HLA DQB1*06:02 Negative Narcolepsy with Hypocretin/Orexin Deficiency

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Study Objectives: To identify rare allelic variants and HLA alleles in narcolepsy patients with hypocretin (orexin, HCRT) deficiency but lacking DQB1*06:02.

Settings: China (Peking University People's Hospital), Czech Republic (Charles University), Denmark (Golstrup Hospital), Italy (University of Bologna), Korea (Catholic University), and USA (Stanford University).

Design: CSF hypocretin-1, DQB1*06:02, clinical and polysomnographic data were collected in narcolepsy patients (552 with and 144 without cataplexy) from 6 sites. Numbers of cases with and without DQB1*06:02 and low CSF hypocretin-1 were compiled. HLA class I (A, B, C), class II (DRBs, DQA1, DQB1, DPA1, and DPB1), and whole exome sequencing were conducted in 9 DQB1*06:02 negative cases with low CSF hypocretin-1. Sanger sequencing of selected exons in DNMT1, HCRT, and MOG was performed to exclude mutations in known narcolepsy-associated genes.

Measurements and Results: Classic narcolepsy markers DQB1*06:02 and low CSF hypocretin-1 were found in 87.4% of cases with cataplexy, and in 20.0% without cataplexy. Nine cases (all with cataplexy) were DQB1*06:02 negative with low CSF hypocretin-1, constituting 1.7% [0.8%-3.4%] of all cases with cataplexy and 1.8% [0.8%-3.4%] of cases with low CSF hypocretin independent of cataplexy across sites. Five HLA negative subjects had severe cataplexy, often occurring without clear triggers. Subjects had diverse ethnic backgrounds and HLA alleles at all loci, suggesting no single secondary HLA association. The rare subtype DPB1*0901, and homologous DPB1*10:01 subtype, were present in 5 subjects, suggesting a secondary association with HLA-DP. Preprohypocretin sequencing revealed no mutations beyond one previously reported in a very early onset case. No new MOG or DNMT1 mutations were found, nor were suspicious or private variants in novel genes identified through exome sequencing.

Conclusions: Hypocretin, MOG, or DNMT1 mutations are exceptional findings in DQB1*06:02 negative cases with hypocretin deficiency. A secondary HLA-DP association may be present in these cases. These represent particularly difficult diagnostic challenges.

Keywords: HLA, MHC, narcolepsy, cataplexy, hypocretin, orexin, MOG, DNMT1, exome sequencing

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INTRODUCTION

Following the report of Nishino et al.,[1] it is well established that narcolepsy with cataplexy is strongly associated with hypocretin (orexin) deficiency and human leukocyte antigen (HLA) DQB1*06:02. Hypocretin deficiency can be tested by measuring cerebrospinal fluid (CSF) levels of hypocretin-1, with one-third of normal values or 110 pg/mL being an optimal diagnostic cutoff.[2] Mutation screening of hypocretin and hypocretin receptor (HCRTR1 and HCRTR2) genes in patients with hypocretin deficiency has shown that hypocretin gene mutations are not a common cause of narcolepsy.[3] Rather, the disorder is autoimmune mediated, as recently demonstrated through genome wide association studies.[4] A likely trigger involves exposure to and molecular mimicry with flu epitopes, notably those of the pandemic H1N1 2009 influenza A strain,[4] explaining increases in incidence reported in 2010.[5-7] Based on the fact narcolepsy onset in children was also seasonal prior to 2010, other mimics involving other upper airway infections were likely involved prior to 2009.[5] Based on the strong genetic and pathophysiological homogeneity, these cases are called "type 1 narcolepsy" or "narcolepsy/hypocretin deficiency" in recent classifications.

