Rapid carrier screening using short tandem repeats in the phenylalanine hydroxylase gene

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التحرّي السريع لحملة جين بيلة الفينيل كيتون باستخدام متكرّرات مترادفة من الجين PAH

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الخلاصة: بيلة الفينيل كيتون مرض وراثي ينتقل كخلّة جسدية متنحية، ينجم عن عيب في منظومة إنزيم هيدروكسيلاز الفينيل ألانين. وكان الهدف من عملنا تحرّي موضع الجين PAH بحثاً عن متكرّرات مترادفة قصيرة قد تكون مفيدة كواسمات لكشف حملة الجين في العائلات المصابة ببيلة الفينيل كيتون في مصر. ولتحديد مستوى تغاير الزّيجوت في جين PAH بين السكان في مصر. وتشتمل منظومة إنزيم هيدروكسيلاز الفينيل ألانين على ما يزيد على ثمانية ألائل مستقلة، تنتقل وفق النمط المندلي، وقد أدّت الاختلافات في عدد المتكرّرات القصيرة المترادفة في العائلات الست عشرة المدروسة إلى تعدّدية في الأشكال، جعلتها واسمات مناسبة لكشف حَمَلَة جين بيلة الفينيل كيتون وتشخيص حالاتهم قبل الولادة. وكان أكثر حجوم الحَتّات الأليلية تواتراً في مرض بيلة الفينيل كيتون هو الحَتّة التي تتكون من 246 من أزواج القواعد (35.7٪)، والتي تؤلّف مع الحَتّة التي تتكون من 254 زوجاً من أزواج القواعد ما يقرب من 60.7٪ من الصبغيات الطافرة.

ABSTRACT Phenylketonuria (PKU) is an autosomal recessive genetic disorder caused by defects in the phenylalanine hydroxylase (PAH) system. Our work aimed to screen the PAH locus for the presence of potentially useful short tandem repeats (STR) as markers for carrier detection in PKU families in Egypt, and to determine the level of PAH heterozygosity within the Egyptian population. The system contains at least eight independent alleles in the Egyptian population, transmitted in a Mendelian fashion. Variations in the number of STR in the 16 families studied gave rise to polymorphisms that proved to be suitable markers for PKU carrier detection and prenatal diagnosis. The most frequent allelic fragment size in PKU patients was 246 bp (35.7%), which together with a fragment of 254 bp accounted for 60.7% of the mutant chromosomes.

Dépistage rapide des porteurs à l'aide des séquences courtes répétées en tandem sur le gène de la phénylalanine-hydroxylase

RESUME La phénylcétonurie est une maladie génétique autosomique récessive due à des anomalies du système de la phénylalanine-hydroxylase (PAH). Notre étude consistait à rechercher dans le locus PAH la présence de séquences courtes répétées en tandem pouvant être utiles en tant que marqueurs pour la détection de porteurs dans les familles atteintes de phénylcétonurie en Egypte, et de déterminer le niveau d'hétérozygotisme de la PAH dans la population égyptienne. Le système contient au minimum huit allèles indépendants dans la population égyptienne, transmis sur un mode mendélien. Les variations du nombre de séquences répétées en tandem dans les 16 familles étudiées ont produit des polymorphismes qui se sont avérés être des marqueurs adaptés pour la détection des porteurs de phénylcétonurie et le diagnostic prénatal. La taille du fragment allélique la plus fréquente chez les patients atteints de phenylcetonurie est 246 bp (35,7 %), qui avec un fragment de 254 bp était à l'origine de 60,7 % des chromosomes mutants.

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Introduction

Classic PKU is an autosomal recessive disorder caused by mutations in the phenylalanine hydroxylase gene. Approximately 1%–2% of individuals institutionalized for mental retardation have PKU [*1*].

