RAPID PUBLICATION

Anti-Tribbles Homolog 2 Autoantibodies in Japanese Patients with Narcolepsy

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Study Objectives: Narcolepsy is a sleep disorder characterized by excessive daytime sleepiness and cataplexy. The association with human leukocyte antigen *(HLA)-DQB1*0602* and T-cell receptor alpha locus suggests that autoimmunity plays a role in narcolepsy. A recent study reported an increased prevalence of autoantibodies against Tribbles homolog 2 (TRIB2) in patients with narcolepsy. To replicate this finding, we examined anti-TRIB2 autoantibodies in Japanese patients with narcolepsy.

Design: We examined anti-TRIB2 autoantibodies against a full-length [^{35}S]-labeled TRIB2 antigen in Japanese patients with narcolepsy-cataplexy (n = 88), narcolepsy without cataplexy (n = 18), and idiopathic hypersomnia with long sleep time (n = 11). The results were compared to Japanese healthy controls (n = 87). Thirty-seven healthy control subjects were positive for *HLA-DRB1*1501-DQB1*0602*. We also examined autoantibodies against another Tribbles homolog, TRIB3, as an experimental control.

Measurements and Results: Autoantibodies against TRIB2 were found in 26.1% of patients with narcolepsy-cataplexy, a significantly higher prevalence than the 2.3% in healthy controls. We found that anti-TRIB3 autoantibodies were rare in patients with narcolepsy and showed no association with anti-TRIB2 indices. No significant correlation was found between anti-TRIB2 positivity and clinical information.

Conclusions: We confirmed the higher prevalence and specificity of anti-TRIB2 autoantibodies in Japanese patients with narcolepsy-cataplexy. This suggests a subgroup within narcolepsy-cataplexy might be affected by an anti-TRIB2 autoantibody-mediated autoimmune mechanism.

Keywords: Narcolepsy, autoantibody, Tribbles homolog 2

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NARCOLEPSY IS A SLEEP DISORDER CHARACTERIZED BY EXCESSIVE DAYTIME SLEEPINESS AND CATAPLEXY. NARCOLEPSY AFFECTS 0.16% TO 0.18% of the general population in Japan and 0.02% to 0.06% of the population in the United States and Europe.[1] Several reports have demonstrated that the level of hypocretin (HCRT) is reduced to undetectable levels in the cerebrospinal fluid (CSF) of more than 90% of patients with narcolepsy-cataplexy[2-4] and the reduction of HCRT neurons in the postmortem hypothalami of patients with narcolepsy.[5,6] Narcolepsy is associated with the human leukocyte antigen *(HLA)-DQB1*0602* allele[7] and a single-nucleotide polymorphism located in the T-cell receptor alpha-locus,[8] suggesting an immunological abnormality in narcolepsy. Therefore, several studies exploring the pathophysiology of narcolepsy have been based on the hypothesis that an autoimmune-mediated process targets hypothalamic HCRT neurons. However, no definitive answers have been obtained so far.[9]

Recently, genetically engineered mice were utilized to identify the expression profiling of genes enriched in mice hypothalamic Hcrt neurons.[10] Twenty-three genes were found to be specifically expressed in Hcrt neurons. Among them, Tribbles homolog 2 (*TRIB2*) was the only gene found to be an autoantigen. *TRIB2* was previously reported to be associated with human autoimmune uveitis.[11] Using an enzyme-linked immunosorbent assay (ELISA) method, higher anti-TRIB2 titers were detected in sera from patients with narcolepsy compared to healthy controls.[10]

This finding prompted us to re-evaluate the presence of anti-TRIB2 autoantibodies in Japanese narcolepsy patients. We have developed a radioligand binding assay that uses recombinant full-length human [^{35}S]-TRIB2 produced by *in vitro* transcription/translation as an antigen. The radioligand binding assay is well known to detect autoantibodies more effectively than the ELISA system, especially autoantibodies against conformational epitopes.[12,13]

