RESEARCH NOTE

Screening of three Mediterranean phenylketonuria mutations in Tunisian families

SAMEH KHEMIR[1], HAJER SIALA[3], SAMEH HADJ TAIEB[1], WAFA CHERIF[4], HATEM AZZOUZ[2], RYM KÉFI[4], SONIA ABDELHAK[4], NAZIHA KHOUJA[5], NEJI TEBIB[2], TAIEB MASSAOUD[3], MARIE FRANÇOISE BEN DRIDI[2] and NAZIHA KAABACHI[1]*

[1]*Laboratory of Biochemistry and* [2]*Department of Paediatrics, Rabta Hospital, 1007 Jebbari, Tunis, Tunisia*
[3]*Laboratory of Biochemistry and Molecular Biology, Children's Hospital, 1007 Jebbari, Tunis, Tunisia*
[4]*Research Unit UR04/SP03, Pasteur Institute, 1002 Tunis-Belvedere, Tunis, Tunisia*
[5]*Department of Paediatrics, Neurology Institute, 1007 Jebbari, Tunis, Tunisia*

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Introduction

Phenylketonuria (PKU; OMIM 261600) is an autosomal recessive disease caused by the liver phenylalanine hydroxylase (PAH) enzyme (EC1.14.16.1) deficiency. If untreated, causes mental retardation. The incidence in Caucasian population is approximately 1:10,000 (Scriver and Kaufman 2001). In Tunisia, it seems to be more frequent with a prevalence of 1 in 7631 (Khemir *et al.* 2011).

To this date, more than 530 mutations in the *PAH* gene (12q22-q24) have been described (http://www.pahdb.mcgill.ca). The characterization of PKU mutations has been made in many countries, all over the world; nevertheless, to date few studies have been reported on the North African populations.

In Mediterranean countries, several mutations have been reported. The most common: IVS10–11G>A seems to be widespread. The G352Vfs delG was reported in Algerian, Italian, French Canadian, Croatian and Lebanese populations (Lyonnet *et al.* 1989); in Morocco, it was described as the most frequent mutation (Dahri *et al.* 2010). The E280K mutation was also reported in Mediterranean populations (Guldberg *et al.* 1993). Since Tunisia is a Mediterranean country, patients with PKU are presumed to have these mutations.

The aim of this study was to assess prevalence of the three above mutations among PKU patients collected from paediatric departments of hospitals in Tunis.

Materials and methods

A total of 55 patients belonging to 38 families with PAH deficiency were included in the study. These patients came to our laboratory (Laboratory of Biochemistry, La Rabta Hospital, Tunis) for metabolic evaluation: the metabolic unit of La Rabta Hospital serves as a reference centre, accepting patients suspected with metabolic diseases from all over the country.

Patients were recruited from the two paediatric departments involved in monitoring the metabolic diseases (Department of Paediatrics, La Rabta Hospital and Department of Neuropaediatrics of the National Institute of Neurology in Tunis).

The diagnosis age ranged from 4 days to 16 years with an average of 3 years 6 months (the median age was 24 months). Only seven out of 55 patients were diagnosed during the neonatal period (4 days to 1 month), and they had antecedent familial history of PKU. Majority of the cases presented with mental retardation and motor delays. PKU diagnosis was confirmed after exclusion of tetrahydrobioterin deficiency for patients showing high values of phenylalanine serum level (Phe $>$ 240 μmol/L). On the basis of blood Phe concentrations prior to treatment, the PAH deficiency can be classified into classical PKU (Phe $>$ 1200 μmol/L), moderate PKU (600 $<$ Phe $<$ 1200 μmol/L) and mild hyperphenylalaninaemia (HPA) (mild HPA $<$ 600 μmol/L) (Abadie *et al.* 2005).

After obtaining informed consent, genomic DNA was extracted from peripheral blood leucocytes using the salting out method (Miller *et al.* 1988). To study the frequency of each mutation in the PKU Tunisian patients, we considered only one case per related family and thus

*For correspondence. E-mail: naziha.kaabachi@rns.tn; naziha.kaabachi@gmail.com.

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analysed 35 unrelated patients (70 alleles). Since the clinical phenotype depended on several environmental parameters, the total number of patients was investigated (55 patients; 110 alleles) for the analysis of genotype/phenotype correlation. Three exons 7, 10 and 11 were amplified using specific primers designed using Primer3 software (http://frodo.wi.mit.edu/primer3/input.htm).

The PCR mixture (total volume of 50 μL) contained 200 ng DNA, in 1× buffer: KCl (50 mM), Tris-HCl (10 mM), $MgCl_2$ (5 mM), 2 mM of each dNTP, 1 U *Taq* polymerase and 10 pmol of each of the primers. Cycle conditions used for amplification were 5 min at 95°C, then 40 cycles of 1 min at 95°C, 1 min at an appropriate melting temperature and 1 min at 72°C, ended by a prolonged extension step of 7 min at 72°C.

