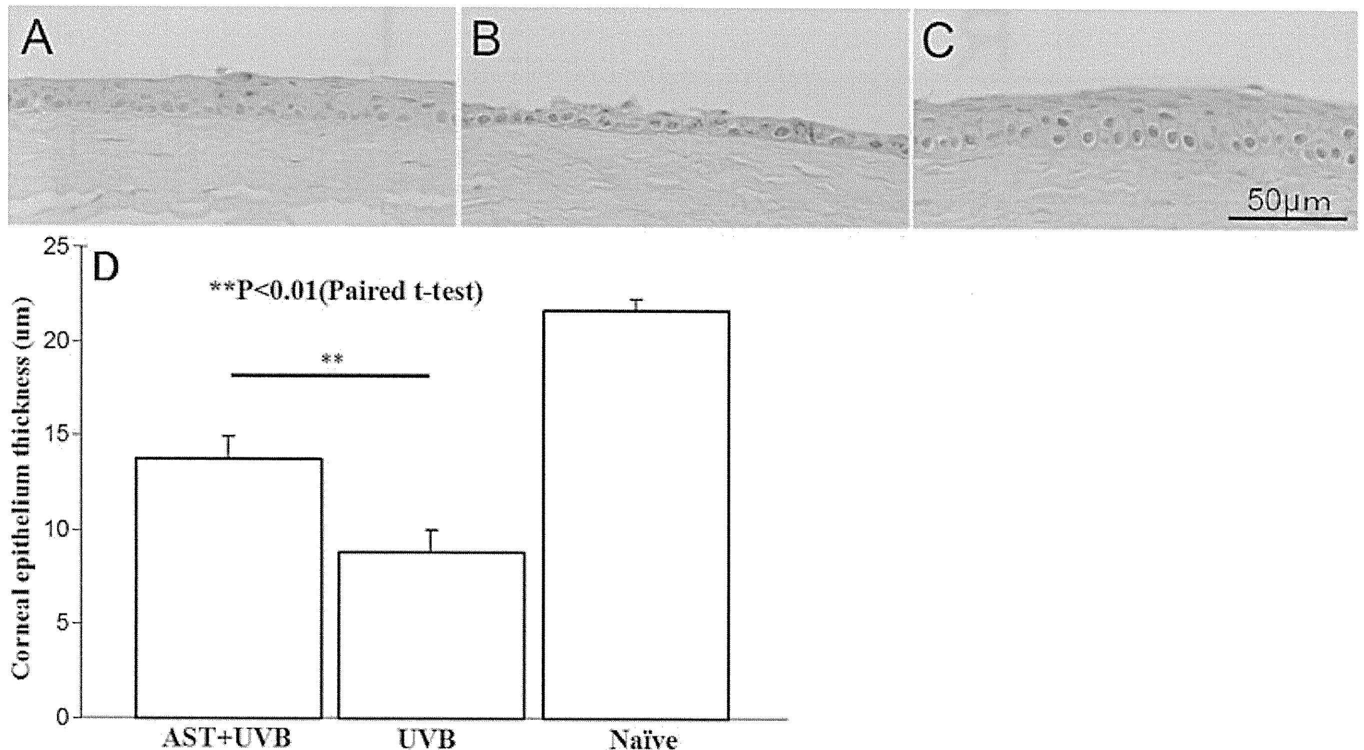

Figure 2. Morphological properties of ultraviolet (UV)-irradiated corneas treated with AST after irradiation. Eyes were treated with AST eye drops within 5 min after UVB exposure (**A**: 1 mg/ml, **B**: 0.1 mg/ml). Control subjects were not irradiated with UVB (**C**). The mean values of corneal epithelial thickness are summarized (**D**). Epithelia were significantly thicker when treated with 1 mg/ml AST compared to fellow eyes as controls (p<0.01). The protective effects of AST remained, even if eyes were treated after irradiation.

maximum LDH release were assumed to be 100%. All experiments were performed in triplicate with 5 or more wells for each group in each experiment.

