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Brief Research Communication

Novel Polymorphism in the Promoter Region of the Tumor Necrosis Factor Alpha Gene: No Association With Narcolepsy

Tadafumi Kato,[1]* Makoto Honda,[2] Shouji Kuwata,[3] Takeo Juji,[4] Hiroshi Kunugi,[5] Shinichiro Nanko,[5] Masato Fukuda,[1] and Yutaka Honda[6]

[1]*Department of Neuropsychiatry, Faculty of Medicine, University of Tokyo, Tokyo, Japan*
[2]*Matsuzawa Hospital, Tokyo, Japan*
[3]*The Third Department of Internal Medicine, Ichihara Hospital, University of Teikyo School of Medicine, Ichihara, Japan*
[4]*Japan Red Cross Central Blood Center, Tokyo, Japan*
[5]*Department of Psychiatry, University of Teikyo School of Medicine, Tokyo, Japan*
[6]*Neuropsychiatric Research Institute, Tokyo, Japan*

The striking evidence of almost 100% association of narcolepsy with human leukocyte antigens (HLA) DR2(DR15) antigen is an important clue to elucidate the molecular basis of this sleep disorder. The gene for tumor necrosis factor α (TNF α) is located in the HLA class II gene cluster. Recent studies have indicated that TNF α plays an important role in the regulation of normal human sleep, and regulation of this cytokine may be disturbed in narcolepsy. We searched for a mutation associated with narcolepsy in the promoter region of the TNF α gene by single-strand conformation polymorphism analysis. A novel polymorphism, C-850T, was found in narcoleptic patients. Genotype frequency was examined by restriction fragment length polymorphism method. No significant difference of genotype distribution was found between 92 patients with narcolepsy and 91 normal controls. These results do not support our hypothesis that genetic abnormality of TNF α production is pathogenetic for narcolepsy. Am. J. Med. Genet. (Neuropsychiatr. Genet.) 88:301–304, 1999.

KEY WORDS: sleep disorders; human leukocyte antigens (HLA); cytokines; molecular genetics

INTRODUCTION

Narcolepsy is a sleep disorder characterized by recurrent day-time sleep episodes and cataplexy. Since the striking discovery by Juji, Honda, and colleagues, who found 100% association of narcolepsy with HLA (human leukocyte antigen) DR2 antigen [Honda et al., 1984; Juji et al., 1984; Honda and Juji, 1988], the pathophysiological basis of this interesting relationship has been investigated. Their subsequent studies revealed that narcolepsy is also associated with Dw2 antigen [Honda et al., 1986]. Moreover, development of an HLA typing method revealed that the HLA associated with narcolepsy was DR15, subtype of DR2. Subsequently, these HLA antigens were investigated at the molecular level. The HLA class II genes associated with narcolepsy were found to be DRB1*1501/DQB1*0602 [Kuwata et al., 1992]. Mignot and colleagues, who have been studying this matter extensively, reported that not DRB1*1501 but DQB1*0602 is associated more closely with narcolepsy in African Americans, and concluded that DQB1*0602 could be a susceptibility gene for narcolepsy [Mignot et al., 1997; Rogers et al., 1997], although association of DQB1*0602 with narcolepsy is not 100%. They also examined polymorphic markers surrounding this locus and found that the haplotype found in narcoleptic patients was similar to those in normal subjects, which also suggested that normal HLA DQ6 antigen itself is associated with narcolepsy [Ellis et al., 1997]. These results suggest that (1) a specific HLA itself plays a pathophysiological role in narcolepsy or (2) a mutation of a gene closely linked with DQB1*0602 is a necessary condition in the pathogenesis of narcolepsy.

Tumor necrosis factor alpha (TNF α) is a cytokine with a wide variety of biological activities, whose gene is in the HLA gene cluster, about 1,000 kb or 1 cM downstream of the HLA DQB1 locus and 850kb of the HLA DR locus. Recent studies by Krueger and colleagues have revealed that TNF α also acts as a neu-

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*Correspondence to: Tadafumi Kato, M.D., Department of Neuropsychiatry, Faculty of Medicine, University of Tokyo, Hongo 7-3-1, Bunkyo, Tokyo, 113-8655, Japan.
E-mail: tadafumi-tky@umin.ac.jp

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TABLE I. Primers for TNF α Promoter*

Primers	Sequence	Length	Size
TP–0020F	5–GAGGCCGCCAGACTGCTGCAG	21 mer	
TP0306R	5–CCCCAGTGTGTGGCCATATCTTCTT	25 mer	325 bp
TP0256F	5–CCAGGTATGGAATACAGGGGACGTT	25 mer	
TP0602R	5–AAAACGGGGTTGGAGGGAAAAGCTG	25 mer	346 bp
TP0553F	5–CAAACACAGGCCTCAGGACTCAACA	25 mer	
TP0923R	5–CTGCACCTTCTGTCTCGGTTTCTTC	25 mer	370 bp
TP0860F	5–TGTGTCCCCAACTTTCCAAATCCCC	25 mer	
TP1178R	5–AGAACCTGCCTGGCAGCTTGTCA	23 mer	319 bp
TP203FM	5–AAGTCGAGTATGGGGACCCCCC**G**TTAA	27 mer	

