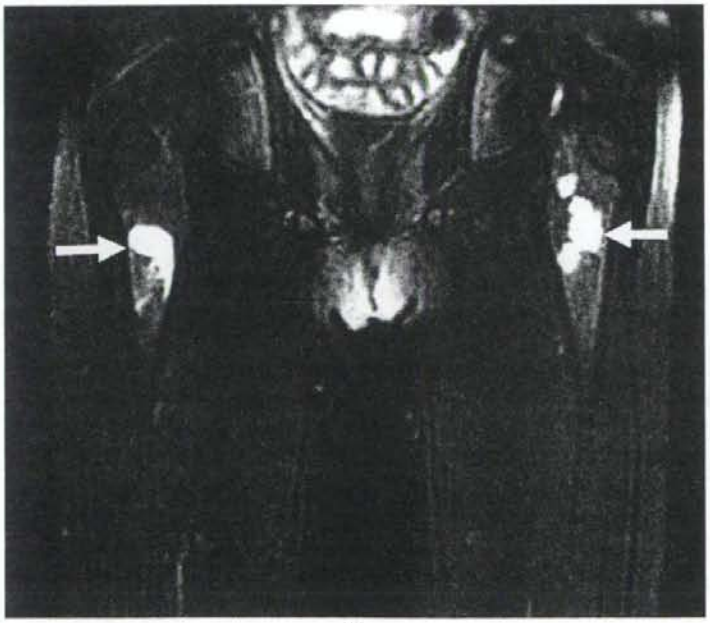
DISCUSSION

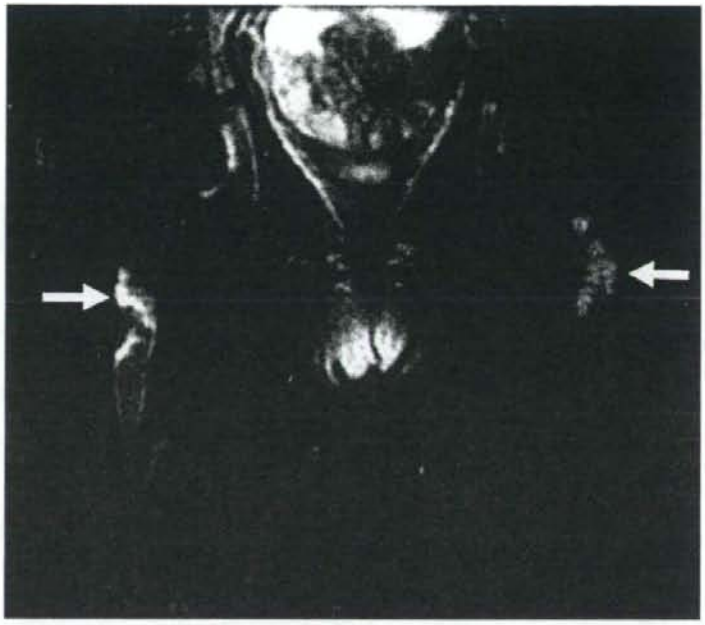
We have presented a case with a rare, progressive, diffuse lymphangiomatosis treated with pegylated IFN alfa-2b. This agent markedly regressed pulmonary effusion caused by thoracic lesion and also regressed multiple bone lesions.

The theoretical concept for using IFN alfa-2b to treat this disease was based on laboratory and clinical findings that have evolved over the past decade. IFN alfa is a cytokine with multiphasic cellular functions, including antiviral, antiproliferative, immunomodulatory, and antiangiogenic activities [6]. The delivery of IFN alfa represents a significant challenge and has potential implication in multiple therapeutic indications, including patients with hepatitis B and C infections [7], malignant melanoma [8], renal cell carcinoma [9], and chronic myelogenous leukemia [10]. Laboratory findings demonstrated that IFN alfa had antiangiogenic effects in 1980. It was discovered that interferon inhibits endothelial cell motility in vitro and that both IFN alfa and IFN beta can inhibit tumor-induced angiogenesis in murine animal models [11]. This led to the first successful application



(a)

FIGURE 2 Femoral MRI showed a multiple high-intensity areas on T2-weighted images as indicated by arrows (a). These lesions were apparently reduced by IFN treatment for 3 months (b). *(Continued)*



(b)

FIGURE 2 (Continued)

of IFN alfa-2b, based on its antiangiogenic activity in a 7-year-old boy with diffuse pulmonary hemangiomatosis; this was the first demonstration of antiangiogenic therapy in humans [12]. There have been several reports documenting successful therapy with IFN alfa-2b for complicated hemangiomas [13]. IFN alfa has also been used with effective results in the treatment of lymphangiomatosis. In our review of the literature (Table 1) [14–21], patients aged between 1 and 15 years (10 males and 4 females) who had recurrent, intractable, progressive lymphangiomatosis and received IFN alfa were treated with IFN alfa. All but patient 8 [19] had pulmonary involvement. Patient 8 was affected in bone, liver, and spleen. The clinical symptoms were coughing, wheezing, dyspnea, and chest pain. Bone lesions exisited in 64.2% (9/14) of the cases. Almost all of them experienced clinical improvement of disease to a greater or lesser extent and several continued that therapy. The most commonly reported side effects in these patients were fever, neutropenia, thrombocytopenia, and nausea. Two cases were unable to tolerate these side effects, leading to the discontinuation of IFN alfa therapy.

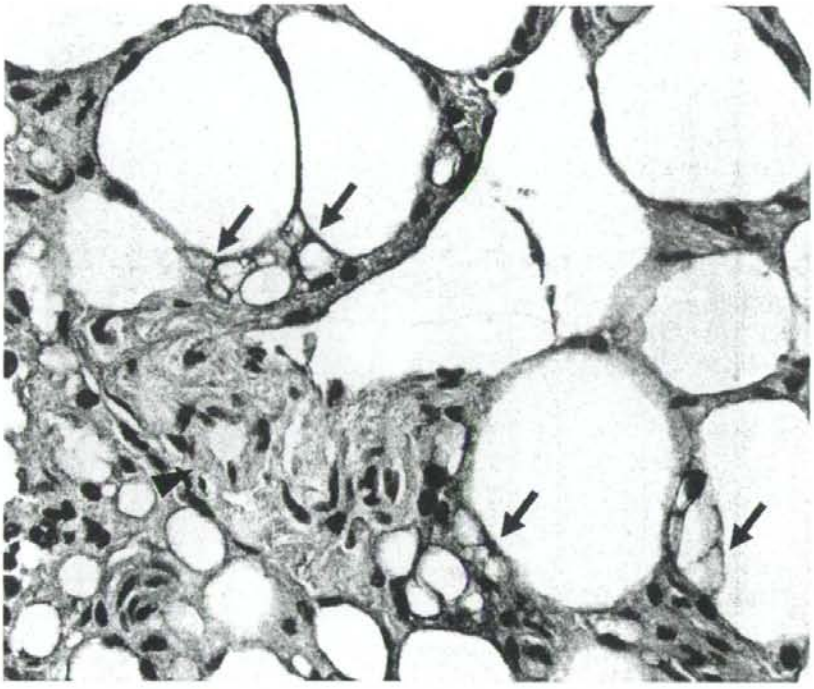


FIGURE 3 Specimen of bone biopsy from left upper forelimb. Dilated endothelial cells are strongly labeled by D2-40 (arrows). In contrast, those of adjacent small blood vessels and capillaries are D2-40 negative (an arrowhead).

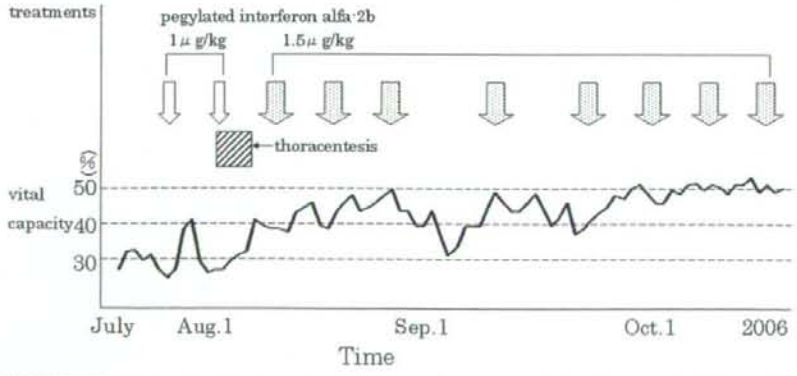


FIGURE 4 Treatments and patient's vital capacity levels during the course of pegylated interferon alfa-2b therapy. For our patient, a compact electronic spirometer was used for measuring vital capacity every day.