MATERIALS AND METHODS

Subjects

This study was approved by the ethics committee of all collaborative organizations. Written informed consent was obtained from all study participants. Diagnosis was made according to the International Classification of Sleep Disorders, 2nd edition[1] at the Neuropsychiatric Research Institute (Tokyo, Japan). Blood samples and data related to sleep conditions were collected at the Tokyo Institute of Psychiatry (Tokyo, Japan) and Neuropsychiatric Research Institute (Tokyo, Japan). Control subjects were excluded if they had excessive daytime sleepiness or any signs of immunologic abnormalities based on responses to a questionnaire at the time of blood collection. Five milliliters of venous blood were drawn, and separated sera were stored at −80°C until use. Genotyping for HLA-DRB1 and DQB1 loci were done at NPO HLA Laboratory (Kyoto, Japan) using polymerase chain reaction (PCR)-sequence specific oligonucleotide probes coupled with the Luminex bioassay detection system (Wakunaga, Hiroshima, Japan). Patients with narcolepsy-cataplexy were all positive for the *HLA-DRB1*1501-DQB1*0602* allele. Thirty-seven of 87 healthy controls, 9 of 18 patients with narcolepsy without cataplexy, and

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Table 1—Age, sex, and anti-TRIB2 positive reaction in patients with hypersomnia and healthy controls

Subjects	Number examined	Age (years) (mean ± SD)	Female/ Male	Number of positive reaction (%) over mean+2SD	Number of positive reaction (%) over mean+3SD	TRIB2 index (mean ± SD)
Narcolepsy with cataplexy	88	43.5 ± 18.3	40/48	23 (26.1)[a]	14 (15.9)[b]	16.0 ± 3.6[c]
Narcolepsy without cataplexy	18	34.8 ± 10.0	7/11	1 (5.6)[†]	0 (0.0)[†]	13.0 ± 2.0[†]
Idiopathic hypersomnia	11	34.3 ± 7.1	6/5	0 (0.0)[†]	0 (0.0)[†]	14.0 ± 1.9[†]
Healthy control	87	38.6 ± 10.3	42/45	2 (2.3)	0 (0.0)	14.7 ± 1.5

Distribution of age and sex of the patient groups was not significantly different from those of healthy controls. [a]$P = 4.8 \times 10^{-6}$. [b]$P = 7.0 \times 10^{-5}$. [c]$P = 0.01$. [†]No significant differences between healthy controls and patients with narcolepsy without cataplexy or healthy controls and patients with idiopathic hypersomnia were found. SD, standard deviation.

2 of 11 patients with idiopathic hypersomnia with long sleep time were positive for the *HLA-DRB1*1501-DQB1*0602* allele. All subjects were unrelated Japanese individuals living in Tokyo or in neighboring areas. We collected the following data to analyze potential relationships with the autoantibody index: age, sex, body mass index, disease duration, Epworth Sleepiness Scale at the time of blood collection, presence of nocturnal sleep disruption (subjective report), and past history of autoimmune disorders. CSF was not collected from the subjects for analysis. The mean age and sex distributions are summarized in Table 1. Distribution of age and sex of the patient groups was not significantly different from that of the healthy controls by the Mann-Whitney *U* test.

Construction of TRIB2 and TRIB3 Expression Vectors

The open-reading frame of *TRIB2* or *TRIB3* was obtained by reverse transcription-polymerase chain reaction (RT-PCR) amplification using the human hypothalamic poly-A RNA (Takara Bio, Kyoto, Japan) or human lymphocytic total RNA.[14] *TRIB3* expression was found in human hypothalamus, though its expression was weak (Figure S1, available online only at www.journalsleep.org). Therefore, we also examined anti-TRIB3 autoantibodies as experimental controls. The following primer pairs with either a *BamH*I or a *Xho*I site for *TRIB2* or an *EcoR*I or a *Xho*I site for *TRIB3*, 5'-CGCGGATCCATGAACATACACAGGTCTA-3' (*TRIB2* forward) and 5'-CCGCTCGAGATTCTTGGCCAACTGTTCCTT-3' (*TRIB2* reverse) (the *BamH*I and *Xho*I sites are underlined) or 5'-GGAATTCATGCGAGCCACCCCTCTGGCTGC-3' (*TRIB3* forward) and 5'-CCGCTCGAGACAGACCCTGCCTAATCCTGT (*TRIB3* reverse) (the *EcoR*I and *Xho*I sites are underlined), were used for each amplification. *TRIB2* or *TRIB3* amplification product was ligated into the pET28a (+) expression vector (Novagen, Madison, WI, USA).

