Molecular Genetics and Metabolism 94 (2008) 343–346

Contents lists available at ScienceDirect

Molecular Genetics and Metabolism

journal homepage: www.elsevier.com/locate/ymgme

Characterization of two missense variants in the hydroxymethylbilane synthase gene in the Israeli population, which differ in their associations with acute intermittent porphyria

Xiaoye Schneider-Yin [a,1], Dana Ulbrichova [b,1], Rivka Mamet [c], Pavel Martasek [b], Christopher C. Marohnic [d], Avner Goren [e], Elisabeth I. Minder [a], Nili Schoenfeld [c,f,*]

[a] Zentrallabor, Stadtspital Triemli, Zürich, Switzerland
[b] Department of Pediatrics and Center for Applied Genomics, 1st School of Medicine, Charles University, Prague, Czech Republic
[c] National Laboratory for the Biochemical Diagnoses of Porphyrias, Rabin Medical Center, Beilinson Hospital, Petah-Tikva, Israel
[d] Department of Biochemistry, The University of Texas Health Science Center at San Antonio, San Antonio, TX, USA
[e] Department of Pediatrics, Safra Children's Hospital, Sheba Medical Center, Tel Hashomer, Israel
[f] Department of Human Molecular Genetics and Biochemistry, The Sackler Faculty of Medicine, Tel Aviv University, Tel Aviv, Israel

ARTICLE INFO

Article history:
Received 6 March 2008
Accepted 6 March 2008
Available online 11 April 2008

Keywords:
Acute intermittent porphyria
Hydroxymethylbilane synthase
Missense mutation
In vitro expression

ABSTRACT

Acute intermittent porphyria (AIP) is an autosomal dominant disorder of heme biosynthesis caused by molecular defects in the hydroxymethylbilane synthase (HMBS) gene. In this study, we report two novel missense sequence variations in the HMBS gene, T59I (C176T) and V215M (G643A), in two patients with clinical symptoms compatible with acute attacks of porphyria. However, only the patient who carried V215M presented with full AIP-affirming biochemical evidence. Both variant proteins were expressed in a prokaryotic system and characterized in vitro. Recombinant T59I and V215M had residual activity of 80.6% and 19.4%, respectively, of that of the wild type enzyme. Moreover, changes in K_m, V_{max} and thermostability observed in the recombinant V215M suggest a causal relationship between V215M and AIP. The association between the T59I substitution and AIP is less obvious. Based on our investigation, substitution T59I is more likely to be a mutation with a weak effect than a rare form of polymorphism. This study demonstrates that in vitro characterization of missense variations in the HMBS gene can provide valuable information for the interpretation of clinical, biochemical and genetic data, for establishing a diagnosis of AIP. It also highlights the fact that there are still many aspects to be investigated concerning AIP and corroborates the need to report new data that can help to clarify the genotype–phenotype relationship.

Introduction

Acute intermittent porphyria (AIP, MIM #176000) is an autosomal dominantly inherited disorder of heme biosynthesis resulting from an ~50% deficiency of hydroxymethylbilane synthase activity (EC 4.3.1.8; HMBS). This enzyme, also known as porphobilinogen deaminase, catalyzes the head-to-tail condensation of four molecules of porphobilinogen (PBG) to form hydroxymethylbilane. Clinically, AIP is characterized by acute intermittent neurovisceral attacks that can be provoked by various factors such as drugs, hormones and alcohol [1]. Biochemical diagnosis of AIP is based on measurement of the urinary porphyrin precursors, δ-aminolevulinic acid (ALA) and PBG, in combination with the determination of erythrocyte HMBS activity. Molecular analysis of the HMBS gene has been shown to be more efficient than enzymatic analysis in detecting latent AIP patients who do not excrete excess amounts of ALA and PBG in the urine [2,3].

The locus for this disorder has been mapped on chromosome 11q24.1–q24.2 [4]. The length of the HMBS gene is ~10 kb, and the cDNA, encoded by 15 exons, is 1.4 kb, with a single open reading frame of 1038 bp [5,6]. Two distinct promoters, located in the 5′ flanking region and in intron 1, respectively, generate housekeeping (contains exon 1 and 3–15) and erythroid-specific (contains exon 2–15) transcripts by alternative splicing of exon 1 and 2 [7].

