this disease is believed to be a complex combination of multiple genetic and environmental factors which lead to heterogeneous phenotypes such as variable degrees of atopic involvement and severity.[2] Early family and twin studies support the role of genetics in the development of the disease.[3,4] A large number of association and linkage studies have identified over 100 genes related to asthma. However, less than a dozen of these are associated with asthma, according to a large number of independent reports.[5] This group of highly promising candidates, discovered through genome-wide linkage analysis, consists of known pathogenesis-related genes and also novel genes of unprecedented linkage to asthma.

The first positionally-cloned asthma susceptibility gene is a member of the *ADAM* (A Disintegrin And Metalloprotease) gene family, *ADAM33*, which is located on human chromosome 20p.[6] Ge-

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netic linkage analysis and association studies of families with asthma across diverse ethnic backgrounds support a relationship between *ADAM33* polymorphisms and asthma phenotypes and airway hyperreactivity (AHR).[7-10] The mouse orthologue of *ADAM33* also lies in the region linked to AHR.[11] The precise mechanisms behind polymorphic variations in *ADAM33* or their encoded proteins in contributing to asthma development remain under intense investigation. Selective expression of the *ADAM33* mRNA and its protein in adult bronchial smooth muscle and human embryonic bronchi and surrounding mesenchyme indicate specific roles of the gene in the observed phenotypes of AHR and airway remodeling.[12-14]

Despite the above notion, negative associations between *ADAM33* polymorphisms with asthma have been reported from ethnically different populations.[15-17] Among various factors, a heterogeneity of allelic frequencies at certain loci among samples from these groups may be yielding discordant results. There is an increased prevalence of asthma in Thailand which may be following similar trends observed in many Western and Asian countries. However, the differences in the genetic background of the Thai population may account for different asthma susceptibility genes.[18] Therefore, we sought to determine *ADAM33* polymorphisms in Thai asthmatics and compared them with non-asthmatic counterparts.

MATERIALS AND METHODS

Subjects

One hundred control subjects and 200 asthmatics of Thai nationality were recruited from pediatric and adult allergy and immunology clinics from two tertiary care centers in Bangkok, Thailand. Asthma was diagnosed based on symptoms and on spirometry assessments using the criteria outlined by the American Thoracic Society. Degrees of asthma severity were determined according to guidelines provided by the Global Initiative for Asthma.[19] Control subjects were asymptomatic for asthma and were devoid of atopic or pulmonary diseases. Pregnant or lactating female subjects were excluded. The study was in accordance with the Helsinki Declaration and approved by the local institutional review boards, and all subjects provided informed, written consent.

Polymorphism genotyping

ADAM33 polymorphisms were genotyped in the 3′ region, from exon 19 to exon 22, by direct sequencing of genomic DNA extracted from peripheral blood leukocytes. The PCR primers designed by the authors are shown in Table 1. They were used to amplify gene segments that spanned eight previously reported SNP locations, which are also known to be associated with the asthma phenotype across ethnically diverse populations.[7] These SNPS are rs3918396/S1, rs528557/S2, rs44707/ST+4, rs574174/ST+7, rs2280091/T1, rs2280090/T2, rs543749/V-1 and rs2787094/V4. PCR was performed in a 25-µl reaction volume containing 1x buffer, 1.0 mM $MgCl_2$, 200 µM dNTPs, 1x Q-solution, 1.0 µM of each primer, 1.0 U *Taq* polymerase (Qiagen, Hilden, Germany) and 50 ng of genomic DNA. Reactions consisted of 30 cycles of denaturation at 94ºC for 30 seconds, annealing at the optimal melting temperature for 30 seconds, and extension at 72ºC for 1 minute on a PTC-100 Programmable Thermal Controller (MJ Research, Waltham, USA). PCR products were

