

Genomewide association study identifies *HAS2* as a novel susceptibility gene for adult asthma in a Japanese population

Y. Yatagai¹, T. Sakamoto¹, H. Yamada¹, H. Masuko¹, Y. Kaneko¹, H. Iijima², T. Naito², E. Noguchi³, T. Hirota⁴, M. Tamari⁴, S. Konno⁵, M. Nishimura⁵, N. Hizawa¹

¹Department of Pulmonary Medicine, Faculty of Medicine, University of Tsukuba, Ibaraki, Japan

²Tsukuba Medical Center, Ibaraki, Japan

³Department of Medical Genetics, Faculty of Medicine, University of Tsukuba, Ibaraki, Japan

⁴Laboratory for Respiratory Diseases, Center for Genomic Medicine, The Institute of Physical and Chemical Research (RIKEN), Kanagawa, Japan

⁵First Department of Medicine, Hokkaido University School of Medicine, Hokkaido, Japan

Correspondence: Yohei Yatagai, Department of Pulmonary Medicine, Faculty of Medicine,

This article has been accepted for publication and undergone full peer review but has not been through the copyediting, typesetting, pagination and proofreading process, which may lead to differences between this version and the Version of Record. Please cite this article as doi: 10.1111/cea.12415

This article is protected by copyright. All rights reserved.

University of Tsukuba, Tennodai 1-1-1, Tsukuba, Ibaraki 305-8575, Japan

E-mail: s1130465@u.tsukuba.ac.jp

Running title: *HAS2* for susceptibility to asthma

Keywords: asthma, genomewide association study, hyaluronan, hyaluronan synthase 2

Summary

Background It is increasingly clear that asthma is not a single disease, but a disorder with vast heterogeneity in pathogenesis, severity, and treatment response. To date, 30 genomewide association studies (GWASs) of asthma have been performed, including by our group. However, most gene variants identified so far confer relatively small increments in risk and explain only a small proportion of familial clustering.

Objective To identify additional genetic determinants of susceptibility to asthma using a selected Japanese population with reduced tobacco smoking exposure.

Methods We performed a GWAS by genotyping a total of 480,098 single-nucleotide polymorphisms (SNPs) for a Japanese cohort consisting of 734 healthy controls and 240

asthma patients who had smoked for no more than 10 pack-years. The SNP with the strongest association was genotyped in 2 other independent Japanese cohorts consisting of a total of 531 healthy controls and 418 asthma patients who had smoked for no more than 10 pack-years. For the hyaluronan synthase 2 (*HAS2*) gene, we investigated SNP-gene associations using an expression quantitative trait loci (eQTL) database and also analyzed its gene expression profiles in 13 different normal tissues.

Results In the discovery GWAS, a SNP located upstream of *HAS2*, rs7846389, showed the strongest statistical significance ($P = 1.43 \times 10^{-7}$). In the 2 independent replication cohorts, rs7846389 was consistently associated with asthma (nominal $P = 0.0152$ and 0.0478 in the first and second replication cohorts, respectively). In the meta-analysis, association of rs7846389 with susceptibility to asthma reached the level of genomewide significance ($P = 7.92 \times 10^{-9}$). This variant was strongly correlated with *HAS2* mRNA expression. The strongest expression of the gene was detected in the lung.

Conclusions Our study identified *HAS2* as a novel candidate gene for susceptibility to adult asthma.

Introduction

A substantial proportion of individual differences in asthma susceptibility are known to be due to genetic factors; therefore, understanding this genetic variation may contribute to better prevention, diagnosis, and treatment of asthma. To date, more than 100 genes associated with asthma have been reported in 2 or more independent candidate gene association studies or demonstrated by genomewide association studies (GWASs) [1]. Our group also has identified 5 loci associated with susceptibility to adult asthma through a GWAS and a replication study consisting of 7171 individuals with adult asthma and 27,912 controls in a Japanese population [2].

Although these previous reports provided valuable information on the genetic architecture of asthma, most variants so far identified confer relatively small increments in risk, and they explain only a small proportion of familial clustering. Potential sources of this so-called missing heritability include the heterogeneity of asthma caused by complex interactions between multiple genetic and environmental factors [3]. Indeed, the extremely large numbers of patients used in recent genetic studies of asthma likely lead to a broad phenotypic variability and introduce substantial nongenetic heterogeneity into the analysis. Phenotypic filtering may reduce the number of phenocopies and perhaps the genetic heterogeneity, thereby increasing the power of a GWAS for a disease with heterogeneous

phenotypes such as asthma even if the sample size is much smaller than that of the original population [4]. Selecting more homogeneous phenotypes for GWAS, therefore, could lead to identification of novel genetic factors in the development of asthma. On the basis of this concept, we have been conducting genetic association studies and have reported associations between polymorphisms of several candidate genes and specific asthma phenotypes [5-7].

