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Genomewide Association Analysis of Human Narcolepsy and a New Resistance Gene

Minae Kawashima, Gen Tamiya, Akira Oka, Hirohiko Hohjoh,* Takeo Juji, Takashi Ebisawa, Yutaka Honda, Hidetoshi Inoko, and Katsushi Tokunaga

Human narcolepsy is a hypersomnia that is affected by multiple genetic and environmental factors. One genetic factor strongly associated with narcolepsy is the *HLA-DRB1*1501-DQB1*0602* haplotype in the human leukocyte antigen region on chromosome 6, whereas the other genetic factors are not clear. To discover additional candidate regions for susceptibility or resistance to human narcolepsy, we performed a genomewide association study, using 23,244 microsatellite markers. Two rounds of screening with the use of pooled DNAs yielded 96 microsatellite markers (including 16 markers on chromosome 6) with significantly different estimated frequencies in case and control pools. Markers not located on chromosome 6 were evaluated by the individual typing of 95 cases and 95 controls; 30 markers still showed significant associations. A strong association was displayed by a marker on chromosome 21 (21q22.3). The surrounding region was subjected to high-density association mapping with 14 additional microsatellite markers and 74 SNPs. One microsatellite marker (*D21S0012m*) and two SNPs (*rs13048981* and *rs13046884*) showed strong associations ($P < .0005$; odds ratios 0.19–0.33). These polymorphisms were in a strong linkage disequilibrium, and no other polymorphism in the region showed a stronger association with narcolepsy. The region contains three predicted genes—*NLC1-A, NLC1-B,* and *NLC1-C*—tentatively named "narcolepsy candidate-region 1 genes," and *NLC1-A* and *NLC1-C* were expressed in human hypothalamus. Reporter-gene assays showed that the marker *D21S0012m* in the promoter region and the SNP *rs13046884* in the intron of *NLC1-A* significantly affected expression levels. Therefore, *NLC1-A* is considered to be a new resistance gene for human narcolepsy.

Narcolepsy (MIM 161400) typically appears, without sexual difference, in early adulthood and affects 0.16%–0.18% of the general population of Japan.[1,2] The disorder is characterized by excessive daytime sleepiness, cataplexy, and pathological manifestation of rapid eye movement (REM) sleep, including hypnagogic hallucinations, sleep paralysis, or sleep-onset REM sleep. Most cases are sporadic, but the risk of the disorder for first-degree relatives of patients with narcolepsy is 1%–2%, ~10 times greater than the general risk of developing narcolepsy. Only about a third of MZ twins are concordant for narcolepsy.[2] Therefore, human narcolepsy is considered to be a multifactorial disorder, involving multiple genetic and environmental factors.

A genetic susceptibility factor associated with the disorder has been found in the human leukocyte antigen (HLA) class II region: the *HLA-DRB1*1501-DQB1*0602* haplotype (*HLA-DRB1* [MIM 142857] and *HLA-DQB1* [MIM 604305]). Although almost all Japanese patients with narcolepsy carry this haplotype, ~10% of the general Japanese population also carries it, suggesting that this haplotype is neither necessary nor sufficient for the development of narcolepsy.[1,3–5] This conclusion is also supported by another line of reasoning. The penetrance and population frequency of *HLA-DRB1*1501* were estimated with the formula described by Ohashi et al.,[8] based on the prevalence of narcolepsy in the Japanese population (0.16%–0.18%)[1,2] and the results of a case-control association study of this haplotype.[7] On the basis of these values and with the formula described by James[9] and by Risch,[10] the expected λs value for *HLA* of Japanese patients with narcolepsy was calculated to be 5.15, much less than the λs of 12 reported for narcolepsy.[6] Therefore, genes other than *HLA* are also expected to contribute to the disease susceptibility.

Several candidate regions[11–13] and genes[14,15] other than *HLA* have been investigated for association with human narcolepsy involving cataplexy (narcolepsy-cataplexy) and daytime sleepiness. Nevertheless, replicated associations are few, except for tumor necrosis factor-α (*TNFA* [MIM 191160]) and TNF-receptor 2 (*TNFR2* [MIM 191191]).[16–20] In autosomal recessive canine models that develop narcolepsy-cataplexy with full penetrance, an insertion in the hypocretin (orexin)–receptor type 2 gene (*HCRTR2* [MIM 602393]) was found to be responsible for the disorder,[21] and preprohypocretin-knockout mice exhibit a phenotype similar to narcolepsy-cataplexy.[22] For human narcolepsy, which shows multifactorial inheritance, as described above, the hypocretin concentration in cerebrospinal fluid was reduced or undetectable in spo-

From the Departments of Sleep Disorder Research (Alfresa) (M.K.; T.E.) and Human Genetics (M.K.; H.H.; K.T.), Graduate School of Medicine, University of Tokyo, the Japanese Red Cross Central Blood Center (T.J.), and the Neuropsychiatric Research Institute (Y.H.), Tokyo; and the Department of Molecular Life Science, Tokai University School of Medicine, Kanagawa, Japan (G.T.; A.O.; H.I.)

Received January 20, 2006; accepted for publication April 27, 2006; electronically published June 13, 2006.

Address for correspondence and reprints: Dr. Kastushi Tokunaga, Department of Human Genetics, Graduate School of Medicine, University of Tokyo, 7-3-1 Hongo Bunkyo-ku, Tokyo, 113-0033, Japan. E-mail: tokunaga@m.u-tokyo.ac.jp

* Present affiliation: National Institute of Neuroscience, Tokyo.

Am. J. Hum. Genet. 2006;79:252–263. 0002-9297/2006/7902-0008$15.00

radic narcolepsy,[23] and the number of hypothalamic hypocretin neurons was decreased in postmortem narcoleptic brains.[24,25] Although the preprohypocretin (MIM 602358) and hypocretin-receptor genes have been examined for possible association with human narcolepsy, variants in these genes were not detected in most human patients with narcolepsy.[26–28] Therefore, human narcolepsy cannot be explained by mutations in preprohypocretin and hypocretin-receptor genes.

There is evidence for a role of autoantibodies in narcolepsy. Recently, mice were injected with purified immunoglobulin G (IgG) fraction from the serum of nine patients who have narcolepsy-cataplexy with the *HLA-DQB1*0602* haplotype. These mice exhibited stresslike behaviors, such as crouching posture and piloerection, and narcoleptic-like behavior, such as brief behavioral pauses lasting from a few seconds to a minute during periods of activity.[29] Another group revealed that IgG in the cerebrospinal fluid of *HLA-DQB1*0602*–positive patients with narcolepsy-cataplexy binds to rat hypothalamic proteins.[30] These two reports suggested that cerebrospinal fluid and serum from patients with narcolepsy contain functional autoantibodies that contribute to the pathogenesis of narcolepsy. However, the pathophysiological mechanism and genetic factors underlying human narcolepsy remain unknown.

