A novel double nucleotide variant in the ferritin-L iron responsive element in a Finnish patient with hereditary hyperferritinaemia-cataract syndrome

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Abstract

Purpose: To present a novel Finnish double nucleotide variant in the iron-responsive element (IRE) of the ferritin L-chain gene (*FTL*) leading to hyperferritinaemia-cataract syndrome (HHCS).

Methods: Genomic DNA extracted from peripheral blood leucocytes and synthetized with three different primers flanking the IRE in the *FTL* 5'-untranslated region of the *FTL* were used in PCR. Thereafter, Sanger Sequencing was performed on the 487-bp and 602-bp PCR amplification products with specific primers to reveal *FTL* IRE mutations.

Results: A 58-year-old female patient with elevated serum ferritin level (1339 µg/l) was diagnosed with HHCS after extensive workup. Genetic testing identified a novel double point mutation g.48965355G>C (chr19, hg19) and g.48965356G>T (chr19, hg19) in the lower stem region of the IRE canonical structure of the *FTL*.

Conclusions: After excluding other causes, elevated serum ferritin level in a person with early onset cataract is indicative for HHCS, a genetic disorder caused by mutation in the IRE of the *FTL*.

Key words: ferritin, iron-responsive element, point mutation, cataract

Introduction

Hereditary hyperferritinaemia-cataract syndrome (HHCS; OMIM #600866) is a rare autosomal, dominantly inherited disease with significantly increased serum ferritin level and early onset of bilateral cataract (Bonneau et al. 1995, Girelli et al. 2001). The syndrome was first described in 1995 (Bonneau et al. 1995, Girelli et al. 1995b) and was shown to be caused by a mutation in the L-subunit coding gene of ferritin (*FTL*), more precisely in the segment of iron responsive element (IRE) where iron responsive element binding protein (IRE-BP) binds to (Girelli et al. 1995a, Luscieti et al. 2013). Under steady state conditions iron deficiency causes IRE-BP to bind to IRE, resulting in inhibition of the expression of the L-subunit of ferritin, thus post-transcriptionally regulating its expression (Anderson et al. 2012). Mutations prevent IRE-BP from binding to the IRE, causing excessive production of the L-subunit of ferritin and subsequently its accumulation in the circulation and in different cells (Kohgo et al. 2008). Accumulation of ferritin L-subunit in the lens of the eye causes clouding of the lenses, i.e. cataract, in a yet unknown mechanism. These patients are not clinically or with laboratory parameters diagnosed with iron overload, inflammatory reactions, malignancy or liver diseases. Thus, the only known clinical symptom of the HHCS is the early onset of bilateral cataract (Cosentino et al. 2016). Nevertheless, HHCS is still unknown to many ophthalmologists and remains undiagnosed even after cataract surgery of young patients (Millonig et al. 2010). The fact that high serum ferritin level may be associated with HHCS is not known for most physicians.

We present a Finnish female patient with considerably elevated serum ferritin level who was diagnosed with HHCS. Her syndrome was shown to be due to a previously unpublished double nucleotide variant g.48965355G>C (chr19, hg19) and g.48965356G>T

(chr19, hg19) in the IRE of the *FTL*. The variant has now been named as Pori +47 G>C and +48 G>T.

Case report

A 58-year-old woman was diagnosed with high blood pressure (200/100 mmHg) in December 2015 and losartan-hydrochlorothiazide medication was started. The patient's general condition was good, but her serum ferritin concentration was found to be significantly increased (1339 µg/l, reference value 10-125 µg/l). Her weight was almost normal (BMI 26.4 kg/m^2), and her liver function tests were normal (Table 1). The consumption of alcohol by the patient was very minimal. After starting losartan-hydrochlorothiazide medication, also her blood pressure returned to normal (142/73 mmHg).

