astaxanthin reduces gastric inflammation, bacterial load and modulates cytokine release by splenocytes. Immunol Lett 1999; 70:185-9. [PMID: 10656672]

22. Mortensen A, Skibsted LH, Sampson J, Rice-Evans C, Everett SA. Comparative mechanisms and rates of free radical scavenging by carotenoid antioxidants. FEBS Lett 1997; 418:91-7. [PMID: 9414102]

23. Ohgami K, Shiratori K, Kotake S, Nishida T, Mizuki N, Yazawa K, Ohno S. Effects of astaxanthin on lipopolysaccharide-induced inflammation in vitro and in vivo. Invest Ophthalmol Vis Sci 2003; 44:2694-701. [PMID: 12766075]

24. Izumi-Nagai K, Nagai N, Ohgami K, Satofuka S, Ozawa Y, Tsubota K, Ohno S, Oike Y, Ishida S. Inhibition of choroidal neovascularization with an anti-inflammatory carotenoid astaxanthin. Invest Ophthalmol Vis Sci 2008; 49:1679-85. [PMID: 18385091]

25. Sasaki M, Ozawa Y, Kurihara T, Kubota S, Yuki K, Noda K, Kobayashi S, Ishida S, Tsubota K. Neurodegenerative influence of oxidative stress in the retina of a murine model of diabetes. Diabetologia 2010; 53:971-9. [PMID: 20162412]

26. Okada N, Kawakita T, Mishima K, Saito I, Miyashita H, Yoshida S, Shimmura S, Tsubota K. Clusterin promotes corneal epithelial cell growth through upregulation of hepatocyte growth factor by mesenchymal cells in vitro. Invest Ophthalmol Vis Sci 2011; 52:2905-10. [PMID: 21282577]

27. Kawakita T, Shimmura S, Hornia A, Higa K, Tseng SC. Stratified epithelial sheets engineered from a single adult murine corneal/limbal progenitor cell. J Cell Mol Med 2008; 12:1303-16. [PMID: 18318692]

28. Baeuerle PA, Henkel T. Function and activation of NF-kappa B in the immune system. Annu Rev Immunol 1994; 12:141-79. [PMID: 8011280]

29. Yamamoto Y, Gaynor RB. Therapeutic potential of inhibition of the NF-kappaB pathway in the treatment of inflammation and cancer. J Clin Invest 2001; 107:135-42. [PMID: 11160126]

30. Schreck R, Meier B, Mannel DN, Droge W, Baeuerle PA. Dithiocarbamates as potent inhibitors of nuclear factor kappa B activation in intact cells. J Exp Med 1992; 175:1181-94. [PMID: 1314883]

31. Németh ZH, Hasko G, Vizi ES. Pyrrolidine dithiocarbamate augments IL-10, inhibits TNF-alpha, MIP-1alpha, IL-12, and nitric oxide production and protects from the lethal effect of endotoxin. Shock 1998; 10:49-53. [PMID: 9688091]

32. Liu SF, Ye X, Malik AB. Inhibition of NF-kappaB activation by pyrrolidine dithiocarbamate prevents In vivo expression of proinflammatory genes. Circulation 1999; 100:1330-7. [PMID: 10491379]

33. Lauzurica P, Martinez-Martinez S, Marazuela M, Gomez del Arco P, Martinez C, Sanchez-Madrid F, Redondo JM. Pyrrolidine dithiocarbamate protects mice from lethal shock induced by LPS or TNF-alpha. Eur J Immunol 1999; 29:1890-900. [PMID: 10382751]

34. Schulze-Osthoff K, Los M, Baeuerle PA. Redox signalling by transcription factors NF-kappa B and AP-1 in lymphocytes. Biochem Pharmacol 1995; 50:735-41. [PMID: 7575632]

35. Muñoz C, Pascual-Salcedo D, Castellanos MC, Alfranca A, Aragones J, Vara A, Redondo JM, de Landazuri MO. Pyrrolidine dithiocarbamate inhibits the production of interleukin-6, interleukin-8, and granulocyte-macrophage colony-stimulating factor by human endothelial cells in response to inflammatory mediators: modulation of NF-kappa B and AP-1 transcription factors activity. Blood 1996; 88:3482-90. [PMID: 8896414]

36. Kawada H, Kojima M, Kimura T, Natori S, Sasaki K, Sasaki H. Effect of 5-S-GAD on UV-B-induced cataracts in rats. Jpn J Ophthalmol 2009; 53:531-5. [PMID: 19847611]

RHEUMATOLOGY

Concise report

Replication of a microsatellite genome-wide association study of Behçet's disease in a Korean population

Yukihiro Horie[1], Akira Meguro[2], Nobuyoshi Kitaichi[3,4], Eun Bong Lee[5], Atsuhiro Kanda[4], Kousuke Noda[1], Yeong Wook Song[5], Kyung Sook Park[6], Kenichi Namba[1], Masao Ota[7], Hidetoshi Inoko[8], Nobuhisa Mizuki[2], Susumu Ishida[1] and Shigeaki Ohno[4]

Abstract

Objective. Behçet's disease is one of the major aetiologies of uveitis causing blindness in Asian countries. A genome-wide association study identified six microsatellite markers as disease susceptibility loci for Japanese patients with Behçet's disease. To confirm our recent results, these microsatellite markers were examined in a Korean population as a replication study.

Methods. Study participants included 119 Behçet's disease patients and 141 controls. All were enrolled in Korea. Association between the six reported microsatellite markers (D3S0186i, D6S0014i, D6S0032i, 536G12A, D12S0645i and D22S0104i) and Behçet's disease was analysed. HLA-B was genotyped by sequence-based typing methods.

Results. A microsatellite marker located near the *HLA-B* region demonstrated significant association with Behçet's disease ($P = 0.028$). The genotype and phenotype frequencies of the *HLA-B*51* gene were significantly increased in patients (23.1 and 39.5%, respectively) compared with healthy controls (11.2 and 20.1%, respectively; $P < 0.001$).

Conclusion. Microsatellite analysis revealed that the *HLA-B*51* gene was strongly associated with Behçet's disease in a Korean population.

Key words: microsatellite, GWAS, Behçet's disease, replication study, HLA-B, Korean.

[1]Department of Ophthalmology, Hokkaido University Graduate School of Medicine, Sapporo, [2]Department of Ophthalmology, Yokohama City University School of Medicine, Yokohama, [3]Department of Ophthalmology, Health Sciences University of Hokkaido, Sapporo, [4]Department of Ocular Inflammation and Immunology, Hokkaido University Graduate School of Medicine, Sapporo, Japan, [5]Department of Internal Medicine and Graduate Program in Immunology, Medical Research Center, Seoul National University Hospital, [6]Department of Biology, College of Natural Sciences, Sungshin Women's University, Seoul, Korea, [7]Department of Legal Medicine, Shinshu University School of Medicine, Matsumoto and [8]Department of Molecular Life Science, Division of Molecular Medical Science and Molecular Medicine, Tokai University School of Medicine, Isehara, Japan.

Correspondence to: Nobuyoshi Kitaichi, Department of Ocular Inflammation and Immunology, Hokkaido University Graduate School of Medicine, Sapporo, Kita-15, Nishi-7, Kita-ku, Sapporo 060-8638, Japan. E-mail: nobukita@med.hokudai.ac.jp

Introduction

Behçet's disease is a chronic, systemic, inflammatory disorder characterized by four major symptoms consisting of oral aphthous ulcers, genital ulcers, skin lesions and recurrent ocular inflammation [1]. The disease is occasionally associated with inflammation in the vascular and/or central nervous system and joints. Behçet's disease is found predominantly between East Asia and the Mediterranean basin along the historic Silk Road; however, it is uncommon in the American continents, Oceania and sub-Saharan Africa [2]. The distribution of intra-ocular inflammation and uveitis also differ in different regions of the world [3, 4].

In 1973, the first genetic factor was reported between Behçet's disease and HL-A5 [5]. The nomenclature of HL-A5 was later changed to HLA-B5. Today, HLA-B5 comprises three subantigens: HLA-B51, HLA-B52 and HLA-B53.

Susceptibility to Behçet's disease is strongly associated with *HLA-B*51*, as reported in different ethnic groups [2, 5, 6]. Populations with a high prevalence of *HLA-B*51* lie predominantly north of the equator, spanning Japan and Western Europe between 30° and 45° N [7]. The area is completely consistent with the countries where Behçet's disease is common. Meanwhile, many other susceptibility genes, such as *HLA-A, HLA-E, HLA-F and HLA-G; TNF-α; Toll-like receptor (TLR) 4; IL-1, IL-8, IL-10, IL-12 and IL-18; IL23R* and *CD28*, have been reported in relation to Behçet's disease [8–13]. Most recently, reports from ourselves and others of genome-wide association studies (GWASs) using 500 000 single nucleotide polymorphism (SNP) microarrays have identified two new disease susceptibility loci for Behçet's disease on Chr.1p31.3 and 1q32.1, aside from the HLA class I region, in Japanese, Turkish, Korean, UK Caucasian and Greek populations [11, 12]. These results are compatible with the fact that approximately one-half of Behçet's disease patients are *HLA-B*51* negative, and most *HLA-B*51* carriers never suffer from Behçet's disease in their lifetime [2, 14].

However, there is little doubt that the *HLA-B* region still demonstrates the strongest association with Behçet's disease among all ethnic groups. A GWAS using microsatellite markers demonstrated that six (D3S0186i, D6S0014i, D6S0032i, 536G12A, D12S0645i and D22S0104i) of 23 465 markers differed significantly between patients and healthy subjects by using pooled DNA and individual typing methods in the Japanese population [8]. One of these six significant markers is located near the *HLA-B* region. Therefore it is critically important to examine these indicated microsatellite markers among the people along the historic Silk Road, considering the prominent high prevalence of both the disease and the *HLA-B*51* gene there. In the present study, these microsatellite markers were investigated in a Korean population as a replication study, and the association was also examined between clinical features and gene frequency.

Methods

Participants

In the present study, 119 Behçet's disease patients and 141 healthy controls were enrolled. All the patients were from Seoul National University Hospital, Seoul, Korea, and fulfilled the diagnostic criteria of the International Study Group for Behçet's Disease (ISGBD). Informed consent was obtained from all participating individuals. The ethical committee of Seoul National University Hospital approved the study. The procedures used conformed to the tenets of the Declaration of Helsinki. All of the patients and control subjects were Korean.