Smoking is a significant environmental risk factor for the development of asthma [8]. Several studies have indicated that the effects of asthma susceptibility genes were influenced by smoking behavior [9-12]. Furthermore, a genomewide linkage analysis of asthma clearly showed that the presence or absence of exposure to tobacco smoke strongly modified the genetic impacts of many loci on the development of asthma [13]. Therefore, given that our original GWAS did not take smoking status into consideration [2], in the present study, we performed a GWAS of susceptibility to asthma by limiting participants to those with a smoking history of no more than 10 pack-years.

Methods

Ethics statement

This study was approved by the Human Genome Analysis and Epidemiology Research Ethics Committee of the University of Tsukuba and by the Human Genome/Gene Analysis Research Ethics Review Committees of the Tsukuba Medical Center, RIKEN, and the Hokkaido

This article is protected by copyright. All rights reserved.

University School of Medicine. Written informed consent was obtained from each participant before the investigation in accordance with the principles of the Declaration of Helsinki.

Study participants

The discovery cohort (Tsukuba cohort), consisting of 974 individuals of Japanese ethnicity (734 healthy volunteers and 240 asthma patients), is described in detail elsewhere [14]. Briefly, we recruited healthy adults without respiratory diseases such as asthma and COPD from the general population who visited the Tsukuba Medical Center for an annual health checkup [15]. The asthma patients were recruited from the University of Tsukuba Hospital [2]. Asthma was diagnosed by pulmonary physicians according to the American Thoracic Society criteria, as previously described [16]. In the present study, we excluded individuals with a smoking history of more than 10 pack-years to eliminate the environmental effects of smoking. Specific serum IgE antibody was measured using the multiple allergen simultaneous test (MAST)-26 chemiluminescent assay [17]. We defined atopy as a positive response (> 4.40 lumicount) to at least 1 of 14 common inhaled allergens [14]. All asthma patients in the discovery cohort were included in our original GWAS [2].

For the replication studies, we analyzed 2 independent Japanese cohorts (Tsukuba and Hokkaido replication cohorts). Both of these 2 cohorts were not included in our original

GWAS [2]. We excluded individuals with a smoking history of more than 10 pack-years from both cohorts. The Tsukuba replication cohort consisted of 351 healthy adults without respiratory diseases and 277 adult patients with asthma. The healthy adults were also recruited from the general population who visited the Tsukuba Medical Center for an annual health checkup [15]. The asthma patients were originally recruited from the University of Tsukuba Hospital for phenotypic and genetic analyses of asthma [18]. The Hokkaido replication cohort consisted of 180 healthy adults and 141 adult patients with asthma recruited at the Hokkaido University Hospital. This population was originally recruited for a case-control genetic association study of asthma and atopy in Japan [14].

Genotyping

Genomic DNA was extracted from whole blood by an automated DNA extraction system (QuickGene-610L; Fujifilm, Tokyo, Japan). Genotyping of the Tsukuba cohort was performed using Illumina HumanHap 550v3 / 610-Quad BeadChips (Illumina, San Diego, CA, USA) as described previously [14]. Quality control (QC) checks for the SNPs were performed using PLINK version 1.07 software [19]. We excluded individuals with a missing genotype rate > 0.02. We excluded SNPs with a missing genotype rate > 0.01, minor allele frequency < 0.01, or Hardy-Weinberg equilibrium P value < 1.0×10^{-6} .

In the replication analyses, genotyping of rs7846389 for each participant was performed using predesigned TaqMan[®] allele-specific polymerase chain reaction (PCR) assays (Applied Biosystems, Foster City, CA, USA).

Gene expression analysis

We investigated SNP-gene associations by investigating expression quantitative trait loci (eQTL) using the Genevar (GENe Expression VARiation) database (<http://www.sanger.ac.uk/resources/software/genevar/>) [20]. A database of lymphoblastoid cell lines from 82 HapMap3 JPT (Japanese in Tokyo) individuals [21] was used. The probe used for hyaluronan synthase 2 (*HAS2*) expression analysis was ILMN_1777562 on Illumina HumanWG 6 v3 (Illumina). Correlation between the number of the rs7846389 allele and the *HAS2* mRNA levels was studied by simple linear regression analysis.

