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Use of PCR with Sequence-specific Primers for High-Resolution Human Leukocyte Antigen Typing of Patients with Narcolepsy

Hye In Woo, M.D.[1], Eun Yeon Joo, M.D.[2], Seung Bong Hong, M.D.[2], Kyung Wha Lee, Ph.D.[3], and Eun-Suk Kang, M.D.[1]

Departments of Laboratory Medicine and Genetics[1] and Neurology[2], Samsung Medical Center, Sungkyunkwan University School of Medicine, Seoul; Hallym Institution for Genome Application[3], Hallym University Sacred Heart Hospital, Hallym University, Anyang, Korea

Background: Narcolepsy is a neurologic disorder characterized by excessive daytime sleepiness, symptoms of abnormal rapid eye movement (REM) sleep, and a strong association with *HLA-DRB1*1501, -DQA1*0102,* and *-DQB1*0602*. Here, we investigated the clinico-physical characteristics of Korean patients with narcolepsy, their HLA types, and the clinical utility of high-resolution PCR with sequence-specific primers (PCR-SSP) as a simple typing method for identifying *DRB1*15/16, DQA1,* and *DQB1* alleles.

Methods: The study population consisted of 67 consecutively enrolled patients having unexplained daytime sleepiness and diagnosed narcolepsy based on clinical and neurological findings. Clinical data and the results of the multiple sleep latency test and polysomnography were reviewed, and HLA typing was performed using both high-resolution PCR-SSP and sequence-based typing (SBT).

Results: The 44 narcolepsy patients with cataplexy displayed significantly higher frequencies of *DRB1*1501* (P_C=0.003), *DQA1*0102* (P_C=0.001), and *DQB1*0602* (P_C=0.014) than the patients without cataplexy. Among patients carrying *DRB1*1501-DQB1*0602* or *DQA1*0102*, the frequencies of a mean REM sleep latency of less than 20 min in nocturnal polysomnography and clinical findings, including sleep paralysis and hypnagogic hallucination were significantly higher. SBT and PCR-SSP showed 100% concordance for high-resolution typing of *DRB1*15/16* alleles and *DQA1* and *DQB1* loci.

Conclusions: The clinical characteristics and somnographic findings of narcolepsy patients were associated with specific HLA alleles, including *DRB1*1501, DQA1*0102*, and *DQB1*0602*. Application of high-resolution PCR-SSP, a reliable and simple method, for both allele- and locus-specific HLA typing of *DRB1*15/16, DQA1,* and *DQB1* would be useful for characterizing clinical status among subjects with narcolepsy.

Key Words: HLA, Genotype, Narcolepsy, Cataplexy

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Corresponding author: Eun-Suk Kang
Department of Laboratory Medicine and Genetics, Samsung Medical Center, 50 Ilwon-dong, Gangnam-gu, Seoul 135-710, Korea
Tel: +82-2-3410-2703
Fax: +82-2-3410-2719
E-mail: eskang@skku.edu

Co-corresponding author: Seung Bong Hong
Department of Neurology, Samsung Medical Center, 50 Ilwon-dong, Gangnam-gu, Seoul 135-710, Korea
Tel: +82-2-3410-3592
Fax: +82-2-3410-0032
E-mail: seungbong.hong@samsung.com

INTRODUCTION

Narcolepsy is a neurologic disorder characterized by excessive daytime sleepiness and the clinical manifestations of abnormal rapid eye movement (REM) sleep such as cataplexy (sudden episodes of loss of postural muscle tone triggered by emotions). The close association between narcolepsy and HLA has been consistently reported in the literature. The HLA types that have been associated with narcolepsy include *HLA-DQB1*0602, DQA1*0102,* and DR2 [1-3]. Previous studies have shown the disorder

to be primarily associated with alleles of the linked *DQ* loci (i.e., *DQB1*0602* and *DQA1*0102*), while DR2 association is a secondary effect [4, 5]. A higher prevalence of *DQB1*0602* homozygotes and a stronger correlation of this genotype with narcolepsy were observed in the Japanese compared to those in African-Americans and white Americans [1]. *DQB1*0602* has been shown to be closely associated with the presence of cataplexy. Previous studies have demonstrated that the presence of *DQB1*0602* is reduced among patients in whom cataplexy is absent or ill defined [6, 7]. Differences in the frequency of certain HLA alleles have been observed in previous reports, and these differences have been suggested to be associated with ethnic origin and other factors, including the diagnostic criteria used to identify narcolepsy [2, 8, 9]. More specifically, the frequency and influence of DR2 has been reported to differ according to ethnic origin and the resolution of the HLA typing method. *DRB1*1501* has been shown to be a risk factor, while *DRB1*1502* has been shown to be a protective factor against the development of narcolepsy [7].

