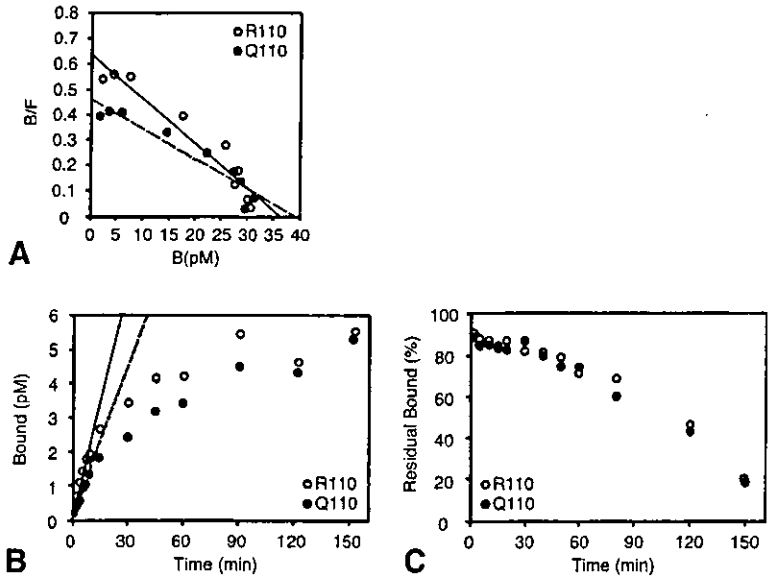

FIG 2. Binding assay of R110 and Q110 to IL-13Rα2. A, Scatchard plot with IL-13Rα2–expressing DND-39 cells (clone 18) incubated with 2 types of ^{125}I-labeled recombinant IL-13 (*solid line*, R110; *dashed line*, Q110) is depicted. The same experiments were performed 3 times, and the representative data are depicted. B, k_{on} rate of R110 and Q110. Specifically bound radioactivity of ^{125}I-labeled IL-13 with IL-13Rα2–expressing DND-39 cells (clone 15) at various times is plotted versus time. C, Dissociation kinetic study of R110 and Q110. Specifically bound radioactivity of preoccupied ^{125}I-labeled IL-13 with IL-13Rα2–expressing DND-39 cells (clone 15) at various times is plotted versus time.

tor with IL-13 and is capable of transducing the IL-13 signal, including STAT6 activation.[28-33] To address whether the binding activities of R110 and Q110 are different, we first used a human B-cell line, DND-39, transfected with IL-13Rα1. Scatchard analysis showed that this cell line expressed high- and low-affinity receptors, which are composed of IL-13Rα1 and IL-4Rα, and IL-13Rα1 alone, respectively (Fig 1, *A* and *B*). The equilibrium dissociation constants of R110 and Q110 with the high-affinity receptor were 21.0 ± 7 and 23.3 ± 3 pmol/L, respectively (mean ± SD, n = 3), which is consistent with a previous report,[28] and there was no difference between R110 and Q110. Furthermore, the transcription activities of the Iε promoter and STAT6 activation by R110 and Q110 were similar (Fig 1, C, and data not shown). Thus the substitution does not affect the binding activity of the IL-13R composed of IL-13Rα1 and IL-4Rα.

Binding activity of the IL-13 variant with IL-13Rα2

Another IL-13–binding molecule, IL-13Rα2, can generate a high-affinity receptor by itself.[30] To analyze the binding activity of the *IL13* variant with IL-13Rα2, we performed a binding assay using DND-39 cells on which IL-13Rα2, but not IL-13Rα1, was transfected. Scatchard analysis showed that IL-13Rα2 formed only a high-affinity receptor, as previously reported.[30] The equilibrium dissociation constants of R110 and Q110 with IL-13Rα2 were 54.7 ± 1.6 and 83.7 ± 3.1 pmol/L, respectively (mean ± SD, n = 3), which meant that Q110 had slightly lower affinity with IL-13Rα2 than did R110 (Fig 2, *A*). To find out what causes the difference in dissociation constants, we next investigated the rate constants for association (k_{on} rate) and dissociation (k_{off} rate) of each IL-13. It turned out that the k_{on} rate of Q110 was slower than that of R110 (Fig 2, *B*: 3.4 ± 0.51 vs 5.3 ± 0.89 × 10^8 × $mol^{-1} \cdot min^{-1} \cdot L$ [mean ± SD, n = 3]). In contrast, there was no apparent difference in k_{off} rates between R110 and Q110 (Fig 2, *C*). These results demonstrated that the affinity of Q110 with IL-13Rα2, which was slightly lower than that of R110, depends on the slower k_{on} rate of Q110.

Clearance of IL-13 by IL-13Rα2

IL-13Rα2 is assumed to act as a decoy receptor because it has a very short cytoplasmic domain. However, the role of IL-13Rα2 in vivo is not explicitly known. One plausible in vivo role for IL-13Rα2 is clearance of local IL-13, and the 2 IL-13 proteins may exert a different activity through this function. To explore this possibility, after either Q110 or R110 was absorbed by IL-13Rα2, we measured the residual IL-13 level by the transcription activity of the Iε promoter in IL-13Rα1–transfected cells (Fig 3). When either Q110 or R110 was preincubated with mock-transfected cells, no decrease of the transcription activity was seen; however, when IL-13 was preincubated with IL-13Rα2–transfected cells, the transcription activities were attenuated in a time-dependent manner, and Q110 caused a slightly smaller decrease than R110. These results apparently demonstrated that IL-13Rα2 is capable of its postulated role; they also indicate that Q110 may be less cleared by IL-13Rα2 than R110.

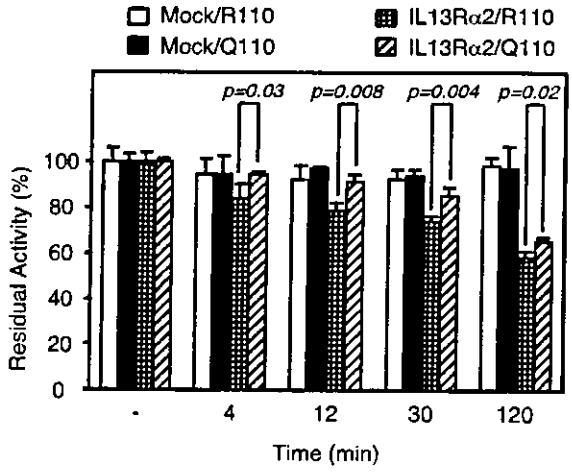

FIG 3. Clearance of R110 and Q110 by IL-13Rα2. Culture medium containing 0.5 ng/mL R110 and Q110 was preincubated with either mock-transfected (clone 1) or IL-13Rα2–transfected (clone 18) DND-39 cells for the indicated period. The removed medium was then incubated with an equal volume of IL-13Rα1–transfected DND-39 cells, and luciferase activity was measured. Fold induction was estimated, comparing the value without the preincubation. Each experiment was done with 3 samples, and the mean values are shown.

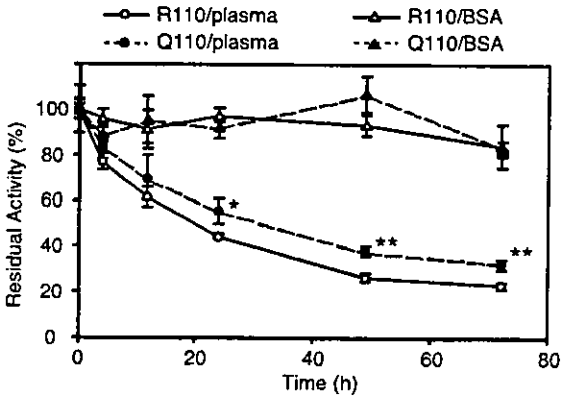

FIG 4. Stability of R110 and Q110 in human plasma. Twenty nanograms per milliliter of R110 and Q110 was incubated with either 100% human plasma or 120 mg/mL BSA for the indicated period and then incubated with IL-13Rα1–transfected DND-39 cells for the luciferase activity assay. The luciferase activities in the representative data are depicted (*solid line,* R110; *dashed line,* Q110). *Statistically significant difference at $P < .05$; **Statistically significant difference at $P < .005$.

Stability of the *IL13* variant

To test the possibility that the amino acid substitution in the variant affects stability of the protein, we first investigated it in both the presence and the absence of human plasma. When R110 and Q110 were incubated with human plasma, Iε transcription activities induced by both proteins declined in a time-dependent manner. The half-life of Q110 in plasma was estimated to be longer than that of R110 (experiment 1: 30.3 hours for Q110 and 18.9 hours for R110 [Fig 4]; experiment 2: 19.0 hours for Q110 and 13.3 hours for R110). The amount of endogenous IL-13 in human plasma was too small to be detected by the transcription activity of this system (data not shown). Substituting serum for plasma showed the same tendency (data not shown), whereas attenuation was not seen with incubation with BSA (Fig 4).

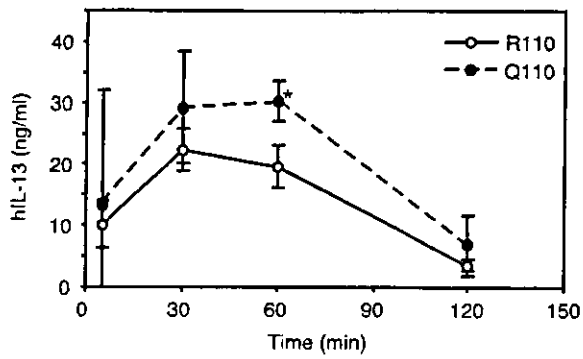

FIG 5. Stability of R110 and Q110 in mice. One microgram of R110 and Q110 was administered into BALB/c mice, and then blood samples were collected at the indicated times. The average values of IL-13 levels in plasma of 4 mice for each are depicted (*solid line,* R110; *dashed line,* Q110). *Statistically significant difference at $P < .05$.