As found in other autoimmune diseases, the HLA association of narcolepsy cannot be summarized by the simple dominant effect of a single allele. Although the presence of DQ0602 dimer is almost a prerequisite to developing narcolepsy, additional HLA-DQ alleles encoded in *trans* (from the other chromosome) of the DQA1*01:02-DQB1*06:02 haplotype appear to modulate susceptibility.[8-15] Homozygosity for the DQA1*01:02-DQB1*06:02 haplotype increases risk twofold, suggesting that the number of DQ0602 molecules on antigen presenting cells is important for predisposition.[8,15,16] Similarly, DQA1 and DQB1 alleles that have high structural sequence homology to DQA1*01:02 or DQB1*06:02 (i.e., belonging to the DQ1 group) have a protective effect. This is presumed to be due to a decrease the amount of available DQ0602 molecules caused by dimerization of these chains with the trans-encoded

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DQA1*01:02 or DQB1*06:02, a phenomenon we call "allele competition."[10] In addition to effects on DQ0602 availability, a predisposing effect of DQB1*03:01 independent of the DQA1 type has been found in multiple ethnic groups and remains unexplained, with a recent studies in Chinese suggesting a strong effect of the allele on age of onset.[7] Contrasting with most other autoimmune diseases, the HLA effects in narcolepsy are conserved across ethnic groups and countries,[9-15] strongly suggesting relative homogeneity of causal antigen presentation.

The association of hypocretin deficiency with DQ0602 in the context of narcolepsy is very strong; only four cases with low CSF hypocretin-1 but without DQB1*0602 have been reported. In almost all cases where HLA DQB1*06:02 is absent, CSF hypocretin-1 is normal,[2,17-22] which has led clinicians to recommend testing DQB1*06:02 prior to conducting a lumbar puncture. Whereas aggressive treatment of narcolepsy with hypocretin deficiency is easily justifiable,[23] caution is required for other patients,[24] many of whom receive lifelong therapy with potentially addictive medications. Indeed, some may have false positive results on the MSLT[25-28] in the presence of a subjective report of cataplexy.

Mutation screening of the hypocretin (HCRT) gene has only identified one potential disease causing mutation.[3] This case was unusual at several levels: the patient was DQB1*06:02 negative, one of only two such cases with low CSF hypocretin-1 at the time of the study. Onset was also unusually early and severe, with cataplexy onset at 6 months of age (very rare before 4 years of age).[29] Sequencing revealed substitution of a polar amino acid in the signal peptide that displayed abnormal trafficking in cell lines, consistent with a dominant phenotype. Mutation screening in other early onset cases has never revealed another plausible hypocretin gene mutation.[3,30-32]

More recently, Hor et al.[33] reported an intriguing Spanish family that included 13 cases with narcolepsy and cataplexy (plus obesity and type II diabetes in most cases). One family branch had DQB1*06:02 positive cases, but cases in the other branch were DQB1*06:02 negative yet DQB1*0201 positive, two of whom had undetectable CSF hypocretin-1. A missense variant in exon 2 of the myelin oligodendrocyte (MOG) gene (~3 Mb from HLA DQ) co-segregated in the pedigree. The variant was predicted to yield a misfolded protein, and abnormal MOG aggregates were seen in the cytoplasm of transfected cell lines. Interestingly, taiep rats, a model with oligodendrocyte cytoskeleton defects and decreased myelin binding protein[34-36] reportedly have narcolepsy-like symptoms together with additional features including absence seizures.

Of special interest is also the recent finding that exon 21 mutations in the DNA methylase gene DNMT1 cause autosomal dominant cerebellar ataxia, deafness and narcolepsy (ADCA-DN) in multiple families and in sporadic cases carrying de novo mutations.[37] Onset of narcolepsy may be the earliest sign, occurring in late adolescence and without cataplexy. Around age 40, central deafness also occurs, rapidly followed by cataplexy and cerebellar ataxia, and later by polyneuropathy and dementia. These cases have hypocretin deficiency, although levels below 110 pg/mL occur only at a very late disease stage.

In parallel with novel familial instances, which have more typically presented narcolepsy as part of a syndrome, only four HLA DQB1*06:02-negative narcolepsy cases with low CSF hypocretin-1 ($\leq$ 110 pg/mL) have been reported.[2,3,29,38-40] To further understand this rare phenotype, we gathered a case series from four centers, and conducted exome sequencing and full HLA class I and II typing. Sequencing of key exons in DNMT1 (exon 20 and 21), preprohypocretin (exon 2), and MOG (exon 2) was also performed to exclude pathogenic mutations in known narcolepsy-associated genes.