For this study, we performed a genomewide association study, using 23,244 microsatellite markers for the detection of susceptibility/resistance regions to narcolepsy. This strategy is expected to be effective in the search for candidate regions throughout the whole genome, because of the high detection power of case-control association studies.[31,32] Microsatellite markers are abundant and interspersed throughout the human genome. Compared with SNPs, microsatellite markers display higher degrees of polymorphisms: multiple alleles exhibit high levels of heterozygosity, so a smaller number of microsatellite markers may provide a reasonable statistical power in association analyses.[33,34] Moreover, to reduce the genotyping cost and labor, genomic DNA samples were pooled[33,35] in the first and second screenings. We demonstrated elsewhere that this strategy can detect the known association with the *HLA* region; using 1,265 microsatellite markers on chromosome 6, we detected strong associations between multiple microsatellite markers in the *HLA* region and human narcolepsy.[36] Here, we extend the strategy to the other chromosomes, using 21,979 additional microsatellite markers.

Material and Methods

Patients and Unaffected Individuals

All patients and unaffected individuals were unrelated Japanese adults living in Tokyo or neighboring areas. Genomic DNAs were obtained from 370 patients given a diagnosis of narcolepsy-cataplexy at the Sleep Disorders Clinic of Seiwa Hospital. All patients with narcolepsy carried the *HLA-DRB1*1501-DQB1*0602* haplotype. These 370 genomic DNAs were divided randomly into three sets (the first and second sets with 110 samples each and the third set with the remaining 150 samples). The control group comprised 610 unrelated unaffected individuals and an additional 125 individuals positive for *HLA-DRB1*1501*. The 610 control samples were also divided into three sets (210 samples each in the first and second sets and the remaining 190 samples in the third set). Genomic DNAs were purified from peripheral blood, with the use of a commercial kit (QIAamp Blood Kit [Qiagen]). This study was approved by the research ethics review committees of the University of Tokyo and the Neuropsychiatric Research Institute, which runs Seiwa Hospital.

Preparation of Pooled DNA Samples

Genomic DNA concentration was measured in triplicate, in accordance with the methods of Collins et al.,[37] with the use of a double-stranded DNA quantification kit (PicoGreen [Molecular Probes]) with a microtiter plate reader (SF600 Corona Electric). Genomic DNAs were adjusted to 8 ng/μl. DNAs from 110 patients with narcolepsy and from 210 controls were then mixed, for the first set of case and control pools, named "case-1" and "control-1," respectively.[38] The second set of pooled DNA (case-2 and control-2) was also prepared from another 110 cases and 210 controls.

Analyses of Microsatellite Polymorphisms for Genomewide Screening

All microsatellite markers and the methods for microsatellite analysis used in this study are described by Tamiya et al.[33] In brief, PCR primers were designed for amplifying fragments that include the microsatellite polymorphisms. All PCR primers were designed to have an annealing temperature of 57°C. Forward primers were labeled at the 5′ end with fluorescent reagent (6-FAM or HEX [Applied Biosystems]). PCR on pooled DNAs was performed in 20-μl reactions containing 48 ng of pooled DNA, 0.5 units of DNA polymerase (AmpliTaq [Applied Biosystems]), 1 × reaction buffer with 1.5 mM $MgCl_2$ provided by the manufacturer (Applied Biosystems), 5 μM of each primer, and 0.25 mM of each deoxyribonucleotide triphosphate (dNTP) in 96- or 384-well plates. The amplification condition consisted of initial denaturation at 96°C for 5 min (hot start), annealing at 57°C for 1 min, and extension at 72°C for 1 min, followed by 40 cycles of denaturation at 96°C for 45 s, annealing at 57°C for 45 s, and extension at 72°C for 1 min, with use of a thermal cycler (GeneAmp PCR system 9700 [Applied Biosystems]).

For microsatellite typing of individual samples, PCR was performed in 12-μl reactions containing 2 ng of genomic DNA, 0.25 units of DNA polymerase (AmpliTaq Gold [Applied Biosystems]), 1 × reaction buffer with 1.5 mM $MgCl_2$ provided by the manufacturer, 5 μM of each primer, and 0.2 mM of each dNTP in 96- or 384-well plates. The amplification conditions were essentially the same as described above.

The PCR products were denatured in formamide (Hi-Di [Applied Biosystems]) at 95°C for 3 min and were separated by electrophoresis, with the use of an automated DNA sequencer with size standards (ABI Prism 3700 Genetic Analyzer, ROX size standard [Applied Biosystems]). The fragment size and the electrophoretograms were analyzed by GeneScan and Genotyper software (Applied Biosystems).

Additional Microsatellite Markers for High-Density Association Mapping

To obtain additional microsatellite markers in the narcolepsy candidate–region 1 (NLC1), the sequence of the candidate region was obtained from the University of California–Santa Cruz (UCSC) Genome Browser database (November 2002 version, based on NCBI Build 31). Then, the sequence was searched for repeated elements with the RepeatMasker program. Dinucleotide repeats with repeat number >12, trinucleotide repeats >8, and tetranucleotide to hexanucleotide repeats >5 were chosen. PCR primers were designed as described above, and we evaluated the polymorphism of each microsatellite with pooled DNA, searching for multiple peaks in the electrophoresis.

SNP Analyses

SNPs within the candidate region were selected from the Celera database at average intervals of ~5 kb, and specific PCR primers were designed. To confirm the polymorphisms of these SNP sites in the Japanese population, we examined 16 samples from patients with narcolepsy by direct sequencing, using a PCR cycle-sequencing kit and an automated DNA sequencer (BigDye Terminator v.3.1 Cycle Sequencing Kit and ABI PRISM 3730 DNA sequencer [Applied Biosystems]). The association analyses with these polymorphic sites were performed by direct sequencing of case and control samples.

The sequence of the entire region of *NLC1-A, NLC1-B,* and *NLC1-C* genes (based on NCBI Build 35 chromosome 21: 45234058–45250151) was also screened for polymorphisms with 16 samples, and polymorphic sites were subjected to association analyses by direct sequencing of case and control samples. Newly detected polymorphisms have been registered in the dbSNP database.

Expression Analysis by RT-PCR

The expression of the predicted genes in candidate region NLC1 was examined by RT-PCR, with the use of poly(A)$^+$ RNA from the human brain, hypothalamus, peripheral blood, sperm, and several organs (i.e., heart, liver, spleen, pancreas, lung, kidney, and skeletal muscle [Bio Chain]). To discriminate PCR products derived from reverse-transcribed mRNA from those derived from genomic DNA, we designed specific forward and reverse primers in the predicted exon 1 and 2 regions, respectively. The primer sets for the predicted genes were as follows: 5′-CTAGGAGGGGAAACTGAGTCC-3′ and 5′-CAGCACAGTTGGAGACATCACT-3′ for *NLC1-A,* 5′-CCTCACAGCATCCCACATT-3′ and 5′-TTTCTGGAAACAGCCAGGAG-3′ for *NLC1-B,* and 5′-GCTGAACTGCCTGGACTTTC-3′ and 5′-ACATGTGCTCCCCACCTAAG-3′ for *NLC1-C*. The thermal cycling profile consisted of initial denaturation at 96°C for 10 min, followed by 35 cycles of denaturation at 96°C for 45 s, annealing at 57°C for 45 s, extension at 72°C for 1 min, and a final extension at 72°C for 5 min, with the use of AmpliTaq Gold polymerase (Applied Biosystems). The PCR products were separated by electrophoresis on 2% agarose gels and were stained with ethidium bromide. The sequences of the amplified products were confirmed by direct sequencing.