Statistical analysis: All data were expressed as the mean ±standard deviation (SD) from the respective test or control groups of data. Statistical significances were determined by the paired *t*-test and non-parametric Mann–Whitney U-test. P values less than 0.05 were considered significant.

RESULTS

Morphological properties: We examined the morphological properties of UVB-irradiated and control corneas using H&E staining. At 24 h after UVB irradiation at a dose of 400 mJ/cm^2, thinning and ulceration of the corneal epithelial layer were observed (Figure 1). The corneal epithelial thicknesses were 25.6±2.9, 18.8±3.5, and 8.2±3.6 μm in eyes treated with 1, 0.1, and 0.01 mg/ml of AST, respectively. The mean corneal epithelial thickness in eyes untreated with AST was 8.91±5.3 μm after UVB-exposure. The mean corneal epithelium thickness of non-irradiated eyes was 29.6±0.5 μm. Corneal epithelium was well preserved, and the thickness of the epithelium remained close to normal in the right eyes treated with 1 mg/ml of AST instillation (Figure 1A). Its protective effect decreased gradually in a concentration-dependent manner (Figure 1B,C). The corneas of mice administered with AST without UVB irradiation (Figure 1D) showed no differences from naïve corneas (Figure 1D). The mean values of the epithelium thickness of corneas were calculated and summarized (Figure 1E). Corneal epithelia were significantly thicker in eyes treated with 1 and 0.1 mg/ml AST eye drops compared with vehicle-treated eyes (p<0.01). Irradiated corneas treated with 1 mg/ml AST showed corneal epithelium thickness close to naïve cornea. AST protected corneal epithelium in a dose-dependent manner (Figure 1E).

We also examined the morphological properties of UVB-irradiated and control corneas using H&E staining when AST was applied to corneas at a concentration of 1 mg/ml 5 min after UVB irradiation at a dose of 400 mJ/cm^2. Twenty-four hours later, the corneal epithelial thickness was 13.7±0.8 μm (Figure 2A). The mean corneal epithelial thickness of vehicle-given corneas was 8.7±1.1 μm after UVB-exposure (Figure 2B). Non-irradiated corneal epithelium was 21.6±0.6 μm thick (Figure 2C). Even when AST was administered after UVB irradiation, the corneal epithelium was significantly thicker than in fellow eyes treated with vehicle (p<0.01, Figure 2D).

TUNEL staining: We compared TUNEL staining in the corneas of AST-treated and untreated eyes of the mice 24 h after UV exposure. Few TUNEL-positive nuclei were detected in corneas of mice treated with AST at 1 and 0.1 mg/ml compared to untreated mice after UV irradiation (Figure

