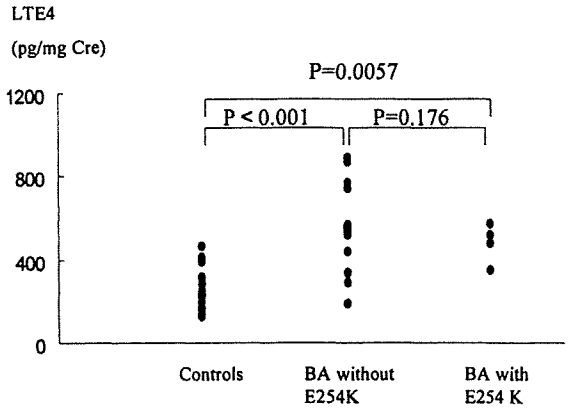

Figure 3. Urinary LTE4 production in individuals with bronchial asthma (BA) without E254K (n=13) and with E254K (n=3), and non-allergic subjects (n=14). The difference between individuals with bronchial asthma and non-allergic subjects was tested using a two-sample t-test. The urinary LTE4 productions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

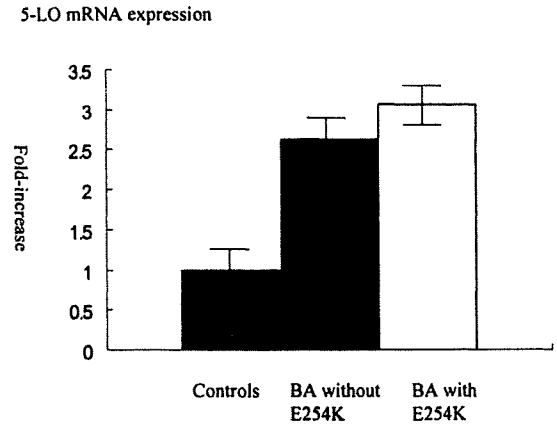

Figure 5. Relative expression of 5-lipoxygenase mRNA in individuals with bronchial asthma (BA) without E254K (n=13) and with E254K (n=3) and non-allergic subjects (n=14). The Y-axis on the left indicates the fold-increase compared to the mRNA expression in non-allergic subjects shown by the open bars.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

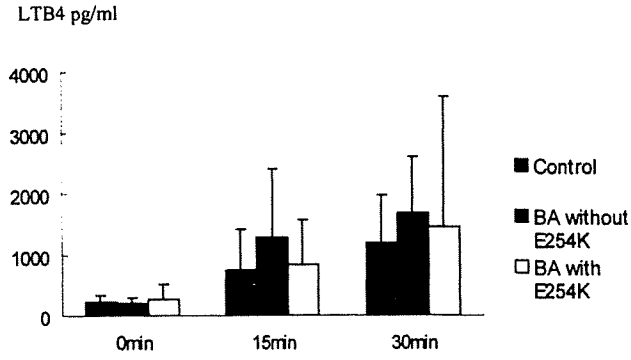

Figure 4. LTB4 production of ionomycin stimulation in neutrophils from individuals with bronchial asthma (BA) without E254K (n=13) and with E254K (n=3), and non-allergic subjects (n=14). The significance of difference between groups of LTB4 was tested using a two-sample-t-test. We detected no difference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

