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DETECTION OF E280K MUTATION AND ITS ASSOCIATION WITH VNTR IN PKU LIBYAN PATIENTS

Nafisah Bakhit[1]*, Ahmed Atia[2] and Abdulla Bashein[3]
[1]Department of Pharmacy, University of Tripoli Alahlia, Janzur, Libya
[2]Department of anesthesia and intensive care, Faculty of Medical technology, Tripoli University, Tripoli, Libya.
[3]Department of Biochemistry, Faculty of Medicine, Tripoli University, Tripoli, Libya.
Corresponding Author: *dr.nafisaabdalla@gmail.com*
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ABSTRACT

Phenylketonuria (PKU) is an autosomal recessive inborn error of phenylalanine (Phe) metabolism caused by phenylalanine hydroxylase (PAH) deficiency. In the Eastern Mediterranean and North Africa region, C.1055del.G and E280K mutations in PAH gene are one of the most frequent mutations observed in PKU patients. The aim of this study was the identification of E280K mutation and its association with the VNTR in PKU Libyan patients. The study included twenty PKU patients from two Libyan families detected by newborn screening program from February to August 2014. Plasma amino acid analyses for all patients exhibited excess phenylalanine and little tyrosine levels consistent with the diagnosis of PKU. PAH mutation was detected by genomic DNA extraction from blood samples of PKU patients from Gharian and Misurata cities in Libya, and analyzed by direct sequencing of PCR products of the promoter region and 7 exon region of PAH gene. One confirmed positive PKU child from Gharian hospital (mutant E280K/E280K), with their parents were carried (E280K/ wild type) and their relatives were mixed (wild type and carriers). While in Misurata hospital, two PKU children detected and their parents were carriers (E280K/wild type).The distribution of E280K mutation in this study were 15% mutant, 55% carriers, and 30% wild type. VNTR units were observed in the affected children, and was associated with affected alleles in both families. The results suggest that VNTR can be used as a marker for the presence of E280K mutation in the studied population.
Keywords: PKU, E280, Mutation, PAH, gene.

INTRODUCTION

Inborn errors of metabolism (IEMs) include a class of disorders in which a single gene defect can lead to a clinically substantial block in a metabolic pathway, resulting either in accumulation of substrate behind the block or deficit of the product [1]. Phenylketonuria (PKU; OMIM 261600) is the most common inborn error of amino acid metabolism and displays autosomal recessive inheritance [2]. This disorder caused by mutations in the gene coding for the hepatic enzyme phenylalanine hydroxylase (PAH), rendering it nonfunctional, and subsequently causes inability of the body to metabolize the vital amino acid phenylalanine [3]. More than 400 PKU mutations in different populations have been identified so far, and are available in the public-domain PAH gene mutation database [4]. The frequency of PKU varies considerably worldwide. The occurrence of

PKU in Europe is 1/10,000 [5], in Iran 1/3627 [6], in China 1/11,000, and in Japan 1/120,000.3 [7]. While, in Libya, there are two mutations have been identified as a molecular cause of PKU, C.1055del.G and E280K mutations [8].

Numerous studies have analyzed the molecular basis of PAH gene, and described the distribution of PAH mutation and their association with variable number of tandem repeat (VNTR). Lyonnet et al [9] studied the molecular genetics of phenylketonuria in Mediterranean countries and a mutation associated with partial phenylalanine hydroxylase deficiency. They found that mutation Glu280Lys (E280K) by sequencing a mutant cDNA clone derived from a needle biopsy of the liver in a child with variant form of phenylketonuria. They concluded a strict concordance between homozygosity for the mutation and this particular phenotype. E280K mutation is linked to an original and rare RFLP haplotype at the PAH locus found in south Europe and North Africa [9].

Previous studies have reported that the most common mutations in the PAH gene in the Libyan population were E280K in the exon 7 and 10del5510 in the exon 10. Therefore, the current study aimed at identifying the occurrence of E280K mutation and its association with the VNTR among Libyan family members of the children suffering from PKU.

