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Polymorphism of *IFN-γ* gene and Vogt-Koyanagi-Harada disease

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Purpose: Interferon-γ (IFN-γ) is a key cytokine in inflammatory disorders. Elevated aqueous and serum levels of IFN-γ levels have been reported to be elevated in patients with Vogt-Koyanagi-Harada (VKH) disease. The aim of this study was to determine the *IFN-γ* gene polymorphisms in VKH disease.
Methods: The study involved 136 VKH patients and 176 healthy controls, who were genotyped for functional single nucleotide polymorphism (SNP; rs2430561; A/T) and functional microsatellite (CA) repeats (rs3138557) in the first intron of the *IFN-γ* gene. Moreover, clinical manifestations of the patients were also analyzed.
Results: Diffuse choroiditis/staining of fluorescein angiography was seen in all VKH patients in this study. Sunset glow fundus and nummular chorioretinal depigmented scars were observed in 83.9%, and 36.1% of the patients, respectively. Neurological and auditory disorders were observed in 90.1% of the patients: meningismus (79.8%), tinnitus (53.0%), and cerebrospinal fluid pleocytosis (70.0%). Dermatologic manifestations were observed in 22.9% of the patients, manifesting as alopecia (6.9%), poliosis (17.6%), and vitiligo (13.0%). In addition, 22.1% of the patients were classified as having complete VKH disease, while 65.4% as having incomplete VKH disease, and 12.5% as having probable VKH disease. There were no significant differences in the allele and genotype frequencies between VKH patients and healthy controls. In addition, we found no association between each clinical manifestation and SNP (re2430561) in the healthy control subject. A strong linkage disequilibrium (LD) was found in the functional SNP T allele and functional microsatellite 12 (CA) repeats (D'=0.96-0.99).
Conclusions: The functional SNP T allele and microsatellite 12 (CA) repeats were found to have a strong LD, although a genetic susceptibility for the *IFN-γ* gene could not be demonstrated among the Japanese VKH patients.

Vogt-Koyanagi-Harada (VKH) disease is one of the most frequent forms of uveitis in Japan [1]. It is characterized as a panuveitis, and is often accompanied by headache, pleocytosis of the cerebrospinal fluid, inner ear disturbances, and skin lesions, such as vitiligo and alopecia [2,3]. VKH disease is considered an autoimmune disease against melanocytes [2-7], and is strongly associated with HLA-DRB1*0405 is strongly associated with VKH disease [8,9]. Interferon-γ (IFN-γ) is significantly elevated in the aqueous humor and sera of VKH patients [10-14]. IFN-γ may be associated with the development of predominant Th1-dominant, cell mediated immune responses which may thereby enhance the expression of HLA class II antigens [15,16].

The *IFN-γ* gene on chromosome 12q24.1 spans approximately 5.4 kb and contains four exons. Like other cytokines, the IFN-γ coding region is invariant, with no reported polymorphisms [17]. Single nucleotide polymorphism (SNP; rs2430561) and microsatellite (rs3138557) within the first intron of the *IFN-γ* gene correlate with a high amount of in vitro production of IFN-γ and are associated with disease severity or resistance to drug therapy in various autoimmune diseases [18-22]. This allele is associated with a higher or a lower risk of a variety of diseases including autoimmune and chronic inflammatory conditions [23]. The association between SNP (rs2430561) alleles T to A with a low (AA), medium (AT), and high (TT) production of cytokines has been reported in vitro [24,25].

We hypothesized that a common allelic variation in these potential functional polymorphisms may be involved in Th1-mediated autoimmune diseases, such as VKH disease. In this case-control association study of Japanese subjects, we investigated whether the aforementioned polymorphisms in the *IFN-γ* gene contribute to the development risk of VKH disease and to some of the clinical features of the disease.

METHODS

We recruited 136 VKH patients and 176 healthy controls from the Uveitis Survey Clinic of the Hokkaido University and Yokohama City University. VKH patients were approached as they visited the clinic for previously scheduled visits and were enrolled after consenting to participate in the study. The control subjects were healthy volunteers who were unrelated to each other or to the patients and had no history of any inflammatory disease. All the participants were Japanese, and they all gave their informed consent. The patients were diagnosed at the Uveitis Survey Clinic of the Hokkaido Univer-

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sity Hospital, Sapporo, Japan, according to the "Revised Diagnostic Criteria for VKH Disease" [2,3]. The procedures used in this study conformed to the tenets of the Declaration of Helsinki.