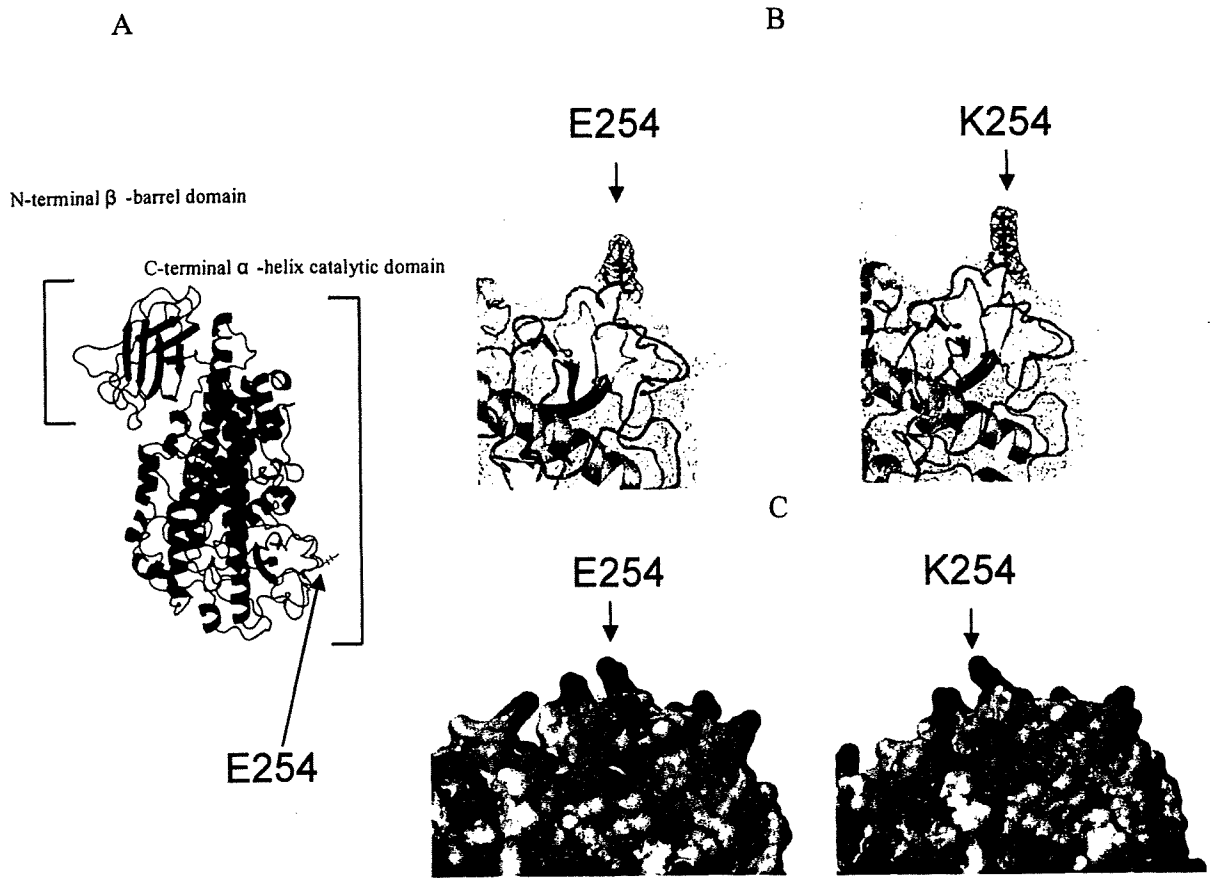

Figure 6. (A) Homology model structure of 5-lipoxygenase. The arrow indicates the part of the E254K polymorphism. (B) Comparison of the orientation of the side chains of glutamine acid 254 and lysine 254. (C) The focuses of the surface electrostatic potential of 5-lipoxygenase structures. Electrostatic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

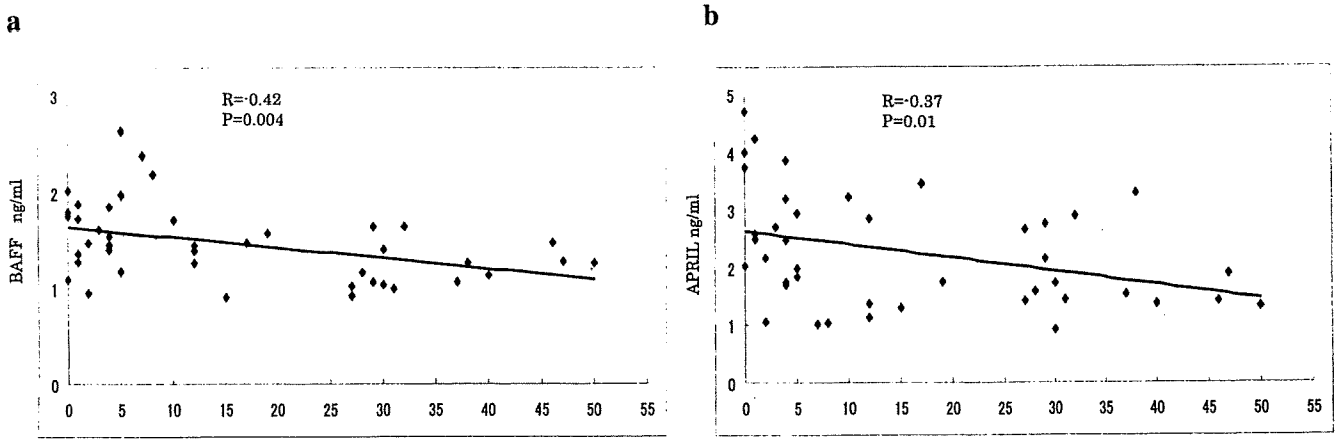

Figure 1. Inverse correlation of BAFF and APRIL plasma levels with age. BAFF and APRIL levels were determined in the same plasma samples (n=43). (a) Inverse correlation of BAFF plasma levels with age (R= -0.42, P=0.004); (b) Inverse correlation of APRIL plasma levels with age (R= -0.37, P=0.01).

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

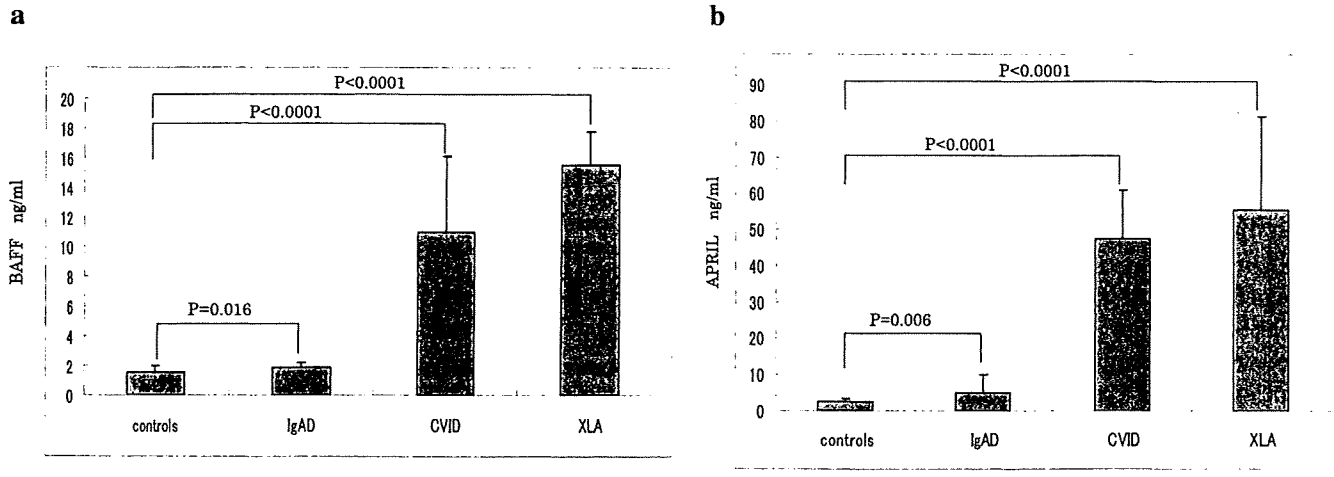

Figure 2. BAFF and APRIL protein expression levels of patients with IgAD, CVID and XLA. BAFF and APRIL levels were determined in the same plasma samples: plasma samples from 3 patients with CVID (mean age 12.3 years), 11 patients with IgAD (mean age 8.6 years), 4 patients with XLA (mean age 29 years)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

