Table 5. Genotype frequencies and case-control analysis of the *IL12RB1* −111 SNP in AD patient subgroups and childhood asthma

	−111 genotype			Genotype AA + AT versus TT		
	AA (%)	AT (%)	TT (%)	OR (95%CI)	χ^2	*P*
AD (Total)	221 (59.7)	113 (30.5)	36 (9.7)	2.46 (1.47–4.13)	12.4	0.00044
IgE ≤ 250 IU/ml	27 (64.3)	11 (26.2)	4 (9.5)	2.41 (0.80–7.23)	2.6	0.11
IgE > 250 IU/ml	194 (59.1)	102 (31.1)	32 (9.8)	2.47 (1.45–4.20)	11.8	0.00059
IgE > 400 IU/ml	183 (58.8)	97 (31.2)	31 (10.0)	2.53 (1.48–4.32)	12.3	0.00046
IgE > 1800 IU/ml	128 (57.1)	71 (31.7)	25 (11.2)	2.87 (1.63–5.06)	14.3	0.00015
Blood eosinophil count >500/μl	85 (52.8)	56 (34.8)	20 (12.4)	3.24 (1.77–5.94)	15.9	0.000068
Early age of disease onset ≤3year	115 (56.9)	62 (30.7)	25 (12.4)	3.23 (1.83–5.70)	17.9	0.000024
History of asthma in childhood	53 (54.6)	31 (32.0)	13 (13.4)	3.54 (1.76–7.12)	14.0	0.00018
History of allergic rhinitis	123 (64.4)	49 (25.7)	19 (9.9)	2.52 (1.37–4.65)	9.4	0.0022
Childhood asthma ($n = 304$)	162 (53.3)	114 (37.5)	28 (9.2)	2.32 (1.34–4.01)	9.5	0.0020

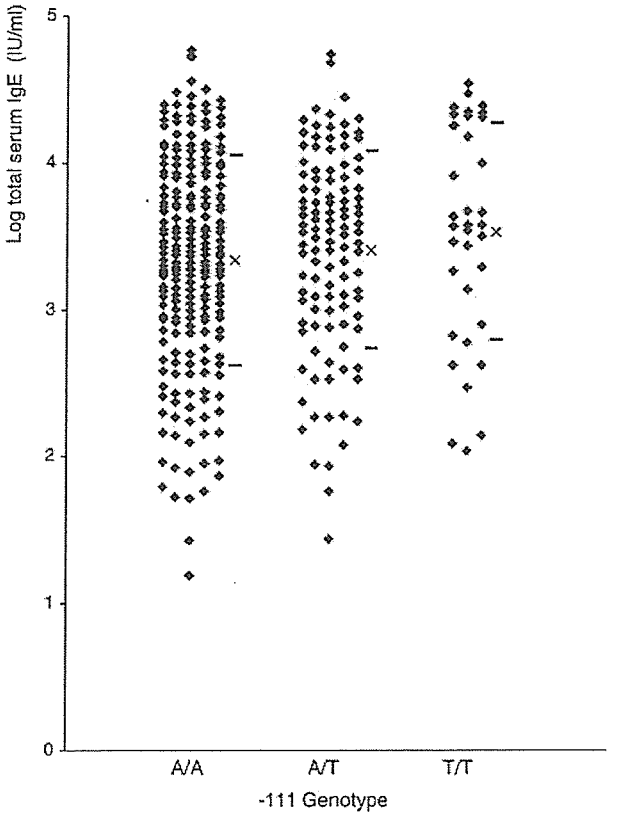

Figure 2. Relationship of *IL12RB1* −111 promoter genotype with total serum IgE levels in AD patients. Log-transformed individual IgE values are plotted, with the mean (×) and SD (bar) shown for each genotype group.

in the majority of affected patients, giving rise to the so-called 'atopic march', which suggests that AD is an initial step or entry point for subsequent allergic diseases (3,28). Therefore, early intervention in AD-susceptible individuals may be an effective strategy in preventing the atopic march. For this reason, *IL12RB1* genotype may be an important genetic marker.

The lymphocytes infiltrating unaffected skin or acute skin lesions in AD patients tend to be Th2-type T cells that produce IL-4, IL-5 and IL-13, whereas expression of IL-12 and the Th1 cytokine IFN-γ are increased in chronic eczematous AD skin lesions (8,29). In addition, AD is known to be associated with a high prevalence of skin infections, particularly involving *Staphylococcus aureus*. Recent studies have shown that at both the mRNA and protein levels, antibacterial peptides such as β-defensins and cathelicidin are decreased or deficient in skin lesions from AD patients when compared with those from psoriasis patients and that the combination of IL-4 and IL-13 inhibited the production of these antimicrobial peptides from keratinocytes (30,31). Although the exact mechanisms by which IL-12Rβ1 regulates these pathophysiological disease features remain unknown, one possibility is that excess Th2 cytokines block a pathway of innate immune activation, leading to an increased susceptibility to skin infections. This in turn facilitates the continued activation of the adaptive immune system, including the recruitment and activation of atopic Th2 cells and perpetuation of the lesions (32). Therefore, our findings suggest that *IL12RB1* SNPs or haplotypes, which appear to affect protein expression or function, may predispose an individual toward the initiation or development of Th2-mediated immune responses in the skin. Further biological and population studies will be required to confirm the role of *IL12RB1* SNPs.

In contrast to AD, the immune response in psoriasis is Th1-mediated and is associated with local neutrophil infiltration. Recent data have indicated that expression of IL-23 and the IL-23-promoting cytokine IL-17 is increased in lesional skin samples of patients with psoriasis vulgaris. As IL-23 utilizes IL-12Rβ1 as part of its receptor, it is reasonable to speculate that the same promoter SNPs in *IL12RB1* that cause susceptibility to AD might be involved in psoriasis vulgaris as well. Our findings were in accordance with a recent study in Morocco that found an association of pulmonary tuberculosis (TB) with two promoter *IL12RB1* SNPs at −111 and −2 (24). Moreover, an association between heterozygous mutations of the *IL12RB2* gene and reduced IFN-γ production by PBMC following stimulation by IL-12 in some Japanese atopic subjects has also been reported (33) and *IL12B* has been identified as a susceptibility gene in patients with AD (18), asthma (34) and type 1 diabetes (35). These studies along with our present data suggest that functionally relevant SNPs in the IL-12/IL-12R and IL-23/IL-23R systems may be associated with the genetic susceptibility to a variety of diseases, including AD and TB.

Table 6. Structure and frequencies of two-locus haplotype in *IL12RB1*

Haplotypes (−111; −2)	−111	−2	AD ($n = 382$)	Childhood asthma ($n = 304$)	Controls ($n = 658$)	P^a	P^b
1	A	C	0.742	0.717	0.787	0.021	0.00081
2	T	T	0.227	0.238	0.188	0.035	0.011
3	T	C	0.027	0.043	0.025	0.741	0.028
4	A	T	0.004	0.002	0.001	0.095	0.57

[a]AD versus controls (global $P = 0.06$).
[b]Childhood asthma versus controls (global $P = 0.005$).

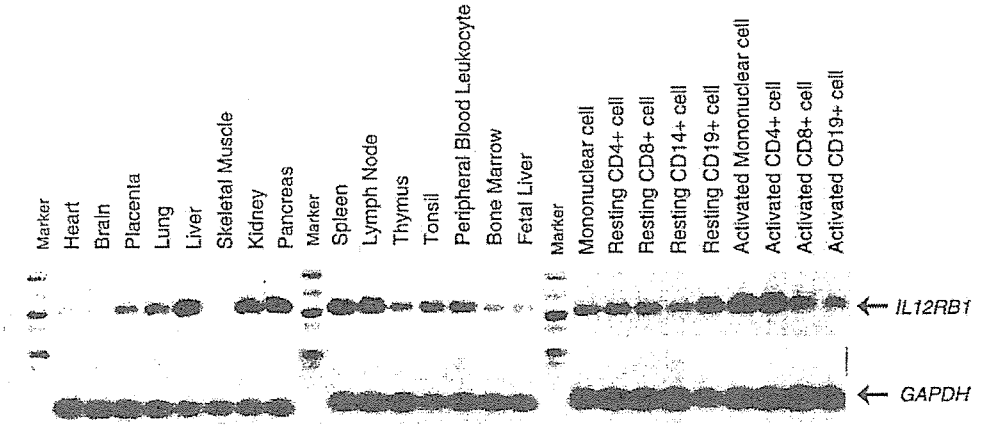

Figure 3. Expression of *IL12RB1* mRNA in multiple tissue cDNA panels. PCR amplification between *IL12RB1* exons 10 and 13 is shown in the upper panel. The *GAPDH* control is shown in the lower panel.

The human *IL12RB1* gene is located on chromosome 19p13.1. Although this region has not been previously implicated by AD linkage studies, a recent study suggested that chromosome 19p13 might harbor a genetic determinant of IgE-related traits (36). Another report showed modest evidence of linkage for atopic phenotypes on chromosome 19p13.3 in an Italian population (37), and a genome-wide scan of a large cohort of German families revealed significant evidence for a psoriasis-susceptibility locus on 19p13 (38). Thus, chromosome 19p13 may indeed contain immunoregulatory genes that influence inflammatory skin diseases such as AD and/or atopy-related phenotypes. In addition, the *IL12RB1* gene is a novel and attractive candidate susceptibility gene for skin inflammatory diseases such as AD, on the basis of its location as well as its function. However, further genetic analyses and biological studies will be required to address whether other atopic disease-related genes or SNPs are also present on 19p13.

In conclusion, we have identified 48 variants (10 novel) of the human *IL12RB1* gene. Our studies demonstrated that the −111T/T *IL12RB1* genotype was associated with high total serum IgE levels and AD susceptibility in a Japanese population. Furthermore, we showed that the −111A/T polymorphism affected the *IL12RB1* gene transcriptional activity and may contribute to low IL-12Rβ1 expression levels. In individuals with the −111T/T genotype, reduced IL-12Rβ1 expression may lead to increased Th2 cytokine production in the skin and contribute to the development of AD and other subsequent allergic diseases. Our findings also highlighted the importance of the IL-12–IFN-γ and/or IL-23–IL-17 pathway in the pathogenesis of AD and regulation of IgE. Although the exact functional role of IL-12Rβ1 in AD remains to be elucidated, the identification of SNPs in *IL12RB1* as a risk factor for AD may provide a strategy to prevent disease onset in susceptible individuals and to represent an attractive target for future therapies for this disorder.

MATERIALS AND METHODS

Study subjects

A total of 382 AD patients (mean age 30.2 years, range 16–65 years; 193 females and 189 males; mean total serum IgE level 2815 IU/ml) were recruited from Yokohama City University Hospital and Kyoto Takao Hospital. All patients with AD were diagnosed by dermatologists according to the criteria of Hanifin and Rajka (39). Controls for the SNP association study were 658 unrelated healthy individuals with an age range of 18–83 years, were from the same geographical areas as the AD patients and had no symptoms or personal and family histories of AD, asthma or allergic rhinitis. We also recruited 304 patients with childhood asthma with an age range of 4–15 years, who have been included in a previous study (40). All subjects in this study were ethnically

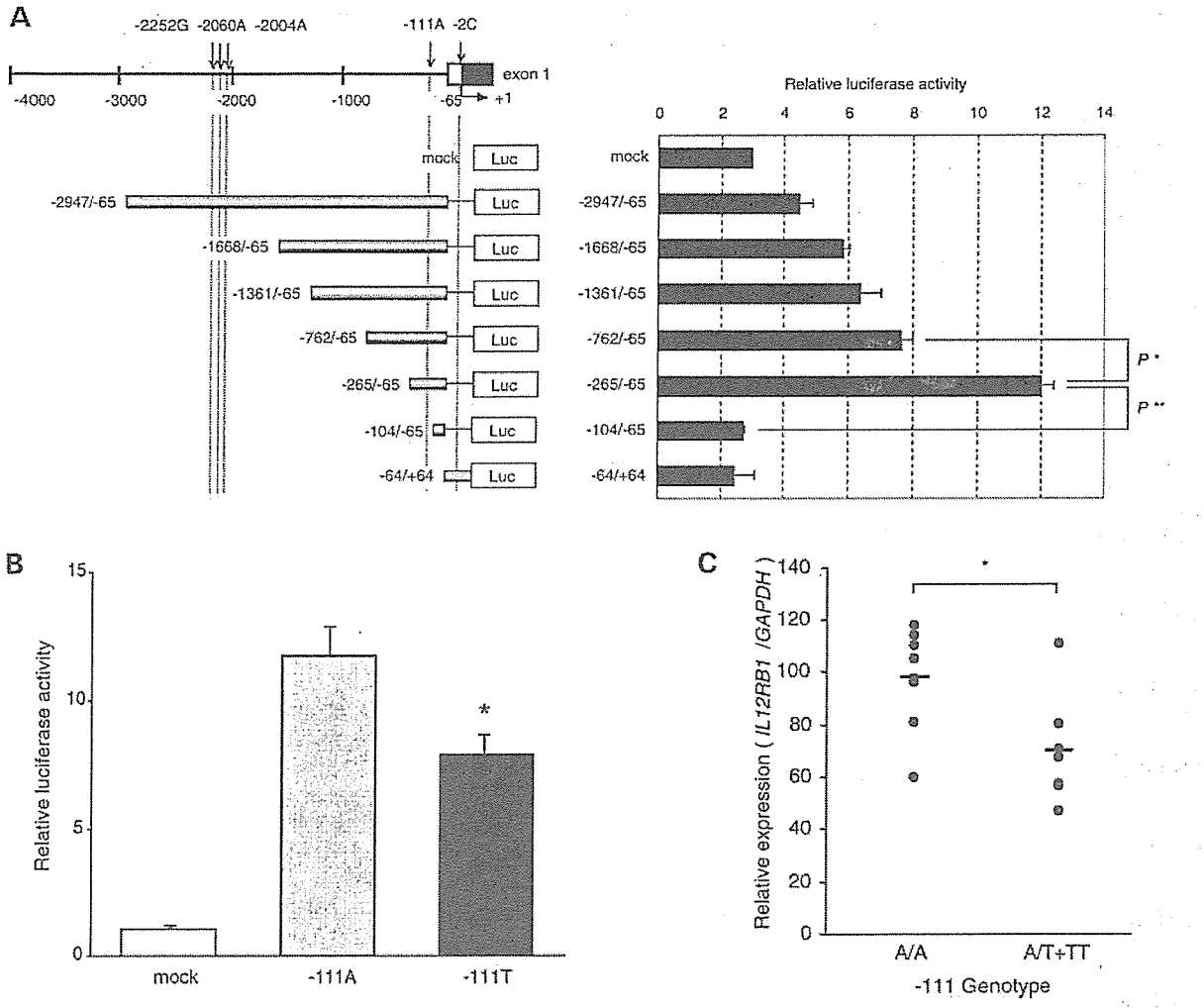

Figure 4. Identification of regulatory elements in the *IL12RB1* promoter, transcriptional effect of the −111A/T SNP and association of the −111 genotype with *IL12RB1* mRNA expression. (**A**) The indicated *IL12RB1* gene promoter fragments derived from wild-type allele sequence (−2252G/−2060A/−2004A/−111A/−2C) were cloned into the pGL3-basic vector and transiently cotransfected with pRL-TK vector as internal control. $^{*}P < 0.01$; $^{**}P < 0.001$. (**B**) Relative luciferase activities of constructs containing the human *IL12RB1* gene fragments (from −265 to −65 bp), with −111A or −111T, were compared in transient transfection assays using Jurkat cells. The relative luciferase activity of the *IL12RB1* reporter constructs is represented as the ratio of firefly luciferase activity to that of *Renilla*. Data are expressed as mean ± SD of three independent experiments performed in triplicate. $^{*}P < 0.01$. (**C**) Quantitative *IL12RB1* mRNA expression in stimulated PBMCs from healthy volunteers (A/A, $n = 8$; A/T, $n = 6$; T/T, $n = 1$). *IL12RB1* mRNA levels in cells from $IL12RB1^{-111A/A}$, $IL12RB1^{-111A/T}$ and $IL12RB1^{-111T/T}$ individuals following anti-CD3 stimulation are shown. Relative mRNA levels were defined as the net intensity of *IL12RB1* and *GAPDH* (*IL12RB1/GAPDH*). $^{*}P$ (A/A versus A/T+ T/T) = 0.019.

