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A Novel Ceruloplasmin Splicing Mutation Causes Early-Onset Aceruloplasminemia with Refractory Microcytic Anemia and Iron Overload

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Abstract

Anemia is a widespread global health issue, with iron deficiency being the most common cause. However, rare genetic conditions, such as aceruloplasminemia, also contribute to unexplained cases. Aceruloplasminemia, a rare autosomal recessive disorder characterized by systemic iron accumulation, is typically diagnosed in adulthood at advanced disease stages when treatment efficacy is limited. Here, we report a novel case of an 11-year-old girl-the youngest confirmed pediatric patient to date-presenting with persistent microcytic hypochromic anemia, low serum iron, markedly elevated serum ferritin, and evidence of hepatic and cerebellar iron deposition. After common causes of anemia were excluded, whole-exome sequencing revealed a homozygous splicing mutation in the ceruloplasmin gene (NM_000096.3: c.147-2A > G), which was classified as likely pathogenic according to ACMG/AMP guidelines. Pedigree analysis revealed that the parents of the affected child are carriers of the mutation. Functional validation confirmed that this mutation led to exon 2 skipping, disrupting the ceruloplasmin structure and abolishing its copper-binding sites. Following iron chelation therapy, the patient remained clinically stable over a three-year follow-up period, with no progression of organ iron overload. This case underscores the importance of considering aceruloplasminemia in pediatric patients with refractory microcytic anemia, particularly when accompanied by low serum iron and disproportionately elevated ferritin levels. Early genetic screening and intervention may help prevent irreversible complications associated with this rare disorder.

Keywords: Ceruloplasmin (CP), Homozygous mutation, Anemia, Acinetobacter pneumoniae

Introduction

Aceruloplasminemia is a rare autosomal recessive disorder caused by mutations in the Ceruloplasmin (CP) gene, leading to the absence or defect of ceruloplasmin ferroxidase activity. The resulting impairment of iron export from cells causes systemic iron overload, particularly in the brain, liver, and pancreas, which contributes to a wide spectrum of clinical manifestations, including retinal degeneration, diabetes mellitus, and progressive neurological disorders. Typically, patients first present with anemia-often microcytic and refractory to conventional treatment-long before the onset of diabetes or neurological symptoms, emphasizing the critical importance of early diagnosis and intervention.[1,2] Neurological manifestations, such as movement disorders (including blepharospasm, grimacing, facial and cervical dystonia, tremors, and chorea) and ataxia (gait disturbance, dysarthria),

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closely correlate with iron accumulation in specific brain regions. Moreover, cognitive dysfunction-manifesting as apathy, memory impairment, and reduced executive function-occurs in more than half of affected individuals.[3-5] While most reported cases are diagnosed in adulthood, often when irreversible organ damage has occurred, pediatric cases with early-stage diagnoses remain exceedingly rare.[4]

In this study, we report a unique case of an 11-year-old girl who presented with recurrent microcytic hypochromic anemia, physical stunting, and cognitive impairment. After comprehensive exclusion of common causes of anemia-including iron deficiency anemia, α- and β-thalassemia, hematological malignancies, and peptic ulcers-a homozygous splicing mutation in the CP gene (NM_000096.3: c.147-2A > G) was identified *via* whole-exome sequencing. Pedigree analysis revealed that the parents of the affected child were carriers of the mutation. The pathogenicity of this variant was confirmed through bioinformatics analysis and functional validation *via* a minigene splicing assay. Notably, early diagnosis allows timely initiation of iron chelation therapy, leading to clinical stabilization over a three-year follow-up without progression of organ iron deposition. This case highlights the diagnostic significance of considering aceruloplasminemia in pediatric patients with unexplained refractory microcytic anemia, particularly when accompanied by paradoxically low serum iron and elevated ferritin. Our findings underscore the value of early genetic screening and intervention in altering the disease course and preventing severe complications associated with systemic iron overload.

