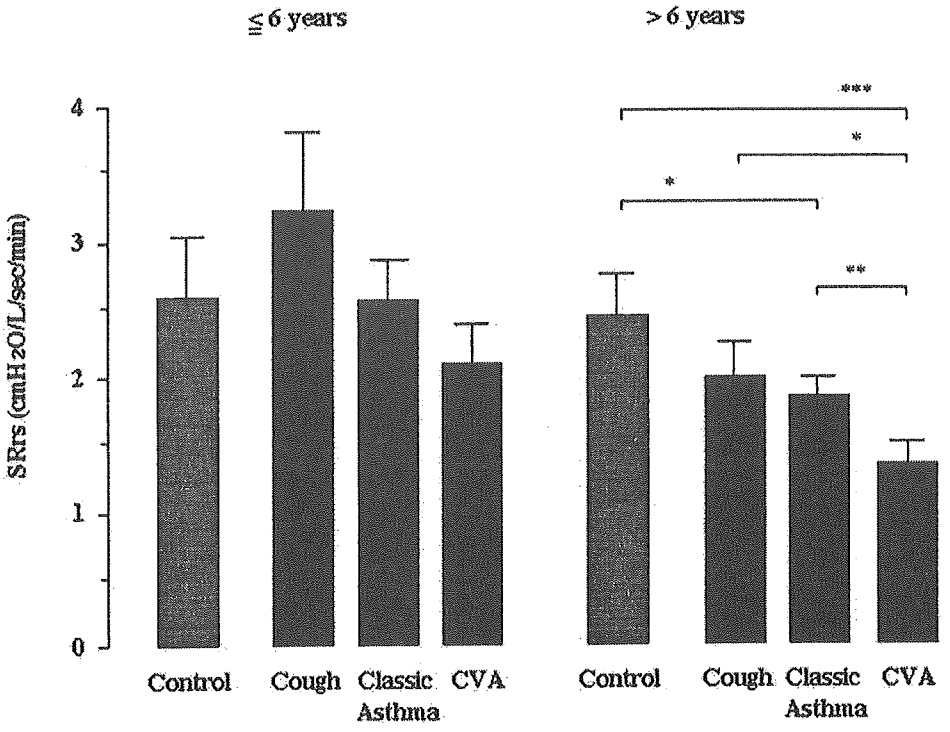

FIGURE 5. Comparison of SRrs between younger children and older children in the four groups. *p < 0.05, **p < 0.02, ***p < 0.01.

and BHR. One of the most common mechanisms, which exacerbates both BHR and hypersensitivity of cough receptors, is airway inflammation. CVA and classic asthma may appear to share a common pattern of inflammation in terms of both cellular infiltration and inflammatory cytokine gene expression. Airway inflammation has been detected in BAL fluid[33] and bronchial biopsies.[34] Also, CVA patients are responsive to inhaled corticosteroid[35] and leukotoriene antagonist.[36]

Niimi and coworkers[37] demonstrated that airway wall thickness was significantly greater in patients with asthma than in the control subjects and in patients with classic asthma than in those with CVA. Although it is not easy to make comparisons between adults with CVA and children with CVA, airway remodeling, which induces a decrease in SRrs[16] and which may also exist in children with CVA, induces a lower value of SRrs in patients with CVA than in control subjects. However, we cannot clearly explain the mechanism that induces the difference in the value of SRrs between classic asthma and CVA. Airway inflammation can induce persistent cough in children with CVA without outstanding wheezing and/or bronchoconstriction. The difference between CVA and classic asthma may depend on variations in cytokine production or cytokine sensitivity, which induce airway remodeling. Further investigation is needed to clarify this matter.

There was no difference in SRrs level between boys and girls or between younger children and older children. Some reports[13,38] have shown that gender has no significant effect on BHR; others[39] have shown that gender is not a determinant of bronchial responsiveness to methacholine in the overall population except in girls aged 11 to 15 years, who have a statistically higher mean methacholine area than their male counterparts. However, our data show that SRrs in the girls with CVA is significantly lower than in the girls with asthma. In the boys, this difference was not so clear. Also, SRrs in the older children with CVA is significantly lower than in the older children with asthma; this difference was not so significant in the younger children.

Although there is no statistical difference in the value of SRrs in the children with CVA between boys and girls, or between younger children and older children, we speculate that there may be a mechanism that induces low bronchial reactivity in older girls with CVA. Previous reports[40,41] have suggested that girls show an increased frequency of BHR compared to boys, and the specificity of adolescent girls, such as the effects of hormones and body size, has been discussed. Forastiere et al[42] reported a longitudinal study conducted to evaluate the determinants of the FEV_1-indicated bronchial responsiveness to methacholine in children and adolescents, and demonstrated that girls had greater bronchial

responsiveness than boys during the adolescent period. Considering these results, adolescent girls with asthma and control subjects may have a raised SRrs, so the lower SRrs in the girls with CVA may stand out. However, we cannot explain why only older girls with CVA have lower SRrs in this report.

Previously, Hills and Chen[43] proposed the unmasking of receptor sites secondary to disruption of the phospholipid barrier as a mechanism for the sensitization of reflexes. They suggested that various agents, especially infectious inflammation, can strip away subepithelial cough receptors, thereby increasing cough receptor sensitivity. Generally, airway infection is more common in infants, and the occurrence of respiratory tract infections declines with increasing age. These findings suggest that persistent cough occurs more frequently in infants than in older children. In this study, however, this pathologic weakness in infants does not explain the decrease of bronchial reactivity in childhood CVA.

Consequently, we have demonstrated that, rather than bronchial sensitivity, it is bronchial reactivity that significantly decreased in the children with CVA compared with the control subjects and the children with classic asthma, and that the decrease of bronchial reactivity induces either slower or less bronchoconstriction, resulting in the clinical feature of CVA: persistent cough without wheezing. Considering the factors that influence the degree of the slope of the response, not only airway remodeling but also smooth-muscle activity may have a potent effect on the slope of the response. However, in this report, we cannot define the mechanisms by which airway remodeling increases or airway smooth-muscle reactivity decreases; further investigation is needed.

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Bronchial Sensitivity and Bronchial Reactivity in Children With Cough Variant Asthma

Hiroyuki Mochizuki, Hirokazu Arakawa, Kenichi Tokuyama and Akihiro Morikawa

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ORIGINAL ARTICLE

IL-12B Promoter Polymorphism Associated with Asthma and *IL-12B* Transcriptional Activity

Koji Tatebayashi[1], Eiko Matsui[1], Hideo Kaneko[1], Toshiyuki Fukao[1], Kimiko Kasahara[1] and Naomi Kondo[1]

ABSTRACT

Background: The interleukin-12B gene (*IL-12B*) encodes the p40 chain of interleukin-12 (IL-12), which promotes cell-mediated Th1 responses and the production of interferon-gamma (IFN-γ) that downregulates IgE production. Chromosome 5q31-q33 near the *IL-12B* locus is significantly linked to asthma, as determined by a genome-wide search in the Japanese population.

Methods: We sequenced exons 1-8 including parts of the introns and promoter region of *IL-12B* in asthmatic patients and healthy controls. We examined plasma IL-12 concentrations, IL-12 production by Derf1-stimulated peripheral blood mononuclear cells (PBMCs) and the *IL-12B* transcriptional activity.

Results: *IL-12B* promoter polymorphism existed as $^{-2703}$CTCTAA/GC and $^{-2403}$T/C alleles, which were linked to each other. Homozygosity for haplotype 1 ($^{-2703}$CTCTAA /$^{-2403}$T) was associated with asthma susceptibility in Japanese children ($P < 0.001$). Both plasma IL-12 concentrations and IL-12 production by Derf1-stimulated PBMCs in the subjects with homozygotes for haplotype 1 were lower than those with homozygotes for haplotype 2 ($^{-2703}$GC /$^{-2403}$C) ($P < 0.001$). The transcriptional activity of the construct with haplotype 1 was lower than that of the construct with haplotype 2, and the *IL-12B* transcriptional activity was influenced by the $^{-2403}$T/C allele rather than by the $^{-2703}$CTCTAA/GC allele.

