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Therapeutic implication of genetic variants of *IL13* and *STAT4* in airway remodelling with bronchial asthma

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Summary

Background Several gene variants identified in bronchial asthmatic patients are associated with a decrease in pulmonary function. The effects of this intervention on pulmonary function have not been fully researched.

Objective We determined the effects of high-dose inhaled corticosteroids (ICSs) on decreased pulmonary function in asthmatic Japanese patients with variants of *IL13* and *STAT4* during long-term treatments with low to mild doses of ICS.

Methods In this study, 411 patients with bronchial asthma who were receiving ICSs and living in Japan were recruited, were genotyped, and underwent pulmonary function tests and fiberoptic examinations. The effects of two years of high-dose ICSs administered to asthmatic patients who were homozygous for *IL13* AA of rs20541 or *STAT4* TT of rs925847 and who progressed to airway remodelling were investigated.

Results High-dose ICS treatment increased the pulmonary function of patients homozygous for *IL13* AA of rs20541 but not of patients homozygous for *STAT4* TT of rs925847. The increased concentrations of the mediators IL23, IL11, GM-CSF, hyaluronic acid, IL24, and CCL8 in bronchial lavage fluid (BLF) were diminished after high-dose ICS treatment in patients homozygous for *IL13* AA of rs20541.

Conclusion and Clinical Relevance *IL13* AA of rs20541 and *STAT4* TT of rs925847 are potential genomic biomarkers for predicting lower pulmonary function. The administration of high-dose ICSs to asthmatic patients with genetic variants of *IL13* AA may inhibit the advancement of airway remodelling. The genetic variants of *STAT4* TT did not respond to high-dose ICSs. Therefore, using medications other than ICSs must be considered even during the initial treatment of bronchial asthma. These genetic variants may aid in the realization of personalized and phenotype-specific therapies for bronchial asthma.

Clinical trial registered with
https://dbcentre3.jmacct.med.or.jp/JMACTR/Default_Eng.aspx (JMA-IIA00045
IL13STAT4-ICS).

Keywords asthma, genetic variation, SNP, IL13, STAT4, bronchial biopsy, airway remodelling

Introduction

Bronchial asthma is a disorder of the conducting airways that leads to variable airflow obstructions in association with airway hyperresponsiveness. Bronchial asthma exhibits a wide clinical spectrum that ranges from mild disease to a severe and intractable state. The severe form of bronchial asthma is characterized by a significant loss of lung function and by a frequent dependence on high-dose inhaled corticosteroids (ICSs). The observed airway obstruction in bronchial asthma is thought to be secondary to airway remodelling [1]. The histopathological features of this process include epithelial changes, subepithelial basement membrane thickening, inflammatory cell infiltration, bronchial smooth muscle hypertrophy, and mucus hypersecretion [2-3]. Interleukin 13 (IL13) promotes IgE isotype switching by B cells and is involved in the recruitment of inflammatory cells, including T cells and eosinophils. IL13 is a major effector of Th2 inflammation and tissue remodelling [4]. Many recent studies confirmed that single nucleotide polymorphisms (SNPs) in the *IL13* promoter and coding regions influence baseline lung function and airway remodelling in asthmatics [5-9]. SNPs in the *IL13* and *IL4Ra* genes were associated with lung function in a study of 264 African Americans with asthma and healthy control subjects [5]. We demonstrated an association between a variant of the *IL13* gene (rs20541 G/A: major allele is G), in which an arginine residue at amino acid 110 is substituted with glutamine, and pulmonary function and hyperresponsiveness in Japanese people [7-8]. We identified increases in IL13, IL23, IL11, GM-CSF, hyaluronic acid and CCL8 in bronchial lavage fluid (BLF) from *IL13* homozygotes. Airway remodelling is also induced by asthma exacerbation, which may be initiated by a respiratory tract virus infection. We may assume that weaker Th1 responses to viral infections are associated with asthma exacerbation and suggest that viral infections exacerbate pre-existing Th2-dominated lung diseases [10-11]. We associated this possibility with data that showing that decreased IFN- γ production in response to viral infection of monocytes at birth predicts the susceptibility to respiratory tract illness during the first year of life. A study of acute asthma exacerbation in children demonstrated that the expression of genes involved in Th1 pathways decreases during exacerbation, which leads to deficits in baseline lung function [12]. Genetic variants in Th1 genes (*STAT4*, *IL12A*, *IL12RB1*, and *IRF2*) affect lung function as shown by a meta-analysis of genome-wide association studies in 4 European American asthmatic populations [13]. Among these genes, *STAT4* is essential to IL12 transcription and function and thus plays an important role in IL12-mediated IFN- γ production and the differentiation of Th1 cells [14, 15]. Associations have been found between reduced IFN- γ neonate production and increased frequency and severity of childhood wheezing and respiratory diseases [16, 17] and between reduced IFN- γ neonate production and increased cold symptoms after viral infection [18]. Moreover, associations have been shown between *STAT4* genotype and viral illness, asthma exacerbations, and childhood wheezing [19-22]. These associations may be due to defects in IFN- γ response profiles or to a skewing of T-helper cell differentiation toward a Th2 phenotype, implicating *STAT4* in Th2 inflammation and airway remodelling [23-25]. However, even in airways where ICSs diminished Th2 cells, inflammation proceeded to induce airway remodelling in the patients who had no symptoms but had genetic variants. The most likely explanation is that sufficient doses of ICSs to control asthma symptoms were not adequate to improve

airway remodelling in subjects with genetic variants in *IL13* and *STAT4* who used ICSs regularly. Some evidence for dose-related differences in the effects of corticosteroids on the bronchial mucosa basement membrane thickness also exists.

We hypothesized that (1) *IL13* and *STAT4* SNPs are associated with low lung function in patients and that (2) phenotyping patients based on *IL13* and *STAT4* SNPs would identify populations that are associated with a differential rate of FEV1 decrease despite the use of high doses of ICSs. To eliminate the possibility of patients with asthma-COPD overlap syndrome registering for the study, we used many screening questionnaires and a more in-depth clinical history for patient selection. This method enabled more strict determination of bronchial asthma than our previous studies [7] and excluded asthmatic patients with a history of smoking tobacco more than 10-pack-years and with exposure to environmental hazards. We selected postbronchodilator percent predicted FEV1 and bronchial biopsy specimens to evaluate airway structural changes. No placebo group was included in this study because randomizing patients with moderate asthma to receive a placebo for several years was not considered ethical.