To date, a total of 301 different mutations have been identified in the HMBS gene [8]. Among them, 97 mutations (32%), were missense. Most of the missense mutations were documented only at the DNA level, while only a small fraction was expressed and characterized in vitro [9–12]. The availability of the crystal structure of *Escherichia coli* HMBS made it possible to postulate the molecular

* Corresponding author. Address: National Laboratory for the Biochemical Diagnoses of Porphyrias, Rabin Medical Center, Beilinson Hospital, Petah-Tikva 49100, Israel. Fax: +972 3 9377768.
E-mail address: nschoenfeld@clalit.org.il (N. Schoenfeld).
[1] Both authors contributed equally to this work.

1096-7192/$ - see front matter © 2008 Elsevier Inc. All rights reserved.
doi:10.1016/j.ymgme.2008.03.001

and functional consequences of missense mutations in the human enzyme because the *E. coli* and human HMBS amino acid sequences have 35% homology and more than 70% similarity [13]. Nevertheless, in vitro expression remains a powerful tool in studying HMBS gene mutations in the absence of information on the tertiary structure of the human enzyme.

In this study, we report on mutation analysis and in vitro characterization of HMBS variants identified in two individuals who were suspected of having AIP. One of the two subjects, however, presented with biochemical features that were not fully compatible with the AIP diagnosis.

Materials and methods

Subjects and clinical presentations

Two Ashkenazi Jewish females were studied. The first subject aged 17, has been experiencing menstruation-related recurrent episodes of severe abdominal pains accompanied by vomiting, tachycardia and hypertension, for the last three years. Biochemical analyses failed to show elevations in urinary PBG (Table 1) and ALA (not shown) levels during three latent and four acute phases.

Normal fecal porphyrin profile and fluorometric plasma scan were recorded (not shown). However, erythrocyte HMBS activity, measured in three independent occasions, showed an average activity of 60% of that of normal (the enzyme activity among our AIP patients ranged from 38% to 61% of that of normal). Since no other diagnosis besides AIP was established, the patient was treated with either glucose or heme-arginate (Normosang®) for acute attacks. Both substances were proven to be effective. Genetic testing was performed on the patient in order to clarify the conflicting situation between clinical symptoms and biochemical analyses.

The second subject aged 30, was suffering from recurrent acute attacks of abdominal pain, hyponatremia and urinary retention for 12 years. She was diagnosed as having AIP during an acute attack three years ago, treated with glucose, and has remained symptom-free since then. The diagnosis was established on the basis of increased urinary ALA and PBG, and reduced erythrocyte HMBS activities to 61% and 50% of normal, measured two months and three years after her last acute attack, respectively (Table 1).

Peripheral blood samples were collected from both subjects and their family members, as well as from 50 non-porphyric subjects of Jewish origin (Ashkenazi) with informed consent. This study was approved by the Helsinki committee of the Israeli Ministry of Health.

Mutation analysis

Genomic DNA was isolated from peripheral blood by using the QIAamp Blood Kit (Qiagen). The 15 coding exons of the HMBS gene, along with more than 60 bp of their flanking regions, were amplified by PCR using specific primers (Genbank Accession Numbers, HMBS gene, M95623; HMBS cDNA, NM000190) as we published previously [14,15]. Sequence analysis was performed on an ABI Prism 310 genetic analyzer (Applied Biosystems).

Plasmid construction and mutagenesis

An EDTA-anticoagulated blood sample from a healthy volunteer was used for RNA extraction. Total RNA was reverse transcribed into cDNA by RT-PCR using an oligo(dT) primer. cDNA of the HMBS gene was obtained by PCR using sequence-specific primers with restriction sites BamHI and XhoI attached to the 5′- and 3′-end, respectively (forward: 5′ata tgg atc cat gtc tgg taa cgg 3′ and reverse: 5′tat act cga gtt aat ggg cat cgt taa 3′. The HMBS cDNA was ligated into an expression vector, pGEX-4T-1 (Amersham Pharmacia Biotech) and then transformed into *E. coli BL21* (*DE3*) (Stratagene). Plasmid DNA containing the HMBS sequence was purified by using the QIAprep Spin Miniprep Kit (Qiagen). Site-directed mutagenesis was performed by using the QuikChange® Site-directed Mutagenesis Kit (Stratagene). Primers 5′agt ttg aaa tca ttg cta tgt cca cca **t**ag ggg aca aga ttc 3′ (forward) and 5′gaa tct tgt ccc ct**a** tgg tgg aca tag caa tga ttt caa act 3′ (reverse) were used to generate variant T59I; primers 5′cct gag aaa tgc atg tat gct **a**tg ggc cag ggg 3′ (forward) and 5′ccc ctg gcc ca**t** agc ata cat gca ttt ctc agg 3′ (reverse) were used to generate variant V215M (the sites of mutagenesis are *in bold* and *underlined*). The results of the mutagenesis were confirmed by sequencing.

