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A NOVEL RNA SPLICING MUTATION IN JAPANESE PATIENTS WITH WILSON DISEASE

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Deletion/insertion mutation of Wilson disease (*WD*) gene in 16 Japanese patients with Wilson disease was studied. A truncated size in a region of exon 4 to 6 was found by reverse transcription-polymerase chain reaction (RT-PCR) covering entire 21 exons except exon 1 for liver cDNA of one patient with a late onset neurologic type. Sequence analysis of the cDNA revealed that this truncation was occurred by skipping of exon 5, though any mutation in exon 5 of genomic DNA was failed to detect. T to G transversion in 5 bp upstream from a junction of intron 4 and exon 5 was found in genomic DNA of the patient. Further, results obtained by RT-PCR and the sequence analysis in intron 4 indicate that the mutation of the patient is homozygous. Since same mutation in one allele of another patient out of 15 patients was found, allele frequency of the splicing mutation in Japanese patients is 9.4%. These results suggest that the point mutation in intron 4 of *WD* gene causes the skipping of exon 5 and the splicing mutation affects the phenotype of Wilson disease.

Wilson disease is an autosomal recessive disorder of copper metabolism. A worldwide frequency is between one in 35,000 and one in 100,000 live births[1]. The trias of the disease are liver dysfunction, extra pyramidal signs and Kayser-Fleischer ring. These symptoms are due to copper deposition in various tissues, such as liver, brain, kidney, cornea and others. The copper toxicity is believed to result from the loss ability to excess copper from liver to bile and to incorporate copper into ceruloplasmin in the liver.

Wilson disease gene (*WD* or *ATP7B*), consisting of 21 exons, is coded for a putative copper-transporting P-type ATPase, a closely related Menkes disease gene[2-4]. Totally, 25 mutations in *WD* gene have been reported[3-5] Though major mutations were one or two base pairs insertion or deletion, which lead to nonsense and missense mutations, three types of splicing mutation were reported[5]. Most of them are in European and North American patients. Two patients of Oriental origin, Chinese in Hong Kong, was analyzed[5]. No mutation study in Japanese has been reported.

In this study, we analyzed the genetic disorder of Japanese patients with Wilson disease and demonstrate that one of mutations is exon skipping that is due to point mutation of splicing acceptor site at 5 bp upstream of a junction of intron 4 and exon 5.

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Materials and Methods

Patient materials. Unrelated 16 Japanese patients with Wilson disease were examined in this study. Total RNA was extracted from liver tissue and genomic DNA was from peripheral blood leukocytes. Tissue and leukocyte samples were collected after informed consent. The patient HF first examined in this study was currently a 32-year old male. He was a late onset neurologic type and his onset was about 29-year old with mainly dysarthria. He has also developed writing disorder and flapping tremor. Kayser-Fleischer ring was detected. There is no history of consanguinity present in the family.

Preparation of cDNA. Liver tissues of the patients with Wilson disease were obtained by open liver biopsy. Normal liver tissue was normal region of another liver disease but not Wilson disease. The tissues were rinsed in phosphate-buffered saline (10 mM Na_2HPO_4, 150 mM NaCl, pH 7.2) and frozen in liquid nitrogen as described[6]. Tissues were homogenized in guanidinium isothiocyanate and total RNA was isolated by cesium chloride gradient centrifugation[7]. The nucleic acid content was determined with a spectrophotometer (DU65, Beckman, Fullerton, CA). cDNA was prepared by a cDNA cycle kit (Invitrogen, San Diego, CA) using an oligo dT primer and specific primers for *WD* gene listed in Table 1.

Isolation of genomic DNA. Genomic DNA was isolated from peripheral blood leukocytes of patients and 25 normal healthy volunteers as described[8].

PCR. Eleven sets of oligonucleotide primers covering full length mRNA of *WD* gene except region corresponding to exon 1 were specific for parts of the gene sequence (EMBL accession L25442, L25591, U03464) (Table 1). A set of oligonucleotides was also prepared to amplify exon 5 as described[5]. Reaction mixture contained a set of 0.1 μM each primer, 200 μM each dNTP, 2.5 U *Taq* DNA polymerase, and amplification buffer consisting of 10 mM Tris-Cl, 50 mM KCl, and 1.5 mM $MgCl_2$, pH 8.8, either cDNA or 100 ng of genomic DNA in a total volume of 100 μl. Thermal condition was 35 cycles of denaturing at 96°C for 1 min, annealing at 55°C for 2 min, and extension at 72°C for 3 min, and final extension for 10 min. Aliquots of each sample were subjected to nondenaturing electrophoresis on a 5% polyacrylamide gel. PCR products were analyzed by the ethidium bromide staining followed by visualization with shortwave UV illumination.

Sequencing analysis. PCR-amplified DNA fragment was electrophoresed on a 0.8% SeaKem GTG agarose gel (FMC BioProducts, Rockland, ME), purified with a Gene Clean II kit (BIO 101 Inc., La Jolla, CA), and used for sequencing analysis. DNA sequencing was performed using a *Taq* DyeDeoxy Terminator Cycle Sequence kit (Perkin-Elmer, Foster City, CA) as recommended by the supplier. The reaction mixture was analyzed on an automated sequencer (Perkin-Elmer, Applied Biosystems Division, model 373S).

