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A Novel Mutation of the Ceruloplasmin Gene in a Patient with Heteroallelic Ceruloplasmin Gene Mutation (HypoCPGM)

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Daimon, M., Susa, S., Ohizumi, T., Moriai, S., Kawanami, T., Hirata, A., Yamaguchi, H., Ohnuma, H., Igarashi, M. & Kato, T. *A Novel Mutation of the Ceruloplasmin Gene in a Patient with Heteroallelic Ceruloplasmin Gene Mutation (HypoCPGM).* Tohoku J. Exp. Med., 2000, **191** (3), 119-125 —— We found a novel missense mutation in the ceruloplasmin (Cp) gene in a patient with the heteroallelic Cp gene mutation (HypoCPGM). The patient was a 72-year-old woman who came to our hospital with a 1-year history of postural tremor of the hands. The diagnosis was made based on serum Cp and copper readings which were about half the normal levels, as well as MRI tests of her brain which showed charcteristics for hereditary ceruloplasmin deficiency (HCD), known to be caused by the homoallelic Cp gene mutation. Polymerase chain reaction (PCR)-direct sequencing analysis of the Cp gene of the patient revealed a novel point mutation, A to T, at nucleotide position 82 in Exon 1. This mutation changes the Ile^{28} codon (ATT) to a Phe codon (TTT) (missense mutation). PCR-restriction analysis with restriction enzyme Tsp EI for the mutation revealed that both the patient and her son were heterozygotes for the mutation. ———— ceruloplasmin; HCD; HypoCPGM; gene mutation; missense mutation

Ceruloplasmin (Cp) is a blue multi-copper oxidase which is found in the plasma of vertebrate species. The protein, which contains greater than 95% of copper (Cu) in the plasma, is synthesized mainly in the liver as a single-chain polypeptide and secreted into the plasma as an α_2-glycoprotein (Kingston et al. 1977; Samokyszyn et al. 1989). Most of the protein present in plasma is copper-saturated holoceruloplasmin with oxidase activity (Danks 1995). The homoallelic ceruloplasmin gene mutation is known to be the cause of hereditary Cp

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deficiency (HCD), which is characterized by complete Cp deficiency, diabetes mellitus, neurological deficits and excessive storage of iron in the brain and other viscera (Daimon et al. 1995; Harris et al. 1995, 1996; Yoshida et al. 1995; Okamoto et al. 1996; Yazaki et al. 1998; Miyajima et al. 1999). A patient with the heteroallelic Cp gene mutation (HypoCPGM) has been believed to be an asymptomatic carrier for HCD (Logan et al. 1994; Morita et al. 1995). However, recently we reported two cases of heteroallelic Cp gene mutation (Daimon et al. 1999) who showed, through magnetic resonance imaging (MRI) testing of the brain, characteristics of HCD, although the signs were mild compared to those seen in HCD. Therefore, we believe that a person with HypoCPGM is not a carrier but a patient with a mild form of HCD. Here we report a novel missense mutation of the Cp gene in a Japanese family with HypoCPGM. Polymerase chain reaction (PCR) restriction analysis with restriction enzyme Tsp EI successfully detected this mutation.

Materials and Methods

Patient

A 72-year-old Japanese woman came to our hospital with a 1-year history of postural tremor of the hands. She had no other symptoms or signs. Her serum levels of Cp and Cu were about half normal (0.12 mg/ml and 0.46 mg/ml, respectively). MRI showed low signal intensity in the putamina on T2-weighted echo-planar images, which is characteristic for HCD (Fig. 1). These data indicated that she had HypoCPGM rather than Wilson's disease. Her son also showed a low level of serum Cp (0.11 mg/ml), indicating that he also had HypoCPGM. She and her son gave their informed consent prior to their inclusion in this study.