Materials and Methods

Patient evaluation and diagnostic workup

An 11-year-old girl with a four-year history of persistent microcytic hypochromic anemia (hemoglobin 81–96 g/L) was admitted for evaluation. The patient had no history of surgery, trauma, or infectious diseases. Physical examination revealed pallor of the skin and sclera; normal skin elasticity and temperature; and no signs of rash, hemorrhage, or lymphadenopathy. Laboratory investigations included a complete blood count, serum iron studies, iron metabolism marker analysis, and hemoglobin electrophoresis. To exclude common causes of anemia, including iron deficiency anemia, *α*- and *β*-thalassemia, hematologic malignancies, and peptic ulcer disease, a comprehensive diagnostic panel was performed. *α*- and *β*-Thalassemia gene testing was carried out, along with bone marrow aspiration cytology, to assess hematopoietic activity and iron status. Abdominal Magnetic Resonance Imaging (MRI) and Computed Tomography (CT) were performed to evaluate potential organ iron deposition. Additionally, cranial MRI, ophthalmologic examinations, blood glucose measurements, and plasma ceruloplasmin quantification were conducted. Whole-exome sequencing was performed to identify potential genetic abnormalities associated with the patient's clinical presentation.

Whole exome sequencing

Genomic DNA was extracted from the peripheral blood mononuclear cells of patients *via* a Qiagen DNA Mini Blood Kit (Qiagen, Hilden, Germany) according to the manufacturer's instructions. The Roche KAPA HyperExome probe set captured exons and adjacent splicing regions for sequencing on the MGISEQ-2000 platform. The raw data were processed and aligned to the hg19 reference genome *via* standard bioinformatics tools (SOAPnuke, BWA, and GATK). Variants were screened and annotated *via* public databases (ClinVar, OMIM, HGMD, 1000 Genomes Project, gnomAD, and ExAC) and classified according to ACMG/AMP guidelines.

Sanger sequencing

Mutations were analyzed *via* direct sequencing of all coding exons and exon–intron boundaries of the Ceruloplasmin (CP) gene, including at least 50 base pairs of flanking intronic sequences. PCR primers were designed *via* Primer3 version 0.4.0 (https://bioinfo.ut.ee/primer3-0.4.0/primer3, last accessed July 10, 2025) Supplementary Table 1. The PCR products were purified and subjected to bidirectional Sanger sequencing. The sequencing results were aligned and compared to the reference sequence of CP (GenBank accession number NM_000096.3) and reviewed manually to identify potential pathogenic variants.

Bioinformatics and splicing analysis

Pathogenicity was predicted *via* bioinformatics tools (PolyPhen-2, MutationTaster, SIFT, and CADD). Splicing effects were evaluated *via* an RNA splicing prediction model from the Guangzhou Rare Disease Gene Therapy Alliance. Protein structure changes due to mutation were modeled *via* SWISS-MODEL. Protein structure visualization was performed *via* PyMol (v3.1.0a0 Open-Source, https://github.com/cgohlke/pymol-open-source-wheels/releases).

Minigene analysis

To evaluate the effect of the CP c.147-2A > G splicing variant on pre-mRNA processing, we performed splicing assays *via* a pCAS2-based minigene system containing CP exon 2 with its flanking intronic regions. Wild-type and mutant constructs were generated and transiently transfected into HEK293T cells. Total RNA was extracted after 48 hours, followed by RT-PCR to assess splicing outcomes. Aberrant transcripts were analyzed *via* agarose gel electrophoresis and verified *via* Sanger sequencing. The primer sequences are provided in Table SI.