HAS2 expression was evaluated in 13 human tissues. Total RNA of 12 tissues (bone marrow, whole brain, heart, kidney, liver, whole lung, trachea, skeletal muscle, spleen, thymus, small intestine, and peripheral leukocytes) was purchased from Clontech (Mountain View, CA, USA). Total RNA of skin was purchased from Biochain (Newark, CA, USA). cDNA was synthesized with SuperScript[®] III Reverse Transcriptase (Life Technologies Japan, Tokyo, Japan) according to the manufacturer's instructions. Real-time quantitative PCR for

HAS2 was performed using SYBR[®] premix II Ex Taq[™] (Takara Bio, Shiga, Japan) according to the manufacturer's instructions and with the following primers: 5'-CTCCGGGACCACACAGAC-3' (forward) and 5'-TCAGGATACATAGAAACCTCTCACA-3' (reverse). Fold changes were normalized to glyceraldehyde-3-phosphate dehydrogenase.

Statistical analysis

In the discovery Tsukuba cohort, QC-filtered SNPs were estimated for association with asthma affection status by using the Cochran–Armitage trend test in PLINK version 1.07 [19]. This test considers only diploid genotypes. Odds ratios (ORs) and 95% confidence intervals (CIs) were calculated in a 2 x 2 allele frequency table. The genomic inflation factor and quantile-quantile (Q-Q) plots were calculated by the multidimensional scaling method in PLINK version 1.07. In the replication studies, the association was examined by the same methods as for the discovery cohort. Replication was confirmed if $P < 0.05$ and if the direction of the effect was the same as in the discovery GWAS. Combined analysis of the discovery GWAS with the replication studies was performed by the Mantel-Haenszel method. The fixed-effect meta-analysis P value was estimated.

Results

Characteristics of the study cohorts

The characteristics of the discovery Tsukuba cohort and of the Tsukuba and Hokkaido replication cohorts are shown in Table 1. Compared with the control groups, lower pulmonary function and higher levels of total serum IgE were consistently noted in the case groups.

GWAS and replication study

After QC filtering, none of the participants was removed from the study, and we retained 480,098 SNPs for further association analysis. The genomic inflation factor λ was 1.024, indicating a low possibility of type I error. The Q-Q plot is shown in Figure 1. Figure 2 shows Manhattan plots of the distribution of $-\log_{10}(P \text{ values})$ across chromosomes; SNPs with a P value $< 1 \times 10^{-6}$ were detected only on chromosome 8 as a single peak. The fine plots in this genomic region (chromosome 8q24) obtained using LocusZoom (<http://csg.sph.umich.edu/locuszoom/>) [22] demonstrated that a SNP located upstream of *HAS2*, rs7846389, had the strongest statistical significance ($P = 1.43 \times 10^{-7}$) (Fig. 3). Because the functional relevance of *HAS2* makes it a good candidate for asthma susceptibility, we focused solely on this SNP in the subsequent analysis. The rs7846389 major A allele was significantly associated with increased risk of asthma (OR = 1.74; 95% CI: 1.41 - 2.15) (Table 2). In both the Tsukuba and the Hokkaido replication cohorts, the rs7846389 A allele

was significantly associated with asthma (Cochran-Armitage trend test, $P = 0.0152$ and 0.0478 ; OR = 1.31 and 1.38; 95% CI: 1.05 - 1.65 and 1.01 - 1.90, respectively). Meta-analysis of the discovery and replication cohorts demonstrated that rs7846389 was associated with asthma at the level of genomewide significance (Mantel-Haenszel test, $P = 7.92 \times 10^{-9}$; OR = 1.50; CI: 1.31 - 1.72).

HAS2 expression

As rs7846389 was associated with susceptibility to asthma and is located upstream of *HAS2*, we investigated the functional significance of rs7846389 in *HAS2* expression by using the mRNA expression profiling data of lymphoblastoid cell lines from HapMap3 JPT individuals. *HAS2* mRNA expression was significantly correlated with the number of nonsusceptible alleles C of rs7846389 ($r = 0.291$; $P = 0.0081$) (Fig. 4). The SNP with the strongest association, rs279661 ($P = 0.0074$) (Fig. 5), was in tight linkage disequilibrium with rs7846389 ($r^2 = 0.965$). These findings suggest that allele C of rs7846389 is correlated with increased expression of *HAS2*, which is associated with decreased risk of asthma. Therefore, we analyzed the mRNA expression of *HAS2* in 13 human tissues (Fig. 6). The strongest *HAS2* expression was detected in the lung.