Reporter-Gene Assay

Reporter-gene assays were performed using constructs containing microsatellite marker *D21S0012m* and SNP *rs13046884* alleles. For *D21S0012m,* genomic DNAs were obtained from four homozygotes for alleles with AC repeat numbers 8, 9, 10, and 12. A 908-bp fragment within the promoter region including *D21S0012m* was amplified by PCR, with use of the specific primers 5′-CAAAGGTACCTCCAGTCCACACCCACC-3′ and 5′-GTTTGAGCTCTTTGGCCTGTCCATCAG-3′. Genomic DNA for SNP *rs13046884* alleles was obtained from one *rs13046884* heterozygote. A 297-bp fragment within *NLC1-A* intron 1, which includes *rs13046884,* was amplified using primers 5′-CAAAGGTACCAGGGTTGGACTCCAAAGGGA-3′ and 5′-GTTTGAGCTCGGGTGACTTCTTCACACCCA-3′. PCR was performed (TaKaRa LA *Taq* [TaKaRa]) with the following thermal cycling profile: denaturation at 96°C for 5 min, followed by 35 cycles at 96°C for 30 s, 60°C for 30 s, and 72°C for 1.5 min. PCR products were digested with *Sac*I and *Kpn*I restriction endonucleases and then were inserted upstream of a firefly luciferase gene in the pGL3-control vector (Promega), with the use of T4 DNA ligase (TaKaRa). Inserted sequences were confirmed by direct sequencing with primers specific to the pGL3-control vector (5′-CATACGCTCTCCATCAAAACAA-3′ and 5′-AAGCCTCCTCACTACTTCTGGA-3′). The neuroblastoma cell line NB-1 and HeLa cells were maintained in accordance with published recommendations (Human Science Research Resources Bank). Then, 0.2 μg of each construct was introduced into the cells by a lipofection method (Effectene Transfection Reagent [Qiagen]), along with 0.02 μg of pRL-SV40 (Promega) as an internal control. Luciferase levels were determined using the DUAL-Luciferase Reporter Assay System (Promega), and firefly luciferase levels were normalized to the levels of renilla luciferase from pRL-SV40.

Statistical Analyses

Disease associations with polymorphisms were assessed by Fisher's exact test, with the use of 2×2 contingency tables for each allele. The smallest P value for each marker was selected. Allele frequencies in pooled-DNA typing were estimated from the height of peaks: each allele frequency was determined by dividing the height of each allele by the summed height of all alleles. In individual typing, the significance was evaluated by Fisher's exact test, with the use of $2 \times m$ (where m is the number of alleles) and 2×2 contingency tables. The significance level was set at .05 throughout this study, except for homogeneity among samples used in the first, second, and third set, which was tested by means of the Q statistic and was considered significant for $P < .10$.[39]

To assess the extent of pairwise linkage disequilibrium (LD) between polymorphisms, Lewontin's[40] D' and r^2 were calculated using a commercial software package (SNPAlyze-3.2 pro [Dynacom]) based on the expectation-maximation algorithm. D' and r^2 were calculated only for polymorphisms with a minor-allele frequency (MAF) >6%. Pairwise D' and r^2 were plotted at the Cartesian coordinate corresponding to the polymorphism location on the physical map with the use of the GOLD program, as described by Abecasis and Cookson.[41]

Results

Genomewide Association Study

The 23,244 microsatellite markers used in the genomewide association study are summarized in table 1. To reduce the cost and the technical burden of genomewide association analysis, the DNA-pooling method was applied in the first and second screenings (fig. 1*A*). Allele

Table 1. Numbers and Mean Intervals of Microsatellite Markers on Each Chromosome

The table is available in its entirety in the online edition of *The American Journal of Human Genetics*.

frequencies were estimated from the height of individual peaks. To avoid false-negative associations, we performed no correction for multiple comparisons. Figure 1*B* shows the results of the association analyses in the first screening; the results for the 1,265 microsatellite markers on chromosome 6, which includes the *HLA* region, were described elsewhere.[36] A total of 2,686 markers (202 of which were on chromosome 6) showed significantly different frequencies between cases and controls. These 2,686 markers were further analyzed in the second screening with pooled DNA samples from different sets of cases and controls, and 96 markers (16 on chromosome 6) remained significantly different between cases and controls and had similar peak patterns between first and second case pools and between first and second control pools.

To confirm the associations observed, we subjected the 80 microsatellite markers located outside of chromosome 6 to individual typing with 95 case and 95 control samples, which were randomly chosen from the samples used in the first and second pools. Thirty markers still showed statistically significant differences by Fisher's exact test, with the use of 2×2 contingency tables (fig. 1*A* and 1*B* and table 2). In particular, 11 microsatellite markers also showed differences in allele frequencies with the use of $2 \times m$ contingency tables. The remaining samples that were used in the first and second screenings (125 cases and 325 controls) were also genotyped for those 11 markers, and all the markers still reached a significant level. In addition, the smallest *P* values calculated by Fisher's exact test, with the use of 2×2 contingency tables in the pooled-DNA genotyping (first and second screenings), were reflected by the smallest *P* values in the individual genotyping with 220 cases and 420 controls.

One of the strong associations with narcolepsy was displayed by the microsatellite marker *D21S0241i,* located on 21q22.3 (based on NCBI Build 35 chromosome 21: 45169766–45169825; $[AAGG]_{7-17}$). The frequency of the $(AAGG)_{10}$ allele was 8.6% among cases and 4.0% among controls ($P = .0012$; odds ratio [OR] 2.24; 95% CI 1.40–3.58). To further confirm the association, we tested *D21S0241i* for association in a third set of individuals not used in either of the pools (150 cases and 190 controls). Significant association was not observed in this set ($P = .12$; OR 2.11; 95% CI 0.88–5.06), but no heterogeneity was detected among the three sets of cases or controls by the Q statistic ($P = .92$), and the allele frequencies in the third set displayed the same general pattern among cases compared with controls as they did in the first and second sets. Moreover, the significant association was still observed when the association analysis was performed by individual genotyping of the three sets of case and control samples ($P = .00064$; OR 2.08; 95% CI 1.38–3.13) (table 3). Taken together, these observations indicate that marker *D21S0241i* is associated with human narcolepsy.

