DNA TYPING OF HLA IN THE PATIENTS WITH MOYAMOYA DISEASE

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Summary Moyamoya disease is a clinical entity demonstrating a chronic occlusion of the cerebrovascular system. Although some possible etiological factors have been postulated, the etiology of this disease is still unknown. So far, some investigations have suggested the association between moyamoya disease and HLA in the serological typing. However, DNA typing of HLA have not been performed yet. Thus, we performed DNA-typing of HLA in the unrelated Japanese patients with definite moyamoya disease, using the polymerase chain reaction-sequence specific oligonucleotide probe (PCR-SSOP) technique. In the total patients, *DQB1*0502* had a positive association with the disease. On the other hand, *DRB1*0405* and *DQB1*0401* showed a negative association. In comparing the early-onset and late-onset groups, two groups did not share the same disease associated alleles at all. Thus, the etiology of moyamoya disease seem to have a genetic background. Furthermore, different genetic factors might also be involved in the difference between the early-onset and late-onset groups.

Key Words moyamoya disease, genetic, HLA typing

INTRODUCTION

Moyamoya disease is a clinical entity with chronic occlusion of cerebrovascular system characterized by progressive steno-occlusive changes at the terminal portion of the bilateral internal carotid arteries (Kudo, 1968; Nishimoto and Takeuchi, 1968). An unusual vascular network at the base of the brain represents secondary collateral formation due to ischemic changes caused by a progressive occlusion of the circle of Willis (Suzuki and Takaku, 1969).

The estimated number of patients in Japan in 1994 was 3,900 (95% confidence

Received October 6, 1997; Accepted October 22, 1997.

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interval; 3,500–4,400). The prevalence and incidence rates are calculated to be 3.16 and 0.35 per 100,000 population, respectively (Wakai *et al.*, 1996). The onset age distribution of this disease is bimodal. The incidence of the disease is highest during the first decade of life, while the second peak occurs in the third decade (Fukui and Kawano, 1996).

Several hypotheses have been put forward to explain the etiology of moyamoya disease, such as an upper respiratory tract infection (Suzuki and Kodama, 1983), a disorder of the autoimmune system (Kitahara *et al.*, 1982), thrombogenesis on the intima (Ikeda and Hosoda, 1993; Ikeda *et al.*, 1993), an abnormality of vascular smooth muscle cells (Masuda *et al.*, 1993; Aoyagi *et al.*, 1993; Suzuki *et al.*, 1994).

Furthermore, there is some evidence for a genetic background: 1) There are remarkable ethnic differences in the frequency of moyamoya disease in the world. Asians, such as Japanese and Koreans, have a higher rate of disease. A total of 1,063 cases of moyamoya disease have been reported, occurred worldwide excluding Japan and 625 of these cases were observed in Asians (Goto and Yonekawa, 1992; Ikezaki *et al.*, 1997); 2) The familial occurrence rate is about 10% in patients with moyamoya disease. Ten pairs of monozygotic twins have also been registered as having the disease in Japan. The incidence rate of parents and siblings in patients with moyamoya disease is from 30 to 40-fold higher than that in the general population (Kanai and Fukuyama, 1992); 3) Angiographic findings similar to moyamoya disease have been seen in well-known genetic chromosomal disorders, such as neurofibromatosis (Woody *et al.*, 1992), Fanconi anemia (Pavlinkis *et al.*, 1995) and Down syndrome (Gadoth, 1993). The responsible gene of neurofibromatosis is located on chromosome 17q or 22q. A complementation group of Fanconi anemia is related to chromosome 9q, while Down syndrome is known to demonstrate 21-trisomy; and 4) Previous serological typing of HLA in moyamoya disease revealed associations between the disease and *Aw24*, *Bw46* and *Bw54* antigens in 18 patients (Kitahara *et al.*, 1982), or *B51*, *B67*, *DR1* and *Cw1* antigens in 32 patients (Aoyagi *et al.*, 1995).

The purpose of this study is, thus, to elucidate the genetic factors that may control the susceptibility to moyamoya disease by performing DNA typing of HLA genes in a large number of unrelated Japanese patients with this disease.

