

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 cardiorespiratory failure 12 days after surgery
11		13 Y/F	Pe, bone	Cylothorax	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 lymphangiomas 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 lymphangiomas of infants and children.

REFERENCES

- [1] Ofelia AA, Ingrid K, Craig WZ. Thoracic lymphangiomas in a child. *J Pediatr Hematol Oncol*. 2004;26:136-141.
- [2] Kandil A, Rostom AY, Mourad WA, Khafaga Y, Gershuny AR, El-Hosseiny G. Successful control of extensive thoracic lymphangiomas by irradiation. *Clin Oncol*. 1997;9:407-411.
- [3] Giguere CM, Banman NM, Yutaka S, et al. Treatment of lymphangiomas with OK-432 (Picibanil) sclerotherapy: a prospective multi-institutional trial. *Arch Otolaryngol Head Neck Surg*. 2002;128:1137-1144.
- [4] Timke C, Krause MF, Oppermann HC, Leuschner I, Claviez A. Interferon alfa 2b treatment in an eleven-year-old boy with disseminated lymphangiomas. *Pediatr Blood Cancer*. 2007;48(1):108-111.
- [5] Glue P, Fang JW, Regine RP, et al. Pegylated interferon-alfa 2b: pharmacokinetics, pharmacodynamics, safety, and preliminary efficacy data. *Clin Pharmacol Ther*. 2000;68:556-567.
- [6] Stark GR, Kerr IM, Williams B, Silverman RH, Schreiber RD. How cells respond to interferons. *Annu Rev Biochem*. 1998;67:227-264.
- [7] Luxon BA, Grace M, Brassard D, Bordens R. Pegylated interferons for the treatment of chronic hepatitis C infection. *Clin Ther*. 2002;24:1363-1383.
- [8] Atzpodien J, Neuber K, Kamanabrou, et al. Combination chemotherapy with or without s.c. IL-2 and IFN-alfa: results of a prospectively randomized trial of the cooperative advanced malignant melanoma chemoimmunotherapy group (ACIMM). *Br J Cancer*. 2002;86:179-184.
- [9] Axel Bex, et al. A phase-II study of pegylated interferon alfa-2b for patients with metastatic renal cell carcinoma and removal of the primary tumor. *Cancer Immunol Immunother*. 2005;54:713-719.
- [10] Pigneux A, Tanguy ML, Michallet M, et al. Prior treatment with alpha interferon does not adversely affect the outcome of allogeneic transplantation for chronic myeloid leukaemia. *Br J Haematol*. 2002;116:193-201.
- [11] Dvorak H, Gresser I. Microvascular injury in pathogenesis of interferon-induced necrosis of subcutaneous tumors in mice. *J Natl Cancer Inst*. 1989;81:497-502.
- [12] Folkman J. Successful treatment of an angiogenic disease. *N Engl J Med*. 1989;320:1211-1212.
- [13] White CW, Sondheimer HM, Crouch EC, Wilson H, Fan LL. Treatment of pulmonary hemangiomas with recombinant interferon alfa-2a. *N Engl J Med*. 1989;320:1197-1200.
- [14] Kelso JM, Kerr DJ, Lie JT, et al. Unusual diffuse pulmonary lymphatic proliferation in a young boy. *Chest*. 1991;100:556-560.
- [15] Tazelaar HD, Kerr D, Yousem SA, Saldana MJ, Langston C, Colby TV. Diffuse pulmonary lymphangiomas. *Hum Pathol*. 1993;24:1313-1322.
- [16] Margaf LR. Thoracic lymphangiomas. *Pediatr Pathol*. 1996;16:155-160.
- [17] Reinhardt MA, Nelson SC, Sencer SF, Bostrom BC, Kurachek SC, Nesbit ME. Treatment of childhood lymphangiomas with interferon-alfa. *J Pediatr Hematol Oncol*. 1997;19:232-236.

- [18] Patton DF, Kaye R, Dickman P, Blatt J. Partial splenic embolization for treatment of disseminated intravascular coagulation in lymphangiomatosis. *J Pediatr*. 1998;132:1057–1060.
- [19] Maki DD, Nesbit ME, Griffiths HJ. Diffuse lymphangiomatosis of bone. *Australas Radiol*. 1999;43:535–538.
- [20] Laverdiere C, David M, Dubois J, Russo P, Hershon L, Lapierre JG. Improvement of disseminated lymphangiomatosis with recombinant interferon therapy. *Pediatr Pulmonol*. 2000;29:321–324.
- [21] Watkins RG, Reynolds R, McComb JG, Tolo VT. Lymphangiomatosis of the spine. *Spine*. 2003;28:E45–E50.
- [22] Harris JM, Martin NE, Modi M. Pegylation; a novel process for modifying pharmacokinetics. *Clin Pharmacokinet*. 2001;40:539–551.
- [23] Hicklin DJ, Ellis LM. Role of the vascular endothelial growth factor pathway in tumor growth and angiogenesis. *J Clin Oncol*. 2005;23:1011–1027.
- [24] Fukunaga M. Expression of D2-40 in lymphatic endothelium of normal tissues and in vascular tumor. *Histopathology*. 2005;46:396–402.

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

CHUNYING BAI, EIKO MATSUI, HIDENORI OHNISHI, KAORI KIMATA, KIMIKO KASAHARA, HIDEO KANEKO, ZENICHIRO KATO, TOSHIYUKI FUKAO and NAOMI KONDO

Department of Pediatrics, Graduate School of Medicine, Gifu University, Gifu, Japan

Received September 24, 2007; Accepted November 13, 2007

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.

Correspondence to: Eiko Matsui, Department of Pediatrics, Graduate School of Medicine, Gifu University, 1-1 Yanagido, Gifu 501-1193, Japan

E-mail: eikom@gifu-u.ac.jp

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'GTGCCACAGCAGCATACT3'	401 bp	55
c.270 G>A RP	5'CCTGCACAGCAGTGTTCATT3'		
c.760 G>A (E254K) FP	5'CCTGGTAGAGCGGGTCATGAATC3'	179 bp	62
c.760 G>A (E254K) RP	5'ACCTCTGCTCCAAGGGGAGCT3'		
c.1728 A>G FP	5'GAAAGAGGATGGACGGACTG3'	295 bp	55
c.1728 A>G RP	5'CTCGTTTTCTGGAAGTGGC3'		

FP, forward primer; RP, reverse primer.

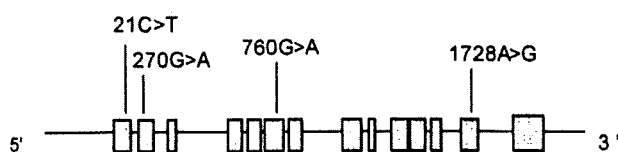


Figure 1. Gene structure and polymorphisms investigated in the 5-LO. The positions marked were found in polymorphisms in the Japanese population.

