*Libyan Academy of Graduate Studies*

*School of Engineering and Applied Sciences*

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# *Detection of E280K mutation and its association with the VNTR in PKU Libyan families.*

A thesis submitted to the school of engineering and applied sciences at the Libyan Academy of Graduate Studies, in partial fulfillment of the requirements of Master Science Degree in Biomedical Engineering.

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بسم الله الرحمن الرحيم

"وقل ربي زدني علما"

صدق الله العظيم

# Dedication

*To my lovely parents*

*To my great family*

*To all who likes me to success*

# Acknowledgement

I thank **Dr. Abdulla Bashein** for his acceptance to supervise me. I thank him for his supervision, suggestions, care and patience in following up all the stages of this research.

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# Abstract

**P**henylketonuria (PKU), is one of inborn errors of metabolism, is an autosomal recessive inborn error of phenylalanine (Phe) metabolism resulting from deficiency of phenylalanine hydroxylase (PAH). Most forms of PKU are caused by mutations in the PAH gene on chromosome 12q23.2. Untreated PKU can lead to significant abnormal phenotypes.

*Till now, p*revious studies showed that the most common mutations in the PAH gene in the Libyan population were E280K in exon 7 and 10del5510 in exon 10.

The aims of the study were to:1. Study the distribution of the E280K ($280^{g1u\text{-}lys}$) mutation among the family members of children suffering from PKU.

2. Draw the pedigree chart of PKU in the families of these children.

3. Study the association of this mutation with a VNTR in PAH gene.

4. Assess the usefulness of using VNTR as a quick screening tool for E280K mutation in PKU patients.

We used the hydrolysis probe real-time PCR for the detection and distribution of E280K mutation and conventional PCR for the detection of VNTR and the association of E280K mutation with VNTR.

Our results showed that the E280K was detected and distributed in 19 out of 40 alleles. Only VNTR repeats 3 and 8 were identified in the studied families and found that the E280K mutation is associated with VNTR 3 only.

In conclusion, E280K mutation is relatively common among the family members of both families. It was detected in 19 out of 40 alleles (47.5%) in both families. The spread of the disease among the two families was attributed to the consanguineous marriage between families carrying the mutation. The present study also clarify that there is a correlation between E280K mutation and VNTR in the PAH gene. So VNTR 3 can be used for screening for PKU.

# الخلاصة


يعتبر مرض الفينيل كيتونيوريا **PKU** من الأمراض الأيضية الشائعة وهو مرض وراثي جسمي متنحي ناتج عن طفرات لجين الفينيل الأنين هيدروكسيلازعلى الكروموسوم 12q23.2 مما يؤدى إلى نقص أنزيم الفينيل الأنين هيدروكسلاز. مرض الفينيل كيتونيوريا يؤدي إلي مضاعفات خطيرة أذا لم يعالج مبكرا.الى حد الآن تم الكشف علي اثنين من الطفرات الوراثية في جين فينيل الأنين هيدروكسيلازعند الليبين وهي **E280K** في الإكسون **7** و**C.1055 del G** في الإكسون **10** .

**الدراسة الحالية تهدف إلى**:-1- دراسة توزيع الطفرة E280K بين افراد اسرتين لديهم اطفال مصابين بمرض الفينيل كتيونيوريا.
2-رسم خريطة لمرض الفينيل كتيونيوريا عند الأسرتين.

3-دراسة الترابط بين الطفرة الجينية E280K مع تعدد أشكال المتكررات المترادفة المتغيرة العدد (VNTR) لجين فينيل الأنين هيدروكسلاز.

4- تحديد مدى الاستفادة من كشف الحاملين لطفرة الفينيل كيتونيوريا بتحليل تعدد أشكال المتكررات المترادفة المتغيرة العدد (VNTR).

تم استخدام تقنية التفاعل البلمرة المتسلسل الأنى Real-time PCR لدراسة توزيع طفرة (E280K) وتقنية التفاعل البلمرة المتسلسل PCR لتحليل تعدد أشكال المتكررات المترادفة المتغيرة العدد (VNTR).

**نتائج هذه الدراسة عند الأسرتين أوضحت أن الطفرة** E280K تعتبر عالية نسبيا في كلتا الأسرتين 19 من أصل 40 أليل بنسبة 47.5% كما أوضحت وجود تعدد أشكال المتكررات المترادفة المتغيرة العدد (VNTR) 3 و 8 وان طفرة E280K مرتبطة فقط مع تعدد أشكال المتكررات المترادفة المتغيرة العدد 3 (VNTR) في جين الفينيل الأنين هيدروكسيلاز.

نستخلص مما سبق دراسته أن طفرة E280K منتشرة عند أفراد الأسرتين فقد وجدت نسبتها 47.5%في كلتا الأسرتين. كما إن انتشار هذا المرض عند الأسرتين قد يعزى إلي زواج الأقارب بين الأسرتين الحاملة لمرض الفينيل كيتونيوريا . **كما أن هذه الدراسة قد أوضحت** ارتباط طفرة E280K مع تعدد أشكال المتكررات المترادفة المتغيرة العدد 3 (VNTR) وبالتالي يمكن استخدامه لعمل مسوحات عن مرض الفينيل كيتونيوريا وبالتالي الكشف عن حاملي طفرة E280K.

# List of abbreviations

| Abbreviation | Description |
|---|---|
| Arg | Argenine |
| BH4 | Tetrahydrobiopterin |
| BHQ1 | Black hole quencher1 |
| Bp | Base pair |
| Ct | Cycle threshold |
| DGGE | Denaturing gradient gel electrophoresis |
| DNA | Deoxyribonucleic acid |
| EDTA | Ethylene diamine tetra acetic acid |
| FAM | 6-carboxyfluorescein |
| G | Guanine |
| Glu | Glutamatic acid |
| HPA | Hyperphenylalaninemia |
| IEMs | Inborn Error of Metabolisms |
| Lys | Lysine |
| MHP | Mild Hyperphenylalaninemia |
| MM | Master mix |
| NCDC | National Centre of Disease Control |
| PAH | Phenylalanine hydroxylase |
| PCR | Polymerase chain reaction |
| Phe | Phenylalanine |
| PKU | Phenylketoneuria |
| QPCR | Quantitative polymerase chain reaction |
| RFLP | Restriction Fragment Length Polymorphism |
| SSCP | Single-Strand Conformation Polymorphism |
| TBE | Tris borate EDTA |
| Trp | Tryptophan |
| Tyr | Tyrosine |
| VNTR | Variable number of tandem repeats |

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# Chapter One

# Introduction

# Introduction

## 1.1 Inborn Errors of Metabolism (IEMs)

Inborn Errors of Metabolism (IEMs) comprise a group of disorders in which a single gene defect causes a clinically significant block in a metabolic pathway resulting either in accumulation of substrate behind the block or deficiency of the product. All IEMs are genetically transmitted typically in an autosomal recessive or X-linked recessive fashion. IEMs are now often referred to as congenital metabolic diseases or inherited metabolic diseases (Oh, et al. 2004).

One of the major categories of inherited metabolic diseases is disorder of amino acid metabolism, phenylketoneuria is one example of this disorder.

## 1.2 Phenylketoneuria

**Phenylketoneuria (PKU)** is the most common congenital metabolic disorder which is inherited as an autosomal recessive metabolic genetic disorder, which means it is passed down through families. Both parents must have at least one defective allele of the gene for phenylalanine hydroxylase (PAH), and the child must inherit two defective alleles, one from each parent (Fig. 1.1) (Oh, et al. 2004).

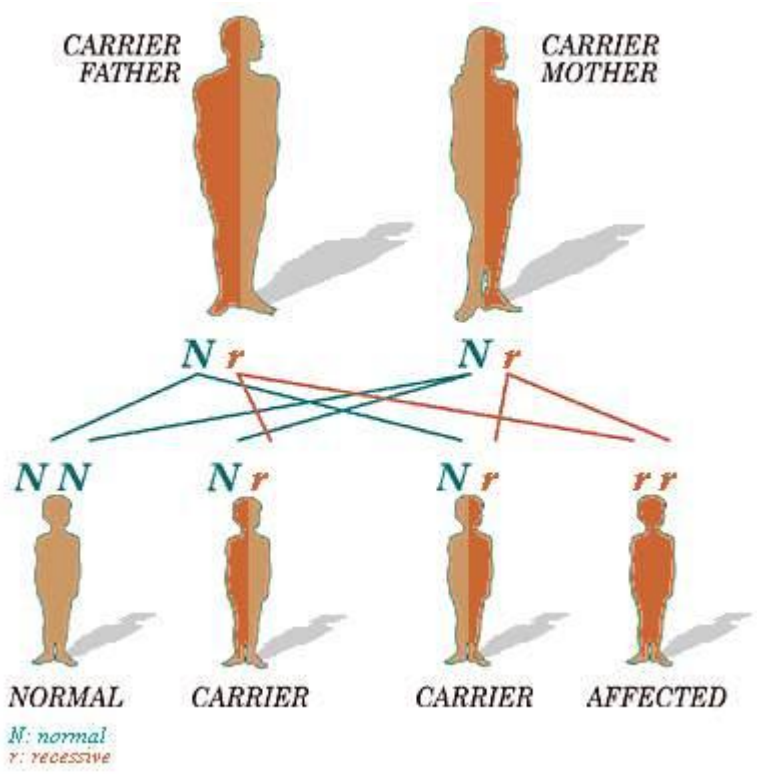


**Figure 1.1:** Inheritance of PKU in an autosomal recessive fashion (Guttler and Guldberg, 2000).

PKU was discovered and described by the Norwegian physician Ivar Asbjorn Folling in 1934 when he noticed that hyperphenylalanimia (HPA) associated with mental retardation (Folling, 1934), and later PKU was identified as an autosomal recessive genetic disorder (Penrose et al. 1937).