Synthesis of Recombinant [^{35}S]-TRIB2 and [^{35}S]-TRIB3 Antigens and the Radioligand Binding Assay

The antigen-antibody reaction was conducted with 1 μL of serum sample obtained from subjects and 20,000 cpm of [^{35}S]-labeled recombinant antigen (50 μL in total). The precise methods for synthesis of recombinant [^{35}S]-antigens and radioligand binding assay were described previously.[15] To avoid inter-assay variation, the results were expressed by an autoantibody index calculated as follows: 100 × cpm of the sample serum / cpm of mouse monoclonal anti-TRIB2 or anti-TRIB3 antibody (diluted in 1:500). As a result, the intra-assay coefficient of variation ranged from 2.6% to 9.6%, and the inter-assay coefficient of variation ranged from 1.5% to 9.0%. The cut-off value was set at the mean + 2SD in healthy controls. The antibodies used as an internal control were commercially available [mouse anti-TRIB2 (sc-100878) or mouse anti-TRIB3 (sc-100879), Santa Cruz Biotechnology, Inc., Santa Cruz, CA, USA].

Statistical Analyses

The positive reactions against TRIB2 and TRIB3 were compared between patient groups and healthy controls by Fisher exact test. Distribution of TRIB2/3 antibody indices was compared by the Mann-Whitney *U* test (one-tailed test) between patient groups and healthy controls. The Pearson correlation coefficient was used to calculate correlations between the antibody indices and the following clinical information: body mass index, disease duration, and Epworth Sleepiness Scale as an indicator for severity of sleepiness. A P-value < 0.05 was considered statistically significant.

RESULTS

Using a radioligand binding assay, we found autoantibodies against TRIB2 in sera from Japanese patients and compared the levels with those of healthy controls. Demographic data and percentages of positive reactions are summarized in Table 1. With the cutoff value at 2 SD above the mean in healthy controls, sera from 23 of 88 patients with narcolepsy-cataplexy (26.1%) and 2 of 87 healthy controls (2.3%) showed a positive reaction (Fisher exact test, $P = 4.8 \times 10^{-6}$) against TRIB2 antigen (Figure 1, Table 1). One of 18 patients with narcolepsy without cataplexy and no patients with idiopathic hypersomnia showed a positive reaction against TRIB2 antigen in their sera. Even with the cutoff value at 3SD, the anti-TRIB2 autoantibody positive rate was significantly higher in patients with narcolepsy-cataplexy (14 of 88 patients with narcolepsy-cataplexy versus 0 of 87 healthy controls; Fisher's exact test, $P = 7.0 \times 10^{-5}$) (Table 1). Indices of anti-TRIB2 autoantibodies in patients with narcolepsy-cataplexy were also significantly higher compared to healthy controls (Mann-Whitney *U* test, P = 0.01) (Table 1).

We also examined autoantibodies against TRIB3 using the same subjects as experimental controls. While a positive reaction against TRIB3 antigen was detected in one patient with narcolepsy-cataplexy, in contrast to TRIB2, no significant differences between groups were found (Figure S2, available online only at www.journalsleep.org).

We analyzed correlations between anti-TRIB2 positive reactions and clinical information, body mass index, disease dura-

tion, and Epworth Sleep Scale score. No significant correlation was found when examining either the entire patient cohort, or just the 26.1% of the narcolepsy-cataplexy patients with high titers (> 2 SD) of anti-TRIB2 antibodies.