Blood samples were collected from each subject (7 ml), and genomic DNA was extracted with a QIAamp DNA blood mini kit (Qiagen, Tokyo, Japan). SNP and microsatellite repeats in the first intron of the *IFN-γ* gene were amplified using two primers: forward 5'-TGA TTC TGG CTA AGG AAT GT-3' and reverse 5'-AAT TGC AAT GTC ACA AAT GA -3'. Polymerase chain reactions (PCRs) were performed in a total volume of 10 μl containing PCR buffer, genomic DNA, 0.2 mM dinucleotide triphosphates, 0.5 μM primers, and 0.25 U Taq polymerase. The reaction mixture was denatured for 2 min at 94 °C, and then subjected to 35 cycles of denaturation at 94 °C for 1 min, annealing at 55 °C, 2 min for extension at 72 °C, and 7 min for final elongation at 72 °C using a PCR thermal cycler, GeneAmp System 9700 (Applied Biosystems, Foster City, CA). The purified PCR products were used as templates for direct sequencing using BigDye terminator v3.1 Cycle Sequencing Kit (Applied Biosystems). The sequencing primer was 5'-TGA TTC TGG CTA AGG AAT GT-3', same as that used previously for amplification. The BigDye XTerminator Purification kit was used for purification of DNA sequencing reactions. Nucleotide sequences were determined by an ABI3130 Genetic Analyzer (Applied Biosystems). Microsatellite repeats were calculated by direct counting (Figure 1).

Statistical analysis: The significance of associations was tested using the chi-square test in a single-point analysis. Pairwise linkage disequilibrium (LD; D' value) between SNP (rs2430561) and microsatellite (rs3138557), and haplotypes frequency were estimated by the expectation-maximization (EM) algorithm using the SNPAlyze program (version 5.1 software; Dynacom, Yokohama, Japan). Haplotype frequencies of the patients and controls were compared using the chi-square test and by permutation p values based on 10,000 permutations.

RESULTS

The characteristics of the VKH patients are summarized in Table 1. The age of the patients ranged from 15 to 80 years (51.9±13.7, mean±SD). The study group included 65 men (47.8%) and 71 women (52.2%). Based on the diagnostic cri-

Table 1. Characteristics of the study patients

	Percentage	(n/N)
Age (years)		
Mean±SD(range)	51.9±13.7(15-80) yo	
Sex		
Male	65 47.8	
Female	71 52.2	
Complete VKH disease	22.1	(30/136)
Incomplete VKH disease	65.4	(89/136)
Probable VKH disease	12.5	(17/136)
Bilateral ocular involvement	100	(136/136)
Diffuse choroiditis	100	(125/125)
Sunset glow fundus	83.9	(99/118)
Depigmented scars	36.1	(44/122)
Neurological auditory findings	90.1	(118/131)
Meningismus (headache, fever etc)	79.8	(103/129)
Tinnitus	53.0	(62/117)
Cerebrospinal fluid pleocytosis	70.0	(77/110)
Integumentary findings	22.9	(30/131)
Alopecia	6.9	(9/131)
Poliosis	17.6	(23/131)
Vitiligo	13.0	(17/131)

The age of the patients ranged from 15 to 80 years (51.9±13.7, mean±SD). The study group included 65 men (47.8%) and 71 women (52.2%). Based on the diagnostic criteria for VKH disease, 30 patients (22.1%) were classified as having complete VKH disease, 89 (65.4%) as having incomplete VKH disease, and 17 (12.5%) as having probable VKH disease. Diffuse choroiditis/staining of fluorescein on angiography was observed in all VKH patients. Sunset glow fundus and nummular chorioretinal depigmented scars were observed in 83.9% and 36.1% of the patients, respectively. Neurological and auditory disorders were observed in 90.1% of the patients: meningismus (e.g., headache and fever), 79.8%; tinnitus, 53.0%; and cerebrospinal fluid pleocytosis, 70.0%. Dermatologic manifestations were observed in 22.9% of the patients: alopecia (6.9%), poliosis (17.6%), and vitiligo (13.0%). n/N represents the number of patients with the characteristics/number of patients with available information. SD represents standard deviation; yo represents years old.

