Characterization of Variegate Porphyria Mutations Using a Minigene Approach

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Abstract Porphyrrias are a group of metabolic diseases that affect the skin and/or nervous system. In 2008, three unrelated patients were diagnosed with variegate porphyria at the CIPYP (Centro de Investigaciones sobre Porfirinas y Porfirias). Sequencing of the protoporphyrinogen oxidase gene, the gene altered in this type of porphyria, revealed three previously undescribed mutations: c.338+3insT, c.807G>A, and c.808-1G>C. As these mutations do not affect the protein sequence, we hypothesized that they might be splicing mutations. RT-PCRs performed on the patient's mRNAs showed normal mRNA or no amplification at all. This result indicated that the aberrant spliced transcript is possibly being degraded. In order to establish whether they were responsible or not for the patient's disease by causing aberrant splicing, we utilized a minigene approach. We found that the three mutations lead to exon skipping; therefore, the abnormal mRNAs are most likely degraded by a mechanism such as nonsense-mediated decay. In conclusion, these mutations are responsible for the disease because they alter the normal splicing pathway, thus providing a functional explanation for the appearance of disease and highlighting the use of minigene assays to complement transcript analysis.

Introduction

Porphyrias are a group of metabolic diseases that arise from deficiencies in the heme biosynthetic pathway. There are seven different types of porphyria, depending on which enzyme is affected, after the first one, in this pathway. They are categorized in terms of the main tissue where the enzyme deficiency is expressed or by the clinical symptoms. Specific patterns of accumulation of the heme precursors δ -aminolevulinic acid (ALA), uroporphobilinogen (PBG), and porphyrins are associated with characteristic clinical features such as acute neurovisceral attacks, skin lesions, or both (Pietrangelo 2010; Puy et al. 2010).

Protoporphyrinogen oxidase (PPOX; EC 1.3.3.4) is the seventh enzyme involved in this pathway, and a partial deficiency leads to variegate porphyria (VP; OMIM 176200). This is a hepatic porphyria and can present itself with skin lesions, acute attacks, or both. Cutaneous photosensitivity is characterized by skin fragility, erosions, blisters, milia, and pigmentary changes in sun-exposed areas. Neurological symptoms include intermittent attacks of abdominal pain, constipation, vomiting, hypertension, tachycardia, fever, and various peripheral and central nervous system manifestations. Acute attacks may frequently result from exposure to diverse porphyrinogenic drugs, alcohol ingestion, reduced calories intake due to fasting or dieting, infections, and hormones which stimulate heme synthesis by delta-aminolevulinic acid synthase (ALA-S) induction thereby increasing the production of the porphyrin precursors ALA and PBG (Batlle 1997; Anderson et al. 2001; Kauppinen 2005; Rossetti et al. 2008).

VP is an autosomal dominant disorder with incomplete penetrance, associated to a 50% decrease of enzymatic activity in heterozygous individuals (Deybach et al. 1981).

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Table 1 Specific primers design for each PCR

Mutation	Construct	Forward primer	Reverse primer
c.338+3insT	pTB E4	<i>ggaattccCATATGgtgggatgtctaggagaggtt</i>	<i>tggaaggCATATGaggatgag</i>
c.807G>A	pTB E7	<i>ggaattcCATATGttcaagcaattctctgcct</i>	<i>ggaattcCATATGggcctaggattctgggtag</i>
c.807G>A	U1snRNA	<i>atggatatccctgcaaagtagggagagatcttggcctctgcccgga</i>	<i>tcggggcagaggcccaagatctctccctactttgcaggggagataccat</i>
c.808-1G>C	pTB E8	<i>ggaattcCATATGgcctgggaaactgagagtga</i>	<i>ggaattcCATATGcttctcactggtaggggttg</i>

Capital letters indicate restriction sites. Italics indicate nucleotide changes. If not indicated, the primers were used for both WT and mutated constructs

Nonetheless, there are reports about homozygous, either compound heterozygous or true homozygous, in which the PPOX activity is even lower than 50% (Hift et al. 1993; Frank et al. 1998; Roberts et al. 1998; Corrigall et al. 2000; Kauppinen et al. 2001; Palmer et al. 2001; Poblete-Gutiérrez et al. 2005; Poblete-Gutiérrez et al. 2006; Pinder et al. 2013).

