

## Molecular Analysis of Prion Protein Gene (PRNP) in Korean Patients with Creutzfeldt-Jakob Disease

Creutzfeldt-Jakob disease (CJD), a relatively uncommon human dementia, is caused by an unconventional slow infectious agent. Several cases of CJD, clinically or histopathologically diagnosed, have been reported in Korea. In order to confirm the diagnosis of CJD and also differential diagnosis of sporadic and familial types of CJD in Korea, we studied two patients who had symptoms of CJD. The histopathological and immunohistochemical studies showed spongiform neurodegeneration and expression of abnormal isoform of prion protein (PrP<sup>Sc</sup>) in astrocytes. Thus, these two patients were diagnosed CJD. To investigate whether these patients were sporadic or familial type of CJD, the molecular analyses of the prion protein gene (PRNP) were done by restriction fragment length polymorphism (RFLP) and DNA sequencing. In the cases of a healthy Korean and two CJD patients, no point mutation was detected in the known hot spots (178, 180, 200, 210, and 232) and they exhibited wild type PRNP sequences. We concluded that both patients have a sporadic type of CJD, but not familial type.

**Key Words :** Creutzfeldt-Jakob disease; Immunohistochemistry; Prion disease, spongiform encephalopathy; Prions, mutation PRNP

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## INTRODUCTION

Prion diseases are neurodegenerative diseases caused by unconventional slow infectious pathogens, designated prions, which are responsible for transmissible and inherited disorders. The definitive diagnosis of human prion disease requires at least one of the four following criteria: presence of prion amyloid plaques, transmission of spongiform encephalopathy to animals, presence of abnormal isoform of prion protein, or presence of a pathogenic PRNP gene mutation(1). Four prion diseases have been identified in humans: kuru, Creutzfeldt-Jakob disease (CJD), Gerstmann-Sträussler-Scheinker syndrome (GSS), and fatal familial insomnia (FFI). CJD is not only characterized clinically by dementia, ataxia, and myoclonus (2, 3), but also pathologically by neuronal loss, astrogliosis, and spongiform neurodegenerations (4-6). Sporadic CJD, which appears worldwide, is found in one person out of a million (7). Familial type CJD comprises 10 to 15 percent of all reported cases of CJD (8), and iatrogenic CJD occurs in small numbers (9). Patients generally develop CJD between the ages of 50 and 70 and die within 1 year. The human prion gene on chromosome 20 encodes a protein designated PrP<sup>C</sup> (10). An abnormal,

protease-resistant isoform (PrP<sup>Sc</sup>) of the prion protein is present in the brain of CJD patients (11). Familial CJD is known to be caused by a point mutation at codon 178 (12, 13), 180 (14), 200 (15-19), 210 (20, 21), or 232 (14), and several insert mutations in the octapeptide repeats of the open reading frame (ORF) of PRNP (22, 23). Recently, several cases of CJD have been reported in Korea (24-27), however all of them were clinically and histopathologically diagnosed. This study was undertaken to confirm the diagnosis of CJD in Korean patients, and to differentiate sporadic or familial types of CJD patients in Korea.

## MATERIALS AND METHODS

### Neuropathology and immunohistochemistry

The biopsy brains (temporal cortex) were fixed in 10% neutral formalin, rinsed with 0.01 M phosphate-buffered saline (PBS, pH 7.0), dehydrated with ethanol, and embedded in paraffin. Coronal sections of the brain (10  $\mu$ m thick) were cut with a microtome. The brain sections were deparaffinized with xylene and hydrated through