Genotyping

Genomic DNA was prepared from peripheral blood specimens using the QIAamp DNA Blood Mini Kit (Qiagen, Tokyo, Japan). Each PCR product for the six microsatellite markers (D3S0186i, D6S0014i, D6S0032i, 536G12A, D12S0645i and D22S0104i) was amplified by PCR

reactions. Each microsatellite marker was amplified using two primers: forward 5'-AGC TCT TCC TAA CTG ATA AGG AAG-3' and reverse 5'-GTA AAG GTT GCT AGG TCC TGT T-3' for D3S0186i, forward 5'-CCA TAT GCT AGA AAT TAT GGT ACT -3' and reverse 5'-GTT TCA CTA TGT TGG CCA G-3' for D6S0014i, forward 5'-TAA GTC TAA GAA TGT GAG ACC AAC-3' and reverse 5'-GTA ATG CTG ATA ACG TTT ACT GTC-3' for D6S0032i, forward 5'-GTG TGC TTG TGT CTG TTA ATT G-3' and reverse 5'-ACA CTA TAT TGT TAG CAA GTT ACT GAA C-3' for 536G12A, forward 5'-GGC AGA GAC AGT GTC TTT CTC-3' and reverse 5'-AGG TCA AGT GCA TGT TTG AC-3' for D12S0645i, and forward 5'-TAA GGC TGA GTA GCA GTC TAC ATA-3' and reverse 5'-TCA TTA AAG AAC TGG ATC TAC CAT-3' for D22S0104i. The reaction mixture was subjected to 5 min at 94°C, followed by 35 cycles of 1 min for denaturing at 94°C, 1 min for annealing at 57°C or 60°C, and 2 min for extension at 72°C, and 10 min for final elongation at 72°C using a PCR thermal cycler GeneAmp System 9700 (Applied Biosystems, Foster City, CA, USA). Each forward primer was labelled at the 5'-end with 6-FAM, NED or VIC to determine the number of microsatellite repeats. Fragment length analysis was performed using an ABI3130 DNA sequencer (Applied Biosystems) and the number of microsatellite repeats was estimated with GeneMapper v3.5 software using GS500(-250)Liz (Applied Biosystems) as a size marker.

Genotyping of the *HLA-B* gene was performed using PCR sequence-based typing methods, and the data were analysed using Assign software. *HLA-B* typing by sequence-based typing methods was performed by using an HLA typing kit (Abbott, Japan).

Statistical analysis

Allele frequencies were calculated by direct counting. The significance of the association was assessed using the chi-square test. The strength of all *P*-values was derived from a two-sided test; $P < 0.05$ was considered statistically significant.

Results

There was a significant difference between patients and healthy controls in one of six microsatellite markers. The allele frequency of 308 of the microsatellite marker D6S0032i was statistically higher among Behçet's disease patients (14.3%) compared with healthy subjects (8.2%) [$P = 0.028$, odds ratio (OR) = 1.86, 95% CI 1.06, 8.66] (Table 1).

Genotype and phenotype frequencies at the *HLA-B* locus were examined, and 23 antigens were identified in 119 patients and 141 controls. The genotype frequency of *HLA-B*51* was significantly higher among patients (23.1%) than among healthy controls (11.2%) [$P = 0.0003$, OR = 2.39, 95% CI 1.48, 9.23]: in particular, *HLA-B*5101* [$P = 0.0006$, OR = 2.31, 95% CI 1.42, 9.12] (Table 1). The phenotype frequency of *HLA-B*51* was significantly higher among patients (39.5%) than among

TABLE 1 Genotype frequencies of D6S0032i and genotyped and phenotyped HLA-B allele frequencies in patients and healthy controls of the Korean population

	Patients, n (%)	Controls, n (%)	Odds (95% CI)	χ^2	P-values
D6S0032i	2n = 238	2n = 280			
304	7 (2.9)	10 (3.6)	0.82 (0.31, 8.80)	0.16	0.69
308	**34 (14.3)**	**23 (8.2)**	**1.86 (1.06, 8.66)**	**4.84**	**0.028**
313	109 (45.8)	163 (58.2)	0.61 (0.43, 7.64)	7.95	0.0048
321	88 (37)	84 (30)	1.37 (0.95, 7.56)	2.82	0.093
Genotype	2n = 238	2n = 278			
B*51	**55 (23.1)**	**31 (11.2)**	**2.39 (1.48, 9.23)**	**13.2**	**0.00028**
B*5101	**52 (21.8)**	**30 (10.8)**	**2.31 (1.42, 9.12)**	**11.73**	**0.00062**
B*5102	3 (1.3)	1 (0.4)	3.54 (0.22, 10.0)	1.35	0.24.
Phenotype	n = 119	n = 139			
B*51	**47 (39.5)**	**28 (20.1)**	**2.59 (1.49, 9.74)**	**11.64**	**0.00064**

Significant P-values are in bold.

healthy controls (20.1%) [P = 0.00064, OR = 2.59, 95% CI 1.49, 9.74] (Table 1).

Discussion

To confirm recent GWASs in Japan [8], it is important to repeat population-specific analysis and other population data analysis. In the present study, we determined the reproducibility of the recent Japanese GWAS results and successfully found the new disease susceptibility gene by using microsatellite markers in a Korean population. Microsatellite polymorphisms have been used in the genetic association study because they are highly polymorphic, relatively simple and inexpensive [15]. Recent technologies have reduced the necessary time and cost to genotype SNPs [16]. However, because of their lower variability, SNPs may be considered as carrying relatively less information at each locus. Furthermore, the microsatellite mutation rate was estimated to be 10^{-2}-10^{-5} each generation; more frequent than that of SNPs (2.5×10^{-8}) [17]. That is to say, although microsatellite analysis has the great advantages of higher polymorphism and sensitivity, it also carries the disadvantage of relatively high genotyping error rates due to its highly polymorphic nature [18]. Therefore it is necessary to replicate the obtained susceptible genes in other ethnic groups by other means. This is why we have organized the present study.

The marker D6S0032i, located at 1.1 Mb telomeric of HLA-B within the HLA class I region, showed statistical significance in Korean Behçet's disease patients. However, there was no significant difference between patients and controls for the other five markers located near the ROBO1 (roundabout, axon guidance receptor, homologue 1), HLA-L and HLA-B, PPIL4 (peptidylprolyl isomerase-like 4), SOX5 (sex determining region Y-box 5) and IGLV1-40 (IGL variable 1-40) genes. The previous Turkish GWAS using fewer than 400 microsatellite markers reported chromosomes 12p12-13 and 6p22-24 as the susceptible loci for Behçet's disease [19]. However, our recent GWAS in Japanese people using 23 465 microsatellite markers disagreed with that Turkish study.

In this type of study, however, there will be sources of bias that may distort the results. In this study we have identified the following possibilities:

(i) classification bias: we adopted International Study Group criteria in this study; however, the Japanese GWAS adopted Japan Behçet Disease Research Committee's Criteria. The use of different standardized criteria may have led to misclassification when comparing the frequencies of systemic features.

(ii) ascertainment bias: all samples in the current work were collected by rheumatologists; however, in the Japanese GWAS, patients were mainly recruited at eye clinics. Patients with ocular lesions were significantly fewer in this study (37.8%) than the recent Japanese GWAS (89.3%) (P = 0.0000067) [9]. It is possible that some patients with quite mild ocular inflammation might be misidentified as no ocular manifestations at rheumatology clinics when they did not consult ophthalmologists.

There are 3.61% of genetic differences between Japanese and Koreans [20]. Additional replication studies in other ethnic groups may be required for better understanding the mechanisms, aetiology and characteristics of Behçet's disease. In conclusion, we performed a microsatellite analysis in a population of Korean Behçet's disease patients as a replication study and demonstrated significant association with the marker located near the HLA-B region.

Rheumatology key messages

- A replication study of microsatellite GWAS was performed.
- The HLA-B*51 gene was strongly associated with Behçet's disease in a Korean population.
- One of the six microsatellite markers was significantly associated with Behçet's disease in a Korean population.

Acknowledgements

References

1 Ohno S, Nakamura S, Hori S *et al*. Efficacy, safety, and pharmacokinetics of multiple administration of infliximab in Behcet's disease with refractory uveoretinitis. J Rheumatol 2004;31:1362-8.

2 Ohno S, Ohguchi M, Hirose S *et al*. Close association of HLA-Bw51 with Behcet's disease. Arch Ophthalmol 1982; 100:1455-8.

3 Kitaichi N, Miyazaki A, Iwata D *et al*. Ocular features of Behcet's disease: an international collaborative study. Br J Ophthalmol 2007;91:1579-82.

4 Kitaichi N, Miyazaki A, Stanford MR *et al*. Low prevalence of juvenile-onset Behcet's disease with uveitis in East/South Asian people. Br J Ophthalmol 2009; 93:1428-30.

5 Ohno S, Aoki K, Sugiura S *et al*. Letter: HL-A5 and Behcet's disease. Lancet 1973;2:1383-4.

6 Ohno S, Asanuma T, Sugiura S *et al*. HLA-Bw51 and Behcet's disease. JAMA 1978;240:529.

7 Verity DH, Marr JE, Ohno S *et al*. Behcet's disease, the Silk Road and HLA-B51: historical and geographical perspectives. Tissue Antigens 1999;54:213-20.

8 Meguro A, Inoko H, Ota M *et al*. Genetics of Behcet disease inside and outside the MHC. Ann Rheum Dis 2010;69:747-54.

9 Meguro A, Ota M, Katsuyama Y *et al*. Association of the toll-like receptor 4 gene polymorphisms with Behcet's disease. Ann Rheum Dis 2008;67:725-7.

10 Horie Y, Meguro A, Ota M *et al*. Association of TLR4 polymorphisms with Behcet's disease in a Korean population. Rheumatology 2009;48:638-42.

11 Mizuki N, Meguro A, Ota M *et al*. Genome-wide association studies identify IL23R-IL12RB2 and IL10 as Behcet's disease susceptibility loci. Nat Genet 2010;42:703-6.

12 Remmers EF, Cosan F, Kirino Y *et al*. Genome-wide association study identifies variants in the MHC class I, IL10, and IL23R-IL12RB2 regions associated with Behcet's disease. Nat Genet 2010;42:698-702.

13 Jiang Z, Yang P, Hou S *et al*. IL-23R gene confers susceptibility to Behcet's disease in a Chinese Han population. Ann Rheum Dis 2010;69:1325-8.

14 Mizuki N, Inoko H, Mizuki N *et al*. Human leukocyte antigen serologic and DNA typing of Behcet's disease and its primary association with B51. Invest Ophthalmol Vis Sci 1992;33:3332-40.

15 Ball AD, Stapley J, Dawson DA *et al*. A comparison of SNPs and microsatellites as linkage mapping markers: lessons from the zebra finch (Taeniopygia guttata). BMC Genomics 2010;11:218.

16 Syvanen AC. Toward genome-wide SNP genotyping. Nat Genet 2005;37(Suppl.):S5-10.

17 Tishkoff SA, Verrelli BC. Patterns of human genetic diversity: implications for human evolutionary history and disease. Annu Rev Genomics Hum Genet 2003;4: 293-340.

18 Pompanon F, Bonin A, Bellemain E *et al*. Genotyping errors: causes, consequences and solutions. Nat Rev Genet 2005;6:847-59.

19 Karasneh J, Gul A, Ollier WE *et al*. Whole-genome screening for susceptibility genes in multicase families with Behcet's disease. Arthritis Rheum 2005;52:1836-42.

20 Miller RD, Phillips MS, Jo I *et al*. High-density single-nucleotide polymorphism maps of the human genome. Genomics 2005;86:117-26.

INFLAMMATORY DISORDERS

Correlation between elevation of serum antinuclear antibody titer and decreased therapeutic efficacy in the treatment of Behçet's disease with infliximab

Daiju Iwata · Kenichi Namba · Kazuomi Mizuuchi ·
Nobuyoshi Kitaichi · Satoru Kase · Yuko Takemoto ·
Shigeaki Ohno · Susumu Ishida

Abstract

Background Infliximab, an anti-TNF-α monoclonal antibody, administered to Behçet's disease (BD) patients in Japan with refractory intraocular inflammation, has shown excellent clinical results. However, some patients demonstrate a decreased response to infliximab during the course of the treatment. In the present study, we investigated the correlation between this reduced therapeutic effect and elevation of the serum antinuclear antibody (ANA) titers in patients with BD who were undergoing infliximab therapy.

