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Spectrum of mutations in Lebanese patients with phenylalanine hydroxylase deficiency☆

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ABSTRACT

Phenylketonuria is an autosomal recessive inborn error of metabolism resulting from phenylalanine hydroxylase deficiency. Genetic basis of phenylalanine hydroxylase deficiency has been reported in various European and Asian countries with few reports available in Arab populations of the Mediterranean region. This is the first pilot study describing phenotype and genotype of 23 Lebanese patients with phenylketonuria. 48% of the patients presented mainly with neurological signs at a mean age of 2 years 9 months, as newborn screening is not yet a nationwide policy. 56.5% of the patients had classical phenylketonuria. Thirteen different mutations were identified: splice site 52%, frameshift 31%, and missense 17% with no nonsense mutations. IVS10-11G>A was found mainly in Christians at high relative frequency whereas Muslims carried the G352fs and R261Q mutations. A rare splice mutation IVS7+1G>T, not described before, was identified in the homozygous state in one family with moderate phenylketonuria phenotype. Genotype–phenotype correlation using Guldberg arbitrary value method showed high consistency between predicted and observed phenotypes. Calculated homozygosity rate was 0.07 indicating the genetic heterogeneity in our patients. Our findings underline the admixture of different ethnicities and religions in Lebanon that might help tracing back the PAH gene flux history across the Mediterranean region.

1. Introduction

Phenylalanine (Phe) is a precursor of many important biomolecules such as melanin, thyroid hormones, and catecholeamines. Alternatively the main pathway of Phe metabolism is its conversion into tyrosine a reaction catalyzed by the enzyme phenylalanine hydroxylase (PAH) (Scriver, 2007; Williams et al., 2008). A deficiency in PAH underlies the autosomal recessive metabolic disorder phenylketonuria (PKU; MIM 261600). Clinical manifestations of this disorder affect mainly the central nervous system with variable psychomotor delay, mental retardation and seizures associated to digestive symptoms as recurrent nausea and vomiting. In some cases, cutaneous manifestations, as eczema, are also observed (Blau et al., 2010; Scriver and Kaufman, 2001). However, with the implementation of newborn screening, most patients are early diagnosed nowadays before clinical symptoms develop (Blau et al., 2010). Treatment of PKU is based classically on Phe dietary restriction and/or tetrahydrobiopterin (BH_4) supplementation with variable responsiveness (Lassker et al., 2002). The human PAH gene maps to 12q23.2 and it comprises 13 exons and 12 intervening sequences (IVS) spanning approximately 90 kb (Scriver et al., 2003). More than 560 mutations have been reported in the PAH database (www.pahdb.mcgill.ca, last accessed November 8, 2012). The various phenotypes show that extensive mutant allelic heterogeneity is the cause of PKU and related hyperphenylalaninemias forms (Scriver, 2007). Molecular basis of PAH deficiency and genotype–phenotype correlation have been reported, so far, in various European Middle Eastern populations. However, few reports are available in Arab populations in the Mediterranean region from Egypt (Effat et al., 2008), Israel (Bercovich et al., 2008a), Morocco (Dahri et al., 2010), and Tunisia (Khemir et al., 2012).

In this pilot study, we report and analyze for the first time the spectrum of mutations and polymorphisms in the PAH gene of 23 Lebanese PKU patients from 20 different families.

2. Materials and methods

2.1. Patients

Twenty-three PKU patients from 20 unrelated families were recruited in this study from the Inborn Errors of Metabolism Clinic at the American University of Beirut–Medical Center which represents the only referral center for such disorders in Lebanon (Khneisser et al., 2008). Demographic data were collected including age, consanguinity, family history, religion, and geographical origin. PKU patients with PAH deficiencies were diagnosed by newborn screening (NBS) or following clinical presentation. Biochemical testing of all subjects included plasma

Abbreviations: AV, Arbitrary Value; BH4, Tetrahydrobiopterin; HR, Homozygosity rate; IVS, Intervening sequences; MHP, Mild Hyperphenylalaninemia; PAH, Phenylalanine hydroxylase; Phe, Phenylalanine; PKU, phenylketonuria; PRA, Predicted residual activity.