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 [³H]-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 2x10⁶ 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

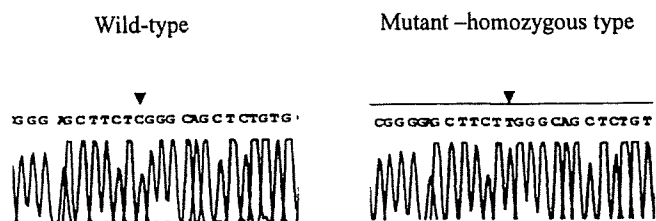


Figure 2. Big Dye terminator DNA sequence data of E254K in the reverse chain of 5-LO. The data of a patient with E254 (wild-type) are shown on the left and the data of a patient with K254 (mutant-homozygous type) are shown on the right.

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'actggaaacacggcaaaaac3' in exon 3 and anti-sense primer 5'tcacgggtaaatccttgtg3' 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^{-ΔΔC_t} 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)	0.824
T	12 (0.1)	10 (0.083)	
c.270 G>A			
G	115 (0.958)	119 (0.992)	0.213
A	5 (0.042)	1 (0.008)	
c.1728 A>G			
A	113 (0.942)	116 (0.967)	0.539
G	7 (0.058)	4 (0.033)	
Genotype frequency			
c.21 C>T			
CC	48 (0.8)	52 (0.867)	0.463
CT	12 (0.2)	6 (0.100)	
TT	0	2 (0.033)	
c.270 G>A			
GG	55 (0.917)	59 (0.983)	0.207
GA	5 (0.083)	1 (0.017)	
AA	0	0	
c.1728 A>G			
AA	53 (0.883)	56 (0.933)	0.529
AG	7 (0.117)	4 (0.067)	
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)	0.0007
A	0	12 (0.033)	
Genotype frequency			
GG	150 (1)	169 (0.9400)	0.0170
GA	0	10 (0.0560)	
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

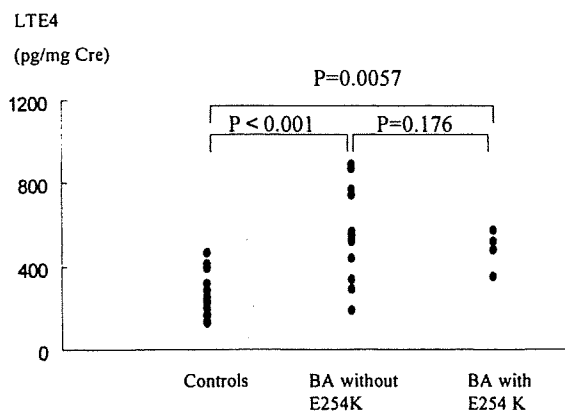


Figure 3. Urinary LTE4 production in individuals with bronchial asthma (BA) without E254K (n=13) and with E254K (n=3), and non-allergic subjects (n=14). The difference between individuals with bronchial asthma and non-allergic subjects was tested using a two-sample t-test. The urinary LTE4 productions 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.

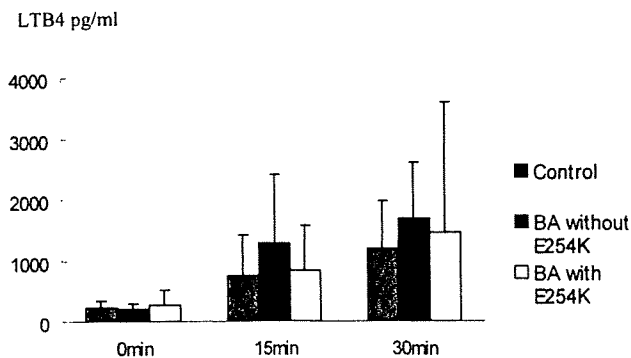


Figure 4. LTB4 production of ionomycin stimulation in neutrophils from individuals with bronchial asthma (BA) without E254K (n=13) and with E254K (n=3), and non-allergic subjects (n=14). The significance of difference between groups of LTB4 was tested using a two-sample-t-test. We detected no difference 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

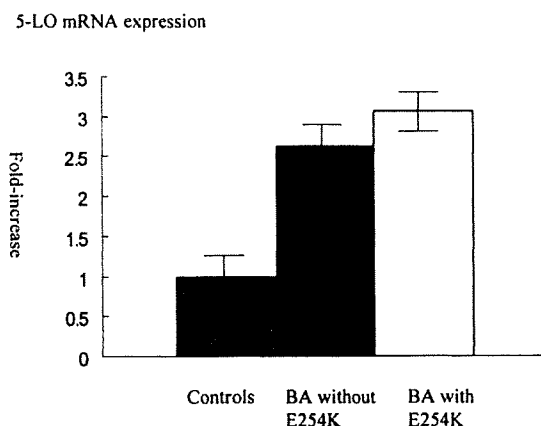


Figure 5. Relative expression of 5-lipoxygenase mRNA in individuals with bronchial asthma (BA) without E254K (n=13) and with E254K (n=3) and non-allergic subjects (n=14). The Y-axis on the left indicates the fold-increase compared to the mRNA expression in non-allergic subjects shown by the open bars.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

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 β -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 glutamic acid 254 and lysine 254 had side chains, which obviously are exposed to