*The names of primers correspond to the base numbers by Takashiba et al. [1993].

romodulator in physiological sleep [Krueger and Majde, 1995]. Injection of TNF α increases non-rapid eye movement (NREM) sleep while it suppresses rapid eye movement (REM) sleep [Kapas et al., 1992; Shoham et al., 1987]. NREM sleep is inhibited by anti-TNF α antibody [Takahashi et al., 1995]. Knockout mice lacking the TNF α receptor have sleep disturbances and TNF α do not induce sleep in these knockout mice [Fang et al., 1997]. TNF α-like immunoreactivity is detected in the brain [Breder et al., 1993].

Recently, it was reported that salidomide, a TNF α inhibitor, worsened cataplexy in narcoleptic dogs [Kanbayashi et al., 1996]. It was also reported that TNF α levels were increased in patients with narcolepsy [Vgontzas et al., 1997]. These results suggest that regulation of TNF α production may be impaired in narcoleptic patients.

Regulation of the TNF α gene has been studied extensively [see Jogeneel, 1994, for review]. Several kinds of polymorphisms in the promoter region of the TNF α gene affecting TNF α production [Rink and Kirchner, 1996] have been reported, of which the G-308A (TNF1/2) polymorphism, which increases TNF α production, may be related to neurological disorders such as cerebral malaria [McGuire et al., 1994], multiple sclerosis [Braun et al., 1996; Huizinga et al.,1997], and bacterial meningitis [Nadel et al., 1996]. The G-238A polymorphism may be related to systemic lupus erythmatosis [D'Alfonso et al., 1996] although it does not affect TNF α production [Pociot et al., 1995].

In this study the promoter region of the TNF α gene was examined by single-strand conformation polymorphism (SSCP) analysis [Orita et al., 1989] in patients with narcolepsy and in normal controls to search for a mutation responsible for narcolepsy.

MATERIALS AND METHODS

Subjects were 92 narcoleptic patients at Tokyo University Hospital or at Seiwa Hospital affiliated with the Neuropsychiatric Research Institute. They were diagnosed to have narcolepsy according to the part I minimum diagnostic criteria for narcolepsy in the International Classification of Sleep Disorders [Diagnostic Classification Steering Committee, 1990]. All of them were positive for HLA DR2 antigen.

Ninety-one control subjects were selected from doctors, nurses, and medical students. They were not screened for narcolepsy and HLA was not examined in these subjects. Informed consent was obtained from all subjects. This study was approved by Ethical Committee of University of Tokyo.

DNA was extracted from these subjects by standard protocols. DNA samples of eight narcoleptic patients and eight controls were used for the SSCP experiment. Four polymerase chain reaction (PCR) fragments, covering the promoter region of the TNF α gene up to 1,000 bp [Takashiba et al., 1993], were amplified by PCR primers (Table I). Primers were purchased from Life Technologies Oriental Inc. (Tokyo, Japan). PCR was performed using Ex-Taq and Takara PCR Thermal Cycler MP (Takara Co. Ltd., Otsu, Japan). The parameters of the PCR reaction were as follows: 94°C for 20 sec, 68°C for 30 sec, 72°C for 60 sec, and 40 cycles. Before the first cycle, heat-denaturation was performed at 94°C for 3 min and final extension was done at 72°C for 10 min.

The PCR product was diluted fivefold by the loading buffer containing 99% deionized formamide, 0.05% bromophenol blue, and 0.05% xylene cyanol. These samples were heat-denatured at 94°C for five minutes and chilled on ice thereafter. These samples were loaded in a 5% nondenaturing polyacrylamide gel containing 5% glycerol with 1:49 ratio of acrylamide and bisacrylamide. Electrophoresis was performed in 0.5× Tris borate buffer (TBE) at 30 W for 2.5 hr at 4°C. After the electrophoresis, the gel was silver stained [Bassam et al., 1991]. These experiments were repeated three times.

A polymorphism was seen only in Fragment 1 am-

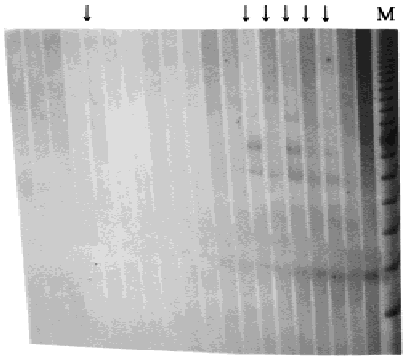

Fig. 1. Single strand conformation polymorphism analysis. Arrows indicate the polymorphic patterns. Right eight lanes were narcoleptic patients, left eight lanes were controls. Five of eight narcoleptic patients and one of eight controls had this polymorphism.

