Detection of a rare mutation in the ferroportin gene through targeted next generation sequencing

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Introduction

Hereditary haemochromatosis (HH) is a disorder characterised by increase of serum iron parameters and gradual iron accumulation in parenchymal organs. Since the discovery of the genetic defects of the most common form of hereditary haemochromatosis (type 1 haemochromatosis [HFE]), many observations have shown that not only rare/familial mutations in *HFE* can be present but also that mutations in other genes (transferrin receptor 2 [*TFR2*], hepcidin [*HAMP*], hemojuvelin [*HJV*], and ferroportin [*SLC40A1*]) can lead to rarer genetic forms of iron overload, referred as non-HFE haemochromatosis[1]. The genetic heterogeneity is particularly evident in the Italian population where only two-thirds of haemochromatosis patients are *HFE* C282Y homozygotes[2], requiring expensive and time-consuming gene specific genotyping to define the molecular diagnosis. Therefore, "second level" genetic tests should be developed for a rapid and simultaneous study of the haemochromatosis genes.

The most common non-HFE haemochromatosis is the autosomal dominant haemochromatosis type 4 (HH4), caused by mutations in the *SLC40A1* gene, encoding for cellular iron exporter ferroportin. Ferroportin acts as receptor of hepcidin, the key regulator of iron metabolism. The hepcidin/ferroportin interaction induces the internalisation of the complex, causing a decrease in iron export and its retention in the cell[3]. HH4 can be phenotypically classified in two groups: patients may have hyperferritinaemia with normal/low transferrin saturation (type-A HH4, ferroportin disease) or both serum iron parameters increased (type-B HH4, non-classical ferroportin disease)[4]. This depends on ferroportin impairment that could be classified as "loss-of-function" or "gain-of-function". In the first case (type-A), the mutated protein is not expressed on the membrane and it is not able to exert its exporting function. In the second case (type-B), the mutated iron exporter becomes resistant to the activity of hepcidin, causing increased iron absorption. Several mutations have been characterised in HH4 patients, spreading along the entire gene sequence[4]. The position of the correspondent amino acidic change is important to define the HH4 clinical phenotype[5].

Among the different ferroportin mutations, the p.A69T variant, despite the causal nucleotide change not being annotated in dbSNP, has been described in a 52-year old Italian woman with diabetes and type-B HH4, on the basis of hepatocyte iron overload[6], but no iron parameters or clinical history of the patient were described. The pathogenic role of this mutation has been subsequently confirmed through *in vitro* experiments in which mutant cDNA has been over-expressed[7]. These studies demonstrated that p.A69T mutated ferroportin has a partial resistance to hepcidin.

Here we report an Italian patient with a severe iron overload phenotype in whom a careful clinical characterisation led to a diagnose of p.A69T non-classical ferroportin disease through the combination of capture of technology with a next generation sequencing (NGS) platform.

Case report and results

In April 2012, a 47-year old man came to the attention of the Department of Transfusion Medicine in Turin for hepatopathy of unknown origin with biochemical signs of iron overload. There was a familial history of chronic liver disease since the father had died of cirrhosis of unknown origin. The patient had severe hyperferritinaemia (6,242 ng/mL), elevated transferrin saturation (95.4%), elevated transaminase levels, increased liver echogenicity mimicking "steatosis" with signs of progression to liver fibrosis at elastography evaluation (9 kPa, F2 Metavir Scale), splenomegaly and arthritis. Red blood cell (RBC) indices were normal (RBC 5.12 million/μL, haemoglobin [Hb] 144 g/L, mean cell volume [MCV] 87 fL). Viral hepatitis and inflammatory diseases were excluded. There was no endocrine or cardiac dysfunction. DNA from mononuclear peripheral blood cells was prepared by standard protocols and a reverse hybridisation assay (Haemochromatosis StripAssay, Nuclear Laser, Settala,

Blood Transfus 2016; 14: 531-4 DOI 10.2450/2016.0286-15

Milan, Italy) was used for the simultaneous detection of 10 *HFE* gene mutations (V53M, V59M, H63D, H63H, S65C, E168X, E168Q, W169X, C282Y, Q283P), 3 *TFR2* mutations (Y250X, E60X, M172K) and 2 *SLC40A1*/ferroportin mutations (N144H, V162del). This first level genetic test showed that the patient was heterozygous for the *HFE* p.H63D polymorphism.