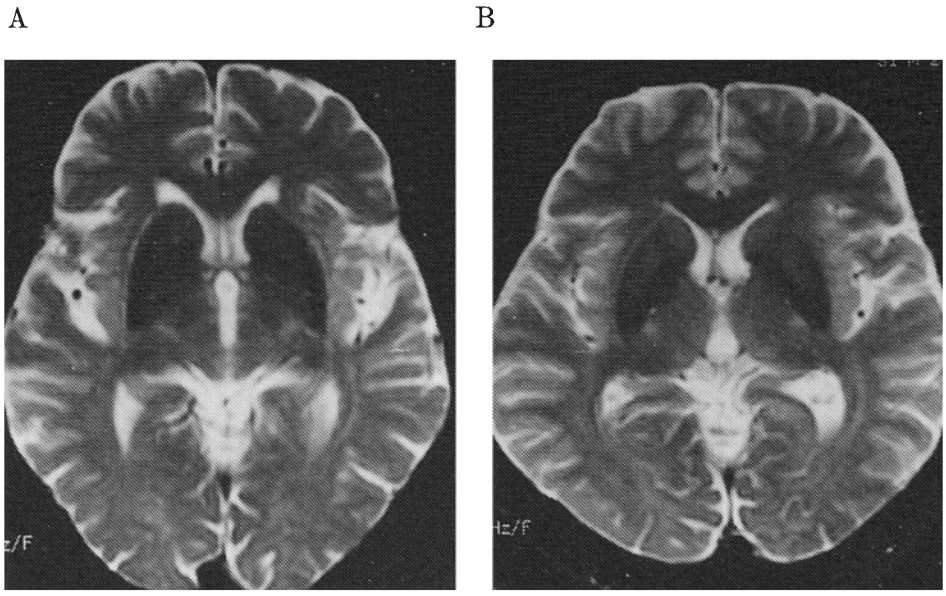

Fig. 1. MRI of the brain of the patient with HypoCPGM (A) and her son (B). T2-weighted echo-planar image shows reduced signal from the putamina.

Molecular analysis

Genomic DNAs of the patient and her son as well as 100 normal control individuals were extracted from peripheral blood leukocytes as described by Sambrook et al. (1989). We made 19 sets of primers ranging from 21 to 26 nucleotides in length to amplify each exon (Exons 1-19). These primers were made to amplify the regions corresponding to all exons and exon-intron splicing sites except Exons 1 and 19. The upstream primer for Exon 1 (Cp-1F) and the downstream primer for Exon 19 (Cp-19R) were made to match the nucleotide sequence of Exon 1 at positions −4 to 19 from the translation initiation codon "ATG" and the nucleotide sequence of Exon 19 at positions 112 to 137 from the translation termination codon "TGA," respectively. All other primers were made to match those of Introns 1 to 18. The sets of primers used are summarized in Table 1. We used the Cycle Sequencing Kit from Pharmacia LKB Biotechnology (Uppsala, Sweden) to perform dideoxy sequencing under thermal cycling conditions using Tth DNA polymerase. The samples obtained were loaded on a 5% LONG RANGER gel (FMC BioProducts, Rockland, ME, USA) containing 7 M urea.

The genomic DNA fragment containing the mutation site was amplified by PCR using primers Cp-1F and Cp-1R. About 100 ng of the PCR amplified genomic DNA fragments were incubated for 4 hours at 65°C with 6 U of Tsp EI restriction enzyme (Toyobo, Osaka) in reaction buffer supplied from the vendor. The restriction enzyme-digested samples were electrophoresed in a 4% agarose gel with Tris-acetate ethylenediaminetetraacetie acid (EDTA) buffer, and the bands were visualized by ethidium bromide staining under UV light.

Results and Discussion

Clinical examination of the patient revealed that she had HypoCPGM. Therefore, it was expected that she would have a Cp gene mutation in one allele. Indeed, direct nucleotide sequencing of the gene in the patient showed double band (A and T) at nucleotide position 82 in Exon 1 (Fig. 2). This result clearly indicated that the patient is heterozygote for normal allele and the mutant allele, which has a single base substitution, A to T. This mutation changes the codon (ATT) for Ile to a codon (TTT) for Phe at amino acid position 28 (missense mutation) (Fig. 2). This mutation may not be responsible for the disease since the Chou-Fasman analysis (Chou and Fasman 1978) for the secondary structure of the protein predicts that the Ile to Phe substitution at amino acid position 28 dose not change the structure (data not shown). Nevertheless, we believe that this is a novel mutation responsible for the disease since we sequenced all regions corresponding to all exons and exon-intron splicing sites and this was the only mutation found in the region. Moreover, PCR restriction analysis of 100 normal control individuals (200 alleles) revealed that none had the mutation (data not