Japanese and gave written informed consent to participate in the study, according to the process approved by the Ethics Committee at the SNP Research Center, Institute of Physical and Chemical Research (RIKEN).

Screening for polymorphisms and genotyping

To identify genetic variants of the human *IL12RB1* gene, we sequenced all 17 exons, adjacent intronic sequence, 4.0 kb 5′ flanking region and 1.5 kb 3′-flanking region in 24 individuals from our study cohort. On the basis of *IL12RB1* genomic and mRNA sequences from the GenBank database (accession nos AC020904 and U03187, respectively), 25 primer sets were designed (Supplementary Material). All PCR products were sequenced using BigDye terminator v3.1 and an ABI Prism 3700 Genetic Analyzer (PE Applied Biosystems, Foster City, CA, USA). The sequences were analyzed and polymorphisms identified using the SEQUENCHER program (Gene Codes Corporation, Ann Arbor, MI, USA). On the basis of information available from the public JSNP database (http://snp.ims.u-tokyo.ac.jp), eight selected SNPs were genotyped by one of two methods: the Invader assay (41) (for −111A/T:IMS-JST063138, −2C/T:IMS-JST063137, 4443C/T:IMS-JST063136, 17183T/C:IMS-JST063134 and 27637T/A:IMS-JST097711) and the TaqMan assay (for 5970G/C, 17369C/T and 25748T/C) on an ABI PRISM 7700 Sequence

Detector Systems (Applied Biosystems), according to the manufacturer's instructions. Probe sets for the Invader assay were designed and synthesized by Third Wave Technologies, and those for the TaqMan assay were obtained from Applied Biosystems.

Tissue expression

We assessed *IL12RB1* expression in a panel of cDNA tissue samples (Human Multiple Tissue, Human Immune System and Human Blood Fractions Multiple Tissues cDNA Panels, Clontech) by PCR amplification of target sequences and Southern blotting. The primer sets were 5′-CAGTGGCTC TGAATATCAGC-3′ and 5′-TGCACCGTGTAGGCTACACC-3′ for *IL12RB1* and 5′-CCCCATGTTCGTCATGGGT-3′ and 5′-GTGATGGCATGGACTGTGG-3′ for *GAPDH*. Southern blotting was performed with a non-radioactive nucleic acid labeling and detection kit (Roche Diagnostic, Basal, Switzerland), according to the manufacturer's instructions. The probes for *IL12RB1* and *GAPDH* were 5′-TGGCAACC TACAGCTGGAGT-3′ and 5′-CCATGAGAAGTATGACAA CAG-3′, respectively.

Luciferase assay

After restriction enzyme digestion with *KpnI* and *XhoI*, luciferase reporter constructs were generated by cloning the different promoter fragments of the *IL12RB1* gene into the pGL3-basic vector (Promega, Madison, WI, USA) between unique *KpnI* and *XhoI* sites. Forward primers used were: −2947/−65, 5′-CCACTTGGGCCTCAGTTTCC-3′; −1668/−65, 5′-CTGACATTTAGAGGCTTTGCC-3′; −1361/−65, 5′-CAAACTCCTGACCTCGTGATC-3′; −762/−65, 5′-CCG TGATTGCACCACTGCAC-3′; −265/−65, 5′-ACCCTGA CTTGCTCCAAAGTC-3′; −104/−65, 5′-TCTCCTTGCTC AGCTTC-3′, with 5′-CCGTCCCCACTCCGGAACAC-3′ used as a common reverse primer. Using plasmid DNA as template, −111T constructs were created using the QuickChange Site-Directed Mutagenesis kit (Stratagene, USA), forward primer (5′-CTTTTTTCTTTTTTTTCTGTCTTTTCTCCTTG CTCAGCTTC-3′), complementary reverse primer and the various pGL3-basic clones. The orientation and integrity of the inserts for each construct were confirmed by DNA sequencing. Jurkat cells were cultured in RPMI-1640 medium supplemented with 10% fetal bovine serum at 37°C in an atmosphere of 5% CO_2. Subconfluent cells cultured in 12-well plates were transiently cotransfected with 2 μg pG L3-basic vector DNA or each reporter construct and 40 ng pRL-TK vector DNA (Promega) as an internal control for transfection efficiency, using DMRIE-C transfection reagent (Invitrogen, San Diego, CA, USA) according to the manufacture's instructions. After 24 h, cells were harvested, and firefly and *Renilla* luciferase activities measured as previously described (40). Data are presented as relative luciferase activity of firefly/*Renilla* luciferase activity.

PBMC preparation

To analyze *IL12RB1* mRNA expression, PBMCs from 16 randomly selected healthy individuals were isolated by Ficoll density gradient centrifugation, after which 1×10^6 cells/ml PBMC were cultured in RPMI-1640 supplemented with 10% fetal bovine serum, 1% HEPES buffer, 1% L-glutamine, 1% sodium pyruvate, 1% non-essential amino acids and 1% penicillin–streptomycin solution. PBMCs were stimulated with 100 μg/ml PHA or 100 μg/ml anti-CD3 antibody for 72 h at 37°C in a 5% CO_2 atmosphere.

Real-time quantitative PCR

Total RNA was isolated from cultured PBMC using the NucleoSpin 96 RNA kit (MACHEREY-NAGEL, Düren, Germany), according to the manufacturer's instructions. cDNA was then synthesized using the SuperScript III First-Strand Synthesis System (Invitrogen). Real-time quantitative PCR was performed on the ABI PRISM 7900 (Applied Biosystems) using an Assay-on-Demand TaqMan probe and primers (Hs00234651_m1 for *IL12RB1*), according to the manufacturer's instructions. Relative expression levels of *IL12RB1* mRNA were normalized according to *GAPDH* expression, using a standard curve method as described by the manufacturer.

Statistical analysis

We calculated allele frequencies and tested agreement with Hardy–Weinberg equilibrium using a χ^2 goodness-of-fit test at each locus. We also compared differences in allele frequencies and genotype distribution of each polymorphism between case and control subjects, using a 2×2 contingency χ^2 test with one degree of freedom or Fisher's exact test. All *P*-values are unadjusted for multiple comparisons unless specifically indicated. We calculated LD coefficients (D' and r^2) using the SNP Alyze statistical package (Dynacom, Chiba, Japan), as described elsewhere (42). We estimated haplotype frequencies using the expectation–maximization algorithm. We calculated ODs with 95% confidence intervals (95%CI) using logistic regression. Comparisons in reporter assays as well as quantitative PCR experiments were performed using Student's *t*-test. Association between log-transformed total serum IgE levels in AD patients and individual genotypes was calculated by the Mann–Whitney *U*-test or the Kruskal–Wallis test. A *P*-value of less than 0.05 was considered to indicate statistical significance.

SUPPLEMENTARY MATERIAL

Supplementary Material is available at HMG Online.

ACKNOWLEDGEMENTS

We thank Miki Kokubo, Aya Jodo and Hiroshi Sekiguchi for their technical assistance. This work was supported by grants-in-aid from the Ministry of Health, Labor and Welfare of Japan, the Japan Science and Technology Corporation and the Japanese Millennium Project.

Conflict of Interest statement. None declared.