grades of ethanol. For observation of histopathological changes, the tissue sections were stained with hematoxylin and eosin (H & E). Double immunostaining for PrP<sup>Sc</sup> and glial fibrillary acidic protein (GFAP) has been performed in the following order: Sections were incubated with proteinase K (10  $\mu$ g/ml) for 7 min, and rinsed with 0.01 M PBS. The sections were treated with 10% normal goat serum in PBS for 1 hour, and incubated with rabbit antiserum against PrP<sup>Sc</sup> (provided by Dr. Richard J. Kascsak) overnight at 4°C. The tissue sections were rinsed in PBS, and incubated for 2 hours in peroxidase-conjugated goat anti-rabbit IgG (Vector, USA) diluted at 1:50 in PBS. The tissue sections were then rinsed in 0.05 M Tris-HCl (pH 7.6). For the detection of horseradish peroxidase, the sections were incubated in a mixture of 0.05% diaminobenzidine (DAB, Sigma, USA) and 0.01% H<sub>2</sub>O<sub>2</sub> in 0.05 M Tris-HCl for 1 min at room temperature. After rinsing in PBS, the sections were treated with 10% normal goat serum in PBS for 1 hour, and incubated overnight at 4°C in rabbit antiserum (Zymed, USA) against GFAP diluted at 1:300 in PBS. The tissue sections were rinsed in PBS, and incubated for 2 hours in gold labeled goat anti-rabbit IgG (Amersham, England) diluted at 1:40 in PBS. The tissue sections were then incubated in silver enhancement mixture (Amersham, England) for 15 min at room temperature. After rinsing in deionized water, the sections were counterstained with hematoxylin.