Discussion

Applying a GWAS to a well-characterized Japanese population, we have identified *HAS2* as a novel candidate gene for susceptibility to adult asthma. Although subanalyses of multiple populations from within large GWASs will find many signals that will probably prove to be artifacts, we thought that the top SNP identified in the current study, very close to *HAS2*, was worth studying for further replication because of its functional importance in relation to asthma [23, 24]. In the current study, to increase the validity of our finding, we also investigated SNP-gene associations using an eQTL database and also analyzed the *HAS2* expression profiles in 13 different normal tissues, both of which studies yielded results supporting the importance of *HAS2* in asthma pathogenesis.

To date, 30 GWASs of susceptibility to asthma in several different ethnicities have reported more than 50 candidate genes (Catalog of Published Genome-Wide Association Studies; <http://www.genome.gov/26525384>). However, none of these studies has identified *HAS2* as a gene linked to asthma susceptibility. Exposure to tobacco smoke increases asthma risk [8]. The prevalence of smokers among adult patients with asthma is similar to that among the general population and smoking asthma has been shown to be a distinct asthma phenotype [25]. Genomewide linkage analysis has clearly shown that the presence or absence of exposure to tobacco smoke strongly modified the genetic impacts of many loci on the

Accepted Article

development of airway hyperresponsiveness and asthma [13]. Therefore, given that the phenotypic heterogeneity of asthma caused by smoking could contribute to the problematic issue of identifying genetic determinants, in the present study, we studied only participants with a smoking history of no more than 10 pack-years. Apparently, studying a rather homogenous population as we did in this study did increase the power to identify the influence of *HAS2* on asthma.

HAS2 is a member of the gene family encoding hyaluronan (HA) synthases. HA is synthesized by HAS1, 2, and 3 on the cytosolic side of the cell membrane and is translocated across the membrane to the interstitial space [26]. HA is a linear nonsulfated glycosaminoglycan and a widely distributed component of the extracellular matrix. HA exists in either a high molecular weight (HMW) form (100 - 600 kDa) or a low molecular weight (LMW) form (10 - 50 kDa). HMW HA is synthesized by HAS1 and 2, whereas LMW HA is produced by direct synthesis by HAS3 [27] or by enzymatic degradation of HMW HA. The biological functions of HA strongly depend on its molecular size. HMW HA has a variety of regulatory functions including the anti-inflammatory response [28-30] and antiangiogenesis [31], as well as a variety of structural functions such as tissue repair [32] and joint lubrication [33]. In contrast, LMW HA induces the proinflammatory response in various kinds of cells including macrophages, T cells, and epithelial cells [34, 35]. LMW HA activates the innate

immune response upon tissue damage and acts as an endogenous danger signal [36]. Klagas et al demonstrated that under the inflammatory condition, human airway smooth muscle cells (ASMCs) from asthma patients produced less HA than did those from healthy individuals [23]. This reduction of HA in ASMCs from asthma patients was due to decreased levels of HAS1 and HAS2 and to increased levels of hyaluronidase-1. Importantly, HA isolated from the ASMCs of the healthy individuals was an HMW form (> 700 kDa), whereas HA isolated from the ASMCs of the asthma patients was an LMW form. Furthermore, it has been shown that HMW HA inhibits proliferation of human ASMCs [37]. Monzón et al revealed increased levels of hyaluronidases in the bronchial epithelia of asthma patients as compared with those of healthy controls and suggested that HA degradation might initiate inflammatory responses in asthma patients [24]. Taken together, these findings indicate that in the lungs of asthma patients, both decreased HMW HA and increased LMW HA contribute to asthmatic airway inflammation. This contention is in line with our findings that the A allele of rs7846389 is significantly associated with both decreased expression of *HAS2* and increased asthma risk.

Several points need further clarification. First, we previously performed a GWAS consisting of 7171 adult asthma patients and 27,192 healthy controls in a Japanese population for susceptibility to asthma and identified 5 loci with *P* values indicative of genomewide significance [2]. Although all asthma patients in the discovery cohort of the present study

Accepted Article

were included in that previous GWAS, association of rs7846389 with asthma was not detected then (nominal $P > 0.05$). We believe that this inconsistency is mainly attributable to the confounding effects of environmental tobacco exposure on the effects of *HAS2*. Since precise information of smoking status in our previous GWAS was available only in the Tsukuba cohort, an association study between *HAS2* and asthma using the whole GWAS population without heavy smokers could not be performed. Second, although rs7846389 (or rs279661, which is very close to rs7846389) might have promoter activity for *HAS2*, the molecular mechanisms underlying the significant correlation between *HAS2* expression and rs7846389 need to be investigated because these SNPs are located more than 200 kb upstream of *HAS2*.