Table 1. Laboratory parameters of the patient

Characteristics	Measured value	Reference values
Blood hemoglobin (g/l)	135	117-155
Erythrocyte MCV (fl)	87	82-98
Plasma CRP (mg/l)	< 5	0-10
Plasma iron (µmol/l)	14.4	9-34
Serum ferritin (µg/l)	1339	10-125
Serum transferrin (g/l)	2.2	1.75-3.13
Transferrin saturation (%)	27	15-45
Plasma ALAT (U/l)	30	< 35
Plasma GT (U/l)	22	< 40
Serum hepcidin (nmol/l)	4.6	0.7-16.8
HFE analysis	H63D -/- , C282Y -/-	

The following abbreviations were used: MCV, mean corpuscular volume; CRP, C-reactive protein; ALAT, alanine aminotransferase; GT, glutamyltransferase; *HFE* analysis refers to C282Y and H63D mutations.

On further examination, mild swelling and tenderness around the metacarpophalangeal (MCP) joints was found in both hands. Otherwise the patient's clinical status was normal. According to the patient, symptoms in her hands had appeared in the past ten years. Because of the swelling of the MCP joints, an X-ray examination was performed. The X-ray examination showed degenerative arthritis in the distal joints of the patient's fingers. Furthermore, a local brighter area and mild swelling of the capsule were seen at the tip of the finger bones, leading to a suspicion of an incipient rheumatoid arthritis. A rheumatologist was consulted but no evidence of an inflammatory rheumatic disease was confirmed.

Repeated examination verified significantly elevated level of serum ferritin (1577 µg/l). Additional laboratory parameters for the differential diagnosis of hemochromatosis including transferrin saturation (27 %, reference value 15-45 %) were normal, and hemochromatosis gene (*HFE*) showed no predisposing main mutations (H63D -/- and C282Y -/-) in genetic testing. The serum hepcidin level was measured to exclude an infection causing the patient's hyperferritinaemia. Patient's serum hepcidin level was normal (4.6 nmol/l, reference value 0.7-16.8 nmol/l) (Itkonen et al. 2012).

A more precise anamnesis of the patient revealed that already at the age of 34 years she had been diagnosed with bilateral cataract exhibiting stellate and punctate lens changes. When she was 43 years old, cataract operation was performed, and at the time of the operation she had bilateral central lens opacities and subcapsular cataract. Also her deceased father had been operated for bilateral cataract when he had been 41 years old. Because patient´s laboratory parameters excluded common diseases causing hyperferritinaemia, the most likely cause of her hyperferritinaemia was HHCS (Bonneau et

al. 1995, Girelli et al. 2001). Genetic testing of the patient and selected family members was performed in order to confirm the diagnosis of HHCS.

Material and methods

Blood samples for DNA testing were obtained from the patient, three affected family members (one sister, one brother, and his son) and two non-affected family members (one sister, and one brother) after informed consent. The family pedigree and the members who were analyzed for the IRE mutation of the *FTL* are shown in Fig. 1. Genomic DNA was extracted from their peripheral blood leucocytes (QiaAmp DNA mini kit, Qiagen). Three different PCR primers (IRE_FRW1: 5'- CCCATTTCACAACACGCTGG -3', IRE-FRW2 5'-CCCATTTCACAACACGCTGG-3' and IRE_REV: 5' - TTACCCGACCGCACAAAGAAGG -3') were synthesized, flanking the IRE in the *FTL* 5'-untranslated region. Two parallel PCR reactions (IRE_FRW1/IRE_REV and IRE_FRW2/IRE_REV) were performed on 50 ng of genomic DNA using 1 U KAPA HiFi (KAPA), 300 nmol/l each primer, and 300 mmol/l each dNTP in a 50 µl final reaction volume. PCR program used was as follows: 95 ^{o}C for 3 min, followed by 32 cycles of 95 ^{o}C for 20 s, 68 ^{o}C for 25 s, 72 ^{o}C for 25 s, and final extension at 72 ^{o}C for 5 min. The 487-bp (IRE_FRW1/IRE_REV) and 602-bp (IRE_FRW2/IRE_REV) PCR amplification products were purified from 1 % agarose gel with NucleoSpin Gel and PCR Clean-up Kit (Macherey-Nagel, Germany). The bidirectional Sanger Sequencing on both amplicons was performed with specific primers (Hetet et al. 2003, Papanikolaou et al. 2006): FRW: 5'-CCGGCGCACCATAAAAGAAGC-3', REV1: GCTCATGGTTGGTTGGCAAG and REV2: AGGTAGGTGTAGGAGGCCTG by using BigDyeTerminator v3.1 Cycle sequencing Kit (Applied Biosystems, USA) (Eurofins Genomics, Germany). The RNA secondary structure of the

sequenced IRE of L-ferritin was modelled with mFold (http://unafold.rna.albany.edu/?q=mfold).