Methods

Study subjects

All study subjects were recruited from the Iwate Medical University Hospital. Patients aged ≥ 18 years were eligible if they had a diagnosis of asthma as defined by the American Thoracic Society criteria for ≥ 5 years and were using ICS at a stable low-mid dose for ≥ 1 year before screening. Well-controlled asthmatic patients who had no other medical disorders, who had smoked less than 10-pack-years and who had not been exposed to environmental hazards were considered for the study. This study was approved by the Iwate Medical University Hospital Ethics Committee (H20-119) and registered with Clinical Trials (JMA-IIA00045 IL13STAT4-ICS). Prospective patients were notified of our desire to include them in our study, and they were asked whether they would be willing to participate. Upon acceptance, the subjects provided written informed consent according to the ethical protocols of our institution. Subjects were assessed for age, sex, asthma duration, smoking history, spirometry, and eosinophil counts. DNA was isolated from lymphocytes using standard procedures. Subjects were genotyped for rs20541 and rs925847 using a 7500 Fast Real-Time PCR System (Life Technologies Japan, Tokyo, Japan) to evaluate the relationship between genotype and airway remodelling. Spirometry was performed, and airway methacholine responsiveness was measured using an Astograph (Jupiter 21, CHEST, Tokyo, Japan) according to the method described by Takishima et al. [26].

Fibreoptic bronchoscopy and specimen handling

BLF collection and bronchial biopsies were performed by inserting a flexible fibreoptic bronchoscope (BF-Q290; Olympus Optical Co Ltd, Tokyo, Japan) under local anaesthesia as described in detail previously [27]. BLF collection was always performed before the biopsy to prevent blood contamination. BLF collection was performed in one of the

subsegmental bronchi of the left lingular division *via* the injection of two 20-ml aliquots of sterile saline pre-warmed to 36.5 °C and gentle aspiration back into polypropylene tubes that were kept on ice. We obtained 20-25 ml of BLF from each subject. Mucus was removed from the fluid by filtration through gauze immediately after lavage, and the fluid was centrifuged at 200 x g for 10 min at 4 °C. The supernatant was decanted and stored at -80 °C. The cell pellet was washed in phosphate-buffered saline (PBS), and a total cell count was performed using a disposable cell counting chamber (Cellometer, Nexcelom Bioscience LLC, Lawrence, MA, USA). Then, the cells were diluted with PBS to a concentration of 1 x 10⁶/mL. Cytocentrifuge slides were prepared and stained with May-Grünwald-Giemsa, and a differential cell count was performed on 300 cells. BLF cells were used for immunocytochemistry, and the 10 mL of BLF supernatant was concentrated to 1.0 mL and subsequently stored at -80 °C until use. Five biopsies were taken using forceps (Olympus) in the segmental bronchi of the right lobe and in the bifurcations of the right bronchi. Biopsied specimens were fixed in 4% paraformaldehyde, immersed in sucrose, and embedded in OCT compound. Five-micrometre tissue sections were affixed to microscope slides and prepared for histology. Then, haematoxylin and eosin (HE) staining was performed. The thickness of the subepithelial layer of the HE-stained biopsies was measured. The thickening of the subepithelial layer in individual biopsy specimens was analysed using an Olympus DP70 system (Olympus) and computer image analysis (Image-Pro Plus 4.1; Media Cybernetics, Rockville, MD, USA), and the results were compared among the three genotypes and before and after treatment in patients homozygous for *IL13* and *STAT4* SNPs. A second round of endobronchial procedures was performed after one year of treatment on the opposite side of the lung, and a third round of procedures was performed after two years of treatment on the left lower lobe of the lung.

Cytokine concentrations of BLF supernatants

We demonstrated that various cytokine concentrations in BLF supernatants were increased in patients homozygous for *IL13* AA (rs20541) compared to wild type and heterozygotes from a previous study using microarray analysis [7]. We also analysed and compared IL23 (LifeSpan BioSciences, Inc., Seattle, WA, USA), IL11 (R&D Systems, Inc., Minneapolis, MN, USA), GMCSF (R&D Systems, Inc.), hyaluronic acid (Echelon Biosciences Inc., Salt Lake City, UT, USA), IL24 (R&D Systems, Inc.), and CCL8 (LifeSpan BioSciences, Inc.) expression. The concentrations of the Th1-type cytokine IFN γ (R&D Systems, Inc.) and Th2-type cytokines IL4 (R&D Systems, Inc.) and IL5 (R&D Systems, Inc.) in BLF were also analysed. SRL Laboratories (Tokyo, Japan) assessed IL13 levels in BLF. The values of *IL13* AA and *IL13* GG obtained by the kit were adjusted based on known proteins as a standard because of the different sensitivities of AA and GG to this assay.

Treatment comparison study design

Eligible subjects were monitored over a 1-month run-in period. Symptoms and lung function stability were examined. Subjects were eligible for the study if they complied with recording information in a home diary and if their asthma was under control. After the clinical and pulmonary function test data of patients homozygous for the SNP rs20541 or rs925847 were assessed, patients homozygous AA for the SNP rs20541 or homozygous TT for the SNP rs925847 were randomly assigned to either 2 years of add-on high-dose ICS treatment or to the continuance of previous treatment using a 1:1 ratio to ensure that the groups contained equal numbers. The subjects were interviewed once every 2 months for 2 years. The subjects recorded the following data twice daily in a home diary: PEFR and symptoms scores, as well as the number of puffs of rescue medication needed. No changes were made to treatments or follow-up procedures during the study, and the prescribed doses of ICSs remained constant until mild exacerbation occurred. In the event of exacerbation, subjects were instructed to contact the investigators immediately and to come to the hospital within 24 h. The investigators assessed each event using pre-established exacerbation criteria, and the dose of ICS was increased threefold for a minimum of 4 days in cases of mild exacerbation or until the PEFR returned to normal values.