We next analyzed the stability of IL-13 in vivo. When R110 and Q110 were administered into mice intraperitoneally, the IL-13 level in mouse plasma reached its peak 30 minutes after the injection and thereafter decreased (Fig 5). Q110 was retained to a greater degree in mouse plasma than R110 (30.3 ± 3.3 ng/mL for Q110 and 19.6 ± 3.5 ng/mL for R110 after 1 hour). We therefore hypothesize that the native structure of the IL-13 protein may be disrupted in plasma and Q110 is less susceptible to such effects of plasma or, alternatively, that Q110 may be metabolized more slowly in vivo by another mechanism.

Relationship between the genotype and serum level of IL-13

The present findings, that Q110 is less cleared by IL-13Rα2 and is more stable than R110, indicated that the variant would affect IL-13 concentrations in vivo. To explore this possibility, we investigated the relationship between the genotype and the serum level of IL-13 (Table I). The serum level of IL-13 in the Q110 homozygote tended to be higher than that in the heterozygote and the R110 homozygote in normal donors, although not statistically significant. By contrast, in asthmatic patients the serum IL-13 level was significantly higher in the Q110 homozygote than in the R110 homozygote. When the same amount of R110 and Q110 was applied to our immunoassay kit for IL-13, R110 showed a slightly higher value (1.3-fold) than Q110, and therefore the difference in serum IL-13 levels was not due to differential assay sensitivity for R110 and Q110. These results suggested that the different clearance rates, stability, or both

TABLE I. Association between the *IL13* genotype and serum IL-13 levels

IL-13 genotype	No. of patients (% frequency)		Median serum IL-13 levels (pg/mL)		
	Nonasthmatic subjects (n = 251)	Asthmatic subjects (n = 39)	Total	Nonasthmatic subjects	Asthmatic subjects
R110/R110	141 (56%)	14 (36%)	5.02 (3.38-9.12)	4.38 (4.12-9.98)	6.05 (3.20-10.8)
Q110/R110	80 (32%)	17 (44%)	6.51 (3.10-10.8)	4.29 (3.32-8.98)	8.81 (3.32-15.8)
Q110/Q110	30 (12%)	8 (20%)	10.2 (3.21-19.8)*	7.21 (3.33-16.8)	12.3 (3.24-21.3)*

The relationship between the *IL13* genotype and serum IL-13 level was shown. Serum IL-13 levels are presented as median (95% confidence interval).
*Significantly greater than in R110/R110 subjects ($P < .05$, median χ^2 test).

would at least partially influence the circulating level of IL-13, although we can not exclude the possibility of other polymorphisms in linkage disequilibrium, with the variant enhancing the transcription activity or mRNA stability of the *IL13* gene.

DISCUSSION

Many attempts have been made to elucidate genetic factors associated with bronchial asthma, with many genes being selected by using positional cloning and candidate gene approaches.[3,42] However, very few genes are reported to be functionally correlated with the pathogenesis of bronchial asthma. It is of great importance to investigate the functional differences of variants that correlated with the disease to exclude the possibility that any other polymorphisms in linkage disequilibrium evoke a genetic influence and to validate the results of genetic analyses. Previously, after we found that an extracellular variant of *IL4RA*, Ile50Val, is associated with atopy, we performed functional analyses, revealing that this variant upregulated IL-4 signals.[43,44] Following the genetic observations that the incidence of the *IL13* variant (Q110) was higher than the wild type (R110) in certain allergic phenotypes,[10-12] we performed functional analyses of the variant in this study.

We first demonstrated its slightly lower affinity with IL-13Rα2, leading to its lesser clearance by IL-13Rα2 (Figs 2 and 3). These results indicated that local IL-13 concentration may be augmented by the variant in vivo. Furthermore, we showed an enhanced stability of the variant in vitro and in vivo (Figs 4 and 5), which meant that on the contrary, the variant may affect the systemic concentration of IL-13 in vivo. As expected, the genotype of *IL13* was positively correlated with the serum level of IL-13 (Table I), although we still can not exclude the possibility that some other polymorphisms in linkage disequilibrium with the variant enhances the transcription activity or the mRNA stability of the *IL13* gene. It was reported that another polymorphism in the 5′ flanking region of the *IL13* gene (−1055 C/T) is associated with allergic asthma and that the variant type (−1055T) augmented binding to nuclear proteins and IL-13 production in T cells.[45,46] Although we could not detect a positive association between this variant and some allergic phenotypes (data not shown), such a polymorphism may also be involved in upregulating serum IL-13 levels in vivo. The findings in this study may suggest a unique mechanism of the variant as a genetic factor of bronchial asthma to upregulate IL-13 concentration in vivo. It is assumed that various triggers for bronchial asthma induce production of IL-13 in vivo and that Q110-bearing individuals may be more susceptible to bronchial asthma or to more severe bronchial asthma as a result of upregulation of IL-13 concentration.

The present finding, that the variant has lower affinity with IL-13Rα2, indicated that the variant would play its role as a genetic factor through IL-13Rα2–expressing cells. It is, however, poorly understood what cell types express IL-13Rα2 and how the expression of IL-13Rα1 and IL-13Rα2 is regulated in each cell. In B cells stimuli for CD40 and IgM receptor augment expression of IL-13Rα1, whereas no expression of IL-13Rα2 was detected in the presence or absence of the stimuli in B cells (data not shown).[29,47] In contrast, in BECs IL-13Rα1 is constitutively expressed and was not influenced on stimulation of IL-4 or IL-13, whereas expression of IL-13Rα2 was augmented by IL-4 and IL-13 (Yuyama N et al, unpublished data). Thus the *IL13* variant would exert its action distinct from the wild type on cells such as BECs but not B cells. This may explain that IgE secretion by the variant and the wild type was invariable (data not shown). Further analyses about the expression mechanism of IL-13Rα1 and IL-13Rα2 in each cell would address this point.

In a previous study computer modeling predicted that the substitution of the amino acid at 110 would affect the binding with IL-4Rα or IL-13Rα1.[10] It has also been recently shown that IL-13, which has aspartic acid instead of arginine at amino acid 110, acted as a potent agonist of IL-13. (In a previous article the same arginine residue was numbered 112[48] because of the difference of the prediction of the signal peptide length.) In this study we demonstrated that the variant has a lower affinity with IL-13Rα2, but not with IL-13Rα1, than the wild type. These results may suggest that amino acid 110 itself or the portion around amino acid 110 faces IL-13Rα2, but not IL-13Rα1. A planned cocrystal structure analysis should elucidate this point.

We further demonstrated that the variant is more stable than the wild type both in vitro and in vivo. There are several possible explanations for these differences. First, it may be due to the difference of degradation of the 2 IL-13 proteins in plasma. The amino acid substitution itself may change the specific recognition site of a certain protease, or the intramolecular conformational change induced by the substitution may influence susceptibility

to a certain protease. In fact, we confirmed that because the sequence containing arginine at amino acid 110 generates a cleavage site for Factor Xa (-Asp-Gly-Arg-), the R110 type was cleaved by it, whereas the Q110 type was not (data not shown). Second, disruption of the intramolecular disulfide bonds of the 2 IL-13 proteins may cause the difference because it has been demonstrated that disruption of the intramolecular disulfide bonds of IL-13 causes it to lose its biologic activity.[49] The intramolecular conformational change induced by the substitution may influence susceptibility to oxidative components. Third, it may be due to the difference of absorption or excretion of the 2 IL-13 proteins in vivo.

In conclusion, we demonstrated here that the *IL13* variant may act as a functional genetic factor of bronchial asthma. This finding is useful not only for diagnosing a risk for susceptibility to bronchial asthma but also for working out a strategy for treating it on the basis of this study. We may be able to remodel the IL-13 protein to act as an antagonist for the IL-13R or remodel the IL-13R proteins to increase their affinities with IL-13 and generate a soluble form blocking IL-13 action with a high affinity.

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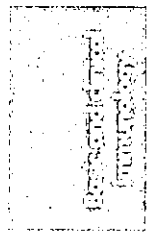

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Identification of an alternative splicing variant of cathepsin C/dipeptidyl-peptidase I

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Abstract

Cathepsin C/dipeptidyl-peptidase I is a papain-like lysosomal cysteine proteinase implicated in the processing of various proenzymes to their active forms. In this study, we identified an alternative splicing variant of cathepsin C in both human and mouse species for the first time. The variant messenger RNA (mRNA) encodes 137 amino acids corresponding to the first and second exons, followed by additional 31 amino acids. The two newly recognized exons are located in the former intron 2. The variant mRNA is distributed ubiquitously, but predominantly in kidney, placenta, and lymph nodes. Furthermore, both interleukin 4 (IL-4) and IL-13, but not a range of cytokines induce expression of the variant in bronchial epithelial cells. These results indicate that the variant may play a role in regulating the biological activities of cathepsin C, involved in the pathogenesis of bronchial asthma.

Keywords: Microarray; Interleukin 4 (IL-4); IL-13; Bronchial epithelial cells

1. Introduction

Cathepsin C/dipeptidyl-peptidase I is a cysteine proteinase, belonging to the papain family (Turk et al., 1997). It is a lysosomal proteinase implicated in the processing to their active forms of various proenzymes such as granzyme A and B (McGuire et al., 1993; Smyth et al., 1995; Kummer et al., 1996; Pham and Ley, 1999), mast cell chymase (Murakami et al., 1995; McEuen et al., 1998; Wolters et al., 2001), granzyme K (Wilharm et al., 1999), and thrombin-cleaved two-chain urokinase-type plasminogen activator (Nauland and Rijken, 1994). It has been furthermore demonstrated that ionophore and substance P induce secretion of cathepsin C in dog mastocytoma cells, indicating the possibility that it could also act extracellularly (Wolters et al., 1998). Cathepsin C is ubiquitously expressed in tissues, particularly highly in lung, kidney, placenta, liver, spleen, and intestines (Pham et al., 1997; Rao et al., 1997). Mutations in the *cathepsin C* gene lead to Papillon-Lefèvre syndrome, characterized by keratosis palmoplantaris and periodontopathia (Toomes et al., 1999).