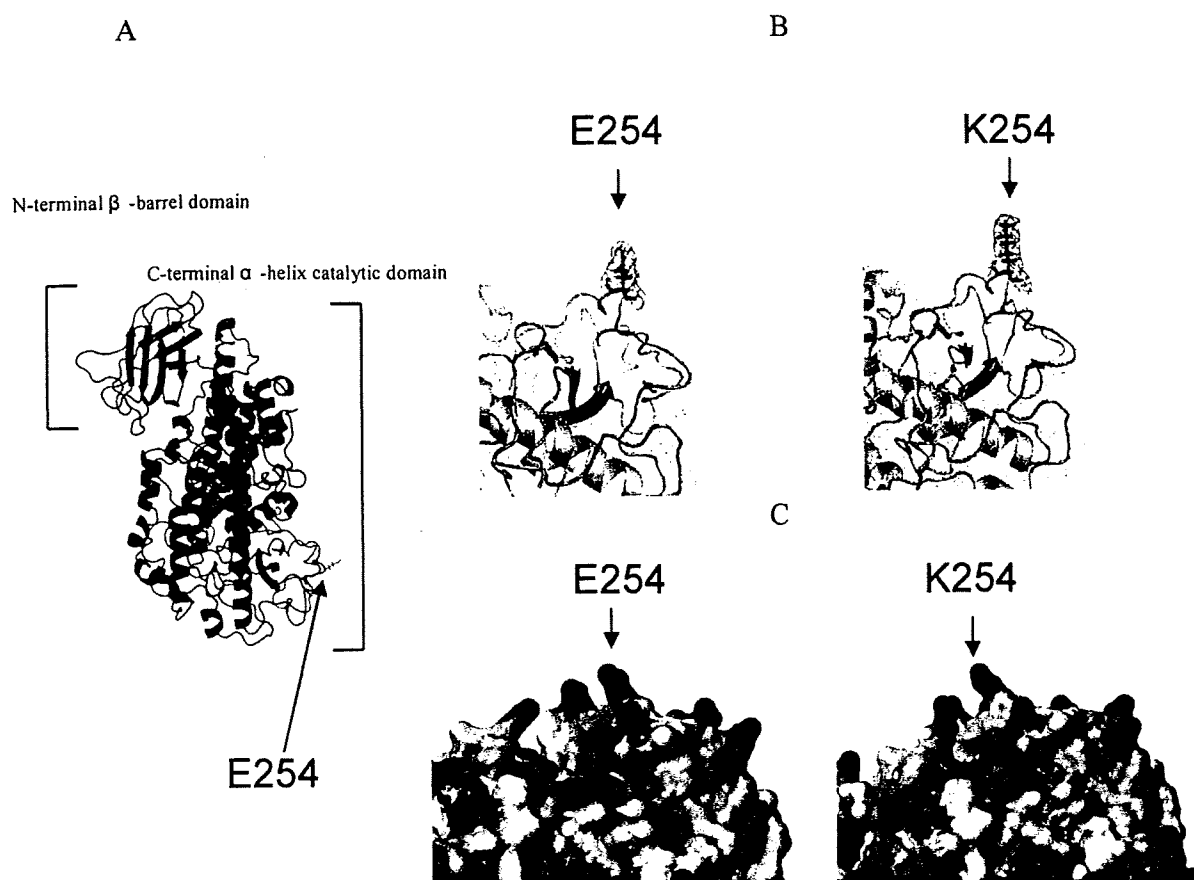


Figure 6. (A) Homology model structure of 5-lipoxygenase. The arrow indicates the part of the E254K polymorphism. (B) Comparison of the orientation of the side chains of glutamine acid 254 and lysine 254. (C) The focuses of the surface electrostatic potential of 5-lipoxygenase structures. Electrostatic 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 β -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

This study was supported by Health and Labour Science Research Grants for Research on Allergic Disease and Immunology from Ministry of Health, Labour and Welfare.

References

- Bochner BS and Busse WW: Allergy and asthma. *J Allergy Clin Immunol* 115: 953-959, 2005.
- Sanak M: Genetic variance of 5-lipoxygenase metabolic pathway in bronchial asthma. *Int Rev Asthma* 4: 70-80, 2002.
- Chen XS, Sheller JR, Johnson EN and Funk CD: Role of leukotrienes revealed by targeted disruption of the 5-lipoxygenase gene. *Nature* 372: 179-182, 1994.
- Samuelsson B: Leukotrienes: mediators of immediate hypersensitivity reactions and inflammation. *Science* 220: 568-575, 1983.
- Samuelsson B, Dahlen SE, Lindgren JA, Rouzer CA and Serhan CN: Leukotrienes and lipoxins: structures, biosynthesis, and biological effects. *Science* 237: 1171-1176, 1987.
- Lewis RA, Austen KF and Soberman RJ: Leukotrienes and other products of the 5-lipoxygenase pathway. *Biochemistry and relation to pathobiology in human diseases*. *N Engl J Med* 323: 645-655, 1990.
- Funk CD, Hoshiko S, Matsumoto T, Radmark O and Samuelsson B: Characterization of the human 5-lipoxygenase gene. *Proc Natl Acad Sci USA* 86: 2587-2591, 1989.
- Drazen JM and Silverman ES: Genetic determinants of 5-lipoxygenase transcription. *Int Arch Allergy Immunol* 118: 275-278, 1999.
- Silverman ES and Drazen JM: Genetic variations in the 5-lipoxygenase core promoter. *Am J Respir Crit Care Med* 161: 77-80, 2000.
- Koshino T, Takano S, Kitani S, *et al*: Novel polymorphism of the 5-lipoxygenase activating protein (FLAP) promoter gene associated with asthma. *Mol Cell Biol Res Commun* 2: 32-35, 1999.
- Koshino T, Takano S, Houjo T, *et al*: Expression of 5-lipoxygenase (5-*LO*) and 5-lipoxygenase-activating protein (FLAP) mRNAs in the peripheral blood leukocytes of asthma. *Biochem Biophys Res Commun* 247: 510-513, 1998.
- Helgadottir A, Manolescu A, Thorleifsson G, *et al*: The gene encoding 5-lipoxygenase activating protein confers risk of myocardial infarction and stroke. *Nat Genet* 36: 233-239, 2004.
- Livak KJ and Schmittgen TD: Analysis of relative gene expression data using real time quantitative PCR and the 2^{- $\Delta\Delta$ C_T} method. *Methods* 25: 402-408, 2001.
- Caterina RD and Zampolli A: From asthma to atherosclerosis - 5-lipoxygenase, leukotrienes, and inflammation. *N Engl J Med* 350: 4-7, 2004.
- Shibata A, Katsunuma T, Tomikawa M, Tan A, Yuki K, Akashi K and Eto Y: Increased leukotriene E4 in the exhaled breath condensate of children with mild asthma. *Chest* 130: 1718-1722, 2006.
- In K-H, Silverman ES, Asano K, *et al*: Mutations in the human 5-lipoxygenase gene. *Clin Rev Allergy Immunol* 17: 59-69, 1999.
- Yuen PH, Ryan EA, Devroe E and Wong PKY: A single Glu (62)-to-Lys (62) mutation in the Mos residues of the R7Delta447Gag-tMos protein causes the mutant virus to induce brain lesions. *Oncogene* 20: 692-703, 2001.
- Berretta F, Butler RH, Diaz G, *et al*: Detailed analysis of the effects of Glu/Lys β 69 human leukocyte antigen-DP polymorphism on peptide-binding specificity. *Tissue Antigens* 62: 459-471, 2003.
- Provost P, Samuelsson B and Radmark O: Interaction of 5-lipoxygenase with cellular proteins. *Biochemistry* 96: 1881-1885, 1999.
- Manev H and Tolga UZ: Primary cultures of rat cerebellar granule cells as a model to study neuronal 5-lipoxygenase and FLAP gene expression. *Ann NY Acad Sci* 890: 183-190, 1999.
- Zimmer JS, Dyckes DF, Bernlohr DA and Murphy RC: Fatty acid binding proteins stabilize leukotriene A4: competition with arachidonic acid but not other lipoxygenase products. *J Lipid Res* 45: 2138-2144, 2004.
- Voelkel NF, Tuder RM, Wade K, *et al*: Inhibition of 5-lipoxygenase-activating protein (FLAP) reduces pulmonary vascular reactivity and pulmonary hypertension in hypoxic rats. *J Clin Invest* 97: 2491-2498, 1996.
- Lepley RA, Muskardin DT and Fitzpatrick FA: Tyrosine kinase activity modulates catalysis and translocation of cellular 5-lipoxygenase. *J Biol Chem* 271: 6179-6184, 1996.
- Abramovitz M, Wong E, Cox ME, Richardson CD, Li C and Vickers PJ: 5-Lipoxygenase-activating protein stimulates the utilization of arachidonic acid by 5-lipoxygenase. *Eur J Biochem* 215: 105-111, 1993.
- In KH, Asano K, Beier D, *et al*: Naturally occurring mutations in the human 5-lipoxygenase gene promoter that modify transcription factor binding and reporter gene transcription. *J Clin Invest* 99: 1130-1137, 1997.
- Israel E: Genetics and the variability of treatment response in asthma. *J Allergy Clin Immunol* 115: 532-538, 2005.