DISCUSSION

A recent study identified elevated levels of autoantibodies against TRIB2 in patients with narcolepsy-cataplexy.[10] In the present study, we re-evaluated this finding by testing for autoantibodies against TRIB2 in Japanese patients with narcolepsy-cataplexy. A significant number of patients with narcolepsy-cataplexy showed a positive reaction for anti-TRIB2 autoantibodies compared to healthy controls.

We also conducted semi-quantitative RT-PCR using human hypothalamic and hippocampus cDNAs (Takara Bio, Kyoto, Japan) and found that *TRIB2* expression in the human hypothalamus is much higher than that in the human hippocampus (Figure S1). The specific reactions against TRIB2 in narcolepsy and its high expression in the hypothalamus are consistent with the hypothesis that anti-TRIB2 autoantibodies target hypothalamic HCRT neurons, resulting in HCRT loss in narcolepsy.

To further confirm the specificity for anti-TRIB2 autoantibodies in patients with narcolepsy, we examined the reactivity against another Tribbles homolog expressed in human hypothalamus, TRIB3 (Figure S1), using the same sera samples. Although the amino acid sequences of human TRIB2 share 52% identity with human TRIB3 overall, the 28 C-terminal amino acids of TRIB2 used as an ELISA antigen[10] have only a low identity with TRIB3. We found a significant incidence of anti-TRIB2 positivity using a full-length TRIB2 antigen containing the C-terminal region, and only one patient with narcolepsy-cataplexy showed a positive reaction against TRIB3. These results suggest that anti-TRIB2 autoantibodies might recognize the TRIB2 C-terminal region, which has a low sequence identity with TRIB3.

Unlike recent reports,[10,16] the relationship between disease duration and anti-TRIB2 autoantibody levels was not replicated in the present study (Figure S3, available online only at www.journalsleep.org). We had a limited number of narcolepsy patients within 5 years of disease onset compared to other groups, although 2 of our 5 patients with recent onset showed higher anti-TRIB2 autoantibody indices (> 2 SD). The number of narcolepsy patients with recent onset might not be sufficient to discuss the relationship between disease duration and anti-TRIB2 autoantibody in our Japanese group.

In conclusion, we confirmed the presence of highly specific autoantibodies against TRIB2 antigen in Japanese patients with narcolepsy-cataplexy as reported in a recent study.[10]

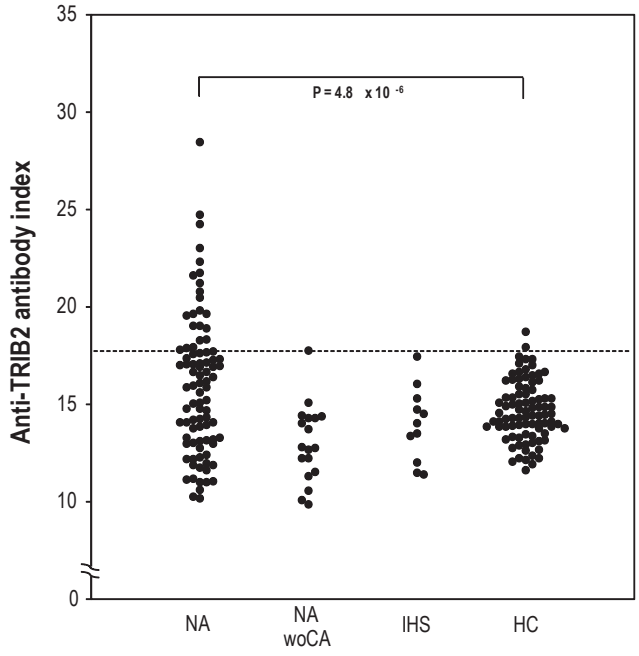

Figure 1—Autoantibody index of human TRIB2. Indices of autoantibodies in patients with narcolepsy-cataplexy, narcolepsy without cataplexy, and idiopathic hypersomnia with long sleep time, and healthy controls are represented. Each dot corresponds to data from one subject. The dotted line indicates the mean + 2SD levels in healthy controls. The indices with subjects above this line were considered to be positive. NA, narcolepsy with cataplexy; NAwoCA, narcolepsy without cataplexy; IHS, idiopathic hypersomnia with long sleep time; HC, healthy control.