The E280K and G352Vfs delG were screened by *Msp*I and *Hph*I (Fermentas, California, USA), respectively and IVS10–11G>A mutation was screened by *Dde*I (Promega, Madison, USA) digestion as recommended by the manufacturers.

Exon 7 was amplified using primers specific to create a restriction site for the *Msp*I enzyme; this site was abolished in the presence of the E280K mutation (normal allele: 272+26 bp and mutant allele 298 bp). The specific primers are: ex7 F: 5′-AGACATCTGAAGCCAAGTCTG-3′ and ex7 R: 5′-GGAGGACAGTACTCACGGTC-3′. The used melting temperature in the PCR programme was 60°C.

For detection of the G352Vfs delG, exon 10 was amplified with following primers; ex10 F: 5′-CCCCAAAATAATGCTTTACTATCT-3′ and ex10 R: 5′-ACGGATACAAATAGGGTTTC-3′, the melting temperature used in the PCR programme was 57°C. In presence of the G352Vfs delG the specific site of the *Hph*I was abolished, the amplified fragment of 300 bp size is cut in the absence of the mutation in three fragments (63+141+96) and in the presence of the mutation in 2 fragments (63 + 237).

Exon 11 was amplified by the following primers; ex11 F: 5′-AATCGGGGTGAGATGAGAG-3′ and ex11 R: 5′-TAGACATTGGAGTCCACTCTC-3′, the melting temperature used in the PCR programme was 60°C. The mutation IVS10–11G>A was highlighted if the fragment of 338 bp which was cut in two by the enzyme *Dde*I (92+246).

For confirmation, one allele for each mutation was sequenced. Statistical analyses were carried out using Epi Info 6 software (http://wwwn.cdc.gov/epiinfo/html/ei6_downloads.htm).

Results and discussions

Molecular analysis

The testing for the mutation (IVS10–11G>A) showed four homozygous (IVS10–11G>A/ IVS10–11G>A) and one heterozygous case (IVS10–11G>A/?) among the 35 studied patients, giving an allele frequency of 0.12 (figure 1). For the mutation G352Vfs delG, five patients were homozygous (G352Vfs delG/G352Vfs delG) and one heterozygous (G352Vfs delG/?) giving an allele frequency of 0.15. The E280K was identified in six patients at homozygous state (E280K/E280K); this mutation with an allele frequency of 0.17 was predominant (12/70) (table 1). Mutations were identified in 32 out of 70 Tunisian PKU alleles, with a mutation detection rate of 0.45.

The results obtained in this study showed that the most frequent mutation observed in Tunisia was the E280K that accounts for 0.17; it is higher than described in north Mediterranean: France (0.06), Greece (0.05) and Spain (0.04) (Zschocke 2003).

The G352Vfs delG mutation was the second most frequent mutation (0.15); it has been reported in Morocco as the most frequent with 0.62 (Dahri *et al.* 2010). In Spain, this mutation is also common, probably originating from the Spanish and Andalusian migration flows during the 7th century. In fact after the reconquista, the Arab Andalusians migrated extensively from Spain to Morocco and Tunisia. In Tunisia, they settled in northeastern cities and villages along Medjerda Valley and Bizerte region.

The IVS10–11G>A, that was described as the most frequent mutation in *PAH* gene in the Mediterranean basin, unexpectedly accounted only for 0.12 of our series. There was a decreasing gradient of the frequency of this mutation from the east to the west of Mediterranean: 0.32 for Turkey (Ozguk *et al.* 1993), 0.25 for Bulgaria (Berthelon *et al.* 1991), 0.17 for Egypt (Goltsov *et al.* 1994), 0.15 for south

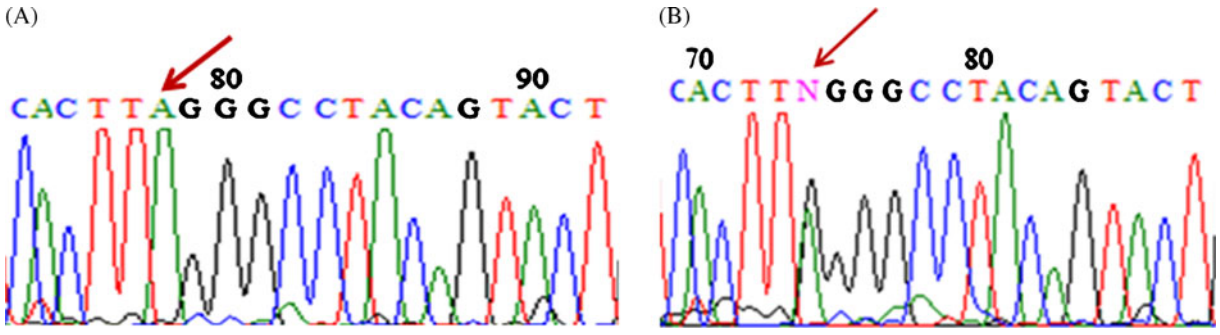

Figure 1. Sequencing results for the mutated region: I10-E11 of the *PAH* gene at homozygous state (A) and heterozygous state (B). The arrow shows the mutation that converts G to A.