MATERIAL AND METHOD

Patients and Methods

A total of 20 patients, 10 cases were from outpatient clinic in Gharian hospital [Group 1] and 10 cases from Misurata hospital [Group 2], were recruited for this study after obtaining informed consent from the parents. Each group of patients where from the same family or their relatives. Three out of the twenty unrelated PKU patients confirmed positive by neonatal screening. The PAH activity was measured by conventional biochemical methods. Most of the patients were identified when they showed mental retardation and few patients were identified during neonatal screening. The subjects were with ages ranging from 1 month to 12 years old, and fulfilled the diagnostic criteria for PKU, with blood phenylalanine concentrations > 20 mg/dL [10]. The study was approved by the ethics committee of Libyan academy of graduate studies.

Molecular characeterization of the PAH locus

A total of 4 ml blood samples were collected in a vacationer tubes with EDTA as an anticoagulant and were transported to the laboratory of National center of disease control (NCDC) and frozen at -20^{o}C until further analysis. Genomic DNA was extracted from peripheral blood according to standard protocols using a DNA extraction kit (QIAGEN Inc., Valencia, CA, USA) with PCR amplification of the 7 PAH exons done using primer sequences as shown in Table 1. The PCR condition were 95 °C denaturation for 2 min, followed by 40 cycles of 95 °C for 15 sec, 58°C for 20 sec, and 65 °C for 30 sec; and a final elongation step at 72 °C for 5 min.Samples were electrophoresed in 1.5 % agarose gel. The PCR products were sequenced by ABI prism 3130 genetic analyzer (Applied Biosystems, USA) and compared with the human genomic DNA sequence in GenBank to identify the mutations.

Table 1: Forward and reverse primer used for PCR amplification

Primer / Probe	Sequence	Annealing Temp.
Forward primer	5’-GTGTACTACTCCACTACCTAAAGGTC-3’	61.3C^{o}
Reverse primer	5’-GAACCCAAACCTCATTCTTG-3’	56.4C^{o}

PCR for VNTR determination

PCR reactions were performed using an automated thermal cycler (TechneProgene, Cambridge, UK). The primers used for determination of the VNTR fragments which are associated with E280K mutation in exon 7 were as designed by Goltsov et al. [11]. PCR reaction conditions were: denaturation at 95 °C for 2 min followed by 35 cycles of amplification at 95 °C for 2 min and 95^0C annealing for 1 min with a final extension at 72 °C for 5 min. The PCR products were run on agarose gel electrophoresis for VNTR detection. All statistical analyses were done using SPSS 22.0 statistical software.

RESULTS AND DISCUSSION

Determination of the E280K distribution

At the present study, the 181bp of exon7 were amplified by using real- time PCR for the determination of the E280K mutation distribution in Libyan patients suffering from PKU. The first group in this study shows one child confirmed positive PKU (mutant E280K/E280K), with their parents were carried (E280K/wild type) and their relatives were mixed (wild type and carriers). The result of *Ct* values for HEX (mutant) and FAM (wild type) of the first group are given in Table 2.

In the second group of patients, which were recruited from Musrata hospital, the results showed that only two children suffering from PKU (mutant E280K\E280K), while their parents were carriers (E280K/wild type). The result of *Ct*values for Hex and Fam of the group No.2 were given in Table 3.

Table 2: Ct values for HEX and FAM of the studied cases of the first group

Sample No.	Family member	Ct value (Hex)	Ct value (Fam)	Status
1	PKU (1)	23.67	No Ct	Mutant
2	Father (1)	25.59	25.59	Carrier
3	Mother (1)	29.29	21.69	Carrier
4	Uncle (1-M)	27.07	27.35	Carrier
5	Uncle (1-M)	No ct	27.27	Wild type
6	Uncle (1-M)	No ct	25.97	Wild type
7	Uncle (1-F)	No ct	25.67	Wild type
8	Uncle (1-F)	26.93	27.32	Carrier
9	Uncle (1-F)	No ct	25.94	Wild type
10	Brother (1)	21.09	22.87	Carrier
11	Sister (1)	26.86	25.82	Carrier
12	Uncle (1-F)	No ct	27.47	Wild type
13	Uncle (1-F)	No ct	28.76	Wild type
14	Uncle (1-M)	25.62	24.40	Carrier
15	Uncle (1-F)	26.27	25.45	Carrier
16	Uncle (1-M)	36.40	29.41	Carrier

Mutant (E280K/E280K); Carrier (E280k/WT); PKU (1); PKU case from family (1); Uncle (1-M): uncle of family No.1 from mother side.Uncle (1-F): uncle of family No.1 from father side.