All known cysteine proteinases of the papain family except cathepsin C are monomers consisting of R- and L-domains (Turk et al., 1997), whereas cathepsin C has a unique structure, consisting of four identical subunits, each composed of three polypeptide chains: the pro-region, the heavy chain, and the light chain (Dolenc et al., 1995; Cigić et al., 1998, 2000). It is controversial whether these chains are joined by a disulfide bond (Dolenc et al., 1995; Cigić et al., 1998); however, it has been postulated that the pro-region contains an intramolecular disulfide bond(s) and that it is glycosylated (Cigić et al., 1998, 2000). For cathepsin C to exert its proteinase activity, it is crucial that the pro-cathepsin C be cleaved by a certain proteinase such as cathepsin L or S (Dahl et al., 2001), followed by forming such an oligomeric structure.

Although the biological activity and the structure of cathepsin C are well characterized, thus far there has been no report of an alternative splicing variant of cathepsin C. In

Abbreviations: Th, T helper; HBECs, human bronchial epithelial cells; cRNAs, complementary RNAs; PCR, polymerase chain reaction; RACE, rapid amplification of cDNA ends; RT, reverse transcription; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; STAT, signal transducer and activator of transcription

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this study, we identified both human and mouse variants for the first time and analyzed their distribution in tissues. Furthermore, we found that expression was augmented by two T helper (Th) 2-type cytokines, interleukin 4 (IL-4) and IL-13.

2. Materials and methods

2.1. Cell culture

Normal human bronchial epithelial cells (HBECs) from single donors were purchased from Clonetics (Walkersville, MD). The cells were cultured in small airway epithelial cell basal medium supplemented with 30 μg/ml bovine pituitary extract, 0.5 μg/ml hydrocortisone, 0.5 ng/ml human epidermal growth factor, 0.5 μg/ml epinephrine, 10 μg/ml transferrin, 5 μg/ml insulin, 0.1 ng/ml retinoic acid, 6.5 ng/ml triiodothyroxine, 50 μg/ml gentamicin, 50 ng/ml amphotericin-B, and 0.5 mg/ml albumin and fatty-acid-free bovine serum (Clonetics) for 8–10 days. For stimulation, cells were cultured in unsupplemented small airway epithelial cell basal medium in the absence or presence of 10 ng/ml human IL-4 (Peprotech, Rocky Hill, NJ) or 50 ng/ml human IL-13 (Peprotech) for the indicated period. Tumor necrosis factor-α, IL-1β, IL-5, IL-6, and IL-9 were purchased from R & D Systems (Minneapolis, MN).

2.2. Probe preparation and microarray analysis

Total RNA was extracted by Isogen (Nippongene, Tokyo, Japan). Single-stranded complementary DNAs (cDNAs) were synthesized by Superscript II Reverse Transcriptase (GIBCO BRL) using T7-$(dT)_{24}$ primer (Amersham Pharmacia, Arlington Heights, IL), and then double-stranded cDNAs were generated by DNA ligase, DNA polymerase I and RNase H (GIBCO BRL). The complementary RNAs (cRNAs) were generated by T7 RNA polymerase using double-stranded cDNA as a template, and were biotin labeled using a bioarray high-yield RNA Transcription Labeling Kit (Affymetrix, Santa Clara, CA). The labeled cRNAs were purified by RNeasy Spin Column (QIAGEN, Valencia, CA), and then fragmented by heat treatment.

Microarray analyses of cRNAs from HBECs were performed with the Affymetrix GeneChip Human 35 K Set. It consisted of four probe arrays (Hu35KsubA, Hu35KsubB, Hu35KsubC, and Hu35KsubD). These covered greater than 35,000 clustered human expressed sequence tag transcripts selected from the UniGene database. Twenty-five μg of cRNA in hybridization cocktail buffer prepared according to the manufacturer's instructions was applied to the array, which was then hybridized at 45°C for 16 h. After washing, the array was stained using Streptavidin Phycoerythrin (Molecular Probes, Eugene, OR), followed by biotin labeled and unlabeled goat immunoglobulin G (Vector Laboratories, Burlingame, CA, Sigma, respectively) and Streptavidin Phycoerythrin, sequentially. Hybridized probe arrays were read using a Hewlett-Packard GeneArray scanner (HP2500 A, Hewlett-Packard, Palo Alto, CA). The data were analyzed using GeneChip software, Suite ver. 3.3 (Affymetrix).

2.3. Molecular cloning of the cathepsin C variant

Polymerase chain reaction (PCR) to identify the human cathepsin C variant was performed with cDNA derived from HBECs as a template with the indicated primers (Table 1) after an initial 2 min denaturation at 94°C, followed by 35 cycles of 94°C for 30 s, 60°C for 2 s, and 74°C for 1 min, using GeneAmp 9700 (Applied Biosystems Japan, Tokyo, Japan). The 5′ and 3′ ends of the human cathepsin C variant were determined by the 5′- and 3′-rapid amplification of cDNA ends (RACE) method using a Marathon™ cDNA amplification kit (Clontech, Palo Alto, CA). The primers used for the 5′- and 3′-RACE were 5′-GGCAAATCATATATCCCCACAGTTCCCAGC-3′ and 5′-GATGTCACTGATTTTATCAGTCATTTGTTCATGC-3′ for the first PCR and 5′-GCATGAACAAATGACTGATAAAATCAGTGACATC-3′ and 5′-GCTGGGAACTGTGGGGATATATGATTTGCC-3′ for the second PCR, respectively.

PCR to identify the mouse cathepsin C variant was performed with mouse lung Marathon-Ready™ cDNA (Clontech) as a template with the indicated primers (5′-GGGCATTTTACCCTCATTTACAACC-3′ and 5′-YTTDATNACNARYTTRTTCCKCARRTGNGG-3′) after an initial 2 min denaturation at 94°C, followed by 35 cycles of 94°C for 15 s, 55°C for 15 s, and 72°C for 15 s, using GeneAmp 9700. The 5′ and 3′ ends of the mouse cathepsin C variant were determined by the 5′- and 3′-RACE method using a Marathon™ cDNA amplification kit. The primers used for the 5′- and 3′-RACE were 5′-CAAATCATACATTCCCACAGTTCCCAGCTG-3′ and 5′-GATGTCACTGATTTTATCAGTCAGTTGTTC-3′ for the first PCR and 5′-GAACAACTGACTGATAAAATCAGTGACATC-3′ and 5′-CAGCTGGGAACTGTGGGAATGTATGATTTG-3′ for the second PCR, respectively.

2.4. Northern blot analysis

Two kinds of probes, recognizing the wild type of human cathepsin C only and both the wild and variant types respec-

Table 1
The primers used for PCR

No. of primer	Oligonucleotide sequence.
#1	5′-GCCTTTGCTATGCTGCTACAGGT-3′
#2	5′-ACAGCCATGAACAGACGTTGG-3′
#4	5′-AATGACTACAAGTGGTTTGCC-3′
#5	5′-TCTTTCCGGTGAAACAAGCC-3′
#7	5′-CCAACTGCACCTATCTTGAC-3′
#8	5′-AGGAATTGGTGTGGCTGCC-3′
#10	5′-GATGTCACTGATTTTATCAGTC-3′
#12	5′-TCTCAGACCCCAATCCTAAGCC-3′
#13	5′-TGCTGCTTCTCTCCGGCG-3′

tively, were prepared by the PCR method using primers #12 and #7 as the forward one respectively, and #8 as the common reverse one (Table 1). The ^{32}P-labeled probes were prepared by Random Primer DNA Labeling Kit Ver. 2 (TAKARA, Kusatsu, Japan).

To generate a riboprobe recognizing only the variant type of human cathepsin C, PCR was performed using primers #1 and #10, and the plasmid coding the antisense nucleotide was selected (Table 1). The ^{32}P-labeled riboprobe was prepared by RiboProbe In Vitro Transcription Systems (Promega, Madison, WI).

A membrane filter blotted with 2 μg of poly(A)$^+$ mRNA from human tissues (Human Immune System MTN Blot II and Human MTN Blot, Clontech) was hybridized with the ^{32}P-labeled probe according to the manufacturer's suggestion. The filter was washed at room temperature for 40 min in 2 × saline-sodium citrate (SSC)/0.05% sodium dodecyl sulfate (SDS) solution, and then at 56°C for 10 min in 0.1 × SSC/0.1% SDS solution for the cDNA probes or at 56°C for 1 h, 60°C for 1 h, and 70°C for 30 min in 0.1 × SSC/0.1% SDS solution for the riboprobe. The filter was exposed to an autoradiogram imaging system using Molecular Imager System™ (Bio-Rad Laboratories, Hercules, CA).

2.5. Quantitative reverse transcription (RT)-PCR analysis

Quantitative analysis of mRNA expression was performed using the ABI PRISM™ 7700 sequence detection system (Applied Biosystems Japan). The primers (#1, #2, Table 1) and TaqMan™ probes used for the analyses (5′-ACACTGTCGCAGGCTGCTCTGTCTCTTA-3′) were designed according to the manufacturer's software, Primer Express (Applied Biosystems Japan). RT was performed using Superscript II Reverse Transcriptase and Random Primers (GIBCO BRL). PCR was performed with cDNA as a template with the appropriate primers after AmpErase UNG treatment at 50°C for 2 min, and then its inactivation at 95°C for 10 min, followed by 50 cycles of 95°C for 15 s, and 60°C for 1 min. To calculate the copy numbers for each gene, standard curves were generated using a plasmid encoding that gene whose copy numbers were known. To normalize the cDNA concentration in each sample, the copy numbers of glyceraldehyde-3-phosphate dehydrogenase (GAPDH) were quantified.