The PPOX gene is located at chromosome 1 (1q22-23), spans a 5.5 kb genomic region, and contains one noncoding and 12 coding exons (Nishimura et al. 1995; Roberts et al. 1995; Taketani et al. 1995; Puy et al. 1996). Its mRNA is 1.8 kb and encodes a 477 amino acid polypeptide with a molecular weight of 50.8 kDa (Roberts et al. 1995).

To date more than 140 different mutations have been identified in the PPOX gene causing VP (Human Gene Mutation Database HGMD, <http://www.hgmd.cf.ac.uk/ac/index.php>).

Previously, we have reported three mutations, two of them are intronic (c.338+3insT and c.808-1G>C) and a missense mutation (c.807G>A) in the PPOX gene of three unrelated patients suffering from porphyria (Rossetti et al. 2008). It was hypothesized that these mutations may affect the splicing process as the coding sequence of the protein would be unaffected. However, the RT-PCR assay performed with each patient's mRNA showed either the correctly processed mRNA or no amplification at all. This may be due to the fact that the abnormal mRNAs can be degraded by a mechanism such as nonsense-mediated decay (NMD), a mechanism of degradation that occurs when a premature stop codon is generated within the transcribed sequence (Maquat 2004). If this were to occur, the transcripts are undetectable in normal RNA analysis (Baralle et al. 2009). The aim of the present work was to study the effect of these mutations using functional minigene splicing assays that would permit us to observe the effect on splicing caused by the mutations. This strategy allowed us to confirm that the three mutations are responsible for the symptoms observed in the patients who carry them.

Materials and Methods

Patients

Written informed consent was obtained from all patients prior to their inclusion in the study. The study protocol was approved by the Ethical Committee of the Centro de Investigaciones sobre Porfirinas y Porfirias (CIPYP – Hospital de Clínicas, CONICET) and was performed in accordance with the 1964 Declaration of Helsinki and its modifications. Three patients were included in this study with biochemical and molecular VP diagnosis, as described previously (Rossetti et al. 2008).

Constructs

Hybrid minigenes were made by amplifying WT and mutated exons of interest together with flanking introns of the PPOX gene. In order to obtain this from patient's genomic DNA, a PCR reaction was carried out with specific primers (Table 1) for each region of the PPOX gene (RefSeq NM_001122764.1) to be studied, flanked by NdeI sites (New England Biolabs). Each amplicon was inserted into the pGEM-T Easy vector (Promega), according to the manufacturer's instructions, and then subcloned into the previously described pTB minigene vector (Baralle and Baralle 2005) using the NdeI restriction sites (Fig. 1a).

The U1 snRNA complementary to the nucleotide change c.807G>A was obtained by means of the QuickChange Site-Directed Mutagenesis Kit (Stratagene), using specific primers (Table 1). The U1 snRNA plasmid has been previously described (Pagani et al. 2002).

The integrity of every clone was confirmed by means of automatic sequencing (Macrogen, ABI3730XL).

Minigenes Expression Assays

The splicing assays were performed by transfecting 0.5 µg of each minigene construct into 2.5×10^5 HeLa cells using

Fig. 1 pTB minigene splicing assay. **(a)** Representation of the three hybrid minigenes designed for each mutation. The vector comprises the β -globin promoter and SV40 enhancer (arrow at the start of the gene), β -globin polyadenylation site, and a series of exonic sequences corresponding to the β -globin (black boxes) and fibronectin (striped boxes). Each hybrid minigene is composed of the exon under study (white boxes) inserted into the NdeI site with and without the mutation together with the flanking introns (IVS). The black arrows above the vector indicate that the specific primers, the position of the mutations, and NdeI site are shown. **(b)** Transfection with pTB constructs. WT = wild-type construct, M = mutated construct. 247 bp = exon exclusion, 308 bp = inclusion of exon 8 (c.808-1G>C), 363 bp = inclusion of exon 4 (c.338+3insT), 438 bp = inclusion of exon 7 (c.807G>A)

Effectene Transfection Reagent (Qiagen). RNA extraction was performed using TriFast Reagent (peqGOLD). RT-PCR analysis was carried out with primers complementary to sequences present in the pTB minigene vector.