The estimated gene prevalence of classic PKU among Egyptians is 0.0067, with an estimated carrier rate for the general population of 1 in 76 [*2*] to 2.3% [*3*]. It has been reported as the most common inborn error of amino acid metabolism (78%) and constituted 8.9% of a study of 450 individuals with mental retardation [*4*]. More than 200 point mutations and microdeletions have been characterized in the PAH gene [*5*]. Mutation analysis of the phenylalanine hydroxylase gene was studied by Hashem et al. [*6*] in 56 Egyptian PKU probands. Four mutations: IVS2nt5g/c (2/16), Y198fs (6/100), G247V, (2/16) and IVS10nt-11g/a (6/112), and five polymorphisms: IVS2nt 19t/c (2/16), Q232Q (10/16), V245V (3/16), L385L (3/16), IVS12nt-16t/g (2/16), were identified. Some association between mutations and polymorphisms, including IVS2nt5g/c and IVS12nt-16t/g, Y198fs and G247V, and Q232Q and V245V, have been identified [*6*]. Nine other mutations were also identified in the 26 alleles detected in the PAH gene in another group of 13 Egyptian PKU patients studied by Effat et al. [*7*]. The commonest mutation in these Egyptian patients was IVS10nt11g (8/26). Six of the mutations so far detected (IVS2nt5-c, R176X, Y198fs, R261Q, S231P, and IVS10nt11g) are also found in other Mediterranean populations. Two mutations, Y206D (2/26) and G46fs (1/26) [*7*], have not previously been reported in the Mediterranean basin.

Short tandem repeats (STR) or microsatellites provide an abundant source of polymorphic markers within the genome [*5*]. These repeats are blocks of di-, tri-, or tetranucleotides. If the number of repeats is large, the locus is often polymorphic. The number of repeat units is inherited as an allele in a Mendelian fashion. This dramatically increases the number of cases that are informative when there is a population, for example Orientals [*8*], characterized by the presence of a single predominant RFLP haplotype.

In a search for an easier method of detecting carriers and providing prenatal diagnosis for families with affected children, we analysed the polymorphism caused by variation in the number of STRs and determined the degree of heterozygosity of this system in the Egyptian population.

Methods

Sixteen unrelated families, each with at least one child with PKU, were included in the study. Patients were evaluated at the Genetics Unit, Department of Paediatrics, Ain Shams University, Cairo. All families were of Egyptian ancestry. The PKU kindreds studied included 16 PKU index cases, four affected siblings and three affected first cousins; 37 PKU carriers and 15 normal individuals were included as normal controls (patients and controls were ethnically homogeneous).

Genomic DNA was prepared from blood samples according to the DNA microextraction protocol of Stratagene. DNA was subjected to PCR amplification using 0.2 mmol/L dNTPs, 67 mmol/L Tris-HCl/pH 8.9, 16.6 mmol/L $(NH_4)SO_4$, 0.46% Triton X-100 and 1.5 mmol/L $MgCl_2$. 500 ng of genomic DNA plus 2.5 units of Taq polymerase were added to a total volume of 100 mL including 0.3 µM of each primer.

The oligonucleotide primers used in this reaction were: 5′-GCCAGAACAACTAC-TGGTTC-3′ and 5′-AATCATAAGTGTT-CCCAGAC-3′. The PCR conditions were as follows: hard denaturation for 5 minutes at 94 °C followed by 35 cycles each composed of denaturation at 94 °C for one minute, annealing at 50 °C for one minute, extension at 72 °C for one minute. Then the PCR product was loaded onto a 5% polyacrylamide gel under denaturing conditions. Each gel was duplicated onto electrophoresis duplication paper (EDP) (Kodak) according to instructions provided by Promega Corporation (Madison Wisconsin, United States of America).

The average level of heterozygosity of the STR system was calculated according to the formula provided by Daiger et al. [9].

Results

The results of the present work are summarized in the tables. Table 1 shows the sizes of STR alleles in 16 PKU index cases. Eight independent alleles, differing only in the number of basic TCTA repeats, were identified. The multiple alleles of the PAH locus were found to segregate in a Mendelian fashion in the 16 families studied (Figure 1).