MATERIALS AND METHODS

Study subjects. The study subjects were all Japanese living in Japan. All patients were diagnosed as having definite moyamoya disease according to the guidelines for the diagnosis of moyamoya disease established in 1988 by the Research Committee on the Spontaneous Occlusion of the Circle of Willis (moyamoya disease) of the Ministry of Health and Welfare, Japan (Kitamura, 1988). Seventy-one unrelated patients with moyamoya disease thus underwent

Table 1. Number of patients analyzed.

	<i>HLA-A</i>		<i>Class II</i> genes	
Age at onset	≤10	>10	≤10	>10
Number of patient (F/M*)	48 (31/17)	20 (16/4)	49 (32/17)	22 (18/4)
Total	68		71	

*F/M=female/male.

DNA typing for *class II* genes. Sixty-eight of these patients underwent *class I* typing. In addition, 525 unrelated healthy Japanese were used as control subjects.

Table 1 shows the number of patients and the sex ratio. Because the onset age distribution of moyamoya disease is bimodal, the patients were divided into two groups according to the onset age, including the early-onset group, with patients 10 years old and under, and the late-onset group, with patients older than 10 years old. Regarding the genotype of *class I* genes, 48 patients were analyzed in the early-onset group and 20 patients in the late-onset group, while for the *class II* genes, 49 patients were analyzed in early-onset group and 22 patients in late-onset group.

This study was approved by the Research Committee and each institutional review board and informed consent was obtained from each patient.

HLA typing. Regarding the *class I* genes of HLA, the *HLA-A* gene was examined, while for the *class II* genes, *HLA-DRB1*, *DQA1*, *DQB1*, *DPA1* and *DPB1* genes were examined. The allele designations are from the WHO HLA Nomenclature Committee (Bodmer *et al.*, 1995). The method of DNA typing of HLA has been described previously (Kimura and Sasazuki, 1992; Kimura *et al.*, 1992; Date *et al.*, 1996).

Genomic DNA was extracted from peripheral granulocytes by the standard sodium dodecyl sulfate (SDS)-proteinase K digestion/phenol-chloroform extraction method. The polymerase chain reaction (PCR) mixture was composed of genomic DNA (0.2 µg), 1 unit of *Taq* DNA polymerase (Takara Taq, Takara Co., Kyoto, Japan), 15 pmol of a 5'-sided primer and a 3'-sided primer, 5 µl of 2 mM deoxyribonucleoside triphosphates (d-NTP), 5 µl of 10×PCR buffer (500 mM KCl, 20 mM MgCl₂, 1 mg/ml Gelatin, 0.2% NP-40, and 100 mM Tris-HCl, pH 8.4) in a final volume of 50 µl. PCR was done in a Thermal Cycler (Perkin Elmer Cetus, Norwalk, CT).

The PCR products were spotted onto nylon filters (Biodyne B; Pall Co., East Hills, NY), immobilized by alkaline denaturation in 0.4 N NaOH, and neutralized in 10×SSPE solution (1.5 M NaCl, 0.1 M sodium phosphate, and 10 mM disodium ethylene-diamine-tetra-acetate-2H₂O (EDTA), pH 7.4). The filters were prehybridized for 1 hr at 54°C in hybridization buffer (50 mM Tris-HCl, 3 M tetramethylammonium chloride, 2 mM EDTA, 5×Denhardt's solution, 0.1% SDS, and 100 µg/ml heat-denatured herring sperm DNA, pH 8.0). After hybridization with ³²P-endlabeled sequence-specific oligonucleotides (SSOPs) at 54°C for 1 hr, the filters were washed twice to remove any excess SSOPs in 2×SSPE and 0.1% SDS

at room temperature for 10 min. The filters were, then, washed once for 10 min at room temperature in TMAC solution (50 mM Tris-HCl, 3 M tetra-methyl-ammonium chloride, 2 mM EDTA, 0.1% SDS, pH 8.0). Another stringent wash was done twice for 10 min at 58°C in TMAC solution. The filters were then exposed to X-ray film for 1 hr at room temperature to detect hybridization signals.

Statistics. The strength of the statistical association between the disease and HLA was expressed by the relative risk as given by Woolf's formula (Woolf, 1955; Miettinen, 1976) with Haldane's modification (Haldane, 1956). The statistical significance was examined by the chi-square test with Yates' correction. When the *p* value was less than 0.05, the association with each individual allele was considered to be significant.

RESULTS

The frequency of the HLA alleles in the patients with moyamoya disease was compared with the distribution of alleles in the control population.