REFERENCES

1. Rudikoff, D. and Lebwohl, M. (1998) Atopic dermatitis. *Lancet*, **351**, 1715–1721.
2. Williams, H., Robertson, C., Stewart, A., Ait-Khaled, N., Anabwani, G., Anderson, R., Asher, I., Beasley, R., Bjorksten, B., Burr, M. *et al.* (1999) Worldwide variations in the prevalence of symptoms of atopic eczema in the International Study of Asthma and Allergies in Childhood. *J. Allergy Clin. Immunol.*, **103**, 125–138.
3. Leung, D.Y., Boguniewicz, M., Howell, M.D., Nomura, I. and Hamid, Q.A. (2004) New insights into atopic dermatitis. *J. Clin. Invest.*, **113**, 651–657.
4. Larsen, F.S., Holm, N.V. and Henningsen, K. (1986) Atopic dermatitis. A genetic–epidemiologic study in a population-based twin sample. *J. Am. Acad. Dermatol.*, **15**, 487–494.
5. Schultz Larsen, F. (1993) Atopic dermatitis: a genetic–epidemiologic study in a population-based twin sample. *J. Am. Acad. Dermatol.*, **28**, 719–723.
6. Novak, N., Bieber, T. and Leung, D.Y. (2003) Immune mechanisms leading to atopic dermatitis. *J. Allergy Clin. Immunol.*, **112**, S128–S139.
7. Hamid, Q., Boguniewicz, M. and Leung, D.Y. (1994) Differential *in situ* cytokine gene expression in acute versus chronic atopic dermatitis. *J. Clin. Invest.*, **94**, 870–876.
8. Hamid, Q., Naseer, T., Minshall, E.M., Song, Y.L., Boguniewicz, M. and Leung, D.Y. (1996) *In vivo* expression of IL-12 and IL-13 in atopic dermatitis. *J. Allergy Clin. Immunol.*, **98**, 225–231.
9. Chan, L.S., Robinson, N. and Xu, L. (2001) Expression of interleukin-4 in the epidermis of transgenic mice results in a pruritic inflammatory skin disease: an experimental animal model to study atopic dermatitis. *J. Invest. Dermatol.*, **117**, 977–983.
10. Watford, W.T., Moriguchi, M., Morinobu, A. and O'Shea, J.J. (2003) The biology of IL-12: coordinating innate and adaptive immune responses. *Cytokine Growth Factor Rev.*, **14**, 361–368.
11. Abbas, A.K., Murphy, K.M. and Sher, A. (1996) Functional diversity of helper T lymphocytes. *Nature*, **383**, 787–793.
12. Presky, D.H., Yang, H., Minetti, L.J., Chua, A.O., Nabavi, N., Wu, C.Y., Gately, M.K. and Gubler, U. (1996) A functional interleukin 12 receptor complex is composed of two β-type cytokine receptor subunits. *Proc. Natl Acad. Sci. USA*, **93**, 14002–14007.
13. Wu, C., Ferrante, J., Gately, M.K. and Magram, J. (1997) Characterization of IL-12 receptor β1 chain (IL-12Rβ1)-deficient mice: IL-12Rβ1 is an essential component of the functional mouse IL-12 receptor. *J. Immunol.*, **159**, 1658–1665.
14. Wu, C., Wang, X., Gadina, M., O'Shea, J.J., Presky, D.H. and Magram, J. (2000) IL-12 receptor β2 (IL-12Rβ2)-deficient mice are defective in IL-12-mediated signaling despite the presence of high affinity IL-12 binding sites. *J. Immunol.*, **165**, 6221–6228.
15. Oppmann, B., Lesley, R., Blom, B., Timans, J.C., Xu, Y., Hunte, B., Vega, F., Yu, N., Wang, J., Singh, K. *et al.* (2000) Novel p19 protein engages IL-12p40 to form a cytokine, IL-23, with biological activities similar as well as distinct from IL-12. *Immunity*, **13**, 715–725.
16. Leung, D.Y. and Bieber, T. (2003) Atopic dermatitis. *Lancet*, **361**, 151–160.
17. Rafatpanah, H., Bennett, E., Pravica, V., McCoy, M.J., David, T.J., Hutchinson, I.V. and Arkwright, P.D. (2003) Association between novel GM-CSF gene polymorphisms and the frequency and severity of atopic dermatitis. *J. Allergy Clin. Immunol.*, **112**, 593–598.
18. Tsunemi, Y., Saeki, H., Nakamura, K., Sekiya, T., Hirai, K., Fujita, H., Asano, N., Kishimoto, M., Tanida, Y., Kakinuma, T. *et al.* (2002) Interleukin-12 p40 gene (IL12B) 3′-untranslated region polymorphism is associated with susceptibility to atopic dermatitis and psoriasis vulgaris. *J. Dermatol. Sci.*, **30**, 161–166.
19. Olesen, A.B., Juul, S., Birkebaek, N. and Thestrup-Pedersen, K. (2001) Association between atopic dermatitis and insulin-dependent diabetes mellitus: a case–control study. *Lancet*, **357**, 1749–1752.
20. Shirakawa, T., Enomoto, T., Shimazu, S. and Hopkin, J.M. (1997) The inverse association between tuberculin responses and atopic disorder. *Science*, **275**, 77–79.
21. Sakai, T., Matsuoka, M., Aoki, M., Nosaka, K. and Mitsuya, H. (2001) Missense mutation of the interleukin-12 receptor β1 chain-encoding gene is associated with impaired immunity against *Mycobacterium avium* complex infection. *Blood*, **97**, 2688–2694.
22. Akahoshi, M., Nakashima, H., Miyake, K., Inoue, Y., Shimizu, S., Tanaka, Y., Okada, K., Otsuka, T. and Harada, M. (2003) Influence of interleukin-12 receptor β1 polymorphisms on tuberculosis. *Hum. Genet.*, **112**, 237–243.
23. van de Vosse, E., Lichtenauer-Kaligis, E.G., van Dissel, J.T. and Ottenhoff, T.H. (2003) Genetic variations in the interleukin-12/interleukin-23 receptor (β1) chain, and implications for IL-12 and IL-23 receptor structure and function. *Immunogenetics*, **54**, 817–829.
24. Remus, N., El Baghdadi, J., Fieschi, C., Feinberg, J., Quintin, T., Chentoufi, M., Schurr, E., Benslimane, A., Casanova, J.L. and Abel, L. (2004) Association of *IL12RB1* polymorphisms with pulmonary tuberculosis in adults in Morocco. *J. Infect. Dis.*, **190**, 580–587.
25. Desai, B.B., Quinn, P.M., Wolitzky, A.G., Mongini, P.K., Chizzonite, R. and Gately, M.K. (1992) IL-12 receptor. II. Distribution and regulation of receptor expression. *J. Immunol.*, **148**, 3125–3132.
26. Grohmann, U., Belladonna, M.L., Bianchi, R., Orabona, C., Ayroldi, E., Fioretti, M.C. and Puccetti, P. (1998) IL-12 acts directly on DC to promote nuclear localization of NF-κB and primes DC for IL-12 production. *Immunity*, **9**, 315–323.
27. Wu, C.Y., Warrier, R.R., Carvajal, D.M., Chua, A.O., Minetti, L.J., Chizzonite, R., Mongini, P.K., Stern, A.S., Gubler, U., Presky, D.H. *et al.* (1996) Biological function and distribution of human interleukin-12 receptor β chain. *Eur. J. Immunol.*, **26**, 345–350.
28. Spergel, J.M. and Paller, A.S. (2003) Atopic dermatitis and the atopic march. *J. Allergy Clin. Immunol.*, **112**, S118–S127.
29. Grewe, M., Gyufko, K., Schopf, E. and Krutmann, J. (1994) Lesional expression of interferon-γ in atopic eczema. *Lancet*, **343**, 25–26.
30. Nomura, I., Goleva, E., Howell, M.D., Hamid, Q.A., Ong, P.Y., Hall, C.F., Darst, M.A., Gao, B., Boguniewicz, M., Travers, J.B. *et al.* (2003) Cytokine milieu of atopic dermatitis, as compared to psoriasis, skin prevents induction of innate immune response genes. *J. Immunol.*, **171**, 3262–3269.
31. Ong, P.Y., Ohtake, T., Brandt, C., Strickland, I., Boguniewicz, M., Ganz, T., Gallo, R.L. and Leung, D.Y. (2002) Endogenous antimicrobial peptides and skin infections in atopic dermatitis. *N. Engl. J. Med.*, **347**, 1151–1160.
32. Kupper, T.S. and Fuhlbrigge, R.C. (2004) Immune surveillance in the skin: mechanisms and clinical consequences. *Nat. Rev. Immunol.*, **4**, 211–222.
33. Matsui, E., Kaneko, H., Fukao, T., Teramoto, T., Inoue, R., Watanabe, M., Kasahara, K. and Kondo, N. (1999) Mutations of the IL-12 receptor β2 chain gene in atopic subjects. *Biochem. Biophys. Res. Commun.*, **266**, 551–555.
34. Morahan, G., Huang, D., Wu, M., Holt, B.J., White, G.P., Kendall, G.E., Sly, P.D. and Holt, P.G. (2002) Association of *IL12B* promoter polymorphism with severity of atopic and non-atopic asthma in children. *Lancet*, **360**, 455–459.
35. Morahan, G., Huang, D., Ymer, S.I., Cancilla, M.R., Stephen, K., Dabadghao, P., Werther, G., Tait, B.D., Harrison, L.C. and Colman, P.G. (2001) Linkage disequilibrium of a type 1 diabetes susceptibility locus with a regulatory *IL12B* allele. *Nat. Genet.*, **27**, 218–221.
36. Laitinen, T., Ollikainen, V., Lazaro, C., Kauppi, P., de Cid, R., Anto, J.M., Estivill, X., Lokki, H., Mannila, H., Laitinen, L.A. *et al.* (2000) Association study of the chromosomal region containing the *FCER2* gene suggests it has a regulatory role in atopic disorders. *Am. J. Respir. Crit. Care Med.*, **161**, 700–706.
37. Venanzi, S., Malerba, G., Galavotti, R., Lauciello, M.C., Trabetti, E., Zanoni, G., Pescollderungg, L., Martinati, L.C., Boner, A.L. and Pignatti, P.F. (2001) Linkage to atopy on chromosome 19 in north-eastern Italian families with allergic asthma. *Clin. Exp. Allergy*, **31**, 1220–1224.
38. Lee, Y.A., Ruschendorf, F., Windemuth, C., Schmitt-Egenolf, M., Stadelmann, A., Nurnberg, G., Stander, M., Wienker, T.F., Reis, A. and Traupe, H. (2000) Genomewide scan in German families reveals evidence for a novel psoriasis-susceptibility locus on chromosome 19p13. *Am. J. Hum. Genet.*, **67**, 1020–1024.
39. Hanifin, J.M. and Rajka, G. (1980) Diagnostic features of atopic dermatitis. *Acta Derm. Venereol.*, **92**(suppl.), 44–47.
40. Akahoshi, M., Obara, K., Hirota, T., Matsuda, A., Hasegawa, K., Takahashi, N., Shimizu, M., Nakashima, K., Cheng, L., Doi, S. *et al.* (2005) Functional promoter polymorphism in the *TBX21* gene associated with aspirin-induced asthma. *Hum. Genet.*, **117**, 16–26.
41. Ohnishi, Y., Tanaka, T., Ozaki, K., Yamada, R., Suzuki, H. and Nakamura, Y. (2001) A high-throughput SNP typing system for genome-wide association studies. *J. Hum. Genet.*, **46**, 471–477.
42. Nakajima, T., Jorde, L.B., Ishigami, T., Umemura, S., Emi, M., Lalouel, J.M. and Inoue, I. (2002) Nucleotide diversity and haplotype structure of the human angiotensinogen gene in two populations. *Am. J. Hum. Genet.*, **70**, 108–123.

Human Molecular Genetics, 2005, Vol. 14, No. 19 *2919–2927*
doi:10.1093/hmg/ddi323
Advance Access published on August 23, 2005

Functional SNPs in the distal promoter of the *ST2* gene are associated with atopic dermatitis

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Received June 15, 2005; Revised and Accepted August 18, 2005

Atopic dermatitis (AD) is a common inflammatory skin disease associated with the local infiltration of T helper type 2 (Th2) cells. The *ST2* gene encodes both membrane-bound ST2L and soluble ST2 (sST2) proteins by alternative splicing. The orphan receptor ST2L is functionally indispensable for Th2 cells. We found a significant genetic association between AD and the −26999G/A single nucleotide polymorphism (SNP) (χ^2-test, raw *P*-value = 0.000007, odds ratio 1.86) in the distal promoter region of the *ST2* gene (chromosome 2q12) in a study of 452 AD patients and 636 healthy controls. The −26999A allele common among AD patients positively regulates the transcriptional activity of the *ST2* gene. In addition, having at least one −26999A allele correlated with high sST2 concentrations and high total IgE levels in the sera from AD patients. Thus, the −26999A allele is correlated with an increased risk for AD. We also found that the −26999G/A SNP predominantly affected the transcriptional activity of hematopoietic cells. Immunohistochemical staining of a skin biopsy specimen from an AD patient in the acute stage showed ST2 staining in the keratinocytes as well as in the infiltrating cells in the dermal layer. Our data show that functional SNPs in the ST2 distal promoter region regulate ST2 expression which induces preferential activation of the Th2 response. Our findings will contribute to the evaluation of one of the genetic risk factors for AD.

INTRODUCTION

Atopic dermatitis (AD) is a chronic inflammatory skin disease associated with the local infiltration of T helper type 2 (Th2) cells that secrete interleukin (IL)-4, IL-5 and IL-13 in the acute stage followed by the infiltration of T helper type 1 (Th1) cells, which is responsible for the chronicity of AD lesions (1). Genetic susceptibility to AD has been suggested by epidemiological and genetic studies (2–4). In one study, monozygotic twin pairs had a concordance rate of 0.72 and dizygotic twin pairs had one of 0.23 for AD (5). The IL-1 receptor (IL1R) gene cluster (2q12–14) has many immunoregulatory genes including IL1R1, IL1R2, IL18R1 and IL18RAP. We and others reported some genetic association studies of atopic diseases investigating this region (6–8). We carried out detailed genetic association studies of the IL1R region and found that genetic polymorphisms within the ST2 (IL1RL1) gene region had a strong association with AD.

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ST2 was originally reported as a gene induced by serum in mouse fibroblasts (9). There are three alternatively spliced variants in ST2 in mammals, membrane-bound ST2L (10), soluble ST2 (sST2) (9) and ST2V (11). ST2L is an orphan receptor which has a conserved cytosolic domain called as the Toll-IL1R (TIR) domain. The functional role of ST2L is relevant to AD because ST2L is expressed in Th2 cells but not in Th1 cells (12), it has critical roles in Th2 effector functions (13) and is considered as a functional marker for Th2 cells. In addition, ST2L expression was also reported in mast cells (14), effector cells in the acute stage of AD (15). In contrast to other members of the TIR family that activate NF-κB signaling pathways (16), ST2L negatively regulates IL1R1 and toll-like receptor (TLR)-4 signals by sequestrating MyD88 and Mal signals (17). In this study, we found single nucleotide polymorphisms (SNPs) associated with increased risk for AD in the promoter region of the *ST2* gene. The high-risk SNPs showed higher ST2 promoter activity and hence increased serum sST2 as well as total IgE levels in AD patients.

RESULTS

Identification of genetic polymorphisms in ST2 and intragenic LD

We discovered 67 genetic variants in the ST2 region (Supplementary Material, Table S2) by resequencing DNA samples from 24 Japanese individuals (12 AD patients and 12 controls). Among the 67 genetic polymorphisms, 34 variants had estimated minor allele frequencies (MAF) of >10% (based on the sequencing of 24 DNA samples). We calculated r^2 as the statistical value for pairwise linkage disequilibrium (LD) between the SNPs (Supplementary Material, Fig. S1). On the basis of location and LD with other sites, we selected seven haplotype tagging SNPs as representative SNPs (Fig. 1A and Table 1) from the 34 common SNPs. Two SNPs (−27639A/G and −26999G/A) were in the 5′-genomic region for exon 1a, one SNP (744C/A) in exon 3 and four SNPs (2992C/T, 5283G/A, 5860C/A, 11147C/T) in the introns. Positions are numbered according to their positions relative to the published *ST2* gene sequence (GenBank accession no. AC007248), and position 1 is the adenine of the first methionine.

Case–control study

A summary of the case–control association study with representative SNPs is shown in Table 1. All seven SNPs were in Hardy–Weinberg equilibrium in both AD and control groups ($P > 0.05$). One −26999G/A SNP showed a significant association under a dominant model [−26999GG versus G/A + A/A, raw P-value = 0.000007, $P = 0.000049$ after Bonferroni correction, odds ratio (OR) = 1.86] (Table 2). The association became stronger (raw P-value = 0.000000038, corrected $P = 0.00000027$, OR = 2.55) for the AD patients with very high serum total IgE levels (IgE > 1700 IU/ml, $n = 290$) (Table 2). Weak association was also observed at −27639A/G SNP (−27639AA versus A/G + G/G, raw P-value = 0.0001, corrected P-value = 0.0007). The −27084G/C SNP was also fully genotyped and we found that the −27084G/C and −26999A/G SNPs were in a state of complete LD.

Haplotype analysis

We also tested the distribution of two- and seven-locus haplotypes in AD and control samples. Among the two-locus haplotypes of the promoter region SNPs (−27639A/G and −26999G/A), haplotype −27639G/−26999A showed an increased risk for AD (Table 3, G, A versus others; $P = 0.0004$, OR = 1.41). We also analyzed the haplotypes of the seven representative SNPs and found that haplotype-A was associated with AD (Table 4, haplotype-A versus others; $P = 0.000028$, OR = 1.45). However, none of these associations was stronger than those observed for the single locus (−26999G/A).

Reporter gene analysis

We made a construct for haplotype-1 (the major haplotype: −27639A, −27084G and −26999G) and haplotype-2 (the common haplotype among AD patients: −27639G, −27084C and −26999A) with pGL3 basic vector. The assay was performed in triplicate, and a representative result of three independent experiments is shown as mean ± SD in Fig. 1B, right. The relative strengths of luciferase activity were 1517 ± 41 (mean ± SD) for haplotype-1 and 3226 ± 84 for haplotype-2, 267 ± 7 for distal-Δ355 (−27639A clone). The distal-Δ355 clone with the −27639G allele showed a result similar to that for the −27639A allele (data not shown). Haplotype-2 induced stronger ST2 promoter activity than haplotype-1.

RT–PCR analysis with a panel of hematopoietic cells, keratinocytes and dermal fibroblasts

For analysis of differential promoter usage, we made specific primer sets to distinguish each promoter and subtype of ST2 expression and performed RT–PCR with cDNA from a human mast cell line (LAD2), human keratinocytes (KC) cultured with serum-free medium and dermal fibroblasts cultured with 10% fetal bovine serum (FBS). For some studies, KC were stimulated with 10% FBS for 24 h. The results showed that only mast cells used both distal and proximal promoters. The other cells (skin fibroblasts and KC) used the proximal promoter exclusively (Fig. 1C). LAD2 cells could express sST2 mRNA using both promoters and ST2L mRNA using the distal promoter, whereas skin fibroblasts and KC could only express sST2 using the proximal promoter (Fig. 1C).