Conclusions: Homozygosity for haplotype 1, which is associated with reduced *IL-12B* transcriptional activity and reduced IL-12 production, is a predisposing factor for asthma susceptibility in Japanese children.

KEY WORDS

asthma, IgE, interferon-gamma, interleukin-12B promoter polymorphism

INTRODUCTION

Interleukin-12 (IL-12) is a heterodimeric molecule that is composed of two disulfide-linked subunits, p35 and p40. It is produced by macrophages, B cells and other antigen-presenting cells (APCs),[1,2] and plays important roles in interferon-gamma (IFN-γ) production by T cells and natural killer (NK) cells.

Genome-wide linkage screens, in which the genetic factors of the diseases can be identified, have been performed for asthma and recognized many regions linked to asthma.[3] Asthma is associated with Th2 cytokines, such as IL-4, IL-5, IL-9, IL-13, which are mapped to chromosome 5q31-q33. Polymorphisms of the IL-4 receptor α chain and IL-13 are associated with asthma.[4-6] Yokouchi *et al.* have reported significant evidence for linkage of asthma to 5 q31-33 near the *IL-12B* locus but not the IL-4 and IL-13 loci in the Japanese population.[7] Therefore, *IL-12B* is one of the candidate genes for asthma. Several polymorphisms have been identified in *IL-12B*,[8,9] including a single-nucleotide polymorphism in the 3' untranslated region, which has been associated with the susceptibility to type 1 diabetes and atopic dermatitis[10,11] but not to asthma and allergic rhinitis in the Japanese population.[12] Recently, it has been reported that the polymorphism exists in the *IL-12B* promoter region.[13,14]

In this study, we sequenced exons 1-8 including parts of the introns and region 3 kb upstream from

[1]Department of Pediatrics, Graduate School of Medicine, Gifu University, Gifu, Japan.
Correspondence: Koji Tatebayashi, Department of Pediatrics, Graduate School of Medicine, Gifu University, 1-1 Yanagido, Gifu, Gifu 501-1194, Japan.
Email: tachi@qj8.so-net.ne.jp
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Table 1 Sequence of oligonucleotides for PCR.

Primer	Sequence	Primer	Sequence	position
1S	5'-GAGAAGCATTCAGAAGCTCT-3'	1A	5'-GTCCCACTTCACAATCCAGA-3'	promoter
2S	5'-GTTTGTCAGCAGACCTTCCT-3'	2A	5'-GGAACAGGGCTCTGAATTGT-3'	promoter
3S	5'-GACAAGTGATTTCACTGCGG-3'	3A	5'-GGGCTAGTCCTATATGAAAG-3'	promoter
4S	5'-GGTATCCAGCTCTCTAACTC-3'	4A	5'-GACTTTGCCTTTTAGCCTTC-3'	promoter
5S	5'-GCAATCTGCTTTGTCCACTT-3'	5A	5'-GCTAAGAGGTATGCAAAGGT-3'	promoter
6S	5'-GCAGGTACATGTTCCTGTTC-3'	6A	5'-GGTTCTTCCCAAGTCAGAGA-3'	promoter
7S	5'-GCCAAGATGGGTGGTAAATA-3'	7A	5'-GAGGAGGGAACATAGACATC-3'	promoter
8S	5'-GCATCTCCATCTCCTTCCTT-3'	8A	5'-GCACACTAACGGTTTCTACA-3'	exon1
9S	5'-GGCTTAAAGGGGCCAAGT-3'	9A	5'-AGGGAGCACTATCCCTCAGC-3'	intron1-1
10S	5'-ATGTTATCTCATTGCCTTTC-3'	10A	5'-AAGTGGTTCTGAAACCACTG-3'	intron1-2
11S	5'-GTATCAGATGGCTTGCCTTA-3'	11A	5'-GTGCATGGTTGCCCATTTCA-3'	exon2
12S	5'-GGGAAGACTAAGCTCTACTG-3'	12A	5'-CAACGAACCAAGACTGTCAT-3'	intron2
13S	5'-GTCTTGTGCTGTTTGCAGTT-3'	13A	5'-GCATCTCCAAACTCTTTGAC-3'	exon3-1
14S	5'-GTGACACCCCTGAAGAAGAT-3'	14A	5'-GAGGCTAAGCATTCAGACTG-3'	exon3-2
15S	5'-GATAGTGTATCACTCTGCAC-3'	15A	5'-GCTGAGAAACCAGAGCAGTT-3'	exon4
16S	5'-TACTTCTGCTGACACCACTA-3'	16A	5'-GAACTAGGATCAAATTGTATAC-3'	intron4-1
17S	5'-GGTTACATAATCATATGTA-3'	17A	5'-GTTAGGATTTCAGGTGTGAG-3'	intron4-2
18S	5'-TCCAGAGACATGTAAGTGC-3'	18A	5'-GAGATGATGCTTGTCAACCA-3'	exon5
19S	5'-GCATCTCTCAGATCCTGCAA-3'	19A	5'-GCACCTGAATCACTTCTTAC-3'	exon6
20S	5'-GCTAGAAAGATGAAAGCTGG-3'	20A	5'-GTTTCTGATTCTGGCAACTG-3'	exon7
21S	5'-TAGCTCATCTTGGAGCGAAT-3'	21A	5'-GCTTGCCAGAGGCTTTCTTG-3'	intron7
22S	5'-GCAAGCTTGCAGGACTCAGA-3'	22A	5'-GATGGATCAGGTCATAAGAG-3'	exon8-1
23S	5'-GCCAGGATGTATGGAATGTT-3'	23A	5'-GACAGGGTCTCATTCTGTCA-3'	exon8-2
24S	5'-GCCTAGGTGACAGAATGAGA-3'	24A	5'-GCAAGCAGAGTACTCAAATC-3'	exon8-3

the transcriptional start site of *IL-12B* in 30 patients. Furthermore, we investigated *IL-12B* promoter polymorphism in 111 asthmatic patients and 78 controls, and examined the relationship between *IL-12B* polymorphism and asthma prevalence, IL-12 production and *IL-12B* transcriptional activity. We showed that *IL-12B* promoter polymorphism is associated with asthma and influences IL-12 production and *IL-12B* transcriptional activity.

METHODS

PATIENTS AND CONTROL SUBJECTS

One hundred and eleven asthmatic patients and 78 controls were enrolled in this study. Asthma was diagnosed on the basis of the American Thoracic Society guidelines. All the asthmatic patients showed total IgE levels above 200 IU/mL or specific sensitization to major allergens such as house dust and mite. The mean age ± SD of the asthmatic patients was 5.6 ± 2.9 years, and their mean IgE ± SD level was 906.8 ± 1347.4 IU/mL. All the controls had a negative history of atopic diseases. Their plasma IgE levels were lower than 150 IU/mL and their specific IgE levels were negative. The mean age ± SD of the controls was 4.7 ± 3.4 years, and their mean IgE ± SD level was 52.3 ± 52.4 IU/mL. Informed consent was obtained from all the subjects or their parents.

DETECTION OF *IL-12B* POLYMORPHISM

Genome DNA was extracted from neutrophils with a Sepa-gene kit (Sanko Junyaku, Tokyo, Japan). Exons 1-8 including parts of the introns and region 3 kb upstream from the transcriptional start site of *IL-12B* were amplified and sequenced using an ABI 3100 DNA sequencer (Applied Biosystems, CA, USA). We also sequenced previously identified polymorphisms in the introns.[9] The conditions for the PCR were 94 ℃ for 1 minute, 60℃ for 1 minute, and 72℃ for 1 minute. The primers used are shown in Table 1.

CELL PREPARATION

Peripheral blood mononuclear cells (PBMCs) were separated from the heparinized blood of the subjects by gradient centrifugation in Ficoll-Paque (Pharmacia, Uppsala, Sweden). PBMCs were suspended at a density of 1 × 10^6/mL in RPMI 1640 medium supplemented with 10% heat-inactivated fetal calf serum (FCS), 2 mmol/L L-glutamine, 100 U/mL penicillin and 100 μg/mL streptomycin.