Allele frequencies in Egyptian individuals were also established by examining the 16 PKU families (Table 2).

The average level of heterozygosity of the STR system was calculated according to the formula provided by Daiger et al. [9]. STR heterozygosity of normal and mutant chromosomes was respectively 0.7441 and 0.7819 (Table 3).

Most of the STR alleles were present on both normal and mutant PAH chromosomes, with a continuous distribution from the smallest (230 bp) to the largest (258 bp)

Table 1 **Size of amplified STR alleles in 16 PKU index cases**

Family	STR alleles (size in bp)
1	238:246
2	246:242
3	254:254
4	230:246
5	238:242
6	246:254
7	246:246
8	234:250
9	238:246
10	230:246
11	254:258
12	250:254
13	246:254
14	242:254
15	230:246
16	234:246

Table 2 **Frequencies of STR alleles on normal and mutant PAH chromosomes in Egyptians**

STR Allele size (bp)	Frequency Normal	Mutant
230	0.045	0.071
234	–	0.053
238	0.1815	0.071
242	0.165	0.107
246[a]	0.065	0.357
250[b]	0.42	0.035
254	0.115	0.25
258	–	0.053
262	–	–

[a]*Most prevalent allele on mutant chromosomes.*
[b]*Most prevalent allele on normal chromosomes.*
Frequencies are based on 46 alleles (23 cases).

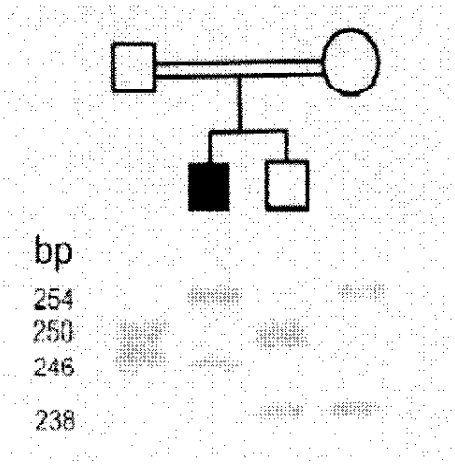

Figure 1 **Case no. 6, classic PKU Mendelian inheritance of STR alleles in PKU families. Amplified DNA from the paternal, maternal and index case samples was resolved on a denaturing acrylamide gel. The observed sizes of the fragments are indicated (father 246/250, mother 238/254, index case 246/254). This figure illustrates the use of the STR system in determining the carrier status where the sib is definitely not a carrier for PKU (238/250).**

allele. The STR allele (262 bp) was absent in this sample population.

There was a statistically significant difference between normal and mutant chromosomes in the distribution of these alleles ($P < 0.001$). This difference was due not only to a higher frequency of the 246 bp allele (35.7%) on mutant chromosomes but also to a higher frequency of the 250 bp allele (42%) on normal chromosomes in Egyptians (Tables 4 and 5).

The two most common normal alleles, 238 bp and 250 bp, together accounted for about 60.15% of normal chromosomes. The two most common mutant alleles, 246 bp and 254 bp, together accounted for

Table 3 **Average level of heterozygosity of the STR system in Egyptians**

Allele size (bp)	Normal P_i	Normal P_i^2	Mutant P_i	Mutant P_i^2
230	0.045	0.0020	0.071	0.0050
234	–	–	0.053	0.0028
238	0.1815	0.0329	0.071	0.0050
242	0.165	0.0272	0.107	0.0114
246	0.065	0.0042	0.357	0.1274
250	0.42	0.1764	0.035	0.0012
254	0.115	0.0132	0.25	0.0625
258	–	–	0.053	0.0028
262	–	–	–	–
$1 - \sum_{i=1} P_i^2$	0.2559		0.2181	
Level of heterozygosity[a]	0.7441		0.7819	