3. Results and discussion

3.1. Identification of the variant type of human cathepsin C

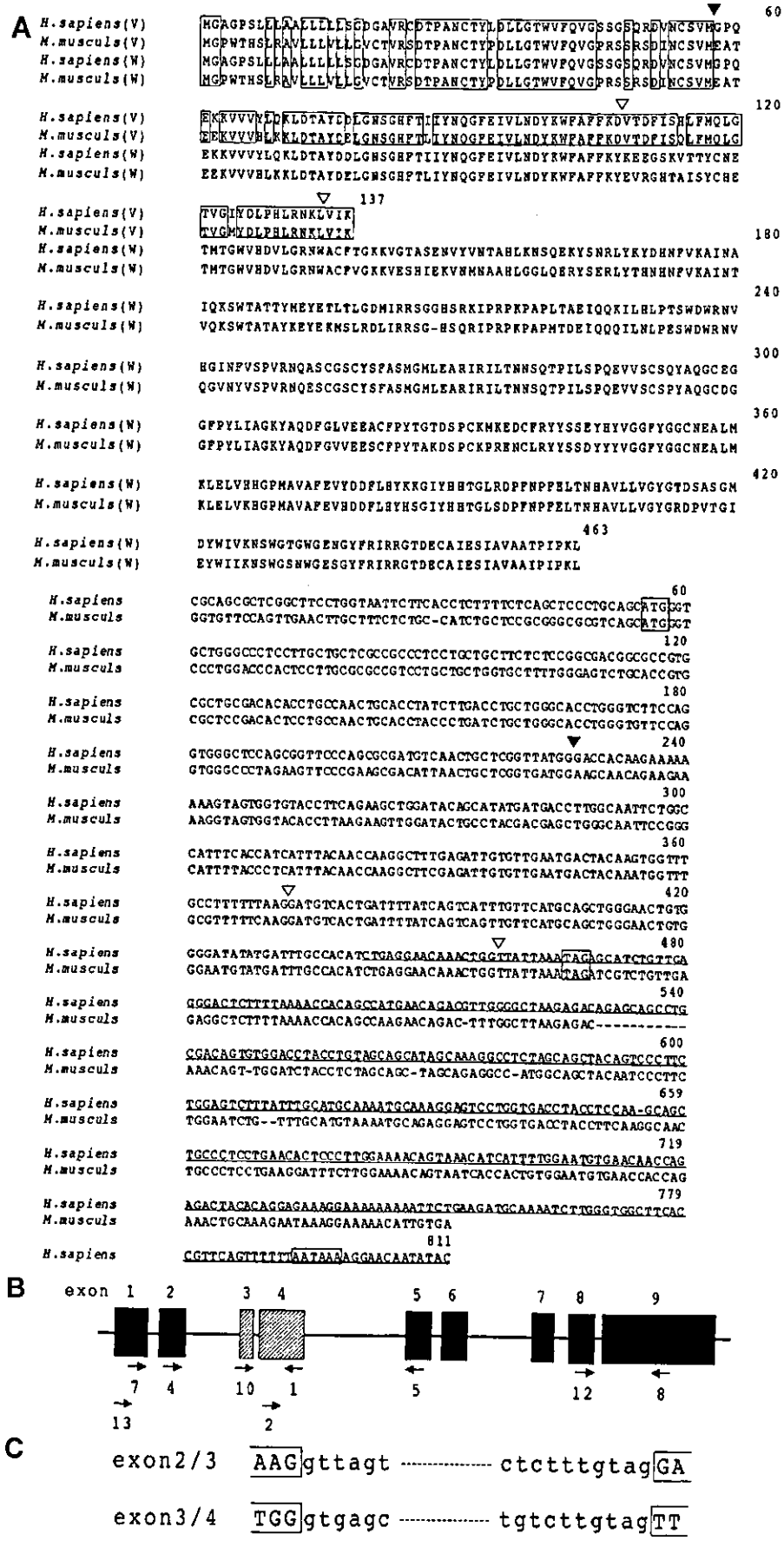
To identify IL-4 or IL-13-inducing genes in HBECs, we subjected cDNA derived from IL-4 or IL-13-stimulated HBECs to microarray analysis. One nucleotide sequence among the inducible genes, named AA011305, was consistently and highly induced by both IL-4 and IL-13 (data not shown, Fig. 1A). We searched this sequence in Human Genome BLAST, finding that it was contained in the second intron of the *cathepsin C* gene. This finding raised the possibility that an alternative splicing variant of cathepsin C existed, and the nucleotide sequence of AA011305 would correspond to its cDNA products.

To explore this possibility, we tried to identify cDNA encoding a cathepsin C variant by PCR method (Table1, Fig. 1B). When PCR was performed by primers #1 and either #4 or #13, short products (~200 and ~500 bp, respectively) were generated, although the sequence corresponding to AA011305 was almost 8 kb away from the second exon. These PCR products encoded amino acids corresponding to the first and second exons, followed by 31 additional amino acids (Fig. 1A). The nucleotide sequence encoding the 31 amino acids was composed of two parts, located at 6741 and 8494 bp downstream of the second exon (Fig. 1B). The newly recognized exon-intron junctions fulfilled the GT-AG rule (Fig. 1C). PCR by primers #4 and #5, #7 and #8 evoked only products corresponding to the wild type, and no product was formed by the reaction using primers #2 and #5 (data not shown). We next determined the 5′ and 3′ ends of cDNA of the cathepsin C variant by the 5′- and 3′-RACE. It turned out that the 5′ end of cDNA was the same as the wild type, and that polyadenylation was added 344 bp after the stop codon in the 3′ end (Fig. 1A). These results indicated that two novel exons existing in the former second intron of the *cathepsin C* gene were implicated in the generation of an alternative splicing variant of cathepsin C.

Thus far, the functional significance of this variant remains uncertain. It has been assumed that the pro-region posttranslationally modified by formation of a disulfide bond(s) and glycosylation is involved in stabilizing the structure of the mature enzyme (Cigić et al., 1998, 2000). Although the first 82 amino acids of the pro-region coded by the first and second exons were reserved in the variant, the variant lost two putative cysteine residues involved in forming the disulfide bonds (Cigić et al., 2000), which may cause an aberrant steric structure of this portion compared to the wild type. It is possible that the splicing variant acts as a negative regulator against the wild pro-region on generating the ternary structure of the mature enzyme.

3.2. Identification of the variant type of mouse cathepsin C

To analyze whether the same variant exists in the mouse *cathepsin C* gene, we performed PCR using a primer corresponding to the second exon and degenerative primers based on the sequence of the human cathepsin C variant and 5′- and 3′-RACE. As a result, it was ascertained that there existed a variant that encoded the first and second exons, followed by 31 amino acids. The region of the mouse counterpart was highly homologous with the human one (Fig. 1A), because only three nucleotides were different from the human gene in this region with 97% homology (342G/T, 369A/G, and 372G/A), resulting in switching two amino


A
H.sapiens(V)
M.musculs(V)
H.sapiens(W)
M.musculs(W)
B
exon 1 2 3 4 5 6 7 8 9
7 4 10 1 5 12 8
13 2
C
exon2/3 AAGgttagt ctctttgtagGA
exon3/4 TGGgtgagc tgtcttgtagTT

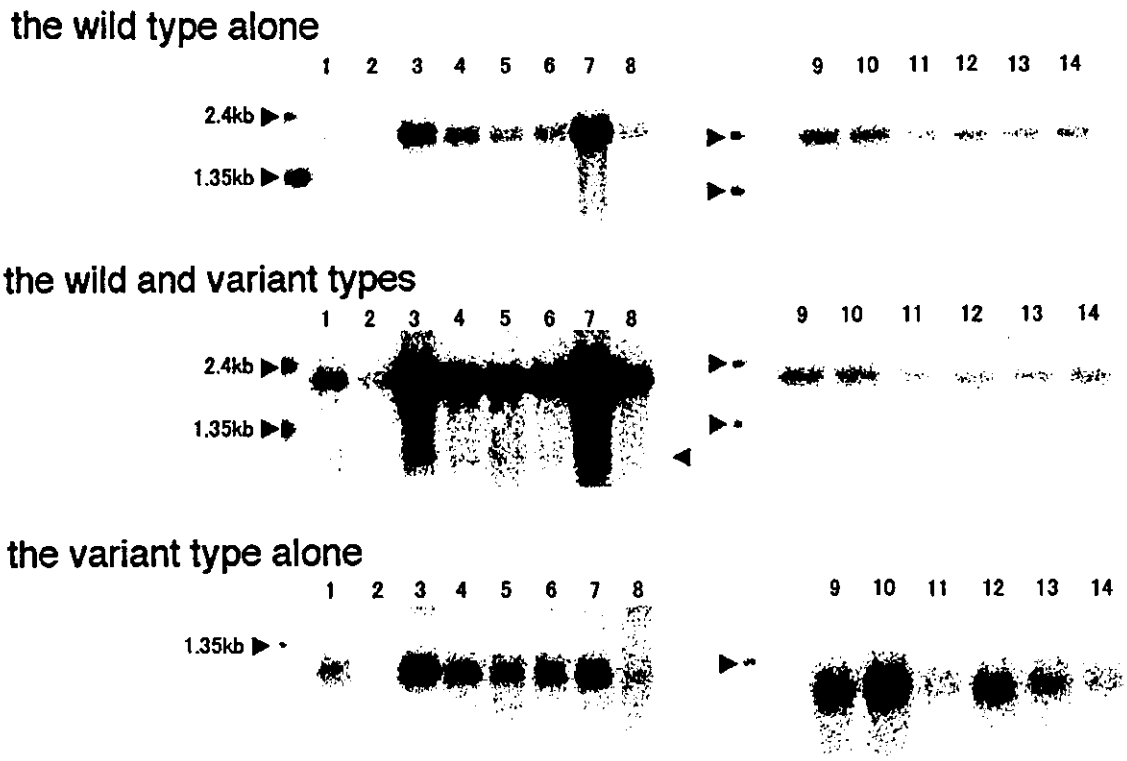


Fig. 2. Tissue distribution of the human cathepsin C variant. Northern blot analyses using either the probe recognizing the wild type of the cathepsin C alone, or the probe recognizing both the wild type and the variant, or the riboprobe recognizing the variant alone are shown. Each lane denotes as follows: 1, heart; 2, brain; 3, placenta; 4, lung; 5, liver; 6, skeletal muscle; 7, kidney; 8, pancreas; 9, spleen; 10, lymph node; 11, thymus; 12, peripheral blood cells; 13, bone marrow; and 14, fetal liver.

acids (Gln114His, Met124Ile). Polyadenylation addition occurred 267 bp after the stop codon. From these results, it was concluded that the mouse counterpart of the cathepsin C variant also existed, and the exon-intron organization for generating the variant was the same between human and mouse *cathepsin C* genes. Such high homology between the mouse and human variants might indicate that the variant has a common role in regulation of the cathepsin C activity.

