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Digital PCR (dPCR) analysis reveals that the homozygous c.315–48T > C variant in the *FECH* gene might cause erythropoietic protoporphyria (EPP)

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ABSTRACT

Alterations in the ferrochelatase gene (*FECH*) are the basis of the phenotypic expressions in erythropoietic protoporphyria. The phenotype is due to the presence of a mutation in the *FECH* gene associated in *trans* to the c.315–48 T > C variant in the intron 3. The latter is able to increase the physiological quota of alternative splicing events in the intron 3. Other two variants in the *FECH* gene (c.1–252A > G and c.68–23C > T) have been found to be associated to the intron 3 variant in some populations and together, they constitute a haplotype (ACT/GTC), but eventually, their role in the alternative splicing event has never been elucidated.

The absolute number of the aberrantly spliced *FECH* mRNA molecules and the absolute expression of the *FECH* gene were evaluated by digital PCR technique in a comprehensive cohort. The number of splicing events that rose in the presence of the c.315–48 T > C variant, both in the heterozygous and homozygous condition was reported for the first time. Also, the percentage of the inserted *FECH* mRNA increased, even doubled in the T/C cases, compared to T/T cases. The constant presence of variants in the promoter and intron 2 did not influence or modulate the aberrant splicing. The results of *FECH* gene expression suggested that the homozygosity for the c.315–48 T > C variant could be considered pathological.

Thus, this study identified the homozygotes for the c.315–48 T > C variant as pathological. By extension, when the samples were categorised according to the haplotypes, the GTC haplotype in homozygosis was pathological.

1. Introduction

Erythropoietic protoporphyria (EPP, MIM#17700) is a genetically determined form of porphyria that is characterised by severe phototoxic manifestations. The patients with EPP experience serious pain, erythaema, and swelling of the exposed skin upon exposure to the sun [2]. In almost 96% of the cases, the clinical manifestations of EPP are due to a decreased activity of ferrochelatase (*FECH*), the enzyme involved in the last reaction of haeme biosynthesis [1]. *FECH* inserts the ferrous iron into the protoporphyrin IX (PPIX) ring, giving rise to protohaeme. As a result of a deficiency of the *FECH* enzyme, PPIX accumulates in the erythrocytes, vascular endothelium, and faeces. Being a highly reactive molecule, PPIX is activated by UV-A of the visible light and generates free radicals, leading to the cellular and tissue injuries and severe pain, characteristic of EPP.

Intron 3 of the *FECH* gene pre-mRNA is subjected to alternative splicing, thereby leading to the insertion of 63 intronic base pairs and the nonsense-mediated decay of this aberrant mRNA molecule. Depending on the genotype of the c.315–48 T > C variant (rs2272783), the percentage of production of this aberrant mRNA varies. In the presence of the wild-type T base, there is a 10–20% physiological production of the inserted *FECH* mRNA, whereas in the presence of the C base – the cause of the so-called 'hypomorphic *FECH* allele' – this percentage increases up to 40% [11]. With the only exception of the subjects with homozygous mutations in the *FECH* gene, a null mutation in the *FECH* gene combined in *trans* to the C base causes the clinical expression of EPP in most of cases. On the other hand, the presence of the T base at the position c.315–48 in *trans* to a mutation usually leads to a symptom-free condition, identifying the carrier subjects of EPP.

Thus, most of the studies about the molecular characterisation of the EPP patients report the presence of the c.315–48C polymorphism in *trans* to a mutant allele of the *FECH* gene. Nevertheless, different studies have also reported the cases of EPP, harbouring only the c.315–418C

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variant in homozygosis [15, 17–19]. One of the studies has reported that patients show only a mild EPP phenotype with slightly high erythrocyte protoporphyrin and mild photosensitivity [18]. Similarly, a recent Japanese study has described three cases of the childhood onset of mild EPP due to the presence of the homozygous C > C variant in intron 3 [15]. Considering these reports, it is not known if the differences in the EPP phenotypes come from a differential expression of the *FECH* gene.

Moreover, there is evidence that other Single Nucleotide Polymorphisms (SNPs) could also be involved in the phenotypic expression or at least be constantly associated to the polymorphism in intron 3. In fact, the c.68–23C > T variant located in intron 1, (SNP, rs2269219) has been studied and it was demonstrated that it is able to alter the secondary structure of the mRNA [16]. In addition, the c.1–252A > G variant in the promoter region, (SNP, rs17063905) has been associated with a reduced expression of the *FECH* gene [9]. Two molecular studies in the Argentine population have reported the constant allelic association of these two SNPs with the c.315–48C variant. In particular, the EPP patients always show the c.1–252G, c.23–68 T, and c.315–48C alleles (GTC haplotype) in *trans* to the mutant allele [6]. Previously, one independent study in two Italian families and another study, involving a single case of EPP in the English Scottish ancestry reported exactly the same association of the GTC haplotype in *trans* to a mutant allele for the expression of clinical EPP [8, 13].

At present, it is still unclear whether these two polymorphisms influence the splicing pattern of intron 3. At the same time, the previously published studies do not give an absolute quantification of the aberrant *FECH* mRNA molecule but give only its percentage, relative to the total *FECH* mRNA.

Recently, a new, highly sensitive, and precise technique called digital PCR (dPCR) has been made available as an alternative to the classical quantitative PCR (qPCR) technique for the absolute quantification of nucleic acids. In this study, the technique is applied to evaluate the alternative splicing events of the *FECH* gene and the absolute expression profile of the *FECH* gene in a cohort, composed of the EPP patients, the carriers of the *FECH* gene mutation, and the normal subjects. The roles of the c.1–252A > G, c.68–23C > T, and c.315–48 T > C variants, when present in the homozygous condition has also been investigated. At the end, the correlations among the expression of the *FECH* gene, the percentage of the inserted *FECH* mRNA, and the altered biochemical parameters are also evaluated in the EPP patients.