METHODS

Subjects

We selected all cases where data for both CSF hypocretin-1 levels and HLA DQB1*06:02 typing were available by (1) searching databases at the Stanford Center for Narcolepsy Research and in China, and (2) by contacting collaborators across the world (China, Czech Republic, Denmark, Korea, Italy; see Table 1). Information extracted for these subjects included demographics, country of origin, referral center, ethnicity, and clinical (notably age of onset of symptoms), biological and polysomnography data. Patients were separated for the presence of cataplexy, per International classification of Sleep Disorders, 2nd edition (ICSD2). A total of 9 HLA DQB1*06:02 negative patients had hypocretin deficiency (67% Caucasian, 33% Asian). Six of these were Caucasians (3 from Italy, 2 from the Czech Republic, 1 from the US), one was Chinese (from China), one was Indian (from Italy), and one was half-Japanese, half-Caucasian (from Denmark, who developed narcolepsy following H1N1 vaccination with Pandemrix). Magnetic resonance imaging (MRI) and extensive evaluations were conducted to eliminate tumors, encephalitis, or other confounding disorders. At least 5 years of follow-up clinical data was available on these subjects, and none had developed any additional pathology suggestive of a secondary etiology. Further, response to therapy was generally typical in these patients.

The ICSD2 was used for diagnosis, separating cases with and without cataplexy. In cases with cataplexy, the symptom had to be clear and definite. Episodes were triggered by strong emotions—most reliably laughing or joking—and were bilateral and brief (< 2 min). Consciousness was preserved, at least at the beginning of the episodes. When checked, a transient and reversible loss of deep tendon reflexes was observed during attacks. Cases without cataplexy all had a positive MSLT (MSL $\leq$ 8 min, $\geq$ 2 SOREMPs), and may have had doubtful or atypical cataplexy. Patients had no positive family history for narcolepsy. We excluded patients with a diagnosis of secondary narcolepsy, multiple sclerosis, or in association any other neurological disorder that could affect CSF hypocretin-1 concentration.[24,41] We also excluded patients (previously reported) with DNMT1 exon 21 ADCA-DN mutations as "secondary" cases.

All subjects gave written informed consent approval, and a Stanford institutional review board approved the study.

Hypocretin-1 Measurement

The methodology for CSF hypocretin-1 measurement has been previously described.[2] In the present study, levels were measured in duplicate in 2 independent assays in each case. Low CSF hypocretin-1 is defined as $\leq$ 110 pg/mL.

Table 1—HLA and CSF hypocretin status in cases with **(A)** and without **(B)** cataplexy across countries *

A

Country	N	06:02+/HCRT low, % (n)	06:02-/HCRT low, % (n)	06:02+/HCRT normal, % (n)	06:02-/HCRT normal, % (n)
US	183	81.0 (149)	0.5 (1)	6.5 (12)	11.4 (21)
Italy	85	90.6 (77)	4.7 (4)	2.4 (2)	2.4 (2)
Denmark	81	87.7 (71)	1.2 (1)	4.9 (4)	6.2 (5)
China	99	92.9 (92)	1.0 (1)	3.0 (3)	3.0 (3)
Korea	52	94.2 (49)	0 (0)	1.9 (1)	3.8 (2)
Czech Rep	22	81.8 (18)	9.1 (2)	4.5 (1)	4.5 (1)
All	522	87.4 (456) [84.2-90.1]	1.7 (9) [0.8-3.2]	4.4 (23) [2.8-6.5]	6.5 (34) [4.6-9.0]

B

Country	N	06:02+/HCRT low, % (n)	06:02-/HCRT low, % (n)	06:02+/HCRT normal, % (n)	06:02-/HCRT normal, % (n)
US	77	10.4 (8)	0.0 (0)	27.3 (21)	62.3 (48)
Italy	9	11.1 (1)	0.0 (0)	0.0 (0)	88.9 (8)
Denmark	14	21.4 (3)	0.0 (0)	7.1 (1)	71.4 (10)
China	22	50.0 (11)	0.0 (0)	18.2 (4)	31.8 (7)
Korea	19	31.6 (6)	0.0 (0)	15.8 (3)	52.6 (10)
Czech Rep	3	0.0 (0)	0.0 (0)	33.3 (1)	66.7 (2)
All	144	20.0 (29) [13.9-27.6]	0.0 (0) [0.0-2.5]	20.8 (30) [14.5-28.4]	59.0 (85) [50.5-67.1]

95% Confidence interval in brackets. * Among cases for whom both HLA typing and CSF hypocretin-1 data were available. Low HCRT defined as CSF hypocretin-1 < 110 pg/mL.