TABLE 1. *Primers used for amplifying the ceruloplasmin (Cp) gene*

Amplified region	Primer	Sequence (5′....3′)	PCR product (bp)
Exon 1	Cp-1F	AGAAATGAAGATTTTGATACTTG	186
	Cp-1R	AATTTTGGTCTATAAACAATAAA	
Exon 2	Cp-2F	AGAAATGATGGCTTCTGATAGAG	354
	Cp-2R	AAGCTAAAAGGCACTTCTACTGA	
Exon 3	Cp-3F	CATCCCCAAGGATCACATAGTAG	388
	Cp-3R	CCTGCCCCTGTCTTTTGGTCATA	
Exon 4	Cp-4F	CATGCTAAAAGTTTAGTCTTGCT	340
	Cp-4R	GCTTTGTTATAAGGACCACAGAC	
Exon 5	Cp-5F	AGGTAACTCTCACTATATCAAGC	362
	Cp-5R	ACCTTTTTCAGCTGACTGCTAAT	
Exon 6	Cp-6F	GTCATCACCCGAGCAGTGTTTAC	315
	Cp-6R	AGTTCCTTTGTGCGGGGGAGAGC	
Exon 7	Cp-7F	GTGAATACCTGCTTTCTGTTCTA	269
	Cp-7R	ATTCTTTCTGAGGTTGATGTAGC	
Exon 8	Cp-8F	GCTCCTAGTAGTTCTTGCCAGAG	319
	Cp-8R	GTGACCAGTACAGTGGGTTATTA	
Exon 9	Cp-9F	GAACTCAGAAGTGGGCTATTC	373
	Cp-9R	CAAATGATCATTTTCAAAGAGAT	
Exon 10	Cp-10F	AGGAACCCTAGTTTACAAAGAGCAAC	443
	Cp-10R	AACCAGTGAGATTACCTTCAGGACAC	
Exon 11	Cp-11F	TAAAGCGTGGGCAAGCCTGAATAGGT	439
	Cp-11R	AAGGGATAAGTTTATCACCCAACACA	
Exon 12	Cp-12F	TCACCTGAGGCCATGATAAGGACTTC	376
	Cp-12R	ACTTCTCTGAAAAGGAAACCTAAAAT	
Exon 13	Cp-13F	TGTTTCAAATGCATTTTTAGATAAGG	206
	Cp-13R	TGACGGTTACTGCAGGTAGCATCACA	
Exon 14	Cp-14F	TGCTGATCTTTGCTTTTATTACTAAG	296
	Cp-14R	CACTTTCTTGGTCCAGACTCTCTATG	
Exon 15	Cp-15F	ACCAGATCCATTAACACTTGATGAAT	335
	Cp-15R	AGCAGCATAGTTGCTAAAGTAGATTG	
Exon 16	Cp-16F	AGCCCTGGGTGCAAAGTCTCAGTTCT	384
	Cp-16R	AAACAAATGAATGGTCTCCAAAATAA	
Exon 17	Cp-17F	GCCTAGAAAAGCAATGTGATA	366
	Cp-17R	AACATTTTCAAACAGAGCAAG	
Exon 18	Cp-18F	CCATTTGTATTAAACACAGAAACGAG	405
	Cp-18R	TTGATGCATCATATTGGGGGAAGTAT	
Exon 19	Cp-19F	AGCAGAAAAATATAAATGAATCAGGA	281
	Cp-19R	GTACAAAGTTGTATGCTTCCAGTCTT	

The first and second primers of each set are presented in the forward and reverse orientations relative to Cp gene transcription, respectively.

shown).

The point mutation that was found abolishes a restriction enzyme Tsp EI recognition site. Restriction analysis of the PCR-amplified genomic DNA frag-