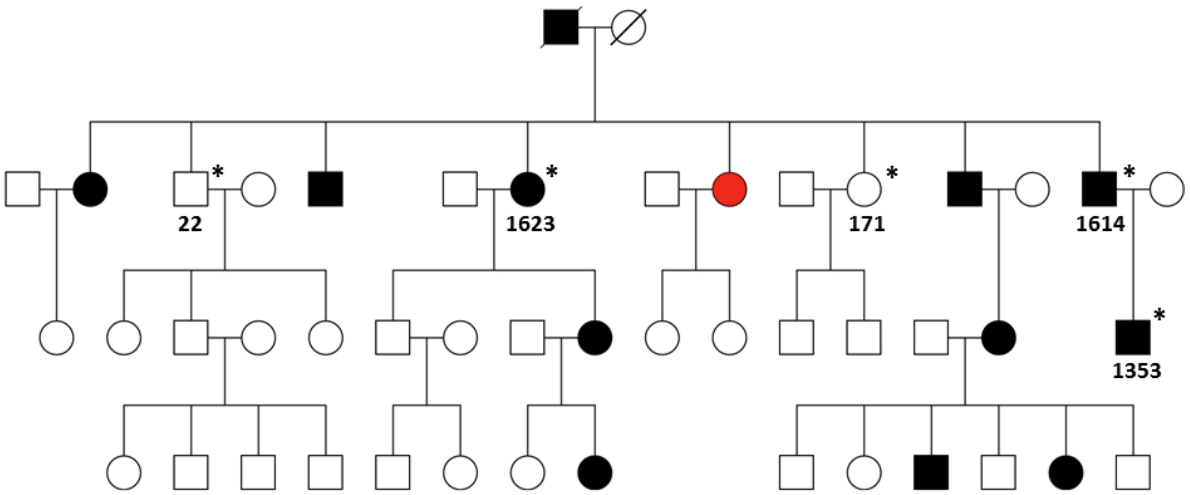

Figure 1.

Results

Sequencing of the IRE of the *FTL* revealed a double nucleotide variant Pori, +47 G>C and +48 G>T, in the patient compared to wild type (her non-affected brother) (Fig. 2). The same double nucleotide variant was also detected in all other affected family members who were analyzed (Fig. 1). The RNA secondary structure of the IRE of ferritin-L was modelled with mFold (http://unafold.rna.albany.edu/?q=mfold) and the mutation was found to locate in the lower stem region of the IRE of the *FTL* (Fig. 3 A). Furthermore, the modelling showed that the identified mutations disrupt the functional domains of the IRE (Fig. 3 B), and no hairpin loop structure is predicted.