HLA Typing

The absence of HLA DQB1*06:02 was first screened using a Sequence Specific PCR assay testing for the presence of codon 9 and codon 70, as described in Mignot et al.[9] When negative and in the presence of low CSF hypocretin-1, HLA DQB1 exon 2 sequencing was performed as reported in Hong et al.[13] Finally, when negativity was confirmed, most HLA exons for HLA, A, B, C, DRB1, DQ, and DP samples were sequenced using a recently developed high throughput sequencing technique involving long range PCR of each HLA locus, sheering of DNA and High Seq sequencing.[42] This technique can detect rare and new subtypes, for example variants in less studied exons. Any unusual typing result (rare allele or haplotype) was also confirmed using commercial PCR-SSO hybridization methods (One Lambda, Canoga Park, CA), with detection in a Luminex platform. Following identification of all alleles at these loci, we tabulated carrier/phenotype frequency for all alleles at each locus, and compared frequency with established values in Caucasian and Asians from the US.

Whole Exome Sequencing, with DNMT1, Hypocretin, and MOG Gene Confirmation

Whole exome sequencing (WES) was performed in all cases using SureSelect XT Human All Exon V4 kit (Agilent) for in-solution enrichment and 100-bp paired-end runs on a HiSeq2500 system (Illumina) with 0.33 lanes per sample for sequencing. Alignment against the human genome assembly hg19 (GRCh37) was done using BWA v0.5.9 with standard parameters. Single-nucleotide variants (SNVs) and small insertions and deletions (indels) were called using SAMtools v 0.1.18. Custom Perl scripts were used for variant annotation of single-nucleotide missense, nonsense, splice-site, stoploss and frameshift variants and indels. Regarding potential dominant effects, we restricted the search-space for putative causal variants to variants with a variant allele frequency ≤ 0.5% in dbSNP-135, the 1000Genomes data, or the Exome Variant Server (EVS) data and an in-house exome database (n = 2,828) of unrelated phenotypes.[37] For potential recessive homozygotes, we checked for putative causal variants of mean allele frequency < 15%, and expected homozygote frequency of 1% to 2%.

Using WES, we also extracted all variants with allele frequencies ≤ 1% in DNMT1, hypocretin, and MOG, but found nothing remarkable. To verify these findings, known pathogenic mutations in these genes were examined through Sanger sequencing of the corresponding exons of these genes. DNMT1 exon 20 and 21 exon sequencing was conducted as described previously.[37,43] MOG exon 2 sequencing was conducted using primers described in Hor et al.[33] HCRT exon 2 sequencing was performed as described in Peyron et al.[3]

Statistical Analysis

The present study is largely descriptive; no statistics were applied, considering that no strong finding emerged. To be considered significant, variants had to be missense mutations, stop codons, frame shifts, or disturbing a splicing site, and never or rarely (≤ 0.5%) reported in the 1000 genomes and our own database. Novel variants with potential functional effects were examined using PolyPhen2, http://genetics.bwh.harvard.edu/pph2/ and sift, sift.jcvi.org/. Regarding HLA allele comparison, sample size was low, and reference allele frequency approximate in such a diverse sample, thus we elected not to conduct formal statistical analysis.

RESULTS

Frequency of the HLA DQB1*06:02 Negative, Low CSF Hypocretin-1 Phenotype in Various Cohorts of Patients

Table 1 summarizes CSF hypocretin and DQB1*06:02 data as derived from the various cohorts with both data points available, separated by cataplexy status. As previously reported, ~90% of patients with and ~20% without cataplexy, respectively, have low CSF hypocretin-1. The occurrence of the HLA DQB1*06:02 negative, low CSF hypocretin-1 phenotype was rare (1.7% of the present sample, n = 9), and was only observed in cases with typical cataplexy (i.e., in response to typical emotional triggers). Among HLA negative patients with clear cataplexy who underwent CSF hypocretin evaluation, this represented 21% of tested subjects. Interestingly, the proportion of DQB1*06:02 negative cases seemed to vary across site, as it was very rare in the US, China, and Korea, and more frequent in Italy and the Czech Republic.