Methods Seventeen patients (14 males and three females) with uveitis in BD who were undergoing treatment with infliximab for 2 years or longer were enrolled. Their blood test results and clinical histories were obtained from medical records.

Results One patient (5.9%) was ANA-positive prior to the initiation of infliximab, and 11 patients (64.7%) developed positive ANA during the therapy. The appearance of ANA was observed 6 months after the initiation of the infliximab therapy, and its titers gradually increased. None of the patients showed lupus symptoms. Five patients (29.4%) have suffered from ocular inflammatory attacks since the sixth month from the initiation of infliximab treatment and all of them were ANA-positive. In contrast, four patients (23.5%) who were ANA-negative experienced no ocular attacks during the follow-up period.

Conclusions Here we report the positive conversion and subsequent elevation of serum ANA titers in some patients with BD after the initiation of infliximab therapy. Since all recurrences of uveitis were shown only in the ANA-positive patients, serum ANA titer may be a helpful biomarker for predicting the recurrence of ocular attacks in BD patients treated with anti-TNF-α antibody therapies.

D. Iwata · K. Namba (✉) · K. Mizuuchi · S. Kase · Y. Takemoto · S. Ishida
Department of Ophthalmology,
Hokkaido University Graduate School of Medicine,
Kita-15, Nishi-7, Kita-ku,
Sapporo 060-8638, Japan
e-mail: knamba@med.hokudai.ac.jp

N. Kitaichi
Department of Ophthalmology,
Health Sciences University of Hokkaido,
Sapporo, Japan

N. Kitaichi · S. Ohno
Department of Ocular Inflammation and Immunology,
Hokkaido University Graduate School of Medicine,
Sapporo, Japan

Introduction

Behçet's disease (BD) is a chronic systemic inflammatory disease characterized by recurrent oral aphthous ulcers, genital ulcers, skin lesions, gastrointestinal involvement, vasculitis, neurological manifestations, and intraocular inflammation. BD is one of the major etiologies of endogenous uveitis in Japan [1], however, its prevalence and clinical features vary among countries and ethnic groups [2, 3]. Recurrent episodes of inflammatory ocular attacks can cause severe visual loss. To prevent the relapse of intraocular inflammation, colchicine and various immunosuppressive

🌊 Springer

agents are administered including cyclosporine A (CyA), which is a selective immunosuppressive agent of T-lymphocytes. However, some patients cannot use these drugs due to intolerable side-effects. Moreover, some patient's diseases are refractory to these agents and can progress to vision loss [4–6].

Infliximab (IFX) is a chimeric monoclonal antibody to TNF-α that can minimize the immunological response when used in humans [7]. It neutralizes both membrane-binding and soluble TNF-α, in addition to suppressing TNF-α production by macrophages. IFX is commonly administered to patients with rheumatoid arthritis [8, 9], Crohn's disease [10], psoriasis [11, 12], and in case of refractory uveitis with non-infectious etiologies including BD [13–20]. IFX is effective for preventing relapse of intraocular inflammations in BD and its efficacy has been well documented in previous studies [13–19]. In Japan, IFX was approved for use in BD patients with refractory uveoretinitis by the Ministry of Health, Labour and Welfare, Japan in January 2007 based on the excellent results from multicenter clinical trials [15, 21]. Though IFX is an excellent agent in the treatment of the BD with refractory uveoretinitis, it has been observed to have decreased efficacy in a subset of BD patients with uveoretinitis [19]. One report showed the development of autoantibodies including antinuclear antibody (ANA) during IFX treatment in BD [22], however, the mechanisms and the meanings of it remain unknown.

In the present study, we investigated ANA titers of the BD patients receiving IFX therapy and examined the correlation between the elevation of ANA and the therapeutic efficacy.

Materials and methods

BD patients with refractory uveoretinitis who had been administered IFX for 2 years or longer were enrolled at Hokkaido University Hospital. The results of their blood tests and clinical histories were obtained from medical records. BD was diagnosed based on the criteria set by the BD Research Committee of Japan, which is part of the Ministry of Health, Labour and Welfare, Japan [23]. The level of ocular inflammation was graded by means of the Standardization of Uveitis Nomenclature (SUN) grading criteria [24]. When a patient showed more than two steps of increase in level of inflammation or increase from grade 3+ to 4+, it was considered an inflammatory ocular attack. Ocular attacks of BD flare up repeatedly and usually disappear within a few weeks. Each ocular attack shows a varying degree of uveitis including only mild iridocyclitis or severe obstructive retinal vasculitis with retinal exudates. The number of ocular attacks was counted regardless of the severity and added both eyes.

Patients were administered 5 mg/kg of IFX intravenously at weeks 0, 2, and 6 as the initial series of infusions and thereafter every 8 weeks. Serum ANA and anti-double-stranded DNA (dsDNA) antibodies were examined prior to IFX infusion. ANA titers were quantified using an indirect immunofluorescence technique using human epithelial (Hep2) cells. Results were classified as positive (ANA≥80) or negative (ANA≤40) according to previous reports [25–31]. Anti-dsDNA antibodies were identified using enzyme-linked immunosorbent assay (ELISA).

Statistical analyses were performed using the Mann–Whitney U test; p values <0.05 were considered to be statistically significant. This study followed the tenets of the Declaration of Helsinki and was approved by the Ethics Committee of Hokkaido University Hospital.

Results

The demographics and clinical characteristics of the 17 Japanese patients, i.e., 14 (82.4%) males and three (17.6%) females ranging in age from 15 to 58 (mean age: 36.9) years, enrolled in the study are listed in Table 1. The rate of ocular inflammatory attacks during 6 months prior to the initiation of IFX was 3.8±2.1 (mean±SD). IFX therapy significantly reduced the rate of ocular attacks to 0.7±1.1 during the first 6 months after the initiation of IFX ($p<0.01$).

Table 1 Characteristics of Behçet's disease patients treated with IFX

Case	Age (years)	Sex	Treatment before IFX initiation	Concomitant treatment with IFX
1	39	M	CyA, Col, PSL	–
2	33	M	Col, PSL	PSL
3	58	M	Col	–
4	42	M	Col	–
5	40	F	PSL	PSL
6	31	M	Col	–
7	44	M	PSL	PSL
8	54	M	CyA, PSL	PSL
9	17	M	CyA, Col	–
10	52	M	Col	–
11	10	M	–	–
12	49	M	CyA	–
13	40	M	CyA	–
14	40	F	CyA	–
15	36	F	PSL	PSL
16	15	M	PSL	–
17	28	M	–	–

IFX – infliximab, CyA – cyclosporine A, Col – colchicine, PSL – prednisolone

Eight patients (47.1%) achieved no relapse of ocular inflammatory attacks between the first infusion and the 24-month visit. Five patients (29.4%) experienced only one ocular inflammatory attack and four patients (23.5%) experienced several ocular attacks during the follow-up period. It was not necessary to administer concomitant drugs with IFX for 12 patients. Three of five patients who were previously administered oral prednisolone (PSL) could decrease and gradually stop their therapy after IFX initiation. Two of these patients required continued PSL administration to control neurological symptoms.

Best-corrected visual acuities (BCVA) were reported 1 year after the initiation of IFX; IFX therapy had successfully maintained their vision acuity (Fig. 1).

ANA profiles and the frequency of ocular attacks in BD patients treated with IFX are shown in Table 2. One patient (5.9%) was ANA-positive prior to the initiation of IFX. Anti-dsDNA antibodies were never detected prior to IFX induction. The change in ANA-positive rates is shown in Fig. 2. The positive conversion of ANA became common 6 months after the initiation of IFX, and the positive titers continued to increase. At the end of the follow-up period, 13 patients (76.4%) were identified positive for ANA (Fig. 2). One patient (5.9%) developed anti-dsDNA antibodies (case #14). However, none of the patients showed lupus symptoms.

The correlation of ocular attacks with elevation of ANA titer is shown in Fig. 3. At the 6th month after the IFX induction, five patients (29.4%) were ANA-positive and 12

(70.6%) were negative. In the ANA-positive group, three patients (60%) had ocular inflammatory attacks during the first 6 months after IFX administrations, whereas in the ANA-negative group, four (33.3%) patients had these attacks. Ocular attacks were much milder than those before IFX therapy both in the ANA-negative group and ANA-positive group.

However, since the 6th month of IFX therapy, all of five patients (29.4%) suffering from a relapse of ocular inflammatory attacks were ANA-positive, and three of five patients had multiple ocular attacks. In two of these three patients, the administration interval was shortened from 8 to 7 weeks, and this successfully led to a lower rate of the ocular attacks. On the other hand, all of four patients (23.5%) with negative ANA had no ocular attacks.

Discussion

ANA appeared in the sera of BD patients 6 months after IFX induction, and its titer gradually increased. It was reported that the development of ANA and anti-dsDNA antibodies is seen during the course of anti-TNF-α therapy in patients with some autoimmune diseases such as rheumatoid arthritis [32–34], psoriasis [35], Crohn's disease [36], and BD [22]. In the present study, 75.0% of the patients converted to ANA-positive during the course of IFX therapy and positive ANA titers (1:80) had been detected in one patient on study enrolment. This patient experienced a twofold increase in

Fig. 1 Visual acuity before and after initiation of IFX. Best-corrected visual acuities (BCVA) 1 year after the initiation of IFX. IFX therapy successfully maintained the visual acuity in these patients
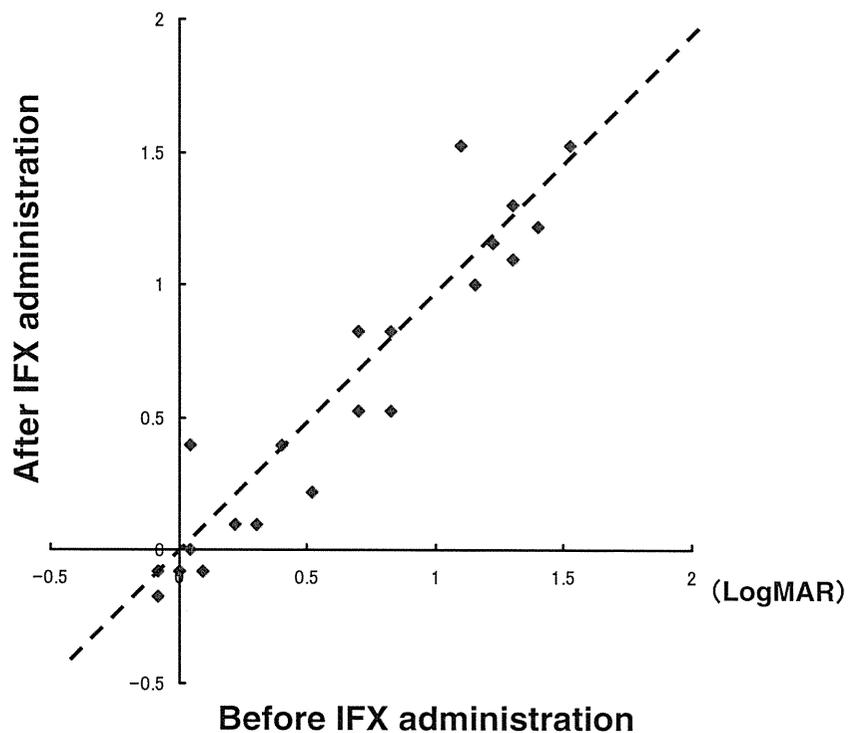
Before IFX administration