PKU is caused by mutations in the gene coding for the hepatic enzyme phenylalanine hydroxylase (PAH), rendering it nonfunctional so the liver will be unable to produce the normal PAH enzyme (Donlon, et al. 2004). PAH enzyme is necessary to metabolize the amino acid phenyalanine (Phe) to the amino acid tyrosine (Tyr). When activity of PAH is reduced, tyrosine is deficient, phenylalanine accumulates and is converted into phenylpyruvates (also known as phenylketones), phenylacetate and

phenylethylamine through the minor route, a transaminase pathway with glutamate, which are detected in the urine ( Nelson, et al. 2000).

Non- classic PKU occurs when there is a deficiency in the cofactor HB4 while, Classic PKU, the most severe form of the disorder, occurs when phenylalanine hydroxylase activity is severely reduced or absent. Buildup of abnormally high phenylalanine concentrations in the blood and brain above normal levels (6mg/dl- 360mmol/L or less) is toxic to the cells that make up the nervous system and causes irreversible abnormalities in brain structure and function in PKU patients (Stemerdink, et al. 2000).

Elevated levels of phenylalanine in the blood and detection of phenylketones in the urine is diagnostic, however most patients are diagnosed via newborn screening.

PKU is associated with an abnormal phenotype which includes growth failure, poor skin pigmentation, microcephaly, seizures, global developmental delay and severe intellectual impairment (kaufman et al. 1975). These abnormal phenotypes are due to an accumulation of phenylalanine in the blood resulting in hyperphenylalaninemia and the abnormal formation of a myelin sheath around neuronal axons in the central nervous system and tyrosine deficiency (kaufman et al. 1975).

Since its discovery, there have been many advances in PKU treatment. Early cases of PKU were treated with a low-phenylalanine diet. Optimal treatment involves maintaining blood Phe levels in a safe range while monitoring diet and cognitive development. There is no cure for PKU, but patients who are diagnosed early and maintain a strict diet can have a normal life span with normal mental development. If, however, the condition is left untreated, it can cause problems with brain development, leading to progressive mental retardation, brain damage, and seizures (Scriver, et al.2001).

## 1.3 Inciedance of PKU in different Populations

The occurrence of PKU varies among ethnic groups and geographic regions worldwide .

PKU is found often in Caucasian populations worldwide, with much lower rates in people of African, Hispanic, and Asian ancestry. As a crossroads of these cultures, the Middle East has comparable rates of PKU to much of the Western world. It is one of the more common genetic diseases, with an estimated incidence of 10 cases of PKU per 100,000 live births in Bahrain (Al-Arrayed,2006), 8/100,000 in Qatar (Lindner et. al, 2007), and 5/100,000 in the UAE (Al-Hosani et. al. 2000). In comparison, the rate of PKU in the United States is about 4-6/100,000. Oman seems to be a regional outlier, with only 2.4 cases of PKU per 100,000 live births (Joshi et. al, 2002), although this is still high enough to be of concern. There have also been documented cases in Algeria, Egypt, Kuwait, and Yemen (Teebi et. al, 1987).

Classic PKU and the other causes of hyperphenylalaninemia affect about one of every 10,000 to 20,000 Caucasian or Oriental births by meaning 1 in 10,000 Caucasian and East Asian babies, 1 in 143,000 Japanese babies, 1 in 2,600 Turkish babies will be born with PKU. The incidence in African Americans is far less. These disorders are equally frequent in males and females . On the other side, the incidence of PKU is about 1 in 15,000 births, but the incidence varies widely in different human populations from 1 in 4,500 births among the population of Ireland (DiLella et al. 1986) to fewer than one in 100,000 births among the population of Finland (Guldberg et al. 1998). Countries with little immigration from Ireland and western Scotland tend to have low PKU

rates. Caucasians in the United States have a PKU incidence of 1 in 8,000, while Blacks have a rate of 1 in 50,000 (Longe 2006).

The higher rates of incidence can be primarily attributed to the high degree of consanguineous marriages in those regions.

## 1.4 Consanguinity and Autosomal Recessive Disorders

In mathematical terms, consanguinity does not alter the allele frequencies of common disorders, but increases the probability of a mating between two individual heterozygotes for the same recessive mutant allele. In this regard, the risk for birth defects in the offspring of first-cousin marriage is expected to increase sharply compared to non consanguineous marriages particularly for rare autosomal recessive disease genes, because for common recessive conditions, there is a high chance that the abnormal gene may be carried by unrelated spouses and may be expressed in their progeny. In Arab society, mutation carriers mostly remain concentrated within the extended family and consanguineous marriages increase the probability of expression of autosomal recessive disorders when both mother and father are carriers of the mutation. Sometimes,

autosomal recessive genes stay hidden within the family for generations and then show on the surface in a new consanguineous marriage within the family (Tadmouri et al. 2009).

## 1.5 Phenylalanine metabolic pathway

Phenylalanine, which is an essential amino acid (building block of proteins) is converted into another a non-essential amino acid tyrosine by phenylalanine hydroxylase PAH enzyme using tetrahydrobiopterin BH4 as an essential cofactor, and any defect in PAH or BH4 will render this normal pathway inactive and phenylalanine metabolism will go to transamination pathway rather than a normal way of metabolism (Michals and Matalon, 1985), (Fig.1.2) illustrate the metabolic pathway and alternative pathways of phenylalanine.

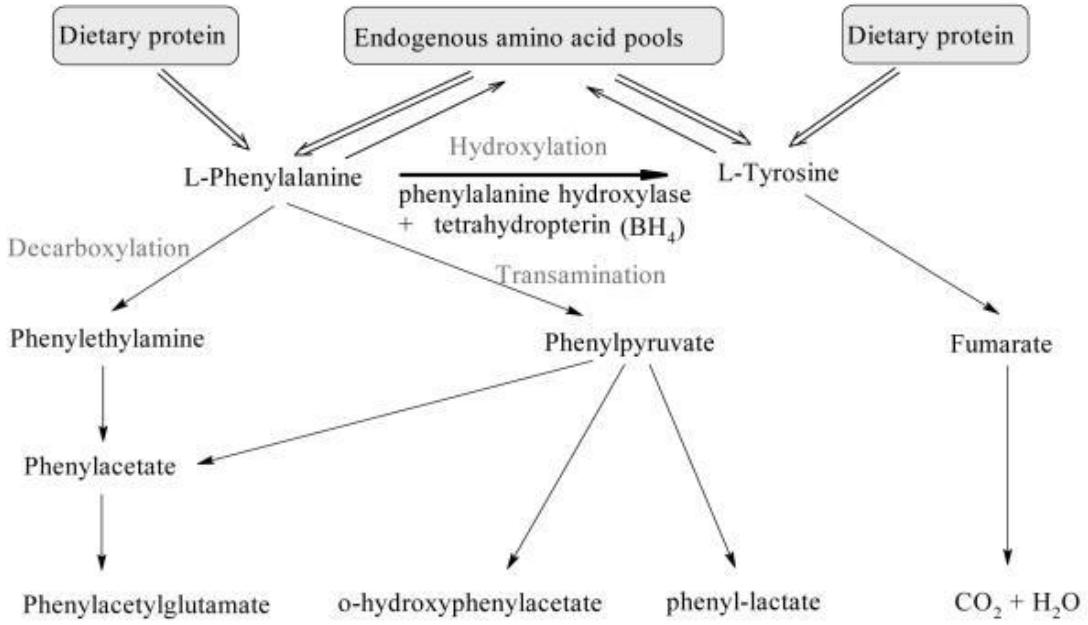


**Figure 1.2:** Phenylalanine metabolic pathway and alternative metabolic pathways ( Russell and Peter 2010).

The normal conversion of Phe to Tyr occurs by a hydroxylating system consisting of:

- PAH
- the unconjugated pterin cofactor, tetrahydrobiopterin *($BH_4$)*

- enzymes which serve to regenerate $BH_4$, namely dihydropteridine reductase and 4α-carbinolamine dehydratase

While the parahydroxylation of Phe is essential for the rupture of the benzene ring, it is not required for further metabolism of the alanine side chain. This alternative pathway of transamination and decarboxylation leads to the formation of metabolites such as phenylpyruvate, phenyllactate, and ***o***-hydroxyphenylacetate which are excreted in urine. Conversion of Phe to Tyr has two outcomes. First, it drives the endogenous production of the non-essential amino acid Tyr. Second, the hydroxylation reaction is the rate limiting step for complete oxidation of Phe to $CO_2$ and $H_2O$ and contributes to the pool of glucose and 2-carbon metabolites (Kaufman et al.1975 ).

Normal metabolic pathway of phenylalanine occur in non PKU while any defect in PAH enzyme will encourage alternative metabolic pathway of phenylalanine which lead to PKU, (Fig.1.3) show the phenylalanine metabolism in non-PKU and PKU.

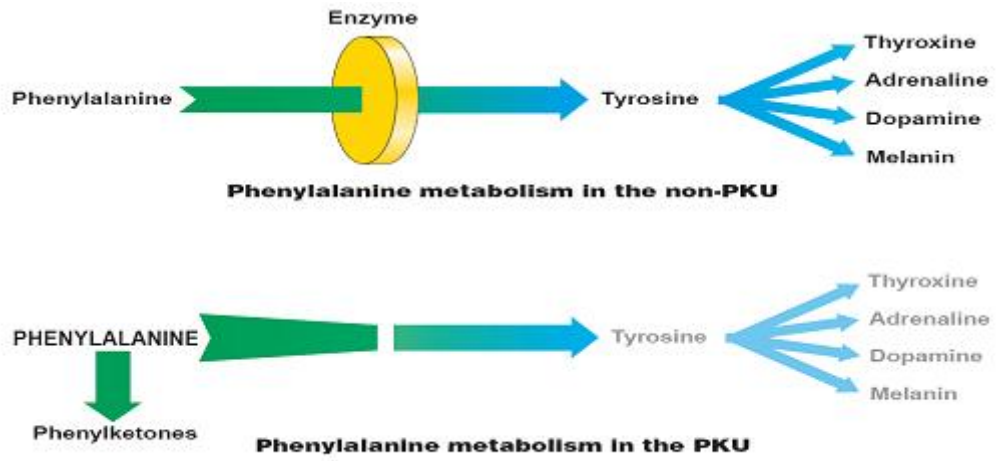


**Figure1.3:** Phenylalanine metabolism in non-PKU and PKU ( Russell and Peter. 2010).

Tyrosine has many functions in the body as it is a precursor for thyroid hormones, catecholamine and melanin, so a tyrosine deficiency could lead to many secondary outcomes like; hypothyroidism as a deficiency in thyroid hormones (thyroxine), neurological abnormality and behavior problems like depression due to decreased levels of catecholamines (dopamine, adrenaline and noradrenalin) and finally decreased melanin which is responsible for pigmentation of skin and hair and that will lead to hypopigmentation (Gelenberg, et al.1982 ).