Results

Clinical findings and diagnostic outcomes

The patient exhibited low serum iron (3.8 μmol/L; reference range: 9.0-32.3 μmol/L), markedly elevated serum ferritin (1585 μg/L; reference range: 12-150 μg/L), and low transferrin

saturation (9.39%; reference range: 20%-50%). She also presented with growth retardation, poor concentration, and low cognitive performance Table SII. Temporary improvement in hemoglobin was noted after iron supplementation, but anemia relapsed repeatedly. α- and β-Thalassemia gene analyses revealed no pathogenic variants. Bone marrow aspiration cytology revealed active granulocytic and erythroid proliferation, with increased numbers of immature and granular megakaryocytes. The intramedullary iron level was elevated, whereas the intramedullary iron level remained within the normal range. Abdominal MRI revealed diffusely prolonged T1 and shortened T2 signals in the liver parenchyma, which was consistent with iron deposition Figure 1. The cranial MR image revealed mild iron deposition in the dentate nucleus of the cerebellum Figure 2. Blood glucose and ophthalmologic assessments were normal. The plasma ceruloplasmin levels significantly decreased (< 20 mg/L; reference range: 210-530 mg/L). Whole-exome sequencing revealed a homozygous splicing mutation in the CP, suggesting that aceruloplasminemia was the underlying cause of the patient's anemia and systemic iron overload. To investigate the molecular and imaging features associated with aceruloplasminemia in the proband, we performed a comprehensive analysis including abdominal imaging, brain Magnetic Resonance Imaging (MRI), and functional validation of the CP gene mutation.

Table SI: Primers used for *in vitro* expression analysis

Primer ID	Sequence(5'to 3')
Primers Used for Pedigree Analysis	
CP-147-2-F	TAAAAACCCAAGCCAGACCGGT
CP-147-2-R	ATGATGCCTTCGCAGATGGTTC
Primers Used for Minigene Plasmid Construction (exon2/intron1-2, exon3/intron2-3)	
CP-147-2-F1	TGGGATTATGCCTCTGACCA
CP-147-2-R1	AAGGTCCGATGAGTCCTGAG
Primers Used for Minigene Assay	
CP-147-2-F2	TGGAATTATTGAAACGACTTGGG
CP-147-2-R2	GTCCTGAGGCAATATCTTTTGGA

Table SII: Wechsler intelligence scale

Subtest Scores	Verbal Test						Performance Test						Full Scale
	Inf	Sim	Arith	Voca	Comp	DS	Pic C	Pic A	Blo D	Obj A	Cod	Maze	
Raw Score	15	15	16	46	13	24	18	15	59	27	75	0	323
Scale Score	3	6	6	4	1	12	8	3	14	14	11	1	83
IQ	75						102						85
Assessment	Borderline Intelligence						Average						Below Average

Abbreviations: Inf: Information; Sim: Similarities; Arith: Arithmetic; Voca: Vocabulary; Comp: Comprehension; DS: Digit Span; Pic C: Picture Completion; Pic A: Picture Arrangement; Blo D: Block Design; Obj A: Object Assembly; Cod: Coding; Maze: Mazes

Table SIII: Variation frequency in multiple public databases and pathogenicity analysis

Database	1000GEA	ESP6500	gnomAD	ExAC	PolyPhen-2	MutationTaster	SIFT	CADD
CP:c.147-2A > G	-	-	0.0001	-	-	1.0 Disease causing	-	23.3 Damaging

Abbreviations: 1,000 GEA: 1,000 Genomes (East Asian); ESP6500: NHLBI GO Exome Sequencing Project; gnomAD_exome (East Asian); ExAC: Exome Aggregation Consortium (East Asian); CADD: Combined Annotation Dependent Depletion; SIFT: Sorting Intolerant from Tolerant

Hepatic and pancreatic iron deposition detected by CT and MRI

Abdominal CT revealed diffusely increased liver parenchymal density in the patient (95 HU), which was markedly greater than that in the healthy control (46 HU), while the pancreatic density remained comparable (49 HU *vs*. 41 HU) Figure 1A,B. On MRI, the patient showed diffusely decreased liver signal intensity on both T1-weighted Figure 1C,D and T2-weighted images Figure 1E,F relative to that of the control, which was consistent with iron overload. Quantitative $R2^*$ mapping by IDEAL-IQ further confirmed significant hepatic iron deposition, with a liver $R2^*$ value of 1033 Hz in the patient and 43.7 Hz in the control. A mildly elevated pancreatic iron content (157.4 Hz *vs*. 26.6 Hz) was also noted Figure 1G–I.