In the total patients, the frequencies of *DRB1*0502* (10% *vs* 3%, phenotype frequency in the patients *versus* that in the controls; chi-sq: chi-square value = 5.485, R.R.: relative risk = 3.27, *p* < 0.025) showed a significantly positive association with the disease. On the other hand, the frequencies of *DRB1*0405* (11% *vs* 27%; chi-sq = 7.142, R.R. = 0.35, *p* < 0.01) and *DQB1*0401* (13% *vs* 26%; chi-sq = 5.663, R.R. = 0.4, *p* < 0.025) were significantly lower in the patients than in the control (Table 2).

In the early-onset group, the frequencies of *A*2602* (13% *vs* 4%; chi-sq = 4.868, R.R. = 3.42, *p* < 0.05), *DRB1*1501* (24% *vs* 12%; chi-sq = 4.663, R.R. = 2.3, *p* < 0.05) and *DQB1*0602* (24% *vs* 12%; chi-sq = 5.337, R.R. = 2.42, *p* < 0.025) showed a significantly positive association with the disease. On the other hand, the frequencies of *DRB1*0405* (12% *vs* 27%; chi-sq = 4.185, R.R. = 0.38, *p* < 0.05) were significantly lower in the moyamoya patients (Table 2).

In the late-onset group, the frequencies of *DQA1*0301* (36% *vs* 15%; chi-sq = 5.503, R.R. = 3.18, *p* < 0.025) and *DQB1*0502* (14% *vs* 3%; chi-sq = 3.865, R.R. = 4.72, *p* < 0.05) showed a positive association with the disease. On the other hand, the frequencies of *DQA1*0302* (32% *vs* 57%; chi-sq = 4.363, R.R. = 0.36, *p* < 0.05) were significantly lower in the moyamoya patients (Table 2). There were no common alleles, which showed significant association in either groups. Furthermore, there were no significant differences in the affected alleles between females and males (data not shown).

The frequencies of the *DR-DQ* haplotypes in moyamoya disease are shown in Table 3. In the total patients, the *DRB1*0802-DQA1*0401-DQB1*0402* haplotype (4.2% *vs* 2.5%; chi-sq = 4.23, R.R. = 1.76, *p* < 0.05) showed a positive association, whereas the *DRB1*0405-DQA1*0302-DQB1*0401* haplotype (11% *vs* 27%; chi-sq = 6.99, R.R. = 0.353, *p* < 0.01) showed a negative association with the disease.

Table 2. HLA alleles which showed significant association with moyamoya disease.

HLA alleles	Early-onset group				Late-onset group				Total patients			
	%pt.*	R.R.	Chi-sq.	p	%pt.	R.R.	Chi-sq.	p	%pt.	R.R.	Chi-sq.	p
Class I												
A*2602	13	3.42	4.868	<0.05	0	0.546	0.127		8.8	2.327	1.935	
Class II												
DRB1*1501	24	2.3	4.663	<0.05	14	1.12	0.024		21	1.9	3.398	
DRB1*0405	12	0.38	4.185	<0.05	9	0.28	2.541		11	0.35	7.142	<0.01
DQA1*0301	14	0.93	0		36	3.18	5.503	<0.025	21	1.49	1.209	15
DQA1*0302	57	1.02	0.01		32	0.36	4.363	<0.05	49	0.74	1.127	57
DQB1*0602	24	2.42	5.337	<0.025	5	0.36	0.497		18	1.71	1.848	12
DQB1*0502	8	2.66	1.845		14	4.72	3.865	<0.05	10	3.27	5.485	3
DQB1*0401	14	0.46	2.896		9	0.28	2.489		13	0.40	5.663	<0.025

*%pt., %patient. R.R., relative risk. Chi-sq, chi-square value. %Cont., %control (N=525).

Table 3. Association of HLA-DRB1-DQA1-DQB1 haplotypes and moyamoya disease.

HLA alleles	Early-onset group				Late-onset group				Total patients			
	%pt.	R.R.	Chi-sq.	p	%pt.	R.R.	Chi-sq.	p	%pt.	R.R.	Chi-sq.	p
DRB1-DQA1-DQB1												
1501-01021-0602	25	2.47	5.58	<0.025	4.5	0.362	0.465		18	1.71	2.00	12
0802-0401-0402	4.1	1.68	1.06		4.5	1.88	0.008		4.2	1.74	4.23	<0.05
0405-0302-0401	12	0.387	4.08	<0.05	9.1	0.278	2.49		11	0.353	6.99	<0.01

In the early-onset group, the *DRB1*1501-DQA1*01021-DQB1*0602* haplotype (25% vs 12%; chi-sq=5.58, R.R.=2.47, $p<0.025$) showed a positive association with the disease, whereas the frequencies of the *DRB1*0405-DQA1*0302-DQB1*0401* haplotype (12% vs 27%; chi-sq=4.08, R.R.=0.387, $p<0.05$) showed a negative association with the disease.