Quantification of sST2 protein and total IgE using the sera from AD patients

The concentration of sST2 in the sera of 124 AD patients was measured with ELISA. The sST2 concentration of the serum of patients with the −26999G/G genotype was 0.225 ng/ml (mean). For the −26999G/A + A/A genotype, it was 0.365 ng/ml (Supplementary Material, Fig. S4A). The sera from −26999G/G genotype patients showed a significantly lower ST2 concentration than those from −26999G/A + A/A patients ($P = 0.0000008$ by Mann–Whitney U-test). All measurements were performed in duplicate. We carried out

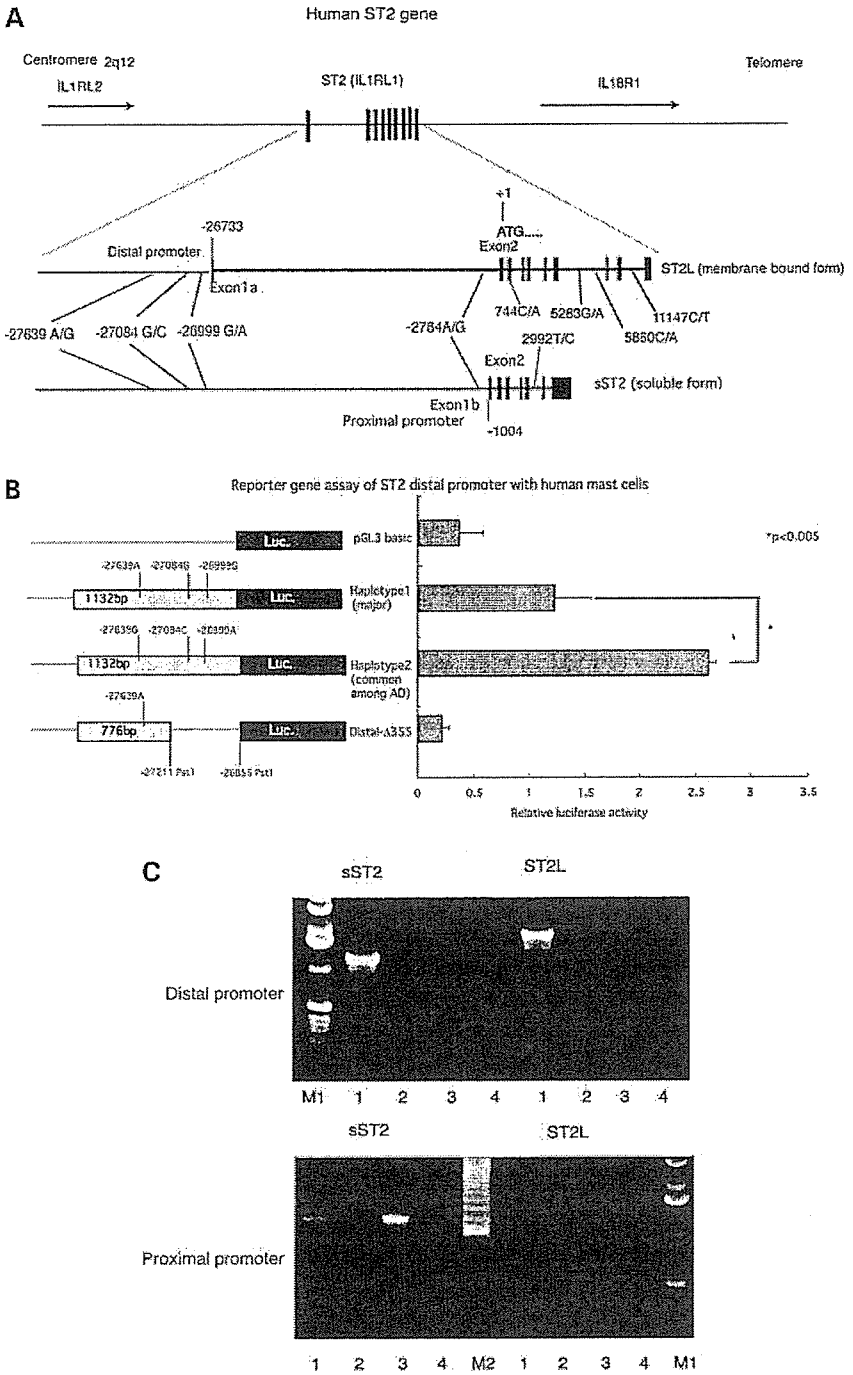

Figure 1. *ST2* gene structures and the roles of promoters in the induction of ST2 transcripts. (**A**) ST2 (IL1RL1) locus SNP map in the genomic region. The complete coding region of ST2, intron/exon boundaries, ~3 kb of 5′-genomic DNA, is shown. The longer variant (ST2L) has 11 exons and the shorter variant (sST2) has eight exons. These exons are indicated by closed rectangles. (**B**) Comparison of allelic variants of the ST2 distal promoter region analyzed by luciferase activity. Allelic differences in luciferase activity were examined using human mast (LAD2) cells. The constructs of the reporter plasmids are shown on the left. Five hundred nanograms of each plasmid was transfected with 10 ng of pRL-TK vector. Transcriptional activity was determined by assaying the *firefly* luciferase activity of cellar extracts prepared 24 h after transfection. Data show the mean ± SD relative activity from a representative experiment done in triplicate. $^*P = 0.004$ by Student's *t*-test. (**C**) RT–PCR with cDNA from various cells in skin using specific primer sets for distinguishing each promoter and subtype (ST2L/sST2) expression. (Top left) Forward primer: exon 1a (distal promoter), reverse primer: sST2 specific region. (Top right) Forward: exon 1a, reverse: ST2L-specific region. (Bottom left) Forward: exon 1b (proximal promoter), reverse: sST2. (Bottom right) Forward: exon 1b, Reverse: ST2L. Lane 1: LAD2 (mast cells), lane 2: KC cultured with serum-free medium (SFM), lane 3: KC cultured with SFM + 10% FBS for 24 h, lane 4: dermal fibroblasts. M1: 1 kb molecular marker, M2: 100 bp molecular marker.

Table 1. Genotype frequencies for ST2 SNPs and AD susceptibility

	SNP number	Location	Control ($n = 636$)					AD ($n = 452$)					P-value[a]	P-value[b]	P-value[c]
			1	2	3	Sum	Minor allele frequency	1	2	3	Sum	Minor allele frequency			
1	14	−27639A/G	205	295	124	624	0.44	99	235	115	449	0.52	0.0026[d]	0.0007[d]	NS
2	18	−26999G/A	223	279	112	614	0.41	106	240	106	452	0.50	0.00024[d]	0.000049[d]	NS
3	41	744C/A	415	182	28	625	0.19	313	123	9	445	0.16	NS	NS	NS
4	49	2992C/T	221	286	113	620	0.41	183	205	57	445	0.36	NS	NS	NS
5	51	5283G/A	272	273	79	624	0.35	204	195	48	447	0.33	NS	NS	NS
6	57	5860C/A	225	284	110	619	0.41	187	205	56	448	0.35	NS	NS	NS
7	67	11147C/T	251	280	91	622	0.37	209	189	47	445	0.32	NS	NS	NS

NS, not significant.
[a]Allele1 versus allele2.
[b]Genotype11 versus 12 + 22.
[c]Genotype11 + 12 versus 22.
[d]P-value statistically significant after Bonferroni correction (raw P-values were multiplied by 7).

Table 2. Association between ST2-26999 G/A SNP and AD

	Controls ($n = 614$)	AD ($n = 452$)	χ^2 (P-value)	OR (95% CI)	AD total IgE>1700 ($n = 290$)	χ^2 (P-value)	OR (95% CI)
−26999G/A			GG:others			GG:others	
GG	223	106	20.20	1.86	53	30.23	2.55
GA	279	240	(0.0000070)[a]	(1.42–2.45)	166	(0.000000038)[a]	(1.81–3.58)
AA	112	106	(0.000049)[b]		71	(0.00000027)[b]	

[a]Raw P-value.
[b]P-value after Bonferroni correction.

Table 3. Haplotype structures and frequencies in ST2 distal promoter

Haplotype −27639, −26999	Haplotype frequency Case	Control	χ^2	P-value	OR
A, G	0.56	0.48	13.00	0.0012	1.37
G, A	0.41	0.50	15.14	0.0004	1.41
G, G	0.025	0.019	0.85	0.35	1.32

two separate experiments and the results were similar. The total IgE concentration in the sera of 428 AD patients was measured with the fluorescence-enzyme immunoassay (FEIA) (Supplementary Material, Fig. S4B). The total IgE concentrations were 5371.9 IU/ml (mean) for the sera from −26999G/G genotype patients and 7898.7 IU/ml for those from −26999 G/A + A/A genotype patients. The serum concentration of total IgE was significantly lower in the sera of −26999G/G patients ($P = 0.0024$ by Mann–Whitney U-test). The correlation between the sST2 and the total IgE concentration was examined among −26999A/A genotype patients (Supplementary Material, Fig. S4C); Pearson's correlation coefficient was 0.28.

ST2L protein expression on the surface of human mast cells

Immunoprecipitation (IP) and subsequent western blotting using LAD2 cell lysate showed a positive band around 90 kDa in the IP samples with an anti-ST2 antibody (clone2A5). Deglycosylation with PNGaseF showed a shift of the band to lower molecular weight, corresponding to the molecular weight of non-glycosylated ST2L protein (Fig. 2A). To further demonstrate the surface expression of ST2L protein, non-stimulated LAD2 cells were stained with the anti-ST2 antibody (with FITC) and analyzed by FACS. The histogram showed a positive shift of the mean FITC intensity of ST2 staining (dotted line, Fig. 2B) compared with that of isotype-matched mouse IgG.

Immunohistochemistry

A paraffin section of the skin biopsy sample from an AD patient in the acute stage was stained with an anti-ST2 monoclonal antibody (clone HB12). Positive staining was observed on the cell surface of KC in the suprabasal layer and infiltrating cells in the dermal layer (Fig. 3A and C). ST2-positive staining was observed only with the infiltrating cells in the dermal layer of the skin of another AD patient in the chronic stage (Fig. 3B). Immunostaining with control mouse IgG_1 did not show positive signals (data not shown).

DISCUSSION

We found an SNP in the distal promoter region of ST2 (−26999G/A) that showed a significant association with AD during our series of genetic association studies within the IL1R gene cluster. This is the first association study for the

Table 4. Haplotype structures and frequencies in ST2

HaplotypeID	Haplotype frequency		−27639	−26999	744	2992	5283	5860	11147
	Case	Control							
Haplotype A	0.41	0.5	G	A	C	T	A	C	C
Haplotype B	0.33	0.32	A	G	C	C	G	A	T
Haplotype C	0.13	0.12	A	G	A	T	A	C	C

Haplotype1/others: $\chi^2 = 17.5$; $P = 0.000028$; OR = 1.45.
Haplotype2/others: $\chi^2 = 0.15$; $P = 0.703$; OR = 1.04.
Haplotype3/others: $\chi^2 = 0.57$; $P = 0.451$; OR = 1.1.

ST2 gene and the results are intriguing, because the SNPs directly affect the expression level of Th2 cell marker ST2. Recent studies have clearly shown essential functional roles of ST2L protein for Th2-mediated immune responses (13,18,19), so it seems reasonable to investigate ST2 genetic polymorphism as a candidate for conferring susceptibility to AD. The result of case–control association studies of seven representative SNPs (Table 1) and haplotype analysis (Tables 3 and 4) showed that the highest association with AD was observed with the −26999G/A SNP as a single locus. There were four other SNPs in the ST2 genomic region that showed tight LD with the −26999G/A SNP. Three SNPs were located distal to the −26999G/A SNP in the distal promoter region. Two SNPs (−28214T/C; 3258 bp distal from the transcription starting site and −29778C/A; 1694 bp distal from the site) were not included in the functional analysis because our series of 5′-deletion promoter assays showed that the critical region for ST2 distal promoter activity was located within 300–500 bp from the transcriptional starting point (20); therefore, these two SNPs seemed to be less functional. Of the remaining two SNPs, one SNP (−27084G/C) was located at 85 bp distal to the −26999G/A SNP (236 bp distal to the transcriptional starting site); therefore, we decided to analyze these two SNPs together by reporter gene assay. The last SNP (−2874A/G) in tight LD with −26999G/A, located in the proximal promoter region of the *ST2* gene, did not affect the proximal promoter activity (Supplementary Material, Fig. S2).

The distal promoter reporter gene assay was performed with two 1132 bp distal promoter constructs, including two major haplotypes −27639A/−27084G/−26999G and −27639G/−27084C/−26999A covering >97% of haplotype frequency (Table 3). In addition to the two SNPs in the state of complete LD, (−27084/−26999), another SNP (−27639 A/G) that also showed a weak association with AD (Table 1) was included for analysis. We have reported that a GATA element commonly observed in both human and mouse *ST2* gene distal promoter region was indispensable for the activation of the promoter activity in Th2 cells (20,21). Therefore, we made another set of promoter assay constructs (Distal-Δ355) deprived of the GATA binding site and two SNP sites, which showed abrogated transcriptional activity (Fig. 1B). From these results, we concluded that this 356 bp region was essential for ST2 transcription and that the two SNPs (−27084G/C and −26999G/A) had major influences on the distal promoter activity among the SNPs with significant associations.

For further analysis of the roles of the genetic polymorphisms, we measured the serum concentrations of sST2 and total IgE and sorted the results by the genotype in the distal promoter. As the association study showed the most significant result under a dominant model (Tables 1 and 2), we compared the results by the genotype −26999G/G (low risk for AD) versus −26999A/G + A/A (high risk for AD). The results matched the results of the reporter gene assay and the association study. Furthermore, the genetic association between the −26999G/A SNP and AD patients for very high serum total IgE (IgE > 1700 IU/ml) became stronger (Table 2). These results suggested that having at least one allele of −26999A was correlated with a high sST2 level and a high total IgE concentration and an increased risk for AD. There is some controversy over the role of IgE in the pathogenesis of AD (22); therefore, it will be useful to genotype intrinsic AD (1) patients in the future.

We found a weak correlation ($r = 0.28$) between the serum sST2 level and the total IgE concentration with the genotype −26999AA patients. This finding was consistent with the recent report that the increase of food-specific IgE is paralleled by elevated sST2 levels, not by serum IL-4, IL-13 and interferon gamma levels (23). These results suggested possible effects of sST2 in IgE production, so further studies seem to be essential.