Based on these reports, it is becoming increasingly important to utilize high-resolution molecular techniques, such as sequence-based typing (SBT), to determine HLA types at the allelic level in narcolepsy patients. SBT is a method that can identify all sequence variations as well as new polymorphisms. This method has been regarded as the standard method for HLA typing at the allelic level. However, SBT has several disadvantages, including high equipment costs, time and labor intensiveness, the need for experts, perceived complexity, and occasional inaccuracy [10]. Low to intermediate resolution PCR-reverse sequence-specific oligonucleotide hybridization (PCR-SSO) and PCR with sequence-specific primers (PCR-SSP) have been used for HLA typing at the serological level in clinical settings. These methods have replaced conventional cytotoxicity-based HLA typing methods; however, these methods has been applied to low to intermediate resolution typing. PCR-SSP that includes more probes and primers for the targeted genes of interest, so called high-resolution PCR-SSP, provides high resolution at the allelic level, a relatively short turn-around time, less complexity in both process and interpretation, and requires fewer resources. Therefore, high-resolution PCR-SSP could be a useful and practical method for the detection of a limited number of target alleles related to specific diseases, including narcolepsy.

In this study, we aimed to make use of high-resolution PCR-SSP to analyze the frequencies of *DRB1, DQA1*, and *DQB1* polymorphisms among patients with narcolepsy in Korea and to investigate the association between HLA allele types and clinical features of the disorder, and thus, demonstrate the usefulness of this simple PCR-SSP method for the clinical assessment of narcolepsy patients.

METHODS

1. Patients and controls

The 67 consecutive Korean patients diagnosed with narcolepsy according to the results of clinical and neurological examinations at the sleep disorder clinic of Samsung Medical Center between July 2009 and January 2010 were enrolled in this study. Written informed consent was obtained from every patient or legal guardian, and the study was approved by the Institutional Ethics Committee. Clinical data, including onset age, the presence or absence of cataplexy, associated features (e.g., sleep paralysis and hypnagogic hallucinations), and the results of the multiple sleep latency test (MSLT) and nocturnal polysomnography were reviewed. The results of polysomnography were available for 64 patients, and the results of the MSLT were available for all 67 subjects. The patients were categorized according to the International Classification of Sleep Disorders (ICSD)-2 [11] into 2 groups: (1) narcolepsy with cataplexy and (2) narcolepsy without cataplexy but with a mean sleep latency of less than or equal to 8 min and the presence of 2 or more sleep-onset REM periods (SOREMPs) during the MSLT. High-resolution PCR-SSP was used to type the *HLA-DRB1*15/16* and *DQB1* alleles in all 67 patients. Simultaneously, SBT was used to type the *DRB1* and *DQB1* alleles in all 67 patients. For *DQA1*, 34 patients were typed using only SBT, and 33 were typed using both PCR-SSP and SBT.

In order to compare the results in patients with narcolepsy with the results in the general population, the prevalence of *HLA-DRB1, DQA1*, and *DQB1* was assessed among 467 randomly selected control participants from previous studies performed in Korea [12, 13].

2. DNA extraction

Genomic DNA was extracted from 200 μL of an EDTA whole blood sample using a QuickGene DNA whole blood kit S and QuickGene-Mini80 (Fujifilm Corp., Tokyo, Japan) according to the manufacturer's instructions. This extracted DNA was used for both high-resolution PCR-SSP and SBT. The concentration and purity of the DNA samples were measured as the optical density at 260 nm and the ratio of the optical densities at 260/280 nm, respectively, using a NanoDrop (Thermo Fisher Scientific, Wilmington, DE, USA). The required concentration and purity were 20-50 ng/μL and 1.7-1.9, respectively.

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3. SBT

To type *DRB1* and *DQB1*, genomic DNA was amplified using SeCore® Sequencing Kits (Invitrogen Corp., Madison, WI, USA). Master mix (20 μL) containing either *DRB1*- or *DQB1*-locus specific primers and FastStart™ Taq DNA polymerase (Roche Diagnostics Korea, Seoul, Korea) was added to the DNA sample (5 μL). The same volume of master mix was added to 5 μL of deionized water as a negative control. The DNA amplification was performed according to the program parameters in an ABI 9700 thermal cycler (Applied Biosystems Inc., Foster City, CA, USA). After the primary amplification, the PCR products were sequenced using 3 separate sequencing primers for exon 2 of *DRB1* (forward, reverse, and codon 86), and 2 separate sequencing primer pairs for *DQB1* (forward and reverse of both exon 2 and exon 3). After 25 cycles of the sequencing reaction, the DNA was purified, denatured, and sequenced using an ABI 3130 genetic analyzer (Applied Biosystems). Sequence data were analyzed using Assign-SBT™ 3.5+ software (Conexio Genomics, Fremantle, Western Australia, Australia).

