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A NONSENSE MUTATION OF THE CERULOPLASMIN GENE IN HEREDITARY CERULOPLASMIN DEFICIENCY WITH DIABETES MELLITUS

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SUMMARY: A novel mutation of the ceruloplasmin (Cp) gene was found in a patient with hereditary ceruloplasmin deficiency (HCD) with diabetes mellitus (DM). The patient had been treated for DM for about 13 years, and then his illness was diagnosed as HCD. One year later, he was found dead in his home. A decrease in insulin-immunostained cells was observed in the islets of the patient's pancreas tissue, which accounted for his DM. The polymerase chain reaction (PCR)-direct sequencing analysis of the Cp gene of his daughter revealed a novel point mutation, G to A, at nucleotide 2630 in exon 15. This mutation changes the Trp^{858} codon (TGG) to a stop codon (TAG) (nonsense mutation). PCR-restriction analysis for the mutation revealed that the patient as well as his daughter was a heterozygote for the mutation, indicating that the patient was a compound heterozygote.

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 (1,2). Although the precise biological roles of Cp are unknown, it may be related to angiogenesis (3), Cu transport (4), iron metabolism (5) and antioxidant defense (6). As an acute-phase protein, the serum level of Cp increases during infection and tissue injury (7). On the other hand, a decrease of the protein in the plasma is observed in some diseases such as Wilson disease (8), hereditary hypoceruloplasminemia (9) and hereditary ceruloplasmin deficiency (HCD) (10).

HCD is an autosomal recessive disease characterized by neurological abnormalities such as progressive cerebral degeneration, complete Cp deficiency and excessive storage of iron in the

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systemic organs such as liver and brain (11,12). In many cases, non-insulin dependent diabetes mellitus (DM) was the first symptom of the disease, and 10 - 20 years later at age 40 - 60 the neurological abnormalities occurred. The defective gene responsible for the disease was suggested to be the Cp gene itself by the genetic linkage analysis of the family with HCD (11). Very recently, two mutations of the Cp gene in two Japanese families with HCD have been reported, indicating that the Cp gene itself is the defective gene (13,14). In both cases reported, the mutation found was observed as homozygote.

Here, we report a novel nonsense mutation of the Cp gene in a Japanese family with HCD. Our patient appeared to be a compound heterozygote. A decrease of insulin-immunostained cells in the patient's pancreas, which accounts for his DM, is also reported.

MATERIALS AND METHODS

Patient: A 59 years old (at the time of death) Japanese man with hereditary ceruloplasmin deficiency, and his wife and a daughter were examined for the mutation of the Cp gene. All other people in his family refused to let us examine their genetical condition. His parents were first cousins. He was in good health until DM was diagnosed at age 45. Two years later from the diagnosis, oral hypoglycemic agent (sulphonylurea) therapy was started. At age 50, he was referred to our university hospital to have better glycemic control. His height was 151.5 cm, and his weight was 48.5 kg. His insulin levels in the fasting state and 2 hr after 75 gr oral glucose loading were 5.7 and 8.7 μU/ml, respectively and fasting blood glucose was 10.9 mmol/l. So, his illness was diagnosed as non-obese and non-insulin-dependent diabetes mellitus with relatively low insulin response to glucose loading. Since then, he had been treated as the patient with DM, and about 3 years later insulin therapy was started. At age 58, he was again referred to us because of depressive mood and forgetfulness. This time, his illness was finally diagnosed as HCD based on the results of the neurological, laboratory and radiological examinations such as measuring IQs (the verbal IQ, 80), and serum Cp and Cu concentration (under the detection level and 7μg/dl, respectively), and head and abdominal CT scan and MRI examination (15). He was found dead in his home at age 59, and an autopsy was performed 20 hrs postmortem. His wife and daughter showed normal and half normal serum Cp and Cu concentrations, respectively. So, his daughter is considered as heterozygote for the gene mutation.

DNA extraction: Genomic DNAs of the wife and a daughter of the patient were extracted from peripheral blood leukocytes as described by Sambrook et al. (16). Genomic DNA of the patient was extracted form paraffin embedded pancreas tissue as reported previously (17).

Histochemistry: Formalin-fixed, paraffin-embedded sections of the pancreas from the present case and an age-matched, non-diabetic patient with a neurodegenerative disease were examined with H & E stain, Perl's stain for Iron and Rodanine stain for copper. In addition, some sections were immunostained with a guinea pig antiserum (1:8000 at dilution) against insulin (18). Labeling was visualized by the avidin-biotin-peroxidase complex (ABC) method (19).