TABLE 1 Cases of Lymphangiomatosis with Interferon Alfa Therapy

No.	Reference	Age/sex	Involvement	Symptom	Therapy	Response to IFN therapy	Reason for stopping IFN	Outcome, follow up
1	Kelso et al. 1991	3 Y/M	Lung, Pe	Cough, dyspnea, hemoptysis	3½ weeks	Regression of symptoms	Thrombocytopenia	Died of hemoptysis 4 weeks after discontinuation of IFN
2	Tazelaar et al. 1993	7 Y/M	Lung, Pe	Unknown	20 months	Clinical improvement	NA	Alive 7 years later
3	Margraf et al. 1996	10 Y/M	Lung, Pe	Chest pain, nausea, dyspnea	Short term	Abate the impending respiratory failure without success	Died	Died of disease
4	Reinhardt et al. 1997	14 Y/M	Thorax, Pe, abdomen	Dyspnea, decrease exercise tolerance	12 months	Improvement, no further thoracetesis, no hospitalization	NA	Alive
5		12 Y/M	Thorax, Pe, bone, spleen	Dyspnea, chest pain	40 months	Slight improvement, then stabilization of effusion	Not reported	Alive
6	Patton et al. 1998	1 Y/M	Lung, pericardium, spleen	Wheezing, fatigue, easy bruising	Several months	Not reported	Responded to splenic embolization	Alive
7	Maki et al. 1999	12 Y/F	Bone, liver, spleen	leg and back pain	About 5 years	clinical improvement, but necessary use of analgesics	NA	Alive
8		12 Y/M	Pe, bone, abdomen	Dyspnea, limitation of motion	3½ years	Clinical and radiological improvement	The dose was reduced due to anemia, hepatic abnormality	Alive, he remains asymptomatic

(Continued)

9	Laverdiere et al. 2000	3 Y/F	Lung, bone, spleen	Dyspnea, degradation of vital capacity	30 months	Clinical and radiological improvement, disease progression during IFN pause	Interrupted for concern of neurological side effects	Alive
10	Robert et al. 2003	15 Y/F	Pe, Med, bone	Dyspnea, shortness of breath	Short term	Unknown	Not reported	Died of cardiorespiratry failure 12 days after surgery
11		13 Y/F	Pe, bone	Chylothorax	About 1 year	Clinical improvement, no progression	NA	Alive, she continue to receive IFN
12	Ofelia et al. 2004	8 Y/M	Pe, lung, Med, bone, DIC	Cough,wheezing, shortness of breath	2 months	20% reduction in size of the mediastinal mass	Not reported	Died of disease
13	Timke et al. 2005	11 Y/M	Lung, Pe, bone	Dyspnea, abdominal pain	2 years	Clinical improvement, no progression. the patient is able to take part in sports	NA	Alive
14	This report	9 Y/M	Pe, lung, Med, bone, spleen	Dyspnea, back pain, lumbago	3 months	Clinical and radiological improvement	NA	Alive, he continue to receive IFN

Note. Pe, pleural effusion; Med, mediastinum; DIC, disseminated intravascular coagulation; ITP, idiopathic thrombocytopenic purpura; IFN, interferon; NA, not available.

Glue et al. reported on the safety, tolerability, pharmacokinetics, and pharmacodynamics of pegylated IFN alfa-2b in 58 patients with chronic hepatitis C [5]. The safety and profiles of pegylated and nonpegylated IFN alfa-2b were similar. Both compounds produced typical interferon-like side effects: flu-like symptoms, myalgia, and headache. The mean serum elimination half-life of pegylated IFN alfa-2b was ~40 h. The mean clearance of pegylated IFN alfa-2b in serum was about one-tenth that of IFN alfa-2b (22 vs. 231 mL/h/kg), with little or no effect on the rate of absorption or volume of distribution. Maximum serum concentrations of pegylated IFN alfa-2b were sustained for 48–72 h before entering a slow elimination phase. The slow elimination phase persisted for 1 week after administration. Reduced frequency of injection may improve a patient's quality of life, especially children. Furthermore, pegylation has the potential to decrease the adverse effects of therapeutic molecules. This may reflect a decrease in the fluctuation of plasma concentrations caused by frequent high doses of unmodified protein, or a reduction in the immunogenic response as a result of fewer administrations of the pegylated protein and steric masking of antigenic sites [22].

The vascular endothelial growth factor (VEGF) pathway is a potent regulator of vascular proliferation, survival, invasion, and permeability [23]. One possible mechanism of the interferon effect on angiogenesis is the downregulation of VEGF expression. In our case, the serum concentration of VEGF was abnormally elevated to 962.0 pg/mL (standard value is under 38.3 pg/mL) at the time of hospitalization and the value was decreased to 57.8 pg/mL during therapy with pegylated IFN alfa-2b. It is therefore possible that pegylated IFN alfa-2b treatment resulted in decreased VEGF expression, and consequently inhibited the growth and permeability of lymphatic vessels in our patient. Another explanation is that the decreased VEGF expression may be resulted from the destruction of lymphatic vessels by IFN alfa-2b.

Histopathologically, differentiation between lymphatics and blood vessels by light microscopy has long been a problem. There are several vasucular endothelial markers, including factor VIII-related antigen, CD34, CD31, and VEGF receptor-3. Recently, podoplanin has been reported as a reliable marker for lymphatic vessel endothelial cells and a useful tool to distinguish them from blood vessel endothelial cells in conventionally processed tissue specimens [24]. It was detected by different groups and otherwise known as M2A. A monoclonal antibody against M2A, D2-40, has now been commercially available. Immunohistochemical analysis of endothelial cells showed extensive D2-40 in our tissue samples; our patient was diagnosed as having diffuse lymphangiomatosis. D2-40 expression has not been examined in previously published cases with lymphangiomatosis. It is considered that D2-40 is very useful to diagnose diffuse lymphangiomatosis.

CONCLUSIONS

Diffuse lymphangiomatosis is an uncommon disorder with poor prognosis, especially when lung involvement is present, in the first two decades of life. From the results of recent studies, IFN alfa seems to be useful and the side effects are few, mild, and reversible. Pegylation now represents a promising sustained-action delivery method. The sustained absorption and reduced clearance of subcutaneously administered pegylated IFN alfa-2b allows for a more convenient dosage schedule. Because of the dramatic response and safety in our patient, we recommend that pegylated IFN alfa-2b be considered as the first-line agent in the treatment of diffuse lymphangiomatosis of infants and children.

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A novel polymorphism, E254K, in the 5-lipoxygenase gene associated with bronchial asthma

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Abstract. Cysteinyl-leukotrienes are important pro-inflammatory mediators in bronchial asthma (BA) and are derived from arachidonic acid by the action of 5-lipoxygenase. We identified a novel polymorphism, c.760 G>A (E254K), in exon 6 of the 5-lipoxygenase gene (*5-LO*). This substitution was detected in 11 out of 180 patients with BA, but not in any of the 150 non-allergic subjects. The frequency of c.760 G>A showed a significant difference between BA and non-allergic subjects (P=0.0007). The c.760 G>A polymorphism existed at the surface edge of the C-terminal catalytic domain, and the E-to-K substitution changed the charge of the side chain from negative to positive. Thus, our results suggest that E254K in the *5-LO* might be associated with BA.

Introduction

Bronchial asthma (BA) is a multifactorial genetic disease (1). Cysteinyl-leukotrienes (cys-LTs) play an important pro-inflammatory role in both early- and late-phase asthmatic responses (2). Cys-LTs constitute a class of potent biological mediators of inflammation and anaphylaxis. 5-lipoxygenase is an essential enzyme which catalyzes the first committed steps in the biosynthetic pathway leading to the production of cys-LTs (3-6). The actions of 5-lipoxygenase result in the sequential conversion of arachidonic acid to 5-hydroperoxy-eicosatetraenoic acid (5-HPETE) and then to leukotriene (LT) A4.