We reported sST2 concentrations of 200 healthy controls and 56 asthmatic patients previously (24). The sST2 concentration of healthy controls was 0–1.65 ng/ml (mean 0.415 ng/ml) and that of asthmatic patients was 0–2.40 ng/ml (mean 0.493 ng/ml). A differential rise of the serum ST2 level that correlated well with the severity of asthma exacerbation was observed (24). The serum concentration of sST2 in AD patients [0–1.02 ng/ml (mean 0.326 ng/ml)] was not significantly higher or lower than that of healthy controls or asthmatics; nonetheless, there was a correlation between the ST2 genotype and the sST2 concentration. We are now investigating the changes of the sST2 concentration during the clinical stages of AD, and the results might further clarify the role of sST2 in AD.

It has been reported that the usage of two different promoters (distal and proximal) depends on the type of cell for the human *ST2* gene (21). Consistent with this report, we showed that only hematopoietic cells utilized the distal promoter and that ST2 transcription of other skin cells (KC, dermal fibroblasts) was initiated from the proximal promoter (Fig. 1C). These results suggested that the significant association of SNPs −27084 and −26999 in the ST2 distal promoter

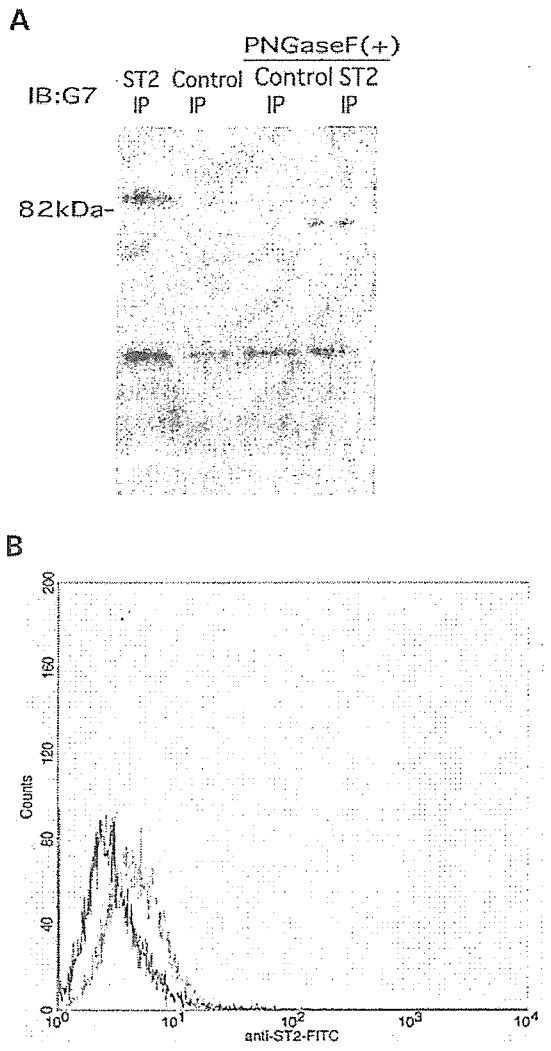

Figure 2. ST2L expression in LAD2 cells. (A) LAD2 cell lysate samples were immunoprecipitated with either an anti-ST2 IgG antibody (2A5) or an isotype-matched control antibody. The immunoprecipitated samples were electrophoresed and immunoblotted with an anti-ST2 IgM antibody (clone: G7). Duplicated samples after IP were treated with PNGaseF for 1 h and then immunoblotted simultaneously. Lane 1: anti-ST2-IP, lane 2: control antibody-IP, lane 3: control antibody-IP-PNGaseF-treated, lane 4: anti-ST2-IP-PNGaseF-treated. (B) Cell surface ST2L protein expression in LAD2 was analyzed with a FACS Calibur. FcR of LAD2 cells were blocked and then stained with an anti-ST2 antibody (2A5). FITC-goat anti-mouse IgG1 was used as the secondary antibody. Staining with control mouse IgG1 is shown with a black line and the anti-ST2 antibody is shown with the dotted red line.

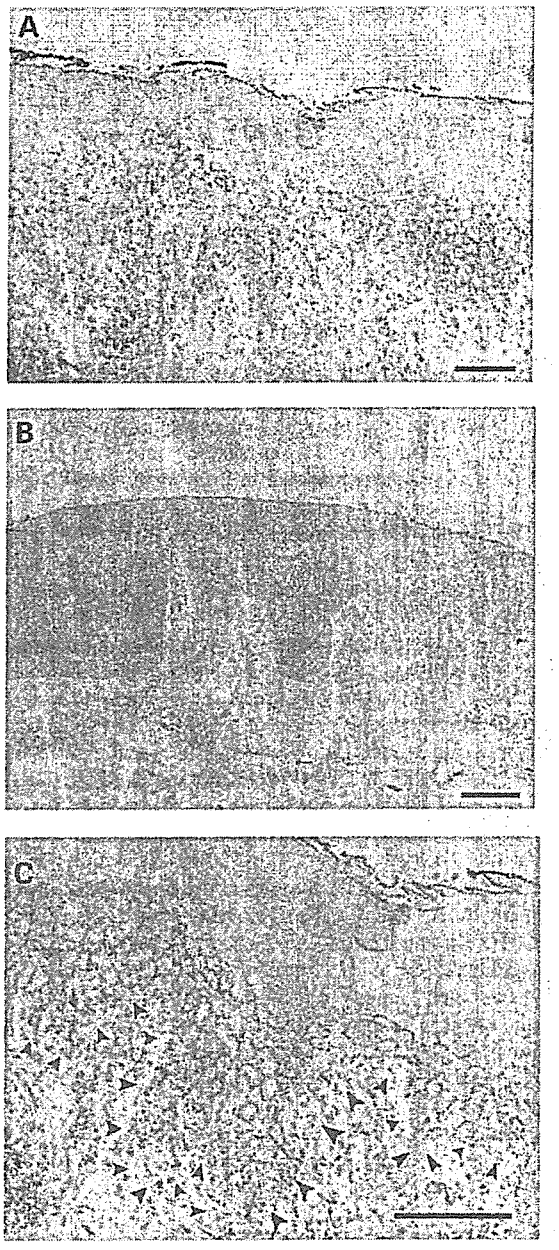

Figure 3. Immunohistochemical staining of human skin samples obtained from AD patients with anti-ST2 monoclonal antibody. Paraffin sections of AD skin biopsies were immunostained with an anti-ST2 antibody (HB12). (A) Skin biopsy from acute stage AD. Suprabasal layers of KC show membranous ST2-positive staining. Sporadic positive staining in the dermal region was also observed. (B) Skin biopsy from chronic stage AD. Some of the infiltrating cells in the dermal layer show positive ST2 staining. (C) High magnification of anti-ST2 immunostaining with acute stage AD. The arrowheads indicate the limit of basement membrane. Bar = 200 μm.

region predominantly affected hematopoietic cells. We found that both ST2L and sST2 mRNAs were expressed most abundantly in mast cells (Supplementary Material, Fig. S3) and confirmed ST2L expression on mast cells at the protein level by western blotting (Fig. 2A) and FACS analysis (Fig. 2B). Moritz *et al.* (14) reported that ST2L was selectively expressed during the development of mast cell lineage, and very recently Chen *et al.* (25) showed that ST2L could be one of the markers for mast cell progenitors in adult mice. These results suggested that abundant ST2L expression might positively affect the number of mature mast cells in the skin, as observed in the AD skin region

(26). The functional roles of ST2L in mast cells will be clarified with an ST2L overexpression system (10), and a study is ongoing.

The positive immunostaining around the cell membrane of suprabasal KC in the acute stage of AD (Fig. 3A and C) reflects the accumulated sST2 in intercellular space because ST2L mRNA expression in KC was not observed in experiments *in vitro*. This is consistent with a previous study that showed intense ST2 protein accumulation in mouse epidermis (27), and we think that serum extravasation during the acute stage of AD may induce sST2 expression in KC as observed in our *in vitro* study (Fig. 1C) (Supplementary Material, Fig. S3). On the other hand, a histological sample from the chronic phase of AD showed slight ST2 staining (Fig. 3B). This might be a reflection of the shift toward the Th1 dominant immunological character observed in the chronic stage of AD (1,28).

Another clinical feature of AD is a reduced skin innate immune response (1). ST2L expression could inhibit the TLR-dependent innate response by sequestering the adaptor molecules Myd88 and Mal (17). Several reports showed that both anti-ST2 antibodies and ST2-immunoglobulin fusion protein could abrogate the Th2 immune response and eosinophilic responses (18,29). Therefore, we consider that sST2/ST2L will be a good therapeutic target of AD and that understanding of the genetic predisposition for high ST2 promoter activity may contribute to the prevention of severe AD.

MATERIALS AND METHODS

Antibodies and cell lines

Anti-ST2 monoclonal antibodies (mouse IgG1; clones 2A5 and HB12) were purchased from MBL (Nagoya, Japan), and an anti-ST2 monoclonal antibody (mouse IgM; clone G7) was generated as previously described (30). Human mast cell line LAD2 was kindly provided by Dr Arnold Kirshenbaum (NIAID, NIH) and maintained as previously described (31). Human neonatal skin fibroblasts were obtained from RIKEN cell bank (Tsukuba, Japan), immortalized human normal keratinocyte cells (PHK16-06b) were obtained from the Japanese Collection of Research Bioresources (JCRB) cell bank (Osaka, Japan).

Subjects

All subjects with AD were diagnosed according to the criteria of Hanifin and Rajka (32). Peripheral blood was obtained from 452 AD patients (mean age 30.0, 11–64 years old at enrollment of the study; mean age 7.1, 0–45 years old at the onset of AD; 236 males and 216 females) from Takao Hospital, Shiga Medical College Hospital and Yokohama City University Hospital. Sera for sST2 ELISA assay were also obtained from some of the patients enrolled in this genetic study. As a control group, we analyzed 636 randomly selected population-based individuals (mean age 42.2, 18–70 years). We excluded the presence of asthma, AD and nasal allergy in the control population via careful interview by physicians. All individuals were Japanese and gave written informed consent to participate in the study (or, for individuals less than 16 years old, their parents gave consent), according to the rules of the process committee at SNP Research Center, RIKEN.

Screening for genetic polymorphisms

The ST2 genomic region targeted for SNP discovery included a 2.5 kb continuous region 5′ to exon 1a (distal promoter region) and a 2.5 kb continuous region 5′ to exon 1b (proximal promoter region) and 11 exons, each with a minimum of 200 bases of flanking intronic sequences (Fig. 1A). Primer sets (Supplementary Material, Table S1) were designed on the basis of the ST2 genomic sequence (GenBank accession no. AC007248). Each polymerase chain reaction (PCR) was carried out with 5 ng of genomic DNA from 24 individuals. Sequence reaction was performed with Big Dye Terminator v3.1 using an ABI 3700 DNA analyzer.

Genotyping

We genotyped a total of seven representative SNPs in the *ST2* gene selected on the basis of the allele frequency (MAF > 10%) and LD (Table 1) (Supplementary Material, Table S2 and Fig. S1). Additional typing was carried out for some SNPs, in relation to the functional assay for *ST2* genes. The SNP typing was carried out either with the invader assay (33) or with the Taqman genotyping assay using an ABI PRISM 7700 sequence detection system. Invader assay was performed with multiplex PCR products as the template. Taqman genotyping assay was carried out according to the manufacturer's protocol.

Statistical analysis

Allele frequencies in AD cases and controls were compared by the contingency χ^2-test. A P-value of less than 0.01, also in the case of multiple comparisons after Bonferroni adjustment, was considered to be statistically significant. ORs and 95% confidence intervals (95% CI) were calculated. Pairwise LD coefficients were calculated and expressed as r^2. Intragenic LD and haplotype analyses were performed using SNPAlyze v2.0 (DYNACOM, Chiba, Japan) as recommended by the manufacturer. We estimated haplotype frequencies using the expectation–maximization algorithm. Comparison in reporter gene assay was performed with Student's t-test. The association between the serum sST2 level or total IgE concentration and the genotype was evaluated by the Mann–Whitney U-test. A P-value of less than 0.05 was considered to be statistically significant.

Reporter gene assay

We subcloned 1131 bp distal promoter sequences continuous to exon 1a into pGL3 basic vector (Promega Corporation, Madison, WI, USA). Two SNPs in this region (−27084G/C and −26999G/A) were in the state of complete LD. We made two haplotype clones 1 (−27639A, −27084G, −26999G) and 2 (−27639G, −27084C, −26999A). Another set of constructs was made by deleting a 355 bp long promoter sequence

between two PstI sites, which contained the −27084G/C and −26999G/A SNPs as well as two putative GATA binding sequences (named distal-Δ355). All subcloned plasmids were verified by direct sequencing. We transfected the pGL3-ST2 promoter plasmid and pRL-TK renilla luciferase vector (Promega) as an internal control for transfection efficiency into human cell line LAD2 with DMRIE-C (Invitrogen, Carlsbad, CA, USA). After 24 h, luciferase activity was measured with a Dual Luciferase Reporter Assay Kit (Promega).

Measurement of sST2 protein and total IgE

The protein level of sST2 in the sera of AD patients was measured using human ST2 ELISA kits (MBL) following the manufacturer's protocol. The total IgE concentration in serum was measured by the FEIA method in a commercial laboratory.

RT–PCR analysis for differential promoter usage

mRNA was isolated from cultured cells (LAD2, KC and human dermal fibroblasts) with a Quick Prep micro-mRNA purification kit (Amersham Bioscience, Little Chalfont, UK). cDNA was made with the Super Script III First-Strand Synthesis System (Invitrogen) using oligo$(dT)_{20}$ primer. To distinguish promoter usage for specific cell types and subtypes (sST2/ST2L) of mRNA, we made sets of specific primers and performed RT–PCR as previously described (21).