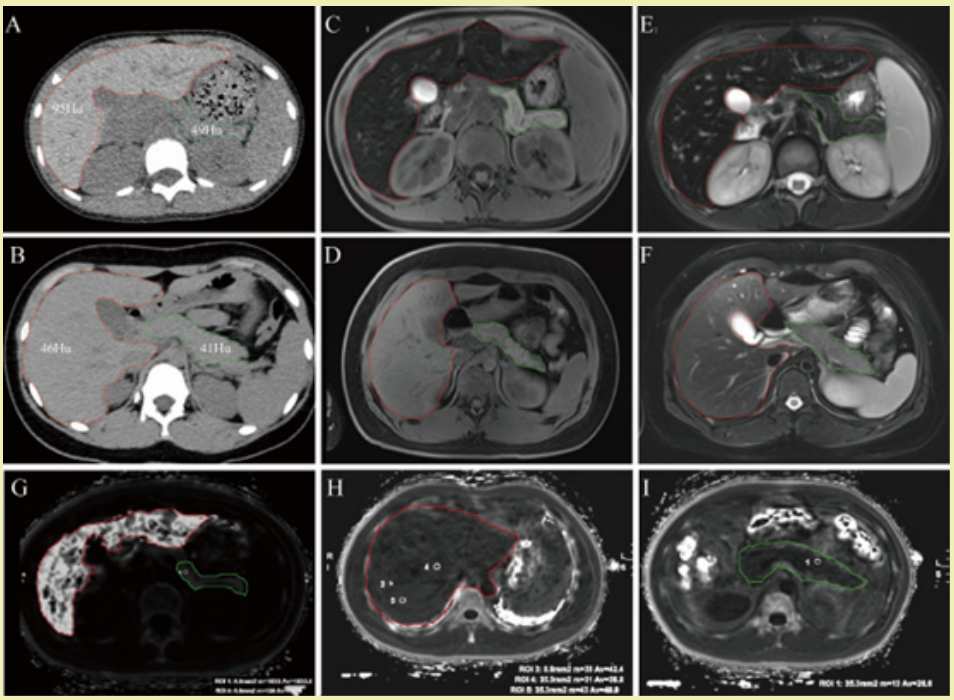

Figure 1: Radiological assessment of hepatic and pancreatic iron deposition in an 11-year-old girl with aceruloplasminemia compared with a healthy control

A, B: Abdominal CT scans. In the patient (A), the liver parenchyma (red outline) shows diffusely increased density (95 HU) compared with the normal control (B, 46 HU), whereas the pancreatic density (green outline) remains comparable (49 HU *vs*. 41 HU)

C, D: Abdominal T1-weighted MR images (T1WI). The patient (C) exhibited diffusely decreased liver signal intensity (red outline) compared with the control (D)

E, F: Abdominal T2-weighted MR images (T2WI). The patient (E) shows diffusely decreased liver signal intensity (red outline) relative to the control (F)

G, H, I: Abdominal IDEAL IQ $R2^*$ mapping. In patient (G), the liver (red outline) has a markedly elevated $R2^*$ value (mean 1033 Hz), which is consistent with severe iron overload, whereas the pancreas (green outline) has a mildly increased $R2^*$ value (157.4 Hz). In contrast, the control liver (H) had a mean $R2^*$ of 43.7 Hz, and the control pancreas (I) had a $R2^*$ of 26.6 Hz, indicating no significant iron deposition

Iron accumulation in the cerebellar dentate nucleus on brain MRI

Brain MRI revealed bilaterally decreased signal intensity in the dentate nuclei on T1-weighted and T2-weighted images in the patient compared with the normal control Figure 2A–D. IDEAL-IQ R^{2*} mapping revealed a markedly elevated $R2^*$ value of 121.4 Hz in the patient's dentate nuclei, which was significantly greater than the control value of 25.7 Hz. The surrounding cerebellar tissue also showed mildly increased $R2^*$ (24.6 Hz *vs*. 19.0 Hz) Figure 2E,F. These findings indicate pathological iron accumulation in the cerebellar dentate nucleus.