The *5-LO* is located on chromosome 10q11.2 (7). In several studies, the addition of an Sp-1 binding motif (-GGG CGG-) or the deletion of one or two Sp-1 binding motifs in the *5-LO* core promoter, has been associated with reduced gene expression (8,9). In addition, the 5-lipoxygenase-activating protein (FLAP) promoter gene polymorphisms (21A repeat and 18A repeat) were reported to be associated with BA (10). Recent evidence demonstrated that up-regulation of *5-LO* and FLAP mRNAs might be involved in the increased leukotriene synthesis and play an important role in the pathogenesis of BA (11).

In this study we identified single-nucleotide polymorphisms (SNPs) in the *5-LO* and researched the relationship between SNPs in the *5-LO* and BA.

Materials and methods

Patients and non-allergic subjects. One hundred and eighty BA patients (105 males and 75 females, mean age 8.4±7.6 years of age) and 150 non-allergic subjects (90 males and 60 females, mean age 9.5±8.2 years of age) were studied. The diagnosis of BA was made according to the criteria of the American Thoracic Society. The non-allergic subjects were healthy and did not have a history of allergic diseases. All of the subjects were randomly selected from patients attending our hospital. Informed consent was obtained from all individuals or from their parents.

Detection of SNPs in 5-LO. Neutrophils were collected from heparinized blood. Genomic DNA was extracted from neutrophils with a Sepagene kit (Sanko Junyaku, Tokyo, Japan). The fourteen exons of *5-LO* were amplified using the PCR technique and sequenced using an ABI 3100 DNA auto-sequencer (Applied Biosystems, CA) in certain individuals with BA (n=16) and certain non-allergic subjects (n=14). For further study, the E254K substitution was detected in all individuals with BA (n=180) and non-allergic subjects (n=150), and the other three silent polymorphisms (c.21 C>T, c.270 G>A, c.1728 A>G) were detected in 60 individuals with BA and 60 non-allergic subjects. The primer details for the PCR used in the detection of these polymorphisms are shown in Table I.

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Abbreviations: SNP, single-nucleotide polymorphism; 5-LO, 5-lipoxygenase gene; FLAP, 5-lipoxygenase-activating protein; BA, bronchial asthma; cys-LTs, cysteinyl-leukotrienes; PBMCs, peripheral blood mononuclear cells; EIA, enzyme immunoassay; ELISA, enzyme-linked immunosorbent assay; 5-HPETE, 5-hydroperoxyeicosatetraenoic acid; ICM, Internal Coordinate Mechanics; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; c., cDNA

Key words: single-nucleotide polymorphism, 5-lipoxygenase gene, bronchial asthma, leukotrienes

Table I. Primer details for PCR used to detect 4 polymorphisms.

Primer	Sequence	Amplified product size	Annealing temp (°C)
c.21 C>T FP	5'CGCCATGCCCTCCTACAC3'	150 bp	56
c.21 C>T RP	5'CCACGCTCGAAGTCGTTGTA3'		
c.270 G>A FP	5'GTGCCACAGCAGCATACCT3'	401 bp	55
c.270 G>A RP	5'CCTGCACAGCAGTGTCATTC3'		
c.760 G>A (E254K) FP	5'CCTGGTAGAGCGGGTCATGAATC3'	179 bp	62
c.760 G>A (E254K) RP	5'ACCTCCTGCTCCAAGGGGAGCT3'		
c.1728 A>G FP	5'GAAAGAGGATGGACGGACTG3'	295 bp	55
c.1728 A>G RP	5'CTCGTTTTCCTGGAACTGGC3'		

FP, forward primer; RP, reverse primer.

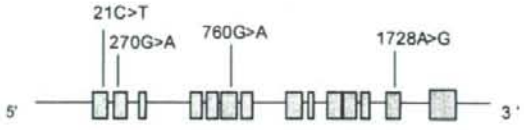

Figure 1. Gene structure and polymorphisms investigated in the *5-LO*. The positions marked were found in polymorphisms in the Japanese population.

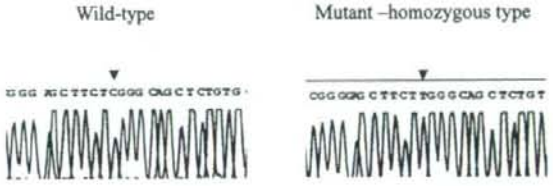

Figure 2. Big Dye terminator DNA sequence data of E254K in the reverse chain of *5-LO*. The data of a patient with E254 (wild-type) are shown on the left and the data of a patient with K254 (mutant-homozygous type) are shown on the right.

Measurement of urinary LTE4. Urine samples from 14 non-allergic subjects and 16 individuals with BA (13 without E254K and 3 with E254K), were stored at -80°C, and analyzed within 1 month of collection. The urinary creatinine level was determined by a creatinine test kit (Pure Auto S CRE-L, Daiichi-kagaku, Tokyo, Japan). Urinary LTE4 concentrations determined by EIA were corrected for recovery of [^{3}H]-LTE4. The urinary LTE4 level was expressed as pg/mg of creatinine.

Measurement of LTB4 production from neutrophils. Neutrophils were collected from heparinized blood in 14 non-allergic subjects and 16 individuals with BA (13 without E254K and 3 with E254K), and $2x10^6$ cells/ml were cultured in an RPMI-1640 medium with 15% fetal calf serum, after stimulation with 1 μM ionomycin. At 0, 15 and 30 min after adding ionomycin, we aspirated 1 ml of the culture medium and stored it at -80°C for the measurement of LTB4. We used the LTB4 Immunoassay (R&D Systems Inc., Minneapolis, MN) to quantify the LTB4 concentration in a supernatant from the cultured ionomycin-stimulation neutrophils (12). The samples were applied to the C18 reverse-phase column, and were measured by ELISA.

Relative expression of 5-lipoxygenase mRNA. PBMCs were isolated from the heparinized blood of the 14 non-allergic subjects and 16 individuals with BA (13 without E254K and 3 with E254K) by gradient centrifugation in Ficoll-Paque (Pharmacia, Uppsala, Sweden) and stored at -80°C for the extraction of mRNA. We quantified the relative expression of 5-lipoxygenase mRNA by real-time PCR. The real-time PCR was carried out with a Light Cycler instrument (Roche, Mannheim, Germany) by using the Light Cycler SYBR-Green I RNA Master Kit (Roche). Each PCR cycle included denaturation at 95°C for 10 sec, primers annealing at 57°C for 10 sec, and a final extension at 72°C for 8 sec. The cDNA was amplified using the following primers: sense primer 5'actgg aaacacggcaaaaac3' in exon 3 and anti-sense primer 5'tcac ggggtaaatccttgtg3' in exon 4. The size of the PCR product was 96 bp and the intron size between these two primers was >26 kb (7). Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) was used as the internal control for real-time quantitative PCR and we analyzed the relative expression of 5-lipoxygenase mRNA by the $2^{-\Delta\Delta Ct}$ method (13).

Homology structural model of 5-lipoxygenase E254 and K254. An initial homology model structure of 5-lipoxygenase was made by a FUGUE (http://www-cryst.bioc.cam.ac.uk/~fugue/) server. Coral 8R-lipoxygenase (2fnq) was selected as the most homologous template of 5-lipoxygenase from the Protein Data Bank (www.rcsb.org/pdb). The target-template identity rate is 39%. On the basis of this initial structural model, we optimized the structure and performed energy minimization using Internal Coordinate Mechanics (ICM) Pro version 3.3 (MolSoft). Furthermore, we generated the mutational form of this structure using the same software package. The validity of these structural models was evaluated by Ramachandran plot. The surface electrostatic potentials of these wild and mutant structures were calculated using Molmol software.

Table II. Allele and genotype frequencies of *5-LO* silent SNPs.