3.3. Tissue expression of the cathepsin C variant

It has been previously demonstrated that cathepsin C is ubiquitously expressed in various tissues, and particularly highly in lung, kidney, placenta, liver, spleen, and intestines (Pham et al., 1997; Rao et al., 1997). We next compared tissue expression of the wild and variant types of cathepsin C. The probe recognizing only the wild type evoked a band with almost 2 kb in size highly in lung, kidney, and placenta, as demonstrated before (Rao et al., 1997; Fig. 2). In contrast, when we used the probe recognizing both the wild and variant types, an additional faint band with almost 1 kb in size appeared by long exposure (Fig. 2; the arrow head). To confirm that this band was derived from the variant type, we subjected the riboprobe recognizing only the variant type to northern blot analysis. Consequently, a band of the same size appeared, highly in kidney, placenta and lymph nodes (Fig. 2), meaning that this band was derived from mRNA of the variant type.

The present results, that expression of the cathepsin C variant was detected in HBECs, support that these cells are one source of cathepsin C in lung tissue. It has been recently demonstrated that the wild type of cathepsin C was localized in lamellar bodies of type II alveolar epithelial cells, and weakly in bronchial epithelial cells (Yayoi et al., 2001). Taken together with these results, it is assumed that cathepsin C may be involved in processing proteins secreted into alveolus/bronchus or cathepsin C itself may be secreted

Fig. 1. The structure of the human and mouse cathepsin C variants and the human *cathepsin C* gene. (A) Amino acid and nucleotide sequences of the human and mouse cathepsin C variants (V) are depicted. Amino acid of the wild type of the human and mouse cathepsin C (W) is aligned. The oligonucleotide sequence that corresponds to AA011305 was underlined. The positions where the known and newly identified introns are inserted, are denoted by closed and open arrowheads, respectively. The translation start and stop codons, and the polyadenylation site are boxed. (B) The structure of human *cathepsin C* gene is depicted. The known exons are denoted by closed boxes and the newly identified exons by oblique boxes. The numbers of new exons and the locations of the primers used in this study are shown. (C) The exon-intron junctions of the newly identified exons are depicted.

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ANALYSIS OF NOVEL DISEASE-RELATED GENES IN BRONCHIAL ASTHMA

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Bronchial asthma is a complex disease characterized by airway inflammation involving interleukin (IL)-4 and IL-13. We have applied microarray analyses to human bronchial epithelial cultures to probe for genes regulated by these cytokines and have identified a subset of disease-relevant genes by comparison with cDNA libraries derived from normal and asthmatic bronchial biopsies. Squamous cell carcinoma antigen-1 (SCCA1) and SCCA2, the cysteine and serine protease inhibitors, respectively, showed the highest expression by IL-4 and IL-13, and particularly, SCCA1 was significantly increased in the asthmatic cDNA library. STAT6 was shown to be involved in expression of SCCA1 and SCCA2 in vitro. Furthermore, serum levels of SCCA were also elevated in asthmatic patients. Taken together, it was supposed that SCCA may play some role in the pathogenesis of bronchia asthma, and measuring its serum level may be relevant for diagnosing or monitoring the status of bronchial asthma. In a complex disorder such as asthma, this combination of in vitro and in vivo genomic approaches is a powerful discriminatory method enabling identification of novel disease-related genes and their mechanisms of regulation.

Bronchial asthma is a complex disorder involving a combination of genetic and environmental factors.[1] Together, these result in infiltration of Th-2 lymphocytes, mast cells and eosinophils into asthmatic airways with downstream mediator release and disordered airway function. Cytokines derived from the Th-2 lymphocytes are considered to orchestrate the asthmatic phenotype, irrespective of atopy.[1]

Among the Th-2 cytokines, considerable evidence supports key roles for IL-4 and IL-13 in the pathogenesis of bronchial asthma. Their expression is higher at baseline and following allergen challenge in bronchial tissues or bronchoalveolar lavage fluids derived from asthma patients, with predominant expression of IL-13.[2,3] Analyses of mice null for components of the IL-4 and IL-13 signal transduction pathways, including IL-4, IL-13, IL-4 receptor α chain (IL-4Rα) and STAT6, have revealed that both cytokines play a pivotal role in induction of airway hyperresponsiveness, a characteristic feature of asthma.[4–6] Several IL-4 and IL-13 signaling molecules have been genetically associated with asthma.[7]

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TABLE 1. Changes in gene expression in three primary HBEC cultures exposed to IL-4 or IL-13 as measured by quantitative PCR analysis

Stimulus	IL-4			IL-13		
Lot	8F1756	8F1548	8F1805	8F1756	8F1548	8F1805
SCCA1	67.5	15.1	8.4	45.5	18.5	11.9
SCCA2	100.3	44.4	23.1	88.8	40.3	40.7
DD96	11.0	11.2	2.1	8.0	10.0	3.5
KAL1	12.3	5.4	3.1	11.9	5.0	5.5
Periostin	27.5	15.0	49.1	17.2	8.5	77.7
Tenascin C	13.2	6.6	2.7	13.4	6.5	4.6
Carboxypeptidase M	12.1	4.9	2.0	10.5	3.5	2.4
IL-13Rα2	11.6	3.1	4.1	10.8	4.2	5.6
Cathepsin C	6.7	4.0	2.5	6.6	3.6	4.7
Dioxin-inducible cytochrome P450	5.4	8.5	4.2	4.2	4.5	8.0
Carbonic anhydrase II	2.6	2.5	4.0	2.1	2.9	4.9
Endothelin-A receptor	6.8	4.4	4.8	7.1	5.3	8.2

especially a variant of *IL4R* (Ile50Val) that up-regulates receptor responses to IL-4 leading to increased STAT6 activation and IgE synthesis,[8] and a functional variant of IL-13 that is positively associated with the incidence of asthma, rather than atopy.[9] Consistent with these findings, a recent clinical trial has shown that inhalation of a soluble decoy IL-4Rα improves both symptoms and lung function in moderate-severe asthma.[10]

It is widely accepted that IL-4 and IL-13 act on immune cells to cause IgE switching, expansion and maintenance of Th-2 type cells and activation of mast cells.[11] However, epithelial expression of an IL-13 transgene or administration of IL-4 or IL-13 in mice has shown that these cytokines induce an asthma-like phenotype independent of T-cells.[12–14] Thus IL-4 and IL-13 are able to act directly on non-immune cells in bronchial tissue and have important roles in asthma beyond their immune and proinflammatory functions.[15] These include goblet cell hyperplasia, sub-epithelial fibrosis and bronchial hyper-responsiveness.[12–14] Consistent with these in vivo observations, direct effects of IL-4 and IL-13 on mucin,[16] TGF-β,[17] IL-8[18] and GM-CSF[19] expression by airway epithelial cells have been reported. To study potential downstream consequences of IL-4 and IL-13 on human airway epithelial cells and to assess their relevance in asthma, we have applied genomic approaches. In the in vitro system, we employed micro-arrays to identify genes induced by IL-4 and IL-13 in human bronchial epithelial cells (HBECs). Evidence for expression of these genes in asthma was then sought using cDNA libraries constructed from bronchial tissues of asthma patients. The significance of these expression data was demonstrated with the identification of squamous cell carcinoma antigen (SCCA) as a potential bronchial asthma-related gene.

RESULTS

Identification of IL-4 and IL-13-inducible genes in HBECs

HBECs derived from three different donors were exposed to IL-4 and IL-13 for 24 h and subjected to microarray analysis. We limited target identification to those genes whose expression was up-regulated >2-fold by IL-4 or IL-13. Of the 5600 annotated genes present on the arrays, IL-4 augmented expression of 46, 46 and 78 genes in each of the three cultures whereas IL-13 affected 47, 63 and 49 genes (data not shown). Amongst these genes, the number affected by both IL-4 and IL-13 were 27, 27, and 18 for the individual cultures (data not shown); these common genes showed a tendency for greater fold changes in expression. To select those genes for which expression was consistently enhanced by IL-4 or IL-13, we set the criterion that the fold change was >2 in at least two out of three of the cultures. Twelve genes emerged fulfilling this criterion (Table 1) and all were up-regulated by both Th-2 cytokines. Among these genes, two members of the serine proteinase inhibitor (serpin) family of protease inhibitors, SCCA1 (SERPINB3) and SCCA2 (SERPINB4), showed the highest increase (~20-fold).

To verify induction of expression of the 12 genes, we used quantitative RT-PCR (Table 1). While the fold change varied between individual cultures, increased expression of each of the 12 genes was confirmed. In two of the cultures, induction of expression was highest for SCCA1 and SCCA2 while periostin showed the highest increase in the other culture. Kinetic analysis showed that the expression profile of each of the 12 genes showed a pattern similar to that shown for SCCA1 and SCCA2 (Fig. 1A). In these two cases, expression was augmented within 6 h of stimulation by IL-4 or IL-13 and continued to be enhanced for up to

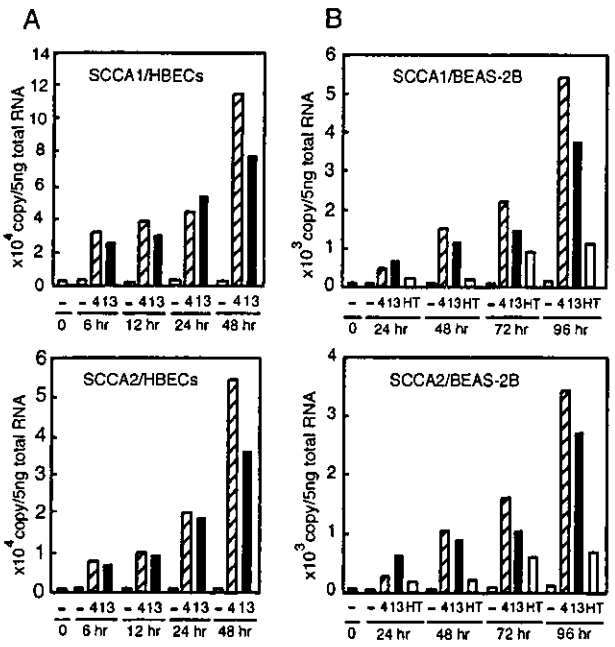

Figure 1. Time course of mRNA expression for SCCA1 and SCCA2.

