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Characterization of a nonsense mutation in the ceruloplasmin gene resulting in diabetes and neurodegenerative disease

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We report here on the characterization of a mutation in the ceruloplasmin gene in a 45 year old woman with insulin-dependent diabetes mellitus who presented with the recent onset of gait disturbance and dysarthria. Physical examination revealed an ataxic gait, scanning speech and retinal degeneration. Magnetic resonance imaging of the brain was consistent with increased basal ganglia iron content and laboratory studies revealed a low serum iron concentration and no detectable serum ceruloplasmin. Nucleotide sequence analysis of the ceruloplasmin gene from this patient revealed a G to A substitution in exon 15 resulting in a nonsense mutation at amino acid 858 (Trp858ter). The patient's younger, neurologically asymptomatic brother was also found to be homozygous for this mutation. Taken together the clinical and genetic data support the concept of an essential and unique role for ceruloplasmin in human iron metabolism. Identification of this kindred extends the spectrum of ceruloplasmin gene mutations resulting in this autosomal recessive, late-onset neurodegenerative disease and highlights the importance of recognizing aceruloplasminemia as a genetic cause of diabetes and neurologic disease.

INTRODUCTION

Ceruloplasmin is an abundant 72-serum glycoprotein which contains >95% of the copper present in human plasma (1). The region of ceruloplasmin gene located in chromosome 3q which spans ~36 kb and is composed of 19 exons. This protein functions as a multi-copper oxidase and is synthesized in hepatocytes with six atoms of copper incorporated prior to secretion (2). Although copper does not affect the synthesis or secretion of the apoprotein, if copper is unavailable during hepatic biosynthesis an unstable protein lacking oxidase activity is secreted (3). In Wilson disease a failure to deliver copper into the hepatocyte secretory pathway impairs both biliary copper excretion and copper availability to newly synthesized ceruloplasmin, resulting secondarily in a decreased serum ceruloplasmin concentration as a result of rapid turnover of secreted apoprotein (4). Consistent with this model, the gene encoding Wilson disease has recently been cloned and shown to be a cation transporting P-type ATPase essential for copper trafficking in hepatocytes (5–7).

The precise biological role of ceruloplasmin has remained unclear despite many decades of investigation. Most recently, molecular genetic analysis of two separate patients with late-onset neurodegenerative disease identified distinct mutations in the ceruloplasmin gene accounting for the clinical symptoms in these individuals (8,9). These studies defined aceruloplasminemia as a novel human genetic disease and revealed an essential and unique role for ceruloplasmin in iron metabolism. We now report on a new ceruloplasmin gene mutation in a kindred with aceruloplasminemia and expand on the clinical spectrum and implications on this disease.

RESULTS

The proband is a 45 year old woman who came to attention after a several month history of difficulty in walking and slurring of her speech. She had previously been in excellent health with the exception of insulin-dependent diabetes mellitus since the age of 31 years. Physical examination revealed a pleasant, apprehensive woman with a normal general physical examination including no evidence of hepatomegaly and a normal dermatologic exam. Neurological examination showed a mildly ataxic gait and dysarthria consisting predominantly of persistent scanning speech without the presence of resting tremors. Ophthalmologic examination was significant for retinal degeneration and the absence of Kayser-Fleischer rings. T2-weighted magnetic resonance imaging revealed decreased signal intensity localized to the basal ganglia, thalamus and dentate nucleus. Biochemical analysis of the patient's

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serum was remarkable for a serum iron concentration of 18 µg/dl (normal 54–200 µg/dl), a serum ferritin concentration of 519 ng/dl (normal 5–100 ng/dl) and a total absence of serum ceruloplasmin detected by oxidase or immunochemical methods. The hemoglobin concentration, hematocrit and liver function tests were all normal. The family history was notable for consanguinity through four generations. The patient's only sibling is a younger brother who also has long-standing diabetes mellitus but is without neurologic symptoms. Consistent with this, he had a normal neurologic exam but was noted to have early retinal degeneration.