	Non-allergic subjects (n=60)	Bronchial asthma patients (n=60)	P-value
Allele frequency			
c.21 C>T			
C	108 (0.9)	110 (0.917)	
T	12 (0.1)	10 (0.083)	0.824
c.270 G>A			
G	115 (0.958)	119 (0.992)	
A	5 (0.042)	1 (0.008)	0.213
c.1728 A>G			
A	113 (0.942)	116 (0.967)	
G	7 (0.058)	4 (0.033)	0.539
Genotype frequency			
c.21 C>T			
CC	48 (0.8)	52 (0.867)	
CT	12 (0.2)	6 (0.100)	0.463
TT	0	2 (0.033)	
c.270 G>A			
GG	55 (0.917)	59 (0.983)	
GA	5 (0.083)	1 (0.017)	0.207
AA	0	0	
c.1728 A>G			
AA	53 (0.883)	56 (0.933)	
AG	7 (0.117)	4 (0.067)	0.529
GG	0	0	

Table III. Allele and genotype frequencies of *5-LO* missense SNP.

	Non-allergic subjects (n=150)	Bronchial asthma patients (n=180)	P-value
Allele frequency			
c.760 G>A			
G	300 (1)	348 (0.967)	
A	0	12 (0.033)	0.0007
Genotype frequency			
GG	150 (1)	169 (0.9400)	
GA	0	10 (0.0560)	0.0170
AA	0	1 (0.0056)	

Statistical analyses. Allele and genotype frequencies were calculated for each locus and tested for Hardy-Weinberg equilibrium. Distribution of the genotype of E254K in the *5-LO* was analyzed by Fisher's exact test. Probability (P) values <0.05 were considered statistically significant. The significance of difference was analyzed by the two-sample t-test.

Results

Polymorphisms in the 5-LO. We identified 4 SNPs in the *5-LO* in individuals with BA (Fig. 1). Three SNPs were silent polymorphisms: c.21 C>T (exon 1), c.270 G>A (exon 2) and c.1728 A>G (exon 13). There were no differences in the frequencies of the three SNPs between individuals with BA and non-allergic subjects (Table II).

One SNP was a missense polymorphism c.760 G>A, and the amino acid at 254 changed from Glu (E) to Lys (K) (Fig. 2). We determined the prevalence of c.760 G>A (E254K) in the *5-LO* of individuals with BA and non-allergic subjects. This SNP was found in 11 (0.061) out of the 180 individuals with BA. One was homozygous AA and 10 were heterozygous GA (2 out of the 10 were brother and sister). The mutant allele frequency was 0.033 in 180 individuals with BA. However, the mutant allele could not be detected in any of the 150 non-allergic subjects (Table III). There was a significant difference in the E254K frequency between

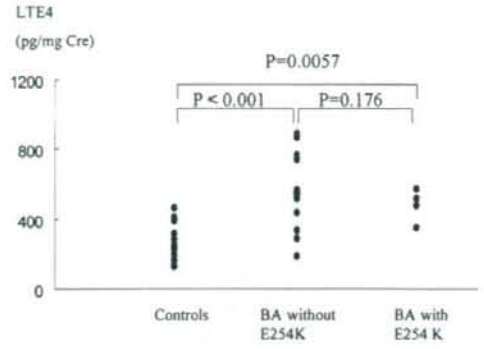

Figure 3. Urinary LTE4 production in individuals with bronchial asthma (BA) without E254K (n=13) and with E254K (n=3), and non-allergic subjects (n=14). The difference between individuals with bronchial asthma and non-allergic subjects was tested using a two-sample t-test. The urinary LTE4 productions were significantly higher in individuals with BA (without or with E254K) than in non-allergic subjects (P<0.05). The red mark is the mean level.

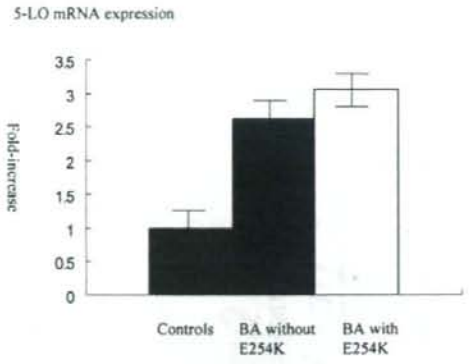

Figure 5. Relative expression of 5-lipoxygenase mRNA in individuals with bronchial asthma (BA) without E254K (n=13) and with E254K (n=3) and non-allergic subjects (n=14). The Y-axis on the left indicates the fold-increase compared to the mRNA expression in non-allergic subjects shown by the open bars. The relative expression of 5-lipoxygenase mRNA in individuals with BA but without E254K increased 2.6-fold above that in non-allergic subjects; in individuals with BA and with E254K it increased 3.1-fold above that in non-allergic subjects.

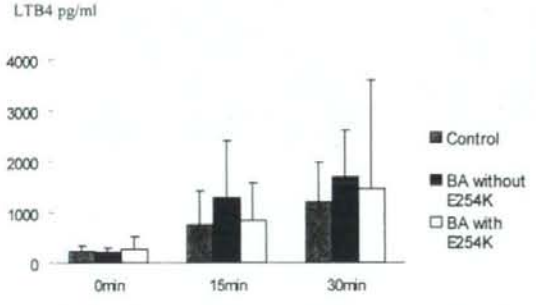

Figure 4. LTB4 production of ionomycin stimulation in neutrophils from individuals with bronchial asthma (BA) without E254K (n=13) and with E254K (n=3), and non-allergic subjects (n=14). The significance of difference between groups of LTB4 was tested using a two-sample-t-test. We detected no difference in LTB4 production in resting neutrophils from individuals with BA compared to non-allergic subjects. The mean level of LTB4 showed a tendency to increase more in individuals with BA (without or with E254K) than in non-allergic subjects after stimulation by ionomycin at 15 and 30 min, but there were no significant differences (P>0.05).

individuals with BA and non-allergic subjects (Fisher's exact test, P=0.0007).

Associations of E254K with urinary LTE4 or LTB4 production in neutrophils. To examine the functional effects of c.760 G>A (E254K) in the *5-LO*, we measured the urinary LTE4 levels in individuals with BA (without or with E254K) and non-allergic subjects. The urinary LTE4 levels were significantly higher in individuals with BA (without or with E254K) than in non-allergic subjects (P<0.05). However, the mean level of urinary LTE4 concentrations showed a tendency to decrease in individuals with BA and with E254K compared to those with BA but without E254K, although there was no significant difference (Fig. 3).

Furthermore, we measured the LTB4 concentrations in neutrophils isolated from individuals with BA (without or with E254K) and non-allergic subjects before and after stimulation by ionomycin. There were no differences in LTB4 production in resting neutrophils from individuals with BA compared to non-allergic subjects. The mean level of LTB4 concentration showed a tendency to increase in individuals with BA (without or with E254K) compared to non-allergic subjects after stimulation by ionomycin at 15 and 30 min (Fig. 4). The mean level of LTB4 concentrations in neutrophils showed a tendency to decrease in individuals with BA and with E254K compared to those with BA but without E254K, although there was no significant difference (P>0.05).

Relative expression of 5-lipoxygenase mRNA. We used the real time PCR (LightCycler 1.5 Instruments and SYBR-Green I system) to quantify the relative expression of 5-lipoxygenase mRNA in individuals with BA (without or with E254K) and non-allergic subjects. GAPDH was used as the internal control for real-time quantitative PCR and the relative expression of 5-lipoxygenase mRNA was analyzed by the $2^{-\Delta\Delta Ct}$ method. The relative expression of 5-lipoxygenase mRNA in individuals with BA but without E254K increased 2.6-fold above that in non-allergic subjects. In individuals with BA and with E254K the relative expression increased 3.1-fold above that in non-allergic subjects. The relative expression of 5-lipoxygenase mRNA was higher in individuals with BA and with E254K than in those with BA but without E254K (Fig. 5).