CELL CULTURE

PBMCs (1 × 10^6/mL) were cultured in the presence or absence of 5 IU/mL recombinant human IL-12 (R &D Systems, Inc, Wiesbaden, Germany) or 5 μg/mL Derf1 (Asahi, Tokyo, Japan) for 24 hours in a final volume of 1 mL in a round-bottom tube (Falcon 2059,

Becton Deckinson Labware, Franklin Lakes, NJ, USA) at 37℃ in a humidified atmosphere containing 5% CO_2.[15]

ASSAYS FOR CYTOKINES

Plasma samples isolated from heparinized blood and the supernatants of the cell culture were stored at -30℃ until assay. IL-12 concentrations in the plasma and the supernatants of the Derf1-stimulated cell culture were measured with a human IL-12+p40 ELISA kit (Bio Source International, CA, USA) ; the minimum detection limit was 7.81 pg/mL. This ELISA kit recognized both natural and recombinant human IL-12, as well as the free p40 subunit. IFN-γ concentrations in the supernatants of the IL-12-stimulated cell culture were measured with a human IFN-γ ELISA kit (Ohtsuka, Tokyo, Japan) ; the minimum detection limit was 15.6 pg/mL.[16]

IgE ASSAY

Plasma IgE levels were determined by chemiluminescent enzyme immunoassay. Specific IgE antibodies for house dust, mite, cedar pollen, cow's milk, and hen's egg were measured by using a Uni-Cap assay kit (Pharmacia, Uppsala, Sweden). A specific IgE level above 3.5 IU/mL was considered positive.

CELL LINES AND REAGENTS

Mouse macrophagic cell line RAW264 (RIKEN Cell Bank, Tsukuba, Japan) was cultured in RPMI 1640 supplemented with 10% FCS, 2 mmol/L L-glutamine, 100 U/mL penicillin and 100 μg/mL streptomycin.

PLASMIDS FOR LUCIFERASE ASSAY

The -2808/-2303 distal enhancer region of *IL-12B* was obtained from genomic DNA by PCR amplification with the primers 5' -ttccggtcgACATGTTGATAA ACCTCTTCTCC-3' and 5' -ttgccggatcCGTAGCTCA CAAGGGGACATCAAAGATGAC-3' . The conditions for the PCR were 94℃ for 1 minute, 60℃ for 1 minute, and 72℃ for 1 minute. *Sal*I and *Bam*HI restriction endonuclease sites embedded in the PCR primers allowed the release of a 512-bp insert. The amplified PCR product was subcloned into the luciferase reporter plasmid PicaGene Promoter Vector 2 (PGV-P2 ; Toyo Ink Mfg. Co., Ltd., Tokyo, Japan). Furthermore, we constructed the mutant vector, which changed the T allele in the reporter vector with a 1.1 genotype into the C allele (mut-1). Circular DNA, which was the vector with a 1.1 genotype, was amplified using the sense primer 5' -GACAAGTGATTTCA CTGCGGGAAGACAATTCAGAGC-3' , and the antisense primer 5' -GCTCTGAATTGTCTTCCCGCAGT GAAATCACTTGTC-3' with native Pfu DNA polymerase (Stratagene, California, USA). These primers were oligonucleotides with one base mismatch (underline). The conditions for the PCR were 95℃ for 1 minute, 55℃ for 1 minute, and 68℃ for 10 minutes. The DNA sequence of PCR product was confirmed with an ABI 3100 DNA sequencer. We constructed another mutant vector, which changed the C allele in the reporter-vector with a 2.2 genotype into the T allele (mut-2) by the same method. The vector with a 2.2 genotype was amplified using the sense primer 5' -GACAAGTGATTTCACTGTGGGAAGACAATTCA GAGC-3' , and the anti-sense primer 5' -GCTCTGAAT TGTCTTCCCACAGTGAAATCACTTGTC-3' .

TRANSFECTION

The *IL-12B* promoter-reporter construct was transfected in RAW264. The cells were collected, resuspended at a concentration of 3×10^6 cells/10 ml in an 80 mm^2 flask, and incubated for 24 hours at 37℃ in a 5% CO_2 atmosphere. DNA (reporter construct 12 μg, CMV control vector 3 μg) was resuspended in 600 μL of OPTI-MEM (Gibco BRL, Grand Island, NY, USA) in a 15 mL Falcon tube, to which 15 μL of lipofectamine 2000 (Gibco BRL, Grand Island, NY, USA) was added ; the mixture was incubated for 20 minutes at 37℃ in a 5% CO_2 atmosphere. The cells were washed once, resuspended in 6 mL of DMEM (Gibco BRL, Grand Island, NY, USA) with 10% FCS and 600 μL of OPTI-MEM including the reporter construct in an 80 mm^2 flask, and incubated for 24 hours at 37℃ in a 5% CO_2 atmosphere. The cells were treated with recombinant murine IFN-γ (1,000 U/mL ; Wako Pure Chemical Industries, Ltd., Osaka, Japan) and 6 mL of the same medium for the stopping lipofectamine reaction for 24 hours before the addition of LPS (1 μg/mL) (Sigma, St. Louis, USA). After stimulation with LPS for 24 hours, cells were harvested and lysed by cell lysis buffer.[17] The lysates were used for luciferase assay with a PicaGene Dual SeaPansy Luminescence kit (Toyo Ink Mfg. Co., Ltd., Tokyo, Japan).

STATISTICAL ANALYSES

The significant difference between two groups was analyzed by the Mann-Whitney *U* test. The significant differences among three groups were analyzed by the Bonferroni/Dunn test. The frequency in *IL-12B* promoter polymorphism between the asthmatic patients and the controls was compared using the chi-square test for independence. Hardy-Weinberg equilibrium was determined by means of chi-square test for independence. Statistical significance was assumed for *p*-values less than 0.05.

RESULTS

DETECTION OF *IL-12B* POLYMORPHISMS

Exons 1-8 including parts of the introns and region 3 kb upstream from the transcriptional start site of *IL-12B* were amplified by PCR. Several polymorphisms have been reported in *IL-12B*.[8,9] We found three novel polymorphisms. The T/C allele (NP2) existed at -2403 from the transcriptional start site of *IL-12B*.

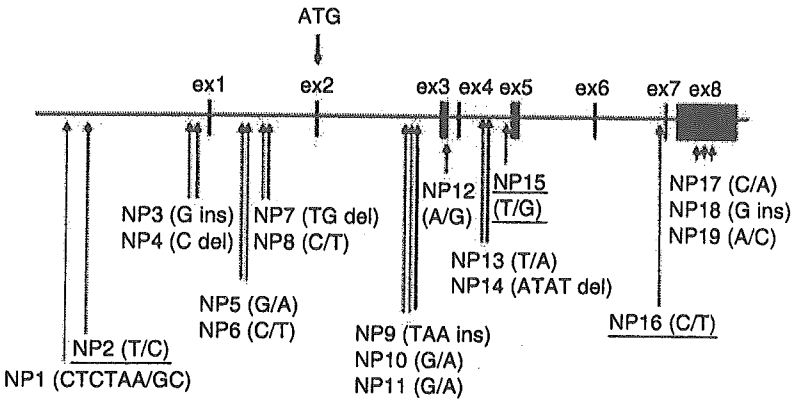

Fig. 1 Genomic organization of human *IL-12B*. By comparing the complete genomic sequence with the published cDNA sequence, the position of exons 1-8 and introns was deduced. Arrows indicate approximate positions of confirmed polymorphisms. NP2, 15 and 16 are novel polymorphisms (underlines). The other described NPs have been reported in the previous study. NP = nucleotide polymorphism

Table 2 Genotype frequencies of *IL-12B* promoter polymorphism in asthmatic patients and controls.