Western blot analysis of the patient's serum revealed a complete absence of ceruloplasmin (data not shown) consistent with the clinical measurements noted above. Southern blot analysis of the ceruloplasmin gene using cDNA probes encompassing the entire ceruloplasmin-coding region failed to distinguish any size differences between the patient's DNA and normal control samples (data not shown). To directly determine the size of each exon of the ceruloplasmin gene, the amplified products were analyzed by agarose gel electrophoresis. In each case the exons amplified from the patient's DNA were of the appropriate size and no differences were seen when directly compared to the identical exon amplified from genomic DNA of normal controls (data not shown).

We next sought to determine the nucleotide sequence of the ceruloplasmin gene in an effort to directly identify a mutation which would not have been detected by the previous methods. When the nucleotide sequence of each amplified exon was examined, a single nucleotide difference was noted between the patient's DNA and the sequence obtained from amplified genomic DNA of normal controls as well as the previously published sequence of human ceruloplasmin. Specifically, a G2630 to A substitution was observed at amino acid 858 in the amplified fragment encompassing exon 15 (Fig. 1). The identical sequence was obtained in multiple clones isolated from the patient's subcloned exon 15 fragment and was never detected in sequence analysis of clones containing exon 15 amplified from genomic DNA isolated from normal individuals (data not shown). The consequences of this point mutation would be to introduce a stop codon at amino acid 858 (Trp858ter) resulting in a nonsense truncation of the open reading frame as depicted (Fig. 1).

The substitution noted in exon 15 of the patient's DNA should result in the loss of an *Nco*I restriction enzyme recognition site (C:CAT**G**G–C:CAT**A**G). To assess the validity of this mutation, oligonucleotide primers were utilized to amplify the 107 bp fragment encoding exon 15 in genomic DNA from the patient's mother and brother and these fragments were subjected to *Nco*I digestion followed by agarose gel electrophoresis. As can be seen in Figure 2 *Nco*I digestion of exon 15 amplified from a normal individual (lane N) results in the appearance of two bands of 72 and 35 bp. In contrast, as anticipated from the nucleotide sequence data, DNA from the patient (lane 2) was not digested with *Nco*I. Furthermore, *Nco*I digestion of amplified exon 15 fragment from the patient's brother also failed to digest with *Nco*I suggesting that he was also homozygous for this mutation (lane 3). As would be expected with an autosomal recessive pattern of inheritance, *Nco*I digestion of the 107 bp fragment from mother's DNA revealed a partial digest with the appearance of both the 107 bp and the 72 and 35 bp fragments (lane 4). Direct sequence analysis of exon 15 confirmed these findings in the patient's mother and brother and further sequencing did not reveal additional abnormalities elsewhere in the ceruloplasmin gene (data not shown). Although previous work has revealed the presence of a ceruloplasmin pseudogene sequence in the human genome which includes exon 15 (10), sequence analysis of the exon 15 sequences examined have confirmed amplification for the authentic ceruloplasmin gene in all cases.