## 1.6 Phenylalanine hydroxylase *(*PAH) gene

The official name of this gene is phenylalanine hydroxylase, PAH is the gene's official symbol. The PAH gene is located on the long (q) arm of chromosome 12 between positions 22 and 24.2.The cytogenic location of PAH gene is (12q22-q24.2) and the molecular location on chromosome 12:base pairs 103,232,103 to 103,311,380 (Fig1.4). The full genomic sequence of the PAH gene spans about 171kb and contains 13 exons. (Hendriksz and Walter, 2004). The full sequence of exon7 of PAH gene is provided in appendix (2).

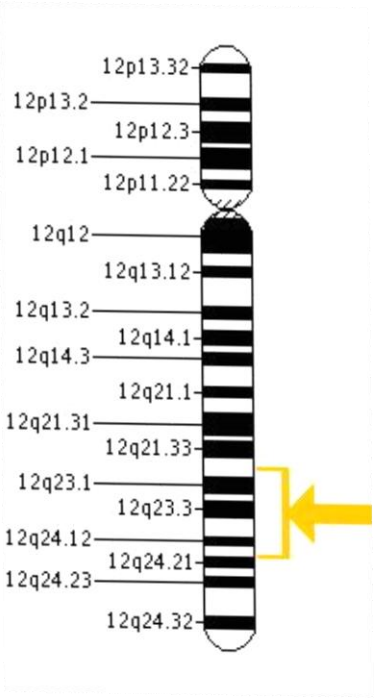


**Figure 1.4:** Location of PAH gene on chromosome 12.

(PAH location is denoted by yellow marker)(Adopted from http:/.ncibi.nlm.nih.gov/mapview/maps),(Accessed on 31. 12.2013).

Mutations in PAH gene will lead to phenylketonuria PKU. More than 500 mutations in the PAH gene have been identified in people in different populations. Most of these mutations change single amino acids in PAH. For example the most common mutation in many populations replaces the amino acid arginine with the amino acid tryptophan at position 408 (written as Arg408Trp or R408W). Other PAH mutations delete small sequences of DNA from the gene or disrupt the way the gene's instructions are used to make phenylalanine hydroxylase (www.pahdb.mcgill.ca/cgibin/pahdb/mutation_statistics-1.cgi -accessed on 20/03/2013 ). However the PAH mutations can be of various types, missense mutations: 62% of PAH alleles, small or large deletions: 13%,

splicing defects: 11% silent polymorphisms: 6%, nonsense mutations: 5%,and insertions: 2% (Kayaalp et al.1997).

Some PAH mutations are more severe than others depending upon their effect on enzyme structure and function, however the effect of PAH mutations on the clinical phenotype is variable (Kayaalp et al.1997).

## 1.7 Variable Number of Tandem Repeat (VNTR) of PAH gene

A **variable number tandem repeat** (**VNTR**) is a location in a genome where a short nucleotide sequence is organized as a tandem repeat. This region contains various multiple of 30-bp tandem repeats and is located 3 kb downstream of the last exon of the gene, and it is an AT-rich (70%) minisatellite region. These can be found on many chromosomes, and often show variations in length between individuals (Jeffreys et al, 1985). Each variant acts as an inherited allele, allowing them to be used for personal or parental identification. Their analysis is useful in genetics and biology research, forensics, and DND fingerprinting. Since the PKU is an outcome of a defect and deficiency in PAH enzyme due to a mutation in PAH gene, so the detection of this mutation may help in monitoring PKU disorder and it is found that the PAH gene mutation is linked to a variable number of tandem repeats (VNTR) region which is located at the 3′-end of the gene and since the VNTR is a highly polymorphic genetic marker and is inherited in a Mendelian fashion, it is used to give a risk estimation of linked defective allele in PAH gene (Scriver, et al.2001). The VNTR for PAH gene were given in appendix (3).

## 1.8 Techniques used to detect DNA mutations

There are many techniques used to detect DNA mutation such as:

### 1.8.1 Restriction Fragment Length Polymorphism Method (RFLP)

RFLP is a sequence of DNA that has a restriction site on each end with a "**target**" sequence in between. A target sequence is any segment of DNA that bind to a **probe** by forming complementary base pairs. A probe is a sequence of single-stranded DNA that has been tagged with radioactivity or an enzyme so that the probe can be detected. When a probe base pairs to its target, the investigator can detect this binding and know where the target sequence is since the probe is detectable. RFLP produces a series of bands when a Southern blot is performed with a particular combination of restriction enzyme and probe sequence. RFLP can be used for paternity, disease status and genetic mapping (Saiki et al. 1985).

### 1.8.2 Polymerase Chain Reaction (PCR)

(**PCR**) is a biochemical technology in molecular biology used to amplify a single or a few copies of a piece of DNA across several orders of magnitude, generating thousands to millions of copies of a particular DNA sequence.

The method relies on thermal cycling, consisting of cycles of repeated heating and cooling of the reaction for DNA melting and enzymatic replication of the DNA. Primers (short DNA fragments) containing sequences complementary to the target region along with a DNA polymerase (after which the method is named) are key components to enable selective and repeated amplification. As PCR progresses, the DNA generated is itself used as a template for replication, setting in motion a

chain reaction in which the DNA template is exponentially amplified. PCR can be extensively modified to perform a wide array of genetic manipulations (Bartlett, et al. 2003).

## 1.8.3 DNA sequencing

**DNA sequencing** is the process of determining the precise order of nucleotides within a DNA molecule. It includes any method or technology that is used to determine the order of the four bases- adenine, guanine, cytosine, and thymine in a strand of DNA. The advent of rapid DNA sequencing methods has greatly accelerated biological and medical research and discovery. Knowledge of DNA sequences has become indispensable for basic biological research, and in numerous applied fields such as diagnostic, biotechnology, forensic biology, and biological systematics. The rapid speed of sequencing attained with modern DNA sequencing technology has been instrumental in the sequencing of complete DNA sequences, or genomes of numerous types and species of life, including the human genome and other complete DNA sequences of many animal, plant, and microbial species. And after the development of fluorescence-based sequencing methods with automated analysis, DNA sequencing has become easier, the most efficient technique and orders of magnitude faster (Olsvik, et al. 1993).

## 1.8.4 Real time PCR

**Real-time PCR**, also called quantitative real time polymerase chain reaction (qPCR) or kinetic polymerase chain reaction is a technique based on the PCR, which is used to amplify and simultaneously quantify a targeted DNA molecule. For one or more specific sequences in a DNA sample, Real Time-PCR enables both detection and quantification. The

quantity can be either an absolute number of copies or a relative amount when normalized to DNA input or additional normalizing genes. The procedure follows the general principle of polymerase chain reaction; its key feature is that the amplified DNA is detected as the reaction progresses in real time. This is a new approach compared to standard PCR, where the product of the reaction is detected at its end. Two common methods for detection of products in real-time PCR are: **(1)** non-specific fluorescent dyes that intercalate with any double-stranded DNA, and **(2)** sequence-specific DNA probes consisting of oligonucleotides that are labeled with a fluorescent reporter which permits detection only after hybridization of the probe with its complementary DNA target; These fluorescent reporter probes detect only the DNA containing the probe sequence; therefore, use of the reporter probe significantly increases specificity, and enables quantification even in the presence of non-specific DNA amplification. Fluorescent probes can be used in multiplex assays—for detection of several genes in the same reaction—based on specific probes with different-colored labels, provided that all targeted genes are amplified with similar efficiency (Van Guilder et al. 2008).

In a real time PCR assay a positive reaction is detected by accumulation of a fluorescent signal. The Ct (cycle threshold), which is defined as the number of cycles required for the fluorescent signal to cross the threshold (ie exceeds background level). Ct levels are inversely proportional to the amount of target nucleic acid in the sample (ie. the lower the Ct level the greater the amount of target nucleic acid in the sample). Cts < 29 are strong positive reactions indicative of abundant target nucleic acid in the sample. Cts of 30-37 are positive reactions indicative of moderate amounts of target nucleic acid. Cts of 38-40 are weak reactions indicative of minimal amounts of target nucleic acid which could represent an

environmental contamination (Fig.1.5) shows the ranges of CT values and their strength (Van Guilder et al. 2008).