Genotype	Control (n=78)(%)	Asthmatic patient (n=111)(%)
1.1	15 (17.9%)	34 (30.6%)
1.2	34 (43.6%)	63 (56.8%)
2.2	29 (37.2%)	14 (12.6%)
P-value		<0.001

and the T/G (NP15) and C/T (NP16) alleles existed in introns 4 and 6 (Fig. 1). We investigated the frequency of *IL-12B* polymorphisms and plasma IL-12 concentrations in 30 subjects. Although the frequencies of all *IL-12B* polymorphisms were not significantly different between the allergic patients and the controls, plasma IL-12 concentrations in the subjects with the homozygotes for the $^{-2703}$CTCTAA/$^{-2403}$T allele (haplotype 1) were significantly different from those in the subjects with homozygotes for the $^{-2703}$GC/$^{-2403}$C allele (haplotype 2) in NP1 + 2 (data not shown). Therefore, we investigated that whether there is an association between NP1 + 2 and asthma, and whether NP1 + 2 affect IL-12 production.

ASSOCIATION BETWEEN *IL-12B* PROMOTER POLYMORPHISM AND ASTHMA

NP1 existed at -2703 from the transcriptional start site in *IL-12B*. We also identified NP2 at -2403 from the transcriptional start site, and this polymorphism was linked to NP1. We designated the homozygotes for haplotype 1, heterozygotes, and homozygotes for haplotype 2 as genotypes 1.1, 1.2, and 2.2, respectively. The frequencies of *IL-12B* promoter polymorphisms in 189 samples are shown in Table 2. Allele frequency of *IL-12B* promoter polymorphism did not deviate from expected Hardy-Weinberg equilibrium ($P > 0.1$). The frequency of a 1.1 genotype in the asthmatic patients was significantly higher than that in the controls ($P < 0.001$).

PLASMA IL-12 CONCENTRATIONS IN THE CONTROLS AND ASTHMATIC PATIENTS

As shown in Figure 2a, plasma IL-12 concentrations in the asthmatic patients were significantly lower than those in the controls. The mean ± SD of plasma IL-12 concentrations in the asthmatic patients and controls were 136.8 ± 62.0 pg/mL and 232.0 ± 84.0 pg/mL ($P < 0.001$).

ASSOCIATION BETWEEN *IL-12B* PROMOTER POLYMORPHISM AND PLASMA IL-12 CONCENTRATIONS

We examined plasma IL-12 concentrations in each genotype in both the controls and asthmatic patients. Plasma IL-12 concentrations in the subjects with a 1.1 genotype were 153.1 ± 70.0 pg/mL, which were significantly lower than those in the subjects with a 2.2 genotype (218.6 ± 85.1 pg/mL) ($P < 0.001$; Fig. 2b). Since reduced IL-12 production in the asthmatic patients might be affected by many factors such as some genetic effects and environmental factors, we also examined plasma IL-12 concentrations in each genotype only in the controls. Plasma IL-12 concentrations in the controls with a 1.1 genotype were 205.2 ± 63.8 pg/mL, which were significantly lower than those in the controls with a 2.2 genotype (255.8 ± 70.8 pg/mL) ($P < 0.05$; Fig. 2c).

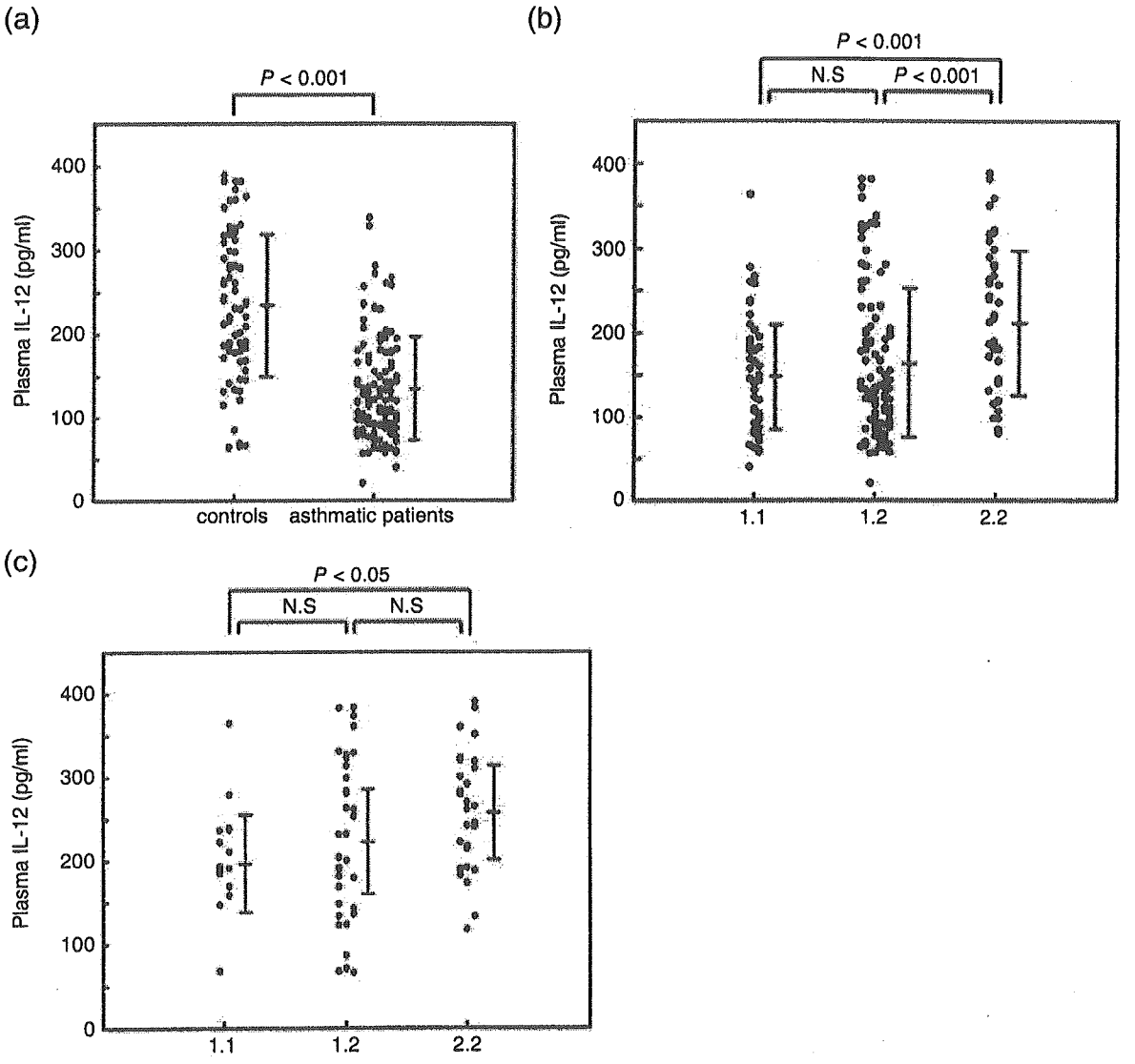

Fig. 2 (**a**) Plasma IL-12 concentrations in asthmatic patients and controls. The plasma IL-12 concentrations in the asthmatic patients were significantly lower than those in the controls (P<0.001). (**b**) Relationship between *IL-12B* promoter polymorphism and plasma IL-12 concentrations in both the controls and asthmatic patients (n=189). The plasma IL-12 concentrations in the subjects with a 1.1 genotype were significantly lower than those in the subjects with a 2.2 genotype (P<0.001). (**c**) Relationship between *IL-12B* promoter polymorphism and plasma IL-12 concentrations in the controls (n=78). The plasma IL-12 concentrations in the controls with a 1.1 genotype were significantly lower than those in the controls with a 2.2 genotype (P<0.05).

IL-12 PRODUCTION BY DERF1-STIMULATED PBMCS IN THE CONTROLS AND ASTHMATIC PATIENTS

Since most asthmatic patients had positive CAP-RAST scores for D farinae (Derf1), their PBMCs were cultured with the specific antigen Derf1 for 24 hours. IL-12 production by Derf1-stimulated PBMCs in the asthmatic patients (378.0 ± 271.4 pg/mL) was significantly lower than that in the controls (663.0 ± 364.2 pg/mL) (P < 0.001 ; Fig. 3a).