Table 1. Geographical distribution of affected PKU alleles.

Mutation	Affected alleles					
	East Tunisia		West Tunisia		Total	
	Number	Frequency	Number	Frequency	Number	Frequency
G352Vfs delG	7	0.15	4	0.16	11	0.15
E280K	10	0.22	2	0.08	12	0.17
IVS10–11G>A	0	0	9	0.36	9	0.12
Total characterized alleles	17	0.37	15	0.6	32	0.45
Total studied alleles	45	–	25	–	70	–

Italy (Dianzani *et al.* 1994) and 0.11 for Spain (Perez *et al.* 1992). Scriver proposed that the distribution of this mutation was suggestive of a Turkish origin and a subsequent spread throughout the Mediterranean basin (Scriver and Kaufman 2001).

In total, the genotypes of 15 patients were definitively identified; all were homozygous (four IVS10–11G>A/IVS10–11G>A, six E280K/E280K and five G352Vfs delG/G352Vfs delG). For two patients although one deficient allele was identified (IVS10–11G>A/? and G352Vfs delG/?) but more thorough investigations are needed to determine the other allele.

Genotype/phenotype correlation

The phenylalanine serum level at diagnosis was measured for the 55 patients and the mean was 1698 μmol/L (28.3 mg/dL) ranging from 810 to 3090 μmol/L (13.5 to 51.5 mg/dL). According to the classification of Abadie *et al.* (2005) based on phenylalanine serum level data before treatment, the total patients (55) were classified into two PKU phenotype classes: 42 patients had classical PKU (76.3%) and 13 had the moderate PKU form (23.6%). The three mutations had occurred in both the phenotypic classes of PKU. The association of PKU phenotype classes with mutated alleles is presented in table 2.

Since the patients having the mutation IVS10–11G>A were more common in the moderate than in classical form (7.1% of classical and 26.9% of moderate forms), this study could suggest that IVS10–11G>A would rather be associated with the moderate forms; this was confirmed by statistical results ($P = 0.006$).

This was also the case for two other mutations; the frequencies of the E280K and the G352Vfs delG mutations in the classical form (21.4% and 27.3% respectively) suggested that the two mutations would associated with the classical form, nevertheless the statistical data do not corroborate these findings since the number of the studied alleles was too small. Both mutations were associated with the severe phenotype of the disease: mental retardation, psychomotors delays, anomalies of pigmentation and mousy odour of urine.

These mutations were reported in different phenotypic classes of PKU: the G352Vfs mutation at the heterozygous state was reported in the two classes of PKU: classical and moderate (Dahri *et al.* 2010). The IVS10–11G>A mutation was present in the three different classes: classical (Dianzani *et al.* 1995), moderate (Perez *et al.* 1994) and mild HPA (Romano *et al.* 1996). The E280K mutation was reported essentially at the homozygote state in classical PKU (Lyonnet *et al.* 1989).

These results could also be explained by the absence of a neonatal screening programme in Tunisia, since the patients were generally diagnosed long after severe and irreversible damage had occurred.

Few studies on the molecular basis of PKU among the North African population have been reported, and they typically studied the immigrant population living in European

Table 2. Distribution of the three mutated alleles in two phenotypic classes of PKU in 55 patients.

Mutation	Affected alleles						
	Classical PKU		Moderate PKU			Total	
	Nb*	(%)	Nb	(%)	*P***	Nb	(%)
G352Vfs delG	23	27.3	4	15.3	0.2	27	24.5
E280K	18	21.4	4	15.3	0.5	22	20
IVS10–11G>A	6	7.1	7	26.9	0.006	13	11.8
Total alleles identified	47	55.9	15	57.6	–	62	56.3
Total affected alleles	84	76.3	26	23.6	–	110	100

* Number; ** comparison between classical and moderate forms of PKU with χ^2 test (chi deux), the comparison is considered significant when $P < 0.05$.

countries (Lyonnet *et al.* 1989; Berthelon *et al.* 1991), and more recently the Moroccan population (Dahri *et al.* 2010). Our results provide preliminary data about *PAH* gene mutations in Tunisia. Since the country received distinct ethnic influences throughout its history, other alleles, including specific mutations are expected.

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