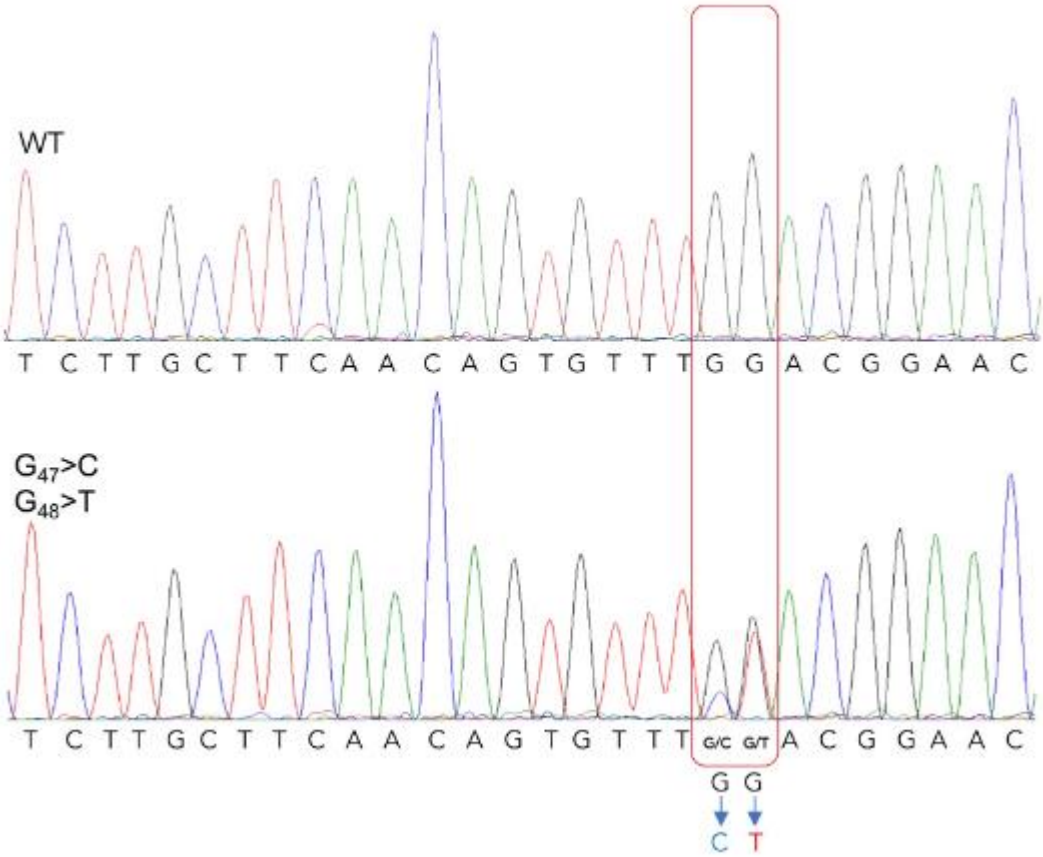
WT
T C T T G C T T C A A C A G T G T T T G G A C G G A A C
G47>C
G48>T
T C T T G C T T C A A C A G T G T T T G/C G/T A C G G A A C
G G
C T

Figure 2.