Table 3: Ct values for Hex and Fam of the studied cases of group No (2)

No. of sample	Family member	Ct value (Hex)	Ct value (Fam)	Status
17	PKU (2)	34.83	No ct	Mutant
18	PKU (2)	25.75	No ct	Mutant
19	Father (2)	39.17	28.99	Carrier
20	Mother (2)	26.14	24.76	Carrier

Mutant (E280K/E280K); Carrier (E280k/WT); PKU(2): PKU case from family No. 2.

Tables 2 & 3 described the Ct values of studied samples for Hex and Fam and the status of PKU case which is the mutant (E280K\E280K) that carries two mutated alleles inherited from both carrier (E280K) parents (autosomal recessive fashion). We observed that Hex probe works as a complementary for mutant alleles and gives the Ct value with them in individuals who have these mutant alleles while Fam probe didn't. The wild type cases react with Fam probe which is complementary for normal allele and gave Ct values with it, while Hex probe didn't react with normal allele. The carrier cases react with Fam probe and Hex probe and gave Ct values with both of them because the carrier case has both normal and mutant alleles. The Ct values which obtained were mostly strong Ct values (< 29) indicating high template concentration. The distribution of E280K mutation in this study showed 15% of the study alleles (six out of forty alleles) were mutant, 55% of them were carriers, and 30% were wild type (Fig. 1).These percentages were gained from studying randomly selected Libyan participants. However these selections cannot be described as fully representatives to the whole Libyan population, due to the fact that the selected participants were few in number and were chosen from only two families.

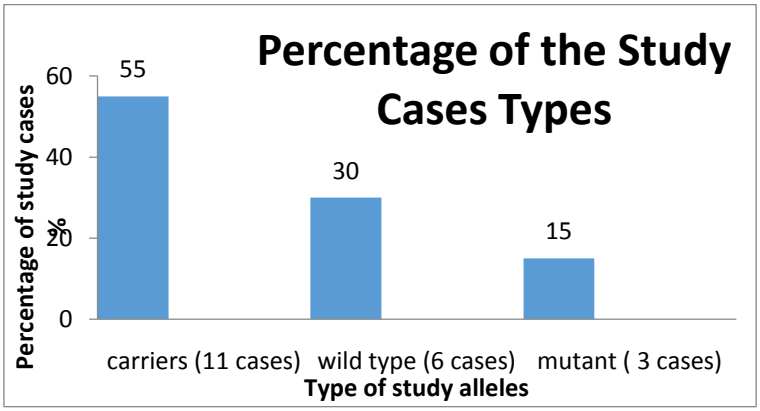

Figure 1: Represents the percentage of the types of study cases

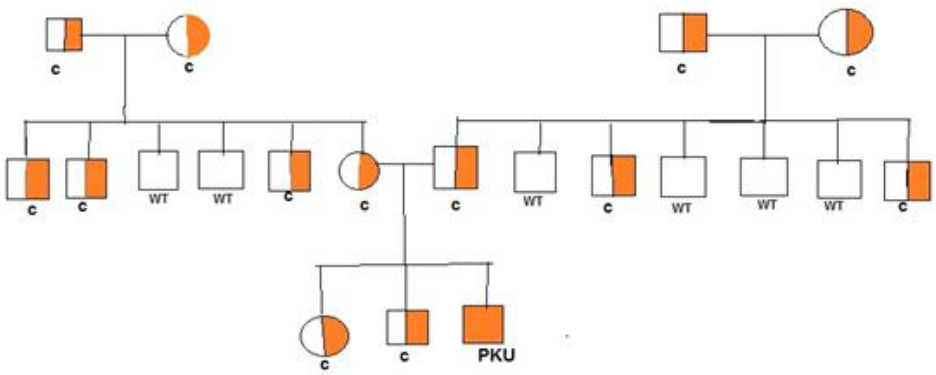

Figure 1(a): Group 1 pedigree tree.