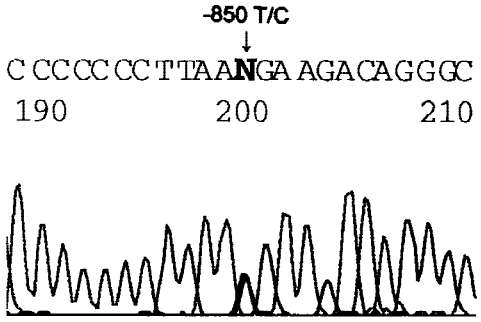

Fig. 2. Sequencing analysis of the promoter region of a patient with narcolepsy. This patient was T/C heterozygote at the position –850 in the promoter region of the TNF α gene.

plified by TP-0020F and TP0306R. In this fragment, five of eight narcoleptic patients and one control subject had a polymorphism (Fig. 1). Other fragments consistently did not show any polymorphism. The PCR products with polymorphisms were sequenced by the cycle-sequence method using an ABI 373S auto sequencer (Perkin Elmer, Japan; Fig. 2). Both forward and reverse primers were used to confirm the sequence. A base substitution C→T at the position of –850 (C-850T), which has never been reported before, was seen (Fig. 2).

The C-850T polymorphism was genotyped by PCR-RFLP (restriction fragment length polymorphism). Because there is no available restriction enzyme to distinguish this base substitution, a mismatched primer TP203FM was designed. In this primer the fifth base from the 3′ end was "G" instead of "C," which makes a restriction site of HincII, GTTAAC, which disappeared when C-850T polymorphism exists. The PCR product amplified by a mismatch primer (TP203FM) and the reverse primer TP306M (Table I) was dissolved in the buffer recommended by the manufacturer for the use of the restriction enzyme. The PCR product was then digested by Hinc II (Takara Shuzo Co. Ltd., Otsu, Japan) at 37°C for 2 hr. These samples were electrophoresed in 4% Metaphor Agarose (Takara Co. Ltd., Otsu, Japan) gels containing in 1% TBE at 4°C for two hr, stained with ethidium bromide, and visualized by ultraviolet trans-illuminator (Fig. 3). When the C-850T base substitution did not exist, HincII digested the 133 bp PCR product to 108 bp.

RESULTS AND DISCUSSION

The genotype and allele frequencies are shown in the Table II. There was no significant difference in genotype or allele frequency between patients with narcolepsy and normal controls. The genotype distributions were in the Hardy-Weinberg equilibrium for both the patients and the controls. Because the genotype distribution of the TNF α promoter in narcoleptic patients having the HLA-DR2 antigen is similar to that in normal controls, there is no linkage disequilibrium between these two loci.

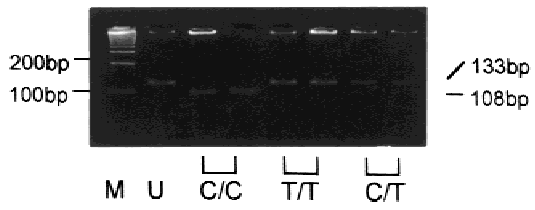

Fig. 3. PCR-RFLP (restriction fragment length polymorphism). The PCR fragment was digested by HincII and electrophoresed in a 4% agarose gel and stained with ethidium bromide. **M:** 100 bp ladder marker. **U:** PCR fragment without enzyme digestion.

TABLE II. C-850T Polymorphism in the TNF α Promoter and Narcolepsy

	Genotype			Allele frequency	
	T/T	T/C	C/C	T	C
Controls (n = 91)	7	24	60	20.9%	79.1%
Narcolepsy (n = 92)	8	28	56	23.9%	76.1%

Contrary to our expectation, a novel polymorphism in the regulatory region of the TNF α gene was not associated with narcolepsy. These results do not support our hypothesis that genetic variation in the promoter region of the TNF α gene plays a pathophysiological role in narcolepsy.

It is not known whether or not this polymorphism alters production of TNF α. If C-850T affects TNF α production, it might be associated with some immunological disorders rather than narcolepsy.

In this study previously reported polymorphisms in the promoter region of TNF α gene were not screened by PCR-RFLP or sequencing in these eight patients. Based on an assumption that detection efficacy of PCR-SSCP is 80%, however, a possibility that a polymorphism associated with narcolepsy was dismissed in this study is less than 1 %. Moreover, all PCR fragments in the promoter region of the TNF α gene have been sequenced in at least one patient with narcolepsy, and no previously reported polymorphism was detected. Therefore, association of other reported polymorphisms with narcolepsy is unlikely.

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