ASSOCIATION BETWEEN *IL-12B* PROMOTER POLYMORPHISM AND IL-12 PRODUCTION BY DERF1-STIMULATED PBMCS

We examined IL-12 production by Derf1-stimulated PBMCs in each genotype in both the controls and asthmatic patients. The subjects with a 1.1 genotype (347.2 ± 229.2 pg/mL) had a lower IL-12 production by Derf1-stimulated PBMCs than those with a 2.2 genotype (690.2 ± 331.1 pg/mL) (P < 0.001 ; Fig. 3b). In the controls, the subjects with a 1.1 genotype

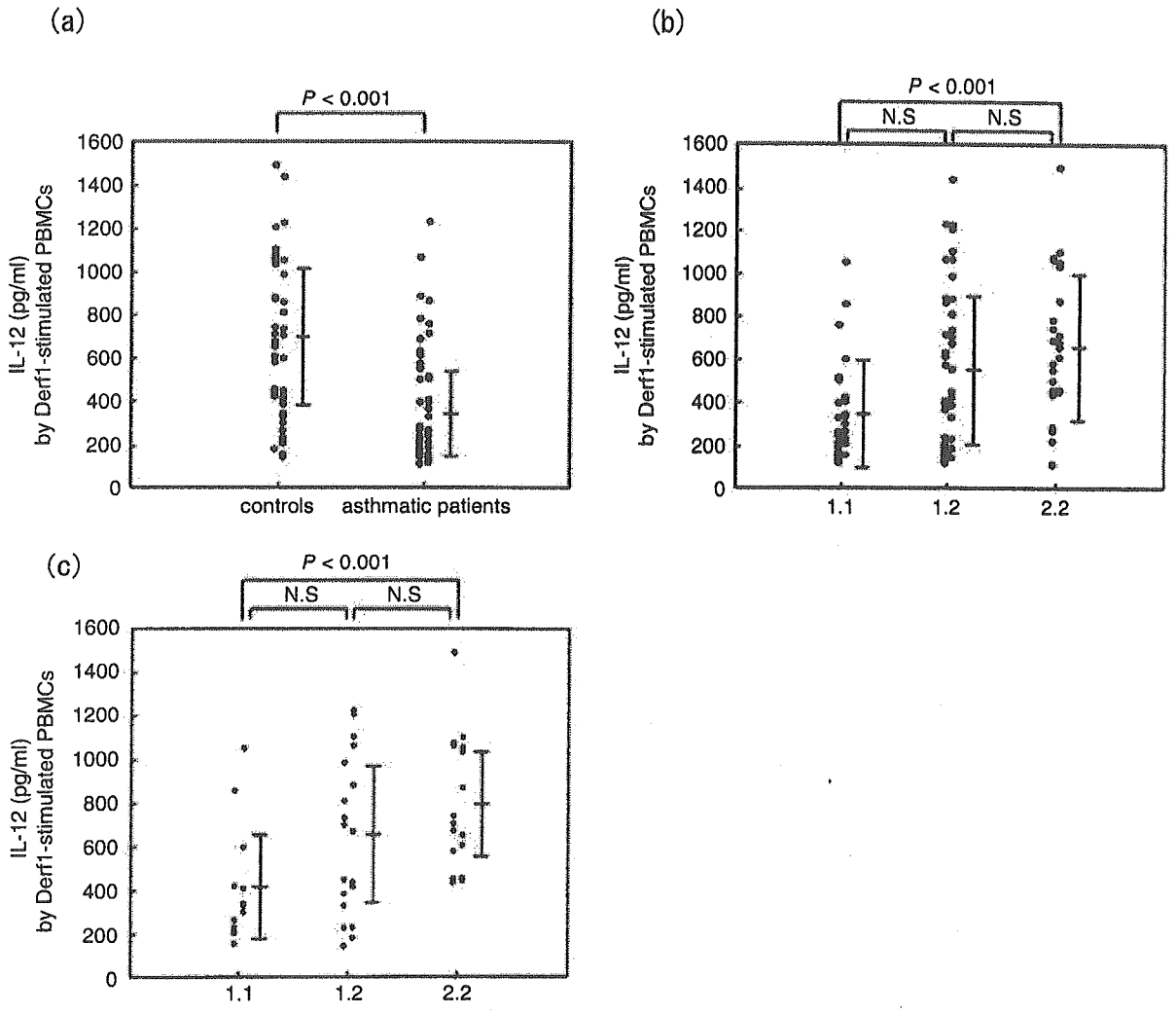

Fig. 3 (**a**) IL-12 production by Derf1-stimulated PBMCs in asthmatic patients and controls. The IL-12 production in the asthmatic patients was significantly lower than that in the controls ($P < 0.001$). (**b**) Relationship between *IL-12B* promoter polymorphism and IL-12 production by Derf1-stimulated PBMCs in both the controls and asthmatic patients ($n = 189$). The IL-12 production in the subjects with a 1.1 genotype was significantly lower than that in the subjects with a 2.2 genotype ($P < 0.001$). (**c**) Relationship between *IL-12B* promoter polymorphism and IL-12 production by Derf1-stimulated PBMCs in the controls ($n = 78$). The IL-12 production in the controls with a 1.1 genotype was significantly lower than that in the controls with a 2.2 genotype ($P < 0.001$).

(412.2 ± 315.0 pg/mL) had a lower IL-12 production by Derf1-stimulated PBMCs than those with a 2.2 genotype (807.2 ± 292.2 pg/mL) ($P < 0.001$; Fig. 3c). IL-12 production by Derf1-stimulated PBMCs positively correlated with IFN-γ production by IL-12-stimulated PBMCs (data not shown). Therefore, the subjects with a 1.1 genotype showed lower IL-12 and IFN-γ productions than those with a 2.2 genotype.

EFFECTS OF PROMOTER POLYMORPHISM ON PROMOTER ACTIVITY

To examine the functional activity of promoter polymorphism, we cloned the -2808/-2303 region of *IL-12B* into the PGV-P2 firefly luciferase reporter plasmid. The activity of the *IL-12B* promoter-reporter constructs was assessed in a transient transfection assay using RAW264 cells. As shown in Figure 4, the luciferase activity of the PGV-P2 plasmid with the *IL-12B* promoter-reporter constructs was significantly higher than that of the PGV-P2 plasmid only. A significantly lower luciferase activity was observed for haplotype 1 construct than for haplotype 2 construct (32.8% decrease ; $P = 0.0083$). Furthermore, we constructed mutant vectors with homozygotes for mut-1 (the CTCTAA/C allele) and mut-2 (the GC/T allele). The luciferase activity of the construct with mut-2 was

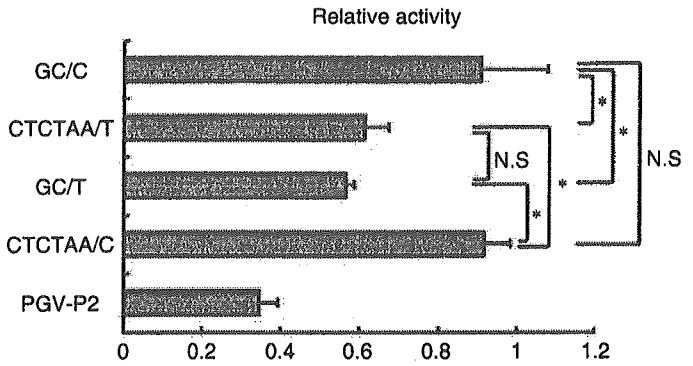

Fig. 4 Effects of *IL-12B* promoter polymorphism on promoter activity. RAW 264 cells were transfected by lipofectamine with reporter-constructs and stimulated with IFN-γ and LPS. To examine whether NP1 or NP2 affect the *IL-12B* transcriptional activity, mutated vectors (mut-1 and mut-2) were constructed. Luciferase activity was measured using the Dual-Luciferase reporter assay system. The presented results are mean ± SD from three independent experiments. * $P < 0.05$.

significantly lower than that of the construct with mut-1 and haplotype 2. This result indicates that *IL-12B* promoter polymorphism, that is, not the CTCTAA/GC allele but the T/C allele, has a major influence on the basal transcription rate of *IL-12B*.