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

RONG JIN, HIDEO KANEKO, HIROKO SUZUKI, TAKAHIRO ARAI, TAKAHIDE TERAMOTO, TOSHIYUKI FUKAO and NAOMI KONDO

Department of Pediatrics, Graduate School of Medicine, Gifu University, Gifu, Japan

Received September 24, 2007; Accepted November 19, 2007

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.

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).

Correspondence to: Dr Hideo Kaneko, Department of Pediatrics, Graduate School of Medicine, Gifu University, 1-1 Yanagido, Gifu, Gifu 501-1194, Japan
E-mail: hideo@gifu-u.ac.jp

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

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'-GCCCCGCGAGGCCTTCCACT-3'	5'-GCAAGCCCCACATCCCAGAGG-3'
2	5'-TTCCCATCAGGGACAAGAGG-3'	5'-CCTTTCCTCAGCCACCTGAC-3'
3	5'-CTTTGTGGTCAAACCCAGAG-3'	5'-CTGGGCTTCATGCATTGTGG-3'
4	5'-CCAGCCTCTCCAGGAGCCAGAC-3'	5'-CCGGGTGCCACTCTCCAGTTA-3'
5	5'-CTGGGTCGGGGGAGAGTG-3'	5'-CTCTTCCCTCTCTGCCTCT-3'
Primers for amplification and sequencing of APRIL genomic DNA		
1	5'-ACCCCACTCTTGAAACCACA-3'	5'-TGCTAACCATCCTCTCCCAG-3'
2	5'-CCTTGACCCTCTTCCATGA-3'	5'-CACGCTGCTTGATCACCTC-3'
3	5'-AGTCAGGGTGAGGGTGGAG-3'	5'-AGCCCGAGTTCCTGGTTATT-3'
4	5'-TCCTGACCGACACTCTCA-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'-TCAGCCTCAGTCCCCGCGAGCTTGT-3'	5'-TGCCACAGGGTCCTTTCAGCCCT-3'
2	5'-TGAAAGGACCCTGTGGGCAG-3'	5'-TCCGTTTCCCCTTAAAGCCC-3'
3	5'-TGCCAGGCCTCTGGACTCA-3'	5'-TGAGGTCTGAAGCCAAAGGCAA-3'
Primers for amplification and sequencing of BCMA genomic DNA		
1	5'-GAAGCAGGCGAAGTTCATTGTT-3'	5'-ATCAAGTTCAGTTCCAAATAATTAC-3'
2	5'-GAGGCAGGAGAATTGTTTGAAC-3'	5'-GCTCACCTCTACCAAGTTCATTT-3'
3	5'-CTTGAGCCAGGAGTTTGAAT-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 GGGAAAGGGTATCCC-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
2	WT	N96S (homo)	WT	P251L (hetero)	WT
3	G67R (hetero)	N96S (hetero)	WT	P251L (homo)	WT
Selective IgAD					
1	G67R (hetero)	N96S (homo)	WT	P251L (hetero)	WT
2	WT	N96S (hetero)	WT	WT	WT
3	WT	N96S (homo)	WT	P251L (homo)	WT
Partial IgAD					
4	G67R (homo)	N96S (homo)	WT	P251L (homo)	WT
5	G67R (hetero)	N96S (hetero)	WT	P251L (hetero)	WT
6	G67R (hetero)	N96S (hetero)	WT	P251L (hetero)	P21R (hetero)
7	G67R (hetero)	N96S (hetero)	WT	P251L (hetero)	WT
8	G67R (hetero)	N96S (homo)	WT	WT	P21R (hetero)
9	G67R (hetero)	N96S (hetero)	WT	WT	P21R (hetero)
10	WT	N96S (homo)	WT	P251L (homo)	WT
11	G67R (homo)	N96S (homo)	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.

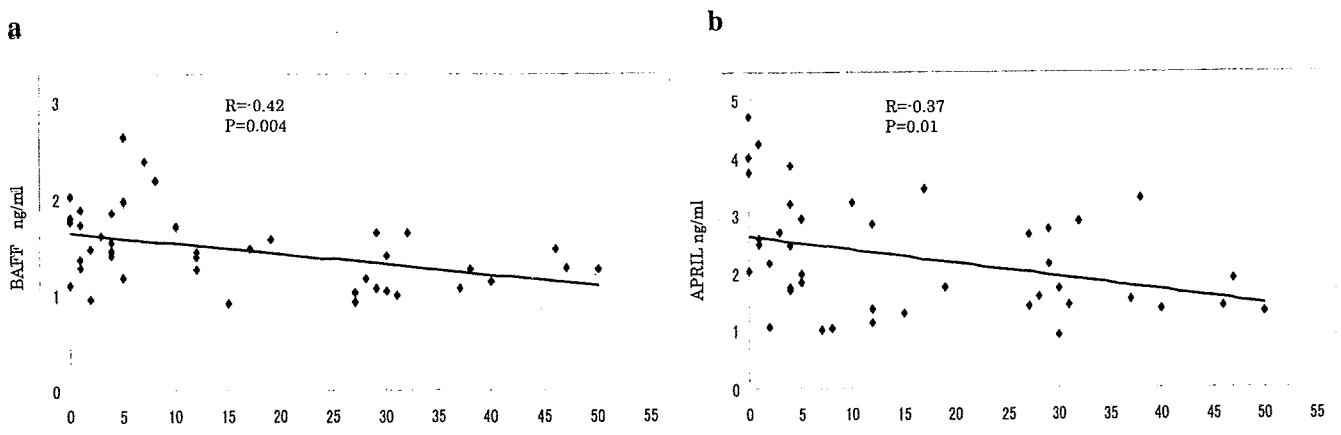


Figure 1. Inverse correlation of BAFF and APRIL plasma levels with age. BAFF and APRIL levels were determined in the same plasma samples (n=43). (a) Inverse correlation of BAFF plasma levels with age (R = -0.42, P=0.004); (b) Inverse correlation of APRIL plasma levels with age (R = -0.37, P=0.01).