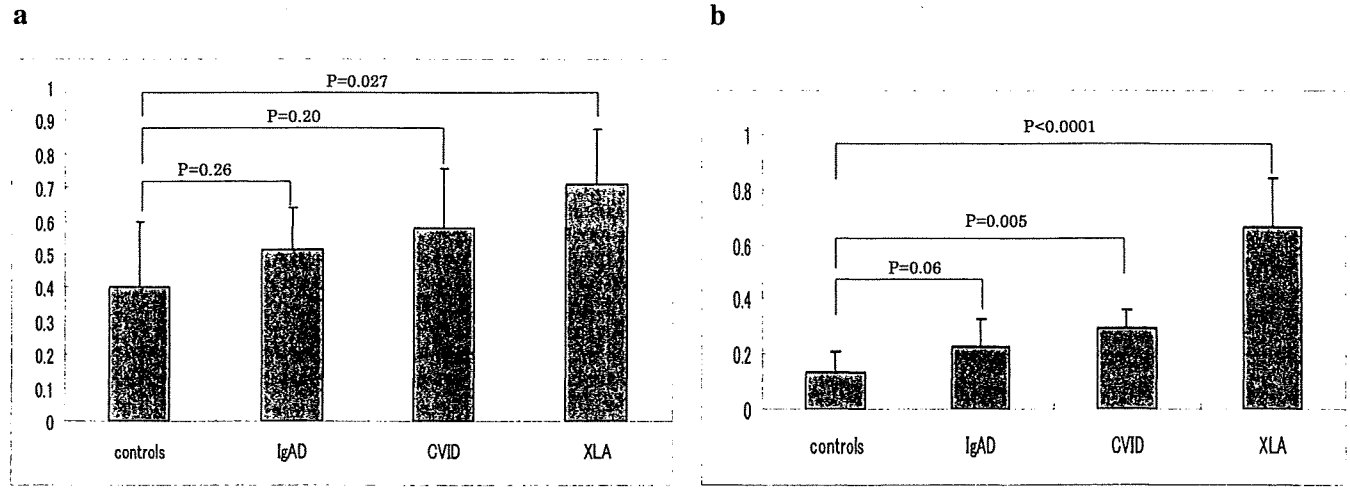

Figure 3. BAFF and APRIL gene expression levels in PBMCs of patients with CVID, IgAD and XLA. BAFF and APRIL gene expression levels of in 3 patients with CVID (mean age 12.3 years), 11 patients with IgAD (mean age 8.6 years), 4 patients with XLA (mean age 29 years) and 28 healthy children (control) (mean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

Acknowledgements

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特集 小児アレルギー疾患の Early Intervention ❹

小児喘息の成人へのキャリオーバーの予防

釣木澤 尚実* 秋山一男*

要旨

小児喘息の30～50%の多くが10歳代に自然治癒傾向を認めるが，一部は成人への持ち越し，成人期においての再発が認められる．思春期に症状が頻回に出現する症例はキャリオーバーの可能性が高い．成人へ持ち越した喘息は，成人発症，成人再発と比較して，吸入ステロイドの反応性やアセチルコリン気道過敏性正常化率も低い．小児喘息の多くは5歳未満で発症し，transient wheezer，non-atopic wheezer，persistent wheezingの状態から喘息への移行の予測が将来，より確実になり吸入ステロイドの早期導入が行われることで成人への持ち越し，予後が改善される可能性がある．

はじめに

筆者らは内科医であり日常臨床では成人喘息を診療しているため，小児喘息で成長とともにアウトグローした症例や小児期に喘息を発症し，思春期を介して成人へ持ち越した症例の全経過を診ることはできない．そのため結果として成人喘息のなかでの小児喘息の持ち越し(キャリオーバー)症例が，成人発症喘息や成人再発症例と比較してどのような臨床像を呈しているかについて当センターでの臨床成績を含めて紹介し，文献的考察を含めて，小児喘息の成人へのキャリオーバーの予防が可能なのかについて私見を述べることにする．

Ⅰ．喘息の発症年齢別分類

1994年に秋山[1]らが報告した成人気管支喘息の分類は発症年齢により，小児喘息が寛解せずに成人まで継続して続いている「小児発症成人移行型喘息」，成人になって初めて発症した「成人発症喘息」，小児喘息が一度寛解し，成人になってから再発した「成人再発喘息」，さらに思春期に発症した「思春期発症喘息」に分類される[1)~3)]．それに小児発症寛解型と小児発症思春期再発型を加え図1に示す．1992年の厚生省成人喘息調査研究班の秋山ら[2)]の報告では32施設の2,790例の年齢発症別の割合は小児発症成人喘息11.1%，成人再発型喘息3.7%，成人発症喘息77.7%であり，小児喘息を有したものは全体の14.8%に過ぎないことが明らかとなった．

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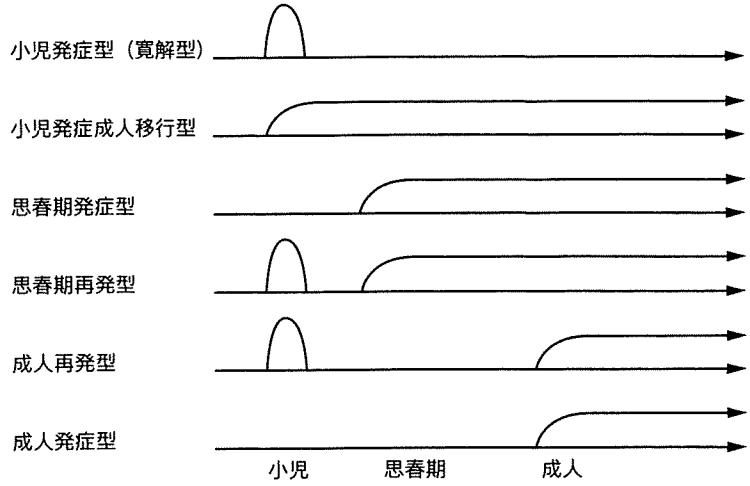