Primers for PCR and sequencing: We have made over 70 PCR primers ranging from 24 to 26 nucleotides in length to analyze the structure of the human ceruloplasmin gene (20). These primer were made to amplify overlapping segments to span the genomic region corresponding to the published human ceruloplasmin cDNA. The 5' and 3' most positions of the published nucleotide sequence of the cDNA were nucleotides 1 and 3456, respectively (20-23). Nucleotide sequences and a part of the sets of the primers, and the PCR products were also reported before (20). We used these primers in this study to find the mutation of the gene.

We describe here only the primers, which were used to detect the mutation found in exon 15 of the gene. Forward primer " M1 (5'-ACCAGATCCATTAACACTTGATGAAT-3') " in intron 14 and reverse primer "8 (5'-AATGATTTTGAACATAGTCCCCTTTT-3')" in intron 16 were used to amplify the genomic DNA fragment. This DNA fragment was used as the template for nucleotide sequencing using primer "M1" as the sequencing primer.

Nucleotide sequencing of the PCR amplified DNA fragments: The genomic DNA fragments of the Cp gene used in this study were amplified as reported previously (20). Excess primers and nucleotides used for the PCR amplification were removed from the PCR preparation by an ultrafree-C3 regenerated cellulose membrane unit (Millipore). The preparation obtained were used as templates for direct DNA sequencing. Three picomoles of the [^{32}P]-End-labeled primer was annealed to 200-500 ng of the corresponding template DNA fragment. We used Cycle Sequencing Kit from Pharmacia LKB Biotechnology to perform dideoxy sequencing under thermal cycling conditions using Tth DNA polymerase. The procedure used in this reaction is that recommended by the supplier. The samples obtained were loaded on a 5 % LONG RANGER gel (AT Biochem) containing 7 M urea.

Restriction analysis of PCR amplified fragment: The genomic DNA fragment containing the mutation site were amplified by PCR using the primers "M3 (5'-CACTTACGTA TGGAAAATCCCAGAAA-3')" and "M2 (5'-GAATTGGACCACAGGAAAACACTAAC-3') (shown in Fig. 3). About 100-300 ng of the PCR amplified genomic DNA fragments were incubated for 2-4 hr at 37 ° C with 10 μU of Nco I restriction enzyme (Takara Shuzo) in reaction buffer supplied from the vendor. The restriction enzyme-digested samples were electrophoresed in a 8 % polyacrylamide gel with Tris-borate EDTA (TBE) buffer, and the bands were visualized by ethidium bromide staining under UV light. Genomic DNAs from the wife and the daughter of the patient were used in this study.

RESULTS

Clinicopathological examinations of our patient (15, unpublished data) were compatible with those reported recently (11,12), and so confirmed the diagnosis of his illness as HCD. The excessive intracytoplasmic deposition of iron, but not of Cu, was observed in his pancreas (data not shown). The size and number of the islets in his pancreas did not appear to be different from those of normal control, but within the islets insulin-immunostained or - containing cells were markedly decreased (Fig.1). So, his DM was accounted to be the consequence of the decrease.

The patient was already dead and paraffin-embedded tissues were the only materials available for us, when we started this project. The quality of the genomic DNA extracted from the tissues was not good enough for sequence analysis. So, genomic DNA from a daughter of the patient were analyzed for the mutation of the Cp gene, since she should be heterozygote for the gene mutation responsible for the disease (the patient should be a homozygote or compound heterozygote). The nucleotide sequences of the PCR amplified genomic DNA fragments of the Cp gene were compared with the previously reported nucleotide sequence of the gene (20-23). A point mutation, G to A, was identified at nucleotide position 2630 in exon 15 (Fig. 2). This mutation changes the Trp^{858} codon (TGG) to a stop codon (TAG) (nonsense mutation), and so should result in the synthesis of a truncated Cp protein (189 amino acids short).