High-Density Association Mapping with Additional Microsatellite Markers and SNPs

We analyzed the region surrounding the marker *D21S0241i* by high-density association mapping with additional microsatellite markers. To develop additional markers, we searched the surrounding genomic sequence for microsatellites. Polymorphisms of individual microsatellites were detected using pooled DNA samples, and 14 new microsatellite markers were established within 652 kb around marker *D21S0241i* (fig. 2*A*). These markers were analyzed in 220 case and 440 control samples (table 4). The new marker, *D21S0012m* (chromosome 21: 45238835–45238862), which is 70 kb from *D21S0241i,* showed a stronger association than *D21S0241i* (frequencies of the $[AC]_{10}$ allele: cases 0.9%, controls 4.6%; $P = .00023$; OR 0.19; 95% CI 0.074–0.48).

Next, to further define the candidate region, we performed high-density mapping with SNPs (listed in appendix A [online only]). To cover a 171-kb region around the markers *D21S0241i* and *D21S0012m,* we selected, from databases, SNPs at ~5-kb intervals. Direct sequencing on 16 cases (8 homozygotes and 8 heterozygotes of *D21S0012m* allele $[AC]_{10}$) was performed to screen for the SNPs. In total, 64 registered SNPs and 10 novel ones were identified and were subjected to association analyses with 190 cases and 190 controls (fig. 2*B*). Of the SNPs near *D21S0241i,* only one (*rs12483718*) showed a significant difference between cases and controls, whereas eight SNPs located close to *D21S0012m* showed significant differences. The strongest association with narcolepsy was exhibited by SNPs *rs13048981* and *rs13046884,* both located close to *D21S0012m* (for *rs13048981,* $P = .0016$, OR 0.17, and 95% CI 0.057–0.51; for *rs13046884,* $P = .0010$, OR 0.16, and 95% CI 0.055–0.48) (see fig. 3). The SNPs *rs13048981* and *rs13046884* were further typed for all the available samples (370 cases and 610 controls). The results for *rs13048981* were $P = .00039$, OR 0.31, and 95% CI 0.16–0.60; the results for *rs13046884* were $P = .00036$, OR 0.33, and 95% CI 0.18–0.62 (table 3). The two SNPs were further analyzed in 125 independently collected *HLA-DRB1*1501*–positive unaffected controls. Both SNPs again exhibited significant associations in the 370 cases compared with these controls (for *rs13048981,* $P = .017$, OR 0.33, and 95% CI 0.14–0.77; for *rs13046884,* $P = .023$, OR 0.36, and 95% CI 0.16–0.83) (table 3). Therefore, we considered this region to be a candidate region for susceptibility/resistance to human narcolepsy and tentatively named the region "narcolepsy candidate–region 1 (NLC1)."

The LD structure in this region was analyzed using the SNPs with MAFs >6%, and one large LD block was found (fig. 3*A* and 3*B*). SNPs *rs13048981* and *rs13046884* and

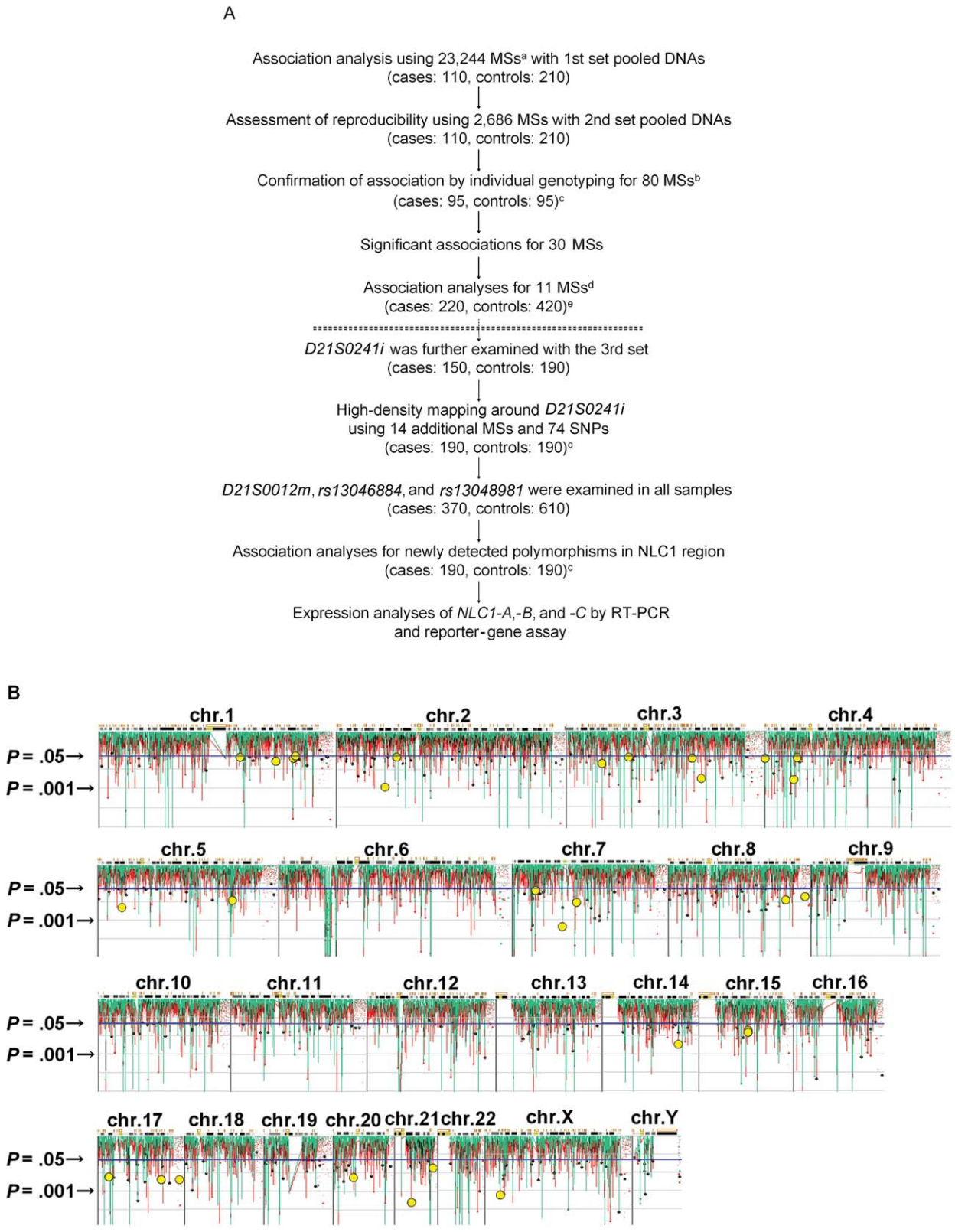

Figure 1. Genomewide association analyses with 23,244 microsatellite markers. *A,* Flow chart of this study. *a,* MS = microsatellite marker. *b,* Sixteen MSs on chromosome 6 and 80 MSs on other chromosomes showing reproducible peak patterns and remaining significantly different between cases and controls. *c,* Selected randomly from the samples used in the first and second screenings. *d,* Eleven MSs reaching significance in both 2×2 and $2 \times m$ contingency tables. *e,* All samples used in first and second screenings. *B,* Results of genomewide screening. The figure shows *P* values by Fisher's exact test based on 2×2 contingency tables (*green line*) or on $2 \times m$ contingency tables (*red line*) in the first screening. Yellow circles indicate 30 MSs that showed significant associations in both first and second screenings and in individual typing.