Homology structural model of 5-lipoxygenase E254 and K254. The human 5-lipoxygenase structural model consisted of the N-terminal ß-barrel domain, thought to interact with lipids, and the C-terminal catalytic domain containing the active site that is the iron-binding site and the substrate-binding cleft. We found that the SNP of *5-LO*, E254K, existed at the surface edge of the C-terminal catalytic domain, but this site was far from the active site of that enzyme (Fig. 6A). However, part of glutaminic acid 254 and lysine 254 had side chains, which obviously are exposed to

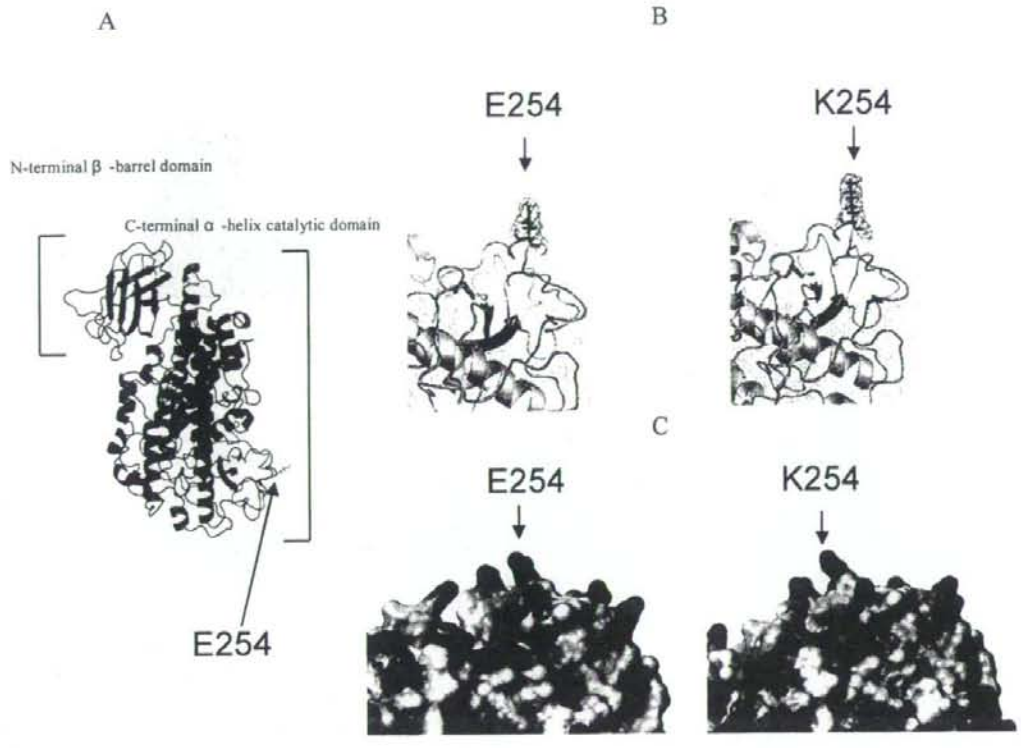

Figure 6. (A) Homology model structure of 5-lipoxygenase. The arrow indicates the part of the E254K polymorphism. (B) Comparison of the orientation of the side chains of glutamine acid 254 and lysine 254. (C) The focuses of the surface electrostatic potential of 5-lipoxygenase structures. Electrostatic potential representations of these two with red indicate areas of negative charge and blue indicate areas of positive charge. Arrows indicate the part of E254 and K254.

the solvent in these structural models (Fig. 6B), and the E-to-K substitution changed the charge of the side chain from negative to positive (Fig. 6C).

Discussion

Clinically similar asthma symptoms may be caused by different mechanisms (14). Chronic airway inflammation is a feature of asthma. Recently, evidence has demonstrated that leukotriene C4, D4 and E4 increase in the serum, urine and exhaled breath condensate (EBC) of asthma patients (11,15). The first committed enzyme in the biosynthetic pathway leading to the production of leukotrienes is 5-lipoxygenase. The addition of an Sp-1 binding motif (-GGGCGG-) or deletion of one or two Sp-1 binding motifs in the *5-LO* core promoter has been associated with reduced gene expression (9). We studied the polymorphisms in the *5-LO* and attempted to clarify the relationship between the novel polymorphism (c.760 G>A) and bronchial asthma.

We found a missense SNP and three silent SNPs in the *5-LO*. All patients who had an E254K substitution suffered from BA. There was a significant difference in the E254K frequency between individuals with BA and non-allergic subjects. Three other silent SNPs (c.21 C>T, c.270 G>A and c.1728 A>G) described previously (16), were also identified, but there were no significant differences in the frequencies between individuals with BA and non-allergic subjects.

To examine the functional effects of c.760 G>A (E254K) in the *5-LO*, we measured the production of urinary LTE4 and LTB4 in neutrophils in individuals with BA (without or with E254K) and non-allergic subjects. We found that urinary LTE4 production was significantly higher in individuals with BA (without or with E254K) than in non-allergic subjects. Furthermore, the mean level of LTB4 production in neutrophils showed a tendency to increase in individuals with BA (without or with E254K) more than in non-allergic subjects after stimulation by ionomycin. These results support the theory that leukotrienes play an important role in BA. The relative expression of 5-lipoxygenase mRNA in individuals with BA (without or with E254K) increased 2.6- or 3.1-fold above that in non-allergic subjects. This result supports the opinion of Koshino *et al* that the up-regulation of 5-lipoxygenase mRNA might be involved in the increased leukotriene synthesis and play an important role in the pathogenesis of asthma (11). In this study, the relative expression of 5-lipoxygenase mRNA was higher in individuals with BA and with E254K than in those with BA but without E254K. As a result, the *5-LO* pathway productions (urinary LTE4 and LTB4 levels in neutrophils) should be higher in individuals with BA and with E254K than in those with BA but without E254K. However, in this study, the urinary LTE4 and LTB4 levels in neutrophils showed a tendency to decrease in individuals with BA and with E254K compared to those with BA but without E254K. This result may be

caused by the change of the E-to-K substitution at amino acid 254. This SNP in the *5-LO*, which changes the charge from negative to positive, may affect the stability of the 5-lipoxygenase. Therefore, this SNP is induced to inhibit the synthesis of cys-LTs.

In order to clarify the functional effect of E254K, we analyzed the structural model of 5-lipoxygenase. The human 5-lipoxygenase structural model consisted of the N-terminal ß-barrel domain, thought to interact with lipids, and the C-terminal catalytic domain containing the active site that is the iron-binding site and the substrate-binding cleft. Our new finding is that the substitution of *5-LO*, E254K, existed at the surface edge of the C-terminal catalytic domain, but this site was far from the active site of that enzyme. However, part of glutamine acid 254 and lysine 254 had side chains, which are obviously exposed to the solvent in these structural models. Also, the E-to-K substitution changed the charge of the side chain from negative to positive, and it has been reported that this type of change can induce certain diseases (17,18). A previous report showed that some of the other cellular proteins interact with 5-lipoxygenase using the yeast two-hybrid screening method (19). Glutamine acid 254 might influence 5-lipoxygenase to interact with some other cellular proteins but not with FLAP or with the substrate of this enzyme (20-25). Pharmacogenetics is the study of how genetic differences influence the variability in patients' responses to therapy (26). Further studies may be necessary to define the relationship between these 4 SNPs and patients' response to therapy.

In conclusion, our study suggested that the c.760 G>A polymorphism, E254K, in the 5-lipoxygenase gene, is associated with bronchial asthma, and our findings can contribute to the evaluation of one of the genetic risk factors for this disease.

Acknowledgements

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Age-related changes in BAFF and APRIL profiles and upregulation of BAFF and APRIL expression in patients with primary antibody deficiency

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Abstract. In some patients with common variable immunodeficiency (CVID) and immunoglobulin (Ig) A deficiency (IgAD), tumor necrosis factor (TNF) family receptor transmembrane activator and calcium-modulator and cyclophilin ligand interactor (TACI) gene mutations have been reported. B cells from individuals with TACI mutations do not produce IgG and IgA in response to the TACI ligand a proliferation-inducing ligand (APRIL) which probably suggests impaired isotype switching. To clarify the pathogenesis of CVID and IgAD of Japanese patients, we investigated the mutations of TNF family members TACI, APRIL, B-cell activating factor (BAFF), B-cell maturation antigen (BCMA) and BAFF receptor (BAFF-R) genes and the expression levels of BAFF and APRIL in patients with CVID, IgAD and X-linked agammaglobulinaemia (XLA). We also investigated the relationship between age and the blood plasma levels of BAFF and APRIL. The causative gene mutations of TNF family members in our patients were not detected. In healthy subjects, the BAFF and APRIL plasma levels correlated inversely with age. The BAFF and APRIL plasma levels of patients with CVID, IgAD and XLA were significantly higher than those of healthy children. Elevated BAFF and APRIL expression levels might partially reflect the common immunological feature of primary antibody deficiency.