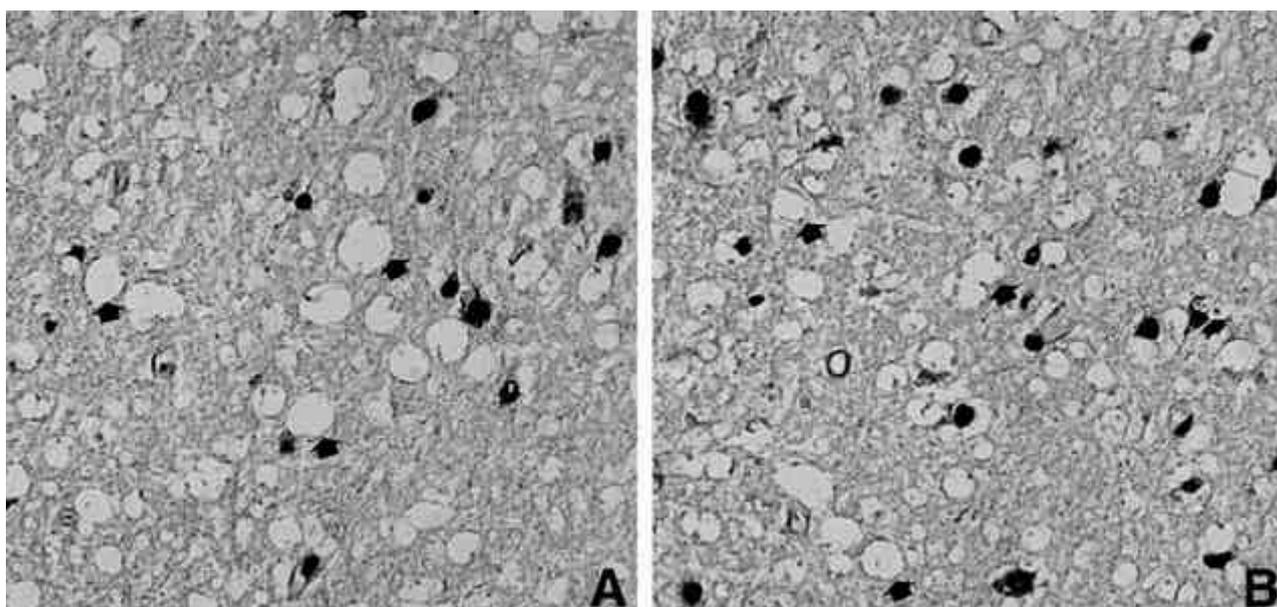
#### RFLP and DNA sequencing

Blood samples were received from a normal person and two cases of CJD patients. Genomic DNA was extracted from peripheral lymphocytes. Polymerase chain reaction (PCR) was performed with T-1 (GATGCTGGTTCTCTTGTGG) and T-2 (CCCACTATCAGGAAGATGAG) primers. The PCR conditions were left in 94°C for 10 min to denature, and 30 cycles of 94°C for 1 min, 62°C for 1 min, and 72°C for 1 min 30 sec. The 714 bp PCR product was digested with *Tth111 I* (Promega, USA) to analyze the codon 178. Restriction products were separated on 1.5% agarose gel, stained with ethidium bromide, and visualized under a lamp. *Tth111 I* cuts the 738 bp PCR product of PRNP into 514 bp and 224 bp fragments in codon 178. The complete DNA sequence was determined by the dideoxy chain termination method using the Sequenase Version 2.0 Kit (USB, USA). The following sequencing primers were used: H-3 (CACCCACAGTCAGTGGAACA), K-4 (GGTCCTCATAGTCAGTGCCG), K-5 (CATGAGCAGGCCCATCATA), K-6 (ACACATCTGCTCAACCACGC), K-7 (GTCACCACAACCACCAAGGG), K-14 (TGTTCCACTGACTGTGGGTG), T-1 (GATGCTGGTTCTCTTTGTGG), T-2 (CCCACTATCAGGAAGATGAG).