Results

To detect abnormal expression of *WD* gene including the relatively large deletion/insertion mutations, RT-PCR using 11 primer sets covering exon 2 to exon 21 (Table 1) was carried out for mRNA extracted from liver tissues of 3 patients. The sizes of PCR products were compared to those of a normal control. Region 3 of the patient was approximately 160 bp less than that of the control (Fig. 1, lanes 1 and 2). The PCR products of other 3 patients in this region was essentially same size as control

Table 1. PCR primer sets used in amplification of the Wilson disease gene

Region	Name	Upstream primer (5'-3')	Name	Downstream primer (3'-5')	PCR product size, bp
1	WD-1	CTGGGAACCAGCAATCAAGAAGA	WD-2	GAGAACTGGAAGACCTGTGATCT	949
2	WD-3	CTTGGAGAACAAAACTGCCCAA	WD-4	GTCATCCCTGTGATTGTCAGCTCA	854
3	WD-5	GCTTCTTACAGATCAAAGGC	WD-6	GGCTCGTTGCTGGGTATCAGCA	567
4	WD-7	CGCTCATCACTTGGACCACAAGAT	WD-8	TGGCTGACCTGTGTCTCAGAGATT	274
5	WD-9	CTTGTGTACCTTTGTCCAGCTCCT	WD-10	CAGGGACCACCCTGACGTAT	407
6	WD-11	TGACCCTTGGTGAGGACAAT	WD-12	GTGATGGACGTCTGGAAAGCA	516
7	WD-13	GATGTCAAAGGCACCCATTCAGCA	WD-14	TGCAGTATCCCCAAGGTCTCTGT	556
8	WD-15	AAGACTGGCACCATTATCCAT	WD-16	GTCCTTTCATCTCGTGGTCTGTCA	450
9	WD-17	CAAAGTCAGCAACGCGGAAGACAT	WD-18	TCCCTTTATTCTGGAGCTCCT	469
10	WD-19	GATGTCAGTGACGCTATGACA	WD-20	CTAGCCACCACATCCAGCAAAT	442
11	WD-21	CGACGTCGTCCTTATCAGAAAT	WD-22	TGCTGGCTGTCCTGCTCAGCT	586

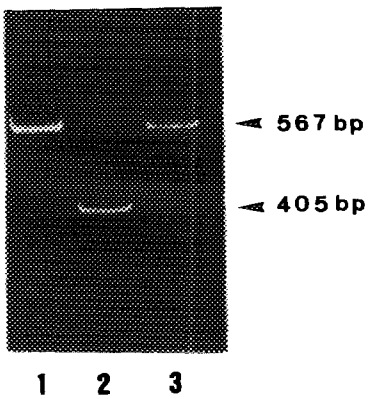

Fig. 1. Electrophoretic analysis of *WD* gene mRNA from 2 Wilson disease patients. RT-PCR was performed with total liver RNA as described under "Materials and Methods".
lane 1, normal subject; lane 2, patient HF; lane 3, patient AS.

(one of these cases shown in Fig. 1, lane 3). There was no significant difference in the sizes of PCR products of other 10 regions of any patients tested (data not shown).

To determine the detail of the deletion found in region 3, which includes exon 4 to 6, the PCR products of the patient and a normal subject were sequenced. Direct analysis of sequence in this region demonstrated the absence of the 162 bp corresponding to exon 5 (Fig. 2), while sequence of normal retained exon 5 (data not shown).

The sequence of around exon 5 of the genomic DNA of the patient was examined by PCR. The size of products derived from the patient and a normal was identical, suggesting that there is no large deletion at a genomic DNA level. The sequencing analysis of these genomic PCR products revealed T to G transversion at position -5 of intron 4 splicing acceptor site (Fig. 3). This transversion was not the result of a PCR artifact because sequence analysis for 3 independent PCR products of this region from the patient showed identical. Further, no such transversion of the sequence of this region of 25 normal individuals (50 alleles) was detected, indicating that T to G transversion in the splicing acceptor site may not be polymorphism.