HBECs (A) or BEAS-2B cells transfected with a conditionally active form of STAT6 (B) were incubated with either IL-4, IL-13 or 4-HT for the indicated time and expression of each gene investigated by quantitative RT-PCR analysis. The copy number was calculated using a standard plasmid, and the concentration of cDNA was normalized by reference to the copy number of GAPDH. The same experiments were performed twice, and the representative data are depicted.

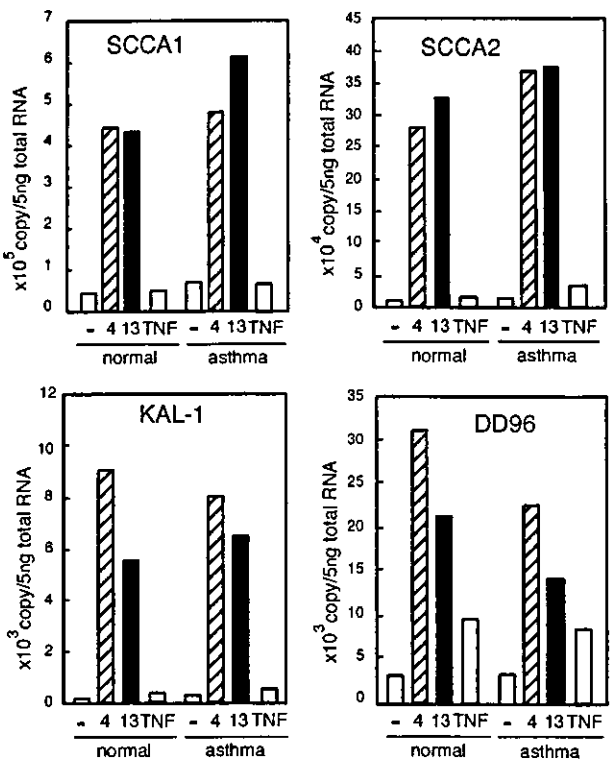

Figure 2. Expression of SCCA, KAL-1 and DD96 in normal and asthmatic primary epithelial cell cultures.

Primary cultures were established from bronchial brushings and exposed to IL-4, IL-13 or TNF-α for 24 h. Induction of gene expression was quantified by RT-PCR as described in Figure 1. The same experiments were performed twice, and the representative data are depicted.

48 h. In BEAS-2B cells transfected with a conditionally active form of STAT6, expression of SCCA1 and SCCA2 was augmented by 4-hydroxytamoxifen (4-HT), but the induced level was below that induced by IL-4 or IL-13 (Fig. 1B). These results indicated that the transcription factor, STAT6, was involved in expression of SCCA1 and SCCA2, however, other transcription factors would be also needed for full induction by IL-4 or IL-13.

Expression of the IL-4 and IL-13-inducible genes in bronchial tissues of asthma patients

We analyzed the expression profiles of cDNA libraries constructed from atopic normal or asthma-derived bronchial biopsies using LifeSeq® Gold from Incyte Genomics Inc. Comparison of the 12 differentially expressed genes identified in the IL-4 or IL-13-treated bronchial epithelial cells with sequence data from these cDNA libraries revealed that four of the genes, SCCA1/2, KAL-1 and DD96 were represented in at least one of the two libraries. The number of SCCA-related clones derived from SCCA was significantly higher in bronchial tissues of asthma patients than those of normal donors (11 clones versus 3 clones; $P<0.005$). Eleven clones among these 14 showed >95% homology with SCCA1, two short clones (one from each library) were highly homologous with both SCCA1 and SCCA2 and could not be distinguished, while one of the three clones derived from the normal library showed sufficient mismatches to suggest that it was more likely to be another SCCA-related molecule. One clone derived from KAL-1 and one from DD96 existed in the atopic asthmatic library but none were detected in the atopic, non-asthmatic library.

The finding that expression of four IL-4 and IL-13-inducible genes was augmented in bronchial tissues of asthma patients suggested that their expression in vivo was a consequence of the activity of IL-4 or IL-13 on the asthmatic bronchial epithelium. To test this directly, we treated primary cultures of asthmatic bronchial epithelial cells in the absence or presence of IL-4 or IL-13 and compared induction of the four genes with that observed in the presence of the pleiotropic cytokine, TNF-α (Fig. 2). Quantitative RT-PCR showed that induction of SCCA1, SCCA2, DD96 and KAL-1 gene expression in the asthma-derived epithelial cell cultures was enhanced by IL-4 or IL-13 while TNF-α was ineffective. The baseline and cytokine-stimulated responses of the asthma cultures were comparable with those obtained from non-asthmatic cell cultures obtained and treated in an identical way (Fig. 2).

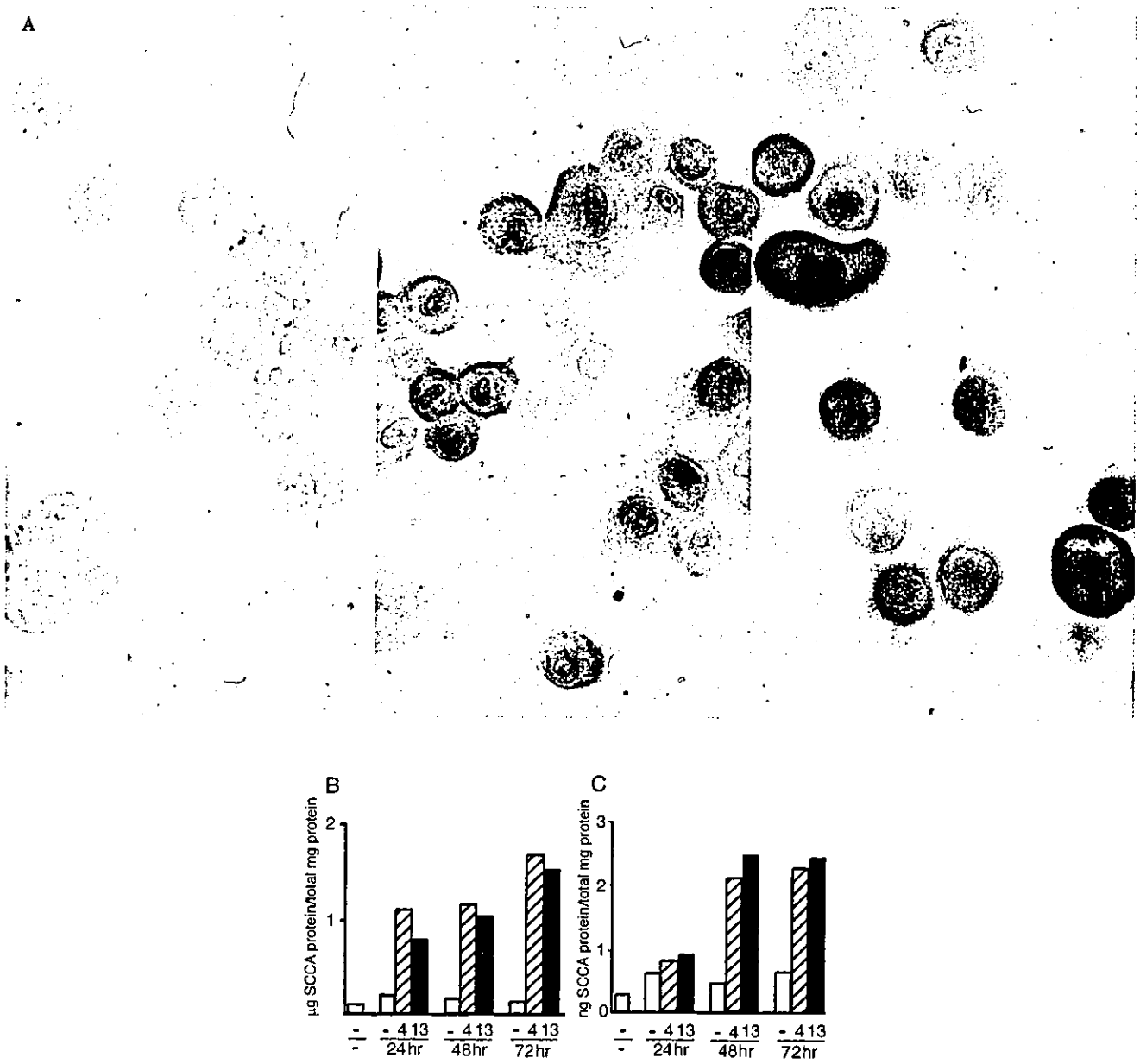

Figure 3. Expression of SCCA protein in HBECs.

(A) HBECs were incubated in the absence (left panel) or presence of either IL-4 (middle panel) or IL-13 (right panel) for 48 h. Immunostaining was performed using an anti-SCCA antibody. (B) and (C) HBECs were incubated in the absence (open bar) or presence of either IL-4 (hatched bar) or IL-13 (closed bar) for the indicated time. Expression of SCCA protein in the cell pellet (B) and culture supernatant (C) is depicted. The same experiments were performed twice, and the representative data are depicted.

Induction of SCCA protein by IL-4 and IL-13 in HBECs

SCCA1 and SCCA2 are serpins with intracellular and extracellular functions.[20] Immunocytochemical analysis using an antibody that recognizes both SCCA1 and SCCA2 showed positive staining only when cells were cultured in the presence of IL-4 or IL-13 (Fig. 3A). Kinetic analysis of cellular extracts and supernatants by ELISA showed that intracellular expression of SCCA protein increased within 24 h of IL-4 or IL-13 stimulation and was slightly augmented thereafter (Fig. 3B); SCCA secretion was not detected at 24 h but was observed 48 h after stimulation and reached a plateau thereafter (Fig. 3C).

