The penetrance of dominant erythropoietic protoporphyria is modulated by expression of wildtype *FECH*

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Erythropoietic protoporphyria (EPP) is an inherited disorder of heme biosynthesis caused by a partial deficiency of ferrochelatase (FECH, EC 4.99.1.1)[1,2]. EPP is transmitted as an autosomal dominant disorder[3] with an incomplete penetrance[1]. Using haplotype segregation analysis, we have identified an intronic single nucleotide polymorphism (SNP), IVS3–48T/C, that modulates the use of a constitutive aberrant acceptor splice site. The aberrantly spliced mRNA is degraded by a nonsense-mediated decay mechanism (NMD), producing a decreased steady-state level of mRNA and the additional FECH enzyme deficiency necessary for EPP phenotypic expression.

We previously reported that the wildtype gene expression level accounts for clinical expression of overt EPP; the EPP phenotype requires a deleterious *FECH* defect on one allele and a common *FECH* variant with low expression on the other[4,5]. Using 25 families with EPP caused by identified mutations, we have unambiguously determined the haplotypes of 19 independent chromosomes bearing a normal-expression allele (Web Table A; haplotype numbers 1–9) and of 23 independent chromosomes bearing a low-expression allele in *trans* to the mutated allele (Web Table A; haplotype numbers 10–17) in asymptomatic carrier parents and individuals with overt EPP, respectively. Low-expression alleles were associated with eight different haplotypes that share a common restricted haplotype ([GGTA] in red; Web Table A) spanning the 5′ noncoding region (–3670A/G) and intron 4 (IVS4–1197C/A). This indicates that a single ancestral event is responsible for the low-expression allele. The [GGTA] subhaplotype was also found on chromosomes bearing a normal-expression allele (Web Table A, number 3), however, indicating that none of the polymorphisms determining this haplotype could be causal. To identify all potential polymorphisms in this interval, we sequenced DNA clones from both *FECH* alleles derived from a subject heterozygous with respect to a low-expression *FECH* allelic variant. Sequence analysis revealed heterozygosity with respect to three loci, including an IVS3–48T/C transition. By subsequent genotyping of 25 family members with EPP, we showed that, in *trans* to a specific *FECH* mutated allele, only the IVS3–48C polymorphism cosegregates with the low-expression *FECH* allele in all individuals with overt EPP. Moreover, the IVS3–48T polymorphism cosegregates with the normal-expression allele in all the asymptomatic carriers. Genotyping of 40 additional unrelated individuals with EPP revealed that 38 had an IVS3–48C allele. The 11% prevalence of the

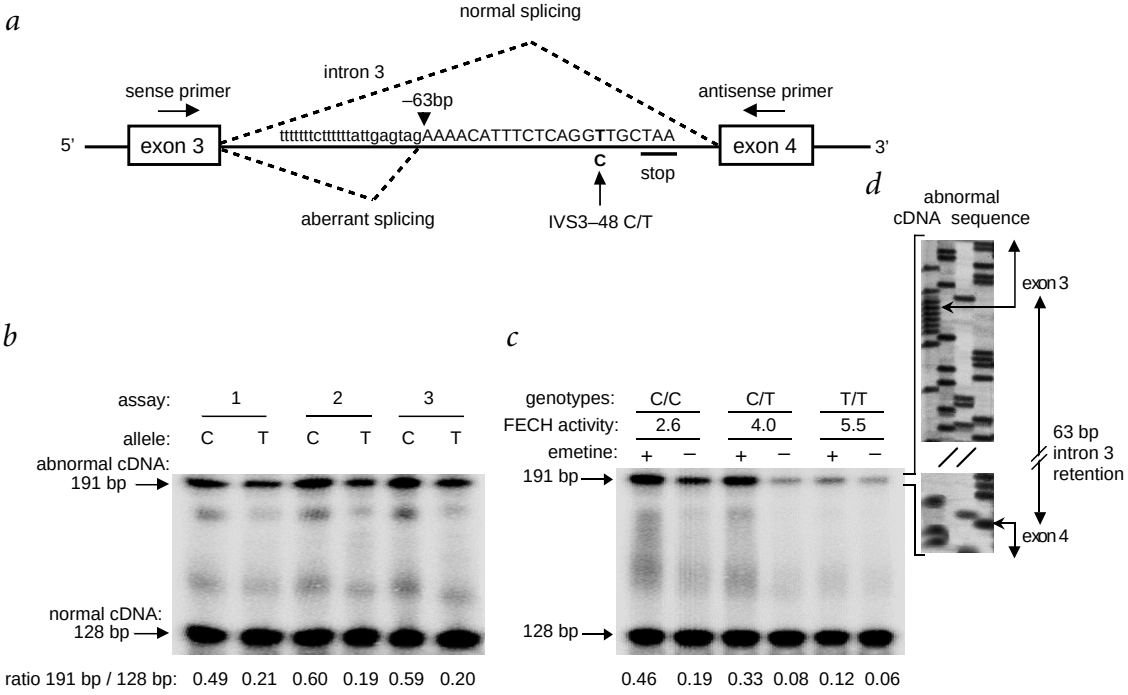

Fig. 1 The IVS3–48T/C transition modulates the splicing efficiency of a constitutive cryptic acceptor splice site. The migration positions of the normal (exon 3–4 128-bp RT–PCR product) and the aberrantly spliced cDNA sequences (191-bp RT–PCR product) are indicated on the left. We calculated the ratios of the 128-bp PCR fragment to the 191-bp PCR fragment during the exponential PCR time (bottom). ***a***, We cloned genomic DNA fragments spanning exons 3–4 from three independent subjects into a eukaryotic expression vector to obtain a minigene carrying either a T or a C at position IVS3–48. ***b***, Transfections of Cos-7 cells using three independent wildtype IVS3–48T and three IVS3–48C minigene preparations. ***c***, Inhibition of *de novo* protein synthesis in three lymphoblastoid cell lines homozygous or heterozygous with respect to the IVS3–48T/C SNP. FECH activities are expressed as nanomoles of Zn-mesoporphyrin per hour per milligram of protein at 37 °C and were carried out in triplicate[10]. +, cell medium culture with emetine; –, cell medium culture without emetine. ***d***, Sequence of the aberrantly spliced 191-bp cDNA fragment showing retention of the 63-bp intron 3.