A weekly phlebotomy treatment was started and well tolerated; no decrease in Hb was seen. After nine months, despite a 12-week combined chelation therapy with deferoxamine, ferritin levels were persistently high (6,519 ng/mL). To accelerate iron depletion, phlebotomies were replaced by erythroapheresis. One month later, ferritin had decreased (3,431 ng/mL) and serum hepcidin (Hep-25), measured by SELDI-TOF-MS[8], was 10.2 nM (normal range 7.02-10.05)[9]. In the following 16 months, ferritin and transaminases levels gradually decreased. Overall, 21 g of iron were removed before iron depletion. Subsequently, monthly phlebotomies were planned as maintenance therapy (Figure 1). During maintenance therapy (ferritin 94 ng/mL) serum hepcidin was lower than normal (0.55 nM).

Due to the genetically unexplained haemochromatosis phenotype, a second level genetic test[10] was performed by combining capture of the five HH genes (*HFE*, *HJV*, *TFR2*, *SLC40A1*/ferroportin, and *HAMP*/hepcidin) through HaloPlex™ Technology (Agilent Technologies, Santa Clara, CA, USA) and sequencing by the IlluminaHiSeq 1000 platform (Illumina, San Diego, CA, USA). Sequence reads were aligned against human reference HG19, and analysed by GoldenHelix™ software to detect all the possible pathogenetic variants. In addition to the known heterozygote *HFE* p.H63D variant, a C>T change in exon 3 of the ferroportin/*SLC40A1* gene at position 190439953 of chromosome 2 (GRCh37/HG19) was identified, leading to the substitution of threonine for alanine at position 69 (p.A69T). The presence of the p.A69T mutation was confirmed by direct sequencing of an amplicon in ferroportin exon 3 using ABI Prism 3130 xl Genetic Analyzer (Applied Biosystem) with the following primers (Fpn1F: 5'CTTCCTGAGTACAATAGACTAG3', Fpn1R: 5'CAGAGGTAGCTCAGGCAT3'). GOR IV Secondary Structure prediction server (http://mig.jouy.inra.fr) was used to predict *in silico* whether the amino acid change would interfere with ferroportin structure; this showed that the substitution causes profound modifications in protein structure (Figure 2A). The mutation was not found in the probands' mother or sister (Figure 2B) (normal iron parameters and slightly decreased serum Hep-25).

Discussion

A total of five haemochromatosis genes and several different mutations, some of which can be very rare or even private familial variants, have been characterised since the discovery of *HFE*[1]. Therefore, genetic diagnosis can be difficult and a traditional approach based on single mutation detection and sequencing of candidate genes can prove unsuccessful or expensive and time consuming, especially in populations in which inherited iron overload disorders are not genetically homogeneous[2].