Exon 2 skipping caused by splicing mutation disrupts the CP structure

A homozygous splicing mutation in the CP gene (NM_000096.3: c.147-2A > G) was identified via whole-exome sequencing. Pedigree analysis revealed that the parents of the affected child were carriers of the mutation Figure 3A, 3B. Variation frequencies in multiple public databases of mutations are extremely rare or absent in the general population (1000 Genomes Project, ESP6500, gnomAD_exome, ExAC). Bioinformatics analysis (PolyPhen-2, MutationTaster, SIFT, and CADD) predicted both mutations as pathogenic Table SIII. RT–PCR analysis of minigene expression demonstrated that the splicing mutation led to a truncated transcript in the mutant compared

with the wild type Figure 3C. Sanger sequencing confirmed that the mutation resulted in exon 2 skipping, causing exon 1 to be spliced directly to exon 3 Figure 3D. Structural modeling of wild-type human ceruloplasmin revealed that its six-domain architecture is stabilized by a trinuclear copper cluster located at the interface between domains 1 and 6 Figure 3E. Deletion of exon 2 resulted in a predicted structural disruption of domain 1, abolishing the trinuclear copper cluster and the associated calcium- and sodium-binding sites Figure 3E-H. This structural alteration likely impairs the ferroxidase activity of ceruloplasmin, contributing to systemic iron overload.