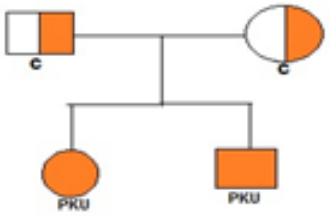

Figure 1(b): Group 2 pedigree tree.

Al Mutawa et al., reported in a study done at Al-Jala pediatric hospital in Tripoli-Libya thatE280K mutation was the most common mutation in Libya [8].In their study, parents of the studied patients were relatives, suggesting that mutation of E280K is strongly related to consanguinity, which is very common in Libya. Therefore it can be predicted that consanguinity in families that have members carriers or disordered is a direct cause to the spread of this mutation in the next generations according to Mendel's Law [12].

E280K mutation reported to be found mostly in Mediterranean populations while it was not detected in any of the Turkish PKU alleles [13].Moreover, previously work screened classical PKU patients for various mutations reportedthat IVS10 mutation was found in 32% of the mutant alleles and comprises 74.5% of the mutations that could be typed: $261^{arg\text{-}gln}$ (6-8%), $158^{arg\text{-}gly}$ (2.3%) $252^{arg\text{-}trp}$ (1.1%), while Glu280Lys (-)[14].In Libya E280K was one of the most common mutations [8], while in Morocco the most frequent mutation was the frame shift change p.G352fsdelG, p.R176X, IVS10nt-11 G>A, p.W120X, p.A165T, p.R243X and p.R243Q), with no E280K mutation present [15]. Another study revealed thatthe most common mutation in Egyptian population was IVS10nt546 followed by R261Q and L48S. They also found missense mutations in exon 7 named (R243P, R261Q, and Y277D) and one silent mutation (V245V) [16]. The most frequent mutation observed in Tunisia was E280K that accounts for 0.17, and this frequency of the mutation was higher than described in north Mediterranean, e.g., France (0.06), Greece (0.05) and Spain(0.04) [17].

Families Pedigrees

As a phenylketonuria is an autosomal recessive ailmentexisting from birth. This means that an affected peoplesmust get a mutant allele from both parents. When deeming the entire family pedigree, it is often siblings who have the disease. This is known as horizontal transmission [18]. Other relatives can be influenced but this only exists in large inherent families. However, when two carriers meet, there is still only a 25% chance to get child with PKU [19]. The disease affects women and men similarly but there are, possibly, moreinfluences for a woman with PKU as she has to take the disease into account before gettingchildren. A recessive trait is more frequentlyexhibited if parents are related, due to the fact that two carriers of the same recessive gene are more possible to unite. This is called consanguinity and it commonly takes place between partners [20].In our study, the first group of family was composed of three children one of them was PKU case. Figure 1 shows the pedigree tree of the first family.The father and mother were carrier so one of their three children was PKU case and the other two children were carriers. It also shows that the two families from the father's side and mother's side had members which were carriers and the others were wild type without any PKU cases. This suggests that the grandfathers and grandmothers for both sides (father and mother) were carriers and wild types.

The pedigree tree of group No.2 shows that this family composed of two PKU cases from carrier parents.

PCR for VNTR detection

An AT-rich(70%) mini-satellite region in PAH gene contains various multiples of 30-bp tandem repeats (VNTR) and is located 3 kb downstream of the final exon of the gene.This may prove useful in studies concerning the origins and distributions of PAH mutations in different human populations [21].This AT-rich minisatellite region was amplified from genomic DNAs of patients and other members of PKU families, both to observe potential heterogeneity in the number of repeats present on chromosomes of various haplotypes in different human populations and to determine their Mendelian segregation. Comparison of this region in Caucasian PKU families demonstrates the presence of at least six alleles that differ in the number of repeated units. These alleles are inherited in a Mendelian fashion and are often associated with specific PAH mutations.