2. Materials and methods

2.1. Study subjects

In this study, a total of 90 subjects, recruited at the Centre of Rare Metabolic Diseases at Fondazione IRCCS Cà Granda from 2014 to 2017 were investigated. The subjects consisted of 32 EPP patients, 12 carriers of the *FECH* gene mutation, 17 carriers of the c.315–48C > T variant (polymorphism), and 29 normal controls. All the EPP patients showed a classical history of skin photosensitivity and high values of free protoporphyrins in the erythrocytes. The carriers of the mutation and of the c.315–48C > T polymorphism were the relatives of the patients as and when they were available. The EPP patients as well as the carriers of the mutation and of the c.315–48C > T polymorphism were already diagnosed genetically and clinically.

Freshly drawn blood samples were collected in the EDTA tubes and immediately processed for the molecular determinations. When possible, the levels of the erythrocyte protoporphyrins and the faecal protoporphyrins were determined.

Written informed consent was obtained from all the subjects in accordance with the ethical guidelines of Fondazione IRCCS Cà Granda Ospedale Maggiore Policlinico and the Declaration of Helsinki by the World Medical Association (WMA).

2.2. The determination of the erythrocyte protoporphyrins and the total faecal protoporphyrins

Whenever possible, the concentrations of the erythrocyte protoporphyrins and the faecal protoporphyrins were determined as a routine analysis. The erythrocyte protoporphyrins were determined as previously reported [5]. The total faecal protoporphyrins were measured according to a previously published method [14]. Briefly, a faecal sample of about 50 mg was weighed and suspended in 1 ml of 37% HCl. Following vigorous mixing, 3 ml of ethyl ether was added and mixed and then 3 ml of water was added and mixed. The mixture was then centrifuged and the upper phase was discarded, while the lower aqueous phase was submitted to a Lambda 25 UV–Vis spectrophotometer from 390 to 425 nm (Perkin-Elmer, Monza, Italy) for the quantification of the total faecal protoporphyrins. The total faecal protoporphyrins were expressed as nmol/g of the faecal dry weight, following the assessment of the content of humidity in the faeces by a Sartorius MA35 moisture analyser balance (Sartorius, Muggiò MB, Italy). The reference range of the total faecal protoporphyrins was up to 200 nmol/g of the faecal dry weight and the limit of quantification of the assay was 30 nmol/g of the faecal dry weight.

2.3. DNA isolation and genotyping

DNA was isolated from the peripheral blood samples with a Maxwell 16 automated extractor (Promega Corporation, Madison, WI, USA) according to the previously published methods [10].

For the control subjects, the c.1–252A > G, c.68–23C > T, and c.315–48 T > C polymorphisms of the *FECH* gene were analysed. An amount of 100 ng of DNA was amplified with the BIOTAQ DNA polymerase (Bioline, London, UK), using the following PCR primer pairs: 5'-AGGGCACGCAACTAGGAGT-3′ and 5'-TCAGGGATCCTGGCCCTG-3′ for the c.1–252A > G polymorphism, 5'-TGCCTGCAGAGAAATGCTAG-3′ and 5′-(GC39) TCTTATTTGTACCTGATGTT-3′ for the c.68–23C > T polymorphism, and 5'-GTGTTGTGTGTCCTGAATCT-3′ and 5-'CGAAA GAACTAATCTAGTTACATGT-3′ for the c.315–48C > T polymorphism, respectively. The PCR products were then subjected to automated direct sequencing on an ABI Prism 310 Genetic Analyser (Thermo Fisher Corporation Inc., San Francisco, CA, USA).

In order exclude a possible allele drop-off, DNA of homozygous patients for the c.315-48 T > C variant was also amplified with a second reverse primer, located in exon 4 (5′- TCCAGCAGCTTCACCAT GCCCT- 3′), and then subjected to direct sequencing.

2.4. RNA isolation, reverse transcription PCR(RT-PCR), mRNA sequencing and digital PCR(dPCR)

For qualitative mRNA analysis purpose, total RNA was retro transcribed as previously described [8]. Two primer pairs allowed sequencing *FECH* mRNA in two different overlapping fragments. The first fragment covered from exon 1 to exon 4 and the second one from exon 2 to exon 11. Thus, 100 ng of cDNA were amplified with the following primers pairs: IP-FW 5'-GGGGATCGCTACCCGGCTC-3′ with IP-RW-5'-TCCAGCAGCTTCACCATGCCCT-3′ and IIP-FW-5'-TCCAGCAGCTGGAG GGTC-3′ with IIP-RW-5'-ACACCCTCTCCACATCGGAGGTATC-3′. The thermal cycling conditions were 94 °C for 5 min, followed by 35 cycles: 92 °C for 30 s, 60 °C for 30 s, 72 °C for 1 min and a final elongation step of 3 min at 72 °C. The two fragments were then subjected to direct sequencing in both orientations.

For digital PCR purpose, total RNA was isolated from freshly drawn buffy coat samples with a Maxwell 16 automated extractor (Promega Corporation, Madison, WI, USA) according to the previously published methods [10].

An amount of 100 ng of total RNA was subjected to Reverse Transcription PCR (RT-PCR), using the ViLo Master Mix (Thermo Fisher Corporation Inc., San Francisco, CA, USA).