A pulmonary function test and endobronchial assessment were performed once annually for 2 years. Subjects had to fulfil the following criteria: no evidence of respiratory tract infection and no asthma exacerbation for ≥ 14 days.

Statistical analysis

Statistical analyses were performed using JMP version 11 (SAS Institute Inc., Tokyo, Japan). All data are expressed as the mean $\pm$ standard error of the mean. Comparisons of the patients' characteristics, concentrations of cytokines of bronchial lavage fluid, and the thickening of the subepithelial layer between the three genotypes were performed using one-way ANOVA. Comparisons of the pulmonary function, mediator concentrations in BLF, and subepithelial layer thickness between two different treatment groups, including the baseline as well as the first- and second-year measurements, were performed using *t*-tests. *Post hoc* multiple comparisons of the differences among all of the groups were performed using the Tukey-Kramer test. *P* values < 0.05 were considered significant. Methods using the Bonferroni correction were performed to avoid multiplicity of primary endpoint analyses.

Results

Subject demographics and enrolment

Of 411 asthmatic patients screened, 387 rs20541 patients and 377 rs925847 patients were successfully genotyped ($\geq 92\%$). We identified 154, 172, and 61 subjects that were GG wild type, GA heterozygotes, and AA homozygotes, respectively, for *IL13* (rs20541)

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during the run-in period. No significant differences in age at study enrolment, sex, smoking history, eosinophil count status, or serum IgE concentration among the three groups (Table 1). However, the %FEV1 exhibited significant differences among the three groups. AA homozygotes of rs20541 had significantly lower %FEV1 compared to other genotype groups ($P < 0.05$) (Table 1). In total, 104 subjects were TT homozygotes of the *STAT4* SNP (rs925847). Similarly, the %FEV1 was significantly lower in TT homozygotes of the *STAT4* SNP between the three genotypes ($P < 0.05$) (Table 1).

Detection of clinically relevant IL23, IL11, GMCSF, hyaluronic acid, IL24, and CCL8 levels and subepithelial layer basement thickness

We performed a bronchoscopy and collected BLF from three groups to confirm whether AA and TT homozygotes of rs20541 and rs925847, respectively, expressed any increased protein levels that were involved with Th1- and Th2-type cytokines and airway remodelling. Seventeen patients who were genotyped as GG, 16 patients as GA, and 25 patients as AA were accepted for the BLF and bronchial biopsy studies. Twenty of the 104 patients who were genotyped as TT homozygotes of rs925847 agreed to undergo fiberoptic bronchoscopy. Twenty-three patients who were genotyped as CC and 15 patients as CT were accepted for the bronchoscopic examination, which was performed following recommended safety procedures and which was well tolerated in all subjects. Patients who were genotyped as double homozygotes of AA and TT were not enrolled. IL13, IL23, IL11, GMCSF, hyaluronic acid, IL24, and CCL8 levels were all significantly increased in patients who were homozygous for AA compared to wild type (Table 2), but no significant difference was found for IL4, IL5, and IFN γ among the three groups. None of these changes differed significantly among the CC, CT, and TT groups in rs925847. The increased concentrations of IL13, IL23, IL11, GMCSF, hyaluronic acid, and CCL8 in AA of *IL13* were also examined in our previous data. Although subjects of asthma without COPD were used in this study, similar tendencies have been shown.

Thickness of the subepithelial layer basement

Subsequently, we investigated the differences in the thickness of the subepithelial layer of the bronchial mucosa. The subepithelial layer was significantly thicker in the biopsies taken from the asthmatic subjects who were homozygous compared to wild type (Fig. 1 and Table 2). Therefore, airway remodelling in homozygotes for AA of rs20541 and TT of rs925847 exhibited increased progression compared to wild type subjects.

Completed patients

Patients who were genotyped as double homozygotes of AA/TT and were not willing to participate were not enrolled in the two-year treatment protocol. The enrolled subjects of *IL13* AA were divided randomly into two groups; the conventional treatment group ($n = 22$) and the high-dose ICS treatment group ($n = 21$). No significant differences were

observed between the two groups for the baseline clinical data (data not shown), and the first-round bronchoscopic examination of 23 patients was divided into two groups: 11 patients in the conventional treatment group and 12 patients in the high-dose ICS group (Fig. 2). Paired clinical data were available for 17 homozygotes for AA of rs20541 who completed for 2 years of treatment, of which 7 patients were randomized to conventional therapy, and 10 patients were randomized to high-dose ICS treatment. In total, 82 patients who were homozygous for TT of rs925847 were randomized to conventional therapy (n = 41) and to high-dose ICS treatment (n = 41). Seventy-four subjects completed the two-year protocol in full. Fourteen subjects underwent a third bronchoscopic examination.

Effects of high-dose ICS treatment on %FEV1 and airway remodelling

Pulmonary function

The group analysis of homozygotes for AA of rs20541 revealed a numerically greater increase from the baseline %FEV1 with high-dose ICS treatment in these patients, whereas no increase in %FEV1 was demonstrated when homozygotes for TT of rs925847 were treated with high-dose ICS or conventional treatment (Fig. 3).

Concentrations of mediators in BLF

We performed bronchoscopies and collected BLF and biopsy sample to determine whether individuals homozygous for AA of rs20541 or for TT of rs925847 expressed decreased protein levels in the airways after treatment. One patient in the conventional treatment group and 2 patients in the high-dose ICS treatment group, all of whom were homozygous for AA of rs20541, had unscheduled hospital visits due to upper respiratory tract infections. Similarly, one patient in the conventional treatment and one patient in the high-dose ICS treatment group, both of whom were homozygous for TT of rs925847, experienced upper respiratory tract infections and could not undergo endobronchial examination after one year of treatment. Seven asthmatic subjects using conventional therapy and ten subjects using high-dose ICS treatment, all of whom were homozygous for AA of rs20541, completed a third bronchial examination. Eight asthmatic subjects using conventional therapy and six subjects using high-dose ICSs, all of whom were homozygous for TT of rs925847, were accepted and completed a third bronchial examination (Fig.2). Bronchoscopic examination with BLF collection was performed with care and was well tolerated in all subjects after treatment. IL13, IL23, IL11, GMCSF, hyaluronic acid, IL24, and CCL8 levels did not decrease significantly after treatment with high-dose ICSs compared with conventional therapy in subjects homozygous for AA of rs20541 (Table 3), whereas no significant difference was found in subjects homozygous for TT of rs925847 (Table 4).