Table 2. Thirty Microsatellite Markers Showing Significant Association in the First and Second Screenings and in the Subsequent Individual Genotyping

		P					
		Pooled DNA[a]		95 Cases and 95 Controls		220 Cases and 420 Controls	
Marker	Chromosome	First	Second	2×2[b]	$2 \times m$[c]	2×2[b]	$2 \times m$[c]
D1S0500i	1q23.1	.046	.00024	.017	.28	…	…
D1S0583i	1q32.2	.038	.049	.018	.11	…	…
D1S1208i	1q32.2	.045	.045	.029	.034	.0010	.0027
D1S1148i	1q31.2	.025	.047	.018	.073	…	…
D2S303	2p14	.045	.017	.021	.19	…	…
D2S0878i	2p16.2	.0011	.025	.0023	.0020	.0010	.00073
D3S0502i	3p22.2	.019	.036	.037	.15	…	…
D3S0971i	3q22.2	.037	.016	.031	.23	…	…
D3S0978i	3q24	.0031	.0047	.0044	.041	.0081	.13
D3S1174i	3p14.1	.044	.027	.022	.0084	.0021	.0013
G08391	4p15.1	.041	.047	.044	.16	…	…
D4S0140i	4p15.1	.0027	.037	.0023	.0067	.0020	.0070
D4S0424i	4p16.3	.038	.036	.049	.63	…	…
D5S0022i	5q32	.012	.0084	.0040	.016	.011	.0017
D5S0565i	5p13.3	.0051	.013	.0031	.052	…	…
D7S0486i	7p15.2	.036	.0020	.049	.68	…	…
D7S0760i	7p11.2	.0044	.0040	.016	.086	…	…
D7S1066i	7q11.22	.0089	.028	.011	.095	…	…
D8S0068i	8	.018	.034	.011	.30	…	…
D8S0584i	8q24.21	.012	.012	.018	.11	…	…
D14S0284i	14q31.3	.0030	.049	.0058	.018	.027	.38
D15S150	15q21.3	.020	.049	.024	.21	…	…
D15S0157i	15q21.3	.019	.0088	.010	.16	…	…
D17S1300	17q24.3	.022	.0071	.036	.13	…	…
D17S0179i	17	.0044	.050	.018	.0090	.0016	.0060
D17S0306i	17p11.2	.0060	.021	.011	.014	.0069	.076
D20S0027i	20p11.21	.0055	.014	.024	.18	…	…
D21S0098i	21q21.1	.023	.0044	.00029	.0051	.00031	.011
D21S0241i	21q22.3	.018	.00043	.0098	.018	.0012	.048
DXS0660i	Xp22.13	.00061	.0080	.0025	.065	…	…

[a] *P* values calculated by Fisher's exact test, based on 2×2 contingency tables with estimated allele frequencies. The smallest *P* value was selected. The alleles that showed the smallest *P* values in the pooled DNA genotypings were reflected in the individual genotyping.

[b] *P* values calculated by Fisher's exact test, based on 2×2 contingency tables. The smallest *P* value was selected.

[c] *P* values calculated by Fisher's exact test, based on $2 \times m$ contingency tables.

microsatellite marker *D21S0012m* were in the same LD block, whereas *D21S0241i* was not. In addition, the *rs13046884* g allele, the *rs13048981* t allele, and the *D21S0012m* $(AC)_{10}$ allele were found to be in strong LD ($r^2 > 0.94$), and the estimated haplotype frequency was 4.5% in the controls.

The region around the three polymorphisms contains three predicted genes registered in the UCSC Genome Browser, each of which is supported by between two and five mRNAs or ESTs (fig. 2*C*). These predicted genes are on the reverse strand on chromosome 21q22.3, with positions as follows: *NLC1-A* 45234209–4523842, *NLC1-B* 45238709–45239923, and *NLC1-C* 45243550–45249070 (Genome Browser accession numbers BC036902, BC009635, and BC027456, respectively). According to the UCSC Genome Browser, *NLC1-A* produces two alternatively spliced transcripts encoding different protein isoforms; the position of the short isoform is 45235619–45238383. The functions of these predicted genes are currently unknown.

We performed further variation screening on the three genes by direct sequencing with 16 samples, and 26 polymorphisms were observed. Fourteen of the polymorphisms were new: eight SNPs in *NLC1-A,* two in *NLC1-B,* and four in *NLC1-C.* Next, these new polymorphisms were subjected to association analysis with 190 cases and 190 controls (fig. 2*C*). Four SNPs reached significance in the analysis, but none was stronger than *rs13048981* or *rs13046884,* indicating that these two SNPs, as well as *D21S0012m,* are associated primarily in this region.

Table 3. Association Analyses of *D21S0241i, D21S0012m, rs13048981,* and *rs13046884* with 370 Patients with Narcolepsy and 610 Unaffected Controls

Marker or SNP and Allele	No. (%) of Individuals		
	Patients with Narcolepsy (n = 370)	Control Individuals (n = 610)	DRB1*1501-Positive Control Individuals (n = 125)
D21S0241i:			
$(AAGG)_7$	0 (.0)	1 (.1)	…
$(AAGG)_8$	0 (.0)	1 (.1)	…
$(AAGG)_9$	1 (.1)	0 (.0)	…
$(AAGG)_{10}$[a]	51 (6.9)	42 (3.4)	…
$(AAGG)_{11}$	65 (8.8)	90 (7.4)	…
$(AAGG)_{12}$	155 (20.9)	258 (21.1)	…
$(AAGG)_{13}$	154 (20.8)	249 (20.4)	…
$(AAGG)_{14}$	195 (26.4)	359 (29.4)	…
$(AAGG)_{15}$	94 (12.7)	180 (14.8)	…
$(AAGG)_{16}$	24 (3.2)	35 (2.9)	…
$(AAGG)_{17}$	1 (.1)	5 (.4)	…
D21S0012m:			
$(AC)_8$	127 (17.2)	168 (13.8)	…
$(AC)_9$	447 (60.4)	762 (62.5)	…
$(AC)_{10}$[b]	12 (1.6)	54 (4.4)	…
$(AC)_{11}$	36 (4.9)	48 (3.9)	…
$(AC)_{12}$	117 (15.8)	188 (15.4)	…
$(AC)_{13}$	1 (.1)	0 (.0)	…
rs13048981:			
Genotype[c,d]:			
CC	360 (97.3)	561 (92.0)	115 (92.0)
CT	10 (2.7)	47 (7.7)	10 (8.0)
TT	0 (.0)	2 (.3)	0 (.0)
Allele[e,f]:			
C	730 (98.6)	1,169 (95.8)	240 (96.0)
T	10 (1.4)	51 (4.2)	10 (4.0)
rs13046884:			
Genotype[g,h]:			
AA	359 (97.0)	559 (91.6)	115 (92.0)
AG	11 (3.0)	49 (8.0)	10 (8.0)
GG	0 (.0)	2 (.3)	0 (.0)
Allele[i,j]:			
A	729 (98.5)	1,167 (95.7)	240 (96.0)
G	11 (1.5)	53 (4.3)	10 (4.0)

[a] OR 2.08; 95% CI 1.4–3.1; $P = .00064$.