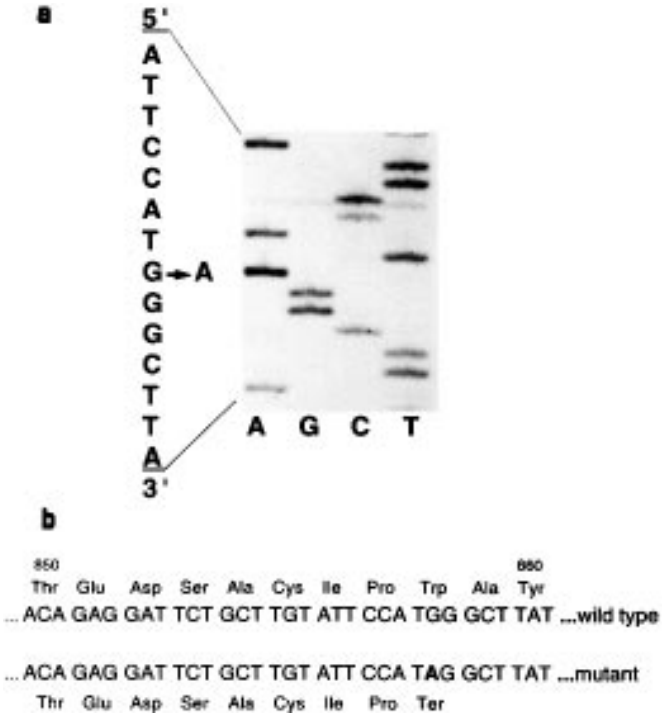

Figure 1. Nucleotide sequence analysis of ceruloplasmin exon 15. (**a**) Sequence ladder of a portion of the amplified exon 15 from the patient. The normal sequence is displayed on the left and the site of the G to A transition is indicated. (**b**) Deduced amino acid sequence of wild-type ceruloplasmin and the predicted result of the aa858 nonsense mutation. Deduced protein size is 857 amino acid in comparison to 1046 amino acids of wild-type.

DISCUSSION

This current study identifies a new kindred with aceruloplasminemia resulting from a nonsense mutation at amino acid 858. The family history of consanguinity, the lack of detection of a normal exon 15 allele sequence in multiple clones and the failure to detect other nucleotide sequence differences all suggest homozygosity for this mutation. Thus far each of the reported mutations in aceruloplasminemia are predicted to result in a truncated open-reading frame (Table 1). Previous studies on ceruloplasmin indicate that copper incorporation results in a conformational change to the active oxidase involving a trinuclear copper cluster encoded by multi-copper oxidase domains in the carboxyl-terminal 50 amino acids (11). Thus ceruloplasmin synthesized without this region would not incorporate copper during biosynthesis, resulting in an apoprotein devoid of oxidase activity and subject to rapid degradation. Consistent with this concept no detectable ceruloplasmin has been found in the sera of any of the patients identified thus far. As noted previously, symptoms of aceruloplasminemia are not found in patients with Wilson disease because extrahepatic production of holoceruloplasmin is sufficient to provide the 5% of normal serum concentration necessary to sustain normal plasma iron turnover rates (8,16).

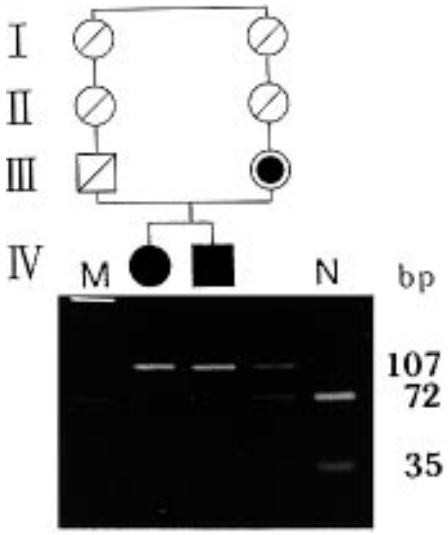

Figure 2. Family pedigree and corresponding *Nco*I restriction enzyme digest of amplified genomic DNA. The amplified exon 15 DNA (107 bp) from the patient (lane 1), her brother (lane 2) and mother (lane 3) as well as that of a control subject (N) were digested with *Nco*I and electrophoresis in 4% agarose. The size and position of the digest products (72 and 35 bp) is indicated. M, molecular size markers are *Hae*III digested ϕx174 DNA.

Table 1. Mutations identified in the ceruloplasmin gene

Mutation	Exon	Predicted effect
Insertion nt 1285 insert TACAC	7	Frameshift
3′ Splice acceptor site nt 3019–1 G–A	Intron 17	Activates cryptic splice acceptor–frameshift
Nonsense aaTrp858ter	15	Truncation[a]

First two mutations reported in refs 8 and 9 respectively.
[a]This report.

The clinical findings in this case illustrate the triad of neurologic disease, diabetes mellitus and retinal degeneration associated with aceruloplasminemia (12,13). Although the onset of diabetes in this kindred is earlier than that reported previously, such heterogeneity is presumably not a primary genetic effect since all patients described thus far have a total absence of detectable ceruloplasmin ferroxidase activity. Clinical heterogeneity is observed in other iron storage disorders and most likely reflects a combination of environmental and genetic influences on both iron metabolism and the host response to iron accumulation (14). The early onset of diabetes in this family strongly suggests that aceruloplasminemia be considered in the differential diagnosis of diabetes in young adults.