DISCUSSION

IL-12 is a key mediator of immune responses. IL-12 is a heterodimeric molecule composed of two disulfide-linked subunits, a 35-kd subunit encoded by *IL-12A* on chromosome 3p12-q13.2 and a 40-kd subunit encoded by *IL-12B* on chromosome 5q31-q33.[18] A previous study has reported the association between *IL-12B* and asthma as determined by a genome-wide search.[7] We sequenced exons 1-8 and region 3 kb upstream from the transcriptional start site of *IL-12B*, and found three novel polymorphisms. In nineteen polymorphisms, NP1 + 2 that existed in the promoter region was shown to have a significant difference between the asthmatic patients and the controls in the genome frequency analysis. The frequencies of *IL-12B* polymorphisms in the coding region (NP3 and NP19) were not significantly different between the controls and asthmatic patients (data not shown). This result is similar to the result of Noguchi *et al.*[12]

Furthermore, the subjects with a 1.1 genotype had significantly lower plasma IL-12 concentrations and the lower IL-12 production by Derf 1-stimulated PBMCs than those with a 2.2 genotype. IL-12 plays an important role in the inhibition of Th2 cytokine production and the promotion of IFN-γ production by binding to plasma membrane receptors on activated T cells or NK cells.[2] IFN-γ inhibits IgE synthesis by human PBMCs *in vitro*.[19-22] Reduced IL-12 production and IL-12-dependent IFN-γ concentrations have been reported in asthmatic patients.[23-25] Therefore, IL-12 was shown to be associated with atopic dermatitis and asthma.[26-28] According to our result, *IL-12B* promoter polymorphism is likely to be associated with asthma prevalence by reducing IL-12 production.

Since the IL-12 levels measured during an exacerbation of asthma or bacterial/viral infection are analyzed in relation to the polymorphism, the functional consequences of the polymorphism cannot be fully explored. Hence, we investigated the functional activity of *IL-12B* promoter polymorphism. The transcriptional activity of the construct with a 1.1 genotype was lower than that of the construct with a 2.2 genotype. The transcriptional activity with mut-2 was significantly lower than that with mut-1 and haplotype 2. These results indicate that *IL-12B* promoter polymorphism, not NP1 but NP2, reduces the *IL-12B* transcriptional activity and IL-12 production. Since the NF-IL6 binding site in the *IL-12B* promoter region is shown to be in the -2405 to -2397 area of the 5'-upstream region, the T/C allele at this point may affect *IL-12B* transcriptional activity.[29] The transcriptional activity of the 296-bp construct from the transcriptional start site was reduced to half compared with that of the 3.3-kb construct.[17] This result shows that there are significant binding sites influencing the transcriptional activity from -296 to -3.3 kb of the transcriptional start site. The sequence near the T/C allele may be one of the binding sites affecting *IL-12B* transcriptional activity.

Morahan *et al.*[13] reported the association between asthma severity and a 4-bp microinsertion, which exists at a region 3 kb upstream from the transcriptional

start site of *IL-12B*. Since we identified only NP1 around the region 3 kb upstream from the transcriptional start site, the 4-bp microinsertion is likely to be NP1. According to their report, heterozygosity for *IL-12 B* promoter polymorphism is associated with asthma severity, reduced *IL-12B* transcription level and decreased IL-12 secretion. Inconsistent results may have occurred due to differences in methods and population. In another study, Morahan *et al.* also reported that a 1.1 genotype is associated with mortality from cerebral malaria and with reduced production of nitric oxide in Tanzanian children, and that the *IL-12B* mRNA expression in the subjects with a 1.1 genotype is lower than that in the subjects with a 2.2 genotype.[30] These results are consistent with our results.

Khoo *et al.*[14] have recently shown that *IL-12B* promoter polymorphism is not associated with asthma prevalence, but that there is an association was between 1.1 genotype and elevated serum IgE levels in male subjects, and reduced pulmonary function in female subjects in childhood. In our data, the subjects with a 1.1 genotype had high IgE levels, however, there was no correlation with sex (data not shown). The subjects with a 1.1 genotype had reduced IL-12 production and *IL-12B* transcriptional activity compared with those with a 2.2 genotype. Therefore, homozygosity for haplotype 1 may elevate serum IgE by reducing IL-12 production.

In conclusion, the frequency of homozygosity for haplotype 1 in asthmatic patients was significantly higher than that in controls. The subjects with a 1.1 genotype had reduced plasma IL-12 concentrations and IL-12 production by Derf1-stimulated PBMCs compared with those with a 2.2 genotype. The *IL-12B* transcriptional activity was reduced by the $^{-2403}$T allele, not by the $^{-2703}$CTCTAA allele. Hence, *IL-12B* promoter polymorphism ($^{-2403}$T/C) can be a risk factor for the development of asthma.

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ORIGINAL ARTICLE

Prevention of Asthma Exacerbation with Vaccination against Influenza in Winter Season

Shin Watanabe[1,2], Yoshiharu Hoshiyama[1], Satoshi Matsukura[2,3], Fumio Kokubu[2], Masatsugu Kurokawa[2], Hideki Kuga[2], Mio Kawaguchi[2], Yosuke Inoue[2], Koushi Ieki[2], Miho Odaka[2], Shintaro Suzuki[2], Mie Aoki[2,3], Tomoko Akabane[2], Hiroko Takeuchi[2], Mitsuru Adachi[2] and Takeshi Kawaguchi[1]

ABSTRACT

Background: Influenza virus is a major trigger of asthma exacerbation. Annual vaccination against influenza should be considered for asthmatics. However, the inoculation rates remain low. One reason may be the lack of evidence supporting the benefit of the vaccination for patients with asthma.

Methods: We studied 115 adults with asthma in 2002–2003 and 67 in 2001–2002 and evaluated the effect of influenza vaccination on the prevention of asthma exacerbation during the winter season (December–March).

Results: The rate of asthma exacerbation per person was significantly lower in asthmatics who received influenza vaccination (0.14 ± 0.4) during the 2002–2003 season than in asthmatics who did not (0.35 ± 0.61) (p = 0.037). During 2001–2002 season, the rate of asthma exacerbation in the vaccinated group (0.08 ± 0.41) appeared to be lower than that in the non-vaccinated group (0.27 ± 0.59). However, the difference between the two groups was not statistically significant (p = 0.143), presumably due to the low number of patients receiving vaccination. Severe adverse effects due to vaccination were not observed in the patients analyzed.

Conclusions: Influenza vaccination during the winter season appears to be effective in the prevention of asthma exacerbation and should be recommended for patients with asthma.

KEY WORDS

asthma, exacerbation, influenza, vaccination, viral Infection

INTRODUCTION

Respiratory infection can exacerbate asthma. Viral pathogens, such as human rhinovirus (HRV), coronavirus, influenza virus, parainfluenza virus, respiratory syncytial virus and adenovirus, are often responsible.[1-3] HRV is an important pathogen leading to asthma attacks. However, influenza epidemics in winter are also a threat. Podosin *et al.* reported asthma exacerbation induced by influenza virus, and since then evidence of the effects of influenza on asthma has been reported.[4,5] Infection with influenza virus renders asthmatics more susceptible to bronchoconstriction, exacerbation of asthma, and prolonged declines in lung function.[6] Influenza-induced severe asthma attacks and hospitalization are associated with needless deaths, particularly in the elderly.[7-9]

Among of the viruses that cause airway infection, influenza is the one for which vaccinations are established and available. The efficacy of influenza vaccination in the prevention of infection has been reported.[10-12] Recent reports support the safety of influenza vaccine for asthmatics.[13,14] However, the numbers of asthmatics that received annual influenza vaccine are reportedly very few.[15] One reason may be that physicians and patients are not aware of the benefit of the influenza vaccine for asthma. Several reviews have indicated that the benefit of influenza vaccination in patients with asthma is modest or unclear.[16,17] There are few studies on the prevention of

[1]Department of Public Health, [2]First Department of Internal Medicine, Showa University School of Medicine, Tokyo and [3]Internal Medicine, Ohta-Fukushima General Hospital, Gumma, Japan.
Correspondence: Shin Watanabe M.D., First Department of Internal Medicine, Showa University School of Medicine, 1-5-8 Hatanodai, Shinagawa-ku, Tokyo 142-8666, Japan.
Email: yphww993@ybb.ne.jp
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Table 1 Characteristics of Subjects