図1　喘息の発症年齢別病型分類

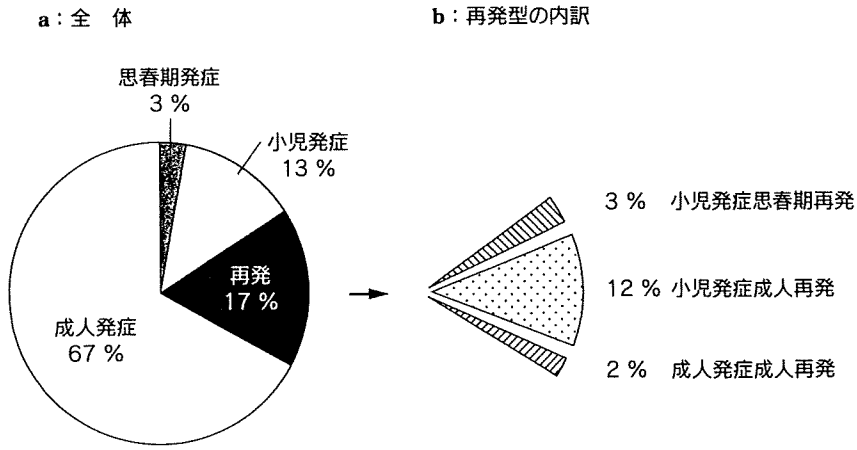

図2　成人喘息患者479例の発症年齢別分類

1999～2003年に当センターに外来通院中の成人喘息患者のなかで，複数回気道過敏性検査を受検した479例の成人喘息患者における発症年齢別の内訳を**図2**に示す[4]．小児発症13%，成人発症67%，成人再発17%，思春期発症3%であり（図2-a），成人喘息における小児発症の頻度は1992年の秋山の報告と近似していた．また成人再発型を3群に分類すると小児発症成人再発12%，小児発症思春期再発3%，成人発症成人再発2%であった（図2-b）．

また発症年齢別に病型を見てみると，今回の母集団の解析では小児発症，思春期発症はすべてアトピー型（**図3-a**）であり，再発型で81%，成人発症で56%がアトピー型であった．再発型では小児発症で成人期に再発する症例はアトピー型が多いのに対し，成人発症成人再発はアトピー型は約半数であった（**図3-b**）．

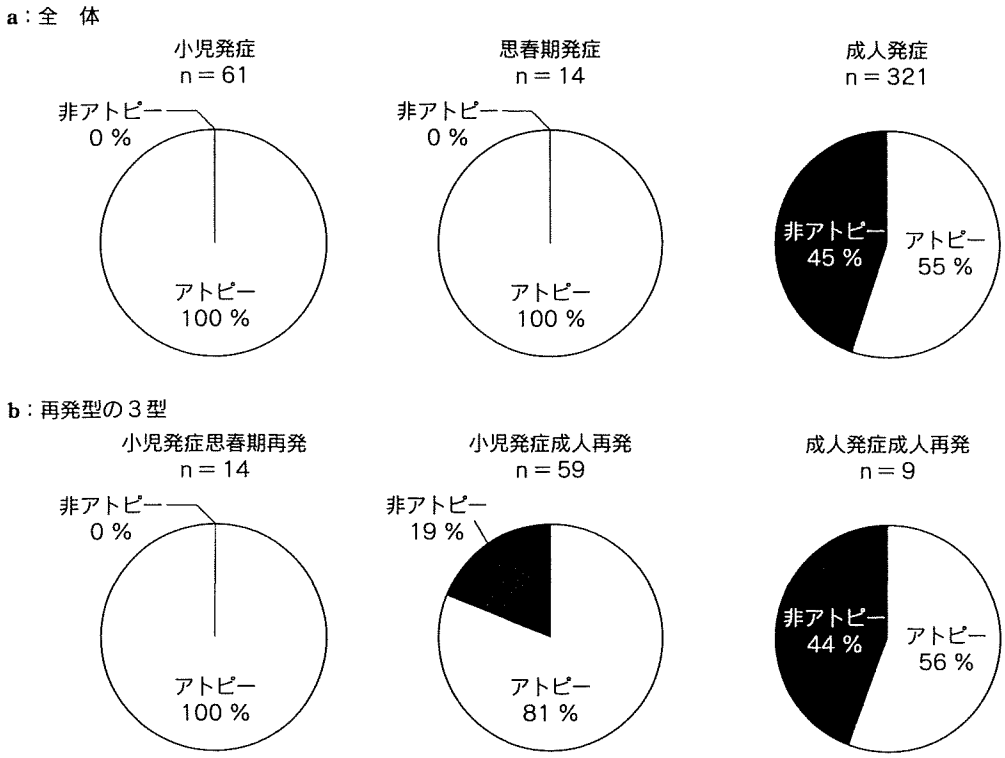

図3 成人喘息の病型分類

II. 小児喘息の予後

GINA（Global Initiative for Asthma）2002には小児喘息の30～50%がいったんは思春期に（特に男子）症状が消失するが，しばしば成人期に再発すること，小児喘息の2/3は思春期，成人期にも喘息症状を有していること，臨床的には喘息症状が落ち着いていても呼吸機能上の問題，すなわち閉塞性障害や気道過敏性の残存することや咳嗽が存在すると記載されている[5]．

