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A 10376 bp deletion of FECH gene responsible for erythropoietic protoporphyria

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Abstract

Erythropoietic protoporphyria (EPP, MIM 177000) is an autosomal dominant disease with incomplete penetrance since the phenotypic expression requires coinheritance of a null allele and a wild-type low expressed allele of Ferrochelatase gene (FECH). In this study, we identify a peculiar mutation in a young Canadian patient of Italian origin. The patient had clinical and biochemical symptoms of EPP, the wild-type low expressed allele but at preliminary analysis no mutation in the promoter, in the entire coding region and in the splice junctions of the gene. Family studies of seven most common polymorphisms along the gene established absence of Mendelian segregation for the promoter polymorphism only. The intron 1 polymorphism appeared in heterozygosis suggesting an hypothetical deletion in the first region of the gene. In order to identify the size of this deletion, single nucleotide polymorphisms (SNPs) analysis was extended to the upstream *N*-asparaginyl-tRNA synthetase gene (NARS). We analyzed two polymorphisms in the last exon of this gene and a dizigous region was found in the patient. A Long-PCR with primers located in previously fixed heterozygous regions showed a 10,376 bp deletion (c.1-7887_67+2422del) that included a portion of the upstream intergenic region, the promoter, the exon 1 and a portion of intron 1. RNA analysis demonstrated that the lack of the entire promoter prevents the expression of the mutated allele, in fact the expression of the Ferrochelatase gene was decreased by half in the subjects carrying only the mutation compared to control.
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Keywords: Heme; Erythropoietic protoporphyria; Ferrochelatase gene; promoter deletion

Introduction

Erythropoietic protoporphyria (EPP, mim 177000) is an inherited disorder caused by molecular abnormalities in the gene coding for the Ferrochelatase (FECH, EC 4.99.1.1) [1]. This enzyme is located in the inner mitochondrial membrane and catalyzes the chelation of ferrous iron into protoporphyrin IX in the terminal step of the heme biosynthetic pathway [2]. A partial deficiency of FECH activity leads to protoporphyrin accumulation into erythrocytes, plasma and stools. Excited protoporphyrins give rise to oxidative damage causing burning sensation, edema and erythema, that are the main clinical manifestations of EPP [3]. In less than 5% of EPP patients, the accumulation of protoporphyrin in the liver leads to cholestasis and unpredictable terminal liver failure. Clinical expression of the disease has a childhood onset and requires co-inheritance of a mutated allele and a wild-type low expressed allele of Ferrochelatase gene (FECH) [4]. The gene maps to chromosome 18q21.3 (NC_000018.8: 53367797–53421484), spans 45 kb for a total of 11 exons and encodes for an inactive pre-protein (423 amino acids) with a cleavable N-terminal mitochondrial leader sequence. At present there is no consensus as to the exact length of the Ferrochelatase leader sequence but recent studies suggested that the first 62 amino acids allow targeting to mitochondria even though they do not contain sufficient information for efficient processing of the protein [5]. The Ferrochelatase is

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transcribed from a single promoter in both erythroid and non-erythroid cells although two poly-adenylation sites produce two mRNAs of different length [6]. A CpG island embracing the 5′ flanking region allows the expression of a single minimal Sp1-driven TATA-less promoter in all cell types by maintaining an open chromatin structure. The recognition of the Sp1-binding sites by either Sp1 or tissue-specific members of the family determines a higher rate of transcription in erythroid cells versus other cell types, a process that may also require the presence of additional cell type-specific factors. A ubiquitously expressed repressor is responsible for the control of erythroid-specific trans-activators that are held in check upstream of the minimal promoter. This repressor only affects the activity of the upstream binding factors, while the activity of the basal promoter remains unaffected. The activity of the repressor seems to be regulated during erythroid differentiation allowing high expression of the FECH gene at the terminal stages of erythroid differentiation [7]. Gouya et al. reported the haplotype c.-251**G**, c.68-23**T** and c.315-48**C** associated to a low expression of the wild-type allele although so far only the c.315-48**C** is recognized as responsible for such mechanism [8]. The low expressed allele, carrying the three polymorphisms (the **GTC** haplotype), is present in 1.8% of the normal Italian population and it is always present in trans to the mutation in our EPP symptomatic patients. To date, more than 90 different mutations have been identified in the FECH gene [9]. Point mutations and small insertions–deletions represent the most common molecular defects but also some large deletions have been reported [10,11]. In this study, we report a new 10,376 bp deletion in a young Canadian patient of Italian origins affected by overt EPP.