Thickness of the subepithelial layer

Significant differences between the subepithelial layer thickness of biopsy specimens taken from asthmatic patients homozygous for AA of rs20541 receiving continuous conventional therapy or high-dose ICS treatment were observed (Table 3), whereas no significant differences in the thickness of the subepithelial layer were observed between treatments in patients homozygous for TT of rs925847 (Table 4).

Discussion

In this study, we investigated genetic variants related to lung function in Japanese asthmatic patients. Several new findings emanated from this study. First, we demonstrated that asthmatic patients with genetic variants of *STAT4* (rs925847) exhibited significantly reduced %FEV1 and bronchial basement membrane thickness. Second, high-dose ICSs did not result in improvements from baseline in lung function and basement membrane thickness in patients homozygous for TT of rs925847, although patients homozygous for AA of rs20541, which conferred asthmatic airway remodelling, displayed improvements in lung function after high-dose ICS treatment. Third, IL13, IL23, IL11, GMCSF, hyaluronic acid, IL24, and CCL8 levels in BLF from *IL13* homozygotes of AA of rs20541 decreased after high-dose ICS treatment, but the levels in BLF from *STAT4* homozygotes of TT of rs925847 did not decrease.

Bronchial asthma is one of the most common chronic diseases that affect 300 million individuals of any age worldwide. The response to treatment is also genetically complex and is characterized by high intraindividual repeatability [28] and high interindividual variability [29], with up to 40% of asthmatic patients exhibiting no response to therapy. Studies in families with conditions other than bronchial asthma demonstrated familial segregation and heritability of responses to glucocorticoid medications [30-31].

Previously, we demonstrated an important role for *IL13* (rs20541) in airway remodelling using pulmonary function tests and bronchial specimens obtained from asthmatic patients [7]. However, because a positive association between *IL13* (rs20541) and COPD and impaired lung function was also found [32], we did not use patients with concurrent asthma and COPD in this study, resulting in differences in the background and characteristics of the patients in the present study compared to those used in previous studies. Therapeutic utility of high-dose ICSs aimed at regulating IL13 and Th2-mediated milieu was examined only in asthmatic patients in this study.

Corticosteroid treatment generally reduced basement membrane thickness in asthmatic patients in both our study and other studies [33-35]. Dose-related differences in the effects of corticosteroids on bronchial mucosal basement membrane thickness are also apparent in some studies [36-37]. Meanwhile, Boulet and Baraket groups did not demonstrate a significant reduction in basement membrane thickness after fluticasone propionate treatment [38-39]. These findings indicate that the diversity of inflammation may reflect a wide repertoire of inflammatory mediators. Whether genetic variants of *STAT4* (rs925847) weaken Th1 responses is not known. Kim et al. demonstrated that *STAT4*-mediated signalling plays a key role in the development of airway

hyperresponsiveness and non-eosinophilic inflammation *via* the actions of Th1 chemokines and macrophages in a Th2 asthma mouse model [40]. One explanation for the lack of a steroid effect on lung function in genetic variants of *STAT4* (rs925847) subjects may be that this SNP has augmented non-eosinophilic inflammation and macrophage activities. In the present study, our examinations demonstrated no significant differences between the rs20541 (*IL13*) and rs925847 (*STAT4*) groups in the levels of PEF and FeNO, in the levels of the Th1 and Th2 cytokines and in the infiltration of inflammatory cells, including eosinophils, neutrophils, and macrophages in the bronchial wall and lavage fluid (data not shown). Further examinations should be conducted to determine whether TT of rs925847 skewed the balance between M1 and M2 macrophage phenotypes and the ability to produce Th1- and Th2-type cytokines from stimulated BAL cells *ex vivo*. The AA of rs20541-induced mediators, such as IL13, IL23, IL11, GM-CSF, hyaluronic acid, and CCL8, are involved in the process of airway remodelling [7]. IL24 was originally termed melanoma differentiation-associated gene 7 and was identified by subtraction hybridization of cDNA libraries from human melanoma cells [41]. The tumour suppressor role of IL24 is well characterized, but its immunomodulatory function and its role in allergic inflammatory diseases remain under scrutiny. Bochkov et al. demonstrated that GM-CSF and IL24 gene expression was induced in rhinovirus-infected primary bronchial epithelial cells [42]. The exact roles of IL24 in epithelial damage and sustainable airway hyperresponsiveness of asthmatic subjects await further experiments. We demonstrated that corticosteroids are less effective in airway remodelling for the endotype *STAT4* TT of rs925847, may be because of the absence of a Th2 process. Non-Th2 asthma likely represents a large proportion of all asthma. However, little is understood regarding this asthma subgroup, the phenotypes underlying it, and the molecular elements that control it compared with Th2 asthma. Non-Th2 asthma may affect more than 50% of corticosteroid-naïve individuals, who exhibit less airway obstruction and hyperreactivity than people with Th2-high asthma do, even though they meet the criteria for asthma.

In conclusion, we demonstrated an important role for *IL13* AA of rs20541 and *STAT4* TT of rs925847 in airway remodelling with bronchial asthma. Further investigations of high-dose ICS utility aimed at regulating these molecules may provide better control of airway remodelling in asthmatic patients homozygous for *IL13* AA of rs20541 but not with patients homozygous for *STAT4* TT of rs925847. The mechanism of this different effect may be mediated *via* the Th1 or Th2 pathways. Thus, *IL13* AA of rs20541 and *STAT4* TT of rs925847 are potential genomic biomarkers that predict lower pulmonary function. Administration of high-dose ICSs to asthmatic patients with genetic variants of *IL13* AA may avoid the advancing of airway remodelling, whereas the genetic variants of *STAT4* TT do not respond to high-dose ICSs. Therefore, the use of medications other than ICSs must be considered even in the initial treatment steps of bronchial asthma. These genetic variants could aid in the realization of personalized and phenotype-specific therapies for bronchial asthma.