In conclusion, using a GWAS, we found a genetic variant near *HAS2* that is a candidate gene for susceptibility to asthma in an adult Japanese population. The mechanisms underlying the strong association between adult asthma and *HAS2* should be further investigated especially in the context of gene-gene and gene-environment interactions. Based on the HapMap database [38], the variant rs7846389 is detected across different ethnicities. Further replication of our finding in different ethnic groups may also help us to understand the importance of *HAS2* on the expression of asthma and asthma-related phenotypes.

Acknowledgements

We would like to thank Ms Flaminia Miyamasu, an associate professor at the Faculty of Medicine, University of Tsukuba, who proofread and commented on this paper. We also thank Ms Takako Nakamura for technical assistance with genotyping.

Funding sources

This study was partly supported by a Grant-in-Aid for Scientific Research (B) (No. 24390206) from the Japan Society for the Promotion of Science. The funders had no role in the study design, data collection and analysis, decision to publish, or preparation of the manuscript. No additional external funding was received for this study.

Conflict of interest

The authors have no conflict of interest to declare.

References

1. Kaneko Y, Yatagai Y, Yamada H, Iijima H, Masuko H, Sakamoto T, Hizawa N, The search for common pathways underlying asthma and COPD. *Int J Chron Obstruct Pulmon Dis* 2013;8: 65-78.
2. Hirota T, Takahashi A, Kubo M, Tsunoda T, Tomita K, Doi S, Fujita K, Miyatake A,

- Enomoto T, Miyagawa T, Adachi M, Tanaka H, Niimi A, Matsumoto H, Ito I, Masuko H, Sakamoto T, Hizawa N, Taniguchi M, Lima JJ, Irvin CG, Peters SP, Himes BE, Litonjua AA, Tantisira KG, Weiss ST, Kamatani N, Nakamura Y, Tamari M, Genome-wide association study identifies three new susceptibility loci for adult asthma in the Japanese population. *Nat Genet* 2011;43: 893-6.
3. Manolio TA, Brooks LD, Collins FS, A HapMap harvest of insights into the genetics of common disease. *J Clin Invest* 2008;118: 1590-605.
 4. Bonnelykke K, Sleiman P, Nielsen K, Kreiner-Moller E, Mercader JM, Belgrave D, den Dekker HT, Husby A, Sevelsted A, Faura-Tellez G, Mortensen LJ, Paternoster L, Flaaten R, Molgaard A, Smart DE, Thomsen PF, Rasmussen MA, Bonas-Guarch S, Holst C, Nohr EA, Yadav R, March ME, Blicher T, Lackie PM, Jaddoe VW, Simpson A, Holloway JW, Duijts L, Custovic A, Davies DE, Torrents D, Gupta R, Hollegaard MV, Hougaard DM, Hakonarson H, Bisgaard H, A genome-wide association study identifies CDHR3 as a susceptibility locus for early childhood asthma with severe exacerbations. *Nat Genet* 2014;46: 51-5.
 5. Osawa R, Konno S, Akiyama M, Nemoto-Hasebe I, Nomura T, Nomura Y, Abe R, Sandilands A, McLean WH, Hizawa N, Nishimura M, Shimizu H, Japanese-specific filaggrin gene mutations in Japanese patients suffering from atopic eczema and asthma. *J Invest Dermatol* 2010;130: 2834-6.

- Accepted Article
6. Isada A, Konno S, Hizawa N, Tamari M, Hirota T, Harada M, Maeda Y, Hattori T, Takahashi A, Nishimura M, A functional polymorphism (-603A --> G) in the tissue factor gene promoter is associated with adult-onset asthma. *J Hum Genet* 2010;55: 167-74.
 7. Hizawa N, Yamaguchi E, Konno S, Tanino Y, Jinushi E, Nishimura M, A functional polymorphism in the RANTES gene promoter is associated with the development of late-onset asthma. *Am J Respir Crit Care Med* 2002;166: 686-90.
 8. Pietinalho A, Pelkonen A, Ryttilä P, Linkage between smoking and asthma. *Allergy* 2009;64: 1722-7.
 9. Ierodiakonou D, Postma DS, Koppelman GH, Gerritsen J, ten Hacken NH, Timens W, Boezen HM, Vonk JM, TGF-beta1 polymorphisms and asthma severity, airway inflammation, and remodeling. *J Allergy Clin Immunol* 2013;131: 582-5.
 10. Liu M, Rogers L, Cheng Q, Shao Y, Fernandez-Beros ME, Hirschhorn JN, Lyon HN, Gajdos ZK, Vedantam S, Gregersen P, Seldin MF, Bleck B, Ramasamy A, Hartikainen AL, Jarvelin MR, Kuokkanen M, Laitinen T, Eriksson J, Lehtimäki T, Raitakari OT, Reibman J, Genetic variants of TSLP and asthma in an admixed urban population. *PLoS One* 2011;6: e25099.
 11. Zhang G, Hayden CM, Khoo SK, Candelaria P, Laing IA, Turner S, Franklin P, Stick S, Landau L, Goldblatt J, Le Souef PN, Beta2-Adrenoceptor polymorphisms and

