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Phenotypic heterogeneity in seven Italian cases of aceruloplasminemia

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ABSTRACT

Introduction: Aceruloplasminemia is an ultra-rare hereditary disorder characterized by iron-restricted microcytic anemia and tissue iron overload associated with diabetes, retinal and progressive neurological degeneration. We describe genotypes and phenotypes at diagnosis, and disease evolution of seven Italian patients.

Methods: Anagraphical, biochemical, genetic, clinical and instrumental data were collected at diagnosis and during a long-term follow-up. Mutations, ferroxidase activity and Western Blot analysis of ceruloplasmin were performed according to standard protocols.

Results: Three mutations were already described (p.Phe217Ser, deletions of exon 11 and 12), p.Ile991Thr is a very rare variant, p.Cys338Ser and IVS6+1G > A were novel mutations. *In silico* analyses suggested they were highly likely or likely to be damaging. At diagnosis, 100% had microcytosis, 86% had mild-moderate anemia, low serum iron and high serum ferritin. Four (57%) had type 1 diabetes or glucose intolerance, 3/7 had neurological manifestations, and only one had early diabetic retinopathy. All but one underwent iron chelation therapy requiring temporary discontinuation because of anemia worsening. At the end of follow-up, three patients aggravated and 2 developed neurological symptoms; only two patients were free of neurological manifestations and showed mild or absent brain iron.

Conclusion: Aceruloplasminemia phenotypes ranged from classical characterized by progressive neurologic derangement to milder in which signs of systemic iron overload prevailed over brain iron accumulation. Within this large heterogeneity, microcytosis with or without anemia, low serum iron and high serum ferritin were the early hallmarks of the disease. Therapeutic approaches other than iron chelation should be explored to reduce morbidity and improve life expectancy.

1. Background

Ceruloplasmin (Cp) is a blue copper-binding (6–7 atoms per molecule) glycoprotein with multi-copper oxidase activity. Cp displays ferroxidase activity, oxidizing Fe^{2+} to Fe^{3+} without releasing reactive oxygen species, thus playing a relevant role in mobilization and oxidation of iron from cells favouring the incorporation of ferric iron into transferrin. The protein is mainly synthesized in hepatocytes and secreted into the plasma as a holo-Cp with 6 atoms of copper incorporated during biosynthesis. The failure to incorporate copper results in the secretion of an unstable apo-Cp that is devoid of oxidase activity and rapidly degraded in the plasma. In the brain, most of the Cp is located on the surface of astrocytes in a glycosylphosphatidylinositol (GPI)-anchored form playing a major role in iron mobilization in the central nervous system. Damaging mutations of the *CP* gene cause reduced if not absent ferroxidase activity leading to aceruloplasminemia (ACP) which occurs

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approximately in 1 in 2 million in non-consanguineous marriages [1–3]. About 100 ACP cases and 70 *CP* mutations, mostly from Japan, have been published to date as reported in HGMD® Professional 2016.2. Pathogenesis of ACP depends on the impairment of iron efflux from cells to serum due to the absence of ferroxidase activity. In patients, the absence of Cp activity leads to a decrease in circulating iron, iron deficient erythropoiesis and iron accumulation in liver, pancreas, retina and brain [3,4]. Classical phenotype is characterized by mild microcytic anemia, low serum iron and transferrin saturation, high serum ferritin, diabetes and retinal degeneration that precede of about ten years the onset of neurological symptoms due to brain iron deposition [3–6]. The neurological symptoms usually appear in the fifth decade of life and include cerebellar ataxia, involuntary movements, parkinsonism, mood and behavior disturbances, and cognitive impairment that usually progress to death [4,5]. A recent report suggested that the spectrum of ACP phenotype could be more heterogeneous than previously believed [6]. In the present paper we describe genetic and clinical characteristics of 7 Italian patients with ACP at diagnosis and during a long-term follow-up with the aim to add further knowledge on this ultra-rare disease.