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Conflict of interests

The authors declare no conflicts of interest.

References

1. Global Initiative for asthma, Global Strategy for Asthma Management and Prevention 2014. <http://www.ginasthma.org/documents/4>
2. Kay AB. Asthma and inflammation. *J Allergy Clin Immunol* 1991; **87**: 893-910.
3. Wenzel SE. Asthma phenotypes: the evolution from clinical to molecular approaches. *Nat Med* 2012; **18**: 716-25.
4. Elias JA, Lee CG, Zheng T, Ma B, Homer RJ, Zhu Z. New insights into the pathogenesis of asthma. *J Clin Invest* 2003; **111**: 291-7.
5. Battle NC, Choudhry S, Tsai HJ *et al*. Ethnicity-specific gene-gene interaction between IL-13 and IL-4R α among African Americans with asthma. *Am J Respir Crit Care Med* 2007; **175**: 881-7.
6. Park HW, Lee JE, Kim SH *et al*. Genetic variation of IL13 as a risk factor of reduced lung function in children and adolescents: a cross-sectional population-based study in Korea. *Respir Med* 2009; **103**: 284-8.
7. Nagashima H, Nakamura Y, Kanno H *et al*. Effect of genetic variation of IL-13 on airway remodeling in bronchial asthma. *Allergol Int* 2011; **60**: 291-8.
8. Utsumi Y, Sasaki N, Nagashima H *et al*. Association of IL-13 gene polymorphisms with airway hyperresponsiveness in a Japanese adult asthmatic population. *Respir Investig* 2013; **51**: 147-52.
9. Beghé B, Hall IP, Parker SG *et al*. Polymorphisms in IL13 pathway genes in asthma and chronic obstructive pulmonary disease. *Allergy* 2010; **65**: 474-81.
10. Sumino K, Tucker J, Shahab M *et al*. Antiviral IFN- γ responses of monocytes at birth predict respiratory tract illness in the first year of life. *J Allergy Clin Immunol* 2012; **129**: 1267-73.
11. Mallia P, Johnston SL. How viral infections cause exacerbation of airway diseases. *Chest* 2006; **130**: 1203-10.

12. Bosco A, Ehteshami S, Stern DA, Martinez FD. Decreased activation of inflammatory networks during acute asthma exacerbations is associated with chronic airflow obstruction. *Mucosal Immunol* 2010; **3**: 399-409.
13. Li X, Hawkins GA, Ampleford EJ *et al*. Genome-wide association study identifies TH1 pathway genes associated with lung function in asthmatic patients. *J Allergy Clin Immunol* 2013; **132**: 313-20.
14. Thierfelder WE1, van Deursen JM, Yamamoto K *et al*. Requirement for Stat4 in interleukin-12-mediated responses of natural killer and T cells. *Nature* 1996; **382**: 171-4.
15. Kaplan MH, Sun YL, Hoey T, Grusby MJ. Impaired IL-12 responses and enhanced development of Th2 cells in Stat4-deficient mice. *Nature* 1996; **382**: 174-7.
16. Gern JE, Brooks GD, Meyer P *et al*. Bidirectional interactions between viral respiratory illnesses and cytokine responses in the first year of life. *J Allergy Clin Immunol*. 2006; **117**: 72-8.
17. Copenhaver CC, Gern JE, Li Z *et al*. Cytokine response patterns, exposure to viruses, and respiratory infections in the first year of life. *Am J Respir Crit Care Med*. 2004; **170**: 175-80.
18. Gern JE, Vrtis R, Grindle KA, Swenson C, Busse WW. Relationship of upper and lower airway cytokines to outcome of experimental rhinovirus infection. *Am J Respir Crit Care Med*. 2000; **162**: 2226-31
19. Li Y1, Wu B, Xiong H, Zhu C, Zhang L. Polymorphisms of STAT-6, STAT-4 and IFN-gamma genes and the risk of asthma in Chinese population. *Respir Med* 2007; **101** 1977-81.
20. Park BL, Cheong HS, Kim LH *et al*. Association analysis of signal transducer and activator of transcription 4 (STAT4) polymorphisms with asthma. *J Hum Genet* 2005; **50**:133-8.
21. Pykäläinen M, Kinoshita R, Valkonen S *et al*. Association analysis of common variants of STAT6, GATA3, and STAT4 to asthma and high serum IgE phenotypes. *J Allergy Clin Immunol*. 2005; **115**: 80-7.
22. Loisel DA, Du G, Ahluwalia TS *et al*. Genetic associations with viral respiratory illnesses and asthma control in children. *Clin Exp Allergy (in press)*
23. Meuronen A, Karisola P, Leino M *et al*. Attenuated expression of tenascin-C in ovalbumin-challenged STAT4^{-/-} mice. *Respir Res* 2011; **12**: 2.
24. Raman K, Kaplan MH, Hogaboam CM, Berlin A, Lukacs NW. STAT4 signal pathways regulate inflammation and airway physiology changes in allergic airway inflammation locally via alteration of chemokines. *J Immunol* 2003; **170**: 3859-65.