Table 2 ANA profile and the rate of ocular attacks of Behçet's disease patients treated with IFX

Case	Frequency of ocular attacks -6~0 month	ANA (titer) 0 month	Frequency of ocular attacks 0~6 month	ANA (titer) 6 month	Frequency of ocular attacks 6~12 month	ANA (titer) 12 month	Frequency of ocular attacks 12~18 month	ANA (titer) 18 month	Frequency of ocular attacks 18~24 month	ANA (titer) 24 month
1	0	0	0	80	0	40	0	80	0	80
2	2	0	0	0	0	0	0	0	0	40
3	8	0	0	0	0	0	0	0	0	40
4	4	0	0	40	0	80	0	80	0	160
5	4	0	0	40	0	80	0	80	0	160
6	5	0	0	40	0	160	0	320	0	640
7	3	0	0	40	0	80	0	160	0	640
8	1	0	0	40	0	40	0	40	0	40
9	4	0	1	0	0	40	0	40	0	80
10	2	0	1	0	0	80	0	80	0	80
11	4	0	1	0	0	0	0	0	0	0
12	4	40	1	80	0	80	0	80	0	80
13	4	0	0	160	1	80	0	160	0	80
14	7	0	1	160	5	160	4	320	3	640
15	2	0	0	40	1	80	1	80	0	40
16	6	80	2	160	1	160	1	160	0	160
17	4	0	4	40	1	80	0	40	0	40

ANA positive: ANA titer≥80, ANA – anti nuclear antibody

the titer (1:160). Only one patient (5.9%) converted to anti-dsDNA antibody-positive during the follow-up period. These findings are consistent with previous studies of other autoimmune rheumatoid diseases, which reported that 25–71% and 4–46% patients became positive for ANA and anti-dsDNA antibodies after IFX initiation in case of psoriasis and rheumatoid arthritis [32, 37, 38]. In these previous studies, a small number of patients had lupus-like symptoms [39–41]. Suhler EB et al. also reported the results of a prospective study in which 23 patients with non-infectious uveoretinitis including four

of BD patients were enrolled [42]. In the report, ANA titers developed in 15 (75.0%) of the 20 patients and two patients with very high titer showed arthritis. Although none of the patients in our study have shown lupus symptoms, we have to observe the patients very carefully.

It is still unknown how ANA and anti-dsDNA antibodies develop during IFX therapy. One possible explanation is that TNF-α may up-regulate cellular expression of the adhesion molecule CD44, which plays a role in the
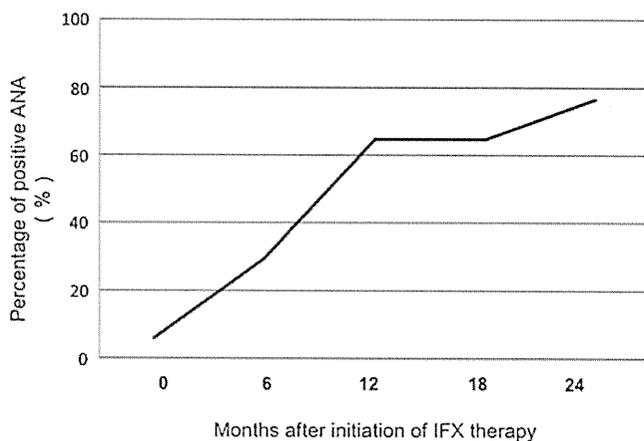

Fig. 2 Frequencies of ANA positivity in BD patients undergoing IFX therapy. The positive conversion of ANA became frequent 6 months after the initiation of IFX, and its positivity rate gradually increased
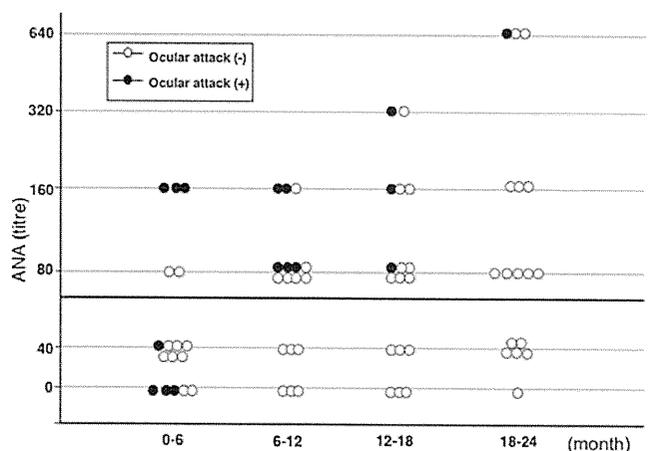

Fig. 3 Correlation of ocular attacks with elevation of ANA titer. Since the 6th month of IFX therapy, all five patients (23.5%) suffering from a relapse of ocular inflammatory attack were ANA-positive, and three of five patients had ocular attacks more than once throughout the observation period

clearance of apoptotic neutrophils by phagocytes [43, 44]. Impaired clearance of apoptotic cells and reduced CD44 expression on leukocytes has been reported in systemic lupus erythematosus (SLE) [45, 46]. IFX may down-regulate CD44 expression and induce an immune reaction toward their own nuclei by impairment of the clearance of apoptotic cells.

In the present study, we also demonstrated the association between the development of ANA and reduced effects of IFX therapy in BD patients. Only a few studies have reported the association of serum ANA development with the effects of IFX [35, 42]. Pink et al. reported that ANA titer was associated with the loss of response to anti-TNF-α therapy in psoriasis. In our study, during 6 months after the initiation of IFX therapy, several patients experienced mild ocular inflammatory attacks, both in the ANA-positive and ANA-negative groups. Presumably, it takes some time for IFX to exert an inhibitory effect on severe ocular inflammation in BD patients. However, after the 6th month IFX therapy, the cases suffering from the relapse of ocular inflammatory attacks were limited to ANA-positive patients. Similar to the study in psoriasis [35], these results suggest that the development of elevated levels of serum ANA may be associated with the reduction of IFX efficacy for BD patients. Suhler EB et al. mentioned no clear relation between the development of ANAs and ocular therapeutic response [42]. The subjects in the report included a variety of uveitis cases, in contrast to our study, which targeted only BD. The tight disease enrollment may be the reason why we could show the relation between recurrences of uveitis and high titer of ANA.

The exact association between ANA and IFX also remains unknown. It has also been found that repeated infusion of IFX leads to induction of antibodies to IFX (ATI) that reduce the efficacy of IFX. This phenomenon has been a serious issue in rheumatoid arthritis [47], Crohn's disease [48], psoriasis [49, 50] and BD [51] therapy. ANA and ATI, both of which appear during the course of IFX treatment, are likely to be involved in the reduced efficacy of IFX; however, the correlation between the two antibodies remains to be elucidated. We speculate that repeated administration of protein agents such as IFX may activate a systemic immune response, leading to the production of various autoantibodies including ANA and ATI. Therefore, detection of high titer of ANA indicates the development of ATI in the patients. According to this theory, the patients, such as cases #6 and #7, with high ANA titer (640×) may have already had ATI. These patients should be monitored closely for further symptoms. If the theory is confirmed that ATI is strongly correlated with the decreased therapeutic efficacy of IFX, we need to consider concomitant use of immune modulatory medicine in BD.

IFX has provided a new way to maintain good vision for a long time in many BD patients with severe uveitis. However, in certain cases IFX becomes less effective while long-term use of IFX. Serum ANA titers may be one of the helpful biomarker to predict IFX ineffectiveness.

References

1. Kitamei H, Kitaichi N, Namba K, Kotake S, Goda C, Kitamura M, Miyazaki A, Ohno S (2009) Clinical features of intraocular inflammation in Hokkaido, Japan. Acta Ophthalmol 87:424–428
2. Kitaichi N, Miyazaki A, Iwata D, Ohno S, Stanford MR, Chams H (2007) Ocular features of Behçet's disease: an international collaborative study. Br J Ophthalmol 91:1579–1582
3. Kitaichi N, Miyazaki A, Stanford MR, Iwata D, Chams H, Ohno S (2009) Low prevalence of juvenile-onset Behçet's disease with uveitis in East/South Asian people. Br J Ophthalmol 93:1428–1430
4. Kaklamani V, Vaiopoulos G, Kaklamanis P (1998) Behçet's disease. Semin Arthritis Rheum 27:197–217
5. Sakane T, Takeno M, Suzuki N, Inaba G (1999) Behçet's disease. N Engl J Med 341:1284–1291
6. Zouboulis C (1999) Epidemiology of Adamantiades-Behçet's disease. Ann Med Interne (Paris) 150:488–498
7. Knight D, Trinh H, Le J, Siegel S, Shealy D, McDonough M, Scallon B, Moore M, Vilcek J, Daddona P (1993) Construction and initial characterization of a mouse-human chimeric anti-TNF antibody. Mol Immunol 30:1443–1453
8. Elliott M, Maini R, Feldmann M, Long-Fox A, Charles P, Bijl H, Woody J (1994) Repeated therapy with monoclonal antibody to tumour necrosis factor alpha (cA2) in patients with rheumatoid arthritis. Lancet 344:1125–1127
9. Maini R, St Clair E, Breedveld F, Furst D, Kalden J, Weisman M, Smolen J, Emery P, Harriman G, Feldmann M, Lipsky P (1999) Infliximab (chimeric anti-tumour necrosis factor alpha monoclonal antibody) versus placebo in rheumatoid arthritis patients receiving concomitant methotrexate: a randomised phase III trial. ATTRACT Study Group. Lancet 354:1932–1939
10. Hanauer S, Feagan B, Lichtenstein G, Mayer L, Schreiber S, Colombel J, Rachmilewitz D, Wolf D, Olson A, Bao W, Rutgeerts P, Group AIS (2002) Maintenance infliximab for Crohn's disease: the ACCENT I randomised trial. Lancet 359:1541–1549
11. Zhang Z, Schmitt J, Wozel G, Kirch W (2009) Treatment of plaque psoriasis with biologics. A meta-analysis of randomized controlled trials. Med Klin (Munich) 104:125–136
12. Nestle F, Kaplan D, Barker J (2009) Psoriasis. N Engl J Med 361:496–509
13. Sfikakis P, Theodossiadis P, Katsiari C, Kaklamanis P, Markomichelakis N (2001) Effect of infliximab on sight-threatening panuveitis in Behçet's disease. Lancet 358:295–296

14. Muñoz-Fernández S, Hidalgo V, Fernández-Melón J, Schlincker A, Martín-Mola E (2001) Effect of infliximab on threatening panuveitis in Behçet's disease. Lancet 358:1644

15. Ohno S, Nakamura S, Hori S, Shimakawa M, Kawashima H, Mochizuki M, Sugita S, Ueno S, Yoshizaki K, Inaba G (2004) Efficacy, safety, and pharmacokinetics of multiple administration of infliximab in Behçet's disease with refractory uveoretinitis. J Rheumatol 31:1362–1368

16. Sfikakis P, Kaklamanis P, Elezoglou A, Katsilambros N, Theodossiadis P, Papaefthimiou S, Markomichelakis N (2004) Infliximab for recurrent, sight-threatening ocular inflammation in Adamantiades-Behçet disease. Ann Intern Med 140:404–406