Material and methods

Patients

The proband and three asymptomatic relatives have been investigated. The patient showed, starting at the age of 6 years: pain, hypersensitivity and swelling on the skin of his hands and his feet after any prolonged exposure to sunlight. At age of 12 years, he had an episode of burning sensation and blistering on his face. He exhibited high free erythrocyte protoporphyrin levels, mild hepatomegaly but not biochemical abnormalities of liver disease. Unfortunately ferrochelatase activity was not performed.

DNA analysis

DNA was isolated from leukocytes by PUREGENE DNA purification system blood kit (Gentra Systems Minneapolis, MN, USA). The promoter and the entire coding region including splicing junctions of the FECH gene were amplified with 7 different specific primer pairs (10 pmol each), in the presence of 0.2 mM dNTPs, 1.5 mM $MgCl_2$, 67 mM Tris–HCl at pH8.8, 16 mM $(NH_4)_2SO_2$, 0.01% Tween20 and 1 U of DNA polymerase (Bioline, London, UK) in a final volume of 50 μl. The polymerase chain reaction (PCR) was carried out in a 9700 thermal cycle (Applied Biosystems, Foster City, CA, USA) under the following conditions: an initial denaturation step at 94 °C for 5 min followed by 30 s at 94 °C, 30 s at 50–63 °C and 30 s at 72 °C for 30 cycles. The PCR products were subsequently submitted to direct sequencing of both strands (Abi-Prism 310 Genetic Analyzer, Applied BioSystems, Foster City, CA, USA). Sequence analysis of genomic DNA can also reveal the status of seven most common polymorphisms along the gene: c.-251A/G (g53405102), c.68-23C/T (g53398452), c.287G/A (g53391503), c.315-48T/C (g53389818), c.798G/C (g53377381), c.921G/A (g53372646), c.1520C/T (g53368694). The SNPs analysis was also extended in last exon of *N*-asparaginyl tRNA synthetase (NARS).

Long-PCRs

Using primers located in heterozygous regions, 500 ng of genomic DNA was amplified with 400 nM of each primer in the presence of 500 μM of dNTPs and 2 U of TripleMaster® Polymerase Mix (Eppendorf, Germany), in a final volume of 50 μl containing Tuning Buffer® 1× and 2.5 mM $MgCl_2$. The Long-PCR was carried out in a 9700 thermal cycler (Applied Biosystems, Foster City, CA, USA) under the following conditions: an initial denaturation step at 93 °C for 3 min followed by 15 s at 93 °C, 30 s at 68 °C and 22 min at 68 °C for 28 cycles. In the last 12 cycles, the time of each elongation step was extended by 20 s. The band corresponding to abnormal fragment was selected reducing the extension time in the Long-PCR protocol.

RNA analysis

Total RNA was isolated from fresh leukocytes by the standard guanidium-isothiocyanate method [12]. One microgram of total RNA was reverse-transcribed using High Capacity cDNA Archive Kit (Applied BioSystems, Foster City, CA, USA) at 25 °C for 10 min and 37 °C for 120 min. For a semi-quantitative analysis, the cDNA spanning exons 2 to 6 was co-amplified

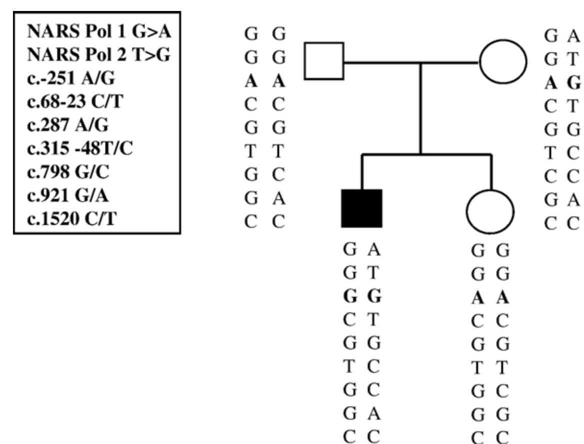

Fig. 1. Family segregation study. The figure reports the sequence analysis in the family members of nine polymorphisms, two NARS and seven FECH polymorphisms listed in the box. In the proband (in black) the c.-251 A>G polymorphism only shows an absence of Mendelian segregation. The asymptomatic relatives are represented in white.

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