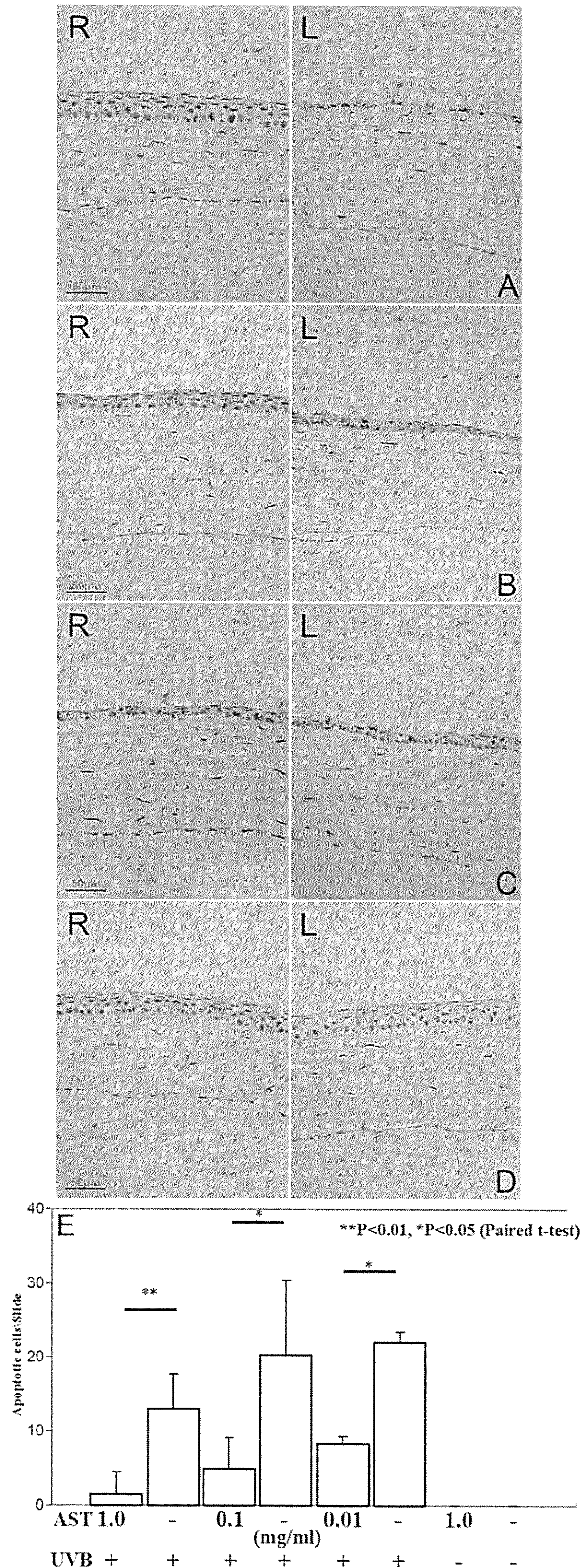

Figure 3. TUNEL labeling at irradiated corneas with and without AST-treatment. Eyes were treated with AST eye drops before UV exposure (**A**: 1 mg/ml, **B**: 0.1 mg/ml, **C**: 0.01 mg/ml AST). Control subjects were not irradiated with UVB (**D**). R: Right eyes were given various concentrations of AST eye drops. L: Left eyes were given vehicle alone as controls. Numbers of apoptotic corneal cells per slide after UVB exposure is shown (**E**). Apoptotic cells were significantly fewer in right eye corneas treated with 1 ($p<0.01$), 0.1 ($p<0.05$), and 0.01 ($p<0.05$) mg/ml AST eye drops compared to the left eyes, which served as controls. There were no apoptotic cells detected in corneas without irradiation.

A
B
C
50µm
DAPI DHE Merge

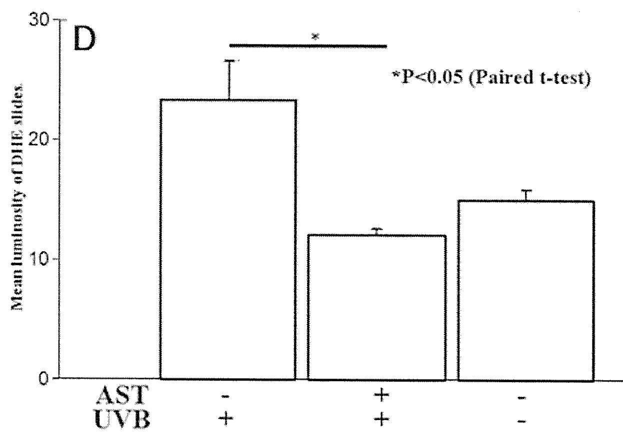

Figure 4. Reactive oxygen species signal expression after UVB exposure. Reactive oxygen species (ROS) were strongly detected (red) in untreated corneal epithelium after UVB irradiation (**A**). The ROS signal was weak in AST treated corneas (**B**) and close to unspecific ROS signaling in naïve corneas (**C**). The mean gray values of the corneal epithelium of DHE stained slides were evaluated by Image J software and summarized (**D**). Mean gray values were significantly lower in corneas treated with 1 mg/ml AST eye drops than in the vehicle-treated eyes ($p<0.05$).