25. Syrbe U, Hoffmann U, Schlawe K, Liesenfeld O, Erb K, Hamann A. Microenvironment-dependent requirement of STAT4 for the induction of P-selectin ligands and effector cytokines on CD4+ T cells in healthy and parasite-infected mice. *J Immunol* 2006 ; **177**: 7673-9.
26. Takishima T, Hida W, Sasaki H, Suzuki S, Sasaki T. Direct-writing recorder of the dose-response curves of the airway to methacholine. Clinical application. *Chest* 1981; **80**: 600-6.
27. Nakamura Y, Hoshino M, Sim JJ, Ishii K, Hosaka K, Sakamoto T. Effect of leukotriene receptor antagonist pranlukast on cellular infiltration in the bronchial mucosa of patients with asthma. *Thorax* 1998; **53**: 835-41.
28. Drasen Jm, Silverman EK, Lee TH. Heterogeneity of therapeutic responses in asthma. *Br Med Bull* 2000; **56**: 1054-70.
29. Szeffler SJ, Martin RJ, King TS *et al*. Significant variability in response to inhaled corticosteroids for persistent asthma. *J Allergy Clin Immunol* 2002; **109**: 410-8.
30. Armaly MF. The heritable nature of dexamethasone-induced ocular hypertension. *Arch Ophthalmol* 1966; **75**: 32-5.
31. Schwartz JT, Reuling FH, Feinleib M, Garrison RJ, Collie DJ. Twin heritability study of the effect of corticosteroids on intraocular pressure. *J Med Genet* 1972; **9**: 137-43.
32. Beghé B, Hall IP, Parker SG, et al. Polymorphisms in IL13 pathway genes in asthma and chronic obstructive pulmonary disease. *Allergy* 2010; **65**: 474-81.
33. Hoshino M, Nakamura Y, Sim JJ *et al*. Inhaled corticosteroid reduced lamina reticularis of the basement membrane by modulation of insulin-like growth factor (IGF)-I expression in bronchial asthma. *Clin Exp Allergy* 1998; **28**: 568-77.
34. Hoshino M, Takahashi M, Takai Y, Sim J. Inhaled corticosteroids decrease subepithelial collagen deposition by modulation of the balance between matrix metalloproteinase-9 and tissue inhibitor of metalloproteinase-1 expression in asthma. *J Allergy Clin Immunol* 1999; **104**: 356-63.
35. Laitinen A, Altraja A, Kämpe M *et al*. Tenascin is increased in airway basement membrane of asthmatics and decreased by an inhaled steroid. *Am J Respir Crit Care Med* 1997; **156**: 951-8.
36. Sont JK, Willems LN, Bel EH *et al*. Clinical control and histopathologic outcome of asthma when using airway hyperresponsiveness as an additional guide to long-term treatment. The AMPUL Study Group. *Am J Respir Crit Care Med* 1999; **159**: 1043-51.
37. Chetta A, Zanini A, Foresi A *et al*. Vascular component of airway remodeling in asthma is reduced by high dose of fluticasone. *Am J Respir Crit Care Med* 2003, **167**: 751-7.

38. Boulet LP, Turcotte H, Laviolette M *et al.* Airway hyperresponsiveness, inflammation, and subepithelial collagen deposition in recently diagnosed versus long-standing mild asthma. Influence of inhaled corticosteroids. *Am J Respir Crit Care Med* 2000; **162**: 1308-13.
39. Baraket M, Oliver BG, Burgess JK, Lim S, King GG, Black JL. Is low dose inhaled corticosteroid therapy as effective for inflammation and remodeling in asthma? A randomized, parallel group study. *Resp research* 2012; **13**: 11.
40. Kim YS, Choi SJ, Choi JP *et al.* IL-12-Stat4-IFN- γ axis is a key downstream pathway in the development of IL-13-mediated asthma phenotypes in a Th2 type asthma model. *Exp Mol Med* 2010; **42**: 533-46.
41. Jiang H, Lin JJ, Su ZZ, Goldstein NI, Fisher PB. Subtraction hybridization identifies a novel melanoma differentiation associated gene, mda-7, modulated during human melanoma differentiation, growth and progression. *Oncogene* 1995; **11**: 2477-86.
42. Bochkov YA, Hanson KM, Keles S, Brockman-Shneider RA, Jarjour NN, Gern JE. Rhinovirus-induced modulation of gene expression in bronchial epithelial cells from subjects with asthma. *Mucosal Immunology* 2010; **3**: 69-80.

Figure legends

Fig. 1 Bronchial biopsy specimens obtained from asthmatic individuals. Representative photomicrographs show bronchial mucosal biopsy specimens stained with haematoxylin and eosin (x 200), derived from (a) GG as a wild type, (b) homozygote AA of rs20541 in *IL13*, (c) CC as a wild type, and (d) homozygote TT of rs925847 in *STAT4*. The thickness of the subepithelial layer is increased in each homozygote compared to the wild type.

Fig 2 Flow diagram of the study population. ICSs, inhaled corticosteroids; BF, performed fiberoptic bronchoscopy; the closed arrows indicate the first year of treatment; open arrows indicate the subsequent one-year treatment that was started after the examination that followed the first year of treatment.

Fig. 3 Changes in %FEV1 before and after 1 and 2 years of treatment according to the conventional and high-dose ICS therapies. A significant increase in %FEV1 was detected in AA of rs20541 when treated with high-dose ICSs after the first and second year compared to those treated with conventional therapy (a). In contrast, no significant differences were observed in the patients of TT of rs925847 who used conventional therapy and high-dose ICSs after the first and second years (b). The open bars and closed bars indicate the means of %FEV1 of the patients with asthma treated with conventional therapy and high-dose ICS treatment, respectively. The whiskers indicate the standard errors of the mean. * $P < 0.05$ and ** $P < 0.01$ compared with the means of %FEV1 when treated with conventional therapy after the first and second years, respectively.

Table 1. Patients characteristics according to the rs20541 genotype of *IL13* and rs925847 of *STAT4*

	rs20541					
	rs925847					
	GG (n=154)	GA (n=172)	AA (n=61)	CC (n=92)	CT (n=181)	TT (n=104)
Age at enrollment (yr)	56.5 ± 1.3	58.2 ± 1.2	60.2 ± 2.2	57.6 ± 1.7	57.9 ± 1.2	57.4 ± 1.5
Sex, M / F	70 / 84	64 / 108	30 / 31	45 / 47	67 / 114	50 / 54
smoking history (never smoker)%	57.6	57.7	55.9	54.4	60.7	51.0
Blood eosinophils / μ L	315 ± 29.3	298 ± 16.9	347 ± 38.1	303 ± 26.2	337 ± 26.8	289 ± 20.9
Serum total IgE IU / μ L	474 ± 89.8	435 ± 120.7	514 ± 121.7	452 ± 68.4	416 ± 79.1	607 ± 204
FEV1, %predicted (%)	89.5 ± 1.8	87.1 ± 1.6	80.5 ± 2.8 [‡]	90.8 ± 2.3	86.7 ± 1.5	82.8 ± 2.2*

Data are expressed as the mean $\pm$ standard error of the mean. FEV1, forced expiratory flow volume in on second

Within-genotype comparison; [‡] $P < 0.05$ compared with GG, * $P < 0.05$ compared with CC