The U1 snRNA constructs were co-transfected along with pTB E7, WT or mutated, following the same procedure described above.

Statistical Analysis

Three independent transfections were made for each experiment, the expression profiles were analyzed using Scion Image Software (Scion Corporation, USA), and the results are shown as mean $\pm$ SD.

Bioinformatic Tools

NNSplice (Reese et al. 1997) was used to evaluate the 5' and 3' splice site strength of the sequence involved in the mutations. This tool compares a set of consensus splicing sites with the input sequence giving a score from 0 (weak site) to 1 (strong site), using a threshold score of 0.1 for donor sites and 0.4 for acceptor sites. ESE Finder (Cartegni et al. 2003; Smith et al. 2006) and Rescue ESE (Fairbrother et al. 2002) served to analyze the possible effect of the mutation on exonic enhancer elements. ESE Finder searches for binding sites for specific serine/arginine-rich (SR) proteins within the given sequence, using the following threshold scores: SF2/ASF, 1.956; SC35, 2.383; SRp40, 2.670; and SRp55, 2.676. Rescue ESE finds these putative ESEs by comparing the input sequence to a large database of hexamers previously identified as such elements.

SeqBuilder (Laser Gene, DNA star) was used to predict the formation of the premature stop codons generated by the frameshift due to the exon skipping.

Results

As a first approach, bioinformatic tools were used to evaluate the possible effects of these mutations on the mRNA. The exonic mutation (c.807G>A) was observed through the use of ESE Finder and Rescue ESE not to affect possible splicing enhancer elements found in exon 7. The most striking outcome was the decrease of the strength assigned to the 5' splice site of exons 4 and 7. In the case of the mutation c.338+3insT in exon 4, this was reduced from 0.96 to 0.25 and in the case of c.807G>A in exon 7 from 0.85 to 0. The 3' splice site of exon 8 in the case of the c.808-1G>C mutation changed from 0.93 to 0.

In order to investigate if the lack of any affect on mRNA in the patients carrying the mutations was indeed being masked by degradation of the mRNA, we created a series of wild-type (WT) and mutated minigenes for each of the exons (Fig. 1a). HeLa cells were transfected with the six pTB minigene constructs, and the mRNA processing analyzed after RT-PCR on RNA extracted from the cells. Figure 1b shows quite clearly the deleterious effect on exon inclusion of all three mutations.

While the WT minigenes used to study the mutations involved with exons 7 and 8 showed a strong inclusion of the corresponding exon ($72 \pm 4.7\%$ and $90 \pm 9.6\%$, respectively), the WT minigene made to study the effect of the mutation c.338+3insT showed only $37 \pm 5.8\%$ of exon inclusion. Notwithstanding, the effect the mutation has on the processing of this exon is still evident, as its introduction results in $100 \pm 0\%$ exon exclusion (Fig. 1b).

Fig. 2 Splicing rescue assay with U1 snRNA. WT = wild type pTB construct for c.807G>A, M = mutated pTB construct, M + U1 = mutated pTB construct and U1 snRNA, M + U1* = mutated pTB construct and snRNA U1 complementary to the c.807G>A mutation. 247 bp = exon exclusion, 438 bp = inclusion of exon 7

However, to elucidate if there was something in the flanking intron sequence at either site of exon 4 that would be rendering the wild-type construct less efficient at including the exon, we redesigned a new larger minigene including exon 3 to exon 5 and the corresponding introns. It was inserted in the eukaryotic vector pcDNA 3, PCR amplified and digested, as well as the vector, with XpnI and XhoI restriction enzymes. The ligated product was used to transform *E. coli* competent cells. Then, following the same protocols described in Materials and Methods for the other minigenes, we again obtained an important exclusion of exon 4 ($98.5 \pm 1.5\%$) in the presence of the mutation. However, despite using a more extended minigene, the wild-type version did not improve the inclusion of this exon ($43.5 \pm 6.5\%$) (data not shown).

In silico analysis of the mutation c.807G>A showed that no ESE is affected, while the analysis of the effect on the splice site was to reduce the strength from 0.85 to 0. However, to further demonstrate that the cause of aberrant splicing in this case is due to the weakening of the complementarity of the 5' splice site to U1snRNA, we created an U1snRNA complementary to the mutation. Co-transfection of this construct with the pTB minigenes containing the exon 7 of the PPOX gene, carrying the c.807G>A mutation, resulted in partial rescue of exon inclusion ($22 \pm 2\%$). This was not observed when WT U1 snRNA was co-transfected along with the mutated pTB minigene ($3 \pm 2\%$ inclusion) (Fig. 2).