In the present study, whole blood samples were collected from two PKU families with at least one affected child. The genomic DNA was extracted from whole blood. A simple and rapid one-step PCR-based procedure was used to find the number of copies of the repeated units of VNTR region linked to the PAH gene, according to the method described by Goltsovet al [22].

For evaluating the polymorphism status, the amplified products were analyzed using 3% agarose gel electrophoresis as shown in Figure 3.

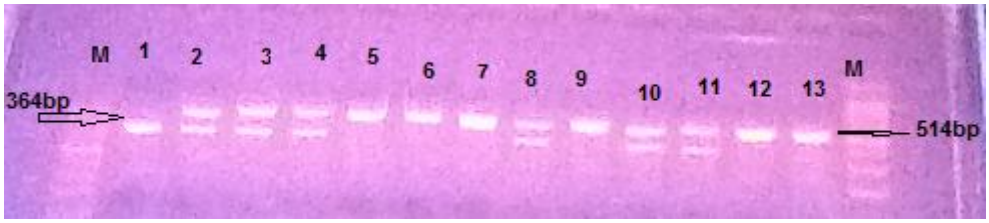

Figure 3: Repeated units of VNTR for the studied cases

DNA fragments with 364 and 514 bp length corresponded to 3 and 8 copies of VNTR units, respectively are shown in the [Table 4 & 5] for two studied families. DNA fragments of 364 bp corresponding to the 3 copies of the VNTR units were observed in the affected children of both families. Therefore, the reported VNTR 8 was associated with normal alleles in both families. This indicating that VNTR 3 is completely associated with the mutant alleles in both families, while the VNTR 8 is completely associated with the wild type alleles. This result suggest that VNTR 3 can be used as a marker for the presence of E280K mutation in the studied population.

Table 4. VNTR (The length of fragment with the number of repeats) for the study cases of family No (1).

Sample No.	Case status	VNTR	
		Length of fragment	No. of repeats
1	Mutant	1 band (364bp)	3
2	Carrier	2bands(364bp, 514bp)	3, 8
3	Carrier	2bands(364bp, 514bp)	3, 8
4	Carrier	2bands(364bp, 514bp)	3, 8
5	Wild type	1 band (514bp)	8
6	Wild type	1 band (514bp)	8
7	Wild type	1 band (514bp)	8

8	Carrier	2bands(364bp, 514bp)	3, 8
9	Wild type	1 band (514bp)	8
10	Carrier	2bands(364bp, 514bp)	3, 8
11	Carrier	2bands(364bp, 514bp)	3, 8
12	Wild type	1 band (514bp)	8
13	Wild type	1 band (514bp)	8
14	Carrier	2bands(364bp, 514bp)	3, 8
15	Carrier	2bands(364bp, 514bp)	3, 8
16	Carrier	2bands(364bp, 514bp)	3, 8

Table 5. VNTR (The length of fragment with the number of repeats) for the study cases of family No (2)

Sample No.	Case status	VNTR	
		Length of fragment	**No. of repeats**
17	Mutant	1 band (364bp)	3
18	Mutant	1 band (364bp)	3
19	Carrier	2bands(364bp, 514bp)	3, 8
20	Carrier	2bands(364bp, 514bp)	3, 8

Units of less than 3 or more than 8 repeats have not been observed in Libyan population, while a 13 units of VNTR has recently reported in Iran, who suggested that the alleles of VNTR are produced by the gain or loss of a single VNTR unit, possibly by slippage during DNA replication [23].

CONCLUSION

The present results confirm the distribution of the E280K mutation among our samples were 55% carriers, 30 % wild type and 15% mutant cases. Offspring who can be disordered by PKU, and both parents could be carriers or disordered. Detection of VNTR in high risk PKU subjects will aid in early screening of E280K mutation. Lastly, by decreasing consanguinity it may help in decreasing the occurrence of PKU, since it was found that the occurrence of the PKU disorder is highly spread in consanguineous families, which is a very common tradition in Libya.

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