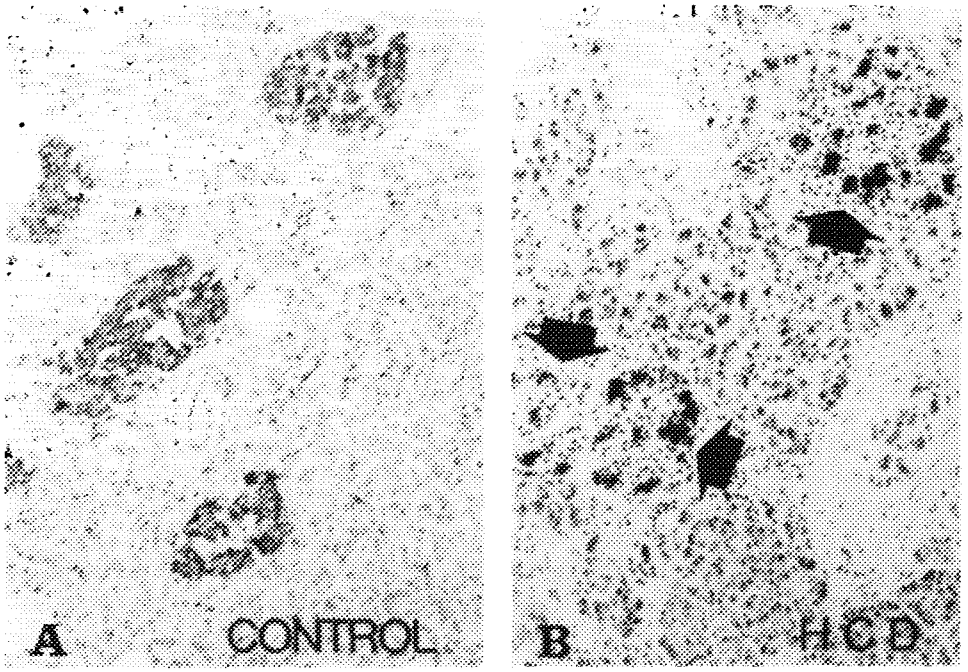

Figure 1. *Immunostaining of pancreas tissue with an antiserum against insulin.* The number of immunoreactive cells was markedly decreased in the patient's islets (**B**) compared to those of normal control (**A**). The arrows point to the islets (**B**). (Magnification: x146)

The point mutation found abolishes the restriction enzyme, Nco I, recognition site (Fig. 3). So, restriction analysis of the PCR amplified genomic DNA fragment containing the mutation site enable us to analyze the mutant and normal alleles in the genome of the family members.

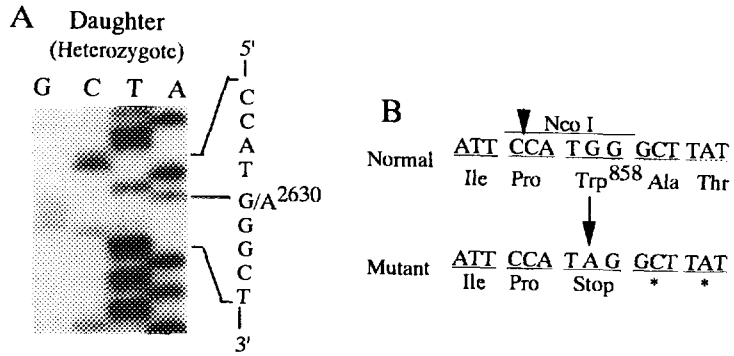

Figure 2. *Identification of point mutation in the patient's daughter.* **A:** Direct sequencing analysis of amplified genomic DNA fragment around the mutation in exon 15 are shown. **B:** The normal and the mutant nucleotide sequences, and the amino acid sequences corresponding to each nucleotide sequence around the the mutation site are presented. The single base substitution and the codon change corresponding to the nucleotide substitution are indicated by the *arrow.* The *Nco I* recognition site abolished by the mutation is over lined, and the cleavage site of the enzyme is indicated by the *arrowhead.*