Discussion

Previously, the PPOX gene from the three unrelated patients presenting VP symptoms was screened for mutations, finding a single base insertion at the exon 4 (c.338+3insT), a transition in the last base of exon 7 (c.807G>A), and a transversion in the last base of intron 7 (c.808-1G>C) (Rossetti et al. 2008). These mutations were thought to affect splicing as c.338+3insT could affect 5' ss definition, c.808-1G>C affect one of the universally conserved dinucleotides of the 3' ss, and c.807 G>A, the only exonic alteration, does not lead to an amino acid change. To verify this, these alterations were studied by RT-PCR using the patient's mRNA; however, we found only the normal transcript or no amplification occurred (Rossetti et al. 2008). A possible explanation why we were not able to find the mutant transcript would be that the mRNA was being degraded through a process such as nonsense-mediated decay (NMD), as in all three cases skipping of the exon leads to a premature stop codon. In order to test whether these mutations are affecting splicing or not, we used a minigene approach.

The mutation c.808-1G>C was shown to result in the skipping of the exon 8 of the PPOX gene. Considering that this alteration is affecting directly the 100% conserved AG dinucleotide present at the intron-exon junction (Zhang 1998), that NNSplice program predicts a total loss of the acceptor site, and that the experimental evidence shows that in fact the exon is being skipped, there is no doubt that the effect of this mutation on the PPOX gene is exon skipping.

With regard to the mutation c.338+3insT, although the WT minigene shows only $37 \pm 5.8\%$ and $43.5 \pm 6.5\%$ inclusion of the exon for the short and large versions, respectively, as opposed to the 100% expected under normal conditions, the effect the mutation has on this sequence is clear as the mutant constructs show nearly $100 \pm 0\%$ exon exclusion.

In the case of the mutation c.807G>A, the results show quite clearly that its effect is to cause exon skipping. Taking into account that there is no ESE affected by this mutation, as predicted by Rescue ESE and ESE Finder, the mutation might be disrupting U1 snRNA binding site. We decided to further investigate this latter hypothesis by making an U1snRNA complementary to the mutation, which lead to a partial rescue of the exon. Taken together, the bioinformatics and experimental data indicate that the mutation is disrupting the U1 snRNA binding site.

In the cases of the three mutations resulted in exon skipping, a premature stop codon is generated in the following exon. As previously described (Maquat 2004), a premature stop codon leads the mRNA to NMD, a mechanism which selectively degrades them explaining the difficulty experienced in obtaining an amplicon

corresponding to these alleles from the patient's mRNA. Through the use of minigenes, we have now however demonstrated that these three mutations are responsible for VP in the patients who carry them through alterations in the normal splicing pathway.

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Synopsis

Characterization of splicing mutations by a minigene approach

Details of Contribution of Individual Authors

Granata BX: carried out the experimental studies and analysis of data and draft the manuscript
Baralle M: supervision of experimental studies in Italy and revising the manuscript
De Conti L: helped in experimental studies
Parera VE: supervision of biochemical studies in Argentina
Rossetti MV: guarantor; conception, design, and supervision of the whole work; and revising the manuscript

Conflict of Interest

Bárabra X Granata, Marco Baralle, Laura De Conti, Victoria E Parera, and María V Rossetti declare that they have no conflict of interest.

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The authors confirm independence from the sponsors; the content of the article has not been influenced by the sponsors.

Details of Ethic Approval

Written informed consent was obtained from all patients prior to their inclusion in the study. The study protocol was approved by the Ethical Committee of the Centro de

Investigaciones sobre Porfirinas y Porfirias (CIPYP – Hospital de Clínicas, CONICET) and was performed in accordance with the Helsinki Declaration of 1964 and its modifications (Tokio, Japón 1975; Venecia, Italia, 1983; Hong Kong, 1989; SudÁfrica, 1996; Edimburgo, Escocia, 2000; Washington 2002; Tokio, 2004; Seúl, Corea, 2008; Fortaleza, Brasil, 2013).

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