Table 1 Oligonucleotide primers used for resequencing the 3' region of *ADAM33*

Position	Forward primer 5'→3'	Reverse primer 5'→3'
Exon 19	5'-TGACTGCCTGCCACAGCCAC-3'	5'-TCTGAGGAGGGGAACCGCAG-3'
Intron 19-1	5'-GCAGTGGGTAGGCTCCGAGC-3'	5'-AGAGTGCCTGCCCTGCCTAG-3'
Intron 19-2	5'-GCGGAGTGGGGAGTCACATAATAC-3'	5'-GGCTGGCACCTCCTCTCTCTAG-3'
Exon 20, 21	5'-AGGTTCTTTGGAAGCTGAGCG-3'	5'-ACTGAGGGGTGGGAGAGGTG-3'
Intron 21	5'-GGCAGGGACCTGGATTCAAAG-3'	5'-CACACCAGACTCCCAGGACAGAG-3'
Exon 22	5'-GTCCCAGAAGCAAAGGTCACAC-3'	5'-TGCGGTGTCTTGCTGTGTTG-3'

purified by the PCR purification kit (Qiagen, Hilden, Germany) and were used as DNA template for cycle sequencing. Direct DNA sequencing was performed using a DYEnamic ET Terminator cycle sequencing kit (Amersham Bioscience, UK). Sequencing reactions were prepared in 10-µl volumes containing 1.0 µM primer, 5 pmol DYE-ET and 30 ng of DNA template and subjected to 25 cycles of denaturation at 95ºC for 20 seconds, annealing at 50ºC for 15 seconds, and extension at 60ºC for 1 minute on a PTC-100 Programmable Thermal Controller.

Statistical analysis

The distribution of allele frequencies was tested for conformity to the Hardy-Weinberg equilibrium by χ^2 test. The degree of linkage disequilibrium between loci were measured using Lewontin's D'.[20] Each SNP was analyzed by comparing differences in genotype frequencies between those in the asthmatic and control groups stratified by population, and χ^2 tests for association were performed.[21] In order to analyze the association between the severity of asthma and *ADAM33* polymorphisms, we subcategorized asthmatic subjects into two groups, termed the high and low severity groups. The high severity group included subjects suffering from moderate to severe persistent asthma, whereas the low severity group consisted of subjects with intermittent and mild persistent asthma. The degree of asthma severity was classified as per the guidelines provided by the Global Initiative for Asthma.

Haplotype association analysis

Haplotype association analysis was carried out in two steps. An optimal SNP set for association was first identified by single marker analysis. Then, in the second step, genetic models for haplotype association based on the optimal SNP set were identified using HAPSTAT.[22-24] Likelihood ratio tests were performed for each genetic model.

RESULTS

One hundred control subjects (46 men and 54 women, mean age 26 years) and 200 individuals suffering from asthma (116 men and 84 women, mean age 29.86 years) were recruited and genotyped for *ADAM33* polymorphisms. The control group had significantly higher lung function parameters than the asthmatic group. Asthmatic subjects showed a mean FEV_1 (forced expiratory volume in 1 second) of 1.83 l (66.34% of predicted) and a mean FVC (forced vital capacity) of 2.89 l (80.14% of predicted). The mean duration of asthma was 10.68 years. Table 2 shows that subjects in the high severity group were significantly older and suffered from asthma for longer durations than those in the low severity group. The mean FEV_1 was significantly lower in the high severity group as compared to those of the low severity group.

Genotyping analyses revealed eight SNPs in the 3′ region of *ADAM33*, being distributed in Hardy-Weinberg equilibrium. Only one SNP, rs528557/S2, was located on exon 19, while the other seven SNPs (rs2853209, rs598418, rs44707/ST+4, rs597980, rs11905233, rs2787094/V4 and rs3746631) were located on intron 19 and 3' UTR. Genotype descriptions for each SNP are outlined in Table 3.