Genotyping of the *DQA1* locus was performed using the method previously described by Voorter et al. [14]. In total, 16 separate sequencing and amplification primers (forward and reverse of exons 1-4) were used. DNA amplification was performed in an ABI 9700 thermal cycler (Applied Biosystems). For the sequencing reactions, a solid phase sequencing approach was performed using an Autoread Sequencing kit (Amersham Biosciences, Roosendaal, Netherlands) according to the supplier's protocol. Sequence data were analyzed using Pharmacia Typing Software (Pharmacia Biotech, Uppsala, Sweden).

4. High-resolution PCR-SSP

High-resolution PCR-SSP was performed using AllSet+™ Gold SSP Typing Kits (Invitrogen Corp., Madison, Wi, USA). High-resolution PCR-SSP provided allelic-level genotyping of *DRB1*15/16* and locus-specific allelic-level genotyping of *DQB1* and *DQA1*. A master mix containing dNTPs, gel loading buffer, and Taq DNA polymerase was added to the contamination control tube and the wells of the AllSet+™ Gold SSP tray containing allele- and/or group-specific primers (23, 28, and 31 pairs for *DRB1*15/16*, DQA1 and *DQB1*, respectively) with a control primer pair matching the non-allelic sequence. Genomic DNA (2 μL) was added to each well, and DNA amplification was performed according to the program parameters using an ABI 9700 thermal cycler. The PCR products were transferred to 1.8% agarose gels containing ethidium bromide, and electrophoresed for 10 min at 15 V/cm. The positive lanes were visually determined and analyzed using UniMatch Software 4.0 (Invitrogen Corp., Madison, Wi, USA).

5. Statistical analysis

Data were analyzed by sorting patients according to the presence or absence of cataplexy, sleep paralysis, hypnagogic hallucinations, the results of polysomnography and MSLT based on ICSD-1 and -2 criteria [11], and HLA types. Categorical variables were used for the frequency analysis. Significance testing was conducted using Fisher's exact test for independence. *P*-values were corrected using the Bonferroni method (*Pc*) by multiplying the probability value by the number of alleles compared. *P* values less than 0.05 were considered statistically significant. The frequency and concordance for HLA alleles were compared for both the SBT and high-resolution PCR-SSP methods. The *HLA-DRB1*1501-DQA1*0102-DQB1*0602* haplotype, sleep paralysis, hypnagogic hallucinations, and a mean REM sleep latency less than 20 min and a mean sleep latency less than 5 min based on ICSD-1 and -2 criteria were included as confounding variables in the multiple logistic regression. Haplotypes were constructed by giving priority to combinations that showed higher association in previously reported Korean data [15]. The test for Hardy-Weinberg equilibrium (HWE) in control subjects was performed with a permutation test using SAS/Genetics version 9.1.3 (SAS Institute Inc., Cary, NC, USA).

RESULTS

1. The frequency of HLA phenotypes in patients with narcolepsy

Among the 67 patients, there were 51 males and 16 females. The median age of the subjects was 34.8 yr (range; 13-69 yr). Of the 67 patients, 44 (65.7%) were diagnosed to have narcolepsy with cataplexy, and 23 (34.3%) were diagnosed to have narcolepsy without cataplexy based on the ICSD-2 criteria.

Phenotype frequencies of HLA alleles in controls and narcolesy patients are shown in Table 1. *HLA-DRB1*1501, DQA1*0102,* and *DQB1*0602* were present in 46 (68.7%), 48 (71.6%), and 46 (68.7%) patients with narcolepsy, respectively, regardless of having cataplexy. All patients with *DRB1*1501* were also typed as *DQB1*0602*, and *DRB1*1501* and *DQB1*0602* were more frequently detected in patients with cataplexy than in patients without cataplexy (86.4% vs. 34.8%, *Pc*=0.003 for *DRB1*1501* and *Pc*=0.001 for *DQB1*0602*). The frequency of *DQA1*0102* was also significantly higher in patients with cataplexy than in patients without cataplexy (86.4% vs. 43.5%, *Pc*=0.014). In healthy controls, the frequencies of *DRB1*1501* (14.8%) and *DQB1*0602* (14.1%) were significantly lower than the frequen-

Table 1. Phenotype frequencies of HLA class II alleles in controls and narcolepsy patients with or without cataplexy