[b] OR 0.36; 95% CI 0.2–0.7; $P = .00068$.

[c] $P = .00095$ (patients with narcolepsy compared with controls, by Fisher's exact test based on a 2 × 3 contingency table).

[d] $P = .016$ (patients with narcolepsy compared with DRB1*1501-positive controls, by Fisher's exact test based on a 2 × 3 contingency table).

[e] OR 0.31; 95% CI 0.16–0.60; $P = .00039$ (patients with narcolepsy compared with controls, by Fisher's exact test based on a 2 × 2 contingency table).

[f] OR 0.33; 95% CI 0.14–0.77; $P = .017$ (patients with narcolepsy compared with DRB1*1501-positive controls, by Fisher's exact test based on a 2 × 2 contingency table).

[g] $P = .0011$ (patients with narcolepsy compared with controls, by Fisher's exact test based on a 2 × 3 contingency table).

[h] $P = .022$ (patients with narcolepsy compared with DRB1*1501-positive controls, by Fisher's exact test based on a 2 × 3 contingency table).

[i] OR 0.33; 95% CI 0.18–0.62; $P = .00036$ (patients with narcolepsy compared with controls, by Fisher's exact test based on a 2 × 2 contingency table).

[j] OR 0.36; 95% CI 0.16–0.83; $P = .023$ (patients with narcolepsy compared with DRB1*1501-positive controls, by Fisher's exact test based on a 2 × 2 contingency table).

Expression Analyses

We assessed the expression of these three predicted genes in the human brain, hypothalamus, and other organs by RT-PCR, using specific primers (fig. 4*A*). Products with the expected size were amplified for *NLC1-A* and *NLC1-C* in whole brain and hypothalamus (fig. 4*B* and 4*C*). Moreover, direct sequencing of the products confirmed that the correct sequence was amplified. However, for *NLC1-B,* the amplified band was from genomic DNA, not from cDNA. These observations indicate that *NLC1-A* and *NLC1-C* were expressed in human whole brain and hypothalamus, whereas *NLC1-B* was not. Notably, *NLC1-A* was also expressed in human spleen, lung, kidney, and skeletal muscle, and *NLC1-C* was also expressed in human spleen, pancreas, lung, and sperm (fig. 4*C*), but neither was expressed in peripheral blood (data not shown). SNP *rs13046884* is in *NLC1-A* intron 1, and *D21S0012m* is 424 bp upstream of the transcriptional start site of *NLC1-A*, which suggests that *NLC1-A* may be a susceptibility/resistance gene for human narcolepsy. SNP *rs13048981* is located 2,602 bp upstream of *NLC1-B,* which was not expressed in human brain, and its position, 4,164 bp upstream of *NLC1-A,* suggests that its association with narcolepsy resulted merely from the LD with *rs13046884* and *D21S0012m.* Thus, it is unlikely that *NLC1-B* is a susceptibility/resistance gene for human narcolepsy.

Table 4. Association Analyses with High-Density Microsatellite Markers around Marker *D21S0241i*

The table is available in its entirety in the online edition of *The American Journal of Human Genetics*.

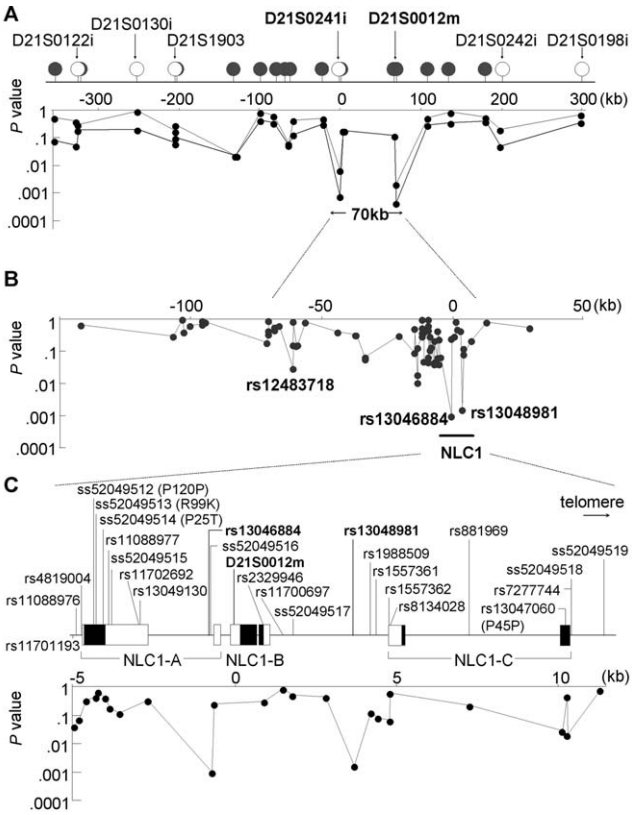

Figure 2. High-density mapping with additional microsatellite markers and SNPs. *A,* Association analyses using high-density microsatellite markers with 220 cases and 420 controls. Unblackened circles indicate microsatellite markers used in the first and second screenings. Blackened circles indicate microsatellite markers newly developed for the high-density mapping. The dark line shows the *P* values calculated by Fisher's exact test based on 2 × 2 contingency tables, whereas the lighter line shows those from 2 × *m* contingency tables. *B,* Association analyses using SNPs with 190 cases and 190 controls. The *X*-axis indicates the distance from *D21S0012m*. SNP *rs12483718* is located near *D21S0241i*. The *Y*-axis shows the *P* values calculated by Fisher's exact test based on 2 × 2 contingency tables. Two SNPs, *rs13048981* and *rs13046884,* showed the strongest associations in the NLC1 region. *C,* Variation screening and high-density association analyses in the NLC1 region. *Top,* Exon-intron structures of *NLC1-A, NLC1-B,* and *NLC1-C*. Boxes indicate exons, with unblackened boxes indicating untranslated regions and blackened boxes indicating coding regions. Predicted gene regions and 1 kb of upstream region were screened for sequence variations. Fourteen additional polymorphisms, including two nonsynonymous substitutions, were detected and were examined for possible associations, but no polymorphisms showed stronger association than *D21S0012m, rs13048981,* and *rs13046884*. *Bottom, P* values for individual SNPs.