小児喘息の予後についての疫学調査はすでに多くの報告がなされている．Strachanら[6]は7歳までに小児喘息，喘息様気管支炎と診断された880例を対象とし，7～33歳まで追跡し16～23歳の間に喘息症状を有していた症例は19%（寛解なし症例が8%，15歳までの再発症例が7%，16歳以降の再発症例が4%）であり，33歳での有症率は27%（寛解なし症例が5%，再発症例が15%，33歳時での再発症例が7%）であり，7～33歳までの完全寛解率は35%であると報告している（図4）．

Searsら[7]はある1年間で出生した児を3～26歳までprospectiveに追跡した613名を検討し，出生後まったく喘鳴を認めなかった児は27.4%，喘鳴が発症後26歳まで持続した症例が14.5%，喘鳴が一度は寛解した症例は27.4%，そのうち45.3%はその後26歳までに再発を認めたと報告している．さらに小児喘息の成人期への移行，成人期での再発のリスクは小児期のダニ，HD感作，気道過敏性の亢進，女性，21歳時の喫煙，発症年齢が低年齢であることなどであり，成人喘息への移行はすでに幼少期早期に

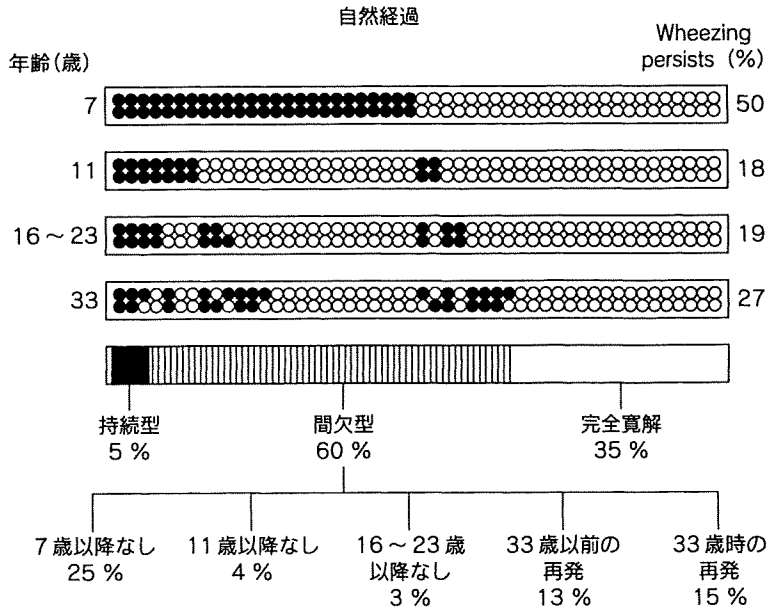

図4 7歳までに喘息または喘息様気管支炎と診断された小児の予後
●は前年度に喘息または喘息様気管支炎があった症例

決定されている可能性があると述べられている.

さらに本報告では9歳時の喘息の頻度は9%, 喘鳴は21.7%であるのに対し, 26歳時にはそれぞれ20.7%, 36.1%と成人期により高くなっており, Bronnimann ら[8]の小児期の喘息の寛解年齢が10歳代に多いという報告も併せて考えると, 寛解症例の再発症例の存在をうかがわせる結果となっている.

またToelle ら[9]は小児期から成人期への移行の可能性を検討し, 危険因子としてアトピー素因, 過去1年間の喘息症状の有無, 女性, 気道過敏性の残存, 閉塞性障害などが挙げられている. この要因のなかでも特に気道過敏性の残存, 閉塞性障害はmultivariate likelihood ratioが高値 (それぞれ2.56, 2.88) であることが示されている. Taylor ら[10]もまた小児喘息の予後を検討し, 15歳時の気道過敏性消失例からの再発症例は少ないと報告している.

III. 小児期から成人期における呼吸機能や気道過敏性の経年的変化

小児期に喘鳴, 気道過敏性を有する症例ではその後10年間の経過でFEV 1% (FEV 1/FVC) が経年的に低下する[11]という報告や, 7～42歳時までの経過を追跡し喘息, 重症喘息児は健常対照群, 喘息様気管支炎と比較して低値で推移するがその程度は経年的に変化がないという報告もある[12].

また気道過敏性に関しては, Vonk ら[13]の5～14歳の小児喘息119例の30年後(32～42歳時) を追跡した調査では, ICS (inhaled corticosteroid) を使用せず臨床症状が消失しているいわゆる臨床的寛解症例は51.7%存在し, そのうち%FEV 1が90%以下またはヒスタミン気道過敏性残存症例は30%存在し, さらに%FEV 1が90%以上, 気道過敏性が正常域である寛解症例は22%に存在していると報告している.

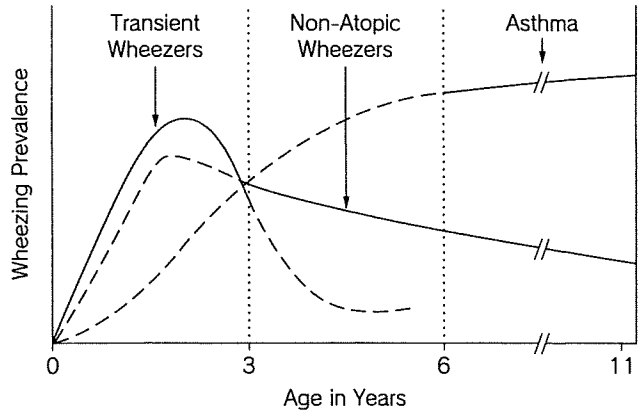

図5 乳幼児喘息の3つの異なるタイプ（仮説）

またGerritsenら[14]の101例の小児喘息を対象とした調査では20年後の臨床的寛解率は57%であり，そのうち93.1%がヒスタミン気道過敏性が消失していたと報告している．

前述したVonkら[13]の報告では，小児期の%FEV 1が高値である症例，初回時より21～33歳時での呼吸機能の改善がより良好である症例が，その後の完全寛解，臨床的寛解と関連していたと述べられている．