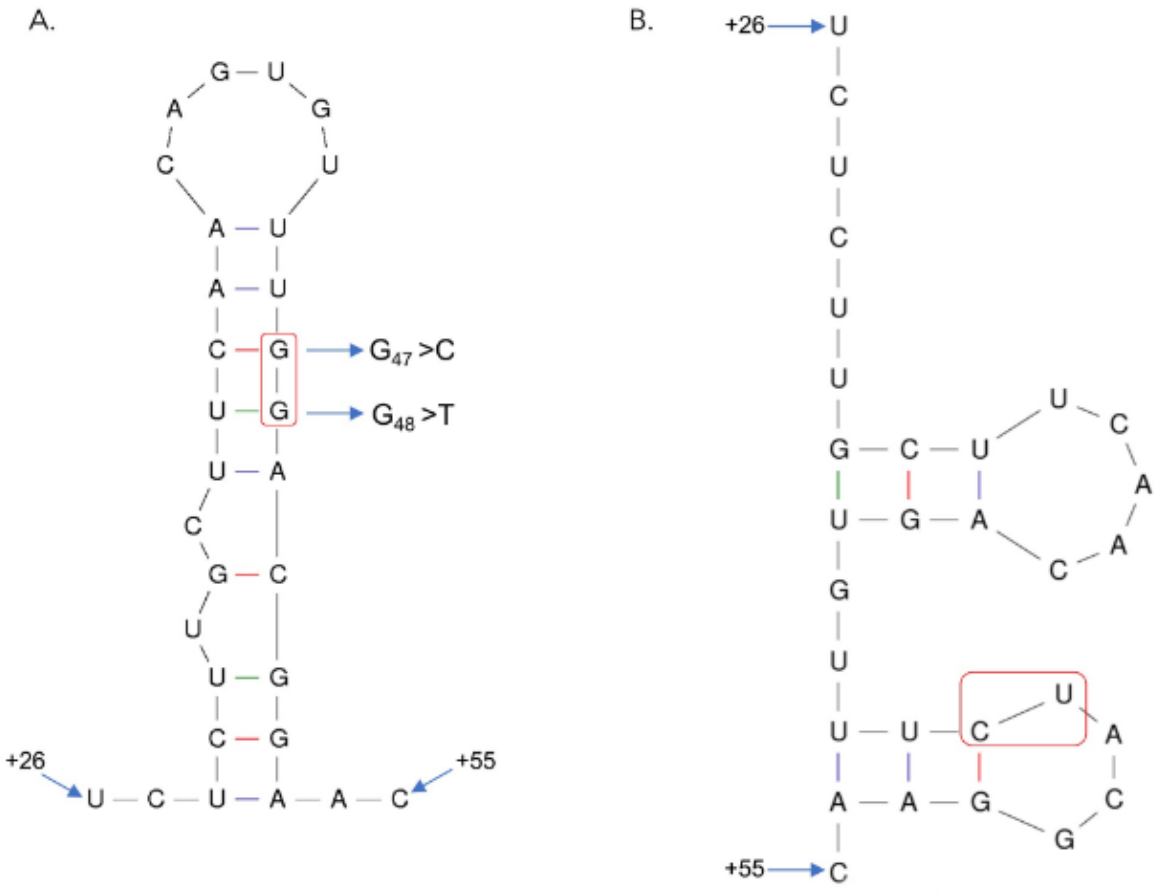

Figure 3.

Discussion

Elevated serum ferritin level is a common finding in clinical practice. Most often it evokes a suspicion of hemochromatosis, and this should be next examined by measuring transferrin saturation. Normal transferrin saturation does not necessarily exclude hemochromatosis, because up to 30 % of the patients with homozygosity for a predisposing mutation, *HFE* C282Y +/+, have been shown to have normal transferrin saturation (Adams et al. 2007). Our patient's transferrin saturation was 27 % and *HFE* analysis revealed that she

had neither H63D nor C282Y mutations that are known to predispose to hemochromatosis. Besides being an iron storage protein, ferritin is also one of the so-called acute phase proteins (Gabay & Kushner 1999). Thus, inflammatory diseases such as rheumatic diseases and infections, malignancy, non-alcoholic fatty liver disease (NAFLD), and an abundant use of alcohol has to be kept in mind when investigating the cause of hyperferritinaemia (Ong et al. 2016, Sackett et al. 2016). The clinical examination of the patient evoked a suspicion of an incipient rheumatoid arthritis. However, that was ruled out after laboratory tests and the consultation of a rheumatologist.

With our patient, a more precise anamnesis evoked suspicion of HHCS. To confirm the diagnosis undoubtedly, mutation analysis of the IRE of the *FTL* was performed and a new, unpublished heterozygous double mutation was detected. This is now named as the "Pori +47 G>C and +48 G>T" mutation following the traditional nomenclature for *FTL* IRE mutations (Luscieti et al. 2013). The same double nucleotide variant was detected in all three affected family members who were analyzed for the IRE of the *FTL* indicating that the double mutation is inherited, and not a de novo mutation.

Previously, at least 31 point mutations and 6 deletions of different sizes have been reported to cause HHCS (Hetet et al. 2003, Bowes et al. 2014, Luscieti et al. 2013, Cosentino et al. 2016). Previously detected point mutations causing HHCS are shown in Table 2. To the best of our knowledge, our patient is the first reported case of HHCS in Finland. The identified double nucleotide variant (Pori +47 G>C and +48 G>T) is located in the lower stem region of the IRE of the *FTL*. This region directly affects the binding affinity of IRE-BP to IRE and hence the functionality of the regulatory element (Goforth et al. 2010, Ke et al. 2000). Modelling of the mutated IRE showed (Fig. 3 B), that the identified double nucleotide variant forces the IRE into the conformation lacking the conserved stem structure with

CAGUG terminal loop which is required for the full IRE functionality (Ke et al. 2000). This explains the deregulated *FTL* expression causing elevated serum ferritin levels and HHCS phenotype. There has been considerable variation in the descriptions of HHCS cataracts, some of the primary characteristics being axial and peripheral white flecks in the lenses as well as translucent cortical vacuoles and crystalline aggregates (Millonig et al. 2010). Our patient had had stellate and punctate lens changes when she was 34 years old, and at the time of cataract operation, when she was 43 years old, she had bilateral central lens opacities and subcapsular cataract.