Aceruloplasminemia results in iron accumulation in both the reticuloendothelial system and parenchymal tissues (15). These observations are consistent with the known role of ceruloplasmin as a plasma ferroxidase and with the presumed mechanisms of iron accumulation in other iron storage diseases (16,17). The mechanisms of tissue injury in aceruloplasminemia are unknown, but presumably relate directly to the excess iron accumulation. For example, 3-cells in pancreatic islets are susceptible to the cytotoxic effects of oxidative stress from a variety of agents and desferrioxamine, an iron-chelating agent, has been shown to ameliorate such injury (18). These data are also consistent with studies indicating that the antioxidant role of ceruloplasmin is related to ferrous iron oxidation (19,20). The pancreatic islets do accumulate excess iron in aceruloplasminemia and the resulting diabetes is thus analogous to that observed in primary hemochromatosis (15).

The neurodegenerative disease observed in aceruloplasminemia is unique amongst iron storage diseases (21,22). The neurological symptoms are those seen in extrapyramidal movement disorders and as such reflect the sites of iron deposition observed on MRI as well as the specific sites of neurodegeneration seen at autopsy (15). The implication of these findings is that ceruloplasmin plays an essential role in brain iron metabolism and the findings of retinal degeneration would thus include this neural tissue in such a process. In each case this is likely to occur locally since ceruloplasmin does not cross the blood-brain barrier and as such this may have implications for any proposed therapeutic intervention. The lack of neurologic symptoms in our patient's brother underscores the importance of early diagnosis since observations of younger family members in other kindred reveals that neurodegenerative disease does inevitably develop in all affected individuals. Although the mechanisms of this late-onset neurodegeneration are not known, such findings may have broad implications for our understanding of the role of transition metals and free radical tissue balance in the generation of brain injury in a variety of important human neurodegenerative diseases (23).

MATERIALS AND METHODS

Freshly isolated serum samples were subjected to electrophoresis in 5–15% SDS-polyacrylamide gels under reducing conditions, transferred to nitrocellulose membranes and immunoblotted for the detection of ceruloplasmin using avidin-biotinylated horseradish peroxidase as previously described (2). The primary antibody was a rabbit polyclonal antisera prepared against ceruloplasmin (Dako Corporation, Carpinteria, CA).

For the isolation of genomic DNA, freshly isolated peripheral blood samples were centrifuged on Hypaque solution (Sigma Chemicals, St Louis, MO) at 400 *g* for 10 min. Isolated leukocytes were lysed in hypotonic saline following buffy coat separation and DNA was then precipitated in ethanol and purified as described (8). Oligonucleotide primers were synthesized according to previously published sequences flanking each of the ceruloplasmin gene exons and used for polymerase chain amplification of isolated DNA. Amplification reactions were performed using 100 pmol of each primer and 100 ng of DNA for 30 cycles at the annealing and extension temperatures previously indicated for each primer pair (8). In all cases the reactions were completed with a final extension cycles at 72°C for 10 min. Following amplification, an aliquot of each sample was analyzed by electrophoresis in 3% agarose gels followed by ethidium bromide staining and visualization under shortwave ultraviolet illumination.

In each case the amplified genomic DNA fragments of appropriate size were isolated following electrophoresis into DEAE membranes and subcloned into the pCRII plasmid (Invitrogen, San Diego, CA). Individual colonies were isolated and the amplified genomic DNA corresponding to each of the

human ceruloplasmin gene exons was sequenced by dideoxynucleotide chain termination using Sequenase (United State Biochemicals, Cleveland, OH). For Southern blot analysis, genomic DNA was digested with a series of restriction enzymes, electrophoresed in agarose, transferred to nitrocellulose and subsequently hybridized with a full-length ceruloplasmin cDNA probe as described (8). *Nco*I restriction site analysis was performed on amplified DNA fragments which were then analyzed following electrophoresis in 4% agarose gels.

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