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Abbreviations: CVID, common variable immunodeficiency; Ig, immunoglobulin; IgAD, IgA deficiency; TNF, tumor necrosis factor; TACI, transmembrane activator and calcium-modulator and cyclophilin ligand interactor; APRIL, a proliferation-inducing ligand; BAFF, B-cell activating factor; BCMA, B-cell maturation antigen; BAFF-R, BAFF receptor; XLA, X-linked agammaglobulinaemia; BTK, Bruton tyrosine kinase; SLE, systemic lupus erythematosus; PBMCs, peripheral blood mononuclear cells; ELISA, enzyme-linked immunosorbent assay

Key words: common variable immunodeficiency, IgA deficiency, B-cell activating factor, a proliferation-inducing ligand, transmembrane activator and calcium-modulator and cyclophilin ligand interactor, B-cell activating factor receptor, B-cell maturation antigen, polymorphism, expression

Introduction

Common variable immunodeficiency (CVID) is a primary immunodeficiency disease characterized by absence of terminal B lymphocyte differentiation into plasma cells, resulting in hypogammaglobulinaemia, antibody deficiency, and recurrent bacterial infections (1-3). IgAD is the most common form of primary immunodeficiency. IgAD is characterized by absence or a very low level (<5 mg/dl) of serum IgA. Individuals with IgAD can be asymptomatic or predisposed to recurrent infections, particularly recurrent sinopulmonary and gastrointestinal infections (4-6).

The molecular bases of IgAD and most cases of CVID remain unknown, whereas X-linked agammaglobulinaemia (XLA) is caused by a defective BTK protein, which is indispensable for the development of mature B cells (7,8). Some individuals initially present with IgAD and then develop CVID later. IgAD and CVID are often observed in members of the same family. These observations suggest that some cases of IgAD and CVID have a common etiology (9). In fact, some patients with CVID and some patients with IgAD show a TACI mutation, as well as a mutation in one of the BAFF and APRIL receptors, associated with CVID and IgAD (10). These results suggest that BAFF and APRIL play crucial roles in B-cell-related immunodeficiency diseases such as CVID and IgAD.

BAFF and APRIL are two closely related cytokines; they share two receptors, TACI and BCMA, which are found mainly on B cells and plasma cells. The third receptor specific for BAFF, BAFF-R, is also found mainly on B cells and plasma cells, but also on some subsets of T cells (11,12). Thus far, no specific receptor for APRIL has been found, but it has been shown to bind to proteoglycans (13). BAFF and APRIL are produced constitutively by monocytes, macrophages, neutrophils, dendritic cells and osteoclasts. The binding of BAFF and APRIL to their receptors induces class switch recombination to IgG and IgA in human B cells (14,15).

Table I. Immunological characteristics of patients with CVID and IgAD.

	Sex	Age (years)	Serum Ig (mg/dl)			Lymph cell (%)		
			IgG	IgA	IgM	CD3	CD19	CD14
CVID								
1	F	8	679	8	15	67.0	9.0	3.2
2	F	9	761	7	11	69.0	22.0	7.1
3	F	20	378	7	5	64.0	22.0	6.6
Selective IgAD								
1	M	10	1363	<5	146	74.6	15.4	8.0
2	F	11	1640	<5	117	71.3	19.3	3.3
3	F	17	1261	<5	137	85.9	10.3	6.1
Partial IgAD								
4	F	4	869	27	106	81.1	11.3	7.3
5	M	4	887	45	101	71.2	17.9	9.0
6	M	4	1223	17	120	61.2	20.0	5.2
7	F	3	1644	15	150	48.1	24.8	5.6
8	M	4	1624	8	100	64.8	10.8	6.2
9	M	14	717	5	184	63.0	22.9	6.2
10	F	7	913	12	105	69.0	21.6	5.7
11	M	13	705	9	39	80.0	12.2	8.5
Controls								
	M/F	Adults	639-1344	70-312	40-240	51-83	5-21	

APRIL is highly expressed in tumors of various origins and poorly expressed in normal cells (16). The serum levels of BAFF and APRIL are elevated in patients with systemic lupus erythematosus (SLE) (17).

In this study, we report the increased BAFF and APRIL expression levels of patients with primary antibody deficiency. We found that the BAFF and APRIL expression levels are inversely correlated with age.

Materials and methods

Subjects. Forty-three healthy individuals (0-50 years of age) were enrolled as normal controls. The other subjects were three patients with primary CVID from different families, diagnosed on the basis of low immunoglobulin serum levels and the presence of circulating B cells, three patients with selective IgAD with serum IgA levels below the detection limit (<5 mg/dl), eight patients with partial IgAD with serum IgA levels >5 mg/dl but with 2 SD below normal levels, and four XLA patients with a BTK mutation. All of the patients were Japanese. The immunological characteristics of the patients are shown in Table I.

Mutation analysis. Genomic DNA was extracted from whole blood and purified with a Sepa Gene kit (Sanko Junyaku, Tokyo, Japan). We determined all the exons of the BAFF and APRIL genes and the genes of their receptors, namely, TACI, BAFF-R and BCMA, in the patients with CVID and IgAD. Primers were designed for regions flanking each exon of the genes of TNF family members, including the splice donor and acceptor recognition sites; the primer sequences are shown in Table II. The PCR conditions were as follows: denaturation at 94˚C for 5 min, followed by 40 cycles at 94˚C for 1 min, annealing at 54-60˚C for 1 min, and 72˚C for 1 min, and a final extension at 72˚C for 7 min. The BAFF-R gene was difficult to amplify because the percentages of G and C in the BAFF-R sequence were too high. We used the Takara LA Taq (Takara Bio Inc., Japan) with GC buffer enzyme for good performance of DNA amplification. The amplified fragments were subjected to direct sequencing.

Blood plasma BAFF and APRIL levels. The collected blood plasma was assayed for BAFF and APRIL by an enzyme-linked immunosorbent assay (ELISA). Peripheral blood was obtained by venipuncture from patients with CVID, IgAD and XLA, and from 43 healthy subjects. The plasma samples were stored at -20˚C until use. Soluble BAFF was quantitated in blood plasma diluted 1:4 (plasma of XLA patients were diluted 1:9) using the ELISA kit from R&D Systems, Inc. (Minneapolis, MN, USA). Enzyme activities were determined at an optical density of 450 nm. APRIL was determined in the same plasma samples. The plasma was diluted 1:1 (plasma of CVID and XLA patients were diluted 1:4) using the ELISA kit from Bender MedSystems GmbH (Vienna, Austria). Enzyme activities were determined at an optical density of 450-650 nm. Both standard and samples were analysed in duplicate.

Table II. Primer sequences used in this study.