IP and western blotting analysis

First, 1×10^7 LAD2 cells were solublized with lysis buffer [1% Triton X-100 in 20 mM Tris–HCl, pH 7.6, 150 mM NaCl with Complete Mini protease inhibitor cocktail tablets (Roche, Penzberg, Germany)]. The cell lysate was centrifuged at 20 000*g* for 15 min at 4°C. The supernatant was taken and pre-cleared with Protein-A Sepharose (Amersham) for 30 min. The sample was reacted with 2 μg of the anti-ST2 antibody (2A5) or control mouse IgG1 for 1 h and then Protein-A Sepharose was added. After 3 h rotation at 4°C, the Sepharose was washed with the lysis buffer and finally suspended with SDS sample buffer (50 mM Tris–HCl, pH 6.8, 2% SDS, 20% glycerol, 0.4% bromophenol blue, 50 mM DTT). To check the glycosylation status of ST2L protein, aliquots of the IP samples were treated with PNGaseF (New England Biolaboratory, Bevery, MA, USA). SDS–PAGE and subsequent immunoblotting were essentially performed as previously described (34). In brief, samples were subjected to SDS–PAGE using 4–20% Tris–glycine polyacrylamide gels and then electrophoretically transferred onto a PVDF membrane (Millipore). The membrane was incubated with the mouse anti-human ST2 IgM antibody (G7) overnight at 4°C. After washing with PBS, the membrane was reacted with a horseradish peroxidase (HRP)-conjugated anti-mouse IgM antibody for 30 min. The membrane was developed onto X-ray film with ECL plus (Amersham).

Flow cytometric analysis

Flow cytometric analysis was carried out using the anti-ST2 monoclonal antibody (2A5). LAD2 cells were washed with PBS, and Fc receptors (FcR) were blocked with FcR blocking reagent (Miltenyi Biotec, Gladbach, Germany). Cells were reacted with 4 μg of the anti-ST2 IgG monoclonal antibody in a volume of 40 μl for 15 min at room temperature. As a control, an isotype-matched mouse IgG1 antibody was used. After washing with PBS, the cells were reacted with an FITC-conjugated anti-mouse IgG antibody (Dako Japan, Kyoto). The stained cells were analyzed with a FACS Caliber (BD Japan).

Immunohistochemistry

ST2 immunohistochemistry was performed essentially as described previously (35). In brief, formaldehyde-fixed paraffin sections of the skin biopsies from AD patients were deparaffinized, then the endogenous peroxidase activity was quenched with 0.3% H_2O_2 in methanol for 20 min. Non-specific staining was blocked with blocking buffer (10% normal goat serum, 1% BSA in PBS) for 30 min. The anti-ST2 monoclonal antibody (clone HB12) was applied and reacted overnight at 4°C. After washing with PBS, slides were incubated with HRP-conjugated anti-mouse IgG for 30 min. The slides were developed with DAB (Dojindo, Kumamoto, Japan).

SUPPLEMENTARY MATERIAL

Supplementary Material is available at HMG Online.

ACKNOWLEDGEMENTS

We thank Dr Arnold Kirshenbaum for providing LAD2 cells, Professor Julian M. Hopkin for continuous supports and valuable comments, Miki Kokubo and Hiroshi Sekiguchi for their excellent technical assistance. This work was supported by a grant from the Japanese Millennium Project.

Conflict of Interest statement. None declared.

REFERENCES

1. Leung, D.Y., Boguniewicz, M., Howell, M.D., Nomura, I. and Hamid, Q.A. (2004) New insights into atopic dermatitis. *J. Clin. Invest.*, **113**, 651–657.
2. Lee, Y.A., Wahn, U., Kehrt, R., Tarani, L., Businco, L., Gustafsson, D., Andersson, F., Oranje, A.P., Wolkertstorfer, A., Von Berg, A. *et al.* (2000) A major susceptibility locus for atopic dermatitis maps to chromosome 3q21. *Nat. Genet.*, **26**, 470–473.
3. Bradley, M., Soderhall, C., Luthman, H., Wahlgren, C.F., Kockum, I. and Nordenskjold, M. (2002) Susceptibility loci for atopic dermatitis on chromosomes 3, 13, 15, 17 and 18 in a Swedish population. *Hum. Mol. Genet.*, **11**, 1539–1548.
4. Cookson, W.O., Ubhi, B., Lawrence, R., Abecasis, G.R., Walley, A.J., Cox, H.E., Coleman, R., Leaves, N.I., Trembath, R.C., Moffatt, M.F. *et al.* (2001) Genetic linkage of childhood atopic dermatitis to psoriasis susceptibility loci. *Nat. Genet.*, **27**, 372–373.
5. Schultz Larsen, F. (1993) Atopic dermatitis. A genetic-epidemiologic study in a population-based twin sample. *J. Am. Acad. Dermatol.*, **28**, 719–723.
6. Mao, X.Q., Kawai, M., Yamashita, T., Enomoto, T., Dake, Y., Sasaki, S., Kataoka, Y., Fukuzumi, T., Endo, K., Sano, H. *et al.* (2000) Imbalance production between interleukin-1beta (IL-1beta) and IL-1 receptor antagonist (IL-1Ra) in bronchial asthma. *Biochem. Biophys. Res. Commun.*, **276**, 607–612.

7. Karjalainen, J., Hulkkonen, J., Pessi, T., Huhtala, H., Nieminen, M.M., Aromaa, A., Klaukka, T. and Hurme, M. (2002) The IL1A genotype associates with atopy in nonasthmatic adults. *J. Allergy. Clin. Immunol.*, **110**, 429–434.
8. Wjst, M., Fischer, G., Immervoll, T., Jung, M., Saar, K., Rueschendorf, F., Reis, A., Ulbrecht, M., Gomolka, M., Weiss, E.H. *et al.* (1999) A genome-wide search for linkage to asthma. German Asthma Genetics Group. *Genomics*, **58**, 1–8.
9. Tominaga, S. (1989) A putative protein of a growth specific cDNA from BALB/c-3T3 cells is highly similar to the extracellular portion of mouse interleukin 1 receptor. *FEBS Lett.*, **258**, 301–304.
10. Li, H., Tago, K., Io, K., Kuroiwa, K., Arai, T., Iwahana, H., Tominaga, S. and Yanagisawa, K. (2000) The cloning and nucleotide sequence of human ST2L cDNA. *Genomics*, **67**, 284–290.
11. Tominaga, S., Kuroiwa, K., Tago, K., Iwahana, H., Yanagisawa, K. and Komatsu, N. (1999) Presence and expression of a novel variant form of *ST2* gene product in human leukemic cell line UT-7/GM. *Biochem. Biophys. Res. Commun.*, **264**, 14–18.
12. Yanagisawa, K., Naito, Y., Kuroiwa, K., Arai, T., Furukawa, Y., Tomizuka, H., Miura, Y., Kasahara, T., Tetsuka, T. and Tominaga, S. (1997) The expression of *ST2* gene in helper T cells and the binding of ST2 protein to myeloma-derived RPMI8226 cells. *J. Biochem. (Tokyo)*, **121**, 95–103.
13. Lohning, M., Stroehmann, A., Coyle, A.J., Grogan, J.L., Lin, S., Gutierrez-Ramos, J.C., Levinson, D., Radbruch, A. and Kamradt, T. (1998) T1/ST2 is preferentially expressed on murine Th2 cells, independent of interleukin 4, interleukin 5, and interleukin 10, and important for Th2 effector function. *Proc. Natl Acad. Sci. USA*, **95**, 6930–6935.
14. Moritz, D.R., Rodewald, H.R., Gheyselinck, J. and Klemenz, R. (1998) The IL-1 receptor-related T1 antigen is expressed on immature and mature mast cells and on fetal blood mast cell progenitors. *J. Immunol.*, **161**, 4866–4874.
15. Wollenberg, A. and Bieber, T. (2000) Atopic dermatitis: from the genes to skin lesions. *Allergy*, **55**, 205–213.
16. Trajkovic, V., Sweet, M.J. and Xu, D. (2004) T1/ST2—an IL-1 receptor-like modulator of immune responses. *Cytokine Growth Factor Rev.*, **15**, 87–95.
17. Brint, E.K., Xu, D., Liu, H., Dunne, A., McKenzie, A.N., O'Neill, L.A. and Liew, F.Y. (2004) ST2 is an inhibitor of interleukin 1 receptor and toll-like receptor 4 signaling and maintains endotoxin tolerance. *Nat. Immunol.*, **5**, 373–379.
18. Coyle, A.J., Lloyd, C., Tian, J., Nguyen, T., Erikkson, C., Wang, L., Ottoson, P., Persson, P., Delaney, T., Lehar, S. *et al.* (1999) Crucial role of the interleukin 1 receptor family member T1/ST2 in T helper cell type 2-mediated lung mucosal immune responses. *J. Exp. Med.*, **190**, 895–902.
19. Xu, D., Chan, W.L., Leung, B.P., Huang, F., Wheeler, R., Piedrafita, D., Robinson, J.H. and Liew, F.Y. (1998) Selective expression of a stable cell surface molecule on type 2 but not type 1 helper T cells. *J. Exp. Med.*, **187**, 787–794.
20. Hayakawa, M., Yanagisawa, K., Aoki, S., Hayakawa, H., Takezako, N. and Tominaga, S. (2005) T-helper type 2 cell-specific expression of the *ST2* gene is regulated by transcription factor GATA-3. *Biochim. Biophys. Acta*, **1728**, 53–64.
21. Iwahana, H., Yanagisawa, K., Ito-Kosaka, A., Kuroiwa, K., Tago, K., Komatsu, N., Katashima, R., Itakura, M. and Tominaga, S. (1999) Different promoter usage and multiple transcription initiation sites of the interleukin-1 receptor-related human *ST2* gene in UT-7 and TM12 cells. *Eur. J. Biochem.*, **264**, 397–406.
22. Halbert, A.R., Weston, W.L. and Morelli, J.G. (1995) Atopic dermatitis: is it an allergic disease? *J. Am. Acad. Dermatol.*, **33**, 1008–1018.
23. Untersmayr, E., Bakos, N., Scholl, I., Kundi, M., Roth-Walter, F., Szalai, K., Riemer, A.B., Ankersmit, H.J., Scheiner, O., Boltz-Nitulescu, G. *et al.* (2005) Anti-ulcer drugs promote IgE formation toward dietary antigens in adult patients. *FASEB J.*, **19**, 656–658.
24. Oshikawa, K., Kuroiwa, K., Tago, K., Iwahana, H., Yanagisawa, K., Ohno, S., Tominaga, S.I. and Sugiyama, Y. (2001) Elevated soluble ST2 protein levels in sera of patients with asthma with an acute exacerbation. *Am. J. Respir. Crit. Care Med.*, **164**, 277–281.
25. Chen, C.C., Grimbaldeston, M.A., Tsai, M., Weissman, I.L. and Galli, S.J. (2005) Identification of mast cell progenitors in adult mice. *Proc. Natl Acad. Sci. USA*, **102**, 11408–11413.
26. Damsgaard, T.E., Olesen, A.B., Sorensen, F.B., Thestrup-Pedersen, K. and Schiotz, P.O. (1997) Mast cells and atopic dermatitis. Stereological quantification of mast cells in atopic dermatitis and normal human skin. *Arch. Dermatol. Res.*, **289**, 256–260.
27. Rossler, U., Thomassen, E., Hultner, L., Baier, S., Danescu, J. and Werenskiold, A.K. (1995) Secreted and membrane-bound isoforms of T1, an orphan receptor related to IL-1-binding proteins, are differently expressed *in vivo*. *Dev. Biol.*, **168**, 86–97.
28. Grewe, M., Bruijnzeel-Koomen, C.A., Schopf, E., Thepen, T., Langeveld-Wildschut, A.G., Ruzicka, T. and Krutmann, J. (1998) A role for Th1 and Th2 cells in the immunopathogenesis of atopic dermatitis. *Immunol. Today*, **19**, 359–361.
29. Walzl, G., Matthews, S., Kendall, S., Gutierrez-Ramos, J.C., Coyle, A.J., Openshaw, P.J. and Hussell, T. (2001) Inhibition of T1/ST2 during respiratory syncytial virus infection prevents T helper cell type 2 (Th2)-but not Th1-driven immunopathology. *J. Exp. Med.*, **193**, 785–792.
30. Yoshida, K., Arai, T., Yokota, T., Komatsu, N., Miura, Y., Yanagisawa, K., Tetsuka, T. and Tominaga, S. (1995) Studies on natural *ST2* gene products in the human leukemic cell line UT-7 using monoclonal antihuman ST2 antibodies. *Hybridoma*, **14**, 419–427.
31. Kirshenbaum, A.S., Akin, C., Wu, Y., Rottem, M., Goff, J.P., Beaven, M.A., Rao, V.K. and Metcalfe, D.D. (2003) Characterization of novel stem cell factor responsive human mast cell lines LAD 1 and 2 established from a patient with mast cell sarcoma/leukemia; activation following aggregation of FcepsilonRI or FcgammaRI. *Leuk. Res.*, **27**, 677–682.
32. Hanifin, J.M. and Rajka, R.G. (1980) Diagnostic features of atopic dermatitis. *Acta Derm. Venereol.*, **92** (suppl.), 44–47.
33. Ohnishi, Y., Tanaka, T., Ozaki, K., Yamada, R., Suzuki, H. and Nakamura, Y. (2001) A high-throughput SNP typing system for genome-wide association studies. *J. Hum. Genet.*, **46**, 471–477.
34. Matsuda, A., Tagawa, Y., Matsuda, H. and Nishihira, J. (1996) Identification and immunohistochemical localization of macrophage migration inhibitory factor in human cornea. *FEBS Lett.*, **385**, 225–228.
35. Tsuchiya, T., Ohshima, K., Karube, K., Yamaguchi, T., Suefuji, H., Hamasaki, M., Kawasaki, C., Suzumiya, J., Tomonaga, M. and Kikuchi, M. (2004) Th1, Th2, and activated T-cell marker and clinical prognosis in peripheral T-cell lymphoma, unspecified: comparison with AILD, ALCL, lymphoblastic lymphoma, and ATLL. *Blood*, **103**, 236–241.

Human Molecular Genetics, 2005, Vol. 14, No. 19 2779–2786
doi:10.1093/hmg/ddi311
Advance Access published on August 22, 2005

Coding SNP in tenascin-C Fn-III-D domain associates with adult asthma

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Received June 20, 2005; Revised and Accepted August 9, 2005

The extracellular matrix glycoprotein tenascin-C (TNC) has been accepted as a valuable histopathological subepithelial marker for evaluating the severity of asthmatic disease and the therapeutic response to drugs. We found an association between an adult asthma and an SNP encoding TNC fibronectin type III-D (Fn-III-D) domain in a case–control study between a Japanese population including 446 adult asthmatic patients and 658 normal healthy controls. The SNP (44513A/T in exon 17) strongly associates with adult bronchial asthma (χ^2 test, $P = 0.00019$, Odds ratio = 1.76, 95% confidence interval = 1.31–2.36). This coding SNP induces an amino acid substitution (Leu1677Ile) within the Fn-III-D domain of the alternative splicing region. Computer-assisted protein structure modeling suggests that the substituted amino acid locates at the outer edge of the beta-sheet in Fn-III-D domain and causes instability of this beta-sheet. As the TNC fibronectin-III domain has molecular elasticity, the structural change may affect the integrity and stiffness of asthmatic airways. In addition, TNC expression in lung fibroblasts increases with Th2 immune cytokine stimulation. Thus, Leu1677Ile may be valuable marker for evaluating the risk for developing asthma and plays a role in its pathogenesis.