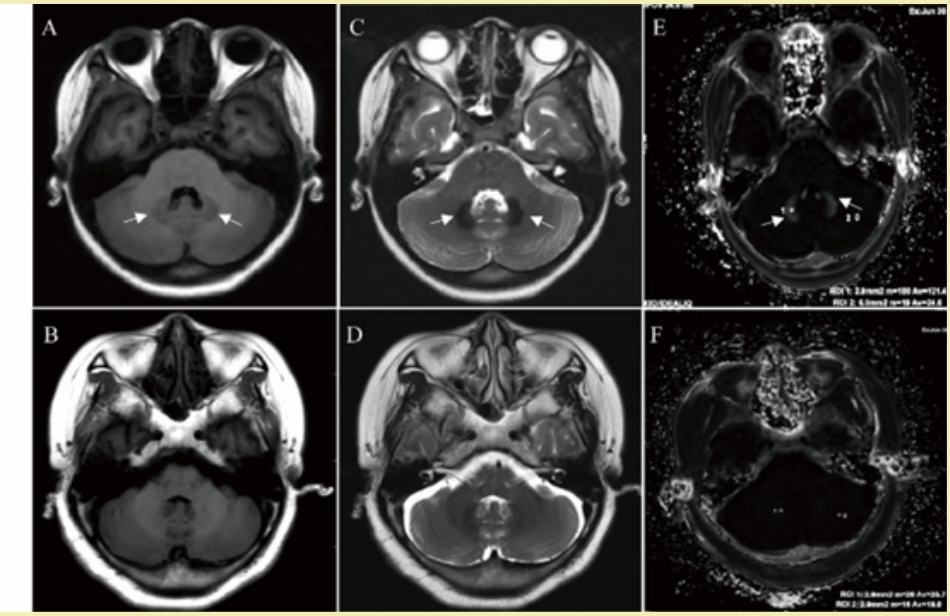

Figure 2: Brain MRI assessment of dentate nucleus iron deposition in an 11-year-old girl with aceruloplasminemia compared with a healthy control
A, B: Axial T1-weighted images (T1WI). In patient (A), the dentate nuclei (arrows) presented decreased signal intensity compared with those in healthy controls (B)
C, D: Axial T2-weighted images (T2WIs). The patient (C) shows decreased signal intensity in the dentate nuclei (arrows) relative to the control (D)
E, F: Axial IDEAL IQ R2* mapping. Patient (E) demonstrated a markedly elevated $R2^*$ value of 121.4 Hz in the dentate nucleus (ROI 1), which was significantly greater than that of the control (F), which was 25.7 Hz. The surrounding cerebellar tissue in the patient (ROI 2) showed a mildly increased $R2^*$ of 24.6 Hz, which was slightly above the control value of 19.0 Hz, indicating localized iron accumulation

Treatment and prognosis

The child was started on oral deferasirox dispersible tablets at 20 mg/kg/day. A follow-up visit 2 weeks after discharge revealed the following: hemoglobin 98 g/L (increased from baseline); mean corpuscular hemoglobin 20.2 pg; mean corpuscular hemoglobin concentration 277 g/L; mean corpuscular volume 73.1 fL; reticulocyte percentage 0.82%; and ferritin 1,255 ng/mL (decreased from baseline). All the values improved, and the patient was under regular outpatient follow-up at our hospital. The current dose of deferasirox dispersible tablets has been reduced to 10 mg/kg/day for treatment.

Discussion

We report an 11-year-old girl with genetically confirmed aceruloplasminemia-the youngest documented to date. She presented with refractory microcytic hypochromic anemia, growth delay, and mild cognitive impairment. Laboratory studies revealed paradoxically low serum iron and markedly elevated ferritin. Whole-exome sequencing revealed a homozygous splice-site variant, c.147-2A > G, in the Ceruloplasmin Gene (CP). Pedigree analysis revealed that the parents of the affected child are carriers of the mutation. CP has six structural domains with a three-copper catalytic center, one Ca^+ binding site, and one Na^+ binding site, all of which are essential for its oxidative function[6] and protein stability.[6,7] The c.147-2A > G variant disrupts the canonical acceptor splice site. Minigene assays revealed complete skipping of exon 2 Figure 3C, 3D. Structural modeling revealed that this deletion disrupts the trinuclear copper cluster bridging domains 1 and 6, eliminates both Na^+- and Ca^{2+}-binding sites Figure 3E-H, abolishes ferroxidase activity, and explains the undetectable serum ceruloplasmin (< 20 mg/L) and pronounced early iron loading Figure 1,2.

A summary of the clinical manifestations and age of onset in 71 Japanese individuals revealed that 80% of patients under 20 years of age presented iron-restricted microcytic anemia. With advancing age, approximately 76% of individuals experience retinal degeneration, 70% are diagnosed with diabetes mellitus, and approximately 90% of patients with ceruloplasmin deficiency exhibit neurological symptoms after an average of 12.5 years of

insulin-dependent diabetes.[1] The clinical presentation of anemia prior to the onset of diabetes or neurologic symptoms is common in patients with aceruloplasminaemia, highlighting the importance of early genetic screening for accurate diagnosis.

Our report advances the earliest age of confirmed diagnosis by at least a decade and positions aceruloplasminemia within the differential diagnosis of pediatric anemia. Although symptoms are typically described between the third and eighth decades of life.[8] this case demonstrates that symptomatic disease can manifest in early childhood. Under physiological conditions, ceruloplasmin-or its intestinal counterpart hephaestin-oxidizes ferrous iron exported by ferroportin; this reaction is required both for safe loading onto transferrin and for the surface stability of ferroportin itself.[6,9-12] In the absence of functional ceruloplasmin, ferroportin is rapidly internalized and degraded.[13,14] Ferrous iron accumulates within hepatocytes, reticuloendothelial macrophages and eventually the central nervous system, whereas transferrin saturation decreases.[15-17] These pathophysiological changes generate an iron profile that is easily misread as common iron deficiency anemia, with one crucial exception.