Exon	Forward primers	Reverse primers
Primers for amplification and sequencing of TACI genomic DNA		
1	5'-GCCCGGCAGGCCTTCCACT-3'	5'-GCAAGCCCCACATCCCAGAGG-3'
2	5'-TTCCCATCAGGGACAAGAGG-3'	5'-CCTTTCCTCAGCCACCTGAC-3'
3	5'-CTTTGTGGTCAAACCCAGAG-3'	5'-CTGGGCTTCATGCATTGTGG-3'
4	5'-CCAGCCTCTCCAGGAGCCAGAC-3'	5'-CCGGGTGCCACTCTCCCAGTTA-3'
5	5'-CTGGGTCGGGGGAGAGTG-3'	5'-CTCTTTCCCTCTCTGCCTCT-3'
Primers for amplification and sequencing of APRIL genomic DNA		
1	5'-ACCCCACTCTTGAAACCACA-3'	5'-TGCTAACCATCCTCTCCCAG-3'
2	5'-CCTTGACCCTCTTTCCATGA-3'	5'-CACGCTGCTTGATCACCTC-3'
3	5'-AGTCAGGGTGAGGGTGGAG-3'	5'-AGCCCGAGTTCCTGGTTATT-3'
4	5'-TCCTGACCGACACACTCTCA-3'	5'-CTCAGTAGGGGGCCAAAGAG-3'
5	5'-GGCCATCCTGTTTTCTTCAA-3'	5'-TAGCTCCCTGCACTGCTACC-3'
6	5'-CTGTGCTTCACTGCGAATCT-3'	5'-ATGTACCCACCCTGGTCTTC-3'
Primers for amplification and sequencing of BAFF genomic DNA		
1	5'-TGCCAGCAAACCTACTGTACAGT-3'	5'-GGCACGCTTATTTCTGCTGTTC-3'
2	5'-ACCACGCGGAGAAGCTGCCA-3'	5'-CAGCGCTGGGGCTTTGCTCTA-3'
3	5'-TCAATGGGCAAATATAAAGTAACT-3'	5'-AGCTTGCTGAGAATGATGGTTTC-3'
4, 5	5'-GTGCAGTAATGTGACTTGTATTC-3'	5'-ACAGACTAGCTTATTATTCAAGAT-3'
6	5'-TAGGCTAAGATAATTGCAATGGTT-3'	5'-TGGTATTTTCAGTTAGATTCTTTC-3'
Primers for amplification and sequencing of BAFF-R genomic DNA		
1	5'-TCAGCCTCAGTCCCCGCAGCTTGT-3'	5'-TGCCCACAGGGTCCTTTCAGCCCT-3'
2	5'-TGAAAGGACCCTGTGGGCAG-3'	5'-TCCGTTTCCCCTTAAAGCCC-3'
3	5'-TGGCCAGGCCTCTGGACTCA-3'	5'-TGAGGTCTGAAGCCAAAGGCAA-3'
Primers for amplification and sequencing of BCMA genomic DNA		
1	5'-GAAGCAGGCGAAGTTCATTGTT-3'	5'-ATCAAGTTCAGTTCCAAATAATTAC-3'
2	5'-GAGGCAGGAGAATTGTTTGAACT-3'	5'-GCTCACCTCTACCAAGTTCATTT-3'
3	5'-CTTGAGCCCAGGAGTTTGAAT-3'	5'-CCATTAAGCTCCCAACAGTAAC-3'

Analyses of BAFF and APRIL gene expression. We carried out real-time PCR analysis to determine the levels of BAFF and APRIL mRNA in peripheral blood mononuclear cells (PBMCs). Total RNA was purified using an Isogen kit (Nippon Gene, Tokyo, Japan), subjected to DNase I treatment (RNA-free), and stored at -80˚C. The primer set for human TNSF13B (GmbH Heidelberg) was used for BAFF. The primers for APRIL were as follows: forward 5-AGAATG GGGAAGGGTATCCC-3 and reverse 5-AGGTGCAGG ACAGAGTGCTG-3. Real-time PCR conditions were as follows: denaturation at 95˚C for 10 min, followed by 40 cycles at 95˚C for 10 sec, 64˚C for 10 sec, and 72˚C for 10 sec. The reaction was carried out using the LightCycler FastStart DNA Master SYBR-Green I kit (Roche GmbH, Mannheim, Germany) according to the manufacturer's instructions. The BAFF or APRIL copy number was standardized relative to GAPDH, and expressed relative to the amount of GAPDH mRNA as an n=fold difference (18). No amplification of nonspecific products was observed.

Statistical analysis. Differences between the BAFF and APRIL protein and mRNA levels of the patients with CVID, IgAD, and XLA, and the controls were analyzed using the Student's t-test. The correlations between age and plasma levels of BAFF and APRIL were determined using Pearson's correlation coefficient test. $P<0.05$ was considered statistically significant.

Results

We found polymorphism variants in the APRIL and TACI genes. The results are shown in Table III. Nucleotide sequencing revealed two variants at codon 67 in exon 1 and at codon 96 in exon 2 of the APRIL gene. Both variants were caused by a single nucleotide substitution. At amino acid residue 67, the first nucleotide G was replaced by A, which resulted in an amino acid change from Gly to Arg (G67R). At codon 96, the second nucleotide A was replaced by G, which resulted in an amino acid change from Asn to Ser (N96S). These variants had the same frequencies in the control subjects. There were no other polymorphisms in the coding region of the human APRIL gene. Moreover, nucleotide sequencing revealed one variant at codon 251 in exon 5 of the TACI gene, which results in the amino acid substitution

Table III. Summary of variants found in patients during the screening of TNF family members.

	APRIL		BAFF	TACI	BAFF-R	BCMA
CVID						
1	G67R (homo)	N96S (homo)	WT	P251L (hetero)	WT	WT
2	WT	N96S (homo)	WT	P251L (hetero)	WT	WT
3	G67R (hetero)	N96S (hetero)	WT	P251L (homo)	WT	WT
Selective IgAD						
1	G67R (hetero)	N96S (homo)	WT	P251L (hetero)	WT	WT
2	WT	N96S (hetero)	WT	WT	WT	WT
3	WT	N96S (homo)	WT	P251L (homo)	WT	WT
Partial IgAD						
4	G67R (homo)	N96S (homo)	WT	P251L (homo)	WT	WT
5	G67R (hetero)	N96S (hetero)	WT	P251L (hetero)	WT	WT
6	G67R (hetero)	N96S (hetero)	WT	P251L (hetero)	P21R (hetero)	WT
7	G67R (hetero)	N96S (hetero)	WT	P251L (hetero)	WT	WT
8	G67R (hetero)	N96S (homo)	WT	WT	P21R (hetero)	WT
9	G67R (hetero)	N96S (hetero)	WT	WT	P21R (hetero)	WT
10	WT	N96S (homo)	WT	P251L (homo)	WT	WT
11	G67R (homo)	N96S (homo)	WT	WT	WT	WT
Control	WT (n=24)	WT (n=5)		WT (n=9)	WT (n=20)	
	G67R (hetero) (n=20)	N96S (hetero) (n=21)		P251L (hetero) (n=5)		
	G67R (homo) (n=6)	N96S (homo) (n=24)		P251L (homo) (n=3)		

homo, homozygous; hetero, heterozygous; WT, wild-type.

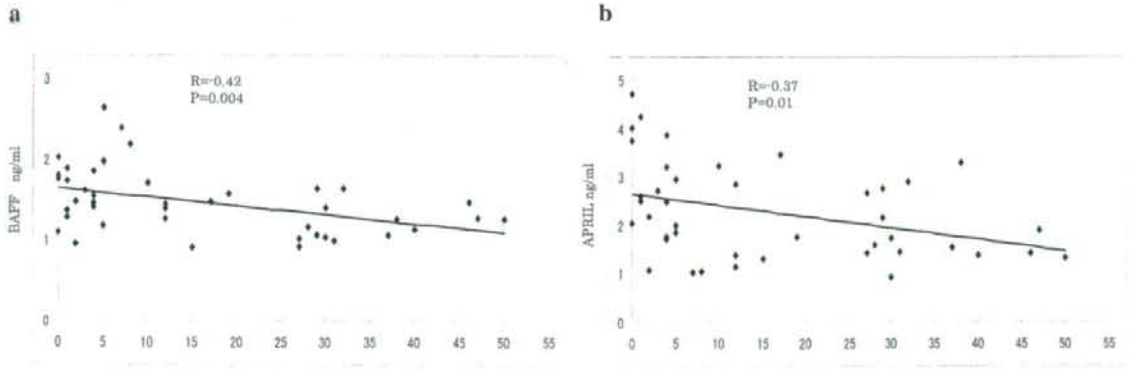

Figure 1. Inverse correlation of BAFF and APRIL plasma levels with age. BAFF and APRIL levels were determined in the same plasma samples (n=43). (a) Inverse correlation of BAFF plasma levels with age (R= -0.42, P=0.004); (b) Inverse correlation of APRIL plasma levels with age (R= -0.37, P=0.01).