また徳山ら[15]は小児喘息患者の臨床的無症状の状態が続く患児において，β_2刺激薬吸入前後においてフローボリューム曲線の変化を吸入後のPEFあるいは$\dot{V}$50，$\dot{V}$25の改善度から4型に分類し，①無変化型（PEFあるいは$\dot{V}$50，$\dot{V}$25ともに改善率20%未満），②末梢気道閉塞改善型（PEFの改善率20%未満，$\dot{V}$50あるいは$\dot{V}$25の改善率が20%以上），③中枢気道閉塞改善型（PEFの改善率20%以上，$\dot{V}$50と$\dot{V}$25の改善率が20%未満），④全般改善型（PEFあるいは$\dot{V}$50，$\dot{V}$25ともに改善率20%以上）に分類し，②の末梢気道閉塞改善型では無変化型と比較してより重症であり，寛解症例が少ないと報告している．

IV. 小児喘息キャリオーバーへのリモデリングの関与

慢性的な気道炎症は気道リモデリング，すなわち気道上皮細胞の剝離，基底膜の肥厚，気道平滑筋の肥大や増生，気管支腺の増大を惹起，促進し，喘息の基本的な気道過敏性の機序として，また難治性喘息の成因の一つとして重視されている．リモデリングの研究の多くはその倫理性，技術面の困難さから主に成人喘息を対象としたものが多かったが，最近では小児においても研究され，Barbatoら[16]は小児喘息の病理所見では，軽症，中等症の段階からすでに基底膜の有意な線維化が認められると報告している．ICSを含めたステロイド薬が気道リモデリングの進展を抑制，阻止，改善が可能なのか，肯定的[17][18]，否定的[19]報告が両方存在し，現在のところ明確な結論は出ていない．

乳幼児の喘鳴には気道ウイルス感染により誘発されるtransient wheezer，non-atopic wheezer（late-onset wheezing），persistent wheezing（asthma）などがあり（図5[20]），初期には将来の喘息への移行を予測しがたいことも多い．Castro-Rodriguezら[21]は，3歳以下の喘鳴患児では親の喘息またはアトピー性皮膚炎のうち1

項目陽性，もしくはアレルギー性鼻炎，感冒以外の喘鳴，好酸球増多（4%）のうち2項目陽性に加え繰り返す喘鳴を生じる場合では76%，少なくとも一度の喘鳴を生じる場合では59%の確率をもって喘息への移行を予測できると報告している．これらの判定項目において判断に苦慮する場合もあるが，喘息への移行がある程度予測できればICSの早期導入が可能になり，将来のリモデリング抑制，阻止へ寄与するかもしれない．

Pauwelsら[22]は，7,165例の発症早期の喘息患者（うち5～10歳の小児喘息は27.6%，11～17歳の思春期喘息は17.0%）を対象とし，無作為二重盲検で低用量のbudesonideを使用し，3年後の経過で急性発作の減少，FEV 1%が改善すると報告し，early interventionの重要性を示している．

V．当センターでの成人喘息における小児発症の特徴

前述したように筆者らは内科医であるため，実際には成人喘息の診療を行っている．そこで成人から診た小児喘息，すなわちキャリオーバーした寛解なし群が成人発症や成人再発と比較してどのような特徴があるのかについて，当センターの日常臨床から得られた臨床成績を紹介し考察する．

当センターに外来通院中の成人喘息患者479例の発症年齢別の外来初診時期の気道過敏性を検討した[4]．小児発症（寛解なし）の発症年齢は平均6.4±4.3歳であるが，初診時年齢は平均28.9±13.3歳であり（**図6-a**），気道過敏性検査は成人期において施行されたものであり，その時点ですでに罹病期間が22.5±14.0年間存在することを表している．その結果ではアセチルコリン気道過敏性では思春期発症が4群間のなかでもっとも亢進していた．小児発症は成人発症より亢進していたが，成人再発とは同程度であった（**図6-b**）．ヒスタミン気道過敏性に関しては小児発症，思春期発症が成人発症，成人再発と比較して有意に亢進していた（**図6-c**）．

今回の結果からも小児発症，思春期発症がほぼアトピー型であり，成人発症では非アトピー型が半数近く占めることから（図3），すでに多くの報告で示されているように気道過敏性の亢進とアトピー素因が関連があることが示唆される．しかもこの結果はヒスタミン気道過敏性で特に顕著であった．また長期間の罹病期間により成人としての初診時の気道過敏性がより亢進していた可能性，さらに今回検討した症例がいわゆる小児期にはICSが普及されていない時代だったため，抗炎症薬によるearly interventionは行われていなかったことの可能性も考えられる．

VI．成人喘息のICSの反応性

当センターでは成人喘息を対象としICS療法を行い，日常臨床の一環として，気道過敏性の経過を追跡している．その臨床成績の一つとして過去にICS治療後の気道過敏性の治療前後での比（post $AchPC_{20}$/pre $AchPC_{20}$）について検討した[23]．その結果，ICSを使用しない場合は小児発症，成人再発，成人発症ともにアセチルコリン気道過敏性の有意な改善を認めなかった．またICS（主にCFC-BDP）を使用した場合では，成人発症および成人再発ではアセチルコリン気道過敏性が約7倍に改善したが小児発症では約3倍の改善であり，臨床的に有意な改善（臨床的な改善とは4倍以上の改善を意味する）を認めなかった．またヒスタミン気道過敏性については，成人発症，成人再発型および小児発症ともにICS治療後も有意な改善を認めなかった．

しかし最近ではFP-DPI，BUD，HFA-BDP

a：発症年齢と初診時年齢

	小児発症	思春期発症	成人再発	成人発症
発症年齢（歳）	6.4 ± 4.3	16.9 ± 1.5	37.1 ± 13.1	43.4 ± 13.6
初診時年齢（歳）	28.9 ± 13.3	31.4 ± 14.6	42.4 ± 14.1	47.6 ± 13.2