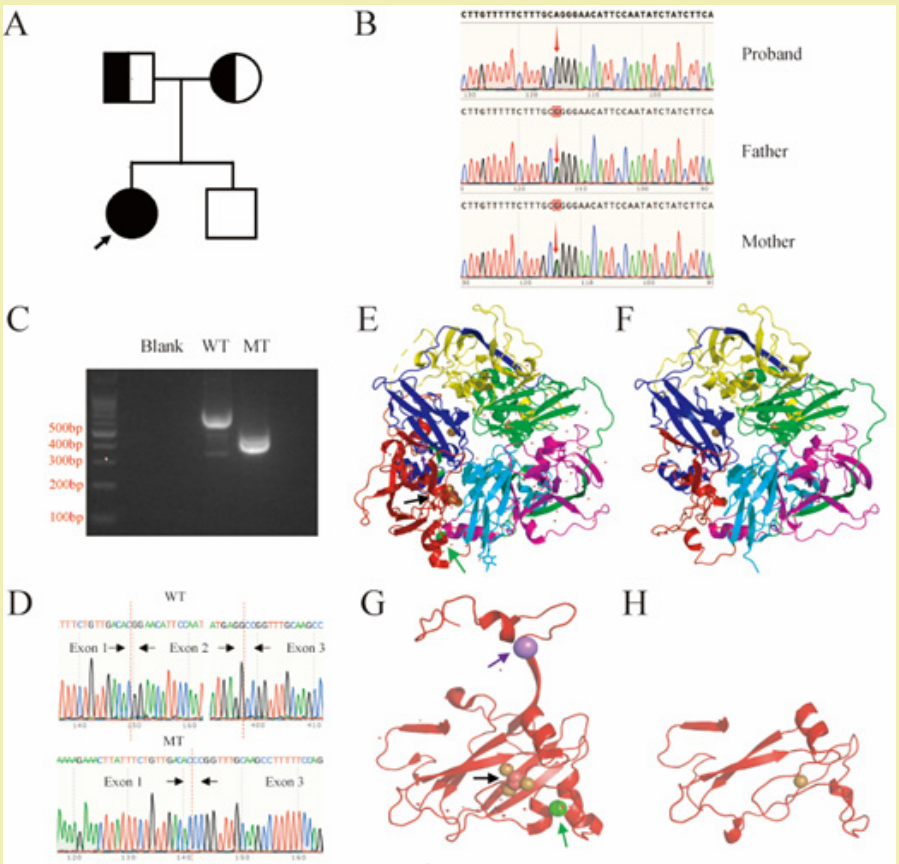

Figure 3: Pedigree analysis of the effects of CP mutation and exon 2 skipping on the ceruloplasmin transcript and protein structure

A: Pedigree of the family carrying CP mutations

B: Sanger sequencing chromatograms illustrating the CP mutations in the family. The red arrows indicate the position of the CP mutations

C: RT–PCR analysis of minigene expression products. Compared with the Wild Type (WT), the Mutant (MT) generated a shorter truncated product, indicating an alternative splicing event

D: Sanger sequencing of the minigene products confirmed that the splicing mutation caused skipping of exon 2, resulting in exon 1 being directly spliced to exon 3 in the Mutant (MT) transcript

E: Predicted wild-type human ceruloplasmin structure, consisting of six domains shown in different colors (domain 1, red; domain 2, dark blue; domain 3, yellow; domain 4, green; domain 5, purple; and domain 6, cyan). The interface between domains 1 and 6 contains a trinuclear copper cluster (type 2 and type 3 binuclear copper centers) essential for catalytic activity, where oxygen is reduced to water

F: Predicted structure of ceruloplasmin after exon 2 deletion. Domain 1 is structurally altered

G: Structural detail of wild-type domain 1 of ceruloplasmin, showing the trinuclear copper cluster (arrow), calcium ion (green sphere), and sodium ion (purple sphere)