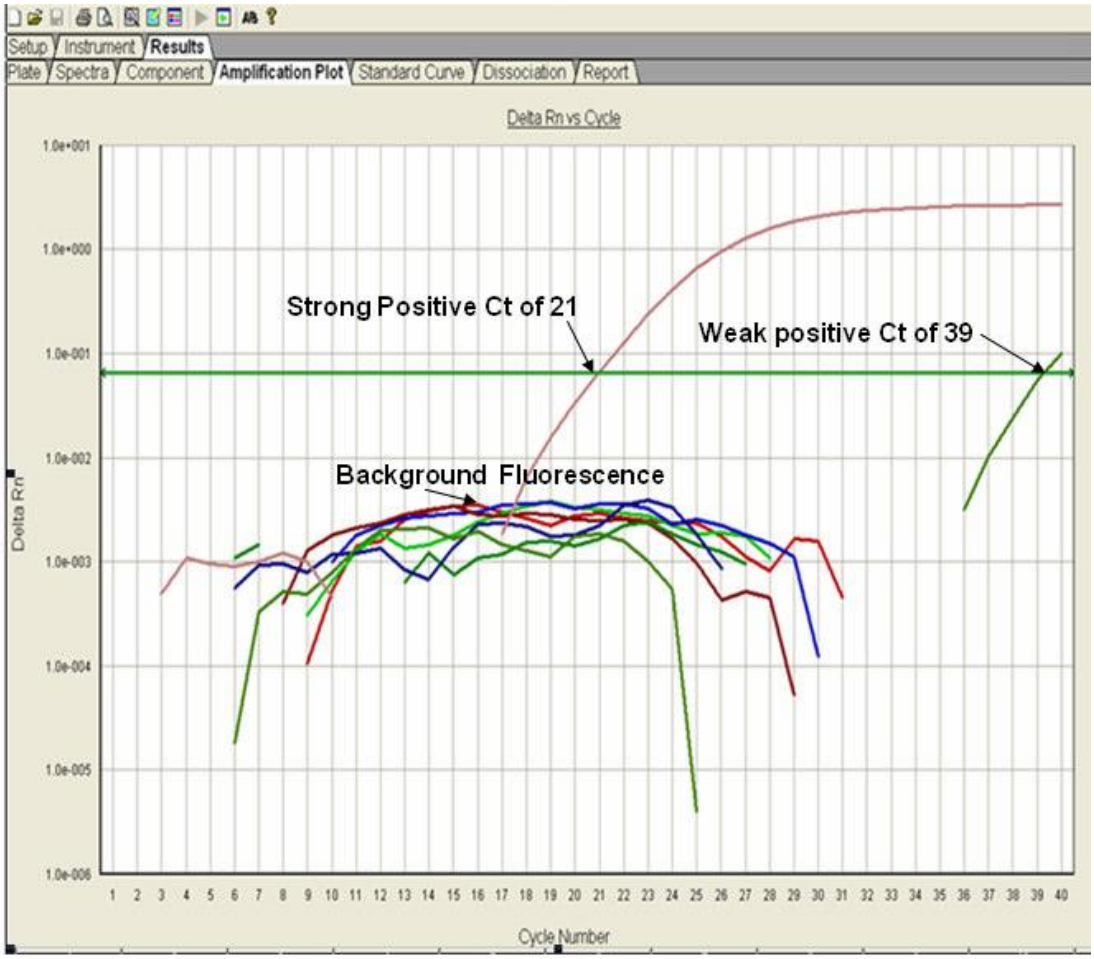


**Figure 1.5:** Ranges of CT values and their strength (Van Guilder et al. 2008).

In other words the threshold cycle is the cycle at which system begins to detect the increase in the fluorescent signal associated with an exponential growth of ampilcon during the log-linear phase. This phase provides the most useful information about the reaction (certainly more important than the end-point).

A number of variables can affect the efficiency of the PCR. These factors include length of the amplicon, secondary structure and primer quality (Van Guilder et al. 2008).

TaqMan probes are oligonucleotides longer than the primers (20-30 bases long with a Tm value of 10 $^{o}$C higher) that contain a fluorescent dye usually on the 5' base, and a quenching dye (usually TAMRA) typically on the 3' base, (TaqMan MGB probes have a non-fluorescent quencher and minor groove binder at the 3' end). When irradiated, the excited fluorescent dye transfers energy to the nearby quenching dye molecule rather than fluorescing. Thus, the close proximity of the reporter and quencher prevents emission of any fluorescence while the probe is intact. TaqMan probes are designed to anneal to an internal region of a amplicon. When the polymerase replicates a template on which a TaqMan probe is bound, its 5' exonuclease activity cleaves the 5' end of probe which contains the reporter dye 22 (Holland et al. 1991).

This ends the activity of quencher and the reporter dye starts to emit fluorescence which increases in each cycle proportional to the rate of probe cleavage. Accumulation of amplicons is detected by monitoring the increase in fluorescence of the reporter dye (note that primers are not labeled). TaqMan assay uses universal thermal cycling parameters and *PCR* reaction conditions. Because the cleavage occurs only if the probe hybridizes to the target, the origin of the detected fluorescence is specific amplification. The process of hybridization and cleavage does not interfere with the exponential accumulation of the product. One specific requirement for fluorogenic probes is that there be no G at the 5' end.

A 'G' adjacent to the reporter dye quenches reporter fluorescence even after cleavage. Well-designed TaqMan probes require very little optimization. In addition to qualification, hydrolysis probe real-time PCR

can be used to detect allelic variation using allele specific probes labeled with different fluorescent dyes (Holland et al. 1991).

### 1.8.4.1 Applications of real-time PCR

Since the PCR reaction starts to generate copies of the target sequence exponentially, Only during the exponential phase of the PCR reaction it is possible to extrapolate back to determine the starting quantity of the target sequence contained in the sample. Because of inhibitors of the polymerase reaction found in the sample, reagent limitation, accumulation of pyrophosphate molecules, and self-annealing of the accumulating product, the PCR reaction eventually ceases to amplify target sequence at an exponential rate and a "plateau effect" occurs, making the end point quantification of PCR products unreliable. This is the attribute of PCR that makes Real-Time Quantitative RT-PCR so necessary to be applied especially in researches, molecular biology, disease status and DNA mutation detection (Purves, 2001).

## 1.9 Literature review of PKU and its association with VNTR

Several studies conducted the molecular analysis of PAH gene and described the distribution of PAH mutation and their association with VNTR. Some of these studies were presented in this section.

Lyonnet and colleagues (1989) studied the Molecular Genetics of Phenylketonuria in Mediterranean Countries and a mutation associated with partial phenylalanine hydroxylase deficiency.

They reported the characterization of a mutation in the phenylalanine hydroxylase (PAH) gene associated with partial residual activity of the enzyme. This point mutation ($280^{glu\text{-}Iys}$) was found by sequencing a mutant cDNA clone derived from a needle biopsy of the liver in a child with variant form of phenylketonuria.

There was a strict concordance between homozygosity for the mutation and this particular phenotype. The ($280^{glu\text{-}lys}$) mutation is linked to an original and rare RFLP haplotype at the PAH locus found in south

Europe and North Africa. So far, this genotype-haplotype association is both inclusive and exclusive (Lyonnet et. al. 1989).

Popescu and colleagues (1998) studied the mutation spectrum and phenylalanine hydroxylase RFLP/VNTR background in 44 Romanian phenylketonuric alleles. The mutation spectrum and polymorphic haplotype background in 22 Romanian families have been analyzed in this study using the restriction digestion of phenylalanine hydroxylase (PAH) regions specifically amplified or the DGGE/direct sequencing methods. Eleven *PAH* mutations specifically associated with six mutant haplotypes were detected. In spite of the relative heterogeneity of the molecular defects in the PAH gene, three mutations covered almost 70% of all alleles: R408W, 47.72%, 21/44; K363fsdelG 13.63%, 6/44; and

P225T 6.81%, 3/44. Among these, R408W,is the most frequent mutation in this population, represented 50% of all the phenylketonuric (PKU) chromosomes. Splice mutation IVS12G>A affected two *PAH* alleles (4.54%); the remaining seven mutations were rare, each having an effect on just one chromosome (1/44), resulting in a relative frequency of 2.27%. A high frequency was observed in the PKU samples for the relatively uncommon mutations, K363fsdelG and P225T mutation, suggesting a possible founder effect at origin. Within the investigated panel, these mutations, both very rare among other Caucasians were exclusively linked to haplotype 5.8 and 1.7, respectively (Popescu et. al. 1998) .

Sueoka and colleagues (1999) studied the mutation screening of phenylketonuria in far east of Russia. They analyzed mutant genotypes at the human phenylalanine hydroxylase (PAH) locus among phenylketonuria (PKU) patients in the far east of Russia. A total of 60 variant alleles from 30 PKU families were analyzed for prevalent Caucasian mutations and restriction fragment length polymorphism\variable number of tandem repeats (RFLP\VNTR) haplotypes. 78% of all variant alleles carried 6 mutations. The most prevalent mutation was R408W (63%), with a haplotype background of 2.3. It also showed a very high degree of homozygosity (43%). The other 5 mutations (R158Q, R261Q, R252W, R261X, and IVS12nt-1) accounted for 1.7%-6.7% of all PKU alleles, and a single haplotype was associated with each genotype, except for R261Q. The genetic of PKU patients in the Far East of Russia seems to be relatively homogeneous, compared with that in the other Slavic and Oriental populations of surrounding countries (Sueoka et. al. 1999).

Acosta and colleagues (2001) studied the Mutations of the Phenylalanine Hydroxylase (PAH) Gene in Brazilian Patients With Phenylketonuria.

In that study, 115 Brazilian families with phenylketonuria (PKU), mainly from the Southeast of the country, were studied using three laboratory methods (DGGE, SSCP, and sequencing).

All 13 exons of the PAH gene were analyzed, including the splicing sites and the promoter region.

They identified 50 distinct mutations and characterized 91% of the mutant alleles. The five most prevalent mutations of the 50 mutations identified (50% of the PKU alleles) were IVS10nt-11G>A (17.4%), followed by R261Q (12.2%), V388M (9.1%), R252W (6.5%), and R270K (4.8%). The other mutations were rare. The mutation spectrum included 10 novel mutations (IVS5nt-54A>G, IVS6nt17G>T, E205A, F240S, K274E, I318T, L321L, C357G, IVS11nt17G>A, and S411X).

To characterize the origin and distribution of the PAH alleles they determined the association between the detected mutations and the PCR/RFLP haplotypes and VNTR alleles located on the PAH gene. For those patients whose mutant alleles were detected, they calculated the correlation with pretreatment phenylalanine levels, thus establishing a genotype/phenotype correlation. The present results confirm the marked heterogeneity observed at the PAH locus and contribute to the understanding of the distribution and frequency of PKU mutations in the Brazilian population (Acosta et. al. 2001).

Hechyporenko and colleagues (2001) studied the analysis of mutations and VNTR-polymorphism in the phenylalanine hydroxylase gene in Ukraine.

The data on 5 PAH gene mutations analysis are presented. The most common mutation observed in Ukrainian population was determined to

be R408W (66.6%). In addition two minor mutations R158Q (2.5%) and Y414C (1.25%) were identified. The allelic variation of the VNTR-polymorphism in 470 healthy volunteers and 39 *PKU*-patients were analyzed. 7 allelic variants and 15 haplotypes were found. The linkage disequilibrium was displayed between mutation R408W and VNTR-haplotypes 3 (Hechyporenko et. al. 2001).