	2002/2003 season	
	Vaccinated Subject	Not-vaccinated Subjects
Number of subjects (%)	57 (49.6%)	58 (50.4%)
Age	62.3 (24-89)	51.1 (15-84)
Male/Female	16/41	27/31
Number of Asthma type		
Allergic	21	20
Non-allergic	36	38
Severity		
Intermittent	1 (1.8%)	0 (0%)
mild persistent	5 (8.8%)	8 (13.8%)
moderate persistent	25 (43.9%)	24 (41.4%)
severe persistent	26 (45.6%)	26 (44.8%)

	2001/2002 season	
	Vaccinated Subject	Not-vaccinated Subjects
Number of subjects (%)	24 (35.8%)	43 (64.2%)
Age	64.3 (31-89)	52.0 (15-84)
Male/Female	8/16	16/27
Number of Asthma type		
Allergic	10	15
Non-allergic	14	28
Severity		
Intermittent	1 (4.2%)	0 (0%)
mild persistent	3 (12.5%)	5 (11.6%)
moderate persistent	8 (33.3%)	13 (30.2%)
severe persistent	12 (50.0%)	25 (58.1%)

asthma exacerbation by influenza vaccination are few.[18] We studied the effect of influenza vaccination on asthma and investigated whether influenza vaccination could prevent asthma exacerbation during the winter season in Japan.

METHODS

We studied 115 adult patients with asthma (age 15–89 years) in 2002–2003 and 67 (age 15–89) in 2001–2002. Asthma was defined as recurrent episodes of airway obstruction that resolved on treatment. Diagnosis was made by a clinical specialist with follow-up every 14–28 days at the Respiratory Division of Showa University Hospital or Ohta-Fukushima General Hospital. Most of the patients self-checked their peak expiratory flow (PEF) and self-recorded their symptoms. Patients who also had chronic obstructive pulmonary disease (COPD) were excluded. Among these patients, we studied all who received influenza vaccination from November to December in 2001 or 2002 and those with similar characteristics who did not receive vaccination were chosen randomly. We excluded patients who might have received vaccination at other hospitals. Then we retrospectively analyzed the data.

We evaluated "influenza-like illness" symptoms associated with influenza infection according to patients' symptoms using a scoring method.[3,5,11] Each symptom, such as sore throat or rhinorrhea, fever, general fatigue, cough, and sudden onset, was assigned one point, and a total of three or more points were defined as influenza-like illness. Some patients who were evaluated as influenza-like illness were positively diagnosed with influenza using an influenza antigen detection kit. However we could not check all patients using this kit, therefore the symptom score was used. Asthma attacks accompanied by wheezing or a decrease of more than 20% in PEF was defined as exacerbation. Patients with constant wheezing were excluded from this study. Exacerbations of asthma were evaluated by specialists according to the guidelines of the Japanese Society of Allergology.

To investigate the effect of influenza vaccination on the prevention of asthma exacerbation, we followed patients from December 1 to March 31 because these four months comprise the epidemic season of influenza in Japan. The number of exacerbation of asthma accompanied by influenza-like illness were calculated

Table 2 Effect of Influenza vaccination on the asthma exacerbations

	2002/2003 season		
	Vaccinated Subject	Not-vaccinated Subject	*p* Value
Person	57	58	
No. of exacerbations	8	20	
Mean±S.D.	0.14±0.4	0.35±0.61	0.037
	2001/2002 season		
	Vaccinated Subject	Not-vaccinated Subject	*p* Value
Person	24	43	
No. of exacerbations	2	12	
Mean±S.D.	0.08±0.41	0.27±0.59	0.143

Table 3 Effect of Influenza vaccination on the exacerbations of asthma who were controlled within Green-Zone before winter season in 2002/2003.

	Vaccinated Subjects	Not-vaccinated Subjects	*p* Value
Patients controlled within Green-zone	32	31	
No. of exacerbations	2	10	
Mean±S.D.	0.06±0.25	0.32±0.65	0.049

per person. Data analysis was performed with the use of Stat-View IV (Abacus Concepts, Inc., Berkeley, CA, USA). The data are expressed as mean ± standard deviation (SD). Statistical differences were determined by analysis of variance with Fisher protected least significant difference.

RESULTS

Characteristics of the study population are shown in Table 1. The severity of asthma was assessed by the use of daily medications according to the guidelines of the Global Initiative for Asthma (GINA). The characteristics were similar between the group that received influenza vaccination and the group that did not.

In the 2002–2003 season, the number of asthma exacerbations per person accompanied by influenza-like illness was significantly lower in the group with vaccination (0.14 ± 0.4, mean ± SD) than in the group without vaccination (0.35 ± 0.61) ($p = 0.037$). In the 2001–2002 season, the number of asthma exacerbations per person accompanied by influenza-like illness tended to be lower in the vaccination group, but the difference was not statistically significant ($p = 0.143$) (Table 2).

To minimize the effects of factors other than respiratory infection triggering exacerbations, we also assigned some patients to the green zone group which included patients whose asthma was stably controlled (PEF was kept within 80% of the highest value) for 3 months before the winter season. In the green zone group, the number of asthma exacerbations per person was lower in those patients receiving vaccination (0.06 ± 0.25) than in those not receiving vaccination (0.32 ± 0.65) ($p = 0.049$) during the 2002–2003 season (Table 3).

DISCUSSION

In this study, we showed that influenza vaccination could reduce the number of asthma exacerbations caused by influenza-like illness during the winter season.

In Japan, vaccination rates have decreased since the law mandating vaccination of school children in 1994. Needless deaths caused by influenza infection have increased since then,[19] and the Japanese government has attempted to increase the rate of influenza vaccination again. The government amended the law and provided influenza vaccination financial support for the elderly in 2001. Vaccination rates have increased since 2001, but they remain low compared to those in Western countries. Therefore, the number of patients studied in the 2001–2002 season was less than that in 2002–2003. The lack of statistically significant effects of vaccination in the 2001–2002 season may be due to insufficient numbers of patients. According to National Institute of Infectious Disease, the Number of Influenza infection in 2001–2002 was relatively lower than that in 2002–2003. The difference of prevalence between the seasons may also influence the results.

The influenza season in Japan is from December through March. We did not detect statistically significant effects of the influenza vaccine when asthma exacerbation triggered by factors other than influenza-like illness were included (data not shown). However,

influenza vaccination clearly prevented asthma exacerbation that occurred when symptoms of influenza-like illness were present. These findings indicate that influenza infection may be just one factor that triggers asthma attacks, and other factors, such as cold air, changes in weather, stress, pollen, or antigens, may also be important. The effect of influenza vaccination in the prevention of total exacerbations of asthma appears to be minor. However, influenza infection can be prevented or its virulence may be weakened by immunization. Therefore, influenza infection should be given serious consideration, and its prevention by vaccination should be recommended for patients with asthma.

In our study, even patients in green zone group had asthma attacks in the presence of influenza-like illness or respiratory infection. Influenza vaccination was effective in preventing asthma exacerbation in these patients. Recent therapies for asthma have been improved, and medications such as inhaled corticosteroids are standard treatments. However, our data indicate that prevention of viral infection or minimization of virulence could improve the control of asthma.

Although GINA recommends influenza vaccination for asthmatics, the number of asthmatics receiving vaccination is low. One reason may be that physicians and patients doubt the safety of the influenza vaccine. Previous reports indicated that influenza vaccination could cause a decrease in PEF.[17,18] However, recent well designed and placebo-controlled trials showed the safety of the inactivated split-virus influenza vaccine, which is commonly used in Japan. The American Lung Association Asthma Clinical Research Centers studied 2032 patients with asthma and reported that the frequency of exacerbation of asthma was similar between groups injected with the influenza vaccine or with placebo.[14] A previous report, which studied a smaller number of asthmatics, indicated that pulmonary function abnormalities might occur within 3 days of influenza vaccination. However, the authors suggested that this effect was minor and outweighed by the advantages of vaccination.[13] We also did not detect prominent systemic side effects or asthma exacerbation in patients who received influenza vaccination (data not shown).