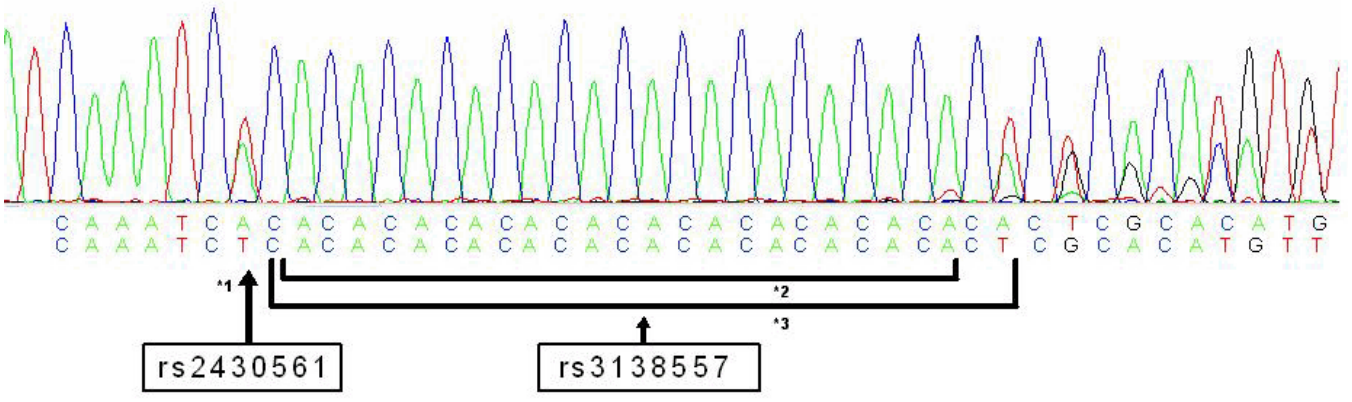

Figure 1. DNA sequences of rs2430561 and rs3138557 in the *IFN-γ* gene. The number of functional microsatellite (CA) repeats was calculated by direct counting. Asterisk (*) 1 indicates the position of the SNP alleles A and T. *2 and *3 indicate functional microsatellite alleles with 12 (CA) repeats and 13 (CA) repeats, respectively. Complement sequence data is available at the NCBI database.

teria for VKH disease, 30 cases (22.1%) were classified as complete VKH disease, 89 cases (65.4%) as having incomplete VKH disease, and 17 cases (12.5%) as having probable VKH disease. Both eyes were affected in all patients. Diffuse choroiditis/staining of fluorescein on angiography was observed in all patients. Sunset glow fundus and nummular chorioretinal depigmented scars were observed in 83.9% and 36.1% of the patients, respectively. Neurological and auditory disorders were observed in 90.1% of the patients: meningismus (e.g., headache and fever) in 79.8%, tinnitus in 53.0%, and cerebrospinal fluid pleocytosis in 70.0%. Dermatologic manifestations were observed in 22.9% of the patients: alopecia (6.9%), poliosis (17.6%), and vitiligo (13.0%; Table 1).

We examined the allele and genotype frequencies of the SNP and microsatellites in the VKH patients and healthy controls (Table 2). Six microsatellite alleles were observed in the study group. There was no significant difference in the allele and genotype frequencies between total the VKH patients and healthy controls.

Overall haplotype frequencies that were greater than 1% are shown in Table 3. The degree of the LD between SNP (rs2430561) and microsatellite (rs3138557) was D'=0.96 in the healthy controls and D'=0.99 in the VKH patients.

DISCUSSION

A positive association has been reported between allele 12 (CA) repeats and several autoimmune diseases, such as rheumatoid arthritis, type 1 diabetes mellitus, and aplastic anemia [19-21,26]. These reports have suggested that either the (CA) repeat sequence itself has a regulatory function or there is an allelic linkage between the CA repeat and functional polymorphism, which would account for the differences in the IFN-γ production [27]. Pravica, et al., in the United Kingdom reported that the presence of the SNP T allele was closely correlated with the 12 CA repeats [18]. In the present study, we showed that a high-IFN-γ productive T allele had strong LD with the 12 CA repeats in the Japanese population (D'=0.96-0.99). Therefore, our results regarding the correlation between with the T allele and 12 CA repeats in the Japanese patients were consistent with the study of Pravica et al. Because this SNP lies within the a binding site for the transcription factor nuclear factor-α-B (NF-α-B) it is believed that, SNP T allele causes an increased production in serum IFN-γ levels [18]. This transcription factor induces IFN-γ expression, and the T allele correlates with a high expression IFN-γ.