17. Tugal-Tutkun I, Mudun A, Urgancioglu M, Kamali S, Kasapoglu E, Inanc M, Gül A (2005) Efficacy of infliximab in the treatment of uveitis that is resistant to treatment with the combination of azathioprine, cyclosporine, and corticosteroids in Behçet's disease: an open-label trial. Arthritis Rheum 52:2478–2484

18. Accorinti M, Pirraglia M, Paroli M, Priori R, Conti F, Pivetti-Pezzi P (2007) Infliximab treatment for ocular and extraocular manifestations of Behçet's disease. Jpn J Ophthalmol 51:191–196

19. Niccoli L, Nannini C, Benucci M, Chindamo D, Cassarà E, Salvarani C, Cimino L, Gini G, Lenzetti I, Cantini F (2007) Long-term efficacy of infliximab in refractory posterior uveitis of Behçet's disease: a 24-month follow-up study. Rheumatology (Oxford) 46:1161–1164

20. Al-Rayes H, Al-Swailem R, Al-Balawi M, Al-Dohayan N, Al-Zaidi S, Tariq M (2008) Safety and efficacy of infliximab therapy in active Behçet's uveitis: an open-label trial. Rheumatol Int 29:53–57

21. Yamada Y, Sugita S, Tanaka H, Kamoi K, Kawaguchi T, Mochizuki M (2010) Comparison of infliximab versus ciclosporin during the initial 6-month treatment period in Behçet disease. Br J Ophthalmol 94:284–288

22. Elezoglou A, Kafasi N, Kaklamanis PH, Theodossiadis PG, Kapsimali V, Choremi E, Vaiopoulos G, Sfikakis PP (2007) Infliximab treatment-induced formation of autoantibodies is common in Behçet's disease. Clin Exp Rheumatol 25:S65–S69

23. Mizushima Y (1988) Recent research into Behçet's disease in Japan. Int J Tissue React 10:59–65

24. Jabs DA, Nussenblatt RB, Rosenbaum JT, Group SoUNSW (2005) Standardization of uveitis nomenclature for reporting clinical data. Results of the First International Workshop. Am J Ophthalmol 140:509–516

25. Fritzler MJ, Pauls JD, Kinsella TD, Bowen TJ (1985) Antinuclear, anticytoplasmic, and anti-Sjögren's syndrome antigen A (SS-A/Ro) antibodies in female blood donors. Clin Immunol Immunopathol 36:120–128

26. de Vlam K, De Keyser F, Verbruggen G, Vandenbossche M, Vanneuville B, D'Haese D, Veys EM (1993) Detection and identification of antinuclear autoantibodies in the serum of normal blood donors. Clin Exp Rheumatol 11:393–397

27. Forslid J, Heigl Z, Jonsson J, Scheynius A (1994) The prevalence of antinuclear antibodies in healthy young persons and adults, comparing rat liver tissue sections with HEp-2 cells as antigen substrate. Clin Exp Rheumatol 12:137–141

28. Tan EM, Feltkamp TE, Smolen JS, Butcher B, Dawkins R, Fritzler MJ, Gordon T, Hardin JA, Kalden JR, Lahita RG, Maini RN, McDougal JS, Rothfield NF, Smeenk RJ, Takasaki Y, Wiik A, Wilson MR, Koziol JA (1997) Range of antinuclear antibodies in "healthy" individuals. Arthritis Rheum 40:1601–1611

29. Fernandez SA, Lobo AZ, Oliveira ZN, Fukumori LM, AM Prigo, Rivitti EA (2003) Prevalence of antinuclear autoantibodies in the serum of normal blood donors. Rev Hosp Clin Fac Med Sao Paulo 58:315–319

30. Watanabe A, Kodera M, Sugiura K, Usuda T, Tan EM, Takasaki Y, Tomita Y, Muro Y (2004) Anti-DFS70 antibodies in 597 healthy hospital workers. Arthritis Rheum 50:892–900

31. Al Jabri AA, Al Buloshi MS (2004) Anticardiolipin and antinuclear antibodies in the adult healthy Omani individuals. Saudi Med J 25:313–317

32. De Rycke L, Kruithof E, Van Damme N, Hoffman IE, Van den Bossche N, Van den Bosch F, Veys EM, De Keyser F (2003) Antinuclear antibodies following infliximab treatment in patients with rheumatoid arthritis or spondylarthropathy. Arthritis Rheum 48:1015–1023

33. Elkayam O, Burke M, Vardinon N, Zakut V, Yitzhak RB, Paran D, Levartovsky D, Litinsky I, Caspi D (2005) Autoantibodies profile of rheumatoid arthritis patients during treatment with infliximab. Autoimmunity 38:155–160

34. Eriksson C, Engstrand S, Sundqvist KG, Rantapää-Dahlqvist S (2005) Autoantibody formation in patients with rheumatoid arthritis treated with anti-TNF alpha. Ann Rheum Dis 64:403–407

35. Pink AE, Fonia A, Allen MH, Smith CH, Barker JN (2010) Antinuclear antibodies associate with loss of response to antitumour necrosis factor-alpha therapy in psoriasis: a retrospective, observational study. Br J Dermatol 162:780–785

36. Nancey S, Blanvillain E, Parmentier B, Flourié B, Bayet C, Bienvenu J, Fabien N (2005) Infliximab treatment does not induce organ-specific or nonorgan-specific autoantibodies other than antinuclear and anti-double-stranded DNA autoantibodies in Crohn's disease. Inflamm Bowel Dis 11:986–991

37. Smith CH, Jackson K, Bashir SJ, Perez A, Chew AL, Powell AM, Wain M, Barker JN (2006) Infliximab for severe, treatment-resistant psoriasis: a prospective, open-label study. Br J Dermatol 155:160–169

38. Charles PJ, Smeenk RJ, De Jong J, Feldmann M, Maini RN (2000) Assessment of antibodies to double-stranded DNA induced in rheumatoid arthritis patients following treatment with infliximab, a monoclonal antibody to tumor necrosis factor alpha: findings in open-label and randomized placebo-controlled trials. Arthritis Rheum 43:2383–2390

39. Cairns AP, Duncan MK, Hinder AE, Taggart AJ (2002) New onset systemic lupus erythematosus in a patient receiving etanercept for rheumatoid arthritis. Ann Rheum Dis 61:1031–1032

40. Debandt M, Vittecoq O, Descamps V, Le Loët X, Meyer O (2003) Anti-TNF-alpha-induced systemic lupus syndrome. Clin Rheumatol 22:56–61

41. Favalli EG, Sinigaglia L, Varenna M, Arnoldi C (2002) Drug-induced lupus following treatment with infliximab in rheumatoid arthritis. Lupus 11:753–755

42. Suhler EB, Smith JR, Wertheim MS, Lauer AK, Kurz DE, Pickard TD, Rosenbaum JT (2005) A prospective trial of infliximab therapy for refractory uveitis: preliminary safety and efficacy outcomes. Arch Ophthalmol 123:903–912

43. Osada A, Nakashima H, Furue M, Tamaki K (1995) Up-regulation of CD44 expression by tumor necrosis factor-alpha is neutralized by interleukin-10 in Langerhans cells. J Invest Dermatol 105:124–127

44. Hart SP, Dougherty GJ, Haslett C, Dransfield I (1997) CD44 regulates phagocytosis of apoptotic neutrophil granulocytes, but not apoptotic lymphocytes, by human macrophages. J Immunol 159:919–925

45. Herrmann M, Voll RE, Zoller OM, Hagenhofer M, Ponner BB, Kalden JR (1998) Impaired phagocytosis of apoptotic cell material by monocyte-derived macrophages from patients with systemic lupus erythematosus. Arthritis Rheum 41:1241–1250

46. Cairns AP, Crockard AD, McConnell JR, Courtney PA, Bell AL (2001) Reduced expression of CD44 on monocytes and neutrophils in systemic lupus erythematosus: relations with apoptotic neutrophils and disease activity. Ann Rheum Dis 60:950–955

47. Finckh A, Dudler J, Wermelinger F, Ciurea A, Kyburz D, Gabay C, Bas S, SCQM PO (2010) Influence of anti-infliximab antibodies and residual infliximab concentrations on the occurrence of acquired drug resistance to infliximab in rheumatoid arthritis patients. Joint Bone Spine 77: 313–318

48. Yamada A, Sono K, Hosoe N, Takada N, Suzuki Y (2010) Monitoring functional serum antitumor necrosis factor antibody level in Crohn's disease patients who maintained and those who lost response to anti-TNF. Inflamm Bowel Dis 16:1898–1904

49. Reich K, Nestle FO, Papp K, Ortonne JP, Evans R, Guzzo C, Li S, Dooley LT, Griffiths CE, investigators Es (2005) Infliximab induction and maintenance therapy for moderate-to-severe psoriasis: a phase III, multicentre, double-blind trial. Lancet 366: 1367–1374

50. Adişen E, Aral A, Aybay C, Gürer MA (2010) Anti-infliximab antibody status and its relation to clinical response in psoriatic patients: a pilot study. J Dermatol 37:708–713

51. Sugita S, Yamada Y, Mochizuki M (2010) Relationship between serum infliximab levels and acute uveitis attacks in patients with Behçet disease. Br J Ophthalmol 95:549–552

Genome variability of human adenovirus type 8 causing epidemic keratoconjunctivitis during 1986-2003 in Japan

Xue-Hai Jin,[1] Koki Aoki,[1] Nobuyoshi Kitaichi,[2,3] Toshihide Ariga,[3] Susumu Ishida,[1] Shigeaki Ohno[2]

[1]Department of Ophthalmology, Hokkaido University Graduate School of Medicine, Sapporo, Japan; [2]Department of Ocular Inflammation and Immunology, Hokkaido University Graduate School of Medicine, Sapporo, Japan; [3]Department of Ophthalmology, Health Sciences University of Hokkaido, Sapporo, Japan

Purpose: Epidemic keratoconjunctivitis (EKC) is a contagious acute conjunctivitis associated with community-acquired infection. Human adenovirus type 8 (HAdV-8) is one of the major serotypes isolated from patients with EKC. DNA restriction enzyme analyses were performed to investigate the genetic characteristics of the isolates and their chronological pattern.
Methods: Viral samples were taken from 11 strains isolated from sporadic cases of EKC and identified as HAdV-8 by the neutralization method with type-specific antiserum against HAdV-8 between 1986 and 2003 in Japan. DNA restriction enzyme analysis included six restriction enzymes: BamHI, HindIII, PstI, SacI, SalI, and SmaI.
Results: The restriction patterns revealed that the genome types were HAdV-8A and HAdV-8B in 1986, HAdV-8K in 1991, and HAdV-8E in 1996. HAdV-8K was a new genome type revealed with the enzyme SacI. Two strains isolated in 2003 exhibited identical restriction patterns as HAdV-54, which was described in 2008 and collected from Japanese patients in 2000.
Conclusions: Genetic changes might occur chronologically in HAdV-8. HAdV-8 displays considerable variability. The investigations of these variants might be helpful for defining the evolutionary tendency and to predict future outbreaks of HAdV infection.