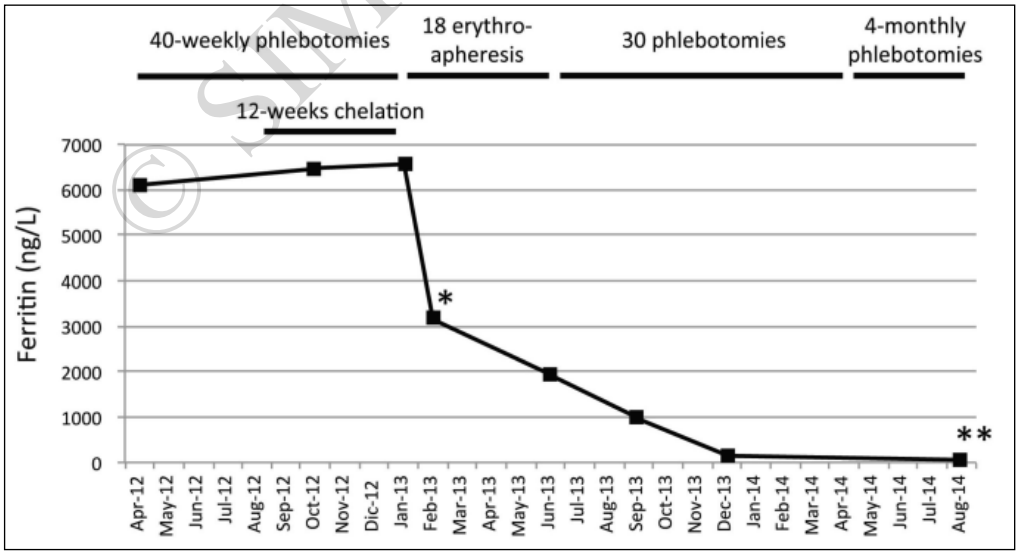

Figure 1 - Decline of ferritin concentration during the iron removal treatments.
Phlebotomy was carried out by removing approximately 400 mL of blood. Asterisks indicate the serum Hepc dosage points (*Hep25=10.2 nM; **Hep25=0.55 nM).