Table 2 Clinical characteristics of high and low severity groups

Characteristics*	Low severity group (n = 95)	High severity group (n = 105)	p-value
Age (years)	22.94 (17.99)	35.78 (17.06)	< 0.0001
Gender (M/F ratio)	1.21	1.56	NS
Duration of asthma (years)	8.91 (6.91)	12.20 (8.23)	0.002
FVC (% of predicted)	81.33 (21.15)	79.19 (16.57)	NS
FEV_1 (% of predicted)	72.27 (16.27)	61.26 (15.83)	< 0.0001

*For age, duration of asthma, FVC and FEV_1 characteristics, the displayed values are means while the numbers in brackets are SDs. For gender characteristics, the displayed values are the ratios between males and females. p-values are obtained from t-tests except for the gender characteristic where the p-value is calculated using a Fisher's exact test. FVC, forced vital capacity; FEV_1, forced expiratory volume in 1 second; NS, non-significant.

Single SNP analysis

Table 4 indicates that there are significant differences in the genotypes observed in asthmatics and in control groups for the SNPs rs528557/S2 (p = 0.012), rs598418 (dominant model: p = 0.017, multiplicative model: p = 0.049) and rs44707/ST+4 (p = 0.049). The asthmatic patients were divided into high and low severity subgroups based on clinical characteristics, as previously described. There was a significant difference in allele frequencies between the low severity and control groups for the SNP rs528557/S2 (p = 0.012). Differences in allelic distribution were also significant between the high severity and control groups for the SNPs rs598418 (p = 0.017) and rs44707/ST+4 (p = 0.045). No statistically significant differences in allele and genotype frequencies between high and the low severity groups were observed.

Haplotype association analysis

The frequency of the haplotype CT at the SNPs rs528557/S2 and rs598418 of asthmatic patients was significantly higher than that of controls (p = 0.036), as shown in Table 5A. Fig. 1 shows that these two SNPs are in strong LD (D' = 0.93995). At the SNPs rs598418 and rs44707/ST+4, a significantly higher frequency of the haplotype CC in the control group when compared to the high severity group was observed (p = 0.046) whereas the haplotype TA was demonstrated to be an at-risk haplotype for the high severity group (p = 0.046), as shown in Table 5B. The SNPs rs598418 and rs44707/ST+4 are also in strong LD (D' = 0.98251) (Fig. 1). No significant differences in the haplotype frequencies between the low severity group and the control group were observed.

Table 3 Genotype description for *ADAM33* SNPs in a Thai population

SNP ID	SNP type	Genotype, case (n = 200)/control (n = 100)		
		Homozygous wild-type	Heterozygous	Homozygous variant
rs528557/S2	Exon	GG (114/72)	GC (77/21)	CC (9/7)
rs2853209	Intron	AA (67/42)	AT (106/45)	TT (27/13)
rs598418	Intron	CC (46/36)	CT (102/42)	TT (52/22)
rs44707/ST+4	Intron	CC (63/43)	CA (103/42)	AA (34/15)
rs597980	Intron	CC (91/53)	CT (97/38)	TT (12/9)
rs11905233	3′ UTR	GG (193/100)	GA (6/0)	AA (1/0)
rs2787094/V4	3′ UTR	CC (87/40)	CG (94/47)	GG (19/13)
rs3746631	3′ UTR	AA (163/88)	AG (37/12)	GG (0/0)

Table 4 Association of SNPs in *ADAM33*

SNP ID	Genetic model	Genotype	Allele	Case-control	High severity-control	Low severity-control
				p-value	*p*-value*	*p*-value*
rs528557/S2	Dominant	GC + CC GG		0.012	NS	0.012
rs598418	Dominant	CT + TT CC		0.017	0.017	NS
rs598418	Multiplicative		T C	0.049	NS	NS
rs44707/ST+4	Dominant	CA+AA CC		0.049	0.045	NS

*NS, non-significant.