Clinical Characteristics of HLA DQB1*06:02 Negative Patients with Low CSF Hypocretin-1

Table 2 reports on these subjects at the clinical level. All had a positive MSLT, and for 5 patients cataplexy was somewhat atypical and was so severe it often occurred spontaneously in addition to being triggered by clear emotions. The clinical picture was very atypical in only one case: patient 2, who had a very early disease onset, and was previously reported to carry an HCRT mutation (pR16L, G47T). Clinical and polysomnographic findings for all other cases were unremarkable. Secondary causes were excluded—all patients had extensive work-up and have been followed up for several years. No additional neurological features suggestive of DNMT1 mutations were seen in any case, 2 of whom (patients 1 and 3) were already older than typical ADCA-DN onset (< 50 years old). Response to usual therapy was always favorable.

WES and Targeted Sequencing of HCRT, DNMT1, MOG Genes

Successful WES was conducted in 8 cases. For one individual, DNA quality was not sufficient for WES. At most 4 of the 8 cases shared private rare mutations (≤ 0.5%) in the same gene and all genes were large and variants of low quality (TTN, LAMA5, MUC4, and PCDHA1), suggesting no common genetic basis. Similarly, at most 4 of 8 cases shared rare potentially functional homozygous variants, and in all cases the corresponding homozygotes were found at an expected frequency of 1% to 2% in our control samples. Analysis of the gene functions and of the effects of the associated polymorphisms using polyphen2 and sift were not revealing, suggesting chance findings. Exon sequencing and analysis of the 3 genes previously associated with hypocretin deficiency, HCRT, MOG, and DNMT1 were also unremarkable. As expected, the previously known c47T > G, p.Leu16Arg HCRT was noted in patient 2. No other significant polymorphisms were found.

Sequencing of HLA-A, B, C, DR, DQ, and DP genes

Table 3 shows the HLA alleles carried by these 9 subjects. No DQ1 or non-DQ1 *cis* or *trans* DQα/DQβ compatible heterodimer was clearly overrepresented, as the subjects were both DQ1 (i.e., carrying DQA1*01 and DQB1*05 or 06) and non-DQ1. Comparing carrier/phenotype frequency for all alleles at each locus with frequencies of these alleles in Caucasians and Asians from the US, and in a sample that would be 72% Caucasian and 28% Asian, no clear difference emerged, although increased DPB1*09:01 and B*38:01 was notable (see Table 3 legend for some selected frequencies). The sample also did not contain unusually rare alleles. Formal statistical analysis was not possible considering the small sample and multiple testing.

DISCUSSION

This study reports on the largest number of narcolepsy cases with hypocretin deficiency and without the predisposing allele HLA DQB1*06:02. In this case series, all HLA DQB1*06:02 negative patients with hypocretin deficiency had cataplexy, facilitating identification. Half of these cases had unusually severe cataplexy, although this could reflect a bias of ascertainment. Using only cases where CSF hypocretin-1 levels had been evaluated, we found that this phenotype was rare, representing 1.3% of narcolepsy patients for whom both HLA typing and hypocretin-1 measurements were available (1.7% with cataplexy) and 1.9% of patients with low hypocretin-1. The low rate of hypocretin deficiency among DQB1*06:02 negative patients justifies the rationale behind conducting HLA testing prior to a lumbar puncture, although it should be clear that exceptions are possible. Notably, in this sample, 21% of 43 cases with clear cataplexy and HLA negativity (all with a positive MSLT) had low CSF hypocretin-1. In contrast, none of the 85 HLA negative subjects without cataplexy (all with a positive MSLT) had low CSF hypocretin-1. CSF evaluation may thus be useful for differential diagnosis in cases with clear/severe cataplexy and a positive MSLT, but not in cases without cataplexy.