In the late onset group, none of the haplotype disease association showed statistical significance.

DISCUSSION

The present study demonstrated that several HLA alleles had a significant association with moyamoya disease ($p<0.05$ with Yates' correction). Furthermore, a significant difference was seen in the affected alleles between the early-onset and the late-onset groups.

The HLA plays an important role in controlling the immune system and is known to be associated with various diseases such as auto-immune diseases (Todd *et al.*, 1988; Sasazuki *et al.*, 1983), infectious diseases (Hirayama *et al.*, 1987; Nishimura and Sasazuki, 1987) and virus-associated tumors (Burt *et al.*, 1994).

The frequency of *DRB1*0405* is lower than that in the controls, both in patients with Takayasu arteritis (10.9%) (Dong *et al.*, 1992) and moyamoya disease (11%) as demonstrated in the current study. Furthermore, the frequency of the *DRB1*1501-DQA1*01021-DQB1*0602* haplotype is higher in patients with systemic lupus erythematoses (32.1%) (Dong *et al.*, 1993) and moyamoya disease (25%) than in the controls.

There are two possibilities which may explain the association of HLA alleles with the disease. One is that these alleles directly control the susceptibility to moyamoya disease and the other is that other responsible genes have linkage disequilibrium with disease-associated HLA alleles (Achord *et al.*, 1982; Hugot *et al.*, 1994).

If these alleles directly control the susceptibility to moyamoya disease, it may be that moyamoya disease in some way is related to autoimmune vasculitis. However, histopathological investigations have not revealed any evidence of active inflammation in the terminal portion of the internal carotid arteries (Hosoda, 1984; Yamashita *et al.*, 1991). Since most histopathological examinations of this disease have only been conducted in adult bleeding cases, it remains unclear as to what kind of pathological changes exist in the most active state in younger aged patients. There has only been one case in which inflammatory change caused angiographic findings similar to those seen in moyamoya disease, while T-cell dominant inflammatory changes were also shown at the terminal portion of the internal carotid artery (Panegyres *et al.*, 1993).

Previous reports have shown associations between the disease and either 1) *Aw24*, *Bw46*, or *Bw54* antigens in 18 patients (Kitahara *et al.*, 1982) and 2) *B51*,

B67, *DR1* or *Cw1* antigens in 32 patients (Aoyagi *et al.*, 1995). In contrast to these reports, no such counterparts of these alleles were seen in this present study. This may be because of the small number of patients with a low specificity of serological typing reported in previous studies.

Interestingly, the affected alleles in the early-onset group were different from those in the late-onset groups. In this respect, it is important to note that the clinical features are different between the early- and the late-onset groups (Suzuki and Kodama, 1983). Thus, the initial symptoms are cerebral ischemia and cerebral hemorrhage in the early-onset and the late-onset groups, respectively. These observations suggest a different etiology for these two groups, in spite of their similar angiographical findings.

If associated alleles do not directly control the susceptibility to this disease, then some other responsible genes may be in linkage disequilibrium with these associated HLA alleles. Based on the above findings, moyamoya disease is considered to have some intrinsic factors in its etiology. To specify genetic backgrounds of moyamoya disease, further studies regarding linkage analysis are thus called for.

Acknowledgments Dr. Ikezaki, Dr. Fukui and Dr. Sasazuki were supported by research grants from the Ministry of Health and Welfare, Japan. We thank the following doctors who kindly helped in collecting the samples, Dr. Yoshiharu Sakurai (Sendai National Hospital), Prof. Hiromu Yamada (Gifu University), Prof. Kazuo Yamada (Nagoya City University), Prof. Toru Hayakawa (Osaka University), Prof. Satoshi Ueda (Kyoto Prefectural University of Medicine), Prof. Takashi Ohmoto (Okayama University). We also thank Ms. Chiharu Noguchi for her secretarial assistance.

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