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

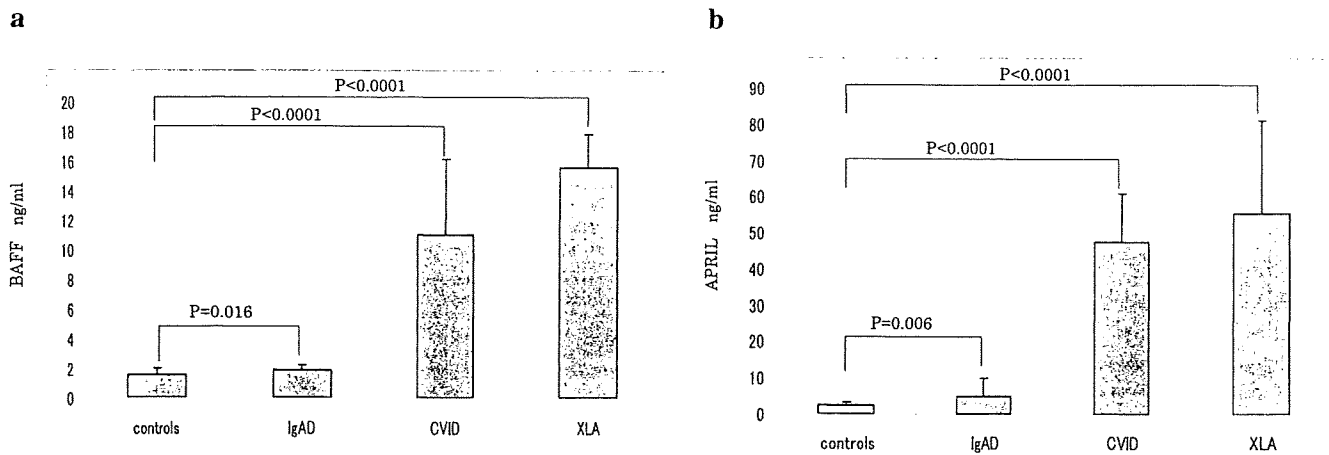


Figure 2. BAFF and APRIL protein expression levels of patients with IgAD, CVID and XLA. BAFF and APRIL levels were determined in the same plasma samples: plasma samples from 3 patients with CVID (mean age 12.3 years), 11 patients with IgAD (mean age 8.6 years), 4 patients with XLA (mean age 29 years)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

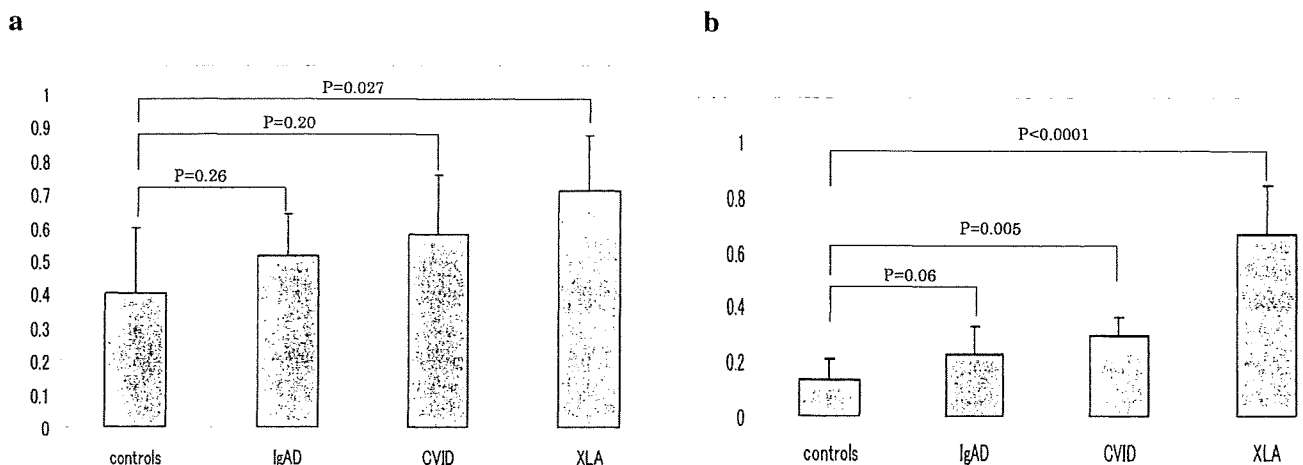


Figure 3. BAFF and APRIL gene expression levels in PBMCs of patients with CVID, IgAD and XLA. BAFF and APRIL gene expression levels of in 3 patients with CVID (mean age 12.3 years), 11 patients with IgAD (mean age 8.6 years), 4 patients with XLA (mean age 29 years) and 28 healthy children (control) (mean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

Acknowledgements

This study was partly supported by a grant from the Ministry of Health, Labor and Welfare of Japan.