In vitro expression and purification of variants and wild type enzyme

Variants and wild type enzyme were expressed as GST-fusion proteins in *BL21* cells using a standard technique. Bacterial cells were subjected to protein purification using a Glutathione Sepharose 4B column (Amersham Biosciences). The purity of the proteins was confirmed on SDS–PAGE.

Enzymatic assay in recombinant enzymes

The HMBS activity assay was carried out according to a previously published method [16]. One microgram of purified enzyme was diluted with an incubation buffer containing 50 mM Tris, 0.1% BSA, 0.1% Triton, pH 8.2, to a final volume of 360 μl. After pre-incubation of the sample at 37 °C for 3 min, 40 μl of 1 mM PBG (ICN Biomedicals) was added. The sample tube was further incubated in the dark for 1 h before 400 μl of 25% TCA were added to terminate the reaction. The samples were subjected to photooxidation under daylight for 60 min and were then centrifuged for 10 min at 1500*g*. Fluorescence intensity was measured in the supernatant immediately after centrifugation using a Perkin-Elmer LS 55 spectrofluorometer. The fluorescence wavelengths were set at 405 nm for excitation and 599 nm for emission. Uroporphyrin I (URO I) purchased from ICN Biomedicals, was used as a standard for calculation of enzyme activity. Protein concentration was determined by the Lowry method. One unit of HMBS activity was defined as 1 nmol URO/h/μg protein.

To determine K_m values of the various recombinant enzymes, a series of substrate concentrations was used, i.e., 1, 2.5, 5, 10, 20, 25, 50, 75, 100 and 150 μM of PBG in the final reaction mixture. The time of incubation varied from 1 to 9 min. To determine optimal pH, HMBS activity was measured within a range from pH 7.0 to pH 9.0. To study thermostability, enzymes were pre-incubated at 65 °C at pH 8.2 for 0, 30, 60, 90, 120, 180 and 240 min, respectively, before activity measurements. K_m and V_{max} values were calculated by Sigma Plot using a single rectangular hyperbolic curve (Michaelis–Menten curve).

Results

Direct sequencing of PCR-amplified HMBS gene fragments from the first subject unveiled C to T transition at nucleotide position 176 in exon 5 resulting in the substitution of Thr59 by an isoleucine residue (T59I). This missense variation was absent in the HMBS gene of her mother, the only family member available for testing. A different missense variation G643A (V215M) was identified in the second subject, as well as in members of her family, including her father, her 3-year-old son, and two of her paternal cousins. Both subjects were heterozygous for the respective sequence variations. Both T59I and V215M were absent in the HMBS gene of 50 non-porphyric Ashkenazi Jewish subjects.

To examine the effect of these missense variations on the HMBS enzyme, T59I and V215M were expressed in a prokaryotic system and purified to near homogeneity based on SDS–PAGE analysis (data not shown). A series of experiments were then carried out using purified enzymes in order to further characterize the two variants.

HMBS activity was measured in triplicate in all three recombinants enzymes, T59I, V215M and wild type. The mean activity of

Table 1
Biochemical, enzymatic and genetic data

	Biochemical analyses			Genetic analysis	In vitro enzymatic studies		
	Urinary PBG (μmol/24 h)[a]		Erythrocyte HMBS activity (% normal)[b]	Sequence variation in HMBS gene	K_m (μM)	V_{max} (nmol/min)	Optimal pH
	Latent	Acute					
Subject 1	6.2 ± 1.7 (n = 3)	8.4 ± 1.4 (n = 4)	60[c]	C176T, T59I	6.6 ± 0.3	1.3 ± 0.1	7.8
Subject 2	92.8	287.3	50[c]	G643A, V215M	70.0 ± 16.3	0.4 ± 0.1	8.0
				Wild type	4.2 ± 0.5	2.1 ± 0.1	8.2

[a] Normal value: <8.8 μmol/24 h.
[b] Normal value: >70%.
[c] Activity measured in the latent phase.