YO-PRO®-1 NF-κB P65 Merge

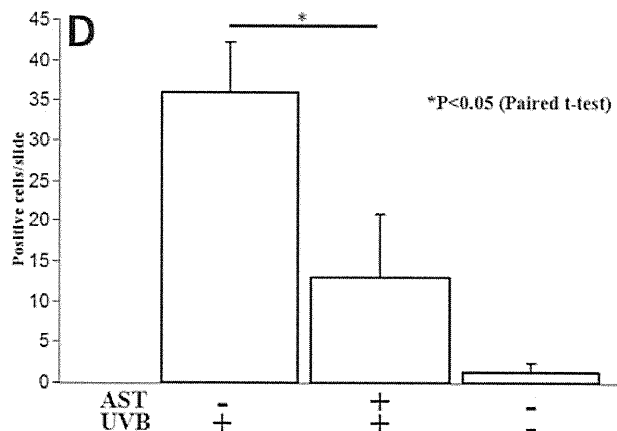

Figure 5. NF-κB expression in corneal epithelia. Eyes were given vehicle alone (**A**) or treated with AST eye drops before UV exposure (**B**); control subjects were not irradiated with UVB (**C**). The mean values of NF-κB positive cells (yellow) in corneal epithelial were summarized (**D**). Expression of NF-κB was significantly downregulated in AST-treated mice after UVB irradiation ($p<0.05$).

3A,B). No significant protective effect was observed in eyes treated with AST 0.01 mg/ml (Figure 3C). There were no apoptotic cells detected in corneas without UVB exposure (Figure 3D).

TUNEL-positive cells were counted, and the numbers were 1.5±3.0, 5.0±4.1, and 8.25±0.9 in eyes treated with 1, 0.1, and 0.01 mg/ml of AST, respectively (Figure 3E). The mean number of apoptotic cells was 18.4±4.7 in vehicle-given irradiated corneas. The apoptotic cells were significantly fewer in corneas treated with AST than those with vehicle only ($p<0.01$).

Detection of reactive oxygen species (ROS): Confocal microscopic images was examined to quantify reactive oxygen species (ROS) production in mouse corneal tissue. ROS production was determined by conversion of DHE to ethidium bromide (EtBr). All images were made in parallel at identical settings (Figure 4). ROS were strongly detected in untreated corneal epithelium after UVB irradiation (Figure 4A). However, the ROS signal was downregulated in AST-treated corneas (Figure 4B) and close to unspecific ROS signaling in naïve corneas (Figure 4C). The mean gray values of the corneal epithelium of DHE stained slides were evaluated by Image J software and summarized in Figure 4D. The mean gray value in vehicle-given irradiated corneas was 23.3±3.3. In eyes treated with 1 mg/ml of AST, after UVB irradiation, the mean gray value was 12.08±0.5. Non-irradiated corneal epithelium showed a 15.04±0.8 mean gray value. AST-treated epithelium showed significantly ($p<0.05$) lower mean gray values than irradiated corneas did.

NF-κB downregulation by AST: Previously, we reported that AST decreased NF-κB expression in endotoxin-induced uveitis (EIU) and choroidal neovascularization [23,24]. To examine whether AST administration effects NF-κB in corneal epithelium, we immunohistochemically analyzed AST expression in the collected corneal tissues and examined it with confocal microscope. NF-κB positive nuclei (yellow) were found to be 13.0±7.9 in corneal epithelial cells in UV-irradiated mice treated with AST at 1.0 mg/kg (Figure 5A). However, 36.0±6.2 (Figure 5B) of multiple NF-κB positive cells were found in the corneal epithelial cells in UV-irradiated mice untreated with AST. Naïve mice corneal tissues showed weak response to anti-NF-κB antibodies, and only 1.3±1.1 positive nuclei were found (Figure 5C). Expression of NF-κB was significantly downregulated in AST-treated mice after UVB irradiation ($p<0.05$, Figure 5D).