Table 2. BLF concentrations of cytokines associated with the Th1, Th2-type, and airway remodeling

	rs20541					
	rs925847					
	GG (n=17)	GA (n=16)	AA (n=25)	CC (n=23)	CT (n=18)	TT (n=20)
IL4 (pg/mL)	7.9 ± 1.5	9.6 ± 1.7	6.1 ± 1.1	7.6 ± 1.2	8.9 ± 2.1	6.6 ± 1.0
IL5 (pg/mL)	1.5 ± 0.2	1.3 ± 0.2	1.4 ± 0.1	1.5 ± 0.2	1.3 ± 0.1	1.3 ± 0.2
IL13 (pg/mL)	9.2 ± 1.1	11.3 ± 2.2	18.7 ± 2.4 [†]	15.5 ± 2.7	16.1 ± 3.6	10.3 ± 1.4
IFN γ (pg/mL)	10.1 ± 1.4	10.9 ± 1.4	11.4 ± 2.1	10.9 ± 1.9	11.0 ± 2.5	10.8 ± 1.3
IL23 (pg/mL)	11.5 ± 1.7	12.3 ± 2.4	22.3 ± 3.0 [†]	15.6 ± 2.7	20.3 ± 3.7	14.4 ± 2.5
IL11 (pg/mL)	8.3 ± 1.1	12.7 ± 2.4	20.7 ± 2.6 [†]	17.0 ± 2.8	16.6 ± 3.0	11.0 ± 1.7
GMCSF (pg/mL)	23.9 ± 2.0	19.7 ± 2.5	36.1 ± 4.1 [†]	33.9 ± 4.0	24.1 ± 3.9	24.1 ± 2.9
hyaluronic acid (ng/mL)	74.6 ± 16.1	81.3 ± 11.0	175.6 ± 24.1 [†]	137.8 ± 25.5	109.9 ± 28.3	107.0 ± 14.0
IL24 (pg/mL)	9.2 ± 1.5	11.0 ± 1.5	15.1 ± 1.5 [†]	13.3 ± 1.6	11.4 ± 1.7	11.7 ± 1.6
CCL8 (pg/mL)	12.4 ± 2.6	20.2 ± 3.8	24.6 ± 3.0 [†]	20.3 ± 2.7	19.3 ± 4.5	19.6 ± 3.5
Subepithelial layer thickness (μ m)	8.2 ± 0.7	7.7 ± 0.7	10.0 ± 0.5 [†]	8.4 ± 0.6	7.7 ± 0.7	10.4 ± 0.6*

Data are expressed as the mean $\pm$ standard error of the mean. BLF, bronchial lavage fluid.
Within-genotype comparison; [†] $P < 0.05$ compared with GG, * $P < 0.05$ compared with CC

Table 3. BLF concentrations of cytokines from AA of rs20541 at baseline and after treatment

	AA of rs20541 of <i>IL13</i>		
	Baseline (n = 23)	1yr (n = 20)	2yr (n = 17)
IL4 (pg/mL)			
conventional	5.1 ± 0.9	5.6 ± 0.9	5.7 ± 0.5
high-dose ICS	7.5 ± 2.1	6.8 ± 1.2	6.2 ± 1.1
IL5 (pg/mL)			
conventional	1.3 ± 0.2	1.2 ± 0.1	1.4 ± 0.2
high-dose ICS	1.6 ± 0.2	1.5 ± 0.2	1.3 ± 0.2
IL13 (pg/mL)			
conventional	19.9 ± 2.9	23.3 ± 3.1	25.2 ± 4.3
high-dose ICS	19.6 ± 4.0	13.6 ± 2.7	11.2 ± 0.7 [‡]
IFN γ (pg/mL)			
conventional	12.5 ± 3.2	9.6 ± 2.0	11.7 ± 2.8
high-dose ICS	11.5 ± 3.4	10.0 ± 2.4	9.8 ± 2.1
IL23 (pg/mL)			
conventional	17.1 ± 4.1	21.9 ± 5.2	22.9 ± 5.0
high-dose ICS	25.4 ± 4.5	15.4 ± 3.3	12.2 ± 1.4 [‡]
IL11 (pg/mL)			
conventional	20.7 ± 3.8	19.8 ± 3.7	25.4 ± 5.1
high-dose ICS	24.1 ± 4.6	16.2 ± 3.6	14.0 ± 2.7 [‡]

GMCSF (pg/mL)

conventional	32.9 ± 7.1	28.3 ± 6.1	27.8 ± 5.7
high-dose ICS	37.6 ± 5.7	18.9 ± 1.9	14.0 ± 2.0 [‡]

hyaluronic acid (ng/mL)

conventional	137.0 ± 24.8	167.4 ± 31.9	162.9 ± 43.7
high-dose ICS	210.9 ± 43.6	102.7 ± 13.3	84.5 ± 14.0 [‡]

IL24 (pg/mL)

conventional	15.8 ± 2.6	18.6 ± 3.8	20.3 ± 5.7
high-dose ICS	14.4 ± 2.0	10.1 ± 1.1 [†]	9.2 ± 1.1 [‡]

CCL8 (pg/mL)

conventional	25.8 ± 5.1	28.9 ± 4.9	28.4 ± 4.3
high-dose ICS	24.6 ± 3.8	17.7 ± 2.6 [†]	18.9 ± 3.0 [‡]

Subepithelial layer thickness (μm)

conventional	9.3 ± 0.8	10.3 ± 0.8	10.5 ± 0.8
high-dose ICS	9.8 ± 0.7	8.7 ± 0.7	7.9 ± 0.5 [‡]

Data are expressed as the mean ± standard error of the mean. BLF, bronchial lavage fluid. ICS, inhaled corticosteroid. Between the treatment comparison; [†] $P < 0.05$ compared with conventional treatment for 1yr; [‡] $P < 0.05$ compared with conventional treatment for 2 yr

Table 4. BLF concentrations of cytokines from TT of rs925847 at baseline and after treatment