	Control*, N=467	Narcolepsy with cataplexy, N=44			Narcolepsy without cataplexy, N=23		
	N (%)	N (%)	*P*c-value†	OR† (95% CI)	N (%)	*P*c-value‡	OR‡ (95% CI)
*DRB1*0101*	58 (12.4)	1 (2.3)			2 (8.7)		
*DRB1*0301*	28 (6.0)	2 (4.5)			1 (4.3)		
*DRB1*0401*	7 (1.5)	0 (0)			1 (4.3)		
*DRB1*0403*	29 (6.2)	1 (2.3)			1 (4.3)		
*DRB1*0404*	19 (4.1)	0 (0)			1 (4.3)		
*DRB1*0405*	81 (17.3)	3 (6.8)			3 (13.0)		
*DRB1*0406*	38 (8.1)	2 (4.5)			1 (4.3)		
*DRB1*0407*	6 (1.3)	1 (2.3)			0 (0)		
*DRB1*0408*	1 (0.2)	0 (0)			1 (4.3)		
*DRB1*0410*	12 (2.6)	1 (2.3)			0 (0)		
*DRB1*0701*	62 (13.3)	2 (4.5)			3 (13.0)		
*DRB1*0802*	18 (3.9)	0 (0)			2 (8.7)		
*DRB1*0803*	73 (15.6)	2 (4.5)			4 (17.4)		
*DRB1*0901*	83 (17.8)	6 (13.6)			2 (8.7)		
*DRB1*1001*	14 (3.0)	0 (0)			0 (0)		
*DRB1*1101*	41 (8.8)	2 (4.5)			1 (4.3)		
*DRB1*1106*	1 (0.2)	0 (0)			0 (0)		
*DRB1*1201*	30 (6.4)	5 (11.4)			4 (17.4)		
*DRB1*1202*	30 (6.4)	3 (6.8)			1 (4.3)		
*DRB1*1205*	0 (0)	1 (2.3)			0 (0)		
*DRB1*1301*	9 (1.9)	0 (0)			1 (4.3)		
*DRB1*1302*	78 (16.7)	6 (13.6)			2 (8.7)		
*DRB1*1307*	0 (0)	1 (2.3)			0 (0)		
*DRB1*1401*	28 (6.0)	3 (6.8)			1 (4.3)		
*DRB1*1404*	0 (0)	1 (2.3)			0 (0)		
*DRB1*1405*	32 (6.9)	0 (0)			1 (4.3)		
*DRB1*1406*	7 (1.5)	0 (0)			0 (0)		
*DRB1*1407*	2 (0.4)	0 (0)			1 (4.3)		
*DRB1*1410*	2 (0.4)	0 (0)			0 (0)		
*DRB1*1412*	2 (0.4)	0 (0)			0 (0)		
*DRB1*1501*	69 (14.8)	38 (86.4)	<0.001	36.5 (7.7-332.6)	8 (34.8)	0.003	11.9 (1.2-173.8)
*DRB1*1502*	32 (6.9)	2 (4.5)			2 (8.7)		
*DRB1*1602*	6 (1.3)	1 (2.3)			0 (0)		
*DQA1*0101*	63 (13.5)	1 (2.3)			2 (8.7)		
*DQA1*0102*	142 (30.4)	38 (86.4)	<0.001	14.5 (3.4-114.0)	10 (43.5)	0.014	8.2 (1.0-98.8)
*DQA1*0103*	94 (20.3)	4 (9.1)			6 (26.1)		
*DQA1*0104*	58 (12.4)	4 (9.1)			3 (13.0)		
*DQA1*0105*	14 (3.0)	0 (0)			0 (0)		
*DQA1*0201*	62 (13.3)	2 (4.5)			3 (13.0)		
*DQA1*0301*	85 (18.2)	4 (9.1)			3 (13.0)		

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Table 1. *(Continued from the previous page)* Phenotype frequencies of HLA class II alleles in controls and narcolepsy patients with or without cataplexy

	Control*, N=467	Narcolepsy with cataplexy, N=44			Narcolepsy without cataplexy, N=23		
	N (%)	N (%)	*Pc*-value†	OR† (95% CI)	N (%)	*Pc*-value‡	OR‡ (95% CI)
*DQA1*0302*	93 (19.9)	6 (13.6)			3 (13.0)		
*DQA1*0303*	110 (23.6)	4 (9.1)			5 (21.7)		
*DQA1*0401*	15 (3.2)	1 (2.3)			2 (8.7)		
*DQA1*0501*	28 (6.0)	2 (4.5)			1 (4.3)		
*DQA1*0503*	11 (2.4)	1 (2.3)			0 (0)		
*DQA1*0505*	55 (11.8)	6 (13.6)			2 (8.7)		
*DQA1*0506*	1 (0.2)	1 (2.3)			1 (4.3)		
*DQA1*0507*	6 (1.3)	0 (0)			0 (0)		
*DQA1*0508*	7 (1.5)	0 (0)			1 (4.3)		
*DQA1*0601*	40 (8.6)	3 (6.8)			1 (4.3)		
*DQB1*0201*	28 (6.0)	2 (4.5)			1 (4.3)		
*DQB1*0202*	57 (12.2)	2 (4.5)			3 (13.0)		
*DQB1*0301*	116 (24.8)	11 (25.0)			7 (30.4)		
*DQB1*0302*	92 (19.7)	4 (9.1)			4 (17.4)		
*DQB1*0303*	99 (21.2)	6 (13.6)			3 (13.0)		
*DQB1*0401*	78 (16.7)	3 (6.8)			3 (13.0)		
*DQB1*0402*	34 (7.3)	2 (4.5)			2 (8.7)		
*DQB1*0501*	75 (16.1)	1 (2.3)			2 (8.7)		
*DQB1*0502*	21 (4.5)	2 (4.5)			1 (4.3)		
*DQB1*0503*	44 (9.4)	3 (6.8)			2 (8.7)		
*DQB1*0601*	87 (18.6)	4 (9.1)			6 (26.1)		
*DQB1*0602*	66 (14.1)	38 (86.4)	<0.001	38.5 (8.8-300.6)	8 (34.8)	0.001	11.9 (1.4-146.6)
*DQB1*0603*	9 (1.9)	0 (0)			0 (0)		
*DQB1*0604*	45 (9.6)	3 (6.8)			2 (8.7)		
*DQB1*0609*	35 (7.5)	3 (6.8)			1 (4.3)		