The frequency of HLA-DQB1*06:02 negative cases with hypocretin deficiency is approximate, as this study suffers from multiple ascertainment biases. At Stanford, subjects most recently diagnosed with narcolepsy only undergo a lumbar puncture if the diagnosis is in doubt, either because the clinical picture is not completely clear or because the clinical picture is clear but the subject is unexpectedly HLA negative. In Italy, China, and Denmark, CSF hypocretin-1 was performed in all persons with narcolepsy who agreed to the procedure, and independently of HLA typing data, but it is impossible to exclude that subjects undergoing lumbar punctures are somehow not representative of all narcolepsy subjects. For example, typical patients with cataplexy, more likely to be HLA positive, may be less likely to be asked or accept a CSF evaluation, inflating the percentage of HLA negative cases in subjects with CSF samples. Nonetheless, this is our best approximation. It is also somewhat surprising that so many more cases came from Italy and the Czech Republic than in other parts of the world—a finding that may reflect differences in genetic background, protocols, or environmental factors. In contrast, very few cases were reported in China, Korea, and the US.

We next evaluated potential pathophysiological mechanisms that could underlie hypocretin deficiency in DQB1*06:02 negative subjects, by first investigating a possible second HLA association. In most other HLA associated autoimmune diseases, residual HLA association with secondary alleles in the same loci can be found in subjects not carrying the main

Table 2—Clinical characteristics of the 9 HLA negative subjects with low CSF hypocretin-1

#	Referrer	Race	Gender	Age (current)	BMI (diagnosis)	Age of Onset			
						Sleepiness	Cataplexy	HH	SP
1	Dr. S. Nevsimalova (Czech Rep)	Eur Cauc	F	26.1	27.1	11	11	15	15
2*	Dr. S. Nevsimalova (Czech Rep)	Eur Cauc	M	31.8	36.6	0.5	0.5	12	12
3	Dr. C. Guilleminault (USA)	Eur Cauc	F	79.6	26.6	46	48	48	48
4	Dr. G. Plazzi (Italy)	Eur Cauc	M	12.8	17.8	6	6.5	no	no
5	Dr. G. Plazzi (Italy)	Ind As	F	18.2	25.0	14	14	14	no
6	Dr. G. Plazzi (Italy)	Eur Cauc	F	15.6	24.0	11	11	11	11
7	Dr. P. Jennum (Denmark)	Eur Cauc, Jap As	M	23.8	22.4	21	21	21	21
8***	Dr. G. Plazzi (Italy)	Eur Cauc	M	36.0	31.1	26	26	26	26
9	Dr. F. Han (China)	Chin As	F	54.9	27.5	49	49	no	25

#	Sleep Study Results								
	MSLT MSL	#SOREM	AHI	PSG REML	TST	SE	PLMI	HCRT	Prior Publication
1	2.2	3 of 5	n.a.	22	n.a	n.a.	n.a.	0	Mignot et al., 2002[2]
2*	1.1	5 of 5	n.a.	n.a.	n.a	n.a.	n.a.	0	Peyron et al., 2000[3]
3	0	5 of 5	40**	450.5	232	45	33.4	0	Guilleminault and Grumet, 1986[39]
4	3.1	4 of 5	0	3	623	91	n.a.	0	Unpublished
5	1.9	5 of 5	0	160	380	68	9.47	17	Unpublished
6	1.2	5 of 5	0	3	347	81	n.a.	34	Unpublished
7	2.1	4 of 5	0	4.3	n.a	n.a.	n.a.	0	Knudsen et al., 2012[40]
8***	1.9	5 of 5	12	1	450	87	31.5	0	Unpublished
9	3.5	2 of 5	10.4	11.3	n.a	n.a.	n.a.	77	Unpublished

#	Clinical Features	Response to Treatment
1	Mild cataplexy, triggered by usual emotions	Good response to modafinil and clomipramine,scheduled naps
2*	Severe cataplexy, classical triggers, occasional falls with injury, behavioral problems, depression	Partial response to sodium oxybate, scheduled naps and modafinil
3	Unusual late onset, severe long attacks, no clear triggers, sometimes triggered by anger or disgust	Partial response with antidepressants, very positive response with sodium oxybate
4	Severe cataplexy, classical triggers, behavioral problems	Polytherapy, partial response, modafinil, venlafaxine and sodium oxybate
5	Severe cataplexy, classical triggers	Polytherapy, partial response, modafinil, venlafaxine and sodium oxybate
6	Severe cataplexy, classical triggers	Polytherapy, partial response, modafinil, venlafaxine and sodium oxybate
7	Severe cataplexy, classical triggers but also long attacks without triggers	Polytherapy, excellent response to sodium oxybate and imipramine.
8	Severe cataplexy, classical triggers but also long attacks without triggers, depression	Polytherapy, partial response, modafinil, venlafaxine and sodium oxybate
9	Unusually late onset, relatively mild cataplexy	Good response to clomipramine but stopped due to RLS