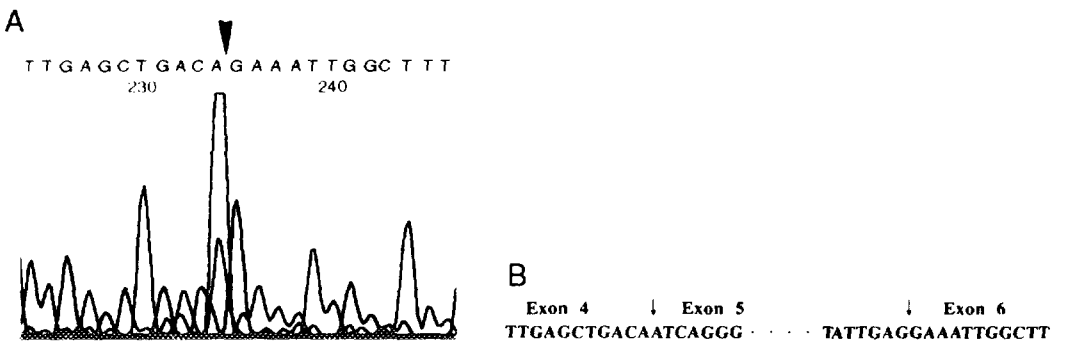

Fig. 2. Sequencing analysis of cDNA from liver of the patient HF. Panel A is an electrophoregram of automated sequencer (ABI model 373S). Arrowhead indicates the junction of exon 4 and exon 6. Panel B shows normal sequence of the junctions of exon 4 & 5 and 5 & 6.

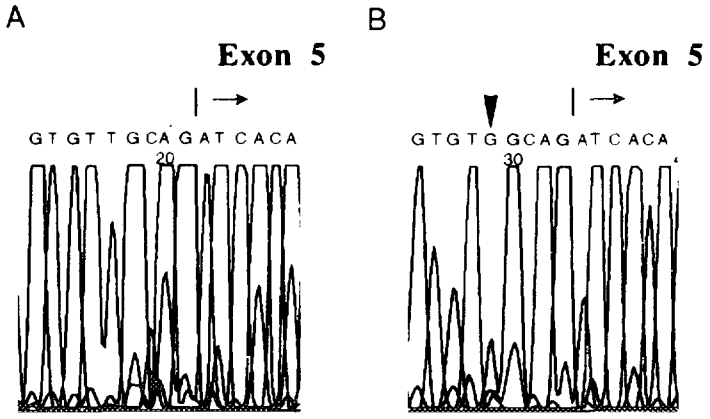

Fig. 3. Electrophoregram of genomic sequencing for the junction of intron 4 and exon 5. A, normal ; B, patient HF; arrowhead, a point mutation in the splicing acceptor site.

The results obtained by RT-PCR (Fig. 1, lane 2) and sequence analysis of genomic DNA (Fig. 3) strongly suggest that *WD* gene of the patient is homozygous. To know allele frequency, the transversion in another 15 Japanese patients was examined. The transversion was found in one allele out of 30 alleles. Allele frequency is approximately 9.4%.

Discussion

Major finding in this study is a new mutation in a Japanese patient with Wilson disease, which is exon 5 skipping in an mRNA level and T to G transversion at 5 bp upstream from the junction of intron 4 and exon 5 of *WD* gene in genomic DNA. The mutation analysis for *WD* gene has been performed mainly in Europe and North America and demonstrated 25 mutations including 3 kinds of splice site mutation [3-5]. These split site mutation has been found a nucleotide in either 5' or 3' terminal end of introns, which is consistent with consensus sequence defined by the GT-AG rule[12]. The Exon 5 skipping mutation, 1 of 3 splice site mutations, was speculated to be a result in G to C transversion at the 3' end of intron 4[5]. A lot of studies for mechanism of splicing have been reported[9-14] and concluded that 2 nucleotide sequence of both ends of an intron is most important[12]. However, a case of a point mutation detected at 5th nucleotide in an intron was demonstrated a G to T change of 5 bp downstream from 3' end of skipped exon of Type II collagen gene[13]. The sequence of 3' end of intron 4 of *WD* gene[5] (Fig. 3a) is consistent with 5 nucleotide consensus sequence, PyNCAG[12]. In the mutated *WD* gene, a pyrimidine nucleotide (T) was converted to G (Fig. 3b). The nature of this T to G change may be altered the pyrimidine-rich nature of the splicing acceptor site. Thus, we conclude that the exon skipping is due to the T to G mutation. This conclusion is also supported by evidences that no such transversion was detected in 50 normal alleles and one allele with same transversion was found in other 30 Wilson disease alleles.

The onset age of the patient HF was significantly higher than the average for a neurologic type of Wilson disease. Correlation between genotype and phenotype has been investigated and discussed,

though details remain unclear[5]. The reported case who had exon 5 skipping with a homozygous mutation was early onset hepatic type of Wilson disease[5]. Although this and our cases had different mutation in genomic DNA, same affected protein must be synthesized because of identical mRNA structure. For explanation of the different phenotypes, there is tow possibilities : 1) This kind of mutation does not tightly correlate with phenotype of the disease; 2) Since analysis of liver mRNA was performed only our case, the G to C mutation may lead to frame shift and termination, instead of the complete skipping of exon 5 as described[5]. The truncated protein makes severe impairment of *WD* gene and leads to early onset, and skipping of exon 5 including 6th copper binding site does not remove function and leads to late onset. The study of correlation between genotype and phenotype is very important to investigate the etiology of onset for each clinical types of Wilson disease.

Totally 3 alleles of the T to G transversion in 32 alleles were found at present. Frequency is approximately 9.4%, though this percentage will change in future when the number of patients increased.

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