H: Predicted structure of domain 1 after exon 2 deletion, showing the loss of the trinuclear copper cluster, calcium ions (green), and sodium ions (purple)

In classic iron deficiency, serum ferritin is the first marker to decrease, followed sequentially by serum iron and transferrin saturation.[18] In aceruloplasminemia, serum iron is low (reflecting impaired export), but ferritin is paradoxically elevated because intracellular iron cannot be mobilized. Thus, any child presenting with microcytic anemia, low serum iron and high or normal ferritin-especially if unresponsive to oral iron-should undergo immediate CP gene analysis. Early recognition not only averts unnecessary and potentially harmful iron supplementation but also allows timely institution of iron-chelation therapy and positions the patient for emerging enzyme- or gene-replacement strategies, thereby reducing the long-term risks of diabetes, retinal degeneration and irreversible neurodegeneration.

Iron chelation therapy is currently the cornerstone treatment for aceruloplasminemia. The small lipophilic chelator deferiprone crosses the blood–brain barrier to capture deposited Fe^{2+} and promote its urinary or biliary excretion.[19] whereas the hydrophilic chelator deferoxamine or oral deferasirox primarily clears hepatic and splenic iron.[20,21] In this case, the child received oral iron chelation therapy. The short-term response has been favorable: anemia has improved, and serum ferritin has decreased. However, the duration of treatment remains brief, and the long-term efficacy is still uncertain; therefore, repeated reassessment of this patient is needed.

Given that this was a single case, the generalisability of our findings is limited; multicenter pediatric registries are needed to validate the full genotype–phenotype–imaging–treatment pathway. In addition, functional validation was restricted to an *in vitro* minigene assay and in silico modeling; patient-derived cells or animal models would provide *in vivo* confirmation. Finally, long-term neurocognitive follow-up remains preliminary. Future studies should incorporate standardized neuropsychological batteries and quantitative brain iron imaging to assess the true impact of early intervention on neurological outcomes.

In summary, aceruloplasminemia caused by CP, c.147-2A > G, can present in childhood with isolated anemia. Early genetic diagnosis coupled with iron chelation therapy effectively halts systemic iron accumulation. We recommend including CP in routine genetic panels for children with unexplained microcytic anemia who have disproportionately elevated ferritin and depressed serum iron levels to enable early diagnosis, timely treatment, and improved long-term outcomes.

Availability of Data and Materials

The data generated in the present study may be requested from the corresponding author.

Authors' Contributions

Xin Tan, Qin Huang, Li Bai and Jin Su are responsible for the clinical work, including patient recruitment, obtaining informed consent, and sample collection. Yuxin Li conducted the CT and MRI examinations. Xin Tan conceived and designed the experiments, whereas Ying Wu and Xiao Liu executed the experiments. Ying Wu, Xiao Liu, Jin Su and Li Bai drafted the primary manuscript. Xin Tan reviewed and revised the manuscript. Funding was obtained from Xin Tan, Yuxin Li and Jin Su. All the authors read and approved the final manuscript. Xin Tan confirmed the authenticity of all the raw data.

Ethics Approval and Consent to Participate

This study was approved by the institutional ethics committee of The Affiliated Changsha Hospital of Xiangya School of Medicine, Central South University, with the approval number [(2025) Ethical Rapid Review NO.22] and the approval date [20250916].

All adults participating in this study provided written informed consent. For minors, written informed consent was provided by their parents.

Patient Consent for Publication

Patient consent for publication was obtained from all participants. For minors, written informed consent was provided by their parents.

Privacy Statement

The privacy rights of human subjects are strictly protected in this study. All personal information of the subjects has been anonymized.

Declaration of Generative AI and AI-assisted Technologies in the Manuscript Preparation Process

No generative AI or AI-assisted technologies were used in the preparation of this manuscript.

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Conflict of Interest Statement

The authors declare that they have no conflicts of interest.

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