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DISCLOSURE STATEMENT

This was not an industry supported study. The authors have indicated no financial conflicts of interest.

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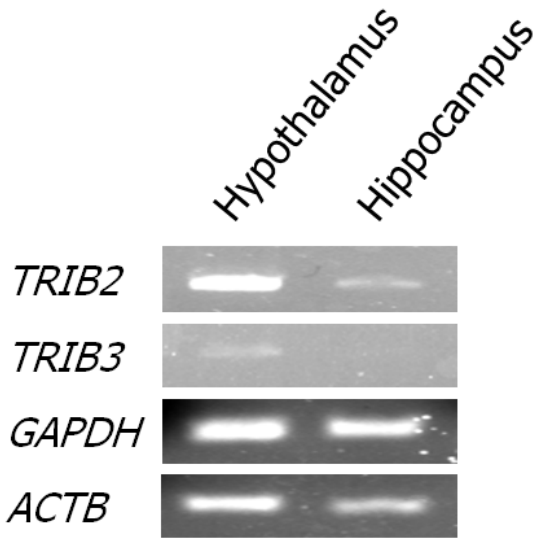

Figure S1—Expression of *TRIB2* and *TRIB3* in human hypothalamus and hippocampus. Expression of genes were analyzed by RT–PCR. *GAPDH* and *ACTB* were used as loading controls. *GAPDH*, glyceraldehyde-3-phosphate dehydrogenase; *ACTB*, actin-beta.

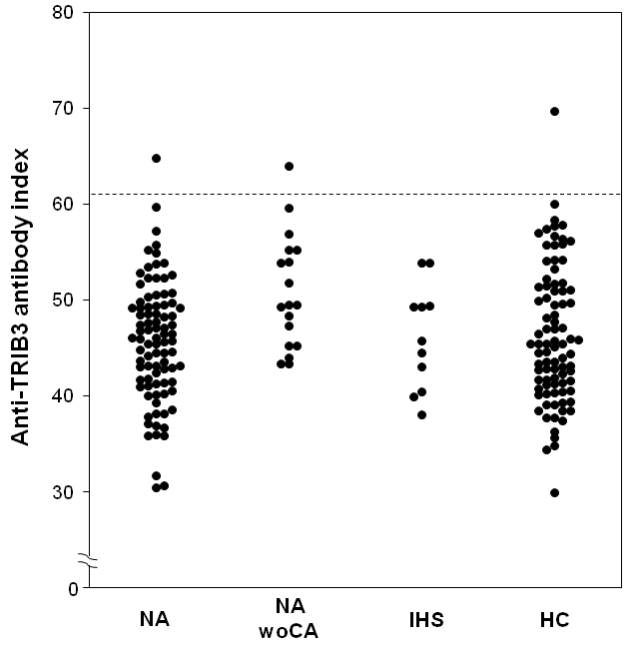

Figure S2—Autoantibody index of human TRIB3. Indices of autoantibodies in patients with narcolepsy-cataplexy, narcolepsy without cataplexy, and idiopathic hypersomnia with long sleep time and healthy controls are represented. Each dot corresponds to data from one subject. The dotted line indicates the mean + 2SD levels in healthy controls. The indices with subjects above this line were considered to be positive. NA, narcolepsy with cataplexy; NAwoCA, narcolepsy without cataplexy; IHS, idiopathic hypersomnia with long sleep time; HC, healthy control.