Pc-values greater than 0.05 are not displayed.
*The control data were obtained from previously published studies in Korea [12-13]; †*Pc*-value and OR from comparison of narcolepsy with cataplexy group to the control group; ‡*Pc*-value and OR from comparison of narcolepsy with cataplexy group to narcolepsy without cataplexy group.
Abbreviations: OR, odds ratio; *Pc*-value, Bonferroni corrected *P* value.

cies among narcolepsy patients with cataplexy (both, $Pc<0.001$). *DQA1*0102* was detected in 30.4% of the controls, which was also significantly lower than the percentage observed in narcolepsy patients with cataplexy ($Pc<0.001$). Compared with the control group, higher frequencies of *DRB1*1501, DQA1*0102*, and *DRB1*0602* were observed among narcolepsy patients without cataplexy; however, this effect did not reach statistical significance. In addition, differences in the frequencies of all other alleles except *DRB1*1501, DQB1*0602*, and *DQA1*0102* were also observed among the groups; however, none of these differences reached statistical significance between the controls and narcolepsy patients with or without cataplexy (Table 1).

All individuals who were homozygous for *DRB1*1501* or *DQB1*0602* were *DRB1*1501-DQB1*0602* homozygotes, and all *DRB1*1501-DQB1*0602* homozygotes were also *DQA1*0102* homozygotes. There were 5 patients (7.5%) who were homozygous for both *DRB1*1501* and *DQB1*0602*, and 4 of these patients (80%) had cataplexy. There were 13 patients (19.4%) who were homozygous for *DQA1*0102*, and these patients also had cataplexy more frequently than *DQA1*0102* heterozygotes, although this difference did not reach statistical significance (25.0% vs. 8.7%, $P=0.191$). Only 2 control individuals (0.4%) were *DRB1*1501-DQA1*0102-DQB1*0602* homozygotes.

In the 69 control subjects with *DRB1*1501-DQA1*0102-DQB1**

0602, there were 26 heterozygous haplotypes carried in trans of *DRB1*1501-DQA1*0102-DQB1*0602*. When we compared the haplotype combinations of all alleles carried in trans of *DRB1*1501*-DQA1*0102-*DQB1*0602* in the control subjects with those in the 46 patients according to the presence or absence of cataplexy, there were 25 haplotypes with frequencies of 2.2-10.9%. However, no statistically significant differences in haplotype frequency were observed between the controls and patients with narcolepsy, and between patient groups with or without cataplexy (data not shown). There was no significant difference from HWE at any locus in the 467 randomly selected controls (P=0.310 for *DRB1*; P=0.337 for *DQA1*; P=0.162 for *DQB1*).

2. High-resolution molecular typing of narcolepsy-associated HLA alleles

There were no discordant results observed in the *DRB1*15/*16* and *DQB1* typing of the 67 patients and the *DQA1* typing of 33 patients using the SBT and high-resolution PCR-SSP methods. Using the high-resolution PCR-SSP technique, *DRB1*1501*, *DRB1*1502*, and *DRB1*1602* alleles were easily distinguished according to their band patterns. A total of 14 *DQB1* and 15 *DQA1* alleles, including *DQB1*0602* and *DQA1*0102*, which have been shown to be associated with narcolepsy, were correctly identified. During an initial interpretation, one SBT and one PCR-SSP result for DQA1 were incorrectly assigned; however, these initial assignments were corrected after a final interpretation. In one case, *DQA1*0401* was assigned as *DQA1*0102* during the initial reading because a weak positive band was ignored in the PCR-SSP. The other was an incorrect assignment of *DQA1*0506* as *DQA1*0503*, due to an overlapped peak not recognized in the SBT. In addition to the comparable performance of PCR-SSP and SBT, the PCR-SSP method saved not only significant time, since the testing time is less than 3 hr compared to more than 6 hr for the SBT method, but also resources, such as an expensive sequencing analyzer and labor for testing and interpretation.