DISCUSSION

We successfully amplified gene segments covering eight SNPs previously reported in other populations.[7] We found only three SNPs in the studied samples that matched those previously reported, i.e. rs528557/S2, rs44707/ST+4 and rs2787094/V4. The SNP rs528557/S2 exhibited significant association with asthma in a dominant model (p = 0.012) whereas the SNP rs44707/ST+4 was marginally associated to the disease according to a dominant model (p = 0.049). The SNP rs528557/S2 has been linked to asthma within UK,[6] German,[8] African-American, US Caucasian and US Hispanic[7] populations. The SNP rs44707/ST+4 was also linked to asthma in the UK and in combined US and UK populations.[6] The SNP rs598418 was associated with asthma in both multiplicative and dominant models (p = 0.049 and 0.017, respectively). Other SNPs, including rs2853209, rs597980, rs11905233 and rs3746631, were not significantly associated with the prevalence of asthma in this Thai population. However, there is a report on the SNP rs597980 as having a positive association with asthma and BHR in a German population.[8] Association between *ADAM33* SNPs and asthma has also been demonstrated in other Asian populations, such as Korean[9] and Japanese.[25,26] A meta-analysis study involving eight populations confirmed the positive association be-

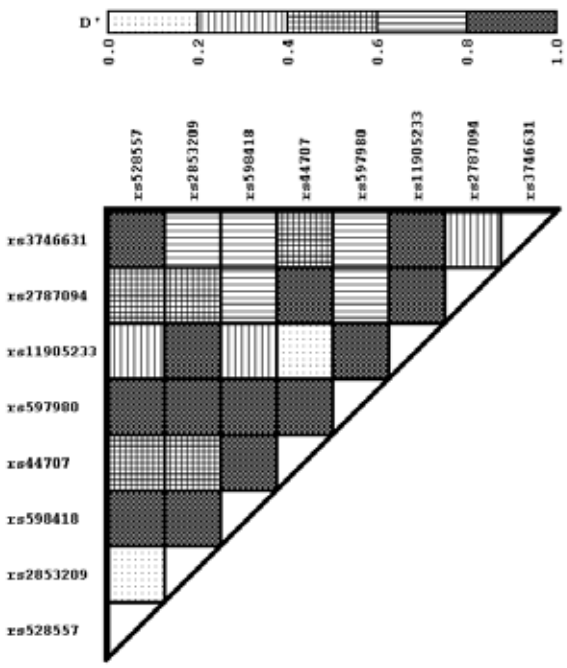

Fig. 1 Pair-wise linkage disequilibrium analysis in the 3' region of *ADAM33* estimated in a Thai population.

Table 5 Haplotype analysis of *ADAM33* (two-SNP haplotype)

A. Case *vs.* control (rs528557/S2 and rs598418)

Haplotype	Haplotype frequency			*p*-value*
	Control (n = 100)	**Case** (n = 200)	**Combined** (n = 300)	
GC	0.56	0.48	0.50	NS
GT	0.26	0.28	0.28	NS
CC	0.01	0.01	0.01	NS
CT	0.17	0.23	0.21	0.036 (dominant)

*NS, non-significant.

B. High severity *vs.* control (rs598418 and rs44707/ST+4)

Haplotype	Haplotype frequency			*p*-value*
	Control (n = 100)	**High severity** (n = 105)	**Combined** (n = 205)	
CC	0.57	0.49	0.53	0.046 (recessive)
CA	0.00	0.00	0.00	NS
TC	0.07	0.09	0.08	NS
TA	0.36	0.42	0.39	0.046 (joint dominant)

*NS, non-significant.

tween *ADAM33* polymorphisms and asthma susceptibility.[10] In contrast, a lack of association between *ADAM33* polymorphisms and asthma have been reported from ethnically different populations.[15-17] This may be influenced by sample sizes, heterogeneity of populations, and different environmental exposures. It is interesting to note that only the SNP rs528557/S2 is located on exon 19 while the other seven SNPs are located on intron 19 and 3' UTR. A previous study has detailed the mechanisms behind the regulation of the 3' UTR of *ADAM33* involving mRNA localization and processing, as well as protein maturation.[27]