Serum levels of SCCA in asthma patients

Since secreted SCCA1 and SCCA2 can be detected in serum of certain cancer patients,[20] we explored the possibility that expression of SCCA in bronchial tissue of asthmatic patients was also reflected in the circulation. Assessment of serum concentrations of SCCA in asthmatic and non-asthmatic children by

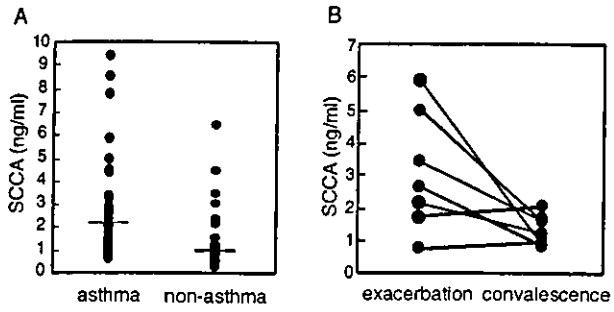

Figure 4. Serum levels of SCCA in asthma patients.

Serum levels of SCCA in asthmatic and non-asthmatic children (A) or in asthmatic children during an asthma exacerbation and in the following convalescence stage (B) are shown. The medians are depicted as bars.

ELISA showed that levels were significantly higher in the asthmatic subjects (2.1 (1.4–3.3) ng/ml, median (interquartile range), $n=32$) than in control, non-asthmatic subjects (1.1 (0.6–1.9) ng/ml, $n=23$, $P<0.005$) (Fig. 4A). In a small group of patients ($n=8$) who were studied during, and after 4–5 days after, an excerbation, SCCA levels were found to be higher during the attack phase (2.4 (1.7–3.8) ng/ml) than during recovery (1.4 (0.9–1.7) ng/ml), although this failed to reach statistical significance ($P=0.09$) (Fig. 4B).

DISCUSSION

The asthmatic phenotype is modified by a variety of factors and chemical mediators. In mice, knock out or transgenic technologies can be used to clarify the functional role of molecules such as IL-4 and IL-13, but clearly this cannot be applied to humans. To correlate these animal studies with human disease, we employed microarray technology which enabled identification of 12 genes regulated by IL-4 and IL-13 in vitro and combined this with in vivo gene analysis which showed four genes to be present in both analyses, with SCCA being significantly increased in both systems. Recognizing that bronchial tissue biopsies are comprised of both structural and inflammatory cells, it would be expected that those epithelial genes identified in the microarray analysis would represent only a subset of the genes present in the biopsy libraries. Also, the cDNA libraries that we generated might not cover all of the expressing genes in asthma patients by technical problems or diversity of asthma patients. Even so, by combining the two approaches it has been possible to assign disease relevance to a subset of genes identified in the in vitro study and, conversely, to attribute detailed regulatory mechanisms to a subgroup of genes identified in vivo. This dual approach may yield further information through a more comprehensive sequence analysis of the normal and asthma cDNA expression libraries. Furthermore, the utility of such an approach may be refined through the use of bronchial brushing to yield highly pure samples of epithelial cells for comparative in vivo and in vitro genomic analysis. During the preparation of this article, the results about analyses of IL-13-inducible genes in primary bronchial epithelial cells using the microarray technology have been published.[21] Some genes such as SCCA2, carbonic anhydrase II, cathepsin C, and tenascin-C were overlapped with ours, however, the others were not. This may be due to the difference of the incubation time in the presence of IL-13, or the diversity of each sample.

From the four genes identified, we focused on expression of the two serpins, SCCA1 and SCCA2. These genes are members of the ovalbumin-serpin (ova-serpin) proteinase inhibitor family.[22] They are 92% identical at the amino acid level and have probably arisen by gene duplication. SCCA was originally isolated from human cervical squamous carcinoma cells and is produced by various squamous tumors, as well as normal bronchial epithelium.[23] In our analysis, SCCA1 and SCCA2 showed highest fold induction amongst the IL-4 and IL-13-inducible genes using microarray and quantitative RT-PCR analyses (Table 1). Consistent with the potent effect of IL-4 and IL-13 in vitro, SCCA genes were also significantly augmented in bronchial tissues of atopic asthma patients. We can not exclude the possibility that other mediators are also involved in expression of SCCA genes in vivo; however, considering the findings of high IL-4/IL-13 expression in asthma lesions,[2,3] it is assumed that IL-4 and IL-13 would be main mediators to induce SCCA expression also in vivo. Unlike squamous tumor cells which constitutively express high levels of SCCA,[23] in vitro studies with asthmatic bronchial epithelial cells confirmed that they maintained a requirement for IL-4 or IL-13 for SCCA expression. Furthermore, the involvement of STAT6 activation for induction of SCCA1 and SCCA2 is consistent with our findings that STAT6 is strongly expressed in airway epithelium and is further increased in severe asthma.[18] In the present study, we demonstrated that increased SCCA gene expression in vitro was accompanied by enhanced SCCA protein synthesis and secretion. These functional responses were also mirrored in vivo with the demonstration that SCCA levels were elevated in the sera of asthmatic patients, particularly at the attack stage. This finding is similar to that reported for sera taken from squamous tumor-bearing patients,[20] however, in this case, a preferential increase in the SCCA2/SCCA1 ratio predicts for disease recurrence.[24] Since the clones identified in the asthma biopsy library were predominantly SCCA1, these data may suggest that an immunoassay

that discriminates SCCA1 will be useful to diagnose bronchial asthma from malignancies.

Increased serum levels of SCCA have also been observed in children suffering from atopic dermatitis (with or without asthma) with levels correlating with disease severity but not with serum IgE or eosoinophils.[25] Asthma children showing high SCCA level in our investigation did not show atopic dermatitis at all, excluding the possibility that high SCCA level was due to complication of atopic dermatitis. Furthermore, since our comparative analysis of gene expression in vivo utilized bronchial biopsies taken from atopic normal and atopic asthmatic subjects, we can exclude the possibility that atopy alone was sufficient to cause increased SCCA expression. These results suggested that measuring serum level of SCCA may be relevant for diagnosing or monitoring the status of bronchial asthma. The finding that atopic asthma and atopic dermatitis manifest the same SCCA response is suggestive of a common mechanism that is dependent on conditions created by the local tissue microenvironment rather than on a systemic immune response. Since atopic dermatitis strongly predisposes for the development of asthma, it is possible that these two diseases share a subset of genes that influence the tissue susceptibility to, or its ability to support, Th-2 type inflammation.

At present, it remains to be determined how SCCA1 and SCCA2 are involved in modifying the asthmatic phenotype. SCCA1 and SCCA2 are homologous with serpins such as plasminogen activator inhibitor type 2, elastase inhibitor and placental thrombin inhibitor.[22] However, recent analyses have revealed that they have distinct properties and substrates: SCCA1 inhibits cysteine proteinases such as cathepsin K, L, S, whereas SCCA2 inhibits serine proteinases including cathepsin G and mast cell chymase.[22] It follows that SCCA1 and SCCA2 may exert a protective role in the airways in asthma by inhibiting endogenous proteases associated with the inflammatory reaction and they may also act on inhaled proteases such as those intrinsic to dust mite allergens, which exhibit both cysteine and serine protease activities. While most serpins are secreted proteins that work extracellularly, the ov-serpins also function as intracellular proteinase inhibitors.[22] Overexpression of SCCA1 and SCCA2 in tumor cells suppresses apoptosis by preventing caspase activation, a phenomenon linked to inhibition of the p38 MAPK pathway.[26] In contrast, both in asthma[27] and atopic dermatitis[28] there is evidence for increased epithelial apoptosis. Thus, during allergic inflammation, enhanced release of SCCA from the epithelium in response to IL-4 or IL-13 may be detrimental to cell survival.

Two other genes, KAL-1 and DD96 were induced by IL-4 and IL-13 and one instance of each gene was found in the asthmatic library, but not in the atopic nonasthmatic library. KAL-1 is responsible for the X-chromosome linked form of Kallman's syndrome. It is a secreted protein that is associated with the cell surface via heparan-sulphate and has been proposed to play a role in neuronal development and in morphogenetic events in non-neuronal structures.[29] DD96 (also known as MAP17) was originally described as a membrane associated protein in human kidney tubules[30] and as an inducible gene in human keratinocytes following their activation by detachment.[31] Most significantly, DD96 expression has been linked to induction of apoptosis[30] and is increased around skin blisters in pemphigus vulgaris and bullous pemphigus[32] and in ulcerative colitis,[33] diseases known to involve substantial epithelial disruption. The presence of DD96 (and SCCA) is in accord with our observation of increased epithelial apoptosis[27] and epithelial shedding as Creola bodies,[34] a characteristic feature of the asthmatic bronchial epithelium.

In summary, we combined in vitro and in vivo genomic approaches to pinpoint a subset of novel disease-related genes in bronchia asthma. This approach would be beneficial for such purpose, and also for clarifying the molecular mechanism of bronchial asthma.

MATERIALS AND METHODS

Cell culture

HBECs were purchased from Clonetics (Walkersville, MD, USA). The cells were cultured in small airway epithelial cell basal medium supplemented with 30 μg/ml bovine pituitary extract, 0.5 μg/ml hydrocortisone, 0.5 ng/ml human EGF, 0.5 μg/ml epinephrine, 10 μg/ml transferrin, 5 μg/ml insulin, 0.1 ng/ml retinoic acid, 6.5 ng/ml triiodothyroxine, 50 μg/ml gentamicin, 50 ng/ml amphotericin-B, and 0.5 mg/ml albumin and fatty acid free bovine serum (Clonetics) for 8–10 days. For stimulation, cells were cultured in unsupplemented small airway epithelial cell basal medium in the absence or presence of 10 ng/ml human IL-4 (Peprotech, Rocky Hill, NJ, USA) or 50 ng/ml human IL-13 (Peprotech) for the indicated period. For microarray and quantitative RT-PCR analyses, the cells were incubated with these cytokines for 24 h.