**Abuglela,** (2007) studied 16 Libyan patients suffering from PKU for the presence of common PAH mutations (R252W, R261Q, R261X, IVS10nt11, R408W, and IVS12nt1) in PAH gene of exons 7, 11 and 12. The study involved DNA extraction and PCR-RFLP analysis and it showed the absence of these mutations in the studied Libyan patients.

Stojiljkovic and colleagues (2007) studied the utilization of mutations in the PAH gene as a tool for population genetics in Serbia.

In the Serbian population, 19 different PAH mutations have been identified. They used PAH mutations as molecular markers for population genetics study. The low homozygosity value of the PAH gene (0.10) indicates that PKU in Serbia is heterogeneous, reflecting numerous migrations throughout Southeast Europe. The strategy for molecular diagnostics of PKU was designed accordingly. To elucidate the origin of the most common (L48S) PKU mutation in Serbia, they performed haplotype analysis by PCR-RFLP. Their results suggest that the L48S mutation was imported into Serbia from populations with different genetic backgrounds (Stojiljkovic et. al. 2007).

Khemir and colleagues (2008), studied Phenylketonuria in Tunisian institutions for the mentally handicapped. The incidence of the disease is unknown in Tunisia and North Africa. The study aimed to assess the incidence of PKU among institutionalized patients with a mental handicap. A total of 833 patients from 17 different centers for the

mentally handicapped in Tunisia were included. Patients with a known genetic syndrome (e.g. Down syndrome) were excluded. The included patients were 6–46 years of age, and the sex ratio was 2:19 male(s)/females. Blood spots were collected using Guthrie cards and phenylalanine was analyzed using the fluorimetric method.

The patient was considered to have PKU when the level of phenylalanine was higher than 4 mg/100 ml**.** Eleven (1.32%) of 805 included patients were diagnosed as having PKU. These patients were divided into two groups according to the French classification , eight patients (0.96% of the total sample) had the classical form of PKU and three patients (0.36% of the total sample) had the moderate form of *PKU*. Patients were fairly evenly distributed across the country with three from the north, three from the middle and five from the south of Tunisia.

This frequency of 1.32% is close to the frequency of 1.6% observed in the two largest Kuwaiti institutions for the mentally handicapped and to the frequency of 1.9% reported in 1934 by Asbjorn Folling in Norwegian institutions for the mentally handicapped before neonatal screening for PKU was routinely carried out. In an Iranian study, approximately 2.2% of 1541 patients in eight different institutions for the mentally handicapped in the province of Isfahan had PKU. In contrast, biochemical screening for inherited metabolic disease in mentally handicapped patients in the Alexandra institute in Cape Town revealed only three patients with PKU, giving a frequency of 0.2% which is considerably lower than those reported in similar studies elsewhere.

The screening data clearly indicate the relatively high frequency of the disease among patients in Tunisian centers for the mentally handicapped, and highlight the need for neonatal screening in the Tunisian population in light of the high incidence of PKU (Khmir et. al. 2008).

Effat and colleagues **(**2008) studied the presence of 6 Mediterranean Mutations in 90 Egyptian patients with PKU. They aimed to assess the prevalence of 6 common mutations in the Mediterranean basin & Turkey among a large group of Egyptian PKU cases.

90 untreated patients 180 alleles were screened for 6 mutations (IVS10-11G>A, R261Q, R252W, Y277D, E221G, & G272S using PCR-RFLP.

IVS10-11G>A was found in 30 alleles (17%), R261Q was found in 12 allels (7%), & R252W in 3 (1.6%) while Y277D, E221G & G272S were not found in this group. They concluded that screening of six Mediterranean mutations identified a heterogeneous pattern among Egyptian PKU patients with a high frequency of IVS10-11G>A (Effat et. al. 2008).

Hosseini –Mazinani and colleagues ( 2008 ) studied 171 people of Iranian families (45 unrelated PKU subjects, and their parents and unaffected siblings) assessed the usefulness of carrier detection of PKU by variable number tandem-repeat(VNTR) polymorphism analysis. Of 342 chromosomes (131non-PKU and 211PKU), 5VNTR alleles were identified. This VNTR system would yield a polymorphism information content of 66%, comparable to that in Europeans and higher than in Chinese. Carrier detection by segregation analysis of VNTR was informative in 89.5% of siblings. They concluded that this polymorphism is highly informative in carrier detection of PKU in the Iranian population (Hosseini –Mazinani et. al. 2008).

Brenner and colleagues (2008) studied the independent origin of rare Y168H mutation of human phenylalanine hydroxylase gene in Russia. This mutation determining phenylketonuria was described only twice: in a patient from Catalonia (Spain) and in a patient from Western Siberia (Russia). The association of Y168h in these families with allelic variants

of STR and VNTR repeats and a number of neural point polymorphisms of *PAH* gene (IVS3nt-22C> T,Q232Q, V245V, L385L) was studied in this work. The Y168H mutation in these families was found to be associated with different haplotypes. Strong linkage of the selected markers and the mutation region excludes recommendation as a possible cause of association of Y168H with different haplotypes. It was concluded that Y168H occurred independently in different populations (Brenner et. al. 2008).

Dahri and colleagues (2010), studied Mutation analysis of phenylketonuria patients from Morocco: High prevalence of mutation G352fsdelG and detection of a novel mutation p.K85X.

They aimed to the knowledge of the molecular basis of the Phenylketonuria (PKU) in different countries provides relevant information for undertaking specific and rational mutation detection strategies in each population and for the implementation of adequate dietary and cofactor treatment. There were no data available in Moroccan population. They described the genetic analysis by mutation scanning using denaturing gradient gel electrophoresis (DGGE) and subsequent direct sequencing of 20 different PKU families from Morocco. They have also included the study of 7 Moroccan PKU patients living in Spain detected by the Spanish newborn screening program. They concluded that, the mutational spectrum in the first sample included eight different changes, one of them, K85X, was novel. The most common mutation was the frame shift change G352fsdelG identified in 62.5% of the mutant chromosomes studied. Other changes include (R176X, IVS10nt-11 g>a, W120X, A165T, R243X and R243Q) were identified, respectively, in 2 or 3 mutant alleles. All detected mutations were severe according to the classical phenotype of the patients. In the 7 patients living in Spain they

have detected 4 severe mutations (p.G352fs, p.R176X, Y198fs and Exon3del) and also milder changes such as p.A403V, p.S196T, p.D145V and p.R408Q detected in 3 mild hyperphenylalaninemia. MHP patients and a novel p.L258P found in a mild PKU patient (Dahri et. al. 2010).

Al Mutawa and colleagues (2010) studied the molecular analysis of phenylalanine hydroxylase PAH gene from dried spots from Libyan phenylketoneuria patients.They described the distribution of PAH mutations in nine probands from Libya with the diagnosis of phenylketonuria and hyperphenylalaninemia. Molecular genetics screening was done at the Shafallah Medical Genetics Center laboratory Doha, Qatar, by resequencing and analysis of the entire coding sequences, exon flanking regions and splice sites of PAH. The 13 exons , exon-intron boundaries and splice sites of the PAH were amplified by polymerase chain reaction using in-house designed primers and optimized conditions. The DNA sequencing reactions were carried out by automated sequencer using BigDye Terminator chemistry.

Two homologous PAH mutations were found in 7/9 probands and were C.838G>A/p. E280K (missense) and c.1055delG (frame shift). The frame shift mutation produces a truncated protein of 399 amino acid length. Two Probands were homozygous for the missense mutation, three were homozygous for the frame shift mutation and two were compound heterozygous for both mutations (Al Mutawa et. al. 2010).

## 1.10 Objectives of the study

The objectives were ,

1.To study the distribution of the E280K ($280^{g1u\text{-}lys}$) mutation among the family members of children suffering from PKU.

2.To draw the pedigree chart of PKU in the families of these children.

3.To study the association of this mutation with a VNTR in PAH gene.

4.To assess the usefulness of using VNTR as a quick screening tool for E280K mutation in PKU patients.

# Chapter Two

## Materials and Methods

## 2.1 Study population subjects (*selection of samples*)

The patients enrolled in this study were selected from the patients follow up in out patient clinic in Gharian hospital and some of them from out patient clinic in Misrata hospital in Libya.

A total of 20 individuals were included in this study which are members from two different Libyan families who have children suffering from PKU.

Each 10 samples were selected from different members of one family which include the child (children) suffering from PKU and their father, mother, brothers, sisters, uncles, aunts, grandfather and\or grandmother. They were divided into two groups, patients and normal individuals. Personal information about the participants and their willingness to participate in the study were ensured by distributing a written questionnaire to every participant which included questions about personal information such as name, age,…..etc this questionnaire is provided in appendix 1.

A number of 1 child from first family and 2 children from the second family were suffering from PKU, the rest of members from these two different families were unknown carriers or wildtype.

Finally, these 20 individuals were tested after agreeing to the written informing consent by signing it.

## 2.2 Blood samples collection and storage

A 4 ml blood samples were collected in a vacationer tubes with EDTA as an anticoagulant and were transported to the laboratory of National centre of disease control- Tripoli (NCDC) then lyses and stored at -20 $^{o}$C until needed for DNA extraction and subsequently real-time PCR and PCR analysis.

## 2.3 Materials

### 2.3.1 chemicals

All chemicals used in this study were of molecular grade and are listed in table (2.1).

**Table 2.1:** List of chemicals and their sources.

| Chemicals | Source |
|---|---|
| Ethanol 96-100% | Sigma |
| Agarose powder | Sigma |
| Ethidium bromide 10mg\ml solution | Sigma |
| Tris borate EDTA (TBE) buffer | Sigma |
| Loading dye | Sigma |
| Proteinase k | QIAGEN |
| DNA Extraction Kit | QIAGEN |

### 2.3.2 Instruments and devices

- Centrifuge.
- Micro centrifuge.
- Vortex.
- Spinner.
- Block heater.
- Refrigerator.
- Microwave.
- Electrophoresis apparatus.
- UV Transilluminator & gel documentation system.
- TC-412 Thermocycler (Techne).
- Real-time PCR machine Mx3005p (Stratagen).