* Putative causative hypocretin gene mutation. ** Prior PSG indicated mild sleep apnea, see Guilleminault and Grumet, 1986. *** Exome sequencing not available.

susceptibility alleles. In ankylosing spondylitis, for example, a disease extremely strongly associated with subtypes of B*27 in all ethnic groups, a secondary association with HLA-B*60 and HLA-B*39 is found in B27 negative Taiwanese patients.[44] Similarly, in celiac disease a strongly HLA-DQ2-associated disorder, ~90% of patients carry DQ2.5 heterodimers, encoded by DQA1*05 and DQB1*02 alleles both in *cis* or *trans* configurations. Most of the remaining patients (~6%) carry DQ8 molecules, encoded by DQB1*03:02, and always paired with the presence of an allele of the DQA1*03 group.[45] The underlying hypothesis for these residual HLA associations is heterogeneity of the antigen presented by each DQα/β heterodimer; for example, DQ2 and DQ8 could bind slightly different gluten-derived peptides in celiac disease.[46]

In our study, surprisingly we did not find any DQ residual association. This is surprising as in other strongly HLA DQ associated diseases, for example, celiac disease and DQA1*05/DQB1*02 (95% of cases), a residual HLA DQ association is found, in this case with DQA1*03/DQB1*03:02 (5% remaining).[46] It was notable that although many patients were

Table 3—HLA genotypes of 9 DQB1*06:02 negative subjects

		HLA class II									
#	Race	DRB1/1	DRB1/1	DQA1/1	DQA1/2	DQB1/1	DQB1/2	DPA1/1	DPA1/2	DPB1/1	DPB1/2
1	Eur Cauc	DRB1*13:01	DRB1*14:54	DQA1*01:03	DQA1*01:04	DQB1*05:03	DQB1*06:03	DPA1*01:03	DPA1*02:01	DPB1*02:01	DPB1*10:01
2†	Eur Cauc	DRB1*04:02	DRB1*07:01	DQA1*02:01	DQA1*03:01	DQB1*03:02	DQB1*03:03	DPA1*01:03	DPA1*01:03	DPB1*04:01	DPB1*04:02
3	Eur Cauc	DRB1*01:01	DRB1*04:05	DQA1*01:01	DQA1*03:02	DQB1*03:02	DQB1*05:01	DPA1*01:03	DPA1*01:03	DPB1*04:01	DPB1*04:01
4	Eur Cauc	DRB1*11:02	DRB1*13:02	DQA1*01:02	DQA1*05:05	DQB1*03:19	DQB1*06:09	DPA1*01:03	DPA1*01:05	DPB1*04:01	DPB1*01:03
5	Ind As	DRB1*04:03	DRB1*07:01	DQA1*02:01	DQA1*03:01	DQB1*03:02	DQB1*03:03	DPA1*01:03	DPA1*02:01	DPB1*02:01	DPB1*09:01
6	Eur Cauc	DRB1*01:01	DRB1*11:01	DQA1*01:01	DQA1*05:05	DQB1*03:01	DQB1*05:01	DPA1*01:03	DPA1*02:01	DPB1*02:01	DPB1*09:01
7	Eur Cauc, Jap As	DRB1*04:01	DRB1*15:02	DQA1*01:03	DQA1*03:03	DQB1*03:01	DQB1*06:01	DPA1*01:03	DPA1*02:01	DPB1*02:01	DPB1*09:01
8	Eur Cauc	DRB1*03:01	DRB1*03:01	DQA1*05:01	DQA1*05:01	DQB1*02:02	DQB1*02:02	DPA1*01:03	DPA1*02:01	DPB1*02:01	DPB1*09:01
9	Chin As	DRB1*08:03	DRB1*10:01	DQA1*01:03	DQA1*01:05	DQB1*05:01	DQB1*06:01	DPA1*01:03	DPA1*02:02	DPB1*04:01	DPB1*05:01

Expected phenotype frequency of HLA class II subtypes found in at least 4 subjects (44%) in this sample: DPA1*01:03~89%, DPA1*02:01~26%, DPB1*0201~29%, DPB1*0401~52%, DPB1*09:01~5%.