The figure is available in its entirety in the online edition of *The American Journal of Human Genetics*.

Figure 3. LD block structure. The legend is available in its entirety in the online edition of *The American Journal of Human Genetics*.

To test whether polymorphisms *rs13046884* and *D21S0012m* directly influence the transcription level of *NLC1-A,* we performed reporter-gene assays. Six constructs carrying different alleles of *D21S0012m* or *rs13046884* were prepared from individuals with *D21S0012m* $(CA)_8$, $(CA)_9$, $(CA)_{10}$, and $(CA)_{12}$ repeats or the *rs13046884* a/g genotype. These constructs were introduced into NB-1 or HeLa cells, and the expression of luciferase was examined in three independent experiments. The differences of transcriptional activity were assessed by *t* test. The luciferase activities of each construct were divided by the ones of empty vector. These values were used for the *t* test. Figure 5 shows that the luciferase activities of reporters carrying the resistance alleles (g allele of *rs13046884* and $[AC]_{10}$ allele of *D21S0012m*) were 1.5- to 2-fold lower than those of other reporters in both NB-1 and HeLa cells, and the differences assessed by *t* test reached statistical significance (for NB-1 cell, $t = 2.4–6.7$ and $P = .039–.0010$; for HeLa cell, $t = 6.9–74.7$ and $P = .0034–.000000096$). Thus, the promoter activity of *NLC1-A* is likely to be reduced in individuals who possess the haplotype *D21S0012m* $(AC)_{10}$–*rs13046884* g.

Discussion

We have systematically performed the first genomewide association analyses, to our knowledge, for detecting susceptibility or resistance genes to human narcolepsy, using 23,244 microsatellite markers. After two separate screenings with pooled DNA samples, followed by individual genotyping with 95 case and 95 control samples of 80 initial candidate markers located outside chromosome 6, 30 microsatellite markers remained as candidates for association with narcolepsy. Among them, one marker (*D21S0241i*) was further analyzed with a third set of cases and controls, to confirm the association. Although the difference between cases and controls in the third set did not reach statistical significance, the allele frequencies were similar to those in the first and second sets. Moreover, a significant association was detected in an analysis of all the available samples (370 cases and 610 controls). In an analysis of the region surrounding *D21S0241i,* one microsatellite marker (*D21S0012m*) and eight nearby SNPs, all located ~70 kb from *D21S0241i,* were significantly associated with narcolepsy. *D21S0012m* and two of the SNPs were the markers most strongly associated with narcolepsy (all $P < .0005$); these three polymorphisms are in strong LD. The genomic region including these three polymorphisms is, therefore, a candidate region for human narcolepsy, which we tentatively designated "NLC1." For each of the three strongly associated polymorphisms, a minor allele displayed significantly reduced frequency in patients with narcolepsy compared with controls (OR 0.19–0.33), which suggests that these alleles confer resistance to narcolepsy.

NLC1 is located on 21q22.3, 2.6 Mb away from a locus recently reported as a candidate region for French familial narcolepsy.[12] According to the SNP genotype data of 45 unrelated Japanese living in the Tokyo area registered in the HapMap project database, there is no LD between NLC1 and the region reported in the French family study. Therefore, the association of NLC1 with human narcolepsy is considered a novel observation.

The NLC1 region contains no known genes, but data-

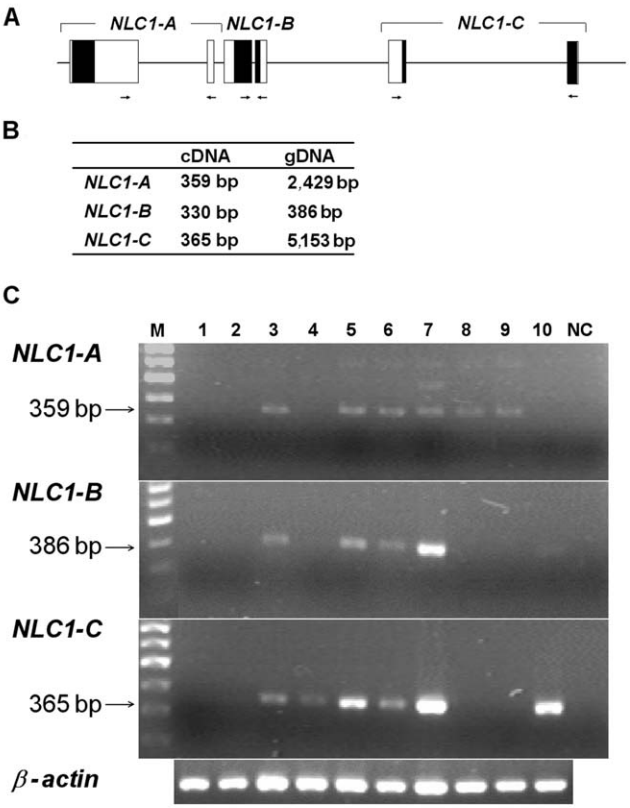

Figure 4. Expression analysis for *NLC1-A, NCL1-B,* and *NCL1-C,* with the use of RT-PCR. *A,* Schematic drawing of the specific primers for RT-PCR. *B,* Expected size of RT-PCR products from cDNA or genomic DNA. On the basis of the UCSC Genome Browser, products with the expected size were amplified from cDNA for *NLC1-A* and *NLC1-C* in samples of whole brain, hypothalamus, and several other organs, but, for *NLC1-B,* only the products from genomic DNA were observed (*C*). Amplified products were confirmed by direct sequencing. *Lane 1,* Heart; *lane 2,* liver; *lane 3,* spleen; *lane 4,* pancreas; *lane 5,* lung; *lane 6,* whole brain; *lane 7,* hypothalamus; *lane 8,* kidney; *lane 9,* skeletal muscle; *lane 10,* sperm. NC = negative control. M = 100-bp ladder size marker.