## 2.4 Methods for genotyping

In the present study, the aforementioned mutation was studied in 20 members from two different Libyan families who have children suffering from PKU. The genotyping genetic analysis included:

1. The DNA extraction using the QIAGEN DNA purification kit.
2. For the E280K mutation frequency, the real-time PCR protocol designed by Dr. Abdulla Bashein (Bashein, et al in preparation) was used.
3. To detect the VNTR, the method described by Goltsov, et. al. (1992) was used.

### 2.5 DNA extraction protocol

DNA was extracted and purified from lyses whole blood by using QIAGEN KIT according to the specific protocol.

The buffers used in this protocol were prepared as following:

i. QIAGEN protease stock solution was prepared by pipetting 1.2ml protease solvent into the vial containing lyophilized QIAGEN protease. (stored at 2-8$^{o}$ C).

ii. Buffer AL was prepared by mixing buffer AL thoroughly by shaking before use. (stored at RT).

iii. Buffer AW1 was supplied as a concentrate (95%) and was prepared by adding the appropriate amount of ethanol (96-100%) (125ml) to obtain the final volume of 220ml. (stored at RT).

iv. Buffer AW2 was supplied as a concentrate (66%) and was prepared by adding the appropriate amount of ethanol (96-100%) (160ml) to obtain the final volume of 226ml. (stored at RT).

After QIAGEN buffers were prepared , the procedure of DNA extraction and purification protocol were done as following:

1) 20µl QIAGEN protease (proteinase k) were pipetted into a bottom of a 1.5ml micro centrifuge tube.
2) 200µl blood sample were added to the 1.5 micro centrifuge tube.
3) 200µl buffer AL was added to the sample and mixed by pulse-vortexing for 15sec.
4) Then, the previous homogeneous solution was incubated at 56$^{o}$C for 10 min in a block heater.
5) The sample in 1.5ml micro centrifuge tube was briefly centrifuged to remove drops from the inside of the lid.
6) 200µl ethanol (96-100%) were added to the sample and mixed again by pulse-vortexing for 15 sec. after mixing the 1.5ml micro centrifuge tube was centrifuged briefly to remove the drops from the inside of the lid.
7) The mixture from step 6 was applied carefully to the QIAamp mini spin column (in a 2ml collection tube), the cap was closed and centrifuged at 8000rpm for 1 min. then the QIAamp mini spin column was placed in a clean 2ml collection tube and the tube containing the filtrate was discarded.
8) QIAamp mini spin column was opened carefully and 500µl buffer AW1 were added. The cap was closed and centrifuged at 8000rpm for 1 min. the QIAamp mini spin column was placed in a clean 2ml collection tube and the collection tube containing the filtrate was discarded.

9) QIAamp mini spin column was opened carefully and 500µl buffer AW2 were added. The cap was closed and centrifuged at full speed 14000 rpm for 3 min.

10) QIAamp mini spin column was placed in a new 2ml collection tube, and the old collection tube with the filtrate was discarded, and centrifuged at full speed 14000rpm for 1 min.

11) QIAamp mini spin column was placed in a clean 1.5ml micro centrifuge tube and the collection tube containing the filtrate was discarded.

12) To elute DNA 100µl buffer AE or distilled water were added and incubated at room temperature (25$^{o}$C) for 1 min, then centrifuged at 8000rpm for 1 min.

**2.6 Determination of genomic DNA quality by agarose gel electrophoresis**

To determine that the DNA samples extracted from previous procedure are suitable for PCR reaction, they were analyzed by agarose gel electrophoresis according to the following procedure:

1) 1.5 gm agarose powder (Sigma) was weighed and dissolved in 150 ml 0.5X TBE buffer by microwave.
2) After the agarose was completely melted to solution , it was left to cool at room temperature until its temperature reached about 65$^{o}$C then 5µl 10 mg/ml ethidium bromide was added and mixed well with agarose solution.
3) Then the agarose mixture was poured carefully into a gel casting tray with comb in place and left to solidify at room temperature, then the gel was putin the electrophoresis

apparatus and the TBE 0.5x buffer was poured to the maximum limit .

4) The comb was removed and extracted DNA samples were injected into the gel wells.
5) The power supply of gel electrophoresis was adjusted to 75 volt and 80 Amber and started the run.
6) After 30min the power supply of gel electrophoresis was turned off and the DNA bands were detected using the gel documentation system .
7) Extracted DNA product samples were stored at -20ºC for subsequent analysis reactions.

## 2.7 Hydrolysis probe Real-time PCR (TaqMan probe)

Real time polymerase chain reaction was done for exon 7 of the PAH gene for the detection of the E280K mutation.

The primers were designed to amplify a fragment of 181 bp of exon 7 and the probes were designed to detect both the wildtype and the mutant alleles. The wildtype probe was labeled with the FAM dye at 5' end and BHQ1 at 3' end, and the mutant probe was labeled with HEX dye at 5' end and BHQ1 at 3' end. The PCR reagents used were from *meta***bio**n (Germany), and the Mx3005p real- time PCR machine (Stratagen) was used.

The reaction volume was 25µl, and the components of this reaction are described in table (2.2).

**Table 2.2:** PCR reaction components for amplification of exon 7 of PAH gene fragments.

| Reagent | Stock concentration | Volume (µl) for 1 reaction | Final concentration |
|---|---|---|---|
| 2X real time MM | 2X | 12.5 | 1X |
| Primer each | 10µM | 0.75 | 0.3mM |
| Probe (Hex) | 10µM | 0.5 | 0.2mM |
| Probe (Fam) | 10µM | 0.5 | 0.2mm |
| DNA sample | 25ng/µl | 2 | 25ng/µl |
| $ddH_2O$ | | 8 | |
| Total volume | | 25µl | |

The sequence of these primers (forward and reverse), the probes (Fam and Hex) and their annealing temp. are shown in (Bashein et al. in press) and are given in table (2.3).

**Table 2.3:**The sequence of primers and probes and their annealing Temp used in real time PCR.

| Primer / Probe | Sequence | Annealing Temp. |
|---|---|---|
| Forward primer | 5'-GTGTACTACTCCACTACCTAAAGGTC-3' | 61.3C° |
| Reverse primer | 5'-GAACCCAAACCTCATTCTTG-3' | 56.4C° |
| Probe (Fam)wildtype | CATGTATACCCCCGAACCGTGAG | 66.4C° |
| Probe (Hex)mutant | CATGTATACCCCCAAACCGTGAG | 64.6C° |

HEX probe works as a complementary for mutant alleles, while Fam probe works as a complementary for normal alleles.

The PCR tube containing the components of 25µl was then placed in a thermo cycler and *PCR* amplification was started according to the thermal profile described in table (2.4).

**Table 2.4:** Thermal cycle program for real time PCR.

| No. of cycles | Description | Temperature (C$^{o}$) | Time |
|---|---|---|---|
| 1 | Initial denaturation and polymerase activation | 95 | 2min |
| 40 | Denaturation | 95 | 15sec |
| | Annealing | 58 | 20sec |
| | Extension | 65 | 30sec |

## 2.8 PCR for VNTR determination

*PCR* amplifications were performed using a thermal cycler TC-412 (Techne). 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. 1992). And sequences of forward and reverse primers for this reaction are given in table (2.5).

**Table 2.5:** Sequence of primers used in PCR reaction for VNTR.

| Primer | Sequence | Melting temp. ($^{o}$C) |
|---|---|---|
| Forward | *GCTTGAAACTTGAAAGTTGC* | 54.3 |
| Reverse | *GGAAACTTAAGAATCCCATC* | 54.3 |

The total volume reaction was 25µl, and the reaction components were obtained from Metabion and are given in table (2.6).

**Table 2.6:** PCR reaction components for VNTR amplification.

| Reagent | Stock concentration | Volume (µl) for 1 reaction | Final concentration |
|---|---|---|---|
| 2X mi Hot Taq Mix | 2X | 12.5 | 1X |
| Primer each | 10µM | 0.75 | 0.3µM |
| DNA | 25ng/µl | 2 | 50ng/µl |
| dd$H_2O$ | | 9 | |
| Total volume | | 25 | |

### 2.8.1 Primers preparation:

1) The primers were prepared by adding 5µl TE to every 1nmol lyophilized powder of the primer to give the final concentration of 100µM.
2) Total reaction volume of 25 µl contained 2 µl of genomic DNA plus 23 µl of MM was prepared.
3) PCR reaction was performed for 40cycles each consisting of 1min at 95$^o$C, 2min denaturation at 92$^o$C,1min annealing at 55$^o$C, and 5min extension at 72$^o$ C.
4) PCR products were kept at 4-8$^o$C for VNTR detection by agarose gel electrophoresis.

## 2.9 Detection of VNTR by agrose gel electrophoresis

Detection of VNTR, by using agarose gel electrophoresis was as follows:

1. 3gm agarose powder (Sigma) were weighed and dissolved in 150 ml 0.5X TBE buffer using microwave.
2. After the agarose was completely melted, leave it to cool at room temperature until its temperature reached about 65$^o$C then 5µl

10mg/ml ethidium bromide were added and mixed well with melted agarose.

3. Then the agarose mixture was poured carefully into a gel casting tray with comb in place and left to solidify at room temperature, then the gel was put in the electrophoresis apparatus and the TBE 0.5X buffer was poured to the maximum limit .
4. The comb was removed and the extracted DNA samples were injected into the gel wells.
5. The mi-50 bp DNA Marker Go (*meta***bio**n), which consists of 10 DNA fragments ranging from 50 to 500 bp, was injected in the first well and mi-100 bp+ DNA Marker Go (*meta***bio**n), which consists of 11 discrete fragments ranging from 100 to 1500 bp, was injected in the last well (these two markers are supplied in a ready- to- use format and they are ideal for size determination of double stranded DNA fragments on agarose gels).
6. The power supply of gel electrophoresis was adjusted to 75volt and 80 Amber and started the run.
7. After 30min the power supply of gel electrophoresis was turned off and the DNA bands were detected for VNTR using the gel documentation system.