Human adenoviruses (HAdVs) cause ocular infections. The most severe disease among ocular infections is epidemic keratoconjunctivitis (EKC), which is characterized by bilateral, acute, severe keratoconjunctivitis and known for frequent intrafamilial infection [1]. EKC is commonly caused by HAdV-8, followed by HAdV-19 and HAdV-37, members of species D of human adenovirus [2,3]. HAdV-8 was first described in the United States in 1955, and the virus was isolated from a sailor (Trim) who had EKC and had arrived from the Orient [4]. Since that time, HAdV-8 has been isolated all over the world from typical cases of EKC. Using restriction enzyme analysis, the serotypes are subclassified into genome types, nominated according to the chronology reported in the literature. HAdV-8A and HAdV-8B were shown to have been circulating in the population of Sapporo, Japan, between 1975 and 1981 [5]. HAdV-8C, D, E, F, G, and H were detected in Kaohsiung, Taiwan from 1980 to 1994 [2,6,7]. The genome type HAdV-8E was also found in South Korea [8]. In Australia and the Philippines, only the prototype strain of HAdV-8 was found [9]. HAdV-8I was isolated from an outbreak of EKC in 1995 and from sporadic cases until 1997 in Hiroshima, Japan [10]. In Europe, HAdV-8 strains isolated

in Germany were classified HAdV-8/D1 to HAdV-8/D6, and substitution of the fastidious Trim strain by the well growing strain D1 as a prototype was suggested [11]. Later, additional genome types HAdV-8/D7 to HAdV-8/D10 were reported [12]. Following this nomenclature system, genome types HAdV-8/D11 and HAdV-8/D12 were isolated in Brazil [13].

To date, HAdV-8A, B, E, and I have been found in Japan as variants of HAdV-8. Recently, two novel HAdV types causing nosocomial EKC were reported from Japan [14-16]. One of them has sometimes been mistyped as HAdV-8, because it is similar to HAdV-8 according to neutralization test (NT) and phylogenetic analyses. However, the virus showed completely different restriction patterns from those of other published HAdV-8 genome types, revealing it is a novel serotype. It is named as HAdV-54 today [14].

In the present study, using HAdV-8 strains isolated between 1986 and 2003 in Japan, we reconfirmed the HAdV type by NT and phylogeny-based classification of partial hexon sequences. Moreover, the genetic differences among the isolates were analyzed by DNA restriction enzyme analysis.

METHODS

Viral strains: Eleven strains of HAdV-8 were isolated from sporadic cases of EKC in Japan (Table 1). Strains number 1, 2, and 3 were isolated in 1986, number 4, 5, and 6 in 1991, and number 7, 8, and 9 in 1996 in Sapporo, northern part of Japan. number 10 was isolated in 2003 in Itoman, the Okinawa

Correspondence to: Nobuyoshi Kitaichi, M.D., Ph.D., Department of Ocular Inflammation and Immunology, Hokkaido University Graduate School of Medicine, Kita-15, Nishi-7, Kita-ku, Sapporo 060-8638, Japan; Phone: +81-11-706-5944; FAX: +81-11-706-5948; email: nobukita@med.hokudai.ac.jp

TABLE 1. SUMMARY OF GENOME TYPE OF 11 HAdV STRAINS ISOLATED IN JAPAN DURING 1986–2003.

Strains of samples	Genome types	Years collected	Enzyme code*						Neutralization test titer	Samples collected in
			BamHI	HindIII	PstI	SacI	SalI	SmaI		
	HAdV-8P	1955	1	1	1	1	1	1	>64	(ATCC)
1	HAdV-8A	1986	2	2	2	2	2	2	>64	Sapporo
2,3	HAdV-8B	1986	2	2	2	2	3	2	>64	Sapporo
4,5,6	HAdV-8K	1991	2	3	2	3	2	2	>64	Sapporo
7,8,9	HAdV-8E	1996	2	3	2	2	2	2	>64	Sapporo
10	HAdV-54	2003	3	4	3	4	4	3	32	Itoman
11	HAdV-54	2003	3	4	3	4	4	3	16	Matsuyama

*Enzyme codes are displayed in alphabetical order: BamHI, HindIII, PstI, SacI, SalI, SmaI. The restriction patterns of HAdV-8P for each enzyme are called number 1. The other patterns are consecutively numbered in chronological order of appearance. The numbering in this study was performed according to the methods of previous report [25]. HAdV-8P: HAdV-8 prototype.

region, and number 11 was isolated in 2003 in Matsuyama, both are southweste area of Japan. All isolates were propagated in A549 cells and identified as HAdV-8 using NT. The HAdV-8 prototype strain was purchased from the American Type Culture Collection (Manassas, VA).

Serological analysis: Those 11 samples of strains were serologically analyzed by a quantitative serum NT with HAdV-8 type-specific antisera purchased from Denka Seiken Co., Ltd. (Tokyo, Japan) to confirm our previous classification. NT was performed in A549 cells on 96-well microplates. The 50% tissue culture infective dose (TCID50) of each HAdV that caused a cytopathic effect after 7 days of incubation at 37 °C was calculated, and 100 TCID50s was used for the challenge virus. Duplicates of the serially twofold diluted antisera were used in the NT.

Virus propagation and DNA extraction: All of the strains were inoculated into culture tubes containing a subconfluent monolayer of A549 cells. The inoculated tubes were maintained for 1 h at 35 °C for viral absorption, and then 2 ml of maintenance medium was added. The inoculated cultures were incubated at 35 °C with medium changes at intervals of 3 or 4 days and examined daily until the appearance of cytopathic effects. The viral DNA was extracted following a previously described protocol with some modifications [17]. The cells were pelleted and rinsed twice with phosphate-buffered saline and then suspended in 1 ml of Hirt lysis solution (10 mM Tris, 1 mM EDTA, 0.6% SDS, pH 8.0). Proteinase K was added to a final concentration of 50 μg/ml and the samples incubated at 37 °C for 1 h. Cellular DNA was precipitated with NaCl (1 M) overnight at 4 °C and discharged. The supernatant was cleaned with a mixture of RNases A and T1, proteinase K (200 μg/ml), and phenol-chloroform extraction. Viral DNA was precipitated with ethanol and suspended in 50 μl of TE buffer (1 mM Tris-HCl, 0.1 mM EDTA, pH 8.0).

Phylogeny-based classification for HAdV typing: For HAdV typing, nucleotide sequences in the partial hexon were amplified and subjected to phylogenetic analysis as described

previously [18]. The nucleotide sequences of the PCR products were determined using a CEQ 2000XL DNA analysis system with a DyeTerminator cycle sequencing kit (Beckman Coulter, Fullerton, CA) and compared with those of all HAdV types using SINCA (Fujitsu Limited, Tokyo, Japan). The evolutionary distances were estimated using Kimura's two-parameter method [19], and unrooted phylogenetic trees were constructed using the neighbor-joining method [20]. Bootstrap analyses were performed with 1,000 resamplings of the data sets.

DNA restriction enzyme analysis: Aliquots of viral DNA (2 μl, approximately 1 μg) were digested with 10 units of restriction enzymes BamHI, HindIII, PstI, SacI, SalI, and SmaI under conditions specified by the manufacturer (Takara Shuzo Co., Kyoto, Japan). Restriction enzyme digests were loaded onto 1.5% agarose gels and run for approximately 2 h at 100 V in Tris-acetate buffer (pH 8.0) with 1 mM EDTA. After staining with ethidium bromide (1 μg/ml), the fragments were visualized under UV transilluminator and photographed with a PolaroidTM camera. Subgenus and genome type identification was performed by comparing the resulting patterns with the published restriction patterns of the prototypes and genome types.

RESULTS

Serological analysis: Eleven strains of samples were specifically neutralized with the antiserum against HAdV-8. However, strains of number 10 and 11 isolated in 2003 reacted to HAdV-8 antiserum only at 1:32 and 1:16 of homologous titer respectively, a weak reaction for the immunological distinctiveness of serotype (Table 1). No other prototype-specific antiserums reacted with these 2 strains. These strains of number 10 and 11 collected in Itoman and Matsuyama were considered as one of the HAdV-8s at that time.

HAdV typing of phylogeny-based classification: The nucleotide sequences of the partial hexon gene were compared with those of all HAdV prototype strains. Strains of number 1 to 9 were clustered in HAdV-8, however number 10 and
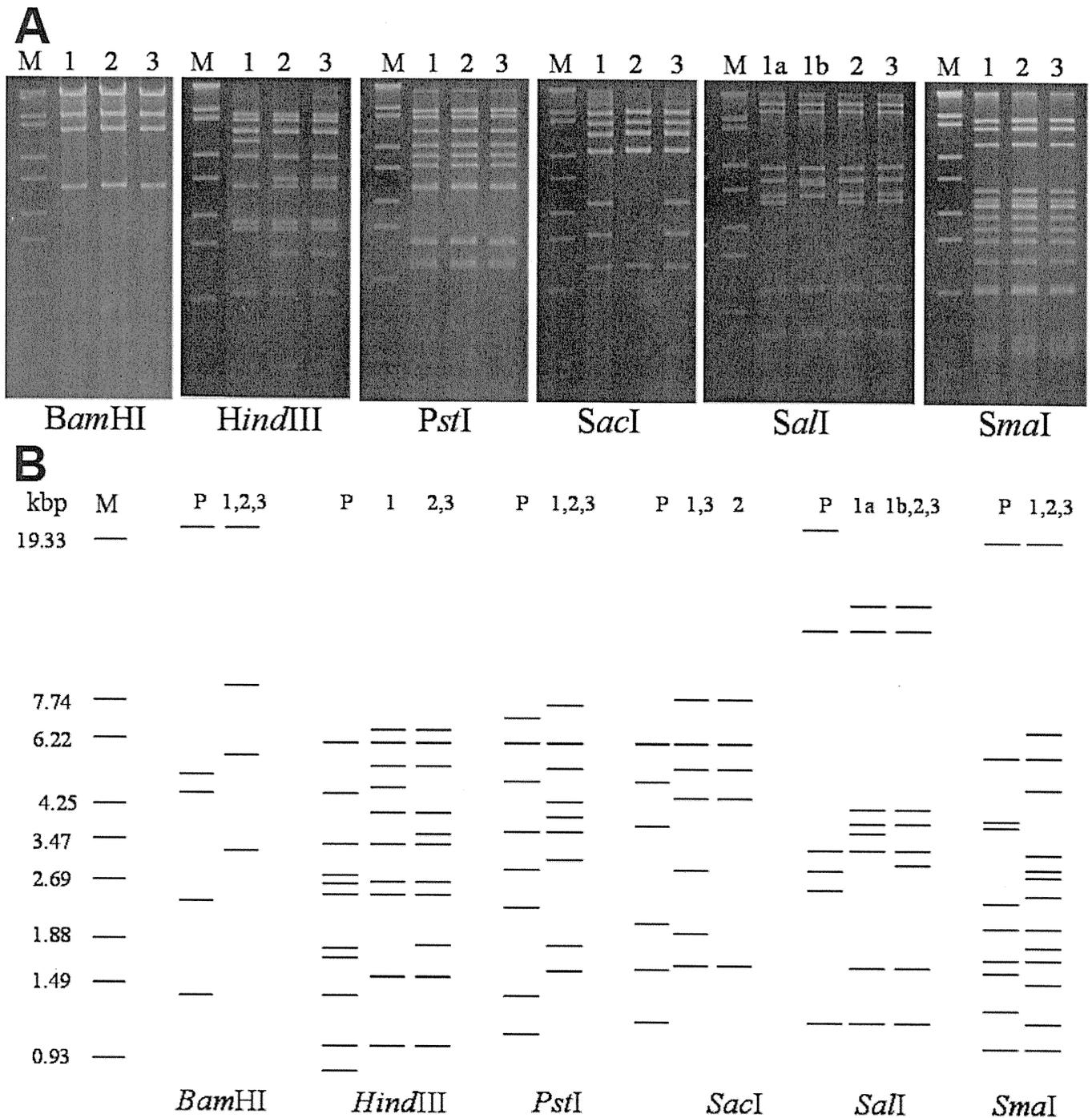
3122

Figure 1. Restriction patterns of strains number 1–9. **A**: Restriction patterns of 9 strains isolated in 1986, 1991, and 1996. Lane M: molecular weight marker (lambda DNA digested with EcoT14I). Lane 1: representative strains number 1–3 isolated in 1986. Lanes 1a and 1b represent the two kinds of restriction patterns for SalI (HAdV-8A and HAdV-8B). Lane 2: representative strains number 4–6 isolated in 1991 (HAdV-8K). Lane 3: representative strains number 7–9 isolated in 1996 (HAdV-8E). **B**: Schematic presentation of the restriction patterns of strains number 1–9 and HAdV-8 prototype.