AST suppressed phototoxicity in corneal epithelial cell cultures after UVB irradiation: Next, cytotoxicity was examined in UVB-irradiated TKE2 cells, murine corneal epithelium-derived progenitor cell line, treated or untreated with AST in vitro (Figure 6). The percentages of cytotoxicity after irradiation were 17.6±2.0, 29.5±2.2, 31.7.2±2.8, and 32.7±1.7% in wells containing 1, 0.1, 0.01, and 0 mg/ml of AST, respectively. Cytotoxicity was significantly suppressed by 1 mg/ml ($p<0.01$) and 0.1 mg/ml ($p<0.05$) AST administration. The effect was AST concentration-dependent.

DISCUSSION

The corneal epithelium serves to protect corneal structures against UV damage by absorbing a substantial amount of UV energy. Though the energy of UV is much less than that of ionizing radiation rays, the injuries on cells and DNA are critical. Excessive UVB irradiation induces various changes of DNA, proteins and cells, through activation of pro-inflammatory mediators including NF-κB. AST can form stable resonance structures by attachment of the carbonyl and hydroxyl groups to its β-ionone ring. It can also remove the chain-carrying lipid peroxyl radicals in the liposomal suspension, protecting cells from oxidative and free radical damages. NF-κB activates proinflammatory cytokines, chemokines, and enzymes that generate mediators of inflammation. It can also activate adhesion molecules that play a key role in the initial recruitment of leukocytes to sites of inflammation. Therefore, activation of NF-κB leads to a coordinated upregulation of many genes whose products mediate the inflammatory loop and perpetuate local inflammatory responses. In our previous studies, AST inhibited the in vivo activation of NF-κB in endotoxin-induced uveitis (EIU) and choroidal neovascularization [23, 24]. Therefore, reactive oxygen species-induced oxidative stress may play an important role in NF-κB activation and proinflammatory cytokine production. Our immunohistochemical finding shows that AST has the same effects of reducing NF-κB expression in corneal epithelia. The anti-inflammatory effects of AST, through its suppression of NF-κB activation, may be based on its antioxidant activity,

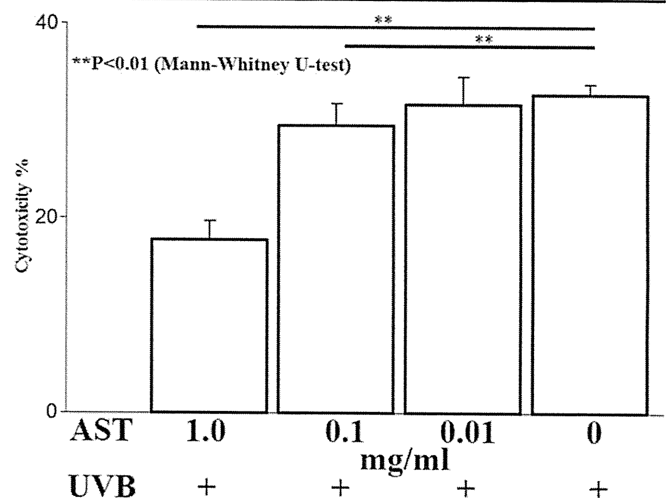

Figure 6. UVB-induced cytotoxicity in TKE2 cells in vitro. AST was added to a culture of UVB-irradiated TKE2 cells. Cytotoxicity after UVB exposure in keratinocyte cultures was significantly decreased by AST in a dose-dependent manner (1 mg/ml: $p<0.01$, 0.1 mg/ml: $p<0.05$, and 0.01 mg/ml: not significant).

as previous reports showed that several antioxidants efficiently inhibit NF-κB activation induced by lipopolysaccharides (LPS) in cell systems [28-33]. Therefore, these effects appear to be mediated by the powerful radical scavenger properties of the antioxidants, which apparently counteract reactive oxygen intermediates generated by NF-κB activation [30,34,35].