- asthma phenotypes: interactions with passive smoking. *Eur Respir J* 2007;30: 48-55.
12. Bouzigon E, Corda E, Aschard H, Dizier MH, Boland A, Bousquet J, Chateigner N, Gormand F, Just J, Le Moual N, Scheinmann P, Siroux V, Vervloet D, Zelenika D, Pin I, Kauffmann F, Lathrop M, Demenais F, Effect of 17q21 variants and smoking exposure in early-onset asthma. *N Engl J Med* 2008;359: 1985-94.
13. Meyers DA, Postma DS, Stine OC, Koppelman GH, Ampleford EJ, Jongepier H, Howard TD, Bleecker ER, Genome screen for asthma and bronchial hyperresponsiveness: interactions with passive smoke exposure. *J Allergy Clin Immunol* 2005;115: 1169-75.
14. Yatagai Y, Sakamoto T, Masuko H, Kaneko Y, Yamada H, Iijima H, Naito T, Noguchi E, Hirota T, Tamari M, Imoto Y, Tokunaga T, Fujieda S, Konno S, Nishimura M, Hizawa N, Genome-Wide Association Study for Levels of Total Serum IgE Identifies HLA-C in a Japanese Population. *PLoS One* 2013;8: e80941.
15. Masuko H, Sakamoto T, Kaneko Y, Iijima H, Naito T, Noguchi E, Hirota T, Tamari M, Hizawa N, Lower FEV1 in non-COPD, nonasthmatic subjects: association with smoking, annual decline in FEV1, total IgE levels, and TSLP genotypes. *Int J Chron Obstruct Pulmon Dis* 2011;6: 181-9.
16. Harada M, Obara K, Hirota T, Yoshimoto T, Hitomi Y, Sakashita M, Doi S, Miyatake A, Fujita K, Enomoto T, Taniguchi M, Higashi N, Fukutomi Y, Nakanishi K,

- Nakamura Y, Tamari M, A functional polymorphism in IL-18 is associated with severity of bronchial asthma. *Am J Respir Crit Care Med* 2009;180: 1048-55.
17. Miller SP, Marinkovich VA, Riege DH, Sell WJ, Baker DL, Eldredge NT, Dyminski JW, Hale RG, Peisach JM, Burd JF, Application of the MAST Immunodiagnostic System to the determination of allergen-specific IgE. *Clin Chem* 1984;30: 1467-72.
18. Kaneko Y, Masuko H, Sakamoto T, Iijima H, Naito T, Yatagai Y, Yamada H, Konno S, Nishimura M, Noguchi E, Hizawa N, Asthma phenotypes in Japanese adults - their associations with the CCL5 and ADRB2 genotypes. *Allergol Int* 2013;62: 113-21.
19. Purcell S, Neale B, Todd-Brown K, Thomas L, Ferreira MA, Bender D, Maller J, Sklar P, de Bakker PI, Daly MJ, Sham PC, PLINK: a tool set for whole-genome association and population-based linkage analyses. *Am J Hum Genet* 2007;81: 559-75.
20. Yang TP, Beazley C, Montgomery SB, Dimas AS, Gutierrez-Arcelus M, Stranger BE, Deloukas P, Dermitzakis ET, Genevar: a database and Java application for the analysis and visualization of SNP-gene associations in eQTL studies. *Bioinformatics* 2010;26: 2474-6.
21. Stranger BE, Montgomery SB, Dimas AS, Parts L, Stegle O, Ingle CE, Sekowska M, Smith GD, Evans D, Gutierrez-Arcelus M, Price A, Raj T, Nisbett J, Nica AC, Beazley C, Durbin R, Deloukas P, Dermitzakis ET, Patterns of cis regulatory variation