a

	genotypic frequency			allelic frequency	
	T/T	C/T	C/C	T	C
controls *n*=350	0.888	0.095	0.017	0.89	0.11

b

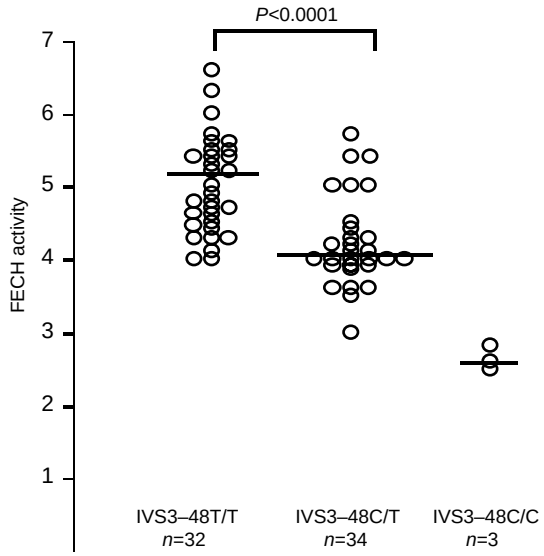

Fig. 2 Correlation between FECH activity and the IVS3–48T/C genotype. ***a***, Genotypic and allelic frequency of the IVS3–48T/C dimorphism for 350 white French people of European descent (controls). ***b***, Lymphocyte FECH activities for 69 unrelated individuals without EPP, according to the IVS3–48T/C genotype. FECH activities were significantly higher (t-test, $P<0.0001$) in control individuals with the IVS3–48 T/T genotype ($n=34$, mean 5.0; 95% C.I., 4.8–5.3) as compared with individuals with the IVS3–48T/C SNP ($n=32$, mean 4.2; 95% C.I., 4.0–4.4). The three persons with the IVS3–48C/C genotype show the lowest FECH values.

IVS3–48C polymorphism in white French people of European descent (Fig. 2*a*) correlates well with the 10% prevalence of the low-expression *FECH* variant, as previously estimated[5]. This shows that the most prevalent genetic status associated with the disease is the co-inheritance of a severe *FECH* defect together with a common low-expression variant IVS3–48C allele, although other situations may be found in rare instances[6,7].

We tested whether the presence of the C nucleotide at position IVS3–48 causes low *FECH* expression. Analysis of the intron 3–exon 4 sequence revealed the presence of a cryptic acceptor splice site 63 bp upstream from the one that is normally used (Fig. 1*a*). To test the effect of the IVS3–48T/C transition on splicing efficiency, we subcloned in a eukaryotic expression vector a 1,936-bp genomic fragment spanning exons 3–4 that differed only at the T/C nucleotide (Fig. 1*a*). Transfection of the IVS3–48C and T minigenes showed that in both cases the physiological and the predicted cryptic acceptor sites were used, but with different efficiency (Fig. 1*b*). The IVS3–48C minigene gave rise to 40% aberrantly spliced mRNA, and the IVS3–48T minigene to only 20% (Fig. 1*b*). The sequence of the abnormal 191-bp cDNA fragment demonstrates the use of the cryptic acceptor splice site (Fig. 1*d*). The use of the cryptic splice site yields an mRNA with a premature termination codon (Fig. 1*a*).

To investigate whether the abnormally spliced mRNA was degraded by NMD, we carried out an *in vivo* assay. We established three lymphoblastoid cell lines from subjects either homozygous or heterozygous with respect to the IVS3–48T/C polymorphism. We detected the synthesis of the aberrantly spliced product after inhibition of *de novo* protein synthesis using emetine in the culture medium (Fig. 1*c*). The progressively increasing proportion of abnormally spliced mRNA from the homozygous 'T' line to the homozygous 'C' line was strictly correlated with a progressively reduced FECH activity and is compatible with a codominant effect of the IVS3–48T/C SNP (Fig. 1*c*). Consistent with these data obtained in lymphoblastoid cell lines, FECH activity in freshly circulating lymphocytes isolated from unaffected people was significantly higher in those with the IVS3–48T/T phenotype as compared with those with the IVS3–48T/C SNP; the three individuals with the IVS3–48C/C phenotype had the lowest FECH values (Fig. 2*b*).

In some dominantly inherited disorders, the clinical expression of the disease results from the diminution of the gene product below a critical threshold. In such diseases, as shown here for EPP and as suspected in Hirschsprung disease[8,9], slight variations in expression of the normal allele that are due to common polymorphisms may have a key role in the clinical expression of the disease by setting the amount of gene product above or below the threshold.

Note: Supplementary information is available on the Nature Genetics web site (http://genetics.nature.com/supplementary_info/).

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Competing interests statement. *The authors declare that they have no competing financial interests.*

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