11were in HAdV-54 (data not shown). Strains of number 10 and 11 were reclassified as HAdV-54.

DNA restriction enzyme analysis: After restriction enzyme digestion, the 11 strains were electrophoresed in two gels. Strains of number 1 to 9 isolated in 1986, 1991, and 1996 that

reacted strongly to HAdV-8 antiserum were run in one gel (Figure 1A). The remaining 2 strains of number 10 and 11 isolated in 2003 showing weak reaction to HAdV-8 antiserum were run in another gel (Figure 2A). To make the restriction patterns of these strains are easy to read, they were also shown
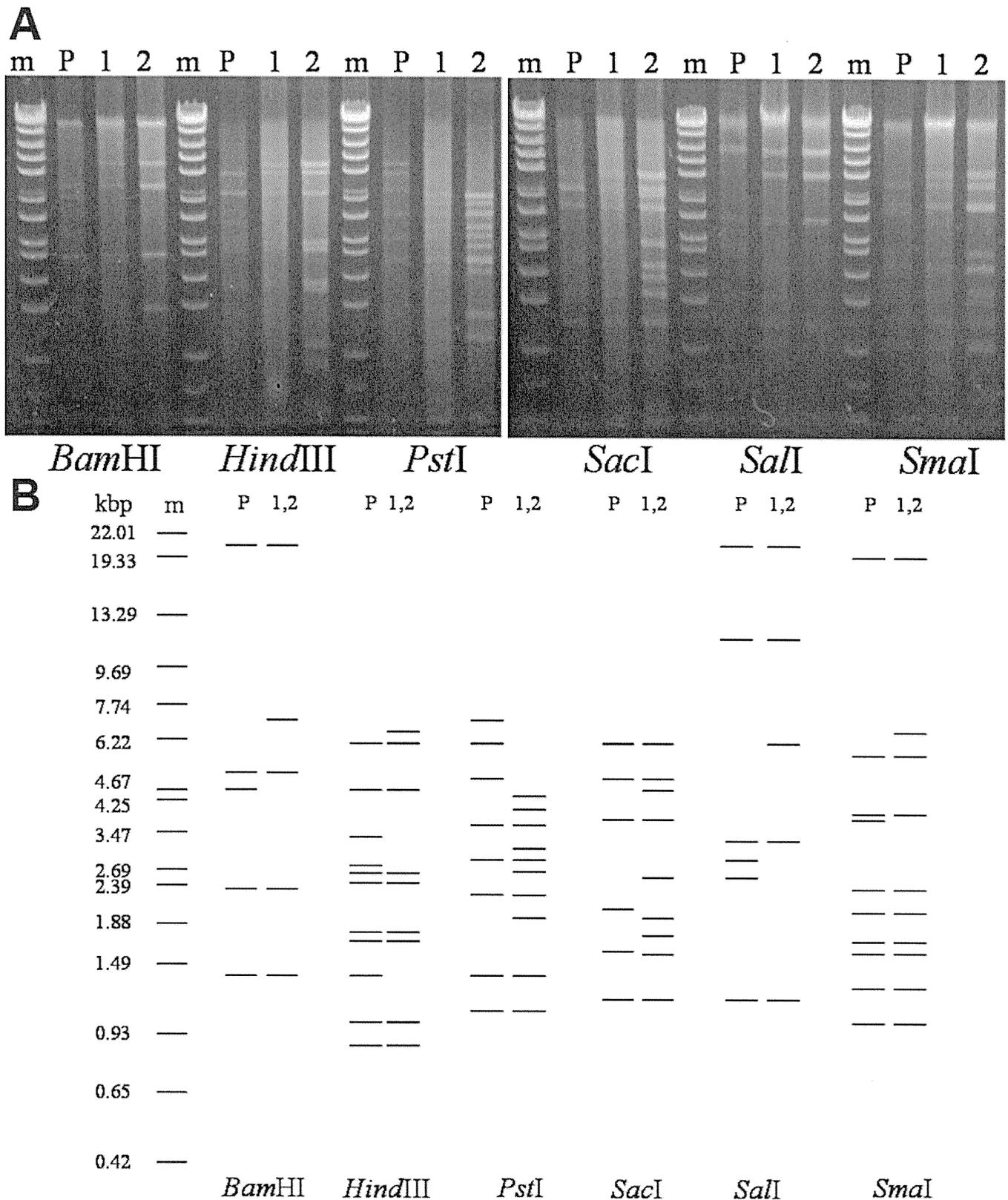
3123

Figure 2. Restriction patterns of strains number 10 and 11. **A**: Restriction patterns of 2 strains isolated in 2003. Lane m: molecular weight marker (lambda DNA digested with EcoT14I plus BglII). Lane P: HAdV-8 prototype. Lane 1: representative strains number 10 isolated in Itoman in 2003 (HAdV-54). Lane 2: representative strains number 11 isolated in Matsuyama in 2003 (HAdV-54). **B**: Schematic presentation of the restriction patterns of strains HAdV-8 prototype and samples number 10 and 11.

as schematic presentations (Figure 1B and Figure 2B). It was revealed that the 11 strains analyzed in this study were divided into five genome types according to the profile of restriction patterns (Table 1). All of 11 strains showed different restriction patterns from those of the HAdV-8 prototype. Of the 3 strains isolated in 1986, number 1 showed restriction patterns similar to HAdV-8A and number 2 and 3 were similar to HAdV-8B. number 4 to 6 isolated in 1991, and number 7 to 9 isolated in 1996 showed identical restriction patterns with restriction endonucleases BamHI, HindIII, PstI, SalI, and SmaI. With SacI, the strains number 4 to 6 isolated in 1991 exhibited different patterns from those of the strains isolated in 1996 and any other previously known HAdV-8 genome types. This new pattern revealed a new genome type of HAdV-8 that we designated as HAdV-8K in accordance with the serial studies in the Asia Pacific area. The restriction patterns of strains number 7 to 9 isolated in 1996 corresponded to HAdV-8E. The restriction patterns of strains number 10 and 11 isolated in 2003 were similar to those of HAdV-54. The results of DNA restriction enzyme analysis are summarized in Table 1.

DISCUSSION

To investigate the genetic characteristics of HAdV-8 isolates and their chronological pattern, we demonstrated the molecular biologic characteristics of 11 HAdV strains isolated from sporadic cases of EKC in Japan over an 18-year period in the present study. All of the strains were identified as HAdV-8 by the neutralization method. In the present study, a new variant strain, HAdV-8K, was identified by the DNA restriction method. The DNA restriction method for genome typing identified two strains isolated in 2003 as HAdV-54, not HAdV-8. These were also confirmed as HAdV-54 by phylogenetic analysis of partial hexon gene. HAdV-54 was not detected before the early 1990s, but replaced HAdV-8 in Japan in the 2000s [21]. HAdV-54 in samples collected in 2003 is consistent with that study. However, major causes of EKC vary in different countries, and HAdV-8 is still the most common pathogenic virus in the Middle East [22].

As HAdV-8 has a much higher tropism for conjunctival cells and produces more severe clinical manifestations and pathological alterations in EKC than HAdV-19 or HAdV-37, HAdV-8 has been the target of extensive study [10]. After HAdV-8 was first isolated as an etiology of EKC, many HAdV-8 genome types have been identified by applying restriction endonucleases and cleavage pattern analysis [7]. Distinct nomenclature systems have been used for these genome types. A nomenclature system using a numerical code to denote adenovirus genome types has been proposed, and HAdV-8/D1 to HAdV-8/D12 have been described depending on the chronological order of the respective isolates [11-13]. Another nomenclature system was also proposed in which HAdV-8 genome types are denoted by alphabetical order [5]. According to the nomenclature system, HAdV-8 genome

types are classified as HAdV-8A to HAdV-8K [5-7,10,23]. Although both systems are commonly used, we adopted the latter nomenclature system here in accordance with a series of collaborative studies of HAdV-8. We compared the restriction patterns of the strains in this study with those of all previously known genome types of HAdV-8. One new genome type was discovered and designated as HAdV-8K.

The cleavage patterns of 2 strains isolated in 2003 with any of the 6 restriction endonucleases were different from those of any other previously known genome type of HAdV-8. The unique restriction patterns indicated that an uncommon mutation or recombination event might have occurred. The restriction patterns of these 2 strains were consistent with those of HAdV-54, which was first reported in 2008 [14]. Furthermore, these strains were neutralized weakly by the antiserum against HAdV-8. Similarly, HAdV-54 responds weakly to the antiserum for HAdV-8. Because of the absence of serotype specific antiserum, the weak reaction with the antiserum against HAdV-8 might be a clue for detecting HAdV-54 at the current time. The strains of number 10 and 11 were also confirmed as HAdV-54 by phylogeny-based classification, which is an effective tool for the rapid identification of HAdVs.

Although HAdV-8 seems to display considerable variability and adenoviral ocular infection is common in East Asian countries, few studies on the circulation of HAdV-8 genome types have been reported in Japan. Thus, this is a report even more focused on the genetic characteristics of HAdV-8 isolates and their chronological patterns than the analysis of a new genome type. Two studies reported the isolation of HAdV-8A and HAdV-8B in Sapporo [5], and the circulation of HAdV-8 genome types in Hiroshima over a 15-year period [10]. In this study, restriction endonuclease analyses revealed a new genome type, HAdV-8K, in Sapporo, where HAdV-8A and HAdV-8B are endemic. The strains isolated in 1986 were HAdV-8A and HAdV-8B, and HAdV-8K strains were isolated from samples collected in 1991. The strains isolated in 1996 exhibited a similar restriction pattern as that of HAdV-8E, which was first isolated in Kaohsiung, Taiwan [6]. HAdV-8 was reported as being prevalent in 1986, 1991, and 1996 in Sapporo [2,24]. Our present results show that different genome types of HAdV-8 appeared in the three prevalence periods, which may indicate that a change in genome type resulted in the next prevalence period of HAdV-8. On the other hand, high frequency of infection in the population is regarded as a probable reason for the diverse evolution of HAdV-8 [7]. As HAdV-8 has become less common in Japan since 1997, a follow-up study of HAdV-8 should be completed in Japan and why we conducted this study.