References

- Salzer U and Grimbacher B: Common variable immunodeficiency: The power of co-stimulation. *Semin Immunol* 18: 337-346, 2006.
- Hammarstrom L, Vorechovsky I and Webster D: Selective IgA deficiency (SIgAD) and common variable immunodeficiency (CVID). *Clin Exp Immunol* 120: 225-231, 2000.
- Salzer U, Chapel HM, Webster AD, Pan-Hammarstrom Q, Schmitt-Graeff A, *et al*: Mutations in TNFRSF13B encoding TACI are associated with common variable immunodeficiency in humans. *Nat Genet* 37: 820-828, 2005.
- Asano T, Kaneko H, Terada T, *et al*: Molecular analysis of B-cell differentiation in selective or partial IgA deficiency. *Clin Exp Immunol* 136: 284-290, 2004.
- Cunningham-Rundles C: Physiology of IgA and IgA deficiency. *J Clin Immunol* 21: 303-309, 2001.
- Burrows PD and Cooper MD: IgA deficiency. *Adv Immunol* 65: 245-276, 1997.
- Weston SA, Prasad ML, Mullighan CG, Chapel H and Benson EM: Assessment of male CVID patients for mutations in the Btk gene: how many have been misdiagnosed? *Clin Exp Immunol* 124: 465-469, 2001.
- Kaneko H, Kawamoto N, Asano T, *et al*: Leaky phenotype of X-linked agammaglobulinemia in a Japanese family. *Clin Exp Immunol* 140: 520-523, 2005.
- Castigli E and Geha RS: Molecular basis of common variable immunodeficiency. *J Clin Immunol* 117: 740-746, 2006.
- Castigli E, Wilson SA, Garibyan L, Rachid R, Bonilla F, Schneider L, Geha RS, *et al*: TACI is mutant in common variable immunodeficiency and IgA deficiency. *Nat Genet* 37: 829-834, 2005.
- Bossen C and Schneider P: BAFF, APRIL and their receptors: Structure, function and signaling. *Semin Immunol* 18: 263-275, 2006.
- Vallerskog T, Heimbürger M, Gunnarsson I, Zhou W, Wahren-Herlenius M, *et al*: Differential effects on BAFF and APRIL levels in rituximab-treated patients with systemic lupus erythematosus and rheumatoid arthritis. *Arthritis Res Ther* 8: R167, 2006.
- Sakurai D, Hase H, Kanno Y, Kojima H, Okumura K, *et al*: TACI regulates IgA production by APRIL in collaboration with HSPG. *Blood* 109: 2961-2967, 2007.
- Mackay F and Ambrose C: The TNF family members BAFF and APRIL: the growing complexity. *Cytokine Growth Factor Rev* 14: 311-324, 2003.
- Matsushita T and Sato S: The role of BAFF in autoimmune diseases. *Jpn J Clin Immunol* 28: 333-342, 2005.
- Tangye SG, Bryant VL, Cuss AK and Good KL: BAFF, APRIL and human B cell disorders. *Semin Immunol* 18: 305-317, 2006.
- Koyama T, Tsukamoto H, Miyagi Y, Himeji D, Otsuka J, *et al*: Raised serum APRIL levels in patients with systemic lupus erythematosus. *Ann Rheum Dis* 64: 1065-1067, 2005.
- Knight AK, Radigan L, Marron T, Langa A, Zhang L, *et al*: High serum levels of BAFF, APRIL, and TACI in common variable immunodeficiency. *Clin Immunol* 124: 182-189, 2007.
- Losi CG, Silini A, Fiorini C, Soresina A, Meini A, *et al*: Mutational analysis of human BAFF receptor TNFRSF13C (BAFF-R) in patients with common variable immunodeficiency. *J Clin Immunol* 25: 496-502, 2005.
- Koyama T, Tsukamoto H, Masumoto K, Himeji D, Havashi K, *et al*: A novel polymorphism of the human APRIL gene is associated with systemic lupus erythematosus. *Rheumatology* 42: 980-985, 2003.
- Schneider P: The role of APRIL and BAFF in lymphocyte activation. *Curr Opin Immunol* 17: 282-289, 2005.
- Lesley R, Xu Y, Kalled SL, Hess DM, Schwab SR, *et al*: Reduced competitiveness of autoantigen-engaged B cells due to increased dependence on BAFF. *Immunity* 20: 441-453, 2004.
- Seyler TM, Park YW, Takemura S, Bram RJ, Kurtin PJ, *et al*: BLYS and APRIL in rheumatoid arthritis. *J Clin Invest* 115: 3083-3092, 2005.
- Roschke V, Sosnovtseva S, Ward CD, Hong JS, Smith R, *et al*: BLYS and APRIL form biologically active heterotrimers that are expressed in patients with systemic immune-based rheumatic diseases. *J Immunol* 169: 4314-4321, 2002.
- Mackay F, Sierro F, Gery ST and Gordon TP: The BAFF/APRIL system: an important player in systemic rheumatic diseases. *Curr Dir Autoimmun* 8: 243-265, 2005.
- Matsushita T, Hasegawa M, Yanaba K, Kodera M, Takehara K, *et al*: Elevated serum BAFF levels in patients with systemic sclerosis: enhanced BAFF signaling in systemic sclerosis B lymphocytes. *Arthritis Rheum* 54: 192-201, 2006.
- Kawasaki A, Tsuchiya N, Fukazawa T, Hashimoto H and Tokunaga K: Analysis on the association of human BLYS (BAFF, TNFSF13B) polymorphisms with systemic lupus erythematosus and rheumatoid arthritis. *Genes Immun* 3: 424-429, 2002.
- Nardelli B, Belvedere O, Roschke V, *et al*: Synthesis and release of B-lymphocyte stimulator from myeloid cells. *Blood* 97: 198-204, 2001.
- Scapini P, Nardelli B, Nadali G, *et al*: G-CSF-stimulated neutrophils are a prominent source of functional BLYS. *J Exp Med* 197: 297-302, 2003.
- Colonna-Romano G, Aquino A, Bulati M, Di Lorenzo G, Listi F, *et al*: Memory B cell subpopulations in the aged. *Rejuvenation Res* 9: 149-152, 2006.

Protein-Losing Enteropathy Associated With Egg Allergy in a 5-Month-Old Boy

M Kondo,¹ T Fukao,^{1,2} K Omoya,¹ N Kawamoto,¹ M Aoki,¹ T Teramoto,¹
H Kaneko,¹ N Kondo¹

¹Department of Pediatrics, Graduate School of Medicine, Gifu University, Gifu, Japan

²Medical Information Science Division, United Graduate School of Drug Discovery and Medical Information Sciences, Gifu University, Gifu, Japan

Abstract

Protein-losing enteropathy (PLE), the manifestation of a diverse set of disorders, is characterized by excessive loss of plasma proteins into the affected portions of the gastrointestinal tract, and this results in hypoalbuminemia. A 5-month-old breastfed boy presented severe PLE with hypogammaglobulinemia, hypocalcemia, and hypomagnesemia induced by an egg allergy. He developed hypocalcemic convulsions. The diagnosis of PLE was confirmed by elevated fecal α_1 -antitrypsin clearance and a positive finding on a protein-losing scintigram. His allergy to egg delivered through maternal milk was confirmed as the cause of PLE, since the mother's elimination of egg from her diet improved his condition and maternal egg challenge provoked symptoms of diarrhea, vomiting, and elevated α_1 -antitrypsin clearance. At the time of writing, he is 22 months old and has experienced no further episodes after the elimination of egg-containing food.

Key words: Protein-losing enteropathy (PLE). Hypocalcemia. Egg allergy. Food challenge.

Resumen

La enteropatía de pérdida de proteínas (EPP), la manifestación de varios conjuntos de trastornos, se caracteriza por la pérdida excesiva de proteínas séricas en los tramos afectados del aparato gastrointestinal, dando lugar a la hypoalbuminemia. Un niño de 5 meses alimentado con lactancia materna presentó EPP grave con hipogammaglobulinemia, hipocalcemia e hipomagnesemia inducidas por una alergia al huevo. El niño presentó convulsiones por hipocalcemia. El diagnóstico de EPP se confirmó mediante la elevada eliminación de α_1 -antitripsina en las heces y el resultado positivo en una gammagrafía de pérdida de proteínas. La alergia al huevo, que procedía de la ingesta de la leche materna, se confirmó como la causa de la EPP, ya que cuando la madre eliminó el huevo de su dieta el estado del niño mejoró, pero la prueba de provocación con huevo de la madre ocasionaba la aparición de los síntomas de diarrea, vómitos y una elevada eliminación de α_1 -antitripsina. En el momento en que se escribe este documento, el niño tiene 22 meses y no ha experimentado ningún otro episodio después de la eliminación de alimentos que contuvieran huevo.