3. Clinico-physical and somnographic characteristics of narcolepsy patients

Sleep paralysis and hypnagogic hallucinations were observed in 52.2% and 37.3% of patients, respectively. The frequencies of sleep paralysis and hypnagogic hallucinations were significantly higher among patients with cataplexy than among patients without cataplexy (63.6% vs. 30.4%, P=0.010 for sleep paralysis;

Table 2. Demographic, clinical, and somnographic characteristics of narcolepsy patients according to the presence of cataplexy

	Narcolepsy with cataplexy	Narcolepsy without cataplexy	*P* value†	OR (95% CI)†	OR (95% CI)‡
Total, N (%)	44 (65.7)	23 (34.3)			
Age, median (range), yr	29 (13-69)	37 (18-62)	0.668	1.01 (0.97-1.04)	
M : F ratio	32:12	19:4	0.548	0.56 (0.16-1.99)	
Age of onset <20 yr, N (%)	29 (65.9)	14/17 (82.4)*	0.348	2.41 (0.60-9.73)	
Clinical features					
Sleep paralysis, N (%)	28 (63.6)	7 (30.4)	0.010	4.00 (1.36-11.78)	1.68 (0.44-6.45)
Hypnagogic hallucinations, N (%)	23 (52.3)	2 (8.7)	<0.001	11.50 (2.40-55.08)	5.02 (0.87-29.00)
Nocturnal polysomnography					
Sleep latency <10 min	40 (90.9)	20 (87.0)	0.684	1.50 (0.31-7.36)	
Mean REM sleep latency <20 min	32 (72.7)	8/20 (40.0)	0.024	5.00 (1.69-14.79)	2.12 (0.54-8.28)
Multiple sleep latency test					
MSL <5 min	40 (90.9)	17 (73.9)	0.080	3.53 (0.88-14.12)	1.55 (0.29-8.36)
MSL ≤8 min	43 (97.7)	21 (91.3)	0.269	4.10 (0.35-47.77)	
SOREMP ≥2 times	42 (95.5)	23 (100)	0.542	-	
HLA haplotype					
*DRB1*1501-DQA1*0102-DQB1*0602*	38 (86.4)	8 (34.8)	<0.001	11.88 (3.52-40.05)	4.74 (1.14-19.81)§

*Age of onset in narcolepsy without cataplexy patient group was available for only 17 patients; †Univariate analysis; ‡Multivariate analysis, analyzed with sleep paralysis, hypnagogic hallucinations, a mean REM sleep latency less than 20 min, a mean sleep latency less than 5 min according to ICSD-1 and -2 diagnostic criteria, and the *DRB1*1501-DQA1*0102-DQB1*0602* haplotype; §In multivariate analysis, only the *HLA-DRB1* 1501- DQA1*0102-DQB1*0602* haplotype had statistical significance (P=0.033).
Abbreviations: OR, odds ratio; CI, confidence interval; REM, rapid eye movement; MSL, mean sleep latency; SOREMP, sleep-onset REM periods.

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Table 3. Demographic, clinical, and somnographic characteristics of narcolepsy patients according to HLA types

	*DRB1*1501**				*DQA1*0102*			
	Pos	Neg	*P* value	OR (95% CI)	Pos	Neg	*P* value	OR (95% CI)
Total, N (%)	46 (68.7)	21 (31.3)			48 (71.6)	19 (28.4)		
M : F ratio	34 : 12	17 : 4	0.758	0.67 (0.19-2.38)	35 : 13	16 : 3	0.526	0.51 (0.13-2.02)
Clinical features								
Sleep paralysis, N (%)	29 (63.0)	6 (28.6)	0.017	4.27 (1.39-13.07)	30 (62.5)	5 (26.3)	0.014	4.67 (1.44-15.13)
Hypnagogic hallucinations, N (%)	23 (50.0)	2 (9.5)	0.002	9.50 (1.98-45.55)	23 (47.9)	2 (10.5)	0.005	7.82 (1.63-37.62)
Nocturnal polysomnography Sleep latency < 10 min	42 (91.3)	18 (85.7)	0.669	1.75 (0.36-8.63)	44 (91.7)	16 (84.2)	0.395	2.06 (0.42-10.24)
Mean REM sleep latency < 20 min	35/45 (77.8)	5/19 (26.3)	< 0.001	10.18 (3.03-34.19)	35/47 (74.5)	5/17 (29.4)	0.003	7.54 (2.26-25.11)
Multiple sleep latency test MSL < 5 min	42 (91.3)	15 (71.4)	0.060	4.20 (1.04-16.96)	44 (91.7)	13 (68.4)	0.025	5.08 (1.24-20.76)
MSL ≤ 8 min	45 (97.8)	19 (90.5)	0.229	4.74 (0.41-55.42)	47 (97.9)	17 (89.5)	0.192	5.53 (0.47-64.96)
SOREMP ≥ 2 times	45 (97.8)	20 (95.2)	0.532	2.25 (0.13-37.80)	47 (97.9)	18 (94.7)	0.490	2.61 (0.16-44.01)

*Identical to *DQB1*0602*, that is, all individuals were *DRB1*1501-DQB1*0602*.
Abbreviations: Pos, positive; Neg, negative; OR, odds ratio; CI, confidence interval; REM, rapid eye movement; MSL, mean sleep latency; SOREMP, sleep-onset REM periods.