INTRODUCTION

Asthma is a chronic inflammatory disease characterized by smooth muscle hypertrophy, excess mucus secretion and increased deposition of extracellular matrix (ECM) around the basement membrane (1–3). Many asthmatic patients also have an atopic tendency characterized by a Th2 dominant cytokine profile including interleukin (IL)-4 and IL-13 (4). Several studies showed genetic associations between asthma and proteinases like ADAM33 (5) or Th2 cytokine receptors (4,6), but to the best of our knowledge, there is no report of an association between asthma and ECM genes. The hexametric

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ECM glycoprotein tenascin-C (TNC) has been accepted as a histopathological marker, beneath the asthmatic airway, for evaluating the severity and the therapeutic effects of drugs in bronchial asthma (7,8) because of its tightly controlled expression pattern. TNC expression is prominently increased around airway basement membranes of asthmatic patients (8), and two independent microarray experiments, including our own, identified TNC as one of the IL-4- or IL-13-induced genes in human bronchial epithelial cells (9,10). Recent studies showed that the fibronectin-III (Fn-III) domain of TNC has molecular elasticity (11) and mechanical strain can induce TNC expression (12), so we consider TNC to be more than just a marker for asthmatic pathology.

In the present study, we show the genetic association between an adult asthma and an SNP in exon 17 (44513A/T) causing amino acid substitution in the fibronectin type III-D (Fn-III-D) domain region of TNC gene (13). We carried out protein structure modeling of the Fn-III-D domain and found that the amino acid replacement Leu1677Ile could affect the structural stability of the Fn-III-D domain, which might affect the elasticity of the domain. In addition, TNC expression in lung fibroblasts was increased with IL-4 or IL-13 stimulation. The aim of our study was to test the association between the coding SNP in the TNC Fn-III-D domain and asthma and to determine how the SNP may affect the pathophysiology of asthma.

RESULTS

Identification of TNC genetic polymorphisms and selection of representative SNPs

We detected 62 genetic polymorphisms within the TNC region (Supplementary Material, Table S1) by resequencing samples from 24 Japanese individuals (12 asthmatics and 12 controls). Of these, we selected 23 SNPs whose minor allele frequency (MAF) was >20%. To check the intragenic linkage disequilibrium (LD) pattern in the TNC gene, pairwise LD was measured by r among the 23 SNPs (Fig. 1A). We selected 10 representative SNPs on the basis of location and LD with other sites; the positions of the 10 SNPs are shown in Figure 1B.

Case–control association study using asthmatic patients

We carried out a case–control association study using a Japanese asthmatic population. Clinical characteristics of the bronchial asthma patients are presented in Table 1. The severity of asthma before treatment was classified by the Global Initiative for Asthma Guideline (14). All 10 investigated SNPs were within the Hardy–Weinberg equilibrium. The overall success rate for genotyping was 99.1%. Of these 10, an SNP in exon 17 (44513A/T) had a significant association with adult bronchial asthma in our Japanese cohort under a recessive model [χ^2 test, 44513TT versus AT + AA, raw P-value 0.00019, Odds ratio (OR) 1.76, 95% confidence interval (95% CI) = 1.31–2.36] (Table 2). Stronger association was observed when we limited case subjects to non-smoking asthmatics (44513TT versus AT + AA, raw P-value 0.000025, OR 2.06, 95% CI = 1.45–2.87). There was no correlation between the severity of asthma and the TNC genetic association (data not shown).

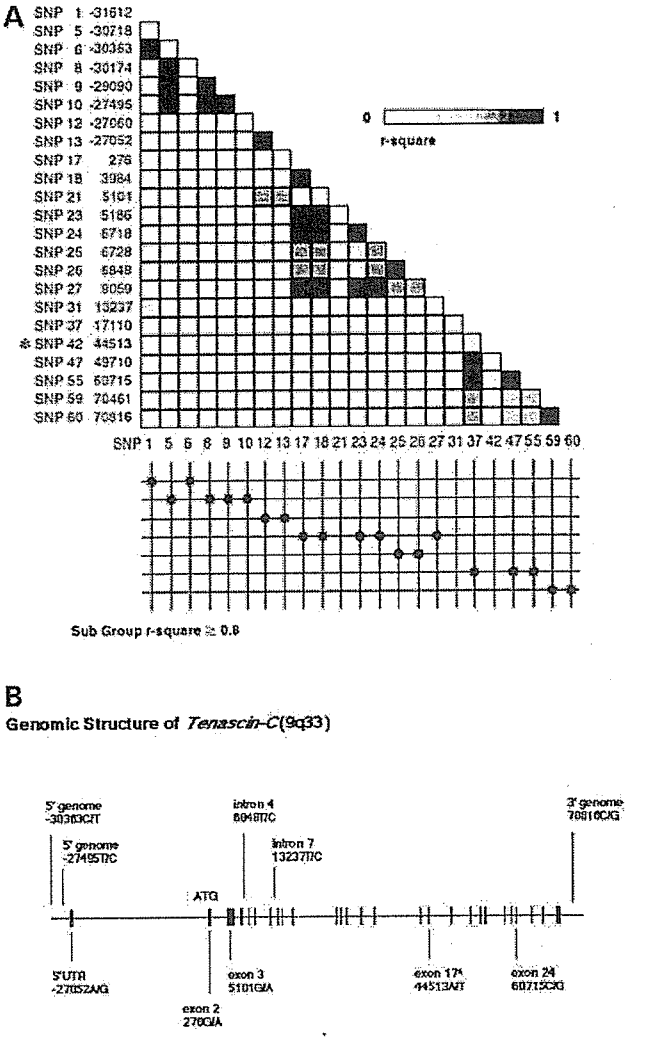

Figure 1. Pairwise LD map and SNP map in the TNC genomic region. (A) Pairwise LD in the TNC gene, as measured by the r^2 value between all pairs of SNPs examined. The position of 44513A/T is indicated with an asterisk and the remaining nine SNPs genotyped were indicated with red color. (B) The complete coding region of TNC, intron/exon boundaries, the intronic sequence, ~3 kb of 5′ genomic DNA and 1 kb of 3′ genomic DNA are shown. Twenty-seven exons are indicated by closed squares. Position 1 is the start codon of the TNC gene. An asterisk indicates the 44513A/T SNP.

LD mapping around the TNC gene

To exclude the possibility that our results reflected the association of other genes near the TNC locus with asthma, we constructed an LD map around the TNC gene locus using 48 SNPs (MAF > 10%). The results indicated that 44513A/T (indicated by an asterisk in Fig. 2) was located in the LD block extended from intron 8 of the TNC gene to the 3′ genome region of TNC (30 kb upstream and 20 kb downstream of this SNP) and that there were no other genes in this block (Fig. 2).

Table 1. Clinical characteristics of the bronchial asthma patients

Pretreatment: severity of disease[a]	Frequency of attack before treatment	Mean age	Mean age at onset	Mean duration of asthma (years)
Class 1: intermittent	Less than once a week	31.1	14.4	14.0
Class 2: mild persistent	More than once a week, less than once a day	45.9	30.6	16.0
Class 3: moderate persistent	Symptoms daily	47.1	36.5	19.8
Class 4: severe persistent	Symptoms daily, frequent nocturnal asthma	54.2	40.7	15.2

[a]Severity of disease was classified by Global Initiative for Asthma Guideline (14).

Table 2. Genotype frequencies for TNC SNPs and asthma susceptibility

SNP location	Control ($n = 658$) (%)			Bronchial asthma ($n = 446$) (%)			P-value[a]	P-value[b]	P-value[c]
	1	2	3	1	2	3			
−30363C/T	464 (71)	178 (27)	16 (2)	313 (71)	124 (29)	5 (0)	NS	NS	NS
−27495T/C	268 (41)	306 (47)	79 (12)	182 (41)	205 (47)	55 (12)	NS	NS	NS
−27052A/G	254 (39)	318 (49)	79 (12)	171 (39)	207 (47)	64 (14)	NS	NS	NS
276G/A	302 (46)	275 (42)	73 (11)	213 (48)	191 (43)	37 (9)	NS	NS	NS
5101G/A	301 (46)	299 (46)	56 (9)	197 (44)	200 (45)	49 (12)	NS	NS	NS
6848T/C	212 (32)	323 (49)	118 (18)	164 (37)	203 (46)	75 (17)	NS	NS	NS
13237T/C	340 (52)	269 (41)	46 (7)	229 (52)	180 (41)	29 (7)	NS	NS	NS
44513A/T	169 (26)	303 (46)	183 (28)	125 (29)	233 (53)	79 (18)	0.037	NS	0.0019
60715C/G	237 (36)	301 (46)	116 (18)	134 (30)	218 (49)	90 (20)	NS	NS	NS
70816C/G	279 (43)	284 (44)	87 (13)	178 (41)	193 (45)	61 (14)	NS	NS	NS

P-value adjusted with Bonferroni correction (raw P-values were multiplied by 10); NS, not significant.
[a]Allelel versus allele 2.
[b]Genotypell versus genotype 12 + 22.
[c]Genotype11 + 12 versus genotype 22.

Haplotype analysis

We carried out haplotype analysis of four representative SNPs in the LD block containing the 44513A/T SNP. Estimated frequencies of the four-locus haplotype were compared between cases and control subjects. The results of association studies for each haplotype showed a significant association between haplotype 1 and asthma (Table 3) (raw P-value = 0.004); however, the association was not stronger than that observed for the single locus (44513A/T).

Immunohistochemistry of TNC

Paraffin sections of asthmatic lungs were immunostained with a rat anti-TNC monoclonal antibody. Subepithelial deposition of TNC protein was observed beneath the bronchial epithelium in the asthmatic lung of a 65-year-old male (Fig. 3A). No apparent TNC staining was observed in the control lung of a 68-year-old male (Fig. 3B).

Computer modeling of the TNC Fn-III-D domain structure

We derived a protein structure model of the TNC Fn-III-D domain with MOE software (Fig. 4) to examine the possible effects of the substitution of the 1677th amino acid. The major allele in the normal population 44513-T encodes 1677Leu, whereas 44513-A, common in asthmatic patients, encodes 1677Ile. The 1677th amino acid is located at the beta-strand, which makes up the outermost side of the beta-sheet (Fig. 4A and B). The amino acid faces to the inside of the beta-sheet structure and there is a hydrophobic interaction between Phe1636, Leu1638, Leu1652, Ile1654 and Leu1680 (Fig. 4C, shaded region). The substitution of Leu1677Ile could result in steric hindrance with Phe1636 because of its side chain (Fig. 4D).

Identification of TNC variant expression in normal human lung fibroblasts by RT–PCR and western blotting

To confirm the expression of the TNC mRNA variant containing SNP 44513A/T in exon 17, RT–PCR (reverse transcription–polymerase chain reaction) was performed with a forward primer in exon 10 and a reverse primer in exon 19. The PCR results showed bands of 1969, 607 and 331 bp with normal human lung fibroblasts (NHLF) cDNA (Fig. 5A, left). The PCR products were subcloned and then sequenced. Larger bands (1969 and 607 bp) contained the Fn-III-D domain, including SNP 44513A/T. The cell lysate of NHLF was electrophoresed and immunoblotted with the rat anti-TNC antibody. A 250 kDa variant of TNC, corresponding to the largest mRNA, was dominantly expressed in NHLF, and both IL-4 and IL-13 could upregulate the 250 kDa TNC protein expression (Fig. 5B).

DISCUSSION

In the present study, asthmatic patients were recruited on the basis of the clinical asthma findings (14). We selected well-controlled cases after asthma treatments, (for class 2, 3 and

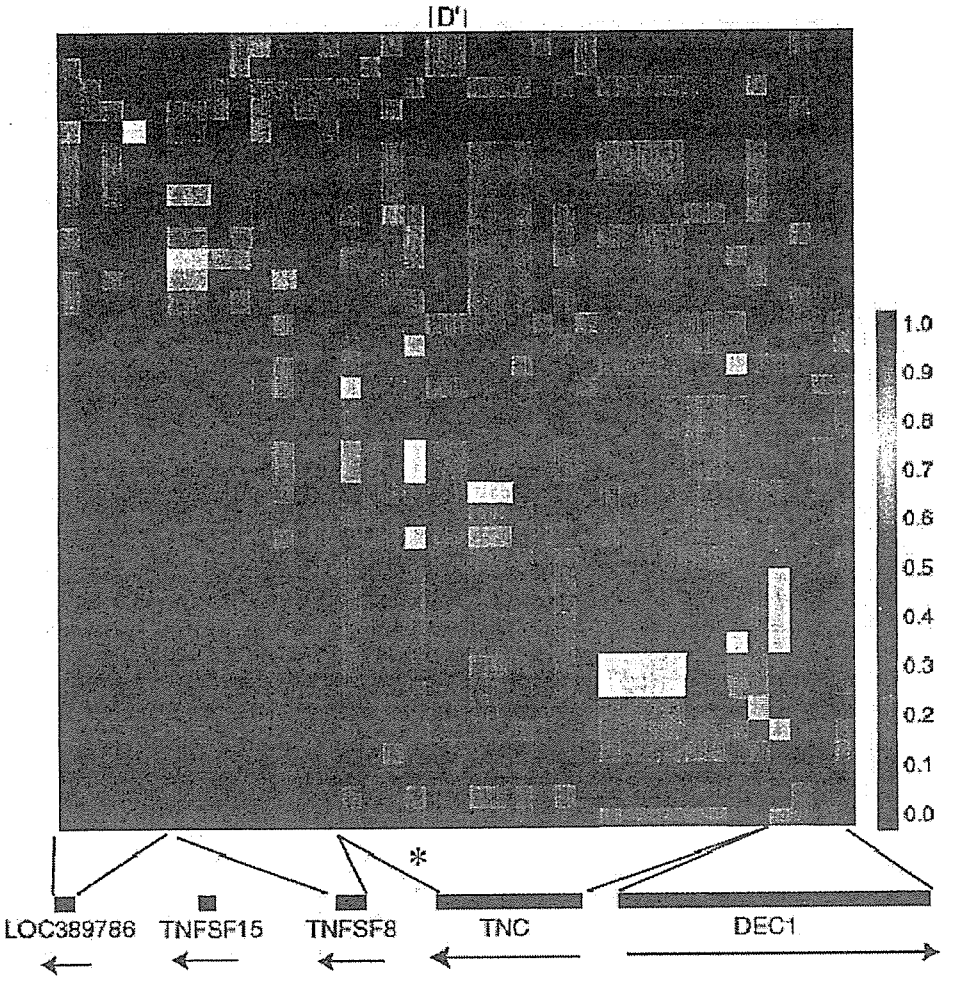

Figure 2. Pairwise LD map around TNC locus, as measured by D' value or r value. Pairwise LD map around the TNC locus, as measured by D' value or r value between all pairs of SNPs examined. The position of 44513A/T is indicated with an asterisk. Arrows indicate the direction of transcription of the genes.