	TT of rs925847 of <i>STAT4</i>		
	Baseline (n = 18)	1yr (n = 16)	2yr (n = 14)
IL4 (pg/mL)			
conventional	6.4 ± 1.2	4.3 ± 0.7	6.5 ± 0.9
high-dose ICS	7.7 ± 2.1	7.2 ± 2.5	8.4 ± 1.9
IL5 (pg/mL)			
conventional	1.4 ± 0.2	1.3 ± 0.2	1.6 ± 0.1
high-dose ICS	1.8 ± 0.4	1.5 ± 0.2	1.3 ± 0.3
IL13 (pg/mL)			
conventional	11.2 ± 2.2	11.8 ± 3.2	12.4 ± 2.5
high-dose ICS	10.2 ± 2.1	10.1 ± 2.2	11.4 ± 2.7
IFN γ (pg/mL)			
conventional	9.7 ± 1.5	14.6 ± 2.1	12.7 ± 3.3
high-dose ICS	7.2 ± 1.7	10.1 ± 3.8	12.5 ± 2.7
IL23 (pg/mL)			
conventional	13.1 ± 3.2	14.0 ± 4.5	10.7 ± 3.9
high-dose ICS	11.3 ± 2.2	12.5 ± 2.1	13.0 ± 3.9
IL11 (pg/mL)			
conventional	7.7 ± 1.3	8.9 ± 1.1	9.3 ± 1.9
high-dose ICS	9.9 ± 2.1	11.2 ± 2.8	9.9 ± 2.7

GMCSF (pg/mL)

conventional	21.4 ± 3.8	19.0 ± 3.1	19.0 ± 1.9
high-dose ICS	22.2 ± 3.6	17.0 ± 3.6	16.3 ± 3.7

hyaluronic acid (ng/mL)

conventional	68.2 ± 11.7	75.8 ± 18.3	67.4 ± 17.5
high-dose ICS	50.0 ± 13.2	58.7 ± 13.5	57.9 ± 13.5

IL24 (pg/mL)

conventional	13.1 ± 2.1	13.8 ± 2.9	15.1 ± 2.6
high-dose ICS	8.8 ± 2.5	12.5 ± 3.2	10.3 ± 4.5

CCL8 (pg/mL)

conventional	15.5 ± 3.8	15.9 ± 2.9	13.7 ± 2.7
high-dose ICS	14.3 ± 4.1	14.9 ± 3.7	13.5 ± 3.8

Subepithelial layer thickness (μm)

conventional	10.4 ± 0.6	9.8 ± 1.0	9.4 ± 1.1
high-dose ICS	9.3 ± 0.8	8.8 ± 0.8	9.5 ± 1.2

Data are expressed as the mean ± standard error of the mean. BLF, bronchial lavage fluid. ICS, inhaled corticosteroid.

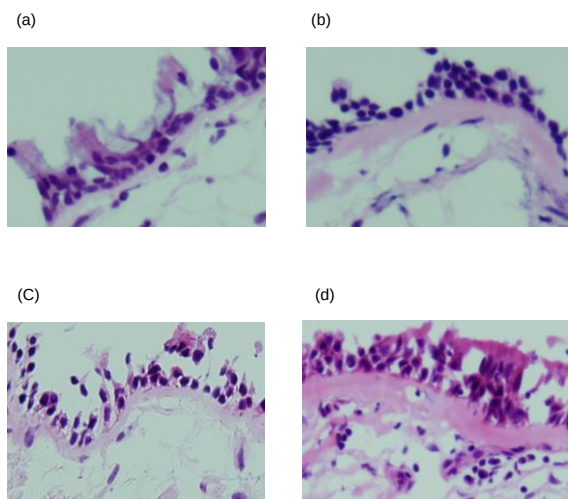


Figure 1

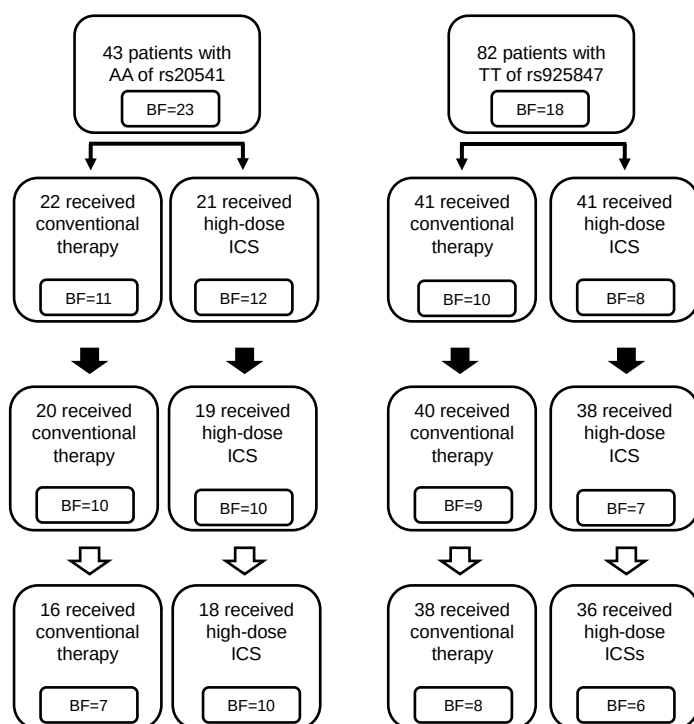


Figure 2

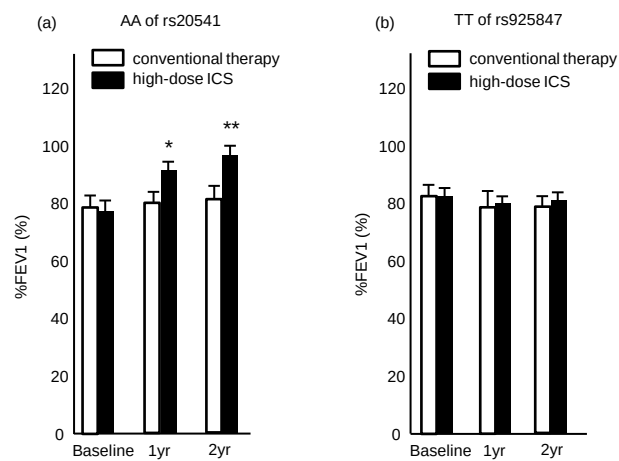


Figure 3