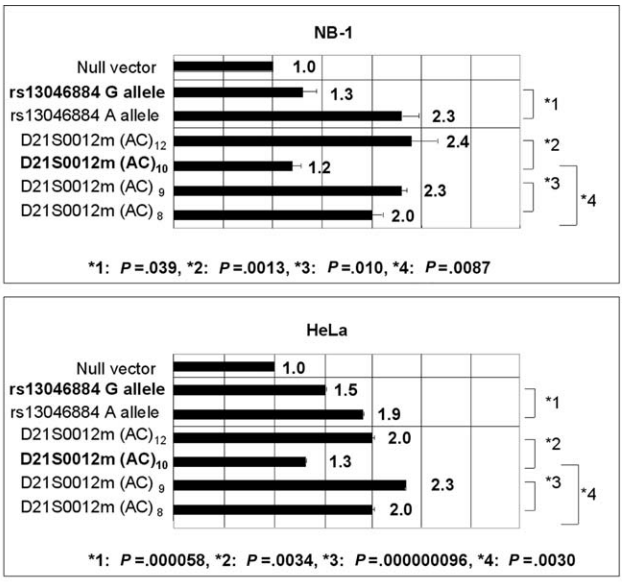

Figure 5. Effects of the microsatellite marker *D21S0012m* in the promoter region and of SNP *rs13046884* in the intron 1 of *NLC1-A* on transcriptional activity. Reporter-gene constructs contained the sequences from IVS1+31 to IVS1+327 for *rs13046884* or 80–987 nt upstream of the transcription initiation site for *D21S0012m*. The chart shows luciferase expression from each reporter in transfected HeLa cells or NB-1 cells, relative to empty vector. Data are means of at least three independent experiments. Error bars represent SDs.

bases show three predicted genes, which we tentatively named "*NLC1-A*," "*NLC1-B*," and "*NLC1-C*." Because of the locations of the three most strongly associated polymorphisms (*D21S0012m* in intron 1 of *NLC1-A*, *rs13046884* 424 bp upstream of *NLC1-A* and in the 3′ UTR of *NLC1-B*, and *rs13048981* 2,602 bp upstream of *NLC1-B*), we focused on *NLC1-A* and *NLC1-B*. In RT-PCR analysis, *NLC1-A*, but not *NLC1-B*, was expressed in human hypothalamus, which also expresses preprohypocretin,[42] a protein important in orchestrating the sleep-wake cycle.[43] Therefore, we finally focused on *NLC1-A*, and we tested whether the *D21S0012m* and *rs13046884* polymorphisms affect gene expression. In a reporter-gene assay, *NLC1-A* fragments containing the alleles for narcolepsy resistance (*D21S0012m* $[CA]_{10}$ allele and *rs13046884* g allele) were less transcriptionally active than were those of other alleles. This finding supports the hypothesis that the polymorphisms of *NLC1-A* may be directly involved in resistance to human narcolepsy.

A motif search of the putative NLC1-A protein, with the use of MOTIF (GenomeNet) and Motif-Finder (RIKEN), revealed a domain known as "binding-protein–dependent transport systems inner membrane component." Binding-protein–dependent transport systems have been characterized as members of a superfamily of transporters found not only in bacteria but also in humans, and they include both import and export systems.[44] Therefore, NLC1-A might function as a transporter of certain substances (amino acids, sugars, large polysaccharides, or proteins). A motif search of the cDNA sequence of *NLC1-A* was also performed using MOTIF and Motif-Finder, and NLC1-A includes domains known as integrin β-chain cysteine-rich domain, anaphylatoxin domain, and epidermal growth factor–1 domain signatures. Furthermore, the amino acid sequence of *NLC1-A* was subjected to secondary structure prediction (SOSUI program). NLC1-A has a long loop (residues 78–125) with high hydrophilicity, flexibility, and surface probability, which suggests that NLC1-A may be a membrane protein. No carbohydrate-modification region was predicted. The UCSC Genome Browser showed a chimpanzee gene with 98% sequence identity to *NLC1-A*. In contrast, there was no homologous gene in rodent or canine genomes. Thus, *NLC1-A* is likely to exist only in primates.

Recently, genomewide association analysis with hundreds of thousands of SNPs has become realistic, but such a systematic product was not available when we started the present study. Therefore, we took a unique approach—genomewide association analyses with highly polymorphic microsatellite markers that were selected every ~100 kb throughout the human genome.[33] Because pooled DNAs were used in the first and second screenings, the typing cost was reasonable, even when 23,244 markers were used.

Because human narcolepsy is a multifactorial disorder for which the relative risks of individual associated genes may not be particularly high, we hypothesize that several more susceptibility/resistance genes remain to be elucidated. Thirty microsatellite markers displayed association with human narcolepsy in both first and second screenings. The observed associations of the microsatellite markers were not strong, and the markers were similar to each other in the strength of association. Therefore, the remaining 29 uncharacterized regions may include other susceptibility/resistance loci for narcolepsy. Some false-positive results may still survive after both screenings with the use of pooled DNA samples, but most of them can be excluded in subsequent high-density mapping and association analysis with additional cases and controls. An association study with an entirely separate set of cases and controls or replication studies in other populations and transmission disequilibrium test may be preferred to completely eliminate false-positive associations, although the detection power is decreased because additional association studies lead to an increase in false-negative associations.

In conclusion, a genomewide association study with the use of a dense set of microsatellite markers and pooled DNA can be useful for the systematic search for candidate regions of multifactorial disorders—such as human narcolepsy, rheumatoid arthritis (RA [MIM 180300]), type II diabetes (NIDDM [MIM 125853]), hypertension (MIM 145500), psoriasis (MIM 177900), and schizophrenia

(SCZD [MIM 181500])—for which pathophysiological mechanisms remain unclear. We were able to detect 30 candidate microsatellite markers, among which one narcolepsy resistance gene, *NLC1-A,* was identified successfully. Functional analyses of *NLC1-A* are in progress, and the remaining 29 candidate markers will be further analyzed.

Acknowledgments

We sincerely thank the patients with narcolepsy who participated in this study. We are indebted to Dr. Jun Ohashi (Department of Human Genetics, Graduate School of Medicine, University of Tokyo), for helpful discussion on statistical analysis, and to Drs. Tomoki Ikuta, Minori Shinya, and Satoshi Makino (Department of Molecular Life Science, Tokai University School of Medicine), for technical help in the genomewide screening. This work was supported by a Grant-in-Aid for Science Research on Priority Areas (Comprehensive Genomics) from the Japanese Ministry of Education, Culture, Sports, Science, and Technology and by a grant from the Kato Memorial Trust for Nambyo Research.

Web Resources

Accession numbers and URLs for data presented herein are as follows:

Celera database, http://www.celera.com/
dbSNP, http://www.ncbi.nlm.nih.gov/SNP/
GOLD program, http://www.sph.umich.edu/csg/abecasis/GOLD/
HapMap, http://www.hapmap.org/
MOTIF, http://motif.genome.ad.jp/
Motif-Finder, http://gibk26.bse.kyutech.ac.jp/jouhou/HOMOLOGY/dbsearch/pdb/pdb_seq.html
Online Mendelian Inheritance in Man (OMIM), http://www.ncbi.nlm.nih.gov/Omim/ (for narcolepsy, *HLA-DRB1, HLA-DQB1, TNFA, TNFR2, HCRTR2,* prepohypocretin, RA, NIDDM, hypertension, psoriasis, and SCZD)
RepeatMasker program, http://www.repeatmasker.org/
SOSUI program, http://sosui.proteome.bio.tuat.ac.jp/sosuiframe0.html
UCSC Genome Browser (November 2002 version, based on NCBI Build 31), http://genome.ucsc.edu/ (for *NLC1-A* [accession number BC036902], *NLC1-B* [accession number BC009635], and *NLC1-C* [accession number BC027456])

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