4 cases with known amounts of an inhaled steroid: beclomethasone dipropionate; BDP), to ensure the reversibility of lung functions (Table 1). We took care to exclude possible COPD (chronic obstructive pulmonary disease) cases by spirometric analysis to check the reversibility of airflow obstruction and by X-ray/CT examinations. We found a genetic association between SNP 44513A/T in exon 17, coding the 1677th amino acid in the Fn-III-D domain, and adult bronchial asthma (Table 2). The genetic association between 44513A/T and asthma became stronger when we limited the analysis to the non-smoker asthmatic subpopulation. This result suggested that the association was not the consequence of secondary impairment of lung function due to smoking. The LD map around the TNC gene region showed that SNP 44513A/T was located in the LD block that extended from intron 8 to the 3′ genomic region of the TNC gene, and there were no other genes in the block (Fig. 2). Therefore, we concluded that the strong association observed with the SNP 44513A/T originated from the TNC gene itself. We intensively searched SNPs around the 44513A/T by resequencing and using public SNP databases, but we could not find any SNPs showing tight LD with 44513A/T. Weak LD was observed with 60715C/G (a coding SNP in exon 24) but there was no association between that SNP and bronchial asthma (Table 2), and the association between the four-SNP haplotypes including these two SNPs and asthma was not stronger than that of the 44513A/T single locus (Table. 3). Therefore, we selected SNP 44513A/T as the target for further analysis.

The TNC gene was chosen as a candidate gene for asthma on the basis of our previous GeneChip experiment (9). According to the results, TNC was one of the few genes constantly upregulated in bronchial epithelial cells in response to Th2 cytokines. We analyzed several candidate genes on the basis of the GeneChip results and found a significant

Table 3. Haplotype structure and frequency in TNC

Haplotype	SNP position				Haplotype frequency		P-value[a]	OR
	13237T/C	44513A/T	60715C/G	70816C/G	Controls	Cases		
Haplotype 1	T	T	C	C	0.33	0.27	0.004	1.33
Haplotype 2	T	A	G	G	0.26	0.27	0.725	1.04
Haplotype 3	C	T	C	C	0.17	0.17	0.816	1.03
Haplotype 4	C	A	G	G	0.08	0.08	0.933	1.01

OR, odds ratio.
[a]Analysis using a 2×2 table for each haplotype against all others combined in cases and controls.

association with the TNC gene. Furthermore, one previous genome-wide linkage study by Wjst *et al.* (15) showed that D9S1784 and D9S195 markers at chromosome 9q33 could be linked to asthma. TNC genes were located between these two markers (~9.7 Mb to D9S1784 and 5 Mb to D9S195). On the basis of these results, TNC seemed to be a good candidate gene for affecting susceptibility to asthma.

Our immunohistochemical staining of asthmatic airways showed TNC deposition around the basement membrane (Fig. 3A). Both bronchial epithelial cells and lung fibroblasts under the basement membrane may produce TNC. *In situ* hybridization experiments with the developing human lung (16) and respiratory distress syndrome (17) have shown that myofibroblasts under the epithelium express TNC mRNA. Therefore, we suppose that TNC in the asthmatic lung is predominantly produced by lung fibroblasts. It should be noted that the TNC Fn-III domain has both molecular elasticity (11) and essential roles for airway branching (18,19). We considered that TNC around the airway might have homeostatic roles for maintaining the integrity of airways in stressed conditions like bronchial asthma.

The structural model of the TNC Fn-III-D domain showed that the Ile1677 variant caused instability of the beta-sheet in the domain (Fig. 4D). Thus, Ile1677, a common variant among adult asthmatic patients, may alter the molecular elasticity of the TNC Fn-III domain. Airway resistance measurements of the asthmatic patients with or without allele 44513-A to investigate genotype–phenotype association are now ongoing.

It is known that a part of the TNC Fn-III domain, Fn-III-A1 through Fn-III-D, (Fig. 5A), is alternatively spliced (13). We checked the alternative splicing exon–intron junction for SNPs that might affect the splicing sites (20), but we could not find any SNPs that showed a significant association with asthma. Previous reports showed that the large form of TNC, including the alternative splicing region, was the predominant form in developing rat lung (19). Thus, it is likely that the large form of TNC is the main variant in the lung. Our monoclonal antibody could not distinguish between the large and small forms of TNC in immunohistochemistry, so we further analyzed the TNC variants by RT–PCR and by western blotting using NHLF. We showed that 250 and 190 kDa TNC variants contained the alternatively spliced Fn-III-D domain in NHLF (Fig. 5A) and either IL-4 or IL-13 treatment could preferentially induce the 250 kDa variant (Fig. 5B). We also found that the induction of TNC mRNA by IL-4 and IL-13 was not the consequence of non-specific inflammation because STAT6 activation could upregulate TNC mRNA expression (Supplementary Material, Fig. S3). From these findings, we conclude that it is highly likely that SNP 44513A/T in the TNC Fn-III-D domain is functional, especially under the influence of Th2 cytokines.

There are a few studies analyzing the role of TNC in pathologic conditions, some of which showed homeostatic roles of TNC protein (21,22). Habu snake-venom toxin induces glomerulonephritis phenotype in TNC knockout mice with more severe disease than that in congenic control mice (23). We suppose that TNC is a molecule with homeostatic functions emergent under stressful conditions. The TNC molecule may also have homeostatic roles in asthmatic conditions and the instability of the Fn-III-D structure caused by this SNP may hence affect the pathophysiology of asthma.

In conclusion, we found a genetic association between the SNP encoding the Fn-III-D domain of the TNC molecule and the adult bronchial asthma. The coding SNP causes instability of the Fn-III-D domain structure. Under the influences of Th2 cytokines, the expression and functional impact of the TNC molecule increase. The coding SNP might be a useful marker for evaluating the risk for adult asthma and provides insights into the precise functional roles of TNC in the pathogenesis of asthma. Further study is needed.

MATERIALS AND METHODS

Materials

The rat anti-human TNC monoclonal antibody (clone 3–6) was described previously (24). A horseradish peroxidase (HRP)-conjugated goat anti-rat IgG antibody and precast Tris–glycine polyacrylamide gels were purchased from Invitrogen (Carlsbad, CA, USA). Recombinant human IL-4 and IL-13 were purchased from Peprotec (London, UK).

Cell culture

NHLF were purchased from BioWhittaker (Walkersville, MD, USA) and cultured with the fibroblast basal medium from the same company according to the manufacturer's protocol.

Subjects

The adult asthmatic patients were recruited from approximately 4000 outpatients who were diagnosed as having bronchial asthma at the Miyatake Asthma Clinic or at the Osaka Prefectural Habikino Hospital by asthma specialists using

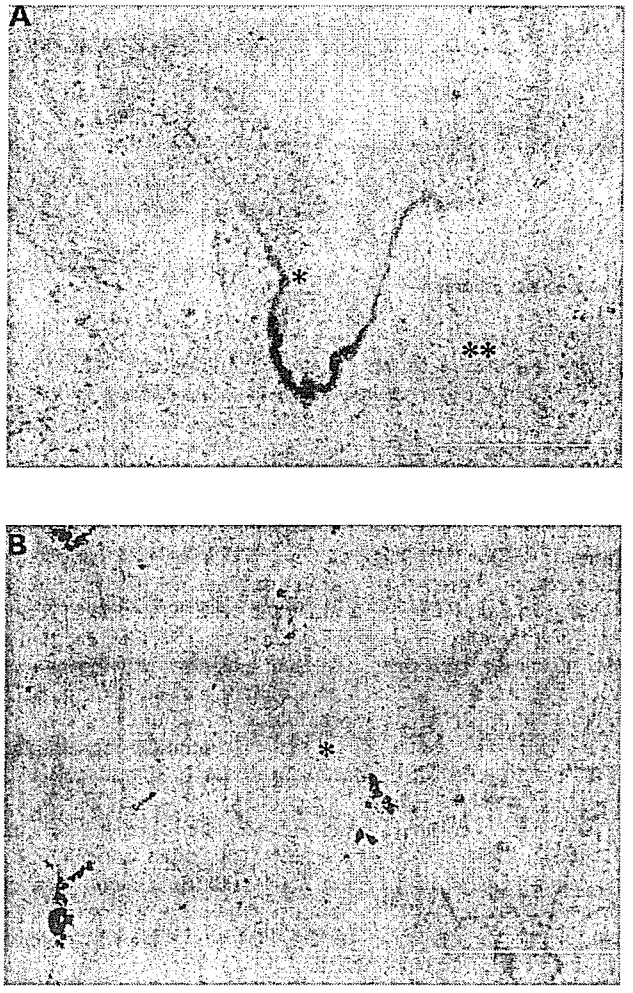

Figure 3. Immunohistochemical analysis of asthmatic lung with TNC antibody. Paraffin sections of asthmatic and control lungs were immunostained with the anti-TNC monoclonal antibody and visualized with indirect immunoperoxidase staining. Intense subepithelial staining is observed in the asthmatic lung (**A**) but not in the control lung (**B**) * indicates bronchial epithelium and ** indicates airway smooth muscle. Dark black spots in the control lung are foreign particles in the lung.

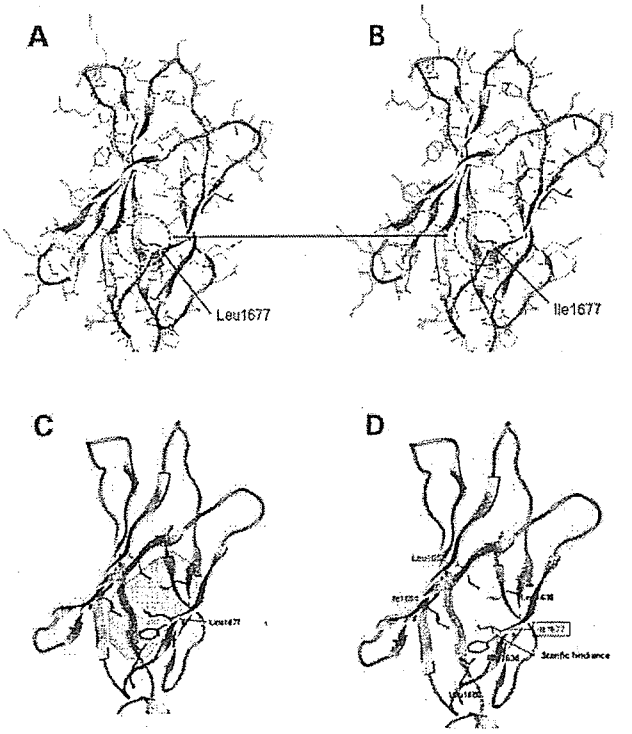

Figure 4. Computer modeling of TNC Fn-III-D domain and effect of 1677 Leu-Ile substitution. The 2.25 Åcrystal structure of chicken TNC (PDB accession no. P24821) was used as a template for homology modeling of the human TNC Fn-III-D domain. (**A**) The amino acid Leu1667 located in the fifth beta-sheet (yellow arrow, downward, indicated by black arrow) of the TNC Fn-III-D domain is shown with a green bar. (**B**) Amino acid Ile1677 located in the fifth beta-sheet of the TNC Fn-III-D domain is shown with a red bar. (**C**) Leu1677 makes a hydrophobic interaction plane (shaded region) among the hydrophobic amino acids. (**D**) The change of the amino acid from Leu to Ile caused steric hindrance with Phe1636 inside the beta-sheet.

the American Thoracic Society criteria as previously described (25,26). We selected 446 adult bronchial asthma patients (mean age 46.9, 16–70 years; male:female ratio, 1.0:1.2; mean serum IgE level, 741.3 U/ml; mite RAST positive 64.9%) satisfying the following symptoms and physical examination criteria: (i) those who showed episodic breathlessness, wheezing and chest tightness before treatment, (ii) the asthmatic symptoms were well controlled with known amounts of inhaled steroids. Among them, 105 patients were smokers or ex-smokers but not heavy smokers judged by the Fagerstrom Tolerance Questionnaire (26). Detailed information about the patients, including the severity of asthma (14) is summarized in Table 1. Peak expiratory flow analysis, spirometry, chest X-ray and CT scan were performed for the patients in need of differential diagnosis for COPD. Bronchial hyper-responsiveness was not tested. Peripheral blood was obtained from these 505 adult bronchial asthma patients. As a healthy control group, we analyzed 625 randomly selected population-based individuals (mean age 42.0, 18–69 years; male:female ratio, 2.5:1.0). We excluded the presence of asthma, atopic dermatitis and nasal allergies in the control population through careful interviews by physicians. All individuals were of Japanese origin and gave written informed consent to participate in the study, according to the process committee at SNP Research Center, RIKEN.

SNP discovery and genotyping in TNC gene

The TNC region targeted for SNP discovery included a 5 kb continuous region 5′ to the gene and 28 exons, each with a minimum 200 bp of a flanking intronic sequence. Forty primer sets were designed on the basis of TNC genomic sequences (Supplementary Material, Table S2). Each PCR was performed with 5 ng of genomic DNA from 24 individuals (12 asthmatic patients and 12 controls). The PCR product was reacted with BigDye Terminator v3.1 (Applied