Palabras clave: Enteropatía de pérdida de proteínas (EPP). Hipocalcemia. Alergia al huevo. Prueba de provocación alimentaria.

Introduction

Protein-losing enteropathy (PLE) has been associated with many disorders occurring at different anatomic sites in the gastrointestinal tract. Any process that leads to a profound leakage of proteins from the gastrointestinal tract can lead to PLE but there are 3 main mechanisms that are responsible: 1) lymphatic obstruction, such as primary intestinal lymphangiectasia, cardiac

diseases, and lymphoma; 2) erosions and ulcerations, such as erosion of the esophagus and ulcerative colitis; and 3) intestinal permeability (leakage), such as is found in celiac disease and Henoch-Schönlein purpura, [1-3]. Food-allergies are attributable to the adverse immune response to dietary proteins and account for numerous gastrointestinal disorders of childhood [3]. Eosinophilic gastroenteritis and dietary protein enteropathy have been reported to cause PLE [3].

We report on a 5-month-old boy who had PLE caused by allergy to egg proteins that had arrived through maternal milk. He developed hypocalcemic convulsions.

Case Description

A 5-month-old boy was hospitalized with the chief complaint of afebrile convulsions 3 times a day. He had been exclusively breastfed until he started eating rice porridge 10 days earlier. His grandmother had pointed out his loose stools 2 weeks before hospitalization and his mother reported that he had had diarrhea (3 times per day) and regurgitated milk (several times per day) for 3 days prior to admission. He drank well and had been cheerful. His parents did not recognize his change, which included edema. His developmental milestones were within the normal range. His height was 69.5 cm and body weight was 7.8 kg. On admission, he was conscious, slightly edematous with pretibial pitting edema, periorbital edema, and was mildly hypotonic. He had no apparent dermatitis. Laboratory data on admission (table) showed remarkable hypoproteinemia including hypogammaglobulinemia (total protein, 2.8 g/dL; albumin, 1.9 g/dL; immunoglobulin [Ig] G, 31 mg/dL; IgM, 19 mg/dL; IgA 7 mg/dL). In addition, serum calcium (3.8 mg/dL; corrected for serum albumin, 5.4 mg/dL) and magnesium (0.8 mg/dL) levels were very low; serum sodium, potassium, and chloride levels were within normal ranges. Lymphocytopenia (<1000/L) was noted. The C-reactive protein level was within the normal range. Urine analysis showed neither proteinuria nor hematuria.

Analyses of cerebrospinal fluid, an electroencephalogram, computed tomography and magnetic resonance imaging of the brain showed no abnormalities. Thus, his recurrent convulsions were considered to be due to severely low calcium and magnesium levels. Convulsions occurred twice after admission. The patient was given intravenous calcium, magnesium, and albumin; after 7 days, calcium and magnesium levels became normal, after which convulsions did not recur.

Since proteinuria was not observed, a loss of proteins into the gastrointestinal tract was suspected as the cause of hypoproteinemia. Fecal occult blood was repeatedly positive and α_1 -antitrypsin clearance was 400 mL/d (normal range, below 13 mL/d). His stool culture was negative and a rotavirus antigen test was negative. May-Giemsa staining of the stool revealed no eosinophils. A protein-losing scintigram using technetium 99m (^{99m}Tc)-labeled human serum albumin demonstrated leakage into the small intestine. Based on these findings, we diagnosed PLE.

The infant's serum IgE level was slightly high (22 U/mL). The egg-white and egg-yolk-specific IgE levels measured by CAP system radioallergen sorbent testing (RAST) (Phadia, Uppsala, Sweden) were 1.2 kU/L and 1.1 kU/L, respectively, whereas other allergen-specific IgE antibodies (to milk, wheat, rice, soybean, mites, house dust) were not detected. Prick tests for egg-white and egg-yolk were positive but tests for other foods (milk, soybean, rice, wheat) were negative. In intracellular interleukin (IL) 4 and interferon (IFN) staining of CD4⁺ cells, the percentages of type 1 helper T cells (T_H1) (IFN- positive, IL-4 negative) and

T_H2 cells (IFN- negative, IL-4 positive) were both highly elevated to 28.5% and 4.8%, respectively, on admission in comparison with age-matched controls (T_H1 , 2.02% [SD, 1.12 %]; T_H2 , 0.33% [SD, 0.18%]) [4]. The T_H1 and T_H2 percentages decreased gradually to 5.23% and 0.28%, respectively, on discharge.

After admission, oral food intake including breastfeeding was stopped and total parenteral nutrition was undertaken for 10 days. Four days before breastfeeding was restarted, elimination of egg-containing foods from his mother's diet was started. Then maternal milk and additional formula milk feeding was restarted. He had no vomiting or diarrhea, and his serum protein levels increased gradually (on the 50th hospital day: total proteins, 5.3 g/dL; albumin, 4.1 g/dL; IgG, 197 mg/dL; IgM, 71 mg/dL; and IgA 28, mg/dL). Fecal α_1 -antitrypsin clearance decreased to the normal range. The second protein-losing scintigram (1 month after the first scintigram) did not detect leakage of the labeled albumin into the intestine.

A food challenge test for egg through maternal milk was started on the 50th hospital day. His mother took 1 whole boiled egg every 2 days and continued to breastfeed. The boy was well until the second challenge when he vomited 3 times. Egg was again eliminated from the mother's diet. Positive occult blood in the stool was noted for 3 days after the second challenge. α_1 -antitrypsin clearance in the stool was elevated again to 360 mL/d; however the clearance became normal within 2 weeks after the challenge. He was discharged at 120 days.

At age 22 months, he thrives with egg-containing foods eliminated from his diet. No other food restriction has been necessary. He has experienced no further episodes of PLE and has had no apparent symptoms of gastrointestinal food allergy.

Discussion

We have presented a case of PLE caused by egg allergy. Diagnosis of PLE was confirmed by hypoalbuminemia, elevated α_1 -antitrypsin clearance in stool, and a positive protein-losing scintigram using ^{99m}Tc -labeled human serum albumin. Egg allergy was confirmed as the cause of PLE by 1) improvement of the boy's condition with the first period of elimination of eggs from the mother's diet; 2) diarrhea and vomiting, with elevated α_1 -antitrypsin clearance, provoked by the egg challenge through maternal milk; 3) further improvement with re-elimination of egg-containing foods from the mother's diet.