- in diverse human populations. PLoS Genet 2012;8: e1002639.
22. Pruim RJ, Welch RP, Sanna S, Teslovich TM, Chines PS, Gliedt TP, Boehnke M, Abecasis GR, Willer CJ, LocusZoom: regional visualization of genome-wide association scan results. Bioinformatics 2010;26: 2336-7.
23. Klagas I, Goulet S, Karakiulakis G, Zhong J, Baraket M, Black JL, Papakonstantinou E, Roth M, Decreased hyaluronan in airway smooth muscle cells from patients with asthma and COPD. Eur Respir J 2009;34: 616-28.
24. Monzón ME, Manzanares D, Schmid N, Casalino-Matsuda SM, Forteza RM, Hyaluronidase expression and activity is regulated by pro-inflammatory cytokines in human airway epithelial cells. Am J Respir Cell Mol Biol 2008;39: 289-95.
25. Kim TB, Jang AS, Kwon HS, Park JS, Chang YS, Cho SH, Choi BW, Park JW, Nam DH, Yoon HJ, Cho YJ, Moon HB, Cho YS, Park CS, Identification of asthma clusters in two independent Korean adult asthma cohorts. Eur Respir J 2013;41: 1308-14.
26. Philipson LH, Schwartz NB, Subcellular localization of hyaluronate synthetase in oligodendrogloma cells. J Biol Chem 1984;259: 5017-23.
27. Itano N, Sawai T, Yoshida M, Lenas P, Yamada Y, Imagawa M, Shinomura T, Hamaguchi M, Yoshida Y, Ohnuki Y, Miyauchi S, Spicer AP, McDonald JA, Kimata K, Three isoforms of mammalian hyaluronan synthases have distinct enzymatic properties. J Biol Chem 1999;274: 25085-92.

- Accepted Article
28. Campo GM, Avenoso A, Nastasi G, Micali A, Prestipino V, Vaccaro M, D'Ascola A, Calatroni A, Campo S, Hyaluronan reduces inflammation in experimental arthritis by modulating TLR-2 and TLR-4 cartilage expression. *Biochim Biophys Acta* 2011;1812: 1170-81.
 29. Bollyky PL, Lord JD, Masewicz SA, Evanko SP, Buckner JH, Wight TN, Nepom GT, Cutting edge: high molecular weight hyaluronan promotes the suppressive effects of CD4+CD25+ regulatory T cells. *J Immunol* 2007;179: 744-7.
 30. Day AJ, de la Motte CA, Hyaluronan cross-linking: a protective mechanism in inflammation? *Trends Immunol* 2005;26: 637-43.
 31. Deed R, Rooney P, Kumar P, Norton JD, Smith J, Freemont AJ, Kumar S, Early-response gene signalling is induced by angiogenic oligosaccharides of hyaluronan in endothelial cells. Inhibition by non-angiogenic, high-molecular-weight hyaluronan. *Int J Cancer* 1997;71: 251-6.
 32. Noble PW, Hyaluronan and its catabolic products in tissue injury and repair. *Matrix Biol* 2002;21: 25-9.
 33. Wang M, Liu C, Thormann E, Dedinaite A, Hyaluronan and phospholipid association in biolubrication. *Biomacromolecules* 2013;14: 4198-206.
 34. McKee CM, Penno MB, Cowman M, Burdick MD, Strieter RM, Bao C, Noble PW, Hyaluronan (HA) fragments induce chemokine gene expression in alveolar

- macrophages. The role of HA size and CD44. *J Clin Invest* 1996;98: 2403-13.
35. Boodoo S, Spannhake EW, Powell JD, Horton MR, Differential regulation of hyaluronan-induced IL-8 and IP-10 in airway epithelial cells. *Am J Physiol Lung Cell Mol Physiol* 2006;291: L479-86.
36. Scheibner KA, Lutz MA, Boodoo S, Fenton MJ, Powell JD, Horton MR, Hyaluronan fragments act as an endogenous danger signal by engaging TLR2. *J Immunol* 2006;177: 1272-81.
37. Kanabar V, Hirst SJ, O'Connor BJ, Page CP, Some structural determinants of the antiproliferative effect of heparin-like molecules on human airway smooth muscle. *Br J Pharmacol* 2005;146: 370-7.
38. The International HapMap Project. *Nature* 2003;426: 789-96.

Figure legends

Fig. 1. Quantile-quantile (Q-Q) plot of observed versus expected *P* values of the GWAS results. The straight line in the Q-Q plot indicates the distribution of SNPs under the null hypothesis.

Fig. 2. Manhattan plots of the distribution of $-\log_{10}$ (P values) for association of 480,098 SNPs with susceptibility to asthma. P values were estimated using the Cochran–Armitage trend test. The x-axis represents the chromosomal position.

Fig. 3. Fine genetic association plots for susceptibility to asthma in the 8q24 chromosomal region. The rs7846389 SNP, located upstream of *HAS2*, showed the strongest statistical significance ($P = 1.43 \times 10^{-7}$).