Our study also demonstrated a significant association between *ADAM33* SNPs and asthma severity. When compared to the control group, the SNP rs528557/S2 was associated with the low severity group (p = 0.012) whereas the SNPs rs598418 and rs44707/ST+4 were associated with the high severity group (p = 0.017 and 0.045, respectively). Subjects in the high severity group were significantly older and suffered longer durations with asthma than those in the low severity group. This finding was in accordance with previous work revealing that lung function in asthmatic patients may decline over time.[28] Haplotypes can reveal information about hitherto unobserved predisposing variants in the region.[29] According to a dominant model, the haplotype CT at the SNPs rs528557/S2 and rs598418 was found to be a disease-predisposing variant in the Thai population. Upon comparison of the high severity and the control group, significant haplotypes were found at the SNPs rs598418 and rs44707/ST+4 (CC, p = 0.046; TA, p = 0.046). This result supports a previous study demonstrating significant global haplotypic association with asthma and asthma severity.[30] Notably, the χ^2 tests (with the application of Bonferroni correction to the single-marker analysis) failed to show any association of *ADAM33* and asthma in this study. Nevertheless, all reported p-values in our haplotype analysis were corrected by HAPSTAT[22-24] and the results confirmed statistical significance. This suggests that both single-marker and haplotype analyses should be performed in association studies, since haplotype-based methods incorporate linkage disequilibrium data from multiple markers and are more powerful for gene mapping than single SNP-based methods.

We did not find significant differences in the distribution of the SNPs and haplotypes between the high and low severity groups, possibly due to the rather small sample sizes. However, a cohort of 200 asthma patients monitored over a 20 year period showed that *ADAM33* SNPs could be linked to a decline in FEV_1, suggesting that the gene may not only affect asthma prevalence but also its severity and disease progression.[31] A potential role of *ADAM33* in asthma pathogenesis may involve the mRNA and protein expression in mesenchymal cells, airway smooth muscle cells and fibroblasts.[12,27] Previous studies have shown a link between *ADAM33* regulation and the expression of TGF-β, the important cytokine produced by bronchial epithelial cells for repair processes.[32] ADAM33 protein levels were significantly elevated in patients with asthma[14] and, in turn, would also involve BHR and airway remodeling.

It is possible that the Thai subjects in the present study are of other ethnic origins. However, we believe that our findings were not affected by population stratification, for two reasons. Firstly, all eight SNPs in control samples were in Hardy-Weinberg equilibrium. Secondly, all SNPs were in strong linkage disequilibrium. Previous studies in other ethnic groups including German,[8] Korean,[9] and Japanese[25] have also indicated that SNPs within the 3′ region of *ADAM33* gene are in strong linkage disequilibrium. The evidence that recombination does not occur frequently in this genomic region among various ethnic groups implies that if population stratification occurs, deviation from Hardy-Weinberg equilibrium would be easily detectable.

In summary, we have identified a positive relationship between *ADAM33* polymorphisms and asthma in a Thai population. The results are supportive of previous studies involving other populations worldwide. The precise roles of *ADAM33* gene in asthma pathogenesis, however, remain undefined and require further investigation.

ACKNOWLEDGEMENTS

This study was supported by a research grant from the Faculty of Medicine Siriraj Hospital, Mahidol University (to TT) and Mahidol Research Grant (to CL). AA was supported by the Thailand Research

Fund (TRF) through the Royal Golden Jubilee Ph.D. Program (Grant No. PHD/4.I.MU.45/C.1) and the National Center for Genetic Engineering and Biotechnology (BIOTEC), the National Science and Technology Development Agency (NSTDA). NC was supported by the Thailand Research Fund through the Research Career Development Grant (Grant No. RSA5180006).

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