# Chapter Three

## Results and Discussion

## 3.1 Testing of extracted DNA quality

The amounts of genomic DNA obtained from the blood samples were relatively high and their quality was good which were determined by agarose gel and these amounts were enough for the subsequent reactions, real-time PCR and conventional PCR for determination of VNTR.

Fig. (3.1) shows the extracted genomic DNA.

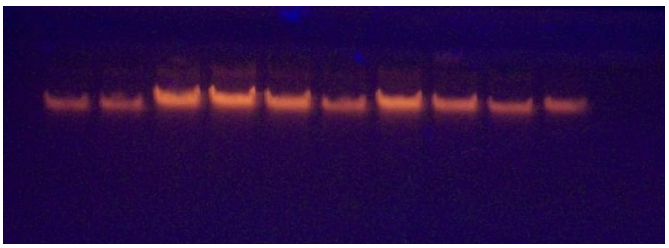

**Figure 3.1**: Extracted genomic DNA products for the study cases.

## 3.2 Determination of the E280K distribution

181bp of exon7 were amplified by using real-time PCR for the determination of the E280K mutation distribution in two different Libyan families which have child (children) suffering from PKU.

The family No. (1) have one child suffering from PKU (mutant E280K/E280K), their parents were carriers (E280K/WT) and their relatives were mixed (wild type and carriers).

The result of Ct. values for HEX and FAM of the family No. (1) are given in table (3.1).

**Table 3.1:** Ct values for HEX (mutant) and FAM (wild type) of the studied cases of family No. (1).

| 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 |

The family No. (2) have 2 children suffering from *PKU* (mutant E280K\E280K), and their parents were carriers (E280K/WT).

The result of Ct. values for HEX and FAM of the family No. (2)were given in table (3.2).

**Table 3.2:** Ct values for HEX (mutant) and FAM (wild type) of the studied cases of family 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 |

**Notes:**

- Mutant (E280K/E280K).
- Carrier (E280k/WT).
- PKU (1): PKU case from family (1).
- Uncle (1-M): family No. (1) uncle from mother side.
- Uncle (1-F): family No. (1) uncle from father side.
- PKU(2): PKU case from family No. (2).

The tables (3.1 and 3.2) described the samples No., Ct values for Hex and Fam and the status. *PKU* case which is the mutant (E280K\E280K) that carries two mutated alleles inherited from both carrier (E280K) parents (autosomal recessive fashion). From the given results 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 not. Wild type cases react with FAM probe which is complementary for normal allele and give Ct values with it and HEX probe doesn't react with normal allele. The carrier cases react with FAM probe and HEX probe and give Ct values with both of them because the carrier case has both normal and mutant alleles. The Ct values which

obtained are mostly strong Ct values (< 29) indicating high template concentration (Fig.3.2)

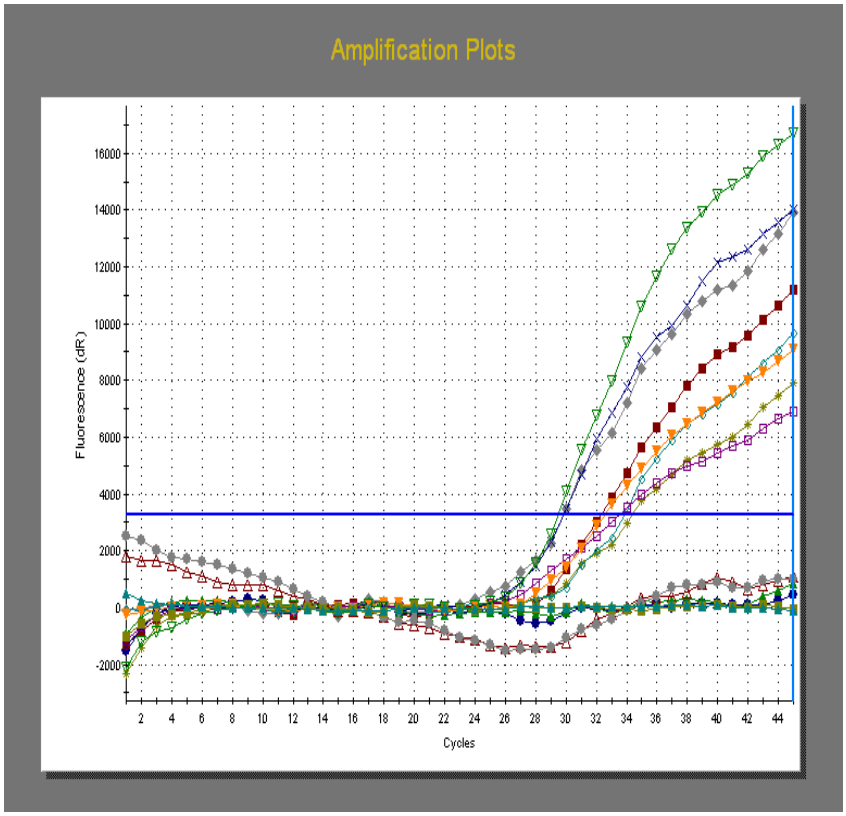


**Fig 3.2:** Amplification curve of the mutant and wild type probes.

The sample under the threshold line considered as negative, and the samples with curves crossing the threshold line are considered as positive.

The distribution of E280K mutation in this study was found that 15% of the study cases (six out of twenty cases) were mutant, 55% of them were carriers and 30% were wild type (Fig.3.3).

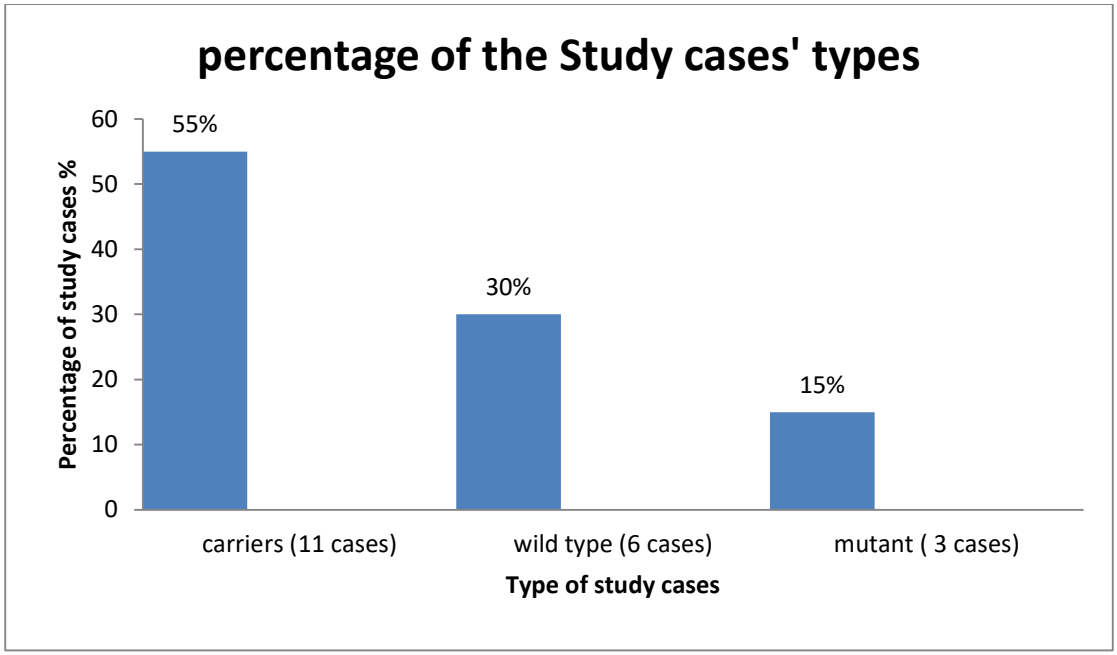


**Figure 3.3:** Chart represents the percentage of the study cases' types.

These percentages were gained from studying selected Libyan participants. However these selections cannot be described as fully representatives to the whole Libyan population. This is due to the fact that the selected participants were few in number and were chosen from only two families from small regions in Libya; i.e. Ghiryan and Musrata. Still, according to a previous study (Al Mutawa et al. 2010) that was carried out in Jala pediatric hospital in Libya, Tripoli, E280K mutation was found to be one of the most common mutation in Libya.

The parents of the cases that were studied were relatives. According to this, it can be said that the mutation of E280K is strongly related to consanguinity, which is very common tradition in Libya. Therefore it can be predicted that consanguinity in families that have members that are carriers or disordered is a direct cause to the spread of this mutation in the coming generations according to Mendel's Law (Tadmouri et al. 2009).

One of the Turkish screened classical PKU patients for various mutations found that IVS10 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 $280^{glu\text{-}lys}$ (-) ( Ozgfil et al. 1993).

In Libya E280K was one of the most common mutations (Al Mutawa et al. 2010), while in Morocco the most common mutation was the frame shift change p.G352fsdelG. Other changes were (p.R176X, IVS10nt-11 G>A, p.W120X, p.A165T, p.R243X and p.R243Q) and they didn't identify any E280K mutation in the studied cases (Dahri et al. 2010). While in Egyptian population the most common mutation was IVS10nt546 followed by R261Q and L48S. In the exon 7 they found 3 missense mutations named (R243P, R261Q, Y277D) and one silent mutation (V245V) (Shawky et al. 1998). 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 et al. 1999).

E280K mutation at codon 280 was identified in endogenous North Africa families and it was later demonestrated to be the most frequent in the whole Maghreb. Thus mutation was also identified in a French family thus raising the question of a relation with the Arab invasion during the seventh century AD (Lyonnet et al. 1989).

The real-time PCR technique which used in this study has many advantages compared with other techniques such as traditional PCR, RFLP and/or sequencing. Some of these advantages involve that; it is accurate, rapid, simple and non expensive.