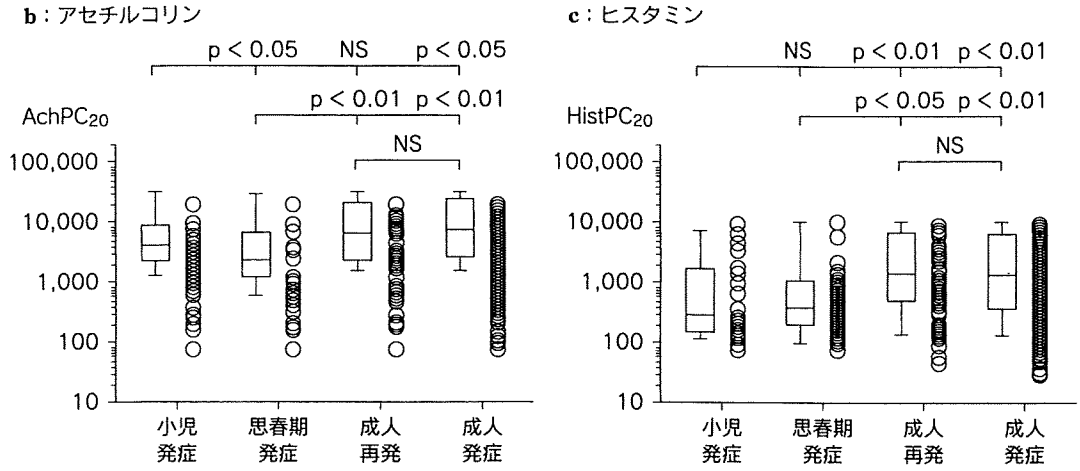

図6　発症年齢別の気道過敏性

などICSの質や吸入効率が改善され，臨床症状の改善だけでなく，アセチルコリン気道過敏性が正常域（すなわち $AchPC_{20}>20,000\ \mu g/ml$）まで改善する症例が増加した．そこでICSを使用しアセチルコリン気道過敏性が正常域まで改善した94例と治療後も気道過敏性が残存した110例（残存群：$AchPC_{20}<5,000\ \mu g/ml$）について発症別（小児発症，成人発症）に分けて背景因子を解析した[24]．

成人発症では治療後気道過敏性が正常域まで改善した群は過敏性残存群と比較して，初診時の肺機能(FEV 1%)が高値であり，初診時のアセチルコリン気道過敏性が軽度であり，発症からICS導入までの期間が短く，ICS一日使用量が多いことが明らかとなった（図7）．しかし，小児発症では初診時FEV 1%，発症からICS導入までの期間，ICS一日使用量は治療後気道過敏性正常域群と残存群では有意差を認めず，初診時のアセチルコリン気道過敏性が亢進しているほど気道過敏性が改善しにくいという結果であった．

この結果から成人発症では気流制限が軽度であり，気道過敏性が比較的軽度である症例ではICSの早期導入，十分量の使用によりアセチルコリン気道過敏性が正常域まで改善する症例も多いことを示すと同時に，小児発症では気道過敏性が正常域まで改善する，しないはICSの治療量，導入時期などに影響されないことを示唆している．

また治療後気道過敏性正常域群と残存群の喘息発症年齢型別の検討では，正常域群では成人発症が93%であり，小児発症は7%に過ぎなかった．しかし，過敏性残存群では小児発症は39%にも及んだ（図8）．これは成人喘息全体の小児発症の割合（19.2%）と比較してもかなり多いといえるであろう．小児発症では治療後も