Further study is necessary to determine the mechanisms by which influenza vaccination prevents asthma exacerbation . Several mechanisms can be hypothesized. First, influenza vaccination may prevent influenza infection, resulting in reduced triggers of asthma exacerbation. In this study, we could not compare the rates of prevention of influenza infection between asthmatics who received and those who did not receive vaccination. However, it has been reported that the efficacy of the influenza vaccine in the prevention of infection is approximately 60%–80%.[10-12] Second, influenza vaccination may interfere with cytokine production induced by influenza infection. Influenza virus is known to induce several kinds of cytokines that are known to cause airway inflammation, including asthma exacerbation.[20-22] Several investigators have reported that the influenza vaccination reduces the production of cytokines, such as TNFα, IL-1, IL-6, and IL-8, which may be induced by influenza infection. In those in whom influenza vaccine does not prevent infection, it may decrease cytokine production and minimize airway symptoms, including asthma attacks.[23,24]

Taken together, these data indicate that vaccination against influenza is beneficial for asthmatics. Even though it only protects against influenza-induced symptoms, vaccination may be clinically important and should be recommended. More efforts to increase the vaccination rates of asthmatics will likely improve the control of asthma overall.

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REVIEW ARTICLE

Clinical Reality of Asthma Death and Near-fatal Cases, in a Department of Pediatrics of a Japanese Chest Hospital

Hiroshi Odajima[1] and Ken Nishio[2]

ABSTRACT
Asthmatic deaths have shown a tendency to decrease in National Fukuoka Hospital. According to our analysis of asthma death and near-fatal asthma, the risk factor for death was considered as follows : low compliance, underestimation of severity (by patient or family), becoming used to attacks through experiencing many attacks, insufficient knowledge about attacks, overuse of β-stimulant metered-dose inhaler, etc. One of the reasons for the decrease in asthma death was thought to be the regular usage of many anti-asthmatic drugs especially inhaled corticosteroid.

KEY WORDS
asthma death, bronchial asthma, inhaled corticosteroid, near-fatal case, puberty, β-agonist

INTRODUCTION

The most important thing in the treatment of childhood asthma is to prevent asthma death. Although recently developed anti-asthma drugs help many patients control their symptoms, some patients still died from asthma attacks or experience near-fatal events. In this paper, we reported the clinical reality concerning asthma deaths and near-fatal cases in our hospital, in an attempt to learn methods of preventing death from asthma.

CLINICAL REALITY OF ASTHMA DEATH IN OUR HOSPITAL

The National Fukuoka Hospital is located in Fukuoka City, in Kyushu, in the south-west of Japan. In the last year, about 1200 cases of asthmatic attack patients were admitted to pediatric wards which contain 50 beds for children. 25,000 outpatients a year consulted about asthma. We have 10 pediatricians, with a 24-hour emergency asthmatic attack service.

Since the pediatric wards opened in 1975, there have been 22 cases of death of asthmatic patients. We retrospectively classified the time, place and cause of death. Table 1 gives the background of these cases. Delay in visiting the outpatient clinic was considered one of the reasons for death in 11/22 cases.

Before 1988, there were no deaths in patients aged 17 or more, but after 1988 there was a tendency for death to occur in patients aged 17 or more (Fig. 1, upper graph).

We held a summer camp for out-patients with asthma. In the camp, the drugs used by patients were closely examined (Fig. 1, lower graph). After 1988, there was an increasing tendency throughout Japan to use prescribed drugs, especially inhaled corticosteroids (ICS) and round the clock therapy of bronchodilators (theophylline and β_2 agonist) and leukotriene receptor antagonist.[1,2] There was also an increasing tendency of more frequent deaths in cars, after 1988 (Fig. 2). Figure 3 shows asthma deaths in relation to calendar months, and there were no deaths from November through April.

Therefore we should pay attention to severe cases of attack from May to October. We examined the number of the patients visiting our emergency room (ER), and hospitalized via the ER in 1992 and 2002 (Fig. 4).

[1]Department of Pediatrics, National Fukuoka Hospital and [2]Department of Pediatrics, Fukuoka University, Fukuoka, Japan.
Correspondence: Hiroshi Odajima, M.D., Ph.D. Department of Pediatrics, National Fukuoka Hospital, 4-39-1 Yakatabaru, Minami-ku, Fukuoka City, Fukuoka 811-1394, Japan.
Email: odajih@mqc.biglobe.ne.jp
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Table 1 Asthmatic Deaths in the Patients treated at our Hospital (JAN.1975 ~)

Case		No.	Sex	Age (yrs)	Date of death	Place of death
	‡	1	f	3	'75. 10. 7	Hospital
†		2	f	11	'76. 7. 26	Home (entrance)
	‡	3	m	5	'77. 9. 28	Hospital
†		4	m	14	'77. 10. 29	Privately-owned car
†		5	f	12	'78. 10. 30	Home
†		6	m	13	'80. 8. 24	Home
		7	f	5	'80. 9. 3	Home
†		8	m	15	'81. 9. 13	Home (lavatory)
†	‡	9	f	16	'84. 5. 25	Taxi
		10	m	10	'85. 8. 4	OPD (clinic)
		11	m	12	'85. 10. 25	OPD (clinic)
		12	f	14	'87. 8. 15	Home
		13	m	16	'87. 10. 4	Privately-owned car
†		14	f	26	'88. 5. 6	Home
†	‡	15	m	14	'88. 9. 24	OPD (University Hospital)
†		16	f	10	'89. 5. 5	Privately-owned car
†		17	m	26	'90. 8. 11	Privately-owned car
†		18	m	9	'91. 7. 30	Privately-owned car
†		19	m	24	'93. 8. 13	Home
†		20	f	25	'93. 11. 30	Hospital
†		21	m	23	'95. 5. 17	Privately-owned car
†		22	m	19	'96. 6. 15	Home

male 14, female 9, average 15.5±7.2 years old † : institutional therapy, ‡ : autopsy, OPD: outpatient department

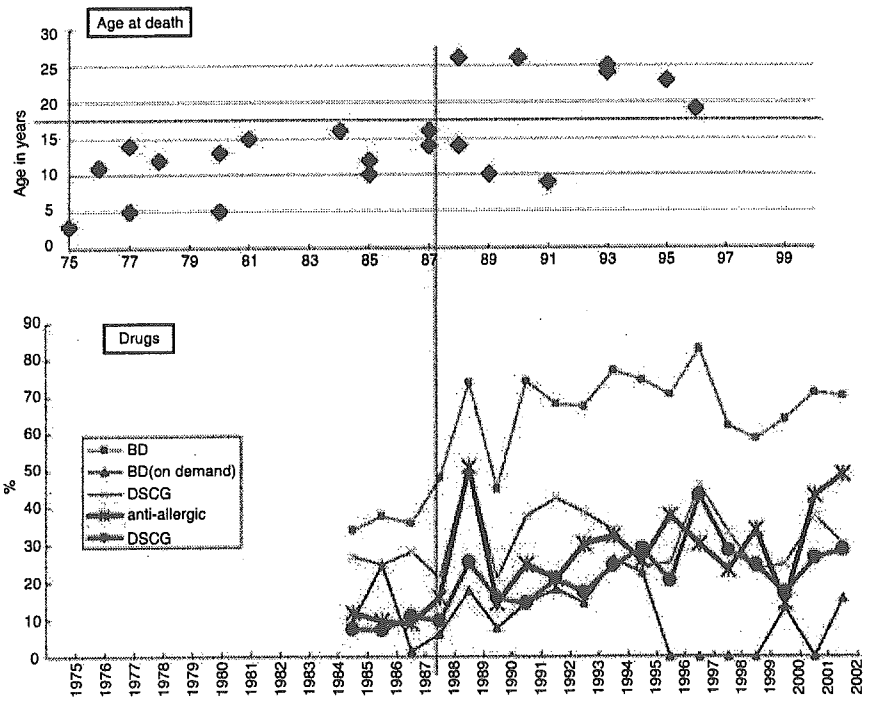

Fig. 1 Age at death and drugs prescribed in asthmatic children who had attended summer camp. BD : bronchodilators (round the clock), DSCG : disodium cromoglycate