Table 2. Previously detected point mutations causing HHSC

Location based on transcription start site	Trivial name	Reference
+ 7 C > G, +40 A > G & + 49 A > C		(Castiglioni et al. 2010)
+ 14 C > G		(Cremonesi et al. 2001)
+ 18 C > A, + 22 U > G, + 24 U > C & + 26 U > G		(Lenzhofer et al. 2015)
+ 18 C > U & + 22 U > G	Pavia-2	(Cazzola et al. 1997)
+ 29 C > G	Torino	(Bosio et al. 2004)
+ 32 G > A	Pavia-1	(Cazzola et al. 1997)
+ 32 G > C	Baltimore	(Kato & Casella 1999)
+ 32 G > U	Paris-2	(Martin et al. 1998)
+ 33 C > A	Paris	(Giansily et al. 2001)
+ 33 C > U	Madrid	(Balas et al. 1999)
+ 34 U > C	Paris	(Hetet et al. 2003)
+ 36 C > A	London-2	(Mumford et al. 1998)
+ 36 C > G	Milano	(Cremonesi et al. 2003)
+ 36 C > U	Badalona	(Luscieti et al. 2013)
+ 37 A > U	Zaragoza	(Garcia Erce et al. 2006)
+ 37 A > G	Milano	(Cremonesi et al. 2003)
+ 39 C > A	Geelong	(Craig et al. 2003)
+ 39 C > G	Paris	(Garderet et al. 2004)
+ 39 C > U	London-1	(Mumford et al. 1998)
+ 40 A > G	Paris-1/ Montpellier-1	(Beaumont et al. 1995)
+ 40 A > G & +41 G > C		(Cremonesi et al. 2001)
+ 41 G > C	Verona-1	(Girelli et al. 1995a)
+ 43 G > A	Salt Lake City	(Phillips et al. 2005)
+ 46 U > G		(Luscieti et al. 2013)
+ 47 G > A	Paris	(Hetet et al. 2003)
+ 49 A > C		(Luscieti et al. 2013)
+ 49 A > U		(Kato et al. 2001)
+ 50 C > A		(Gonzalez-Huerta et al. 2008)
+ 51 G > C	Torino	(Camaschella et al. 2000)
+ 52 G > C	Heidelberg	(Luscieti et al. 2013)
+ 56 A > U	Paris	(Luscieti et al. 2013)

It is noteworthy, that although HHCS is a rare genetic disorder, physicians should be aware of it in order to avoid unnecessary treatment to deplete iron (Yin et al. 2014).

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Legends to the figures

Fig. 1. The family pedigree. Circles denote female family members and squares male family members. The patient is indicated using red circle. Symbols with diagonal line denote deceased family members. Black symbols indicate individuals with HHCS. Open symbols indicate non-affected individuals. Family members analyzed for the IRE of the *FTL* are indicated with asterisk and their ferritin levels (µg/l) are also provided.

Fig. 2. Sequence trace of the IRE of the *FTL*, showing wild-type (WT) allele (top) and mutant allele (below) with specified nucleotide changes g.48965355G>C (chr19, hg19) and g.48965356G>T (chr19, hg19). Red rectangle denotes the position of the mutations. Note, that the other three affected family members who were analyzed for the IRE of the *FTL*, had the same mutant allele as the patient. Furthermore, both of the examined non-affected family members had the WT allele.

Fig. 3. A) The RNA secondary structure of the IRE of the *FTL*. The structure was modelled with mFold (http://unafold.rna.albany.edu/?q=mfold). The identified novel double point mutation Pori +47 G>C and +48 G>T in the lower stem region of the IRE canonical structure is denoted within the sequence. Numbering is based on the transcription start site. **B)** Double point mutation disrupts the functional domains of IRE, and no hairpin loop structure is predicted. The location of the mutated nucleotides is denoted within the structure.