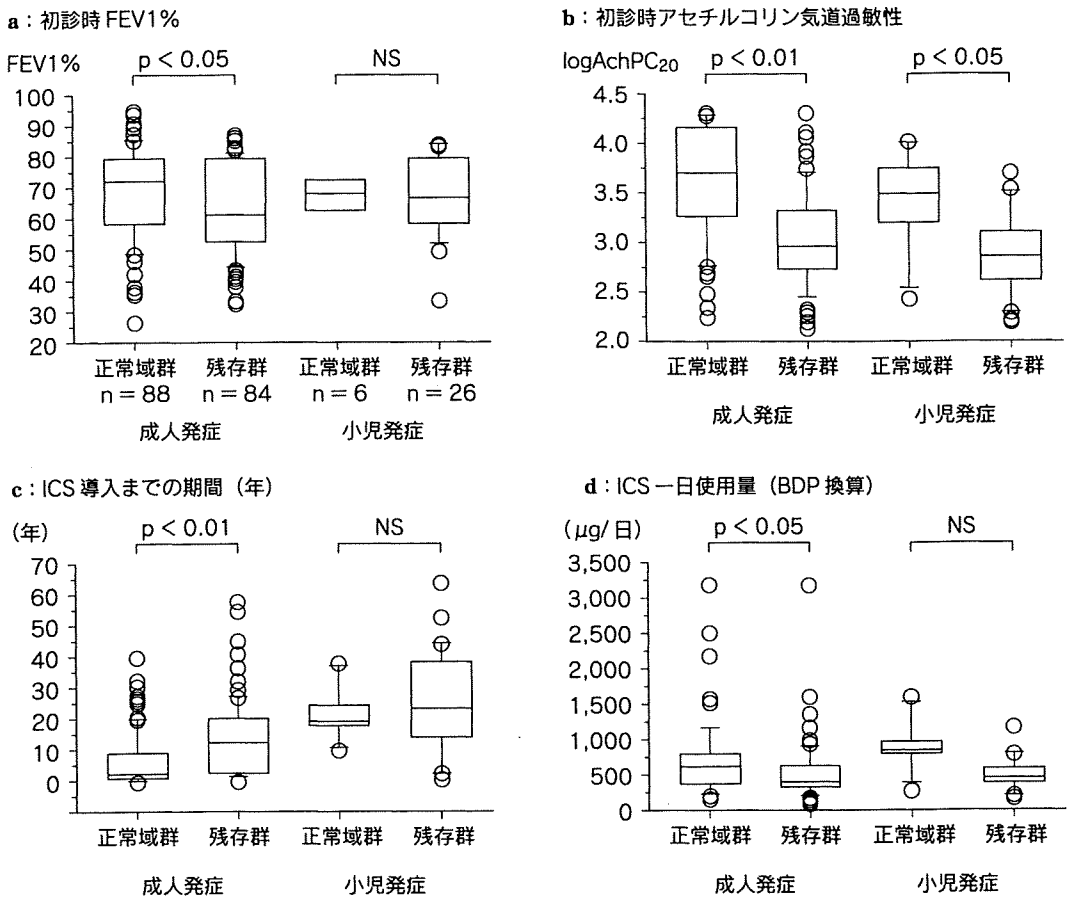

図7　治療後アセチルコリン気道過敏性正常域群と過敏性残存群の小児，成人発症別の差異

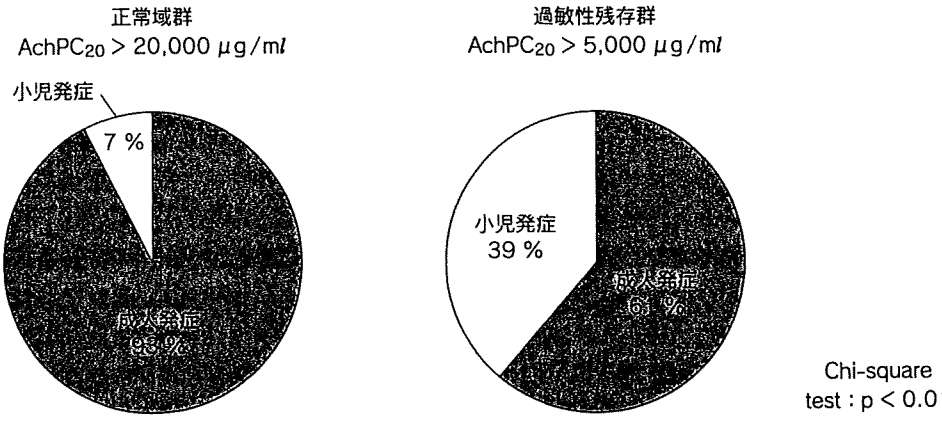

図8　治療後アセチルコリン気道過敏性正常域群と過敏性残存群の喘息発症年齢

気道過敏性が残存する症例が多く，アトピー素因の強さか，過敏性に対する遺伝性が背景に存在するのか，罹病期間の長さによるリモデリングによるのか，いまだに解明されていない．

Ⅶ. 小児喘息が成人へキャリオーバーするということとは

小児喘息が寛解する年代は10歳代が多いと考えられており[6]，その年代には当然思春期も含まれている．また成人に持ち越す小児喘息(小児発症寛解なし）も思春期を経過する．成人へキャリオーバーを考えるうえで思春期は非常に重要である．思春期喘息は難治性で死亡率も高いと考えられており，松井ら[25]の小児喘息発症22～35年後の長期予後調査では，思春期，若年成人に治癒する症例もあるが，思春期までに治癒しなかった症例では成人では中等症以上の重症が多くなると報告している．思春期では受診率，服薬コンプライアンスなど小児，成人と比較して治療内容が不十分であることも重症化の一因と考えられる．

当センターでは過去に小児発症成人喘息患者を対象とし，アンケート形式により思春期（中学生，高校生，19～20歳）における治療内容，臨床症状，小児期および現在からみた印象について調査した[26]．その結果，小児期からみた思春期の印象が寛解，改善と回答した症例は全体の50～60%であるのに対し，成人からみた思春期の印象が症状なし，よかったと回答した症例は34～42%であり，逆に悪かったと回答した症例は約44%存在した．この結果は小児期から思春期にかけて軽症化するものの症状が持続し，成人へと移行後もさらに軽症化する症例が存在することを示している．軽症化した時期にさらに十分量のICS治療が行われれば，治癒・寛解率も変化する可能性があるかもしれない．

Ⅷ. Early interventionはキャリオーバーを防げるか

これまでの数々の報告より小児喘息の成人期への移行に関する要因はアレルギー素因の有無，呼吸機能検査での閉塞性障害，気道過敏性の有無，臨床症状の頻回反復症例，女性であることなどが危険因子であることが示唆される．その多くはすでに遺伝的に，あるいは幼少期早期の環境によって定められているということになる．Waalkensら[27]はbudesonideで治療した小児喘息患者を対象とし，28～36カ月間の無症状期間を確認しても中止によって臨床症状の増悪を認めると報告し，CAMP study[28]では長期間の治療を行っても治療中止によってplacebo群と同等までに悪化するという報告もある．Warkeら[29]は臨床的寛解に至った小児喘息の気管支肺胞洗浄を行い，寛解症例においても好酸球増多を示す症例が存在することを報告している．

気道の好酸球性炎症の鎮静化が閉塞性障害の改善，さらには気道過敏性の消失へとつながるのであれば，症状が軽症化している小児喘息に対してのICS導入，診断できた時点での小児喘息に対してのearly interventionとしてのICS導入が，成人期への持ち越しを予防する可能性も十分に期待できるのではないかと考える．今後のprospectiveな研究が期待される．

おわりに

小児喘息の予後を考えるうえでは遺伝素因(アトピー，気道過敏性)，罹病期間，抗炎症薬としてのICS治療内容，治療効果など多岐にわたって検討する必要がある．transient wheezer，non-atopic wheezer，persistent wheezingから喘息への移行への予測が可能になり，ICSの早期導入が可能になり，治癒・寛解率が増加する可能性があると期待したい．

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