## 3.3 PKU Families Pedigrees

As a phenylketonuria is an autosomal recessive disorder present from birth. This means that an affected individual must have gained mutant alleles from both parents.

When considering a whole family pedigree it is often siblings who have the disease. This can be referred to as Horizontal Transmission. Other relatives can be affected but this usually only occurs in large inbred families. However, when two carriers meet there is still only a 25% chance of them giving any one child with PKU. The disease affects women and men equally but there are, perhaps, greater implications for a woman suffering from PKU as she has to take the disease into account before she has children. A recessive trait is more commonly expressed if parents are related. This is because 2 carriers of the same recessive gene are more likely to unite. This is termed consanguinity and it usually takes place between cousins. This is more likely to be relevant if the condition is rare (Woolf L. 1986).

As mentioned before the first family was composed of three children one of them was PKU case, figure (3.4) shows the pedigree tree of the first family.

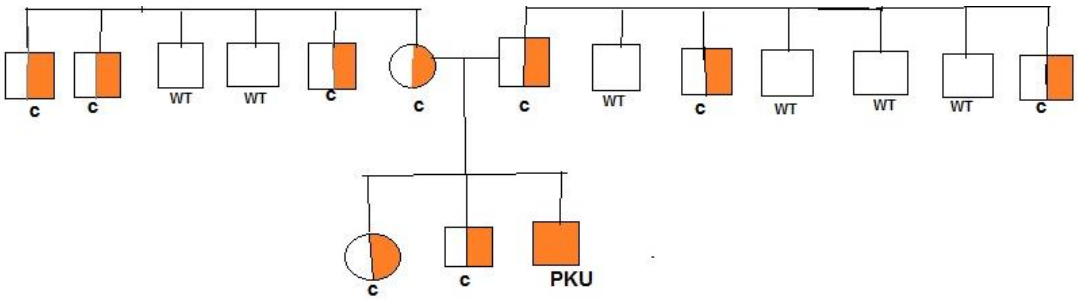


**Figure 3.4**: Family (1) pedigree tree.

Figure (3.4) shows that 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 suggest that the both grandfathers and grandmothers or one of them at least form both sides (father and mother) were carriers and wild types, (Actually we had no data about them in this study).

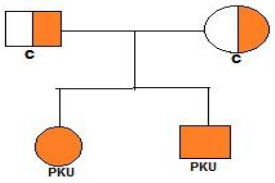


**Figure 3.5**:Family(2) pedigree tree.

The pedigree tree of family No.(2) shows that this family composed of two PKU cases and their carrier parents.

## 3.4 Detection of VNTR

An AT-rich (70%) minisatellite 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 (Jeffreys et al. 1985), this AT-rich minisatellite region was amplified from genomic DNAs of patients and other members of PKU families, both to examine possible 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 (Jeffreys et al. 1985).

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 (Goltsov et al. 1992).

For evaluating the polymorphism status, the amplified products were analyzed using 3% agarose gel electrophoresis as shown in (Fig.3.6 a & b).


**Figure 3.6a :** Repeated units of VNTR for the some studied cases for family No.(1).

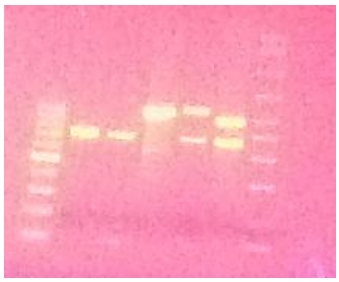

**Figure 3.4b :** Repeated units of *VNTR* for the studied cases for family No.(2).

DNA fragments with 364 and 514 bp length corresponded to 3 and 8 copies of VNTR units, respectively are reported in the table (3.3 and 3.4) for two studied families.

**Table 3.3:** 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, 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, 8 |
| 6 | Wild type | 1 band (514bp) | 8, 8 |
| 7 | Wild type | 1 band (514bp) | 8, 8 |
| 8 | Carrier | 2bands(364bp, 514bp) | 3, 8 |
| 9 | Wild type | 1 band (514bp) | 8, 8 |
| 10 | Carrier | 2bands(364bp, 514bp) | 3, 8 |
| 11 | Carrier | 2bands(364bp, 514bp) | 3, 8 |
| 12 | Wild type | 1 band (514bp) | 8, 8 |
| 13 | Wild type | 1 band (514bp) | 8, 8 |
| 14 | Carrier | 2bands(364bp, 514bp) | 3, 8 |
| 15 | Carrier | 2bands(364bp, 514bp) | 3, 8 |
| 16 | Carrier | 2bands(364bp, 514bp) | 3, 8 |

**Table 3.4:** 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 fragments** | **No. of repeats** |
| 17 | Mutant | 1 band (364bp) | 3, 3 |
| 18 | Mutant | 1 band (364bp) | 3, 3 |
| 19 | Carrier | 2bands(364bp, 514bp) | 3, 8 |
| 20 | Carrier | 2bands(364bp, 514bp) | 3, 8 |

As shown in tables 3.3 and 3.4, 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.

Units of less than 3 or more than 8 repeats have not been observed in Libyan population, while a unit less than 3 or more than 12 repeats has not observed in other studied populations of United State of America, Brazil, Italy, Russia, Poland, Sweden, England, Turkey, Ireland, Spain, Switzerland, and Denmark, but 13 units of VNTR has recently reported in Iran. It is suggested that the alleles of VNTR are produced by the gain or loss of a single VNTR unit, possibly by slippage during DNA replication (Kamkar et al.2003).

## 3.5 Conclusion

## We can concluded that,

- Offsprings who can be disordered by PKU, both parents should be carriers or disordered (autosomal recessive disease).
- The distribution of the E280K mutation among the subjects that were studied is 55% carriers, 30 % wildtype and 15% mutant cases.
- By using traditional PCR it was found that the mutant alleles is directly related to repeat 3 , while the wild type allele is related to repeat 8. For the carrier cases, it follows that they should possess both repeats; repeat 3 and repeat 8.
- Detection of VNTR in high risk PKU subjects will aid in early screening of E280K mutation.
- According to the findings of this study and from the social perspective, it can be expected that, decreasing consanguinity will help lower 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.

## 3.6 Recommendations

- PKU is a good candidate for neonatal screening because the blood test is easily performed, the treatment is well known and available, and the consequences of missing the diagnosis early and not treating the disease are serious. Rather than caring for patients who are severe physically and mentally disabled, it is very cost effective and highly recommended to screen for and treat the disease at birth.
- The use of VNTR will aid prenatal testing in addition, association of specific VNTR alleles with mutations in the PAH gene can shed light on the origin and diffusion of various mutant alleles.
- Methodologically, it is recommended that a bigger sample have to be tested.
- For a 100% generalization of the findings, it is recommended that the entire Libyan population have to screened for PKU. A good way to do this is to include a test at birth.
- Besides, it is recommended that a sample is chosen from every geographic region in Libya to be tested for *PKU* so as to correlate the occurrence and frequency of PKU to the environmental conditions, such as climate conditions, pollution, diet habits and more important, ethnicities.
- It is recommended in the procedure that many trials are carried out to study more than one sample from *every* participant to eliminate the possibility that any systematic or random errors have taken place throughout the study.
- It is also recommended that any participants chosen to be tested in similar future studies undergo tests for other mental or behavioral

disorders to make sure that there are no extraneous factors that act as independent variables that affect the DNA sequences studied.

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# Appendix 1: The questionnaire

الاسم:.....
اللقب:.....
العمر:.....
المنطقة:.....
اسم الأب ثلاثي:.....
اسم الأم ثلاثي:.....
هل الأب والأم أقارب:.....
هل تعانى من اى أمراض أخرى: نعم ..... لا.....

أوافق واقر أنا الموقع أدناه اننى بطوعي واختياري وبكامل قواي العقلية على اخذ عينة دم واستعمالها فيما يخص البحث العلمي لمرض الفينيل كيتونيوريا .
الاسم:.....
التوقيع:.....
التاريخ:.....

# Appendix 2: Full sequence of exon 7 of PAH gene

*GCCTAGCGTCAAAGCCTATGTCCCTGGGCAGTTATGTGTACTACTCCACTACCTAAAGGTCTCCTAGTGC*

*CTCTGACTCAGTGGTGATGAGCTTTGAGTTTTCTTTCTTCTTTTCATCCCAGCTTGCACTGGTTTCCGCC*

*TCCGACCTGTGGCTGGC**CTGCTTTCCTCTCGGGATTTC**TTGGGTGGCCTGGCCTTCCGAGTCTTCCACTG*

*CACACAGTACATCAGACATGGATCCAAGCC**CATGTATACCCCCGAACCGTGAG**TACTGTCCTCCAGCTAC*

*CAGTTGCCAGGCACAATGAGCGCCATCTTTTCCTGCTGCAAGAATGAGGTTTGGGTTCATTGCTGGCTGG*

# Appendix 3: VNTR for PAH gene

CACACACAGATGGTCACTGTCTTTTAATAGATTTCCTGTGAAGTTTCGAAAGTAAAATTGCTTGAAACTT

GAAAGTTGCTAAGAGTAGATTTTAATGTTCTCACCCGCCAAAAATATAAATATGTTATGTGATGGATATG

CTAATTACCTTGATTTAATCATTTTACAATGTGTGTGCACATATATGTATATGCATATGTACGTATGCAC

ATATATGTATATGCATATGTACGTATGCACATATATGTATATGCATATGTACGTATGCACATATATGTAT

ATGCATATGTACGTATGCACATATATGTATATGCATATGTACGTATGCACATATATGTATGTGCATATGT

ACGTATGCACATATATGTATGTGCATATGTACATAGGCACATATATGTATGTGCATATGTATGTATACAT

ATAAATGCATATATGTGCATATCTATATATGTATTGCACACATATATATACACCACATTATACACCATAA

ATATATACAATTTGTATTTGTGAATTTTAAAACTTTCTTTTAAAAAGATGTGCTTCTCTGAGAGATGGGA

TTCTTAAGTTTCCAAGAAAACATTACTAAAGAAATCATAACAGCCTTTTAATTTTTTAATTTGCCTAAAT