HAdV-54 has recently been the major cause of EKC in Japan. The strain has occasionally been identified as HAdV-8 due to its weak reaction to HAdV-8 antiserum. On the basis

3125

of the nucleotide sequence identities and phylogenetic analysis, HAdV-54 was much closer to the HAdV-8 strains over the entire genome than other HAdVs [15].

Our results show that genetic changes in HAdV-8 occur chronologically. HAdV-8 displays considerable variability. Because continued investigations of these variants might be helpful for defining the evolutionary tendency and to predict future outbreaks of HAdV infection, further genetic, epidemiological, and clinical surveillance of HAdVs should be performed.

ACKNOWLEDGMENTS

REFERENCES

1. Aoki K, Kaneko H, Kitaichi N, Ohguchi T, Tagawa Y, Ohno S. Clinical features of adenoviral conjunctivitis at the early stage of infection. Jpn J Ophthalmol 2011; 55:11-5. [PMID: 21331686]
2. Aoki K, Tagawa Y. A twenty-one year surveillance of adenoviral conjunctivitis in Sapporo, Japan. Int Ophthalmol Clin 2002; 42:49-54. [PMID: 12189615]
3. Tanaka-Yokogui K, Itoh N, Usui N, Takeuchi S, Uchio E, Aoki K, Usui M, Ohno S. New genome type of adenovirus serotype 19 causing nosocomial infections of epidemic keratoconjunctivitis in Japan. J Med Virol 2001; 65:530-3. [PMID: 11596089]
4. Jawetz E, Kimura S, Nicholas AN, Thygeson P, Hanna L. new type of APC virus from epidemic keratoconjunctivitis. Science 1955; 122:1190-1. [PMID: 13274081]
5. Fujii SI, Nakazono N, Sawada H, Ishii K, Kato M, Aoki K, Ohtsuka H, Fujinaga K. Restriction endonuclease cleavage analysis of adenovirus type 8: two new subtypes from patients with epidemic keratoconjunctivitis in Sapporo, Japan. Jpn J Med Sci Biol 1983; 36:307-13. [PMID: 6328068]
6. Fujii S, Nakazono N, Ishii K, Lin CC, Sheu MM, Chen CW, Fujinaga K. Molecular epidemiology of adenovirus type 8 (Ad 8) in Taiwan: four subtypes recovered during the period of 1980–1981 from patients with epidemic keratoconjunctivitis. Jpn J Med Sci Biol 1984; 37:161-9. [PMID: 6098752]
7. Chang C, Sheu M, Chern C, Lin K, Huang W, Chen C. Epidemic keratoconjunctivitis caused by a new genotype of adenovirus type 8 (Ad8)-a chronological review of Ad8 in Southern Taiwan. Jpn J Ophthalmol 2001; 45:160-6. [PMID: 11313048]
8. Ishii K, Nakazono N, Fujinaga K, Fujii S, Kato M, Ohtsuka H, Aoki K, Chen CW, Lin CC, Sheu MM, Lin KH, Oum BS, Lee SH, Chun CH, Yoshii T, Yamazaki S. Comparative studies on aetiology and epidemiology of viral conjunctivitis in three countries of East Asia–Japan, Taiwan and South Korea. Int J Epidemiol 1987; 16:98-103. [PMID: 3032816]
9. Guo DF, Shinagawa M, Aoki K, Sawada H, Itakura S, Sato G. Genome typing of adenovirus strains isolated from conjunctivitis in Japan, Australia, and the Philippines. Microbiol Immunol 1988; 32:1107-18. [PMID: 2851695]
10. Adhikary AK, Numaga J, Kaburaki T, Kawashima H, Araie M, Ikeda Y, Ogino T, Suzuki E, Ushijima H, Mukoyama A, Matsuno S, Inada T, Okabe N. Genetic characterisation of adenovirus type 8 isolated in Hiroshima city over a 15 year period. J Clin Pathol 2003; 56:120-5. [PMID: 12560390]
11. Adrian T, Wolf U, Lauer HJ, Wigand R. Restriction site mapping of adenovirus type 8 genome types. Res Virol 1990; 141:611-24. [PMID: 2087599]
12. de Jong JC, Demazure M, Legrand-Quillien MC, Le Lay G, Colin J, Wermenbol AG, Verweij-Uyterwaal MW, van der Avoort HG, Chastel C. New developments in the molecular epidemiology of adenovirus 8 keratoconjunctivitis. J Med Virol 1992; 38:102-7. [PMID: 1334124]
13. Tanaka K, Itoh N, Saitoh-Inagawa W, Uchio E, Takeuchi S, Aoki K, Soriano E, Nishi M, Junior RB, Harsi CM, Tsuzuki-Wang L, Durigon EL, Stewien KE, Ohno S. Genetic characterization of adenovirus strains isolated from patients with acute conjunctivitis in the city of Sao Paulo, Brazil. J Med Virol 2000; 61:143-9. [PMID: 10745247]
14. Ishiko H, Shimada Y, Konno T, Hayashi A, Ohguchi T, Tagawa Y, Aoki K, Ohno S, Yamazaki S. Novel human adenovirus causing nosocomial epidemic keratoconjunctivitis. J Clin Microbiol 2008; 46:2002-8. [PMID: 18385435]
15. Kaneko H, Iida T, Ishiko H, Ohguchi T, Ariga T, Tagawa Y, Aoki K, Ohno S, Suzutani T. Analysis of the complete genome sequence of epidemic keratoconjunctivitis-related human adenovirus type 8, 19, 37 and a novel serotype. J Gen Virol 2009; 90:1471-6. [PMID: 19264666]
16. Aoki K, Ishiko H, Konno T, Shimada Y, Hayashi A, Kaneko H, Ohguchi T, Tagawa Y, Ohno S, Yamazaki S. Epidemic keratoconjunctivitis due to the novel hexon-chimeric-intermediate 22,37/H8 human adenovirus. J Clin Microbiol 2008; 46:3259-69. [PMID: 18701656]
17. Wadell G, de Jong JC. Restriction endonucleases in identification of a genome type of adenovirus 19 associated with keratoconjunctivitis. Infect Immun 1980; 27:292-6. [PMID: 6247272]
18. Miura-Ochiai R, Shimada Y, Konno T, Yamazaki S, Aoki K, Ohno S, Suzuki E, Ishiko H. Quantitative detection and rapid identification of human adenoviruses. J Clin Microbiol 2007; 45:958-67. [PMID: 17229856]
19. Kimura M. A simple method for estimating evolutionary rates of base substitutions through comparative studies of nucleotide sequences. J Mol Evol 1980; 16:111-20. [PMID: 7463489]
20. Saitou N, Nei M. The neighbor-joining method: a new method for reconstructing phylogenetic trees. Mol Biol Evol 1987; 4:406-25. [PMID: 3447015]
21. Kaneko H, Suzutani T, Aoki K, Kitaichi N, Ishida S, Ishiko H, Ohashi T, Okamoto S, Nakagawa H, Hinokuma R, Asato Y, Oniki S, Hashimoto T, Iida T, Ohno S. Epidemiological and virological features of epidemic keratoconjunctivitis due to new human adenovirus type 54 in Japan. Br J Ophthalmol 2011; 95:32-6. [PMID: 20530657]
22. Tabbara KF, Omar N, Hammouda E, Akanuma M, Ohguchi T, Ariga T, Tagawa Y, Kitaichi N, Ishida S, Aoki K, Ishiko H, Ohno S. Molecular epidemiology of adenoviral keratoconjunctivitis in Saudi Arabia. Mol Vis 2010; 16:2132-6. [PMID: 21139691]

23. Adhikary AK, Banik U, Okabe N, Fujimoto T. Molecular characterization of human adenovirus type 8 (HAdV-8), including a novel genome type detected in Japan. Jpn J Infect Dis 2011; 64:493-8. [PMID: 22116328]

24. Saitoh-Inagawa W, Aoki K, Uchio E, Itoh N, Ohno S. Ten years' surveillance of viral conjunctivitis in Sapporo, Japan. Graefes Arch Clin Exp Ophthalmol 1999; 237:35-8. [PMID: 9951639]

25. de Jong TA, Wermenbol AG, van der Avoort HG, Wigand R. Genome type analysis of adenovirus 37 isolates. J Med Virol 1988; 25:77-83. [PMID: 2842449]

BASIC SCIENCE

Astaxanthin increases choroidal blood flow velocity

**Michiyuki Saito · Kazuhiko Yoshida · Wataru Saito ·
Akio Fujiya · Kazuhiro Ohgami · Nobuyoshi Kitaichi ·
Hiroki Tsukahara · Susumu Ishida · Shigeaki Ohno**

Abstract

Purpose Previous studies have reported that astaxanthin (AXT) has antioxidative and anti-inflammatory effects in addition to its ability to shorten blood transit times. As laser speckle flowgraphy (LSFG) can noninvasively visualize the hemodynamics of the choroidal circulation, we used the technique to evaluate whether continuous ingestion of 12 mg of AXT per day could increase quantitative blood flow velocity.

Methods In this randomized, double-blind, placebo-controlled study, we examined 20 healthy volunteers who ingested 12 mg AXT or placebo capsules over a 4-week period. LSFG was measured in the right eyes of all subjects at pre-ingestion, and at 2 and 4 weeks after the treatment of AXT. LSFG values were used to calculate the square blur rate (SBR), which is a quantitative index of relative blood flow velocity.

Results A significant increase of the macular SBR was seen 4 weeks after AXT ingestion when compared to the pre-ingestion values (Wilcoxon signed-rank test, $P=0.018$). In contrast, no statistical difference in the macular SBR was detected in the placebo group (Friedman test, $P=0.598$). No subjective or objective adverse events were found after the 12-mg AXT ingestion.

Conclusions Results suggest that administration of AXT over a 4-week period can elevate the choroidal blood flow velocity without any adverse effects.

M. Saito · K. Yoshida (✉) · W. Saito · A. Fujiya · K. Ohgami ·
N. Kitaichi · S. Ishida
Department of Ophthalmology,
Hokkaido University Graduate School of Medicine,
Nishi-7-chome, Kita 15-jou, Kita-ku,
Sapporo 060-8638, Japan
e-mail: kyoshida@med.hokudai.ac.jp

N. Kitaichi
Department of Ophthalmology,
Health Sciences University of Hokkaido,
Sapporo, Japan

H. Tsukahara
Fuji Chemical Industry Co., Ltd,
Tokyo, Japan

S. Ohno
Department of Ocular Inflammation and Immunology,
Hokkaido University Graduate School of Medicine,
Sapporo, Japan

Introduction

Astaxanthin (AXT) is one of the most common carotenoids, and is found in the red pigment of crustacean shells (e.g., crabs and shrimp), salmon, salmon roe, and members of Asteroidea (e.g., starfish). The chemical structure of this carotenoid has been shown to be modified, with the long-chain conjugate double bond and the b-ionone ring at both ends replaced by a keto- and hydroxyl-group respectively. Discovery of AXT's strong antioxidant effect has led to a large number of studies focusing on the compound, with these studies demonstrating a wide variety of biological activities that have included antioxidant [1], anti-tumor [2], anti-Helicobacter pylori effects [3], anti-arteriosclerosis [4], anti-inflammatory [5], or even perhaps control diabetic nephropathy (kidney disease) [6, 7].

Additionally, it has been reported that AXT ingestion has the effect of improving the microcirculation [8, 9]. A

🖄 Springer