52.3% vs. 8.7%, $P<0.001$ for hypnagogic hallucinations, Table 2). The difference in the frequency of a mean REM sleep latency less than 20 min between patients with and without cataplexy was statistically significant (72.7% vs. 40.0%, $P=0.024$). In multivariable analyses, only the *HLA-DRB1*1501-DQA1*0102-DQB1*0602* haplotype still maintained statistical significance ($P=0.033$).

A majority of the subjects (70.5%) had onset of disorder before adolescence. Although the frequency of cataplexy was lower, and the frequencies of sleep paralysis and hypnagogic hallucinations were slightly higher among patients with onset before adolescence, these differences did not reach statistical significance (67.4% vs. 83.3%, $P=0.207$ for cataplexy; 58.1% vs. 50.0%, $P=0.559$ for sleep paralysis; 44.2% vs. 33.3%, $P=0.432$ for hypnagogic hallucinations). The frequencies of polysomnography and the MLST diagnostic criteria were similar across groups (data not shown).

In the context of HLA type, the frequency of sleep paralysis and hypnagogic hallucinations was significantly higher among patients with *DRB1*1501-DQB1*0602*, (63.0% vs. 28.6%, $P=0.017$ for sleep paralysis; 50.0% vs. 9.5%, $P=0.002$ for hypnagogic hallucinations; Table 3). Similar results were observed among patients typed as DQA1*0102 (62.5% vs. 26.3%, $P=0.014$ for sleep paralysis; 47.9% vs. 10.5%, $P=0.005$ for hypnagogic hallucinations). According to the polysomnography findings, a mean REM sleep latency of less than 20 min was observed more frequently among *DRB1*1501-DQB1*0602* (77.8% vs. 26.3%, $P<0.001$) and *DQA1*0102* (74.5% vs. 29.4%, $P=0.003$) positive patients than among negative patients; furthermore, *DQA1*0102* was associated with a mean sleep latency of less than 5 min (91.7% vs. 68.4%, $P=0.025$).

DISCUSSION

*HLA-DRB1*15/*16, DQA1*, and *DQB1* typing was performed in narcolepsy patients using both the SBT and high-resolution PCR-SSP methods. The SBT method is used for the identification of sequence variations, as well as the direct detection of new polymorphisms [16]. However, this method is demanding in terms of the resources, technique, time, and labor required. PCR-SSP is a powerful method for detecting genetic variability, including single base pair mismatches. It is a relatively quick and easy technique, and the post-amplification processing is simple, rapid, and consists only of determining whether amplification has occurred [17, 18]. Additionally, HLA typing using PCR-SSP has been shown to be a relatively easy method with a higher detection rate than both PCR-SSO and PCR-restriction fragment length polymorphism (PCR-RFLP) methods [16-18]. Furthermore, when the PCR-SSP method was used, the results of the HLA typing were quickly obtained at a relatively low cost. In this study, when comparing the results using the high-resolution PCR-SSP and SBT methods, there were no discordant results observed for the typing of *DRB1*15/*16, DQA1*, and *DQB1*. However, there were potential errors in both methods during the interpretation process. The weak intensity of the positive bands in the PCR-SSP method, and the small or overlapped peaks in

the SBT method could result in the incorrect assignment of HLA types. However, in nearly all cases, the exclusion of the weak band during the interpretation of PCR-SSP could result in correct HLA types; thus, it was possible to avoid misinterpretation. Both of the initial misinterpretations that occurred in this study were made in *DQA1* typing, which generally requires the sequencing of more exons than most of the other HLA class II types in order to distinguish alleles [13, 14]. Therefore, extra caution seems to be required for *DQA1* typing. Additionally, high-resolution PCR-SSP cannot detect new alleles or alleles not provided by manufacturer. Therefore, the application of the high-resolution PCR-SSP would be appropriate for testing of certain HLA types known to be related to a specific disease, but not for general use (e.g., HLA typing for transplantation).

This study provides additional population data that confirms the association of the *HLA-DRB1*1501, DQA1*0102*, and *DQB1*0602* alleles with narcolepsy and cataplexy among Korean patients [8, 10]. In previous reports, *DRB1*1501-DQB1*0602* and *DQA1*0102-DQB1*0602* have been shown to display a high degree of linkage disequilibrium in the Korean population [12, 13, 15]. There were some observed differences in the frequency of *DRB1*1501, DQA1*0102*, and *DQB1*0602* among previous studies on narcolepsy patients [2, 8, 9]. These differences have been proposed to be due to differences in the diagnostic criteria, including the presence of cataplexy, the ethnic origin of the patient groups, and the resolution of the HLA-typing method [2, 19-21]. Even the frequency of *DQB1*0602* in Korean narcoleptic patients with cataplexy from previous reports and this study varied from 86.4% to 100% [9]. The relatively lower frequencies of *DRB1*1501* and *DQB1*0602* observed in this study compared to that observed by Hong et al. [8] and Roh et al. [9] might be due to the differences in conducting the polysomnography and the MLST or distinguishing between atypical and typical cataplexy.