☆ Conflict of interest: The authors declare no conflict of interest.

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amino acid levels determination using ion exchange chromatography, dehydropteridine reductase activity in blood and urinary biopterin and neopterin ratio. Biopterin metabolism testing was performed in France (Biochemistry Laboratory-Philbert Hospital). Only patients with Phe level > 120 μmol/L and normal biopterin metabolism testing were included in this study.

The study was approved by the Institutional Review Board of the American University of Beirut (Protocol # PED.PK.01).

2.2. Genetic screening of the PAH gene

Blood samples were collected from the patients and genomic DNA was extracted following standard procedures. Primers spanning the exons, including the splice sites in the intronic regions, were designed using PRIMER3 software (http://frodo.wi.mit.edu/cgi-bin/primer3/primer3) and used to amplify the various 13 exons by PCR, in addition to intron 10 (Table 1). Amplified amplicons were separated by agarose electrophoresis, extracted and purified using nucleospin Extract II (Machery-Nagel) extraction kits. The purified amplicons were then sequenced using Avant Genetic Analyzer, AB3130 A, machine. Three databanks were accessed: the PAH database, NCBI: Blast (http://www.ncbi.nlm.nih.gov/BLAST), and BLAT at UCSC (http://www.genome.ucsc.edu/cgi-bin/hgBlat, last accessed November 8, 2012) to check for mutations and polymorphisms in the PAH gene.

2.3. Calculation of homozygosity rate (HR)

Homozygosity rate, an indicator of the extent of genetic variation, was calculated based on the allele frequency of the studied patients (Guldberg et al., 1998).

2.4. Clinical classification

Phenotypes of PKU patients were categorized according to their pre-treatment plasma Phe level at diagnosis and their daily dietary Phe tolerance into four categories (as per Guldberg et al., 1998): classic PKU (Phe level > 1200 μmol/l and dietary Phe tolerance less than 250–350 mg/day); moderate PKU (600 μmol/l < Phe level < 1200 μmol/l and dietary Phe tolerance between 350 and 400 mg/day); mild PKU (360 μmol/l < Phe level < 600 μmol/l and dietary Phe tolerance between 400 and 600 mg/day); and mild hyperphenylalaninemia (MHP) in patients with Phe < 360 μmol/l on a normal diet. Alternatively, phenotype was predicted by applying the Guldberg system to patients where two mutations have been identified (Guldberg et al., 1998). Each mutation was assigned an arbitrary value (AV): AV = 1 for classic PKU mutation, AV = 2 for moderate PKU mutation, AV = 4 for mild PKU mutation, and AV = 8 for MHP mutation. The choice of the arbitrary values was based on the least positive whole number that allows the differentiation between the different mutation combinations. In heterozygote state, the less severe of the two mutations determines the phenotype of the patient, and two mutations with similar severity will cause a milder phenotype than if each mutation was expressed alone, i.e. in homozygote state.

Predicted residual activity (PRA), based on the in vitro expression studies according to PAH database (http://www.pahdb.mcgill.ca) and Biopku database (http://www.biopku.org, last accessed November 8, 2012), and the predicted phenotype were correlated to the observed phenotype for each mutation (Berchovich et al., 2008b; Daniele et al., 2009; Mallolas et al., 1999; Santos et al., 2010; Stojiljkovic et al., 2006; Zurflüh et al., 2008).

2.5. Tetrahydrobiopterin responsiveness

The optimized 48-h tetrahydrobiopterin loading protocol was adopted (Blau et al., 2009). BH_4 was administered at a dose of 20 mg/kg/day for two consecutive days and plasma Phe level was taken at $T = 0$, 8, 16, 24 and 48 h. The simplified criteria to define BH_4 responsiveness proposed by Karacic et al., 2009 were applied. A patient was considered as "responder," if there was a reduction of blood Phe by ≥30% within 24 h and "slow responder," if a reduction of blood Phe by <20% at T_8 and ≥20% but <30% at T_{24} was observed.