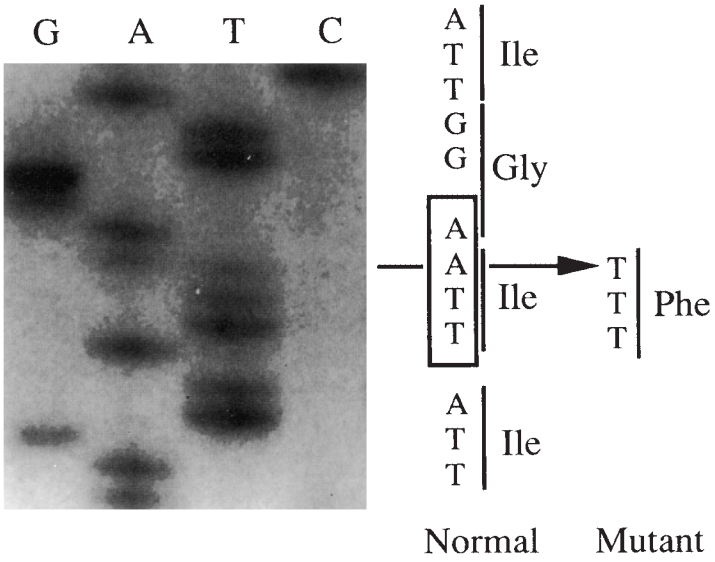

Fig. 2. Identification of point mutation in the patient. Direct sequencing analysis of the amplified genomic DNA fragment around the mutation in Exon 1 is shown. The normal and the mutant nucleotide sequences and the amino acid sequences corresponding to each nucleotide sequence around the mutation site are presented. The single-base substitution and the codon change corresponding to the nucleotide substitution are indicated by the arrow. The Tsp EI recognition site abolished by the mutation is boxed.

ment containing the mutation site enabled us to analyze the mutant and normal alleles in the genomes of the family members. Digestion of a 186-bp PCR-amplified fragment (one from the patient and one from her son) produced an undigested fragment and two smaller size fragments (102 and 84 bp), but digestion of a PCR-amplified fragment from healthy individuals produced two smaller size fragments (Fig. 3). These results indicate that the patient and her son are heterozygotes for the mutation reported here.

The serum levels of ceruloplasmin are very low in such diseases as Wilson's disease and HCD. The defective gene responsible for Wilson's disease has been found not to be the ceruloplasmin gene (Bull et al. 1993; Tanzi et al. 1993). The defective gene for HCD is now known to be the Cp gene itself since seven different mutations of the gene responsible for HCD have been reported (Daimon et al. 1995; Harris et al. 1995, 1996; Yoshida et al. 1995; Okamoto et al. 1996; Yazaki et al. 1998; Miyajima et al. 1999). Two of the mutations are insertions (Harris et al. 1995; Okamoto et al. 1996), two are deletions (Harris et al. 1996; Miyajima et al. 1999) and three are single-base substitutions (Daimon et al. 1995; Yoshida et al. 1995; Yazaki et al. 1998). These single-base substitutions are either nonsense (Daimon et al. 1995) or splicing mutations (Yoshida et al. 1995; Yazaki et al. 1998). These mutations were found in Exons 3, 6, 7, 13 14 15 and 17. Serum level of Cp of a patient with HCD and HypoCPGM is null and about half normal, respectively. Therefore, gene mutations responsible for the diseases seem not to produce mature Cp protein in the sera, and indeed the mutations reported so far

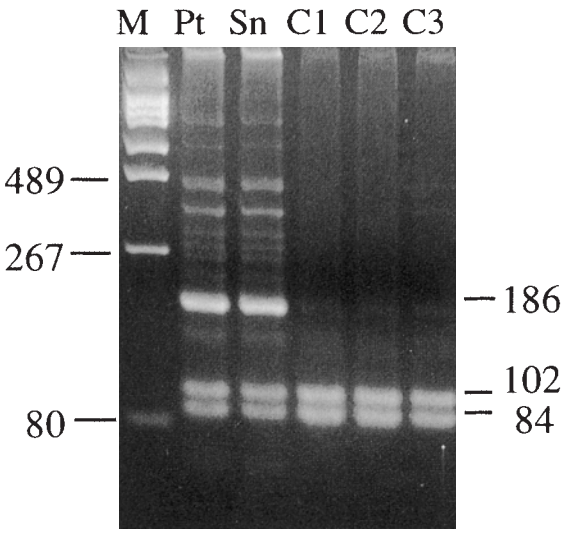

Fig. 3. Restriction analysis of the PCR-amplified genomic DNA fragments. Agarose gel electrophoresis of the PCR-amplified fragments treated with Tsp EI is shown. Samples from the patient (Pt), her son (Sn) and control normal individuals (C1-3) are shown in the lane indicated on the top of the panel. In Lane M, the DNA molecular weight standard pHY size marker (Toyobo) is shown. The numbers on the left and right indicate the size of each fragment and the DNA size markers, respectively.

produced substantial changes of the structure of the protein as mentioned above. The novel mutation of the Cp gene reported here was a point mutation, A to T, in Exon 1, resulting in an amino acid substitution (missense mutation). This gene mutation seems to be responsible for HypoCPGM, or decreased serum level of Cp, although the structural change of the protein produced by the mutation is not expected to be substantial. Therefore, this mutation may cause an unstability of the mRNA, a decreased translation rate from the mRNA and/or an unstability of the protein. This is the first missense mutation reported for the gene and in the Exon 1.

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