In the present study, no significantly different allelic or genotypic distributions of the analyzed *IFN-γ* gene polymorphisms were found between Japanese VKH patients and healthy controls. In our clinically stratified analysis, we investigated the onset of VKH disease and the presence of some of its clinical features such as diffuse choroiditis, sunset glow fundus, depigmented scars, meningismus, tinnitus, cerebrospinal fluid pleocytosis, and integumentary manifestations. None of these clinical findings were significantly associated with SNP (data not shown).

VKH disease is a multifactorial disease that results from interactions between susceptibility genes, environmental factors, and immunological responses. If IFN-γ production by the T allele is sufficient for disease onset, then VKH disease may not require the high-IFN-γ polymorphism allele. In this study, most of the patients were categorized as having incomplete VKH disease. We attributed the low number of patients with complete VKH disease to early detection. Early detection and a rapid cure can affect the prognosis of VKH disease. If the treatment of VKH is delayed, systemic complications, such as vitiligo and alopecia, will frequently arise [28]. Yang et al. recently classified 66.6% of the Chinese VKH patients in their study as having complete VKH. Most of these patients (82.2%) were referred to the uveitis center two months or later after the disease onset [29]. The clinical features are quite different between these two neighboring countries [2,29]. It is therefore still possible that the IFN-γ polymorphism is associated with disease severity and a poor prognosis. If the patients could be followed without therapy, as some of the Chinese patients were, we might have confirmed this hypothesis. Microsatellite markers are often used as a tool for sequencing disease candidate genes because they have many polymorphisms, and their LD is approximately in the 100 kb range [30]. The results of the present study indicate that the

Table 3. Haplotype distribution

	VKH patients			Healthy controls	
	SNP	MS	Percentage	Percentage	p-value
-	---	---	---	---	---
1	A	13	55.2	52.3	0.50
2	A	15	27.9	32.1	0.26
3	T	12	9.6	9.1	0.80
4	A	16	3.3	1.7	0.23
5	A	17	1.8	2.0	0.77
6	A	14	0.7	2.3	0.12

We examined the allele and genotype frequencies of the SNP and microsatellites in the VKH patients and healthy controls. Six microsatellite alleles were observed in the study group. There was no significant difference in the allele and genotype frequencies between the VKH patients and healthy controls. RR represents relative risk.

Table 2. Genotype and allele frequencies of rs2430561 (A/T) and rs3138557 functional microsatellites

	VKH patients		Healthy controls			
rs2430561 A/T	(n=136)	(%)	(n=176)	Percentage	RR	p-value
Genotype frequency	AA 111	81.6	146	83.0	0.91	0.76
	AT 24	17.6	27	15.3	1.16	0.63
	TT 1	0.7	3	1.7	0.43	0.45
T allele frequency (%)		9.6		9.3	1.02	0.94
rs3138557 CA	(2n=272)	(%)	(2n=352)	Percentage		
Allele frequency	12 30	11.0	33	9.3	1.20	0.50
	13 150	55.1	184	52.3	1.12	0.48
	14 2	0.7	8	2.3	0.32	0.13
	15 76	27.9	114	32.4	0.81	0.23
	16 9	3.3	6	1.7	1.97	0.19
	17 5	1.8	7	2.0	0.92	0.89

We examined the allele and genotype frequencies of the SNP and microsatellites in the VKH patients and healthy controls. Six microsatellite alleles were observed in the study group. There was no significant difference in the allele and genotype frequencies between the VKH patients and healthy controls. RR repesents relative risk.

IFN-γ gene and the genes surrounding it are not truly susceptible loci associated with VKH disease [31]. (4) All patients in this study had normal levels of IFN-γ. Although we did quantify IFN-g levels in the present study, some previous studies reported that serum and aqueous IFN-γ levels were elevated in VKH patients [11,14]. There was a slight possibility that only our patients had normal IFN-γ levels in the sera and aqueous humor.

In conclusion, no significant correlation was observed between the IFN-γ gene polymorphism and VKH disease onset. Because all the patients in the present study received systemic corticosteroids immediately after diagnosis, the association between polymorphisms and disease prognosis and/or complications is still unclear. Further studies are required to clarify the genetic mechanisms underlying VKH disease.

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