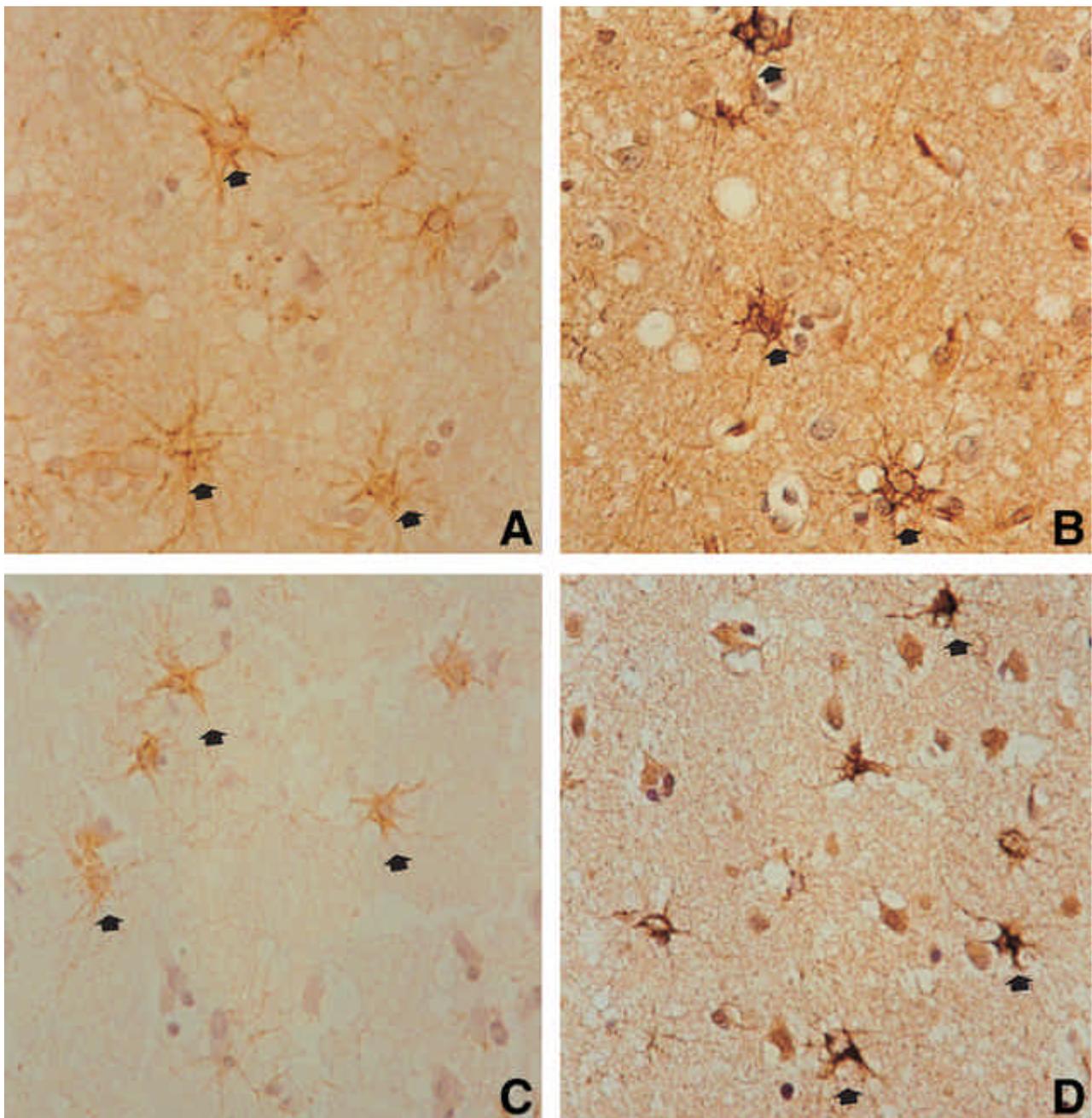
## RESULTS

### Neuropathological and immunohistochemical diagnoses of CJD

Spongiform changes are one of the typical neuropath-



**Fig. 1.** Neuropathological findings. Case 1 (A) and Case 2 (B) in temporal cortex stained with hematoxylin and eosin show spongiform degeneration (arrows) ( $\times 400$ ).



**Fig. 2.** Immunohistochemical changes. Case 1 (A) and case 2 (C) show temporal cortex stained with anti-GFAP showing intense reaction astrocytosis and case 1 (B) and case 2 (D) stained with double immunostaining for PrP<sup>Sc</sup> (brown: DAB) and GFAP (black: silver) ( $\times 400$ ).

ological findings of prion diseases including CJD. To determine the spongiform changes in these cases, we performed H & E staining. Spongiform changes were observed predominantly in the temporal cortex in both cases (Fig. 1. A, B arrows). And then we used GFAP immunohistochemistry to determine the astrocytosis, we observed remarkable reactive astrocytosis in the temporal cortex of these cases (Fig. 2. A, C arrows). Furthermore, to

determine the immunoreactivity and the distribution of infectious prion proteins (PrP<sup>Sc</sup>), we used the double immunohistochemistry of PrP<sup>Sc</sup> and GFAP antiserum. In these cases, we found that PrP<sup>Sc</sup> immunoreactivity was detected in astrocytes, not in neuronal cells (Fig. 2. B, D arrows). These results demonstrate that the two patients were diagnosed with CJD.

TT TGC AGA GCA GTC ATT ATG GCG AAC CTT GGC TGC TGG ATG CTG GTT CTC TTT GTG GCC 60  
Met T-1 sense primer

ACA TGG AGT GAC CTG GGC CTC TGC AAG AAG CGC CCG AAG CCT GGA GGA TGG AAC ACT GGG 120

GGC AGC CGA TAC CCG GGG CAG GGC AGC CCT GGA GGC AAC CGC TAC CCA CCT CAG GGC GGT 180

GGT GGC TGG GGG CAG CCT CAT GGT GGT GGT GGC TGG GGG CAG CCT CAT GGT GGC TGG GGG 240

CAG CCC CAT GGT GGT GGC TGG GGT CAA GGA GGT GCC ACC CAC AGT CAG TGG AAC AAG CCG 300  
H-3 sense, K-14 antisense primer 102  
GSS : Pro→CTG(Leu)

AGT AAG CCA AAA ACC AAC ATG AAG CAC ATG GCT GGT GCT GCA GCA GCT GGG GCA GTG GTG 360  
GSS : Pro→CTA(Leu) 117  
GSS : Ala → GTG(Val), GCG(Ala)

GGG GGC CTT GGC GGC TAC ATG CTG GGA AGT GGC ATG AGC AGG CCC ATC ATA CAT TTC GGC 420  
CJD : Met → GTG(Val) 129  
K-5 sense primer K-4 antisense

AGT GAC TAT GAG GAC CGC TAC TAT CGT GAA AAC ATG CAC CGT TAC CCC AAC CAA GTG TAC 480  
145  
primer GSS : Tyr→TAG(stop)

TAC AGG CCC ATG GAT GAG TAC AGC AAC CAG AAC AAC TTT GTG GTG GAC TGC GTC AAT ATC 540  
178 180  
CJD : Asp→AAC(Asn) Val→ATC(Ile)