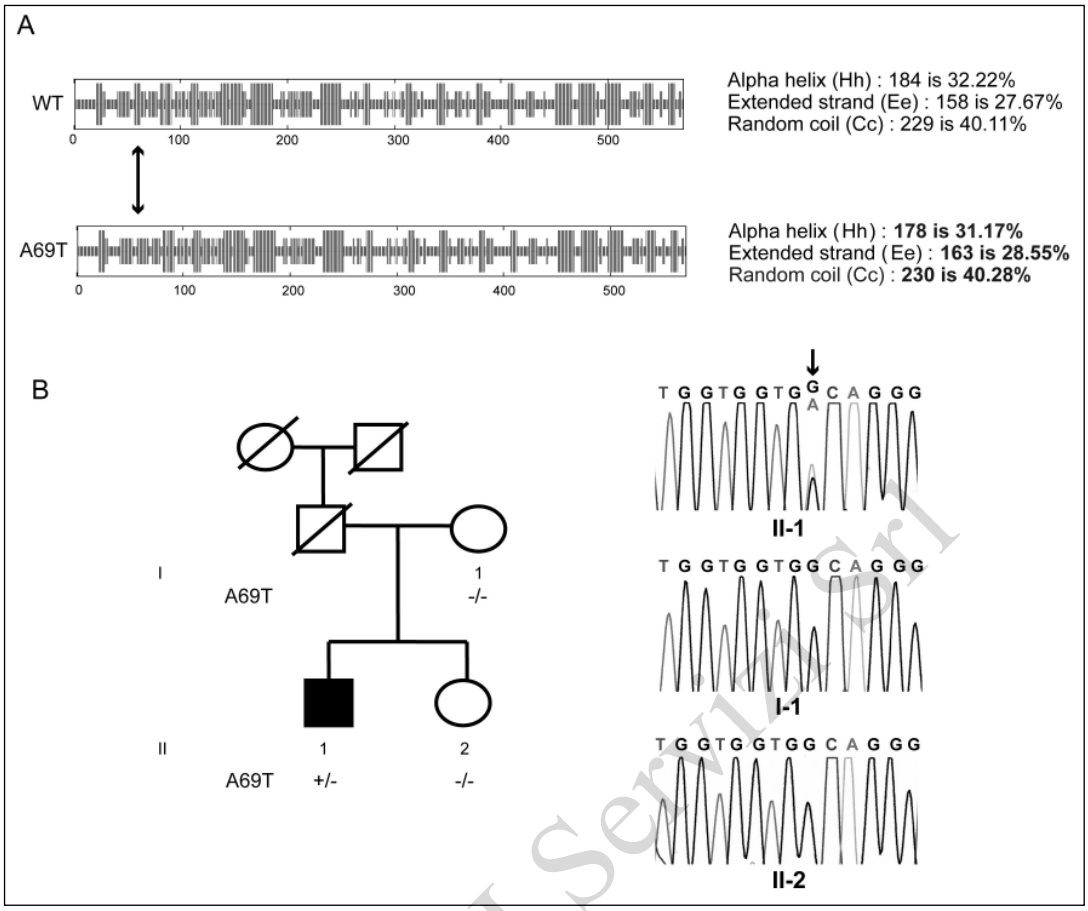

Figure 2 - Molecular results in study patients and relatives.
(A) Secondary structure prediction of p.A69T mutated ferroportin protein according to GOR IV server (http://mig.jouy.inra.fr). Double-headed arrow indicates the loss of an alpha-helix motif. Percentages of main amino acidic motifs are reported on the right; altered values in mutated protein are highlighted in red. (B) (Left) Genealogical tree of patient (II-1). (Right) Electrophoretograms of patient's antisense sequence, encompassing the C>T genomic mutation (indicated by an arrow), and of the 2 available relatives.

Large-scale analysis based on massively parallel DNA-sequencing systems has recently overcome this problem. Moreover, hybridisation-based capture and PCR-based amplification of targeted sequence followed by NGS allows only the genomic regions of interest to be studied, making genetic analysis cost-effective. This approach has been successfully used to identify causal mutations in many Mendelian and complex neurological conditions[11], in primary immunodeficiencies[12], and to screen for recurrently mutated genes in chronic lymphocytic leukaemia[13]. Moreover, NGS approaches have been recently advocated to identify rare haemochromatosis variants when iron overload is well documented and initial screening approaches are negative[14].

In this study, we performed a HaloPlex™ capture with NGS platform analysis to simultaneously screen 5 haemochromatosis genes in a patient with a clear haemochromatosis phenotype (on the basis of age of onset, high serum ferritin, high transferrin saturation and hepatopathy) but without genetic diagnosis. This approach allowed identification of a rare mutation (p.A69T) in the ferroportin gene that had led to a type B HH4, non-classical ferroportin disease. The patient's phenotype is in accordance with the "gain-of-function" of p.A69T mutant ferroportin, recently described as partially resistant to hepcidin *in vitro*[7]. In agreement with this, and in contrast with the most types of haemochromatosis, no hepcidin deficiency was seen in our iron-overloaded patient (despite the fact that phlebotomy/erythroapheresis treatment was ongoing). Few studies have assessed the serum hepcidin level in haemochromatosis due to ferroportin mutations. Two reports have shown that patients carrying ferroportin variants (either abolishing hepcidin binding to ferroportin[15] or leading to classical ferroportin disease[16]) have high serum hepcidin. These and our data confirm that the cause for HH4 is not a hepcidin deficiency as

in other types of haemochromatosis (related to *HFE*[17], *TFR2*[18] or *HJV*[19] mutations), but the lack of functional ferroportin. However, once an iron depletion state was achieved and a monthly phlebotomy maintenance treatment started, hepcidin concentration decreased, as already demonstrated in other haemochromatosis types[20]. This might be related to a regulatory effect by depleted iron stores and/or by stimulated erythropoiesis in response to long-term phlebotomy treatment.

In conclusion, we provide a clear description of the iron overload phenotype, including serum hepcidin monitoring, of a patient with a rare ferroportin mutation. Our study also demonstrates that, after careful clinical characterisation, NGS of targeted capture may be a useful approach for variant detection in cases of haemochromatosis. This could represent a good option for second level genetic testing in referral centres.

Acknowledgements

This work was supported in part by the *Progetto di Ricerca di Ateneo Università di Torino - Compagnia San Paolo* 2012 TO_Call1_2012_0088 and *Ricerca Locale 2013 Università di Torino* DEGMRILO13 FERRO.

Authorship contributions

LF, MPM, SDA, DG, AR and MDG conceived, designed and performed the study. LF performed DNA extraction. LF and SB performed genetic tests. AF and MD performed bioinformatic analysis. NC was responsible for hepcidin measurement. MPM, TF, VT, AV, AMDO and SDA collected clinical data. LF, MPM, DG, AR and MDG analysed and reviewed the data. MPM, DG, AR and MDG wrote the paper.

Keywords: haemochromatosis, ferroportin, hepcidin, next generation sequencing.

The Authors declare no conflicts of interest.

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Arrived: 29 October 2015 - Revision accepted: 4 February 2016
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