Among Japanese subjects, it was determined that a significant proportion of patients with narcolepsy were typed as *DRB1*1501-DQA1*0102-DQB1*0602* homozygotes (15.2% of the patients). Other HLA types were also suggested as being associated with narcolepsy, *DQB1*0301* as a susceptibility allele, and *DQB1*0601/DQB1*0602* and *DQB1*0601/DQB1*0501* heterozygotes as having protective effects [1, 8]. In addition, the *DQB1*0602* homozygote had a 2- to 4-fold increased risk of developing narcolepsy with cataplexy when compared to the heterozygote. In this study, 5 patients (7.5%) were *DRB1*1501-DQA1*0102-DQB1*0602* homozygotes, and among these patients, 4 presented with cataplexy. Heterozygous *DQB1*0601/DQB1*0602* and *DQB1*0601/DQB1*0501* were detected in only a few patients (3 and 1 patients, respectively); therefore, we could not assess the clinical significance. A total of 18 patients (26.9%) had *DQB1*0301*, which was similar to the previously reported frecuency in Korean patients (27.8%); and this proportion was relatively high compared to that observed among Japanese, European-American, and African-American patients (10% to 14.9%) [1, 8]. However, there was no significant difference observed in the frequency of *DQB1*0301* between the control and patient groups. With the exception of the *DRB1*1501, DQB1*0602*, and *DQA1*0102*, no significant differences in the frequency of other HLA alleles were observed between the patient and control groups, or among the patients according to the presence or absence of cataplexy.

Previous research conducted in Korea by Hong et al. revealed significant differences in *DQA1*0303* and *DQB1*0301* as susceptibility alleles, and in *DQA1*0103* and *DQB1*0601* as protective alleles in the context of a *DQA1*0102* or *DQB1*0602* bearing haplotype [8]. In our study, we compared the haplotype combinations for all alleles carried in trans of *DRB1*1501-DQA1*0102-DQB1*0602* in the control subjects and narcolepsy patients. However, the differences in the frequencies did not reach statistical significance. Therefore, additional large-scale studies would be needed in order to evaluate the difference in the haplotype distribution according to the presence or absence of cataplexy.

Sleep paralysis and hypnagogic hallucinations were frequently observed among narcolepsy patients with cataplexy, and a significant number of these patients had *DRB1*1501, DQA1*0102,* and *DQB1*0602*. However, we could not confirm the significance of these findings in a multivariate analysis between the presence or absence of cataplexy and other categorized variables. The clinical features associated with narcolepsy, such as sleep paralysis and hypnagogic hallucinations, have been considered to have low diagnostic value. These criteria was removed from the ICSD-2 diagnostic criteria due to insufficient specificity for narcolepsy [11, 23]. Previous studies have shown that the frequency of hypnagogic hallucinations and sleep paralysis does not appear to differ significantly between patients with and without cataplexy [20, 22, 24]. In this study, a mean REM sleep latency of less than 20 min in nocturnal polysomnography was frequently observed among narcolepsy patients with *DRB1*1501, DQA1*0102,* and *DQB1*0602*, and a mean sleep latency of less than 5 min in the MLST was frequently observed in patients with *DQA1*0102*. Watson et al. has reported the associations between somnographic findings and *DQB1*0602* [22]. They showed that sleep latency was significantly shorter in patients with *DQB1**

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0602 than in patients without *DQB1*0602* but did not evaluate mean REM sleep latency. In this study, we categorized and analyzed the somnographic findings according to ICSD-1 and -2 criteria, whereas in the previous study these were analyzed as continuous variables. Although we considered that differences in sleep latency may exist between patients with and without *DRB1*1501, DQA1*0102,* and *DQB1* 0602*, this study might be not sufficient to confirm the significance of these differences due to limitation in number of patients. The relationship of HLA alleles with clinical and somnographic findings in narcolepsy patients should be confirmed through further studies.

In summary, this study confirmed the association between specific HLA alleles and narcolepsy among Korean patients using both the high-resolution PCR-SSP and SBT methods. Differences in the clinical and somnographic findings according to the presence of particular HLA alleles, as well as the presence or absence of cataplexy, were shown. In addition to the high level of concordance with SBT methods for identifying *DRB1* 15/16, DQA1*, and *DQB1* alleles, the high-resolution PCR-SSP was simple and cost-effective method for the testing of *HLA-DRB1, -DQA1*, and *-DQB1* alleles known to be related with narcolepsy.

Authors' Disclosures of Potential Conflicts of Interest

No potential conflict of interest relevant to this article was reported.

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