ACA ATC AAG CAG CAC ACG GTC ACC ACA ACC ACC AAG GGG GAG AAC TTC ACC GAG ACC GAC 600  
K-7 Sense primer GSS: Phe→TCC(Ser) CJD: Glu→AAG(Lys) 198 200

GTT AAG ATG ATG GAG CGC GTG GTT GAG CAG ATG TGT ATC ACC CAG TAC GAG AGG GAA TCT 660  
210 217  
CJD : Val→ATT(Ile) K-6 Antisense primer GSS : Gln→CGG(Arg)

CAG GCC TAT TAC CAG AGA GGA TCG AGC ATG GTC TTC TTC TCC TCT CCA CCT GTG ATC GTC 720  
232  
CJD : Met→AGG(Arg)

CTG ATC TCT TTC CTC ATC TTC CTG ATA GTG GGA TGA GGA GGT GTT CTG TTT TCA CCATCTT 783  
T-2 Antisense primer End

Fig. 3. Nucleotide sequence of PrP open reading frame in a healthy Korean. The underlined sequences indicate DNA sequencing primer and the site of point mutation with familial CJD and GSS patient.

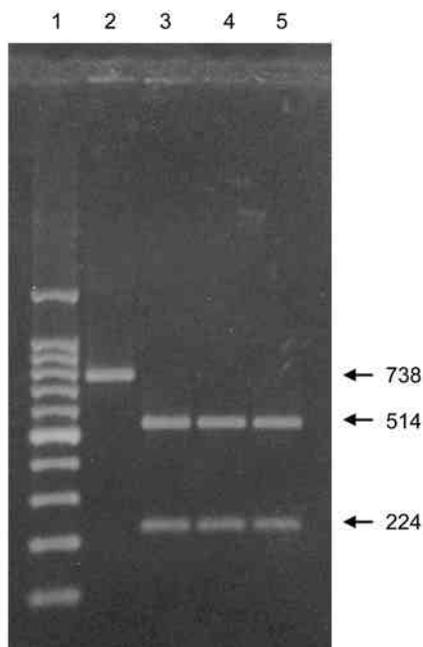
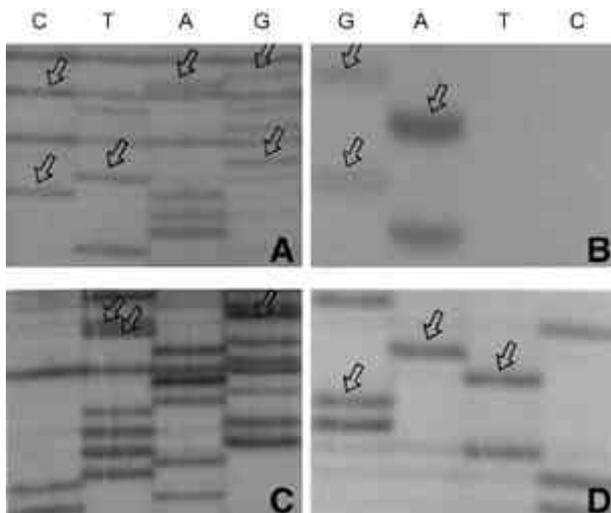


Fig. 4. Restriction analysis of PrP ORF with *Tth1111*-digestion. The enzyme *Tth1111* cleaves the 738bp PCR fragments of normal allele into two smaller fragments of 514bp and 224bp. Lane 1, 100bp DNA ladder marker. Lane 2, PCR product. Lane 3, the *Tth1111*-digested PCR product from control. Lane 4, 5, the *Tth1111*-digested PCR products from the two patients.

Analysis of DNA sequence of PrP ORF in a healthy Korean and two clinically diagnosed CJD patients