[a] *Level of heterozygosity* = $1 - \sum_{i=1} P_i^2$ *where* P_i *is the population frequency of the i allele [9].*

Table 4 **Comparison of the frequency of STR alleles on normal PAH chromosomes in Caucasians (C), Orientals (O) and Egyptians (E)**

Allele size (bp)	C [8]	O [8]	E
230	0.025	–	0.045
234	0.11	0.065	–
238	0.098	0.097	0.1815
242	0.245	0.226	0.165
246[a]	0.325	0.452	0.065
250[b]	0.16	0.129	0.42
254	0.025	–	0.115
258	0.012	0.032	–
262	–	–	–

[a] *Commonest normal allele in Caucasians and Orientals.*
[b] *Commonest normal allele in Egyptians. Frequencies are based on 163 alleles (Caucasians), 31 alleles (Orientals) and 65 alleles (Egyptians).*

Table 5 **Comparison of the frequency of STR alleles on mutant PAH chromosomes in Caucasians (C), Orientals (O) and Egyptians (E)**

Allele size (bp)	C [8]	O [8]	E [8]
230	0.012	0.121	0.071
234	0.048	0.091	0.053
238	0.12	–	0.071
242[a]	0.416	0.333	0.107
246[b]	0.271	0.364	0.357
250	0.048	0.091	0.035
254	0.066	–	0.25
258	0.006	–	0.053
262	0.012	–	–

[a] *Commonest mutant allele in Caucasians and Orientals.*
[b] *Commonest mutant allele in Egyptians.*
Frequencies are based on 166 alleles (Caucasians), 33 alleles (Orientals) and 65 alleles (Egyptian).

about 60.7% of mutant chromosomes in the Egyptian sample studied. Tables 4 and 5 compare the frequency of STR alleles on normal and mutant PAH chromosomes in Caucasians, Orientals [8] and Egyptians.

Discussion

The spectrum of mutations of the PAH gene in PKU Egyptian probands has previously been investigated by Hashem et al. [6] and Effat et al. [7]. Therefore, we focused our attention on carrier detection and on prenatal diagnosis of PKU families using an informative rapid diagnostic strategy. This study verifies the use of the STR-based polymorphic system of the human PAH gene in carrier detection and prenatal diagnosis among PKU families in Egypt. The STR system contains at least nine independent alleles that are transmitted in a Mendelian fashion [8]. Among Egyptians only eight alleles were identified. These alleles differ only in the number of the basic TCTA repeat, i.e. they vary by 4 bp increments located in intron 3 of the PAH gene [10,11]. This suggests that the polymorphic nature of this system is a consequence of insertions or deletions of 4 bp repeat units.

Most STR alleles were present on both normal and mutant PAH chromosomes, with a continuous distribution from the smallest (230 bp) to the largest (258 bp). There was a statistically significant difference between normal and mutant chromosomes in the distribution of these alleles ($P < 0.001$). The 250 bp allele was the commonest on normal chromosomes (42%), while the 246 bp allele was the commonest on mutant chromosomes (35.7%). The 262 bp allele, which has been detected in other studies [8,10,11], was absent in this sample population, possibly due to the small sample size.

As in Caucasians and Orientals, the distribution of STR alleles between the normal and mutant chromosomes of Egyptians was different. The average probability of heterozygosity of this system was 78% for mutant chromosomes compared to 80% and 75% respectively for Caucasians and Orientals [8].

The STR system has two advantages over traditional RFLP haplotype analysis. First, in populations characterized by the presence of a single predominant RFLP haplotype, it increases the number of cases that are informative. Second, despite the recent advances in PCR methods for the analysis of other polymorphic markers in the human PAH gene, the simplicity of the STR system will produce a greater degree of heterozygosity than any combination of other markers [8].

To conclude, the STR system has the advantage of a high degree of polymorphism among Egyptian PKU patients and strong Mendelian segregation. It is therefore useful for prenatal diagnosis and carrier screening in PKU families in Egypt.

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