A positive egg-white and egg-yolk IgE RAST and positive prick test for those foods indicated that the patient was sensitized to egg through maternal milk since he had never taken egg-containing food directly. Egg allergens, as well as cow's milk and wheat allergens, have been detected in breast milk as little as 2 to 6 hours after maternal intake and can be detected as long as 4 days later [5-7]. We therefore considered egg allergy as one of the causes of PLE. There are several gastrointestinal food-allergic disorders of infancy and childhood [3]. Our patient's PLE could have been dietary protein enteropathy [3], but an endoscopic examination and biopsy were not performed in this case. Usually, these gastrointestinal food-allergic disorders,

Table. Comparison of the Present Protein-Losing, Enteropathy Case With a Previously Reported Case [9]

	Present Case	Previous Case [9]
Onset	5 months	4 months
Feeding	breastfed	milk-formula-fed ^a
Diarrhea		
Onset	4.5 months	3 months
Frequency	3 times/d	5-6 times/d
Weight gain	good	good
General condition	good	good
Convulsions	present	present
Laboratory data on admission		
Total protein, g/dK	2.8	2.8
Albumin, g/dL	1.9	1.6
IgG, mg/dL	31	69
IgA, mg/dL	7	11.8
IgM, mg/dL	19	35
IgE, U/mL	22	220
Sodium, mEq/L	136	125
Potassium, mEq/L	3.9	3.2
Chloride, mEq/L	108	99
Calcium, mg/dL	3.8	4.6
Magnesium, mg/dL	0.8	0.7
White blood cells, per L	6440	NR
Lymphocytes, per L	943	NR
CD3 ⁺ , per L	366	1366
CD4 ⁺ , per L	134	528
CD8 ⁺ , per L	269	1157
CD19 ⁺ , per L	359	NR
T _H 1, % in CD4 ⁺ cells	28.5%	NR
T _H 2, % in CD4 ⁺ cells	4.8%	NR
IgE CAP-RAST		
Egg white, kU/L	1.4	markedly increased ^b
Egg yolk, kU/L	1.1	markedly increased ^b
Milk, kU/L	<0.34	markedly increased ^b
Histamine releasing test	no information	egg positive
Prick test	egg positive	NR
Protein-losing scintigram	positive	positive
Lymphoscintigram	not done	dilated
Egg elimination	effective	effective
Egg challenge	positive	not done

Abbreviations: Ig, immunoglobulin; T_H1 and T_H2, types 1 and 2 helper T cells; NR, not reported.

^a This patient breastfed for the first few days.

^b In their report, Hamada et al [9] did not report exact values; they used the phrase "RAST values for milk and egg were markedly increased" (p. 687).

except for immediate gastrointestinal hypersensitivity, are non-IgE-mediated but are associated with T cell responses [8]. We consider that the presence of a positive IgE antibody and prick test for egg protein does not exclude the presence of non-IgE-mediated enteropathy with T cell responses. In fact, activation of T_H1 and T_H2 cells were suspected by the fact that the percentages those cells in peripheral blood CD4⁺ lymphocytes were very high on admission and were normalized on discharge from the hospital.

The boy presented not only hypoalbuminemia but also hypogammaglobulinemia, lymphocytopenia, and hypocalcemia/

hypomagnesemia. In general, a low immunoglobulin level and lymphocytopenia are common in PLE that is caused by lymphatic obstruction but rare in PLE caused by other mechanisms. In this context, this patient was suspected of having PLE caused by lymphatic obstruction.

We located a case report of a 4-month-old boy whose clinical presentation was very similar to our patient's (table) [9]. That infant also had hypogammaglobulinemia, hypocalcemia, and hypomagnesemia with recurrent convulsions. He was diagnosed as having intestinal lymphangiectasia accompanying PLE by means of a protein-losing scintigram and lymphoscintigram.

However, although tests for IgE antibodies were positive for both egg and milk, a histamine-releasing test was positive only for egg. He was on hyperalimentation for the first month and thereafter elimination of egg from his diet was continued. His PLE condition, including laboratory findings, normalized completely 6 months later. A challenge test for egg was not performed in that case, but the elimination of egg as well as medium-chain triglyceride milk improved his condition. We did not perform a lymphoscintigram for our patient, but the laboratory data were consistent with those for lymphatic obstruction, and in general our case is consistent with the previous report. We speculate that some patients with dietary protein enteropathy may have lymphatic obstruction or a similar pathological condition which excretes gammaglobulin, calcium, and magnesium into the intestinal tract.

In summary, we report a rare case of infantile egg allergic enteropathy through maternal milk which caused severe PLE with hypocalcemia and hypomagnesemia resulting in afebrile convulsions in an infant.

Acknowledgment

The immunological studies referred to in this report were in part funded by the Research and Development Program for New Bio-industry Initiatives (2005-2009) of the Bio-oriented Technology Research Advancement Institution (BRAIN), Japan.

References

1. Gracey M, Anderson CM. Intestinal lymphangiectasia and other causes of protein-losing gastroenteropathies. In: Gracey M, Burke V, eds. *Pediatric Gastroenterology and Hepatology*. Oxford: Blackwell Scientific Publications. 1993. p. 439-40.
2. Strober W, Wochner RD, Carbine PP, Waldmann TA. Intestinal lymphangiectasia. A protein-losing gastroenteropathy with hypogammaglobulinemia, lymphocytopenia and impaired homograft rejection. *J Clin Invest*. 1967;46:1643-56.
3. Sicherer SH. Clinical aspects of gastrointestinal food allergy in childhood. *Pediatrics*. 2003;111:1609-16.
4. Kawamoto N, Kaneko H, Takemura M, Seishima M, Sakurai S, Fukao T, Kasahara K, Iwasa S, Kondo N. Age-related changes in intracellular cytokine profiles and Th2 dominance in allergic children. *Pediatr Allergy Immunol*. 2006;17:125-33.
5. Cant A, Marsden RA, Kilshaw PJ. Egg and cows' milk hypersensitivity in exclusively breast fed infants with eczema, and detection of egg protein in breast milk. *Br Med J (Clin Res Ed)*. 1985;291:932-5.
6. Stuart CA, Twiselton R, Nicholas MK, Hide DW. Passage of cows' milk protein in breast milk. C into human breast milk. *Acta Paediatr Scand*. 1987;76:453-6.
8. Hauer AC, Breese EJ, Walker-Smith JA, MacDonald TT. The frequency of cells secreting interferon-gamma and interleukin-4, -5, and -10 in the blood and duodenal mucosa of children with cow's milk hypersensitivity. *Pediatr Res*. 1997;42:629-38.
9. Hamada A, Kondoh T, Kamei T, Tomiyama N, Tsuru A, Matsumoto T, Matsuzaka T, Moriuchi H. Protein-losing enteropathy complicated with recurrent convulsions and developmental delay in a 4-month-old boy. *Pediatr Int*. 2002; 44:686-9.

Manuscript received June 21, 2007; accepted for publication August 20, 2007.

Toshiyuki Fukao

Department of Pediatrics
Graduate School of Medicine, Gifu University
Yanagido I-1, Gifu 501-1194, Japan
E-mail: toshi-gif@umin.net