We also showed that the relieving effect of AST on UVB photokeratitis is not derived from light-interception (sunglasses effect) but its direct pharmacological effect (Figure 2). In fact, a previous study showed that AST eye drops did not delay the progression of UVB-induced cataract in rats when AST was not on lens [36]. In addition, UVB irradiation improved AST accumulation in green microalgae [8]. These results strongly suggest that AST can produce a protective effect against UV, and organisms have been using AST to save themselves from harmful UV light.

AST is found abundantly in the red-orange pigment of marine animals such as salmon (and salmon roe) and the shells of crabs and shrimp. Humans have taken in these food products since ancient times, and AST is now available as an oral supplement. Therefore, AST may be free of any harmful side effects. In the present study, we demonstrated that topical AST, instead of systemic administration, is effective in protecting the ocular surface against UV exposure with no adverse effects. Thus, in conclusion, AST might be a promising naturally-derived substance for protecting ocular surfaces from the damages caused by ultraviolet radiation.

ACKNOWLEDGMENTS

This work was supported by grants from the Japan Society for the Promotion of Science (JSPS); the Ministry of Education, Culture, Sports, Science, and Technology (MEXT); and the Mishima Memorial Foundation, Japan.

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Articles are provided courtesy of Emory University and the Zhongshan Ophthalmic Center, Sun Yat-sen University, P.R. China. The print version of this article was created on 10 February 2012. This reflects all typographical corrections and errata to the article through that date. Details of any changes may be found in the online version of the article.

Rheumatology Advance Access published January 18, 2012
RHEUMATOLOGY

Concise report

doi:10.1093/rheumatology/ker444

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

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CONCISE REPORT

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.

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Submitted 16 May 2011; revised version accepted 29 November 2011.

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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.

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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

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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	2*n* = 238	2*n* = 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	2*n* = 238	2*n* = 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.

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Acknowledgements

The authors thank clinical staff, patients and their families for their participation in and dedication to the project.

Funding: This work was supported by grants-in-aid from the Ministry of Education, Culture, Sports, Science and Technology of Japan, and the Ministry of Health, Labour and Welfare, Japan.

Disclosure statement: The authors have declared no conflicts of interest.

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Graefes Arch Clin Exp Ophthalmol
DOI 10.1007/s00417-011-1908-1

INFLAMMATORY DISORDERS

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

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Received: 2 September 2011 / Revised: 28 November 2011 / Accepted: 13 December 2011
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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.

Keywords Behçet's disease · Retinal vasculitis · Uveitis · Antinuclear antibody · Infliximab · Biomarker · Anti-TNF-α monoclonal antibody

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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

Published online: 11 January 2012

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 ($ANA \geq 80$) or negative ($ANA \leq 40$) 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