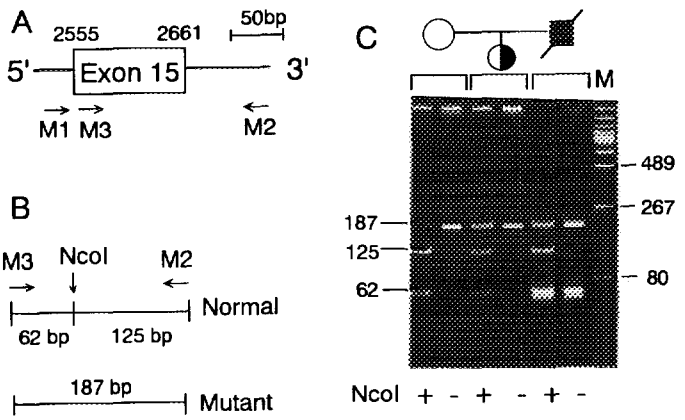

Figure 3. *Restriction analysis of the PCR amplified genomic DNA fragments.* **A:** Positions of primers used for PCR amplification and direct sequencing to find out the mutation found were shown in relation to the Cp gene. The *box* indicates exon 15 and the solid lines indicate introns 14 and 15. The numbers written above the exon indicate the 5' and 3' most nucleotide positions of the exon. The nucleotide sequences of these primers were written in "Materials and Methods". **B:** Restriction map of the fragments amplified by PCR with primers M3 and M2. The Nco I restriction enzyme recognition site is indicated by a vertical *arrow*. The numbers with "bp" indicate the size of the expected fragments. **C:** Acrylamide gel electrophoresis of the PCR amplified fragments treated (+) or not treated (-) with Nco I. The individuals in the patient's family examined are indicated on the top of the panel. Solid square and open circle indicate the patient and his wife, respectively. In the lane indicated by "M" , DNA molecular weight standard pHY size marker (Toyobo) was loaded. The numbers on the left and the right indicate the sizes of each fragment and DNA size markers, respectively.

The 187-bp PCR amplified fragment from his wife was digested completely into two smaller size fragments (125 and 62 bp), but digestion of the PCR amplified fragment from the patient and his daughter produced undigested fragment as well as two smaller size fragments (Fig. 3). These results indicated that the patient and his daughter were heterozygotes for the mutation reported here. Therefore, the patient was considered to be a compound hetrozygote, since a patient with an autosomal recessive disease should have gene mutations in both alleles. Another mutation in the patient could not be found, since the quality of the genomic DNA extracted from paraffin-embedded tissue of the patient was not good enough for sequencing analysis and genomic DNAs of other members of his family were not available at all. The invariant 60 - 70 bp PCR amplified fragments, observed only in the sample of the patient, were considered as artifacts (Fig. 3). The bad quality of the genomic DNA of the patient may account for the amplification of the extra fragments.

DISCUSSION

The serum levels of ceruloplasmin are very low in such diseases as Wilson disease, hereditary hypoceruloplasminemia and HCD. The ceruloplasmin gene was therefore thought to be defective in these diseases. The defective gene responsible for Wilson disease was concluded not to be the ceruloplasmin gene (24,25). But, very recently, the defective gene for

HCD was revealed as the Cp gene, since two mutations of the gene responsible for the disease were reported in two different families (13,14). One mutation reported was a point mutation, G to A, at the splice acceptor site of the intron 17, resulting in a premature stop codon at amino acid position 991 by defective splicing (13). The other mutation reported was a 5-bp insertion at amino acid 410, resulting in a frame-shift and so a truncated open reading frame (14). Their patient were homozygote for each corresponding mutation. We found a novel mutation in another family with the disease. The mutation found was a point mutation, G to A, in exon 15, resulting in a premature stop codon at amino acid position 858 (nonsense mutation). Furthermore, our patient was a heterozygote for the mutation reported here and so considered as a compound heterozygote. These results indicates that mutations of the Cp gene may not be quite few and the number of the defective Cp gene carrier (heterozygote) may not be so little, although only few cases of the disease (homozygote) have been reported by now. Our patient should carry another mutation as he was considered as a compound heterozygote. But, this mutation was not able to be determined as described in "Result".

In many patients with HCD, the diagnosis was made when the neurological abnormalities were manifest at age 40 - 60. However, all but one family (10) had a history of DM, which occurred 10 - 20 years prior to the occurrence of the neurological symptoms. So, DM can be considered as a symptom of the disease, although no direct evidence to prove a relation between DM and HCD has been reported yet. Cp catalyses the oxidation of Fe(II) to Fe(III), and is believed to play an important role to maintain a normal supply of iron from tissu to transferrin, an iron-transport protein (26). So, iron deposits in systemic organs observed in the disease is considered as the consequence of the lack of the Cp protein. Iron deposition in the pancreas was observed in our patient and also have been reported previously in a case (12). In our patient, in addition, a decrease of insulin-immunostained cells was disclosed in the pancreas, accounting at least in part for his DM. Iron deposition in the pancreas may be blamed for the decrease of the cells, but other functions such as antioxidant protection could also contribute to the etiology of the decrease of insulin-immunostained cells in the pancreas. So, emphasis of DM as the first symptom of the disease may be important for future research to find out more cases, and to reveal the mechanisms involved in the decrease of the insulin-imunostained cells.

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