P251L. This variant had the same frequency in the control subjects. Thus, the above nucleotide substitutions probably represent polymorphisms. In addition, we found a variant of the BAFF-R gene in three patients with partial IgAD. The variant was a heterozygous G-to-C substitution at position 62 in exon 1 (62C>G/wt), resulting in the replacement of the wild-type proline with an arginine (P21R/wt), as previously reported (19). The sequence analysis showed that causative gene mutations of TNF family members, namely, TACI, BAFF-R, BCMA, and their ligands, namely, BAFF and APRIL were not detected in the patients with CVID and IgAD.

To compare the BAFF and APRIL levels of patients with primary antibody deficiency with those of healthy subjects, we measured the blood plasma levels of BAFF and APRIL in

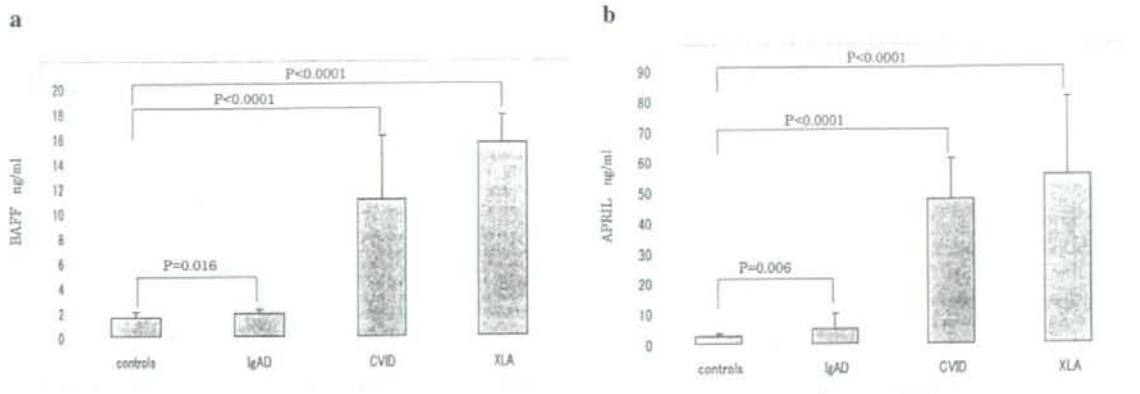

Figure 2. BAFF and APRIL protein expression levels of patients with IgAD, CVID and XLA. BAFF and APRIL levels were determined in the same plasma samples: plasma samples from 3 patients with CVID (mean age 12.3 years), 11 patients with IgAD (mean age 8.6 years), 4 patients with XLA (mean age 29 years) and 28 healthy children (control) (mean age 5.6 years). (a) BAFF protein expression levels of patients with CVID, IgAD and XLA. (b) APRIL protein expression levels of patients with CVID, IgAD and XLA.

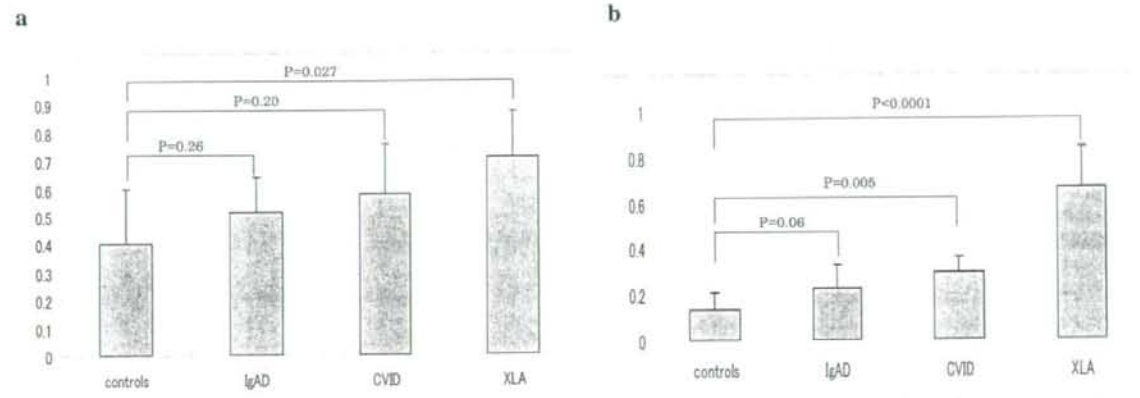

Figure 3. BAFF and APRIL gene expression levels in PBMCs of patients with CVID, IgAD and XLA. BAFF and APRIL gene expression levels of in 3 patients with CVID (mean age 12.3 years), 11 patients with IgAD (mean age 8.6 years), 4 patients with XLA (mean age 29 years) and 28 healthy children (control) (mean age 5.6 years). (a) BAFF mRNA. (b) APRIL mRNA.

43 healthy subjects of different ages. We found that the plasma levels of BAFF and APRIL were inversely correlated with age (BAFF: R= -0.42, P=0.004; APRIL: R= -0.37, P=0.01) (Fig. 1a and b). The inverse correlation of BAFF and APRIL gene expression levels with age was not detected by semi-quantitative PCR analysis (data not shown).

Next, the BAFF and APRIL protein levels of 11 patients with IgAD, 3 patients with CVID, and 4 patients with XLA were measured. The patients with CVID, IgAD and XLA showed significantly higher plasma levels of BAFF and APRIL than 28 normal children (Fig. 2a and b). The median plasma levels of BAFF were 1.92 ng/ml for IgAD, 11.04 ng/ml for CVID, 15.59 ng/ml for XLA and 1.59 ng/ml for the controls. The median plasma levels for APRIL were 5.2 ng/ml for IgAD, 47.39 ng/ml for CVID, 55.59 ng/ml for XLA and 2.46 ng/ml for the control.

The BAFF gene expression levels of the patients with XLA and the APRIL gene expression levels of the patients with CVID and XLA were significantly higher than those of the healthy subjects (Fig. 3a and b). The median levels of BAFF mRNA were 0.52 for IgAD, 0.58 for CVID, 0.71 for XLA, and 0.40 for the controls. The median levels of the APRIL mRNA were 0.23 for IgAD, 0.30 for CVID, 0.66 for XLA and 0.14 for the controls.

Discussion

Two studies have shown that coding variants in the TACI gene are associated with primary immunodeficiencies in humans (3,10). We found a P251L substitution in the TACI gene in patients with CVID and IgAD, and in the healthy subjects, which was consistent with previous findings (10). Moreover,

the G67R and N96S variants in the APRIL gene were detected at the same ratio as that in the healthy subjects, as previously reported (20). In the BAFF-R gene, three patients with IgAD showed the P21R variant, whereas no healthy subjects presented this variant. However, these variants have been reported to have no effect on the BAFF-R function (19). Therefore, we concluded that no causative mutation of the genes of TNF family members was detected in our patients.

We showed that the BAFF and APRIL levels were upregulated in the plasma of patients with CVID, IgAD and XLA. These findings indicate that BAFF and APRIL are involved in the common pathogenesis of primary antibody deficiencies. If fewer B cells are present than can be sustained by BAFF, the circulating pool of BAFF becomes augmented, and in the case of a mouse with total B-cell deficiency, BAFF was found to reach its highest levels (21-23). Patients with XLA have the lowest numbers of B cells associated with primary antibody deficiencies, that is, CD19-positive B cells were <0.1% (8). We showed that XLA is associated with the highest BAFF protein and gene expression levels. The low B-cell numbers might induce high BAFF gene expression levels to increase the number of B cells.

Recently, Knight *et al* have shown that CVID patients show a marked increase in the serum levels of BAFF, APRIL and TACI (18). Consistent with the results of their study, our findings revealed that primary immunodeficiency patients, including those with CVID, IgAD and XLA, showed increased plasma levels of BAFF and APRIL. It was suggested that primary antibody deficiency might have common immunological features such as monocyte activation (24-27). It has been reported that inflammation and cytokines such as IFNγ or G-CSF in particular enhance BAFF production (28,29).

In addition we found that the BAFF and APRIL plasma levels correlated inversely with age. It was also reported that the number of B cells is inversely correlated with age (30). In humans as well as in mice, the BAFF level might determine the size of the peripheral B-cell pool.

Although BAFF and APRIL are produced by many cells of the hematopoietic system, it is unclear how the expression and production of these cytokines are regulated. To understand the pathogenesis of primary antibody deficiency, it is necessary to elucidate the mechanism of the upregulation of BAFF and APRIL expression.

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