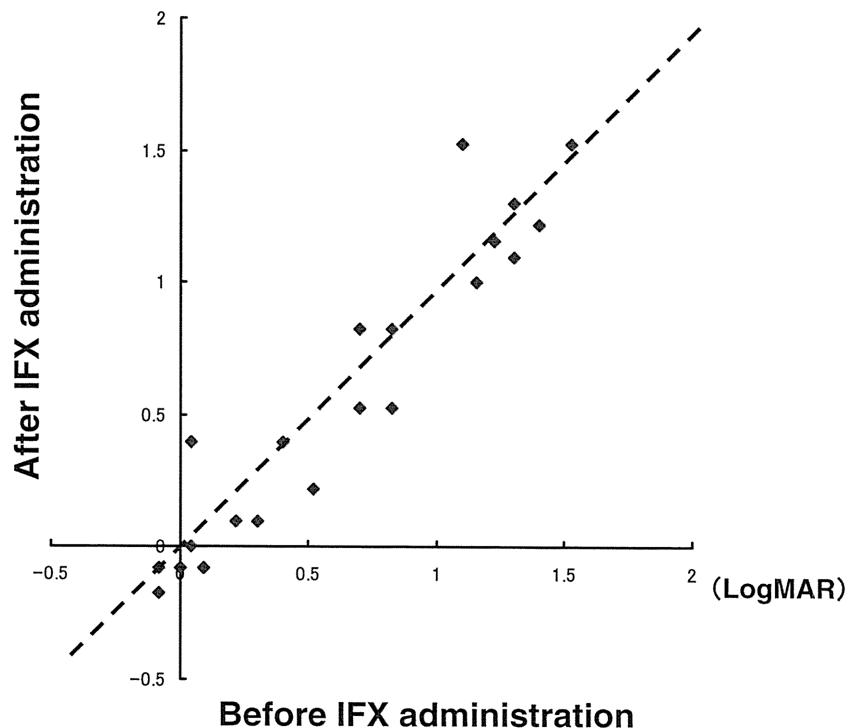

Fig. 1 Visual acuity before and after initiation of IFX. Best-corrected visual acuities (BCVA) 1 year after the initiation of IFX. IFX therapy successfully maintained the visual acuity in these patients

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

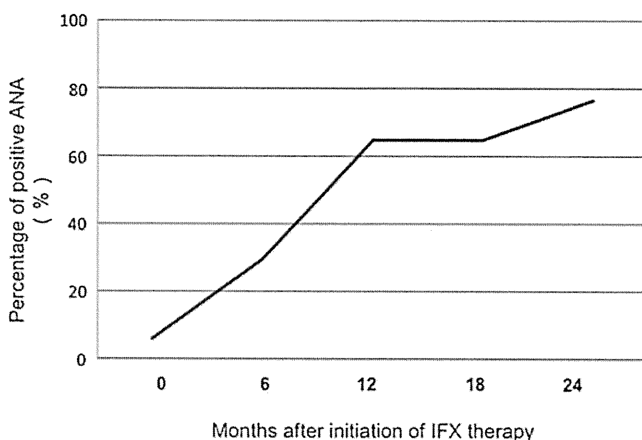

Fig. 2 Frequencies of ANA positivity in BD patients undergoing IFX therapy. The positive conversion of ANA became frequent 6 months after the initiation of IFX, and its positivity rate gradually increased

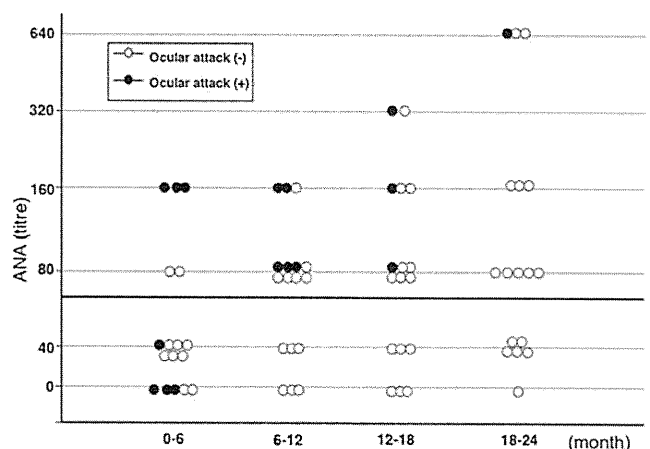

Fig. 3 Correlation of ocular attacks with elevation of ANA titer. Since the 6th month of IFX therapy, all five patients (23.5%) suffering from a relapse of ocular inflammatory attack were ANA-positive, and three of five patients had ocular attacks more than once throughout the observation period

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.

Competing interests None.

Funding None.

Ethics approval This study was approved by the institutional Ethics Committee of Hokkaido University

Patient consent Obtained.

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Molecular Vision 2011; 17:3121-3127 <http://www.molvis.org/molvis/v17/a336>
Received 3 September 2011 | Accepted 26 November 2011 | Published 30 November 2011
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Genome variability of human adenovirus type 8 causing epidemic keratoconjunctivitis during 1986-2003 in Japan

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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.

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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

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* BamHI	HindIII	PstI	SacI	SalI	SmaI	Neutralization test titer	Samples collected in
	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