		HLA class I					
#	Race	A/1	A/2	C/1	C/2	B/1	B/2
1	Eur Cauc	A*02:01	A*02:01	C*03:04	C*12:03	B*15:01	B*38:01
2†	Eur Cauc	A*26:01	A*66:01	C*12:03	C*12:03	B*08:01	B*38:01
3	Eur Cauc	A*02:05	A*03:01	C*04:01	C*07:01	B*35:01	B*47:01
4	Eur Cauc	A*03:01	A*24:02	C*02:02	C*07:02	B*07:02	B*35:03
5	Ind As	A*11:01	A*26:01	C*06:02	C*07:04	B*15:18	B*57:01
6	Eur Cauc	A*02:01	A*33:01	C*05:01	C*12:03	B*38:01	B*44:02
7	Eur Cauc, Jap As	A*11:01	A*24:02	C*03:04	C*12:02	B*40:01	B*52:01
8	Eur Cauc	A*02:01	A*29:01	C*03:02	C*07:01	B*08:01	B*58:01
9	Chin As	A*01:01	A*11:01	C*06:02	C*08:03	B*37:01	B*48:01

Expected phenotype frequency of HLA class I subtypes found in at least 3 subjects (33%) in this sample: A*02:01-~37%, C*12:03~15%, B*38:01~4%. † With mutation in the hypocretin gene.

DQ1 heterozygous, some were non-DQ1. As trans encoded DQ1 and non-DQ1 α and β chains are not known to pair into functional dimers,[47,48] the hypothesis of a secondary, particular DQ molecule resulting from DQA1-DQB1 trans-complementation is not plausible. One additional DQB1*06:02 negative patient with detailed HLA typing was reported by Dalal et al.[38] (sample not available for this study), and displayed HLA DRB1*04:07, DRB1*12:01, and DQB1*03:01 (presumably homozygous), a combination not present in any of our other subjects (see Table 3).

Other loci such as HLA A, B, C, and DP were also explored and did not show a single residual association, although DPB1*09:01 and B*38:01, two rare alleles (phenotype frequency expected below 5%) were present in 4 (44%) and 3 (33%) subjects, respectively. The DPB1*09:01 finding is likely significant, as it occurred in four of the eight subjects without the hypocretin mutation, and one more such subject was DPB1*10:01, a closely related subtype to DPB1*09:01 differing only at amino acid 57 (D > E). DPB1*09:01 or DPB1*10:01 in conjunction with DPA1*02:01 may thus represent a secondary association in DQB1*06:02 negative cases. Whether or not this is a chance finding is difficult to assess considering ethnic diversity of the sample and the large amount of multiple testing that was carried out. To confirm this observation will require studying larger samples of these exceptionally rare subjects.

Seeking alternative explanations for hypocretin deficiency in these patients, we next conducted exome sequencing, hypothesizing that rare mutations at other genes could mediate hypocretin deficiency independent of HLA effects. No novel common gene alterations were identified through whole exome sequencing, nor did we identify suspicious variants in a careful survey of MOG and DNMT1, two genes previously implicated in rare DQB1*06:02-negative (multiplex) narcolepsy cases. The exception was the confirmation (through exome sequencing) of a previously reported mutation in preprohypocretin in one atypical case. These results indicate that most DQB1*06:02 negative subjects with hypocretin deficiency do not share a single genetic basis either in known HLA genes, in previously implicated loci, or in exons of other genomic loci. Importantly, none of our cases was part of a multiplex family, in contrast to cases previously reported with MOG and DNMT1 mutations.

In summary, we confirm that DQB1*06:02-negative subjects with hypocretin deficiency exist, but found that they are extremely rare. CSF evaluation of hypocretin in HLA negative

cases may be justified in cases with clear cataplexy and strongly positive polysomnographic findings. In contrast, HLA negative patients without cataplexy do not benefit from this evaluation, as CSF is always normal in this group. Further examination of the patients' HLA types found a possible residual DP association. Whether or not the cause of the hypocretin deficiency in these cases is related to an autoimmune process with presentation of autoantigen epitopes by DP, as in more classic, DQB1*06:02 positive cases is unknown and will need exploration.

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