Fig. 4. Association of the rs7846389 genotype with *HAS2* mRNA levels. *HAS2* mRNA expression was significantly correlated with the number of nonsusceptibility C alleles of rs7846389.

Fig. 5. Fine plots of the distribution of $-\log_{10}$ (P values) for association of SNPs with *HAS2* mRNA expression levels. Rs279661, close to rs7846389, showed the strongest statistical significance ($P = 0.0074$). These 2 SNPs were in tight linkage disequilibrium ($r^2 = 0.965$).

Fig. 6. *HAS2* mRNA expression in 13 different kinds of normal human tissues. Fold changes were normalized to glyceraldehyde-3-phosphate dehydrogenase (*GAPDH*). The strongest expression was detected in the lung.

Table 1. Characteristics of the study cohorts

	Asthma Cases	Controls
Tsukuba cohort (N = 974)		
Number of samples	240	734
Female sex	59.2%	68.0%
Age, y (mean $\pm$ SD)	51.2 $\pm$ 15.1	49.3 $\pm$ 9.3
Smoking status		
Never-smoker	82.5%	82.7%
0 < pack-years $\leq$ 10	17.5%	17.3%
FEV ₁ % predicted (mean $\pm$ SD)	89.9 $\pm$ 20.1%	94.6 $\pm$ 12.3%
FEV ₁ /FVC (mean $\pm$ SD)	74.0 $\pm$ 11.2%	83.7 $\pm$ 5.3%
Atopy ^a	70.4%	56.1%
Serum IgE, log (mean $\pm$ SD)	2.23 $\pm$ 0.61 IU/mL	1.68 $\pm$ 0.55 IU/mL
Tsukuba replication cohort (N = 628)		
Number of samples	277	351
Female sex	74.7%	65.0%
Age, y (mean $\pm$ SD)	58.6 $\pm$ 18.1	49.3 $\pm$ 9.9
Smoking status		
Never-smoker	83.0%	82.6%
0 < pack-years $\leq$ 10	17.0%	17.4%
FEV ₁ % predicted (mean $\pm$ SD)	83.9 $\pm$ 18.2%	91.4 $\pm$ 12.5%
FEV ₁ /FVC (mean $\pm$ SD)	70.6 $\pm$ 11.1%	82.9 $\pm$ 6.3%
Atopy ^a	73.0%	68.4%
Serum IgE, log (mean $\pm$ SD)	2.16 $\pm$ 0.65 IU/mL	1.92 $\pm$ 0.62 IU/mL
Hokkaido replication cohort (N = 321)		
Number of samples	141	180
Female sex	78.7%	64.4%
Age, y (mean $\pm$ SD)	58.6 $\pm$ 15.0	48.8 $\pm$ 8.7
Smoking status		
Never-smoker	68.8%	83.9%
0 < pack-years $\leq$ 10	31.2%	16.1%
FEV ₁ % predicted (mean $\pm$ SD)	104.5 $\pm$ 21.7%	106.0 $\pm$ 13.7%
FEV ₁ /FVC (mean $\pm$ SD)	71.0 $\pm$ 12.4%	83.9 $\pm$ 6.5%
Atopy ^a	70.2%	53.3%
Serum IgE, log (mean $\pm$ SD)	2.17 $\pm$ 0.71	1.67 $\pm$ 0.65

^aAtopy was defined as a specific positive IgE response (> 4.40

lumicount) to at least 1 of 14 common inhaled allergens obtained with the multiple allergen simultaneous test (MAST)-26 chemiluminescent assay [14].

FEV₁, forced expiratory volume in 1 second; FVC, forced vital capacity.

Table 2. Results of the discovery GWAS and replication studies

SNP	Allele (risk allele)	Sample	Asthma Cases		Controls		<i>P</i> value	OR (95% CI) ^b
			Total	RAF	Total	RAF		
rs7846389	A/C (A)	Tsukuba cohort	240	0.62	734	0.48	1.43E-07 ^a	1.74 (1.41 - 2.15)
		Tsukuba replication cohort	277	0.57	351	0.50	0.0152 ^a	1.31 (1.05 - 1.65)
		Hokkaido replication cohort	141	0.60	180	0.52	0.0478 ^a	1.38 (1.01 - 1.90)
		Combined	658	0.59	1265	0.49	7.92E-09	1.50 (1.31 - 1.72)

RAF, risk allele frequency; OR, odds ratio; CI, confidence interval.

^aCochran-Armitage trend test.

^bORs and 95% CIs were calculated using a nonsusceptibility allele as the reference.