2. Methods

2.1. Subjects

All the patients were diagnosed at the Centre for disorders of iron metabolism at the ASST-Monza, S.Gerardo Hospital. Proband 1, 4, and 5 have been previously described as single case reports [7–9]. Diagnosis of ACP was based on biochemical, instrumental, clinical and molecular grounds. Blood cell count, serum iron, transferrin and ferritin were measured by standard methods; serum hepcidin was measured by SELDI-TOF at the University of Verona as previously described [10]. Liver iron concentration (LIC) was calculated by magnetic resonance imaging (MRI) performed on a 1.5T whole-body scanner (Achieva 1.5T SE; Philips Medical Systems, Best, the Netherlands) as previously reported [11]. Liver biopsy was performed in one patients with associated metabolic syndrome. Brain iron was evaluated by MR and imaging studies were obtained as previously reported [7]. On admission and every 1–3 years, all patients were examined by neurologists including tests to assess cognitive and motor performances and check for behavioral abnormalities. Ferroxidase activity was measured in 36 healthy subjects (blood donors at their first donation) with normal red blood cell count and iron indices.

2.2. DNA analysis

Genomic DNA was extracted from peripheral blood leukocytes using Wizard® genomic DNA purification kit (Promega, Madison, WI, USA) and amplified by PCR. PCR products were directly sequenced by ABI Prism 3130 DNA-sequencer (Applied Biosystems, Foster City, CA, USA) and compared with GenBank reference sequence (NM_000096.3). Written informed consent for genetic study were obtained from all patients according to the Institution's guidelines.

2.3. In silico studies

Different available online tools were used to analyse mutations at genomic and protein levels (http://www.ebi.ac.uk/Tools/msa/clustalw2; http://genetics.bwh.harvard.edu/pph; http://www.umd.be/HSF; http://www.cbs.dtu.dk/services/NetGene2).

2.4. Ferroxidase activity

According to Erel O [12], 3 μl of serum were incubated (1 min at 20 °C) in 350 μl of buffer solution (0.45 mol/L acetate, pH 5.8), then, 75 μl of substrate solution [thiourea and ammonium iron(II) sulfate hexahydrate] were added and the mixture was incubated at 37 °C for 3 min and 40 s. The reaction was then activated by adding 30 μl of chromogen solution [3-(2-pyridyl)-5,6-bis(2-[5-furylsulfonic acid])-1,2,4-triazine].

2.5. Immunoblotting

Analyses were performed on serum samples in all the patients and on the single sample of liver biopsy available. Once collected, samples were immediately frozen and stored at −80 °C. To remove albumin, Affi-Gel blue resin (BIO-RAD, Hercules, CA, USA) was added to the sample. Proteins separation was conducted on NuPage® Novex 4–12% gel (Invitrogen, Carlsbad, CA, USA) and electro-transferred by I-Blot® Gel Transfer Device (Invitrogen, Carlsbad, CA, USA). The blot was blocked in 5% milk solution (TBS containing 0.1% Tween 20, TBST) and incubated with rabbit anti-human ceruloplasmin (ab48614, AbCam, Cambridge, UK) diluted 1:1000 in 0.1% TBST. Finally the membrane was incubated with horseradish peroxidise-conjugated donkey anti-rabbit Ig (GE Healthcare, Buckinghamshire, UK) diluted 1:10000 in 0.1% TBST. Ceruloplasmin bands were visualized by using enhanced chemiluminescence (GE Healthcare, Amersham Biosciences Europe GmbH, Freiburg, Germany) and X-ray film.

2.6. Statistical analysis

A paired *t*-test was used to compare serum parameters at baseline and during or at the end of follow-up. All tests were two sided and with a significance level of α equal to 0.05. Analyzes were carried out by the GRAPHPAD PRISM statistical analysis software (version 3.02) (GraphPad Software, Inc., La Jolla, CA, USA).

3. Results

3.1. Clinical evaluation at diagnosis

Table 1 shows the age of the first reported manifestation possibly related to ACP. In six patients (85.7%) the first symptoms were microcytosis with or without anemia and alteration of serum iron indices. For patient 4, we considered diabetes as the first manifestation because microcytic anemia, which was known since his childhood, was attributed concomitantly to Thalassemia syndrome. A mean of 15.7 years (SD: +9.8; range: 2–31 years) passed between the first manifestation and time of diagnosis. Anagraphical and biochemical data of the 7 patients collected at time of diagnosis and at study endpoint are reported in Table 2, and

Table 1
First clinical manifestations and age of onset.

Patient	First clinical manifestation(s)	Age (Yrs)
1	Microcytic anemia	22
2	Microcytic anemia and hyperferritinemia	36
3	Microcytosis and hyperferritinemia	52
4	Diabetes type 1 ([a])	20
5	Microcytic anemia	15
6.1	Microcytic anemia, low serum iron and hyperferritinemia	37
6.2	Low serum iron	20

[a] Since childhood he showed mild-moderate microcytic anemia related to β-thal trait [9].

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