The complete structure and full sequence of the human PrP gene have been determined (10). Familial type of CJD was linked to several point mutations at codon 178 (GAC→AAC), 180 (GTC→ATC), 200 (GAG→AAG), 210 (GTT→ATT) and 232 (ATG→AAG). Thus, we examined the first full sequence of PrP ORF in a healthy Korean using DNA sequencing. PrP gene in the Korean exhibited octapeptide repeat sequence and observed same sequences compared to data of foreigners (Fig. 3). RFLP and DNA sequencing of PRNP ORP were carried out in two Korean patients who were clinically diagnosed with CJD. At first, to identify the codon 178 mutation, we performed a *Tth1111* I digestion of the PCR product. A wild type of the PCR product was then cut into 514 and 224 bp, while a mutant type abolished the *Tth1111* I restriction site. Patterns of *Tth1111* I digestion (Fig. 4) showed that both patients had no mutations at codon 178. We sequenced the ORF of PrP gene from the two patients to identify the familial type of CJD. The point mutations at codon 178, 180, 200, 210, and 232 were not observed: GAC (Asp) at codon 178, GTC (Val) at codon 180, GAG (Glu) at codon 200, GTT (Val) at co-



**Fig. 5.** DNA sequence pattern of the subcloned PrP gene of patients at codon 178 (A), 180 (A), 200 (B), 210 (C) and 232 (D). Point mutations at codon 178 (GAC→AAC), codon 180 (GTC→ATC), codon 200 (GAG→AAG), codon 210 (GTT→ATT), and codon 232 (ATG→AAG) were linked to familial CJD.

don 210, and ATG (Met) at codon 232 (Fig. 5). According to these results, point mutations of PrP ORF were not found in the two Korean patients. These results indicate that both patients had the sporadic type of CJD, but not familial type.

## DISCUSSION

Recently, five cases of CJD have been reported in Korea (24-27). All of them were based on only clinical symptoms, electroencephalograph (EEG), and pathological findings. In this study, we described the clinical features and histopathological and genetic evaluation of sporadic CJDs. The clinical features of the two patients are rapid course, rapidly progressive dementia, visual disturbance, gait disturbance, myoclonus, mute, and bed confinement. Clinical findings in our cases are similar to those of sporadic or familial type previously reported (6, 28). The detailed histopathological studies demonstrate the presence of a lesion characterized by spongiform neurodegeneration and reactive astrocytosis. PrP<sup>Sc</sup> is synthesized from the normal cellular isoform, PrP<sup>C</sup>, by posttranslational process. PrP<sup>Sc</sup> is partially digested by proteases to form a fragment designated PrP27-30. On the other hand, PrP<sup>C</sup> is completely degraded under the same condition (29). And PrP<sup>Sc</sup> selectively accumulates in astrocytes during the progress of prion disorders (30). The etiology of CJD remains unclear, but it may involve spontaneous conversion of PrP<sup>C</sup> to PrP<sup>Sc</sup> or PRNP mutations. In immunohistochemistry, our cases show the expression of PrP<sup>Sc</sup> in

astrocytes, however these findings were not exhibited in normal controls (data not shown). Recently, PrP gene sequences were well known in humans. We analysed the first ORF of PRNP in Koreans. The result showed the same sequences found in published data (10). There are several reports of mutations in the familial types of human prion diseases. For example, familial type of CJD was linked to insertion of variable numbers of octapeptide repeats and mutations at codon 178, 180, 200, 210, or 232. These two kinds of mutations have been reported in many families of CJD. The first group is insertion mutation of octapeptide repeats in a Japanese (31) and two Americans (32, 33). The second group is the point mutations: codon 178 in a Finnish, two French, a Dutch, a Canadian (34), and a Hungarian (35); codon 180 in two Japanese families (14); codon 200 in a English (36), three Japanese (17), and a German (37); codon 210 in five Italians (20) and a French family (21); codon 232 in three Japanese families (14). In our studies, point mutations of codon 178, 180, 200, 210, and 232 and insertion mutation of octapeptide repeat were not found in these patients. FFI, another familial prion disorder, was clinically distinguishable from CJD because of the presence of insomnia. Point mutation of PRNP at codon D178N-M129 was genetically diagnosed as FFI, while point mutation of PRNP at codon D178N-V129 was diagnosed as familial CJD (13). PrP gene of Korean patients appears at D178-M129. Thus, this result indicates that both patients were not FFI and familial CJD. GSS was related to mutation at codon P102L, P105L, A117V, T145Stop, P198S, or G217A (14, 38-42). Also, point mutation of these were not present in Korean patients. These results show that both patients were a sporadic CJD, but not familial CJD, nor FFI, GSS.

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