The transformed human bronchial epithelial cell line, BEAS-2B was from ATCC (CRL-9609). Cells were cultured with F-12 nutrient mixture (Ham's F-12, GIBCO BRL, Rockville, MD, USA) containing 10% FCS. The murine STAT6-murine estrogen receptor (ER) fusion gene, pMXGSTAT6:ER,[35] was transfected into the packaging cell line Phoenix (provided by Dr G. Nolan, Stanford University, Stanford, CA, USA) using lipofectamine (Life Technologies, Gaithersburg, MD, USA). After 2 days, viral supernatants were used for infection of BEAS-2B cells. Cells expressing the plasmid were selected using enhanced green fluorescent protein as a marker. The transfected cells were treated in the

absence or presence of 1 μM 4-HT (Sigma, St Louis, MO, USA) for the indicated times. When the transfectants were treated with 4-HT, activated STAT6 could be detected by the electrophoretic mobility shift assay.

Primary cultures of asthmatic and normal bronchial epithelial cells were grown from bronchial brushings obtained by fibreoptic bronchoscopy as previously described.[18] Written informed consent was obtained prior to inclusion in the study, and the study was approved by the combined Southampton University and Hospital's Ethics Committee. Cell cultures were grown in bronchial epithelial growth medium and were used at passage 2. Cells were seeded in 24-well plates (2×10^5/well) and were grown to 80% confluence before being rendered quiescent by incubation in bronchial epithelial basal medium for 24 h. The cells were then exposed to IL-4 (20 ng/ml), IL-13 (20 ng/ml) or TNF-α (10 ng/ml) for 24 h and RNA extracted using Trizol (Life Technologies). Equal amounts of RNA from cultures derived from five asthmatic subjects and four normal subjects were combined from each treatment for RT-PCR analysis.

Probe preparation and microarray analysis

Total RNA was extracted by Isogen (Nippongene, Tokyo, Japan). Single-stranded cDNAs were synthesized by Superscript II reverse transcriptase (GIBCO BRL) using T7-(dT)24 primer (Amersham Pharmacia, Arlington Heights, IL, USA), and then double-stranded cDNAs were generated by DNA ligase, DNA polymerase I and RNase H (GIBCO BRL). The complementary RNAs (cRNAs) were generated by T7 RNA polymerase using double-stranded cDNA as a template, and were biotin-labeled using bioarray high yield RNA transcription labeling kit (Affymetrix, Santa Clara, CA, USA). The labeled cRNAs were purified by RNeasy Spin Column (QIAGEN, Valencia, CA, USA), and then fragmented by heat-treatment.

The microarray analyses of cRNAs from HBECs were performed with human 7129 high-density oligonucleotide probe arrays (HuGeneFL Array) representing approximately 5600 full-length, non-redundant genes supplied by Affymetrix. 12.5 μg of cRNA in the hybridization cocktail buffer prepared according to the manufacturer instructions was applied to the array, which was then hybridized at 45°C for 16 h. After washing, the array was stained using streptavidin phycoerythrin (Molecular Probes, Eugene, OR, USA), followed by biotin-labeled and unlabeled goat IgG (Vector Laboratories, Burlingame, CA, Sigma, USA, respectively) and streptavidin phycoerythrin, sequentially. Hybridized probe arrays were read using a Hewlett-Packard GeneArray scanner (HP2500A, Hewlett-Packard, Palo Alto, CA, USA).

The data were analyzed using Gene Chip software, Suite ver.3.3 (Affymetrix). Briefly, the presence of a specific gene was determined by comparing the average fluorescent intensities of sixteen to twenty pairs of perfect-match and single nucleotide-mismatch oligonucleotides. Average difference was defined as an average of fluorescent intensity differences between perfect-matched and single nucleotide-mismatched oligonucleotides. Fold change was depicted as the ratio of the average differences between unstimulated and stimulated samples.

Quantitative RT-PCR analysis

Quantitative analysis of mRNA expression was performed using the ABI PRISMTM 7700 sequence detection system (Perkin-Elmer Japan, Urayasu, Japan). The primers and TaqMan® probes used for the analyses were designed according to the manufacturer's software, Primer Express (Perkin-Elmer Japan, Table 2). Reverse transcription was performed using reverse transcriptase and random hexamers (GIBCO BRL). PCR reactions were performed with cDNA as a template with the appropriate primers after AmpErase UNG treatment at 50° for 2 min, and then its inactivation at 95°C for 10 min, followed by 50 cycles of 95°C for 15 s and 60°C for 1 min. To calculate the copy numbers for each gene, standard curves were generated using a plasmid encoding that gene whose copy numbers were known. To normalize the cDNA concentration in each sample, the copy numbers of β-actin and GAPDH were quantified.

Production of cDNA libraries from bronchial biopsies of normal and asthmatic subjects

Four atopic normal and three atopic asthmatic subjects with mean (± SD) ages of 24.0 ± 4.7 and 38.0 ± 11.5 years respectively were recruited for bronchoscopy and bronchial biopsy. The atopic normals had a mean FEV1 of 103 ± 9% of predicted, PC20 methacholine >8 mg/ml and geometric mean serum IgE of 24.2 IU/ml. For the atopic asthmatics, mean FEV1 was of 95.3 ± 24.4% of predicted, PC_{20} methacholine was 2.1 mg/ml (geometric mean) and serum IgE was 92.4 IU/ml (geometric mean). All the subjects were atopic as determined by positive skin prick testing to a panel of common aero-allergens (ALK, Horsholm, Denmark). The asthmatic subjects experienced asthma symptoms every 1–2 days, used inhaled bronchodilators for symptom relief between 1–7 times per week and were not receiving inhaled corticosteroids. Written informed consent was obtained prior to inclusion in the study, and the study was approved by the combined Southampton University and Hospital's Ethics Committee. Bronchial biopsies were obtained from the volunteers by fibreoptic bronchoscopy in accordance with standard published guidelines.[36] Biopsies were taken from sub-carinae of segmental bronchi of the right lower lobe and were immediately snap frozen in liquid nitrogen.

Non-normalized expression libraries were prepared from the frozen bronchial biopsies after pooling samples obtained from the non-asthmatic or asthmatic subject groups. Total RNA (20 μg and 40 μg respectively) was isolated using Trizol and acid-phenol extraction. PolyA mRNA (400 ng and 600 ng respectively) was purified using Oligotex (Qiagen) and cDNA synthesis initiated using a NotI-oligo(dT) primer. To generate the libraries, double-stranded cDNA was blunted, ligated to EcoRI adaptors, digested with NotI, size-selected, and cloned into the NotI and EcoRI sites of pINCY vector (Incyte Genomics Inc., Palo Alto, CA, USA). A total of 4311 and 4080 randomly selected clones from the normal and asthmatic libraries were sequenced and subtractions performed in silico. Sequencing was performed by Incyte Genomics Inc. and the sequence data compared using LifeSeq® Gold.

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Short Report

IL-4 receptor alpha chain genetic polymorphism and total IgE levels in the English population: two-locus haplotypes are more informative than individual SNPs

Bottini N, Borgiani P, Otsu A, Saccucci P, Stefanini L, Greco E, Fontana L, Hopkin JM, Mao X-Q, Shirakawa T. IL-4 receptor alpha chain genetic polymorphism and total IgE levels in the English population: two-locus haplotypes are more informative than individual SNPs.
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The IL-4RA locus encodes for the alpha chain of the IL-4 receptor, and is both a functional and positional candidate gene for atopy and allergic disease. Recently Ober et al. have shown that the study of haplotypes at multiple loci in the IL-4RA gene could be more informative than the separate study of single nucleotide polymorphisms (SNPs).
One hundred and fifty subjects affected by atopic asthma and 150 healthy control subjects were studied in the English population (Oxford district). Subjects and controls were genotyped for the Ile50Val, Ser478Pro and Gln551Arg polymorphism of the IL-4 receptor alpha chain. The distribution of haplotypes 50–478 shows a highly significant association with IgE levels. In particular, the haplotype Val50/Pro478 is much less frequent in subjects with IgE levels $>100\,U\,mL^{-1}$ than in those with IgE levels $<100\,U\,mL^{-1}$. Furthermore, the distribution of haplotype 50-551 shows a weak association with IgE levels that is lacking for 478-551 haplotypes. A lower frequency of the Val50/Pro478 haplotype is also observed among asthmatic subjects as compared to healthy controls. With regard to individual SNPs (50 478 and 551), no significant association has been observed with IgE levels or with asthma, thus confirming the higher informative value of the haplotype analysis as compared to separate study on SNPs.

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Key words: allergy – asthma – IgE – IL-4 receptor

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The alpha chain of the IL-4 receptor (IL-4RA) constitutes part of receptors for both IL-4 and IL-13, which are important cytokines in mediating the isotype switching to IgE, and finally, in the pathogenesis of atopy and allergic diseases (1). The IL-4RA gene has been suggested as a candidate gene for atopy based on linkage between atopic phenotypes and the marker D16S401 that lies near the gene on chromosome 16p12 (2, 3). Several association studies have been published on the relationship between single nucleotide polymorphisms (SNPs) of the IL-4RA, and atopy and allergic diseases: the present authors found that the Ile50 allele was strongly associated with atopy in the Japanese population (odds ratio ≈ 7), and showed a functional effect of this allele on IL-4 signal transduction in transfection experiments (4, 5). A protective effect of the Pro478 allele on atopic phenotypes has been shown (6), and both protective and predisposing effects have been found for the Arg551 allele in Caucasian populations (7): the 551 polymorphism has been also shown to interact with the 478 polymorphism in influencing total IgE levels and IL-4 signal transduction. In general, it has been difficult to confirm association studies performed on single IL-4RA SNPs in more than one population (8). Recently Ober et al. (9) have reported data suggesting that the study of haplo-

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