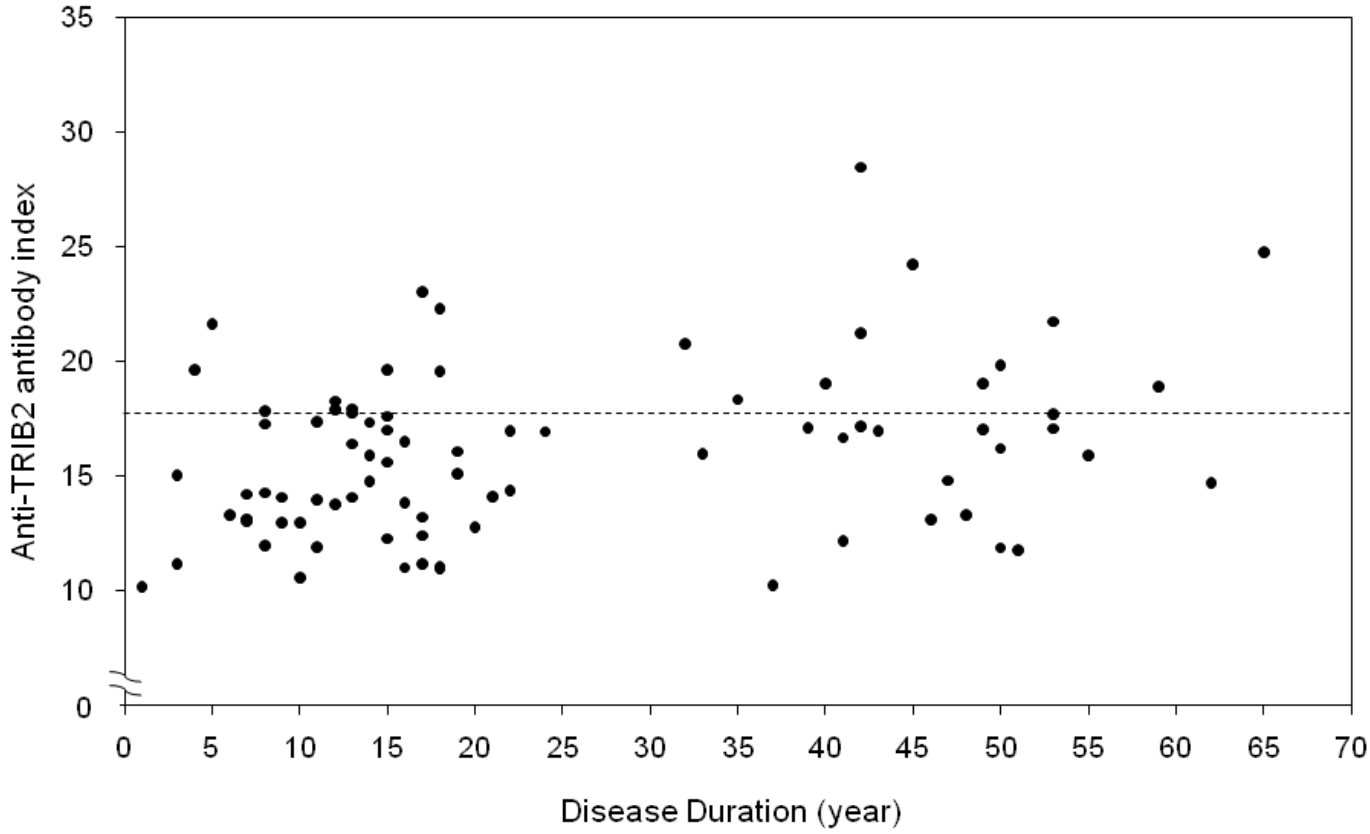

Figure S3—Indices of anti-TRIB2 autoantibodies in Japanese patients are not associated with disease duration. Indices of autoantibodies in patients with narcolepsy-cataplexy are plotted. Each dot corresponds to data from one subject. The dotted line indicates the mean + 2SD levels in healthy controls.