Table 1
Primers used in amplifying the different exons comprising the phenylalanine hydroxylase gene, size and annealing temperature (TM) for each set of primers. *: amplified amplicons between exons 10 and 11, including intron 10, Bp: base pair, F/R: forward/reverse, (°C): degree Celsius.

Exon	Length (bp)	Primers (F/R)	TM (°C)
E1	60	F: 5′ TTAAAACCTTCAGCCCCACG 3′ R: 5′ TGGAGGCCCAAATTCCCCTAACTG 3′	65
E2	108	F: 5′ GAGGTTTAACAGGAATGAATTGCT 3′ R: 5′ TCCTGTGTTCTTTTCATTGC 3′	55
E3	183	F: 5′ GCCTGCGTTAGTTCCAGTGA 3′ R: 5′ CTTATGTTGCAAAATTCCTC 3′	60
E4	90	F: 5′ ATGTTCTGCCAATCTGTACTCAGGA 3′ R: 5′ CATCCTACGGGCCATGGACT 3′	65
E5	68	F: 5′ TCATGGCTTGAGAGCCCCCA 3′ R: 5′ AAGCAGGCTAGGGGTGTGTTTTTC 3′	60
E6	191	F: 5′CCGACTCCCTCTGCTAACCT 3′ R: 5′ CAATCCTCCCCCAACTTTCT 3′	60
E7	136	F: 5′ GGTGATGAGCTTTGAGTTTTCTTTC 3′ R: 5′ CAGCAATGAACCCAAACCTC 3′	65
E8	70	F: 5′ TGGCTTGGCTTAAACCTCCTCCCCT 3′ R: 5′ CTGGGCTCAACTCATTTGAG 3′	65
E9	57	F: 5′ ATGGCCAAGTACTAGGTTGG 3′ R: 5′ GAGGGCCATAGCCTATAGCA 3′	60
E10–E11*	786	F: 5′ TTAACCATCATAGAGTGTGC 3′ R: 5′ GCCAACCACCCACAGATGAG 3′	60
E12	116	F: 5′ ATGCCACTGAGAACTCTCTT 3′ R: 5′ GATTACTGAGAAACCGAGTGGCCT 3′	60
E13	41	F: 5′ GACACTTGAAGAGTTTTTGC 3′ R: 5′ TTTTCGGACTTTTTCTGATG 3′	60

3. Results

3.1. Demographic and clinical data

Patients were recruited from different regions in Lebanon: North Lebanon (34, 7%), Beirut (17.3%), South Lebanon (17.3%), Mount Lebanon (13%) and Bekaa valley (13%).

Phenotypic classification of the patients revealed 56.5% classical PKU, 26% moderate PKU, 4.5% mild PKU and 13% mild HPA (Table 2). The largest proportion of these patients, 56.5%, was Muslims; Christians and Druze were affected to a lower extent, 39.5% and 4% respectively. First degree consanguinity rate was 43%. However, no consanguinity was noted in Christian families. A positive family history of PKU was found in 47.8%.

Around half of the recruited patients were asymptomatic, diagnosed by NBS during the first 2 weeks of life, while 48% were symptomatic presenting at different ages (mean age 2 years 9 months). All late-diagnosed patients presented with neurological signs: mainly psychomotor delay in 54%, associated to intractable seizures in 36%, whereas variable mental retardation was observed in 45%. Poor feeding, recurrent vomiting and nausea were observed in 73% and eczema in 18% (Table 2).

Upon diagnosis, Phe dietary restriction was implemented with variable outcome. Two of the late diagnosed patients presenting with moderate psychomotor delay at 2 years of age showed marked improvement in motor tone after Phe dietary restriction (Table 2: patients number 2 and 16).

3.2. Spectrum of mutations and polymorphisms

A total of 23 patients from 20 different families were screened for mutations in the *PAH* gene: 34.7% were homozygous, 26% compound